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Ing. Jana Hinďoš Hřebačková

Působení kyseliny valproové a její účinek v kombinaci s cytostatiky na nádorové buňky *in vitro*

Effects of valproic acid and its combinations with cytostatic agents on tumor cells *in vitro*

Disertační práce

Školitel: Prof. Ing. Jan E. Dyr, DrSc.

Konzultant: Prof. MUDr. Tomáš Eckschlager, CSc.

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OBSAH

Abstract

Cancer is one of the most challenging problems the modern medicine is facing today. An increasing incidence and a great variability of tumor cells are the main reasons those drive the research to develop better diagnostics and therapeutic protocols. Histone deacetylase inhibitors, a group of epigenetic chemotherapeutics, are able to improve the performance of currently used anticancer agents. Vaplroic acid that is commonly used as antiepileptic drug exhibits a remarkable anticancer activity by itself as well as it is capable of therapy potentiation based on other therapeutic agents. Its effect to inhibit growth of tumor cells and induce apoptotic cell death seems to be even greater under hypoxic conditions (<1% O2).

This study is focused on effect of valproic acid on neuroblastoma cell lines in vitro under normoxic and hypoxic conditions. We observed significantly greater efficacy of valproic acid in hypoxia compared to normoxia. The mechanism of induction of apoptotic cell death is based on disruption of the balance between pro- and antiapoptoic proteins. Intrinsic apoptotic pathway is probably initiated by the action of 19 kDa variant of proapoptotic protein Bax on mitochondrial membrane. Moreover, we examined the efficiency of a combined treatment of neuroblastoma cells with valproic acid and DNA-targeting chemotherapeutics – cisplatin and ellipticine. Combination with cisplatin showed synergistic effect only under certain conditions depending on the cell line and experimental conditions. On the other hand, combination treatment with valproic acid and ellipticine was significantly more effective than treatment with each drug separately. The synergy mechanism is based on direct cytotoxic effect of valproic acid, changes in chromatin structure and accessibility of DNA to ellipticine and cisplatin and in case of ellipticine on changes in levels of cytochromes P450 responsible for its metabolization. The future perspective of valproic acid as an anticancer agent is further supported by many research studies as well as by a number of ongoing clinical trials.

Abstrakt

Nádorová onemocnění představují v dnešní společnosti jeden z nejzávažnějších problémů moderní medicíny. Jejich zvyšující se incidence a velká variabilita nádorových buněk jsou hlavními důvody pro zlepšování diagnostických a terapeutických postupů. Perspektivní skupinou látek zesilujících účinky konvenční chemoterapie jsou inhibitory histondeacetylas, které lze zařadit mezi epigenetická chemoterapeutika. Nejdéle používanou látkou z této skupiny je antiepileptikum kyselina valproová, která má navíc protinádorové účinky a podporuje působení řady chemoterapeutik. Významným se jeví zejména její účinek na růst a buněčnou smrt nádorových buněk za hypoxických podmínek (<1% O_2).

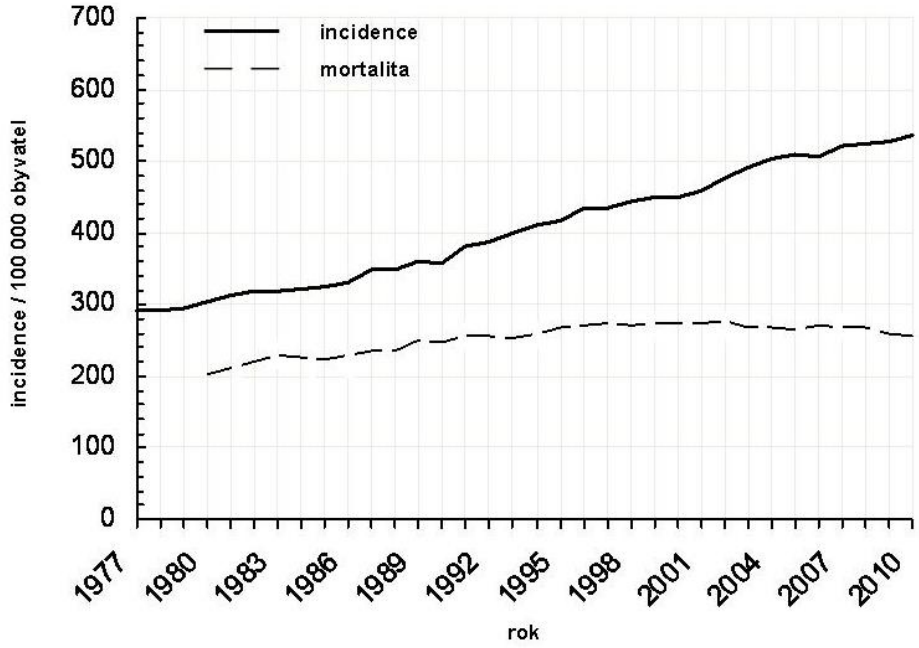
Tato práce je zaměřena na sledování účinků kyseliny valproové na neuroblastomové buněčné linie *in vitro* v normoxii a hypoxii. V hypoxii vykazovala významně vyšší účinek než v normoxii. Spouštěcím mechanismem apoptotické buněčné smrti je narušení rovnovážného stavu pro- a anti apoptotických proteinů. Vnitřní apoptotická dráha je pravděpodobně zahájena narušením mitochondriální membrány 19kDa variantou proapoptotického proteinu Bax. V dalších experimentech jsme testovali účinek kombinace kyseliny valproové a DNA-poškozujících chemoterapeutik – cisplatiny a ellipticinu – na neuroblastomové buňky. Kombinace s cisplatinou vykazovala synergistický účinek pouze v některých případech v závislosti na typu buněčné linie a podmínkách experimentu. U ellipticinu byl účinek kombinace obou látek významně vyšší než účinek jednotlivých preparátů u všech testovaných linií. Mechanismus synergie je založen na přímém účinku kyseliny valproové na nádorové buňky a na ovlivnění chromatinové struktury a přístupnosti DNA pro cisplatinu a ellipticin. U ellipticinu je to také vliv na expresi cytochromů P450 podílejících se na jeho nitrobuněčné metabolisaci. Perspektivu kyseliny valproové pro kombinační protinádorovou terapii potvrzují i mnohé probíhající klinické studie a další výzkumné práce.

1. SEZNAM POUŽITÝCH ZKRATEK A SYMBOLŮ

AIF	faktor indukující apoptosu
An/PI	annexin/propidiumjodid
Bcl -2	"B-cell lymphoma 2" protein
CA9	karbonanhydrasa 9
CDDP	cisplatina
CDK	cyklin dependentní kinasa
COX-1	cyklooxygenasa 1
CYP	cytochrom P450
elli	ellipticin
eNOS	endotheliální NO synthasa
FIH	faktor inhibující HIF
HAF	faktory asociované s hypoxií
HAT	histonacetyltransferasy
HDAC	histondeacetylasy
HDACi	inhibitory histondeacetylas
HIF	hypoxií indukovatelné faktory
HRE	elementy odpovídající na hypoxii
IAP	inhibitor apoptotických proteinů
LPO	laktoperoxidasa
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 difenyltetrazolium bromid
NGF	nervový růstový faktor
NK	nukleové kyseliny
NSE	neuron specifická enolasa
ODDD	degradační doména závislá na kyslíku
PARP	poly(ADP-ribosa)-polymerasa
ROS	reaktivní kyslíkové formy
SAHA	suberoylanilidhydroxamová kyselina
TrkA	neurotropní tyrosinkinasový receptor typu 1
TSA	trichostatin A
TUNEL	z angl. Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	vaskulární endoteliální růstový faktor
VHL	von Hippel-Lindau faktor
VPA	valproát, kyselina valproová

2. ÚVOD

Nádorová onemocnění představují v dnešní společnosti jeden z nejzávažnějších problémů medicíny. Vzhledem k ekonomické náročnosti terapie a ke kvalitě života nemocných jsou navíc také značným socioekonomickým problémem. V České republice incidence novotvarů podle Ústavu zdravotnických informací a statistiky (ÚZIS) dlouhodobě narůstá. Naproti tomu je poměrné zastoupení úmrtí spojených s novotvary relativně stabilní, viz Obr. 1, a činí přibližně 25 % ze všech úmrtí.



Obrázek 1: Incidence a mortalita u nádorových onemocnění v ČR (ÚZIS, www.svod.cz).

Vysvětlení trendu rostoucí incidence lze hledat ve změnách týkajících se životního stylu obyvatelstva, stárnutí populace a částečně také ve zlepšující se diagnostice nádorových onemocnění. Mortalita se výrazněji nemění zejména díky časné diagnostice, použití adekvátní terapie podle biologických vlastností nádorů a dalších souvisejících faktorů. Hledání nových léčebných postupů a nových léků je proto mimořádně důležité.

2.1. NÁDOROVÁ ONEMOCNĚNÍ A JEJICH TERAPIE

2.1.1 ETIOLOGIE A PATOGENEZE NÁDOROVÉHO PROCESU

Snahy o odhalení příčin nádorového onemocnění trvají již řadu desetiletí. Díky rozvoji molekulární biologie a cytogenetiky se v současnosti na zhoubné nádory pohlíží jako na genetické onemocnění vznikající na podkladě mutací (Klener, 2002). Mutace v genomu buňky probíhají jak spontánně (s frekvencí cca 10^{-9} na jedno buněčné dělení), tak působením exogenních vlivů. Protože je frekvence spontánních mutací vzhledem k incidenci nádorových onemocnění příliš nízká, aby vysvětlovala beze zbytku původ všech novotvarů, lze považovat exogenní vlivy a formu životního stylu za významnou složku v patogenezi nádorů.

Rizikovými faktory souvisejícími s životním stylem, které lze do určité míry individuálně ovlivnit, patří: kouření, nadměrné požívání alkoholu, dieta s nízkým zastoupením ovoce a zeleniny, nedostatek pohybu, obezita a některé infekce. Zvýšený výskyt nádorových onemocnění také souvisí s delší dobou dožívání, resp. se zvyšujícím se průměrným věkem obyvatelstva.

Ke kancerogenním faktorům vnějšího prostředí – mutagenům, řadíme: faktory fyzikální (ionizující a ultrafialové záření, lokální tepelné změny, mechanické dráždění a další), chemické (aromatické a polycyklické uhlovodíky, aromatické aminy, nitrosloučeniny, léky, anorganické látky) a biologické (onkogenní viry – retroviry, papilomaviry, herpesviry, hepadnaviry; bakterie – *H. pylori*; parazity - Schistosoma).

Kancerogeneze/onkogeneze popisuje vznik a rozvoj nádorového onemocnění. Tento proces má vícestupňový charakter a zahrnuje několik etap (Klener, 2002). *Iniciace* je počáteční fází, kdy je z endogenních či exogenních příčin porušen buněčný genom. Tento stav však může bez funkčních důsledků dlouhodobě přetrvávat. Druhou fází je *promoce,* ve které za účasti dalších podnětů dochází k poruchám buněčné diferenciace. Při *konverzi* – transformaci je fenotyp buněk již významně pozměněn a vzniká maligní klon, který má mj. pozměněnou schopnost reagovat na signály regulující proliferaci či diferenciaci. Následuje lokální růst nádorové masy, tzv. *progrese*. Poslední fází je *metastazování*, tedy šíření nádoru mimo prvotní ložisko.

Za fyziologického stavu probíhá v organismu iniciace s určitou frekvencí. Další fáze rozvoje onemocnění jsou ve většině případů zastaveny zejména díky imunitnímu dozoru, kdy je nádorová buňka eliminována imunitním systémem. V případě, že v organismu vzniká velký

počet iniciovaných nádorových buněk, imunitní dozor může být pro odstranění pozměněných buněk nedostatečný. Rychle mutující a množící se nádorové buňky, které eliminační proces přežijí, vstupují do fáze rovnováhy. V této fázi se selekčním tlakem stává část buněk vůči eliminaci rezistentní. Ty vstupují do fáze úniku a onemocnění prograduje (Burnet, 1957; Dunn et al., 2002; Kim et al., 2007a).

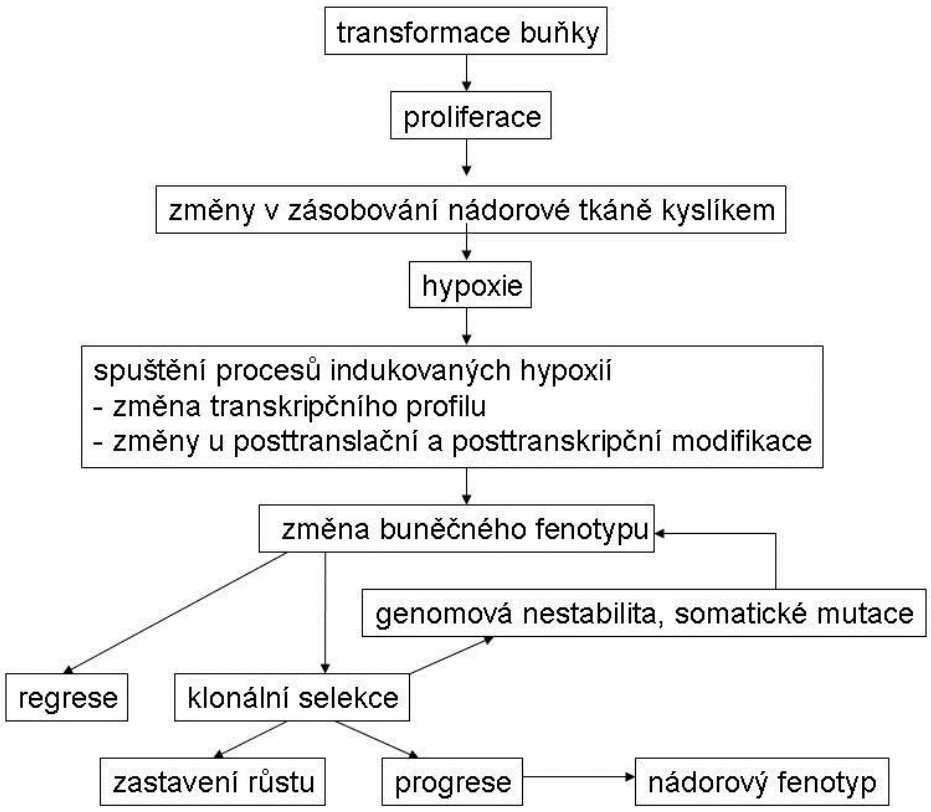
Pro transformaci jsou charakteristické změny zejména na úrovni genomu – mutace. Mutace postihují genetickou výbavu buňky na různé úrovni. Od bodových mutací, insercí a delecí postihujících promotor nebo kódující či nekódující část genu až po rozsáhlejší změny, např. genovou amplifikaci, chromosomální translokaci (např. Philadelphský chromosom s produkcí bcr-abl fuzního proteinu – onkogenní tyrosin kinasa) či aneuploidii. Geny, které jsou zodpovědné za transformaci buněk, lze rozdělit do dvou základních skupin: onkogeny a tumor supresorové geny. Jedná se o geny podílející se na kontrole a regulaci buněčného cyklu, diferenciaci, nitrobuněčné signalizaci, opravě poškozené DNA, regulaci apoptosy a mnoho dalších. Celkem se na transformaci buněk může podílet velké množství genů, kterých bylo u člověka identifikováno přes 900 (Venter et al., 2001). Z toho plyne velké množství různých vlastností nádorových buněk a vysoká heterogenita novotvarů, a to i v rámci jedné nádorové masy.

Nádorové buňky tak na podkladě řady mutací získávají charakteristické vlastnosti (Hanahan, Weinberg, 2000; Cipro et al., 2012), jako jsou:

- nezávislost na růstových faktorech či související signalizaci,
- snížení citlivosti vůči signálům inhibujícím proliferaci,
- zvýšená odolnost vůči proapoptotickým signálům a následná proliferace navzdory chybám v genomu buňky,
- pomalejší senescence (např. aktivace telomerasy),
- schopnost stimulace angiogeneze a tudíž podpora nutrice a zásobování buněk kyslíkem,
- zvýšená odolnost vůči hypoxii (aktivace genů umožňujících překonat oxidační stres),
- zvýšená exprese povrchových molekul potlačujících funkci imunokompetentních buněk (FasL) nebo snížená syntéza povrchových struktur, které buňky imunitního systému stimulují (HLA I. třídy s abnormálními peptidy).

Transformace buňky obvykle vyžaduje současnou přítomnost několika mutací (Knudson, 2001). Výjimkou však mohou být změny v genech, které jsou pro regulaci buněčného dělení, apoptosy či opravy DNA klíčové a tudíž jejich izolovaná mutace postačuje pro buněčnou transformaci. V konečném důsledku tak kumulace určitých genetických mutací může vést k nekontrolovanému buněčnému dělení a eventuelně s přispěním hypoxie ke vzniku

zhoubného nádoru, viz Obr. 2. V rámci nádoru nemusí mít nutně všechny buňky schopnost proliferace či sebeobnovy. Ta může být omezena na menší počet buněk, tzv. kmenových nádorových buněk, které při dělení produkují dceřiné, již částečně diferencované buňky s omezenou schopností replikace (Cho and Clarke, 2008).



Obrázek 2: Schéma rozvoje nádorového procesu v hypoxických podmínkách.

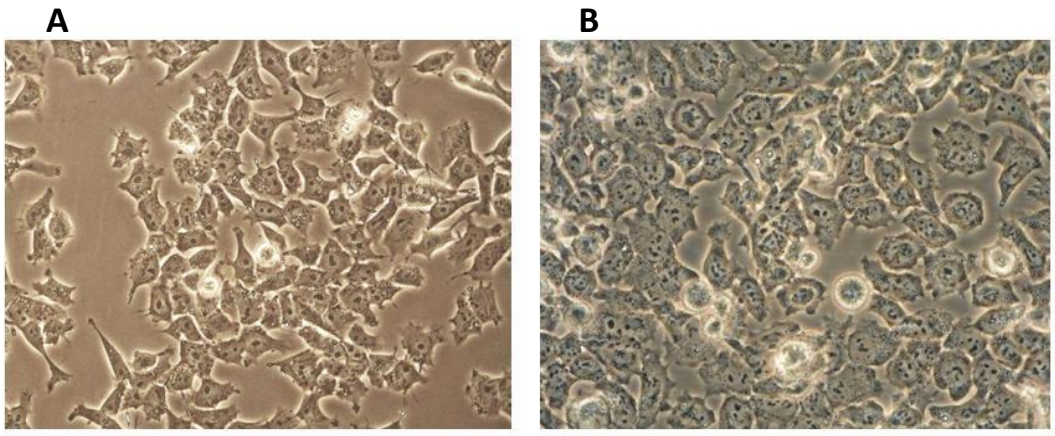
2.1.2 NEUROBLASTOM

Neuroblastom je embryonální nádor vycházející z buněk primitivní neurální lišty – sympatogonií, které v průběhu embryogeneze osídlují dřeň nadledvin, sympatická ganglia a paraganglia. Tomu odpovídá lokalizace neuroblastomů – dřeň nadledviny, malá pánev, retroperitoneum, zadní mediastinum a vzácně oblast krku. Je nejčastěji se vyskytujícím extrakraniálním solidním nádorem dětského věku. Prevalence neuroblastomů je odhadována na 1:10000 živě narozených dětí. Průměrný věk při stanovení diagnózy se pohybuje kolem 2 let (Gains et al., 2012).

Na patogenezi neuroblastomů se podílejí jak vlivy okolního prostředí, tak vlivy genetické. Z faktorů zevního prostředí jsou to především látky chemické – předpokládá se

užívání hydantoinátů, fenobarbitalu, event. alkoholu během gravidity (Klener, 2002). Genetické vlivy podílející se na patogenezi neuroblastomů jsou v praxi významné zejména pro prognózu onemocnění. Téměř u 80 % vysoce rizikových neuroblastomů je prokázána ztráta heterozygozity na 1. chromosomu (delece na krátkém raménku). Ostatní riziková stádia vykazují četné delece nebo translokace a mají diploidní nebo triploidní karyotyp. Obvyklá bývá také amplifikace onkogenu N-myc (krátké raménko 2. chromosomu), která je známkou velmi špatné prognózy (tzv. high risk neuroblastoma). Studována je i role nervového růstového faktoru (NGF – nerve growth factor), který po vazbě na příslušný receptor indukuje diferenciaci sympatických neuronů. Poruchy v této signalizační dráze se zřejmě rovněž podílejí na patogenezi onemocnění a souvisejí se sníženou expresí vysokoafinitního neutrofinového receptoru pro NGF – neurotropní tyrosinkinasový receptor typu 1 (TrkA). Jeho exprese obvykle inverzně koreluje s amplifikací N-myc a je spojena s příznivou prognózou onemocnění (Nakagawara et al., 1993; Westermark et al., 2011). Pro buňky neuroblastomů je dále charakteristická produkce neuron-specifické enolasy (NSE).

Podle fenotypového projevu rozlišujeme tři typy neuroblastomů: N-typ (neuroblastický), S-typ (adherující k substrátu) a I-typ (přechodný), viz Obr. 3, str. 15. Buňky neuroblastomu N-typu jsou malé, nediferencované, mají menší množství cytoplasmy a krátké, někdy početné neurity. Při pěstování *in vitro* k sobě buňky adherují lépe než k substrátu a vytvářejí shluky připomínající pseudoganglia. Pro S-typ jsou charakteristické větší, ploché buňky s oválným jádrem a velkým množstvím cytoplasmy. *In vitro* k substrátu dobře adherují, vytvářejí na povrchu média jednobuněčnou vrstvu a vykazují kontaktní inhibici růstu. Neurity zde nebývají přítomny. Fenotypový projev neuroblastomu I-typu vykazuje prvky, jak N-, tak S-typu. Buňky adherují dobře k substrátu i sobě samým, mají okrouhlé jádro s větším množstvím cytoplasmy, než je tomu u buněk neuroblastomu N-typu. Neurity zde mohou a nemusí být přítomny. Při růstu *in vitro* vytvářejí více buněčných vrstev s tvorbou agregátů. Tento typ obsahuje většinou nejvíce kmenových buněk (Jeanette et al., 2004; Acosta et al., 2009).



Obrázek 3: Neuroblastomové nádorové linie. A: UKF-NB-3 (N-typ), B: UKF-NB-4 (S-typ)

Mikroskopicky je neuroblastom složen z malých uniformních buněk obsahujících hyperchromatická jádra obklopená velmi malým množstvím cytoplasmy. Histochemicky se od ostatních nádorů z „malých tmavých buněk“ odlišuje pozitivitou NSE, vimentinu a elektron-mikroskopicky lze prokázat přítomnost neurofilament a neurosekrečních granul na membráně.

Primární tumor bývá nejčastěji lokalizován v retroperitoneu (70 %), v hrudníku a krku (25 %) a v malé pánvi (5 %). Onemocnění bývá v době diagnózy lokalizované pouze u 40 % nemocných. Přibližně 60 % pacientů má již detekovatelné metastázy, obvykle v lymfatických uzlinách, kostech, kostní dřeni, játrech nebo kůži.

Neuroblastomy mají nejčastěji nespecifické příznaky (únava, nechutenství, teploty, atd.) a symptomy vyvolané růstem nádoru nebo jeho metastázami. Kromě anamnézy a fyzikálního vyšetření jsou důležitou součástí diagnostického procesu zobrazovací metody – RTG (skelet, hrudník), CT (hrudník, břicho), USG (břicho, malá pánev, mediastinum), scintigrafie (kosti). Další testy zahrnují cytologické vyšetření punktátu kostní dřeně (event. trepanobiopsii), laboratorní vyšetření séra (laktátdehydrogenasa, NSE, ferritin) a metabolitů katecholaminů v moči (kyselina vanilmandlová (VMA) a homovanilová (HVA)). Rozhodující pro diagnózu je histopatologické vyšetření vzorku odebraného z nádorové tkáně doplněné molekulárně cytogenetickým a někdy i klasickým cytogenetickým vyšetřením.

Na základě stádia onemocnění, věku pacienta, lokalizace nádoru a výsledků laboratorních vyšetření lze formulovat prognostická kritéria. S věkem nad 18 měsíců, vysokou hladinou NSE, ferritinu a poměrem VMA/HVA>1, pokročilým stádiem onemocnění, nepříznivou histologií, amplifikací N-myc, delecí 11p a nízkou expresí trkA bývá spojena horší prognóza. Dvouleté bezpříznakové období bývá na základě prognózy dosažené u 95-98 %

pacientů s nízkým stupněm rizika. U nemocných se středním rizikem je to kolem 75 % a u vysoce rizikových forem 10-30 % (Klener, 2002).

Terapie nemocných s neuroblastomem je značně variabilní a její výběr se řídí podle prognostických skupin. Neuroblastomy mohou mít formu schopnou spontánní regrese nebo vyzrávání v benigní ganglioneuroblastom, dobře léčitelnou nebo také velmi obtížně léčitelnou s častým vznikem chemorezistentních relapsů.

V léčbě se uplatňují všechny základní druhy protinádorové terapie – chirurgická, radioterapie a chemoterapie.

Chirurgická terapie je rozhodující u lokalizovaných forem onemocnění, ale uplatňuje se i u generalizovaných a vysoce rizikových forem ve druhém sledu k odstranění rezidua nádoru po neoadjuvantní chemoterapii.

Radioterapie je využívána k léčbě makroskopických reziduálních lézí nebo při léčbě metastáz. Používá se teleradioterapie, ale lze zvolit i systémovou radioterapii podáním ^{123}I-metajodbenzylguanidinu, který má k neuroblastomovým buňkám velkou afinitu a emitované záření je usmrcuje (Matthay et al., 2012).

Chemoterapie se u lokalizovaných onemocnění někdy uplatňuje jako adjuvantní léčba. U pokročilých forem je léčbou neoadjuvantní. Používané jsou kombinace obsahující cyklofosfamid, ifosfamid, cisplatinu, karboplatinu, etopozid a antracykliny (doxorubicin, epirubicin). U vysoce rizikových forem je vzhledem k častým recidivám u nemocných po dosažení kompletní či parciální remise zařazena vysokodávková chemoterapie s následnou autologní transplantací kostní dřeně (Klener, 2002). Jako dlouhodobá udržovací léčba je testována terapie protilátkami proti disialogangliosidu a podávání retinoidů (kyseliny 13-*cis*-retinové) (Matthay et al., 2012).

2.1.3 HYPOXIE A JEJÍ VLIV NA BUŇKU

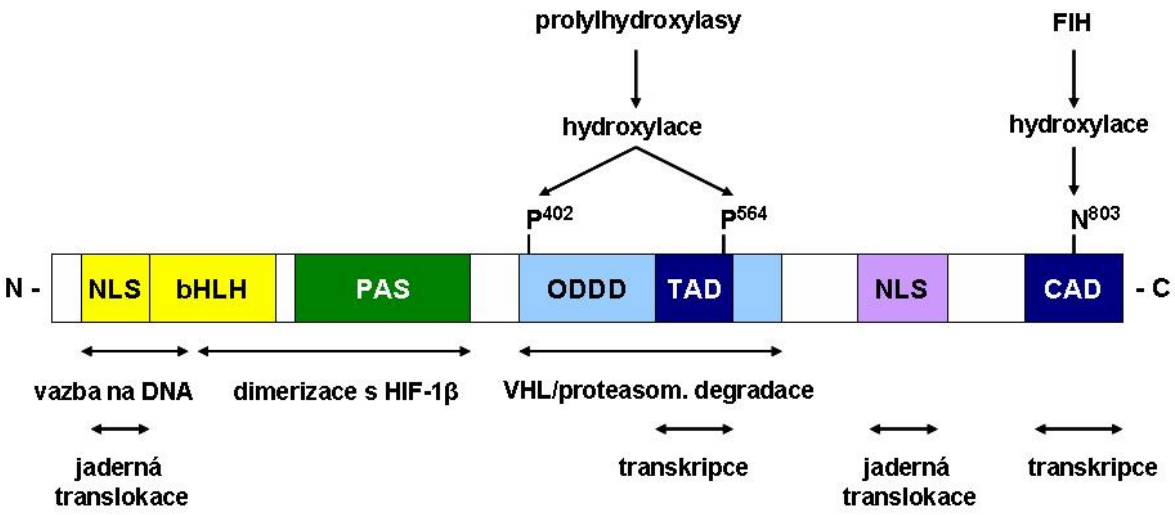
Přístup ke kyslíku nebo případná adaptace nádorových buněk na anaerobní podmínky je pro progresi a invazivitu nádoru spolu s dalšími podmínkami velmi důležitá. Při nekontrolovatelném nádorovém růstu není vaskularizace tkáně organizovaná, takže v rámci nádorové masy se vytvářejí hypoxická ložiska. Aby buňky v prostředí s nedostatkem kyslíku přežily, aktivují geny umožňující zachovat buněčné funkce potřebné k přežití.

Klíčovými regulátory, které adaptaci na hypoxii zajišťují, jsou transkripční faktory indukované hypoxií – hypoxia inducible facotrs – HIF. Jsou to heterodimerické komplexy

složené z řetězců alfa a beta. V současnosti jsou známy tři různé funkční formy HIF charakterizované prostřednictvím odpovídajícího α řetězce: HIF-1, HIF-2 a HIF-3. HIF-1 a HIF-2 jsou aktivovány v počáteční fázi hypoxie a jejich aktivita je pro další osud buňky kritická (Koh, Powis, 2012). Úloha HIF-3 není ještě zcela objasněna a je pravděpodobně regulační ve smyslu inhibice transkripční aktivity HIF-1 (Makino et al., 2001; Maynard et al., 2007). Mechanismus působení HIF-1 a HIF-2 je velmi podobný, obě formy se spolu s transkripčními koaktivátory váží na tzv. hypoxia responsive elements (HRE) elementy DNA a aktivují transkripci daného genu. Některé cílové geny pro HIF-1 a HIF-2 jsou stejné, další skupina je odlišná, což je pravděpodobně způsobeno odlišnostmi ve struktuře α podjednotek a schopností vazby odlišných transkripčních kofaktorů (Talks et al., 2000; Bracken et al., 2005).

Nejlépe prozkoumaným faktorem indukovaným hypoxií je HIF-1. Jak již bylo zmíněno, je jedním z klíčových regulátorů buněčného metabolismu v prostředí chudém na kyslík (Semenza, 1999; Pugh, Ratcliffe, 2003; Wang a Si, 2013). Byl poprvé popsán Semenzou a Wangem při studiu indukce transkripce genu pro erytropoetin v peritubulárních buňkách ledvin *in vitro* jako reakce na snížený parciální tlak kyslíku v krvi (Semenza, Wang, 1992). Později byla funkce induktoru erytropoetinu *in vivo* popsána u HIF-2 (Morita et al., 2003; Scortegagna et al., 2005; Mastrogiannaki et al., 2009; Kapitsinou et al., 2010).

Řetězce α i β tvořící HIF-1 jsou v buňce exprimovány konstitutivně. Podjednotka β je stabilní za normoxických i hypoxických podmínek. Specifickou aktivitu pro genovou transkripci zajišťuje α podjednotka. Je tvořena 826 aminokyselinami s celkovou hmotností asi 120 kDa, které vytvářejí několik domén: domény helix-loop-helix (bHLH) a NLS (z angl. nuclear localisation sequence), které jsou odpovědné za vazbu na DNA a translokaci do jádra buňky, doménu Per/Arnt/Sim (PAS), která je nutná pro dimerizaci s β podjednotkou, degradační doménu závislou na kyslíku (ODDD – oxygen-dependent degradation domain), zodpovědnou za regulaci hladiny HIF-1α v buňce a dvou transaktivačních domén TAD a CAD, které odpovídají za specifitu a regulaci genové transkripce (Dayan et al., 2006; Hu et al., 2007; Semenza, 2013; Wang a Si, 2013), viz Obr. 4.



Obrázek 4: Doménová struktura HIF-1α a základní funkce jednotlivých domén.

Za normoxických podmínek je α podjednotka degradována mechanismem ubikvitin-proteasom, který je spuštěn hydroxylací prolinových zbytků 402 a 564 na ODDD molekuly (Jaakkola et al., 2001; Ivan et al., 2001). Proces hydroxylace α řetězce je řízen enzymaticky HIF-1α prolylhydroxylasami a je přímo závislý na dostupnosti kyslíku (Jaakkola et al., 2001; Berra et al., 2003). Hydroxylací je umožněna jeho interakce s faktorem von Hippel-Lindau (VHL). Následné připojení E3-ubikvitin ligasového komplexu způsobí ubikvitinylaci HIF-1α a jeho proteasomální degradaci (Maxwell et al., 1999; Ohh et al., 2000; Semenza, 2013). V případě nedostatku kyslíku je degradace α podjednotky potlačena a současně nedochází k hydroxylaci C koncového asparaginu (Asn-803) hydroxylasou FIH (faktor inhibující HIF), která inhibuje interakci s transkripčním koaktivátorem p300 a tudíž zamezuje správné funkci HIF-1 (Hewitson et al., 2002; Lando et al., 2002). Inhibiční funkce FIH je navíc posílena její schopností interakce s VHL (Mahon et al., 2001).

Po translokaci do jádra podjednotka HIF-1α dimerizuje s podjednotkou β. Vytvořený heterodimer HIF-1 je pak schopen vazby na HRE elementy genů, u kterých dojde k aktivaci exprese v přímé závislosti na množství kyslíku v buňce (Semenza et al., 1996; Caro, 2001). Některé práce navíc pro stabilizaci HIF-1α za hypoxických podmínek postulují nutnou přítomnost funkčních mitochondrií. Reaktivní kyslíkové metabolity mitochondriálního původu se tak zatím ne zcela jasným způsobem podílejí na inhibici hydroxylace HIF-1α prolylhydroxylasami (Sena a Chandel, 2012). Pravděpodobným mechanismem je vyčerpávání kapacity antioxidačních látek podílejících se na redukci atomu železa v katalytickém centru prolylhydroxylas, která je pro jejich aktivitu nezbytná (Gao et al.,2007; Semenza, 2013).

Stabilita a transkripční aktivita HIF-1 je kromě koncentrací kyslíku ovlivněna i některými růstovými faktory a cytokiny (Wenger, 2002). Úlohu v regulaci hladin HIF-1 hrají i exprese faktoru asociovaného s hypoxií – hypoxia associated factor – HAF (Koh et al., 2008), interakce s receptorem aktivované proteinkinasy C (RACK1) (Liu et al., 2007) a dalšími proteiny (HDAC4, HDAC6, Hsp90) (Qian et al., 2006). Rovněž ji ovlivňuje posttranslační modifikace HIF-1 – acetylace, sumoylace (Berta et al., 2007; Bae et al., 2004) a fosforylace (Richard et al., 1999). Příkladem je deacetylace N-koncového lysinu ODD domény, která vede ke zvýšené stabilitě HIF-1α. Stabilizace se učastní histondeacetylasy HDAC1 a HDAC3, které s ODD doménou přímo interagují (Kim et al., 2007b). V případě, že je rovnovážný stav acetylace-deacetylace ovlivněn směrem ke zvýšené acetylační aktivitě, je N-koncový lysin acetylován acetylasou ARD1. Tato forma HIF-1α pak snadněji interaguje s faktorem VHL a je poté degradována v proteasomu mechanismem popsaným výše (Jeong et al., 2002; Yoo et al., 2006; Chang et al., 2006). Inhibice HDAC1 a/nebo HDAC3 potlačuje deacetylační aktivitu a tudíž vede ke zvýšené degradaci HIF-1α. Valproát (VPA), inhibitor HDAC1/3, ovlivňuje hladinu HIF-1α v buňce pravděpodobně i tímto mechanismem.

Kromě regulace transkripce erytropoetinu, u které byl HIF-1 objeven, se HIF-1 podílí na regulaci mnoha dalších buněčných procesů indukovaných hypoxií zahrnujících metabolickou adaptaci, angiogenezi, buněčný růst a diferenciaci, viabilitu, apoptosu a mnoho dalších (viz Tab. 1.). Existuje více než 100 genů, u kterých byl popsán regulační vliv HIF-1 (Maxwell et al., 2001). U celé skupiny faktorů HIF je regulovaných genů několikanásobně více (Tsai a Wu, 2012).

V případě nádorových onemocnění je častým jevem aktivace HIF-1 v důsledku hypoxie s následnou transkripcí cílových genů (Talks et al., 2000; Zhong et al., 1999; Semenza, 2013). Regulace aktivity HIF-1 v nádorových buňkách bývá často velmi složitá a zahrnuje vlivy onkogenů, tumor supresorových genů, cytokinů, růstových faktorů a nádorového mikroprostředí. Výsledkem změn v expresi genů může být v případě přežití buněk v těchto nepříznivých podmínkách různý fenotypový projev, jako je tomu i u neuroblastomů (N-, S- a I-typ), viz Obr. 3, str 15.

Tabulka 1: Vybrané cílové geny HIF-1. Upraveno podle (Ke a Costa, 2006).

Funkce	Gen	Reference
Erytropoesa/ metabolismus železa	Erytropoetin	Semenza et al., 1991
	Transferin	Rolfs et al., 1997
	Transferinový receptor	Bianchi et al., 1999
	Ceruloplasmin	Lok a Ponka, 1999
Angiogeneze a metabolismus mezibuněčné hmoty (matrix)	VEGF	Levy et al., 1995
	Receptor pro VEGF	Gerber et al., 1997
	Endokrinní VEGF (EG-VEGF)	LeCouter et al., 2001
	Leptin	Grosfeld et al., 2002
	CXCR-4, CXCL12 (SDF-1)	Andrikopoulou et al., 2011
	CX3CR1	Zhao et al., 2012
	Transformující růstový faktor β3	Scheid et al., 2002
	Matrixové metaloproteinasy a jejich regulátory	Ben-Yosef et al., 2002; Fukuda et al., 2007, Forsythe et al., 1996
	Receptory inhibitoru aktivátoru plasminogenu	Kietzmann et al., 1999
	Kolagenprolylhydroxylasa	Takahashi et al., 2000
Svalový tonus	NO syntasa	Melillo et al., 1995
	Hemoxygenasa	Lee et al., 1997
	Endotelin 1	Hu et al., 1998
	Adrenomedulin	Nguyen a Claycomb, 1999
	α_{1B}-adrenergní receptor	Eckhart et al., 1997
Metabolismus glukosy	Adenylátkinasa	O'Rourke et al., 1996
	Aldolasa-A,C	Semenza et al., 1996
	Enolasa	Semenza et al., 1996
	Glukosový transportér	Chen et al., 2001
	Glyceraldehydfosfátdehydrogenasa	Graven et al., 1999
	Hexokinasa (HK1,2)	Mathupala et al., 2001
	Laktátdehydrogenasa	Semenza et al., 1996
	Pyruvátkinasa	Semenza et al., 1994
	Fosfofruktokinasa	Semenza et al., 1994
	Fosfoglycerátkinasa	Semenza et al., 1994
	Fosfofruktokinasa 2	Minchenko et al., 2002
	Cytochrom c oxidasa	Fukuda et al., 2007
	Pyruvátdehydrogenasakinasa	Kim et al., 2006; Papandreou et al., 2006
Regulace pH	Monokarboxylátový transportér	Ullah et al., 2006
	Na^+/H^+ transportér	Shimoda et al., 2006
	Karbonanhydrasa 9 a 12	Chiche et al., 2009; Wykoff et al., 2000
Buněčná proliferace a přežívání	"Insulin-like" růstový faktor-2	Feldser et al., 1999
	Transformující růstový faktor-α	Krishnamachary et al., 2003
	Adrenomedulin	Cormier-Regard et al., 1998
Apoptosa	BNip3	Carrero et al., 2000
	Nip3-like protein X	Bruick, 2000

Příkladem mechanismu aktivace HIF-1 nezávislé na koncentraci kyslíku, je snížená proteasomální degradace podjednotky α v důsledku potlačení exprese tumor supresorového genu kódujícího VHL (Ivan a Kaelin, 2001; Robinson a Ohh, 2014), nebo inhibice HIF-1α

prolylhydroxylas fumarátem jakožto meziproduktem Krebsova cyklu u některých renálních karcinomů (Pollard et al., 2007). Dalšími tumor supresorovými geny, které ovlivňují funkci HIF-1α jsou p53 a PTEN (z angl. phosphatase and tensin homolog**)**, které inhibují aktivaci cílových genů pravděpodobně vazbou na HIF-1 či modulací aktivity proteinkinasy AKT (An et al., 1998; Zundel et al., 2000; Jiang a Liu, 2009). Na aktivitu HIF-1 mají vliv i růstové faktory, cytokiny (insulin, PDGF, růstový faktor podobný insulinu, NO) a další látky (Zelzer et al., 1998; Richard et al., 2000; Chowdhury et al., 2012). Vliv na aktivitu a expresi HIF-1 je patrný i u chemoterapie a radioterapie, které je obvykle zvyšují. Vysoká aktivita HIF-1 v rámci nádorové masy bývá zpravidla spojena s horší prognózou onemocnění, protože HIF-1 se kromě regulace buněčného metabolismu a angiogeneze podílí i na procesech souvisejících s agresivitou tumoru. Negativně ovlivňuje expresi adhezní molekuly E-kadherinu, který zprostředkovává mezibuněčnou interakci (Cheng et al., 2013). Následkem je snížená vzájemná adherence buněk související se zvýšenou nádorovou invazivitou. Migrace a usazování – homing – nádorových buněk při metastazování nádoru jsou do jisté míry HIF-1 také ovlivněny, jelikož HIF-1 přímo ovlivňuje expresi receptoru CXCR4 a jeho ligandu SDF-1, receptoru CXCR3, enzymů účastnících se metabolismu mezibuněčné hmoty a dalších proteinů ovlivňujících adhezivitu a migrační schopnosti buněk, viz Tab. 1, str. 20.

Komplexní účinek a centrální postavení HIF-1 při adaptaci buněk na hypoxii jsou současnou medicínou využívány i terapeuticky. Látky inhibující aktivitu HIF-1, kam patří i inhibitory histondeacetylas (HDACi), jsou podávány u některých nádorových onemocnění k usmrcení či oslabení nádorových buněk v hypoxickém prostředí. Terapeutická aktivace HIF-1 může být naproti tomu prospěšná u chorob, kdy je podpora hypoxického metabolismu a angiogeneze žádoucí (inhibitory prolylhydroxylas). Příkladem je podpora reparace poškozené srdeční tkáně po ischemické příhodě srdeční (Pan a Zhou, 2012; Ong a Hausenloy, 2012).

2.1.4 PROTINÁDOROVÁ TERAPIE

V současnosti je k dispozici velké množství informací popisujících iniciaci a rozvoj nádorového procesu, biologické vlastnosti transformovaných buněk a spolu s dalšími poznatky jsou podkladem pro rozvoj protinádorové terapie. I když je úroveň znalostí o biologii nádorové buňky v některých oblastech ještě stále značně omezená, máme v současnosti k dispozici relativně širokou škálu terapeutických možností.

K základním druhům terapie patří – *chirurgická terapie, chemoterapie a radioterapie.* Doplňuje je léčba hormonální, imunoterapie a cílená terapie (u nás někdy ne zcela správně nazývaná biologická) a další.

Jednotlivé terapeutické přístupy mohou mít v rámci léčby účinek profylaktický, diagnostický, kurativní nebo paliativní. Profylaktický a diagnostický účinek představuje především léčbu chirurgickou odstraněním tkáně, která je zatížená vysokým rizikem vzniku nádoru (například kolektomie u mnohočetné adenomatózní polypózy), resp. pro získání bioptického materiálu a pomoci při zpřesnění stadia pokročilosti onemocnění (například odběr lymfatických uzlin). Kurativní terapie je možná většinou dostupných metod – chirurgické odstranění lokalizovaných solidních nádorů, radioterapie a chemoterapie u vybraných nádorů. Paliativní účinek lze očekávat u všech druhů terapie, ať už se jedná o chirurgické odstranění nádorové masy utlačující okolní tkáň, analgetickou radioetrapii kostních metastáz, paliativní chemoterapii vedoucí ke zlepšení kvality a prodloužení života nemocných nebo hormonální léčbu u hormonálně senzitivních nádorů v pokročilém stádiu atd. (Klener, 2002).

Velkým problémem současné onkologie je *rezistence/odolnost* nádorů vůči terapii. Ta může být nádoru vlastní – primární, nebo sekundární – indukovaná (zpravidla vyvolaná předchozí terapií). Pravděpodobně největší váhu při rozvaze o možné rezistenci ke konkrétnímu druhu terapie má histopatologický typ buňky. Při radioterapii se možná rezistence k léčbě odvíjí navíc od orgánové lokalizace, klinického stádia onemocnění a objemu nádorové masy. U chemoterapie je povaha primární a především sekundární rezistence více komplexní. Podílejí se na ní mnohé faktory, zejména změny farmakokinetiky, cytokinetiky a metabolismu buněk. V praxi je pak rezistence nádorových buněk výsledkem kombinace dále uvedených faktorů. Změny farmakokinetiky zahrnují snížení resorpce, urychlení katabolismu, inaktivaci nebo vylučování léčiva. Cytokinetické změny nastávají s narůstající populací nádoru, kdy větší část buněk přechází do klidového stavu, fáze G0, v němž je citlivost k většině cytostatik omezená. S přibývajícím počtem nádorových buněk se také kumulují změny v jejich genomu (Klener, 2002). Tyto mutace mohou diverzifikovat nádorovou masu z hlediska jejího vztahu k terapii. V případě, že mutace ovlivňuje rezistenci buňky k chemoterapii, jsou vlivem léčby usmrceny citlivé klony a selektují se klony k terapii rezistentní.

Podstatou rezistence k chemoterapii způsobené metabolickými a funkčními změnami jsou indukované alterace v expresi genů kódujících proteiny, podílející se na omezení/zamezení funkce cytostatika (nitrobuněčné aktivaci, katabolismu, transportu z buňky), snížení citlivosti buněk k apoptose (oprava poškozené DNA, zvýšená resp. snížená exprese antiapoptotických resp. proapoptotických proteinů), přizpůsobení buněčného metabolismu hypoxickým

podmínkám. Tyto změny patří k nejčastějším příčinám rezistence nádorových buněk k chemoterapii.

Významným mechanismem vzniku rezistence k chemoterapii je zvýšení exprese povrchových transportních proteinů. Nejznámějším zástupcem této skupiny je P glykoprotein, ATP-závislá membránová pumpa, zodpovědná za urychlené vypuzování cytostatik z buňky a vznik mnohočetné lékové rezistence (MDR – multiple drug resistance). Dalšími transportéry, které mohou při vysoké expresi navodit rezistenci k některým cytostatikům, jsou např. TAP (transporter of antigenic peptides) a proteiny patřící do skupiny MVP (major vaults proteins). Citlivost buněk k proapoptotickým stimulům je ovlivnitelná změnou exprese proteinů účastnících se zahájení, progrese a regulace apoptotické signalizace. Nárůst koncentrace inhibitorů apoptosy vede k tomu, že i když buňka není chráněna před přímým účinkem cytostatika, nedochází k jejímu usmrcení. Po odeznění účinku chemoterapie pak pokračuje v proliferaci. Ovlivnění rovnovážného stavu pro- a antiapoptotických proteinů je také výsledkem hypoxie, která je pro většinu nádorů charakteristická.

2.1.4.1 CHEMOTERAPIE A JEJÍ POSTAVENÍ MEZI OSTATNÍMI DRUHY TERAPIE

Chemoterapie je terapeutická metoda, která využívá chemických látek – cytostatik – schopných zastavit růst nádorových buněk. Základním předpokladem pro její použití je citlivost nádorů k podávanému léčivu, která bývá různá u různých typů nádorů, a to i v rámci jedné nádorové masy. Nádorová onemocnění lze podle citlivosti k chemoterapeutické léčbě rozdělit do čtyř skupin. První skupinu tvoří nádory, které jsou k chemoterapii velmi citlivé a chemoterapií vyléčitelné – kurativní účinek. Z celkového počtu nádorů tvoří asi 7-8 %. U druhé skupiny, kterou tvoří přibližně jedna čtvrtina nádorů, působí chemoterapeutická léčba paliativně, nicméně zde ve vysokém počtu případů vede k remisi onemocnění a prodloužení doby přežití. Třetí skupinu – přibližně 30 % nádorů – tvoří novotvary, u kterých je citlivost k chemoterapii nejednoznačná. Zde se tato terapie používá především jako terapie doplňková. Do zbývající skupiny patří nádory s vysokou rezistencí na dostupná cytostatika. Nicméně s objevy nových cytostatik se některé nádory dostávají do příznivějších skupin.

Chemoterapie bývá v praxi běžně využívána v kombinaci s dalšími metodami – chirurgickou terapií a radioterapií. Jelikož je celkový výsledek terapie ovlivněn nejen druhem použitých terapeutických modalit, ale také jejích sekvencí, jsou v současnosti k dispozici

mezinárodně platné léčebné protokoly a doporučení. Cytostatika se používají i při léčbě dalších chorob, např. Bechtěrevovy a Crohnovy nemoci a autoimunitních onemocnění.

Konvenční chemoterapeutika působí v terapeutických dávkách pouze na proliferující buňky a umožňují je zasáhnout na různých místech a odlišným způsobem. Podle povahy účinku je lze rozdělit do několika základních skupin: inhibitory syntézy nukleových kyselin (NK), látky poškozující strukturu a funkci NK, přípravky inhibující průchod buněčným cyklem, inhibitory proteosyntézy a epigenetická chemoterapeutika, viz Tab. 2. Některá terapeutika mají účinek kombinovaný a působí na cílové buňky různými mechanismy (Klener, 2002).

Tabulka 2.: Základní skupiny chemoterapeutik a mechanismus jejich účinku.

Povaha účinku	Mechanismus účinku
Inhibice syntézy NK	Analoga kyseliny listové, analoga dusíkatých bazí, inhibice ribonukleotidreduktasy
Poškození struktury NK	Alkylace NK, interkalační činidla, inhibice topoisomerasy, látky štěpící DNA
Inhibice průchodu buněčným cyklem	Inhibice polymerizace nebo depolymerizace mikrotubulů, inhibitory cyklin-dependentních kinas
Inhibice proteosyntézy	Deplece aminokyselin využívaných nádorem, poškození ribosomů
Epigenetický účinek	Inhibice histondeacetylas a DNA methyltransferas

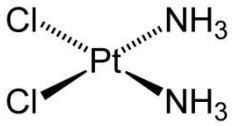
2.1.4.1.1 Charakteristika vybraných cytostatik

Cisplatina

Cisplatina, anorganická sloučenina platiny - *cis*-diamindichloroplatnatý komplex, je jedno z nejstarších chemoterapeutik. Poprvé byla popsána v roce 1844 Peyronem, ovšem její účinky na buněčnou replikaci byly popsány o více než sto let později (Peyrone, 1844; Rosenberg et al., 1965).

Mechanismus jejího účinku spočívá ve vazbě na DNA a vytváření kovaletních můstků mezi bázemi. V důsledku této modifikace je pozměněna struktura DNA. Na DNA se v místě poškozeném cisplatinou váží proteiny obsahující tzv. „high mobility group“, nebo-li HMG doménu. Tímto způsobem je bráněno opravě poškozeného úseku DNA vystřižením poškozené báze. Dochází tak k inhibici transkripce a v průběhu buněčného cyklu k zablokování replikační

fáze v místě poškození (Hughes et al., 1992; Ohndorf et al., 1999; Rruggiero et al., 2013). V závislosti na podané dávce cisplatiny jsou buňky buďto schopny cisplatinu tolerovat, resp. opravit poškozenou DNA, nebo v důsledku rozsáhlého poškození DNA zahájit apoptosu (Siddik, 2003). S tím souvisí i problematika rozvoje rezistence k léčbě. Rezistentní nádorové buňky tak obvykle vykazují zvýšenou aktivitu oprav DNA nebo vyšší odolnost vůči proapoptotickým signálům.

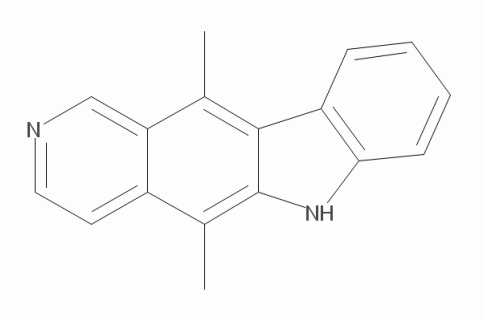


Obrázek 5: Struktura cisplatiny.

Tak jako všechna cytotoxická léčiva i cisplatina má řadu vedlejších účinků. Nejvíce závažná je nefrotoxicita, která může v extrémních případech pacienta ohrozit na životě (Arany, Safirstein, 2003; Pabla, Dong, 2008; Ruggiero et al., 2013). Omezení nežádoucích účinků je možné použitím cisplatinových derivátů, které mají nižší nefrotoxicitu, ovšem i celkově nižší protinádorový účinek – karboplatiny a oxaloplatiny. Důležitým prvkem pro redukci vedlejších účinků terapie je zvýšení její efektivity. Potenciace účinku DNA-poškozujících léčiv je možná i prostřednictvím epigenetických chemoterapeutik, kam patří i inhibitory histondeacetylas. Jejich působením je chromatinová struktura rozvolněna a terapeutika mají k DNA lepší přístup.

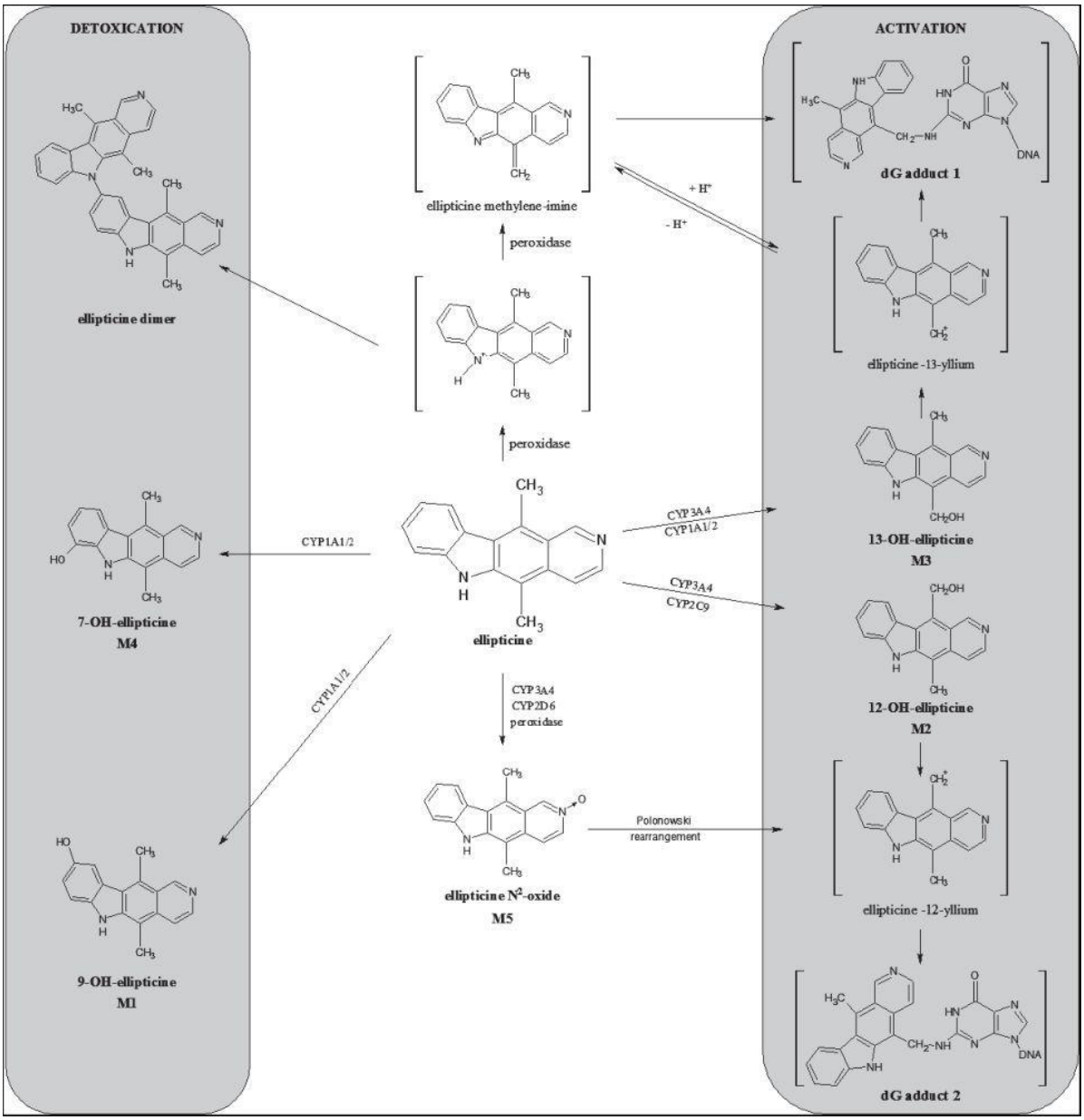
Ellipticin

Ellipticin (5,11-dimethyl-6H-pyrido[4,3-b]karbazol) je rostlinný alkaloid, který byl poprvé izolován z tropického stromu *Ochrosia elliptica* v roce 1959. Mechanismus účinku na nádorové buňky je různý. Inhibuje topoisomerasu II, je schopen interkalace do DNA a tvorby DNA-kovalentních aduktů, zasahuje do progrese buněčného cyklu, ovlivňuje metabolismus mitochondrií a expresi proteinů podílejících se na apoptotické signalizaci, navozuje stres endoplasmatického retikula a ovlivňuje buněčnou signalizaci (Schwaller et al., 1995; Kuo et al., 2005A; Hägg et al., 2004; Kuo et al., 2005B; Kuo et al., 2005C). Tyto mechanismy potlačují replikaci DNA, narušují energetický metabolismus a navozují apoptosu. Jedním z důležitých mechanismů účinku ellipticinu je tvorba DNA aduktů po metabolické aktivaci cytochromy P450 a peroxidasami, viz Obr. 7, str 25. Tyto adukty významným způsobem poškozují DNA a jejich přítomnost v buňce indukuje apoptózu (Stiborova et al., 2004; Stiborova et al., 2007; Stiborova et al., 2012a; Poljakova et al., 2011).



Obrázek 6: Struktura ellipticinu.

V praxi byly vyzkoušeny jeho polárnější deriváty, jako je např. 2-methyl-9-hydroxyellipticin. Vedlejší účinky léčby byly oproti ostatním protinádorovým léčivům méně časté a přítomny jsou asi u třetiny pacientů (Paioletti et al., 1980), nefrotoxicita bývá spíše vzácná (Klener, 2002). Omezení případných nežádoucích účinků nebo posílení účinnosti ellipticinu je založeno na podobném principu jako u cisplatiny. První variantou je hledání derivátů, u kterých je rozsah vedlejších účinků na pacienta menší. Další možností je podání podpůrného preparátu, který zvýší efektivitu působení. Podobně jako u cisplatiny jsou inhibitory histondeacetylas účinek ellipticinu schopny potencovat (Poljakova et al. 2011).



Obrázek 7: Schéma metabolismu ellipticinu indukovaného cytochromy P450 a peroxidasami ukazuje charakteristické detoxikační metabolity a metabolity podílející se na vzniku kovalentních aduktů s DNA. V závorkách jou uvedeny přechodné aktivované formy a jejich vazba na DNA (převzato z Poljakova et al., 2011)

2.1.4.2 MOŽNOSTI ZVÝŠENÍ ÚČINNOSTI CHEMOTERAPEUTICKÉ LÉČBY

V rámci nádoru často vykazuje proliferační aktivitu pouze část buněk. Jelikož většina chemoterapeutik účinkuje pouze na buňky v některé z fází buněčného cyklu, může být tedy tato léčba méně efektivní. Existuje několik způsobů, kterými lze účinnost terapie zvýšit (Klener, 2002).

Ovlivnění buněk v klidové (G0) fázi

- přímý chemoterapeutický účinek na buňky v G0 fázi je umožněn podáváním vysokých dávek cytostatik spolu s antidotem nebo podpůrnou léčbou. Nevýhodou u tohoto léčebného postupu je především poškození frakce zejména normálních hemopoetických kmenových buněk ve fázi G0, které jsou důležité pro repopulaci všech krevních řad po terapeutickém cyklu.
- převedení buněk z klidového stavu do stavu proliferace. V praxi se provádí nepřímo, tj. redukcí proliferující části nádorové masy. V důsledku toho přechází část klidových buněk do reprodukční fáze a stává se citlivou k chemoterapii.
- zablokování buněk v G0 fázi. Spočívá v blokádě buněčného cyklu normálních buněk. Tím získávají vyšší odolnost vůči chemoterapii, což umožňuje použití efektivnějších dávek chemoterapeutik, resp. agresivnější léčbu onemocnění.

Sladění probíhajících buněčných cyklů nádorové populace

- podání fázově specifického terapeutika, které působí pouze v některé fázi buněčného cyklu. Část nádorových buněk je usmrcena a buněčný cyklus u přežívající proliferující populace je zastaven. Po následném uvolnění blokády probíhá buněčný cyklus u dělící se populace synchronizovaně. To umožňuje efektivní použití fázově specifických cytostatik, které tak působí na podstatně větší nádorovou populaci než v případě podání bez předchozí synchronizace.

Vhodné časování podávání léčby

- volba vhodného intervalu mezi jednotlivými dávkami. Vychází z poznatku, že čas pro obnovu stejného počtu buněk je pro nádorovou populaci obvykle delší, než je tomu u buněk normálních. Příliš krátká doba mezi jednotlivými cykly omezuje regeneraci normálních buněk a naopak příliš dlouhá doba dovoluje nádorové populaci větší prostor pro obnovu. Optimálním časováním jednotlivých dávek tak lze docílit vyšší efektivity léčby.
- terapie ve vhodnou dobu. Již delší dobu je známo, že cirkadiánní rytmus má vliv na výsledek léčby chemoterapií. Vhodné načasování podání může dokonce až desetinásobně ovlivnit toleranci organismu vůči cytostatiku. V případě, že je léčivo podané v denní době, kdy je organismem nejlépe tolerováno, bývá efektivita terapie vyšší, a to z důvodu narušeného cirkadiánního rytmu u nádorové populace a zachovaného u buněk normální tkáně (Lévi et al., 2010).

Vyjma zvýšení efektivity chemoterapie úpravou léčebného režimu způsobem popsaným výše, máme k dispozici další poznatky, které umožňují lépe využít potenciál cytostatik, tj. posílit jejich účinek.

Podávaní látek, které mají pro organismus ochranný účinek – antidot a protektiv

Zde spočívá posílení účinku chemoterapeutik v možnosti podání vyšší dávky. V některých případech může zvýšení dávky na dvojnásobnou hodnotu zvýšit účinnost léčby až o jeden řád, tj. usmrcení 10-násobného počtu nádorových buněk v porovnání s dávkou původní (Klener, 2002).

Mechanismus působení jednotlivých protektivních látek je mezi jednotlivými preparáty značně odlišný (obcházení cytostatikem blokované enzymatické reakce v normálních buňkách – leukovorin, snižování oxidačního stresu zdravých tkání – Dexrazoxan, Amifostin, blokování vazby léčiva na DNA ve zdravých buňkách – Amifostin, štěpení cytostatika – karboxypeptidasa G2 – nebo inaktivace jeho metabolitů – Mesna, a další).

Sloučeniny, které zvyšují citlivost nádorových buněk k chemoterapii

Další skupinou jsou látky – chemosenzibilizátory, které je možné použít pro zvýšení citlivosti nádorových buněk k chemoterapii. Uplatnění nacházejí také při resenzibilizaci po předchozí léčbě.

Používají se například inhibitory poly(ADP-ribosa)-polymerasy (PARP) – enzymu, který se účastní opravy DNA (zejména mechanismem vystřižení „base excision repair“). Zvýšená aktivita tohoto enzymu je asociována s vyšší odolností buněk vůči cytostatikům poškozujícím DNA. Jeho cílená inhibice tedy vede ke zvýšení citlivosti nádorových buněk k chemoterapii. Inhibitory PARP mají také samy o sobě protinádorový účinek a lze je v některých případech použít dokonce ve formě monoterapie (Tentori et al., 2013).

K dalším chemosenzibilizačním látkám patří např. látky bioreduktivní, jejichž účinek se odvíjí od aktivace v hypoxickém prostředí, které je pro solidní tumory charakteristické. Alespoň částečné ovlivnění mnohočetné lékové rezistence umožňují inhibitory kalciových kanálů, antagonisté kalmodulinu a steroidní hormony.

Epigenetická chemoterapeutika

Mezi epigenetická terapeutika se řadí látky, které jsou schopny měnit genovou expresi cílových/nádorových buněk prostřednictvím ovlivnění methylace DNA, acetylace histonů a dalších jaderných proteinů a remodelace chromatinu. Jak již bylo zmíněno, pro nádorové buňky je typická aberantní genová exprese. Obvykle bývají negativně ovlivněny tumor supresorové geny a dále geny, jejichž produkty určují citlivost buňky k apoptose a mnohé další. Utlumení transkripce bývá provázeno methylací promotorové části genu nebo deacetylací histonů vedoucích k prostorovým změnám ve struktuře chromatinu. Zvýšená exprese bývá u genů, které umožňují např. přežívání buněk navzdory nepříznivým podmínkám (např. hypoxie, malnutrice), kumulaci genomových aberací (enzymy opravující DNA) a proapoptotických stimulů (Bcl-2).

Úlohou epigenetické terapie je zvrácení pozměněné genové exprese transformovaných buněk a navození exprese žádoucích genů – tumor supresorové geny, geny kódující proteiny, které jsou vhodným cílem pro následnou terapii a další. V současnosti jsou k dispozici epigenetická terapeutika ovlivňující aktivitu DNA-methyltranferas a histondeacetylas. Mezi histondeacetylasové inhibitory patří také kyselina valproová.

2.2 HISTONDEACETYLASY A JEJICH INHIBITORY

Acetylace a deacetylace histonů a dalších proteinů v buňce hraje důležitou roli v regulaci genové transkripce a mnoha buněčných procesů. Stav acetylace/deacetylace je výsledkem rovnovážného stavu mezi aktivitou histonacetyltranferas (HAT) a histon-deacetylas (HDAC).

2.2.1 HISTONDEACETYLASY

V současné době je u lidí identifikováno 18 různých histondeacetylas (Barneda-Zahonero a Parra 2012; Marks a Dokmanovic, 2005). Při deacetylaci lysinových zbytků na dusíku postranního řetězce se uplatňují dva různé mechanismy, na základě kterých se HDAC rozdělují do dvou skupin. První je skupina Zn^{2+} dependentních HDAC, kde Zn^{2+} přímo interaguje s acetylovaným substrátem a napomáhá jeho stabilizaci v aktivním místě enzymu a polarizaci karbonylového uhlíku acetylové skupiny pro nukleofilní molekuly vody (Finnin et al., 1999). Druhou skupinou jsou NAD^{+}-dependentní HDAC, kde po deacetylaci dochází k rozpadu acetylovaného kofaktoru na O-acetyl ADP ribosu a nikotinamid (Trapp a Jung,

2006). Další rozdělení, které se při klasifikaci HDAC používá častěji, vychází z homologie HDAC a jejich analogů u kvasinek. Tímto způsobem lze HDAC rozdělit do čtyř tříd, viz Tab.3.

Histondeacetylasy nejsou ve své katalytické aktivitě redundantní (Rosato a Grant, 2005). Například HDAC třídy I jsou umístěné v jádru buňky a jejich exprese je ubikvitní napříč všemi typy buněk. Naproti tomu HDAC patřící do třídy II jsou lokalizovány jak v jádře, tak v cytoplasmě buněk a jejich exprese se v rámci různých tkání odlišuje.

Fylogenetické studie, které byly prováděny na HDAC bakteriálního původu naznačují, že tyto enzymy jsou původem starší, než samotné histony podle kterých jsou pojmenovány (Gregoretti et al., 2004). Lze tedy usuzovat, že původními substráty HDAC byly nehistonové acetylované proteiny, viz Tab. 3. Do této skupiny patří široká škála buněčných proteinů – nehistonové proteiny chromatinu, transkripční aktivátory, koaktivátory jaderných receptorů, transkripční faktory, enzymy podílející se na opravě DNA a další. Acetylačním stavem je pak ovlivňována jejich stabilita (pozitivně i negativně), interakce s dalšími proteiny (podpora nebo zamezení) nebo DNA a u transkripčních faktorů jejich aktivita (Kim a Bae, 2011).

Tabulka 3: Klasifikace HDAC, jejich lokalizace v buňce a některé nehistonové substráty. M: mitochondrie; J: jádro; C: cytoplasma; CH: chaperon; CR: protein remodelující chromatin; JR: jaderný receptor; RE: enzymy opravující DNA; SM: signální molekula; SP: strukturní protein; TR: transkripční regulátor; TF: transkripční faktor.

Třída	HDAC	Umístění	Nehistonové substráty
Třída I Zn^{2+}	HDAC1	J	JR: androgenový receptor, SHP; SM: Stat3, Smad7; TF: p53, YY-1, GCMa, Myo-D, E2F-1
	HDAC2	J	JR: glukokortikoidový receptor; SM: Stat3, Smad7; TF: Bcl-6, YY-1
	HDAC3	J	JR: SHP; SM: Smad7,Stat3; TF: YY-1, GMCa, GATA-1, GATA-3, MEF2D, RelA (NFκB)
Třída IIa Zn^{2+}	HDAC8	J	SP: aktin
	HDAC4	J/C	CR: HP-1; TF: GCMa, GATA-1
	HDAC5	J/C	CR: HP-1; SM: Smad7; TF: GCM, GATA-1
	HDAC7	J/C	TF: PLAG1, PLAG2
Třída IIb Zn^{2+}	HDAC9	J/C	TF: Bcl-6, N-CoR, TEL
	HDAC6	hlavně C	CH: Hsp90; JR: SHP; SM: Smad7; SP: α-tubulin
	HDAC10	hlavně C	TF: SMRT
Třída III NAD^+	Sirt1	J/C	RE: Ku70; TR: Rb; TF: p53, p73, p300, Bcl-6, Myo-D, FOXOs, NFkB, N-CoR, E2F-1;
	Sirt2	C	SP: α-tubulin; TF: FOXO
	Sirt3	M	AcCoA syntetasa
	Sirt4	M	GDH
	Sirt5	M	Cytochrom c
	Sirt6	J	Telomerický chromatin; WRN
	Sirt7	J	RNA pol I; TF: p53
Třída IV Zn^{2+}	HDAC11	J/C	Neznámé

2.2.2 INHIBITORY HISTONDEACETYLAS

Jedním z charakteristických znaků transformovaných buněk je transkripční profil odlišný od normální buněčné populace, z které nádorová buňka vznikla. Jsou to změny, jak pozitivního (zvýšení transkripce), tak negativního charakteru. Transkripční aktivita bývá právě na podkladě deacetylace histonů často omezena u tumor supresorových genů, které odpovídají za klíčové procesy, např. za správný průběh a regulaci buněčného dělení a diferenciace, opravu poškozené DNA, či za programovanou buněčnou smrt. Inhibicí histondeacetylas by bylo možné do určité míry aktivitu těchto genů a tím i regulační pochody proliferace, buněčnou diferenciaci

či citlivost k apoptotickým stimulům alespoň částečně obnovit. Velkou výhodou histondeacetylas je, že působí jak na dělící se buňky, tak na buňky v klidové fázi.

V současné době je k dispozici poměrně široká škála inhibitorů histondeacetylas (HDACi) jak přírodního tak i syntetického původu, viz Tab. 4. Podle struktury je lze rozdělit alespoň do čtyř skupin: deriváty kyseliny hydroxamové, cyklické peptidy, alifatické kyseliny a benzamidy. Prvním objeveným HDACi byl trichostatin A – TSA (Yoshida et al., 1990). Vorinostat (suberoylanilidhyroxamová kyselina – SAHA) a depsipeptid byly před několika lety jako první uvolněné FDA ke klinickému použití jako protinádorová léčiva u kožního T-lymfomu.

Tabulka 4: Inhibitory histondeacetylas. Upraveno podle (Eot-Houllier et al., 2009; Lee a Workman, 2007; Eyal et al., 2005).

Strukturní skupina	**HDACi**	**Cíl**
deriváty kyseliny hydroxamové	TSA	Třída I a II
	Vorinostat – SAHA	Třída I a II
	LAQ824	Třída I a II
	LBH589	Třída I a II
alifatické kyseliny	VPA	Třída I a IIa
	butyrát sodný, fenylbutyrát	Třída I a IIa
	AN-9	Třída I a IIa
	OSU-HDAC42	Třída I a IIa
cyklické peptidy	depsipeptid, apicidin	Třída I a IIb (HDAC1, 6)
benzamidy	MS-275	Třída I (HDAC 1, 2, 3)
	MGCD0103	Třída I a IIb (HDAC 1, 2, 4, 6)
thioláty	NCH-51	Třída IIb (HDAC6)
nikotinamid a jeho deriváty	nikotinamid, 2-anilinobenzamid	SIRT 1 a SIRT1-7
analoga NAD$^+$	karba-NAD$^+$	SIRT1-7
deriváty hydronaftaldehydu	sirtinol, para-sirtinol, cambinol	SIRT1, 2
splitomiciny	splitomicin, 5-benzyloxy-splitomicin	SIRT2
indoly	EX-527	SIRT1
analoga adenosinu	Ro31-8220	SIRT1, 2

2.2.2.1 Mechanismus působení inibitorů histondeacetylas

Mechanismy působení HDACi zahrnují

- alteraci genové exprese v důsledku hyperacetylace histonů,
- inhibici buněčné odpovědi na stresové podmínky (hypoxie) (Jeong et al., 2002; Hrebackova et al., 2010),
- narušení rovnováhy pro- a antiapoptotických signálů směrem k apoptose vnější nebo vnitřní cestou (Richon et al., 2000; Sandor et al., 2000; Ruefli et al., 2001; Zhao et al., 2005; Minucci a Pelicci, 2006; Zhang et al., 2006),
- inhibici buněčného cyklu blokováním cyklin/CDK komplexů zvýšením exprese p21 a dalšími mechanismy,
- stabilizaci a aktivaci p53 (Espinosa a Emerson, 2001; Xu, 2003),
- navození mitotické katastrofy/smrti (Qiu et al., 2000; Xu et al., 2005),
- buněčnou smrt způsobenou autofagií a senescencí (Shao et al., 2004),
- metabolický rozvrat a buněčnou smrt v důsledku hromadění reaktivních kyslíkových metabolitů (reactive oxygen species – ROS) (Rosato a Grant, 2005; Ungerstedt et al., 2005),
- další.

Buněčná odpověď na HDACi závisí kromě koncentrace inhibitoru na jeho typu, délce expozice a hlavně na typu buněk, které jsou jeho působení vystaveny.

2.2.2.1.1 Alterace genové exprese navozená hyperacetylací histonů

Histony jsou bazické proteiny s vysokým obsahem argininu a lysinu, které tvoří základní kámen nukleosomů a jaderného chromatinu. Rozlišuje se pět skupin histonů: H1/H5, H2A, H2B, H3 a H4. Vždy dvě kopie H2A, H2B, H3 a H4 vytvářejí oktamerovou jadernou strukturu nukleosomu, která stabilizuje DNA v délce 147 bp. Jedna kopie H1/H5 histonu stabilizuje strukturu mezi dvěma nukleosomovými částicemi tvořenou 10-90 bp DNA a je podstatná pro tvorbu kompaktního chromatinu (Mariño-Ramírez et al., 2001; Bhasin et al., 2006). Mimo výstavbové funkce mají i funkci regulační, jelikož se podílejí na zpřístupňování/znepřístupňování cílových sekvencí DNA pro některé proteiny, zejména transkripční faktory.

N-terminální konce histonů jsou místem jejich posttranslační modifikace – acetylace, methylace, ubikvitinylace, fosforylace a sumoylace. Modifikací histonu se mění jeho vlastnosti a může dojít k interakcím se sousedními nukleosomy nebo proteiny asociovanými

s chromatinem. Pomocí těchto interakcí je alespoň zčásti regulována struktura chromatinu (Chakravarthy et al., 2005). První experimenty z poloviny devadesátých let zaměřené na vliv HDACi na genovou expresi ukázaly, že pouze 2 % genů (z 340 zkoumaných) byly inhibicí HDAC ovlivněny (Van Lint et al., 1996). S využitím novějších metodických přístupů (cDNA array) bylo později zjištěno, že počet ovlivněných genů může být až 7-10 % z celkového počtu, přičemž za relevantní změnu byl považován dvojnásobný nárůst nebo pokles exprese na úrovni mRNA (Chambers et al., 2003; Glaser et al., 2003; Mitsiades et al., 2004; Sasakawa, 2005). Na počet ovlivněných genů má přímý vliv délka kultivace a koncentrace HDACi. Krátká doba expozice a nízké koncentrace HDACi jsou schopny ovlivnit menší počet genů. S vyšší koncentrací a prodloužením expoziční doby počet genů s pozměněnou expresí až do určitého rozsahu narůstá. Počet genů, jejichž exprese je inhibicí HDAC pozitivně ovlněna, je přibližně stejný jako počet genů, jejichž aktivita je naopak utlumena (Kachhap et al., 2010).

Pravděpodobně nejvíce studovanou posttranslační modifikací histonů je acetylace. Tato modifikace umožňuje navázání proteinů nesoucích tzv. bromodoménu vyznačující se afinitou k acetyllysinu (histonacetyltransferasy a další proteiny podílející se na remodelaci chromatinu). Rozpoznání acetylovaného histonu je tak důležitým mechanismem regulace interakcí protein-protein u mnoha buněčných procesů, např. remodelace chromatinu a aktivace transkripce (Zheng, Zhou, 2002; Khorasanizadeh, 2004; Eberharter et al., 2005). Ovlivnění těchto interakcí spolu s účinky na funkci nehistonových proteinů účastnících se různých buněčných pochodů, včetně genové transkripce (viz Tab. 3, str. 32), je pravděpodobně nosným mechanismem protinádorového účinku HDACi.

Míra acetylace N-konců histonů a lysinových zbytků dalších uvedených proteinů je dána rovnovážným stavem mezi aktivitou komplexů histonacetyltransferas a histondeacetylas. Následkem převažující acetylace histonů je chromatinová struktura DNA rozvolněna a zpřístupněna pro transkripční faktory, což vede ke zvýšené transkripční aktivitě příslušného genomového lokusu. Hypoacetylace naopak chromatin kondenzuje a transkripční aktivita je snížena (Kuo, Allis, 1998; Davie, 1998; Backs a Olson, 2006). Uvedený obecný mechanismus ale neplatí vždy. Převažující acetyltransferasová aktivita nemusí nutně vést ke zvýšené transkripční aktivitě navzdory rozvolněné chromatinové struktuře. Příčina tohoto jevu spočívá pravděpodobně v negativním vlivu acetylace na aktivitu nebo stabilitu nehistonových proteinů podílejících se přímo na transkripci DNA (Ma et al., 2009).

2.2.2.1.2 Inhibice buněčné odpovědi na stresové podmínky (hypoxie)

Růst solidních nádorů je obvykle omezen přístupem nádorových buněk ke kyslíku, který je podmíněn stupněm vaskularizace nádorové tkáně. V případě snížené koncentrace kyslíku jsou v buňkách aktivovány signalizační dráhy, které buňku adaptují na tyto podmínky (Jang et al., 2013). Jedním z klíčových regulátorů adaptace k hypoxii je transkripční faktor HIF-1 (viz kapitola Hypoxie a její vliv na buňku, str. 16). HIF-1 se podílí mj. i na neovaskularizaci nádoru aktivací genové exprese řady proangiogenních faktorů.

I když je u nádorů cévní síť strukturně i funkčně abnormální, inhibice její tvorby vede k omezení nádorového růstu a je tudíž vhodným cílem protinádorové terapie. Inhibitory histondeacetylas potlačují nádorovou angiogenezi ovlivněním stability HIF-1, který je v buňce jejich působením degradován. Následně se snižuje exprese jeho cílových genů, především VEGF, endoteliální NO synthasy (eNOS) a receptorů pro VEGF (VEGFR-1), viz Tab. 1, str. 20 (Deroanne et al., 2002; Bolden et al., 2006; Liang et al., 2006; Zupkovitz et al., 2006; Montgomery et al., 2007). Aktivita eNOS je navíc kyselinou valproovou negativně ovlivněná díky indukci transkripce proteinu destabilizujícího mRNA pro eNOS (Rössig et al., 2002). Některé HDACi také inhibují angiogenezi zvýšením exprese antiangiogenních faktorů jako je trombospondin-1 a aktivin A, a snížením exprese proangiogenního růstového faktoru fibroblastů (basic fibroblast growth factor – bFGF) (Cinatl et al., 2002).

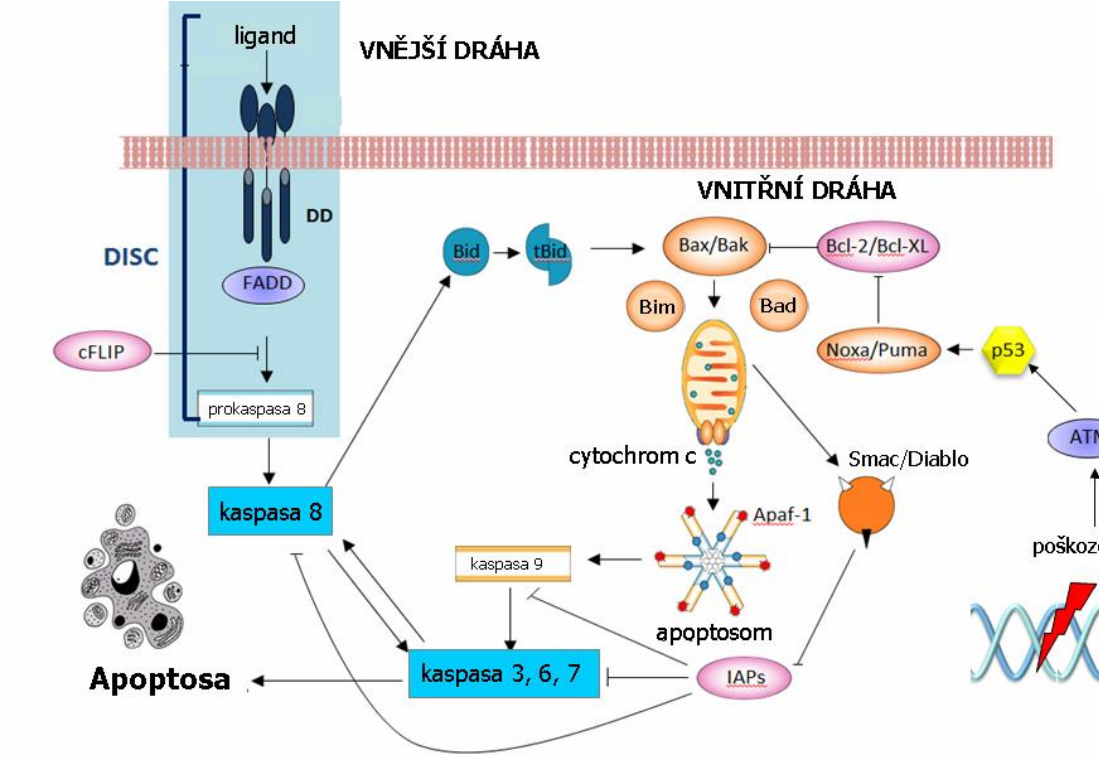
2.2.2.1.3 Ovlivnění rovnovážného stavu pro- a antiapoptotických stimulů

Inhibitory histondeacetylas ovliňují transformované buňky také prostřednictvím změn týkajících se apoptotické rovnováhy, a to ve smyslu posunu rovnovážného stavu mezi pro- a antiapoptotickými stimuly směrem k apoptose. Apoptosa je proces naprogramované buněčné smrti, při kterém je spuštěna kaskáda biochemických procesů vedoucích k smrti buňky provázené charakteristickými morfologickými změnami. Změny zahrnují: smrštění buňky související s degradací cytoskeletu, fragmentaci jaderného obsahu a v konečné fázi vytvoření apoptotických tělísek. Ty mohou být následně odstraněny fagocytujícími buňkami. Při apoptotické smrti tak není buněčný obsah vylit do mezibuněčného prostoru a okolní tkáně nejsou poškozeny.

