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Disertační práce

Molekulární mechanismy indukce apoptózy taxany u buněk nádorů prsu

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Michael Jelínek

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Klíčová slova: taxany, buňky nádorů prsu, kaspázy, proteiny rodiny Bcl-2, rezistence k taxanům, receptor ABCB1

Key words: taxanes, breast cancer cells, caspases, Bcl-2 family proteins, taxane resistance, ABCB1 receptor

Obsah

Abstrakt

Taxany jsou cytostatika rutinně používaná k léčbě solidních nádorů prsu, vaječníků, prostaty, hlavy a krku a dalších typů nádorů. Rezistence nádorových buněk k účinkům taxanů představuje vážnou komplikaci v použití taxanů k léčbě nádorů. Tato rezistence může být mimo jiné spojena se sníženou mírou indukce apoptózy v nádorových buňkách a nebo také se zvýšenou hladinou transportérů, které transportují taxany ven z buňky.

V této disertační práci jsme se pokusili: (1) Přispět k objasnění úlohy molekulárních mechanismů indukce apoptózy taxany v buňkách lidských nádorů prsu. Konkrétně to znamenalo přispět k objasnění úlohy iniciačních kaspáz 8, 9, a hlavně potom iniciační kaspázy 2. Dále přispět k objasnění úlohy exekučních kaspáz 3, 6 a 7 a vybraných proteinů rodiny Bcl-2. (2) Přispět k objasnění molekulárních mechanismů rezistence buněk lidských nádorů prsu k taxanům. Konkrétně to znamenalo popsat úlohu vybraných funkčních skupin struktury taxanů v navození a překonávání rezistence k taxanům a dále přispět k objasnění úlohy P-glykoproteinu (ABCB1 transportér) v rezistenci k jednotlivým taxanům.

(1) Zjistili jsme, že apikální kaspázou v indukci apoptózy taxany v buňkách nádorů prsu s původem v mléčné žláze je kaspáza 2. V aktivaci kaspázy 2 v těchto buňkách nehraje zásadní úlohu protein p53 ani komplex PIDDosom. Je však možné, že se kaspáza 2 aktivuje v důsledku transportu do cytosolu po rozpadu jaderného obalu. Aktivovaná kaspáza 2 poté aktivuje exekuční kaspázy 3 a 7. Důležitá je také vzájemná aktivace kaspáz 3 a 7, která vede k amplifikaci proapoptotického signálu. Kaspáza 9 je také aktivovaná kaspázou 2. Nezdá se tedy pravděpodobné, že je zde mitochondriální dráha pro indukci apoptózy klíčová. V buňkách s původem v duktu se aktivuje pouze iniciační kaspáza 8 a exekuční kaspázy 7 a 6. V těchto buňkách je tedy apoptóza pravděpodobně indukována alternativním mechanismem. Pokud jde o proteiny rodiny Bcl-2, indukce apoptózy taxany je v buňkách nádorů prsu spojena se zvýšením hladiny proapoptotického proteinu Bad. Dále jsme pozorovali jisté zvýšení hladin proapoptotických proteinů Bim a Bok. Naopak hladina antiapoptotického proteinu Bcl-2 klesá.

(2) Ukázali jsme, že rezistenci k taxanům lze u buněk nádorů prsu navodit jenom proti takovým taxanům, které mají v pozicích C3´ a C3´N fenylové skupiny (např.

klinicky používaný paclitaxel). Pokud je alespoň jedna fenylová skupina v pozicích C3´ a C3´N nahrazena nearomatickou skupinou, nelze k takovému taxanu rezistenci navodit. Tyto deriváty překonávají rezistenci nádorových buněk k taxanům s fenyly v obou pozicích C3´ a C3´N. Dále jsme zjistili, že taxany s fenyly v obou zmíněných pozicích mají vysokou afinitu k vazebnému místu ABCB1 transportéru a jsou tak ABCB1 transportérem účinně transportovány ven z buňky. Pokud je ve zmíněných pozicích alespoň jedna nearomatická skupina, je vazebná afinita taxanu k ABCB1 transportéru nižší a taxan není účinně transportován ven z buněk. K takovému taxanu pak nelze navodit rezistenci.

Můžeme tedy shrnout, že indukce apoptózy taxany se může lišit mezi jednotlivými typy buněk nádorů prsu v závislosti na jejich původu. V některých pak hraje zásadní úlohu kaspáza 2 jako apikální kaspáza a důležitou úlohu zde hrají některé proteiny rodiny Bcl-2. Rezistenci lze navodit pouze k takovým taxanům, které mají v obou pozicích C3´ a C3´N fenyl. Pouze takové taxany jsou účinně transportovány ven z buněk ABCB1 transportérem, který má tak zásadní úlohu v navozené rezistenci k těmto taxanům.

Abstract

Taxanes are cytostatic routinely used for the treatment of solid breast, ovarian, prostate, head and neck tumors and other types of tumors. Resistance of tumor cells to the effect of taxanes represents serious obstacle for the employment of taxanes in the treatment of tumors. This resistance can be associated, among other things, with lower rate of apoptosis induction in cancer cells or also with increased level of transporters transporting taxanes out of the cell.

In this PhD thesis we tried: (1) to contribute to elucidation of the role of molecular mechanisms of apoptosis induction by taxanes in cells of human breast cancer. Specifically, it meant to contribute to elucidation of the role of initiator caspase -8 a -9 and mainly of initiator caspase-2. Next, to contribute to elucidation of the role of executioner caspase -3 - 6, and -7 and selected proteins of the Bcl-2 family. (2) To contribute to elucidation of molecular mechanisms of resistance of human breast cancer cells to taxanes. Specifically, it meant to describe the role of selected functional groups in taxane structure in bringing about and overcoming resistance to taxane and next to contribute to elucidation of the role of P-glycoprotein (ABCB1 transporter) in the resistance to individual taxanes.

1) We found that caspase-2 represents apical caspase in apoptosis induction by taxanes in breast cancer cells originated from mammary gland. Protein p53 as well as PIDDosome complex do not play crucial role in the activation of caspase-2 in these cells. It is possible that caspase-2 is activated due to its transport into the cytosol after nuclear envelope disintegration. Activated caspase-2 subsequently activates executioner caspase -3 and -7. Mutual activation of caspase -3 a -7 is important for the amplification of pro-apoptotic signal. Caspase-9 is also activated by caspase-2. Thus, it does not seem probable that the mitochondrial pathway is crucial for apoptotic induction. In cells with ductal origin, only inititiator caspase-8 and executioner caspase-7 and -6 are activated. Therefore, apoptosis is probably induced by an alternative mechanism in these cells. Concerning Bcl-2 family proteins, apoptosis induction by taxanes in breast cancer cells is associated with increased level of pro-apoptotic protein Bad. Next, we observed some increase in levels of pro-apoptotic proteins Bim a Bok. On the contrary, the level of anti-apoptotic protein Bcl-2 decreases.

2) We showed that the resistance to taxanes could be brought about in breast cancer cells only to such taxanes that have phenyl groups at C3´ a C3´N positions (e.g. clinically used paclitaxel). If at least one of phenyl groups at C3´ a C3´N positions is replaced by a non-aromatic group, it is impossible to bring about resistance to such taxane. These derivates overcome resistance of cancer cells to taxanes with phenyl groups at both C3´ a C3´N positions. Next we found that taxanes with phenyl groups at both mentioned positions have high affinity to binding site of ABCB1 transporter and thus they are effectively transported by ABCB1 transporter out of the cell. When there is at least one non-aromatic group at mentioned positions, the binding affinity of taxane to ABCB1 transporter is lower and the taxane is not effectively transported out of the cells. Then it is impossible to bring about resistance to such taxane.

We can summarize that the induction of apoptosis by taxanes can differ between individual breast cancer cell types depending on their origin. In some types, caspase-2 plays crucial role as an apical caspase, and some proteins of the Bcl-2 family play an important role here. Resistance can be brought about only to those taxanes having phenyl groups at both C3´ and C3´N positions. Only these taxanes are effectively transported out of cells by ABCB1 transporter, which has key role in bringing about resistance to these taxanes.

Seznam zkratek

ABCA - ATP binding cassete subfamily A
ABCB1 - ATP binding cassete subfamily B member 1
ABCBE - ATP binding cassete subfamily E
AIDS - acquired immune deficiency syndrome
AIF - apoptosis inducing factor
Apaf-1 - apoptotic protease activating factor 1
ANT - adenine nucleotide translocator
ATP - adenosine triphosphate
Bad - Bcl-2-associated death promoter
Bax - Bcl-2-associated X protein
Bak - Bcl-2 homologous antagonist killer
Bcl-2 - B-cell lymfoma-2
Bcl-xL - B-cell lymphoma-extra large
BH - Bcl-2 homology
Bik - Bcl-2 interacting killer
Bid - BH3 interacting-domain death agonist
tBid - truncated Bid
BIR - baculoviral IAP repeats
Bok - Bcl-2 related ovarian killer
BRCA1 - breast cancer coding protein 1
BRCA2 - breast cancer coding protein 2
CAD - caspase-activated DNase
iCAD - inhibitor of CAD
Cdk2 - cyclin-dependent kinase 2
Chk1 - checkpoint kinase 1
CARD - caspase activation and recruitment domain
CDC25 - cell division cycle protein 25
CYP3A4 - cytochrome P450 3A4
DISC - death-inducing signaling complex
DNA - deoxyribonucleic acid
DR3 - death receptor 3
ER - endoplazmatické retikulum
FADD - Fas-associated protein death domain
Fas - first apoptosis signal
FasL - Fas ligand