Hlavními efektorovými enzymy apoptosy jsou kaspasy. Tyto cysteinové proteasy jsou v buňce konstitutivně exprimovány v neaktivní formě v podobě proenzymu – zymogenu. Proenzymy jsou při dostatečném apoptotickém stimulu štěpeny do aktivní formy

s kaskádovitým šířením signálu. Jsou popsány dvě dráhy aktivace apoptosy – vnitřní a vnější, viz Obr. 8, str. 38. Vnější apoptotická dráha je spuštěna aktivací tzv. receptorů smrti (z angl. death receptors). Patří sem Fas (Apo-1, CD95), TNFα receptor 1 (TNFR-1, CD120a), receptory DR-3 (Apo-3), DR-4 (TRAIL-R1, CD261), DR-5 (TRAIL-R2, CD262), DR-6 (CD358) a receptor ektodysplasinu A (EDAR). Za normálních okolností jsou tyto receptory aktivovány po navázání příslušných ligandů – FasL (Fas), TNF (TNFR-1), TRAIL/Apo2-L (DR-4, DR-5) a TL1A (DR-3) (Schneider-Brachert et al., 2013). Vytváří se signální komplex DISC (death-induced signalling complex), který aktivuje tzv. aktivátorové kaspasy – kaspasu 8 a 10. Ty po aktivaci štěpí efektorové kaspasy – např. kaspasu 3 a 7. Tento proces je regulován prostřednictvím proteinu c-FLIP a snad i dalšími mechanismy, kterými jsou regulátory aktivace prokaspasy 8 a 10 (Chang et al., 2002; Schneider-Brachert et al., 2013), a inhibitorem apoptotických proteinů – IAP (inhibitor of apoptosis proteins). IAP působí inhibičně jak na aktivátorové, tak na efektorové kaspasy.

HDACi mohou mít selektivní účinek na expresi receptorů smrti a tím zvýšit citlivost buněk k apoptose, resp. navodit apoptosu. Pro receptor DR-5 byla pozorována na různých buněčných liniích jeho zvýšená exprese v návaznosti na dávku HDACi pouze u nádorově transformovaných buněk, zatímco nenádorové buňky nebyly ovlivněny (Nakata et al., 2004). U leukemických a neuroblastomových buněk byly působením HDACi na jejich povrchu ve zvýšené míře exprimovány Fas a FasL (Insinga et al., 2005; Glick et al., 1999). Vnější apoptotická dráha je tedy HDACi ovlivněna především na úrovni exprese receptorů smrti, jejich ligandů a také regulátorů procesu apoptosy. Výsledný efekt na úrovni vnější apoptotické dráhy je značně závislý na modelové buněčné linii, koncentraci a typu použitého HDACi a dalších podmínkách (Cipro et al., 2012).



Obrázek. 8: Základní schéma drah apoptotické buněčné smrti. Upraveno podle (Goncharenko-Khaider et al., 2013).

Vnitřní apoptotická dráha je zahajována na úrovni mitochondrií. Při její aktivaci je narušena mitochondriální membrána a dochází k uvolnění proteinů z mezimembránového prostoru. Proteiny vnitřní dráhy zahrnují cytochrom c, faktor indukující apoptosu (apoptosis inducing factor – AIF), Smac/DIABLO, HtRA2 a další. Po uvolnění do cytoplasmy vzniká navázáním cytochromu c na cytoplasmatický faktor Apaf-1 (apoptotic protease activation factor-1) tzv. apoptosom. Apoptosom aktivuje prokaspasu 9, prostřednictvím které se aktivují efektorové prokaspasy 3, 6 a 7. Smac/DIABLO, resp. HtRA2 se podílí na inhibici IAP mechanismem přímé interakce resp. interakce s následným enzymatickým štěpením (Wang, 2001; Suzuki et al., 2001). Funkce faktoru AIF v apoptotickém procesu je na kaspasach nezávislá. Po jeho vazbě na DNA je spuštěna kondensace chromatinu a fragmentace DNA (Kitagawa a Niikura, 2008).

Permeabilizace mitochondriální membrány, která předchází uvolnění uvedených faktorů do cytosolu, je regulována prostřednictvím pro- a antiapoptoticky působících proteinů rodiny Bcl-2. Proapoptotické proteiny (Bax, Bad) jsou aktivovány prostřednictvím proteinu t-Bid, který vzniká při aktivaci vnitřní apoptotické dráhy z prekursoru Bid štěpením především kaspasou 3. Další alternativou, která vede k aktivaci Bax a Bad, je interakce proapoptotických

proteinů (např. Noxa, Puma, Bim) s antiapoptotickými Bcl-2 proteiny – Bcl-2, Bcl-X_L (Adams, Cory, 1998; Huang, Strasser, 2000) vedoucí k potlačení jejich antiapoptotického vlivu na Bax a Bad. Jelikož je Bid možné aktivovat také kaspasou 8, představuje určitou formu propojení mezi vnitřní a vnější apoptotickou dráhou a slouží zároveň jako amplifikátor apoptotického signálu (Green, 2000). Aktivátory vnitřní apoptotické dráhy tak mohou pozitivně ovlivňovat i citlivost buněk k apoptotickým stimulům z vnějšího prostředí.

Zdrojem signálu pro aktivaci vnitřní apoptotické dráhy bývají různé stresové situace – poškození DNA, hypoxie, nedostatek růstových faktorů, oxidační stres a další. Jako biologický sensor buněčného stresu, který je při aktivaci vnitřní apoptotické dráhy velmi důležitý, slouží p53 (Lowe, Lin, 2000). Aktivní forma p53 ovlivňuje řadu genů souvisejících s vnitřní i vnější apoptotickou dráhou. A to ve smyslu zvýšení exprese, která se týká proapoptotických proteinů – např. Bax, Bak, PUMA, Noxa a recetorů smrti CD95 a DR-5. Naproti tomu syntéza antiapoptotických faktorů (IAP, Bcl-2, Bcl-X_L) je aktivovaným p53 potlačena (Bartke et al., 2001; Hoffman et al., 2002; Ryan et al., 2001; Wu et al., 2001; Herr, Debatin, 2001). p53 aktivuje i další geny, které citlivost buněk k apoptose ovlivňují – PTEN, Apaf-1, PERP, p53AIP1 a geny ovlivňující metabolismus reaktivních metabolitů kyslíku (Hwang et al., 2001; Moroni et al., 2001; Ryan et al., 2001; Stambolic et al., 2001).

Inhibitory histondeacetylas jsou schopny aktivovat i vnitřní apoptotickou dráhu. Děje se tak několika mechanismy. Jedním z nich je zvýšení exprese proapoptotických proteinů – Bim, Bmf, Bax, Bak a Bid (Ververis et al., 2013). Mechanismus tohoto zvýšení je znám pouze u některých z uvedených faktorů. Příkladem je aktivace vnitřní apoptotické dráhy proapoptotickým proteinem Bim, který je po kultivaci buněk s HDACi exprimován ve zvýšené míře. Na jeho transkripci se po inhibici histondeacetylas pomocí HDACi podílí transkripční faktor E2F1, jehož aktivita bývá u mnohých nádorových onemocnění zvýšená a je jedním z mechanismů deregulace buněčného cyklu. Zvýšená koncentrace Bim potlačuje antiapoptotickou aktivitu Bcl-2 a tím iniciuje vnitřní apoptotickou dráhu. (Zhang et al., 2004; Zhao et al., 2005). HDACi ovlivňují také aktivitu regulátorů vnitřní apoptotické dráhy – jejich transkripci a/nebo posttranslační modifikaci. Příkladem je proteolytické štěpení Bid. Mechanismus působení HDACi na jeho štěpení není ještě zcela pochopen. Pravděpodobně se ho neúčastní kaspasy. Není zcela vyloučena možnost, že HDACi indukují ještě neidentifikované proteasy, i když některé práce poukazují u štěpení Bid na možnou účast kalpainů (Ruefli et al., 2001; Mitsiades et al., 2003; Peart et al., 2003; Willis a Adams, 2005). HDACi navíc zatím neznámými mechanismy navozují uvolnění cytochromu c, AIF a Smac z mitochondrií s následnou aktivací kaspasy 9. Navíc mohou také zvyšovat jejich expresi

(Marks a Dokmanovic, 2005; Lu et al., 2005; Ruefli et al., 2001; Rosato et al., 2006; Xu et al., 2006).

Druhým mechanismem, kterým HDACi ovlivňují vnitřní apoptotickou dráhu, je snížení exprese antiapoptotických proteinů rodiny Bcl-2 – Bcl-2, Bcl-X_L, Bcl-w a Mcl-1, a inhibitorů apoptosy (IAP) (Rosato et al., 2006; Xu et al., 2006). Zvýšená exprese antiapoptotických proteinů Bcl-2 a Bcl-X_L může být jedním z mechanismů ochrany nádorových buněk před indukcí apoptosy. V těchto konkrétních případech je navození apoptotické buněčné smrti působením HDACi nutné podpořit dodatečnou chemickou inhibicí Bcl-2 (Xu et al., 2006).

Účinek histondeacetylasových inhibitorů na apoptotickou signalizaci nádorových buněk tedy souvisí s jejich aktivitou na úrovni ovlivnění genové exprese (receptory smrti a jejich ligandy, pro- a antiapoptotické faktory), regulací aktivity proapoptotických proteinů (indukce štěpení Bid) a dalšími, ne zcela pochopenými mechanismy (uvolnění spouštěčů vnitřní apoptotické dráhy z mezimembránového prostoru mitochondrií). To, jakým způsobem nádorové buňky reagují, závisí na jejich typu, na použitém HDACi, jeho koncentraci, době expozice, prostředí (např. normoxie/hypoxie) a dalších podmínkách.

2.2.2.1.4 Ovlivnění buněčného cyklu

Inhibitory histondeacetylas zastavují buněčný cyklus transformovaných buněk. Při nižších koncentracích je obvykle zastaven ve fázi G1 (Ververis et al., 2013). U vyšších koncentrací HDACi bývá kromě G1 fáze zablokován i přechod G2/M (Richon et al., 2000).

Zastavení buněčného cyklu ve fázi G1 a G2 bývá spojováno s indukcí p21 (WAF1/CIP1). p21 je inhibitor kinas závislých na cyklinu (cyclin-dependent kinase – CDK). Je jedním z cílových genů, jejichž exprese je inhibicí HDAC zvýšena. Zvýšení exprese koreluje se zvýšenou acetylací histonů H3 a H4 v oblasti promotoru pro p21 (Richon et al., 2000). Navíc je ovlivněno i složení proteinového komplexu, který je asociován s promotorem pro p21. Komplex za normálních okolností obsahuje HDAC1, HDAC2, Myc, BAF155, Brg-1, GCN5, p300 a SP1. Inhibice HDAC (vorinostat/SAHA) vede ke snížení obsahu HDAC1 a Myc v tomto komplexu a ke zvýšení schopnosti vazby RNA polymerasy II. Ztráta HDAC1 v promotorovém komplexu není asociována se snížením jejího množství v buněčném jádru, ovlivněna je zřejmě pouze interakce proteinů (Gui et al., 2004). Na zvýšení koncentrace p21 se může podílet také p53, který je v případě inhibice histondeacetylas acetylován a následně stabilizován (Nakamura et al., 2000; Rodriguez et al., 2000). Indukcí p21 tak dochází k inhibici CDK kontrolujících progresi G1 fáze (CDK4, CDK6), přechod G1/S (CDK2) a G2/M (CDK1) a podjednotky DNA

polymerasy delta – proliferujícího nukleárního antigenu (PCNA) (Vidal, Koff, 2000). Některé HDACi (TSA) byly schopny progresi buněčného cyklu inhibovat i u buněk s vyřazeným genem pro p21. Inhibiční účinek je zde pravděpodobně způsobený indukcí inhibitoru cyklin C-dependentních kinas p15 (INK4b) (Hitomi et al., 2003). Dalším inhibičním mechanismem může být aktivace exprese p27, který inhibuje aktivitu CDK2 a CDK4 (Vidal a Koff, 2000; Nimmanapalli et al., 2003).

Obecně lze tedy říci, že inhibitory histondeacetylas zvýšují expresi inhibitorů cyklin-dependentních kinas se současným omezením exprese cyklinů. To vede k defosforylaci proteinu pRb, zablokování aktivity transkripčního faktoru E2F a zastavení progrese cyklu. U některých buněk může navíc zablokování buněčného cyklu ve fázi G1 vést k diferenciaci (Marks et al., 1996). U vorinostatu/SAHA byla tato vlastnost zjištěna dokonce dříve než jeho inhibiční účinek na HDAC (Marks a Breslow, 2007).

2.2.2.1.5 Stabilizace a aktivace p53

p53 je nejčastěji mutovaný tumor supresorový gen u lidských nádorů. Je to transkripční faktor, který se váže na specifickou sekvenci DNA. Je tvořen několika doménami s různými funkcemi, viz obr.9. N-koncová doména (TA) je zodpovědná za samotnou transkripční aktivitu a regulaci hladiny p53 v buňce, C-koncovou část tvoří lokalizační signální doména pro jádro (NLS), tetramerizační doména (TET) a C-koncová regulační doména (REG) (Ko a Prives, 1996). p53 se aktivuje ve stresových situacích, například při poškození DNA nebo aktivaci onkogenní signalizace. Při poškození DNA se aktivují specifické kinasy (ATR, ATM), které následně fosforylují a tím aktivují kinasy Chk1 a Chk2, které fosforylují a stabilizují p53 inhibicí jeho proteasomální degradace narušením interakce mezi p53 a Mdm2 (s E3 ubikvitin ligasovou aktivitou) (Khanna a Jackson, 2001). Druhý způsob aktivace p53 nezávisí na poškození DNA. Stabilizaci p53 napomáhá protein $p14^{ARF}$, který je syntetizován při použití alternativního čtecího rámce genu pro inhibitor CDK INK4a/ARF u onkogenní signalizace. Vazbou na Mdm2 inhibuje interakci s p53 a tím i jeho proteasomální degradaci (Palmero et al., 1998, de Stanchina et al., 1998; Zindy et al., 1998). Na stabilizaci p53 se kromě fosforylace podílejí i další posttranslační modifikace jako jsou acetylace a sumoylace, které ovlivňují interakci mezi p53 a Mdm2.

Protein vážící CREB (CREB-binding protein, CBP) a p300 jsou schopny koaktivace řady transkripčních faktorů včetně p53 (Avantaggiati et al., 1997; Gu a Roeder, 1997; Lill et al., 1997). Jak p300, tak faktor PCAF, asociovaný s p300/CBP, mají histonacetyltransferasovou

aktivitu a při poškození DNA acetylují p53 na regulační a lokalizační signální doméně (Sakaguchi et al., 1998; Liu et al., 1999). Fosforylace p53 po poškození DNA působí pozitivně na interakci p53 a PCAF/p300 komplexu s následnou acetylací p53 (Sakaguchi et al., 1998; Dumaz a Meek, 1999). Acetylace C-koncové domény p53 je důležitá pro nasměrování CBP/p300 k promotorům genů řízených p53 (Barlev et al., 2001), protože transkripční aktivita p53 je závislá na nasměrování p300 do promotorové oblasti řízených genů (Espinosa a Emerson, 2001).

Acetylace p53, kromě regulace jeho transkripční aktivity, hraje roli i při regulaci jeho stability. Zatímco Mdm2 se na p53 váže v oblasti N-koncové domény, C-koncová regulační doména je nutná k degradaci p53 zprostředkované Mdm2 (Kubbutat et al., 1998). Ubikvitinylace p53 zprostředkovaná Mdm2 probíhá na lysinových zbytcích 370, 372, 373, 381, 382 a 386. Stejné aminokyselinové zbytky jsou cílem histonacetylas, které acetylací p53 stabilizují. Mechanismem této ochrany p53 před proteasomální degradací je znemožnění ubikvitinylace na cílových aminokyselinových zbytcích (Nakamura et al., 2000; Rodriguez et al., 2000). Samotná vazba Mdm2 na p53 není acetylací ovlivněna.

Aktivní forma p53 aktivuje nebo inaktivuje řadu genů. Ty souvisí s různými procesy: apoptotickou dráhou (vnější i vnitřní aktivace), zastavením buněčného cyklu, opravou poškozené DNA, metabolismem reaktivních forem kyslíku, angiogenezí a produkcí chemotaktických faktorů. Stabilizace p53 může být podpořena inhibitory histondeacetylas inhibicí jeho proteasomální degradace na podkladě acetylace C-koncové domény.

Obrázek. 9: Doménová struktura p53 a základní funkce vybraných domén. TA-transaktivační doména, Pr-doména bohatá na prolin, DBD-doména vázající DNA, NLS-lokalizační signální doména, TET-tetramerizační doména, REG-regulační doména.

2.2.2.1.6 Navození mitotické katastrofy/smrti

Mitotická katastrofa byla poprvé popsána u kvasinek jako smrtí končící fenotyp, který je spojený s abnormalitami segregace chromosomů. (Molz et al., 1989). Současná definice mitotické katastrofy ji popisuje jako mechanismus, který je citlivý na mitotické poruchy, a který

v případě jejich výskytu vede k nevratnému procesu končícímu buněčnou smrtí – apoptosou, nekrózou nebo senescencí (Lanz et al., 2013).

Na mitotickou katastrofu u savčích buněk lze pohlížet jako na poruchu průběhu kompletní mitosy po poškození DNA. Druhou variantou je neschopnost buněk zastavit progresi buněčného cyklu při takovém poškození, které za normálních okolností zastaví vstup buňky do mitotické fáze. Porucha mitosy vede k tetraploidii po jednom buněčném cyklu, (Andreassen et al., 2001; Margottin-Goguet et al., 2003) nebo endopolyploidii po několika cyklech dělení, která je spojena s rozsáhlým poškozením DNA s následnou opravou. V druhém případě dochází k aberantní mitose s poruchami segregace chromosomů nebo k jejich ztrátám. Buňky tento stav v závislosti na rozsahu poškození buďto nepřežijí, nebo může dojít k selekci populace, která je vůči daným apoptotickým stimulům rezistentní (Ivanov et al., 2003). Schopnost uniknout buněčné smrti mitotickou katastrofou je považována za jeden z mechanismů uplatňujících se při nádorové transformaci (Vitale et al., 2011).

Inhibitory histondeacetylas hyperacetylací histonů v heterochromatinu a v blízkosti centromér navozují mitotické defekty u nádorových buněk. Mechanismem je narušení struktury a správné funkce centroméry a pericentrického heterochromatinu, u kterého je porušena interakce s dalšími proteiny (Taddei et al., 2001; Cimini et al., 2003). Inhibice HDAC navíc ovlivňuje i fosforylaci proteinů regulujících průchod buňky mitotickou fází (BubR1, hBUB1 a dalších). V důsledku toho buňky pravděpodobně podstupují hůře regulovanou mitosu spojenou s aberantní chromosomovou segregací a chromosomálními ztrátami (Dowling et al., 2005; Robbins et al., 2005). U specifické inhibice HDAC6 vedoucí ke zvýšené acetylaci α-tubulinu, komponenty mitotického tělíska, nebyl vliv na progresi buněčného cyklu pozorován (Haggarty et al., 2003).

2.2.2.1.7 Buněčná smrt způsobená autofagií a senescencí

Autofagie je proces, který pomáhá buňkám udržet homeostasu ve stresových podmínkách. Cytoplasmatické proteiny a organely jsou v jeho průběhu degradovány a následně slouží jako zdroj energie (Klionsky a Emr, 2000). U eukaryotických buněk jsou popsány tři typy autofagie: makroautofagie, mikroautofagie a chaperony zprostředkovaná autofagie (Mizushima a Klionsky, 2007).

Mikroautofagií jsou degradovány proteiny cytoplasmy po jejich obklopení lysosomální membránou. Autofagie zprostředkovaná chaperony degraduje proteiny obsahující motiv KFERQ, který je rozeznáván chaperonem Hsc70. Tyto proteiny jsou transportovány

k lysosomální membráně a pomocí Lamp-2 translokovány do lumen lysosomu, kde jsou degradovány (Chiang et al., 1989; Cuervo a Dice, 1996; Cuervo a Dice, 2000; Massey et al., 2006). Hlavní cestou degradace intracelulárních komponet za účelem získání energie je makroautofagie. U tohoto procesu jsou buněčné součásti, jako proteinové agregáty nebo organely, obklopeny a obaleny membránou, a vzniká struktura s dvojitou membránou – autofagosom (Mizushima et al., 2002). Autofagosomy fúzují s lysosomy a jejich obsah je degradován.

Aberantní regulace autofagického procesu může vést k některým onemocněním – neurodegenerativním chorobám nebo zhoubným nádorům (Shintani a Klionsky, 2004; Levine a Kroemer, 2008). Roli hraje také při imunitních procesech, protože prostřednictvím autofagie je možná efektivní eliminace intracelulárních patogenů (Nakagawa et al., 2004; Gutierrez et al., 2004; Xu et al., 2007a).

Buněčná senescence byla poprvé popsána roku 1961 (Hayflick a Moorhead, 1961). U kultivovaných buněk, které zpočátku prochází fází rychlé proliferace, jsou chromosomové telomery zkracovány. To vede k postupnému prodloužení generační doby a končí stavem, kdy dělení ustane. Tento stav, kdy jsou buňky ve fázi trvale zastaveného buněčného cyklu, se nazývá replikační senescence.

Inhibitory histondeacetylas navozují u nádorových buněk autofagickou smrt nebo senescenci. Na příkladu HeLa buněk bylo ukázáno, že inhibitory histondeacetylas (butyrát, vorinostat/SAHA) jsou schopny navodit buněčnou smrt u buněk, které jsou rezistentní k apoptotickým stimulům. Usmrcené buňky vykazovaly jasné známky autofagické smrti (Shao et al., 2004). V jiné studii byl senescenční fenotyp nádorových buněk pozorován při kultivaci buněk s vorinostatem/SAHA. Ten byl navíc spojen s obrazem polyploidních buněk, pravděpodobně v důsledku mitotických defektů (Xu et al., 2005).

2.2.2.1.8 Metabolický rozvrat v důsledku hromadění reaktivních kyslíkových metabolitů

Reaktivní kyslíkové metabolity zahrnují molekuly obsahující nestabilní formu kyslíku (ROS). Pro tyto struktury je charakteristická přítomnost nepárových elektronů nebo peroxidové formy kyslíku, což je činí extrémně reaktivními. Interagují s různými molekulami, jako jsou proteiny, DNA, lipidy a sacharidy (Buonocore et al., 2010). Jejich hlavní funkcí je ochrana organismu – systémy generující ROS jsou součástí vrozené imunity a tvoří první linii obrany před patogeny (syntesa baktericidních látek při oxidativním vzplanutí). Aktivní kyslíkové

metabolity jsou navíc produkovány ve všech buňkách enzymy v cytosolu (xantinoxidasou, cyklooxygenasou a dalšími) a v mitochondriích. Kromě imunity se podílejí i na dalších důležitých fyziologických procesech jako jsou buněčná signalizace a hojení ran (odstraňování apoptotických či nekrotických buněk) a pravděpodobně i na adaptaci buněk k hypoxii. Pro stabilizaci HIF-1α za hypoxických podmínek je potřebná přítomnost funkčních mitochondrií, respektive produkce ROS mitochondriemi. Ty se zatím neznámým způsobem podílejí na inhibici hydroxylace HIF-1α prolylhydroxylasami a zvyšují aktivitu HIF-1 omezením jeho proteasomální degradace (Sena a Chandel, 2012). Z hlediska signalizace jsou ROS důležité například při regulaci signalizace cytokiny, insulinem a růstovými faktory (Finkel, 1998). Negativní dopad ROS je patrný z jejich účasti na rozvoji některých chorob, jako jsou například kloubní a neurodegenerativní onemocnění, astma a nádory (Storz, 2005; Bryan et al., 2012). Zde se ROS poškozováním buněk tkáně podílejí na rozvoji onemocnění jako takového. U nádorových onemocnění může produkce ROS přispívat v iniciační fázi k buněčné transformaci a dále pak ke kumulaci mutací v důsledku ROS-zprostředkovaným poškozením DNA. V případě, že jsou kyslíkové metabolity produkovány ve větším rozsahu, než je buňka schopna tolerovat, dochází k metabolickému rozvratu nebo aktivaci signalizace vedoucí k buněčné smrti.

Inhibitory histondeacetylas navozují akumulaci ROS v transformovaných buňkách, která pravděpodobně vede k jejich usmrcení. Tento proces probíhá v kultivovaných buňkách několik hodin před rozpadem mitochondrií a zahájením aktivace vnitřní apoptotické dráhy. Vychytávače volných radikálů, jako např. N-acetylcystein, jsou schopny HDACi navozenou buněčnou smrt do jisté míry potlačit (Ruefli et al., 2002; Rosato et al., 2003). Účinek kumulace ROS je dále posílen vlivem na expresi proteinů, regulujících aktivitu vychytávačů radikálů. HDACi zvyšují expresi proteinu 2 vážícího thioredoxin (thioredoxin (Trx) binding protein 2 – TBP2) a tím potlačují správnou funkci Trx v transformovaných buňkách (Butler et al., 2002; Ungerstedt et al., 2005). Ten se kromě jiného podílí na inhibici kinasy regulující apoptotické signály (apoptosis signal regulating kinase – ASK1), která je v přítomnosti HDACi rovněž exprimována ve zvýšené míře (Saitoh et al., 1998). ASK1 se podílí na proapoptotické signalizaci zvýšením exprese proteinu Bim, pravděpodobně prostřednictvím aktivace transkripčního faktoru E2F (Tan et al., 2006). Je diskutabilní, zda je zvýšená produkce nebo akumulace kyslíkových metabolitů v buňce po působení HDACi příčinou její smrti, nebo jestli se jedná pouze o důsledek aktivace signalizačních drah, které spouští mechanismy vedoucí k buněčné smrti.

2.2.2.1.9 Další mechanismy působení inhibitorů histondeacetylas

Kromě uvedených mechanismů působení na nádorové buňky jsou HDACi schopny zasahovat i do dalších buněčných procesů, např: ovlivňují funkci chaperonového proteinu Hsp90 (protein teplotního šoku 90, heat shock protein 90), signalizaci pomocí proteinkinas, aktivitu proteinfosfatasy 1 a správnou funkci agregosomu. Hsp90 je ATP-dependentní chaperonový protein o molekulární hmotnosti 90 kDa. Podílí se na svinování, aktivaci a stabilizaci „klientských" proteinů. Samotná aktivita Hsp90 je sérií konformačních změn a interakcí s klientským proteinem a tzv. kochaperony, která je závislá na hydrolýze ATP (Workman et al., 2007). Klientské proteiny jsou definovány jako bílkoviny, které jsou schopné vazby na Hsp90, a jejichž množství v buňce po inhibici Hsp90 klesá, pravděpodobně v důsledku nesprávného svinutí s následnou proteasomální degradací. V současnosti je známo více než 100 klientských proteinů Hsp90. Jejich funkce je různá, ovšem mnohé z nich jsou proteiny účastnící se buněčné signalizace a transkripce DNA – kinasy a transkripční faktory. Některé z nich jsou v podstatě onkoproteiny využívané nádorovými buňkami při onkogenní signalizaci (EGFR, CDK4, AKT, bcr-abl, estrogenové a androgenové receptory, HIF-1, katalytická podjednotka telomerasy hTERT a další) (Workman et al., 2007; Pearl et al., 2008). Hsp90 je ve vysoké míře exprimován také v buňkách zdravé tkáně, kde se svojí funkcí podílí na udržování homeostasy. U transformovaných buněk je Hsp90 v porovnání se zdravými buňkami přítomen obvykle ve vyšších koncentracích a ve formě, tzv. multichaperonového komplexu (Kamal et al., 2003). Ten je využíván zejména k podpoře labilních forem aktivovaných onkoproteinů a tím pádem k redukci buněčného stresu způsobeného maligním fenotypem (Workman et al., 2007; Trepel et al., 2010).

Inhibitory histondeacetylas jsou schopny inhibicí HDAC6 navodit akumulaci acetylované formy Hsp90 v buňce. Hsp90 a HDAC6 spolu fyzicky interagují a navzájem se regulují prostřednictvím acetylace a deacetylace (Aoyagi a Archer, 2005). Acetylovaná forma Hsp90 je inaktivní pravděpodobně v důsledku ovlivnění vazby ATP (Bali et al., 2005; Kovacs et al., 2005). HDACi se mohou uplatnit, pokud jsou transformované buňky závislé na signalizaci, jejíž funkčnost je ovlivněná Hsp90 (signalizace pomocí hormonálních receptorů, bcr-abl, Akt). Po inhibici HDAC6 je tato signalizace narušena a tím může být omezena např. proliferační schopnost nádorových buněk nebo jejich odolnost k apoptose (Kaliszczak et al., 2013).

Jednou ze základních vlastností všech organismů je schopnost reagovat na podněty z vnějšího prostředí. Reakce se odehrávají jak na úrovni celého organismu, tak na úrovni

buněčné. V rámci buňky je mnoho procesů, které jsou všechny méně nebo více složitým způsobem regulovány, a které zajišťují udržení homeostasy. K regulačním mechanismům patří také buněčná signalizace založená na kinasach aktivovaných mitogenem – MAPK (mitogen-activated protein kinase), dále na proteinkinase C a Wnt signalizaci. Tyto dráhy regulují důležité procesy jako je buněčný růst, diferenciace a motilita, apoptosa, angiogeneze a další (Leppä et al., 1998; Carter, 2000; Kermorgant et al., 2001; Leppä et al., 2001; Teicher et al., 2001a; Teicher et al., 2001b). Inhibitory histondeacetylas tyto signalizační dráhy ovlivňují – aktivují MAPK/ERK, potlačují signalizaci pomocí proteinkinasy C a Wnt signalizaci. Výsledkem je buněčná diferenciace a omezení nádorového růstu (Leppä et al., 1998; Chen et al, 2000; Leppä et al., 2001; Chen et al., 2004; Zhu et al., 2004). Naproti tomu některé práce ukazují spojitost mezi aktivací MAPK a rezistencí na některé HDACi (Ozaki et al., 2006; Matsubara et al., 2009). Přesné mechanismy účinku HDACi na uvedené signalizační pochody zatím nejsou zcela objasněny a vzhledem k částečně protichůdným nálezům vyžadují další zkoumání.

Proteinová fosfatasa 1 (protein phosphatase 1 – PP1) je jedna z nejvíce zastoupených fosfatas v buňce. Podílí se na regulaci mnoha buněčných pochodů: metabolismu glykogenu, transkripce, vezikulárního transportu, odpovědi na poškození DNA a progresi buněčného cyklu. Substrátovou specifitu katalytické podjednotky PP1 ovlivňuje řada regulačních podjednotek, které s ní interagují (Ceulemans a Bollen, 2004; Virshup a Shenolikar, 2009). Jedním z interagujících proteinů jsou i histondeacetylasy. Komponenty fosfatasových komplexů jsou HDAC6, HDAC1 a HDAC10. HDACi jsou schopny interakci HDAC s těmito komplexy narušit a tím PP1 aktivovat (Brush et al., 2004; Chen et al., 2005). Vzhledem k řadě funkcí, které v buňce PP1 zastává (mimo jiné defosforyluje a inaktivuje Akt, která se podílí na antiapoptotické signalizaci), se lze domnívat, že její ovlivnění může být jedním z mechanismů protinádorových účinků HDACi.

Nově syntetizované proteiny jsou po translaci svinovány do funkční podoby. Malé proteiny se svinují přes sérii meziproduktů do konečné proteinové formy. Během tohoto procesu mají obvykle částečně svinuté proteiny pro přechodnou dobu do okolního prostředí vystaveny hydrofobní úseky svého řetězce. To představuje pro syntetizované proteiny určité riziko proteinové agregace bez zaujmutí správné funkční konformace. Na svinování proteinů má vliv pH, teplota, iontová síla roztoku a oxidačně-redukční vlastnosti prostředí (Gottesman et al., 1997; Wickner et al., 1999). Tvorba agregátu probíhá obvykle kotranslačně a je pro buňky toxická. Špatně svinuté nebo agregované proteiny jsou v buňce odtraňovány buďto opakovaným svinutím proteinů pomocí chaperonů, proteasomální degradací nebo

prostřednictvím tzv. agresomů (Garcia-Mata et al., 2002). Jednotlivé agresomální částice, složené z většího množství nesprávně svinutých proteinů, jsou v buňce aktivně transportovány a hromaděny na jednom místě ve formě agresomu. Transport probíhá pomocí dyneinu a vyžaduje intaktní tubulární strukturu. Agresom je v buňce částečně redukován chaperony a proteasomy. Jeho kompletní odstranění probíhá autofagií.

HDAC6 je pro formování agresomu důležitá, protože se podílí na transportu polyubikvitinylovaných proteinů k dyneinu s následným transportem do agresomu (Kawaguchi et al., 2003). HDAC6 také aktivuje expresi chaperonových proteinů v závislosti na přítomnosti proteinových agregátů a deacetylací aktivuje Hsp90 (Boyault et al., 2007). Inhibicí HDAC6 dochází ke zvýšení citlivosti buněk na stres způsobený hromaděním nesprávně svinutých proteinů, který může navodit jejich usmrcení (Kawaguchi et al., 2003).

2.2.3 REZISTENCE NA INHIBITORY HISTONDEACETYLAS

Rezistence nádorů k léčbě představuje velký problém klinické onkologie. Obdobně jako je tomu u ostatních protinádorových léků, vyvíjí se rezistence i k inhibitorům histondeacetylas. Mechanismy ochrany nádorových buněk proti účinku HDACi byly identifikovány *in vitro* na buněčných liniích, které byly selektovány na základě dlouhodobého působení HDACi nebo zkoumáním transfekovaných buněčných linií, které tak ve zvýšené míře exprimovaly proteiny zprostředkující rezistenci. Na rezistenci k HDACi se tak podílí: transportní proteiny vylučující léčivo z buňky, proteiny podílející se na regulaci buněčného cyklu, mechanismy zajišťující zvládání oxidačního stresu, antiapoptotické proteiny, změněná exprese histondeacetylas, modulace buněčné signalizace a zvýšená aktivita některých transkripčních faktorů. Tyto mechanismy vycházejí z popsaných účinků HDACi (viz kapitola Mechanismus působení inibitorů histondeacetylas, str. 34) a do jisté míry tak omezují efekt působení jak samotných HDACi, tak i jejich kombinaci s dalšími terapeutiky.

Jednou z nejčastějších změn v buněčném fenotypu, která bývá spojena s mnohočetnou lékovou rezistencí nádorových buněk, je zvýšená exprese transportních proteinů, které jsou schopny aktivně transportovat léčivo z buňky – ABC transportéry (ATP binding cassette transporter). Mezi tyto transportéry patří i P-glykoprotein. Jeho syntéza bývá zvýšená působením různých HDACi – butyrátu, trichostatinu A, romidepsinu (depsipeptid) a VPA (Mickley et al., 1989; Frommel et al., 1993; Lee et al., 1994; Jin a Scotto, 1998; Cerveny et al., 2007). V současnosti je z uvedené skupiny HDACi prokázaným substrátem pro P-glykoprotein pouze romidepsin. Zvýšená exprese P-glykoproteinu nebo dalších proteinů zajišťujících

vylučování léčiva z buňky se proto jeví spíše jako důsledek jejich působení než jako ochranný mechanismus buněk. To platí pro HDACi patřící do skupiny derivátů kyseliny hydroxamové a k alifatickým kyselinám, viz Tab. 4, str. 33.

HDACi ovlivňují buněčný cyklus navozením exprese inhibitorů cyklin-dependentních kinas se současným omezením exprese cyklinů. Indukce cílového genu pro p21, který je zodpovědný za zastavení buněčného cyklu ve fázi G1, je jedním z mechanismů, kterým HDACi ovlivňují růst nádorových buněk. Některé práce ovšem ukazují, že právě indukce p21 představuje pro buňku určitou formu ochrany před usmrcením. p21 deficitní buňky nebo buňky ošetřeny antisense RNA komplementární k mRNA p21 byly k působení některých HDACi citlivější než buňky, které byly schopny normální exprese p21 (Vrana et al., 1999; Sandor et al., 2000; Nguyen et al., 2003). Zvýšení citlivosti buněk k HDACi bylo dosažené působením flavopiridolu i temisirolimu, které omezují zvýšenou expresi p21 (Almenara et al., 2002; Rosato et al., 2004; Yazbeck et al., 2008).

HDACi jsou schopny navodit v nádorových buňkách akumulaci reaktivních metabolitů kyslíku – ROS, která vede v konečném důsledku k jejich usmrcení. Existují mechanismy, které omezují léčivem navozený oxidační stres v buňce. Jak bylo popsáno výše, HDACi zvyšují expresi proteinu 2 vážícího thioredoxin a tím potlačují funkci thioredoxinu v buňce (Butler et al., 2002; Ungerstedt et al., 2005). Zvýšená exprese thioredoxinu a dalších proteinů, které se podílejí na metabolismu ROS tak zřejmě představuje ochranný mechanismus buněk před oxidačním stresem způsobeným HDACi (Ungerstedt et al., 2005; Xu et al., 2006; Powis a Kirkpatrick, 2007; Chen et al., 2008).

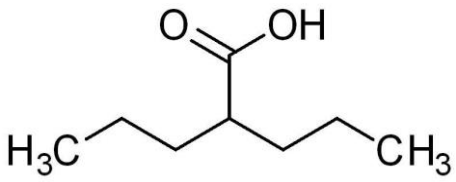
Dalším mechanismem, kterým se nádorové buňky brání účinkům HDACi a dalších terapeutik, je zvýšená exprese antiapoptotických proteinů. Vyšší hladiny Bcl-2 a Bcl-X_L navozují rezistenci nádorových buněk k apoptose po vystavení vorinostatu, dacinostatu, panobinostatu a romidepsinu. Současné působení HDACi a inhibitoru funkce Bcl-2 proapoptotickou signalizaci obnovuje (Vrana et al., 1999; Newbold et al., 2008; Ellis et al., 2009). Omezení proapoptotické signalizace po působení HDACi může být u nádorových buněk způsobeno také sníženou expresí proapoptotických proteinů (Yang et al., 2009).

Rezistence k HDACi byla rovněž zjištěna u buněk, které exprimují zvýšené množství cílů HDACi – histondeacetylas nebo u buněk, které mají tyto enzymy mutací pozměněny (Yoshida et al., 1990; Bandyopadhyay et al., 2004; Ropero et al., 2006). Přímá souvislost mezi klinickým účinkem HDACi a úrovní exprese HDAC ještě ale nebyla definitivně prokázána. Snížený efekt HDACi na nádorové buňky je dáván do souvislosti také s aktivitou některých transkripčních faktorů. Patří sem transkripční faktory rodiny STAT (signal transducer and

activator of transcription) a transkripční komplex NF-κB (Dai et al., 2005; Duan et al., 2007; Fantin et al., 2008; Diaz et al., 2011). Omezení jejich funkce pomocí inhibitorů – v případě STAT i NF-κB inhibitorů jejich fosforylace – vede ke zvýšení citlivosti buněk k HDACi.

2.2.4 KYSELINA VALPROOVÁ

Kyselina valproová (obr. 10) je malá, osmiuhlíkatá rozvětvená mastná kyselina. Poprvé byla syntetizovaná ve snaze nalézt nová organická rozpouštědla Burtonem (Burton, 1882). Její antiepileptické vlastnosti byly objeveny až roku 1963 (Meunier et al., 1963). Koncem šedesátých a začátkem sedmdesátých let byla schválena pro klinické použití jako antiepileptikum ve Francii a Spojených státech amerických (Blaheta a Cinatl, 2002; Blaheta et al., 2005).



Obrázek 10: Struktura kyseliny valproové

Struktura a chemické vlastnosti kyseliny valproové umožňují její snadné podávání. Obvykle bývá podávána orálně ve formě hořečnaté nebo sodné soli. Kromě léčby epilepsie je rovněž používána při terapii bipolárních poruch a migrén. Její účinek pravděpodobně spočívá v podpoře funkce neurotransmiteru GABA, ovlivnění NMDA-zprostředkované excitace a blokování napětím řízených sodných kanálů (Rosenberg, 2007; Martella et al., 2008; Chateauvieux et al., 2010). Poločas VPA v séru je 7-16 hodin, efektivní koncentrace se pohybuje v rozmezí 0,5 – 1,0 mM. Metabolizace probíhá především v játrech a ve formě glukuronátu je vylučována močí. Vedlejší účinky terapie jsou velmi mírné (závratě, útlum, třes), gastrointestinální toxicita a další (polycystická ovária, encefalopatie) (Wood et al., 2005; Gerstner et al., 2008). Pouze ve výjimečných případech u pacientů s hemoragickou pankreatitidou, koagulopatií nebo myelosupresí mohou nastat vážné komplikace (selhání jater). VPA má navíc teratogenní účinky (defekty neurální trubice, kraniofaciální, kardiovaskulární a skeletální defekty) (Nau et al., 1991; Gurvich et al., 2005).

Protinádorový účinek kyseliny valproové

Již dlouhou dobu je známo, že transformované buňky vykazují aberantní expresní profil. Existuje několik způsobů, které dokáží tyto změny alespoň částečně farmakologicky korigovat. Jeden z nich je podávání inhibitorů histondeacetylas (HDACi), mezi které patří i VPA. U VPA byla v poslední dekádě popsána protinádorová aktivita v *in vitro* a *in vivo* experimentech. Inhibiční účinek na proliferaci gliomových buněk byl poprvé popsán již dříve v práci publikované Martinem a Reganem (Martin a Regan, 1991). VPA i další HDACi byly identifikovány jako látky účinně potlačující růst a podporující diferenciaci nádorových buněk. Účinnost VPA byla dále popsaná *in vitro* u neuroblastomů (Cinatl et al., 1997), leukémií (Kawagoe et al., 2002; Tang et al., 2004), meduloblastomů (Li et al., 2005), nádorů prsu (Olsen et al., 2004; Chavez-Blanco et al., 2006) a prostaty (Iacopino et al., 2008). Tyto informace byly dále potvrzeny i v preklinických studiích (Bolden et al., 2006; Carey a La Thangue, 2006; Marchion, Munster, 2007; Chateauvieux et al., 2010).

Přesný mechanismus účinku VPA na nádorové buňky není zcela znám. Možných vysvětlení je více a zahrnují: inhibici HDAC skupin I a IIa (HDAC1 a HDAC3) (Gottlicher et al., 2001; Phiel et al., 2001) a aktivaci jejich proteasomální degradace (Kramer et al., 2003), ovlivnění Wnt signalizace, inhibici proteinkinasy C (Blaheta a Cinatl, 2002) a demethylaci DNA (Detich et al., 2003; Carew et al., 2008). Dále byl popsán také antiangiogenní účinek (Michaelis et al., 2004; Michaelis et al., 2006) a vliv na sestřih, transport a integritu mRNA nehistonových proteinů, ovlivnění translace, aktivity a stability syntetizovaného proteinu a jeho interakcí s ostatními buněčnými složkami (Spange et al., 2009). Navození hyperacetylace histonů H3 a H4 a dalších proteinů v důsledku inhibice HDAC se změnou expresního profilu buněk je pravděpodobně nosným mechanismem protinádorového působení VPA (Phiel et al., 2001).

2.2.5 CHEMOTERAPIE KOMBINACÍ INHIBITORŮ HISTONDEACETYLAS A KONVENČNÍCH CYTOSTATIK

Většina chemoterapeutik používaných v protinádorové terapii nemá v tolerovatelných dávkách schopnost eliminace nádorových buněk, a navíc se u monoterapie zpravidla záhy vyvíjí chemorezistence. Z tohoto důvodu bývá při terapii podávaná kombinace více cytostatik a součástí protinádorové terapie je i podpůrná terapie, což zvyšuje efektivitu léčby.

Situace u HDACi je podobná. I když samy o sobě vykazují protinádorové účinky, jejich efektivita je omezena pouze na některé nádory. Na základě zkušeností se současným použitím různých druhů terapie, resp. chemoterapeutik, HDACi byly a jsou zkoumány v kombinaci s dalšími protinádorovými léčivy nebo ostatními druhy terapie. V současnosti probíhá řada klinických studií, u kterých jsou zkoušeny HDACi ve formě monoterapie nebo častěji v kombinaci s dalšími cytostatiky a/ nebo s chemoterapií (Ma et al., 2009). HDACi jsou kombinovány s léčivy s různým terapeutickým mechanismem účinku (pro přehled viz. Xu et al., 2007b; Hrebackova et al., 2010; Kim a Bae, 2011; Stiborova et al., 2012b), nebo dalšími terapeutickými postupy:

- epigenetická terapeutika (inhibitory methylace DNA – azacytidin, deoxycytidin; inhibitor demethylace histonů – tranylcypromin),
- látky podoporující tvorbu ROS (adaphostin, β-fenylethyl isothiokyanát),
- stabilizátory mikrotubulů (taxany),
- proteasomální inhibitory (monoklonální protilátky – bortezomib, marizomib, carfilzomib),
- látky poškozující DNA (doxorubicin, epirubicin, cisplatina, 5-fluorouracil, ellipticin a další) a radioterapie, viz kapitola Inhibitory histondeacetylas a látky poškozující DNA, str. 54,
- další (antimetabolity – fluorodeoxyuridin a gemcitabin; transkripční regulátory – trans-retinová kyselina, vitamin D3 a jeho analoga, inhibitory NFκB – I Kappa B Alfa; inhibitor Hsp90 – 17-alylaminodemethoxygeldanamycin; trastuzumab – monoklonální protilátka proti Her2/Neu receptoru; inhibitory signálních drah – protilátka proti tyrosinkinase bcr-abl – imatinib).

V Tab. 5 je uveden výběr probíhajících studií testujících VPA v kombinaci s dalšími cytostatiky.

Tabulka 5: Probíhající klinické studie VPA v kombinaci s dalšími cytostatiky nebo léčbou (převzato a upraveno podle www.clinicaltrials.gov)

Léčivo/léčiva v kombinaci s VPA	**Terapeutický účinek**	**Cílové onemocnění**	**Fáze**
Decitabin	Inhibice methylace DNA	Akutní myeloidní leukémie dospělých, lymfom z malých lymfocytů / chronická lymfatická leukémie	1
Imatinib	Inhibice signalizace BCR/Abl	Chronická myeloidní leukémie	2
Karboplatina + azacytidin	Poškození DNA, inhibice methylace DNA	Solidní nádory (ovariální)	1
Karenitecin	Inhibice topoisomerasy	Maligní melanom	1,2
^{131}I	Systémová radioterapie	Thyroidální nádory	2
Decitabin	Inhibice methylace DNA	Nemalobuněčný karcinom plic	1
Azacytidin	Inhibice methylace DNA	Pokročilé nádory různého původu	1
Bevacizumab + temsirolimus	Inhibice VEGF-A, inhibice kinasy mTOR	Pokročilé nádory různého původu	1
Etoposid	Inhibice topoisomerasy	Pokročilé neuroektodermální nádory, nádory s mozkovými metastázemi	1
Decitabin	Inhibice methylace DNA	Burkittův lymfom, difuzní velkobuněčný lymfom, lymfom z plášťových buněk	1
Bevacizumab	Inhibice VEGF-A	Pokročilé nádory různého původu	1
Sorafenib tosylát + sildenafil citrát	Inhibice tyrosin proteinkinas (VEGFR, PDGFR), inhibice P-glykoproteinu	Glioblastom	2
Vinorelbin, cisplatina	Chemoradioterapie, poškození DNA, depolymerizace mikrotubulů	Pokročilý neoperabilní nemalobuněčný karcinom plic	1,2
Cisplatina, karbo-platina, oxaloplatina	Chemoradioterapie, poškození DNA	Nádor hlavy a krku	2
Azacytidin	Inhibice methylace DNA	Akutní myeloidní leukémie, myelodysplastický syndrom	2
Dasatinib, erlotinib, lapatinib, lenalidomide, sorafenib, sunitinib	Inhibice BCR/Abl, tyrosinkinasy EGFR, EGFR+Her2/Neu, anti-angiogenní účinek + apoptosa, inhibice tyrosin proteinkinas (VEGFR, PDGFR)	Solidní nádory	1
Radioterapie, pak bevacizumab	Radiační usmrcení, inhibice VEGF-A	Gliomy vyššího stupně	2
Gemcitabin, beva-cizumab, docetaxel	Inhibice replikace DNA (analog), inhibice VEGF-A, stabilizace mikrotubulů	Různé formy sarkomů	1,2
Cladribin	Inhibice syntézy DNA (purinový analog)	Chronická lymfocytární leukémie	1,2
Radiační terapie + temozolomide	Radiační usmrcení, alkylace DNA	Nádory s mozkovými metastázemi	1
Temsirolimus	Inhibice kinasy mTOR	Nádory CNS, sarkomy, solidní nádory dětského věku	1
Epirubicin, epirubicin + 5-fluorouracil + cyklofosfamid	Inhibice topoisomerasy + interkalace do DNA, inhibice syntézy DNA (pyrimidinový analog), poškození DNA (alkylace)	Pokročilé nádory různého původu	1
Adriamycin, cyclofosfamid, vindesin	Poškození DNA (interkalace, navázání), alkylace DNA, inhibice tubulinové polymerizace	Malobuněčný karcinom plic	2
Hydralazin	Inhibice methylace DNA	Nádory plic	1

2.2.5.1 Inhibitory histondeacetylas a látky poškozující DNA

HDACi jsou schopny potencovat účinek terapie zaměřené na poškození DNA nádorových buněk – chemoterapie a radioterapie. Jak již bylo zmíněno v kapitole Mechanismus působení inibitorů histondeacetylas, jedním z důsledků působení HDACi je navození hyperacetylace histonů. Zvýšenou acetylací je struktura chromatinu ovlivněna ve smyslu rozvolnění jeho struktury. DNA – poškozující chemoterapeutika tak mají pro svou funkci lépe přístupnou molekulu DNA (Kim et al., 2003; Thurn et al., 2011). Tím je chemoterapie a radioterapie efektivnější, a umožňuje účinnou aplikaci chemoterapeutik v nižších dávkách tam, kde je potřeba omezit nežádoucí účinky léčby. Další mechanismus, kterým HDACi zvyšují účinek chemoterapie a radioterapie, je inhibice/oddálení oprav zlomů DNA. Tato hypotéza je podporována zjištěním, že při kombinované terapii HDACi a látkami poškozujícími DNA je v buňce prodloužená přítomnost fosforylované formy histonu H2A – γ-H2AX, který je časným ukazatelem dvouvláknových zlomů DNA (Geng et al., 2006). Ovlivněna je také aktivace proteinkinasy kontrolního bodu buněčného cyklu Chk2 (Chen et al., 2009). HDACi mohou účinek dalších terapeutických přístupů ovlivňovat také prostřednictvím pozměnění genové exprese buněk. A to zejména zásahem do exprese proteinů podílejících se na opravách poškozené DNA a proteinů buněčné signalizace, které rozhodují o přežití buňky nebo spuštění apoptosy (Zhang et al., 2006; Hrebackova et al., 2010; Cipro et al., 2012).

Synergistický efekt HDACi a chemoterapeutik, které poškozují DNA, byl prokázan v mnoha studiích. K preparátům, které jsou schopné HDACi podpořit, patří: interkalační látky, inhibitory topoisomerasy, látky kovalentně se vážící na DNA a inhibitory DNA replikace (např. doxorubicin, epirubicin, cisplatina, ellipticin a další). Kromě chemoterapie HDACi potencují i účinek radioterapie. (Deb et al., 2001; Munshi et al., 2005; Karagiannis et al., 2005, 2006, 2007; Friedmann et al., 2006; Munshi et al., 2006; Schuchmann et al., 2006; Zhang et al., 2006; Catalano et al., 2007; Chen et al., 2007; Harikrishnan et al., 2008; Chen et al., 2009; Munster et al., 2009; Mutze et al., 2010; Nadmar et al., 2010; Poljakova et al., 2011; Shoji et al., 2012; Wang et al., 2012; Xie et al., 2012; Zhang et al., 2012; Eriksson et al., 2013).

3. CÍLE PRÁCE

Tato práce je zaměřena na studium účinku kyseliny valproové na neuroblastomové buněčné linie *in vitro*. Jejím cílem je ověřit a specifikovat působení VPA za podmínek normoxie i hypoxie a přispět k objasnění mechanismu takto indukované buněčné smrti. Věnuje se rovněž možnostem využití VPA pro potenciaci účinku konvenční chemoterapie – DNA poškozujících preparátů – při léčbě nádorových onemocnění. Výsledky a jejich diskuse jsou podrobněji zpracovány v přiložených publikacích.

4. MATERIÁL A METODIKA

4.1 POUŽITÉ CHEMIKÁLIE

Abcam (Velká Británie): protilátky: anti-BID (králičí monoklonální), anti-CYP1B1 (králičí polyklonální), anti-cytochrom b5 (králi.čí polyklonální), anti-COX-1 (myší monoklonální), anti- LPO (králičí polyklonální)

AbD Serotec (Velká Británie): anti-CYP3A4 protilátka (králičí polyklonální)

Bio-Rad (USA): Nitrocellulose transfer membrane, standardy pro SDS-elektroforesu „Precision Plus Protein Prestained Standards“, odtučněné sušené mléko, protilátky:
anti-králičí HRP (polyklonální kozí), anti-myší HRP (polyklonální kozí)

Cell Signaling (USA): anti- Bax protilátka (králičí polyklonální)

EBEWE Pharma (Rakousko): cisplatina (CDDP, 0,5mg/ml)

Fluka (Švýcarsko): akrylamid, dodecylsulfát sodný (SDS), 2-merkaptoethanol, N,N-methylen-bis-akrylamid (BIS), methanol, ethylacetát

GIBCO^{TM} (Velká Británie): sodný fosfátový pufr s obsahem NaCl - izotonický roztok (Phosphate Buffer with Salt, PBS), trypsin

Invitrogen (USA): TRIzol

Millipore (USA): protilátky: anti-CYP 1A1 (králičí polyklonální), anti-Bcl2 (myší monoklonální), anti-GAPDH (myší monoklonální), anti-HIF-1α (myší monoklonální), anti-acetylované histony H3 a H4 (králičí polyklonální)

PAA laboratories (Rakousko): Iscove's modified Dulbecco's medium (IMDM), fetální hovězí sérum, streptomycin/penicilin

R&D Systems, Inc. (USA): Z-IETD-FMK (inhibitor kaspasy 8)

Serva (Německo): N, N´- methylen-bis-akrylamid (BIS), Triton X-100, N,N,N´,N´-tetramethyl-ethylendiamin (TEMED), Coomassie Briliant Blue R-250

Sigma Aldrich(USA): valproát sodný, trichostatin A, 5-bromo-4-chloro-3 indolylfosfát/nitro-blue tetrazolium (BCIP/NBT), hydrogenuhličitan sodný, dimethylsulfoxid (DMSO), dodecylsulfát sodný (SDS), L-glutamin, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), 2-merkaptoethanol, standardy pro SDS-elektroforesu „Wide range“, trypanová modř, anti-aktin protilátka (myší monoklonální)

4.2 KULTIVACE BUNĚČNÝCH LINIÍ

4.2.1 BUNĚČNÉ LINIE

Nádorové buněčné linie UKF-NB-3 a UKF-NB-4 jsou odvozeny z metastáz v kostní dřeni neuroblastomu vysokého rizika s MYCN amplifikací; původní mateřské linie a linie rezistentní na cisplatinu byly darem Prof. Jindřicha Činátla, DrSc. z J. W. Göethe Universitate Frankfurt am Main. Linie UKF-NB-4 rezistentní na ellipticin byla připravena RNDr. Jitkou Poljakovou, Ph.D. na pracovišti KDHO UK 2.LF Motol, Praha (Poljaková et al., 2009). IMR-32 – neuroblastomová linie vysokého rizika s MYCN amplifikací – zakoupena u LGC Promochem, Wesel, Německo. SK-N-AS – neuroblastomová linie odvozená z metastáz v kostní dřeni, normální diploidní MYCN status, zakoupena u Evropské kolekce buněčných kultur (ECACC, Velká Británie). Linie SK-N-AS rezistentní na cisplatinu byla připravena v naší laboratoři inkubací původní mateřské linie s postupně se zvyšující koncentrací cisplatiny (konečná koncentrace cisplatiny byla 1μg/ml media).

4.2.2 KULTIVACE

Buněčné kultury byly pěstovány v Iscove's modified Dulbecco's mediu (IMDM) doplněného 0,3% w/w hydrogenuhličitanem sodným, 4mM L-glutaminem, 10% fetálním hovězím sérem a 100 U/ml penicilinem a 100 μg/ml streptomycinem při 37 °C, 5% CO_2 a 95% vlhkosti pro „normoxii". Buňky v „hypoxii" byly kultivovány za pomocí hypoxické komory (firma Billups-Rothenberg (USA)) v atmosféře obsahující 1% O_2, 5% CO_2, a 94% N_2 při 37 °C. Buňky byly z povrchu kultivačních lahviček odstraňovány trypsinací po předchozím promytí PBS (pH 7,2) a resuspendovány obvykle v 10 ml media do 25 cm^2 lahviček. Pasážování buněk probíhalo podle potřeby.

4.2.3 VYSTAVENÍ BUNĚK ELLIPTICINU ZA ÚČELEM ANALÝZY DNA ADUKTŮ

Přibližně 1 x 10^5 buněk/ml bylo inkubováno 24 hodin před přidáním ellipticinu v inkubátoru (Jouan TGO 150) při 37°C, 5% CO_2 a 95% vlhkosti v 75 cm^2 kultivačních lahvičkách o celkovém objemu 20 ml IMDM media. Po 24 hodinách byl přidán ellipticin o výsledné koncentraci 0,01; 0,1; 1,0; 2,0; 5,0 a 10,0 μM (koncentrace organického rozpouštědla ≤1 obj. %) a buňky byly pěstovány jak za podmínek „hypoxie", tak i „normoxie". Pro sledování účinnosti kombinace ellipticinu s VPA nebo TSA byly buňky preinkubovány po dobu 24 hodin s VPA nebo TSA a poté byl přidán ellipticin. Po 48 hodinách byly buňky

mechanicky sejmuty z povrchu kultivačních lahviček, centrifugovány při 3000 rpm (Megafuge 1.0R, Heraeus Instruments) 5 minut. Dále byly promyty 10 ml PBS a centrifugovány za stejných podmínek, pelety byly rozsuspendovány v 1 ml PBS a centrifugovány při 3000 rpm 5 minut, pelety byly uchovávány při -20 °C.

4.3 TESTOVÁNÍ VIABILITY BUNĚK

Viabilita buněk byla testována pomocí 3-(4,5-dimethylthiazol-2-yl)-2,5 difenyltetrazolium bromidového (MTT) testu, ve kterém živé buňky metabolizují žlutý MTT na modrý formazan. Do 1. sloupce 96 jamkové mikrotitrační destičky bylo pipetováno 100 µl IMDM media. Do 2. až 12. sloupce bylo pipetováno 50 µl media. Do 3. sloupce bylo přidáno 50 µl media s určitou koncentrací histondeacatylasových inhibitorů a/nebo CDDP. Výsledná koncentrace DMSO v mediu nepřesáhla 0,02 %. Výsledná koncentrace histondeacatylasového inhibitoru a/nebo cytostatika ve třetím sloupci byla poloviční. Ředěním tzv. dvojkovou řadou byl histondeacatylasový inhibitor a/nebo CDDP pipetováno do všech dalších sloupců destičky. Dále bylo do 2. až 12. sloupce pipetováno 50 µl buněk o koncentraci přibližně 4 x 10^5/ml. Destička byla inkubována 72 hodin v inkubátoru (Jouan TGO 150) při 37 °C, 5% CO_2 a 95% vlhkosti za podmínek „normoxie“, v „hypoxii“ byly buňky inkubovány v prostředí 1% O_2, 5% CO_2, a 94% N_2. Poslední 4 hodiny byla destička inkubována s 50 µl MTT (2 mg/ml). Nakonec bylo přidáno 100 µl 50% (v/v) *N*,*N*-dimethylformamidu, 10% SDS, pH 4,7 bylo upraveno kyselinou octovou. Po rozpuštění sraženiny formazanu byla absorbance měřena čtečkou mikrodestiček VERSA max (Molecular Device Inc., USA) při vlnové délce 570 nm a vyhodnocena softwarovým programem SOFT max PRO. Vynesením naměřených hodnot do grafu byly určeny IC_{50} pro daná cytostatika.

4.4 ISOLACE DNA, ^{32}P-POSTLABELING DNA ADUKTŮ A NÁSLEDNÁ HPLC ANALÝZA

DNA byla isolována fenol-chloroformovou extrakcí (Frei et al., 2002; Poljaková et al., 2009). Po hydrolyse dochází za použití P1 nukleasy k obohacení aduktů a následnému ^{32}P-postlabelingu. Adukty byly autoradiograficky detekovány a poté kvantifikovány (Frei et al, 2002; Poljaková et al., 2011).

4.5 EXPRESE PROTEINŮ V BUNĚČNÝCH LINIÍCH

Buňky, připravené různými postupy, byly mechanicky sejmuty z povrchu kultivačních lahviček, centrifugovány při 3000 rpm 5 minut, potom 2x promyty 10 ml PBS a centrifugovány za stejných podmínek. Pelety byly po promytí zamraženy a uchovávány při teplotě -80 °C. Pro stanovení acetylovaných histonů musely být buňky zpracovány odlišným způsobem – tzv. kyselou extrakcí podle instrukcí v návodu při užití protilátek proti acetylovaným histonům H3 a H4. Po promytí PBS byla peleta resuspendována v lyzačním pufru (10mM HEPES, pH 7,9; 1,5mM $MgCl_2$; 10mM KCl; 0,5mM DTT; 1,5mM PMSF), poté byla přidána kyselina chlorovodíková do finální koncentrace 0,2 M. Po 30 minutové inkubaci na ledu byly vzorky centrifugovány při 11000 g, 4 °C po dobu 10 minut. Supernatant byl dialyzován 2x po dobu 1 hodiny proti 0,1M kyselině octové a 3x proti H_2O po dobu 1 hodiny, 3 hodin a nakonec přes noc.

Pro stanovení proteinů byly pelety rozsuspendovány v RIPA pufru (ve stejném objemu jako peleta), který obsahoval proteasový inhibitor (Complete, Roche). Po hodinové inkubaci na ledu byly vzorky centrifugovány po dobu 20 minut na 15000 g při 4 °C. V supernatantu byly stanoveny proteiny za použití DC protein assay (Bio-Rad) s hovězím sérovým albuminem jako standardem. Elektroforéza vzorků v potřebné koncentraci proteinů probíhala po smíchání se vzorkovým pufrem v přítomnosti SDS na polyakrylamidovém gelu při použití 10 % separačního (pro stanovení acetylace histonů byl použit 16 %) a 6 % zaostřovacího gelu. Pro elektroforézu byla použita aparatura Mini-PROTEAN® (Bio-Rad). Elektromigrace probíhala ve vertikálním uspořádání pH=8,8 a konstantním proudu 40 mA. Přenos proteinů na PVDF membránu (Millipore) probíhal za použití aparatury Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) při konstantním proudu 350 mA po dobu 80 minut. Membrána s přenesenými proteiny byla blokována v blokovacím roztoku (5% sušené mléko v PBS/Tween) po dobu 1 hodiny. Inkubace se specifickou primární protilátkou probíhala přes noc při 4 °C za stálého míchání. Po promytí roztokem PBS/Tween (5 x 2 minuty) byla membrána vložena do roztoku sekundární protilátky (ředění 1:3000, kozí IgG proti králičí IgG - s navázanou peroxidasou, ev. kozí IgG proti myší IgG s navázanou peroxidasou, Bio-Rad). Po promytí byl komplex protilátky a antigenu vizualizován chemiluminiscenční detekcí dle instrukcí výrobce (Immun-Star HRP Substrate, Bio-Rad). Filmy (MEDIX XBU, Foma, ČR) byly skenovány a vyhodnoceny pomocí systému ElfoMan 2.0 (Ing. Semecký, ČR).

4.6 STANOVENÍ APOPTOSY

4.6.1 STANOVENÍ APOPTOSY A VIABILITY BUNĚK POMOCÍ ZNAČENÍ ANNEXIN V/PROPIDIUM JODID

Annexin V byl použit pro měření apoptosy a také viability buněk. Stanovení apoptotických buněk bylo prováděno za pomoci Annexin V-FITC Apoptosis Detection kitu podle instrukcí výrobce (Biovision, USA). Buňky byly promyty v PBS a resuspendovány v „binding“ pufru po inkubaci s různými látkami (VPA, TSA, cisplatina atd.) za normoxie a/nebo hypoxie. Buňky byly inkubovány po dobu 10 minut s annexinem V a propidium jodidem při laboratorní teplotě a poté analyzovány průtokovým cytometrem (FACSCalibur, BD, USA). Získaná data byla vyhodnocena pomocí metody popsané Bossy-Wetzelem (Bossy-Wetzel a Green, 2000).

4.6.2 STANOVENÍ APOPTOSY METODOU TUNEL

ApoDirect DNA Fragmentation Assay kit (Biovision, USA) byl použit pro měření apoptosy dle instrukcí výrobce. Buňky byly fixovány 1% paraformaldehydem a poté inkubovány s terminální deoxynucleotidyltransferasou a FITC-dUTP 60 minut př 37 °C a „obarveny“ propidium jodidem. Buňky byly poté analyzovány průtokovou cytometrií (FACSCalibur, BD, USA).

4.7 ANALÝZA BUNĚČNÉHO CYKLU

Pro analýzu buněčného cyklu byly buňky, ošetřené různými způsoby, trypsinovány a fixovány 70% ledovým ethanolem, promyty v PBS a resuspendovány v 1 ml PBS obsahujícím 1mg/ml RNAsy a 50 mg/ml propidium jodidu. Inkubace probíhala ve tmě po dobu 30 minut při laboratorní teplotě a analýza byla provedena průtokovou cytometrií na přístroji FACSCalibur (BD, USA) a zastoupení jednotlivých fází buněčného cyklu bylo stanoveno za využití softwaru ModFit LT (Verity Software House).

4.8 STANOVENÍ AKTIVITY KASPAS

Aktivita kaspasy 8 byla stanovena za použití Caspase-8 assay kit (Biovision) podle návodu výrobce. Vzorky byly připraveny shodným způsobem jako při stanovení exprese proteinů až po stanovení proteinů. Definované množství proteinu (200 μg) bylo přidáno do reakčního pufru, který obsahoval IETD-pNA kolorimetrický substrát, a inkubováno po dobu

2 hodin při 37 °C. Hydrolyzovaný *p*-nitroanilid (pNA) byl detekován pomocí VersaMax plate reader (Molecular Device Inc., USA) při vlnové délce 405 nm.

4.9 REAL-TIME PCR

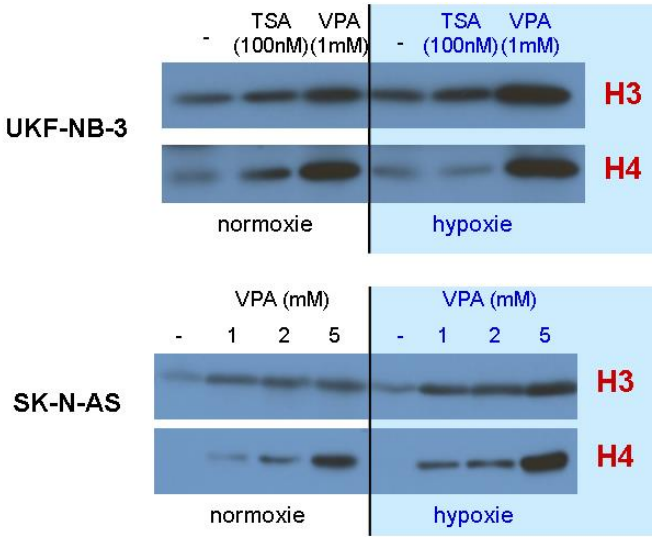
RNA byla extrahována z buněčných linií za použití TRIzolu® (Invitrogen, USA). Kvalita izolované RNA byla ověřena elektroforézou na agarozovém gelu a kvantita RNA byla měřena pomocí BioMate 3 UV-Vis Spectrophotometer (Thermo Scientific, USA). Komplementární DNA byla syntetizována z 500 ng RNA použitím tzv. „random hexamerů" a MultiScribe™ reverzní transkriptázy (Applied Biosystems, USA). RT-PCR byla provedena pro VEGF, anhydrázu kys. uhličité (CA-9) a β-2-mikroglobulin (B2M) za využití primerů a sondy připravených v Generi Biotech (ČR). B2M byl použit jako referenční gen. Relativní exprese a statistická významnost byly určeny za použití REST-MCS software (DrMichaelPfaffl, Německo) použitím techniky popsané Pfafflem (Pfaffl et al., 2002).

5. VÝSLEDKY

5.1 PŮSOBENÍ KYSELINY VALPROOVÉ NA NEUROBLASTOMOVÉ LINIE

5.1.1 VPA NAVOZUJE HYPERACETYLACI HISTONŮ A V HYPOXII OVLIVŇUJE AKTIVITU HIF-1

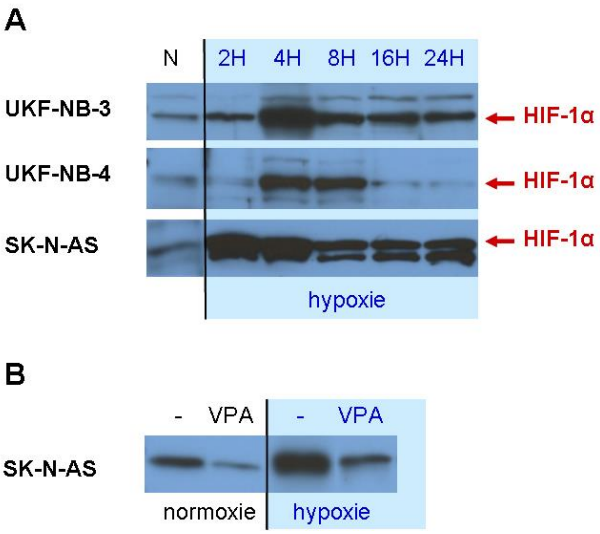
Za jeden z hlavních mechanismů působení HDACi je považována acetylace histonů. Pro průkaz vlivu histondeacetylasových inhibitorů VPA a TSA na lidské neuroblastomové buněčné linie UKF-NB-3 a SK-N-AS byla použita metoda Western blot. Buňky byly inkubovány po dobu 24 hodin v normoxii nebo hypoxii (<1 % O_2). Při použitých koncentracích, tj. 100nM pro TSA a 1, 2 a 5mM pro VPA, byla acetylace histonů H3 a H4 v porovnaní s kontrolou výrazně zvýšena. V hypoxii byl v porovnání s normoxií stupeň acetylace ještě vyšší a narůstal se zvyšující se koncentrací VPA, viz Obr. 11.



Obrázek 11: Acetylace histonů po působení TSA a VPA po dobu 24 hodin v podmínkách normoxie nebo hypoxie u linií UKF-NB-3 a SK-N-AS.

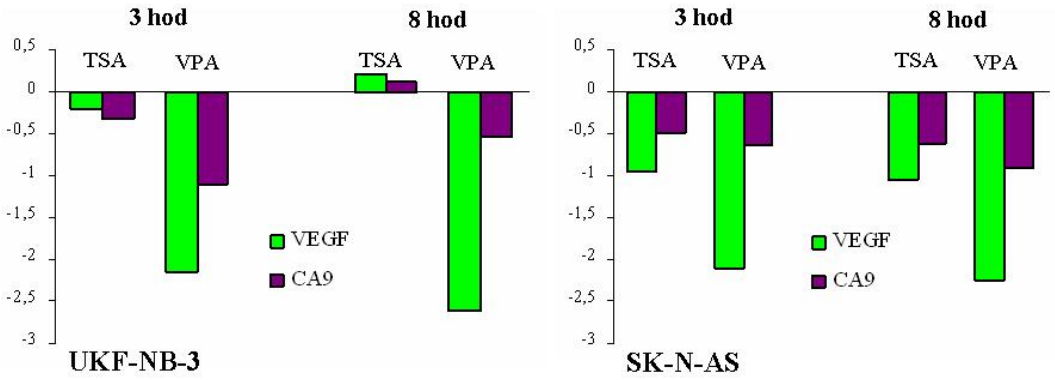
Změna acetylační a deacetylační aktivity v buňce ovlivňuje kromě uspořádání chromatinu prostřednictvím modifikace histonů i její další vlastnosti. Jedná se zejména o narušení adaptace buňky k prostředí s omezenou dostupností kyslíku. Mechanismem je snížení aktivity transkripčního faktoru HIF-1 zvýšeným odbouráváním podjednotky HIF-1α. Její koncentrace je za normoxických podmínek nízká a přísně regulována. V hypoxii ovšem narůstá již od okamžiku omezení dostupnosti kyslíku a v závislosti na typu buňky či použité

buněčné linii kulminuje přibližně po 4 hodinách nebo i dříve, Obr 12A. VPA množství HIF-1α v buňkách snižoval v normoxických i hypoxických podmínkách, viz Obr. 12B.



Obrázek 12: Vliv hypoxie na hladinu HIF-1α u neuroblastomových buněčných linií v závislosti na trvání hypoxie (A). Ovlivnění hladiny HIF-1α po 4 hodinové inkubaci s 1mM VPA (B).

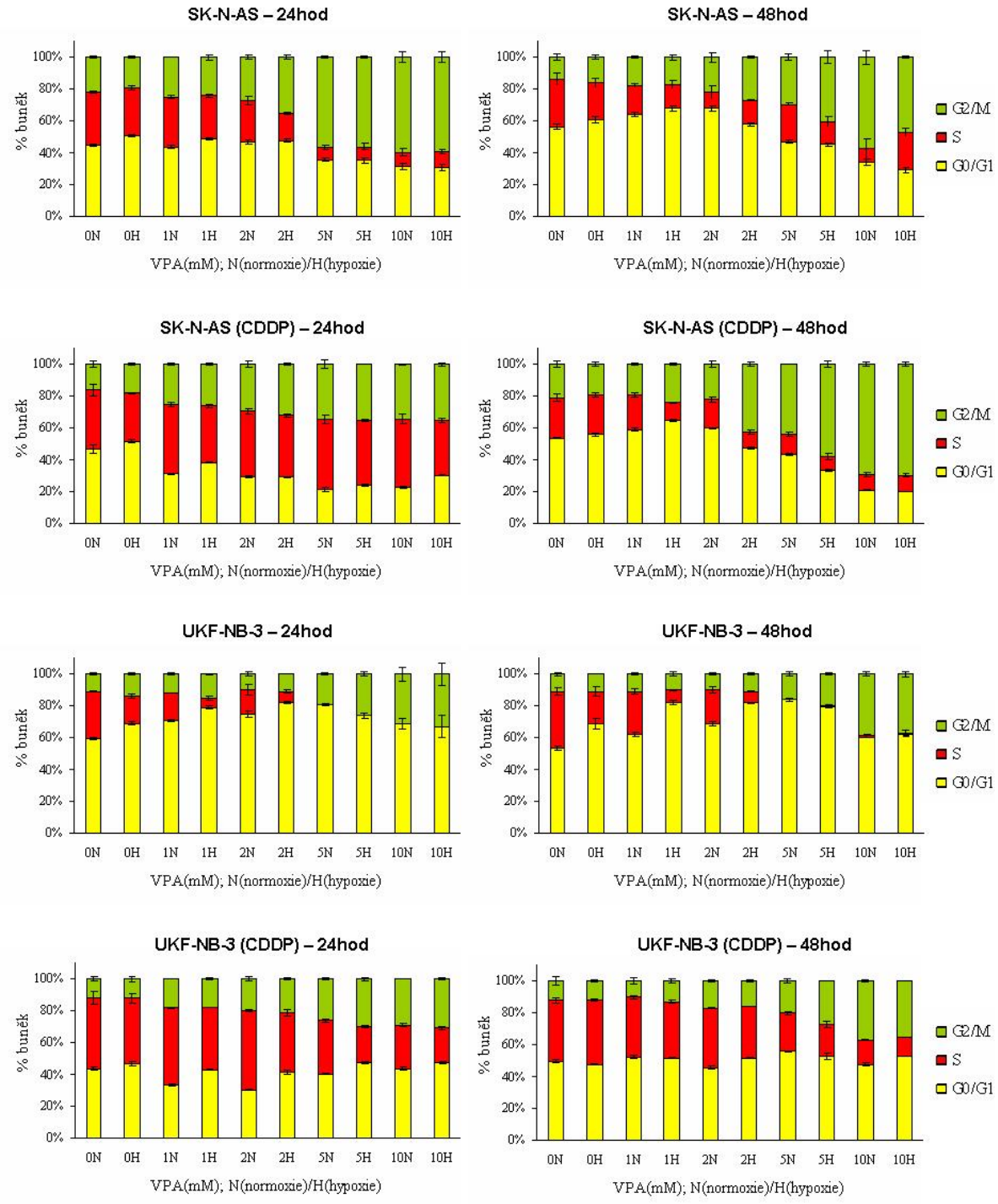
K potvrzení snížené aktivity HIF-1 působením VPA a TSA na úrovni mRNA byla využita real-time PCR cílových genů HIF-1: VEGF a karboanhydrasy 9, viz Obr. 13. Buňky UKF-NB-3 a SK-N-AS byly preinkubovány s 100nM TSA nebo 2mM VPA 24 hodin a poté umístěny do hypoxie na dobu 3 a 8 hodin. Exprese obou HIF-1 cílových genů po inkubaci s VPA významně poklesla u obou testovaných linií. U VEGF byla snížená zhruba dvojnásobně a u karboanhydrasy 9 téměř pětinásobně u linie UKF-NB-3 i SK-N-AS po 3 hodinách v hypoxii.



Obrázek 13: Vliv VPA a TSA na množství mRNA HIF-1 cílových genů VEGF a karboanhydrasy 9.

5.1.2 VPA ovlivňuje buněčný cyklus neuroblastomových buněk

Ovlivnění buněčného cyklu VPA v normoxii i v hypoxii (1% O_2) bylo hodnoceno průtokovou cytometrií u neuroblastomových linií UKF-NB-3, SK-N-AS a od nich odvozených rezistentních linií – UKF-NB-3(CDDP) a SK-N-AS(CDDP). Použité koncentrace VPA byly od 1 do 10 mM a doba působení 24 a 48 hodin. Při nižších koncentracích VPA (do 2 mM) byl pozorován mírný nárůst počtu buněk ve fázi G0/G1 na úkor buněk v S fázi. Trend byl patrný zejména u 48 hodinové inkubace jak v normoxii, tak v hypoxii. S rostoucí koncentrací VPA se v normoxii i hypoxii u všech linií postupně zvyšoval počet buněk ve fázi G2/M až několikanásobně a dosahoval hodnot až kolem 70 % u linie SK-N-AS(CDDP), viz Obr. 14, str 65.

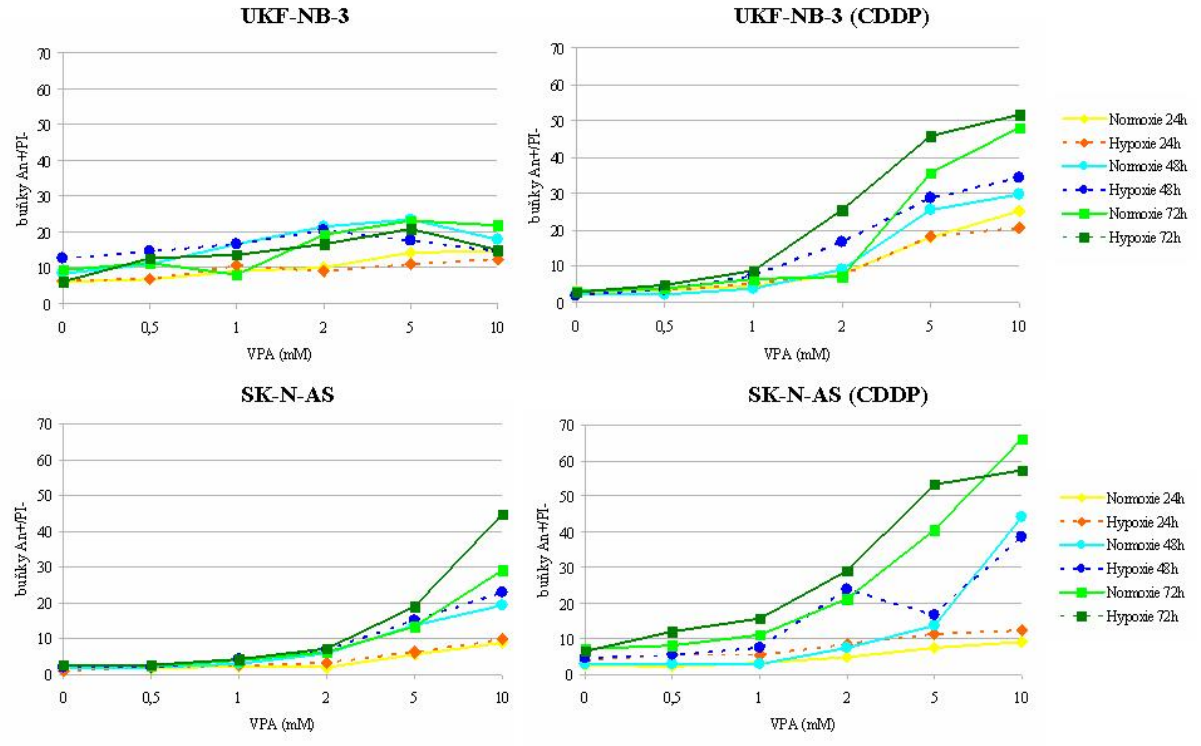


Obrázek 14: Vliv VPA, času a koncentrace kyslíku na buněčný cyklus vybraných neuroblastomových linií.

5.1.3 VPA NAVOZUJE APOPTOSU NEUROBLASTOMOVÝCH BUNĚK

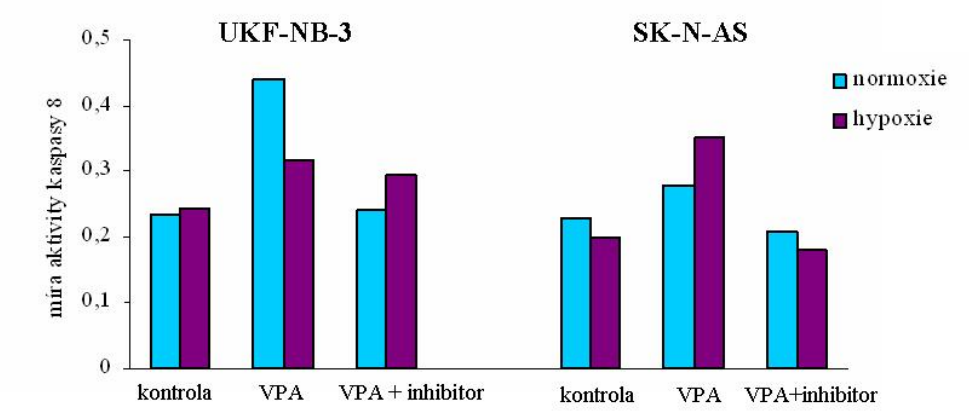
Efekt VPA na apoptosu neuroblastomových buněk byl zkoumán v závislosti na dávce, inkubační době a přístupu ke kyslíku (normoxie, hypoxie (<1 % O_2)). Buňky byly kultivovány

24 hodin v normoxii, poté byl do média přidán VPA, a misky byly umístěny do hypoxické komůrky nebo kultivovány dále v normoxii. Použitá koncentrace VPA byla od 0,5 do 10 mM a doba působení 24 až 72 hodin. Apoptosa byla stanovena cytometricky pomocí značeného annexinu V a propidium jodidu, viz Obr. 15. Hypoxií indukovanou rezistenci k apoptose po působení VPA jsme nepozorovali. Počet apoptotických buněk v hypoxii byl naopak mírně vyšší než v normoxii, rozdíl ovšem nebyl statisticky významný.