c-FLIP - cellular FADD-like-interleukin-1β converting enzyme-inhibitory protein
GDP - guanosine diphosphate
GTP - guanosine triphosphate
Her2/neu - human epidermal growth factor receptor 2/neu
HtrA2/Omi - high-temperature requirement factor A2/Omi
IAPs - inhibitors of apoptotic proteins
IAP1 - inhibitor of apoptotic proteins 1
IAP2 - inhibitor of apoptotic proteins 2
IBM - IAP binding motif
JNK - c-Jun NH_2-terminal kinase
MC - mitotic catastrophe
Mcl-1 - myeloid cell leukemia-1
MHCI - major histocompatibility complex I
MMP - mitochondriální membránový potenciál
NAIP - nucleotide-binding apoptosis inhibitory protein
NCCD - Nomenclature Committee on Cell Death
NK - natural killers
PARP - poly (ADP-ribose) polymerase
PCD - programmed cell death
PIDD - p53-induced death domain protein
PKB - protein kinase B
Plk1 - polo-like kinase 1
PTP - permeability transition pore
Puma - p53 upregulated modulator of apoptosis
Raf - rapidly accelerated fibrosarcoma
RAIDD - RIP-associated Ich-1/Ced-3-homologue protein with a death domain
Smac - second mitochondria-derived activator of caspases
TM - transmembrane
TNF - tumor necrosis factor
TRADD - TNF receptor type 1-associated death domain protein
TRAIL - TNF-related apoptosis-inducing ligand
VDAC - voltage-dependent anion channel
XIAP - X-linked inhibitor of apoptosis

1. Úvod

Nádory prsu jsou v rozvinutých zemích jedním z nejčastěji diagnostikovaných nádorových onemocnění. Přes stále se zlepšující úroveň protinádorové prevence a úspěchy v chirurgickém odstranění nádorů však někdy neexistuje jiná možnost, než přikročit k systémové terapii. Tou může být u některých typů nádorů antihormonální terapie, nebo protilátková terapie. V ostatních případech je běžně aplikována nespecifická chemoterapie, která je často jedinou možností léčby nádoru prsu v pokročilejších stádiích.

Pro chemoterapii nádorů prsu se obvykle používají dvě skupiny látek: 1) látky, které blokují replikaci DNA (*deoxyribonucleic acid*), např. anthracykliny (doxorubicin, epirubicin) a 2) mitotické jedy, které buňce nedovolí zrealizovat proces mitózy. Mezi nejdůležitější rostlinné mitotické jedy patří vincaalkaloidy (např. vinblastin) a taxany (např. paclitaxel).

Taxany jsou původně přírodní, dnes však už i syntetizované látky, které se používají v léčbě mnoha typů solidních nádorů, např. prsu, vaječníků, plic nebo prostaty. Častým problémem spojeným s používáním taxanů je rezistence nádorových buněk k účinkům taxanů, a to vrozená nebo získaná v důsledku opakované aplikace taxanů. Rezistence k taxanům je obvykle podmíněna produkcí určitých proteinů, které ve výsledku neutralizují účinek taxanů. Jedním z nejčastěji studovaných proteinů, který je zapojen do rezistence k taxanům, je ABCB1 (*ATP binding cassete subfamily B member 1*) transportér (P-glykoprotein). Tento protein transportuje taxany ven z buněk, přičemž se spotřebovává ATP (*adenosine triphosphate*).

Významnou příčinou rezistence k taxanům může také být narušený mechanismus indukce apoptózy. Změna exprese nebo aktivity proapoptotických a antiapoptotických proteinů (např. kaspáz, proteinů rodiny Bcl-2, *B-cell lymfoma-2*) je totiž často spojená s rezistencí k taxanům. Jedním z těchto proapoptotických proteinů, u kterého byla popsána úloha v indukci buněčné smrti, je i kaspáza 2.

Cílem přípravy syntetických derivátů klasického taxanu paclitaxelu je získání takového taxanového preparátu, který bude účinně indukovat apoptózu i v buňkách rezistentních k účinku klasických taxanů. Zatím stále nedořešenou otázkou zůstává, zda jsou mechanismy indukce apoptózy nově syntetizovanými taxanovými deriváty stejné jako u klasických taxanů.

Je zřejmé, že pro účinnou indukci apoptózy taxany v nádorových buňkách je znalost podrobných molekulárních mechanismů indukce apoptózy a mechanismů rezistence k indukci apoptózy velmi důležitá.

2. Cíle

Disertační práce má dva hlavní experimentální cíle:

1) Přispět k objasnění molekulárních mechanismů indukce apoptózy taxany v buňkách lidských nádorů prsu:

- **Přispět k objasnění úlohy kaspázy 2**
- **Podrobněji charakterizovat úlohu iniciačních kaspáz 8 a 9 a exekučních kaspáz 3, 6 a 7**
- **Přispět k objasnění úlohy některých proteinů rodiny Bcl-2**

2) Přispět k objasnění molekulárních mechanismů navozené rezistence buněk lidských nádorů prsu k účinkům taxanů:

- **Popsat úlohu vybraných funkčních skupin struktury taxanů v navození a překonávání rezistence k taxanům**
- **Přispět k objasnění úlohy P-glykoproteinu (ABCB1 transportér) v rezistenci k jednotlivým taxanům**

3. Literární přehled

3.1 Nádory prsu

Nádory prsu jsou diagnostikovány až milionu žen ročně a stovky tisíc jich také tomuto onemocnění podlehnou. Po nádoru plic se jedná o nejčastější nádorové onemocnění u žen v rozvinutých zemích ***(Brufsky et al. 2015, Cerk et al. 2016)***.

Pro ilustraci, v České republice byl v roce 2012 nádor prsu diagnostikován u 7000 žen. To je sice relativně vysoká hodnota ve srovnání s jinými evropskými zeměmi, vždy je ale nutné vzít úvahu úroveň screeningu v daných zemích ***(Altobelli et al. 2017)***.

3.1.1 Charakterizace nádorů prsu

Nádory prsu se tradičně dělí do čtyř základních typů podle toho, jaké membránové receptory produkují nádorové buňky. Typy I a II jsou tvořené buňkami, které produkují receptor pro estrogen nebo receptor pro progesteron (až 70%). Typ III, jehož buňky produkují receptor Her2/Neu (*Human epidermal growth factor receptor 2/neu*), (cca 20%) a Typ IV, který tvoří buňky, které neprodukují ani jeden z těchto receptorů, tj. „triple negative" nádory ***(Brufsky et al. 2015)***. V posledních letech byly ale navrženy také alternativní možnosti třídění nádorů prsu, které jsou založeny na analýze většího souboru proteinů ***(Cerk et al. 2016)***.

3.1.2 Terapie nádorů prsu

Pro léčbu nádorů produkujících receptory pro steroidní hormony (typ I a typ II) se používá tzv. antihormonální terapie. Ta spočívá v podávání inhibitorů aromatáz, které inhibují produkci steroidních hormonů, nebo modulátorů biologické aktivity hormonů, které se na hormony vážou, a tak je inhibují. Další možností je podávání látek, které snižují expresi receptorů pro hormony apod. ***(Brufsky et al. 2016)***. Uvádí se, že při včasném odhalení nádorového onemocnění je tento způsob léčby poměrně účinný.

Na druhou stranu, léčba nádorů produkujících Her2/neu (typ III) je poněkud problematická a ne tak účinná jako v předchozím případě. Nejčastěji se, obvykle

v kombinaci s dalšími chemoterapeutiky, používají komerčně syntetizované protilátky pertuzumab® a trastuzumab®, které inhibují receptory Her2/neu, a tím zastavují proliferační aktivitu nádorových buněk ***(Hurvitz et al. 2017)***.

Triple-negative nádory prsu (typ IV) jsou primárně léčeny nespecifickou chemoterapií, nejčastěji doxorubicinem a taxany ***(Visconti a Grieco 2017)***. Nespecifická chemoterapie se také používá jako sekundární volba, pokud jsou nádory I. až III. typu k výše popsaným typům léčby rezistentní.

3.1.3 Buněčné linie odvozené z nádorů prsu

Pro testování účinku chemoterapeutik, včetně účinku taxanů, jsou používány různé linie buněk nádoru prsu ***(Holliday and Speirs 2011)***.

Kromě svého původu (buňky mléčné žlázy vs buňky duktu) se modelové buněčné linie nádorů prsu liší především produkcí specifických proteinů. Jedná se o estrogenové a progesteronové receptory, receptor pro Her2/neu **(viz výše)**, produkty tumor supresorových genů BRCA1 (*breast cancer coding protein 1*) a BRCA2 (*breast cancer coding protein 2*), protein p53, ABCB1 transportér **(viz 3.5.1)**, proteiny účastnící se indukce apoptózy apod. ***(Pavlíková et al. 2014 a,b, Němcová-Furstová et al. 2016, Takaoka et al. 2018)***.

3.2 Apoptóza jako programovaná buněčná smrt

Smrt buňky může probíhat v rámci regulovaného procesu spuštěného na základě vnitřního stavu buňky, nebo jako odpověď na signál přicházející z vnějšího prostředí buňky.

Pokud se buňka na procesu buněčné smrti aktivně nepodílí, hovoříme o neregulované buněčné smrti neboli nekróze. Nekróza je obvykle způsobena náhlou změnou fyzikálních nebo chemických podmínek (teplota, osmotické podmínky, hypoxie), případně některými jedy. V nekrotické buňce se zvětšuje celkový objem buňky, zatímco chromatin a jádro zůstává intaktní. Nakonec dochází k porušení plazmatické membrány a intracelulární látky se uvolňují do okolí, kde mohou vyvolávat zánětlivou odpověď ***(shrnuto ve Furuse et al. 2015)***.