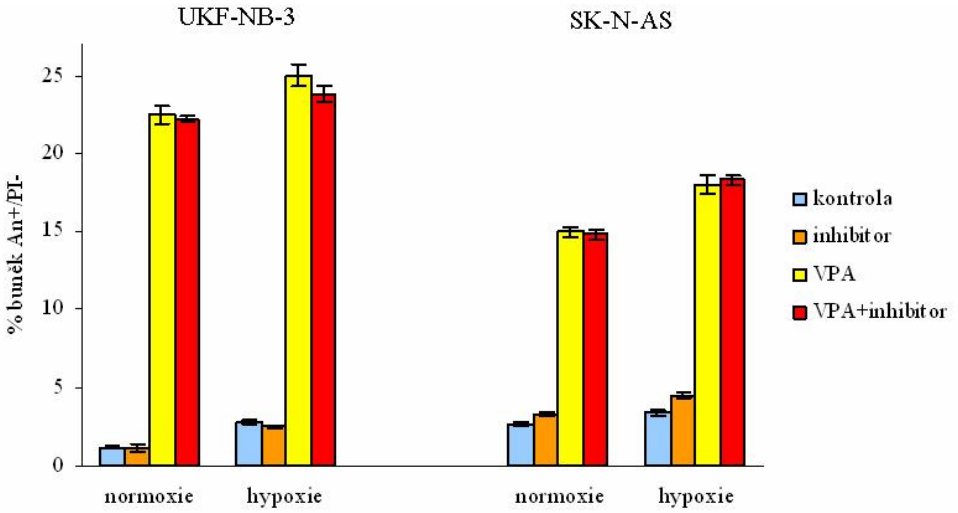
Obrázek 15: Vliv VPA na časnou apoptosu měřený jako počet An^+/PI^- buněk v závislosti na koncentraci VPA, čase a koncentrace kyslíku u neuroblastomových linií.

Pro určení apoptotické dráhy spuštěné působením VPA byla měřena aktivita kaspasy 8, která se podílí na iniciaci apoptotického signálu vnější drahou. Inkubace v normoxii nebo hypoxii byla 48 hodin. U buněčných linií SK-N-AS a UKF-NB-3 VPA zvyšoval aktivitu kaspasy 8, viz Obr. 16, str. 67. Přidáním inhibitoru kaspasy 8 (Z-IETD-FMK) 15 minut před VPA se hodnoty aktivity vrátily téměř na úroveň kontrolních buněk. Pro ověření účinku zvýšené aktivity kaspasy 8 na viabilitu buněk byla cytometricky pomocí značeného annexinu V a propidium jodidu měřena apoptosa.

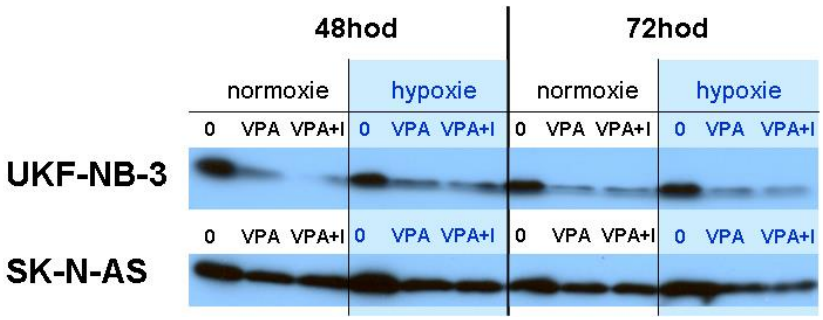


Obrázek 16: Míra aktivity kaspasy 8 po inkubaci s VPA (2mM pro UKF-NB-3 a 5mM pro SK-N-AS) a s inhibitorem kaspasy 8 (Z-IETD-FMK) po 48 hodinách v normoxii a hypoxii.

Na množství apoptotických buněk po působení VPA u linií UKF-NB-3 a SK-N-AS neměl inhibitor kaspasy 8 vliv, viz Obr. 17. To naznačuje spíše usmrcení buněk vnitřní apoptotickou drahou. Její zapojení bylo potvrzeno štěpením proapoptotického proteinu Bid po inkubaci s VPA v přítomnosti inhibitoru kaspasy 8, na kterém se tak pravděpodobně podílí efektorové kaspasy (kaspasa 3) aktivované právě vnitřní drahou, viz Obr. 18, str. 68.

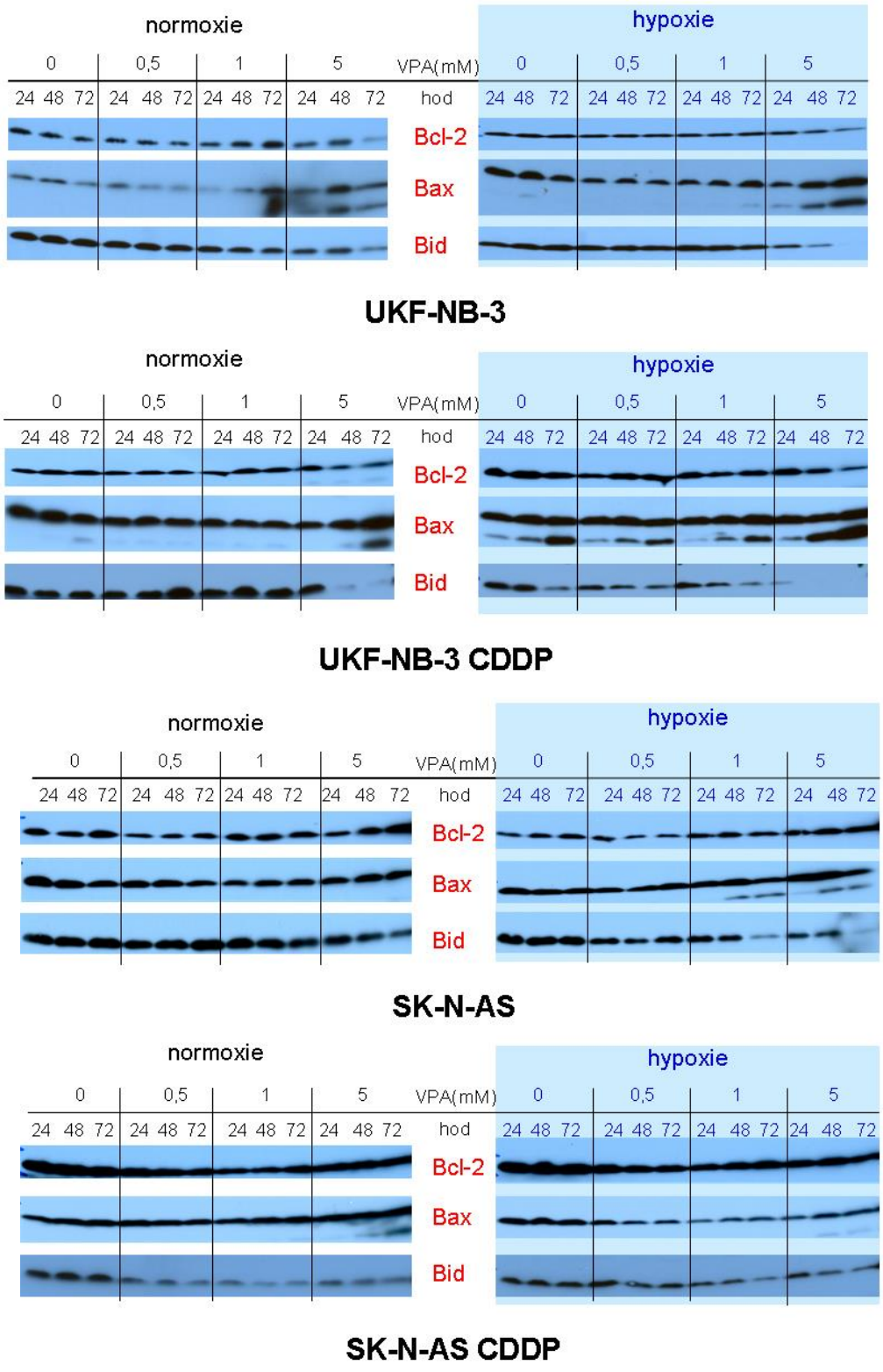


Obrázek 17: Apoptosa u linií UKF-NB-3 a SK-N-AS po inkubaci s VPA a ihibitorem kaspasy 8. Buňky byly 15 min. preinkubovány s 2μM inhibitorem, po přidání VPA (5mM) byly inkubovány 48 hodin v normoxii nebo hypoxii.



Obrázek 18: Exprese Bid po působení VPA a VPA s inhibitorem kaspasy 8 (I). Koncentrace VPA byla 5mM pro UKF-NB-3 a 10mM pro SK-N-AS. Doba inkubace: 48 a 72 hodin.

Na regulaci signalizace vnitřní apoptotické dráhy se podílí řada proteinů, mezi které patří Bcl-2, Bcl-X_L, Bid, Bax, Bak, Bad, Bim a další. Pomocí Western blotu byly sledovány změny v expresi Bcl-2, Bid, Bax u linií UKF-NB-3, UKF-NB-3(CDDP), SK-N-AS a SK-N-AS(CDDP). Exprese antiapoptotického proteinu Bcl-2 se při nižší koncentraci VPA neměnila a její změny – mírný nárůst nebo pokles – byly pozorovány až u koncentrace 5 mM. Změny hladin proapoptotických proteinů v závislosti na době inkubace byly detekovány již při nízkých hladinách VPA (od 0,5 mM) u linií UKF-NB-3(CDDP) a SK-N-AS(CDDP), kde byl pozorován pokles koncentrace Bid v normoxii, související zřejmě s jeho aktivací proteolytickým štěpením. Při vyšších koncentracích VPA byl tento účinek pozorován u všech linií a s výjimkou linie SK-N-AS(CDDP) byl v hypoxii výraznější než v normoxii. Hladina exprese Bax (21 kDa) byla rostoucí koncentrací VPA ovlivněna pouze mírně až při 5 mM. Alternativní varianta Bax (19 kDa) s vyšší afinitou k mitochondriální membráně byla detekována při vysokých koncentracích VPA (5 mM) v normoxii. V hypoxii již byla ovšem přítomna i při nižších koncentracích VPA a u linie UKF-NB-3 byla dokonce indukována samotnou hypoxií, viz Obr. 19, str. 69.

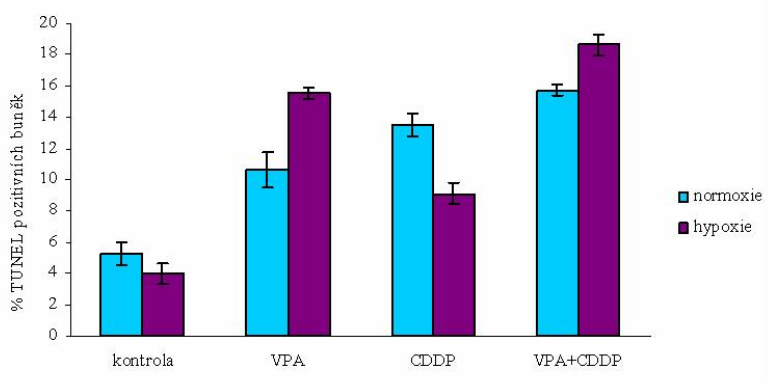


Obrázek 19: Změny v hladinách vybraných antiapoptotických a proapoptotických proteinů u linií UKF-NB-3, SK-N-AS, UKF-NB-3(CDDP) a SK-N-AS(CDDP) v závislosti na koncentraci VPA, době inkubace a koncentraci kyslíku.

5.2 PŮSOBENÍ KOMBINACE KYSELINY VALPROOVÉ A VYBRANÝCH CYTOSTATIK NA NEUROBLASTOMOVÉ LINIE

5.2.1 NEUROBLASTOMOVÉ LINIE JSOU CITLIVÉ NA VPA, CISPLATINU A JEJICH KOMBINACI V NORMOXII I HYPOXII

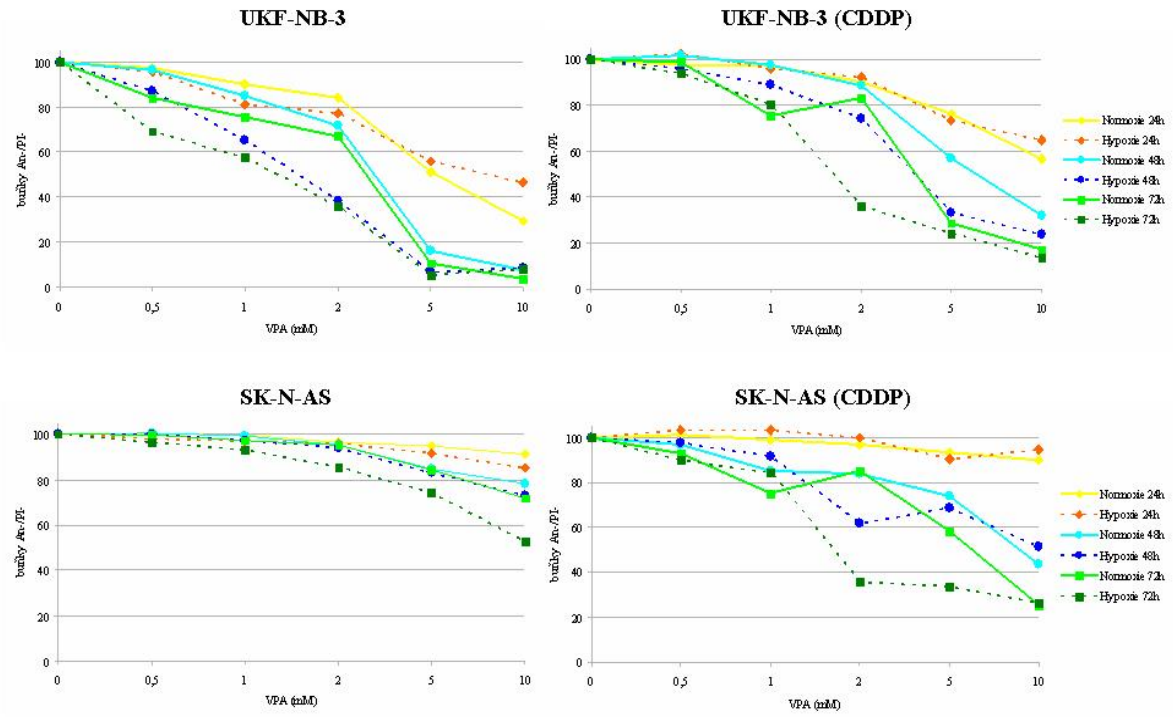
Citlivost na VPA, cisplatinu a jejich kombinaci byla vyhodnocena u neuroblastomových buněčných linií IMR-32, UKF-NB-3, UKF-NB-4 a SK-N-AS. Dále u linií rezistentních k cisplatině, které jsou odvozeny z mateřských linií, pěstovaných v médiu s postupně rostoucí koncentrací cisplatiny – UKF-NB-3(CDDP), UKF-NB-4(CDDP) a SK-N-AS (CDDP). Ke stanovení byl využit MTT test po 72 hodinové inkubaci (viz kapitola Testování viability buněk, str. 58). Hodnoty koncentrace potřebné pro usmrcení poloviny buněk (IC_{50}) jsou uvedeny v Tab. 6, str. 72. Účinnost kombinace VPA a cisplatiny byla u linie UKF-NB-3 ověřena průtokovou cytometrií využitím metody TUNEL, detekující pozdní fázi apoptosy na základě průkazu fragmentace DNA, viz Obr. 20.



Obrázek 20: Účinek kombinace VPA a cisplatiny na linii UKF-NB-3 v normoxii a hypoxii vyjádřený detekcí % apoptotických (Tunel pozitivních) buněk. Koncentrace VPA a cisplatiny byly 1mM a 1 μM. Doba inkuace: 24 hodin.

Testované linie byly na VPA, cisplatinu a jejich kombinaci citlivé. Hodnota IC_{50} pro VPA a cisplatinu se v normoxii pohybovala v závislosti na linii v rozmezí od 0,76 mM VPA u linie IMR-32 a 0,99 μM cisplatiny u UKF-NB-3, po 13,09 mM VPA u linie SK-N-AS a 13,92 μM cisplatiny pro linii UKF-NB-4(CDDP). V hypoxii byly hodnoty IC_{50} podobně variabilní: pro VPA od 1,19 mM u IMR-32 po 10,45 mM u SK-N-AS, pro cisplatinu od 1,01 μM u linie UKF-NB-3 po 30,70 μM u UKF-NB-4(CDDP). Linie UKF-NB-3, SK-N-AS a zejména pak buňky s navozenou rezistencí k cisplatině byly k VPA v hypoxii v porovnání s mateřskými

liniemi významně citlivější. S rostoucím časem inkubace účinek VPA v normoxii i hypoxii přetrvával, jak ukazuje s časem klesající podíl živých (An^-/PI^-) buněk, viz Obr. 21.



Obrázek 21: Viabilita stanovená průtokovou cytometrií jako počet An^-/PI^- buněk v závislosti na koncentraci VPA, čase a koncentraci kyslíku u vybraných neuroblastomových linií.

Zjištěné hodnoty IC_{50} pro VPA u většiny z testovaných linií přesahovaly terapeuticky dosažitelné hladiny. Pro testování synergistického účinku cisplatiny a VPA byla proto zvolena klinicky relevantní koncentrace VPA 1 mM. Synergistický účinek kombinace 1 mM VPA s cisplatinou v porovnání se samotnou cisplatinou byl zjištěn pouze v některých případech - u linie UKF-NB-3 v noxmoxii a u linie UKF-NB-4(CDDP) v hypoxii. V ostatních případech nebyl rozdíl statisticky významný, nebo synergistický účinek nebyl pozorován, viz Tab. 6.

*Tabulka 6: Citlivost neuroblastomových linií k VPA (A), cisplatině (B) a jejich kombinaci (C). Data byla hodnocena oboustranným t-testem na hladině α=0,05. * Označuje statisticky významný rozdíl mezi normoxií a hypoxií, # rozdíl citlivosti na VPA mezi mateřskou linií a linií rezistentní k cisplatině a $ signifikantní rozdíl citlivosti na cisplatinu a VPA v kombinaci s cisplatinou (u všech pro p < 0,05). Ke stanovení IC_{50} byl použit MTT test. Doba inkubace: 72 hodin.*

A

	IC_{50} VPA (mM)	
Buněčná linie	Normoxie	Hypoxie
IMR-32*	0,76 ± 0,13	1,19 ± 0,13
UKF-NB-3*	2,40 ± 0,16	1,85 ± 0,12
UKF-NB-3 (CDDP)*	3,82 ± 0,12#	1,46 ± 0,06#
UKF-NB-4*	2,77 ± 0,08	3,14 ± 0,09
SK-N-AS*	13,09 ± 0,60	10,45 ± 0,87
SK-N-AS (CDDP)*	6,29 ± 0,07#	1,71 ± 0,25#

B

	IC_{50} CDDP (μM)	
Buněčná linie	Normoxie	Hypoxie
UKF-NB-3	0,99 ± 0,10	1,01 ± 0,15
UKF-NB-3 (CDDP)*	7,67 ± 0,60	6,27 ± 0,61
UKF-NB-4	6,27 ± 1,28	7,32 ± 1,58
UKF-NB-4 (CDDP)*	13,92 ± 1,55	30,70 ± 2,58
SK-N-AS*	3,71 ± 0,30	3,12 ± 0,07
SK-N-AS (CDDP)*	11,68 ± 1,52	18,23 ± 1,91

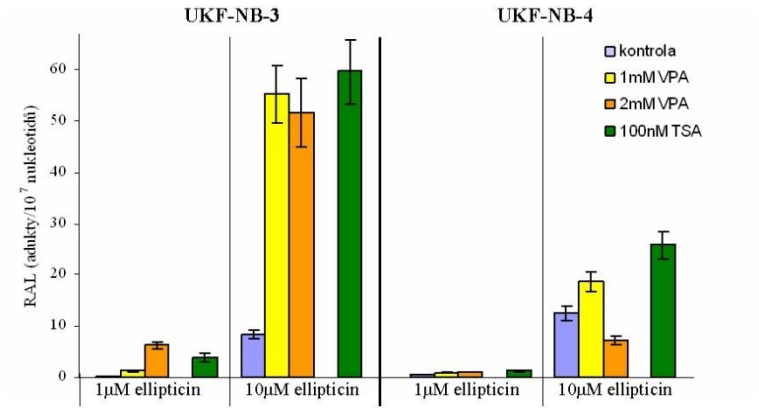
C

	IC_{50} 1 mM VPA + CDDP (μM)	
Buněčná linie	Normoxie	Hypoxie
UKF-NB-3	0,52 ± 0,18$	0,79 ± 0,04
UKF-NB-3 (CDDP)	6,58 ± 0,67	6,66 ± 0,52
UKF-NB-4*	4,87 ± 0,34	7,32 ± 0,62
UKF-NB-4 (CDDP)*	14,13 ± 0,36	20,35 ± 1,83$

5.2.2 NEUROBLASTOMOVÉ LINIE JSOU CITLIVÉ K ELLIPTICINU A JEHO KOMBINACI S VPA I TSA

Citlivost k ellipticinu a kombinaci VPA a ellipticin byla měřena u neuroblastomových linií IMR-32, UKF-NB-3, UKF-NB-4 a od ní odvozené UKF-NB-4(elli) rezistentní k ellipticinu, připravené z mateřské linie pěstováním v médiu s postupně rostoucí koncentrací ellipticinu. Ke stanovení byl využit MTT test. Doba inkubace byla 72 hodin (viz kapitola Testování viability buněk, str. 58). Hodnoty IC_{50} elipticinu jsou uvedeny v Tab. 7 A. Testované linie byly k ellipticinu a ke kombinaci VPA a ellipticin citlivé. Hodnota IC_{50} pro ellipticin se pohybovala v rozmezí od 0,27 μM u linie IMR-32 v normoxii po 1,57 μM u linie UKF-NB-4(elli) v hypoxii. S výjimkou linie UKF-NB-3, kde byla IC_{50} stejná, způsobila hypoxie významný nárůst hodnot IC_{50}, tj. zvýšenou rezistenci buněk k ellipticinu (Poljaková et al., 2008).

Kombinace VPA a ellipticinu měla v normoxii v porovnání se samotným ellipticinem významně vyšší účinek, viz Tab. 7 B, str. 74. Příznivý efekt koreloval se zvýšenou hladinou aduktů ellipticinu a DNA, viz Obr. 22.



Obrázek č. 22: Tvorba aduktů po působení ellipticinu po 24hod preinkubaci s VPA nebo TSA u neuroblastomových linií UKF-NB-3 a UKF-NB-4 v normoxii. Celková doba inkubace byla 72 hodin.

*Tabulka 7: Citlivost neuroblastomových linií k ellipticinu (A) a ke kombinaci VPA a ellipticinu (B). Data byla hodnocena oboustranným t-testem na hladině α=0,05. * Označuje statisticky významný rozdíl mezi normoxií a hypoxií, # významný rozdíl citlivosti na ellipticin a ellipticin v kombinaci s VPA nebo TSA (u všech pro p < 0,05). K stanovení byl použit MTT test. Doba inkubace: 72 hodin.*

A

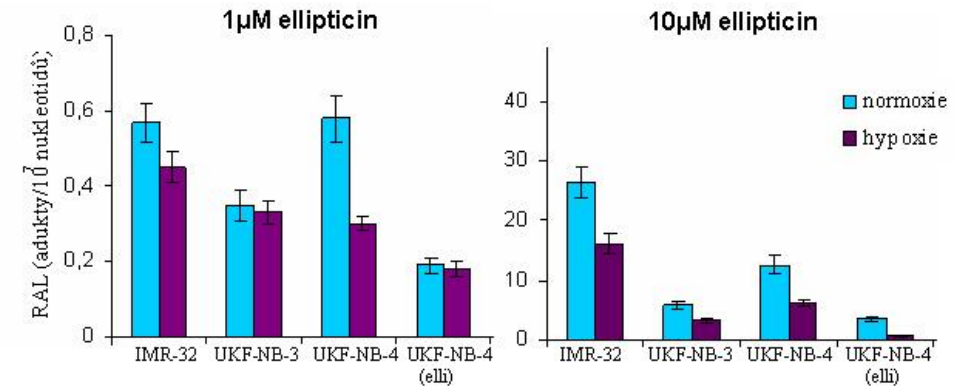
	IC_{50} ellipticin (μM)	
Buněčná linie	Normoxie	Hypoxie
IMR-32*	0,27 ± 0,02	0,43 ± 0,02
UKF-NB-3	0,44 ± 0,03	0,44 ± 0,03
UKF-NB-4*	0,44 ± 0,03	0,77 ± 0,04
UKF-NB-4(elli)*	1,17 ± 0,07	1,57 ± 0,08

B

	IC_{50} pro ellipticin za normoxie	
Kombinace \ linie	UKF-NB-3	UKF-NB-4
bez VPA	0,440 ± 0,030	0,490 ± 0,035
1mM VPA + ellipticin	$0,199 \pm 0,011^{\#}$	$0,389 \pm 0,029^{\#}$
2mM VPA + ellipticin	$0,047 \pm 0,003^{\#}$	$0,252 \pm 0,021^{\#}$
100nM TSA + ellipticin	$0,303 \pm 0,030^{\#}$	$0,389 \pm 0,030^{\#}$

Hlavním mechanismem účinku ellipticinu je poškozování DNA kovalentní vazbou jeho aktivovaných forem. Pomocí chromatografie na tenké vrstvě s autoradiografickou detekcí byly nalezeny adukty ellipticinu s DNA u linií IMR-32, UKF-NB-3, UKF-NB-4 a UKF-NB-4(elli). Buňky byly inkubovány v normoxii nebo hypoxii s ellipticinem o koncentraci 1 a 10 μM po dobu 48 hodin. Ellipticin tvořil s DNA dva hlavní adukty vznikající interakcí DNA s aktivovanými formami – ellipticin-13-ylium a ellipticin-12-ylium. Zdrojem reaktivních forem je 13-hydroxyellipticin a 12-hydroxyelipticin, které v buňce vznikají metabolisací ellipticinu, viz Obr. 7, str. 27. Kromě hlavních aduktů jsou tvořeny další dva minoritní adukty. V porovnání s majoritními formami je jejich hladina několikanásobně nižší. Charakterizace/identifikace a stanovení relativní hladiny aduktů bylo provedeno pomocí ^{32}P-postlabelingu. Hladina byla v normoxii u všech linií pro obě koncentrace ellipticinu stejná nebo vyšší než v hypoxii, viz Obr. 23. S výjimkou linie UKF-NB-3 korelovaly pro jednotlivé linie hladiny aduktů s hodnotou IC_{50} měřenou za různých podmínek u maternálních i odvozených linií (Stiborova et al., 2009).

Analýzou aduktů aktivovaného ellipticinu a DNA byl potvrzen synergistický účinek VPA i TSA a ellipticinu v normoxii zjištěný pomocí MTT testu (Poljakova et al., 2011). Hladina aduktů u linie UKF-NB-3 byla pro kombinaci 10μM ellipticin a 1 nebo 2mM VPA několikanásobně vyšší v porovnání s účinkem samotného ellipticinu. U linie UKF-NB-4 byla hladina detekovaných aduktů u kombinace v porovnání se samotným ellipticinem slabě zvýšena, viz Obr. 22, str.73.

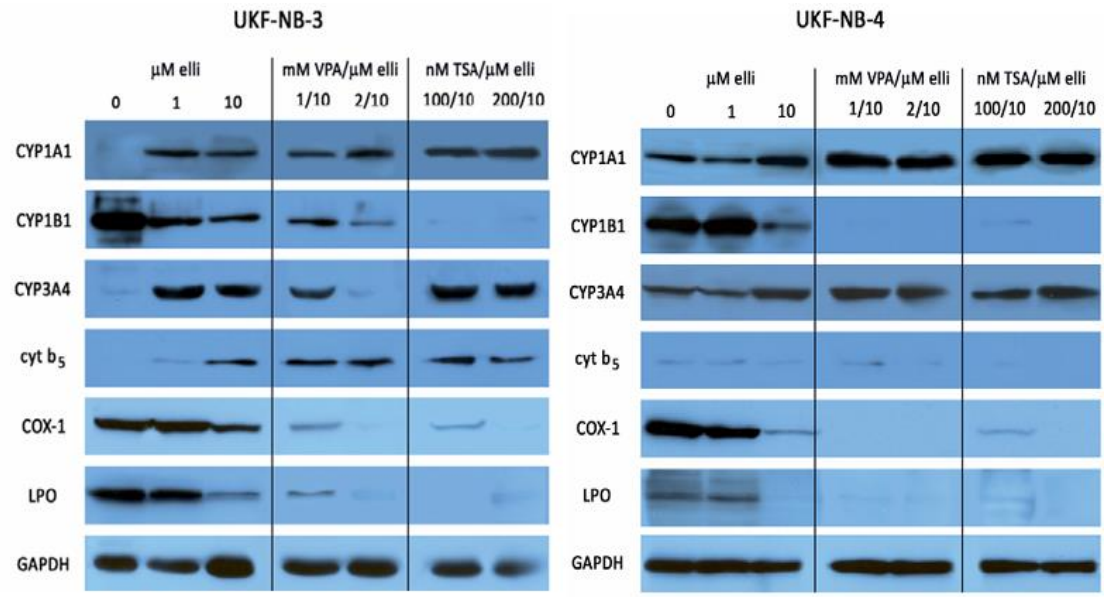


Obrázek 23: Hladina aduktů DNA a ellipticinu u linií IMR-32, UKF-NB-3, UKF-NB-4 a UKF-NB-4(elli) po 48h inkubaci s ellipticinem (1 μM a 10 μM) v normoxii a hypoxii.

5.2.2.1 VPA i TSA v kombinaci s ellipticinem ovlivňují expresi enzymů podílejících se na jeho metabolisaci

Na metabolisaci ellipticinu se podílí řada enzymů, zejména cytochromy rodiny P450 a peroxidasy. Pomocí Western blotu byla stanovena exprese cytochromů P450 CYP1A1, 1B1, 3A4 a cytochromu b_5, laktoperoxidasy (LPO) a cyklooxygenasy-1 (COX-1) u neuroblastomových linií UKF-NB-3 a UKF-NB-4. VPA i TSA samostatně jsou schopny expresi cytochromů P450 ovlivnit (Hřebačková et al., 2009). Zde byl sledován vliv ellipticinu a kombinace VPA i TSA s ellipticinem na hladinu jednotlivých enzymů, viz Obr. 24. Hladina CYP1A1 byla u obou linií indukována již samotným ellipticinem. Tento efekt byl dále výrazně posílen VPA i TSA. Množství CYP3A4 bylo ovlivněno pouze ellipticinem, kde byl pozorován nárůst u obou linií. Kombinace ellipticinu s HDACi expresi CYP3A4 v porovnání se samotným ellipticinem téměř neovlivnila s výjimkou linie UKF-NB-3, kde byl při kombinaci s VPA pozorován pokles exprese. Exprese cytochromu CYP1B1 byla ellipticinem negativně ovlivněna u obou linií. Kombinace s HDACi hladiny CYP1B1 dále snižovala. U linie UKF-NB-4 byla exprese úplně potlačena po kombinaci ellipticinu s VPA i TSA. Buňky UKF-NB-3 vykazovaly stejný efekt pro kombinaci ellipticinu a TSA. VPA s ellipticinem zde

hladinu CYP1B1 pouze snížili. Cytochrom b_5, regulující aktivitu dalších cytochromů (Schenkmann, Jansson, 2003; Stiborova et al. 2006), byl indukován ellipticinem o vyšší koncentraci (10 μM) pouze u linie UKF-NB-3, kombinace ellipticinu a HDACi nevedla k dalším změnám. Hladina studovaných peroxidas metabolisujících ellipticin byla jak ellipticinem, tak jeho kombinací s HDACi snížena a u vyšších koncentrací VPA i TSA již nebyly použitou metodikou detekovatené.



Obrázek 24: Western blotová analýza působení ellipticinu a jeho kombinace s VPA nebo TSA na expresi CYP1A1, CYP1B1, CYP3A4, cytochromu b_5, LPO a COX-1. Exprese glyceraldehyd fosfát dehydrogenasy (GADPH) byla použita jako kontrola.

6. DISKUSE

6.1 ÚČINEK KYSELINY VALPROOVÉ A CISPLATINY NA NEUROBLASTOMOVÉ BUŇKY *IN-VITRO*

Nádorové buňky mají expresní profil odlišný od normálních buněk. Jeho příčinou mohou být mimo jiné i epigenetické změny jako je acetylace histonů. Acetylační stav histonů, který je výsledkem rovnovážného stavu mezi aktivitou histondeacetylas a histonacetyltranferas, ovlivňuje mnoho buněčných procesů. Kromě histonů jsou jejich substráty proteiny podílející se na remodelaci chromatinu, receptory a signální molekuly, enzymy, transkripční faktory a další, viz Tab. 3, str. 32. Výsledkem aberantní exprese histondeacetylas se současnými změnami v aktivitě histonacetyltranferas je potlačení exprese genů s antiproliferační aktivitou (Ropero a Esteller, 2007; Sharma et al., 2010) a ovlivnění řady procesů, které se podílejí na rozvoji onemocnění.

Ověřili jsme, že VPA navozuje hyperacetylaci histonů u neuroblastomových linií v normoxii. Tento mechanismus se podílí na protinádorovém účinku VPA také v hypoxii, kde byla acetylace histonů ještě výraznější. Celkové ovlivnění genové exprese je tak v hypoxii pravděpodobně větší než v normoxii. Navozená remodelace chromatinu spojená s rozvolněním struktury DNA může jednak obnovit expresi tumor supresorových genů, které byly v důsledku nádorové transformace umlčeny, ale také umožnit efektivnější působení chemoterapeutik i radioterapie (Teufel et al., 2006; Catalano et al., 2007; Chen et al., 2007; Harikrishnan et al., 2007; Munster et al., 2009; Cipro et al., 2012; Shoji et al., 2012; Xie et al., 2012). Naše experimenty dále potvrzují schopnost VPA zamezit adaptaci buněk na hypoxii. Přístup nádorových buněk ke kyslíku je nezbytný pro progresi nádorového onemocnění. Ve stavu hypoxie buňky svůj metabolismus adaptují na vzniklé podmínky. Klíčovým regulátorem těchto pochodů je transkripční faktor HIF-1. Jeho aktivace u solidních nádorů bývá spojena s horší prognózou a koreluje s vyšším stupněm malignity, rezistencí k chemoterapii i radioterapii a celkově horším výsledkům léčby (Shannon et al., 2003; Um et al., 2004; Cheng et al., 2013). U neuroblastomových linií jsme prokázali pokles hladiny HIF-1α po VPA, který jsme potvrdili také sledováním exprese vybraných cílových genů HIF-1 – VEGF a karboanhydrasy 9. Výrazný pokles exprese genů řízených aktivitou HIF-1, kam kromě uvedených patří řada dalších, viz Tab. 1, str. 20, navozuje buněčný stres, který vede k omezení proliferace, zastavení růstu a apoptose.

Všechny testované neuroblastomové linie byly na VPA citlivé. IC_{50} valproátu se pohybovaly ve značném rozmezí v závislosti na buněčné linii, viz Tab. 6 A, str. 72. Buněčný cyklus neuroblastomových buněk byl VPA ovlivněn u všech testovaných linií po 24 i 48 hodinové inkubaci v normoxii i hypoxii. S rostoucí koncentrací VPA jsme zaznamenali mírně zvýšený počet buněk ve fázi G0/G1, která u vyšších koncentrací přecházela do kumulace ve fázi G2/M. Zastavení buněčného cyklu ve fázi G0/G1 souvisí pravděpodobně s indukcí exprese genu pro p21 (WAF/CIP1), který inhibuje cyklin dependentní kinasy. Při dlouhodobé kultivaci buněk s HDACi je p21 navíc nutný k ireverzibilnímu zastavení buněčného cyklu a navození buněčné senescence (Richon et al., 2000; Romanov et al., 2010). Detailní mechanismus zastavení buněčného cyklu ve fázi G2/M není zatím zcela objasněn a je spojován s inhibicí HDAC3 (Wilson et al., 2006) a zvýšenou expresí regulátorů přechodu G2/M (Noh et al., 2009).

Apoptosu neuroblastomových buněk jsme hodnotili průtokovou cytometrií vazbou AnexinuV a metodou TUNEL, kdy jsme potvrdili citlivost testovaných linií k VPA. U linie UKF-NB-3 byl počet apoptotických buněk v normoxii i hypoxii již od nižších koncentrací VPA, která byla vztažená k IC_{50}, velmi nízký. Tento jev je způsobený zvýšeným počtem buněk v pozdní apoptose (An^{+}/PI^{+}). Stanovením aktivity kaspasy 8, která je hlavním efektorovým enzymem zahajujícím vnější apoptotickou dráhu, a množství jejího substrátu, proapoptotického proteinu Bid, jsme testovali zapojení vnější apoptotické dráhy do působení VPA. Protein Bid je štěpením aktivován (forma t-Bid) a interakcí s dalšími proteiny – Bax a Bak – zprostředkovává uvolnění cytochromů z mezimembránového prostoru mitochondrií. Tyto výsledky naznačovaly při inkubaci buněk s VPA zapojení právě vnější apoptotické dráhy. Aktivita kaspasy 8 byla po přidání VPA zvýšená a tento jev byl zamezen použitím specifického inhibitoru, viz Obr. 16, str. 67. Množství proteinu Bid v buňkách po inkubaci s VPA klesalo, což potvrzuje jeho štěpení. U buněk kultivovaných v normoxii byl pokles patrný při vyšších koncentracích VPA (5mM). V hypoxii byl tento efekt značný již od nízkých koncentrací (0,5mM) a v případě linie UKF-NB-3(CDDP) byl indukován samotnou hypoxií. Stanovení počtu apoptotických buněk po inkubaci s VPA bez inhibitoru a s inhibitorem ovšem nepotvrdilo vnější apoptotickou dráhu jako hlavní cestu buněčné smrti navozené VPA, jelikož byl počet apoptotických (An^{+}/PI^{-}) buněk v obou případech stejný, viz Obr. 17, str. 67. Zapojení vnitřní cesty aktivace jsme ověřili pomocí western blotové analýzy proteinů Bcl-2 a Bax. Množství antiapoptotického proteinu Bcl-2 v buňkách se působením valproátu měnilo až u vyšších koncentrací VPA. V závislosti na buněčné linii jsme zaznamenali jeho pokles v normoxii i hypoxii (UKF-NB-3, UKF-NB-3(CDDP)), a nezměněné nebo mírně zvýšené množství u SK-N-AS a SK-N-AS(CDDP). Zvýšení koncentrace některé z variant proapototického

proteinu Bax (21 a 19 kDa) bylo pozorováno u všech linií v normoxii a hypoxii s výjimkou buněk SK-N-AS v normoxii. Zjistili jsme, že k největším změnám docházelo především u 19kDa varianty, která se v porovnání s 21kDa formou Bax vyznačuje vyšší afinitou k mitochondriální membráně a je při indukci apoptosy mnohem efektivnější (Cartron et al., 2002; Westphal et al., 2011). Nárůst Bax (19kDa) jsme v závislosti na kultivačních podmínkách a buněčné linii pozorovali již při nižších koncentracích VPA (<5mM). Při 5 mM VPA byly změny detekovatelné u všech linií s výjimkou buněk SK-N-AS, která je k VPA nejméně citlivá, v normoxii i hypoxii. Tyto výsledky naznačují, že apoptosa neuroblastomových buněk způsobená VPA zřejmě probíhá především aktivací vnitřní dráhy. Na této aktivaci se podílí obě formy proapoptotického proteinu Bax, zejména pak jeho 19kDa varianta. Aktivací vnitřní apoptotické dráhy lze také vysvětlit pokles množství Bid v buňkách, jelikož je tento protein substrátem i pro kaspasu 3, která je aktivována až v pozdějších fázích po narušení mitochondriální membrány. Štěpením Bid je pak apoptotická signalizace dále zesílena. Za důležité považujeme především zjištění, že v hypoxii je účinek VPA stejný nebo často dokonce větší než v normoxii.

Významným zjištěním je, že v hypoxických podmínkách byly buňky k VPA ve většině případů výrazně citlivější než v normoxii. Změny v citlivosti byly nejvíce patrné v hypoxii u buněk s navozenou rezistencí k cisplatině. Vysvětlení může souviset s narušením adaptace k hypoxii odbouráváním HIF-1α a tím, že buňky rezistentní k jednomu typu terapie mohou mít ostatní mechanismy rezistence oslabeny. Účinek VPA v normoxii i hypoxii navíc s rostoucím časem inkubace přetrvával, kdy jsme v závislosti na čase pozorovali pokles počtu intaktních (An^-/PI^-) buněk. Podání VPA by tak mohlo být indikováno zvláště u chemorezistentních recidiv. Značné rozdíly v efektu VPA mezi jednotlivými buněčnými liniemi souvisí patrně s rozdílnou expresí HDAC, dalších enzymů, s úrovní exprese proapoptotických a antiapoptotických proteinů, indukcí ABC transportérů a stabilizací p53.

Většina prací zabývajících se účinkem kombinace HDACi a dalších preparátů zkoumala efekt kombinace pouze v normoxických podmínkách. Vzhledem k častému výskytu hypoxických oblastí v solidních nádorech jsme testovali v normoxii a hypoxii kromě samotné VPA i účinek cisplatiny a jejich kombinace. Všechny testované linie byly k cisplatině citlivé. Hodnoty IC_{50} se v závislosti na buněčné linii podobně jako u VPA značně odlišovaly, viz Tab. 6 B, str. 72. Synergistický účinek VPA a cisplatiny jsme prokázali pouze v některých případech (linie UKF-NB-3 a UKF-NB-4(CDDP)), kdy v porovnání se samotnou cisplatinou postačovala k usmrcení stejného počtu buněk jenom poloviční koncentrace cisplatiny. U ostatních linií nebyl synergistický účinek buď vůbec pozorován, nebo rozdíl nebyl statisticky

významný. Potenciace efektu cisplatiny VPA je zřejmě způsobena hyperacetylací histonů spojené s rozvolněním chromatinové struktury. DNA je tak pro cisplatinu přístupnější a rozsah poškození větší. Na zvýšené efektivitě kombinace se dál může podílet i VPA samotným ovlivněním exprese proteinů podílejících se na opravě poškozené DNA, apoptotické signalizaci, stabilizaci p53 acetylací a dalšími mechanismy. To, že synergistický efekt nebyl pozorován ve všech případech, případně pouze za určitých podmínek kultivace nebo jenom u některé buněčné linie, naznačuje, že mechanismus potenciace nemusí být pouze jeden. Výsledný účinek tak závisí na použité buněčné linii, kultivačních podmínkách a zřejmě i na mechanismu rezistence k léčivu, které je v kombinaci použito. Důležité je také pořadí, ve kterém jsou terapeutika podávána (Groh et al., 2012).

6.2 KYSELINA VALPROOVÁ A ELLIPTICIN A JEJICH ÚČINEK NA NEUROBLASTOMOVÉ BUŇKY *IN-VITRO*

Všechny testované neuroblastomové linie byly k ellipticinu citlivé. Koncentrace IC_{50} se pohybovaly v rozmezí od 0,27 po 1,57 μM v závislosti na podmínkách a buněčné linii, viz Tab. 7 A, str. 74. S výjimkou linie UKF-NB-3 jsme v porovnání s normoxií u všech linií pozorovali nárůst hodnot IC_{50} v hypoxii. Zvýšená odolnost buněk v hypoxickém prostředí negativně korelovala s hladinou aduktů ellipticinu a DNA. Poškození DNA zprostředkované ellipticinem je tak zřejmě hlavním mechanismem jeho cytotoxického účinku (Poljaková et al., 2007, 2009, 2011; Martinkova et al., 2009). Zvýšená rezistence vůči ellipticinu v hypoxii může souviset s pozměněným expresním profilem buněk, týkajícím se jak enzymů metabolisujících ellipticin, tak enzymů opravujících poškozenou DNA nebo i s dalšími hypoxií indukovanými změnami, například zpomalením buněčného cyklu.

U linie UKF-NB-3 se s hypoxií citlivost k ellipticinu nezměnila. Jelikož korelace mezi hladinou DNA aduktů a IC_{50} měřenou u této linie i odvozených variant za různých podmínek nebyla prokázána, na buněčné smrti způsobené ellipticinem se zde zřejmě podílejí další mechanismy. U ellipticinu je to inhibice topoisomerasy II, interkalace do DNA nebo ovlivnění oxidativní fosforylace s narušením energetického metabolismu buňky (Schwaller et al., 1995; Stiborova et al., 2009). Nicméně zůstává otázkou, co způsobuje rozdílnou reakci různých typů neuroblastomových buněk na ellipticin. Roli zde hrají zřejmě genetické odchylky mezi jednotlivými buněčnými typy (Bedrnicek et al., 2005) a další odlišnosti.

Účinek kombinace VPA i TSA s ellipticinem na neuroblastomové linie prokázal podpůrný účinek HDACi na cytotoxicitu ellipticinu. Na celkovém účinku kombinace se

HDACi podílejí kromě přímého cytotoxického účinku i ovlivněním exprese enzymů metabolisujících ellipticin (Hřebačková et al., 2009) a změnou struktury chromatinu a jeho zpřístupněním pro ellipticin. Prokázali jsme významně lepší účinek ellipticinu v kombinaci s VPA nebo TSA v normoxii. Účinnost ellipticinu v kombinaci s 1mM nebo 2mM VPA byla u sledovaných linií několikanásobně vyšší v porovnání se samotným ellipticinem. Nejvyšší účinek jsme pozorovali u linie UKF-NB-3, kde byl u kombinace VPA a ellipticinu zaznamenán pokles hodnoty IC_{50} až na desetinu hodnoty pro ellipticin. Zvýšená cytotoxicita byla doprovázena u linie UKF-NB-3 několikanásobně vyššími hladinami aduktů ellipticinu s DNA, viz Obr. 22, str. 73. Hladiny aduktů detekované u buněk UKF-NB-4 byly nižší a 2mM VPA tvorbu aduktů v porovnání s kontrolními buňkami dokonce omezil. Rozdíly v hladinách DNA aduktů, resp. v citlivosti obou linií mohou souviset s rozdílným buněčným genotypem i fenotypem – invazivní N-typ UKF-NB-3 byl ke kombinaci VPA a ellipticinu citlivější než méně agresivní linie S-typu UKF-NB-4. Na odlišné reakci těchto linií na testovanou kombinaci se podílí především rozdíly v expresi cytochromů P450 metabolisujících ellipticin a proteinů, které jejich aktivitu modulují. Význam zde může mít i odlišná exprese P-glykoproteinu, která je u linie UKF-NB-4 několikanásobně vyšší než u buněk UKF-NB-3.

Analýzy ellipticin metabolisujících enzymů pomocí western blotu ukázaly, že množství aduktů DNA a ellipticinu závisí především na cytochromech P450 CYP1A1, CYP3A4 a cytochromu b_5. Roli zde hrají i změny v koncentraci CYP1B1, COX-1 a LPO. U linie UKF-NB-3 je indukce cytochromu b_5 vyšší koncentrací ellipticinu (kombinace s VPA nebo TSA na expresi dodatečný vliv neměla) pravděpodobně klíčová pro tvorbu DNA aduktů. Tento protein ovlivňuje aktivitu jak cytotochromu CYP3A4, který katalyzuje proměnu ellipticinu na 12-hydroxy a 13-hydroxy formy tvořící DNA adukty, tak cytochromu CYP1A1 podílejícího se především na detoxikaci ellipticinu ale částečně i na jeho aktivaci (Stiborova et al., 2004; 2006B; 2011). Omezení detoxikační aktivity CYP1A1 a podpora tvorby cytotoxických forem ellipticinu jsou zprostředkovány právě cytochromem b_5 (Kotrbova et al., 2011). U linie UKF-NB-4 není hladina cytochromu b_5 ovlivněna, zatímco CYP1A1 je ellipticinem indukován a tento efekt je dále posílen VPA i TSA. Proto pravděpodobně u linie UKF-NB-4 převažuje detoxikační efekt nad přeměnou ellipticinu CYP1A1 na aktivované formy. V konečném důsledku vzniká menší množství DNA aduktů a účinek ellipticinu je nižší. Další z cytochromů P450 – CYP1B1, který se podílí na detoxikaci ellipticinu (Stiborova et al., 2004), byl ovlivněn u linie UKF-NB-3 i UKF-NB-4. Jeho pokles jsme pozorovali při inkubaci se samotným ellipticinem i u kombinace s VPA nebo TSA, které hladinu cytochromu CYP1B1 dále snižovaly. Nižší hladiny CYP1B1 se u obou linií mohou projevit poklesem detoxikační aktivity,

který je ovšem u linie UKF-NB-4 částečně kompenzován nárůstem CYP1A1. Aktivita peroxidas COX-1 a LPO podílejících se na aktivaci ellipticinu a tvorbě minoritních DNA aduktů s DNA, byla u linií UKF-NB-3 a UKF-NB-4 účinkem ellipticinu a jeho kombinace s VPA nebo TSA výrazně snížena až úplně omezena. Tyto enzymy se tak zřejmě nepodílí na potenciaci efektu ellipticinu v kombinaci s VPA nebo TSA.

7. ZÁVĚR

Navzdory pokroku v léčbě nádorů je vznik rezistence k terapii trvalým problémem. Protože v praxi nelze jejímu vzniku vždy zabránit, jsou vyvíjeny léčebné postupy usilující zejména o zvýšení efektivity terapie. Perspektivní skupinou látek s protinádorovými účinky, které jsou navíc schopny modulovat účinek chemoterapeutik i radioterapie, jsou inhibitory histondeacetylas, které patří do skupiny epigenetických chemoterapeutik. Působí prostřednictvím navození hyperacetylace histonů, ovlivnění stability řady proteinů, modulace transkripce a dalších mechanismů. Významné účinky vykazují zejména v prostředí s omezeným přístupem ke kyslíku, které je u solidních nádorů velmi časté. Ovlivnění adaptace nádorových buněk k hypoxii se děje prostřednictvím omezení funkce transkripčního faktoru HIF-1. Nejdéle klinicky používaným HDACi je kyselina valproová, používaná od konce šedesátých let minulého století jako antiepileptikum.

V této práci jsme se ukázali, že VPA má na neuroblastomové buňky přímý cytotoxický účinek jak v normoxických, tak v hypoxických podmínkách. Apoptotický proces indukovaný VPA probíhá pravděpodobně aktivací vnitřní apoptotické dráhy na základě ovlivnění exprese proteinů Bcl-2 a zejména Bax. U Bax jsme pozorovali nárůst koncentrace 19kDa varianty vyznačující se vysokou proapoptotickou aktivitou, která může být pro zahájení apoptosy klíčová. Přínosem VPA v protinádorové terapii by mohl být zejména účinek v hypoxických podmínkách, při kterých jsme u většiny testovaných linií pozorovali jeho významné zvýšení v porovnání s normoxií. Největší rozdíly byly zjištěny u linií s navozenou rezistencí k cisplatině a VPA by tak mohl být indikován hlavně u chemorezistentních recidiv.

Zvýšenou cytotoxicitu kombinace VPA a DNA poškozujících cytostatik jsme pozorovali jak u cisplatiny, tak u ellipticinu. Kombinace VPA s cisplatinou byla v porovnání se samotnou cisplatinou účinnější pouze v některých případech, pravděpodobně v závislosti na typu použité buněčné linie a podmínkách experimentu. U ellipticinu bylo použití kombinace s VPA v normoxii efektivnejší u všech testovaných linií. Zlepšení působení DNA poškozujících cytostatik pomocí VPA se děje kromě příspěvku přímého cytotoxického účinku pravděpodobně na základě hyperacetylace histonů a rozvolnění chromatinové struktury umožňující lepší přístup k DNA. VPA účinek ellipticinu posiluje i ovlivněním exprese cytochromů podílejících se na jeho metabolisaci a aktivaci. VPA se zdá nadějný pro použití v kombinaci s dalšími protinádorovými léčivy což dokazují i klinické studie.

8. SEZNAM POUŽITÉ LITERATURY

1. Acosta S, Lavarino C, Paris R, Garcia I, de Torres C, Rodríguez E, Beleta H, Mora J. Comprehensive characterization of neuroblastoma cell line subtypes reveals bilineage potential similar to neural crest stem cells. BMC Dev Biol. 2009; 9:12.
2. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science. 1998; 281(5381):1322-6.
3. Almenara J, Rosato R, Grant S. Synergistic induction of mitochondrial damage and apoptosis in human leukemia cells by flavopiridol and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). Leukemia. 2002; 16(7):1331-43.
4. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature. 1998; 392(6674):405-8.
5. Andreassen PR, Lacroix FB, Lohez OD, Margolis RL. Neither p21WAF1 nor 14-3-3sigma prevents G2 progression to mitotic catastrophe in human colon carcinoma cells after DNA damage, but p21WAF1 induces stable G1 arrest in resulting tetraploid cells. Cancer Res. 2001; 61(20):7660-8.
6. Andrikopoulou E, Zhang X, Sebastian R, Marti G, Liu L, Milner SM, Harmon JW. Current Insights into the role of HIF-1 in cutaneous wound healing. Curr Mol Med. 2011; 11(3):218-35.
7. Aoyagi S, Archer TK. Modulating molecular chaperone Hsp90 functions through reversible acetylation. Trends Cell Biol. 2005; 15(11):565-7.
8. Arany I, Safirstein RL. Cisplatin nephrotoxicity. Semin Nephrol. 2003; 23(5):460-4.
9. Avantaggiati ML, Ogryzko V, Gardner K, Giordano A, Levine AS, Kelly K. Recruitment of p300/CBP in p53-dependent signal pathways. Cell. 1997; 89(7):1175-84.
10. Backs J, Olson EN. Control of cardiac growth by histone acetylation/deacetylation. Circ Res. 2006; 98(1):15-24.
11. Bae SH, Jeong JW, Park JA, Kim SH, Bae MK, Choi SJ, Kim KW. Sumoylation increases HIF-1alpha stability and its transcriptional activity. Biochem Biophys Res Commun. 2004; 324(1):394-400.
12. Bali P, Pranpat M, Bradner J, Balasis M, Fiskus W, Guo F, Rocha K, Kumaraswamy S, Boyapalle S, Atadja P, Seto E, Bhalla K. Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. J Biol Chem. 2005; 280(29):26729-34.
13. Bandyopadhyay D, Mishra A, Medrano EE. Overexpression of histone deacetylase 1 confers resistance to sodium butyrate-mediated apoptosis in melanoma cells through a p53-mediated pathway. Cancer Res. 2004; 64(21):7706-10.
14. Barlev NA, Liu L, Chehab NH, Mansfield K, Harris KG, Halazonetis TD, Berger SL. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol Cell. 2001; 8(6):1243-54.
15. Barneda-Zahonero B, Parra M. Histone deacetylases and cancer. Mol Oncol. 2012; 6(6):579-89.

16. Bartke T, Siegmund D, Peters N, Reichwein M, Henkler F, Scheurich P, Wajant H. p53 upregulates cFLIP, inhibits transcription of NF-kappaB-regulated genes and induces caspase-8-independent cell death in DLD-1 cells. Oncogene. 2001; 20(5):571-80.

17. Bedrnicek J, Vicha A, Jarosova M, Holzerova M, Cinatl Jr J, Michaelis M, Cinatl J, Eckschlager T. Characterization of drug-resistant neuroblastoma cell lines by comparative genomic hybridization. Neoplasma. 2005;52(5):415-9.

18. Ben-Yosef Y, Lahat N, Shapiro S, Bitterman H, and Miller A. Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation. Circ Res. 2002; 90: 784-791.

19. Berra E, Benizri E, Ginouvès A, Volmat V, Roux D, Pouysségur J. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J. 2003; 22(16):4082-90.

20. Berta MA, Mazure N, Hattab M, Pouysségur J, Brahimi-Horn MC. SUMOylation of hypoxia-inducible factor-1alpha reduces its transcriptional activity. Biochem Biophys Res Commun. 2007; 360(3):646-52.

21. Bhasin M, Reinherz EL, Reche PA. Recognition and classification of histones using support vector machine. J Comput Biol. 2006; 13(1):102-12.

22. Bianchi L, Tacchini L, and Cairo G. HIF-1-mediated activation of transferrin receptor gene transcription by iron chelation. Nucleic Acids Res. 1999; 27: 4223-4227.

23. Blaheta RA, Cinatl J Jr. Anti-tumor mechanisms of valproate: a novel role for an old drug. Med Res Rev. 2002; 22:492-511.

24. Blaheta RA, Michaelis M, Driever PH, Cinatl J Jr. Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. Med Res Rev. 2005; 25: 383-97.

25. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov. 2006; 5(9):769-84.

26. Bossy-Wetzel E, Green DR. Detection of apoptosis by annexin V labeling. Methods Enzymol. 2000; 322:15-8.

27. Boyault C, Sadoul K, Pabion M, Khochbin S. HDAC6, at the crossroads between cytoskeleton and cell signaling by acetylation and ubiquitination. Oncogene. 2007; 26(37):5468-76.

28. Bracken CP, Whitelaw ML, Peet DJ. Activity of hypoxia-inducible factor 2alpha is regulated by association with the NF-kappaB essential modulator. J Biol Chem. 2005; 280(14):14240-51.

29. Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci U S A. 2000; 97(16):9082-7.

30. Brush MH, Guardiola A, Connor JH, Yao TP, Shenolikar S. Deactylase inhibitors disrupt cellular complexes containing protein phosphatases and deacetylases. J Biol Chem. 2004; 279(9):7685-91.

31. Bryan N, Ahswin H, Smart N, Bayon Y, Wohlert S, Hunt JA. Reactive oxygen species (ROS)--a family of fate deciding molecules pivotal in constructive inflammation and wound healing. Eur Cell Mater. 2012; 24:249-65.

32. Buonocore G, Perrone S, Tataranno ML. Oxygen toxicity: chemistry and biology of reactive oxygen species. Semin Fetal Neonatal Med. 2010; 15(4):186-90.

33. Burnet M. Cancer; a biological approach. I. The processes of control. Br Med J. 1957; 1(5022):779-86.

34. Burton BS. On the propyl derivatives and decomposition products of ethylacetoacetate. Am Chem J. 1882; 3:385–395.

35. Butler LM, Zhou X, Xu WS, Scher HI, Rifkind RA, Marks PA, Richon VM. The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. Proc Natl Acad Sci USA. 2002; 99(18):11700-5.

36. Carew JS, Giles FJ, Nawrocki ST. Histone deacetylase inhibitors: mechanisms of cell death andpromise in combination cancer therapy. Cancer Lett. 2008; 269:7-17.

37. Carey N, La Thangue NB. Histone deacetylase inhibitors: gathering pace. Curr Opin Pharmacol. 2006; 6:369–75.

38. Caro J. Hypoxia regulation of gene transcription. High Alt Med Biol. 2001; 2(2):145-54.

39. Carrero P, Okamoto K, Coumailleau P, O'Brien S, Tanaka H, and Poellinger L. Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. Mol Cell Biol. 2000; 20: 402-415.

40. Carter CA. Protein kinase C as a drug target: implications for drug or diet prevention and treatment of cancer. Curr Drug Targets. 2000; 1(2):163-83.

41. Catalano MG, Poli R, Pugliese M, Fortunati N, Boccuzzi G. Valproic acid enhances tubulin acetylation and apoptotic activity of paclitaxel on anaplastic thyroid cancer cell lines. Endocr Relat Cancer. 2007; 14(3):839-45.

42. Cerveny L, Svecova L, Anzenbacherova E, Vrzal R, Staud F, Dvorak Z, Ulrichova J, Anzenbacher P, Pavek P. Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways. Drug Metab Dispos. 2007; 35(7):1032-41.

43. Ceulemans H, Bollen M. Functional diversity of protein phosphatase-1, a cellular economizer and reset button. Physiol Rev. 2004; 84(1):1-39.

44. Cimini D, Mattiuzzo M, Torosantucci L, Degrassi F. Histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects. Mol Biol Cell. 2003; 14(9):3821-33.

45. Cinatl J Jr, Kotchetkov R, Blaheta R, Driever PH, Vogel JU, Cinatl J. Induction of differentiation and suppression of malignant phenotype of human neuroblastoma BE(2)-C cells by valproic acid: enhancement by combination with interferon-alpha. Int J Oncol. 2002; 20(1):97-106.

46. Cinatl J Jr., Cinatl J, Driever PH, Kotchetkov R, Pouckova P, Kornhuber B et al. Sodium valproate inhibits *in vivo* growth of human neuroblastoma cells. Anticancer Drugs. 1997; 8:958-63.

47. Cipro Š, Hřebačková J, Hraběta J, Poljaková J, Eckschlager T. Valproic acid overcomes hypoxia-induced resistance to apoptosis. Oncol Rep. 2012; 27(4):1219-26.

48. Cormier-Regard S, Nguyen SV, and Claycomb WC. Adrenomedullin gene expression is developmentally regulated and induced by hypoxia in rat ventricular cardiac myocytes. J Biol Chem. 1998; 273: 17787-17792.

49. Cuervo AM, Dice JF. A receptor for the selective uptake and degradation of proteins by lysosomes. Science. 1996; 273(5274):501-3.

50. Cuervo AM, Dice JF. Unique properties of lamp2a compared to other lamp2 isoforms. J Cell Sci. 2000; 113(24):4441-50.

51. Dai Y, Rahmani M, Dent P, Grant S. Blockade of histone deacetylase inhibitor-induced RelA/p65 acetylation and NF-kappaB activation potentiates apoptosis in leukemia cells through a process mediated by oxidative damage, XIAP downregulation, and c-Jun N-terminal kinase 1 activation. Mol Cell Biol. 2005; 25(13):5429-44.

52. Davie JR. Covalent modifications of histones: expression from chromatin templates. Curr Opin Genet Dev. 1998; 8(2):173-8.

53. Dayan F, Roux D, Brahimi-Horn MC, Pouyssegur J, Mazure NM. The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1alpha. Cancer Res. 2006; 66(7):3688-98.

54. de Stanchina E, McCurrach ME, Zindy F, Shieh SY, Ferbeyre G, Samuelson AV, Prives C, Roussel MF, Sherr CJ, Lowe SW. E1A signaling to p53 involves the p19(ARF) tumor suppressor. Genes Dev. 1998; 12(15):2434-42.

55. Deb AA, Wilson SS, Rove KO, Kumar B, Koul S, Lim DD, Meacham RB, Koul HK. Potentiation of mitomycin C tumoricidal activity for transitional cell carcinoma by histone deacetylase inhibitors *in vitro*. J Urol. 2011; 186(6):2426-33.

56. Deroanne CF, Bonjean K, Servotte S, Devy L, Colige A, Clausse N, Blacher S, Verdin E, Foidart JM, Nusgens BV, Castronovo V. Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling. Oncogene. 2002; 21(3):427-36.

57. Detich N, Bovenzi V, Szyf M. Valproate induces replication-independent active DNA demethylation. J Biol Chem. 2003; 278:27586-92.

58. Diaz T, Navarro A, Ferrer G, Gel B, Gaya A, Artells R, Bellosillo B, Garcia-Garcia M, Serrano S, Martínez A, Monzo M. Lestaurtinib inhibition of the Jak/STAT signaling pathway in Hodgkin lymphoma inhibits proliferation and induces apoptosis. PLoS One. 2011; 6(4):e18856.

59. Dowling M, Voong KR, Kim M, Keutmann MK, Harris E, Kao GD. Mitotic spindle checkpoint inactivation by trichostatin A defines a mechanism for increasing cancer cell killing by microtubule-disrupting agents. Cancer Biol Ther. 2005; 4(2):197-206.

60. Duan J, Friedman J, Nottingham L, Chen Z, Ara G, Van Waes C. Nuclear factor-kappaB p65 small interfering RNA or proteasome inhibitor bortezomib sensitizes head and neck squamous cell carcinomas to classic histone deacetylase inhibitors and novel histone deacetylase inhibitor PXD101. Mol Cancer Ther. 2007; 6(1):37-50.

61. Dumaz N, Meek DW. Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. EMBO J. 1999; 18(24):7002-10.

62. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002; 3(11):991-8.

63. Eberharter A, Ferreira R, Becker P. Dynamic chromatin: concerted nucleosome remodelling and acetylation. Biol Chem. 2005; 386(8):745-51.

64. Eckhart AD, Yang N, Xin X, and Faber JE. Characterization of the alpha1B-adrenergic receptor gene promoter region and hypoxia regulatory elements in vascular smooth muscle. Proc Natl Acad Sci USA. 1997; 94: 9487-9492.

65. Ellis L, Bots M, Lindemann RK, Bolden JE, Newbold A, Cluse LA, Scott CL, Strasser A, Atadja P, Lowe SW, Johnstone RW. The histone deacetylase inhibitors LAQ824 and LBH589 do not require death receptor signaling or a functional apoptosome to mediate tumor cell death or therapeutic efficacy. Blood. 2009; 114(2):380-93.

66. Eriksson I, Joosten M, Roberg K, Ollinger K. The histone deacetylase inhibitor trichostatin A reduces lysosomal pH and enhances cisplatin-induced apoptosis. Exp Cell Res. 2013; 319(1):12-20.

67. Espinosa JM, Emerson BM. Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. Mol Cell. 2001; 8(1):57-69.

68. Fantin VR, Loboda A, Paweletz CP, Hendrickson RC, Pierce JW, Roth JA, Li L, Gooden F, Korenchuk S, Hou XS, Harrington EA, Randolph S, Reilly JF, Ware CM, Kadin ME, Frankel SR, Richon VM. Constitutive activation of signal transducers and activators of transcription predicts vorinostat resistance in cutaneous T-cell lymphoma. Cancer Res. 2008; 68(10):3785-94.

69. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, and Semenza GL. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res. 1999; 59: 3915-3918.

70. Finkel T. Oxygen radicals and signaling. Curr Opin Cell Biol. 1998; 10(2):248-53.

71. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol. 1996; 16(9):4604-13.

72. Friedmann I, Atmaca A, Chow KU, Jäger E, Weidmann E. Synergistic effects of valproic acid and mitomycin C in adenocarcinoma cell lines and fresh tumor cells of patients with colon cancer. J Chemother. 2006; 18(4):415-20.

73. Frei E, Bieler CA, Arlt VM, Wiessler M, Stiborová M. Covalent binding of the anticancer drug ellipticine to DNA in V79 cells transfected with human cytochrome P450 enzymes. Biochem Pharmacol. 2002; 64(2):289-95.s

74. Frommel TO, Coon JS, Tsuruo T, Roninson IB. Variable effects of sodium butyrate on the expression and function of the MDR1 (P-glycoprotein) gene in colon carcinoma cell lines. Int J Cancer. 1993; 55(2):297-302.

75. Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL. HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. Cell. 2007; 129(1):111-22.

76. Gains J, Mandeville H, Cork N, Brock P, Gaze M. Ten challenges in the management of neuroblastoma. Future Oncol. 2012; 8(7):839-58.

77. Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, Bhujwalla ZM, Felsher DW, Cheng L, Pevsner J, Lee LA, Semenza GL, Dang CV. HIF-dependent antitumorigenic effect of antioxidants *in vivo*. Cancer Cell. 2007; 12(3):230-8.

78. Garcia-Mata R, Gao YS, Sztul E. Hassles with taking out the garbage: aggravating aggresomes. Traffic. 2002; 3(6):388-96.

79. Geng L, Cuneo KC, Fu A, Tu T, Atadja PW, Hallahan DE. Histone deacetylase (HDAC) inhibitor LBH589 increases duration of gamma-H2AX foci and confines HDAC4 to the cytoplasm in irradiated non-small cell lung cancer. Cancer Res. 2006; 66(23):11298-304.

80. Gerber HP, Condorelli F, Park J, Ferrara N. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem. 1997; 272(38):23659-67.

81. Gerstner T, Bell N, Konig S. Oral valproic acid for epilepsy- long-term experience in therapy and side effects. Expert Opin Pharmacother. 2008; 9:285-92.

82. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther. 2003; 2(2):151-63.

83. Glick RD, Swendeman SL, Coffey DC, Rifkind RA, Marks PA, Richon VM, La Quaglia MP. Hybrid polar histone deacetylase inhibitor induces apoptosis and CD95/CD95 ligand expression in human neuroblastoma. Cancer Res. 1999; 59(17):4392-9.

84. Goncharenko-Khaider N, Lane D, Matte I, Rancourt C, Piche A. Biological Significance of Apoptosis in Ovarian Cancer: TRAIL Therapeutic Targeting, Ovarian Cancer - A Clinical and Translational Update, Dr. Ivan Diaz-Padilla (Ed.), 2013; ISBN: 978-953-51-1030-9.

85. Gottesman S, Wickner S, Maurizi MR. Protein quality control: triage by chaperones and proteases. Genes Dev. 1997; 11(7):815-23.

86. Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J. 2001; 20:6969-78.

87. Graven KK, Yu Q, Pan D, Roncarati JS, and Farber HW. Identification of an oxygen responsive enhancer element in the glyceraldehyde-3-phosphate dehydrogenase gene. Biochim Biophys Acta. 1999; 1447: 208-218.

88. Green DR. Apoptotic pathways: paper wraps stone blunts scissors. Cell. 2000; 102(1):1-4.

89. Grosfeld A, Andre J, Hauguel-De Mouzon S, Berra E, Pouyssegur J, and Guerre-Millo M. Hypoxia-inducible factor 1 transactivates the human leptin gene promoter. J Biol Chem. 2002; 277: 42953-42957.

90. Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell. 1997; 90(4):595-606.

91. Gui CY, Ngo L, Xu WS, Richon VM, Marks PA. Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. Proc Natl Acad Sci USA. 2004; 101(5):1241-6.

92. Gurvich N, Berman MG, Wittner BS, Gentleman RC, Klein PS, Green JB. Association of valproate-induced teratogenesis with histone deacetylase inhibition *in vivo*. FASEB J. 2005; 19:1166-8.

93. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell. 2004; 119(6):753-66.

94. Hägg M, Berndtsson M, Mandic A, Zhou R, Shoshan MC, Linder S. Induction of endoplasmic reticulum stress by ellipticine plant alkaloids. Mol Cancer Ther. 2004; 3(4):489-97.

95. Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci USA. 2003; 100(8):4389-94.

96. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000; 100(1):57-70.

97. Harikrishnan KN, Karagiannis TC, Chow MZ, El-Osta A. Effect of valproic acid on radiation-induced DNA damage in euchromatic and heterochromatic compartments. Cell Cycle. 2008; 7(4):468-76.

98. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961; 25:585-621.

99. Herr I, Debatin KM. Cellular stress response and apoptosis in cancer therapy. Blood. 2001; 98(9):2603-14.

100. Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, Schofield CJ. Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem. 2002; 277(29):26351-5.

101. Hitomi T, Matsuzaki Y, Yokota T, Takaoka Y, Sakai T. p15(INK4b) in HDAC inhibitor-induced growth arrest. FEBS Lett. 2003; 554(3):347-50.

102. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem. 2002; 277(5):3247-57.

103. Hrebackova J, Hrabeta J, Eckschlager T. Valproic acid in the complex therapy of malignant tumors. Curr Drug Targets. 2010; 11(3):361-79.

104. Hřebačková J, Poljaková J, Eckschlager T, Hraběta J, Procházka P, Smutný S, Stiborová M. Histone deacetylase inhibitors valproate and trichostatin A are toxic to neuroblastoma cells and modulate cytochrome P450 1A1, 1B1 and 3A4 expression in these cells. Interdiscip Toxicol. 2009; 2(3):205-10.

105. Hu CJ, Sataur A, Wang L, Chen H, Simon MC. The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. Mol Biol Cell. 2007; 18(11):4528-42.

106. Hu J, Discher DJ, Bishopric NH, and Webster KA. Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. Biochem Biophys Res Commun. 1998; 245: 894-899.

107. Huang DC, Strasser A. BH3-Only proteins-essential initiators of apoptotic cell death. Cell. 2000; 103(6):839-42.

108. Hughes EN, Engelsberg BN, Billings PC. Purification of nuclear proteins that bind to cisplatin-damaged DNA. Identity with high mobility group proteins 1 and 2. J Biol Chem. 1992; 267(19):13520-7.

109. Hwang PM, Bunz F, Yu J, Rago C, Chan TA, Murphy MP, Kelso GF, Smith RA, Kinzler KW, Vogelstein B. Ferredoxin reductase affects p53-dependent, 5-fluorouracil-induced apoptosis in colorectal cancer cells. Nat Med. 2001; 7(10):1111-7.

110. Chakravarthy S, Park YJ, Chodaparambil J, Edayathumangalam RS, Luger K. Structure and dynamic properties of nucleosome core particles. FEBS Lett. 2005; 579(4):895-8.

111. Chambers AE, Banerjee S, Chaplin T, Dunne J, Debernardi S, Joel SP, Young BD. Histone acetylation-mediated regulation of genes in leukaemic cells. Eur J Cancer. 2003; 39(8):1165-75.

112. Chang CC, Lin MT, Lin BR, Jeng YM, Chen ST, Chu CY, Chen RJ, Chang KJ, Yang PC, Kuo ML. Effect of connective tissue growth factor on hypoxia-inducible factor 1alpha degradation and tumor angiogenesis. J Natl Cancer Inst. 2006; 98(14):984-95.

113. Chang DW, Xing Z, Pan Y, Algeciras-Schimnich A, Barnhart BC, Yaish-Ohad S, Peter ME, Yang X. c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J. 2002; 21(14):3704-14.

114. Chavez-Blanco A, Perez-Plasencia C, Perez-Cardenas E, Carrasco-Legleu C, Rangel-Lopez E, Segura-Pacheco B et al. Antineoplastic effects of the DNA methylation inhibitor hydralazine and the histone deacetylase inhibitor valproic acid in cancer cell lines. Cancer Cell Int. 2006; 6:2.

115. Chen C, Pore N, Behrooz A, Ismail-Beigi F, and Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem. 2001; 276: 9519-9525.

116. Chen CS, Wang YC, Yang HC, Huang PH, Kulp SK, Yang CC, Lu YS, Matsuyama S, Chen CY, Chen CS. Histone deacetylase inhibitors sensitize prostate cancer cells to agents that produce DNA double-strand breaks by targeting Ku70 acetylation. Cancer Res. 2007; 67(11):5318-27.

117. Chen CS, Weng SC, Tseng PH, Lin HP, Chen CS. Histone acetylation-independent effect of histone deacetylase inhibitors on Akt through the reshuffling of protein phosphatase 1 complexes. J Biol Chem. 2005; 280(46):38879-87.

118. Chen G, Bower KA, Ma C, Fang S, Thiele CJ, Luo J. Glycogen synthase kinase 3beta (GSK3beta) mediates 6-hydroxydopamine-induced neuronal death. FASEB J. 2004; 18(10):1162-4. Epub 2004 May 7.

119. Chen G, Li A, Zhao M, Gao Y, Zhou T, Xu Y, Du Z, Zhang X, Yu X. Proteomic analysis identifies protein targets responsible for depsipeptide sensitivity in tumor cells. J Proteome Res. 2008; 7(7):2733-42.

120. Chen G, Masana MI, Manji HK. Lithium regulates PKC-mediated intracellular cross-talk and gene expression in the CNS *in vivo*. Bipolar Disord. 2000; 2(3 Pt 2):217-36.

121. Chen X, Wong P, Radany E, Wong JY. HDAC inhibitor, valproic acid, induces p53-dependent radiosensitization of colon cancer cells. Cancer Biother Radiopharm. 2009; 24(6):689-99.

122. Cheng JC, Klausen C, Leung PC. Hypoxia-inducible factor 1 alpha mediates epidermal growth factor-induced down-regulation of E-cadherin expression and cell invasion in human ovarian cancer cells. Cancer Lett. 2013; 329(2):197-206.

123. Chateauvieux S, Morceau F, Dicato M, Diederich M. Molecular and therapeutic potential and toxicity of valproic acid. J Biomed Biotechnol. 2010;2010. pii: 479364.

124. Chiang HL, Terlecky SR, Plant CP, Dice JF. A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science. 1989; 246(4928):382-5.

125. Chiche J, Ilc K, Laferrière J, Trottier E, Dayan F, Mazure NM, Brahimi-Horn MC, Pouysségur J. Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. Cancer Res. 2009; 69(1):358-68.

126. Cho RW, Clarke MF. Recent advances in cancer stem cells. Curr Opin Genet Dev. 2008; 18(1):48-53.

127. Chowdhury R, Godoy LC, Thiantanawat A, Trudel LJ, Deen WM, Wogan GN. Nitric oxide produced endogenously is responsible for hypoxia-induced HIF-1α stabilization in colon carcinoma cells. Chem Res Toxicol. 2012; 25(10):2194-202.

128. Iacopino F, Urbano R, Graziani G, Muzi A, Navarra P, Sica G.Valproic acid activity in androgen-sensitive and -insensitive human prostate cancer cells. Int J Oncol. 2008; 32:1293-303.

129. Insinga A, Monestiroli S, Ronzoni S, Gelmetti V, Marchesi F, Viale A, Altucci L, Nervi C, Minucci S, Pelicci PG. Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat Med. 2005; 11(1):71-6.

130. Ivan M, Kaelin WG Jr. The von Hippel-Lindau tumor suppressor protein. Curr Opin Genet Dev. 2001; 11(1):27-34.

131. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science. 2001; 292(5516):464-8.

132. Ivanov A, Cragg MS, Erenpreisa J, Emzinsh D, Lukman H, Illidge TM. Endopolyploid cells produced after severe genotoxic damage have the potential to repair DNA double strand breaks. J Cell Sci. 2003; 116(20):4095-106.

133. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science. 2001; 292(5516):468-72.

134. Jang M, Kim SS, Lee J. Cancer cell metabolism: implications for therapeutic targets. Exp Mol Med. 2013; 45:e45.

135. Jeanette D. Walton, David R. Kattan, Sharon K. Thomas, Barbara A. Spengler, Hong-Fen Guoy, June L. Biedler, Nai-Kong V. Cheungy, Robert A. Ross. Characteristics of Stem Cells from Human Neuroblastoma Cell Lines and in Tumors. Neoplasia. 2004; 6:838–845

136. Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, Yoo MA, Song EJ, Lee KJ, Kim KW. Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell. 2002; 111(5):709-20.

137. Jiang BH, Liu LZ. PI3K/PTEN signaling in angiogenesis and tumorigenesis. Adv Cancer Res. 2009; 102:19-65.

138. Jin S, Scotto KW. Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y. Mol Cell Biol. 1998; 18(7):4377-84.

139. Kachhap SK, Rosmus N, Collis SJ, Kortenhorst MS, Wissing MD, Hedayati M, Shabbeer S, Mendonca J, Deangelis J, Marchionni L, Lin J, Höti N, Nortier JW, DeWeese TL, Hammers H, Carducci MA. Downregulation of homologous recombination DNA repair

genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. PLoS One. 2010; 5(6):e11208.

140. Kaliszczak M, Trousil S, Åberg O, Perumal M, Nguyen QD, Aboagye EO. A novel small molecule hydroxamate preferentially inhibits HDAC6 activity and tumour growth. Br J Cancer. 2013; 108(2):342-50.

141. Kamal A, Thao L, Sensintaffar J, Zhang L, Boehm MF, Fritz LC, Burrows FJ. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature. 2003; 425(6956):407-10.

142. Kapitsinou PP, Liu Q, Unger TL, Rha J, Davidoff O, Keith B, Epstein JA, Moores SL, Erickson-Miller CL, Haase VH. Hepatic HIF-2 regulates erythropoietic responses to hypoxia in renal anemia. Blood. 2010; 116(16):3039-48.

143. Karagiannis TC, Harikrishnan KN, El-Osta A. Disparity of histone deacetylase inhibition on repair of radiation-induced DNA damage on euchromatin and constitutive heterochromatin compartments. Oncogene. 2007; 26(27):3963-71.

144. Karagiannis TC, Harikrishnan KN, El-Osta A. The histone deacetylase inhibitor, Trichostatin A, enhances radiation sensitivity and accumulation of gammaH2A.X. Cancer Biol Ther. 2005; 4(7):787-93.

145. Karagiannis TC, Kn H, El-Osta A. The epigenetic modifier, valproic acid, enhances radiation sensitivity. Epigenetics. 2006; 1(3):131-7.

146. Kawagoe R, Kawagoe H, Sano K.Valproic acid induces apoptosis in human leukemia cells by stimulating both caspase-dependent and -independent apoptotic signaling pathways. Leuk Res. 2002; 26:495-502.

147. Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell. 2003; 115(6):727-38.

148. Ke Q, Costa M. Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol. 2006; 70(5):1469-80.

149. Kermorgant S, Aparicio T, Dessirier V, Lewin MJ, Lehy T. Hepatocyte growth factor induces colonic cancer cell invasiveness via enhanced motility and protease overproduction. Evidence for PI3 kinase and PKC involvement. Carcinogenesis. 2001; 22(7):1035-42.

150. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet. 2001; 27(3):247-54.

151. Khorasanizadeh S. The nucleosome: from genomic organization to genomic regulation. Cell. 2004; 116(2):259-72.

152. Kietzmann T, Roth U, and Jungermann K. Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia-inducible factor-1 in rat hepatocytes. Blood. 1999; 94: 4177-4185.

153. Kim HJ, Bae SC. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. Am J Transl Res. 2011; 3(2):166-79.

154. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab. 2006; 3(3):177-85.

155. Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F. Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res. 2003; 63(21):7291-300.

156. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology. 2007A; 121(1):1-14.

157. Kim SH, Jeong JW, Park JA, Lee JW, Seo JH, Jung BK. et al. Regulation of the HIF-1alpha stability by histone deacetylases. Oncol Rep. 2007B; 17: 647-51.

158. Kitagawa K, Niikura Y. Caspase-independent mitotic death (CIMD). Cell Cycle. 2008; 7(8):1001-5.

159. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science. 2000; 290(5497):1717-21.

160. Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer. 2001; 1(2):157-62.

161. Ko LJ, Prives C. p53: puzzle and paradigm. Genes Dev. 1996; 10(9):1054-72.

162. Koh MY, Darnay BG, Powis G. Hypoxia-associated factor, a novel E3-ubiquitin ligase, binds and ubiquitinates hypoxia-inducible factor 1alpha, leading to its oxygen-independent degradation. Mol Cell Biol. 2008; 28(23):7081-95.

163. Koh MY, Powis G. Passing the baton: the HIF switch. Trends Biochem Sci. 2012; 37(9):364-72.

164. Kotrbová V, Mrázová B, Moserová M, Martínek V, Hodek P, Hudeček J, Frei E, Stiborová M. Cytochrome b(5) shifts oxidation of the anticancer drug ellipticine by cytochromes P450 1A1 and 1A2 from its detoxication to activation, thereby modulating its pharmacological efficacy. Biochem Pharmacol. 2011; 82(6):669-80.

165. Kovacs JJ, Murphy PJ, Gaillard S, Zhao X, Wu JT, Nicchitta CV, Yoshida M, Toft DO, Pratt WB, Yao TP. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. Mol Cell. 2005; 18(5):601-7.

166. Kramer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters MA et al. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. EMBO J. 2003; 22:3411-20.

167. Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P, et al. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res. 2003; 63: 1138-1143.

168. Kubbutat MH, Ludwig RL, Ashcroft M, Vousden KH. Regulation of Mdm2-directed degradation by the C terminus of p53. Mol Cell Biol. 1998; 18(10):5690-8.

169. Kuo MH, Allis CD. Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays. 1998; 20(8):615-26.

170. Kuo PL, Hsu YL, Chang CH, Lin CC. The mechanism of ellipticine-induced apoptosis and cell cycle arrest in human breast MCF-7 cancer cells. Cancer Lett. 2005A; 223(2):293-301.

171. Kuo PL, Hsu YL, Kuo YC, Chang CH, Lin CC. The anti-proliferative inhibition of ellipticine in human breast mda-mb-231 cancer cells is through cell cycle arrest and apoptosis induction. Anticancer Drugs. 2005B; 16(7):789-95.

172. Kuo PL, Hsu YL, Kuo YC, Chang CH, Lin CC. The anti-proliferative inhibition of ellipticine in human breast mda-mb-231 cancer cells is through cell cycle arrest and apoptosis induction. Anticancer Drugs. 2005C; 16(7):789-95.

173. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. 2002; 16(12):1466-71.

174. Lanz HL, Zimmerman RM, Brouwer J, Noteborn MH, Backendorf C. Mitotic catastrophe triggered in human cancer cells by the viral protein apoptin. Cell Death Dis. 2013; 4:e487.

175. LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller GA, et al. Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature (Lond). 2001; 412: 877-884.

176. Lee JS, Paull K, Alvarez M, Hose C, Monks A, Grever M, Fojo AT, Bates SE. Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. Mol Pharmacol. 1994; 46(4):627-38.

177. Lee PJ, Jiang BH, Chin BY, Iyer NV, Alam J, Semenza GL, and Choi AM. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J Biol Chem. 1997; 272: 5375-5381.

178. Leppä S, Eriksson M, Saffrich R, Ansorge W, Bohmann D. Complex functions of AP-1 transcription factors in differentiation and survival of PC12 cells. Mol Cell Biol. 2001; 21(13):4369-78.

179. Leppä S, Saffrich R, Ansorge W, Bohmann D. Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. EMBO J. 1998; 17(15):4404-13.

180. Lévi F, Okyar A, Dulong S, Innominato PF, Clairambault J. Circadian timing in cancer treatments. Annu Rev Pharmacol Toxicol. 2010; 50:377-421.

181. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008; 132(1):27-42.

182. Levy AP, Levy NS, Wegner S, and Goldberg MA. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J Biol Chem. 1995; 270: 13333-13340.

183. Li XN, Shu Q, Su JM, Perlaky L, Blaney SM, Lau CC. Valproic acid suppresses cell proliferation and induces apoptosis and senescence in medulloblastoma cells through histone hyperacetylation, p53-independent activation of p21Cip1, restoration of p16Ink4a/CDK4 pathway, and down regulation c-MYC gene expression. Mol Cancer Ther. 2005; 4:1912-22.

184. Liang D, Kong X, Sang N. Effects of histone deacetylase inhibitors on HIF-1. Cell Cycle. 2006; 5(21):2430-5.

185. Lill NL, Grossman SR, Ginsberg D, DeCaprio J, Livingston DM. Binding and modulation of p53 by p300/CBP coactivators.Nature. 1997; 387(6635):823-7.

186. Liu L, Scolnick DM, Trievel RC, Zhang HB, Marmorstein R, Halazonetis TD, Berger SL. p53 sites acetylated *in vitro* by PCAF and p300 are acetylated *in vivo* in response to DNA damage. Mol Cell Biol. 1999; 19(2):1202-9.

187. Liu YV, Baek JH, Zhang H, Diez R, Cole RN, Semenza GL. RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha. Mol Cell. 2007; 25(2):207-17.

188. Lok CN, and Ponka P. Identification of a hypoxia response element in the transferrin receptor gene. J Biol Chem. 1999; 274: 24147-24152.

189. Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis. 2000; 21(3):485-95.

190. Lu ZP, Ju ZL, Shi GY, Zhang JW, Sun J. Histone deacetylase inhibitor Trichostatin A reduces anti-DNA autoantibody production and represses IgH gene transcription. Biochem Biophys Res Commun. 2005; 330(1):204-9.

191. M. Peyrone. 'Ueber die Einwirkung des Ammoniaks auf Platinchlorür. Ann. Chem. Pharm. 1844; 51

192. Ma X, Ezzeldin HH, Diasio RB. Histone deacetylase inhibitors: current status and overview of recent clinical trials. Drugs. 2009; 69(14):1911-34.

193. Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. 2001; 15(20):2675-86.

194. Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature. 2001; 414(6863):550-4.

195. Margottin-Goguet F, Hsu JY, Loktev A, Hsieh HM, Reimann JD, Jackson PK. Prophase destruction of Emi1 by the SCF(betaTrCP/Slimb) ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase. Dev Cell. 2003; 4(6):813-26.

196. Marchion D, Munster P. Development of histone deacetylase inhibitors for cancer treatment. Exp Rev Anticancer Ther. 2007; 7:583–98.

197. Mariño-Ramírez L, Levine KM, Morales M, Zhang S, Moreland RT, Baxevanis AD, Landsman D. The Histone Database: an integrated resource for histones and histone fold-containing proteins. Database (Oxford). 2011; 2011:bar048.

198. Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol. 2007; 25(1):84-90.

199. Marks PA, Dokmanovic M. Histone deacetylase inhibitors: discovery and development as anticancer agents. Expert Opin Investig Drugs. 2005; 14(12):1497-511.

200. Marks PA, Richon VM, Rifkind RA. Cell cycle regulatory proteins are targets for induced differentiation of transformed cells: Molecular and clinical studies employing hybrid polar compounds. Int J Hematol. 1996; 63(1):1-17.

201. Martella G, Costa C, Pisani A, Cupini LM, Bernardi G, Calabresi P. Antiepileptic drugs on calcium currents recorded from cortical and PAG neurons: therapeutic implications for migraine. Cephalalgia. 2008; 28:1315-26.

202. Martin ML, Regan CM. The anticonvulsant valproate teratogen restricts the glial cell cycle at a defined point in the mid-G1 phase. Brain Res. 1991; 554:223–228.

203. Martinkova E, Dontenwill M, Frei E, Stiborova M. Cytotoxicity of and DNA adduct formation by ellipticine in human U87MG glioblastoma cancer cells. Neuro Endocrinol Lett. 2009; 30 Suppl 1:60-6.

204. Massey AC, Zhang C, Cuervo AM. Chaperone-mediated autophagy in aging and disease. Curr Top Dev Biol. 2006; 73:205-35.

205. Mastrogiannaki M, Matak P, Keith B, Simon MC, Vaulont S, Peyssonnaux C. HIF-2alpha, but not HIF-1alpha, promotes iron absorption in mice. J Clin Invest. 2009; 119(5):1159-66.

206. Mathupala SP, Rempel A, and Pedersen PL. Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. J Biol Chem. 2001; 276: 43407-43412.

207. Matsubara H, Watanabe M, Imai T, Yui Y, Mizushima Y, Hiraumi Y, Kamitsuji Y, Watanabe K, Nishijo K, Toguchida J, Nakahata T, Adachi S. Involvement of extracellular signal-regulated kinase activation in human osteosarcoma cell resistance to the histone deacetylase inhibitor FK228 [(1S,4S,7Z,10S,16E,21R)-7-ethylidene-4,21-bis(propan-2-yl)-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo[8.7.6]tricos-16-ene-3,6,9,19,22-pentone]. J Pharmacol Exp Ther. 2009; 328(3):839-48.

208. Matthay KK, George RE, Yu AL. Promising therapeutic targets in neuroblastoma. Clin Cancer Res. 2012; 18(10):2740-53.

209. Maxwell PH, Pugh CW, Ratcliffe PJ. Activation of the HIF pathway in cancer. Curr Opin Genet Dev. 2001; 11(3):293-9.

210. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 1999; 399(6733):271-5.

211. Maynard MA, Evans AJ, Shi W, Kim WY, Liu FF, Ohh M. Dominant-negative HIF-3 alpha 4 suppresses VHL-null renal cell carcinoma progression. Cell Cycle. 2007; 6(22):2810-6.

212. Melillo G, Musso T, Sica A, Taylor LS, Cox GW, and Varesio L. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J Exp Med. 1995; 182: 1683-1693.

213. Meunier H, Carraz G, Neunier Y, et al. Pharmacodynamic properties of N-dipropylacetic acid. Therapie. 1963; 18:435–8.

214. Mickley LA, Bates SE, Richert ND, Currier S, Tanaka S, Foss F, Rosen N, Fojo AT. Modulation of the expression of a multidrug resistance gene (mdr-1/P-glycoprotein) by differentiating agents. J Biol Chem. 1989; 264(30):18031-40.

215. Michaelis M, Michaelis UR, Fleming I, Suhan T, Cinatl J, Blaheta RA et al. Valproic acid inhibits angiogenesis *in vitro* and *in vivo*. Mol Pharmacol. 2004; 65:520-7.

216. Michaelis M, Suhan T, Michaelis UR, Beek K, Rothweiler F, Tausch L et al. Valproic acid induces extracellular signal-regulated kinase 1/2 activation and inhibits apoptosis in endothelial cells. Cell Death Differ. 2006; 13:446-53.

217. Minchenko A, Leshchinsky I, Opentanova I, Sang N, Srinivas V, Armstead V, and Caro J. Hypoxia-inducible factor-1-mediated expression of the 6-phospho-fructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect. J Biol Chem. 2002; 277: 6183-6187.

218. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Hideshima T, Akiyama M, Chauhan D, Munshi N, Gu X, Bailey C, Joseph M, Libermann TA, Richon VM, Marks PA, Anderson KC. Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. Proc Natl Acad Sci USA. 2004; 101(2):540-5.

219. Mitsiades N, Mitsiades CS, Richardson PG, McMullan C, Poulaki V, Fanourakis G, Schlossman R, Chauhan D, Munshi NC, Hideshima T, Richon VM, Marks PA, Anderson KC. Molecular sequelae of histone deacetylase inhibition in human malignant B cells. Blood. 2003; 101(10):4055-62.

220. Mizushima N, Klionsky DJ. Protein turnover via autophagy: implications for metabolism. Annu Rev Nutr. 2007; 27:19-40.

221. Mizushima N, Ohsumi Y, Yoshimori T. Autophagosome formation in mammalian cells. Cell Struct Funct. 2002; 27(6):421-9.

222. Molz L, Booher R, Young P, Beach D. cdc2 and the regulation of mitosis: six interacting mcs genes. Genetics. 1989; 122(4):773-82.

223. Montgomery RL, Davis CA, Potthoff MJ, Haberland M, Fielitz J, Qi X, Hill JA, Richardson JA, Olson EN. Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility. Genes Dev. 2007; 21(14):1790-802.

224. Morita M, Ohneda O, Yamashita T, Takahashi S, Suzuki N, Nakajima O, Kawauchi S, Ema M, Shibahara S, Udono T, Tomita K, Tamai M, Sogawa K, Yamamoto M, Fujii-Kuriyama Y. HLF/HIF-2alpha is a key factor in retinopathy of prematurity in association with erythropoietin. EMBO J. 2003; 22(5):1134-46.

225. Moroni MC, Hickman ES, Lazzerini Denchi E, Caprara G, Colli E, Cecconi F, Müller H, Helin K. Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol. 2001; 3(6):552-8.

226. Munshi A, Kurland JF, Nishikawa T, Tanaka T, Hobbs ML, Tucker SL, Ismail S, Stevens C, Meyn RE. Histone deacetylase inhibitors radiosensitize human melanoma cells by suppressing DNA repair activity. Clin Cancer Res. 2005; 11(13):4912-22.

227. Munshi A, Tanaka T, Hobbs ML, Tucker SL, Richon VM, Meyn RE. Vorinostat, a histone deacetylase inhibitor, enhances the response of human tumor cells to ionizing radiation through prolongation of gamma-H2AX foci. Mol Cancer Ther. 2006; 5(8):1967-74.

228. Munster P, Marchion D, Bicaku E, Lacevic M, Kim J, Centeno B, Daud A, Neuger A, Minton S, Sullivan D. Clinical and biological effects of valproic acid as a histone deacetylase inhibitor on tumor and surrogate tissues: phase I/II trial of valproic acid and epirubicin/FEC. Clin Cancer Res. 2009; 15(7):2488-96.

229. Mutze K, Langer R, Becker K, Ott K, Novotny A, Luber B, Hapfelmeier A, Göttlicher M, Höfler H, Keller G. Histone deacetylase (HDAC) 1 and 2 expression and chemotherapy in gastric cancer. Ann Surg Oncol. 2010; 17(12):3336-43.

230. Nakagawa I, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, Kamimoto T, Nara A, Funao J, Nakata M, Tsuda K, Hamada S, Yoshimori T. Autophagy defends cells against invading group A Streptococcus. Science. 2004; 306(5698):1037-40.

231. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. N Engl J Med. 1993; 328(12):847-54.

232. Nakamura S, Roth JA, Mukhopadhyay T. Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. Mol Cell Biol. 2000; 20(24):9391-8.

233. Nakata S, Yoshida T, Horinaka M, Shiraishi T, Wakada M, Sakai T. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. Oncogene. 2004; 23(37):6261-71.

234. Namdar M, Perez G, Ngo L, Marks PA. Selective inhibition of histone deacetylase 6 (HDAC6) induces DNA damage and sensitizes transformed cells to anticancer agents. Proc Natl Acad Sci USA. 2010; 107(46):20003-8.

235. Nau H, Hauck R S, Ehlers K. Valproic acid-induced neural tube defects in mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanisms. Pharmacol Toxicol. 1991; 69:310–21.

236. Newbold A, Lindemann RK, Cluse LA, Whitecross KF, Dear AE, Johnstone RW. Characterisation of the novel apoptotic and therapeutic activities of the histone deacetylase inhibitor romidepsin. Mol Cancer Ther. 2008; 7(5):1066-79.

237. Nguyen DM, Schrump WD, Tsai WS, Chen A, Stewart JH 4th, Steiner F, Schrump DS. Enhancement of depsipeptide-mediated apoptosis of lung or esophageal cancer cells by flavopiridol: activation of the mitochondria-dependent death-signaling pathway. J Thorac Cardiovasc Surg. 2003; 125(5):1132-42.

238. Nguyen SV and Claycomb WC. Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes. Biochem Biophys Res Commun. 1999; 265: 382-386.

239. Nimmanapalli R, Fuino L, Stobaugh C, Richon V, Bhalla K. Cotreatment with the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) enhances imatinib-induced apoptosis of Bcr-Abl-positive human acute leukemia cells. Blood. 2003; 101(8):3236-9.

240. Noh EJ, Lim DS, Jeong G, Lee JS. An HDAC inhibitor, trichostatin A, induces a delay at G2/M transition, slippage of spindle checkpoint, and cell death in a transcription-dependent manner. Biochem Biophys Res Commun. 2009; 378(3):326-31.

241. Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V, Kaelin WG. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol. 2000; 2(7):423-7.

242. Ohndorf UM, Rould MA, He Q, Pabo CO, Lippard SJ. Basis for recognition of cisplatin-modified DNA by high-mobility-group proteins. Nature. 1999; 399(6737):708-12.

243. Olsen CM, Meussen-Elholm ET, Roste LS, Tauboll E. Antiepileptic drugs inhibit cell growth in the human breast cancer cell line MCF7. Mol Cell Endocrinol. 2004; 213:173-9.

244. Ong SG, Hausenloy DJ. Hypoxia-inducible factor as a therapeutic target for cardioprotection. Pharmacol Ther. 2012; 136(1):69-81.

245. O'Rourke JF, Pugh CW, Bartlett SM, and Ratcliffe PJ. Identification of hypoxically inducible mRNAs in HeLa cells using differential-display PCR. Role of hypoxia-inducible factor-1. Eur J Biochem. 1996; 241: 403-410.

246. Ozaki K, Minoda A, Kishikawa F, Kohno M. Blockade of the ERK pathway markedly sensitizes tumor cells to HDAC inhibitor-induced cell death. Biochem Biophys Res Commun. 2006; 339(4):1171-7.

247. Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney Int. 2008; 73(9):994-1007.

248. Palmero I, Pantoja C, Serrano M. p19ARF links the tumour suppressor p53 to Ras. Nature. 1998; 395(6698):125-6.

249. Pan JY, Zhou SH. The hedgehog signaling pathway, a new therapeutic target for treatment of ischemic heart disease. Pharmazie. 2012; 67(6):475-81.

250. Paoletti C, Le Pecq JB, Dat-Xuong N, Juret P, Garnier H, Amiel JL, Rouesse J. Antitumor activity, pharmacology, and toxicity of ellipticines, ellipticinium, and 9-hydroxy derivatives: preliminary clinical trials of 2-methyl-9-hydroxy ellipticinium (NSC 264-137). Recent Results Cancer Res. 1980; 74:107-23.

251. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab. 2006; 3(3):187-97.

252. Pearl LH, Prodromou C, Workman P. The Hsp90 molecular chaperone: an open and shut case for treatment. Biochem J. 2008; 410(3):439-53.

253. Peart MJ, Tainton KM, Ruefli AA, Dear AE, Sedelies KA, O'Reilly LA, Waterhouse NJ, Trapani JA, Johnstone RW. Novel mechanisms of apoptosis induced by histone deacetylase inhibitors. Cancer Res. 2003;63(15):4460-71.

254. Pfaffl MW1, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002; 30(9):e36.

255. Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and tetratogen. J Biol Chem. 2001; 276:36734-41.

256. Poljaková J, Eckschlager T, Hrabeta J, Hrebacková J, Smutný S, Frei E, Martínek V, Kizek R, Stiborová M. The mechanism of cytotoxicity and DNA adduct formation by the anticancer drug ellipticine in human neuroblastoma cells. Biochem Pharmacol. 2009; 77(9):1466-79.

257. Poljaková J, Eckschlager T, Hřebačková J, Hraběta J, Stiborová M. The comparison of cytotoxicity of the anticancer drugs doxorubicin and ellipticine to human neuroblastoma cells. Interdiscip Toxicol. 2008; 1(2):186-9.

258. Poljakova J, Frei E, Gomez JE, Aimova D, Eckschlager T, Hrabeta J, Stiborova M. DNA adduct formation by the anticancer drug ellipticine in human leukemia HL-60 and CCRF-CEM cells. Cancer Lett. 2007; 252(2): 270–9.

259. Poljakova J, Hrebackova J, Dvorakova M, Moserova M, Eckschlager T, Hrabeta J, Göttlicherova M, Kopejtkova B, Frei E, Kizek R, Stiborova M. Anticancer agent ellipticine combined with histone deacetylase inhibitors, valproic acid and trichostatin A, is an effective DNA damage strategy in human neuroblastoma. Neuro Endocrinol Lett. 2011; 32 Suppl 1:101-16.

260. Pollard PJ, Spencer-Dene B, Shukla D, Howarth K, Nye E, El-Bahrawy M, Deheragoda M, Joannou M, McDonald S, Martin A, Igarashi P, Varsani-Brown S, Rosewell I, Poulsom R, Maxwell P, Stamp GW, Tomlinson IP. Targeted inactivation of fh1 causes proliferative renal cyst development and activation of the hypoxia pathway. Cancer Cell. 2007; 11(4):311-9.

261. Powis G, Kirkpatrick DL. Thioredoxin signaling as a target for cancer therapy. Curr Opin Pharmacol. 2007; 7(4):392-7.

262. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med. 2003; 9(6):677-84.

263. Qian DZ, Kachhap SK, Collis SJ, Verheul HM, Carducci MA, Atadja P, Pili R. Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1 alpha. Cancer Res. 2006; 66(17):8814-21.

264. Richard DE, Berra E, Gothié E, Roux D, Pouysségur J. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem. 1999; 274(46):32631-7.

265. Richard DE, Berra E, Pouyssegur J. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. J Biol Chem. 2000; 275(35):26765-71.

266. Richon VM, Sandhoff TW, Rifkind RA, Marks PA. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA. 2000; 97(18):10014-9.

267. Robbins AR, Jablonski SA, Yen TJ, Yoda K, Robey R, Bates SE, Sackett DL. Inhibitors of histone deacetylases alter kinetochore assembly by disrupting pericentromeric heterochromatin. Cell Cycle. 2005; 4(5):717-26.

268. Robinson CM, Ohh M. The multifaceted von Hippel-Lindau tumour suppressor protein. FEBS Lett. 2014 Feb 25. doi: 10.1016/j.febslet.2014.02.026. [Epub ahead of print]

269. Rodriguez MS, Desterro JM, Lain S, Lane DP, Hay RT. Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. Mol Cell Biol. 2000; 20(22):8458-67.

270. Rolfs A, Kvietikova I, Gassmann M, and Wenger RH. Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. J Biol Chem. 1997; 272: 20055-20062.

271. Romanov VS, Abramova MV, Svetlikova SB, Bykova TV, Zubova SG, Aksenov ND, Fornace AJ Jr, Pospelova TV, Pospelov VA. p21(Waf1) is required for cellular senescence but not for cell cycle arrest induced by the HDAC inhibitor sodium butyrate. Cell Cycle. 2010; 9(19):3945-55.

272. Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. Mol Oncol. 2007; 1(1):19-25.

273. Ropero S, Fraga MF, Ballestar E, Hamelin R, Yamamoto H, Boix-Chornet M, Caballero R, Alaminos M, Setien F, Paz MF, Herranz M, Palacios J, Arango D, Orntoft TF, Aaltonen LA, Schwartz S Jr, Esteller M. A truncating mutation of HDAC2 in human cancers confers resistance to histone deacetylase inhibition. Nat Genet. 2006; 38(5):566-9.

274. Rosato RR, Almenara JA, Grant S. The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of p21CIP1/WAF1 1. Cancer Res. 2003; 63(13):3637-45.

275. Rosato RR, Almenara JA, Yu C, Grant S. Evidence of a functional role for p21WAF1/CIP1 down-regulation in synergistic antileukemic interactions between the histone deacetylase inhibitor sodium butyrate and flavopiridol. Mol Pharmacol. 2004; 65(3):571-81.

276. Rosato RR, Grant S. Histone deacetylase inhibitors: insights into mechanisms of lethality. Expert Opin Ther Targets. 2005; 9(4):809-24.

277. Rosato RR, Maggio SC, Almenara JA, Payne SG, Atadja P, Spiegel S, Dent P, Grant S. The histone deacetylase inhibitor LAQ824 induces human leukemia cell death through a process involving XIAP down-regulation, oxidative injury, and the acid sphingomyelinase-dependent generation of ceramide. Mol Pharmacol. 2006; 69(1):216-25.

278. Rosenberg B, Vancamp L, Krigas T. Inhibition of cell division in Escherichia coli by electrolysis products from a platinum electrode. Nature. 1965; 205:698-9.

279. Rosenberg G. The mechanisms of action of valproate in neuropsychiatric disorders: can we seethe forest for the trees? Cell Mol Life Sci. 2007; 64:2090-103.

280. Rössig L, Li H, Fisslthaler B, Urbich C, Fleming I, Förstermann U, Zeiher AM, Dimmeler S. Inhibitors of histone deacetylation downregulate the expression of endothelial nitric oxide synthase and compromise endothelial cell function in vasorelaxation and angiogenesis. Circ Res. 2002; 91(9):837-44.

281. Ruefli AA, Ausserlechner MJ, Bernhard D, Sutton VR, Tainton KM, Kofler R, Smyth MJ, Johnstone RW. The histone deacetylase inhibitor and chemotherapeutic agent suberoylanilide hydroxamic acid (SAHA) induces a cell-death pathway characterized by cleavage of Bid and production of reactive oxygen species. Proc Natl Acad Sci USA. 2001; 98(19):10833-8.

282. Ruefli AA, Bernhard D, Tainton KM, Kofler R, Smyth MJ, Johnstone RW. Suberoylanilide hydroxamic acid (SAHA) overcomes multidrug resistance and induces cell death in P-glycoprotein-expressing cells. Int J Cancer. 2002; 99(2):292-8.

283. Ruggiero A, Trombatore G, Triarico S, Arena R, Ferrara P, Scalzone M, Pierri F, Riccardi R. Platinum compounds in children with cancer: toxicity and clinical management. Anticancer Drugs. 2013; 24(10):1007-19.

284. Ryan KM, Phillips AC, Vousden KH. Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol. 2001; 13(3):332-7.

285. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J. 1998; 17(9):2596-606.

286. Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, Anderson CW, Appella E. DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev. 1998; 12(18):2831-41.

287. Sandor V, Senderowicz A, Mertins S, Sackett D, Sausville E, Blagosklonny MV, Bates SE. P21-dependent g(1)arrest with downregulation of cyclin D1 and upregulation of cyclin E by the histone deacetylase inhibitor FR901228. Br J Cancer. 2000; 83(6):817-25.

288. Sasakawa Y, Naoe Y, Sogo N, Inoue T, Sasakawa T, Matsuo M, Manda T, Mutoh S. Marker genes to predict sensitivity to FK228, a histone deacetylase inhibitor. Biochem Pharmacol. 2005; 69(4):603-16.

289. Scortegagna M, Ding K, Zhang Q, Oktay Y, Bennett MJ, Bennett M, Shelton JM, Richardson JA, Moe O, Garcia JA. HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. Blood. 2005; 105(8):3133-40.

290. Semenza GL. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. J Clin Invest. 2013; 123(9):3664-71.

291. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, Giallongo A. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem. 1996; 271(51):32529-37.

292. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, and Giallongo A. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem. 1996; 271: 32529-32537.

293. Semenza GL, Nejfelt MK, Chi SM, and Antonarakis SE. Hypoxia-inducible nuclear factors bind to an enhancer element located 3′ to the human erythropoietin gene. Proc Natl Acad Sci USA. 1991; 88: 5680-5684.

294. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem. 1994; 269(38):23757-63.

295. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol. 1992; 12(12):5447-54.

296. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol. 1999; 15:551-78.

297. Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. Mol Cell. 2012; 48(2):158-67.

298. Shannon AM, Bouchier-Hayes DJ, Condron CM, Toomey D: Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies. Cancer Treat Rev. 2003; 29(4): 297-307.

299. Shao Y, Gao Z, Marks PA, Jiang X. Apoptotic and autophagic cell death induced by histone deacetylase inhibitors. Proc Natl Acad Sci USA. 2004; 101(52):18030-5.

300. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. Carcinogenesis. 2010; 31(1):27-36.

301. Shimoda LA, Fallon M, Pisarcik S, Wang J, Semenza GL. HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial myocytes. Am J Physiol Lung Cell Mol Physiol. 2006; 291(5):L941-9.

302. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. Science. 2004; 306(5698):990-5.

303. Shoji M, Ninomiya I, Makino I, Kinoshita J, Nakamura K, Oyama K, Nakagawara H, Fujita H, Tajima H, Takamura H, Kitagawa H, Fushida S, Harada S, Fujimura T, Ohta T. Valproic acid, a histone deacetylase inhibitor, enhances radiosensitivity in esophageal squamous cell carcinoma. Int J Oncol. 2012; 40(6):2140-6.

304. Scheid A, Wenger RH, Schaffer L, Camenisch I, Distler O, Ferenc A, Cristina H, Ryan HE, Johnson RS, Wagner KF, et al. Physiologically low oxygen concentrations in fetal skin regulate hypoxia-inducible factor 1 and transforming growth factor-beta3. FASEB J. 2002; 16: 411-413.

305. Schenkman JB, Jansson I. The many roles of cytochrome b5. Pharmacol Ther. 2003; 97(2):139-52.

306. Schneider-Brachert W, Heigl U, Ehrenschwender M. Membrane trafficking of death receptors: implications on signalling. Int J Mol Sci. 2013; 14(7):14475-503.

307. Schuchmann M, Schulze-Bergkamen H, Fleischer B, Schattenberg JM, Siebler J, Weinmann A, Teufel A, Wörns M, Fischer T, Strand S, Lohse AW, Galle PR. Histone deacetylase inhibition by valproic acid down-regulates c-FLIP/CASH and sensitizes hepatoma cells towards CD95- and TRAIL receptor-mediated apoptosis and chemotherapy. Oncol Rep. 2006; 15(1):227-30.

308. Schwaller MA, Allard B, Lescot E, Moreau F. Protonophoric activity of ellipticine and isomers across the energy-transducing membrane of mitochondria. J Biol Chem. 1995; 270(39):22709-13.

309. Schwaller MA, Allard B, Lescot E, Moreau F. Protonophoric activity of ellipticine and isomers across the energy-transducing membrane of mitochondria. J Biol Chem. 1995; 270(39):22709-13.

310. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene. 2003; 22(47):7265-79.

311. Spange S, Wagner T, Heinzel T, Kramer OH. Acetylation of non-histone proteins modulates cellular signalling at multiple levels. Int J Biochem Cell Biol. 2009; 41:185-98.

312. Stambolic V, MacPherson D, Sas D, Lin Y, Snow B, Jang Y, Benchimol S, Mak TW. Regulation of PTEN transcription by p53. Mol Cell. 2001; 8(2):317-25.

313. Stiborová M, Eckschlager T, Poljaková J, Hraběta J, Adam V, Kizek R, Frei E. The synergistic effects of DNA-targeted chemotherapeutics and histone deacetylase inhibitors as therapeutic strategies for cancer treatment. Curr Med Chem. 2012B; 19(25):4218-38.

314. Stiborová M, Martínek V, Schmeiser HH, Frei E. Modulation of CYP1A1-mediated oxidation of carcinogenic azo dye Sudan I and its binding to DNA by cytochrome b5. Neuro Endocrinol Lett. 2006A; 27 Suppl 2:35-9.

315. Stiborova M, Poljakova J, Eckschlager T, Kizek R, Frei E Analysis of covalent ellipticine- and doxorubicin-derived adducts in DNA of neuroblastoma cells by the ^{32}P-postlabeling technique. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2012A; 156(2):115-21.

316. Stiborová M, Rupertová M, Frei E. Cytochrome P450- and peroxidase-mediated oxidation of anticancer alkaloid ellipticine dictates its anti-tumor efficiency. Biochim Biophys Acta. 2011; 1814(1):175-85.

317. Stiborová M, Poljaková J, Ryslavá H, Dracínský M, Eckschlager T, Frei E. Mammalian peroxidases activate anticancer drug ellipticine to intermediates forming deoxyguanosine adducts in DNA identical to those found *in vivo* and generated from 12-hydroxyellipticine and 13-hydroxyellipticine. Int J Cancer. 2007;120(2):243-51.

318. Stiborova M, Rupertova M, Schmeiser HH, Frei E. Molecular mechanisms of antineoplastic action of an anticancer drug ellipticine. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2006B; 150(1):13-23.

319. Stiborová M, Sejbal J, Borek-Dohalská L, Aimová D, Poljaková J, Forsterová K, Rupertová M, Wiesner J, Hudecek J, Wiessler M, Frei E. The anticancer drug ellipticine

forms covalent DNA adducts, mediated by human cytochromes P450, through metabolism to 13-hydroxyellipticine and ellipticine N2-oxide. Cancer Res. 2004; 64(22):8374-80.

320. Storz P. Reactive oxygen species in tumor progression. Front Biosci. 2005; 10:1881-96.

321. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell. 2001; 8(3):613-21.

322. Taddei A, Maison C, Roche D, Almouzni G. Reversible disruption of pericentric heterochromatin and centromere function by inhibiting deacetylases. Nat Cell Biol. 2001; 3(2):114-20.

323. Takahashi Y, Takahashi S, Shiga Y, Yoshimi T, and Miura T. Hypoxic induction of prolyl 4-hydroxylase α (I) in cultured cells. J Biol Chem. 2000; 275: 14139-14146.

324. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol. 2000; 157(2):411-21.

325. Tan J, Zhuang L, Jiang X, Yang KK, Karuturi KM, Yu Q. Apoptosis signal-regulating kinase 1 is a direct target of E2F1 and contributes to histone deacetylase inhibitor-induced apoptosis through positive feedback regulation of E2F1 apoptotic activity. J Biol Chem. 2006; 281(15):10508-15.

326. Tang R, Faussat AM, Majdak P, Perrot JY, Chaoui D, Legrand O et al. Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. Leukemia. 2004; 18:1246-51.

327. Teicher BA, Menon K, Alvarez E, Galbreath E, Shih C, Faul M. Antiangiogenic and antitumor effects of a protein kinase Cbeta inhibitor in human T98G glioblastoma multiforme xenografts. Clin Cancer Res. 2001B; 7(3):634-40.

328. Teicher BA, Menon K, Alvarez E, Liu P, Shih C, Faul MM. Antiangiogenic and antitumor effects of a protein kinase C beta inhibitor in human hepatocellular and gastric cancer xenografts. In Vivo. 2001A; 15(3):185-93.

329. Tentori L, Muzi A, Dorio AS, Dolci S, Campolo F, Vernole P, Lacal PM, Praz F, Graziani G. MSH3 expression does not influence the sensitivity of colon cancer HCT116 cell line to oxaliplatin and poly(ADP-ribose) polymerase (PARP) inhibitor as monotherapy or in combination. Cancer Chemother Pharmacol. 2013; 72(1):117-25.

330. Thurn KT, Thomas S, Moore A, Munster PN. Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer. Future Oncol. 2011; 7(2):263-83.

331. Trepel J, Mollapour M, Giaccone G, Neckers L. Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer. 2010; 10(8):537-49.

332. Tsai YP, Wu KJ. Hypoxia-regulated target genes implicated in tumor metastasis. J Biomed Sci. 2012;19:102.

333. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem. 2006; 281(14):9030-7.

334. Um JH, Kang CD, Bae JH, Shin GG, Kim DW, Kim DW, Chung BS, Kim SH. Association of DNA-dependent protein kinase with hypoxia inducible factor-1 and its implication in resistance to anticancer drugs in hypoxic tumor cells. Exp Mol Med. 2004; 36(3):233-42.

335. Ungerstedt JS, Sowa Y, Xu WS, Shao Y, Dokmanovic M, Perez G, Ngo L, Holmgren A, Jiang X, Marks PA. Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors. Proc Natl Acad Sci USA. 2005; 102(3):673-8.

336. Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expr. 1996; 5(4-5):245-53.

337. Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A., Gocayne, J.D., Amanatides, P., Ballew, R.M., Huson, D.H., Wortman, J.R., Zhang, Q., Kodira, C.D., Zheng, X.H., Chen, L., Skupski, M., Subramanian, G., Thomas, P.D., et al. The sequence of the human genome. Science. 2001; 291(5507):1304-51.

338. Ververis K, Hiong A, Karagiannis TC, Licciardi PV. Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. Biologics. 2013; 7:47-60.

339. Vidal A, Koff A. Cell-cycle inhibitors: three families united by a common cause. Gene. 2000; 247(1-2):1-15.

340. Virshup DM, Shenolikar S. From promiscuity to precision: protein phosphatases get a makeover. Mol Cell. 2009; 33(5):537-45.

341. Vitale I, Galluzzi L, Castedo M, Kroemer G. Mitotic catastrophe: a mechanism for avoiding genomic instability. Nat Rev Mol Cell Biol. 2011; 12(6):385-92.

342. Vrana JA, Decker RH, Johnson CR, Wang Z, Jarvis WD, Richon VM, Ehinger M, Fisher PB, Grant S. Induction of apoptosis in U937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. Oncogene. 1999; 18(50):7016-25.

343. Wang L, Xiang S, Williams KA, Dong H, Bai W, Nicosia SV, Khochbin S, Bepler G, Zhang X. Depletion of HDAC6 enhances cisplatin-induced DNA damage and apoptosis in non-small cell lung cancer cells. PLoS One. 2012; 7(9):e44265.

344. Wang X. The expanding role of mitochondria in apoptosis. Genes Dev. 2001; 15(22):2922-33.

345. Wang XJ, Si LB. Advances on hypoxia inducible factor-1. Chin Med J (Engl). 2013; 126(18):3567-71.

346. Wenger RH. Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. FASEB J. 2002; 16(10):1151-62.

347. Westermark UK, Wilhelm M, Frenzel A, Henriksson MA. The MYCN oncogene and differentiation in neuroblastoma. Semin Cancer Biol. 2011; 21(4):256-66.

348. Wickner S, Maurizi MR, Gottesman S. Posttranslational quality control: folding, refolding, and degrading proteins. Science. 1999; 286(5446):1888-93.

349. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol. 2005; 17(6):617-25.

350. Wilson AJ, Byun DS, Popova N, Murray LB, L'Italien K, Sowa Y, Arango D, Velcich A, Augenlicht LH, Mariadason JM. Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. J Biol Chem. 2006; 281(19):13548-58.

351. Wood JR, Nelson-Degrave VL, Jansen E, McAllister JM, Mosselman S, Strauss JF 3rd. Valproate-induced alterations in human theca cell gene expression: clues to the association between valproate use and metabolic side effects. Physiol Genomics. 2005; 20:233-43.

352. Workman P, Burrows F, Neckers L, Rosen N. Drugging the cancer chaperone HSP90: combinatorial therapeutic exploitation of oncogene addiction and tumor stress. Ann NY Acad Sci. 2007; 1113:202-16.

353. Wu Y, Mehew JW, Heckman CA, Arcinas M, Boxer LM. Negative regulation of bcl-2 expression by p53 in hematopoietic cells. Oncogene. 2001; 20(2):240-51.

354. Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, et al. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res. 2000; 60: 7075-7083.

355. Xie C, Edwards H, Lograsso SB, Buck SA, Matherly LH, Taub JW, Ge Y. Valproic acid synergistically enhances the cytotoxicity of clofarabine in pediatric acute myeloid leukemia cells. Pediatr Blood Cancer. 2012; 59(7):1245-51.

356. Xu W, Ngo L, Perez G, Dokmanovic M, Marks PA. Intrinsic apoptotic and thioredoxin pathways in human prostate cancer cell response to histone deacetylase inhibitor. Proc Natl Acad Sci USA. 2006; 103(42):15540-5.

357. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action.Oncogene. 2007B; 26(37):5541-52.

358. Xu WS, Perez G, Ngo L, Gui CY, Marks PA. Induction of polyploidy by histone deacetylase inhibitor: a pathway for antitumor effects. Cancer Res. 2005; 65(17):7832-9.

359. Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity. 2007A; 27(1):135-44.

360. Yang Y, Zhao Y, Liao W, Yang J, Wu L, Zheng Z, Yu Y, Zhou W, Li L, Feng J, Wang H, Zhu WG. Acetylation of FoxO1 activates Bim expression to induce apoptosis in response to histone deacetylase inhibitor depsipeptide treatment. Neoplasia. 2009; 11(4):313-24.

361. Yazbeck VY, Buglio D, Georgakis GV, Li Y, Iwado E, Romaguera JE, Kondo S, Younes A. Temsirolimus downregulates p21 without altering cyclin D1 expression and induces autophagy and synergizes with vorinostat in mantle cell lymphoma. Exp Hematol. 2008; 36(4):443-50.

362. Yoo YG, Kong G, Lee MO. Metastasis-associated protein 1 enhances stability of hypoxia-inducible factor-1alpha protein by recruiting histone deacetylase 1. EMBO J. 2006; 25(6):1231-41.

363. Yoshida M, Kijima M, Akita M, Beppu T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J Biol Chem. 1990; 265(28):17174-9.

364. Zelzer E, Levy Y, Kahana C, Shilo BZ, Rubinstein M, Cohen B. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. EMBO J. 1998; 17(17):5085-94.

365. Zeng L, Zhou MM. Bromodomain: an acetyl-lysine binding domain. FEBS Lett. 2002; 513(1):124-8.

366. Zhang QC, Jiang SJ, Zhang S, Ma XB. Histone deacetylase inhibitor trichostatin A enhances anti-tumor effects of docetaxel or erlotinib in A549 cell line. Asian Pac J Cancer Prev. 2012; 13(7):3471-6.

367. Zhang XD, Gillespie SK, Borrow JM, Hersey P. The histone deacetylase inhibitor suberic bishydroxamate regulates the expression of multiple apoptotic mediators and induces mitochondria-dependent apoptosis of melanoma cells. Mol Cancer Ther. 2004; 3(4):425-35.

368. Zhang Y, Adachi M, Kawamura R, Zou HC, Imai K, Hareyama M, Shinomura Y. Bmf contributes to histone deacetylase inhibitor-mediated enhancing effects on apoptosis after ionizing radiation. Apoptosis. 2006; 11(8):1349-57.

369. Zhao T, Gao S, Wang X, Liu J, Duan Y, Yuan Z, Sheng J, Li S, Wang F, Yu M, Ren H, Hao J. Hypoxia-inducible factor-1α regulates chemotactic migration of pancreatic ductal adenocarcinoma cells through directly transactivating the CX3CR1 gene. PLoS One. 2012; 7(8):e43399.

370. Zhao Y, Tan J, Zhuang L, Jiang X, Liu ET, Yu Q. Inhibitors of histone deacetylases target the Rb-E2F1 pathway for apoptosis induction through activation of proapoptotic protein Bim. Proc Natl Acad Sci USA. 2005; 102(44):16090-5.

371. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res. 1999; 59(22):5830-5.

372. Zhu P, Martin E, Mengwasser J, Schlag P, Janssen KP, Göttlicher M. Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. Cancer Cell. 2004; 5(5):455-63.

373. Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ, Roussel MF. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev. 1998; 12(15):2424-33.

374. Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, Giaccia AJ. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev. 2000; 14(4):391-6.

375. Zupkovitz G, Tischler J, Posch M, Sadzak I, Ramsauer K, Egger G, Grausenburger R, Schweifer N, Chiocca S, Decker T, Seiser C. Negative and positive regulation of gene expression by mouse histone deacetylase 1. Mol Cell Biol. 2006; 26(21):7913-28.

9. PŘÍLOHY

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ORIGINAL ARTICLE

The comparison of cytotoxicity of the anticancer drugs doxorubicin and ellipticine to human neuroblastoma cells

Jitka POLJAKOVÁ [1], Tomáš ECKSCHLAGER [2], Jana HŘEBAČKOVÁ [2], Jan HRABĚTA [2], Marie STIBOROVÁ [1]

[1] Department of Biochemistry, Faculty of Science, Charles University, Prague, Albertov 2030, 128 40 Prague 2, CZECH REPUBLIC
[2] Department of Pediatric Hematology and Oncology, 2nd Medical School, Charles University and University Hospital Motol, Prague 5, CZECH REPUBLIC

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ABSTRACT
Ellipticine is an antineoplastic agent, whose mode of action is based mainly on DNA intercalation, inhibition of topoisomerase II and formation of covalent DNA adducts mediated by cytochromes P450 and peroxidases. Here, the cytotoxicity of ellipticine to human neuroblastoma derived cell lines IMR-32 and UKF-NB-4 was investigated. Treatment of neuroblastoma cells with ellipticine was compared with that of these cancer cells with doxorubicin. The toxicity of ellipticine was essentially the same as that of doxorubicin to UKF-NB-4 cells, but doxorubicin is much more effective to inhibit the growth of the IMR-32 cell line than ellipticine. Hypoxic conditions used for the cell cultivation resulted in a decrease in ellipticine and/or doxorubicin toxicity to IMR-32 and UKF-NB-4 neuroblastoma cells.

KEY WORDS: anticancer drug; ellipticine; doxorubicin; human neuroblastoma cells; cytotoxicity

Introduction

Neuroblastoma is the most frequent solid extra cranial tumor in children and is a major cause of death from neoplasia in infancy (Maris and Mathay, 1999). These tumors are biologically heterogeneous, with cell populations differing in their genetic programs, maturation stage and malignant potential (Brodeur, 2003). On the one hand, low risk neuroblastoma is one of the rare human malignancies known to demonstrate spontaneous regression from an undifferentiated state to a completely benign cellular formation (ganglioneuromas). On the other hand, high risk neuroblastoma grows relentlessly and may be rapidly fatal (Brodeur, 2003). Prognosis of high risk tumors is poor, because drug resistance arises in the majority of those patients, initially responding to chemotherapy, in spite of the intensive therapy including megatherapy with subsequent hematopoietic progenitor cell transplantation, biotherapy and immunotherapy (Brodeur, 2003).

Chemotherapy agents used in combination have been found to be effective against neuroblastoma. Agents commonly used are platinum compounds (carboplatin), alkylating agents (cyclophosphamide, ifosfamide, melphalan), topoisomerase II inhibitors (etoposide), anthracycline antibiotics (doxorubicin) and vinca alkaloids (vincristine). Some novel regimen include also topoisomerase I inhibitors (topotecan and irinotecan), which have been found to be effective against recurrent disease (Brodeur, 2003).

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) and several of its more soluble derivatives exhibit significant antitumor and anti-HIV activities (Stiborová *et al.*, 2001) . The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer (Auclair, 1987). Nevertheless, ellipticine is a potent mutagen (for an overview, see Stiborová *et al.*, 2001). Ellipticine is an agent which cause DNA damage by several modes of action. Intercalation and/or inhibition of topoisomerase II was thought to be the major mechanism of ellipticine action. These mechanisms of action do not however, explain the ellipticine selectivity to several cancer diseases. Recently we have showed that ellipticine also covalently binds to DNA after being enzymatically activated (Stiborová *et al.*, 2001; Frei *et al.*, 2002; Stiborová *et al.*, 2003a; Stiborová *et al.*, 2003b). Human cytochrome P450 (CYP) enzymes, CYP3A4, 1A1, and 1B1–enzymes, which are expressed in tumors sensitive to ellipticine (*i.e.*, breast cancer; Murray *et al.*, 1993), were found to be the most efficient CYPs activating ellipticine to form covalent DNA adducts (Stiborová *et al.*, 2001). The formation of these adducts was also detected in V79 lung fibroblast cells transfected with human CYP3A4, 1A1, and 1A2 (Frei *et al.*, 2002), in human

Correspondence address:
Prof. Marie Stiborová, DrSc.
Department of Biochemistry, Faculty of Science, Charles University, Prague, Albertov 2030, 128 40 Prague 2, Czech Republic
E-MAIL: stiborov@natur.cuni.cz

breast adenocarcinoma MCF-7 cells (Borek-Dohalská *et al.*, 2004), and *in vivo* in rats exposed to ellipticine (Stiborová *et al.*, 2003b). On the basis of these data, ellipticine might be considered a drug whose pharmacological efficiencies and/or genotoxic side effects are dependent on its enzymatic activation in target tissues (Stiborová *et al.*, 2001; Frei *et al.*, 2002; Stiborová *et al.*, 2003a; Stiborová *et al.*, 2003b; Borek-Dohalská *et al.*, 2004).

Some improvement in therapeutic options of neuroblasoma has been made in the last decade, but there is still the need for the development of new therapies. Therefore, the present study was undertaken to investigate the cytotoxicity of ellipticine on human neuroblastoma cell lines and to compare the cytotoxicity of ellipticine with that of doxorubicin to these cancer cells. Another aim of the study was to evaluate the effect of hypoxia on cytotoxicity produce by both agents.

Material and methods

Chemicals

Ellipticine was obtained from Sigma (St. Louis, MO, USA). Doxorubicin was obtained from EBEWE Pharma Ges.m.b.H. (Uunterach, Austria). All other chemicals used in the experiments were of analytical purity or better.

Cell cultures

The UKF-NB-4 neuroblastoma cell line, established from bone marrow metastases of high risk neuroblastoma, was a gift of prof. J. Cinatl, Jr. (J. W. Goethe University, Frankfurt, Germany). Cell line UKF-NB-4 was established from infiltrated bone marrow of chemoresistant high risk neuroblastoma recurrency. IMR-32, high risk neuroblastoma derived cell line, was of the commercial source (LGC Promochem, Wesel, Germany). Both used cell lines were derived from a high risk neuroblastoma with MYCN amplification. The doxorubicin and/or ellipticine resistant cell sublines designated IMR-32 (DOXO), UKF-NB-4 (DOXO) and UKF-NB-4 (Elli) were established by incubation of parental cells with increasing concentrations of respective drug by the procedure as described (Kotchetkov *et al.*, 2003). Cells were grown at 37 °C and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) (KlinLab Ltd, Prague, Czech Republic), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicilline and 100 µg/ml streptomycine (PAA Laboratories, Pasching, Austria). For hypoxia experiments, cells were maintained in modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) flushed with 1% O_2, 5% CO_2 and balance N_2 for 4 min (hereafter refered to as hypoxia). This chamber was placed into 37 °C.

MTT assay

The cytotoxicity of ellipticine and doxorubicin was determined by MTT test. For a dose-response curve, DMSO stock solutions of ellipticine (10 mM) and aqueous solutions of doxorubicin (1 mM) were dissolve in culture medium to final concentrations of 0–50 µM were added. Cells in exponential growth were seeded at 1×10^4 per well in a 96-well plate. Briefly, after incubation (96 hours) at 37 °C in 5% CO_2 saturated atmosphere the MTT solution (2 mg/ml PBS) was added, the plates were incubated for 4 hours and cells lysed in 50% N,N-dimethylformamide containing 20% of SDS pH 4.5. The absorbance at 570 nm was measured for each well by multiwell ELISA reader Versamax (Molecular devices, CA). The mean absorbance of medium controls was the background and was subtracted. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control. Each value is the mean of 8 wells with standard deviation. The IC_{50} values were calculated from the linear regression of the dose-log response curves by SOFTmaxPro.

Flow cytometry

Cells were harvested by trypsinization and washed with PBS. FITC labeled antibody against ABCB1 (anti P-gp FITC, Immunotech) was added and samples were incubated for 15 minutes at room temperature. After washing with PBS the cells were resuspended in PBS and analyzed by flow cytometry FACSCalibur (BD).

Results

Cytotoxicity of ellipticine to human neuroblastoma cells

To determine the cytotoxicity of ellipticine to human neuroblastoma cells, these cells were treated with increasing concentrations of ellipticine. We first determined the effect of ellipticine on growth of human neuroblastoma cell lines (IMR-32 and UKF-NB-4) cultured for 96 h in the presence of ellipticine, using MTT assay. As shown in Figure 1, both neuroblastoma cell lines were sensitive to ellipticine.

Table 1.
Cytotoxicity of ellipticine and doxorubicin to neuroblastoma cell lines.

Cells	IC_{50} (µM)	
	for ellipticine	for doxorubicin
IMR-32	0.27 ± 0.02	0.02 ± 0,01
IMR-32 (hypoxic conditions)	0.43 ± 0.02	not measured
IMR-32 (DOXO)	0.53 ± 0.03	0.03 ± 0.01
UKF-NB-4	0.44 ± 0.03	0.70 ± 0.07
UKF-NB-4 (hypoxic conditions)	0.77 ± 0.03	1.61 ± 0.08
UKF-NB-4 (DOXO)	1.14 ± 0.07	3.80 ± 0.38
UKF-NB-4 (Elli)	1.17 ± 0.07	0.70 ± 0.07
UKF-NB-4 (Elli) (hypoxic conditions)	1.57 ± 0.14	1.51 ± 0.08

IC_{50} values were calculated from the linear regression of the dose-log response curves after 96 h exposure to the compound, determined by the MTT assay. Values are mean ± S.D. of at least 3 experiments.

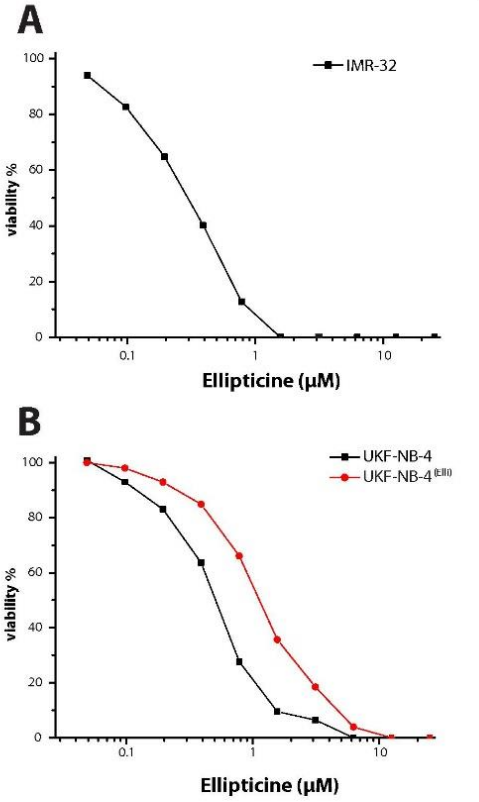

Figure 1. Cytotoxicity (viable cells as percentage of control) of ellipticine to IMR-32 (A), UKF-NB-4 and UKF-NB-4 cells resistant to ellipticine (UKF-NB-4^{ELLI}) (B) after 96 h exposure to the compound, determined by the MTT assay. Values are means of 8 determinations. Standard deviation was ≤ 10%.

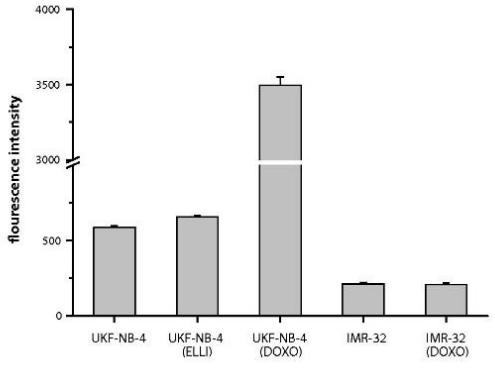

Figure 2. Levels of P-glycoprotein expressed as mean fluorescence intensity in neuroblastoma derived cell lines.

Cytotoxicity of ellipticine was compared with that of doxorubicin, one of the drugs currently used for neuroblastoma treatment (Table 1). Besides the effects of ellipticine or doxorubicin to these parental neuroblastoma cell lines, derived daughter neuroblastoma lines that were resistant to doxorubicin were also investigated. Ellipticine and doxorubicin inhibited the growth of neuroblastoma cell lines in a dose-dependent manner. The IC_{50} values for ellipticine and doxorubicin calculated from the dose-log response curves are shown in Table 1. The toxicity of ellipticine to UKF-NB-4 cells was analogous to that of doxorubicin to these cell lines, while IMR-32 cells were much more sensitive to the treatment with doxorubicin than with ellipticine (see IC_{50} values shown in Table 1).

A decrease in the viability induced by ellipticine and/or doxorubicin in the parental cell lines was compared with that in their variants resistant to doxorubicin. Interestingly, a decrease in sensitivity of the doxorubicin-resistant lines to ellipticine was significantly lower than a decrease in sensitivity of these lines to doxorubicin (Table 1), even though the cross-resistance would be expectable (Kotchetkov *et al.*, 2003). In the case of the UKF-NB-4 cell line, the IC_{50} value for ellipticine in UKF-NB-4 (DOXO) was only 2.6-fold higher than that in the parental UKF-NB-4 cell line, whereas the IC_{50} value of doxorubicin increased by 5.4-fold in the doxorubicin resistant cell line.

Ellipticine induces resistance to this compound in neuroblastoma cells

In order to evaluate the potential of ellipticine to induce the resistance of neuroblastoma cells to this drug, one of the cell lines, UKF-NB-4, was incubated 36 months with increasing concentrations of ellipticine (1–2.5 μM). A 2.6-fold increase in the IC_{50} value for ellipticine was found (Table 1, Figure 1). The induction of resistance of the UKF-NB-4 cell line to ellipticine is not mediated by P-glycoprotein, a member of ABC transporters (Figure 2).

Ellipticine resistance of UKF-NB-4 is not mediated by P-glycoprotein

Lower cytotoxicity of doxorubicin to UKF-NB-4 (DOXO) than to UKF-NB-4 is caused by higher expression of P-glycoprotein in a membrane of the doxorubicin resistant cell line. In contrast to these results, the IMR-32 (DOXO) cell line expresses P-glycoprotein at the same level as parental IMR-32 line. The UKF-NB-4 cell line resistant to ellipticine also does not express P-glycoprotein in the higher levels than the parental cell line. Resistance to ellipticine is therefore mediated by another mechanism of action. The study resolving this mechanism is under the way in our laboratories.

Hypoxia decreases the toxicity of ellipticine and doxorubicin

Hypoxia was found in most tumor malignancies. To determine the effect of hypoxia on the cytotoxicity of ellipticine and/or doxorubicin to IMR-32, UKF-NB-4 and daughter cells resistant to doxorubicin, the cells were treated with increasing concentrations of either cytostatics for 96 h under hypoxic conditions and analyzed using MTT assay. As shown in Table 1, all neuroblastoma cell lines were less sensitive to applied cytostatics under the hypoxic conditions of their cultivation.

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Discussion

The results of this study show that ellipticine is cytotoxic to human neuroblastoma cell lines (both to the parental IMR-32 and UKF-NB-4 lines and their variants resistant to doxorubicin), inhibiting the growth of these cancer cells.

Hypoxia frequently occurs in tumors because of their fast growth and inadequate vascularisation. It strongly correlates with advanced disease and poor outcome caused by chemoresistance. The cytotoxicity of ellipticine or doxorubicin decreased when neuroblastoma cells were cultivated and treated with these agents under the limited concentration of oxygen.

Because drug resistance is general feature of neuroblastoma, and arises in the majority of patients suffering from this cancer, we also investigated whether ellipticine is capable of inducing resistance in neuroblastoma cells. The UKF-NB-4 cell line was used for such a study. The results demonstrate that ellipticine might, to some extent, induce the drug resistance in these cells. Long-term treating this cancer line with increasing concentrations of ellipticine resulted in the morphological changes in the ellipticine-resistant cell line (not shown). The decrease in sensitivity of this cell line to ellipticine was, however, lower than that in sensitivity of the doxorubicin-resistant UKF-NB-4 cell line to doxorubicin. The doxorubicin-induced resistance of these neuroblastoma cells resulted in more than 5-times higher decrease in sensitivity of neuroblastoma to this drug. Moreover, the IC_{50} values for ellipticine in the ellipticine-resistant neuroblastoma cell line is, even under the hypoxic conditions, still one order of magnitude lower than the ellipticine levels in blood in mice, 4 hours after their p.o. treatment with ellipticine in a tolerable dose (Hardesty *et al.*, 1972). The study resolving the reasons of the changes found in the ellipticine-resistant neuroblastoma cells on the molecular levels is under way in our laboratories. The changes in the genetic programs in this cell line after induction of resistance to ellipticine are analyzed at the present time. Our experiments suggest that the ellipticine-mediated resistance is not dependent on expression of P-glycoprotein as it is the case of the UKF-NB-4 cell line resistant to doxorubicin (Bedrníček *et al.*, 2005, present paper).

The results presented in this paper are the first report demonstrating the cytotoxicity of ellipticine in human neuroblastoma cells. Another important result of the study is finding that ellipticine can induce resistance to this agent, which is, however, lower than resistance of neuroblastoma cells to doxorubicin caused by doxorubicin. This finding is the promising result that might be utilized for the development of new neuroblastoma therapies, namely, for the substitution and/or combination of the current anticancer drugs with ellipticine or its derivatives. For the practical use it seems to be important that there is only partial cross-resistance between ellipticine and doxorubicin in neuroblastoma, which is not caused in all cell lines by P-glycoprotein.

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REFERENCES

Auclair C (1987) Multimodal action of antitumor agents on DNA: The ellipticine series. *Arch Biochem Biophys* **259**: 1–14.

Bedrníček J, Vícha A, Jarošová M, Holzerová M, Činátl J Jr, Michaelis M, Činátl J, Eckschlager T (2005) Characterization of drug-resistant neuroblastoma cell lines by comparative genomic hybridization. *Neoplasma* **52**: 415–419.

Borek-Dohalská L, Frei E, Stiborová M (2004) DNA adduct formation by the anticancer drug ellipticine and its hydroxy derivatives in human breast adenocarcinoma MCF-7 cells. *Collect Czech Chem Commun* **69**: 603–615.

Brodeur GM (2003) Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* **3**: 203–216.

Frei E, Bieler CA, Arlt VM, Wiessler M, Stiborová M (2002) Covalent binding of the anticancer drug ellipticine to DNA in V79 cells transfected with human cytochrome P450 enzymes. *Biochem Pharmacol* **64**: 289–295.

Hardesty CT, Chaney NA, Mead JA (1972) The effect of route of administration on the distribution of ellipticine in mice. *Cancer Res* **32**: 1884–1889.

Kotchetkov R, Cinatl J, Blaheta R, Vogel JU, Karaskova J, Squire J, Hernáiz Driever P, Klingebiel T, Cinatl J Jr (2003) Development of resistance to vincristine and doxorubicin in neuroblastoma alters malignant properties and induces additional karyotype changes: a preclinical model. *Int J Cancer* **104**: 36–43.

Lesca P, Beaune P, Monsarrat B (1981) Ellipticines and human liver microsomes: spectral interaction with cytochrome P-450 and hydroxylation. Inhibition of arylhydrocarbon metabolism and mutagenicity. *Chem-Biol Interact* **36**: 299–309.

Maris JM, Mathay KK (1999) Molecular biology of neuroblastomas. *J Clin Oncol* **17**: 2264–2279.

Murray GI, Weaver RJ, Paterson PJ, Ewen SW, Melvin WT, Burke MD (1993) Expression of xenobiotic metabolizing enzymes in breast cancer. *J Pathol* **169**: 347–353.

Stiborová M, Bieler CA, Wiessler M, Frei E (2001) The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts. *Biochem Pharmacol* **62**: 1675–1684.

Stiborová M, Stiborová-Rupertová M, Bořek-Dohalská L, Wiessler M, Frei E (2003a) Rat microsomes activating the anticancer drug ellipticine to species covalently binding to deoxyguanosine in DNA are a suitable model mimicking ellipticine bioactivation in humans. *Chem Res Toxicol* **16**: 38–47.

Stiborová M, Breuer A, Aimová D, Stiborová-Rupertová M, Wiessler M, Frei E (2003b) DNA adduct formation by the anticancer drug ellipticine in rats determined by ^{32}P-postlabeling. *Int J Cancer* **107**: 885–890.

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interdisciplinary toxicology

ORIGINAL ARTICLE

Histone deacetylase inhibitors valproate and trichostatin A are toxic to neuroblastoma cells and modulate cytochrome P450 1A1, 1B1 and 3A4 expression in these cells

Jana HŘEBAČKOVÁ [1], Jitka POLJAKOVÁ [1,2], Tomáš ECKSCHLAGER [1], Jan HRABĚTA [1], Pavel PROCHÁZKA [1], Svatopluk SMUTNÝ [3], Marie STIBOROVÁ [2]

[1] Department of Pediatric Hematology and Oncology, 2nd Medical School, Charles University and University Hospital Motol, Prague 5, Czech Republic
[2] Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic
[3] 1st Department of Surgery, 2nd Medical School, Charles University and University Hospital Motol, V Úvalu 84, 150 06 Prague 5, Czech Republic

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ABSTRACT

Histone deacetylase inhibitors such as valproic acid (VPA) and trichostatin A (TSA) were shown to exert antitumor activity. Here, the toxicity of both drugs to human neuroblastoma cell lines was investigated using MTT test, and IC50 values for both compounds were determined. Another target of this work was to evaluate the effects of both drugs on expression of cytochrome P450 (CYP) 1A1, 1B1 and 3A4 enzymes, which are known to be expressed in neuroblastoma cells. A malignant subset of neuroblastoma cells, so-called N-type cells (UKF-NB-3 cells) and the more benign S-type neuroblastoma cells (UKF-NB-4 and SK-N-AS cell lines) were studied from both two points of view. VPA and TSA inhibited the growth of neuroblastoma cells in a dose-dependent manner. The IC_{50} values ranging from 1.0 to 2.8 mM and from 69.8 to 129.4 nM were found for VPA and TSA, respectively. Of the neuroblastoma tested here, the N-type UKF-NB-3 cell line was the most sensitive to both drugs. The different effects of VPA and TSA were found on expression of CYP1A1, 1B1 and 3A4 enzymes in individual neuroblastoma cells tested in the study. Protein expression of all these CYP enzymes in the S-type SK-N-AS cell line was not influenced by either of studied drugs. On the contrary, in another S-type cell line, UKF-NB-4, VPA and TSA induced expression of CYP1A1, depressed levels of CYP1B1 and had no effect on expression levels of CYP3A4 enzyme. In the N-type UKF-NB-3 cell line, the expression of CYP1A1 was strongly induced, while that of CYP1B1 depressed by VPA and TSA. VPA also induced the expression of CYP3A4 in this neuroblastoma cell line.

KEY WORDS: histone deacetylase inhibitors; valproate; trichostatin A; neuroblastoma; cytotoxicity

Introduction

Neuroblastoma is the major cause of death from neoplasia in infancy (Maris and Mathay, 1999). These solid extracranial tumors are biologically heterogeneous, with cell populations differing in their genetic programs, maturation stage and malignant potential (Brodeur, 2003). Low-risk neuroblastoma is one of the rare human malignancies that are known to demonstrate spontaneous regression in infants from an undifferentiated state to a completely benign cellular formation (ganglioneuroma), whereas high-risk neuroblastoma grows relentlessly and may be rapidly fatal. Prognosis of high-risk form of cancer is poor, because drug resistance arises in the majority of those patients, initially responding to chemotherapy (Brodeur, 2003).

Neuroblastoma consists of two principal neoplastic cells (Voigt *et al.*, 2000; Hopkins-Donaldson *et al.*, 2004): i) neuroblastic or N-type: undifferentiated, round and small scant cytoplasm cells; and ii) stromal or S-type: large hyaline, flattened and adherent differentiated cells. As neuroblastoma cells seem to have the capacity to differentiate spontaneously *in vivo* and *in vitro* (Morgenstern *et al.*, 2004), their heterogeneity could affect treatment outcome, in particular the response to apoptosis induced by chemotherapy.

To achieve the most suitable concept of treatment, drugs are usually used in various combinations. Agents commonly used in neuroblastoma treatment are platinum compounds (cisplatin, carboplatin), alkylating agents (cyclophosphamide, ifosfamide, melphalan), topoisomerase II inhibitors (etoposide), anthracycline antibiotics (doxorubicin) and

Correspondence address:
Prof. Marie Stiborová, DSc.
Department of Biochemistry, Faculty of Science, Charles University,
Albertov 2030, 128 40 Prague 2, Czech Republic
TEL: +420-221951285 • E-MAIL: stiborov@natur.cuni.cz
TEL: +420-221951241 • E-MAIL: jitka.poljakova@seznam.cz

vinca alkaloids (vincristine). Some novel regimen include also topoisomerase I inhibitors (topotecan and irinotecan) that are effective against recurrent disease (Brodeur, 2003).

Because the epigenetic structure of DNA and its lesions play a role in the origin of human neuroblastomas, pharmaceutical manipulation of the epigenome may offer other treatment options also for neuroblastomas (Furchert *et al.*, 2007). Histone deacetylases (HDAC) and histone acetyl transferases modify histone proteins and contribute to an epigenetic code recognized by proteins involved in regulation of gene expression (Marks *et al.*, 2003; 2004; Hooven *et al.*, 2005). Indeed, former studies demonstrated the cytotoxicity of HDAC inhibitors to several neuroblastoma cells, resulting in growth inhibition of these tumor cells (Cinatl *et al.*, 1996; Michaelis *et al*, 2004; 2007; Furchert *et al.*, 2007). In neoplastic cells, where overexpression of different HDACs was frequently detected (for summary see, Bolden *et al*, 2006), the abundance of deacetylated histones is usually associated with DNA hypermethylation and gene silencing (Santini *et al.*, 2007). Treatment with HDAC inhibitors induced the reactivation of growth regulatory genes and consequently apoptosis in these cells. One of the HDAC inhibitors, valproic acid (VPA), inhibits growth and induces differentiation of human neuroblastoma UKF-NB-2 and UKF-NB-3 cells *in vitro* at concentrations ranging from 0.5 to 2 mM that have been achieved in human with no significant adverse effects (Cinatl *et al.*, 1996). However, information on effects of VPA and other HDAC inhibitors on additional neuroblastoma cells are scarce. Therefore, here we extended this study by investigating the effect of VPA and another HDAC inhibitor, trichostatin A (TSA), on other neuroblastoma cell lines. Because heterogeneity of neuroblastoma cells could affect their treatment, two types of neuroblastoma cell lines were tested for their response to VPA and TSA treatment. Besides the effect of VPA and TSA on UKF-NB-3 cells (the invasive N-type), that on the UKF-NB-4 and SK-N-AS cell lines (the non-invasive and less-aggressive S-type) was investigated in this work.

In addition, VPA and TSA are known to be metabolized by cytochrome P450 (CYP) biotransformation enzymes and can increase and/or decrease their activities and/or expression, thereby affecting mechanisms that control drug disposition (Fisher *et al.*, 1991; Rogiers *et al.*, 1992; 1995; Isojärvi *et al.*, 2001; Wen *et al.*, 2001; Bort *et al.*, 2004; Cerveny *et al.*, 2007; Nelson-DeGrave *et al.*, 2004; Hooven *et al.*, 2005; Snykers *et al.*, 2007; Kiang *et al.*, 2006). Because several CYP enzymes metabolizing a variety of drugs (CYP1A1, 1B1 and 3A4) were found to be expressed in neuroblastoma cells (Poljaková *et al.*, 2009), here we also investigated whether their expression is influenced by VPA and TSA in these cells.

Material and methods

Chemicals

Valproate and trichostatin A were obtained from Sigma (St. Louis, MO, USA). All other chemicals used in the experiments were of analytical purity or better.

Cell cultures

The UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines, established from bone marrow metastases of high-risk neuroblastoma, were a gift of prof. J. Cinatl, Jr. (J. W. Goethe University, Frankfurt, Germany). Cell line UKF-NB-4 was established from infiltrated bone marrow of chemoresistant high-risk neuroblastoma recurrence and have high expression of P-glycoprotein. SK-N-AS, derived from bone marrow metastases of neuroblastoma, was of the commercial source (ECACC, Salisbury, UK). Cells were grown at 37 °C and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) (KlinLab Ltd, Prague, Czech Republic), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml of penicilline and 100 µg/ml streptomycine (PAA Laboratories, Pasching, Austria).

MTT assay

The cytotoxicity of valproate and trichostatin A was determined by MTT test. For a dose-response curve, culture medium stock solutions of valproate (200 mM) and DMSO solutions of trichostatin A (1 mM) were dissolved in culture medium to final concentrations of 0 – 50 mM and 0 – 1 µM for valproate and trichostatin A, respectively. Cells in exponential growth were seeded at 1×10^4 per well in a 96-well microplate. After incubation (72 hours) at 37 °C in 5% CO_2 saturated atmosphere the MTT solution (2 mg/ml PBS) was added, the microplates were incubated for 4 hours and cells lysed in 50% N,N-dimethylformamide containing 20% of SDS, pH 4.5. The absorbance at 570 nm was measured for each well by multiwell ELISA reader Versamax (Molecular devices, CA, USA). The mean absorbance of medium controls was subtracted as a background. The viability of control cells was taken as 100% and the values of treated cells were calculated as a percentage of control. The IC_{50} values were calculated from at least 3 independent experiments using linear regression of the dose-log response curves by SOFTmaxPro.

Estimation of contents of cytochromes P450 1A1, 1B1 and 3A4 in neuroblastoma cells by Western blot

To determine the expression of CYP1A1, 1B1 and 3A4 proteins, cells were homogenized in RIPA buffer. Protein concentrations were assessed using the DC protein assay (Bio-Rad, Hercules, CA, USA) with serum albumin as a standard. 10–45 µg of extracted proteins were subjected to SDS-PAGE electrophoresis on a 10% gel. After migration, proteins were transferred to a nitrocellulose membrane and incubated with 5% non-fat milk to block non-specific binding. The membranes were then exposed to specific anti-CYP1A1 (1:1000, Millipore, MA, USA) anti-CYP1B1 (1:500, AbCam, MA, USA) and anti-CYP3A4 (1:5000, AbD Serotec, Oxford, UK) rabbit polyclonal antibodies overnight at 4 °C. Membranes were washed and exposed to peroxidase-conjugated anti-IgG secondary antibody (1:3000, Bio-Rad, Hercules, CA, USA), and the antigen-antibody complex was visualized by enhanced chemiluminiscence's detection system according to the manufacturer's instructions (Immun-Star HRP Substrate, Bio-Rad, Hercules, CA, USA). Films (MEDIX XBU, Foma, Hradec Králové, Czech Republic) were scanned with a computerized image-analyzing system (ElfoMan 2.0, Ing. Semecký, Prague, Czech Republic).

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Results

Cytotoxicity of valproate and trichostatin A to human neuroblastoma cells

To evaluate the cytotoxicity of VPA and TSA to human neuroblastoma cells (UKF-NB-3, UKF-NB-4 and SK-N-AS), these cells were treated with increasing concentrations of both drugs (0–50 mM for VPA and 0–1 μM for TSA). We first determined the effect of VPA and TSA on growth of human neuroblastoma cell lines cultured for 72 hours in the presence of both drugs, using MTT assay. As shown in Figures 1 and 2, all three neuroblastoma cell lines were sensitive to VPA and TSA. Both drugs inhibited the growth of neuroblastoma cell lines in a dose-dependent manner. The IC_{50} values for VPA and TSA calculated from the dose-log response curves are shown in Figures 1B and 2B.

Among the neuroblastoma cell lines tested in this study, the UKF-NB-3 cell line was the most sensitive to both drugs, with IC_{50} values of 1.02 mM and 69.8 nM for VPA and TSA, respectively. The IC_{50} values indicating the toxicity of VPA and TSA to UKF-NB-4 cells were similar to those found for the SK-N-AS cell line, being up to a 2.7-fold lower than for the UKF-NB-3 cell line (Figures 1 and 2). Nevertheless, the curves showing the viability of SK-N-AS cells under treatment with increasing concentrations of VPA and TSA significantly differed from those of UKF-NB-3 and UKF-NB-4 cell lines. At higher VPA and TSA concentrations, the sensitivity of SK-N-AS cells was much lower than that of other two neuroblastoma cell lines analyzed in this work.

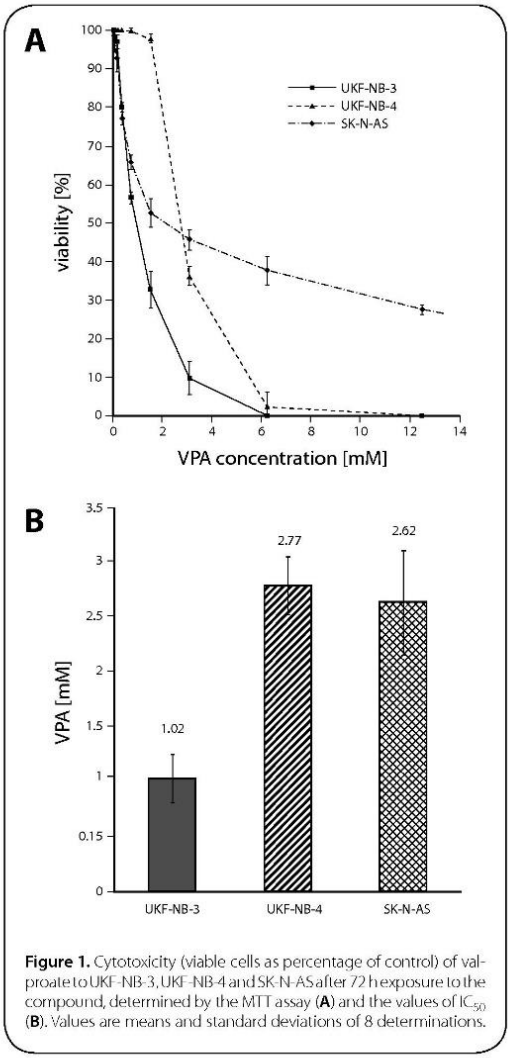

Figure 1. Cytotoxicity (viable cells as percentage of control) of valproate to UKF-NB-3, UKF-NB-4 and SK-N-AS after 72 h exposure to the compound, determined by the MTT assay (**A**) and the values of IC_{50} (**B**). Values are means and standard deviations of 8 determinations.

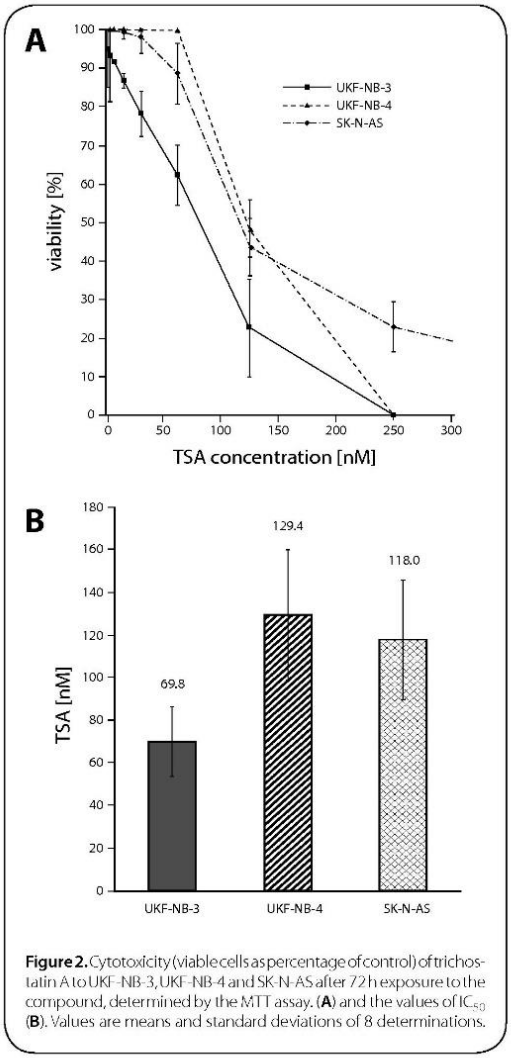

Figure 2. Cytotoxicity (viable cells as percentage of control) of trichostatin A to UKF-NB-3, UKF-NB-4 and SK-N-AS after 72 h exposure to the compound, determined by the MTT assay. (**A**) and the values of IC_{50} (**B**). Values are means and standard deviations of 8 determinations.

Effect of VPA and TSA on expression of cytochrome P450 1A1, 1B1 and 3A4 proteins

Using Western blot analysis with antibodies raised against CYP1A1, 1B1 and 3A4, the effects of VPA and TSA on protein expression levels of these enzymes were analyzed in the tested neuroblastoma cell lines.

Expression of CYP1A1 protein in neuroblastoma UKF-NB-3 and UKF-NB-4 cells was elevated by increasing concentrations of VPA and/or TSA in a dose-dependent manner (Figure 3). A 1.7-, 4.0- and 8.1-fold increase in CYP1A1 expression was caused by treating the UKF-NB-3 cells for 48 hour with 0.5, 1.0 and 2.0 mM VPA, respectively, while lower, only up to a 1.7-fold increase in levels of this CYP was produced by VPA in UKF-NB-4 cells. In the SK-N-AS cells, even no effect of VPA on the CYP1A1 expression was detectable.

Similar effects on CYP1A1 expression in neuroblastoma UKF-NB-3 and UKF-NB-4 cell lines were detected when cells were treated for 48 hours with TSA. Up to a 4.4-fold increase in expression levels of CYP1A1 was produced by 50–200 nM TSA in these cells (Figure 3). No effects of TSA on the expression of CYP1A1 protein in SK-N-AS were found.

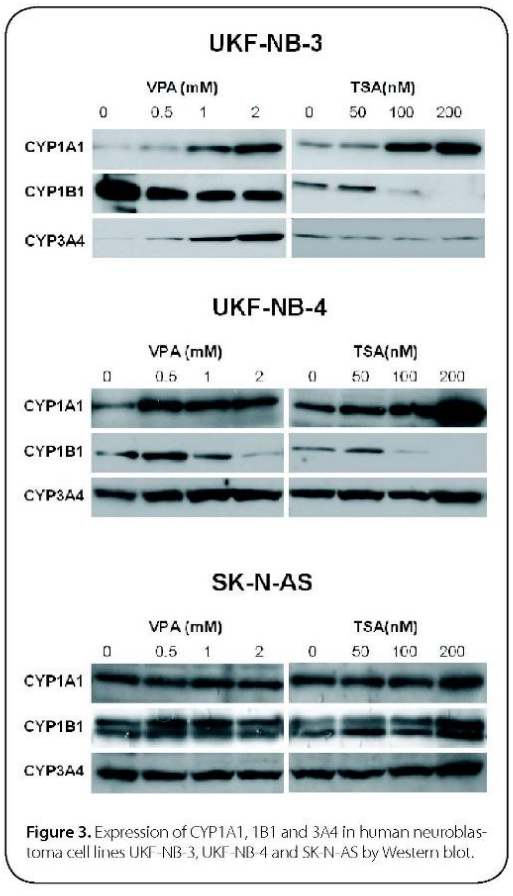

Figure 3. Expression of CYP1A1, 1B1 and 3A4 in human neuroblastoma cell lines UKF-NB-3, UKF-NB-4 and SK-N-AS by Western blot.

Expression of CYP1B1 protein was decreased in UKF-NB-3 and UKF-NB-4 cells after their 48-hour treatment with increasing concentrations of VPA and/or TSA, being decreased in a dose-dependent manner. Similar to CYP1A1, no effect of both HDAC inhibitors on expression of CYP1B1 was produced in SK-N-AS cells (Figure 3). Interestingly, two protein bands detectable by antibody against CYP1B1 were found in SK-N-AS cells.

In the case of the effects of VPA and TSA on expression of CYP3A4 protein in neuroblastoma cells, both these drugs essentially did not influence its expression in S-type UKF-NB-4 and SK-N-AS cell lines. The N-type of neuroblastoma cell line, UKF-NB-3, was only the exception; whereas increased concentrations of VPA increased CYP3A4 expression in this cell line, TSA had no effect (Figure 3).

Discussion

The results of this work show that human neuroblastoma UKF-NB-3, UKF-NB-4 and SK-N-AS cell lines are sensitive to two tested HDAC inhibitors, VPA and TSA. In the case of VPA, its concentrations that were toxic to neuroblastoma cells are clinically applicable, since concentrations between 0.35–0.7 mM in patients serum are commonly therapeutically used (Duenas-Gonzalez *et al.*, 2007). The IC_{50} values for VPA and TSA indicate that a UKF-NB-3 cell line was the most sensitive to both HDAC inhibitors, while their toxicity to the UKF-NB-4 and SK-N-AS cell lines was up to a 2.7-fold lower. Thus, the sensitivity to the two drugs seems to be related to the phenotype, with the S-type cells (UKF-NB-4 and SK-N-AS) being less sensitive than the N-type (UKF-NB-3), probably because of their partly lower capability of undergoing apoptosis (Servidei *et al.*, 2004). However, the results shown here indicate that it seems to be questionable to evaluate the toxic effects of chemicals to cells in culture using only the IC_{50} values. The question arises, whether the IC_{50} value is a real appropriate sensitivity marker. Namely, of the S-type neuroblastoma cells utilized in this study, the SK-N-AS cell line seems to be even less sensitive to VPA and TSA than the second S-type cell line, UKF-NB-4, even though the IC_{50} values for VPA and TSA were similar for both these cell lines. At higher VPA and TSA concentrations, the sensitivity of SK-N-AS cells was much lower than that of UKF-NB-4. This less sensitive SK-N-AS line seems to be, at least in part, capable of overcoming treatment with VPA and TSA at concentrations that cause almost complete eradication of UKF-NB-4 cells. These results suggest that caution should be exerted to sort neuroblastoma cells into their types. Even in one type of neuroblastoma cells (S-type in this case), biological heterogeneity should be taken into account. This suggestion is also supported by further features found in this cell line. Namely, the SK-N-AS cell line behaves differently from the other S-type cell line, UKF-NB-4, from the point of view of the effects of VPA and TSA on CYP expression; no effects of both drugs was found on levels of individual CYP enzymes. Moreover, in this cell line, the two CYP1B1 protein bands

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were detectable by antibody against this CYP. Now, we can only speculate on the origin of the second protein band in SK-N-AS cells. The questions, whether it might follow from a degradation of the CYP1B1 protein in this cell line or it is the artifact caused by the method used (Western blot), remain to be answered.