Pokud se na buněčné smrti podílí buněčné proteiny v rámci více či méně regulovaných mechanismů, označujeme takovou buněčnou smrt jako programovanou

(*programmed cell death, PCD*). Zřejmě nejdůležitější a nejlépe popsanou PCD je apoptóza.

V buňkách, ve kterých je indukována apoptóza, se spotřebovává energie ve formě ATP, dochází zde k produkci a aktivaci mnoha proteinů a chromatin je štěpen pomocí buněčných DNáz na krátké fragmenty. Nakonec se buňka zmenšuje a rozpadá se na apoptotická tělíska, která jsou posléze pohlcena fagocytujícími buňkami, takže nedochází k rozvoji zánětu jako u výše popsané nekrózy ***(shrnuto v Ulukaya et al. 2011).***

3.2.1 Typy programované buněčné smrti

Dle NCCD (*Nomenclature Commitee on cell death*) jsou čtyři nejdůležitější PCD:

1) Apoptóza indukovaná vnější dráhou - K indukci apoptózy dochází v důsledku aktivace specifických receptorů s doménou smrti v plazmatické membráně vazbou příslušných ligandů (FasL, *first apoptosis signal ligand*, TRAIL, *TNF-related apoptosis-inducing ligand*, TNF, *tumor necrosis factor*, apod.), **(viz 3.2.2.1)**.

2) Apoptóza indukovaná vnitřní dráhou - Proces, který je spojený s vytvářením pórů, mimo jiné proapoptotickými proteiny rodiny Bcl-2, ve vnější mitochondriální membráně a uvolněním cytochromu c do cytosolu **(viz 3.2.2.2)**.

3) Autofagická buněčná smrt - makroautofagie **-** Za fyziologických podmínek slouží k degradaci nepotřebných nebo poškozených organel, které jsou obaleny membránou a katalyticky rozloženy v autofagolyzozomech ***(Glick et al. 2010)***. Autofagie chrání v první řadě buňku před vyhladověním, protože umožní přežití buňky i v případě, že je v okolí nedostatek živin. Pokud však úroveň autofagie překročí určitou mez, může vést ztráta organel v buňce k indukci buněčné smrti ***(Codogno a Meijer 2005)***. V případě, že je zvýšena exprese antiapoptotických proteinů a současně snížena exprese proapoptotických proteinů, může být autofagie dokonce preferovaným typem buněčné smrti ***(Shimizu et al. 2004, Yu et al. 2004)***.

4) Mitotická katastrofa (*mitotic catastrophe, MC*) - MC je různými autory popisována buď jako specifický typ buněčné smrti indukovaný během mitózy, nebo jako děj, probíhající před indukcí vnitřní cesty apoptózy. Případně jako proces probíhající paralelně k indukci apoptózy. Důležitým morfologickým znakem mitotické katastrofy je přítomnost mnohojaderných buněk, nebo buněk s mikrojádry (specifické útvary složené vždy z několika chromozomů a zbytků jaderného

materiálu), které mají výrazně odlišnou morfologii od normálních buněk v mitóze i buněk v apoptóze ***(Castedo et al. 2004, Llovera et al. 2012)***. MC může být spuštěna během aberantní mitózy, která se v nádorových buňkách dosti často objevuje po aplikaci chemoterapeutik, např. taxanů ***(Castedo et al. 2004, Galluzzi et al. 2012, Llovera et al. 2012)***.

Byly popsány i další typy PCD probíhající pouze za specifických podmínek. Je to nekroptóza, anoikisis, entosis a i další méně detailně popsané typy PCD, které probíhají pouze v některých typech buněk (parthanatos, pyroptóza, netóza, a kornifikace), (***shrnuto v Galluzi et al. 2012***).

3.2.2 Apoptóza

Apoptóza, indukovaná vnější nebo vnitřní drahou, je v současnosti dosud nejlépe popsaným typem programované buněčné smrti. Můžeme se s ní setkat během vývoje jednodušších i složitějších organismů včetně člověka. Podobně se s ní můžeme setkat při fyziologické obnově buněk různých tkání, jako je epitel tenkého střeva nebo buňky krve. Dále je aktivována v buňkách s poškozenou DNA, nefunkčními organelami a obecně s takovým poškozením, které neumožňuje další přežití buňky. Je aktivována rovněž v nefunkčních buňkách, potenciálně nádorových buňkách, či v buňkách infikovaných intracelulárním patogenem ***(Hengartner 2000)***.

U mnohobuněčných organismů je indukce apoptózy v jednotlivých buňkách striktně regulována. Deregulace apoptózy je příčinou mnoha závažných onemocnění: Zvýšená míra apoptózy je příčinou AIDS (*acquired immune deficiency syndrom*), neurodegenerativních onemocnění, diabetu, hepatitidy, infarktu a dalších onemocnění. Snížená míra apoptózy může vést k vývojovým vadám, autoimunitním onemocněním, nebo k tvorbě nádorů.

3.2.2.1 Vnější dráha indukce apoptózy

Vnější dráha indukce apoptózy je indukována buňkami imunitního systému v infikovaných nebo nádorových buňkách, ale také během vývoje imunitního sytému, po ukončení procesu imunitní odpovědi, apod. Tato dráha může být spuštěna buď systémem perforinů a granzymů, nebo aktivací receptorů s doménou smrti.

Systém perforinů a granzymů využívají zejména T-lymfocyty a NK (*natural killer*) buňky pro indukci apoptózy v infikovaných a nádorových buňkách. Zvláště těch,

které jsou rezistentní k indukci apoptózy přes receptory s doménou smrti, nebo těch, které na svém povrchu neprezentují MHCI (*major histocompatibility complex I*) glykoproteiny. Prvním krokem indukce buněčné smrti je vytvoření perforinových kanálů v membráně cílových buněk. Těmito kanály se do buněk dostávají granzymy (obvykle granzym A a granzym B), které štěpí a aktivují kaspázy nebo přímo substráty smrti **(viz níže)**, ***(Martínez-Lostao et al. 2015, Voskoboinik et al. 2015)***.

Co se týká receptorů s doménou smrti, k nejdůležitějším zástupcům této proteinové rodiny patří Fas (*first apoptosis signal*) receptor, DR3 (*death receptor 3*), TNF receptor a další receptory, příslušné ligandy těchto receptorů jsou např. Fas ligand, TNF, nebo TRAIL ***(Lanni et al. 1997, Siegmund et al. 2017)***.

Receptory s doménou smrti jsou obvykle aktivovány vazbou příslušného ligandu. K aktivovaným receptorům se poté vážou adaptorové proteiny FADD (*Fas-associated protein death domain*) nebo TRADD (*TNF receptor type 1-associated death domain protein*), a dále iniciační prokaspázy 8 a 10, čímž dochází k formování aktivačního komplexu DISC (*death-inducing signaling complex*). V aktivačních komplexech jsou prokaspázy 8 a 10 štěpeny a aktivovány **(viz 3.2.3.1)**, ***(Cohen et al. 1997)***. Přestože je samovolné skládání komplexu DISC, a tedy i aktivace kaspáz 8 a 10 velmi nepravděpodobná, je zde ještě další úroveň regulace aktivace tohoto komplexu realizovaná vazbou proteinu c-FLIP (*cellular-FADD-like-interleukin-1β converting enzyme-inhibitory protein*) na komplex DISC, která tento komplex inhibuje ***(Scaffidi et al. 1999)***.

3.2.2.2 Vnitřní dráha indukce apoptózy

Vnitřní dráha apoptózy může být spuštěna mnoha stimuly: Poškozenou DNA, oxidativním stresem, v důsledku poškození cytoskeletu, infekcí buňky intracelulárním patogenem atd. Indukce vnitřní dráhy apoptózy se často účastní protein p53, ve většině případů se na indukci vnitřní dráhy apoptózy podílí také proteiny z rodiny Bcl-2, nezastupitelnou úlohu v apoptóze mají proteázy z rodiny kaspáz.

3.2.2.2.1 p53

Transkripční faktor p53 je produkt významného tumor-supresorového genu, jehož mutace jsou pozorovány až u 70 % nádorových buněk *in vitro* i *in vivo*. p53 je

v buňkách aktivován aktivačními fosforylacemi v důsledku hypoxie, intracelulární infekce, při snížení hladiny živin a nukleotidů, ale zejména pokud je poškozena buněčná DNA, nebo se v DNA objeví větší počet neopravených mutací.

V takovém případě aktivovaný protein p53 indukuje expresi proteinu p21, který se váže na komplex cyklinu E a cdk2 (*cyklin-dependentní kinázy 2*), a zastavuje tak buněčný cyklus na rozhraní G1 a S fáze ***(Levine et al. 1997)***. Pokud není DNA během G1/S bloku buněčného cyklu opravena, nebo pokud je poškození DNA nevratné, je p53 dále posttranslačně modifikován a jako klíčový proapoptotický transkripční faktor zvyšuje expresi proapoptotických proteinů PIDD (*p53-induced death domain protein*), Bax (*Bcl-2-associated X protein*), Puma (*p53 upregulated modulator of apoptosis*) a Fas receptoru ***(Tinnel a Tschopp 2004, Roos and Kaina 2013)***. Kromě regulace genové exprese proapoptotických proteinů se může p53 také vázat přímo na protein Bcl-2, uvolňovat ho z komplexu Bcl-2/Bax a přispívat tak k uvolňování cytochromu c z mitochondrií a k indukci apoptózy vnitřní cestou **(viz 3.2.2.2.2)**, ***(Deng et al. 2006)***. Výsledně je tedy v buňce indukována apoptóza a případné tumorogenní mutace v DNA tak nejsou přeneseny do další generace buněk.

Pro případnou chemoterapii je důležitý fakt, že v nádorových buňkách s neaktivním p53 je obtížné indukovat apoptózu látkami, které poškozují DNA, neboť v takových buňkách poškození DNA nevede k zastavení buněčného cyklu ani k indukci apoptózy **(viz výše)**, ***(Bacus et al. 2001)***.

3.2.2.2.2 Proteiny rodiny Bcl-2

Proteiny rodiny Bcl-2 (Bcl-2 proteiny) jsou klíčovými regulátory vnitřní cesty indukce apoptózy, neboť aktivita jednotlivých Bcl-2 proteinů rozhoduje o spuštění, dočasném pozastavení nebo inhibici procesu apoptózy. Bcl-2 proteiny mohou mít také další funkce, které s apoptózou přímo nesouvisí: Protein Bax urychluje průběh S fází buněčného cyklu, protein Bcl-2 prodlužuje G0 fázi a protein Mcl-1 (*myeloid cell leukemia-1*) blokuje buněčný cyklus na rozhraní S/G2 fáze ***(Deng et al. 2003, Zinkel et al. 2006, Ajabnoor et al. 2012, Gurkar et al. 2013)***.

Typickým strukturním motivem Bcl-2 proteinů je hydrofobní BH (*Bcl-2 homology*) doména, která slouží k vzájemné interakci jednotlivých Bcl-2 proteinů. Ve struktuře většiny Bcl-2 proteinů se také často nachází transmembránová (*TM*) doména, pomocí

které se Bcl-2 proteiny inkorporují do membrán mitochondrií a endoplazmatického retikula (***Kirkin 2004)***.

Tato proteinová rodina má více než 20 členů, které můžeme podle funkce rozdělit na proapoptotické a antiapoptotické ***(Gustafsson et al. 2007, Rodriguez et al. 2011)***. Proapoptotické Bcl-2 proteiny můžeme rozdělit na ty, jež tvoří kanály ve vnější mitochondriální membráně (mají tři BH domény) a na ty, které snižují aktivitu antiapoptotických proteinů (mají obvykle jednu BH doménu), ***(Rautureau et al. 2010)***.

Antiapoptotické Bcl-2 proteiny jsou klinicky významné protoonkogeny, neboť mutace zvyšující jejich hladinu nebo aktivitu mohou hrát významnou roli v rezistenci nádorových buněk k různým chemoterapeutikům, naopak proapoptotické Bcl-2 proteiny jsou důležité onkosupresory ***(Oakes et al. 2012)***.

Proteiny Bax, Bak (*Bcl-2 homologous antagonist killer*) a Bok (*Bcl-2 related ovarian killer*), které patří do první zmíněné podrodiny proapoptotických proteinů, se (obvykle ve formě monomerů) nacházejí v cytoplazmě nebo jsou inkorporované do vnější mitochondriální membrány. Protein Bok se také může nacházet v jádře nebo v membránách Golgiho systému ***(Youle a Strasser 2008, Westphal et al. 2011, Echeverry et al. 2013)***. Během indukce apoptózy vytváří tyto proteiny transmembránové póry ve vnější mitochondriální membráně ***(Cheng et al. 2001***, ***Yakovlev et al. 2004, Westphal et al. 2011)***. Těmito póry se poté translokuje cytochrom c, Smac (*second mitochondria-derived activator of caspases*) a další proapoptotické proteiny z mitochondrií do cytosolu **(viz 3.2.3.1)**.

Proteiny Bid (*BH3 interacting-domain death agonist*), Bim, Bad (*Bcl-2-associated death promoter*), Puma, Noxa, Bik (*Bcl-2 interacting killer*) a další jsou řazeny do druhé podrodiny proapoptotických proteinů, tzv. BH3-only proteinů. BH3-only proteiny obvykle snižují aktivitu antiapoptotických proteinů vazbou svojí BH3 domény na BH3 doménu těchto proteinů. Některé z nich ale mohou také přímo aktivovat proapoptotické proteiny se třemi doménami, popsané výše ***(Ley et al. 2005, Youle a Strasser 2008)***. Pravděpodobně nejlépe je popsaná funkce BH3-only proteinů Bad, Bid a Bim.

Protein Bad, který je jedním z proteinů účastnících se signální dráhy vedoucí k aktivaci proteinkinázy B (*PKB*), je aktivován např. v důsledku nedostatku živin v okolí buňky nebo při snížené proliferační signalizaci. V takovém případě je PKB

deaktivována, protein Bad je defosforylován a může inhibovat antiapoptotické proteiny Bcl-2 a Bcl-xL (*B-cell lymphoma-extra large*) a spouštět tak apoptózu ***(Chen et al. 2005, Quan et al. 2013)***.

Protein Bid může být aktivován v buňkách, ve kterých byl zastaven buněčný cyklus v důsledku poškození DNA ***(Zinkel et al. 2006)***. Hlavní funkcí proteinu Bid je ale propojení vnější a vnitřní dráhy indukce apoptózy. Bid je totiž obvykle proteolyticky štěpen a tím aktivován iniciačními kaspázami 8 a 10, případně kaspázou 2 ***(Ho et al. 2008, Pozzesi et al. 2014)***. Výsledkem štěpení proteinu Bid je aktivní forma proteinu tBid (*truncated Bid*), která je translokována z cytosolu do vnější membrány mitochondrií, kde se může plně projevit jeho proapoptotická aktivita ***(Kim et al. 2017)***.

Protein Bim je aktivován při nedostatku živin v okolí buňky, během poškození cytoskeletu, nebo při vývoji imunitního systému. Produkuje se obvykle v tzv. krátké formě, ve specifických případech i v delší a extra dlouhé formě. Nejčastěji je regulován různými buněčnými kinázami, např. JNK (*c-Jun NH_2-terminal kinase*) kinázou ***(Ley et al. 2005)***.

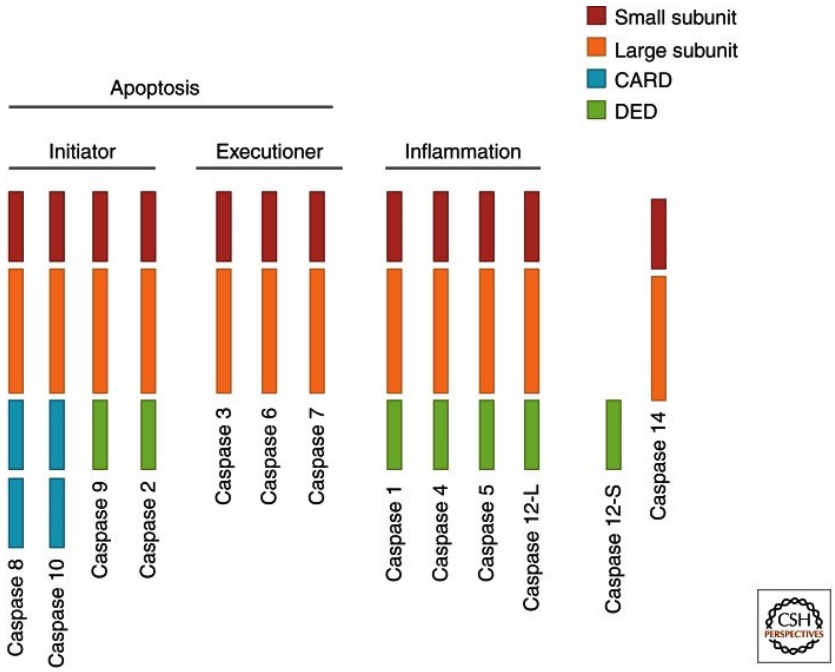
Antiapoptotické proteiny z rodiny Bcl-2, Bcl-2, Bcl-xL a další mají ve své struktuře čtyři BH domény ***(Youle a Strasser 2008)***. Proteiny z této podrodiny zabraňují indukci apoptózy vazbou svých BH domén na BH domény proteinů podrodiny proteinu Bax. Mohou ale využívat i jiné mechanizmy zastavení proapoptotických procesů ***(Kim et al. 2003)***.

Nejvýznamnější antiapoptotické Bcl-2 proteiny jsou Bcl-2 a Bcl-xL, které jsou svojí transmembránovou doménou zakotveny ve vnější membráně mitochondrií, kde brání tvorbě výše popsaných Bax/Bak kanálů. Protein Bcl-2 se nachází také v membránách endoplazmatického retikula (*ER*), kde se podílí na transportu vápenatých iontů ***(Youle a Strasser 2008)***.

3.2.3 Kaspázy

Kaspázy jsou proteázy, které mají v aktivním místě enzymu cystein, a které štěpí proteinový řetězec za aminokyselinou aspartátem. Rodina kaspáz se dělí na podrodinu iniciačních kaspáz (kaspáza 2, 9, 8, 10), exekučních kaspáz (kaspáza 3, 6, 7) a prozánětlivých kaspáz (kaspáza 1, 4, 5), **(*Cohen, 1997*, viz Obr. 1)**. Poněkud nejasná zůstává úloha kaspázy 4 jako mezičlánku stresu ER a apoptózy ***(Szegezdi et***

al. 2006, Rasheva a Domingos 2009). Funkce dalších kaspáz (např. kaspázy 12 a 14) dosud přesně popsána nebyla, hlodavčí kaspáza 12 je ale například schopná aktivovat kaspázu 7 v myších buňkách, a tím indukovat buněčnou smrt v důsledku stresu ER ***(Rao et al. 2004, Di Sano et al. 2006)***.