The expression of all CYP enzymes analyzed in this work was modulated by VPA and TSA only in the N-type UKF-NB-3 cell line. Whereas the CYP1A1 enzyme was induced by both drugs, expression of CYP1B1 was depressed by both drugs. The CYP3A4 enzyme was increased by VPA, but TSA had no influence on the expression of this enzyme. The expression of CYP1A1 and 1B1 was also similarly affected by VPA and TSA in the UKF-NB-4 cell line, but no effect on expression levels of CYP3A4 was produced in this line. Similarity in response of UKF-NB-3 and UKF-NB-4 cells to the effects of VPA and TSA on CYP1A1 and 1B1 expression might probably be caused by their similar effects on state (degree) of acetylation of histones and, therefore, transcription activity. But differences between these two cell lines and particularly SK-N-AS cells in response to CYP enzyme expression and its affecting by VPA and TSA are still valuable. The question whether such differences are caused by the fact that cells vary in the broad spectrum of metabolic and signalling pathways that might also be affected by VPA and TSA in a different way, independently of a cell type (N- or S-type), remains to be answered. Further studies with these and other neuroblastoma cell lines and various HDAC inhibitors and broader spectrum of CYP enzymes have to be performed in order to shed more light on this field.

Since CYP enzymes are involved in biosynthesis and metabolism of many endogenous physiologically active substances and in biotransformation of xenobiotics with pharmacological and/or toxic effects (Myasodeova, 2008), a change in their expression might affect the cells significantly. In the case of oncology, the participation of CYPs in drug metabolism seems to be their most important role. A variety of CYP enzymes is involved in metabolism of a broad spectrum of drugs that can, moreover, either increase or decrease their expression levels. The finding that VPA and TSA are capable of inducing and depressing CYP enzyme expression in neuroblastoma cells (CYP1A1, 1B1 and 3A4 tested in our work) might have great importance. This feature might be utilized mainly in the combination therapy with other drugs whose pharmacological effects are dependent on their CYP-mediated metabolism. Such a study with one of these drugs, ellipticine, is under way in our laboratory.

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REFERENCES

Batty N, Malouf GG, Issa JP. (2009). Histone deacetylase inhibitors as anti-neoplastic agents. *Cancer Lett* **280**: 192–200.

Blaheta RA, Cinatl J Jr. (2002). Anti-tumor mechanisms of valproate: a novel role for an old drug. *Med Res Rev* **22**: 492–511.

Bolden JE, Peart MJ, Johnstone RW. (2006). Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* **5**: 769–784.

Bort R, Gomez-Lechon MJ, Castell JV, Jover R. (2004). Role of hepatocyte nuclear factor 3 gamma in the expression of human CYP2C genes. *Arch Biochem Biophys* **426**: 63–72.

Brodeur GM. (2003). Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* **3**: 203–216.

Cerveny L, Svecova L, Anzenbacherova E, Vrzal R, Staud F, Dvorak Z, Ulrichova J, Anzenbacher P, Pavek P. (2007). Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways. *Drug Metab Dispos* **35**: 1032–1041.

Cinatl J Jr, Cinatl J, Scholz M, Driever PH, Henrich D, Kabickova H, Vogel JU, Doerr HW, Kornhuber B. (1996). Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. *Anticancer Drugs* **7**: 766–773.

Condorelli F, Gnemmi I, Vallario A, Genazzani AA, Canonico PL. (2008). Inhibitors of histone deacetylase (HDAC) restore the p53 pathway in neuroblastoma cells. *Br J Pharmacol* **153**: 657–668.

Duenas-Gonzalez A, Candelaria M, Perez-Plascencia C, Perez-Cardenas E, de la Cruz-Hernandez E, Herrera LA. (2008). Valproic acid as epigenetic cancer drug: preclinical, clinical and transcriptional effects on solid tumors. *Cancer Treat Rev* **34**: 206–222.

Fisher JE, Nau H, Löscher W. (1991). Alterations in the renal excretion of valproate and its metabolites after chronic treatment. *Epilepsia* **32**: 146–150.

Furchert SE, Lanvers-Kaminsky C, Juürgens H, Jung M, Loidl A, Frühwald MC. (2007). Inhibitors of histone deacetylases as potential therapeutic tools for high risk embryonal tumors of the nervous system of childhood. *Int J Cancer* **120**: 787–1794.

Hooven LA, Mahadevan B, Keshava C, Johns C, Pereira C, Desai D, Amin S, Weston A, Baird WM. (2005). Effects of suberoylanilide hydroxamic acid and trichostatin A on induction of cytochrome P450 enzymes and benzo[a]pyrene DNA adduct formation in human cells. *Bioorg Med Chem Lett* **15**: 1283–1287.

Hopkins-Donaldson S, Yan P, Bourloud KB, Muhlethaler A, Bodmer JL and Gross N. (2002). Doxorubicin-induced death in neuroblastoma does not involve death receptors in S-type cells and is caspase-independent in N-type cells. *Oncogene* **21**: 6132–6137.

Isojärvi JI, Turkka J, Pakarinen AJ, Kotila M, Rättyä J, Myllylä VV. (2001). Thyroid function in men taking carbamazepine, oxcarbazepine, or valproate for epilepsy. *Epilepsia* **42**: 930–934.

Kiang TK, Ho PC, Anari MR, Tong V, Abbott FS, Chang TK. (2006). Contribution of CYP2C9, CYP2A6, and CYP2B6 to valproic acid metabolism in hepatic microsomes from individuals with the CYP2C9*1/*1 genotype. *Toxicol Sci* **94**: 261–271.

Marchion DC, Bicaku E, Daud AI, Sullivan DM, Munster PN. (2005). Valproic acid alters chromatin structure by regulation of chromatin modulation proteins. *Cancer Res* **65**: 3815–3822.

Maris JM, Mathay KK. (1999). Molecular biology of neuroblastomas. *J Clin Oncol* **17**: 2264–2279.

Marks PA, Miller T, Richon VM. (2003). Histone deacetylases. *Curr Opin Pharmacol* **3**: 344–351.

Marks PA, Richon VM, Miller T, Kelly WK. (2004). Histone deacetylase inhibitors. *Adv Cancer Res* **91**: 137–168.

Michaelis M, Doerr HW, Cinatl J Jr. (2007). Valproic acid as anti-cancer drug. *Curr Pharm Des* **13**: 3378–3393.

Michaelis M, Suhan T, Cinatl J, Driever PH, Cinatl J Jr. (2004). Valproic acid and interferon-alpha synergistically inhibit neuroblastoma cell growth in vitro and in vivo. *Int J Oncol* **25**: 1795–1799.

Morgenstern BZ, Krivoshik AP, Rodriguez V and Anderson PM. (2004). Wilms' tumor and neuroblastoma. *Acta Paediatr Suppl* **93**: 78–85.

Myasoedova KN. (2008). New findings in studies of cytochromes P450. *Biochemistry (Mosc)* **73**: 965–969.

Nelson DeGrave VL, Wickenheisser JK, Cockrell JE, Wood JR, Legro RS, Strauss JF 3rd, McAllister JM. (2004). Valproate potentiates androgen biosynthesis in human ovarian theca cells. *Endocrinology* **145**: 799–808.

Poljakova J, Eckschlager T, Hrabeta J, Hrebackova J, Smutny S, Frei E, Martinek V, Kizek R, Stiborova M. (2009). The mechanism of cytotoxicity and DNA adduct formation by the anticancer drug ellipticine in human neuroblastoma cells. *Biochem Pharmacol* **77**: 1466–1479.

Rogiers V, Akrawi M, Vercruysse A, Phillips IR, Shephard EA. (1995). Effects of the anticonvulsant, valproate, on the expression of components of the cytochrome-P-450-mediated monooxygenase system and glutathione S-transferases. *Eur J Biochem* **231**: 337–343.

Rogiers V, Callaerts A, Vercruysse A, Akrawi M, Shephard E, Phillips I. (1992). Effects of valproate on xenobiotic biotransformation in rat liver. In vivo and in vitro experiments. *Pharm Weekbl Sci* **14**: 127–131.

Santini V, Gozzini A, Ferrari G. (2007). Histone deacetylase inhibitors: molecular and biological activity as a premise to clinical application. *Curr Drug Metab* **8**: 383–393.

Servidei T, Morosetti R, Ferlini C, Cusano G, Scambia G, Mastrangelo R, Koeffler HP. (2004). The cellular response to PPARgamma ligands is related to the phenotype of neuroblastoma cell lines. *Oncol Res* **14**: 345–354.

Silva MF, Aires CC, Luis PB, Ruiter JP, Ijlst L, Duran M, Wanders RJ, Tavares de Almeida I. (2008). Valproic acid metabolism and its effects on mitochondrial fatty acid oxidation: A review. *J Inherit Metab Dis* **31**: 205–216.

Snykers S, Vanhaecke T, De Becker A, Papeleu P, Vinken M, Van Riet I, Rogiers V. (2007). Chromatin remodeling agent trichostatin A: a key-factor in the hepatic differentiation of human mesenchymal stem cells derived of adult bone marrow. *BMC Dev Biol* **7**: 24–39.

Van Lint C, Emiliani S, Verdin E. (1996). The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr* **5**: 245–253.

Voigt A, Hartmann P and Zintl F. (2000). Differentiation, proliferation and adhesion of human neuroblastoma cells after treatment with retinoic acid. *Cell Adhes Commun* **7**: 423–440.

Wen X, Wang JS, Kivistö KT, Neuvonen PJ, Backman JT. (2001). In vitro evaluation of valproic acid as an inhibitor of human cytochrome P450 isoforms: preferential inhibition of cytochrome P450 2C9 (CYP2C9). *Br J Clin Pharmacol* **52**: 547–553.

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The mechanism of cytotoxicity and DNA adduct formation by the anticancer drug ellipticine in human neuroblastoma cells

Jitka Poljaková [a], Tomáš Eckschlager [b], Jan Hraběta [b], Jana Hřebačková [b], Svatopluk Smutný [c], Eva Frei [d], Václav Martínek [a], René Kizek [e], Marie Stiborová [a,*]

[a] Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic
[b] Department of Pediatric Hematology and Oncology, 2nd Medical School, Charles University and University Hospital Motol, V Úvalu 84, 150 06 Prague 5, Czech Republic
[c] 1st Department of Surgery, 2nd Medical School, Charles University and University Hospital Motol, V Úvalu 84, 150 06 Prague 5, Czech Republic
[d] Division of Molecular Toxicology, German Cancer Research Center, In Neuenheimer Feld 280, 69120 Heidelberg, Germany
[e] Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, 613 00 Brno, Czech Republic

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ABSTRACT

Ellipticine is an antineoplastic agent, whose mode of action is based mainly on DNA intercalation, inhibition of topoisomerase II and formation of covalent DNA adducts mediated by cytochromes P450 and peroxidases. Here, the molecular mechanism of DNA-mediated ellipticine action in human neuroblastoma IMR-32, UKF-NB-3 and UKF-NB-4 cancer cell lines was investigated. Treatment of neuroblastoma cells with ellipticine resulted in apoptosis induction, which was verified by the appearance of DNA fragmentation, and in inhibition of cell growth. These effects were associated with formation of two covalent ellipticine-derived DNA adducts, identical to those formed by the cytochrome P450- and peroxidase-mediated ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine. The expression of these enzymes at mRNA and protein levels and their ability to generate ellipticine-DNA adducts in neuroblastoma cells were proven, using the real-time polymerase chain reaction, Western blotting analyses and by analyzing ellipticine-DNA adducts in incubations of this drug with neuroblastoma S9 fractions, enzyme cofactors and DNA. The levels of DNA adducts correlated with toxicity of ellipticine to IMR-32 and UKF-NB-4 cells, but not with that to UKF-NB-3 cells. In addition, hypoxic cell culture conditions resulted in a decrease in ellipticine toxicity to IMR-32 and UKF-NB-4 cells and this correlated with lower levels of DNA adducts. Both these cell lines accumulated in S phase, suggesting that ellipticine-DNA adducts interfere with DNA replication. The results demonstrate that among the multiple modes of ellipticine antitumor action, formation of covalent DNA adducts by ellipticine is the predominant mechanism of cytotoxicity to IMR-32 and UKF-NB-4 neuroblastoma cells.

1. Introduction

Neuroblastoma, a tumor of the peripheral sympathetic nervous system, is the most frequent solid extra cranial tumor in children and is a major cause of death from neoplasia in infancy [1]. These tumors are biologically heterogeneous, with cell populations differing in their genetic programs, maturation stage and malignant potential [2]. Neuroblastoma may regress spontaneously in infants, mature to benign ganglioneuromas in older children, or grow relentlessly and be rapidly fatal [2]. Prognosis of high risk tumors is poor, because drug resistance arises in the majority of those patients, initially responding to chemotherapy, in spite of intensive therapy including megatherapy with subsequent hematopoietic progenitor cell transplantation, biotherapy and immunotherapy [2]. Little improvement in therapeutic options has been made in the last decade, requiring a need for the development of new therapies.

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. 1), an alkaloid isolated from *Apocyanacea* plants, and several of its more soluble derivatives (9-hydroxyellipticine, 9-hydroxy-N^2-methyl-ellipticinium, 9-chloro-N^2-methyl-ellipticinium and 9-methoxy-N^2-methyl-ellipticinium) exhibit significant antitumor activities (for a summary see [3]). The main reasons for the interest

* Corresponding author. Tel.: +420 221951285; fax: +420 221951283.
E-mail address: stiborov@natur.cuni.cz (M. Stiborová).

Abbreviations: α-NF, α-naphthoflavone; COX, cyclooxygenase; CYP, cytochrome P450; Δc_T, difference of target minus reference cycle threshold; DMSO, dimethyl sulfoxide; IMDM, Iscove's modified Dulbecco's medium; HPLC, high-performance liquid chromatography; LPO, lactoperoxidase; MDR, multidrug resistance; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide; PBS, phosphate buffered saline; PEI-cellulose, polyethylenimine-cellulose; PVDF, polyvinylidene difluoride; RAL, relative adduct labeling; RT, real-time; PCR, polymerase chain reaction; r.t., retention time; SDS, sodium dodecyl sulphate; TLC, thin layer chromatography.

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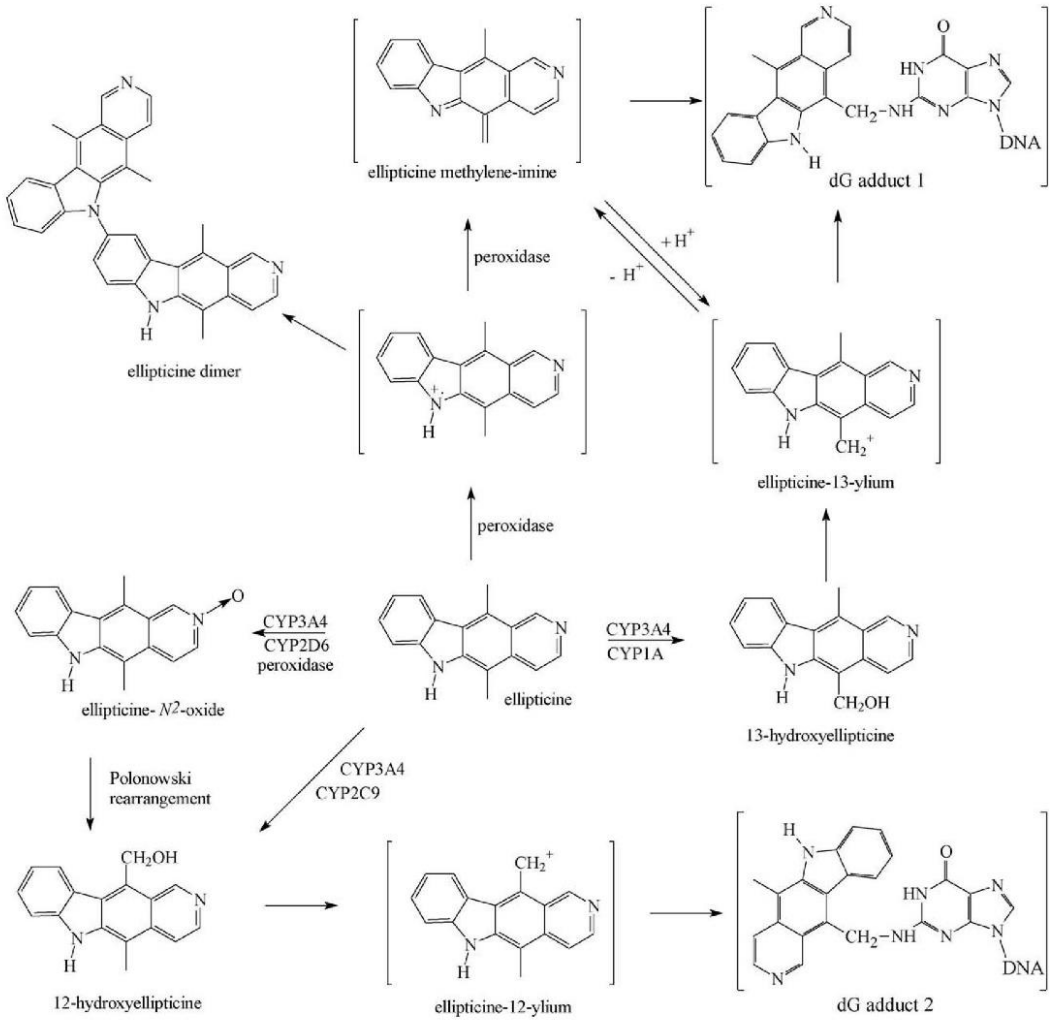

Fig. 1. Metabolism of ellipticine by peroxidases and human CYPs showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and are the electrophilic metabolites postulated as ultimate arylating species or the postulated N^2-deoxyguanosine adducts.

in ellipticine and its derivatives for clinical purposes are their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity [4]. Nevertheless, ellipticine is a potent mutagen.

Ellipticine has been reported to arrest cell cycle progression by regulating the expression of cyclinB1 and Cdc2 as well as phosphorylation of Cdc2 [5,6], to induce apoptotic cell death by the generation of cytotoxic free radicals, the activation of Fas/Fas ligand system, the regulation of Bcl-2 family proteins [5–8], an increase of wild-type p53, the rescue of mutant p53 activity and the initiation of the mitochondrial apoptosis pathway [5,6,8,9]. Ellipticine also uncouples mitochondrial oxidative phosphorylation [10] and thereby disrupts the energy balance of cells. Ellipticine and 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines [11,12], and this correlates with their cytotoxic activity. However, the precise molecular mechanism responsible for these effects has not yet been explained. Chemotherapy-induced cell cycle arrest was shown to result from DNA damages caused by a variety of chemotherapeutics. In the case of ellipticine, it was suggested that the prevalent DNA-mediated mechanisms of their antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA [4,13] and (ii) inhibition of DNA topoisomerase II activity [4,14–16].

We have demonstrated that ellipticine also covalently binds to DNA *in vitro* and *in vivo* after being enzymatically activated with cytochromes P450 (CYP) or peroxidases [3,17–23], suggesting a third possible mechanism of action. Human and rat CYP1A, 1B1 and 3A are the predominant enzymes catalyzing oxidation of ellipticine *in vitro* either to metabolites that are excreted (7-hydroxy- and 9-hydroxyellipticine) or that form DNA adducts (12-hydroxy- and 13-hydroxyellipticine) [3,17–20]. Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine

lactoperoxidase (LPO), human myeloperoxidase (MPO) and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts (Fig. 1) [21,23]. CYP- and/or peroxidase-mediated ellipticine-DNA adducts were detected also in rats and mice *in vivo* [18,22,24]. The same DNA adducts were also detected in cells in culture expressing enzymes activating ellipticine (CYP1A1, COX-1 and MPO), such as human breast adenocarcinoma MCF-7 cells [25], leukemia HL-60 and CCRF-CEM cells [26] and V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 [27]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its activation by CYPs and peroxidases in target tissues.

The aims of this study were to test the cytotoxicity of ellipticine on a panel of human neuroblastoma cell lines [28], including lines resistant to several anticancer drugs [29] and to examine whether DNA adducts are formed in these human cancer cells. The ^{32}P-postlabeling method was used to determine DNA adduct formation by ellipticine and cytotoxicity of ellipticine was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay [30]. Furthermore, other biological parameters including cell-cycle apoptosis and expression of enzymes activating ellipticine were investigated in this study. In addition, we wanted to establish the molecular mechanisms of anticancer action of ellipticine in neuroblastoma cells and to evaluate whether resistance to ellipticine might be induced upon long-term exposure of these cells to this drug.

2. Materials and methods

2.1. Chemicals

Ellipticine, calf thymus DNA, α-naphthoflavone (α-NF), ketoconazole, MTT, N,N-dimethylformamide, agarose, methanol, ethanol, Tris, RNase, proteinase K, propidium iodide, sodium dodecyl sulphate (SDS), polyethylene glycol 6000 and NADPH were from Sigma (St. Louis, MO, USA), dimethyl sulfoxide (DMSO) from Amresco Inc. (Solon, OH, USA), EDTA, Na_2HPO_4, NaH_2PO_4, H_3PO_4, acrylamide, 1% Triton X-100 from Fluka Chemie AG (Buchs, Switzerland), hydrogen peroxide from Merck (Darmstadt, Germany), doxorubicin from Ebewe Pharma (Unterach, Austria), phenol–chloroform from Roth (Karlsruhe, Germany), serum albumin, ammonium hydroxide, isopropanol from PLIVA-Lachema (Brno, Czech Republic). 12-Hydroxy- and 13-hydroxyellipticine were isolated from multiple HPLC runs of ethyl acetate extracts of incubations containing ellipticine and human and/or rat hepatic microsomes as described [19]. All these and other chemicals used in the experiments were of analytical purity or better. Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described [3].

2.2. Cell cultures

The UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines, established from bone marrow metastases of high risk neuroblastoma, were a gift of Prof. J. Cinatl Jr. (J. W. Goethe University, Frankfurt, Germany). The cell line UKF-NB-4 was established from chemoresistant recurrency. IMR-32, high risk neuroblastoma derived cell line, was of commercial source (LGC Promochem, Wesel, Germany). All three cell lines used were derived from high risk neuroblastoma with MYCN amplification, del1p and aneuploidy. The vincristine, doxorubicin, cisplatin and ellipticine resistant cell sublines designated UKF-NB-3 (VCR), UKF-NB-3 (DOXO), UKF-NB-4 (DOXO), UKF-NB-4 (cisplatin) and UKF-NB-4 (Elli) were established by incubation of parental cells with increasing concentrations of the respective drug by a procedure described [31]. Cells were grown at 37 °C and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) (KlinLab Ltd, Prague, Czech Republic), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicilline and 100 μg/ml streptomycine (PAA Laboratories, Pasching, Austria). For hypoxia experiments, cells were maintained in modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) flushed with 1% O_2, 5% CO_2 and balance N_2 for 4 min at 37 °C (hereafter referred to as hypoxia).

2.3. MTT assay

The cytotoxicity of ellipticine and doxorubicin to cells in exponential growth was determined in a 96-well plate. For a dose–response curve, cells in exponential growth were seeded in 100 μl of medium with 10^4 cells per well. Solution of ellipticine and doxorubicin in DMSO (5 μl) in final concentrations of 0.02–50 μM was added. Control cells and medium controls without cells received 5 μl of DMSO without drug. Tumor cell viability was evaluated by MTT test as previously described [30]. Briefly, after incubation (2–4 days) at 37 °C in 5% CO_2 saturated atmosphere the MTT solution (2 mg/ml) was added, the plates were incubated for 4 h and cells lysed in PBS containing 20% of SDS and 50% N,N-dimethylformamide, pH 4.5. The absorbance at 570 nm was measured for each well by multiwell ELISA reader Versamax (Molecular Devices, Sunnyvale, CA, USA). The mean absorbance of medium controls was the background and was subtracted. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control. Each value is the mean of 8 wells with standard deviations. The IC_{50} values were calculated from the linear regression of the dose–log response curves by SOFTmaxPro.

2.4. Cell cycle analysis

To determine cell cycle distribution analysis, 5×10^5 cells were plated in 60 mm dishes and treated with ellipticine (0, 1 and 10 μM) for 48 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in PBS, resuspended in 1 ml of PBS containing 1 μg/ml RNase and 50 mg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by flow cytometry on a FACSCalibur cytometer (BD, San Jose, CA, USA). The data were analyzed using ModFit LT software (Verity Software House, Topsham, ME, USA).

2.5. Apoptosis assay

Cells (5×10^6) were treated with vehicle alone (5 μl DMSO) and increasing concentrations of ellipticine for 48 h, and then collected by centrifugation. Pellets were lysed by DNA lysis buffer (10 mM Tris, pH 7.5, 400 mM EDTA, and 1% Triton X-100) and centrifuged. The supernatant obtained was incubated overnight with proteinase K (0.1 mg/ml) and with RNase (0.2 mg/ml) for 2 h at 37 °C. After extraction with phenol–chloroform (1:1), the DNA was separated in 2% agarose gel (Amresco Inc., Solon, OH, USA) and visualized by UV after staining with ethidium bromide (Top-Bio s.r.o., Prague, Czech Republic).

2.6. Preparation of S9 fractions and assays

S9 fractions were isolated from IMR-32, UKF-NB-3 and UKF-NB-4 cancer cell homogenates by fraction centrifugation as described [32] and the 10,000×*g* for 15 min supernatants were used for incubations.

2.7. Incubations of ellipticine with neuroblastoma S9 fractions

Incubation mixtures used to generate DNA adducts by ellipticine consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, S9 fractions (0.5 mg protein) from IMR-32, UKF-NB-3 and UKF-NB-4 cells, 0.1 mM ellipticine (dissolved in 7.5 μl methanol) and 0.5 mg of calf thymus DNA in a final volume of 750 μl. Incubations were also carried out in the presence of a peroxidase cofactor, hydrogen peroxide (0.1 mM) [33,34]. Incubations were carried out at 37 °C for 30 min; ellipticine-DNA adduct formation was found to be linear up to 30 min of incubation [3]. Control incubations were carried out (i) without S9 fraction, (ii) without NADPH or hydrogen peroxide, (iii) without DNA and (iv) without ellipticine. After the incubation, DNA was isolated by a standard phenol–chloroform extraction method.

2.8. Inhibition studies

The following chemicals were used to inhibit the activation of ellipticine to form DNA adducts in the presence of S9 subcellular fraction of neuroblastoma cells: α-NF, which inhibits CYP1A1 and 1A2 [34–36], and activates oxidation of some substrates by CYP3A4 [36], and ketoconazole, an inhibitor of CYP3A4 [36]. Inhibitors were dissolved in 7.5 μl of methanol, to yield final concentrations of 0.1 mM in the incubation mixtures. Mixtures were then incubated at 37 °C for 10 min with NADPH prior to adding ellipticine, and then incubated for a further 30 min at 37 °C. After the incubation, DNA was isolated as described above, and analyzed for ellipticine-DNA adduct formation.

2.9. Isolation of CYP enzymes and preparation of antibodies against these enzymes

Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA [37]. Human recombinant CYP3A4 was a gift of P. Anzenbacher (Palacky University, Olomouc, Czech Republic).

Leghorn chickens were immunized subcutaneously three times a week with CYP antigens (rat recombinant CYP1A1 and human recombinant CYP3A4 emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for boosters). Immunoglobulin fraction was purified from pooled egg yolks using fractionation by polyethylene glycol 6000 [37,38].

Commercial antibodies, rabbit anti-human CYP1B1 (AbCam, Cambridge, UK) and mouse monoclonal anti-human COX-1 and -2 (Exalpha, Shirley, MA, USA), were used in the experiments.

2.10. Estimation of contents of CYPs, COX-1 and -2 in neuroblastoma cells

Immunoquantitation of CYPs (CYP1A1, 1B1 and 3A4) and COX-1 and -2 in homogenates of neuroblastoma cells was done by Western blot. Protein concentrations in homogenates were assessed using the bicinchoninic acid protein assay (Pierce Rockford, IL, USA) with serum albumin as a standard [18]. Samples containing 10–45 μg proteins were subjected to electrophoresis on SDS/10% polyacrylamide gels [37,38]. After migration, proteins were transferred onto polyvinylidene difluoride (PVDF) (Millipore, Billerica, MA, USA) and/or nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Cellular CYP and COX-1 and -2 proteins were probed with the anti-CYP and anti-COX-1 and -2 rabbit and mouse polyclonal antibodies, respectively, as reported elsewhere [26,37,38]. The antibody against rat recombinant CYP1A1 recognizes both CYP1A1 and 1A2 in rat and human liver microsomes [37–39]. Human recombinant CYP1A1, 1B1 and 3A4 (in Supersomes™, Gentest Corp., Woburn, MA, USA), ovine COX-1 and human recombinant COX-2 (Gentest Corp., Woburn, MA, USA) were used as positive controls to identify the bands of these enzymes in cellular homogenates. The antigen–antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as dye [37,38] and/or with chemiluminescence reagents (Immun-Star HRP Substrate, Bio-Rad, Hercules, CA, USA). Membranes were scanned with a computerized image-analyzing system (Imstar). The detection limit was 0.005 pmol CYP1A per lane (see also literature [37,38]) and 0.01 pmol for the other enzymes.

2.11. Estimation of MPO content in neuroblastoma cells

MPO was analyzed by flow cytometry using anti-human MPO-FITC antibody (Immunotech, Marseille, France). Cultivation was performed in 12-well plates, three samples from every well were prepared and two wells measured. Cells were permeabilized with Fix and Perm kit (Caltag Laboratories, Burlingame, CA, USA) according to the producer's recommendation. The fluorescence intensity of at least 10,000 cells was measured by FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with 488 nm laser and list mode data were analyzed with the CellQuest software [40]. Expression was evaluated as mean intensity of fluorescence. The fluorescence measurements were calibrated for each run by FITC-conjugated bead standards (DAKO Glostrup, Denmark).

2.12. CYP1A1, 1B1, 3A4, COX-1 and -2 mRNA content in neuroblastoma cells

Total RNA was isolated from neuroblastoma cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the procedure supplied by the manufacturer. The quality of isolated RNA was verified by horizontal agarose gel electrophoresis and RNA quantity was assessed by UV–vis spectrophotometry on a Carry 300 spectrophotometer (Varian, Palo Alto, CA, USA). RNA samples (1 μg) were reversely transcribed using random hexamer primers utilizing High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The prepared cDNA was used for real-time (RT) polymerase chain reaction (PCR) under the following cycling conditions: incubation at 50 °C for 2 min and initial denaturation at 95 °C for 10 min, then 50 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The PCR reaction mixtures (20 μl) contained 2 μl cDNA, 10 μl TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) and 2 μl of primer and probe mixture (commercially available unlabeled PCR primers and FAM™ dye-labeled probe for human *CYP1A1*, *1B1*, *3A4*, *COX-1* and *-2* as target genes and *β-actin* as reference internal standard gene) produced by Generi Biotech (Hradec Kralove, Czech Republic). Each sample was analyzed in three parallel aliquots. Negative controls had the same composition as samples but ultrapure water was used instead of cDNA. Samples were analyzed by ABI 7300 cycler (Applied Biosystems, Foster City, CA, USA) and data were evaluated by comparative cycle threshold (c_T) method for relative quantitation of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then Δc_T was evaluated according to the following equations: $\Delta c_T = c_T$ (target) $- c_T$ (internal standard). Δc_T is positive if the target is expressed at a lower level than the internal standard (*β-actin*), and negative if expressed at a higher level. The changes in mRNA expression of studied target genes in UKF-NB-4 (Elli) cells compared to the parental line was calculated with $2^{-(\Delta\Delta c_T)}$.

2.13. Treatment of neuroblastoma cells with ellipticine for DNA adduct analyses

Neuroblastoma cell lines were seeded 24 h prior to treatment at a density of 1×10^5 cells/ml in two 75 cm^3 culture flasks in a total volume of 20 ml of IMDM. Ellipticine was dissolved in 20 μl of DMSO, the final concentration was 0, 0.1, 1 or 10 μM. After 48 h the cells were harvested after trypsinizing by centrifugation at 2000×g for 3 min and two washing steps with 5 ml of PBS yielded a cell pellet, which was stored at −20 °C until DNA isolation. DNA was isolated and labeled as described in the next section.

2.14. DNA isolation and ^{32}P-postlabeling of DNA adducts

DNA from cells was isolated by the phenol–chloroform extraction as described [25,27]. ^{32}P-postlabeling analyses were performed using nuclease P1 enrichment as described previously [3,19,21,27]. From experiments performed earlier, calf thymus DNA incubated with 13-hydroxy- and 12-hydroxyellipticine [19,21], with ellipticine and human recombinant COX-2 [21], and liver DNA of rats treated with ellipticine [17] were labeled with ^{32}P to compare adduct spot patterns.

2.15. HPLC analysis of ^{32}P-labeled DNA adducts

HPLC analysis was performed essentially as described previously [18]. Individual spots detected by ^{32}P-postlabeling were excised from the thin layer and extracted [18]. Cut-outs were extracted with two 800 μl portions of 6 M ammonium hydroxide/isopropanol (1:1) for 40 min. The eluent was evaporated in a Speed-Vac centrifuge. The dried extracts were dissolved in 100 μl of methanol/phosphate buffer (pH 3.5) 1:1 (v/v). Aliquots (50 μl) were analyzed on a phenyl-modified reversed-phase column (250 mm × 4.6 mm, 5 μm Zorbax Phenyl; Säulentechnik Knauer, Berlin, Germany) with a linear gradient of methanol (from 40 to 80% in 45 min) in aqueous 0.5 M sodium phosphate and 0.5 M phosphoric acid (pH 3.5) at a flow rate of 0.9 ml/min. Radioactivity eluting from column was measured by monitoring Cerenkov radiation with a Berthold LB 506 C-1 flow-through radioactivity monitor (500 μl cell, dwell time 6 s).

2.16. Statistical analyses

Statistical associations between IC_{50} values for ellipticine in human neuroblastoma cells and levels of total ellipticine-derived DNA adducts formed in the same cells were determined by the linear regression using Statistical Analysis System software version 6.12. All *P*-values are two-tailed and considered significant at the 0.05 level.

3. Results

3.1. Cytotoxicity of ellipticine to human neuroblastoma cells

To determine the cytotoxicity of ellipticine to human neuroblastoma cells, these cells were treated with increasing concentrations of ellipticine. We first determined the effect of ellipticine on growth of human neuroblastoma cell lines (IMR-32, UKF-NB-3 and UKF-NB-4) cultured for 96 h in the presence of ellipticine, using MTT assay. As shown in Fig. 2, all three neuroblastoma cell lines were sensitive to ellipticine. The cytotoxic effects of ellipticine were also detected to the derived daughter neuroblastoma lines that were resistant to vincristine, doxorubicin or cisplatin (Table 1).

Cytotoxicity of ellipticine was compared with that of doxorubicin (Table 1), one of the drugs currently used for neuroblastoma

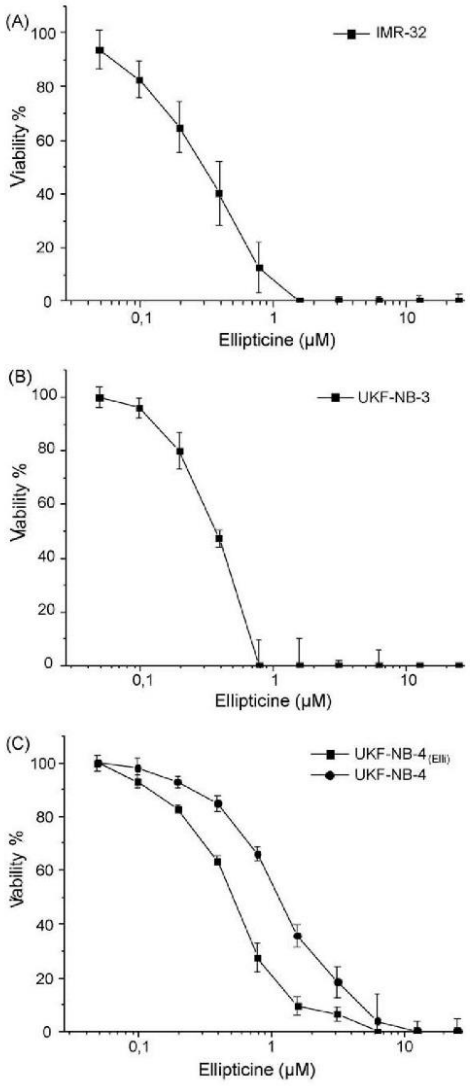

Fig. 2. Cytotoxicity (viable cells as percent of control) of ellipticine to IMR-32 (A), UKF-NB-3 (B), UKF-NB-4 and UKF-NB-4 cells resistant to ellipticine (UKF-NB-4^{Elli}) (C) after 96 h exposure to the compound, determined by the MTT assay. Values are means and standard deviations of 8 determinations.

treatment [2]. Ellipticine and doxorubicin inhibited the growth of neuroblastoma cell lines in a dose-dependent manner. The IC_{50} values for ellipticine and doxorubicin calculated from the dose-log response curves are shown in Table 1. The toxicity of ellipticine to UKF-NB-3 and UKF-NB-4 cells was similar to that of doxorubicin to these cells, while IMR-32 cells were more than 20-times more sensitive to doxorubicin than to ellipticine (see IC_{50} values shown in Table 1).

Cytotoxicity elicited by ellipticine and/or doxorubicin in the parental cell lines was compared to that in their variants resistant to doxorubicin. Neuroblastoma cells resistant to doxorubicin were only slightly resistant to ellipticine (Table 1), even though cross-resistance is expected [31]. The IC_{50} values for ellipticine in IMR-32 (DOXO), UKF-NB-3 (DOXO) and UKF-NB-4 (DOXO) were only 2-,

Table 1
Cytotoxicity of ellipticine and doxorubicin to neuroblastoma cell lines.

Cells	IC_{50}	
	For ellipticine (μM)	For doxorubicin (μM)
IMR-32	0.27 ± 0.02	0.01 ± 0.01
IMR-32 (hypoxic conditions)	0.43 ± 0.02***	Not measured
IMR-32 (DOXO)	0.53 ± 0.03***	0.10 ± 0.03***
UKF-NB-3	0.44 ± 0.03	0.42 ± 0.04
UKF-NB-3 (hypoxic conditions)	0.44 ± 0.03	0.47 ± 0.04
UKF-NB-3 (VCR)	0.64 ± 0.05***	0.83 ± 0.08***
UKF-NB-3 (DOXO)	0.78 ± 0.06***	1.75 ± 0.17***
UKF-NB-3 (cisplatin)	0.46 ± 0.02	0.69 ± 0.05***
UKF-NB-4	0.44 ± 0.03	0.70 ± 0.02
UKF-NB-4 (hypoxic conditions)	0.77 ± 0.04***	0.60 ± 0.05**
UKF-NB-4 (Elli)	1.17 ± 0.07***	0.70 ± 0.05
UKF-NB-4 (Elli) (hypoxic conditions)	1.57 ± 0.08***ΔΔΔ	1.51 ± 0.09***ΔΔΔ
UKF-NB-4 (DOXO)	1.14 ± 0.07***	3.80 ± 0.12***
UKF-NB-4 (cisplatin)	0.62 ± 0.05***	0.22 ± 0.02***

IC_{50} values were calculated from the linear regression of the dose–log response curves. Values are mean ± S.D. of at least three experiments. The data were analyzed statistically by Student's *t*-test. Values significantly different from individual parent cell lines cultivated under the standard conditions: $^{**}P < 0.01$, $^{***}P < 0.001$. Values significantly different from the UKF-NB-4 (Elli) cell line cultivated under the standard conditions: $^{\Delta\Delta\Delta}P < 0.001$.

1.8- and 2.6-fold higher than those in the parental cell lines, respectively, whereas the IC_{50} values for doxorubicin were up to 10-fold higher in the doxorubicin-resistant cell lines. Likewise, neuroblastoma UKF-NB-3 and UKF-NB-4 cell lines resistant to vicristine and cisplatin showed practically no cross-resistance to ellipticine; the IC_{50} values for ellipticine in UKF-NB-3 (VCR) and UKF-NB-4 (cisplatin) were only 1.4-fold higher than those in the parental cell lines (Table 1). The IC_{50} values for ellipticine in UKF-NB-3 and UKF-NB-3 (cisplatin) were even identical (Table 1).

3.2. Ellipticine-induced cell cycle arrest and apoptosis in human neuroblastoma cells

In order to examine the mechanism responsible for ellipticine-mediated cell growth inhibition, we first evaluated cell cycle distribution using flow cytometric analysis. Compared with the vehicle-treated control, treatment cells with 10 μM ellipticine resulted in an appreciable arrest of IMR-32 and UKF-NB-4 cells in S phase of cell cycle after 48 h of treatment with a concomitant decrease in G0/G1 phase (Fig. 3). On the contrary, different effects of ellipticine on the cell cycle of UKF-NB-3 cells were detected; an arrest in G0/G1 and G2/M phases of cell cycle with a concomitant pronounced decrease in S phase was found in this neuroblastoma line.

We next assessed the effect of ellipticine on the induction of apoptosis in neuroblastoma cells by DNA fragmentation assay. The results showed that ellipticine treatment results in the formation of DNA fragments in neuroblastoma cells, as assessed by agarose gel electrophoresis at 48 h (see Fig. 4 for UKF-NB-4 cells).

3.3. Determination of DNA adduct formation by ellipticine in neuroblastoma cells

The three parental neuroblastoma cell lines shown to be sensitive to ellipticine (Table 1) were treated with 0.1, 1, and 10 μM ellipticine for 48 h. Using the nuclease P1 version of ^{32}P-postlabeling assay, which was found to be suitable to detect and quantify DNA adducts formed by ellipticine [3,17–19,21,22], ellipticine-derived adducts were detected in the DNA of these cells (Fig. 5A–C). Two major ellipticine-DNA adducts (spots 1 and 2 in Fig. 5A–C) were formed in all cells (Table 2). No adducts were detected in DNA of control cells treated with solvent only.

Chromatographic properties of the two major adduct spots on PEI-cellulose TLC plates (spots 1 and 2) were similar to those of ellipticine-derived DNA adducts found previously after *in vitro* incubation of calf thymus DNA with ellipticine and isolated CYPs [3,18], or peroxidases [21] or after treatment of cells in culture with this anticancer drug [25–27] or *in vivo* (Fig. 5D), in several organs of rats [17,22] and mice [24] exposed to this agent. Both these adducts were found to be generated from 13-hydroxy- and 12-hydroxyellipticine [19,21,41] (Fig. 5E and F) as confirmed by cochromatographic analysis using TLC and HPLC (data not shown). Both adducts were identified as deoxyguanosine adducts in DNA [18]. Besides these adducts, additional two minor adducts (spots 6 and 7 in Fig. 5C and D) were detected in DNA of UKF-NB-4 cells treated with 10 μM ellipticine (Table 2). Both these minor adducts are known to be generated *in vitro* mainly by peroxidase-catalyzed oxidation [21,23]. The low levels of these adducts prevented HPLC co-chromatographic analysis or their further characterization.

Ellipticine-DNA adduct levels were dose dependent in all cells with an overproportional increase between 1 and 10 μM ellipticine. IMR-32 cells resistant to doxorubicin formed lower levels of ellipticine-DNA adducts as did UKF-NB-4 cells resistant to this drug than the respective parental cells. All resistant daughter cells of these lines except those to cisplatin had lower ellipticine-DNA adduct levels. The highest levels over all were formed in UKF-NB-3 resistant to cisplatin (Table 2).

3.4. Ellipticine-resistant neuroblastoma cells

To induce resistance to ellipticine, UKF-NB-4 cells were incubated for 36 months with increasing concentrations of ellipticine (1–2.5 μM). Morphological changes were seen in these UKF-NB-4 cells (Fig. 6), which did not change during five passages in medium without ellipticine. These cells were indeed resistant, since IC_{50} values of ellipticine increased 2.7-fold (Fig. 2 and Table 1). In addition, the growth rate of the ellipticine-resistant UKF-NB-4 cell line was slower than that of the sensitive line (see doubling times shown in Table 3). The development of resistance of the UKF-NB-4 cells resulted in 3.5-fold lower levels of ellipticine-DNA adducts after treatment of these cells with ellipticine (Table 2). These effects were associated also with changes in ellipticine-induced cell cycle arrest. While 1 and 10 μM ellipticine induced an arrest in the S phase of the cell cycle in the parent neuroblastoma cell line, no significant arrest in this phase was detectable in the ellipticine-resistant UKF-NB-4 cell line at 1 μM ellipticine; only a minor arrest in this phase of the cell cycle ($P < 0.05$) was induced by 10 μM ellipticine. In addition, a low but significant arrest in G2/M phase of cell cycle with a concomitant decrease in G0/G1 phase was caused by ellipticine in the resistant line ($P < 0.001$ at 10 μM ellipticine) (Fig. 3).

3.5. Expression of biotransforming enzymes in human neuroblastoma cells

Using Western blot analysis with polyclonal antibodies raised against CYP1A1, 1B1 and 3A4 or with monoclonal antibodies raised against COX-1 or -2, the protein expression levels of these enzymes were analyzed in the parental neuroblastoma lines and in the cells resistant to ellipticine. Under the experimental conditions used, expression of CYP1A, 1B1 and 3A4 (Fig. 7) was found in all cells, while no expression of COX-1 and -2 was detected. MPO expression was analyzed by flow cytometry, but was not detected in any of the neuroblastoma cells analyzed (data not shown).

Besides protein expression of the enzymes, mRNA expression in neuroblastoma cells was also investigated. Total RNA was isolated

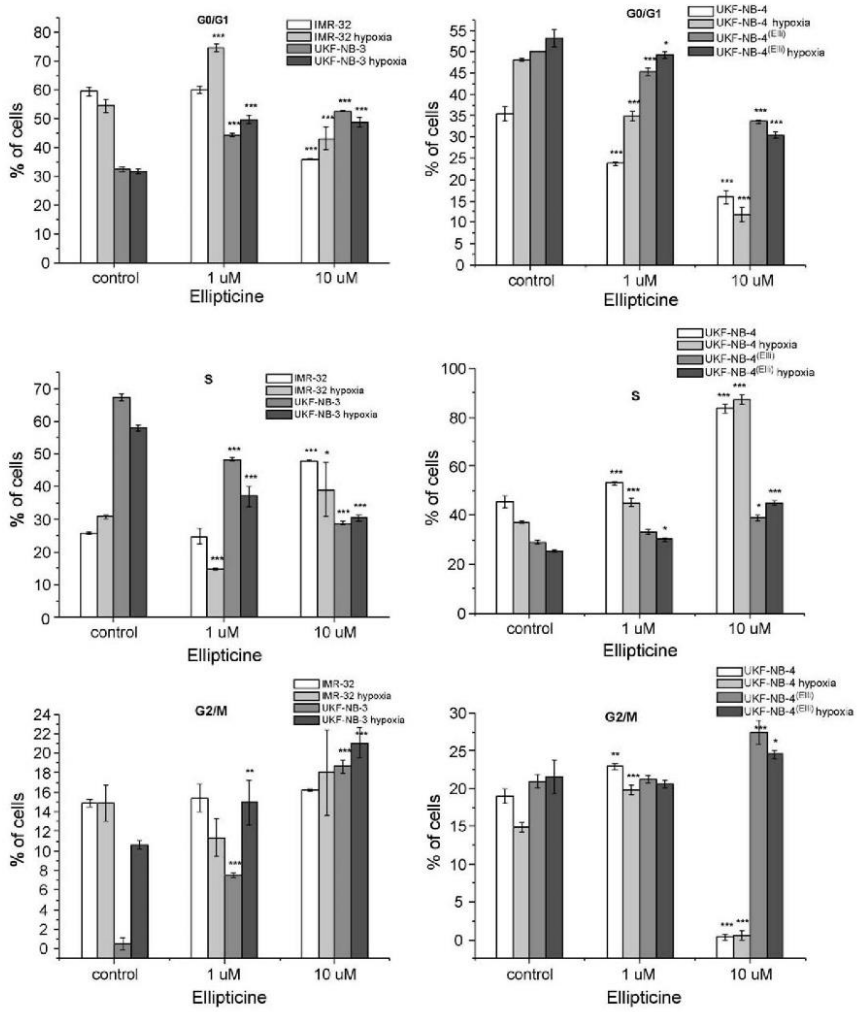

Fig. 3. Effect of ellipticine on cell cycle distribution in neuroblastoma cells. Cell were treated with vehicle or ellipticine for 48 h, and cell cycle distribution was assessed by flow cytometry. $N = 3$, values are means ± S.D.

from frozen cells and the relative amounts of *CYP1A1*, *1B1*, *3A4*, *COX-1* and *-2* mRNAs were measured by real-time PCR. Even though expression of mRNAs of these enzymes was detectable in all neuroblastoma cell lines, levels of *COX-2*, *CYP3A4* and *1A1* mRNAs were extremely low in the three original lines (Table 4). In UKF-NB-4 cell resistant to ellipticine, 0.5- and 0.3-fold lower levels of *CYP3A4* and *1B1* mRNAs, respectively, were found than in the parental line (Table 4). On the contrary, higher levels of *CYP1A1* (10.3-fold) and *COX-2* mRNAs (300.6-fold) were detected in the ellipticine-resistant cells line (Table 4). The changes in mRNA expression of studied target genes in UKF-NB-4 (Elli) cells compared to the parental line were calculated with $2^{-(\Delta\Delta cT)}$. However, if cells of both lines were exposed to 10 μM ellipticine, no difference in expression levels of these mRNAs was found between the ellipticine-resistant and sensitive UKF-NB-4 cell lines; levels of *CYP1A1*, *1B1*, *3A4*, *COX-1* and *-2* mRNAs were essentially the same and similar to those found in unexposed cells (data not shown).

3.6. S9 fractions of neuroblastoma cells are capable of activating ellipticine

Because CYPs and peroxidases, which activate ellipticine [3,17,19,22], were found to be expressed in neuroblastoma cells (see above), we evaluated whether S9 fractions isolated from the parental IMR-32, UKF-NB-3 and UKF-NB-4 cells activate ellipticine to species forming DNA adducts.

The DNA adduct pattern generated by ellipticine in this system was determined by ^{32}P-postlabeling, and again consisted of two adducts, which were identical to adduct spots 1 and 2 formed in intact neuroblastoma cells (Fig. 5A–C and Table 5). Chromatographic analysis of spots 1 and 2 on HPLC confirmed that these adducts are derived from 13-hydroxy- and 12-hydroxyellipticine, respectively, by their coelution with prepared reference compounds (data not shown). Control incubations without S9 subcellular fractions were free of adduct spot 1, but adduct spot 2 was always detected (data not shown). This finding is consistent

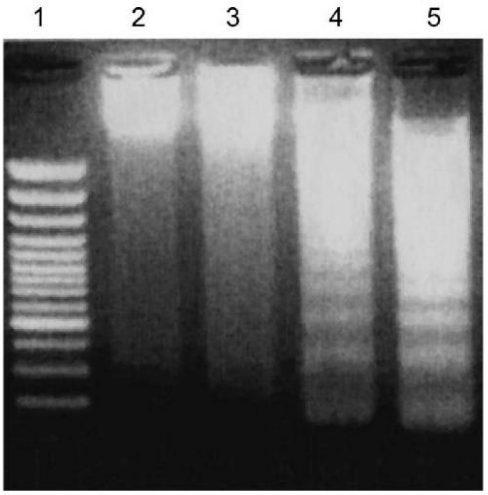

Fig. 4. The induction of apoptosis in ellipticine-treated neuroblastoma UKF-NB-4 cells. The DNA fragmentation was assessed by gel electrophoresis. Cells were treated with vehicle and ellipticine for 48 h, and then the fragmentation of DNA was assessed by agarose gel electrophoresis. (Lane 1) marker, (lane 2) UKF-NB-4 cells treated with vehicle alone (DMSO), (lane 3) 0.1 μM ellipticine, (lane 4) 1 μM ellipticine, and (lane 5) 10 μM ellipticine.

with our previous results showing that this adduct is formed also non-enzymatically [3,18,19,21].

To determine the extent of CYP and peroxidase contribution to ellipticine activation, we added cofactors and selective inhibitors of these enzymes to incubations containing S9 fractions isolated from neuroblastoma cells. In the presence of NADPH, the cofactor of CYP, S9 samples of all cells were capable of activating ellipticine to form DNA adducts (Table 5). The S9 fraction from IMR-32 cells was most active.

α-NF, an inhibitor of CYP1A and an activator of CYP3A4 [36], slightly decreased the levels of ellipticine-DNA adducts formed by the S9 fraction of UKF-NB-3 cells (Table 5), but had no effect in incubation with S9 fraction from UKF-NB-4 cells. In contrast, α-NF added to the S9 fraction of IMR-32 cells led to 3-fold higher levels of ellipticine-DNA adducts, predominantly of adduct 1, suggesting the importance of CYP3A4 in ellipticine activation in these cells (Table 5). This suggestion was confirmed by the effect of ketoconazole, a selective inhibitor of CYP3A [36,42], which significantly decreased the levels of ellipticine-DNA adducts formed by S9 fractions from all three cell lines. Addition of hydrogen peroxide, a cofactor for peroxidases [21,33,34,43], increased ellipticine-DNA adduct levels in S9 fractions of all neuroblastoma cells, most effectively in UKF-NB-4 cells, where 10-fold higher levels than without cofactor were determined (Table 5).

These results demonstrate that CYPs and peroxidases are active to different degree in neuroblastoma cells from different patients, and capable of catalyzing the formation of DNA adducts 1 and 2 from ellipticine.

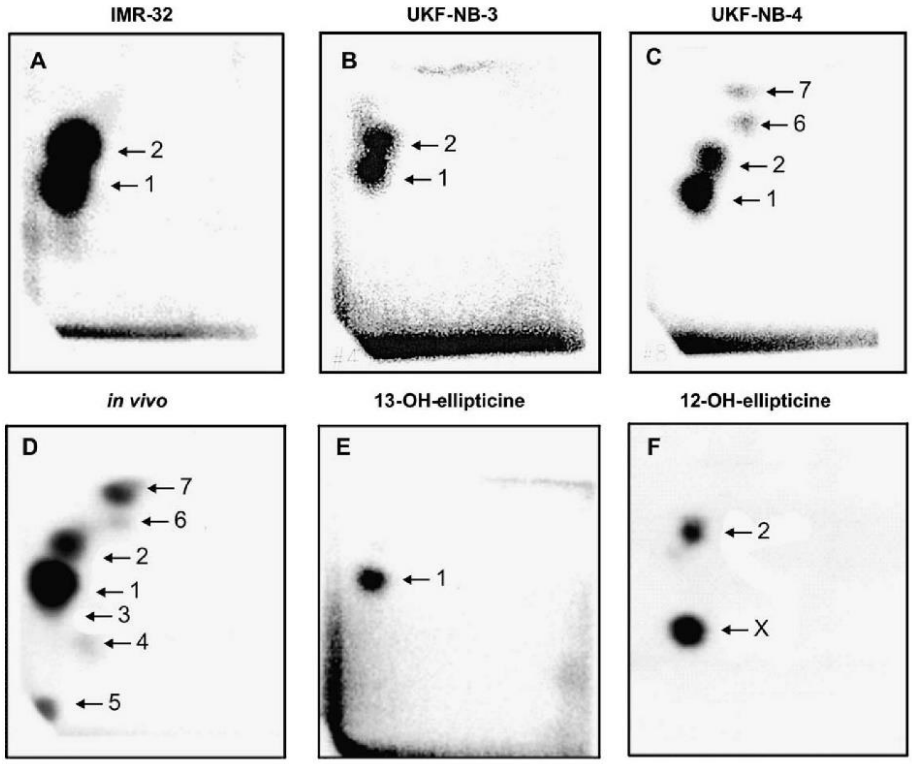

Fig. 5. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of DNA isolated from neuroblastoma IMR-32 (A), UKF-NB-3 (B) and UKF-NB-4 cells exposed to 10 μM ellipticine (C) for 48 h, of liver DNA of rats treated with 40 mg ellipticine per kilogram body weight (D), from calf thymus DNA reacted with 13-hydroxyellipticine (E) and 12-hydroxyelipticine (F). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. (A, B, D) Scans of the plates for 6.5 min from the imager; (C, E, F) autoradiographs of films exposed for 1 h at −80 °C. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

Table 2
DNA adduct formation by ellipticine in human neuroblastoma cell lines.

Cells	Levels of DNA adducts (RAL × 10^{-7})[a]				
	Adduct 1	Adduct 2	Adduct 6	Adduct 7	Total
IMR-32					
+0.1 μM ellipticine	0.10 ± 0.01	0.13 ± 0.01	n.d.	n.d.	0.23 ± 0.02
+1.0 μM ellipticine	0.26 ± 0.02	0.31 ± 0.03	n.d.	n.d.	0.57 ± 0.05
+1.0 μM ellipticine hypoxic conditions	0.22 ± 0.03*	0.23 ± 0.02***	n.d.	n.d.	0.45 ± 0.04**
+10 μM ellipticine	13.15 ± 1.30	13.13 ± 1.30	n.d.	n.d.	26.28 ± 2.60
+10 μM ellipticine hypoxic conditions	8.88 ± 1.01***	7.22 ± 0.82***	n.d.	n.d.	16.10 ± 1.63***
IMR-32 (DOXO)					
+0.1 μM ellipticine	0.02 ± 0.01	0.12 ± 0.01	n.d.	n.d.	0.14 ± 0.01
+1.0 μM ellipticine	0.17 ± 0.02	0.30 ± 0.03	n.d.	n.d.	0.47 ± 0.05
+10 μM ellipticine	2.88 ± 0.29	7.14 ± 0.70	n.d.	n.d.	10.02 ± 1.00
UKF-NB-3					
+1.0 μM ellipticine	0.12 ± 0.01	0.23 ± 0.02	n.d.	n.d.	0.35 ± 0.04
+1.0 μM ellipticine hypoxic conditions	0.18 ± 0.02**	0.15 ± 0.02***	n.d.	n.d.	0.33 ± 0.03
+10 μM ellipticine	3.26 ± 0.32	2.64 ± 0.40	n.d.	n.d.	5.90 ± 0.68
+10 μM ellipticine hypoxic conditions	1.63 ± 0.21***	1.65 ± 0.19***	n.d.	n.d.	3.28 ± 0.42***
UKF-NB-3 (VCR)					
+0.1 μM ellipticine	0.04 ± 0.01	0.04 ± 0.01	n.d.	n.d.	0.08 ± 0.02
+1.0 μM ellipticine	0.27 ± 0.03	0.62 ± 0.06	n.d.	n.d.	0.89 ± 0.09
+10 μM ellipticine	6.49 ± 0.65	10.14 ± 1.00	n.d.	n.d.	16.63 ± 1.70
UKF-NB-3 (DOXO)					
+0.1 μM ellipticine	0.13 ± 0.01	0.22 ± 0.02	n.d.	n.d.	0.35 ± 0.04
+1.0 μM ellipticine	0.18 ± 0.02	0.43 ± 0.04	n.d.	n.d.	0.61 ± 0.06
+10 μM ellipticine	4.02 ± 0.40	6.00 ± 0.60	n.d.	n.d.	10.02 ± 1.00
UKF-NB-3 (cisplatin)					
+0.1 μM ellipticine	0.03 ± 0.01	0.02 ± 0.01	n.d.	n.d.	0.05 ± 0.01
+1.0 μM ellipticine	0.06 ± 0.01	0.08 ± 0.01	n.d.	n.d.	0.14 ± 0.01
+10 μM ellipticine	18.4 ± 8.5	25.50 ± 2.54	n.d.	n.d.	43.99 ± 4.40
UKF-NB-4					
+0.1 μM ellipticine	0.04 ± 0.01	0.01 ± 0.01	n.d.	n.d.	0.05 ± 0.01
+1.0 μM ellipticine	0.20 ± 0.02	0.38 ± 0.04	n.d.	n.d.	0.58 ± 0.06
+1.0 μM ellipticine hypoxic conditions	0.18 ± 0.02	0.15 ± 0.02***	n.d.	n.d.	0.30 ± 0.02***
+10 μM ellipticine	5.40 ± 0.56	6.50 ± 0.81	0.27 ± 0.03	0.37 ± 0.05	12.54 ± 1.51
+10 μM ellipticine hypoxic conditions	1.86 ± 0.21***	3.74 ± 0.42***	0.26 ± 0.03	0.39 ± 0.03	6.25 ± 0.68***
UKF-NB-4 (Elli)					
+1.0 μM ellipticine	0.07 ± 0.01***	0.06 ± 0.01***	0.06 ± 0.01	n.d.	0.19 ± 0.02***
+1.0 μM ellipticine hypoxic conditions	0.08 ± 0.01***	0.06 ± 0.0***	0.04 ± 0.01***	n.d.	0.18 ± 0.02***
+10 μM ellipticine	1.99 ± 0.20***	1.43 ± 0.14***	0.15 ± 0.01***	0.02 ± 0.01***	3.60 ± 0.39***
+10 μM ellipticine hypoxic conditions	0.34 ± 0.03***ΔΔΔ	0.24 ± 0.03***ΔΔΔ	0.12 ± 0.01***Δ	0.02 ± 0.01**	0.72 ± 0.07***ΔΔΔ
UKF-NB-4 (DOXO)					
+0.1 μM ellipticine	0.07 ± 0.01	0.10 ± 0.01	n.d.	n.d.	0.17 ± 0.02
+1.0 μM ellipticine	0.54 ± 0.05	1.02 ± 0.10	n.d.	n.d.	1.56 ± 0.16
+10 μM ellipticine	0.23 ± 0.20	1.22 ± 0.10	n.d.	n.d.	1.45 ± 0.14
UKF-NB-4 (cisplatin)					
+0.1 μM ellipticine	0.02 ± 0.01	0.03 ± 0.01	n.d.	n.d.	0.05 ± 0.01
+1.0 μM ellipticine	0.24 ± 0.02	0.42 ± 0.04	n.d.	n.d.	0.66 ± 0.07
+10 μM ellipticine	5.73 ± 0.58	9.66 ± 0.97	n.d.	n.d.	15.39 ± 1.50

Neuroblastoma cells were exposed to ellipticine for 48 h. DNA adducts were analyzed by the nuclease P1 version of the ^{32}P-postlabeling assay.
The data were analyzed statistically by Student's *t*-test. Values significantly different from individual parent cell lines cultivated under the standard conditions: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Values significantly different from the UKF-NB-4 (Elli) cell line cultivated under the standard conditions: $^{\Delta\Delta\Delta}P < 0.001$.
[a] RAL, relative adduct labeling; averages and S.D. of three experiments. n.d.: not detected (the detection limit of RAL was $1/10^{11}$ nucleotides).

To investigate whether the cytotoxic activity of ellipticine to neuroblastoma cells depends on the CYP- and/or peroxidase-catalyzed formation of ellipticine-DNA adducts, and if so, which is the relative contribution of each enzyme system, two experimental approaches were employed: (i) correlation of the IC_{50} values for ellipticine in each cell line with the levels of ellipticine-DNA adducts in the same line, and (ii) analysis of the effect of hypoxic cell culture conditions on ellipticine cytotoxicity and DNA adduct formation. Evaluation of ellipticine cytotoxicity under anaerobic conditions is also important, because such conditions mimic the environment typical for malignant tumors.

3.7. Cytotoxicity of and DNA adduct formation by ellipticine in neuroblastoma cells cultivated under hypoxic conditions

Growth inhibition and a cell cycle arrest are mediated by ellipticine in neuroblastoma cells even under hypoxic conditions of their cultivation. Ellipticine was less toxic to the cells grown under hypoxic conditions except to UKF-NB-3 cells (Table 1). The cell

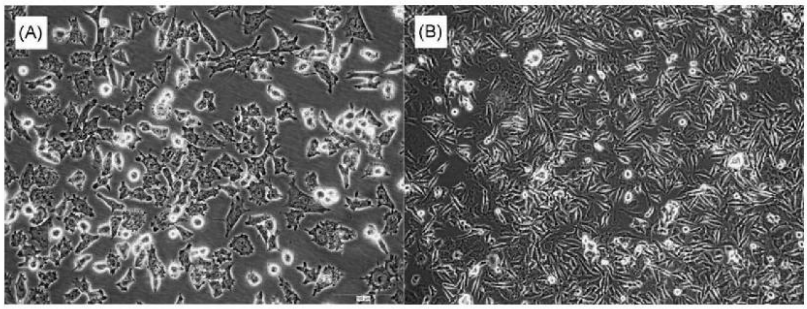

Fig. 6. Neuroblastoma cell lines UKF-NB-4 (A) and UKF-NB-4 resistant to ellipticine (B) (200-fold magnification).

growth rates under the hypoxic conditions were slower in IMR-32 and UKF-NB-4, and in this line resistant to ellipticine (see doubling times shown in Table 3). On the contrary, no changes in the doubling times were found in UKF-NB-3 cell lines (Table 3). The effects of ellipticine on the cell cycle of all original lines tested were not changed significantly by hypoxic conditions, but cultivation of UKF-NB-4 resistant to ellipticine under such conditions resulted in an arrest in S phase of the cell cycle (Fig. 3). Under hypoxic conditions the ellipticine-DNA adduct levels in neuroblastoma cells were lower, predominantly in IMR-32 and UKF-NB-4 cells (Table 2). In parental UKF-NB-4 cells and those resistant to ellipticine, the decrease in levels of ellipticine-DNA adducts by hypoxia was 5-fold and resulted from a decreased formation of adducts 1 and 2, but not of adducts 6 and 7 (Table 2). This finding shows that CYP enzymes, whose activities are dependent on oxygen, are predominantly responsible for formation of adducts 1 and 2 in these neuroblastoma cells, while generation of adducts 6 and 7 is mediated by peroxidases, which are oxygen independent.

Table 3
Doubling times[a] of neuroblastoma cells grown under standard and hypoxic conditions.

Neuroblastoma	Doubling times[a] (h)	
	Standard conditions	Hypoxic conditions
IMR-32	25.18 ± 0.22	$34.69 \pm 0.46^{***}$
UKF-NB-3	29.67 ± 0.28	30.11 ± 0.40
UKF-NB-4	26.68 ± 0.24	$32.56 \pm 0.75^{***}$
UKF-NB-4 (Elli)	$34.56 \pm 0.61^{\Delta\Delta\Delta}$	$44.30 \pm 1.60^{***\Delta\Delta\Delta}$

[a] Results shown are mean ± S.D. from data found of four experiments. The data were analyzed statistically by Student's *t*-test. Values of doubling times significantly different from those of the cells grown under standard conditions: $^{***}P < 0.001$. Values of doubling times significantly different from those of the UKF-NB-4 parent cell lines: $^{\Delta\Delta\Delta}P < 0.001$.

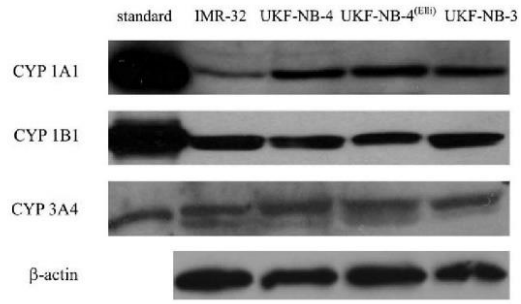

Fig. 7. Immunoblots of CYP1A1, CYP1B1, and CYP3A4 in neuroblastoma cell homogenates. Cell homogenates were subjected to SDS-PAGE, proteins transferred to PVDF membranes and probed with antibodies as described in Material and Methods. Human CYP1A1, CYP1B1 and CYP3A4 were used as standards.

3.8. Correlation of cytotoxicity of ellipticine with DNA adduct formation

We analyzed the relationships between IC_{50} values for ellipticine and levels of ellipticine-DNA adducts. In the case of IMR-32 and UKF-NB-4 cells, high levels of DNA adducts correlated with ellipticine-induced cytotoxicity to these cells (Fig. 8). In contrast to these results, the ellipticine-induced cytotoxicity to the UKF-NB-3 cells showed no correlation to ellipticine-DNA adduct levels.

4. Discussion

The results of this study show that ellipticine is cytotoxic to human neuroblastoma cell lines (both to the parental IMR-32, UKF-NB-3, UKF-NB-4 lines and some of their sublines resistant to several antitumor drugs). In addition, the results shed light on the mechanism of ellipticine cytotoxicity to neuroblastoma cells. The mode of antitumor, cytotoxic and mutagenic action of ellipticine is considered to be based mainly on DNA damage such as intercalation into DNA [4,44], inhibition of topoisomerase II [4,14–16], and formation of covalent DNA adducts mediated by CYPs and peroxidases [3,19,21,24,45]. Intercalation of ellipticine into DNA and inhibition of topoisomerase II occur in all cell types irrespective of their metabolic capacity, because of the general chemical properties of this drug and its affinity to DNA and topoisomerase II protein [4,9,45]. However, the formation of ellipticine-DNA adducts, which is dependent on ellipticine activation by CYPs and peroxidases, has not yet been proven as a general mechanism. This ellipticine action was unambiguously found *in vitro*, using several CYP and peroxidase enzymes for ellipticine activation [3,17,19,21,24,45] and *in vivo* in rats and mice [8,22,24]. Ellipticine-DNA adducts were quantified also in three human cancer cell lines, breast adenocarcinoma MCF-7 cells [25], and the leukemia HL-60 and CCRF-CEM cells [26]. Because ellipticine derivatives target also neurological tumors, we investigated whether this mechanism occurs also in neuroblastoma cells. In addition, a role of this mechanism in ellipticine toxicity to these cancer cells was examined.

Using the ^{32}P-postlabeling assay we clearly demonstrated that ellipticine binds covalently to DNA of neuroblastoma cells; two major ellipticine-DNA adducts, identical with those formed by the CYP- and peroxidase-mediated ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine, were formed in all neuroblastoma cells tested in the study, including the line with induced resistance to ellipticine. These adducts are formed from two

Table 4
Expression of mRNA of CYP1A1, 1B1, 3A4, COX-1 and -2 in neuroblastoma cells.

Neuroblastoma	Δc_T[a]				
	CYP1A1	CYP1B1	CYP3A4	COX-1	COX-2
IMR-32	12.21 ± 0.13	5.19 ± 0.12	15.31 ± 0.73	12.99 ± 0.33	14.86 ± 0.28
UKF-NB-3	10.53 ± 0.15	3.35 ± 0.02	15.52 ± 0.67	4.63 ± 0.09	16.40 ± 0.11
UKF-NB-4	11.00 ± 0.48	6.25 ± 0.28	11.96 ± 0.19	9.04 ± 0.41	16.06 ± 0.30
UKF-NB-4 (Elli)	7.63 ± 0.08***	7.85 ± 0.35**	12.83 ± 0.13*	8.78 ± 0.02	7.80 ± 0.21***

[a] Δc_T was evaluated according to the following equations: $\Delta c_T = c_T$ (target) − c_T (internal standard). Δc_T is positive if the target is expressed at a lower level than the internal standard (*β-actin*), and negative if expressed at a higher level. Results shown are mean ± S.D. from data found for three experiments. The data were analyzed statistically by Student's *t*-test. Values significantly different from the parent UKF-NB-4 cell line: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

reactive species, ellipticine-13-ylium and ellipticine-12-ylium (Fig. 1), which we had suggested earlier to react with one of the nucleophilic centers in the deoxyguanosine residue in DNA (e.g. the exocyclic amino group of guanine, Fig. 1) [19,21,23,24,41]. The low amount of each DNA adduct recovered from digests of DNA treated with 13-hydroxyellipticine or 12-hydroxyellipticine (Fig. 4), however, prevented their further structural characterization. Synthetic approaches are currently being followed in our laboratory to prepare authentic ellipticine-DNA adduct standards [41,46]. Besides these adducts, two additional adduct spots were found in UKF-NB-4 cells.

Hypoxia frequently occurs in tumors because of their fast growth and inadequate vascularization. It strongly correlates with advanced disease and poor outcome caused by chemoresistance. The covalent binding of ellipticine metabolites to DNA decreased when cells were cultivated and treated with ellipticine under hypoxic conditions. The importance of oxygen for the ellipticine-DNA adduct formation suggests that activation of ellipticine to species binding to DNA occurring in neuroblastoma cells is mediated by CYP enzymes. The observed effects of α-NF and ketoconazole upon NADPH-dependent ellipticine-DNA adduct formation in S9 fractions from neuroblastoma cells suggests that CYP1A1 and 3A4 are the relevant CYP enzymes for ellipticine activation. CYP3A4 and, to a lower extent CYP1A1/2 were also found to be the enzymes activating ellipticine in human and rat livers [3,18,19]. Human CYP1A1/2 and 1B1 also oxidize ellipticine to 9-hydroxy- and 7-hydroxyellipticine, which are detoxication products [19]. The expression levels and the activities of these enzymes are, therefore, crucial for the extent of covalent DNA modification by ellipticine in neuroblastoma cells. Indeed, all CYPs relevant for ellipticine metabolism (CYP1A1, 1B1 and 3A4) were found to be expressed both at mRNA and protein levels. Even though expression of peroxidases such as COX-1 and -2 or MPO have only been detected at mRNA levels not by Western blots, hydrogen peroxide stimulated the formation of ellipticine-DNA adducts in *in vitro* incubations with S9 fractions from neuroblastoma cells, particularly from UKF-NB-4 cell line. The pattern of the ellipticine-DNA adducts found in UKF-NB-4 cells also point to peroxidase activation in intact cells, because in addition to DNA adducts 1 and 2, two minor DNA adducts 6 and 7, the typical products of peroxidase activation [21], are formed in these cells. The objective of our future work is to analyze the expression of COX and MPO in more detail in UKF-NB-4 cells with more sensitive antibodies against these enzymes, in addition to investigating the expression of other peroxidases such as LPO, which was not analyzed here.

Table 5
DNA adduct formation by ellipticine activated by S9 fractions from neuroblastoma cells.

S9 fraction of cells	Levels of DNA adducts (RAL × 10^{-8})[a]		
	Adduct 1	Adduct 2	Total
IMR-32			
Without cofactor	0.093 ± 0.010	0.031 ± 0.010	0.124 ± 0.016
+NADPH	0.275 ± 0.021	0.300 ± 0.028	0.575 ± 0.052
+NADPH + α-NF	1.470 ± 0.131***	0.397 ± 0.031***	1.867 ± 0.165***
+NADPH + ketoconazol	0.110 ± 0.010***	n.d.	0.110 ± 0.010***
+H_2O_2	0.413 ± 0.040***	0.071 ± 0.009***	0.484 ± 0.050*
UKF-NB-3			
Without cofactor	0.113 ± 0.010	0.039 ± 0.010	0.152 ± 0.015
+NADPH	0.133 ± 0.011	0.150 ± 0.012	0.283 ± 0.025
+NADPH + α-NF	0.115 ± 0.012	0.090 ± 0.010***	0.205 ± 0.021**
+NADPH + ketoconazol	n.d.	n.d.	n.d.
+H_2O_2	0.230 ± 0.020***	0.068 ± 0.008**	0.298 ± 0.023
UKF-NB-4			
Without cofactor	0.040 ± 0.008	0.100 ± 0.010	0.140 ± 0.014
+NADPH	0.099 ± 0.010	0.190 ± 0.015	0.289 ± 0.026
+NADPH + α-NF	0.083 ± 0.010	0.207 ± 0.020	0.290 ± 0.026
+NADPH + ketoconazol	0.041 ± 0.007***	0.151 ± 0.016*	0.192 ± 0.020***
+H_2O_2	0.809 ± 0.081***	0.843 ± 0.060***	1.652 ± 0.150***

[a] Mean RAL (relative adduct labeling) of four determinations (duplicate analyses of two independent *in vitro* incubations). n.d.: not detected (the detection limit of RAL was $1/10^{11}$ nucleotides). The data were analyzed statistically by Student's *t*-test. Values significantly different from incubations with NADPH: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

A negative correlation was seen between the IC_{50} values for ellipticine and the formation of ellipticine-DNA adducts in IMR-32 and UKF-NB-4 cells. These findings suggest that the cytotoxic activity of ellipticine to these two neuroblastoma cell lines is a consequence of the formation of ellipticine-DNA adducts. The role of ellipticine-DNA adduct formation in cytotoxicity was further supported by the finding that a decrease in the levels of these adducts in IMR-32 and UKF-NB-4 cells under hypoxic conditions corresponded to a decrease in toxicity of ellipticine under these conditions. In addition, the cell growth rates under hypoxic conditions were slower in IMR-32, UKF-NB-4 and ellipticine-resistant UKF-NB-4 cell lines, which might also protect the cells from the cytotoxic effects of ellipticine.

A number of DNA-damaging agents have been shown to inhibit cell growth by arrest at the G1/S boundary of the cell cycle [47–49]. This cell arrest is thought to be an important cellular defense mechanism that prevents replication of damaged DNA. We found that exposure to ellipticine caused an accumulation of IMR-32 and UKF-NB-4 cells in S phase. It is tempting to speculate that the mechanism of the S phase delay is the inability of the DNA polymerase complex to replicate over ellipticine-induced DNA adducts. Namely, it has been shown that DNA damage blocks DNA replication and/or transcription by polymerase [49–51]. In addition, inhibition of DNA replication has also been implicated as a proximate initiator of apoptosis [50], which we found in neuroblastoma cells studied in this work. However, the question whether the ellipticine-modified DNA templates can really block DNA polymerase awaits further investigation.

The predominant role of covalent ellipticine-DNA binding in cytotoxicity has already been found previously in leukemia cell lines; the cytotoxicity of ellipticine in human promyelotic leukemia HL-60 cells and human leukemia CCRF-CEM cells

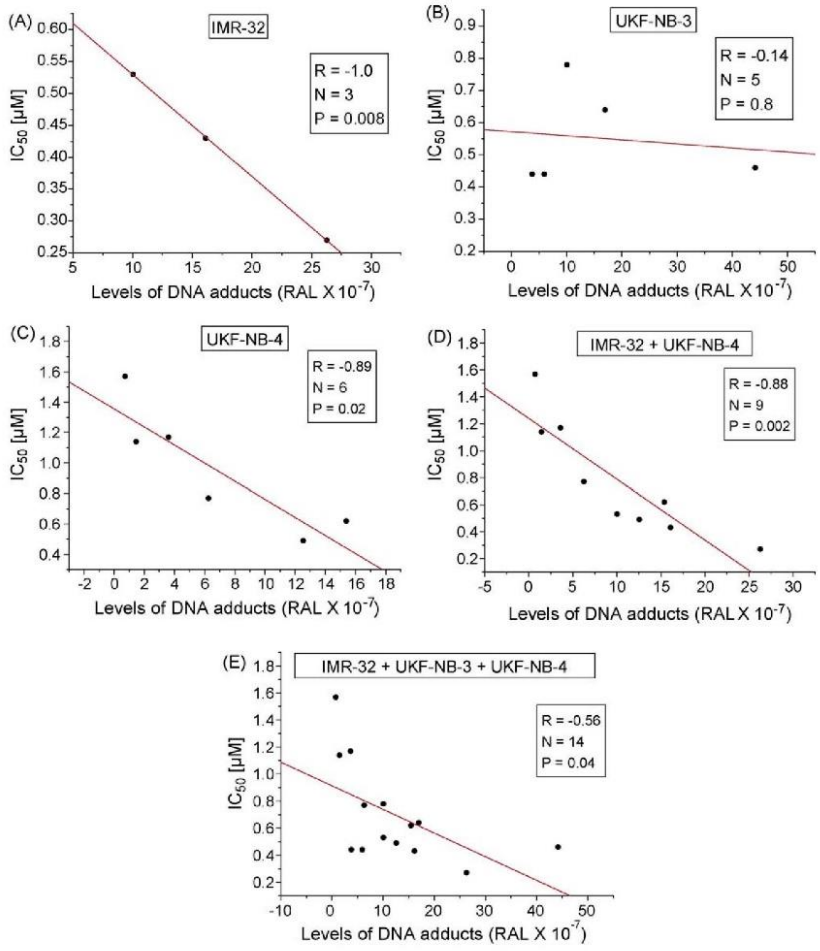

Fig. 8. Correlation of the formation of ellipticine-DNA adducts and IC_{50} values for ellipticine in IMR-32 (A), UKF-NB-3 (B), UKF-NB-4 (C), IMR-32 plus UKF-NB-4 (D) and IMR-32, UKF-NB-3 plus UKF-NB-4 cells (E). *R*, correlation coefficient; *N*, number of data points, *P*, level of significance.

correlated with the levels of ellipticine-derived DNA adducts generated in these cells [26]. The expression levels of CYP and peroxidase enzymes activating ellipticine in leukemia cells were found to be responsible for these results [26].

Likewise, Rekha and Sladek [52] demonstrated that the cytotoxic activity of ellipticine to MCF-7 cells depends on the levels of enzymes activating ellipticine to DNA-binding species, CYP enzymes in this case. The authors showed that MCF-7 cells treated with 3-methylcholanthrene transiently expressed elevated levels of CYP1A and were transiently much more sensitive to ellipticine. The DNA adducts we have observed in MCF-cells [25] might be responsible for the higher sensitivity observed by the above authors. Taken together, the data in the present work and those of previous studies [25,26,52] indicate that the activities and expression levels of CYP and peroxidase enzymes, which effectively activate ellipticine to metabolites forming DNA adducts, may be important factors in the specificity of ellipticine for acute myeloid leukemia, breast cancer and for some neuroblastomas.

However, a different response of the UKF-NB-3 cell line to ellipticine was found. Although ellipticine also induced apoptosis in this cell line, UKF-NB-3 cells were arrested in the G0/G1 and G2/M phase of the cell cycle. This finding indicates that mechanisms of ellipticine-mediated cytotoxicity in UKF-NB-3 cells are different from those in the other neuroblastoma cell lines tested in this study. Moreover, these mechanisms seem not to be associated with the covalent DNA damage caused by ellipticine, because no correlation was found between IC_{50} values for ellipticine and the formation of ellipticine-DNA adducts. Likewise, even though the levels of ellipticine-DNA adducts decreased in UKF-NB-3 cells under hypoxic conditions, no change in toxicity of ellipticine to these cells occurred. Therefore, acute ellipticine toxicity to UKF-NB-3 cells might be caused by its intercalation into DNA and/or inhibition of topoisomerase II, or by uncoupling mitochondrial oxidative phosphorylation and disrupting the cell's energy balance [10]. At the present time, we can only speculate on the mechanism explaining the different responses of UKF-NB-3 to ellipticine. The question whether they result from the biological heterogenicity of neuroblastoma cells, for example from their different genetic programs [29], remains to be answered.

Because drug resistance is a general feature of neuroblastoma, and arises in the majority of patients suffering from this cancer, we also investigated whether ellipticine is able to induce resistance in

neuroblastoma cells. The UKF-NB-4 cell line was used for such a study. The results demonstrate that ellipticine might, to some extent, induce resistance in these cells, but only after a long-term treatment with increasing concentrations of ellipticine. Resistance was accompanied by changes in cell morphology and 3-fold lower levels of ellipticine-DNA adducts. Even though the levels of mRNAs of CYP1A1 and COX-2 enzymes, by which formation of DNA adducts is mediated, were higher in the ellipticine-resistant UKF-NB-4 (Elli) cells, if cells of both lines were exposed to 10 μM ellipticine, mRNA levels of these enzymes were the same in the sensitive and the resistant line. Likewise, the expression of these enzymes at the protein level was not changed in the ellipticine-resistant line; COX-1 and -2 proteins were not detectable by Western blotting in either cell line. Levels of CYP3A4 protein expression were, however, slightly decreased in the ellipticine-resistant UKF-NB-4 line (Fig. 7), which might be responsible for a decrease in DNA adduct levels in these cells. Another mechanism might result from a shift of the ellipticine binding to CYP enzymes, from its binding as a CYP substrate (a type I ligand) to that as a heme ligand (a type II ligand) in the ellipticine-resistant UKF-NB-4 line. It has been found that ellipticine may, depending on several conditions such as pH, temperature [53] and the presence of endogenous CYP substrates [54], bind to some CYPs including the major enzyme activating this drug, CYP3A, as a heme ligand [53]. Because the heme ligands inhibit the initiation of the CYP reaction cycle, by preventing oxygen binding to the heme, ellipticine might in this case decrease its own activation by this mechanism [53].

The resistance factor of this cell line to ellipticine was lower (2.7) than that of the doxorubicin-resistant UKF-NB-4 cell line to doxorubicin (5.4). Likewise, neuroblastoma UKF-NB-3 and UKF-NB-4 cells resistant to vincristine and cisplatin were only slightly resistant to ellipticine. Even though the IC_{50} values for vincristine and cisplatin in these cells were not determined in this study, it is noteworthy that resistance of another neuroblastoma cell line, UKF-NB-2, to vincristine decreased toxicity of this drug significantly (resistance factor of this cell line to vincristine was 274) [31]. Moreover, resistance to doxorubicin, vincristine and cisplatin was induced during much shorter cultivation period with these drugs [29,31]. In addition, the IC_{50} value for ellipticine in the ellipticine-resistant UKF-NB-4 cell line, even under the hypoxic conditions, is still one order of magnitude lower than the ellipticine levels in blood of mice 4 h after their p.o. treatment with ellipticine in a tolerable dose [55]. The resistance mechanisms to ellipticine will be investigated in our laboratories, by analyzing the changes in the genetic programs in this cell line. Preliminary experiments suggest that ellipticine resistance is not dependent on P-glycoprotein expression, which is the case in the UKF-NB-4 cell line resistant to doxorubicin [29].

Collectively, the results presented in this paper are the first report demonstrating the cytotoxicity of ellipticine and formation of ellipticine-DNA adducts in human neuroblastoma cells. One of the most important results of this study is the finding that formation of ellipticine-DNA adducts is the predominant mechanism leading to cytotoxicity. Another important result of the study is finding that neuroblastoma cells can become resistant to ellipticine only after prolonged treatment and to a much lower extent than to doxorubicin or vincristine [31]. This finding is a promising result that might lead to the development of new neuroblastoma therapies, substituting or combining current anticancer drugs with ellipticine or its derivatives.

Acknowledgments

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References

[1] Maris JM, Mathay KK. Molecular biology of neuroblastomas. J Clin Oncol 1999;17:2264–79.
[2] Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 2003;3:203–16.
[3] Stiborová M, Bieler CA, Wiessler M, Frei E. The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts. Biochem Pharmacol 2001;62:675–84.
[4] Auclair C. Multimodal action of antitumor agents on DNA: the ellipticine series. Arch Biochem Biophys 1987;259:1–14.
[5] Kuo PL, Hsu YL, Kuo YC, Chang CH, Lin CC. The antiproliferative inhibition of ellipticine in human breast mda-mb-231 cancer cells is through cell cycle arrest and apoptosis induction. Anti-Cancer Drugs 2005;16:789–95.
[6] Kuo PL, Hsu YL, Chang CH, Lin CC. The mechanism of ellipticine-induced apoptosis and cell cycle arrest in human breast MCF-7 cancer cells. Cancer Lett 2005;223:293–301.
[7] Kovacic P, Ames JR, Lumme P, Elo H, Cox O, Jacson H, et al. Change transfer-oxy radical mechanism for anti-cancer agents. Anti-Cancer Drug Design 1986;1:197–214.
[8] Kuo PL, Kuo YC, Hsu YL, Cho CY, Lin CC. Ellipticine induced apoptosis through p53-dependent pathway in human hepatocellular carcinoma HepG2 cells. Life Sci 2006;78:2550–7.
[9] Garbett NC, Graves DE. Extenting nature's leads: the anticancer agent ellipticine. Curr Med Chem Anti-Cancer Agents 2004;4:149–72.
[10] Schwaller MA, Allard B, Lescot E, Moreau F. Protonophoric activity of ellipticine and isomers across the energy-transducing membrane of mitochondria. J Biol Chem 1995;270:22709–13.
[11] Ohashi M, Sugikawa E, Nakanishi N. Inhibition of p53 protein phosphorylation by 9-hydroxyellipticine: a possible anticancer mechanism. Jpn J Cancer Res 1995;86:819–29.
[12] Sugikawa E, Hosoi T, Yazaki N, Gamanuma N, Nakanishi N, Ohashi M. Mutant p53 mediated induction of cell cycle arrest and apoptosis at G1 phase by 9-hydroxyellipticine. Anticancer Res 1999;19:3099–108.
[13] Singh MP, Hill GC, Peoch D, Rayner B, Inabach JL, Lown JW. High-field NMR and restrained molecular modeling studies on a DNA heteroduplex containing a modified apurinic abasic site in the form of covalently linked 9-aminoellipticine. Biochemistry 1994;33:10271–85.
[14] Monnot M, Mauffret O, Simon V, Lescot E, Psaume B, Saucier JM, et al. DNA-drug recognition and effects on topoisomerase II-mediated cytotoxicity. A three-mode binding model for ellipticine derivatives. J Biol Chem 1991;25:1820–9.
[15] Fossé P, René B, Charra M, Paoletti C, Saucier JM. Stimulation of topoisomerase II-mediated DNA cleavage by ellipticine derivatives: structure–activity relationships. Mol Pharmacol 1992;42:590–5.
[16] Froelich-Ammon SJ, Patchan MW, Osheroff N, Thompson RB. Topoisomerase II binds to ellipticine in the absence or presence of DNA. Characterization of enzyme–drug interactions by fluorescence spectroscopy. J Biol Chem 1995;270:14998–5004.
[17] Stiborová M, Breuer A, Aimová D, Stiborová-Rupertová M, Wiessler M, Frei E. DNA adduct formation by the anticancer drug ellipticine in rats determined by ^{32}P-postlabeling. Int J Cancer 2003;107:885–90.
[18] Stiborová M, Stiborová-Rupertová M, Bořek-Dohalská L, Wiessler M, Frei E. Rat microsomes activating the anticancer drug ellipticine to species covalently binding to deoxyguanosine in DNA are a suitable model mimicking ellipticine bioactivation in humans. Chem Res Toxicol 2003;16:38–47.
[19] Stiborová M, Sejbal J, Bořek-Dohalská L, Aimová D, Poljaková J, Forsterová K, et al. The anticancer drug ellipticine forms covalent DNA adducts, mediated by human cytochromes P450, through metabolism to 13-hydroxyellipticine and ellipticine N^2-oxide. Cancer Res 2004;64:8374–80.
[20] Stiborová M, Bořek-Dohalská L, Aimová D, Kotrbová V, Kukačková K, Janouchová K, et al. Oxidation pattern of the anticancer drug ellipticine by hepatic microsomes—similarity between human and rat systems. Gen Physiol Biophys 2006;25:245–61.
[21] Stiborová M, Poljaková J, Ryšlavá H, Dračínský M, Eckschlager T, Frei E. Mammalian peroxidases activate anticancer drug ellipticine to intermediates forming deoxyguanosine adducts in DNA identical to those found *in vivo* and generated from 12-hydroxyellipticine and 13-hydroxyellipticine. Int J Cancer 2007;120:243–51.
[22] Stiborová M, Rupertová M, Aimová D, Ryšlavá H, Frei E. Formation and persistence of DNA adducts of anticancer drug ellipticine in rats. Toxicology 2007;236:50–60.
[23] Poljaková J, Dračínský M, Frei E, Hudeček J, Stiborová M. The effect of pH on peroxidase-mediated oxidation of and DNA-adduct formation by ellipticine. Collect Czech Chem Commun 2006;71:1169–85.
[24] Stiborová M, Arlt VM, Henderson CJ, Wolf CR, Kotrbová V, Moserová M, et al. Role of hepatic cytochromes P450 in bioactivation of the anticancer drug

ellipticine: studies with the hepatic NADPH:cytochrome P450 reductase null mouse. Toxicol Appl Pharmacol 2008;226:318–27.
[25] Bořek-Dohalská L, Frei E, Stiborová M. DNA adduct formation by the anticancer drug ellipticine and its hydroxy derivatives in human breast adenocarcinoma MCF-7 cells. Collect Czech Chem Commun 2004;69:603–15.
[26] Poljaková J, Frei E, Gomez JE, Aimová D, Eckschlager T, Hraběta J, et al. DNA adduct formation by the anticancer drug ellipticine in human leukemia HL-60 and CCRF-CEM cells. Cancer Lett 2007;252:270–9.
[27] Frei E, Bieler CA, Arlt VM, Wiessler M, Stiborová M. Covalent binding of the anticancer drug ellipticine to DNA in V79 cells transfected with human cytochrome P450 enzymes. Biochem Pharmacol 2002;64:289–95.
[28] Schwab M. Human neuroblastoma: from basic science to clinical debut of cellular oncogenes. Naturwissenschaften 1999;86:71–8.
[29] Bedrnicek J, Vicha A, Jarosova M, Holzerova M, Cinatl Jr J, Michaelis M, et al. Characterization of drug-resistant neuroblastoma cell lines by comparative genomic hybridization. Neoplasma 2005;52:415–9.
[30] Cinatl Jr J, Cinatl J, Driever PH, Kotchetkov R, Pouckova P, Kornhuber B, et al. Sodium valproate inhibits *in vivo* growth of human neuroblastoma cells. Anti-Cancer Drugs 1997;8:958–63.
[31] Kotchetkov R, Cinatl J, Blaheta R, Vogel JU, Karaskova J, et al. Development of resistance to vincristine and doxorubicin in neuroblastoma alters malignant properties and induces additional karyotype changes: a preclinical model. Int J Cancer 2003;104:36–43.
[32] Hakura A, Suzuki S, Sawada S, Sugihara T, Hori Y, Uchida K, et al. Use of human liver S9 in the Ames test: assay of three procarcinogens using human S9 derived from multiple donors. Regul Toxicol Pharmacol 2003;37:20–7.
[33] Eling TE, Thompson DC, Foureman GL, Curtis JF, Hughes MF. Prostaglandin H synthase and xenobiotic oxidation. Annu Re Pharmacol Toxicol 1990;30:1–45.
[34] Stiborová M, Frei E, Hodek P, Wiessler M, Schmeiser HH. Human hepatic and renal microsomes, cytochromes P450 1A1/2, NADPH:cytochrome P450 reductase and prostaglandin H synthase mediate the formation of aristolochic acid-DNA adducts found in patients with urothelial cancer. Int J Cancer 2005;113:189–97.
[35] Arlt VM, Hewer A, Sorg BL, Schmeiser HH, Phillips DH, Stiborová M. 3-Aminobenzanthrone, a human metabolite of the environmental pollutant 3-nitrobenzanthrone, forms DNA adducts after metabolic activation by human and rat liver microsomes: evidence for activation by cytochrome P450 1A1 and P450 1A2. Chem Res Toxicol 2004;17:1092–101.
[36] Rendic S, DiCarlo FJ. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. Drug Metab Rev 1997;29:413–80.
[37] Stiborová M, Martínek V, Rýdlová H, Hodek P, Frei E. Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes. Cancer Res 2002;62:5678–84.
[38] Stiborová M, Martínek V, Rýdlová H, Koblas T, Hodek P. Expression of cytochrome P450 1A1 and its contribution to oxidation of a potential human carcinogen 1-phenylazo-2-naphthol (Sudan I) in human livers. Cancer Lett 2005;220:145–54.
[39] Stiborová M, Dračínská H, Hájková J, Kadeřábková P, Frei E, Schmeiser HH, et al. The environmental pollutant and carcinogen 3-nitrobenzanthrone and its human metabolite 3-aminobenzanthrone are potent inducers of rat hepatic cytochromes P450 1A1 and -1A2 and NAD(P)H:quinone oxidoreductase. Drug Metab Dispos 2006;34:1398–405.
[40] Lanza F, Latorraca A, Moretti S, Castagnari B, Ferrari L, Castoldi G. Comparative analysis of different permeabilization methods for the flow cytometry measurement of cytoplasmic myeloperoxidase and lysozyme in normal and leukemic cells. Cytometry 1997;30:134–44.
[41] Moserová M, Kotrbová V, Rupertová M, Naiman K, Hudeček J, Hodek P, et al. Isolation and partial characterization of the adduct formed by 13-hydroxyellipticine with deoxyguanosine in DNA. Neuro Endocrinol Lett 2008;29:728–32.
[42] Ueng Y-F, Kuwabara T, Chun Y-J, Guengerich FP. Cooperativity in oxidation catalyzed by cytochrome P450 3A4. Biochemistry 1997;36:370–81.
[43] Arlt VM, Henderson CJ, Wolf CR, Schmeiser HH, Phillips DH, Stiborová M. Bioactivation of 3-aminobenzanthrone, a human metabolite of the environmental pollutant 3-nitrobenzanthrone: evidence for DNA adduct formation mediated by cytochrome P450 enzymes and peroxidases. Cancer Lett 2006;234:220–31.
[44] Chu Y, Hsu MT. Ellipticine increases the superhelical density of intracellular SV40 DNA by intercalation. Nucleic Acids Res 1992;20:4033–8.
[45] Stiborová M, Rupertová M, Schmeiser HH, Frei E. Molecular mechanism of antineoplastic action of an anticancer drug ellipticine. Biomed Pap 2006;150:13–23.
[46] Dračínský M, Sejbal J, Rygerová B, Stiborová M. An efficient modification of ellipticine synthesis and preparation of 13-hydroxyellipticine. Tetrahetron Lett 2007;48:6893–5.
[47] Dipple A, Khan QA, Page JF, Pontén I, Szeliga J. DNA reactions, mutagenic action and stealth properties of polycyclic aromatic hydrocarbon carcinogens (review). Int J Oncol 1999;14:103–11.
[48] Khan QA, Vousden KH, Dipple A. Lack of p53-mediated G1 arrest in response to an environmental carcinogen. Oncology 1999;57:258–64.
[49] Simões ML, Hockley SL, Schwerdtle T, da Costa GG, Schmeiser HH, Phillips DH, Arlt VM. Gene expression profiles modulated by the human carcinogen aristolochic acid I in human cancer cells and their dependence on TP53. Toxicol Appl Pharmacol 2008;232:86–98.
[50] Ross WP, Kaina B. DNA damage-induced cell death by apoptosis. Trends Mol Med 2006;12:440–50.
[51] Broschard TH, Wiessler M, von der Lieth CW, Schmeiser HH. Translesional synthesis on DNA templates containing site-specifically placed deoxyadenosine and deoxyguanosine adducts formed by the plant carcinogen aristolochic acid. Carcinogenesis 1994;15:2331–40.
[52] Rekha GK, Sladek NE. Multienzyme-mediated stable and transient multidrug resistance and collateral sensitivity induced by xenobiotics. Cancer Chemother Pharmacol 1997;40:215–24.
[53] Aimová D, Stiborová M. Antitumor drug ellipticine inhibits the activities of rat hepatic cytochromes P450. Biomed Pap 2005;149:437–40.
[54] Chang C-Y, Puga A. Constitutive activation of the aromatic hydrocarbon receptor. Mol Cell Biol 1998;18:525–35.
[55] Hardesty CT, Chaney NA, Mead JA. The effect of route of administration on the distribution of ellipticine in mice. Cancer Res 1972;32:1884–9.

Valproic Acid in the Complex Therapy of Malignant Tumors

J. Hrebackova, J. Hrabeta and T. Eckschlager*

Department of Pediatric Hematology and Oncology, 2nd Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic

Abstract: Valproic acid (VPA) has been used for epilepsy treatment since the 1970s. Recently, it was demonstrated that it inhibits histone deacetylases (HDAC), modulates cell cycle, induces tumor cell death and inhibits angiogenesis in various tumor models. The exact anticancer mechanisms of VPA remains unclear, but HDAC inhibition, extracellular-regulated kinase activation, protein kinase C inhibition, Wnt-signaling activation, proteasomal degradation of HDAC, possible downregulation of telomerase activity and DNA demethylation participate in its anticancer effect. Hyperacetylation of histones, as a result of HDAC inhibition, seems to be the most important mechanism of VPA´s antitumor action.

Preclinical data suggest that the anticancer effect of chemotherapy is augmented when VPA is used in combination with cytostatics. Besides the effects of pretreatment with HDAC inhibitors, which increases the efficiency of 5-aza-2´-deoxycytidine, VP-16, ellipticine, doxorubicin and cisplatin, pre-exposure to VPA increases the cytotoxicity of topoisomerase II inhibitors. There are two suggested cell death mechanisms caused by potentiation of anticancer drugs by HDAC inhibitors that are neither exclusive nor synergistic. The first involves apoptosis and can be both p53 dependent or independent; the second involves mechanisms other than apoptosis. In resistant chronic myeloid leukemia (CML), VPA restores sensitivity to imatinib. We have demonstrated the synergistic effects of VPA and cisplatin in neuroblastoma cells.

VPA can be taken orally, crosses the blood brain barrier and can be used for extended periods. Clinical trials in patients with malignancies are being conducted. The use of VPA prior to or together with anticancer drugs may thus prove a beneficial treatment.

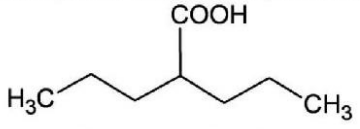
Keywords: Valproic acid, histone deacetylase inhibitors, antiangiogenic effect, anticancer effect, clinical studies.

1. INTRODUCTION

During the last few decades, several approaches have been applied in an effort to discover new and more effective anticancer drugs. As a result, many promising compounds have been investigated. However chemoresistance, one of the major obstacles in cancer treatment, can be present from the start or it can appear after only one cycle of chemotherapy. To achieve the best treatment, drugs are often used in various combinations. Another way of improving treatment effectivity is through addition of support agents. Following this trend, histone deacetylase inhibitors (HDACi) and a member of this group – valproic acid (valproate, VPA) – appears to be very promising. Because VPA has been used as an antiepileptic drug for over forty years, its pharmacology and side-effects have been studied in detail. Therefore, it might be useful in the treatment of patients suffering from malignant tumors [1]. This review summarizes VPA's mechanisms of action on tumor growth, invasion and differentiation, on the intracellular signaling cascade and gene transcription. Potentiation of other cytostatic drugs by VPA is also discussed.

2. VPA CHARACTERIZATION

Valproate is an eight-carbon branched-chain fatty acid (Fig. **1**) that was first synthesized by Burton in 1882. In the late 1960s and in the 1970s it was authorized as an antiepileptic drug in France and in the USA respectively [1, 2]. Usually, it is taken orally in the form of a sodium or magnesium salt. The two preparations were found to be bioequivalent; magnesium valproate appeared to be without bioavailability problems and had reduced inter-subject variability, compared with sodium valproate [3]. It is often used in treatment of certain forms of epilepsy and also to treat bipolar disorders and migraines. VPA appears to reverse the transamination process to form more gamma aminobutyric



2-propylpentanoic acid

Fig. (1). Chemical structure of VPA.

acid in the brain. Other mechanisms of action (attenuation of NMDA-mediated excitation, blockage of voltage-gated sodium channels and T-type calcium channels) in neuropsychiatric disorders have been proposed [4, 5]. The half-life of VPA in serum is approximately 7 - 16 hours. Its chemical properties, small size and relative hydrophobicity, allow easy delivery to the cells. As an antiepileptic, VPA requires a serum concentration between 0.5 - 1.0 mM. It is primarily metabolized in the liver and excreted as glucuronide conjugate in urine. Although toxic side-effects of VPA are low, serious complications may occur rarely in patients suffering from hemorrhagic pancreatitis, coagulopathies or bone marrow suppression. VPA is a well established teratogen associated with elevated risks for neural, craniofacial, cardiovas-

*Address correspondence to this author at the Department of Pediatric Hematology and Oncology, V Uvalu 84, 150 06 Prague 5, Czech Republic; Tel: + 4202 2443 6494; Fax: + 4202 2443 6420;
E-mail: eckschlagertomas@yahoo.com

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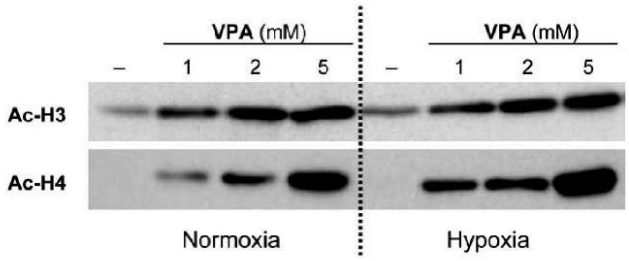

Fig. (2). Acetylation state of histones H3 and H4 after 24 hour incubation in normoxia and hypoxia (<1% O2) at different VPA concentrations in SK-N-AS neuroblastoma cell line.

cular, and skeletal birth defects [6, 7]. Women receiving long-term VPA therapy frequently develop polycystic ovary syndrome with associated increased ovarian androgen production and follicle development defects [8]. Additionally, cases of hepatotoxicity and encephalopathy have been documented [9].

During the past years, it was demonstrated that VPA also exhibits anticancer activity as seen in several *in vitro* and *in vivo* experiments. Hyperacetylation of histones and other proteins as a result of histone deacetylase (HDAC) inhibition appears to be an important mechanism of VPA's antitumor effect [1, 2, 10]. Our experiments have shown that VPA increases H3 and H4 acetylation, both in normoxic and hypoxic conditions *in vitro* (Fig. **2**).

The first experiments were carried out using neuroectodermal tumor, leukemia and lymphoma cell lines. In the first published report about VPA's anticancer effect, Martin and Regan described growth inhibition of glioma cells [11]. VPA's preclinical efficacy was later also demonstrated in neuroblastomas [12], leukemias [13, 14], medulloblastomas [15], breast [16, 17], and prostate cancers [18].

3. MODE OF ANTICANCER ACTION OF VPA

The exact anticancer mechanisms of VPA still remain unclear. They might be tumor-specific and generally it is thought, that they involve a broad spectrum of pathway alterations, including HDAC inhibition, [19, 20] extracellular-regulated kinase and Wnt-signaling activation, [1] protein kinase C inhibition, proteasomal degradation of HDAC [21] and DNA demethylation [22, 23]. In addition, the antiangiogenic effects of VPA have also been described [24, 25]. Hyperacetylation of histones H3 and H4 and non-histone proteins, as a result of HDAC inhibition, where VPA most probably binds to the catalytic center [19, 23], is now hypothesized to be one of the major mechanisms of VPA's antitumor effects [20] (Fig. **3**).

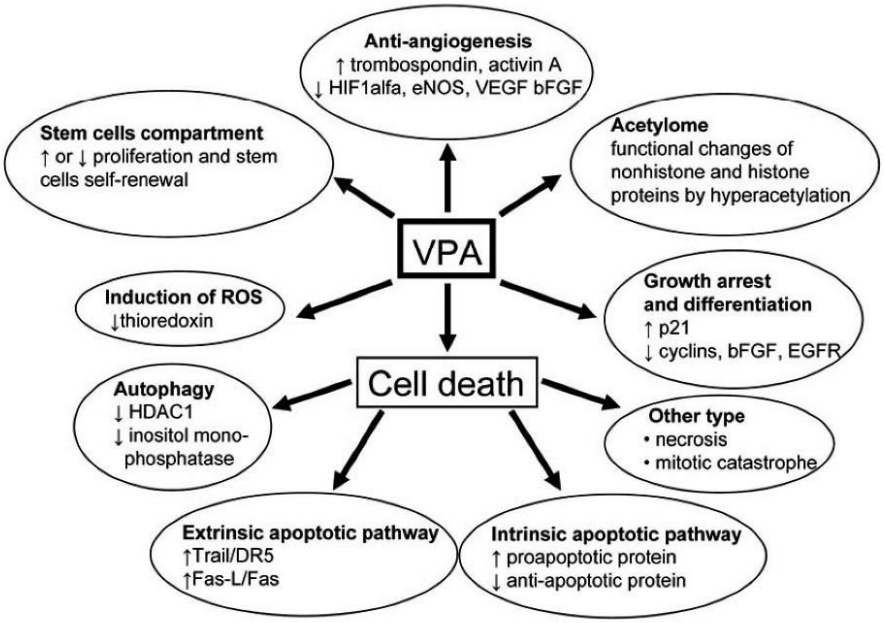

Fig. (3). Valproic acid activates numerous antitumor pathways. The response to valproic acid depends on concentration, the time of exposure, and cell type.

3.1. Acetylation and Deacetylation

Modification of histones by acetylation affects transcription by changing the structure of chromatin and by modulating the accessibility of transcription factors to their target DNA sequences and plays a key role in epigenetic regulation of gene expression [26, 27]. Additionally, acetylation/deacetylation of non-histone proteins modifies many cell functions like mRNA splicing, transport and integrity; translation; protein activity, localization, stability and interactions [28].

The acetylation state of histones and other proteins is maintained by histone acetyltransferases (HAT) and HDAC, the former catalyzing the transfer of an acetyl group from acetyl-CoA to the ε-NH_2 group of lysine residues in proteins and the latter removing it [29]. HDACs utilize two different mechanisms to remove the acetyl group and they are therefore divided into two distinct families. The "classical family" comprises Zn^{2+} dependent HDACs. Zn^{2-} ion directly interacts with acetylated substrate and helps to stabilize it in the catalytic center of the enzyme as well as to polarize the carbonyl group making the carbon a better target for nucleophilic water molecules [30]. The other HDAC family is NAD^+-dependent. Here, O-acetyl ADP ribose and nicotinamide are formed as a result of the acetyl transfer [31]. Furthermore, based on the homology to their yeast analogues, HDACs can be divided into four classes, see Table **1**. Class I, located in the nucleus, includes HDACs 1, 2, 3 and 8. HDACs 4, 5, 7 and 9 constitute class IIa, isoforms 6 and 10 make up class IIb, being located in both the cytoplasm and nucleus. Mammalian NAD^+-dependent homologues 1-7 of the yeast Sir2 proteins (termed sirtuins) are designed as class III and have mono-ADP-ribosyltransferase activity. Finally, class IV which exhibits features of class I and II, includes only one known isoenzyme HDAC11 [32].

HATs, as cellular "opponents" of HDACs, are a diverse set of multi-subunit complexes. They can be divided into Gcn5 N-acetyltransferases (GNATs) and MYST HATs. Gcn5 is the founding member of the former group which includes Gcn5, PCAF, Elp3, Hat1, Hpa2 and Nut1. The latter is an acronym for the members of this family: **M**orf, **Y**bf2 (Sas3), **S**as2 and **T**ip60. Although these two groups of HATs are the main ones, other proteins including p300/CBP (CREB-binding protein), Taf1 and a number of nuclear receptor coactivators possess intrinsic acetylase activity. However, they do not contain true consensus HAT domains and therefore represent an "orphan class" of HAT enzymes [33].

Most of the classic histone deacetylases form large high molecular weight complexes of up to 1–2 MDa [34]. They contain multiple transcriptional co-repressors (such as Sin3, NuRD, NcoR and SMRT) with chromatin remodeling activity. These transcriptional corepressors recruit HDACs, notably class I and II, to sequence specific transcription factors [22]. It has been suggested that HDAC complexes can contribute to gene repression through two mechanisms - specific targeting by repressors and constitutive association with chromatin [35, 36]. The first model disease in which the involvement of HDACs was demonstrated was AML. It is suggested that aberrant recruitment of corepressor complexes

Table 1. HDAC Classification, Localization and Some of the Non-Histone Substrates

Class	HDAC	Localization	Non-Histone Substrates
Class I Zn^{2+}	HDAC1	N	NR: androgen receptor, SHP; SM: Stat3, Smad7; TF: p53, YY-1, GCMa, Myo-D, E2F-1
	HDAC2	N	NR: glucocorticoid receptor; SM: Stat3, Smad7; TF: Bcl-6,YY-1
	HDAC3	N	NR: SHP; SM: Smad7,Stat3; TF: YY-1, GMCa, GATA-1, GATA-3, MEF2D, RelA (NFκB)
	HDAC8	N	SP: actin
Class IIa Zn^{2+}	HDAC4	N/C	CR: HP-1;TF: GCMa, GATA-1
	HDAC5	N/C	CR: HP-1; SM: Smad7; TF: GCM, GATA-1
	HDAC7	N/C	TF: PLAG1, PLAG2
	HDAC9	N/C	TF: Bcl-6, N-CoR, TEL
Class IIb Zn^{2+}	HDAC6	Mostly C	CH: Hsp90; NR: SHP; SM: Smad7; SP: α-tubulin
	HDAC10	Mostly C	TF: SMRT
Class III NAD^+	Sirt1	N/C	RE: Ku70; TC:Rb; TF: p53, p73, p300, Bcl-6, Myo-D, FOXOs, NFkB, N-CoR, E2F-1;
	Sirt2	C	SP: α-tubulin; TF: FOXOs
	Sirt3	M	AcCoAS
	Sirt4	M	GDH
	Sirt5	M	Cytochrome c
	Sirt6	N	Telomeric chromatin; WRN
	Sirt7	N	RNA pol I; TF: p53
Class IV Zn^{2+}	HDAC11	N/C	not known

M: mitochondrion; N: nucleus; C: cytoplasm; CH: chaperone; CR: chromatin remodeling protein; NR: DNA-binding nuclear receptor; RE: DNA repair enzyme; SM: signaling mediator; SP: structural protein; TC: transcription coregulator; TF: DNA-biding transcription factor. Modified according to [33; 34].

is a general mechanism of leukemogenesis. As good example, AML1-ETO fusion protein causes silencing of AML1 target genes, which are important for hematopoietic differentiation, by recruiting HDAC-containing repressor complexes. VPA could therefore be used for targeting of the AML1/ETO-HDAC1 complex in AML with t(12;21). This therapy was shown to be effective, since VPA treatment disrupts AML1/ETO-HDAC1 interaction and stimulates dissociation of the complex from the promoters of AML1/ETO target genes [37]. However, treatment effectivity/HDACi sensitivity seems to be restricted to transformed cells only. Normal myeloid progenitors or premalignant cells with overexpression of fusion oncoprotein PML/RARα or AML1/ETO were not sensitive to HDACi [38]. These findings indicate that HDACi-mediated suppression of transcription repression can restore, by disrupting the AML1/ETO-HDAC1 complex, expression of growth-inhibitory genes [21, 39, 40] and consequently induce a biological response such as differentiation, cell cycle inhibition and cell death; additionally it might be an explanation of tumor specificity of VPA as well as other HDACi.

3.2 HDAC inhibition and its effects

Histone acetylation has been shown to be an important regulatory mechanism that controls transcription of approximately 2-10% of genes [41, 42]. An altered acetylation status of histones causes chromatin condensation or decondensation (increased deacetylation or acetylation). Such changes might result in decreased or increased gene transcription respectively. However, histones are not the only proteins whose activity is affected by acetylation. Throughout the cell there are many more – chromatin remodeling proteins, DNA-binding nuclear receptors, DNA repair enzymes, signaling mediators, structural proteins, transcription coregulators, DNA-biding transcription factors and others (Table **1**). In particular, the function of transcription factors can be positively or negatively affected, which provides an explanation why gene expression, in case of HDAC inhibition, is not always increased even if the chromatin structure is loosened. Taken together, disturbed HAT-HDAC activity balance can (i) lead to an altered gene expression profile as well as to changes at least in some signaling pathways such as the ERK and Wnt pathways, (ii) affect proteasomal degradation, (iii) protein kinase C activity, (iv) DNA demethylation, etc., as discussed later in this review. In various cancer cells, the shift to an increased acetylation/deacetylation ratio by HDAC inhibition was found to have a substantial effect on their fate. Antiangiogenic effect of HDACi in tumor tissues has also been described. ERK, PKC, Wnt signaling pathways and HDAC2 inhibition, as stated above, seems to be specifically influenced by VPA. Other mechanisms might be of use only in certain tumors. Antiangiogenic effects and increased expression of HLA class-I antigens might also be of importance, but only *in vivo*.

There are many cancer types where HDACs overexpression has been detected. Namely, HDAC1 in prostate, gastric, lung, esophageal, colon and breast cancer; HDAC2

Table 2. HDAC Inhibitors (Incomplete List) and their Targets

HDAC Family Inhibited	Structural Class	HDAC Inhibitor	Target
Zinc-dependent	Hydroxamic acid derivatives	TSA	Class I and II
		SAHA	Class I and II
		LAQ824	Class I and II
		LBH589	Class I and II
	Carboxylic acids	VPA	Class I and IIa
		Sodium/phenyl butyrate	Class I and IIa
		AN-9	Class I and IIa
		OSU-HDAC42	Class I and IIa
	Cyclic peptides	Depsipeptide	Class I and IIb (HDAC1, 6)
	Benzamides	MS-275	Class I (HDAC 1, 2, 3)
		MGCD0103	Class I and IIb (HDAC 1, 2, 4, 6)
	Thiolates	NCH-51	Class IIb (HDAC6)
NAD^+-dependent	Nicotinamide	Nicotinamide	SIRT1-7
	Nicotinamide derivatives	2-anilinobenzamide	SIRT1
	NAD^+ analogues	Carba-NAD^+	SIRT1-7
	Hydronaphtaldehyde derivatives	Sirtinol, para-sirtinol, cambinol	SIRT1, 2; to be investigated
	Splitomicins	Splitomicin, 5-benzyloxy-splitomicin, others	SIRT2
	Indoles	EX-527	SIRT1
	Adenosin analogues	Ro31-8220	SIRT1, 2

Modified according to [32; 33; 47].

in colorectal, cervical and gastric cancer; HDAC3 in colon and breast cancer, HDAC6 in breast cancer, HDAC8 in neuroblastoma and HDAC11 in rhabdomyosarcoma [29, 43, 44, 45]. The effect of disruption of the balance between HDACs and HATs was illustrated by Huang *et al.*, who showed that knockdown of overexpressed HDAC2 by siRNA in HeLa cells increases expression of $p21^{Cip1/WAF1}$ and p53 with consequent cytostatic effects [46], showing the potential of HDACi in cancer treatment.

HDAC inhibitory activity is a property of a variety of natural and synthetic compounds. These can be divided into two groups based on the family of HDACs they inhibit and as well as their chemical structure (Table **2**). Interestingly, *in vitro* studies showed that VPA, among various VPA constitutional isomers, had the optimal chemical structure in terms of HDACs inhibition and tumor cells cytotoxicity [47].

One of the most interesting effects of HDACi regarding regulation of gene expression is their ability to modulate the transcription rate of genes that are responsible for cell fate determination. A property of many cancer cells is an altered susceptibility to apoptosis stimuli. With the amount/ratio and the activity of pro- and antiapoptotic proteins dictating the fate of every cell, finding a way to shift the balance towards apoptosis could be of major importance in terms of cancer treatment. Gene-expression profiling of HDACi treated tumor cells shows upregulation of proapoptotic genes involved in the extrinsic (for example, TRAIL, DR5, Fas, Fas-L, and TNF-α) and/or intrinsic apoptotic pathway (Bax, Bak and APAF1), while antiapoptotic genes (e.g. Bcl-2 and XIAP) are downregulated [29]. In some cases, VPA might, through activation of ERK, increase the expression of antiapoptotic proteins, e.g. Bcl-2 [48]. However, the simultaneous affection of activity of proapoptotic factors, such as Bid, is sufficient to induce apoptosis even if Bcl-2 is present in greater amounts [29]. That could explain why HDACi can also induce cell death in apoptosis resistant cells. For example, FK228, an HDAC class I inhibitor, or HDAC1 siRNA induces autophagy in HeLa cells *in vitro* [49]. In addition, the acetylation status of c-jun, E2F-1, p53 and NF-κB also plays an important role in the regulation of cell fate. VPA activates the p53 protein via hyper-acetylation and nuclear re-localization in neuroblastoma cells [50]. The interactions between p53 and its coactivators such as ASPPs (ankyrin-repeat-, SH3-domain- and proline-rich-region-containing proteins), 53BP1, Tip60/hMOF, hCAS/CSE1L, and HZF (hematopoietic zinc finger) might be modulated by acetylation as well [51]. Another feature of HDACi is their ability to cause deregulation of cell cycle checkpoints. For example, expression of p21, a cyclin-dependent kinase inhibitor belonging to the Cip/Kip family is regulated on the transcriptional level by p53. Under stress conditions, p53 directly binds to the p21 promoter at the Sp1 site, competing with HDAC1 that represses p21 transcription [52]. After HDACi treatment, HDAC1 is released from the Sp1 site, thus increasing p21 expression. In addition, acetylated p53, as a result of HDACi action, was found to have a longer half-life as well as higher affinity for the p21 promoter [53]. This, together with increased acetylation of histones around the p21 promoter, which facilitates the access of transcription factors, further increases the effectiveness of HDACi. Additionally, increased stability of p21 mRNA, as a result of HDACi action, has also been reported [54]. Ultimately, the amount of p21 raises and mediates cell cycle arrest or even apoptosis [55, 56, 57].

As mentioned above, VPA seems to affect several cell signaling pathways with a broad spectrum of action (Fig. **4**). Protein kinases are one of the most important regulators of cellular processes such as cell growth, differentiation, development and apoptosis. To date, three distinct mitogen-activated protein kinases (MAPK) have been identified; namely, extracellular-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. An interesting feature of HDACi, which could find its place in cancer therapy, is regulation of cell differentiation through activation of ERK. VPA has been shown to enhance DNA binding and transactivation activity of AP-1 transcription factor by ERK activation. It increases expression of c-Jun and c-Jun phosphorylation in SH-SY5Y neuroblastoma cells [48, 58]. The latter feature is required to direct cellular differentiation of poorly differentiated PC12 rat pheochromocytoma cells [59, 60]. There is a hypothesis that the effect of HDACi on ERK is not caused directly by HDAC inhibition [61], which is supported by observations that the time course for the VPA-induced activation of ERK was much slower than that induced by insulin-like growth factor-I or brain derived neurotrophic factor in SH-SY5Y cells [62, 63]. Also the fact that different HDACi (trichostatin A, HDAC42, MS-275 and suberoylanilide acid (SAHA)) have different effects on the activation of MAPK, which do not correlate with level of HDAC class I inhibition, further supports this hypothesis [61]. Moreover, it is possible that VPA might stimulate synthesis of a still unknown factor, which activates the ERK pathway [48] and/or is incorporated into larger phospholipid molecules which activates ERK through the phosphatidylinositol 3-kinase (PI-3K)/Janus kinase 2 (JAK 2)/MEK-1-dependent pathway in endothelial cells and through the tyrosine kinase-Ras-dependent pathway in mesangial cells [64, 65]. Interestingly, VPA induces the expression of ERK/AP-1 pathway regulated genes, e.g. growth-associated protein-43 (GAP-43) and Bcl-2 and could therefore support tumor progression in SH-SY5Y neuroblastoma cells [48, 66]. However, in neuroblastoma cells, VPA increased expression of Bcl-2 in a concentration dependent manner without impact on the rate of apoptosis (Hrabeta J *et al*, unpublished result). This could be explained, as noted above, by simultaneous activation of proapoptotic factor Bid [29].

Regulation of cell differentiation and cell survival is not restricted only to MAPK and it is shared at least with protein kinase C (PKC) isoenzymes, which also play an important role in the regulation of cell proliferation. Some isoforms of PKC have been shown to be overexpressed in cancer and there is evidence of a probable linkage between increased PKC activity and regulation of angiogenesis, tumor proliferation, invasion and dedifferentiation [67-70]. PKC is processed by phosphorylation and regulated by cofactor binding and subcellular localization. PKC was identified as one of the targets of VPA [71] and it is supposed that the PKC signaling pathway and PKC-mediated gene expression might be an important factor in VPA effectiveness on bipolar disorders. In addition, exposure of glioma cells to VPA for 6 - 7 days decreased the activity of isoenzyme PKC α and ε, but not δ and ζ. Downregulation of PKC activity in the presence of VPA has also been observed in human U937

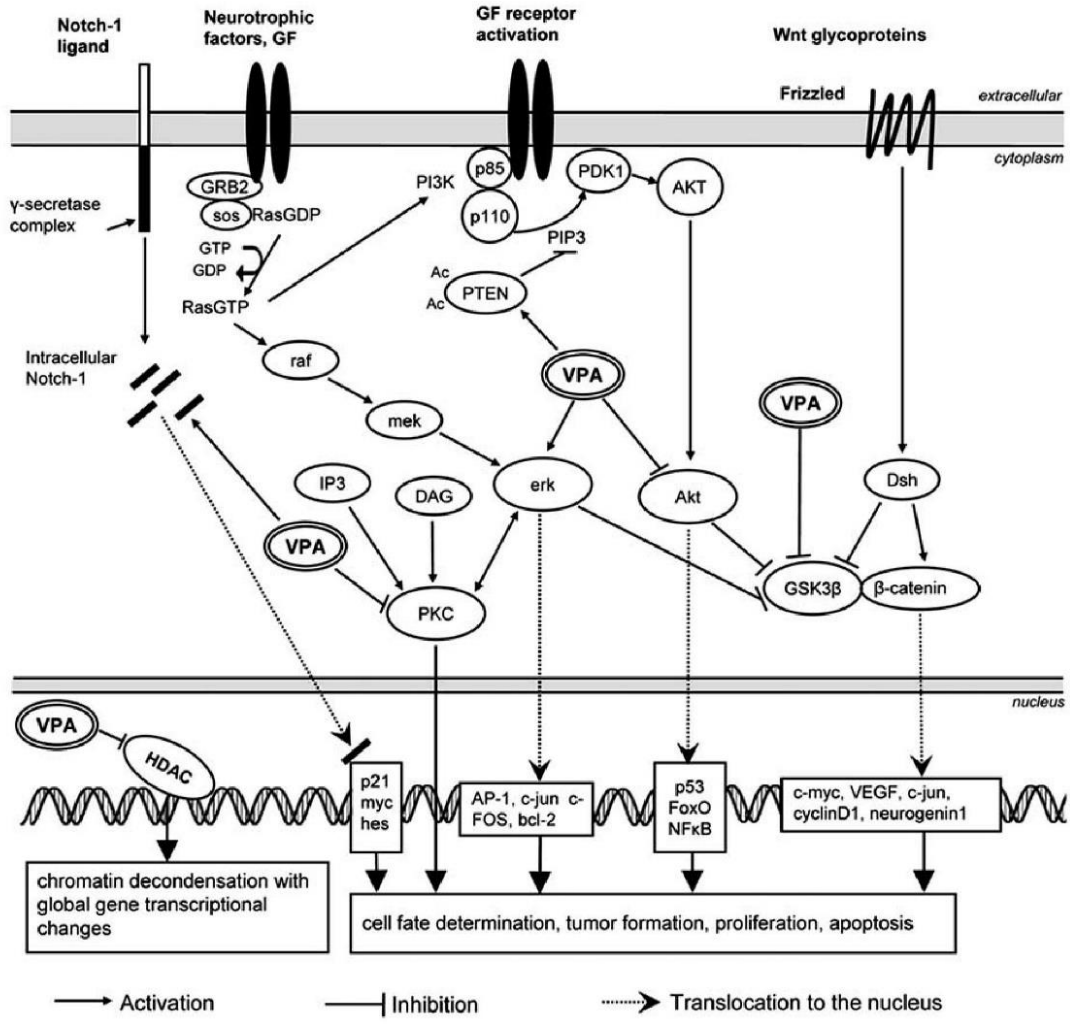

Fig. (4). Effect of VPA on certain cellular signaling pathways

Understanding the anticancer mechanism of action for VPA has been complicated by a number of different cellular targets and their opposite effects. GSK-3β functions as an intermediary in a number of signaling pathways including neurotrophic signaling pathways, and the insulin/PI3 kinase pathway and plays a key inhibitory role in the Wnt signaling. GSK-3β, active in cells under normal conditions, is regulated through inhibition of its activity. Inhibiting GSK-3β is generally antiapoptotic and leads to activation β-catenin, its translocation to the nucleus and activation of the β-catenin/tcf/lef transcription complex. The impact of β-catenin signaling activation depends on the cell type. In neural progenitor cells, β-catenin activation causes differentiation; while in hematologic stem cells it induces self renewal and increases proliferation (see text for more detail). PI3-K induced activation of Akt/PKBα results in phosphorylation of Ser9 on GSK-3β, which inhibits GSK-3β activity. Akt is an essential pro-survival factor in cell proliferation and is often deregulated during tumorigenesis. VPA impedes Akt1/PKBα and Akt2/PKBβ expression, which leads to Akt deactivation and apoptotic cell death in some types of cancer. PTEN functions as a tumor suppressor by negatively regulating the Akt/PKB signaling pathway. Acetylation of PTEN by histone acetyltransferase p300/CBP-associated factor can stimulate its activity. VPA treatment also leads to activation of Notch signaling and an increase in intracellular Notch-1, which participates in cell differentiation and contributes to the anticancer action of VPA in some cell types. Protein kinase C (PKC) is involved in signal transduction associated with cell proliferation, differentiation, and apoptosis. Altered PKC activity has been linked with various types of malignancies. VPA decreases IP3 and DAG, which are endogenous activators of PKC, by decreasing myo-inositol uptake. These perturbations caused by VPA likely contribute to the reduction in PKC activity. Higher levels of PKC and differential activation of various PKC isozymes have been reported in breast tumors, thyroid cancer tissue, leukemic cells, and lung cancer cells. ERK/MAPK kinases are a group of proteins that are activated in response to a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus. Controlled regulation of these cascades is involved in cell proliferation and differentiation, whereas unregulated activation of these MAP kinases can result in oncogenesis. In general, the activation scheme involves stimulation of receptor tyrosine kinases by growth factors, such as EGF, which provides a binding site for the adapter protein Grb2, which in turn localizes Sos to the plasma membrane. Sos activates Ras by exchange of GTP for GDP. The Ras-GTP unit binds directly to serine-threonine kinase, Raf, forming a transient membrane-anchoring signal. ERK si translocated to the nucleus to phosphorylates its target transcription factor.

monocytic leukemia cells [72]. VPA reduces the amount of main cellular PKC substrate myristoylated alanine-rich C kinase substrate [2]. However, PKC downregulation might lead to diminished ERK activity, and vice versa; upregulation of ERK activity might be coupled with enhanced PKC expression [2]. Thus, the beneficial effect of VPA, via inhibition of PKC, might, under certain circumstances, be attenuated by decreased ERK activity.

VPA has been reported to affect the Wnt signaling pathway by phosphorylation of Ser9 of glycogen synthase kinase-3β (GSK-3β) [1], which leads to prevention of subsequent adenomatosis polyposis coli (APC) -dependent degradation of β-catenin [73, 74] (Fig. **4**). Wnt proteins, which regulate many processes including cell fate determination, motility, polarity and organogenesis, have been recently implicated in stem cell renewal [75]. Wnt signaling, with β-catenin being its major effector, is a causative factor for a number of pleiotropic human pathologies. Most notably, these pathologies include colon, breast, ovarian, prostate and endometrial cancers, medulloblastoma, melanoma, skeletal defects and others [76]. In colon carcinoma, frequent oncogenic mutations in the APC gene resulted in accumulation of β-catenin and transcription of TCf-4/Lef1 target genes. Loss of functional APC and activation of β-catenin leads to overexpression of HDAC2, indicating it as a target of APC/β-catenin [77]. Elevated HDAC2 expression prevents cell death in colon carcinoma cell lines. In spite of causing an elevation of β-catenin, VPA also decreases polyp formation in colon carcinoma model, probably in connection with increased HDAC2 proteasomal degradation [77]. In addition, VPA was recently shown to enhance proliferation and self-renewal of normal hematopoietic stem cells by inhibition of GSK-3β, which leads to activation of Wnt signaling [78]. Bug *et al.* showed that VPA also maintains a significantly higher proportion of leukemia progenitor cells and colony forming units compared to control cultures in six AML samples, but selectively reduces leukemia cell numbers in another AML sample with expression of AML1/ETO [79]. In addition, VPA in concentrations of 0.5-1 mM enables reprogramming of primary human fibroblasts with ectopic expression Oct4 and Sox2 to a pluripotent state [80]. Oct4, Sox2, Klf4 and c-Myc stem cells markers have been detected in a set of cancer tissues and could be a sign of pathological self-renewal of cancer stem cells [81]. On the other hand, treatment of mouse neural stem cells with VPA and trichostatin A markedly inhibited self-renewal [82] and in neuroblastoma cells, VPA evoked differentiation with downregulation of stem cell markers [83, 84]. Future studies should elucidate the exact effects of VPA treatment on Wnt signaling in normal and cancer stem cells.

In addition to cell signaling pathways, HDACi and, consequently, the acetylation status of the proteins can also affect their proteasomal degradation either by increasing or preventing it. HDACi have been shown to induce the expression of various enzymes of proteasomal degradation pathways. p53 acetylation abrogates formation of a complex between p53 and Mdm2 E3 ligase and enables the p53-mediated stress response regardless of its phosphorylation status, while hypoacetylation enhances p53 proteasomal degradation and cancels p53-dependent growth arrest and apoptosis [51]. The expression of various enzymes of proteasomal degradation pathways is affected by acetylation. Ubc8 E2 ubiquitin conjugase, the level of which is the limiting factor for proteasomal degradation, and RLIM, a subunit of the SCF E3 family of ubiquitin ligases, is induced by VPA, which suggests the mechanism of HDAC2 degradation [21]. Moreover, it has been proposed that SCF E3 ubiquitin ligase plays an important role in the proteolysis of core components that control cell cycles at G1-S and G2-M transitions [85]. HDAC2 downregulation, besides direct inhibition by VPA, is an additional mechanism for HDAC inhibition and might be significant with regard to anticancer activity, since HDAC2 is one of the isoenzymes overexpressed in tumor cells, e.g. in colorectal and gastric cancer [21, 86]. Proteasomal degradation of the fusion proteins AML1/ETO and PML/RAR, which critically contribute to leukemia pathogenesis, is similarly linked to an increase of Ubc8 E2. VPA can therefore help eliminate leukemia fusion proteins [87] and improve patient prognosis.

One of the characteristic features of cancer cells is an abnormal transcription profile. Activation or repression of transcription might be caused by altered transcription factors activity, genetic and genomic changes, histones acetylation status, DNA methylation and others. In many cancers, gene promoters CpG islands acquire abnormal methylation, which results in hereditable transcriptional silencing. "Methylated genes" are not transcribed because the methyl group interferes with the binding of transcription factors. Secondly, and likely more important, methylated DNA-binding proteins (MBD) recruit repressor complexes to methylated DNA, further blocking the transcription apparatus. Methylation status can only be changed during replication, so it is difficult to reverse DNA methylation in non-dividing cells [22]. One way is the modification of histone acetylation, which may actively change the methylation status [88]. Alonso-Aperte *et al.* showed that VPA can induce hypomethylation in the rat liver in a way that is similar to DNA methylation inhibitor 5-aza-2′-deoxycytidine and that this process is dependent on histone acetylation. H3 hyperacetylation induced by VPA increases the accessibility of demethylases to DNA. MBD2/dMTase, with its expression associated with demethylation of endogenous genes [89, 90], is the competent enzyme *in vitro* [91]. VPA seems to be the first agent which is able to reverse DNA methylation in a replication-independent manner by increasing the accessibility of methylated sites for demethylating enzymes.

Tumor progression and invasion is determined not only by the ability of cancer cells to evade the immune system, apoptosis and other antitumor mechanisms, but also by the accessibility to oxygen and adaptation to its low concentration. Within tumors, new blood vessel formation can occur by sprouting from pre-existing vasculature with possible assistance from circulating cells such as bone marrow derived endothelial cells, macrophages and fibroblasts [92, 93]. It is suggested that one of the most important regulators of tumor angiogenesis is transcription factor HIF-1α (hypoxia inducible factor), which under hypoxic conditions regulates expression of many genes, including those that influence angiogenesis. HDACi is suggested to negatively affect the stability of HIF-1α. For example, inhibition of HDAC6 by trichostatin A results in hyperacetylation of HSP90, accumulation of immature HIF-1α protein/HSP70 complex, and degradation of HIF-1α by the 20S proteasome [94]. HDAC1 and 3 directly interact with the ODD domain

of HIF-1α, resulting in increased HIF-1α stability and transactivation during hypoxia. VPA strongly downregulates HIF-1α levels by decreasing HDAC1/3 activity with consequent hyperacetylation in ODD [95]. Structurally diverse HDACi have different inhibitory effects on HIF-1α, and the different potency may be due to the effectiveness in targeting one or multiple HDACs classes.

In addition, VPA inhibits angiogenesis and vasculogenesis *via* increased expression of thrombospondin-1 and activin A, proteins with antiangiogenic effect and by downregulation of proangiogenic factors like bFGF [96]. Inhibition of cell proliferation, migration and capillary tube formation but not apoptosis has been detected using human umbilical vein endothelial cells (HUVEC) [24, 25, Hrabeta J *et al.*, unpublished results] (Fig. **5**). The antiangiogenic effect of VPA has also been demonstrated using three-dimensional collagen matrix assays in muscle and tumor explants [97]. In neuroblastoma xenografts in nude mice, VPA reduces microvascular density [98] probably due to decreased expression of endothelial nitric oxide synthase (eNOS), which is important for angiogenesis, as has been demonstrated in various experiments as well as studies with eNOS -/- mice [99, 100]. Regulation of expression and activity of eNOS includes its phosphorylation by protein kinase Akt and many hemodynamic and hormonal stimuli [101- 103]. HDACi decreases eNOS mRNA stability by increasing the transcription of RNA-destabilizing protein, which binds to the 5′-untranslated region of eNOS mRNA [104].

Besides of the effects described above, VPA exerts other activities which might significantly affect the state of the cells. However, these are beyond the scope of this review. For example, transcription factors as STAT1, FOXO1 and FOXO4 are inactivated by VPA/acetylation causing changes in the expression profile of the cell; while, overexpression of HDAC1, HDAC2, or HDAC3 enhances STAT1-dependent gene expression [28, 105]. In addition, VPA has been shown to interfere with mitochondrial fatty acid β-oxidation and is probably the cause of microvesicular liver steatosis, which is one of its adverse effects. Whether it also participates in the anticancer effects of VPA is not known [106]. Also a decrease in CYP2C9 enzyme activity has been described in epilepsy patients treated with VPA [107]. Since CYP2C9 is important for drug metabolism (e.g. warfarin, phenytoin, tolbutamide, miconazole, tamoxifen, and certain nonsteroidal anti-inflammatory drugs) the risk of pharmacokinetic interactions should be considered during use of VPA. There may also be a role for VPA in antitumor immunity, since it increases constitutive HLA class-I expression and might therefore serve as an adjuvant for immunotherapy [108]. It also induces downregulation of VLA-4 (CD49d), which is important for the retention of normal hematopoietic progenitor cells and leukemic blast cells in bone marrow [109], on various AML cell lines, on blasts cells from patients with AML and on hematopoietic progenitor cells and peripheral blood mononuclear cells from healthy donors. VPA slightly induces lytic EBV gene expression and significantly increases induction of lytic viral gene expression in EBV-positive epithelial and lymphoid cells. VPA potentiates the effect of chemotherapeutics in lymphoblastoid cells and gastric cells containing wild-type EBV, but does not affect their cytotoxicity in EBV-negative gastric cells [110].

The field of activity of VPA on tumor cells, with HDAC inhibition probably only being the top of the iceberg, also comprises other important features (Fig. **3**). It would be surprising if there were none, since histone acetylation and the affect of various transcription factors are general changes to a fine-tuned system of cellular functions. However, the overall anticancer action of VPA is still unknown and may be specific for different tumors types.

4. PRECLINICAL STUDIES

VPA in doses which are usually applied when treating epilepsy to reach efficient serum concentration (0.5 - 1 mM)

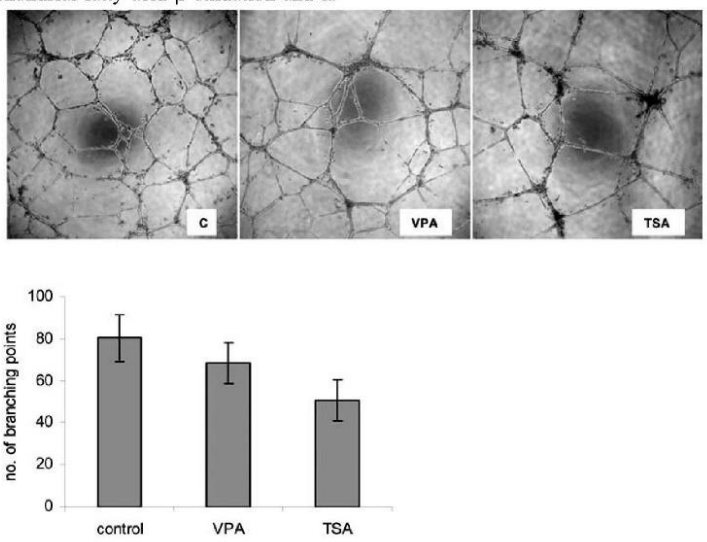

Fig. (5). Capillary tube formation after 24 hour incubation with VPA (1mM) and trichostatin A (100nM) performed on HUVEC cells. VPA and trichostatin A significantly reduce capillary tube formation. Photo: J.Hrabeta.

Table 3. Preclinical Studies of VPA Action on Cancer Cell Lines *in vitro* and *in vivo*

Cell Line	Effect	Refs.
UKF-NB-2, UKF-NB-3 neuroblastoma	Decreased N-myc expression, cell differentiation, increased immunogenicity	[127]
UKF-NB-3 neuroblastoma xenograft	Growth inhibition, cell differentiation, apoptosis induction	[12]
Teratocarcinoma F9, hematopoietic progenitors, leukemic blasts	Induction of differentiation,growth inhibition	[19]
MT-450 breast carcinoma	Reduced tumor growth and metastasis formation	[19]
A172, 85HG66, 86HG39 glioma	Growth inhibition	[111]
HEC-1B, HEC59, Ishikawa, RL95–2, KLE, AN3CA endometrial cancer	Proliferation inhibition, apoptosis	[120]
HuH-7, PLC/PRF/5, HepG2-hepatoma	Viability decrease, apoptosis	[112]
D54 glioma,KB laryngeal carcinoma,MCF-7 breast adenocarcinoma, SW480 colon carcinoma, HeLa cervical cancer, Hep-2 oral carcinoma, HT1080 sarcoma	Growth inhibition	[17]
T24, TCC-SUP, HT1376 bladder cancer	Invasiveness decrease	[117]
T24 bladder cancer xenograft	Growth inhibition	[117]
D283, DAOY, MHH-MED-1 medulloblastoma xenografts	Growth inhibition, apoptosis	[116]
LNCaP, C4-2, DU145 prostate cancer xenograft	Growth inhibition	[118]
SiHa, CasKi, HeLa cervical cancer	Growth inhibition	[115]
GI BON carcinoid cell lines	Growth inhibition, cell cycle arrest	[114]
Kasumi-1, NB-4 leukemia cells	Apoptosis, cell differentiation (Kasumi-1)	[121]
LNCaP prostate cancer	Cell differentiation, apoptosis	[119, 128]
Isolated CLL cells	Apoptosis	[122]
Caki-1, KTC-26, A498 renal carcinoma, Caki-1 xenograft	Cell adhesion blockage, growth inhibition (xenograft)	[125]
MM1, OPM1, DOX-40, INA-6 mutiple myeloma	Growth inhibition, apoptosis (not DOX-40)	[124]
MM1, OPM1 multiple lyeloma xenograft	Growth inhibition	[124]
Follicular thyroid cancer FTC236, papillary thyroid cancer DRO	Growth inhibition	[113]

affects several cancer cells as demonstrated in a variety of *in vitro* and *in vivo* experiments, see Table **3**.

VPA inhibits growth of cervical, colon, breast, head and neck cancer, sarcoma, and glial tumor-derived cell lines *in vitro* as measured using the MTT test [17, 111]. It induces differentiation of teratocarcinoma cells and depresses growth of teratocarcinoma, colon and breast cancer and leukemia blasts isolated from AML patients. It also inhibits growth and formation of metastasis in rat breast cancer model *in vivo* [19]. VPA induces apoptosis in hepatocellular carcinoma cell lines but not in normal human hepatocytes [112]. It inhibits growth of carcinoid cells both *in vitro* and in xenografts. VPA treatment of papillary and follicular thyroid cancer lines suppresses cell growth and induces overexpression of the Notch1 protein, the activation of which seems to play an important role in growth inhibition of carcinoid cells. Notch1 acts as a tumor suppressor that can be induced by HDACi in some cancers. Moreover, knock-out of Notch1 by siRNA suppresses the cytostatic effect of VPA in thyroid cancer cells [113]. High expression of Notch-1 in medullary thyroid and small cell lung cancer cells has been described to result in an increase of p21 and induction of cell cycle arrest [114]. VPA treatment also leads to an activated Notch signaling cascade in neuroblastoma by increasing the levels of intracellular Notch-1 and Hes-1, mimicking the initial phase of differentiation. Therefore, VPA could be potentially used in differentiation-based therapy of neuroblastoma [83]. VPA increases oncoprotein E6 and E7 expression in human papillomavirus positive cervical cancer cell lines *in vitro*. However, in tumor samples from patients treated with VPA, E6 and E7, transcription was unchanged. Cervical cancer cell lines increase expression of p53 and show inhibition of growth when incubated with VPA [115].

Treatment with VPA increases survival of SCID mice with intracerebellar orthotopic human medulloblastoma cells xenografts. Prolonged survival correlated with histone acetylation, cancer cell differentiation and apoptosis and was accompanied by decreased angiogenesis [116]. VPA administration decreases invasivity of bladder cancer cell lines *in vitro* but not of prostate cancer cell lines. Long lasting ingestion of VPA suppresses growth of bladder cancer cell line xenografts in athymic nu/nu mice [117]. Inhibition of tumor growth after long-term administration of VPA has been observed in both androgen receptor-positive and negative prostate cancer xenografts in mice [118]. VPA induces neuroendocrine-like differentiation of prostate cancer cell line together with overexpression of neuron-specific enolase (NSE), and down-regulation of androgen receptor protein and prostate-specific antigen. Furthermore, microarray expression profiling shows that VPA might enhance dihydrotestosterone catabolism *in vitro*. This may indicate a modulation in the responsiveness to hormonal therapy [119].

However, since higher levels of NSE and a lower expression of androgen receptors are usually associated with a poorer prognosis, using of VPA for treatment of this cancer type, even if it is able to induce cell differentiation, should be considered carefully. Other HDACi (SAHA, VPA, trichostatin A, and sodium butyrate) are effective in inhibiting growth of endometrial cancer cells *in vitro* and in nude mice xenografts [120].

VPA disrupts the AML1/ETO-HDAC1 interaction and induces apoptosis in AML1/ETO positive but not in negative AML cell lines [121]. VPA induces apoptosis in chronic lymphocytic leukemia (CLL) cells by activation of caspase 8 and 9 and cleavage of proapoptotic protein BID with its C-terminal part being translocated to mitochondria where it triggers cytochrome c release. In addition, the Bcl-2/Bax ratio is decreased in response to VPA [122]. VPA kills myeloma cell lines and CD138+ myeloma cells collected from patients via both caspase-dependent and independent cell death, but it does not influence CD138 negative bone marrow cells. It suppresses osteoclastogenesis and osteoclast-mediated myeloma cell growth. VPA also suppresses vascular tubule formation in co-cultures of myeloma cells and osteoclasts [123]. VPA inhibits proliferation and induces apoptosis of multiple myeloma cells *in vitro*, inhibits tumor growth and prolongs survival of immunodeficiency mice with multiple myeloma xenografts compared to untreated controls [124].

VPA, when tested *in vitro* and *in vivo* on renal cell carcinoma models, decreases tumor cell adhesion to vascular endothelial cells or to immobilized extracellular matrix proteins and inhibits the growth of subcutaneous xenografts in mice [125]. Other experiments showed that VPA alters integrin α and β subtype expression and blocks integrin-dependent integrin-linked kinase, focal-adhesion kinase signaling as well as the interaction of renal cell carcinoma cells with endothelium and extracellular matrix, and reduced tumor growth *in vitro* [126]. VPA induces differentiation and blocks the growth of neuroblastoma cells. It enhances cell surface adhesion molecules expression and decreases N-myc levels. Experiments on nude mice xenografts of neuroblastoma cells supported the *in vitro* data [12].

In conclusion, many *in vitro* and *in vivo* experiments show that VPA in concentrations that are found in the serum of patients treated for epilepsy (0.5 – 1 mM) has significant anticancer activity. Effects on brain tumors (glioblastoma, medulloblastoma), which usually have a bad prognosis, seems to be very important from a practical point of view, since VPA is effectively delivered to brain tissue. Other promising results have demonstrated the specific effect of VPA on malignant but not on normal cells in hepatocellular carcinoma [112] or in AML1/ETO positive but not in negative AML cell lines [121].

5. COMBINATION OF VPA WITH CYTOSTATICS AND/OR RADIOTHERAPY

It is believed that the main mechanism for HDAC inhibitors in enhancing the cytotoxicity of DNA-targeting drugs lies in acetylation of histones and subsequent relaxation of chromatin, which facilitates drug effects. For example, VPA monotherapy does not induce apoptosis in anaplastic thyroid cancer cell lines CAL-62 and ARO *in vitro*, but it significantly increases doxorubicin-mediated apoptosis in these cells. Moreover, the combination of VPA and doxorubicin has been shown to increase caspase 3 activation and to arrest cells in the G2 phase [129]. Another experiment with using a combination of VPA and anthracyclines showed that exposure to VPA followed by epirubicin causes regression in human breast cancer xenografts in nu/nu mice while the reverse order of drugs was less effective. VPA increases epirubicin- and aclarubicin-induced apoptosis in estrogen-responsive MCF-7 breast cancer cells. This effect is caused by an increased interaction between DNA and the drug. However, this potentiation of apoptosis requires a longer pretreatment period (48 hours) with 2 mM of VPA. One of the possible mechanisms for this synergy is depletion of topoisomerase IIα and therefore this potentiation is limited only to cancer cells expressing topoisomerase IIα [130]. VPA and etoposide, another topoisomerase II inhibitor, independently inhibit the growth of LN18, U251 and U87 glioblastoma cells. Exposure of tumor cells to both drugs for 72 hours significantly increases the cytotoxic effect in all tested cell lines. In this case, pre-exposure to VPA did not yield additional treatment effects for the VPA / etoposide combination [131]. VPA pretreatment of non-small cell lung cancer (H460), esophageal cancer (TE12) and malignant mesothelioma (H513) cell lines increases apoptosis induced by Apo2L/TRAIL [132]. The increased effect of TRAIL might be explained by activation of NF-κB, which increases the expression of death receptor 5 (DR5, TRAIL-R2). Results of several experiments have shown that VPA can increase the effect of many other cytostatics with different modes of action like paclitaxel, hydroxyurea, cisplatin, staurosporin, and 5-aza-2′-deoxycytidine. VPA enhances paclitaxel-induced cytotoxicity caused by binding to the β subunit of tubulin in CAL-62 and ARO anaplastic thyroid carcinoma cell lines by increasing acetylation of tubulin which leads to hyperstabilization of microtubule structures. Hyperstabilization of microtubules adversely affects the process of cell division by preventing restructuralization of microtubules and therefore induces apoptosis [133]. VPA and hydroxyurea, which inhibits production of deoxyribonucleotides, have been shown to synergistically induce apoptosis in SK-Mel-37 melanoma cells *in vitro*. Cells resistant to one compound became sensitive after addition of the other [134]. Preincubation of neuroblastoma cell line UKF-NB-4, with experimentally induced resistance to cisplatin, with 1 mM VPA increases the efficiency of cisplatin [Hrabeta J *et al.*, unpublished results], see Fig (**6**).

Kinase inhibitors staurosporine or its derivative UCN-01 and VPA interact to mediate induction of apoptosis of mesothelioma (H211), esophageal (TE12) and non-small cell lung (H460) cancer cell lines *in vitro*. Sensitizing cancer cells to VPA, by a kinase inhibitor, is caused by suppression of NF-κB transcriptional activity [135]. 5-aza-2′-deoxycytidine, a hypomethylating agent with anti-leukemia effects and VPA are both growth inhibitors and apoptosis inductors which induce the expression of p57KIP2 and p21CIP1 (cyclin dependent kinase inhibitors) in leukemia cell lines HL-60 (promyelocytic leukemia) and MOLT4 (T cell acute lymphoblastic leukemia (ALL)) at doses of 1 μM and 1 mM respectively. In addition, their combination has been found to produce synergistic effects in terms of growth inhibition,

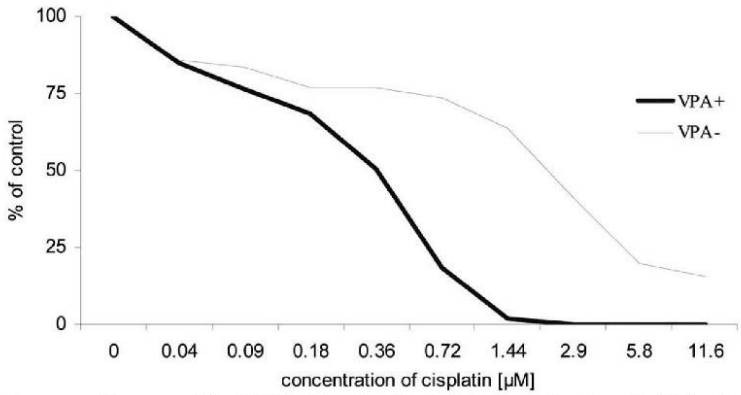

Fig. (6). Effect of cisplatin on neuroblastoma cell line UKF-NB-4 resistant to cisplatin in combination with VPA and without VPA.

induction of apoptosis and on p57KIP2 and p21CIP1 reactivation [136]. Cytotoxicity enhancement of several cytostatics by HDACi has also been verified by experiments of Kim *et al.* using a large panel of cell lines. Preincubation of glioblastoma, breast, and colon cancer cell lines with trichostatin A or SAHA increased the efficiency of etoposide, doxorubicin, ellipticine and cisplatin [137].

Potentiation of 5-aza-2′-deoxycytidine effects by VPA has also been detected in animal models. Heterozygous Ptch knockout mice spontaneously develop medulloblastoma and rhabdomyosarcoma which resembles the corresponding human tumors. Evidence exist that silencing of the Ptch allele contributes to the formation of this tumor. Treatment with a combination of 5-aza-2′-deoxycytidine and VPA, which was more effective than using each drug alone, prevented tumor formation with reactivated Ptch expression, probably because of reduced methylation of the Ptch promoter and histone hyperacetylation [138].

VPA with interferon-α synergistically inhibit cell growth of BE(2)-C neuroblastoma with morphological signs of neuronal differentiation, accompanied by decreased expression of N-myc protein and induction of Bcl-2 and neural cell adhesion molecule. In differentiated cells, upregulation of inhibitors of angiogenesis thrombospondin-1 and activin A has been found [96]. After combination treatment with VPA and interferon-α, growth of UKF-NB-2 and UKF-NB-3 neuroblastoma cells is synergistically inhibited showing an accumulation of cells in the G0/G1-phase. Besides that, this combination inhibits the growth of UKF-NB-3 neuroblastoma xenografts in nude mice [139].

VPA has cytostatic effects on human Philadelphia chromosome-positive ALL and CML cell lines. VPA enhances activity of imatinib mesylate, nilotinib, farnesyl transferase inhibitor SCH66336, interferon-α, and cytosine arabinoside in various leukemia cell lines [140]. In addition, VPA increases the anti-myeloma effects of melphalan and dexamethasone [123].

Besides chemotherapy, VPA was found to have synergistic effect with other treatment methods, such as ionizing radiation. A 24 hour pretreatment of erythroleukemia K562 cells with VPA enhances the effectiveness of radiation therapy, probably due to the arrest of cells in a phase of the cell cycle that makes them more radiosensitive [141]. Radiosensitization by VPA has also been detected in SF539 and U251 brain tumor cells *in vitro*. In *in vivo* experiments, survival of mice with U251 brain tumor xenografts treated with radiotherapy and VPA was longer than that of controls treated with radiotherapy alone [142]. Radiosensitizing properties of VPA have also been found in human promyelocytic leukemia cells HL-60 [143].

All results mentioned above show that VPA potentiates anticancer effect of several drugs and/or radiotherapy. However, further experiments and clinical studies are necessary in order to determine the dosage of the drugs, their combination and optimal sequencing since some trials showed potentiation only when a specific drug sequence was applied, while others failed to demonstrate any potentiation [131, 132].

6. CLINICAL STUDIES

Due to promising results of preclinical experiments with VPA, or its combinations with other drugs and/or radiotherapy, several clinical trials in solid tumors, in particular AML and myelodysplastic syndrome, have been conducted, see Table **4**.

VPA was used orally in a phase II trial to treat patients with castration-resistant prostate cancer. Ten patients were included in this study. Generally, VPA was not well tolerated; the main toxicities were neurological symptoms (dizziness, memory impairment, drowsiness, somnolence and tremor) grade 1 and 2 and fatigue grade 1 and 2. Two of ten patients had PSA responses, one of which was durable and correlated with total VPA levels. However, VPA cannot be reliably administered orally to achieve satisfactory treatment in patients with this type of cancer [144].

Noguchi *et al.* reported a case of successful treatment of anaplastic thyroid carcinoma with a combination of oral valproic acid, cisplatin and doxorubicin, external and intraoperative radiation and surgery. Treatment side effects were no more severe than anticipated and included nausea, taste disturbance, dizziness, smell hypersensitivity and others. Interestingly, even if VPA is sometimes used for headache treatment, unexpected side effects were recurrent headaches that improved after the patient was tapered off VPA.

Table 4. Overview of Chosen Clinical Studies with VPA Alone or in Combination with Other Drugs

Phase	No. of patients	Cancer type	Combinatory treatment	Refs.
case-study	1	anaplastic thyroid carcinoma	cisplation, doxorubicin, radiation surgery	[145]
pilot study	8	MDS (4), AML (3), Ph+ CML	ATRA	[152]
pilot study	11	AML	ATRA	[154]
I	44	melanoma (11), skin (2), colon (2), prostate (2), breast (10), small-cell lung cancer (4), sarcoma (3), others (10)	Epirubicin	[147]
I/II	18	MDS and sAML/MDS	monotherapy; ATRA	[149]
I/II	58	AML (22), sAML/MDS (32), sAML/myeloproliferative syndrome	monotherapy; ATRA	[150]
I/II	54	AML (48), MDS (6)	5-aza-2'-deoxycytidine (decitabine)	[155]
I/II	53	AML (49), MDS (4)	5-aza-2'-deoxycytidine (decitabine), ATRA	[156]
I/II	20	AML	ATRA	[A]
I/II	29; 18 evaluated	melanoma	dacarbazine, interferon-alpha	[158]
II	10	hormonal therapy resistant prostate cancer	monotherapy	[144]
II	17; 15 evaluated	cervix (3), breast (3), lung (1), testis (1), and ovarian (7) carcinomas	cisplatin, carboplatin, paclitaxel, vinorelbine, gemcitabine, pemetrexed, topotecan, doxorubicin, cyclophosphamide, and anastrozole	[146]
II	26	AML (14), sAML/MDS (6), sAML/other (6)	ATRA	[151]
II	20	AML (13), chronic myelomonocytic leukemia (2), MDS (5)	ATRA	[153]
II	119	MDS (58), AML (61)	monotherapy; ATRA	[B]

A- Craddock C *et al.* Predictors of clinical response in patients with high risk acute myeloid leukemia receiving treatment with the histone deacetylase inhibitor sodium valproate. ASH Annual Meeting Abstracts. Blood 2005; 106. Abstract 2791; B- Kuendgen A *et al.* Valproic acid (VPA) achieves high response rates in patients with low-risk myelodysplastic syndromes. ASH Annual Meeting Abstracts. Blood 2005; 106. Abstract 789.

Cytology performed during therapy showed changes in cell types ranging from anaplastic to well differentiated. The patient is alive and disease-free (Ga scintigraphy and whole body PET scan performed six months after the surgery) two years after diagnosis [145]. This case report demonstrates the very promising effect of VPA when used in combination with other cytostatics, especially when considering that anaplastic thyroid carcinoma usually has a very poor prognosis.

In another phase II study, hydralazine and magnesium valproate were added to the same schedule of chemotherapy with different cytostatics on which 17 patients were progressing. Schedules comprised cisplatin, carboplatin, paclitaxel, vinorelbine, gemcitabine, pemetrexed, topotecan, doxorubicin, cyclophosphamide and anastrozole. A clinical benefit was observed in 12 patients: four partial remissions and eight cases of stable disease. The most significant toxicity was hematologic. Reduction in global DNA methylation and histone deacetylase activity, as well as increased promoter demethylation was observed. Based on their clinical benefit, VPA and hydralazine could support the treatment of epigenetically-driven chemoresistant tumors [146].

A clinical trial combining VPA and epirubicin in patients with advanced solid tumor malignancies demonstrated that the combination of VPA followed by epirubicin seems to be active without enhancing epirubicin-induced toxicity. Objective responses were seen in 22% of heavily pretreated patients, and stabilized disease was observed in an additional 39% of patients. Responses were seen across multiple tumor types, including breast, small-cell lung, pancreas, and prostate cancer, as well as in tumor types thought to be anthracycline insensitive, such as melanomas and cervical cancer. A limited phase II trial in patients with metastatic breast cancer, in which fluorouracil and cyclophosphamide are added to the VPA therapy, is ongoing, and a phase II neoadjuvant trial is planned [147].

Results of several clinical studies in patients with AML and myelodysplastic syndrome (MDS) have been published [148, 149]. In a pilot study, 18 MDS or secondary AML/MDS patients were treated with VPA, either alone or in combination with all-trans-retinoic acid (ATRA). VPA monotherapy was more affective than the first-line combination with ATRA. A possible explanation might be that HDAC inhibition is needed to relieve repression of retinoic acid signaling pathways. Therefore, pretreatment with VPA might be necessary for the synergistic effect of both drugs. The response was 44% for VPA monotherapy with a trend toward better responses in patients with low-risk MDS [149]. On the basis of these results, a study with 58 AML or

secondary AML/MDS patients was performed. However, the response rate was only 5% [150]. In another study, 61 patients with AML and 58 patients with MDS were treated with VPA or VPA in combination with ATRA. The response rate in MDS patients was 28% and in AML 15% [Kuendgen A *et al.* Valproic acid (VPA) achieves high response rates in patients with low-risk myelodysplastic syndromes. ASH Annual Meeting Abstracts. Blood 2005;106. Abstract 789]. Bug *et al.* described results from treatment of 26 patients with poor-risk AML with VPA and ATRA. Only one patient had minor hematologic improvement and two patients with secondary AML/MDS achieved a partial remission with the conclusion that VPA did not have meaningful antiproliferative effects in AML [151]. Cimino *et al.* performed a pilot study with VPA followed by ATRA using a sequential schedule in 8 patients with refractory or high-risk AML. Hematologic improvement was detected in 2 patients [152]. Another study with twenty older patients with AML or MDS also investigated VPA and ATRA in a sequential schedule; 6 patients demonstrated hematologic improvement [153]. Raffoux *et al.* used VPA and ATRA to treat 11 elderly AML patients with 3 bone marrow responses including one complete remission. Two additional patients showed hematologic improvement. Moreover, 4 of 5 responders in this study had a normal karyotype [154]. In a Phase I/II study of VPA and ATRA therapy in patients with high-risk AML, 4 of 20 patients, all of them with relapsed AML, had a hematologic response. One patient achieved a complete remission and two had partial remissions [Craddock C *et al.*, Predictors of clinical response in patients with high-risk acute myeloid leukemia receiving treatment with the histone deacetylase inhibitor sodium valproate. ASH Annual Meeting Abstracts. Blood 2005; 106. Abstract 2791].

Both DNA methylation and histone deacetylation were shown to cause inhibition of gene expression. A combination of methyltransferase inhibitors with HDACi could further increase the effectivity of treatment in this field and therefore serve as a valuable therapeutic tool. Most notably in AML and MDS [149] as presented in several clinical studies. In a phase I/II trial, 54 AML patients were treated using a combination of decitabin (5-aza-2′-deoxycytidine) and VPA. Neurologic toxicity, usually grade 1 or 2 was common. Twelve patients had objective responses, including 10 complete remissions and 2 complete remissions with incomplete platelet recovery [155]. In another phase I/II study, the effect of the combination of 5-aza-2′-deoxycytidine, VPA and ATRA in patients with refractory or relapsed AML or high-risk MDS and in untreated patients older than 60 years with AML or high-risk MDS was evaluated; the overall response was 42%. The dose-limiting factor was reversible neurotoxicity [156]. Statistical analyses performed on the basis of data from the Düsseldorf MDS Registry demonstrated that lengthening of survival of MDS patients treated with thalidomide, valproic acid, low-dose cytarabine, antithymocyte globulin, induction chemotherapy, or allogeneic stem cell transplantation was restricted to certain subgroups of patients. With the exception of allotransplantation, MDS treatment was generally palliative [157].

In a phase I/II clinical trial, with a combination of VPA and standard chemoimmunotherapy in patients with advanced melanoma, 29 eligible patients started taking valproate and 18 received chemoimmunotherapy and were evaluated. One patient had a complete response, 2 had partial remissions and 3 had disease stabilization. The conclusion was that the addition of VPA to chemoimmunotherapy did not improve therapeutic results [158].

Several results of clinical studies described above, particularly of studies combining VPA with other drugs, confirm the therapeutic potential of VPA. For example the case report of anaplastic thyroid cancer [145], studies with hydralazine and magnesium VPA [146], VPA and epirubicin [147] both in solid tumors, together with other studies in AML and MDS, especially those combining VPA with ATRA and/ or decitabin [148, 149, 156]. However, more studies with different approaches and treatment procedures need to be conducted in order to obtain further and perhaps more detailed information about the potential for VPA.

7. RESISTANCE TO VPA

Acquired resistance to cytostatic drugs is the main reason for anticancer therapy failure. There is only limited knowledge about resistance of cancer cells to VPA. Its presence and mechanisms might be predicted on the basis of analogic information about other HDACi. Resistance to HDACi might have the same mechanisms as in the case of other anticancer drugs: increased drug efflux, overexpression or modification of target molecules (HDACs), epigenetic modifications or induction of antiapoptotic mechanisms [159]. One of the few reports about resistance to VPA was published by Armeanu *et al.* In their experiments they showed that transfection of Bcl-X(L) into hepatocellular carcinoma-derived cell line suppresses VPA-induced apoptosis [112].

More data is available about resistance to sodium butyrate, which is chemically the closest HDACi to VPA, since both are short-chain carboxylic acids. Expression of multidrug resistance gene 1 (MDR1) mRNA is induced by sodium butyrate in parental and multi-drug resistant (Ad1000) SW620 colon cancer cell lines. Butyrate treatment has no influence on MDR1 mRNA stability, suggesting that mRNA induction is attributable to altered MDR1 transcription. Interestingly, it was found that even if expression of P-glycoprotein is higher, its phosphorylation, which is important for its proper function, was decreased [160].

Another mechanism of resistance to HDACi involves increased amount/activity of HDACs. Sodium butyrate induces apoptosis in UCD-Mel-N, A375 and SB2 melanoma cell lines but cells with increased HDAC1 levels (transfected with HDAC1 plasmid) are apoptosis resistant. On the other hand, antisense HDAC1 vector significantly increases sensitivity to this drug. Described resistance to apoptosis in HDAC1-overexpressing cells is associated with p-53-dependent Bax promotor repression [161]. Treatment of HDACs class II-deficient cells with trichostatin A (inhibitor of HDAC I and II) does not inhibit their proliferation but these cells are sensitive to VPA and sodium butyrate which are selective inhibitors of class I HDACs [162].

Treatment of cancer cells with HDAC inhibitors including vorinostat, SNDX-275, sodium butyrate, panobinostat, and romidepsin results in oxidative stress characterized by overproduction of reactive oxygen species and a decreased ratio of reduced/oxidized glutathione. Oxidative damage

caused by reactive oxygen species participates in the anti-proliferative effects of HDACi. Cellular redox homeostasis is maintained by many systems including the thioredoxin family of proteins. They protect the cells against oxidative damage and stress-induced cell death. It has been suggested that HDACi might, at least in part, disrupt these mechanisms [163].

Upon HDACi treatment, acetylated Ku70 releases Bax, allowing it to translocate to mitochondria and trigger cytochrome c release and apoptosis [164]. Neuroblastoma S-type cells, unlike N-type cells, lack expression of HAT cAMP-responsive element-binding protein (CBP). Depletion of CBP and the impossibility of Ku70 acetylation, contributes to the relative resistance of the S-type neuroblastoma cells to HDACi [165]. We found more than a twofold difference in the IC_{50} for VPA for SK-N-AS neuroblastoma cells (S type) than for UKF-NB-3 (N type) [Hrabeta J and Cipro S, unpublished results]. Treatment of non-small cell cancer cell lines with sodium butyrate and trichostatin A induces NF-κB expression and that helps them to prevent apoptosis induced by HDAC inhibition. Inhibition of NF-κB activation increases HDAC inhibitors efficiency [166]. However, it is known that the effect of NF-κB is different in different cells [167] with the increase of its amount and/or activity not necessarily associated with increased cell survival.