Obr. 1 Přehled kaspáz (převzato z McIlwain et al. 2013)

3.2.3.1 Iniciační kaspázy

Iniciační kaspázy se obvykle aktivují v proteinových komplexech, které se formují v cytoplazmě. Hlavní funkcí iniciačních kaspáz je následná aktivace exekučních kaspáz.

Kaspáza 8, která má klíčovou úlohu v aktivaci vnější dráhy indukce apoptózy, se nachází ve formě neaktivní prokaspázy v cytoplazmě a obvykle se aktivuje v komplexu DISC **(viz 3.2.2.1)**. V komplexu DISC se kromě kaspázy 8 aktivuje i kaspáza 10 ***(Earnshaw et al 1999, Park et al 2004)***. Obě iniciační kaspázy se mohou alternativně aktivovat štěpením exekučními kaspázami ***(van Haefen et al. 2003, Jelínek et al. 2015)***. Aktivní formy kaspázy 8 a 10 štěpí, a tím aktivují kaspázu 3 **(viz 3.2.3.3)** a protein Bid **(viz 3.2.2.2.2), *(Park et al 2004)***.

Pro aktivaci vnitřní dráhy indukce apoptózy je zásadní aktivace kaspázy 9. Tato kaspáza je v buňkách produkována konstitutivně, podobně jako výše zmíněné kaspázy, ve formě neaktivní prokaspázy ***(Würstle et al. 2012)***. K aktivaci kaspázy 9

dochází v komplexu apoptozómu **(viz níže)** v důsledku uvolnění cytochromu c z mitochondrií.

Translokace cytochromu c z mitochondrií úzce souvisí s membránovým potenciálem na vnější mitochondriální membráně (*mitochondrial membrane potential, MMP*). Ten vzniká v důsledku transportu elektronů přes membrány mitochondrií v dýchacím řetězci. Různé proapoptotické stimuly snižují MMP, což může vést k tvorbě pórů ve vnější mitochondriální membráně, kterými mohou relativně volně procházet ionty i další látky. Póry v mitochondriální membráně můžeme rozdělit do dvou hlavních skupin: Póry tvořené proapoptotickými proteiny z Bcl-2 rodiny **(viz 3.2.2.2.2)** a tzv. PTP (*permeability transition pore*) póry. PTP póry se skládají ze dvou podjednotek, VDAC (*voltage-dependent anion channel*) a ANT (*adenine nucleotide transporter*). Proteiny VDAC a ANT nejsou v mitochondriích normálně v kontaktu, ale v důsledku aktivace proapoptotických proteinů Bcl-2 rodiny se k sobě přiblíží a vytvoří kompletní PTP pór ***(Kidd et al. 2002, Sugiyama et al. 2002)***. Vytvoří-li se ve vnější mitochondriální membráně póry, dochází k translokaci cytochromu c, ale také dalších proapoptotických proteinů (Smac, HtrA2/Omi, *high-temperature requirement factor A2/Omi*, endonukleázy G a AIF, *apoptosis inducing factor*) do cytosolu ***(Garrido et al. 2006, Jeong a Seol 2008)***.

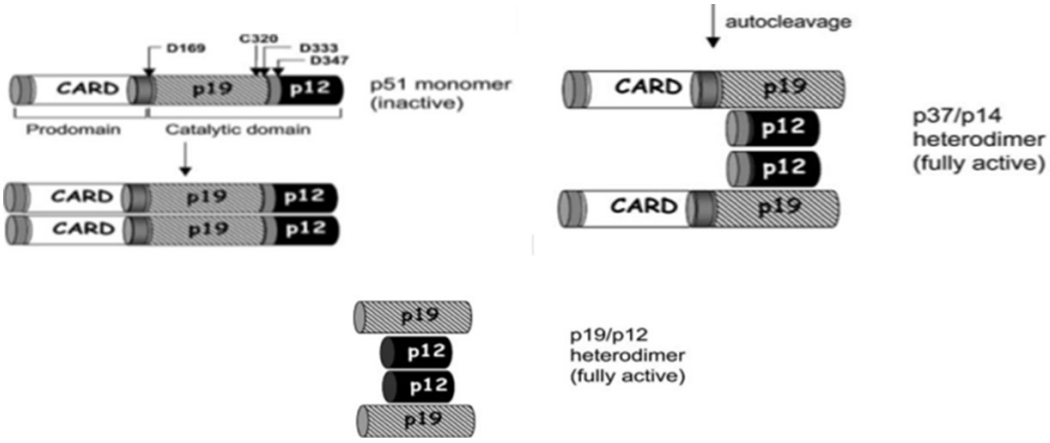
Pro řádnou indukci apoptózy kaspázou 9 je poté nezbytná vazba cytochromu c na cytosolický protein Apaf-1 (*apoptotic protease activating factor-1*), který následně váže svoji CARD doménou CARD doménu prokaspázy 9 ***(Pan a Nelson, 2007)***. Výsledně dochází k tvorbě apoptózomu, heptamerického komplexu skládajícího se ze sedmi molekul Apaf, sedmi molekul cytochromu c a sedmi molekul prokaspázy 9. V tomto komplexu se prokaspáza 9 aktivuje ***(Janssen et al. 2007)***. Aktivovaná kaspáza 9 poté štěpí exekutivní kaspázy 3 a 7, případně i exekutivní kaspázu 6 **(viz 3.2.3.3)**, ***(Bao and Shi, 2007, D´Anneo et al. 2010)***.

3.2.3.2 Kaspáza 2

Kaspáza 2, jedna z evolučně nejstarších kaspáz, je obvykle řazena mezi iniciační kaspázy, avšak může mít i roli v amplifikaci apoptotických signálů ***(Samraj et al. 2007)***. Ale také další, s apoptózou nesouvisející funkce, např. regulaci buněčného cyklu, diferenciaci buněk, nebo obranné reakce buňky na snížené množství živin v okolí ***(Vakifahmetoglu and Zhivotovski 2009, Fava et al. 2012)***.

V buňce je kaspáza 2 produkována ve formě monomeru, který se translokuje do jádra (v prvních 150 aa jsou lokalizovány dva jaderné lokalizační signály), a také do Golgiho aparátu. V cytosolu se ve větší míře objevuje až v pokročilých stádiích apoptózy ***(Mancini et al. 2000, Baliga et al. 2004, Jelínek et al. 2013)***.

Jedním z nejlépe popsaných způsobů aktivace kaspázy 2 během indukce apoptózy je autokatalytické štěpení v komplexu proteinů zvaném PIDDosom. Ten se skládá z proteinu PIDD **(viz 3.2.2.2.1)**, adaptorového proteinu RAIDD (*RIP-associated Ich-1/Ced-3-homologue protein with a death domain*) a prokaspázy 2 ***(Tinel and Tschopp 2004)***. PIDDosom je formován po vazbě CARD (*caspase activation and recruitment domain*) domény kaspázy 2 na CARD doménu proteinu RAIDD a následné vazbě domény smrti proteinu RAIDD na doménu smrti proteinu PIDD ***(Tinel et al. 2007, Baptiste-Okoh et al. 2008)***.



Obr. 2 Struktura kaspázy 2, schéma dimerizace a aktivace kaspázy 2 ***(převzato a upraveno z Baliga et al. 2004)***

Mimo to může být kaspáza 2 aktivována buněčnými kinázami, kdy dochází k dimerizaci a autokatalytickému štěpení dvou molekul prokaspázy 2 bez účasti proteinů RAIDD a PIDD ***(Mhaidat et al. 2008b)***. Kaspáza 2 se také může vázat do komplexu DISC v T a B lymfocytech, kde se posléze aktivuje. V komplexu DISC aktivovaná kaspáza 2 může štěpit proapoptotický protein Bid, a indukovat tak apoptózu, pravděpodobně následnou aktivací kaspázy 8 ***(Lavrik et al. 2006)***.

Prvním krokem aktivace kaspázy 2 je autokatalytické odštěpení p12 domény, poté je odštěpena CARD doména. Dvě velké p19 a dvě malé p12 domény výsledně tvoří

aktivní tetramer, který může štěpit příslušné substráty **(viz Obr. 2)**, ***(Baliga et al. 2004)***.

Aktivovaná kaspáza 2 štěpí nespecificky některé substráty, které štěpí i ostatní kaspázy, např. PARP *(poly ADP-ribose, polymerase*) a spektrin. Specifickým způsobem štěpí protein Golgin 160, což má pravděpodobně úlohu v rozpadu Golgiho aparátu během apoptózy ***(Mancini et al. 2000)***. Důležitým substrátem kaspázy 2 je také kaspáza 3 ***(Jelínek et al. 2015)***.

Kaspáza 2 je v buňkách často aktivována během mitotické katastrofy **(viz 3.2.1)**, ***(Manzl et al. 2009)***, dále v buňkách s poškozeným cytoskeletem nebo s mutovanou DNA ***(Zhivotovski et al. 2005, Ho et al. 2008)***.

3.2.3.3 Exekuční kaspázy

Exekuční kaspázy 3, 6 a 7 jsou primárně aktivovány štěpením iniciačními kaspázami. V rámci amplifikace apoptotického signálu se mohou sekundárně aktivovat vzájemným štěpením ***(Brentnall et al. 2013, Jelínek et al. 2015)***. Lokalizace exekučních prokaspáz je vesměs cytoplazmatická, přičemž aktivní exekuční kaspázy se mohou translokovat do jádra a dalších organel.