Results of our experiments with neuroblastoma and Ewing sarcoma cell lines have shown that VPA is almost as effective in hypoxic as in normoxic conditions, while in the majority of other cytostatics, the relative resistance in hypoxia is at least doubled. We compared the effect of VPA on different neuroblastoma cell lines. The IC_{50} of all tested lines, including a line derived from recurrent disease with high expression of P-glycoprotein and lines with experimentally-induced resistance to doxorubicin and cisplatin, were nearly the same as the serum levels in patients treated by VPA. Sensitivity of neuroblastoma cells to VPA with experimentally induced resistance to doxorubicin or cisplatin does not differ significantly from sensitivity of parental cells [Hrabeta J *et al.*, unpublished results].

Resistance to anticancer drugs including HDACi seems to be multifactorial and dependent on tumor origin and genetic heterogeneity [159]. Identification of these resistance mechanisms is crucial for efficient use of VPA as an anticancer drug in clinical practice.

8. FUTURE DIRECTIONS

On the basis of the investigations that have been conducted in order to reveal the potential of VPA, we suppose that it might have a place in palliative or maintenance therapy in high-risk neuroblastoma, brain tumors, AML and MDS. After further verification, VPA could be used as a curative therapy in combination with cytostatics and/or radiotherapy in certain cancer types. For clinical purposes, enhancement of immunotherapy by overexpression of MHC I and potentiation of TRAIL and/or interferon-α by VPA could be also beneficial.

Since the cooperation between gene hypermethylation and histone deacetylation in silencing of gene transcription was described [168] and a clinical phase II study with VPA and hydralazine was successful [146], one may speculate that a combination of VPA with methyltransferase inhibitors and cytostatic drugs could be the right approach to the treatment of certain malignancies. In addition, there is potential for construction of new VPA-derivatives that might have higher anticancer potency and specificity. For example propyl-4-yn-valproic acid has been shown to significantly reduce glioma cell proliferation [1].

However, optimal drug combinations, their sequencing, dosages and possible combination with radiotherapy have to be found in order to achieve more effective treatment. Another important question is sensitivity to VPA. It is known that sensitivity to VPA, as well as to other drugs could be notably different for tumor cells derived not only from different tissues but also for the ones of similar origin. An example is a study, which demonstrated that different glioma cell lines can vary in their sensitivity to VPA [111]. Susceptibility to VPA depends not only on mitotic activity, and differentiation, but also on the underlying genetic alterations. Therefore, precise patient selection for VPA therapy is necessary. In order to achieve the best selection for a particular therapy, more *in vitro* and *in vivo* experiments and especially clinical trials need to be performed.

For a drug, VPA could be definitely marked as "old". However, now it has revealed a novel set of properties such as induction of tumor cell death, differentiation and cell cycle arrest; affection of malignancies by regulation of the immune response and tumor angiogenesis. This, together with the broad possibilities for use in cancer therapy, where VPA could be used either alone or as a potentiating agent to increase the effect of other anticancer drugs and/or radiotherapy, has made it "new and interesting" once again.

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ABBREVIATIONS

ALL	=	Acute lymphoblastic leukemia
AML	=	Acute myelocytic leukemia
ATRA	=	all-trans-retinoic acid
CBP	=	cAMP-responsive element-binding protein
CLL	=	Chronic lymphocytic leukemia
CML	=	Chronic myeloid leukemia
DR5	=	Death receptor 5 (TRAIL-R2)
EBV	=	Epstein-Barr virus
eNOS	=	Endothelial nitric oxide synthase
ERK	=	Extracellular-regulated kinase
GSK-3β	=	Glycogen synthase kinase-3β

HAT	=	Histone acetyltransferase
HDAC	=	Histone deacetylase
HDACi	=	Histone deacetylase inhibitor
HIF-1α	=	Hypoxia inducible factor 1α
HSP90	=	Heat shock protein 90
HUVEC	=	Human umbilical vein endothelial cells
JNK	=	c-Jun N-terminal kinase
MAPK	=	Mitogen-activated protein kinases
MBD	=	Methylated DNA-binding proteins
MDR1	=	Multi-drug resistance gene 1
MDS	=	Myelodysplastic syndrome
MHC	=	Major histocompatibility complex
NMDA	=	N-methyl-D-aspartic acid
NSE	=	Neuron-specific enolase
PCAF	=	P300/CBP-associated factor, also known as K(lysine) acetyltransferase 2B
PI-3K	=	Phosphatidylinositol 3-kinase
PKC	=	Protein kinase C
SAHA	=	Suberoylanilide acid
SCF	=	Skp, Cullin, F-box containing complex
SCID	=	Severe combined immunodeficiency
TRAIL	=	TNF-related apoptosis-inducing ligand
VPA	=	Valproic acid

REFERENCES

[1] Blaheta RA, Cinatl J Jr. Anti-tumor mechanisms of valproate: a novel role for an old drug. Med Res Rev 2002; 22: 492-511.

[2] Blaheta RA, Michaelis M, Driever PH, Cinatl J Jr. Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. Med Res Rev 2005; 25: 383-97.

[3] Balbi A, Sottofatori E, Mazzi M, Sannita WG. Study of bioequivalence of magnesium and sodium valproates J Pharm Biomed Anal 1991; 9: 317-21.

[4] Rosenberg G. The mechanisms of action of valproate in neuropsychiatric disorders: can we see the forest for the trees? Cell Mol Life Sci 2007; 64: 2090-103.

[5] Martella G, Costa C, Pisani A, Cupini LM, Bernardi G, Calabresi P. Antiepileptic drugs on calcium currents recorded from cortical and PAG neurons: therapeutic implications for migraine. Cephalalgia 2008; 28: 1315-26.

[6] Nau H, Hauck R S, Ehlers K. Valproic acid-induced neural tube defects in mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanisms. Pharmacol Toxicol 1991; 69: 310–21.

[7] Gurvich N, Berman MG, Wittner BS, Gentleman RC, Klein PS, Green JB. Association of valproate-induced teratogenesis with histone deacetylase inhibition *in vivo*. FASEB J 2005; 19: 1166-8.

[8] Wood JR, Nelson-Degrave VL, Jansen E, McAllister JM, Mosselman S, Strauss JF 3rd. Valproate-induced alterations in human theca cell gene expression: clues to the association between valproate use and metabolic side effects. Physiol Genom 2005; 20: 233-43.

[9] Gerstner T, Bell N, König S. Oral valproic acid for epilepsy- long-term experience in therapy and side effects. Expert Opin Pharmacother 2008; 9: 285-92.

[10] Sigalotti L, Fratta E, Coral S, *et al.* Epigenetic drugs as pleiotropic agents in cancer treatment: biomolecular aspects and clinical applications. J Cell Physiol 2007; 212: 330-44.

[11] Martin ML, Regan CM. The anticonvulsant valproate teratogen restricts the glial cell cycle at a defined point in the mid-G1 phase. Brain Res 1991; 554: 223–228.

[12] Cinatl J Jr., Cinatl J, Driever PH, *et al.* Sodium valproate inhibits *in vivo* growth of human neuroblastoma cells. Anticancer Drugs 1997; 8: 958-63.

[13] Kawagoe R, Kawagoe H, Sano K. Valproic acid induces apoptosis in human leukemia cells by stimulating both caspase-dependent and -independent apoptotic signaling pathways. Leuk Res 2002; 26: 495-502.

[14] Tang R, Faussat AM, Majdak P, *et al.* Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. Leukemia 2004; 18: 1246-51.

[15] Li XN, Shu Q, Su JM, Perlaky L, Blaney SM, Lau CC. Valproic acid suppresses cell proliferation and induces apoptosis and senescence in medulloblastoma cells through histone hyperacetylation, p53-independent activation of p21Cip1, restoration of p16Ink4a/CDK4 pathway, and down regulation c-MYC gene expression. Mol Cancer Ther 2005;4:1912-22.

[16] Olsen CM, Meussen-Elholm ET, Røste LS, Taubøll E. Antiepileptic drugs inhibit cell growth in the human breast cancer cell line MCF7. Mol Cell Endocrinol 2004; 213: 173-9.

[17] Chavez-Blanco A, Perez-Plasencia C, Perez-Cardenas E, *et al.* Antineoplastic effects of the DNA methylation inhibitor hydralazine and the histone deacetylase inhibitor valproic acid in cancer cell lines. Cancer Cell Int 2006; 6: 2.

[18] Iacopino F, Urbano R, Graziani G, Muzi A, Navarra P, Sica G. Valproic acid activity in androgen-sensitive and -insensitive human prostate cancer cells. Int J Oncol 2008; 32: 1293-303.

[19] Gottlicher M, Minucci S, Zhu P, *et al.* Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 2001; 20: 6969-78.

[20] Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and tetratogen. J Biol Chem 2001; 276:36734-41.

[21] Kramer OH, Zhu P, Ostendorff HP, *et al.* The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. EMBO J 2003; 22:3411-20.

[22] Detich N, Bovenzi V, Szyf M. Valproate induces replication-independent active DNA demethylation. J Biol Chem 2003; 278: 27586-92.

[23] Carew JS, Giles FJ, Nawrocki ST. Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. Cancer Lett 2008; 269: 7-17.

[24] Michaelis M, Michaelis UR, Fleming I, *et al.* Valproic acid inhibits angiogenesis *in vitro* and *in vivo*. Mol Pharmacol 2004;65:520-7.

[25] Michaelis M, Suhan T, Michaelis UR, *et al.* Valproic acid induces extracellular signal-regulated kinase 1/2 activation and inhibits apoptosis in endothelial cells. Cell Death Differ 2006; 13: 446-53.

[26] Roth SY, Allis CD. Histone acetylation and chromatin assembly: a single escort, multiple dances? Cell 1996; 87: 5-8.

[27] Strahl BD, Allis CD. The language of covalent histone modifications. Nature 2000; 403: 41-5.

[28] Spange S, Wagner T, Heinzel T, Krämer OH. Acetylation of non-histone proteins modulates cellular signalling at multiple levels. Int J Biochem Cell Biol 2009;41:185-98.

[29] Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 2006; 5: 769–84.

[30] Finnin MS, Donigian JR, Cohen A, *et al.* Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature 1999; 401:188-93.

[31] Trapp J, Jung M. The role of NAD+ dependent histone deacetylases (sirtuins) in ageing.Curr Drug Targets 2006; 7: 1553-60.

[32] Eot-Houllier G, Fulcrand G, Magnaghi-Jaulin L, Jaulin C. Histone deacetylase inhibitors and genomic instability. Cancer Lett 2009; 274: 169-76.

[33] Lee KK, Workman JL. Histone acetyltransferase complexes: one size doesn't fit all. Nat Rev Mol Cell Biol 2007; 8: 284-95.

[34] Yang XJ, Seto E. Lysine acetylation: codified crosstalk with other posttranslational modifications. Mol Cell 2008; 31: 449-61.

[35] Karagianni P, Wong J. HDAC3: taking the SMRT-N-CoRrect road to repression. Oncogene 2007; 26: 5439-49.

[36] Li J, Wang J, Wang J, *et al.* Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3.EMBO J 2000; 19: 4342-50.

[37] Liu S, Klisovic RB, Vukosavljevic T, *et al.* Targeting AML1/ETO-histone deacetylase repressor complex: a novel mechanism for valproic acid-mediated gene expression and cellular differentiation in AML1/ETO-positive acute myeloid leukemia cells. J Pharmacol Exp Ther 2007; 321: 953-60.

[38] Insinga A, Monestiroli S, Ronzoni S, *et al.* Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat Med 2005; 11: 71-6.

[39] Marks PA, Richon VM, Breslow R, Rifkind RA. Histone deacetylase inhibitors as new cancer drugs. Curr Opin Oncol 2001; 13: 477-83.

[40] Melnick A, Licht JD. Histone deacetylases as therapeutic targets in hematologic malignancies. Curr Opin Hematol 2002; 9: 322-32.

[41] Daly K, Shirazi-Beechey SP. Microarray analysis of butyrate regulated genes in colonic epithelial cells. DNA Cell Biol 2006; 25: 49-62.

[42] Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expr 1996; 5: 245-53.

[43] Nakagawa M, Oda Y, Eguchi T, *et al.* Expression profile of class I histone deacetylases in human cancer tissues. Oncol Rep 2007; 18: 769-74.

[44] Gao L, Cueto MA, Asselbergs F, Atadja P. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J Biol Chem 2002; 277: 25748-55.

[45] Oehme I, Deubzer HE, Wegener D, *et al.* Histone deacetylase 8 in neuroblastoma tumorigenesis. Clin Cancer Res 2009; 15: 91-9.

[46] Huang BH, Laban M, Leung CH-W, *et al.* Inhibition of histone deacetylase 2 increases apoptosis and $p21^{Cip1/WAF1}$ expression, independent of histone deacetylase 1 Cell Death Differentiation. 2005; 12: 395-404.

[47] Eyal S, Yagen B, Shimshoni J, Bialer M. Histone deacetylases inhibition and tumor cells cytotoxicity by CNS-active VPA constitutional isomers and derivatives. Biochem Pharmacol 2005; 69: 1501-08.

[48] Yuan PX, Huang LD, Jiang YM, Gutkind JS, Manji HK, Chen G. The mood stabilizer valproic acid activates mitogen-activated protein kinases and promotes neurite growth. J Biol Chem 2001; 276: 31674-83.

[49] Oh M, Choi IK, Kwon HJ. Inhibition of histone deacetylase1 induces autophagy. Biochem Biophys Res Commun 2008; 369: 1179-83.

[50] Condorelli F, Gnemmi I, Vallario A, Genazzani AA, Canonico PL. Inhibitors of histone deacetylase (HDAC) restore the p53 pathway in neuroblastoma cells. Br J Pharmacol 2008; 153: 657-68.

[51] Tang Y, Zhao W, Chen Y, Zhao Y, Gu W. Acetylation is indispensable for p53 activation. Cell 2008; 133: 612-26.

[52] Ocker M, Schneider-Stock R. Histone deacetylase inhibitors: signalling towards p21cip1/waf1. Int J Biochem Cell Biol 2007; 39: 1367-74.

[53] Zhao Y, Lu S, Wu L, *et al.* Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21(Waf1/Cip1). Mol Cell Biol 2006; 26: 2782-90.

[54] Hirsch CL, Bonham K. Histone deacetylase inhibitors regulate p21WAF1 gene expression at the post-transcriptional level in HepG2 cells. FEBS Lett 2004; 570: 37-40.

[55] Suzuki T, Yokozaki H, Kuniyasu H, *et al.* Effect of trichostatin A on cell growth and expression of cell cycle- and apoptosis-related molecules in human gastric and oral carcinoma cell lines. Int J Cancer 2000; 88: 992-7.

[56] Mahyar-Roemer M, Roemer K. p21Waf1/Cip1 can protect human colon carcinoma cells against p53-dependent and p53- independent apoptosis induced by natural chemopreventive and therapeutic agents. Oncogene 2001; 20: 3387-98.

[57] Richon VM, Sandhoff TW, Rifkind RA, Marks PA. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA 2000; 97: 10014-19.

[58] Asghari V, Wang JF, Reiach JS, Young LT. Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells. Brain Res Mol Brain Res 1998; 58: 95-102.

[59] Leppä S, Eriksson M, Saffrich R, Ansorge W, Bohmann D. Complex functions of AP-1 transcription factors in differentiation and survival of PC12 cells. Mol Cell Biol 2001; 21: 4369-78.

[60] Leppä S, Saffrich R, Ansorge W, Bohmann D. Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. EMBO J 1998; 17: 4404-13.

[61] Chen CS, Weng SC, Tseng PH, Lin HP, Chen CS. Histone acetylation-independent effect of histone deacetylase inhibitors on Akt through the reshuffling of protein phosphatase 1 complexes. J Biol Chem 2005; 280: 38879-87.

[62] Kim B, Leventhal PS, Saltiel AR, Feldman EL. Insulin-like growth factor-I-mediated neurite outgrowth *in vitro* requires mitogen-activated protein kinase activation. J Biol Chem 1997; 272: 21268-73.

[63] Encinas M, Iglesias M, Llecha N, Comella JX. Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. J Neurochem 1999; 73: 1409-21.

[64] Bassa BV, Roh DD, Vaziri ND, Kirschenbaum MA, Kamanna VS. Lysophosphatidylcholine activates mesangial cell PKC and MAP kinase by PLCgamma-1 and tyrosine kinase-Ras pathways. Am J Physiol 1999; 277: F328-37.

[65] Cieslik K, Abrams CS, Wu KK. Upregulation of endothelial nitric-oxide synthase promoter by the phosphatidylinositol 3-kinase gamma/Janus kinase 2/MEK-1-dependent pathway. J Biol Chem 2001; 276: 1211-19.

[66] Mani S, Shen Y, Schaefer J, Meiri KF. Failure to express GAP-43 during neurogenesis affects cell cycle regulation and differentiation of neural precursors and stimulates apoptosis of neurons. Mol Cell Neurosci 2001; 17: 54-66.

[67] Teicher BA, Menon K, Alvarez E, Liu P, Shih C, Faul MM. Antiangiogenic and antitumor effects of a protein kinase C beta inhibitor in human hepatocellular and gastric cancer xenografts. *In vivo* 2001; 15: 185-93.

[68] Teicher BA, Menon K, Alvarez E, Galbreath E, Shih C, Faul M. Antiangiogenic and antitumor effects of a protein kinase Cbeta inhibitor in human T98G glioblastoma multiforme xenografts. Clin Cancer Res 2001; 7: 34-640.

[69] Kermorgant S, Aparicio T, Dessirier V, Lewin MJ, Lehy T. Hepatocyte growth factor induces colonic cancer cell invasiveness via enhanced motility and protease overproduction. Evidence for PI3 kinase and PKC involvement. Carcinogenesis 2001; 22: 1035-42.

[70] Carter CA. Protein kinase C as a drug target: implications for drug or diet prevention and treatment of cancer. Curr Drug Targets 2000; 1: 163-83.

[71] Chen G, Masana MI, Manji HK. Lithium regulates PKC-mediated intracellular cross-talk and gene expression in the CNS *in vivo*. Bipolar Disord 2000; 2: 21736.

[72] Chen G, Manji HK, Hawver DB, Wright CB, Potter WZ. Chronic sodium valproate selectively decreases protein kinase C alpha and epsilon *in vitro*. J Neurochem 1994; 63: 2361-4.

[73] Chen G, Bower KA, Ma C, Fang S, Thiele CJ, Luo J. Glycogen synthase kinase 3beta (GSK3beta) mediates 6-hydroxydopamine-induced neuronal death. FASEB J 2004; 18: 1162-4.

[74] Rowe MK, Wiest C, Chuang DM. GSK-3 is a viable potential target for therapeutic intervention in bipolar disorder. Neurosci Biobehav Rev 2007; 31: 920-31.

[75] Komiya Y, Habas R. Wnt signal transduction pathways. Organogenesis 2008; 4: 68-75.

[76] Logan CY, Nusse R. The Wnt Signaling Pathway in Development and Disease. Annu Rev Cell Dev Biol 2004; 20: 781-810.

[77] Zhu P, Martin E, Mengwasser J, Schlag P, Janssen KP, Göttlicher M. Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. Cancer Cell 2004; 5: 455-63.

[78] Bug G, Gül H, Schwarz K, *et al.* Valproic acid stimulates proliferation and self-renewal of hematopoietic stem cells. Cancer Res 2005; 65: 2537-41.

[79] Bug G, Schwarz K, Schoch C, *et al.* Effect of histone deacetylase inhibitor valproic acid on progenitor cells of acute myeloid leukemia. Haematologica 2007; 92: 542-5.

[80] Huangfu D, Osafune K, Maehr R, *et al.* Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 2008; 26: 1269-75.

[81] Schoenhals M, Kassambara A, De Vos J, Hose D, Moreaux J, Klein B. Embryonic stem cell markers expression in cancers. Biochem Biophys Res Commun 2009; 383: 157-62.

[82] Moon JH, Yoon BS, Kim B, *et al.* Induction of neural stem cell-like cells (NSCLCs) from mouse astrocytes by Bmi1. Biochem Biophys Res Commun 2008; 371: 267-72.

[83] Stockhausen MT, Sjölund J, Manetopoulos C, Axelson H. Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells. Br J Cancer 2005; 92: 751-9.

[84] Hahn CK, Ross KN, Warrington IM, *et al.* Expression-based screening identifies the combination of histone deacetylase inhibitors and retinoids for neuroblastoma differentiation. Proc Natl Acad Sci USA 2008; 105: 9751-6.

[85] Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer 2006; 6: 369-81.

[86] Yang WM, Yao YL, Sun JM, Davie JR, Seto E. Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. J Biol Chem 272; 1997: 28001-7.

[87] Krämer OH, Müller S, Buchwald M, Reichardt S, Heinzel T. Mechanism for ubiquitylation of the leukemia fusion proteins AML1-ETO and PML-RAR alpha. FASEB J 2008; 22: 1369-79.

[88] Cervoni N, Szyf M. Demethylase activity is directed by histone acetylation. J Biol Chem 2001; 276: 40778-87.

[89] Alonso-Aperte E, Ubeda N, Achon M, Perez-Miguelsanz J, Varela-Moreiras G. Impaired methionine synthesis and hypomethylation in rats exposed to valproate during gestation. Neurology 1999; 52: 750-6.

[90] Hattori M, Sakamoto H, Satoh K, Yamamoto T. DNA demethylase is expressed in ovarian cancers and the expression correlates with demethylation of CpG sites in the promoter region of c-erbB-2 and survivin genes. Cancer Lett 2001; 169: 155-64.

[91] Bhattacharya SK, Ramchandani S, Cervoni N, Szyf M. A mammalian protein with specific demethylase activity for mCpG DNA. Nature 1999; 397: 579-83.

[92] Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol 2007; 8: 464-78.

[93] Kerbel RS. Tumor angiogenesis. N Engl J Med 2008; 358: 2039-49.

[94] Kong X, Lin Z, Liang D, Fath D, Sang N, Caro J. Histone deacetylase inhibitors induce VHL and ubiquitin-independent proteasomal degradation of hypoxia-inducible factor 1alpha. Mol Cell Biol 2006; 26: 2019-28.

[95] Kim SH, Jeong JW, Park JA, *et al.* Regulation of the HIF-1alpha stability by histone deacetylases. Oncol Rep 2007; 17: 647-51.

[96] Cinatl J Jr, Kotchetkov R, Blaheta R, Hernaiz-Driever P, Vogel JU, Cinatl J. Induction of differentiation and suppression of malignant phenotype of human neuroblastoma BE(2)-C cells by valproic acid: enhancement by combination with interferon- alpha. Int J Oncol 2002; 20: 97-106.

[97] Isenberg JS, Jia Y, Field L, *et al.* Modulation of angiogenesis by dithiolethione-modified NSAIDs and valproic acid. Br J Pharmacol 2007; 151: 63-72.

[98] Yang Q, Tian Y, Liu S, *et al.* Thrombospondin-1 peptide ABT-510 combined with valproic acid is an effective antiangiogenesis strategy in neuroblastoma. Cancer Res 2007; 67: 1716-24.

[99] Lee PC, Salyapongse AN, Bragdon GA, *et al.* Impaired wound healing and angiogenesis in eNOS-deficient mice. Am J Physiol 1999; 277: H1600-8.

[100] Fukumura D, Gohongi T, Kadambi A, *et al.* Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc Natl Acad Sci USA 2001; 98: 2604-9.

[101] Kawasaki K, Smith RS Jr, Hsieh CM, Sun J, Chao J, Liao JK. Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis. Mol Cell Biol 2003; 23: 5726-37.

[102] Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature (Lond) 1999; 399: 601-5.

[103] Fulton D, Gratton J-P, McCabe TJ, *et al.* Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature (Lond) 1999; 399: 597-601.

[104] Rossig L, Li H, Fisslthaler B, *et al.* Inhibitors of histone deacetylation downregulate the expression of endothelial nitric oxide synthase and compromise endothelial cell function in vasorelaxation and angiogenesis. Circ Res 2002; 91: 837-44.

[105] Nusinzon I, Horvath CM. Interferon-stimulated transcription and innate antiviral immunity require deacetylase activity and histone deacetylase 1. Proc Natl Acad Sci USA 2003; 100: 14742-7.

[106] Silva MF, Aires CC, Luis PB, *et al.* Valproic acid metabolism and its effects on mitochondrial fatty acid oxidation: A review. J Inherit Metab Dis 2008; 31: 205-16.

[107] Gunes A, Bilir E, Zengil H, Babaoglu MO, Bozkurt A, Yasar U. Inhibitory effect of valproic acid on cytochrome P450 2C9 activity in epilepsy patients. Basic Clin Pharmacol Toxicol 2007; 100: 383-6.

[108] Mora-García Mde L, Duenas-González A, Hernández-Montes J, *et al.* Up-regulation of HLA class-I antigen expression and antigen-specific CTL response in cervical cancer cells by the demethylating agent hydralazine and the histone deacetylase inhibitor valproic acid. J Transl Med 2006; 4: 55.

[109] Mahlknecht U,Schonbein C. Histone deacetylase inhibitor treatment downregulates VLA-4 adhesion in hematopoietic stem cells and acute myeloid leukemia blast cells. Haematologica 2008; 93: 443-6.

[110] Feng WH, Kenney SC. Valproic acid enhances the efficacy of chemotherapy in EBV-positive tumors by increasing lytic viral gene expression. Cancer Res 2006; 66: 8762-69.

[111] Knupfer MM, Pulzer F, Schindler I, Hernaiz DP, Knupfer H, Keller E. Different effects of valproic acid on proliferation and migration of malignant glioma cells *in vitro*. Anticancer Res 2001; 21: 347-51.

[112] Armeanu S, Pathil A, Venturelli S, *et al.* Apoptosis on hepatoma cells but not on primary hepatocytes by histone deacetylase inhibitors valproate and ITF2357. J Hepatol 2005; 42: 210-7.

[113] Xiao X, Ning L, Chen H. Notch1 mediates growth suppression of papillary and follicular thyroid cancer cells by histone deacetylase inhibitors. Mol Cancer Ther 2009; 8: 350-6.

[114] Greenblatt DY, Vaccaro AM, Jaskula-Sztul R, *et al.* Valproic acid activates Notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. Oncologist 2007; 12: 942-51.

[115] de la Cruz-Hernández E, Pérez-Cárdenas E, Contreras-Paredes A, *et al.* The effects of DNA methylation and histone deacetylase inhibitors on human papillomavirus early gene expression in cervical cancer, an *in vitro* and clinical study. Virol J 2007; 4: 18.

[116] Shu Q, Antalffy B, Su JM, *et al.* Valproic Acid prolongs survival time of severe combined immunodeficient mice bearing intracerebellar orthotopic medulloblastoma xenografts. Clin Cancer Res 2006; 12: 4687-94.

[117] Chen CL, Sung J, Cohen M, *et al.* Valproic acid inhibits invasiveness in bladder cancer but not in prostate cancer cells. J Pharmacol Exp Ther 2006; 319: 533-42.

[118] Xia Q, Sung J, Chowdhury W, *et al.* Chronic administration of valproic acid inhibits prostate cancer cell growth *in vitro* and *in vivo*. Cancer Res 2006; 66: 7237-44.

[119] Valentini A, Biancolella M, Amati F, *et al.* Valproic acid induces neuroendocrine differentiation and UGT2B7 up-regulation in human prostate carcinoma cell line. Drug Metab Dispos 2007; 35: 968-72.

[120] Takai N, Desmond JC, Kumagai T, *et al.* Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. Clin Cancer Res 2004; 10: 1141-9.

[121] Liu S, Klisovic RB, Vukosavljevic T, *et al.* Targeting AML1/ETO-histone deacetylase repressor complex: a novel mechanism for valproic acid-mediated gene expression and cellular differentiation in AML1/ETO-positive acute myeloid leukemia cells. J Pharmacol Exp Ther 2007; 321: 953-60.

[122] Bokelmann I, Mahlknecht U. Valproic acid sensitizes chronic lymphocytic leukemia cells to a poptosis and restores the balance between pro- and antiapoptotic proteins. Mol Med 2008; 14: 20-7.

[123] Kitazoe K, Abe M, Hiasa M, *et al.* Valproic acid exerts anti-tumor as well as anti-angiogenic effects on myeloma. Int J Hematol 2009; 89: 45-57.

[124] Neri P, Tagliaferri P, Di Martino MT, *et al. In vivo* anti-myeloma activity and modulation of gene expression profile induced by valproic acid, a histone deacetylase inhibitor. Br J Haematol 2008; 143: 520-31.

[125] Jones J, Juengel E, Mickuckyte A, *et al.* Valproic acid blocks adhesion of renal cell carcinoma cells to endothelium and extracellular matrix. J Cell Mol Med 2008. [Epub ahead of print] PMID: 19067765.

[126] Juengel E, Engler J, Mickuckyte A, *et al.* Effects of combined valproic acid and the epidermal growth factor/vascular endothelial growth factor receptor tyrosine kinase inhibitor AEE788 on renal cell carcinoma cell lines *in vitro*. BJU Int 2009. [Epub ahead of print] PMID: 19594733.

[127] Cinatl J Jr, Cinatl J, Scholz M, *et al.* Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. Anticancer Drugs 1996; 7: 766-73.

[128] Angelucci A, Valentini A, Millimaggi D, *et al.* Valproic acid induces apoptosis in prostate carcinoma cell lines by activation of multiple death pathway. Anticancer Drugs 2006; 17: 1141-50.

[129] Catalano MG, Fortunati N, Pugliese M, *et al.* Valproic acid, a histone deacetylase inhibitor, enhances sensitivity to doxorubicin in anaplastic thyroid cancer cells. J Endocrinol 2006; 191: 465-72.

[130] Marchion DC, Bicaku E, Daud AI, Sullivan DM, Munster PN. Valproic acid alters chromatin structure by regulation of chromatin modulation proteins. Cancer Res 2005; 65: 3815-22.

[131] Das CM, Aguilera D, Vasquez H, *et al.* Valproic acid induces p21 and topoisomerase-II (alpha/beta) expression and synergistically enhances etoposide cytotoxicity in human glioblastoma cell lines. J Neurooncol 2007; 85: 159-70.

[132] Ziauddin MF, Yeow WS, Maxhimer JB, *et al.* Valproic acid, an antiepileptic drug with histone deacetylase inhibitory activity, potentiates the cytotoxic effect of Apo2L/TRAIL on cultured thoracic cancer cells through mitochondria-dependent caspase activation. Neoplasia 2006; 8: 446-57.

[133] Catalano MG, Poli R, Pugliese M, Fortunati N, Boccuzzi G. Valproic acid enhances tubulin acetylation and apoptotic activity of paclitaxel on anaplastic thyroid cancer cell lines. Endocr Relat Cancer 2007; 14: 839-45.

[134] Kramer OH, Knauer SK, Zimmermann D, Stauber RH, Heinzel T. Histone deacetylase inhibitors and hydroxyurea modulate the cell cycle and cooperatively induce apoptosis. Oncogene 2008; 27: 732-40.

[135] Yeow WS, Ziauddin MF, Maxhimer JB, *et al.* Potentiation of the anticancer effect of valproic acid, an antiepileptic agent with histone deacetylase inhibitory activity, by the kinase inhibitor Staurosporine or its clinically relevant analogue UCN-01. Br J Cancer 2006; 94: 1436-45.

[136] Yang H, Hoshino K, Sanchez-Gonzalez B, Kantarjian H,Garcia-Manero G. Antileukemia activity of the combination of 5-aza-2-deoxycytidine with valproic acid. Leukemia Res 2005; 29: 739-48.

[137] Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F. Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 2003; 63: 7291-300.

[138] Ecke I, Petry F, Rosenberger A, *et al.* Antitumor effects of a combined 5-Aza-2´Deoxycytidine and valproic acid treatment on rhabdomyosarcoma and medulloblastoma in Ptch mutant mice. Cancer Res 2009; 69: 887-95.

[139] Michaelis M, Suhan T, Cinatl J, Driever PH, Cinatl J Jr. Valproic acid and interferon-alpha synergistically inhibit neuroblastoma cell growth *in vitro* and *in vivo*. Int J Oncol 2004; 25: 1795-9.

[140] Kircher B, Schumacher P, Petzer A, *et al.* Anti-leukemic activity of valproic acid and imatinib mesylate on human Ph+ ALL and CML cells *in vitro*. Eur J Haematol 2009; 83: 48-56.

[141] Karagiannis TC, Kn H, El-Osta A. The epigenetic modifier, valproic acid, enhances radiation sensitivity. Epigenetics 2006; 1: 131-7.

[142] Camphausen K, Cerna D, Scott T, *et al.* Enhancement of *in vitro* and *in vivo* tumor cell radiosensitivity by valproic acid. Int J Cancer 2005; 114: 380-6.

[143] Rezacova M, Zaskodova D, Vavrova J, Vokurkova D, Tichy A. Antileukemic activity of the combination of ionizing radiation with valproic acid in promyelocytic leukemia cells HL-60. Neoplasma 2008; 55: 519-25.

[144] Sharma S, Symanowski J, Wong B, Dino P, Manno P, Vogelzang N. A Phase II clinical trial of oral valproic acid in patients with castration-resistant prostate cancers using an intensive biomarker sampling strategy. Transl Oncol 2008; 1: 141-7.

[145] Noguchi H, Yamashita H, Murakami T, *et al.* Successful treatment of anaplastic thyroid carcinoma with a combination of oral valproic acid, chemotherapy, radiation and surgery. Endocr J 2009; 56: 245-9.

[146] Candelaria M, Gallardo-Rincón D, Arce C, *et al.* A phase II study of epigenetic therapy with hydralazine and magnesium valproate to overcome chemotherapy resistance in refractory solid tumors. Ann Oncol 2007; 18: 1529-38.

[147] Munster P, Marchion D, Bicaku E, *et al.* Phase I trial of histone deacetylase inhibition by valproic acid followed by the topoisomerase II inhibitor epirubicin in advanced solid tumors: a clinical and translational study. J Clin Oncol 2007; 25: 1979-85.

[148] Kuendgen A, Gattermann N. Valproic acid for the treatment of myeloid malignancies. Cancer 2007; 110: 943-54.

[149] Kuendgen A, Strupp C, Aivado M, *et al.* Treatment of myelodysplastic syndromes with valproic acid alone or in combination with all-trans retinoic acid. Blood 2004; 104: 1266-9.

[150] Kuendgen A, Schmid M, Schlenk R, *et al.* The histone deacetylase (HDAC) inhibitor valproic acid as monotherapy or in combination with all-trans retinoic acid in patients with acute myeloid leukemia. Cancer 2006; 106: 112-9.

[151] Bug G, Ritter M, Wassmann B, *et al.* Clinical trial of valproic acid and all-trans retinoic acid in patients with poor-risk acute myeloid leukemia. Cancer 2005; 104: 2717-25.

[152] Cimino G, Lo-Coco F, Fenu S, *et al.* Sequential valproic acid/ alltrans retinoic acid treatment reprograms differentiation in refractory and high-risk acute myeloid leukemia. Cancer Res 2006; 66: 8903-11.

[153] Pilatrino C, Cilloni D, Messa E, *et al.* Increase in platelet count in older, poor-risk patients with acute myeloid leukemia or myelodysplastic syndrome treated with valproic acid and all-trans retinoic acid. Cancer 2005; 104: 101-9.

[154] Raffoux E, Chaibi P, Dombret H, Degos L. Valproic acid and all-trans retinoic acid for the treatment of elderly patients with acute myeloid leukemia. Haematologica 2005; 90: 986-8.

[155] Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, *et al.* Phase 1/2 study of the combination of 5-aza-20-deoxycytidine with valproic acid in patients with leukemia. Blood 2006; 108: 3271-9.

[156] Soriano AO, Yang H, Faderl S, *et al.* Safety and clinical activity of the combination of 5-azacytidine, valproic acid, and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome. Blood 2007; 110: 2302-8.

[157] Nachtkamp K, Kündgen A, Strupp C, *et al.* Impact on survival of different treatments for myelodysplastic syndromes (MDS). Leuk Res 2009; 33: 1024-8.

[158] Rocca A, Minucci S, Tosti G, *et al.* A phase I-II study of the histone deacetylase inhibitor valproic acid plus chemoimmunotherapy in patients with advanced melanoma. Br J Cancer 2009; 100: 28-36.

[159] Fantin VR, Richon VM. Mechanisms of resistance to histone deacetylase inhibitors and their therapeutic implications. Clin Cancer Res 2007; 13: 7237-42.

[160] Morrow CS, Nakagawa M, Goldsmith ME, Madden MJ, Cowan KH. Reversible transcriptional activation of mdr1 by sodium butyrate treatment of human colon cancer cells. J Biol Chem 1994; 269: 10739-46.

[161] Bandyopadhyay D, Mishra A, Medrano EE. Overexpression of histone deacetylase 1 confers resistance to sodium butyrate-mediated apoptosis in melanoma cells through a p53-mediated pathway. Cancer Res 2004; 64: 7706-10.

[162] Fantin VR, Richon VM. Mechanisms of resistance to histone deacetylase inhibitors and their therapeutic implications. Clin Cancer Res 2007; 13: 7237-42.

[163] Butler LM, Zhou X, Xu WS, *et al.* The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. Proc Natl Acad Sci USA 2002; 99: 11700-5.

[164] Subramanian C, Opipari AW Jr, Bian X, Castle VP, Kwok RP. Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors. Proc Natl Acad Sci USA 2005; 102: 4842-7.

[165] Subramanian C, Jarzembowski JA, Opipari AW Jr, Castle VP, Kwok RP. CREB-binding protein is a mediator of neuroblastoma cell death induced by the histone deacetylase inhibitor trichostatin A. Neoplasia 2007; 9: 495-503.

[166] Mayo MW, Denlinger CE, Broad RM, *et al.* Ineffectiveness of histone deacetylase inhibitors to induce apoptosis involves the transcriptional activation of NF-kappa B through the Akt pathway. J Biol Chem 2003; 278: 18980-9.

[167] Vainer GW, Pikarsky E, Ben-Neriah Y. Contradictory functions of NF-kappaB in liver physiology and cancer. Cancer Lett 2008; 267: 182-8.

[168] Morgan MA, Reuter CWM. Molecularly targeted therapies in myelodysplastic syndromes and acute myeloid leukemia. Ann Hematol 2006; 85: 139-63.

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ORIGINAL ARTICLE

Anticancer agent ellipticine combined with histone deacetylase inhibitors, valproic acid and trichostatin A, is an effective DNA damage strategy in human neuroblastoma

Jitka Poljakova [1], Jana Hrebackova [2], Marketa Dvorakova [1], Michaela Moserova [1], Tomas Eckschlager [2], Jan Hrabeta [2], Marketa Göttlicherova [1], Barbora Kopejtkova [1], Eva Frei [3], Rene Kizek [4], Marie Stiborova [1]

1 Department of Biochemistry, Faculty of Science, Charles University, Prague, Czech Republic
2 Department of Pediatric Hematology and Oncology, Charles University and University Hospital Motol, Prague, Czech Republic
3 Division of Preventive Oncology, National Center for Tumour Diseases, German Cancer Research Center, Heidelberg, Germany
4 Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University, Brno, Czech Republic

Correspondence to: Prof. RNDr. Marie Stiborova, DSc.
Department of Biochemistry, Faculty of Science, Charles University
Albertov 2030, 128 40 Prague 2, Czech Republic.
TEL: +420-221 951; FAX: +420-221 951 283; E-MAIL: stiborov@natur.cuni.cz

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Abstract

OBJECTIVES: Valproic acid (VPA) and trichostatin A (TSA) exert antitumor activity as histone deacetylase inhibitors, whereas ellipticine action is based mainly on DNA intercalation, inhibition of topoisomerase II and formation of cytochrome P450 (CYP)- and peroxidase-mediated covalent DNA adducts. This is the first report on the molecular mechanism of combined treatment of human neuroblastoma UKF-NB-3 and UKF-NB-4 cells with these compounds.
METHODS: HPLC with UV detection was employed for the separation and characterization of ellipticine metabolites formed by microsomes and peroxidases. Covalent DNA modifications by ellipticine in neuroblastoma cells and in incubations with microsomes and peroxidases were detected by ^{32}P-postlabeling. Expression of CYP enzymes, peroxidases and cytochrome b_5 was examined by Western blot.
RESULTS: The cytotoxicity of ellipticine to neuroblastomas was increased by pretreating these cells with VPA or TSA. A higher sensitivity of cells to ellipticine correlated with an increase in formation of covalent ellipticine-derived DNA adducts in these cells. To evaluate the mechanisms of this finding, we investigated the modulation by VPA and TSA of CYP- and peroxidase-mediated ellipticine-derived DNA adduct formation *in vitro*. The effects of ellipticine in the presence of VPA and TSA on expression of CYPs and peroxidases relevant for ellipticine activation and levels of cytochrome b_5 and P-glycoprotein in neuroblastoma cells were also investigated. Based on these studies, we attribute most of the enhancing effects of VPA and TSA on ellipticine cytotoxicity to enhanced ellipticine-DNA adduct formation caused by an increase in levels of cytochrome b_5, CYP3A4 and

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CYP1A1 in neuroblastoma cells. A lower sensitivity of UKF-NB-4 cells to combined effects of ellipticine with VPA and TSA than of UKF-NB-3 cells is also attributable to high levels of P-glycoprotein expressed in this cell line.

CONCLUSION: The results found here warrant further studies and may help in the design of new protocols geared to the treatment of high risk neuroblastomas.

Abbreviations:

AhR	- aryl hydrocarbon receptor
COX	- cyclooxygenase
CYP	- cytochrome P450
DMSO	- dimethyl sulfoxide
GAPDH	- glyceraldehyde phosphate dehydrogenase
HDAC	- histone deacetylase
HPLC	- high-performance liquid chromatography
IMDM	- Iscove's modified Dulbecco's medium
LPO	- lactoperoxidase
MPO	- myeloperoxidase
MTT	- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide
NADP+	- nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
PBS	- phosphate buffered saline
PEI-cellulose	- polyethylenimine-cellulose
RAL	- relative adduct labeling
r.t.	- retention time
SDS	- sodium dodecyl sulphate
TLC	- thin layer chromatography
TSA	- trichostatin A
VPA	- valproic acid

INTRODUCTION

Neuroblastoma, a tumor of the peripheral sympathetic nervous system, is the most frequent solid extra cranial neurological tumor in children and is a major cause of death from neoplasia in infancy (Maris & Mathay 1999; Schwab 1999). These tumors are biologically heterogeneous, with cell populations differing in their genetic programs, maturation stage and malignant potential (Brodeur 2003). Neuroblastoma consists of three principal neoplastic cells (Voigt *et al.* 2000; Hopkins-Donaldson *et al.* 2002): i) neuroblastic or N-type: undifferentiated, round and small cells with scant cytoplasm and neuritic processes; ii) stromal or S-type: large, flattened and adherent differentiated cells; and iii) intermediate or I-type with morphological features of both above mentioned types, i.e. cells with short neurite-like processes such as adherent growth. As neuroblastoma cells seem to have the capacity to differentiate spontaneously *in vivo* and *in vitro* (Morgenstern *et al.* 2004), their heterogeneity could affect treatment outcome, in particular the response to apoptosis induced by chemotherapy. Neuroblastoma may regress spontaneously in infants, mature to benign ganglioneuromas, or grow relentlessly and be rapidly fatal (Brodeur 2003). Prognosis of patients with high risk tumors is poor, in spite of intensive therapy including megatherapy with subsequent hematopoietic progenitor cell transplantation, biotherapy and immunotherapy because drug resistance arises in the majority of those patients who initially responded to chemotherapy (Brodeur 2003). Little improvement in therapeutic options has been made in the last decade, requiring a need for the development of new therapies.

Recently, we have suggested novel treatment of neuroblastomas, utilizing a drug targeting DNA, the plant alkaloid ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*] carbazole, Figure 1). We have found that exposure of human neuroblastoma IMR-32, UKF-NB-3 and UKF-NB-4 cell lines to this agent resulted in strong inhibition of cell growth, followed by induction of apoptosis (Poljakova *et al.* 2009). These effects were associated with formation of two covalent ellipticine-derived DNA adducts, identical to those formed by the cytochrome P450 (CYP)- and peroxidase-mediated ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine (Figure 1). In addition, other mechanisms, such as intercalation into DNA (Singh *et al.* 1994; Auclair 1987), and inhibition of DNA topoisomerase II activity (Auclair 1987; Monnot *et al.* 1991; Fossé *et al.* 1992; Froelich-Ammon *et al.* 1995) resulting in ellipticine toxicity to neuroblastoma cannot be excluded (for a summary see Stiborova *et al.* 2001; 2006c; 2011). The levels of covalent ellipticine-derived DNA adducts correlated with ellipticine toxicity in IMR-32 and UKF-NB-4 cell lines. In addition, hypoxic cell culture conditions resulted in a decrease in ellipticine toxicity to these cells and this correlated with lower levels of DNA adducts. Cells of both lines accumulated in S phase, suggesting that ellipticine-DNA adducts interfere with DNA replication. We therefore concluded that formation of ellipticine-DNA adducts was the predominant DNA-damaging mechanism of ellipticine action, resulting in its strong cytotoxicity to these neuroblastoma cells (Poljakova *et al.* 2009).

Because epigenetic modifications of chromatin play a role in the origin of human neuroblastomas, pharmaceutical manipulation of the epigenome may offer treatment options for this type of cancer (Furchert *et al.* 2007). Histone deacetylases (HDAC) and histone acetyl transferases modify histone proteins and contribute to an epigenetic code recognized by proteins involved in regulation of gene expression (Blaheta & Cinatl 2002; Blaheta *et al.* 2002; Marks *et al.* 2003; 2004; Hooven *et al.* 2005; Hrebackova *et al.* 2010). Indeed, former studies demonstrated the cytotoxicity of HDAC inhibitors to several neuroblastoma cells, resulting in growth inhibition of these tumor cells (Cinatl *et al.* 1996; Furchert *et al.* 2007; Michaelis *et al.* 2004; 2007). In neoplastic cells, where overexpression of different HDACs was frequently detected (for summary see Bolden *et al.* 2006; Hrebackova *et al.* 2010), the abundance of deacetylated histones is usually associated with DNA hypermethylation and gene silencing (Santini *et al.* 2007). Treatment with HDAC inhibitors induced the reactivation

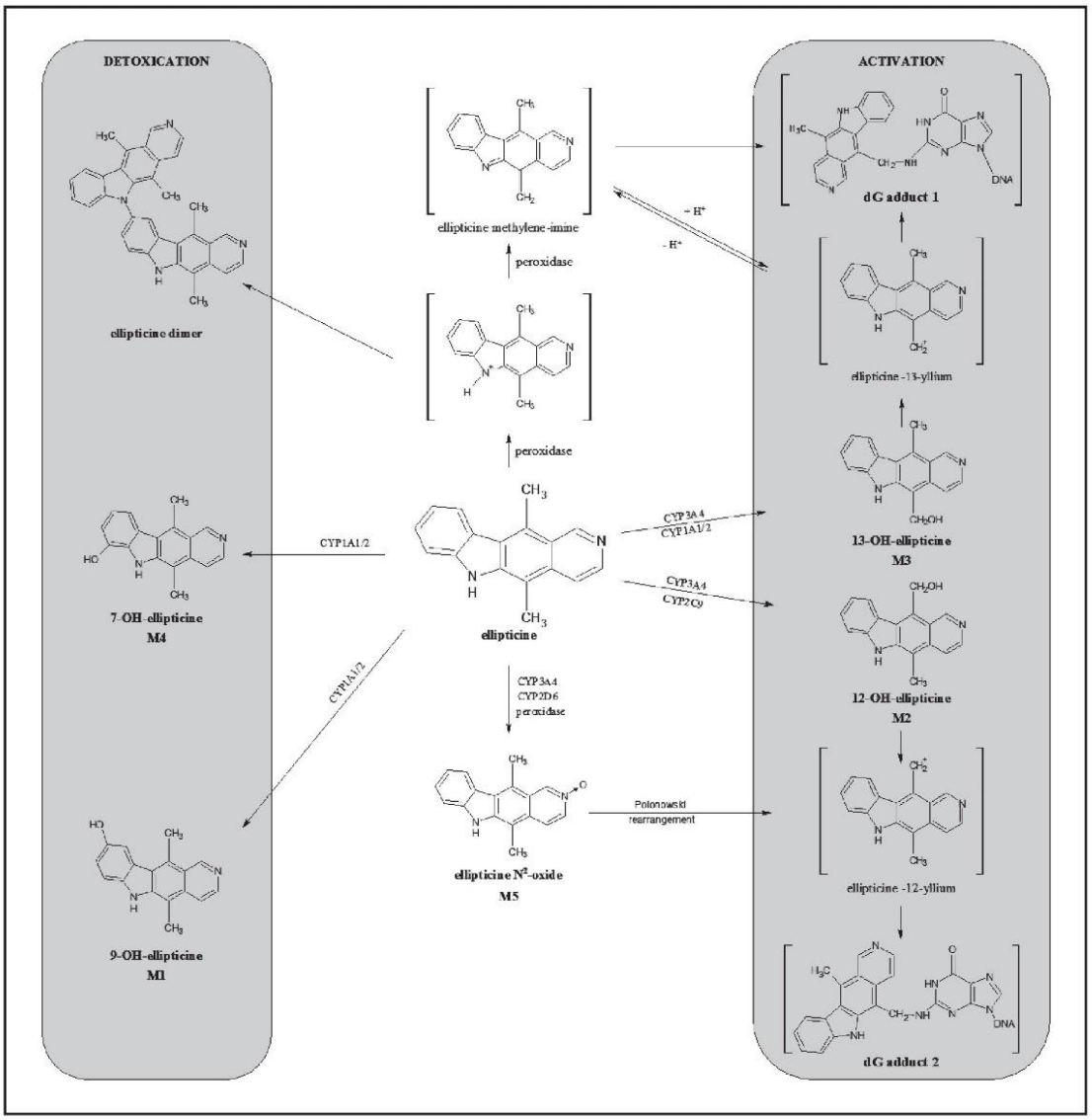

Fig. 1. Scheme of the metabolism of ellipticine by human CYPs and peroxidases showing the characterized detoxication metabolites and those proposed to form DNA adducts. The compounds shown in brackets are the hypothetical electrophilic metabolites postulated as ultimate arylating species or the postulated N^2-deoxyguanosine adducts.

of growth regulatory genes and consequently apoptosis in these cells. The HDAC inhibitor valproic acid (VPA) inhibits growth and induces differentiation of UKF-NB-2 and UKF-NB-3 human neuroblastoma cells *in vitro* at concentrations ranging from 0.5 to 2 mM, plasma concentrations that have been achieved in humans with no significant adverse effects. Nevertheless, it should be noted that common therapeutic concentrations of VPA are lower (Cinatl *et al.* 1996; Hrebackova *et al.* 2009).

Ultrastructural features of VPA-treated cells were consistent with the neuronal differentiation and are associated with decreased expression of N-myc oncoprotein and increased expression of neutral cell adhesion molecule in their membrane. In these cells VPA synergized interferon-alpha treatment leading to a massive accumulation of cells in G0/G1-phase. A synergism which was confirmed *in vivo*, where VPA plus interferon-alpha inhibited growth of UKF-NB-3 xenografts in nude mice and leading to complete cures in two out

of six animals, while single treatments merely inhibited tumor growth (Michaelis *et al.* 2004). Moreover, VPA combined with ABT-510, a peptide derivative of the natural angiogenic inhibitor thrombospondin-1, was even more efficient to inhibit neuroblastoma tumor growth and angiogenesis, suggesting that this combination may be an effective antiangiogenic treatment strategy for children with high-risk neuroblastoma (Yang *et al.* 2007). VPA also influences the resistance of neuroblastoma cells to several chemotherapeutic agents (Blaheta *et al.* 2007). This drug reverts the enhanced adhesion of drug-resistant UKF-NB-2, UKF-NB-6 and SK-N-SH neuroblastoma cells accompanied by diminished N-myc and enhanced p73 protein levels (Blaheta *et al.* 2007).

Besides VPA, another HDAC inhibitor, trichostatin A (TSA), was found to inhibit growth of neuroblastoma cells, at concentration as low as 50–250 nM (Hrebackova *et al.* 2009). This HDAC inhibitor in combination with anticancer drugs that act on DNA such as vepesid, ellipticine, doxorubicin, epirubicin, and cis-platin was recently found to enhance the efficacy of these in several tumor cells (Kim *et al.* 2003). The mechanisms are, however, unknown as well as the activity against neuroblastomas in combination with DNA damaging agents.

In this study we describe that pre-treatment of human neuroblastoma cells with the HDAC inhibitors, VPA or TSA, increases the toxicity of ellipticine to these human neuroblastoma cells and that this higher sensitivity to ellipticine correlates with an increase in formation of ellipticine-derived DNA adducts. In addition, we examined the molecular mechanisms of such effects in these neuroblastoma cells. Because neuroblastoma is heterogenous and this feature could affect its treatment, two types of neuroblastoma cell lines were tested for their response to combined treatment by ellipticine with VPA or TSA, UKF-NB-3 cells (the invasive N-type), and UKF-NB-4 cells (the less-aggressive S-type).

MATERIALS AND METHODS

Chemicals

Ellipticine, $NADP^+$ and NADPH were from Sigma Chemical Co. (St. Louis, MO, USA). Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described (Stiborova *et al.* 2001; 2003a; 2003b; 2004). 12-Hydroxy- and 13-hydroxyellipticine were isolated from multiple HPLC runs of ethyl acetate extracts of incubations containing ellipticine and human and/or rat hepatic microsomes as described (Stiborova *et al.* 2004). The human hepatic microsomal sample was a pooled sample obtained from Gentest corp. (Woburn, MA, USA) (catalog no. H161). Rat hepatic microsomes (0.6 nmol CYP/mg protein) were isolated as described (Stiborova *et al.* 2001; 2006a). The experiments were conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which complies with the Declaration of Helsinki. Bovine lactoperoxidase (LPO; 117 purporogallin units/mg protein, 13 guaiacol units/mg protein), human myeloperoxidase (MPO; 105 purporogallin units/mg protein, 11 guaiacol units/mg protein), and ovine cyclooxygenase 1 (prostaglandin H synthase 1, COX-1; 44371 oxygen consumption units/mg protein, one units consumes one nmole of O_2/min in the presence of 100 µM arachidonic acid, 2 mM phenol and 1 µM hematin) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All these and other chemicals used in the experiments were of analytical purity or better.

Cell cultures

The UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines, established from bone marrow metastases of high risk neuroblastoma, were a gift of Prof. J. Cinatl, Jr. (J. W. Goethe University, Frankfurt, Germany). UKF-NB-4 was derived from recurrent disease. Both two cell lines used were derived from high risk neuroblastoma with MYCN amplification, del1p and aneuploidy. Cells were grown at 37 °C and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) (Lonza Inc, Allndale, NJ, USA), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicilline and 100 µg/ml streptomycine (PAA Laboratories, Pasching, Austria).

MTT assay

The cytotoxicity of ellipticine to cells in exponential growth was determined in a 96-well plate. For a dose-response curve, cells in exponential growth were seeded in 100 µl of medium with 10^4 cells per well. Solution of ellipticine in dimethyl sulfoxide (DMSO) (1 µl) in final concentrations of 0.02–50 µM was added. To investigate the effects of VPA and TSA on ellipticine-cytotoxicity, neuroblastoma cells were pre-treated with 0.5–2.0 mM VPA (dissolved in IMDM) or 0.1–0.2 µM TSA (dissolved in DMSO) 24 h before adding ellipticine. Control cells and medium controls without cells received 1 µl of DMSO without drug. Tumor cell viability was evaluated by MTT test as previously described (Cinatl *et al.* 1997). Briefly, after incubation (3 days) at 37 °C in 5% CO_2 the MTT solution (2 mg/ml PBS) was added, the plates were incubated for 4 hours and cells lysed in solution containing 20% of SDS and 50% N,N-dimethylformamide pH 4.5. The absorbance at 570 nm was measured for each well by multiwell ELISA reader Versamax (Molecular Devices, CA, USA). The mean absorbance of medium controls was the background and was subtracted. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control. Each value is the mean of 8 wells with standard deviations. The IC_{50} values were calculated from at least 3 independent experiments using the linear regression of the dose-log response curves by SOFTmaxPro.

Microsomal incubations

Incubation mixtures used to analyse DNA adduct formation by ellipticine *in vitro* consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, human or rat hepatic microsomes (0.5 mg protein), 0.1 mM ellipticine (dissolved in 7.5 µl methanol) and 0.5 mg of calf thymus DNA in a final volume of 750 µl. To investigate the effect of VPA and TSA on ellipticine-derived DNA adduct formation, 0.1 or 1.0 mM VPA (dissolved in distilled water) or 0.1 µM TSA (dissolved in 5 µl DMSO) were added into the incubation mixtures. Incubations were carried out at 37 °C for 30 minutes; ellipticine-DNA adduct formation was found to be linear up to 30 min of incubation (Stiborova *et al.* 2006a; 2010). Control incubations were carried out (*i*) without microsomes, (*ii*) without NADPH, (*iii*) without DNA, (*iv*) without ellipticine, and (*v*) without VPA and TSA. After the incubation, DNA was isolated by a standard phenol-chloroform extraction method and analyzed for DNA adduct formation by ^{32}P-postlabeling (see below).

Incubation mixtures used to form the ellipticine metabolites contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase (NADPH-generation system), 0.2 mg protein of rat hepatic microsomes and 10 µM ellipticine (dissolved in 5 µl methanol) in a final volume of 500 µl. The reaction was initiated by adding the substrate. To investigate the effect of VPA and TSA on ellipticine oxidation, 0.1, 0.5 or 1.0 mM VPA (dissolved in distilled water) or 50 and 100 nM TSA (dissolved in 5 µl DMSO) were added into the mixtures. In the control incubation, ellipticine was omitted from the incubation mixture. After incubation in open glass tubes (37 °C, 20 min) the reaction was stopped by adding 100 µl of 2 M NaOH. The oxidation of ellipticine is linear up to 30 min of incubation (Stiborova *et al.* 2006a). After incubation, 5 µl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethyl acetate (2 × 1 ml). Analyses of ellipticine metabolites were performed by HPLC as described (Stiborova *et al.* 2004; 2006a). Recoveries of ellipticine metabolites were around 95%.

Peroxidase incubations

Incubation mixtures used to quantify DNA adducts by ellipticine activated with peroxidases (LPO, MPO and COX-1) contained 50 mM potassium phosphate buffer (pH 7.4), 100 µM ellipticine dissolved in 7.5 µl methanol, 10 µg of peroxidase and 1 mg of calf thymus DNA in a final volume of 750 µl. In incubations with LPO and MPO, 100 M hydrogen peroxide was added as a cofactor. In incubations with COX-1, 100 µM arachidonic acid was added as a cofactor, in addition to hematin (1 µM) and $MgCl_2$ (5 mM). To investigate the effects of VPA and TSA on ellipticine-derived DNA adduct formation, 0.1 and 1.0 mM VPA (dissolved in distilled water) or 100 nM TSA (dissolved in 5 µl DMSO) were added into the incubation mixtures. Control incubations were without peroxidases, without hydrogen peroxide or without arachidonic acid, without DNA, without ellipticine or without VPA or TSA. Incubations with peroxidases were carried out at 37 °C for 30 min. All reactions were initiated by adding ellipticine. After the incubation and ethyl acetate extraction, DNA was isolated by a standard phenol-chloroform extraction method and analyzed for DNA adduct formation by ^{32}P-postlabeling (see below).

Incubation mixtures used to analyze ellipticine metabolites generated by peroxidase (LPO was used as a model) with a final volume of 500 µl consisted of 50 mM potassium phosphate buffer (pH 7.4), 10 µM ellipticine (dissolved in 5 µl methanol), 10 µg of LPO and hydrogen peroxide (20 µM). To investigate the effects of VPA and TSA on ellipticine oxidation by LPO, 0.1, 0.5 and 1.0 mM VPA (dissolved in distilled water) or 50 and 100 nM TSA (dissolved in 5 µl DMSO) were added into the incubation mixtures. Control incubations were without peroxidases, without hydrogen peroxide, without ellipticine, or without HDAC inhibitors. Incubations were carried out at 37 °C for 30 min. All reactions were initiated by adding ellipticine dissolved in methanol (final concentration of methanol was 1%). After incubations, 5 µl of 1 mM salicylamide in methanol was added as an internal standard, ellipticine and its metabolite extracted twice with ethyl acetate (2 × 1 ml) as described (Poljakova *et al.* 2005; Stiborova *et al.* 2007a). The extracts were evaporated under nitrogen and dissolved in 50 µl of methanol. Ellipticine and its metabolite were separated by HPLC. The column used was a 5 µm Ultrasphere ODS (Beckman, 4.6 × 250 mm) preceeded by a C-18 guard column. The eluent was 45–90% methanol in 10 mM ammonium acetate (pH 2.8), with flow rate of 0.8 ml/min, detection was at 296 nm (Poljakova *et al.* 2005; Stiborova *et al.* 2007a). Only one product peak with a retention time (r.t.) of 19.95 min corresponding to the ellipticine dimer (Stiborova *et al.* 2007a) and unconverted ellipticine with r.t. of 11.85 min were separated by HPLC. Recovery of the ellipticine dimer was around 95%.

Estimation of contents of CYPs, peroxidases and cytochrome b_5 in neuroblastoma cells

To determine the expression of cytochrome b_5, CYP1A1, 1B1 and 3A4, LPO and COX-1 proteins, cells were homogenized in 25 mM Tris-HCl buffer pH 7.6 containing 150 mM NaCl, 1% detergent NP-40 (Sigma, St. Louis, MO, USA), 1% sodium deoxycholate, 0.1% SDS and with solution of COMPLETE (protease inhibitor cocktail tablet, Roche, Basel, Swizerland) at concentration described by provider. The homogenates were centrifuged for 20 min at 14 000 g and supernatant was used for additional analysis. Protein concentrations were assessed using the DC protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard, and 10–45 µg of extracted proteins were sub-

jected to SDS-PAGE electrophoresis on a 11% gel for analysis of CYP1A1, 1B1 and 3A4, LPO and COX-1 protein expression, and a 17% gel for analysis of cytochrome b_5 protein expression (Stiborova *et al.* 2002; 2005). After migration, proteins were transferred to a nitrocellulose membrane and incubated with 5% non-fat milk to block non-specific binding. The membranes were then exposed to specific rabbit polyclonal anti-cytochrome b_5 (1:750, AbCam, MA, USA), anti-CYP1A1 (1:1000, Millipore, MA, USA), anti-CYP1B1 (1:500, AbCam, MA, USA), anti-CYP3A4 (1:5000, AbD Serotec, Oxford, UK), anti-LPO (2 µg/ml, AbCam, MA, USA) and anti-COX-1 (1:1000, AbCam, MA, USA) antibodies overnight at 4 °C. Membranes were washed and exposed to peroxidase-conjugated anti-IgG secondary antibody (1:3 000, Bio-Rad, Hercules, CA, USA), and the antigen-antibody complex was visualized by enhanced chemiluminiscence's detection system according to the manufacturer's instructions (Immun-Star HRP Substrate, Bio-Rad, Hercules, CA, USA). X-Rays films were from MEDIX XBU (Foma, Hradec Kralové, Czech Republic). Antibody against glyceraldehyde phosphate dehydrogenase (GAPDH) (1:750, Millipore, MA, USA) was used as loading control.

Treatment of neuroblastoma cells with ellipticine for DNA adduct analyses

Neuroblastoma cell lines were seeded 24 h prior to treatment at a density of 5×10^5 cells/ml in two 75 cm^2 culture flasks in a total volume of 20 ml of IMDM. Ellipticine was dissolved in 5 µl of DMSO, the final concentration was 0, 1 or 10 µM. After 48 h the cells were harvested after trypsinizing by centrifugation at 2 000 × g for 3 min and two washing steps with 5 ml of PBS yielded a cell pellet, which was stored at –80 °C until DNA isolation. DNA was isolated and labeled as described in the next section.

An analogous procedure was used to evaluate the effect of pre-treatment of neuroblastoma cells with VPA or TSA prior to adding ellipticine. In this case, cells were pre-treated with 0.5–2 mM VPA (VPA was dissolved in IMDM) or 0.1 µM TSA (dissolved in 5 µl DMSO) for 24 h before adding ellipticine. Further procedures were the same as described above.

DNA isolation and ^{32}P-postlabeling of DNA adducts

DNA from cells was isolated by the phenol-chloroform extraction as described (Frei *et al.* 2002; Borek-Dohalska *et al.* 2004; Poljakova *et al.* 2007; 2009). The ^{32}P-postlabeling of nucleotides using nuclease P1 enrichment procedure, found previously to be appropriate to detect and quantify ellipticine-derived DNA adducts formed *in vitro* (Stiborova *et al.* 2001; 2004; 2007a; Frei *et al.* 2002; Martinkova *et al.* 2009; 2010) and *in vivo* (Stiborova *et al.* 2003a; 2007b; 2008; 2011). From experiments performed earlier, calf thymus DNA incubated with 13-hydroxy- and 12-hydroxyellipticine (Stiborova *et al.* 2004; 2007a) and DNA of breast adenocarcinoma of rats treated i.p. with 4 mg ellipticine per kilogram body weight (Stiborova *et al.* 2011) were labeled with ^{32}P to compare adduct spot patterns.

Animal experiments

Female Wistar rats bearing the *N*-methyl-*N*-nitrosourea induced mammary adenocarcinoma (McCormick *et al.* 1981) were i.p. treated with 4 mg ellipticine per kilogram body weight. Ellipticine was administered dissolved in 1% acetic acid at a concentration of 2.5 mg/ml as described previously (Stiborova *et al.* 2008). One day after ellipticine treatment, the DNA from tumor tissues was isolated and analyzed for formation of DNA adducts using the nuclease P1 version of the ^{32}P-postlabeling assay as described (Stiborova *et al.* 2003a; 2007b; 2008; 2011). All experiments with animal models were conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki.

HPLC analysis of ^{32}P-labeled DNA adducts

HPLC analysis was performed essentially as described previously (Stiborova *et al.* 2003a). Individual spots detected by ^{32}P-postlabeling were excised from the thin layer plates and extracted with two 800 µl portions of 6 M ammonium hydroxide/isopropanol (1:1) for 40 min (Stiborova *et al.* 2003a). The eluent was evaporated in a Speed-Vac centrifuge, and the dried extracts dissolved in 100 µl of methanol/phosphate buffer (pH 3.5) 1:1 (v/v). Aliquots (50 µl) were analyzed on a phenyl-modified reversed-phase column (250 mm × 4.6 mm, 5 µm Zorbax Phenyl; Säulentechnik Knauer, Germany) with a linear gradient of methanol (from 40 to 80% in 45 min) in aqueous 0.5 M sodium phosphate and 0.5 M phosphoric acid (pH 3.5) at a flow rate of 0.9 ml/min. Radioactivity eluting from the column was measured by monitoring Cerenkov radiation on a Berthold LB 506 C-I flow-through radioactivity monitor (500 µl cell, dwell time 6 s) and integrated with the Borwin software (JMBS Developments, Grenoble, France).

Detection of P-glycoprotein by flow cytometry

P-glycoprotein expression was detected by flow cytometry using monoclonal antibody anti CD243 PE labeled (Beckmann Coulter, Nyon, Switzerland). Cultivation was performed in 12-well plates, three samples from every well were prepared and two wells measured. The fluorescence intensity of at least 10,000 cells was measured by FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with 488 nm laser and list mode data were analyzed with the CellQuest software (Lanza *et al.* 1997). Expression was evaluated as mean intensity of fluorescence. The fluorescence measurements were calibrated for each run by FITC-conjugated bead standards (DAKO cat. no. K0110). Results were expressed as mean of six determinations ± standard deviation.

Statistical analyses

The data were analyzed statistically by Student's *t*-test. All *p*-values are two-tailed and considered significant at the 0.05 level.

RESULTS

VPA and TSA increase cytotoxicity of and DNA adduct formation by ellipticine in human neuroblastoma cells

To determine the effect of VPA and TSA on cytotoxicity of ellipticine to human neuroblastoma cells, these were treated with increasing concentrations of ellipticine without or with pre-treatment with HDAC inhibitors. The VPA and TSA concentrations used for cell pre-treatment were close to their respective IC_{50} values to neuroblastoma cells (Hrebackova *et al.* 2009). The pre-treatment of UKF-NB-3 and UKF-NB-4 cells with either HDAC inhibitor made cells more sensitive to ellipticine. The decrease in IC_{50} values for ellipticine caused by both compounds in UKF-NB-3 and UKF-NB-4 cells was dose-dependent, being higher in UKF-NB-3 cells than in UKF-NB-4 cells (Table 1).

Because formation of covalent DNA adducts by ellipticine was found to be one of the most important DNA damaging mechanisms responsible for ellipticine cytotoxicity to neuroblastoma cells, the effect of VPA and TSA on ellipticine-DNA adduct formation was investigated with the nuclease P1 version of ^{32}P-postlabeling assay (Stiborova *et al.* 2001; 2003a; 2003b; 2004; 2007a; 2007b). Two major ellipticine-DNA adducts were formed in both neuroblastoma cells under all conditions tested in this study (spots 1 and 2 in Figure 2A,B). The two adducts are identical to those found previously after *in vitro* incubation of calf thymus DNA with ellipticine and isolated CYPs (Stiborova *et al.* 2001; 2003b), or peroxidases (Stiborova *et al.* 2007a) or after treatment of cells in culture with this anticancer drug (Frei *et al.* 2002; Borek-Dohalska *et al.* 2004; Martinkova *et al.* 2009) or *in vivo*, in several tissues, including mammary adenocarcinoma (Figure 2C) of rats (Stiborova *et al.* 2003a; 2007b; 2011) and mice (Stiborova *et al.* 2008) exposed to this agent. These adducts are generated from ellipticine-13-ylium and ellipticine-12-ylium (Figure 1), the reactive species formed from ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine (Stiborova *et al.* 2004; 2007a; Poljakova *et al.* 2006; Moserova *et al.* 2008) (Figure 2D,E) as confirmed by co-chromatographic analysis using TLC and HPLC (data not shown). Besides these adducts, two additional minor adducts (spots 6 and 7 in Figure 2B) were detected in DNA of UKF-NB-4 cells treated with 10 μM ellipticine (Figure 2B and Table 2). Both minor adducts we have shown to be generated *in vitro* mainly by peroxidase-catalyzed oxidation of ellipticine (Poljakova *et al.* 2006; Stiborova *et al.* 2007a). The low level of these adducts prevented HPLC co-chromatographic analysis or their further characterization. No adducts were detected in DNA of control cells treated with solvent, VPA or TSA only.

Ellipticine-DNA adduct levels were ellipticine dose dependent in both neuroblastoma cell lines with an

Tab. 1. The effect of valproate and trichostatin A on toxicity of ellipticine to UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines.

	IC_{50} for ellipticine (μM)	
	UKF-NB-3 cells	UKF-NB-4-cells
None	0.440 ± 0.030	0.490 ± 0.035
+ 1 mM valproate	0.199 ± 0.011***	0.389 ± 0.029**
+ 2 mM valproate	0.047 ± 0.003***	0.252 ± 0.021***
+ 100 nM TSA	0.303 ± 0.030***	0.389 ± 0.030**

IC_{50} values were calculated from the linear regression of the dose-log response curves. Values are mean ± S.D. of at least 3 experiments. The data were analyzed statistically by Student's *t*-test. Values significantly different from individual cell lines cultivated without VPA or TSA: **p<0.01, ***p<0.001.

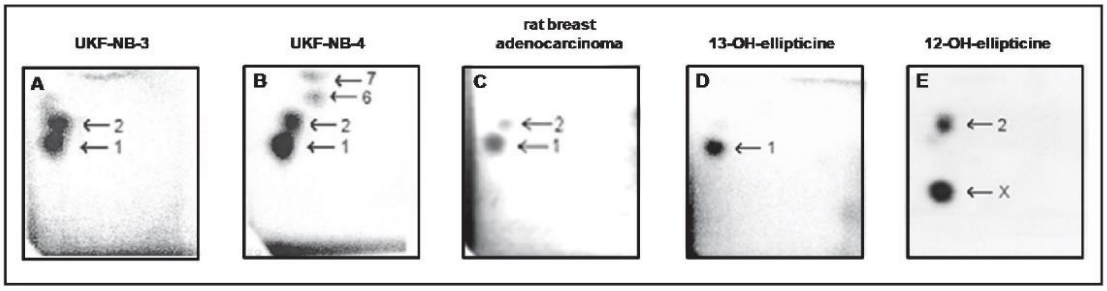

Fig. 2. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of DNA isolated from neuroblastoma UKF-NB-3 (**A**) and UKF-NB-4 (**B**) cells exposed to 10 μM ellipticine for 48 h, of DNA of breast adenocarcinoma of Wistar rats treated i.p. with 4 mg ellipticine per kilogram body weight (**C**), from calf thymus DNA reacted with 13-hydroxyellipticine (**D**) and 12-hydroxyellipticine (**E**). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. (**A,B**) Scans of the plates from the imager for 6.5 min; (**C,D,E**) autoradiographs of films exposed for 1 h at –80 °C. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

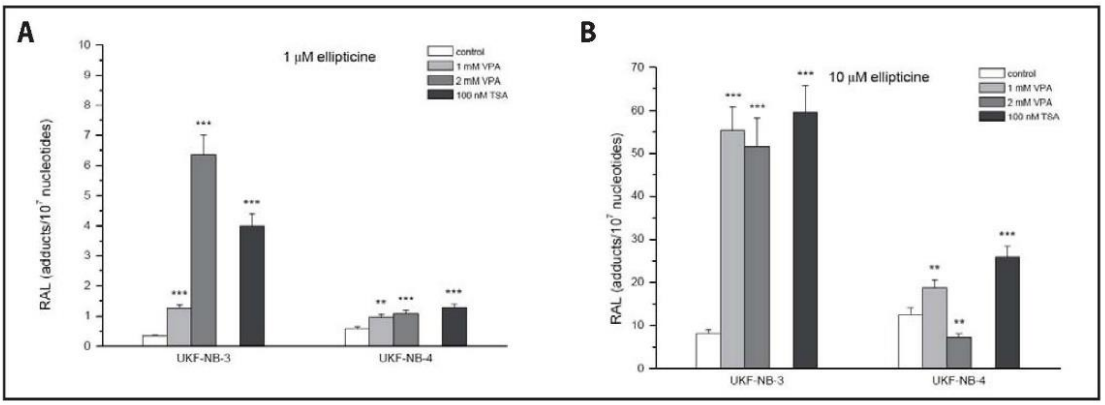

Fig. 3. The effect of valproate and trichostatin A on DNA adduct formation by 1 μM (A) and 10 μM ellipticine (B) in human UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines. *Columns*: Mean RAL (relative adduct labeling) ± standard deviations shown in the figure represent total levels of DNA adducts of three ^{32}P-postlabeling analyses. The data were analyzed statistically by Student's *t*-test. Values significantly different from cells exposed to ellipticine alone. $^{**}p<0.01$, $^{***}p<0.001$.

Tab. 2. The effect of valproate and trichostatin A on DNA adduct formation by ellipticine in human UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines.

Cells	Levels of DNA adducts (RAL × 10^{-7})[a]				
	Adduct 1	Adduct 2	Adduct 6	Adduct 7	Total
UKF-NB-3					
1.0 μM ellipticine	0.12 ± 0.01	0.23 ± 0.02	n.d.	n.d.	0.35± 0.04
+ 1 mM valproate	0.35 ± 0.03***	0.92 ± 0.10***	n.d.	n.d.	1.27 ± 0.10***
+ 2 mM valproate	3.22 ± 0.30***	3.14 ± 0.30***	n.d.	n.d.	6.36 ± 0.65***
+ 100 nM TSA	2.46 ± 0.25***	1.53 ± 0.15***	n.d.	n.d.	3.99 ± 0.40***
10 μM ellipticine	3.27 ± 0.32	5.01 ± 0.50	n.d.	n.d.	8.28 ± 0.8
+ 1 mM valproate	21.71 ± 2.02***	33.60 ± 3.30***	n.d.	n.d.	55.31 ± 5.5***
+ 2 mM valproate	27.59 ± 2.76***	23.92 ± 2.90***	n.d.	n.d.	51.51 ± 6.7***
+ 100 nM TSA	30.86 ± 3.89***	27.60 ± 2.70***	0.59 ± 0.06	0.60 ± 0.06	59.65 ± 6.2***
UKF-NB-4					
1.0 μM ellipticine	0.20 ± 0.02	0.38 ± 0.04	n.d.	n.d.	0.58 ± 0.06
+ 1 mM valproate	0.33 ± 0.03**	0.63 ± 0.07***	n.d.	n.d.	0.96 ± 0.10**
+ 2 mM valproate	0.38 ± 0.04***	0.71 ± 0.07***	n.d.	n.d.	1.09 ± 0.10***
+ 100 nM TSA	0.48 ± 0.04***	0.80 ± 0.08***	n.d.	n.d.	1.28 ± 0.12***
10 μM ellipticine	5.40 ± 0.56	6.50 ± 0.81	0.27±0.03	0.37±0.05	12.54 ± 1.51
+ 0.5 mM valproate	5.49 ± 0.51	5.65 ± 0.62	0.38±0.04	0.57±0.06	12.09 ± 1.24
+ 1 mM valproate	9.04 ± 0.99***	8.73 ± 0.89*	0.60±0.07**	0.39±0.04	18.72 ± 1.95**
+ 1.5 mM valproate	8.66 ± 0.84**	7.97 ± 0.82	0.17±0.02	0.17±0.02	16.97 ± 1.78*
+ 2 mM valproate	3.85 ± 0.41**	3.23 ± 0.32***	0.10±0.02**	0.11±0.02**	7.29 ± 0.89**
+ 100 nM TSA	13.08 ± 1.38***	11.90 ± 1.90***	0.47 ± 0.03**	0.40 ± 0.04	25.85 ± 2.60***

Neuroblastoma cells were treated with VPA and TSA for 24 h before ellipticine was added for another 48 h. [a]RAL, relative adduct labeling; averages and standard deviations of three ^{32}P-postlabeling analysis. n.d. - not detected (the detection limit of RAL was $1/10^{11}$ nucleotides). The data were analyzed statistically by Student's *t*-test. Values significantly different from cells exposed to ellipticine alone $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

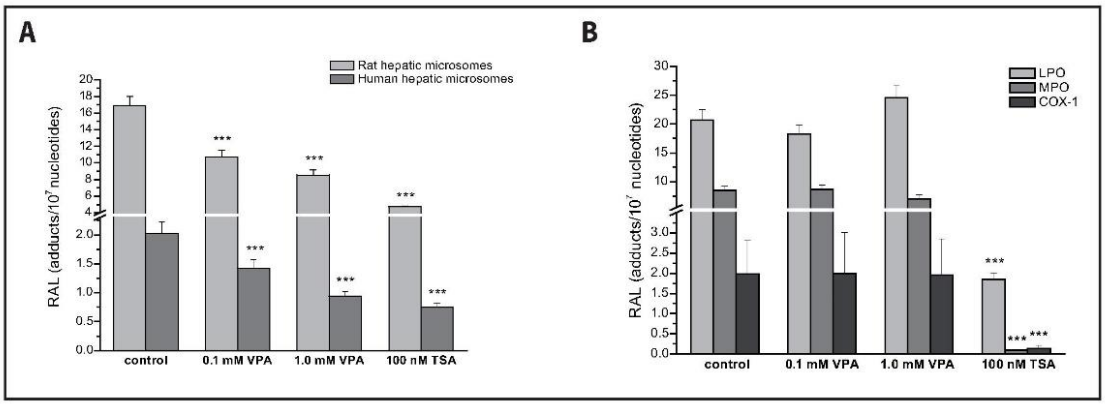

Fig. 4. The effect of VPA and TSA on DNA adduct formation by ellipticine activated with human or rat hepatic microsomes (A) or with peroxidases (B) as determined by TLC ^{32}P-postlabeling. *Columns*: Mean RAL (relative adduct labeling) ± standard deviations shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent *in vitro* incubations). The data were analyzed statistically by Student's *t*-test. Values significantly different from incubations without VPA or TSA: ***P<0.001. Control = without VPA or TSA; VPA = valproic acid; TSA = trichostatin A.

over-proportional increase between 1 μM and 10 μM ellipticine (Figure 3 and Table 2). Pre-treatment of UKF-NB-3 and UKF-NB-4 neuroblastoma cells with VPA and TSA had pronounced stimulatory effects on ellipticine-DNA adduct levels predominantly in UKF-NB-3 cells (Figure 3 and Table 2). TSA significantly increased DNA adducts at both ellipticine concentrations (1 and 10 μM) in both cell lines (up to 10-fold). However, VPA at concentrations of 1 and 2 mM increased only the levels of ellipticine-DNA adducts up to 20-fold at 1 and 10 μM ellipticine in UKF-NB-3 cells, and at 1 μM ellipticine in the UKF-NB-4 cell line (Figure 3 and Table 2). In the case of UKF-NB-4 cells treated with 10 μM ellipticine, even though VPA at 1 mM lead to a 1.5-fold increase in ellipticine-DNA adduct levels, this compound at 2 mM inhibited formation of all four ellipticine-DNA adducts, by 42% (Figure 3 and Table 2).

Because both VPA and TSA have been shown to be metabolized by CYPs and by peroxidases (Fisher *et al.* 1991; Rogiers *et al.* 1992; 1995; Isojärvi *et al.* 2001; Wen *et al.* 2001; Bort *et al.* 2004; Gupta *et al.* 2004; Hooven *et al.* 2005; Eyal *et al.* 2006; Perucca 2006; Cerveny *et al.* 2007) as is ellipticine (Stiborova *et al.* 2004; 2006c; 2007a), three experimental approaches were employed to elucidate their influence on ellipticine activation: (i) analysis of the effect of VPA and TSA on formation of ellipticine-DNA adducts in the CYP- and peroxidase-systems *in vitro*, (ii) analysis of the effect of these HDAC inhibitors on ellipticine oxidation to individual metabolites by both enzymatic systems *in vitro*, and (iii) analysis of the potential of VPA and TSA in the presence of ellipticine to influence expression levels of CYP and peroxidase enzymes responsible for ellipticine metabolism in neuroblastoma cells.

The effect of VPA and TSA on oxidative activation of ellipticine to DNA adducts by human and rat hepatic microsomes and peroxidases

Human and rat hepatic microsomes rich in CYP enzymes and isolated peroxidases (human MPO, bovine LPO and ovine COX-1 as models) that are capable of activating ellipticine to species forming DNA adducts (Stiborova *et al.* 2003b; 2004; 2007a) were used in the experiments. The DNA adduct pattern generated by ellipticine in the CYP-microsomal and peroxidase systems as determined by ^{32}P-postlabeling, again consisted of the two adducts derived from 13-hydroxy- and 12-hydroxyellipticine, spots 1 and 2, respectively, and of two additional adducts (adduct spots 6 and 7) (Stiborova *et al.* 2003b; 2004; 2007a) (Figure 2 and Table 3). In accordance to our former studies (Stiborova *et al.* 2001; 2003b), rat hepatic microsomes activated ellipticine more effectively than microsomes from human liver. In the case of peroxidases, the highest efficiency to activate ellipticine to DNA adducts exhibited LPO, followed by MPO and COX-1 (Figure 4). This finding corresponds to the results found previously, in our study investigating activation of ellipticine with peroxidases (Stiborova *et al.* 2007a).

As shown in Figure 4A and Table 3, VPA and TSA inhibited the activation of ellipticine to species forming DNA adducts mediated by human and rat hepatic microsomes. The peroxidase-catalyzed activation of ellipticine to DNA adducts, including adducts 6 and 7, was significantly inhibited by TSA, but VPA had no such effect (Figure 4B and Table 3).

In order to evaluate whether the lower ellipticine-DNA adduct levels in the presence of VPA and TSA is caused directly by their inhibition of CYPs and peroxidases, the effect of these compounds on formation

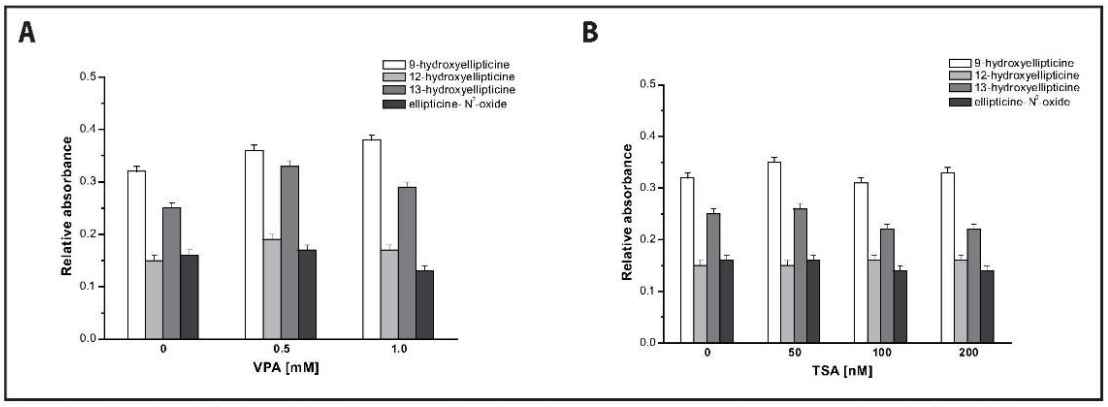

Fig. 5. The effect of VPA (**A**) and TSA (**B**) on ellipticine oxidation by rat hepatic microsomes. *Columns*: Mean values of individual ellipticine metabolites ± standard deviations of three experiments.

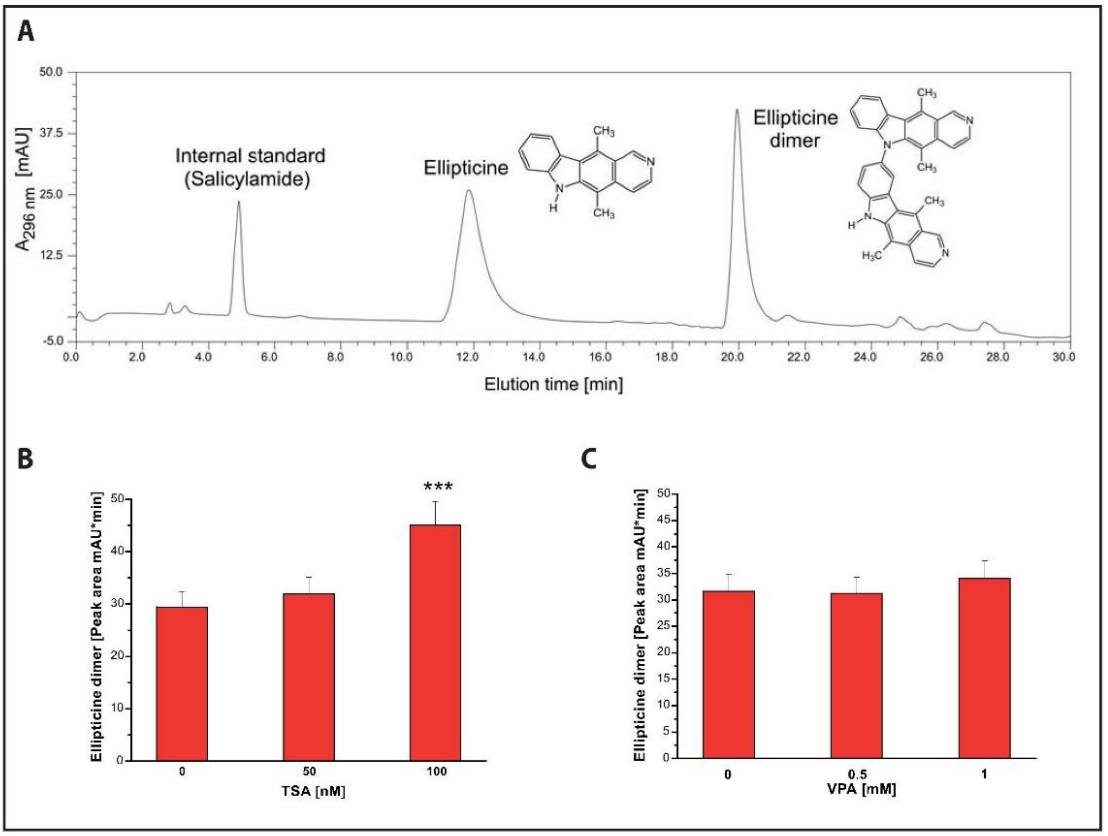

Fig. 6. HPLC separations of ellipticine from its dimer formed by oxidation of ellipticine with LPO (A) and the effect of TSA (B) and VPA (C) on ellipticine oxidation by LPO. *Columns*: Mean values of ellipticine dimer formed by LPO ± standard deviations of three experiments. ***$p<0.001$.

Tab. 3. The effect of valproate and trichostatin A on DNA adduct formation by ellipticine activated with either rat or human hepatic microsomes or peroxidases.

Enzymatic system	Levels of DNA adducts (RAL × 10^{-7})[a]				
	Adduct 1	Adduct 2	Adduct 6	Adduct 7	Total
Rat hepatic microsomes + ellipticine					
Without VPA and TSA	16.10 ± 0.80	0.26 ± 0.02	0.29±0.02	0.27±0.01	16.92 ± 1.10
+ 0.1 mM valproate	10.10 ± 0.80***	0.16 ± 0.01***	0.18±0.01***	0.26±0.01	10.70 ± 0.85***
+ 1.0 mM valproate	8.25 ± 0.54***	0.12 ± 0.05***	0.11±0.01***	0.05±0.01***	8.53 ± 0.65***
+ 100 nM TSA	4.20 ± 0.45***	0.12 ± 0.01***	0.21±0.01*	0.21±0.01*	4.74 ± 0.04***
Human hepatic microsomes + ellipticine					
Without VPA and TSA	1.60 ± 0.12	0.16 ± 0.02	0.18±0.02	0.07±0.01	2.03 ± 0.20
+ 0.1 mM valproate	1.09 ± 0.10**	0.13 ± 0.01	0.14±0.01**	0.06±0.01	1.42 ± 0.15***
+ 1.0 mM valproate	0.67 ± 0.06***	0.12 ± 0.09*	0.11±0.01***	0.04±0.01**	0.94 ± 0.08***
+ 100 nM TSA	0.50 ± 0.05***	0.09 ± 0.01***	0.11±0.01***	0.05±0.01*	0.75 ± 0.07***
Cyclooxygenase-1 + ellipticine					
Without VPA and TSA	1.50 ± 0.09	0.41 ± 0.02	0.02±0.001	0.05±0.002	1.98 ± 0.85
+ 0.1 mM valproate	1.51 ± 0.08	0.42 ± 0.03	0.03±0.	0.04±0.002	2.00 ± 1.01
+ 1.0 mM valproate	1.48 ± 0.08	0.41 ± 0.02	0.02±0.001	0.04±0.002	1.95 ± 0.90
+ 100 nM TSA	0.10 ± 0.01***	0.04 ± 0.01***	n.d.[b]	n.d.	0.14 ± 0.08***
Myeloperoxidase + ellipticine					
Without VPA and TSA	5.75 ± 0.48	1.61 ± 0.13	0.86±0.07	0.27±0.03	8.49 ± 0.74
+ 0.1 mM valproate	5.74 ± 0.42	1.81 ± 0.15	0.84±0.07	0.28±0.03	8.67 ± 0.75
+ 1.0 mM valproate	4.58 ± 0.35	1.16 ± 0.08	0.96±0.08	0.34±0.03	7.04 ± 0.71
+ 100 nM TSA	0.05 ± 0.01***	0.01 ± 0.01***	0.02±0.01***	0.01±0.01***	0.09 ± 0.01***
Lactoperoxidase + ellipticine					
Without VPA and TSA	13.36 ± 1.01	4.56 ± 0.32	2.27±0.18	0.51±0.04	20.70 ± 1.81
+ 0.1 mM valproate	12.12 ± 1.00	3.71 ± 0.29	1.89±0.12	0.54±0.05	18.26 ± 1.59
+ 1.0 mM valproate	16.29 ± 1.32	5.94 ± 0.46	1.78±0.17	0.55±0.05	24.56 ± 2.23
+ 100 nM TSA	1.42 ± 0.11***	0.18 ± 0.02***	0.14±0.01***	0.11±0.01***	1.85±0.16***

[a]Mean RAL (relative adduct labeling) of four determinations (duplicate analyses of two independent *in vitro* incubations).
[b]n.d. - not detected (the detection limit of RAL was $1/10^{11}$ nucleotides). The data were analyzed statistically by Student's *t*-test. Values significantly different from incubations without VPA or TSA: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Tab. 4. P-glycoprotein expression in neuroblastoma cells and its influencing by VPA and TSA.

Cells	P-glycoprotein levels (mean intensity of fluorescence)			
	Without pretreatment	1 mM VPA	2 mM VPA	100 nM TSA
UKF-NB-3	114.4 ± 5.4	165.5 ± 16.8**	135.2 ± 21.0**	136.1 ± 10.7**
UKF-NB-4	1417 ± 11	1710 ± 17***	2122 ± 20***	1542 ± 32*

Values are mean of six determinations ± standard deviation. The data were analyzed statistically by Student's *t*-test. Values significantly different from individual cell lines cultivated without VPA or TSA: *$p<0.05$, **$p<0.01$, ***$p<0.001$

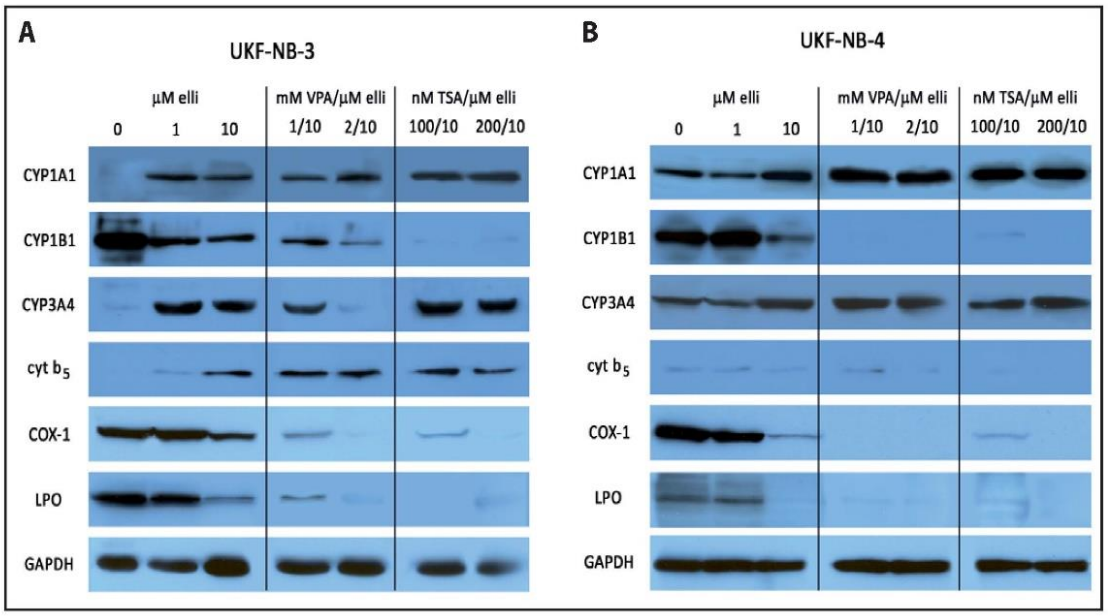

Fig. 7. Immunoblots showing the effects of exposing UKF-NB-3 cells (A) and UKF-NB-4 cells (B) to ellipticine with VPA or to ellipticine with TSA for 48 h on expression of CYP1A1, CYP1B1, CYP3A4, cytochrome b_5, COX-1 and LPO. Cell homogenates were subjected to SDS-PAGE, proteins transferred to nitrocellulose membranes and probed with antibodies as described in Material and Methods. GAPDH was used as loading control.

of individual ellipticine metabolites was investigated. Five ellipticine metabolites, 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide, were produced by microsomal CYPs (see metabolites in Figure 1) (Stiborova *et al.* 2004; 2006a). But because of the low levels of 7-hydroxyellipticine formed by the rat microsomes (Stiborova *et al.* 2006a) that were used as a model in these experiments, they were not quantified in this study. Neither VPA nor TSA inhibited the oxidation of ellipticine by rat hepatic microsomes to any of the ellipticine metabolites (Figure 5A,B). These findings suggest that a decrease in ellipticine-derived DNA adduct levels by VPA and TSA (Figure 4A) was not the result of inhibition of CYP activity but mediated by other mechanisms.

VPA also had no effect on the LPO-catalyzed oxidation of ellipticine (Figure 6C) to the ellipticine dimer (see the peak of the ellipticine dimer in Figure 6A), the compound which is the major detoxification metabolite of ellipticine formed by peroxidases (compare Figure 1) (Stiborova *et al.* 2007a). TSA at 100 nM induced an increase in ellipticine dimer levels catalyzed by LPO (Figure 6B). This finding and the results above showing the inhibition of ellipticine-DNA adduct formation by TSA suggest that TSA alters the peroxidase-mediated activation of ellipticine from the reaction with DNA to the reaction with a second ellipticine molecule to form the dimer.

The effects of ellipticine and VPA or TSA on levels of proteins CYP1A1, 1B1, 3A4, COX-1, LPO and cytochrome b_5

Using Western blot analysis with antibodies raised against CYP1A1, 1B1, 3A4, LPO, COX-1 and cytochrome b_5, the effects of exposure of the cells to ellipticine with and without VPA or TSA on protein levels of these enzymes were analyzed.

Levels of CYP1A1 protein in neuroblastoma UKF-NB-3 and UKF-NB-4 cells were elevated by exposure of the cells to ellipticine; a dose dependent increase was seen in neuroblastoma cells by this compound (Figure 7). The two HDAC inhibitors had stimulation effects on CYP1A1 expression levels induced by ellipticine, predominantly in UKF-NB-4 cells (Figure 7).

Levels of CYP3A4 protein were increased only by ellipticine in both neuroblastoma cells (Figure 7). Treatment of cells with ellipticine and both two HDAC inhibitors had essentially no effects on CYP3A4 protein levels induced by ellipticine, but 2 mM VPA decreased the amounts of CYP3A4, mainly in a UKF-NB-3 neuroblastoma cell line (Figure 7).

In contrast to the increase in CYP1A1 and CYP3A4 protein levels by ellipticine, amounts of CYP1B1 protein were decreased in neuroblastoma cells treated with this drug (Figure 7). The combined treatment of cells with ellipticine and HDAC inhibitors leads to an additional decrease in CYP1B1 protein levels, resulting even in a complete lack of its expression caused by TSA in

UKF-NB-3 cells (Figure 7A) and by both two HDAC inhibitors in UKF-NB-4 cells (Figure 7B).

Amounts of cytochrome b_5, the protein that modulates the enzymatic activities of several CYPs including CYP3A4 (for a summary see Schenkman & Jansson 2003) and CYP1A1 (Stiborova *et al.* 2006b), were elevated in UKF-NB-3 by ellipticine, but VPA or TSA had practically no effect on this ellipticine-mediated induction (Figure 7A). On the contrary, no increase in cytochrome b_5 protein levels by ellipticine was found in UKF-NB-4 cells. In addition, its amounts were even decreased by VPA and TSA in combination with 10 μM ellipticine in these cells (Figure 7B).

The peroxidases metabolizing ellipticine, COX-1 and LPO, are expressed in UKF-NB-3 and UKF-NB-4 cells (Figure 7). Their amounts decreased with 10 μM ellipticine, increasing concentrations of VPA and TSA further decreased COX-1 and LPO levels in both two neuroblastoma cell lines (Figure 7).

P-glycoprotein expression in neuroblastoma cells

Expression levels of P-glycoprotein (encoded by multidrug resistance gene 1) were analyzed in UKF-NB-3 and UKF-NB-4 cell lines. As shown in Table 4, more than 12-fold higher expression levels of P-glycoprotein were found in the UKF-NB-4 line than in the UKF-NB-3 cells. In addition, a 1.5-fold increase in expression of this protein was produced by treating UKF-NB-4 cells with VPA, while only a 1.2-fold increase caused by VPA was found in UKF-NB-3 cell line (Table 4). In the case of pre-treatment of both neuroblastoma cell lines with TSA, only ~1.1-fold increase in P-glycoprotein expression levels were detected (Table 4).

DISCUSSION

The results of this study demonstrate that a combination of ellipticine with the HDAC inhibitors, VPA and TSA, leads to an increase in ellipticine toxicity to UKF-NB-3 and UKF-NB-4 neuroblastoma cells. The sensitivity of the cells to this combination seems to be related to the phenotype, with the invasive N-type (UKF-NB-3) being higher sensitive than the less-aggressive S-type cells (UKF-NB-4). The increase in toxic effects of ellipticine to neuroblastoma cells and the higher sensitivity of the N-type neuroblastoma UKF-NB-3 cells to ellipticine caused by VPA and TSA were associated with an increase in formation of ellipticine-derived DNA adducts, which we have shown to be one of the predominant DNA-damaging mechanisms of ellipticine action in several cancer cells including neuroblastomas (Borek-Dohalska *et al.* 2004; Poljakova *et al.* 2007; 2009; Martinkova *et al.* 2009). Hence, the increase in ellipticine–mediated DNA damage by both HDAC inhibitors should be an important consideration in their use as chemotherapeutic agents of the invasive N-type neuroblastomas.

At least two phenomena might determine the levels of ellipticine-derived DNA adducts in neuroblastoma cells: (i) the expression and activities of CYPs and peroxidases metabolizing (activating and detoxicating) ellipticine and levels of protein(s) influencing their activities, and/or (ii) the expression levels of P-glycoprotein dictating concentrations of drugs in the cells.

In this study, we have found that the CYP-mediated formation of ellipticine-derived DNA adducts in tested neuroblastoma cells depends on expression levels of CYP1A1 and 3A4 enzymes and also on expression of another protein present in the membrane of endoplasmic reticulum, cytochrome b_5. The finding that CYP3A4 is expressed in UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines is interesting, because this CYP is highly expressed mainly in the liver and in the intestine (Rendic & Di Carlo 1997), but not in the brain. The ellipticine–mediated modulations of levels of cytochrome b_5 seem to play a key role in higher formation of ellipticine-derived DNA adducts in UKF-NB-3 cells. This heme protein was found to influence oxidation of ellipticine catalyzed by CYP3A4, the enzyme oxidizing this compound mainly to 12-hydroxy- and 13-hydroxylated metabolites that form DNA adducts (see Figure 1), and by CYP1A1, which predominantly detoxicate ellipticine (Stiborova *et al.* 2006a; 2006c; 2004; 2011). In the case of CYP3A4, stimulating effects of cytochrome b_5 on oxidation of ellipticine to 12-hydroxy- and 13-hydroxyellipticine lead to an increase in ellipticine-DNA adduct formation (Stiborova *et al.* 2004). In the case of CYP1A1, cytochrome b_5 has been recently found to alter the ratio of ellipticine metabolites formed by this CYP, "switching" CYP1A1-mediated oxidation of this anticancer drug from detoxication (9-hydroxy- and 7-hydroxyellipticine) to DNA-forming metabolites (12-hydroxy- and 13-hydroxyellipticine). These changes resulted in an increase in formation of covalent DNA adducts by ellipticine (Kotrbova *et al.* 2011).

In the present study, we have found that ellipticine increases amounts of cytochrome b_5 in UKF-NB-3 cells, but not in UKF-NB-4 cells. The higher levels of cytochrome b_5 and the increase in CYP1A1 levels induced by ellipticine with VPA or TSA in UKF-NB-3 cells, therefore, promote the activation of ellipticine catalyzed by CYP1A1 in these cells. On the contrary, because of low levels of cytochrome b_5 in UKF-NB-4 cells, the elevated expression levels of CYP1A1 caused by exposure to ellipticine in the presence of VPA and TSA results in higher ellipticine detoxication in these cells. Hence, both these induction effects produce concerted regulatory effects of ellipticine, VPA and TSA on ellipticine pharmacological action.

The finding that CYP1A1 is induced by ellipticine is in accordance with data found in the former studies (Fernandez *et al.* 1988; Gasiewicz *et al.* 1996; Aimova *et al.* 2007; Martinkova *et al.* 2009). Expression of CYP1A1 protein as well as its enzymatic activity was

significantly induced by ellipticine in rats treated with this compound (Aimova *et al.* 2007). This induction was explained by several authors (Fernandez *et al.* 1988; Gasiewicz *et al.* 1996) to be predominantly a consequence of the ellipticine binding to aryl hydrocarbon receptor (AhR). Surprisingly, CYP1B1, another CYP enzyme expressed in both two neuroblastoma cell lines metabolizing ellipticine mainly to detoxication metabolites (9-hydroxy- and 7-hydroxyellipticine) (Stiborova *et al.* 2004), whose transcriptional activation (*CYP1B1* gene) is believed to also involve the AhR (Kerzee & Ramos 2001), was, however, not induced by ellipticine in these cells. Its expression was even decreased by treating the cells with ellipticine plus VPA and TSA. The mechanisms of this phenomenon as well as mechanisms of the potential of ellipticine to increase levels of CYP3A4 and cytochrome b_5 in neuroblastoma cells were, however, not evaluated in this work and await further investigation.

In contrast to CYP1A1 and 3A4 enzymes, the peroxidases COX-1 and LPO that are expressed in UKF-NB-3 and UKF-NB-4 cells do not participate in the increased sensitivity of these neuroblastoma cells to ellipticine in the presence of VPA and TSA. Their expression levels are even decreased in neuroblastoma cells exposed to ellipticine plus either HDAC inhibitor.

Another reason for the differences between UKF-NB-3 and UKF-NB-4 neuroblastoma cell's sensitivities to the combined effect of ellipticine with HDAC inhibitors might be their different genetic programs (Cinatl *et al.* 1999; Bedrnicek *et al.* 2005). The UKF-NB-4 line was established from a recurrent disease and, in contrast to UKF-NB-3, the P-glycoprotein causing multidrug resistance, was found previously to be expressed at high levels in this cell line (Cinatl *et al.* 1999). Indeed, here we found that more than 12-fold higher levels of P-glycoprotein are expressed in the UKF-NB-4 line than in UKF-NB-3 cells. This transport protein might efficiently eliminate VPA, TSA and ellipticine (and their metabolites) from cells, thereby decreasing the toxic effects of these compounds. Moreover, because VPA acts as an inducer of P-glycoprotein expression in several human tumor cell lines (Eyal *et al.* 2006; Cerveny *et al.* 2007), including neuroblastoma cells (up to 1.5-fold) (present study), elimination of both these HDAC inhibitors and ellipticine from the UKF-NB-4 cell line might be even higher. In contrast to VPA, ellipticine has not been identified to be a substrate of P-glycoprotein (Huang *et al.* 2005), indicating its suitability to be used in combined therapy with other drugs.

Collectively, the results presented in this paper are the first report demonstrating that VPA and TSA increase sensitivity of neuroblastomas to ellipticine. The higher sensitivity of neuroblastomas to ellipticine caused by VPA and TSA corresponds to the increase in DNA-damage responsible for ellipticine cytotoxicity in these cells (Poljakova *et al.* 2009). This increase is not caused by the direct stimulation effects of these HDAC inhibitors on the enzymes catalyzing reactions leading to formation of ellipticine-DNA adducts; these enzymes were either not influenced by VPA and TSA or even inhibited by these HDAC inhibitors. Therefore, the increase in DNA adduct formation is mediated by elevated levels of CYP3A4 and 1A1 enzymes and/or cytochrome b_5 caused by their induction with ellipticine and/or VPA and TSA.

Concerning formation of ellipticine-derived DNA adducts, the risk of treating children with this compound might be considered. Nevertheless, our *in vivo* studies using the rat experimental model mimicking the fate of ellipticine in human (Stiborova *et al.* 2003b; 2006a) demonstrated that ellipticine-DNA adducts did not persist in healthy tissues of rats treated with ellipticine (Stiborova *et al.* 2007b). Therefore, these results suggest a relatively low risk of the genotoxic side effects of ellipticine during the cancer treatment in human.

It should be also emphasized that besides the above mechanism explaining elevated levels of ellipticine-derived DNA adducts in neuroblastoma cells another mechanism, based on epigenetic modifications of chromatin by VPA and TSA, which was not examined in this work, might modulate ellipticine-induced cytotoxicity in these cells, too. Namely, it was found that loosening-up the chromatin structure by histone acetylation is caused by HDAC inhibitors (Kim *et al.* 2003; Marchion *et al.* 2005a; 2005b; Catalano *et al.* 2006). Such changes in the chromatin structure, leading to chromatin decondensation, might make cell DNA more accessible to the ellipticine–mediated damage such as formation of ellipticine-DNA adducts or intercalation of ellipticine into DNA and inhibition of topoisomerase II activity. Therefore, stimulation effects of VPA and TSA on ellipticine toxic effects to neuroblastoma cells following from these phenomena should also be taken into account. Such effects of VPA and TSA in neuroblastoma cells are, hence, planned to be studied in our further work.

The results of the present study strongly suggest that combined therapy of ellipticine with VPA or TSA might be an effective treatment strategy for children with high risk neuroblastomas.

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REFERENCES

1 Aimova D, Svobodova L, Kotrbova V, Mrazova B, Hodek P, Hudecek J, *et al.* (2007) The anticancer drug ellipticine is a potent inducer of rat cytochromes P450 1A1 and 1A2, thereby modulating its own metabolism. Drug Metab Dispos **35**: 1926–1934.

2 Auclair C (1987) Multimodal action of antitumor agents on DNA: The ellipticine series. Arch Biochem Biophys **259**: 1–14.
3 Bednicek J, Vicha A, Jarosova M, Holzerova M, Cinatl J Jr, Michaelis M, *et al.* (2005) Characterization of drug-resistant neuroblastoma cell lines by comparative genomic hybridization. Neoplasma **52**: 415–419.
4 Blaheta RA and Cinatl J Jr (2002) Anti-tumor mechanisms of valproate: a novel role for an old drug. Med Res Rev **22**: 492–511.
5 Blaheta RA, Michaelis M, Natsheh I, Hasenberg C, Weich E, Relja B, *et al.* (2007) Valproic acid inhibits adhesion of vincristine- and cisplatin-resistant neuroblastoma tumour cells to endothelium. Br J Cancer **96**: 1699–1706.
6 Blaheta RA, Nau H, Michaelis M and Cinatl J Jr (2002) Valproate and valproate-analogues: potent tools to fight against cancer. Curr Med Chem **9**: 1417–1433.
7 Bolden JE, Peart MJ and Johnstone RW (2006) Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov **5**: 769–784.
8 Borek-Dohalska L, Frei E and Stiborova M (2004) DNA adduct formation by the anticancer drug ellipticine and its hydroxy derivatives in human breast adenocarcinoma MCF-7 cells. Collect Czech Chem Commun **69**: 603–615.
9 Bort R, Gómez-Lechón MJ, Castell JV and Jover R (2004) Role of hepatocyte nuclear factor 3 gamma in the expression of human CYP2C genes. Arch Biochem Biophys **426**: 63–72.
10 Brodeur GM (2003) Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer **3**: 203–216.
11 Catalano MG, Fortunati N, Pugliese M, Poli R, Bosco O, Mastrocola R, *et al.* (2006) Valproic acid enhances tubulin acetylation and apoptotic activity of paclitaxel on anaplastic thyroid cancer cell lines. J Endocrinol **191**: 465–472.
12 Cerveny L, Svecova L, Anzenbacherova E, Vrzal R, Staud F, Dvorak Z, *et al.* (2007) Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways. Drug Metab Dispos **35**: 1032–1041.
13 Cinatl J Jr, Cinatl J, Driever PH, Kotchetkov R, Pouckova P, Kornhuber B and Schwabe D (1997) Sodium valproate inhibits in vivo growth of human neuroblastoma cells. Anti-Cancer Drugs **8**: 958–963.
14 Cinatl J Jr, Cinatl J, Kotchetkov R, Vogel JU, Woodcock BG, Matousek J, *et al.* (1999) Bovine seminal ribonuclease selectively kills human multidrug-resistant neuroblastoma cells via induction of apoptosis. Int J Oncol **15**: 1001–1009.
15 Cinatl J Jr, Cinatl J, Scholz M, Driever PH, Henrich D, Kabickova H, *et al.* (1996) Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. Anti-Cancer Drugs **7**: 766–773.
16 Eyal S, Lamb JG, Smith-Yockman M, Yagen B, Fibach E and Altschuler Y (2006) White HS, Bialer M. The antiepileptic and anticancer agent, valproic acid, induces P-glycoprotein in human tumour cell lines and in rat liver. Br J Pharmacol **149**: 250–260.
17 Fernandez N, Roy M and Lesca P (1988) Binding characteristics of Ah receptors from rats and mice before and after separation from hepatic cytosols. 7-Hydroxyellipticine as a competitive antagonist of cytochrome P-450 induction. Eur J Biochem **172**: 585–592.
18 Fisher JE, Nau H and Löscher W (1991) Alterations in the renal excretion of valproate and its metabolites after chronic treatment. Epilepsia **32**: 146–150.
19 Fosse P, Rene B, Charra M, Paoletti C and Saucier JM (1992) Stimulation of topoisomerase II-mediated DNA cleavage by ellipticine derivatives: structure-activity relationships. Mol Pharmacol **42**: 590–595.
20 Frei E, Bieler CA, Arlt VM, Wiessler M and Stiborova M (2002) Covalent binding of the anticancer drug ellipticine to DNA in V79 cells transfected with human cytochrome P450 enzymes. Biochem Pharmacol **64**: 289–295.
21 Froelich-Ammon SJ, Patchan MW, Osheroff N and Thompson RB (1995) Topoisomerase II binds to ellipticine in the absence or presence of DNA. Characterization of enzyme-drug interactions by fluorescence spectroscopy. J Biol Chem **270**: 14998–15004.
22 Furchert SE, Lanvers-Kaminsky C, Juürgens H, Jung M, Loidl A and Frühwald MC (2007) Inhibitors of histone deacetylases as potential therapeutic tools for high-risk embryonal tumors of the nervous system of childhood. Int J Cancer **120**: 1787–1794.
23 Gasiewicz TA, Kende RS, Rucci G, Whitney B and Willey JJ (1996). Analysis of structural requirements for Ah receptor antagonist activity: Ellipticines, flavones, and related compounds. Biochem Pharmacol. **52**: 1787–830.
24 Gupta M, Gupta YK, Agarwal S, Aneja S and Kohli K (2004) A randomized, double-blind, placebo controlled trial of melatonin add-on therapy in epileptic children on valproate monotherapy: effect on glutathione peroxidase and glutathione reductase enzymes. Br J Clin Pharmacol **58**: 542–547.
25 Hooven LA, Mahadevan B, Keshava C, Johns C, Pereira C, Desai D, *et al.* (2005) Effects of suberoylanilide hydroxamic acid and trichostatin A on induction of cytochrome P450 enzymes and benzo[a]pyrene DNA adduct formation in human cells. Bioorg Med Chem Lett **15**: 1283–1287.
26 Hopkins-Donaldson S, Yan P, Bourloud KB, Muhlethaler A, Bodmer JL and Gross N (2002) Doxorubicin-induced death in neuroblastoma does not involve death receptors in S-type cells and is caspase-independent in N-type cells. Oncogene **21**: 6132–6137.
27 Hrebackova J, Hrabeta J and Eckschlager T (2010) Valproic acid in the complex therapy of malignant tumors. Curr Drug Targets **11**: 361–379.
28 Hrebackova J, Poljakova J, Eckschlager T, Hrabeta J, Prochazka P, Smutny S *et al.* (2009) Histone deacetylase inhibitors valproate and trichostatin A are toxic to neuroblastoma cells and modulate cytochrome P450 1A1, 1B1 and 3A4 expression in these cells. Interdisc Toxicol **2**: 205–210.
29 Huang Y, Blower PE, Yang C, Barbacioru C, Dai Z, Zhang Y, *et al.* (2005) Correlating gene expression with chemical scaffolds of cytotoxic agents: ellipticines as substrates and inhibitors of MDR1. Pharmacogenomics J **5**:112–125.
30 Isojärvi JI, Turkka J, Pakarinen AJ, Kotila M, Rättyä J and Myllylä VV (2001) Thyroid function in men taking carbamazepine, oxcarbazepine, or valproate for epilepsy. Epilepsia **42**: 930–934.
31 Kerzee JK and Ramos KS (2001) Constitutive and inducible expression of Cyp1a1 and Cyp1b1 in vascular smooth muscle cells. Circ Res **89**: 573–582.
32 Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Yand Carrier F (2003) Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res **63**: 7291–7300.
33 Kotrbova V, Mrazova B, Moserova M, Martínek V, Hodek P, Hudecek J, *et al.* (2011) Cytochrome b_5 shifts oxidation of anticancer drug ellipticine by cytochromes P450 1A1 and 1A2 from its detoxication to activation metabolites, thereby modulating its pharmacological efficacy. Biochem Pharmacol. **82**(6): 669–80.
34 Lanza F, Latorraca A, Moretti S, Castagnari B, Ferrari L and Castoldi G (1997) Comparative analysis of different permeabilization methods for the flow cytometry measurement of cytoplasmic myeloperoxidase and lysozyme in normal and leukemic cells, Cytometry **30**: 134–144.
35 Marchion DC, Bicaku E, Daud AI, Sullivan DM and Munster PN (2005a) Valproic acid alters chromatin structure by regulation of chromatin modulation proteins. Cancer Res **65**: 3815–3822.
36 Marchion DC, Bicaku E, Daud AI, Sullivan DM and Munster PN (2005b) In vivo synergy between topoisomerase II and histone deacetylase inhibitors: predictive correlates. Mol Cancer Ther **4**: 1993–2000.
37 Maris JM and Mathay KK (1999) Molecular biology of neuroblastomas. J Clin Oncol **17**: 2264–2279.
38 Marks PA, Miller T and Richon VM (2003) Histone deacetylases. Curr Opin Pharmacol **3**: 344–351.
39 Marks PA, Richon VM, Miller T and Kelly WK (2004) Histone deacetylase inhibitors. Adv Cancer Res **91**: 137–168.
40 Martinkova E, Dontenwill M, Frei E and Stiborova M (2009). Cytotoxicity of and DNA adduct formation by ellipticine in human U87MG glioblastoma cancer cells. Neuro Endocrinol Lett. **30** (Suppl): 60–66.

41 Martinkova E, Maglott A, Leger, DY, Bonnet D, Stiborova M, Takeda K, *et al.* (2010). α5β1 integrin antagonists reduce chemotherapy-induced premature senescence and facilitate apoptosis in human glioblastoma cells. Int J Cancer **127**: 1240–1248.
42 McCormick DL, Adamowski CB, Fiks A and Moon RC (1981) Lifetime dose-response relationships for mammary tumor induction by a single administration of N-methyl-N-nitrosourea, Cancer Res **41**: 1690–1694.
43 Michaelis M, Doerr HW and Cinatl J Jr (2007) Valproic acid as anti-cancer drug. Curr Pharm Des **13**: 3378–3393.
44 Michaelis M, Suhan T, Cinatl J, Driever PH and Cinatl J Jr (2004) Valproic acid and interferon-alpha synergistically inhibit neuroblastoma cell growth in vitro and in vivo. Int J Oncol **25**: 1795–1799.
45 Monnot M, Mauffret O, Simon V, Lescot E, Psaume B, Saucier JM, *et al.* (1991) DNA-drug recognition and effects on topoisomerase II-mediated cytotoxicity. A three-mode binding model for ellipticine derivatives. J Biol Chem **25**: 1820–1829.
46 Morgenstern BZ, Krivoshik AP, Rodriguez V and Anderson PM (2004) Wilms' tumor and neuroblastoma. Acta Paediatr Suppl **93**: 78–85.
47 Moserova M, Kotrbova V, Rupertova M, Naiman K, Hudecek J, Hodek P, *et al.* (2008). Isolation and partial characterization of the adduct formed by 13-hydroxyellipticine with deoxyguanosine in DNA. Neuro Endocrinol Lett. **29**: 728–732.
48 Perucca E (2006) Clinically relevant drug interactions with antiepileptic drugs. Br J Clin Pharmacol 61: 246–255.
49 Poljakova J, Dracinsky M, Frei E., Hudecek J and Stiborova M (2006) The effect of pH on peroxidase-mediated oxidation of and DNA-adduct formation by ellipticine. Collect Czech Chem Commun. **71**: 1169–1185.
50 Poljakova J, Eckschlager T, Hrabeta J, Hrebackova J, Smutny S, Frei E, *et al.* (2009) The mechanism of cytotoxicity and DNA adduct formation by the anticancer drug ellipticine in human neuroblastoma cells. Biochem Pharmacol. **77**: 1466–1479.
51 Poljakova J, Forsterova K, Sulc M, Frei E and Stiborova M (2005) Oxidation of ellipticine by peroxidases. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub **149**: 449–453.
52 Poljakova J, Frei E, Gomez JE, Aimova D, Eckschlager T, Hrabeta J, *et al.* (2007) DNA adduct formation by the anticancer drug ellipticine in human leukemia HL-60 and CCRF-CEM cells. Cancer Lett **252**: 270–279.
53 Rendic S and Di Carlo FJ (1997) Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. Drug Metab Rev **29**: 413–580.
54 Rogiers V, Akrawi M, Vercruysse A, Phillips IR and Shephard EA (1995) Effects of the anticonvulsant, valproate, on the expression of components of the cytochrome-P-450-mediated monooxygenase system and glutathione S-transferases. Eur J Biochem **231**: 337–343.
55 Rogiers V, Callaerts A, Vercruysse A, Akrawi M, Shephard EA and Phillips IR (1992) Effects of valproate on xenobiotic biotransformation in rat liver. In vivo and in vitro experiments. Pharm Weekbl Sci **4**:127–131.
56 Santini V, Gozzini A and Ferrari G (2007) Histone deacetylase inhibitors: molecular and biological activity as a premise to clinical application. Curr Drug Metab. **8**: 383–393.
57 Schenkman, J.B., Jansson, I. 2003. The many roles of cytochrome b_5. Pharmacol Ther **97**: 139–152.
58 Schwab M (1999) Human neuroblastoma: from basic science to clinical debut of cellular oncogenes. Naturwissenschaften **86**: 71–78.
59 Singh MP, Hill GC, Peoch D, Rayner B, Inabach JL and Lown JW (1994) High-field NMR and restrained molecular modeling studies on a DNA heteroduplex containing a modified apurinic abasic site in the form of covalently linked 9-aminoellipticine. Biochemistry **33**: 10271–10285.
60 Stiborova M, Arlt VM, Henderson CJ, Wolf CR, Kotrbova V, Moserova M, *et al.* (2008). Role of hepatic cytochromes P450 in bioactivation of the anticancer drug ellipticine: studies with the hepatic NADPH:cytochrome P450 reductase null mouse. Toxicol Appl Pharmacol. **226**: 318–27.
61 Stiborova M, Bieler CA, Wiessler M and Frei E (2001). The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts. Biochem Pharmacol. **62**: 1675–1684.
62 Stiborova M, Borek-Dohalska L, Kotrbova V, Kukackova K, Janouchova K, Rupertova M, *et al.* (2006a). Oxidation pattern of the anticancer drug ellipticine by hepatic microsomes – Similarity between human and rat systems. Gen Physiol Biophys **25**: 245–261.
63 Stiborova M, Breuer A, Aimova D, Stiborova-Rupertova M, Wiessler M and Frei E (2003a). DNA adduct formation by the anticancer drug ellipticine in rats determined by ^{32}P-postlabeling. Int J Cancer **107**: 885–890.
64 Stiborova M, Martinek V, Rydlova H, Hodek P and Frei E (2002). Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes. Cancer Res **62**: 5678–5684.
65 Stiborova M, Martinek V, Rydlova H, Koblas T and Hodek P (2005). Expression of cytochrome P450 1A1 and its contribution to oxidation of a potential human carcinogen 1-phenylazo-2-naphthol (Sudan I) in human livers. Cancer Lett. **220**: 145–154.
66 Stiborova M, Martinek V, Schmeiser HH and Frei E (2006b). Modulation of CYP1A1-mediated oxidation of carcinogenic azo dye Sudan I and its binding to DNA by cytochrome b_5. Neuro Endocrinol Lett. **27** (Suppl 2): 35–39
67 Stiborova M, Moserova M, Mrazova B, Kotrbova V and Frei E (2010) Role of cytochromes P450 and peroxidases in metabolism of the anticancer drug ellipticine: additional evidence of their contribution to ellipticine activation in rat liver, lung and kidney. Neuro Endocrino. Lett **31** (Suppl. 2): 26–35.
68 Stiborova M, Poljakova J, Ryslava H, Dracinsky M, Eckschlager T and Frei E (2007a). Mammalian peroxidases activate anticancer drug ellipticine to intermediates forming deoxyguanosine adducts in DNA identical to those found in vivo and generated from 12-hydroxyellipticine and 13-hydroxyellipticine. Int J Cancer **120**: 243–251.
69 Stiborova M, Rupertova M, Aimova D, Ryslava H and Frei E (2007b) Formation and persistence of DNA adducts of anticancer drug ellipticine in rats. Toxicology **236**: 50–60.
70 Stiborova M, Rupertova M and Frei E (2011) Cytochrome P450- and peroxidase-mediated oxidation of anticancer alkaloid ellipticine dictates its anti-tumor efficiency. Biochim Biophys Acta **1814**: 175–185.
71 Stiborova M, Rupertova M, Schmeiser HH and Frei E (2006c) Molecular mechanism of antineoplastic action of an anticancer drug ellipticine. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub **150**: 13–23.
72 Stiborova M, Sejbal J, Borek-Dohalska L, Poljakova J, Forsterova K, Rupertova M *et al.* (2004) The anticancer drug ellipticine forms covalent DNA adducts, mediated by human cytochromes P450, through metabolism to 13-hydroxyellipticine and ellipticine N^2-oxide. Cancer Res. **64**: 8374–8380.
73 Stiborova M, Stiborova-Rupertova M, Borek-Dohalska L, Wiessler M and Frei E (2003b) Rat microsomes activating the anticancer drug ellipticine to species covalently binding to deoxyguanosine in DNA are a suitable model mimicking ellipticine bioactivation in humans. Chem Res Toxicol. **16**: 38–47.
74 Voigt A, Hartmann P and Zintl F (2000) Differentiation, proliferation and adhesion of human neuroblastoma cells after treatment with retinoic acid. Cell Adhes Commun **7**: 423–440.
75 Wen X, Wang JS. Kivistö KT, Neuvonen PJ and Backman JT (2001) In vitro evaluation of valproic acid as an inhibitor of human cytochrome P450 isoforms: preferential inhibition of cytochrome P450 2C9 (CYP2C9). Br J Clin Pharmacol **52**: 547–553.
76 Yang Q, Tian Y, Liu S, Zeine R, Chlenski A, Salwen HR, *et al.* 2007. Thrombospondin-1 peptide ABT-510 combined with valproic acid is an effective antiangiogenesis strategy in neuroblastoma. Cancer Res **67**: 1716–1724.

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Valproic acid overcomes hypoxia-induced resistance to apoptosis

ŠIMON CIPRO[1*], JANA HŘEBAČKOVÁ[1*], JAN HRABĚTA[1], JITKA POLJAKOVÁ[1,2] and TOMÁŠ ECKSCHLAGER[1]

[1]Department of Pediatric Hematology and Oncology, 2nd Medical School, Charles University and University Hospital Motol, V Úvalu 84, 150 00 Prague 5; [2]Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic

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Abstract. Valproic acid (VPA), a histone deacetylase inhibitor (HDACi), has been shown to be an effective tool in cancer treatment. Although its ability to induce apoptosis has been described in many cancer types, the data come from experiments performed in normoxic (21% O_2) conditions only. Therefore, we questioned whether VPA would be equally effective under hypoxic conditions (1% O_2), which is known to induce resistance to apoptosis. Four neuroblastoma cell lines were used: UKF-NB-3, SK-N-AS, plus one cisplatin-resistant subline derived from each of the two original sensitive lines. All were treated with VPA and incubated under hypoxic conditions. Measurement of apoptosis and viability using TUNEL assay and Annexin V/propidium iodide labeling revealed that VPA was even more effective under hypoxic conditions. We show here that hypoxia-induced resistance to chemotherapeutic agents such as cisplatin could be overcome using VPA. We also demonstrated that apoptosis pathways induced by VPA do not differ between normoxic and hypoxic conditions. VPA-induced apoptosis proceeds through the mitochondrial pathway, not the extrinsic pathway (under both normoxia and hypoxia), since inhibition of caspase-8 failed to decrease apoptosis or influence bid cleavage. Our data demonstrated that VPA is more efficient in triggering apoptosis under hypoxic conditions and overcomes hypoxia-induced resistance to cisplatin. The results provide additional evidence for the use of VPA in neuroblastoma (NBL) treatment.

Introduction

Neuroblastoma (NBL) is the most common extracranial solid tumor in children and a major cause of neoplastic death in infancy. It originates from undifferentiated cells of the sympathetic nervous system. Based on its cellular and biological heterogeneity, NBL behavior can range from low-risk cancers with a tendency toward spontaneous regression or maturation, to high-risk cancers with extensive growth, early metastasis and a poor prognosis (1). Treatment of high-risk neuroblastomas (HR NBL) usually fails despite intensive therapy, which includes megatherapy followed by hematopoietic progenitor cell transplantation, biotherapy and immunotherapy. Treatment failure is due to drug resistance that arises in the majority of patients who initially responded well to chemotherapy. The necessity to develop new treatment modalities is indisputable.

An increasing body of information indicates that epigenetic modifications are associated with cancer onset and progression. This awareness has led to prolific research into drugs that interfere with the epigenome (2,3). Histone deacetylase inhibitors (HDACi) represent such a group of compounds since histones are the main protein components of chromatin and have an indispensable role in gene regulation. Cancer cell histones are frequently hypo-acetylated, due to overexpression of histone deacetylases (HDACs), and are often connected with impaired gene transcription in tumors (4), including dysregulation of genes responsible for growth control and apoptosis. Consequently inhibition of HDACs can reactivate gene transcription and restore the balance between pro- and anti-apoptotic genes and eventually lead to apoptosis (5). HDAC inhibition also decompacts chromatin structure making the DNA structure more available to other cytotoxic agents that target DNA. Despite advances in understanding, the mode of anti-tumor action of HDACi is complex and still not completely understood (6,7).

Valproic acid (VPA) has been studied as an anti-cancer drug excessively over the past years because it can be taken orally, is well tolerated by patients and there is cumulative experience coming from its use as an anti-epileptic drug. Although earlier reports showed the cytotoxic potential of VPA on NBL cells *in vitro* and *in vivo* (8,9), the studies were carried out solely under normoxic conditions and little was known about its anti-tumor activity under hypoxic conditions.

Hypoxic areas are common in solid tumors. Hypoxia arises as a consequence of pathological microcirculation within the tumor. Rapid tumor growth can outstrip its own blood supply

Correspondence to: Dr Tomáš Eckschlager, 2nd Medical School, Charles University, V Úvalu 84, 150 00 Prague 5, Czech Republic
E-mail: eckschlagertomas@yahoo.com

*Contributed equally

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and therefore cancer cells are exposed to oxygen deprivation (chronic hypoxia) (10). Another factor that contributes to tumor hypoxia is the poor quality of the newly developing tumor vessels, which often display severe structural abnormalities. Whereas normal vasculature shows a hierarchical branching pattern, tumor blood vessels are often tortuous in appearance with uneven diameters, branch irregularity and form arterio-venous shunts. These vessels are more susceptible to thrombosis and on occasion collapse, which ultimately leads to acute hypoxia within the tumor mass (11).

Hypoxia also induces adaptational changes in cells that are otherwise physiological, in the sense that they are normal and noncancerous; however, due to regional hypoxia these cells contribute to chemo- and radio-resistance in hypoxic cancer cells (12-14). Notably, hypoxia-induced resistance is not limited to only conventional chemotherapy but it can also decrease the efficiency of targeted therapy, as documented with imatinib in cases of chronic myeloid leukemia (15). Additionally, hypoxia induces genomic instability that leads to progressive transformation of cancer cells into more malignant phenotypes (16). The presence of hypoxic regions within the tumor mass correlates with more aggressive phenotypes, lower response rates and a decline in overall disease survival (17-19).

In our study, we addressed the issue of whether hypoxia promotes resistance to VPA and if apoptosis pathways differ between normoxic and hypoxic conditions, with respect to VPA treatment.

Materials and methods

Cell lines and chemicals. The UKF-NB-3 cell line was established from bone marrow metastases of HR NBL with *MYCN* amplification. The line was kindly provided by Professor J. Cinatl Jr. (Institute for Medical Virology, Hospital of the Johann Wolfgang Goethe University, Frankfurt, Germany). Cells were grown in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal calf serum (PAA Laboratories, Pasching, Austria). The SK-N-AS cell line was derived from bone marrow metastasis of a female patient with HR NBL. SK-N-AS, with normal diploid *MYCN* status, was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and was cultivated according to the manufacturer's instructions. The CDDP-resistant subline, designated UKF-NB-3^{CDDP} was also kindly provided by Professor J. Cinatl Jr. SK-N-AS^{CDDP} was prepared in our laboratory by incubation of parental cells with increasing concentrations of CDDP. Solutions of CDDP (EBEWE Pharma Ges.m.b.H. Nfg. KG, Unterach, Austria) were prepared according to the manufacturer's instructions. CDDP-resistant cell lines were cultivated in a medium containing 1 μg/ml of CDDP. Valproic acid (dissolved in distilled water) and trichostatin A (dissolved in DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The specific caspase-8 inhibitor, Z-IETD-FMK (specific caspase-8 inhibitor), was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). It was dissolved in DMSO and was used at a final concentration of 2 μM, as recommended by producer. All other chemicals used in experiments were of analytical purity or better.

Hypoxic environment. A hypoxia chamber purchased from Billups-Rothenberg (Del Mar, CA, USA) was prepared with an atmosphere containing 1% O_2, 5% CO_2, and 94% N_2. Controls were grown at 5% CO_2 and all samples were grown at 37˚C.

Annexin V/propidium iodide labeling. Annexin V, a phospholipid-binding protein with a high affinity for phosphatidyl serine, was used to measure apoptosis and viability. Apoptosis was determined using an Annexin V-FITC Apoptosis Detection kit according to manufacturer instructions (Biovision, Mountain View, CA, USA). Cells were washed in PBS and resuspended in a 'binding buffer' after incubation with different compounds, under normoxic and/or hypoxic conditions, as described below. Cells were incubated with Annexin V and propidium iodide for 10 min at room temperature and then analyzed using flow cytometry (FACSCalibur, BD, San Jose, CA, USA). Data obtained from flow cytometry were evaluated using the same technique described in a study by Bossy-Wetzel (20).

TUNEL assay. Apoptotic cells were determined using an ApoDirect DNA Fragmentation Assay kit per manufacturer's instructions (Biovision). Cells were fixed with 1% paraformaldehyde and then incubated with terminal deoxynucleotidyl transferase and FITC-dUTP for 60 min at 37˚C and counterstained with propidium iodide. Cells were then analyzed using flow cytometry.

Western blot was used to determine the expression of BID protein. Cells were homogenized in RIPA buffer. Protein concentrations were assessed using the DC protein assay (Bio-Rad, Hercules, CA, USA) with serum albumin as a standard. 10-45 μg of extracted proteins were subjected to SDS-PAGE electrophoresis on a 10% gel. After migration, proteins were transferred to a nitrocellulose membrane and incubated with 5% non-fat milk to block non-specific binding. The membranes were then exposed to specific anti-BID (1:1000, AbCam, Cambridge, UK) rabbit monoclonal antibodies overnight at 4˚C. Membranes were washed and exposed to peroxidase-conjugated anti-IgG secondary antibody (1:3000, Bio-Rad), and the antigen-antibody complex was visualized using an enhanced chemiluminescence detection system according to the manufacturer's instructions (Immun-Star HRP Substrate, Bio-Rad). The resulting films (MEDIX XBU, Foma, Hradec Králové, Czech Republic) were scanned with a computerized image-analyzing system (ElfoMan 2.0, Ing. Semecký, Prague, Czech Republic).

Caspase activity. Caspase-8 activity was measured using a caspases-8 assay kit according to manufacturer's instructions (Biovision). Briefly, cells were lysed in cell lysis buffer after incubation with VPA. Total protein (200 μg) were added to the reaction buffer, which contained IETD-pNA colorimetric substrate, and incubated for 2 h at 37˚C. Hydrolyzed pNA was detected using a VersaMax plate reader (Molecular Device Inc., Sunnyvale, CA, USA) at 405 nm.

Real-time PCR analysis. Total RNA was extracted from cells lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of the isolated RNA was verified using horizontal agarose gel electrophoresis and RNA quantity was measured using a BioMate 3 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Complementary DNA was

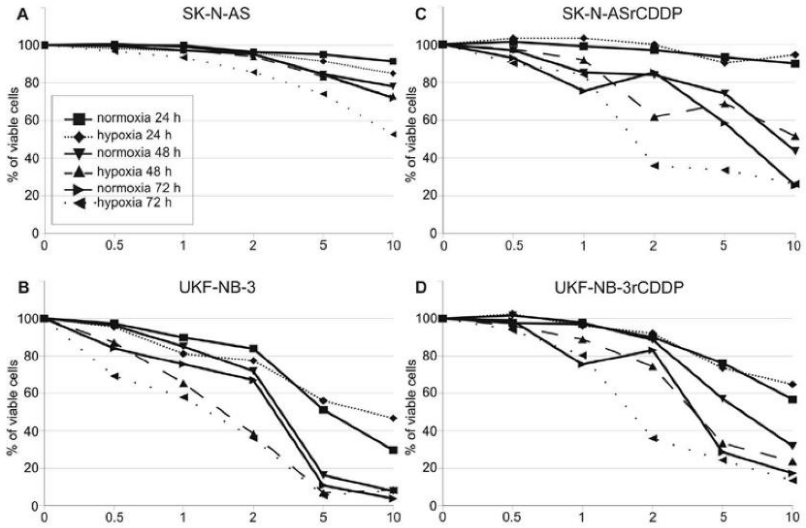

Figure 1. Cell viability measured as An^+/PI^- cells. Maternal cell lines SK-N-AS and UKF-NB-3 (A and B) and cell lines resistant to cisplatin (rCDDP) derived from them (C and D). Cells were grown under normoxic conditions for 24 h before administration of VPA.

Table I. Percentage of apoptotic cells measured as An^+/PI^- cells.

	Control		24 h		48 h		72 h	
	N (%)	H (%)	N (%)	H (%)	N (%)	H (%)	N (%)	H (%)
SK-N-AS (5 mM)	1.6	1.1	5.59	6.63	13.86	14.91	13.37	19.02
SK-N-ASrCDDP (5 mM)	2.59	3.99	7.51	11.51	13.61	16.82	40.63	53.47
UKF-NB-3 (2 mM)	6.3	6.66	10.13	9.29	21.49	20.70	19.39	16.57
UKF-NB-3rCDDP (2 mM)	2.89	2.73	7.81	7.43	9.12	16.60	7.22	25.49

Concentration of VPA was 2 mM for UKF-NB-3 and UKF-NB-3 resistant to cisplatin (rCDDP) and 5 mM for SK-N-AS and SK-N-ASrCDDP. Cells were grown for 24 h under normoxic conditions before administration of VPA and before being placed into a hypoxia chamber. Similar or even lower number of apoptotic cells under hypoxic conditions in UKF-NB-3 was due to shift from An^+/PI^- quadrant to An^+/PI^+ quadrant because of the high sensitivity of this cell line. Data from one representative experiment are shown.

synthesized from 500 ng of RNA using random hexamers and MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed using assays for vascular endothelial growth factor (VEGF), carbonic anhydrase-9 (CA9) and β-2-microglobulin (B2M) purchased from Generi Biotech (Hradec Kralove, Czech Republic). B2M was used as a reference gene. Relative expression and statistical significance were determined using REST-MCS software (Dr Michael Pfaffl, Germany) using the technique described by Pfaffl (21).

Results

VPA induces apoptosis under both normoxic and hypoxic conditions. We set up dose and time course experiments in order to prove efficacy of VPA under hypoxic and normoxic conditions. Concentrations of VPA ranged from 0.5 to 10 mM. Cells were grown under normoxic conditions for 24 h after plating and then VPA was added. Plates were then put into the hypoxia chamber, while control cells stayed under normoxic conditions. Apoptosis was determined using Annexin V (An) and propidium iodide (PI) staining at 24, 48 and 72 h after addition of VPA. We observed time- and dose-dependent apoptosis. UKF-NB-3 showed higher sensitivity to VPA compared to SK-N-AS (Fig. 1A and B). We did not observe any hypoxia induced resistance to VPA. Moreover, slightly more Annexin positive/propidium iodide negative cells (early apoptotic) and Annexin positive/propidium iodide positive cells (late apoptotic or necrotic) were seen under hypoxic conditions in both cell lines (Table I). For instance, 13.4% Annexin V single positive (An^+/PI^-) cells were observed after treatment with 5 mM VPA under normoxic conditions whereas 19.0% An^+/PI^- cells were observed in the hypoxia SK-N-AS cell line. Although the higher number of apoptotic cells, under hypoxic conditions, was not statistically significant, this trend was clearly obvious in all cell lines tested. This result indicates that VPA promotes apoptosis irrespective of oxygen tension and therefore should be equally

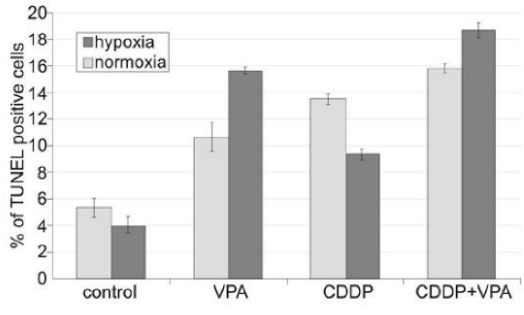

Figure 2. VPA synergizes with cisplatin (CDDP) under hypoxic conditions. UKF-NB-3 cells were exposed to 1 mM VPA and 1 μM CDDP at the same time. One representative experiment is shown.

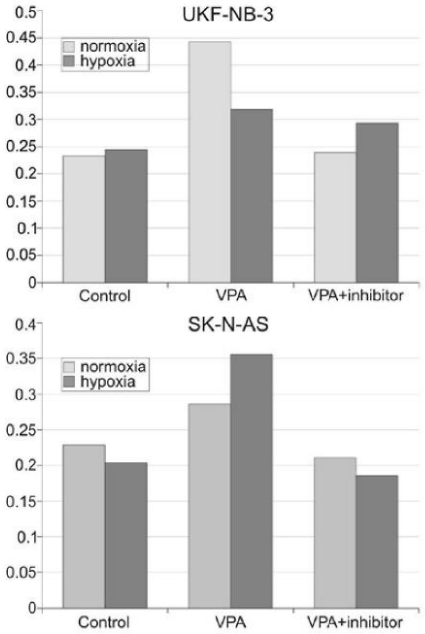

Figure 3. Caspase-8 activity and VPA treatment. VPA increased activity of caspase-8 in both parental cell lines (UKF-NB-3 and SK-N-AS).

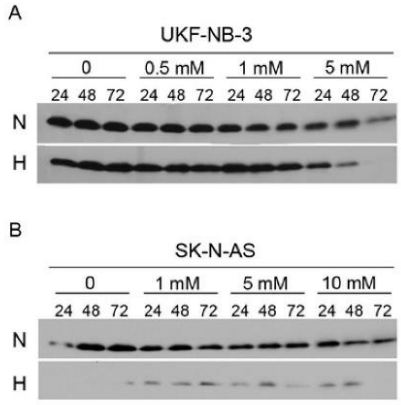

Figure 4. (A) Cells were incubated with different concentrations of VPA (0.5, 1 and 5 mM) for 24-72 h, this led to a decrease of full-length BID in a dose- and time-dependent manner in UKF-NB-3 under normoxic conditions (N), whereas it was cleaved only upon treatment with high concentration of VPA under hypoxic conditions (H). (B) Cleavage of bid was less expressed under normoxic conditions (N) in SK-N-AS. There was almost no detectable amount of bid under hypoxic conditions (H) in SK-N-AS.

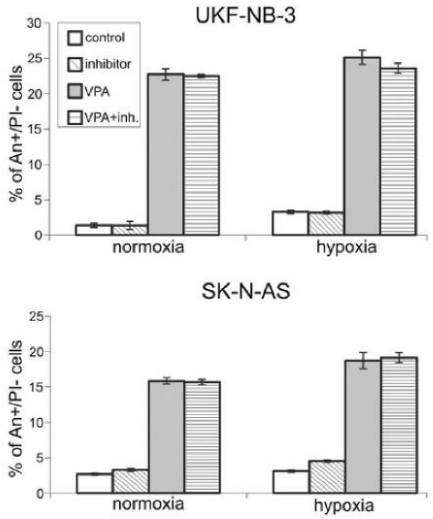

Figure 5. Inhibition of caspase-8 did not influence apoptosis in UKF-NB-3 or in SK-N-AS. Cells were preincubated with 2 μM of caspase-8 inhibitor for 15 min before VPA was added. Graphs shows number of apoptotic cells measured as An^+/PI^- cells.

efficient throughout the entire tumor volume. We performed the same experiments with cell lines resistant to cisplatin, which had been derived from SK-N-AS and UKF-NB-3, and obtained similar results (Fig. IC and D).

We also evaluated apoptosis using TUNEL assay in order to validate the data using an independent method. Both SK-N-AS and UKF-NB-3 cell lines revealed higher number of apoptotic cells (TUNEL positive) under hypoxic conditions than under normoxic conditions. The TUNEL results therefore supported the data obtained using An/PI staining (data not shown).

VPA has a synergistic effect with cisplatin. As mentioned in a previous section, VPA is capable of overcoming hypoxia resistance; however, its overall toxicity to NBL cells is quite poor considering that clinically achievable concentrations are <1 mM. Thus, we addressed the issue of whether small concentrations of VPA, which are clinically well tolerated, could be useful in overcoming hypoxia induced resistance to chemotherapeutic agents, such as cisplatin (CDDP), which are commonly used in HR NBL therapy.

Cells were treated with lower concentrations of VPA (1 mM) or CDDP (1 μM) alone and in combination. Apoptosis was assessed 24 h after administration of the drugs using a TUNEL assay. The degree of apoptosis induced by CDDP alone was diminished by hypoxic conditions, while VPA alone was

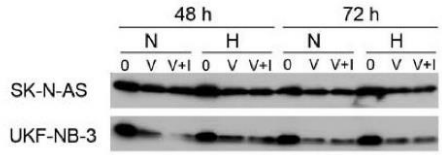

Figure 6. Cleavage of bid upon treatment with VPA (V) was not influenced by caspase-8 inhibitor (I). VPA (5 mM) was used for UKF-NB-3 and 10 mM for SK-N-AS.

more efficient under hypoxic conditions than under normoxic conditions. Cells administered as combination of VPA and CDDP showed a higher degree of apoptosis under hypoxic conditions (Fig. 2), suggesting not merely a synergistic effect for VPA and CDDP, but the added ability of VPA to overcome hypoxia-induced resistance to CDDP.

VPA activates caspase-8. To clarify whether VPA activates the receptor-mediated apoptotic pathway, we determined the activity of caspase-8. Cells were grown for 24 h and then 2 mM VPA was added to UKF-NB-3 cells and 5 mM was added to SK-N-AS cells. Caspase-8 activity was determined after 48 h of treatment. VPA increased the activity of caspase-8 in both cells lines (Fig. 3). Of note, caspase-8 activity was higher under hypoxic conditions in the SK-N-AS line, albeit only slightly. This discovery supports the above mentioned observations that showed VPA to be more effective under hypoxic conditions. This result also suggests that caspase-8 is the first caspase activated in the apoptotic cascade during VPA treatment, which is why we focused on the cleavage of the pro-apoptotic BID protein. Since BID is the substrate for caspase-8, its cleavage would clearly demonstrate the presence of activated caspase-8.

VPA initiates cleavage of BID. We addressed the question whether BID is cleaved to its active form, which could consecutively activate the mitochondrial apoptotic pathway. Cells were treated with different concentrations of VPA (0.5, 1 and 5 mM for UKF-NB-3 and 1, 5 and 10 mM for SK-N-AS) for 24, 48 and 72 h (Fig. 4A). We observed a time- and dose-dependent cleavage of BID in the UKF-NB-3 cell line under normoxic conditions. Whereas under hypoxic conditions BID was cleaved only when treatment with a relatively high concentration of VPA (5 mM). In the case of the SK-N-AS line, corresponding concentrations of VPA also led to a decrease of full-length BID albeit only marginally (Fig. 4B). This is in concert with the lower overall sensitivity of this cell line to VPA. We used 20 mM of VPA to confirm the dose-dependent manner of BID cleavage in SK-N-AS. This enormous concentration of VPA, exceeding IC_{50} values of SK-N-AS, decreased full-length BID, confirmed that BID cleavage caused by VPA was really dose-dependent and also demonstrated the poor sensitivity of this cell line to VPA. Together these data indicate that BID is cleaved upon VPA treatment and can subsequently transfer the apoptotic signal from the receptor-mediated to the intrinsic apoptotic pathway.

Inhibition of caspase-8 does not influence VPA-induced apoptosis. Caspases-8 has been reported as the main effector responsible for BID cleavage (22). We therefore inhibited caspase-8 using a specific inhibitor, z-IETD-fmk, to determine whether its inhibition was capable of blocking apoptosis induced by VPA. Cells were treated with 2 μM z-IETD-fmk for 15 min preceding VPA addition. Cell cultures were then incubated together with caspase-8 inhibitor and VPA for 48 h. We employed Annexin V/PI labeling to detect apoptotic changes. Surprisingly, overall viability measured as Annexin V/PI double negative cells was not increased in samples treated with the caspase-8 inhibitor. This inhibition did not influence the percentage of early apoptotic cells (An^+/PI^-) (Fig. 5) nor the percentage of necrotic/late apoptotic cells (An^+/PI^+). We did not observe a shift of Annexin V/propidium iodide double positive cells to the Annexin V single positive population, which would have signaled that caspase-8 inhibition only delayed apoptotic progress. Moreover, there were no differences between normoxic and hypoxic conditions. WB analysis showed that BID was cleaved regardless of caspase-8 inhibition (Fig. 6), which further points to a non-essential role for caspase-8 in apoptosis induction.

The effectivity of caspase-8 inhibition was also determined by measuring its activity after treatment with z-IETD-fmk. It was found that it was decreased to the level of untreated samples (data not shown); this confirmed that the concentration of z-IEDT-fmk used was sufficient. It is therefore evident that inhibition of caspase-8 has no significant effect on apoptosis and BID cleavage in NBL cell lines.

VPA decreases transcriptional activity of HIF-1. Hypoxia inducible factor 1 (HIF-1) influences the expression of many genes which can directly or indirectly inhibit apoptosis (23,24). HDACi have been described to attenuate stability of HIF-1 hence re-establishing sensitivity to apoptosis. We employed real-time PCR techniques for determination of mRNA levels of two well-described (25,26) HIF-1 target genes, VEGF and carbonic-anhydrase 9 (CA9) in order to assess whether VPA diminish HIF-1 transcriptional activity in NBL cells. Cells were preincubated with 2 mM VPA or 100 μM trichostatin A for 24 h and then placed into a hypoxia chamber for 3 and 8 h, respectively. Expression of both genes was significantly ($P<0.01$) decreased, in a time-dependent manner in both SK-N-AS and UKF-NB-3 cell lines (Fig. 7). VPA attenuated expression of VEGF 2.2-fold and CA9 4.2-fold compared with untreated samples of UKF-NB-3 after 3 h of hypoxia. Similar results were obtained for SK-N-AS cells. These results indicate that inhibition of HIF-1 by VPA participates with higher efficiency of VPA under hypoxic conditions by sensitizing NBL cells to apoptosis as discussed below.

Discussion

Hypoxia is regarded as a negative prognostic factor for solid tumors. It correlates with higher risk of cancer malignancy, resistance to radio- and chemotherapy and poorer patient outcomes (27,28). Hence, agents capable of overcoming hypoxia resistance would be beneficial for cancer treatment. We found that VPA was able to induce apoptosis under hypoxic conditions and moreover, was even more efficient than under normoxic conditions. To our knowledge this is the first observation of increased VPA efficacy under hypoxic conditions. Moderate

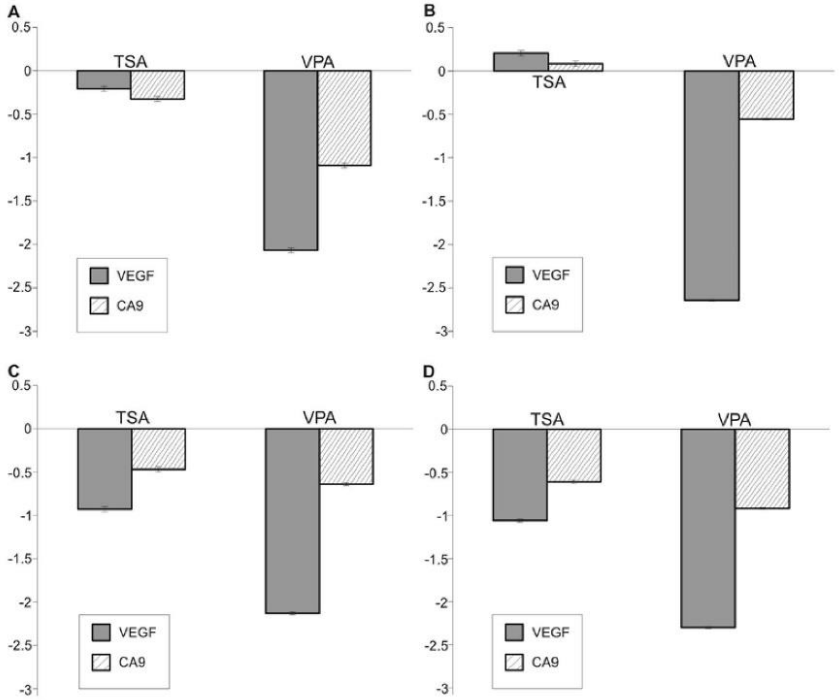

Figure 7. VPA and TSA decreased expression of HIF-1 target genes in both UKF-NB-3 (A and B) and SK-N-AS (C and D) after being cultivated for 3 h (A and C) and 8 h, respectively (B and D) under hypoxic conditions.

hypoxia (1% O_2) caused apoptosis resistance in hypoxic cells (29,30). Resistance can be caused by both HIF-1-dependent and -independent mechanisms. The role of HIF-1 as an anti- or pro-apoptotic transcription factor is still controversial (31). It is dependent on the severity and duration of hypoxia, HIF-1 phosphorylation status and cell type (32). HDACi have been previously reported to attenuate HIF-1 transcription activity (33). In concert with this observation, we showed that two HDACi (VPA and TSA) down-regulate expression of HIF-1 target genes VEGF and CA9 in hypoxic NBL cells. Several mechanisms can be proposed by which inhibition of HIF-1 by VPA promotes apoptosis under hypoxic conditions via attenuation of HIF-1 transcriptional activity.

p53 is usually said to be stabilized by HIF-1 (34) hence promoting apoptosis. However, it has been recently shown that HIF-1 can also antagonize p53 pro-apoptotic function through several mechanisms. First, HIF-1 increases expression of tyrosinase-related protein 2 (TRP2; also called DCT) which then down-regulates p53, thereby impeding apoptosis (35). Second, homeodomain-interacting protein kinase-2 (HIPK2) is an important co-activator of p53. HIF-1 increases proteasomal degradation of HIPK2 under hypoxic conditions, which eventually attenuates p53 pro-apoptotic function (36). Taken together, inhibition of HIF-1 by VPA can promote apoptosis by both re-establishing HIPK2 levels and attenuation of TRP2 expression.

AP-1 is another transcription factor induced by hypoxia. Recent studies showed that induction of AP-1 is also involved in hypoxia induced resistance to apoptosis (37-39). On the other hand, we do not suspect a role for AP-1 regarding the higher efficacy of VPA during hypoxic conditions, since it has been shown that VPA enhances AP-1 mediated gene expression in the SH-SY5Y NBL cell line (40). Therefore, VPA acts, most likely, as an inductor of AP-1 rather than a suppressor. Additionally, lithium chloride (LiCl) also increases transcription activity of AP-1, however, we did not observe higher efficacy of LiCl during hypoxia (data not shown). It is therefore probable that AP-1 has no significant role in VPA induced apoptosis during hypoxia. The actual contribution of different transcription factors to hypoxia-induced apoptosis resistance depends on several things (e.g. cell type, severity and length of hypoxia and/or type of pro-apoptotic stimuli); therefore a substantial role for HIF-1 is very likely in NBL cell lines.

Two points concerning the question of whether VPA should be used as monotherapy or in a combination regimen need to be addressed. First, despite the ability of VPA to overwhelm hypoxia resistance, sensitivity of some NBL cell lines, e.g. SK-N-AS in this study and UKF-NB-4, reported in our previous study (41) is quite low. For example, there was only a 20% induction of apoptotic cells by 5 mM VPA in SK-N-AS after 72 h, whereas 1 mM VPA has been reported to induce apoptosis in >50% of cells in some hematological malignancies (5). Second, plasma levels of VPA, in patients treated for epilepsy, usually do not exceed 0.7 mM and have minimal or no side effects in such concentrations. Serious adverse reactions are seen when the concentration exceeds 3.1 mM (42). It can be argued that unlike epilectic patients, where very long-term therapy is necessary, cancer patients

could tolerate short-term application of higher doses of VPA. Our measurement of apoptosis when cells were treated with VPA and CDDP together demonstrated that low concentration of VPA (1 mM) were enough to overcome hypoxia induced apoptosis resistance to CDDP while still maintaining low VPA toxicity. Based on this we see VPA, in NBL treatment, mainly used in combination regimens in which its low concentration would have minimal side effects, yet it would be able to synergize with other agents even in the hypoxic areas of a tumor.

BID is thought to be cleaved by caspase-8 upon activation of receptor mediated apoptosis. Truncated BID (tBID) then translocates from the cytosol to mitochondria where it promotes release of cytochrome c and caspase-9 which, in turn, forms apoptosome and activates executive caspase-3. However, BID can also be cleaved by caspase-3 and served as a self-amplification loop (22). We suspect that BID cleavage, during VPA treatment, is mediated by caspase-3, since inhibition of caspase-8 neither prevented BID cleavage nor influenced the number of apoptotic cells.

Although HDACi have been described to trigger apoptosis through both receptor mediated (43) and intrinsic pathways, the latter was shown to be dominant in NBL cells during VPA treatment (44). We also demonstrated this in our experimental setting. We further showed that mitochondrial activation is the first event in apoptosis induction and BID cleavage and caspase-8 activation were a consequence of the progressing apoptotic cascade. Notably, there was no difference in the pathway through which apoptosis proceeded relative to normoxic or hypoxic conditions and VPA treatment.

To conclude, we showed that VPA is effective in both normoxic and hypoxic conditions and can overcome hypoxia induced resistance to CDDP-induced apoptosis. Considering all its advantages (i.e. orally applicable, low toxicity, an already approved drug), VPA alone might be beneficial in NBL treatment keeping in mind that VPA alone failed to induce significant apoptosis in some NBL cell lines. However, VPA combined with conventional chemotherapeutic drugs should be much more effective and is worthy of consideration. Additionally, VPA seems to be a very suitable compound for continued research regarding hypoxia-induced resistance. We also presented a possible role for HIF-1 as it relates to the VPA mode of action, but the direct mechanisms by which it acts are unknown and need further elucidation.

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References

1. Brodeur GM: Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 3: 203-216, 2003.
2. Furchert SE, Lanvers-Kaminsky C, Juurgens H, Jung M, Loidl A and Frühwald MC: Inhibitors of histone deacetylases as potential therapeutic tools for high-risk embryonal tumors of the nervous system of childhood. Int J Cancer 120: 1787-1794, 2007.
3. Mossman D and Scott RJ: Long term transcriptional reactivation of epigenetically silenced genes in colorectal cancer cells requires DNA hypomethylation and histone acetylation. PloS One 6: e23127, 2011.
4. Santini V, Gozzini A and Ferrari G: Histone deacetylase inhibitors: molecular and biological activity as a premise to clinical application. Curr Drug Metab 8: 383-393, 2007.
5. Bokelmann I and Mahlknecht U: Valproic acid sensitizes chronic lymphocytic leukemia cells to apoptosis and restores the balance between pro- and antiapoptotic proteins. Mol Med 14: 20-27, 2007.
6. Fullgrabe J, Hajji N and Joseph B: Cracking the death code: apoptosis-related histone modifications. Cell Death Differ 1: 1238-1243, 2010.
7. Hrebackova J, Poljakova J, Eckschlager T, *et al*: Histone deacetylase inhibitors valproate and trichostatin A are toxic to neuroblastoma cells and modulate cytochrome P450 1A1, 1B1 and 3A4 expression in these cells. Interdiscip Toxicol 2: 205-210, 2009.
8. Cinatl J, Scholz M, Driever PH, *et al*: Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. Anticancer Drugs 7: 766-773, 1996.
9. Michaelis M, Suhan T, Cinatl J, Driever PH and Cinatl J: Valproic acid and interferon-α synergistically inhibit neuroblastoma cell growth *in vitro* and *in vivo*. Int J Oncol 25: 1795-1799, 2004.
10. Harris AL: Hypoxia - a key regulatory factor in tumour growth. Nat Rev Cancer 2: 38-47, 2002.
11. Vaupel P, Kallinowski F and Okunieff P: Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 49: 6449-6465, 1989.
12. Hussein D, Estlin EJ, Dive C and Makin GW: Chronic hypoxia promotes hypoxia-inducible factor-1alpha-dependent resistance to etoposide and vincristine in neuroblastoma cells. Mol Cancer Ther 5: 2241-2250, 2006.
13. Lara PC, Lloret M, Clavo B, *et al*: Severe hypoxia induces chemo-resistance in clinical cervical tumors through MVP overexpression. Radiat Oncol 4: 29, 2009.
14. Song X, Liu X, Chi W, *et al*: Hypoxia-induced resistance to cisplatin and doxorubicin in non-small cell lung cancer is inhibited by silencing of HIF-1alpha gene. Cancer Chemother Pharmacol 58: 776-784, 2006.
15. Giuntoli S, Rovida E, Barbetti V, Cipolleschi MG, Olivotto M and Dello Sbarba P: Hypoxia suppresses BCR/Abl and selects imatinib-insensitive progenitors within clonal CML populations. Leukemia 20: 1291-1293, 2006.
16. Huang LE, Bindra RS, Glazer PM and Harris AL: Hypoxia-induced genetic instability - a calculated mechanism underlying tumor progression. J Mol Med (Berl) 85: 139-148, 2007.
17. Brizel DM, Scully SP, Harrelson JM, *et al*: Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res 56: 941-943, 1996.
18. Hockel M, Schlenger K, Aral B, *et al*: Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 56: 4509-4515, 1996.
19. Hockel M, Schlenger K, Hockel S and Vaupel P: Hypoxic cervical cancers with low apoptotic index are highly aggressive. Cancer Res 59: 4525-4528, 1999.
20. Bossy-Wetzel E and Green DR: Detection of apoptosis by annexin V labeling. Methods Enzymol 322: 15-18, 2000.
21. Pfaffl MW, Horgan GW and Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36, 2002.
22. Yin X-M: Bid, a BH3-only multi-functional molecule, is at the cross road of life and death. Gene 369: 7-19, 2006.
23. Baek JH, Jang JE, Kang CM, Chung HY, Kim ND and Kim KW: Hypoxia-induced VEGF enhances tumor survivability via suppression of serum deprivation-induced apoptosis. Oncogene 19: 4621-4631, 2000.
24. Liu X-H, Yu EZ, Li Y-Y and Kagan E: HIF-1alpha has an anti-apoptotic effect in human airway epithelium that is mediated via Mcl-1 gene expression. J Cell Biochem 97: 755-765, 2006.
25. Lee J-W, Bae S-H, Jeong J-W, Kim S-H and Kim K-W: Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions. Exp Mol Med 36: 1-12, 2004.
26. Semenza GL: HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 88: 1474-1480, 2000.
27. Shannon AM, Bouchier-Hayes DJ, Condron CM and Toomey D: Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies. Cancer Treat Rev 29: 297-307, 2003.

28. Um JH, Kang CHD, Bae JH, *et al*: Association of DNA-dependent protein kinase with hypoxia inducible factor-1 and its implication in resistance to anticancer drugs in hypoxic tumor cells. Exp Mol Med 36: 233-242, 2004.
29. Graeber TG, Osmanian C, Jacks T, *et al*: Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379: 88-91, 1996.
30. Liu L, Ning X, Sun L, *et al*: Hypoxia-inducible factor-1 alpha contributes to hypoxia-induced chemoresistance in gastric cancer. Cancer Sci 99: 121-128, 2008.
31. Piret J-P, Mottet D, Raes M and Michielis C: Is HIF-1alpha a pro- or an anti-apoptotic protein? Biochem Pharmacol 64: 889-892, 2002.
32. Suzuki H, Tomida a and Tsuruo T: Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia. Oncogene 20: 5779-5788, 2001.
33. Kim SH, Jeong JW, Park JA, *et al*: Regulation of the HIF-1α stability by histone deacetylases. Oncol Rep 17: 647-651, 2007.
34. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV and Neckers LM: Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature 392: 405-408, 1998.
35. Sendoel A, Kohler I, Fellmann C, Lowe WS and Hengartner OM: HIF-1 antagonizes p53-mediated apoptosis through a secreted neuronal tyrosinase. Nature 465: 577-585, 2010.
36. Nardinocchi L, Puca R and Orazi GD: HIF-1α antagonizes p53-mediated apoptosis by triggering HIPK2 degradation. Aging 3: 33-43, 2011.
37. Flamant L, Notte A, Ninane N, Raes M and Michiels C: Anti-apoptotic role of HIF-1 and AP-1 in paclitaxel exposed breast cancer cells under hypoxia. Mol Cancer 9: 191, 2010.
38. Dong Z, Venkatachalam Ma, Wang J, *et al*: Up-regulation of apoptosis inhibitory protein IAP-2 by hypoxia. Hif-1-independent mechanisms. J Biol Chem 276: 18702-18709, 2001.
39. Piret J-P, Cosse J-P, Ninane N, Raes M and Michiels C: Hypoxia protects HepG2 cells against etoposide-induced apoptosis via a HIF-1-independent pathway. Exp Cell Res 312: 2908-2920, 2006.
40. Chen G, Yuan PX, Jiang YM, Huang LD and Manji HK: Valproate robustly enhances AP-1 mediated gene expression. Brain Res Mol Brain Res 64: 52-58, 1999.
41. Hrebackova J, Hrabeta J and Eckschlager T: Valproic acid in the complex therapy of malignant tumors. Curr Drug Targets 11: 361-379, 2010.
42. Catalano MG, Fortunati N, Pugliese M, *et al*: Valproic acid induces apoptosis and cell cycle arrest in poorly differentiated thyroid cancer cells. J Clin Endocrinol Metab 90: 1383-1389, 2005.
43. Nebbioso A Clarke N, Voltz E, *et al*: Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. Nat Med 11: 77-84, 2005.
44. Muhlethaler-Mottet A, Meier R, Flahaut M, *et al*: Complex molecular mechanisms cooperate to mediate histone deacetylase inhibitors anti-tumour activity in neuroblastoma cells. Mol Cancer 7: 55, 2008.