Aktivace exekučních kaspáz je zásadním nevratným krokem indukce apoptózy (viz níže) a proto je regulována na několika úrovních. V savčích buňkách jednu z velmi důležitých úrovní regulace aktivace představují inhbitory apoptotických proteinů (*inhibitors of apoptotic proteins, IAPs*). Do proteinové rodiny IAPs patří např. proteiny NAIP (*nucleotide-binding apoptosis inhibitory protein*), XIAP (*X-linked inhibitor of apoptosis*), IAP1 (*inhibitor of apoptotic proteins 1*) a IAP2 (*inhibitor of apoptotic proteins 2*) a survivin ***(Dubrez-Daloz et al. 2008)***.

IAP proteiny mají BIR (*baculovirus IAP repeat*) doménu, kterou se vážou na IBM (*IAPs binding motif*) motiv kaspáz 3 a 7, a tak je inhibují. Protein XIAP se navíc může vázat přímo do aktivního místa kaspáz ***(Pop and Salvensen 2009)***. Některé proteiny IAP rodiny mohou také snižovat hladinu proapopoptotických proteinů jejich ubiquitinací a následnou degradací v proteasomech ***(Morizano et al. 2005, Dubrez-Daloz et al. 2008)***.

Zmíněný protein survivin může být důležitým protonkogenem, neboť zvýšení hladiny survivinu je *in vitro* spojeno s rezistencí nádorových buněk k indukci apoptózy. Také v klinických studiích byla zvýšená hladina survivinu špatným

prognostickým znakem ***(Petrarca et al. 2011, Hu et al. 2015)***. Survivin se na rozdíl od většiny IAPs neváže přímo na kaspázy, ale na komplex proteinů IAP a Smac. Vazba survivinu na tento komplex uvolní IAPs z vazby s proteinem Smac a IAPs poté inhibují v nádorových buňkách apoptózu výše popsaným mechanismem ***(Song et al. 2003, Fulda et al. 2007)***.

Aktivované exekuční kaspázy štěpí tzv. substráty smrti. To jsou v první řadě důležité buněčné proteiny, které v apoptotické buňce nejsou dále potřebné: Enzymy, které opravují DNA (protein PARP), cytoskeletální proteiny (štěpení laminu vede k rozpadu jádra na jednotlivé váčky a štěpení aktinu a tubulinu k zakulacení buňky) nebo regulační proteiny (protein Rb), ***(Slee et al. 2001)***.

Do druhé skupiny důležitých substrátů smrti patří inhibitory proteinů, které se podílí na progresi pozdních fází apoptózy (exekuci apoptózy). Jedná se např. o protein iCAD (*inhibitor of CAD*), který je inhibitorem endonukleázy CAD (*caspase-activated DNase*), ***(Enari et al. 1998)***. Aktivace CAD, proteinu AIF a dalších příbuzných proteinů je následována mimo jiné kondenzací a štěpením buněčné DNA, což je nevratný krok, který vždy vede ke smrti buňky ***(Joza et al. 2001)***.

Kaspáza 3 je klíčovou exekuční kaspázou, neboť se podílí na degradaci DNA a strukturních proteinů v exekučních fázích apoptózy. Zásadním způsobem také amplifikuje apoptotické signály, neboť může štěpit, a tak aktivovat iniciační prokaspázy **(viz 3.2.3.1)**. K důležitým substrátům kaspázy 3 patří dále protein PARP, jehož štěpení je mimo jiné rutinně používáno pro detekci apoptózy v buňkách ***(Dawson et al. 2004)***.

Kaspázy 7 a 6 jsou zřejmě často aktivovány kaspázou 3, přestože přesná úloha kaspázy 3 v aktivaci těchto dvou proteáz zůstává nejasná ***(Yang et al. 2006)***. Mezi důležité substráty kaspázy 6 patří jaderný protein lamin, který udržuje jádra kompaktní. Kaspáza 7 se podílí na změnách tvaru buněk v průběhu apoptózy ***(Brentall et al. 2012)***.

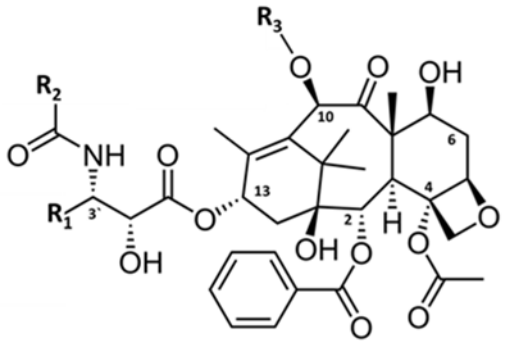
3.3 Taxany

Taxany byly objeveny v 60. letech 20. stol. v dřevinách patřících do čeledi tisovitých (Taxaceae), ***(Rowinsky et al. 1997)***. První taxan, jehož chemická struktura byla

detailně popsána, byl taxan paclitaxel, obsažený v jehlicích a kůře tisu pacifického (Taxus brevifolia), ale i v dalších druzích tisovitých dřevin ***(Wani et al. 1971)***.

3.3.1 Chemická struktura taxanů

Molekula paclitaxelu se skládá ze čtrnáctiuhlíkového baccatinového jádra III a několika funkčních substituentů připojených k různým uhlíkům baccatinového jádra ***(Ojima et al. 1994, Ojima et al. 1996)***. Zvlášť důležité pro navození a překonání taxanové rezistence jsou substituenty v poloze C3´, C3´N a C10 **(viz Obr. 3)**. Paclitaxel má v obou pozicích C3´ a C3´N (viz **R1** a **R2** na **Obr. 3**) fenylové zbytky, docetaxel má v pozici C3´ fenyl a v pozici C3´N terc-butoxycarbonyl. V pozici C10 (**R3** na **Obr. 3**) má paclitaxel acetát a docetaxel hydroxylovou skupinu. Nově připravené deriváty paclitaxelu mají v uvedených pozicích různé funkční substituenty **(viz 3.3.4)**, většina nových taxanů však neobsahuje v pozicích C3´ a C3´N ani jeden fenyl ***(Ojima et al. 1998, Ojima et al. 2000)***.



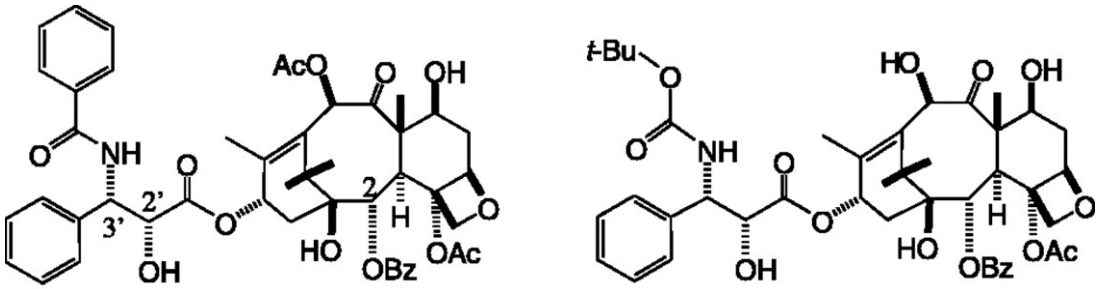
Obr. 3 Obecný vzorec taxanů s vyznačenými substituenty v pozicích C3´(R_1), C3´N (R_2) a C10 (R_3)

3.3.2 Klinické využití taxanů

Paclitaxel, s komerčním názvem Taxol, se používá pro léčbu nádorů vaječníků od roku 1992 a pro léčbu nádoru prsu od roku 1994. Od roku 1996 se pro léčbu nádoru prsu a plic používá také semisyntetický taxan docetaxel ***(Cortes et al. 2012, Joshii et al. 2014)***. Docetaxel byl připraven z přírodního prekurzoru z jehlic tisu *Taxus baccata* a patří k taxanům tzv. druhé generace **(viz Obr. 4)**. Paclitaxel a docetaxel jsou při léčbě nádorových onemocnění obvykle kombinovány s radioterapií a dalšími chemoterapeutiky, dosti často s cis-platinou ***(Choy 2001, Jennewein a Croteau, 2001)***. V současné době je v protinádorové léčbě, zatím převážně nádorů prostaty, u kterých proběhla primární léčba docetaxelem, také používán taxan druhé generace cabazitaxel (Jevtana ®), ***(Paller and Antonarakis 2011)***.

V devadesátých letech 20. stol. byla také připravena celá řada taxanů třetí generace, které jsou účinné v indukci buněčné smrti i u rezistentních nádorových linií. Někdy se tyto taxany třetí generace označují jako taxoidy ***(Ojima et al. 1996)***.

Paclitaxel **Docetaxel**



Obr. 4 Strukturní vzorce paclitaxelu a docetaxelu ***(převzato a upraveno z Hari et al. 2006)***

Aby se snížila cytotoxicita taxanů (nebo rozpouštědla), která bývá značná, jsou taxany kovalentně nebo nekovalentně vázány na různé typy nosičů. Jedná se například o hydroxyapatitové nanopartikule obsahující kyselinu olejovou, albuminové částice, poly-L-glutamát, nebo mastné kyseliny. Komplexy taxanu a nosiče se přednostně dostávají pouze do nádorových buněk a v ideálním případě tedy neohrožují přilehlou zdravou tkáň. Tyto systémy jsou intenzivně zkoumány, pilotní výsledky jsou zatím poměrně slibné ***(Singer 2005, Payne et al. 2006, Miele et al. 2009, Luo et al. 2010)***. Albuminové částice s paclitaxelem s komerčním názvem Abraxan se dokonce začínají používat v léčbě některých solidních nádorů (slinivky, prsu), (***Giordano et al. 2017, Zong et al. 2017***).

3.3.3 Interakce taxanů s mikrotubuly

Taxany se v buňkách reverzibilně vážou na β tubulin, a to jednak na β tubulin ve formě volných dimerů α a β tubulinu, ale především na β tubulin, který se nachází v polymerovaných mikrotubulech. *In vitro* taxany, v závislosti na použité koncentraci, indukují polymeraci volných tubulinových dimérů do tubulinových vláken, svazků, a dalších složitějších struktur ***(Jordan et al. 1993, Diaz a Andreu 1993, Rao et al. 1999)***.

Interakce taxanů a β tubulinu bude dále popsána na příkladu paclitaxelu, interakce taxanů druhé a třetí generace s β tubulinem jsou velice podobné. Vazba paclitaxelu k β tubulinu probíhá se stechiometrií jedna molekula paclitaxelu na jeden dimer α/β tubulinu. Paclitaxel se váže k N-konci β tubulinu jen několik aminokyselin od tzv. M-smyčky. Následně se mění dynamika konformace M-smyčky v několika krocích. Nejprve dochází k přerušení intramolekulárních vazeb v M-smyčce v jednotlivých monomerech β tubulinu, a poté dochází k tvorbě laterálních vazeb mezi jednotlivými tubulinovými monomery mezi M-smyčkou jednoho monomeru a H1-S2 smyčkou následujícího monomeru ***(Mitra a Sept 2008)***.

Klíčová pro indukci apoptózy taxany je vazba taxanů na polymerované mikrotubuly, která vede ke stabilizaci tubulinových vláken a následnému zastavení procesu depolymerace mikrotubulů *in vitro* i *in vivo* ***(Jordan et al. 1993, Jordan a Wilson 2004, Altmann a Gertsch 2007)***. Polymerované mikrotubuly jsou vysoce dynamická vlákna, jejichž polymerace a depolymerace je regulována vazbou GTP (*guanosine triphosphate*) a GDP (*gunosine diphosphate*). Za normálních podmínek dochází v polymerovaných tubulinových filamentech ke štěpení GTP na GDP a protofilamenta s navázaným GDP se následně rozpadají. Vazba paclitaxelu k β tubulinu v určitých ohledech připomíná konformaci β tubulinu s navázaným GTP, je ale ještě stabilnější, takže se jednotlivá tubulinová vlákna nejenom nerozpadají, ale ani nezakřivují a neohýbají ***(Elie-Caille et al. 2007, Mitra a Sept 2008)***. Je zajímavé, že struktura tubulinových filament je v přítomnosti paclitaxelu zřejmě poněkud odlišná od struktury normálních vláken (12 protofilament v přítomnosti taxanů vs 13 protofilament standardně), ***(Andreau et al. 1994)***.

Ne všechny nové taxany se ale chovají stejně jako paclitaxel, nový taxan SB-T-1213 v buňkách indukuje tvorbu nepravidelných tubulinových vláken a vazba jiného nového taxanu, IDN5109, k mikrotubulům vede k formování tubulinových vláken uspořádaných do plošinek. Oba taxany navíc stabilizují mikrotubuly v nižší koncentraci než paclitaxel ***(Ferlini et al. 2000, Jordan et al. 2002).***

Depolymerace mikrotubulů má v buňkách klíčovou funkci, mimo jiné v rozdělení chromozomů do dceřiných buněk během mitózy ***(Derry 1998, Yvon et al. 1999)***. V případě, že je depolymerace mikrotubulů tvořících mitotické vřeténko taxany zastavena, zastavuje se také buněčný cyklus, a to na rozhraní G2 a M fáze ***(Ehrlichová et al. 2005a)***. Přestože vede obvykle dlouhodobější stabilizace

mitotického vřeténka a následné zastavení mitózy k indukci apoptózy ***(Fan 1999)***, byly popsány i dvě alternativní cesty selhání mitotického aparátu ***(Fabbri et al. 2008)***. Výsledkem jedné byl vznik hypodiploidních buněk, výsledkem druhé vznik multijaderných buněk. Na druhou stranu, některé nové taxany, které se také vážou na mitotické vřeténko a indukují buněčnou smrt, buňky v mitóze nezastavují ***(Kovář et al. 2009)***.

3.3.4 Nové taxanové deriváty

Od druhé poloviny devadesátých let 20. stol. jsou syntetizovány a intenzivně testovány taxany druhé a třetí generace **(viz 3.3.2)**. Jedná se buď o taxany, které mají různé funkční skupiny připojené k molekule paclitaxelu, nebo o takové taxanové preparáty, kde jsou původní funkční skupiny paclitaxelu nahrazeny jinými funkčními skupinami ***(Geney et al. 2005, de Bono et al. 2010, Duran et al. 2014)***.

Hlavním cílem přípravy těchto nových taxanových derivátů je příprava takových derivátů, které budou indukovat buněčnou smrt i v nádorových buňkách rezistentních k paclitaxelu, případně k docetaxelu, neboli překonávání rezistence nádorových buněk k taxanům ***(Miller a Ojima 2001, Galletti et al. 2007)***. Mezi nejúčinnější taxany třetí generace patří taxoidy připravené na univerzitě Stony Brook v New Yorku v pracovní skupině prof. Ojimy, SB-T-1213, SB-T-1214, SB-T-1102, SB-T-1216 a další ***(Ojima et al. 1996, Ojima et al. 1998)***. Tyto taxany byly připraveny modifikacemi funkčních skupin na uhlíku 10 (C10) a uhlíků 3´ (C3´) a dusíku 3´ (C3´N) postranního řetězce připojeného k uhlíku 13 (C13) molekuly paclitaxelu, **(viz 3.3.1)**. Případně i dalšími modifikacemi molekuly paclitaxelu (modifikace funkční skupiny na uhlíku 2, viz ***Zheng et al. 2017***). Některé nové taxany obsahují v molekule taxanu heteroatomy fluoru, které by měly snižovat odbourávání taxanů a prodlužovat jejich biologický účinek, jedná se o taxany řady SB-T-12851 až 12854 ***(Pepe et al. 2009, Vobořilová et al. 2011, Ojima et al. 2016)***. Řada studií potvrdila, že mnohé tyto taxanové deriváty byly účinnější v indukci buněčné smrti v senzitivních i rezistentních nádorových buňkách ovárií, plic, střeva, prsu atd. ***(Ojima et al. 1996, 1998, 2000, 2008, Kovář et al. 2009, Vobořilová et al. 2011, Jelínek et al. 2013)***.

Účinky taxanů třetí generace SB-T-1214, SB-T-12854 a IDN5109 ***(Ferlini et al. 2005)*** byly testovány v zajímavých studiích Otové a kol. ***(2012)*** *in vivo* v krysích

lymfomech. Všechny testované taxany účinně snižovaly proliferaci krysích nádorových buněk. Taxany SB-T-1214 a IDN5109 v testovaných buňkách navíc snižovaly hladinu ABCB1 transportéru, který se může zásadně účastnit rezistence nádorových buněk k taxanům **(viz. 3.5.1)**. Nicméně se zde objevil typický problém spojený s používáním nových taxanů, a to je jejich vysoká cytotoxicita *in vivo* ***(Otová et al. 2012),*** **(viz 3.3.2)**.

3.4 Indukce buněčné smrti taxany

Typ PCD indukované taxany závisí na koncentraci použitých taxanů. Pokud jsou taxany k buňkám přidány ve vysoké koncentraci, dochází v buňkách k zastavení dynamiky mikrotubulů a následně k nekróze **(viz 3.2)**. U buněk, které nemají funkční protein Bcl-2 a/nebo funkční kaspázu 3, taxany poměrně často indukují mitotickou katastrofu **(viz 3.2.1)**, ***(Blajeski et al. 2001, Morse et al. 2005, Khongkow et al. 2016)***. V nádorových buňkách byla po aplikaci taxanů popsána i PCD závislá na uvolnění cathepsinů z lyzosomů, pyroptóza a buněčná smrt podobná autofagii **(viz 3.2.1)**, ***(Górka et al. 2005, Liao a Lieu, 2005, Mediavilla-Varela et al. 2009, Salinas et al. 2014)***.

Nejčastěji však taxany zastavují buněčný cyklus v mitóze a indukují apoptózu **(viz níže)**. Apoptóza se však také může spouštět nezávisle na zastavení buněčného cyklu ***(Fan 1999, Mailloux et al. 2001)***, zvláště pokud taxany indukují transport vápenatých iontů z ER do cytosolu a následně aktivují proteiny stresu ER ***(Pan a Gollahon, 2011, Tanimukai et al. 2013)***.

3.4.1 Úloha proteinu p53 v indukci apoptózy taxany

Aplikace taxanů na buňky vede často k aktivaci proteinu p53 **(viz 3.2.2.2.1)**, v některých nádorových buněčných liniích byl p53 aktivován dokonce tak nízkou koncentrací paclitaxelu, která nezastavovala buněčný cyklus na rozhraní G2/M fáze ***(Héliez et al. 2003)***.

Ve většině buněčných linií hraje ale p53 v indukci apoptózy pouze minoritní roli, neboť indukce apoptózy, přímo závislá na aktivaci proteinu p53, byla *in vitro* pozorována výjimečně ***(Drago-Ferrante et al. 2008)***. V buňkách s funkčním p53 spíše docházelo k zastavení buněčného cyklu v G2/M fázi a k indukci apoptózy

takovou koncentrací taxanů, která nebyla dostatečná pro indukci apoptózy v buňkách bez funkčního p53. Funkční p53 tedy v nádorových buněčných liniích snižoval koncentraci použitého paclitaxelu nutnou k indukci apoptózy ***(Das et al. 2001)***.

V řadě testovaných nádorových linií, které funkční p53 produkovaly, se však p53 po aplikaci taxanů výrazněji neaktivoval a buněčná smrt byla spuštěna nezávisle na aktivaci p53. To je v souladu s faktem, že v některých nádorových buňkách byla indukována apoptóza, přestože funkční p53 neprodukovaly ***(Chadderton et al. 2000, Das et al. 2001, Ehrlichová et al. 2005a)***.

Poněkud atypická situace byla pozorována v buněčné linii s původem v nádoru střeva, kde paclitaxel indukoval buněčnou smrt v přítomnosti i nepřítomnosti funkčního proteinu p53, ale buňky bez funkčního p53 vykazovaly odlišnou morfologii ***(Llovera et al. 2012)***.

3.4.2 Úloha kaspáz v apoptóze indukované taxany

Taxany v buňkách aktivovaly výše popsané iniciační i exekuční kaspázy **(viz 3.2.3)**. Ve specifických případech byla pozorována aktivace i dalších kaspáz, např. kaspázy 4, během indukce stresu ER taxany ***(Liao et al. 2008, Tanimukai et al. 2013)***.

3.4.2.1 Úloha iniciačních kaspáz 8, 10 a 9 v apoptóze indukované taxany

Kaspáza 8 se v mnoha typech nádorových buněk po aplikaci taxanů aktivovala. Jelikož se ale taxany dostávají do buněk difúzí, nepředpokládá se jejich interakce s receptory smrti (a tvorba komplexu DISC, **viz 3.2.3.1**), a tudíž musí být kaspázy 8 a 10 aktivovány v alternativních proapoptotických signálních drahách. Jedním z těchto popsaných alternativních mechanismů je aktivace kaspázy 8 v důsledku interakce taxanů s proteinem FADD, dalším je aktivace kaspázy 8 navázané na mikrotubuly (struktury, na kterou se vážou i taxany, **viz 3.3.3)**, ***(Mielgo et al. 2009)***.

V současné době se předpokládá, že indukce apoptózy, pozorovaná v některých nádorových liniích, která byla přímo závislá na aktivaci kaspázy 8, je spíše unikátní vlastností těchto buněk ***(Oyizu et al. 1999, van Haefen et al. 2003)***. Na druhou stranu, v celé řadě testovaných nádorových linií se kaspáza 8 účastnila různých proapoptotických amplifikačních drah ***(Ofir et al. 2002, Wang et al. 2004, Liao et al. 2008, Jelínek et al. 2015)***.

Kaspáza 10 obvykle po aplikaci taxanů aktivována nebývá, nicméně je nutné zmínit, že její aktivace není rutinně testována. Otázkou tedy zůstává, zda je dráha zahrnující aktivaci kaspázy 10, popsaná v leukemických buňkách, pouze unikátním znakem těchto buněk, nebo zda se jedná o rozšířenější jev ***(Park et al. 2004)***.

Naopak, v mnoha nádorových i nenádorových buněčných liniích byla aktivace kaspázy 9 zásadní pro indukci vnitřní dráhy indukce apoptózy taxany. Prvním krokem indukce apoptózy byla v tomto případě translokace cytochromu c a proteinu Smac do cytosolu probíhající v senzitivních nádorových buňkách ***(Carré et al. 2002, Kim et al. 2006, Mhaidat et al. 2007, Luo et al. 2010, Peng et al. 2016)***. Ale také v rezistentních nádorových buňkách po aplikaci paclitaxelu a docetaxelu ve vyšší koncentraci ***(Ehrlichová et al. 2005a)***, nebo v buňkách s aktivovanou dráhou stresu ER ***(Liao et al. 2008)***. Výsledkem translokace proapoptických faktorů z mitochondrií do cytosolu bylo formování apoptozómu a aktivace kaspázy 9 **(viz 3.2.3.1)**, ***(Perkins et al. 2000, Janssen et al. 2007, Liao et al. 2008)***.

U jiných typů nádorových buněk nebyl membránový potenciál mitochondrií narušen a cytochrom c se do cytosolu neuvolňoval ***(Vobořilová et al. 2011)***. Ve specifickém případě taxany indukovaly uvolňování proteinu Smac z mitochondrií nezávisle na poklesu MMP, tj. pravděpodobně jiným mechanismem než je uvolňován cytochrom c. Uvolnění proteinu Smac ale v tomto případě nemělo výrazný vliv na průběh buněčné odpovědi ***(von Haefen et al. 2003)***. V mnoha dalších typech nádorových buněk k indukci apoptózy závislé na kaspáze 9 však vůbec nedocházelo ***(Ofir et al. 2002, Jelínek 2015)***.

Úloha kaspázy 9 v indukci apoptózy taxany se tedy zřejmě velmi liší u jednotlivých typů buněk.

3.4.2.2 Úloha kaspázy 2 v indukci apoptózy taxany

V nádorových buňkách různých typů taxany prokazatelně aktivují kaspázu 2 **(viz 3.2.3.2)**, ***(Wang et al. 2004, Ehrlichová et al. 2005a, Mhaidat et al. 2007, Kovář et al. 2009, Luo et al. 2010, Vobořilová et al. 2011)***. K aktivaci kaspázy 2 docházelo také po koaplikaci paclitaxelu a dalších látek ***(Xu et al. 2011)***. Kaspáza 2 byla aktivována v rámci indukce mitotické katastrofy ***(Mediavilla-Varela et al. 2009)***, nebo během indukce apoptózy, a to pravděpodobně pomocí fosforylace prokaspázy 2 kinázou Jun **(*Mhaidat et al. 2007)***. Otázkou zůstává přesná úloha kaspázy 2, pokud

byla aktivována iniciační kaspázou 9 ***(Yuan et al. 2002)***, nebo zatím poněkud nejasným mechanismem prostřednictvím kaspázy 8 ***(Drago-Ferrante et al. 2008)***.
V každém případě byla v některých nádorových buňkách apoptóza indukovaná taxany na aktivaci kaspázy 2 přímo závislá ***(Fabbri et al. 2008, Jelínek et al. 2013)***. Důležitost kaspázy 2 v indukci apoptózy potvrzuje fakt, že některé nádorové i nenádorové buňky byly významně rezistentnější k účinkům taxanů, pokud neměly funkční kaspázu 2 ***(Mhaidat et al. 2007, Ho et al. 2008, Jelínek et al. 2013)***. Kaspáza 2 má zřejmě důležitou úlohu už v nejčasnějších stádiích apoptózy, neboť může indukovat konformační změny některých proapoptotických proteinů a spouštět tak vnitřní dráhu indukce apoptózy **(*Mhaidat et al. 2007)***.

3.4.2.3 Aktivace exekučních kaspáz 3, 7 a 6 taxany

Aktivace exekuční kaspázy 3 je velmi důležitá pro apoptózu indukovanou taxany v nádorových buňkách původem z Kaposhiho sarkomu, ovárií, močového měchýře, prsu, atd. ***(Yuan et al. 2002, Wang et al. 2004, Liao et al. 2008, Kovář et al. 2009, Flores et al. 2012)***. Kaspáza 3 byla aktivována také během koaplikace taxanů a dalších látek ***(Alvero et al. 2006, Sarkar et al. 2011)***. Poměrně specifická byla aktivace kaspázy 3 bez paralelní aktivace iniciačních kaspáz v nádorových buňkách původem z ovárií ***(Chen et al. 2005)***.
Jako vhodný model pro studium úlohy kaspázy 3 v rezistenci nádorových buněk k taxanům mohou být použity takové buňky, které přirozeně kaspázu 3 neprodukují (např. MCF-7), případně tyto buňky transfekované genem pro funkční kaspázu 3. Zatímco produkce kaspázy 3 v některých takových buňkách opravdu zvyšovala citlivost k indukci apoptózy paclitaxelem ***(Friedrich et al. 2001)***, v jiných nádorových liniích k tomu nedocházelo ***(Friedrich et al. 2001, Ofir et al. 2002)***. Předpokládá se tedy, že účinek taxanu na aktivaci kaspázy 3 je závislý na typu použité buněčné linie.
Zajímavá zjištění jsou popsána v práci von Haefen a kol. ***(2003)***. V leukemických buňkách, kde paclitaxel aktivoval vnitřní dráhu indukce apoptózy, aktivovala kaspáza 3 kaspázu 8, která poté štěpila protein Bid a přispívala tak k amplifikaci proapoptotického signálu. Tato pozitivní zpětná vazba vzájemné aktivace kaspázy 3 a 8 byla nezbytná pro účinnou iniciaci apoptózy v testovaných buňkách ***(von Haefen et al. 2003)***.

Kaspáza 7 se aktivuje v buňkách nádoru děložního čípku, prsu, nebo neuroblastomu ***(Nicolini et al. 2001, Tai et al. 2014, Jelínek et al. 2015)*** a kaspáza 6 v leukemických buňkách ***(Park et al. 2004)***, buňkách retinoblastomu ***(D'Anneo et al. 2010)*** nebo v buňkách nádoru prsu ***(Jelínek et al. 2015)***. Přesná funkce těchto dvou kaspáz v indukci apoptózy taxany zatím ale zcela objasněná nebyla.

3.4.3 Úloha proteinů rodiny Bcl-2 v apoptóze indukované taxany

Taxany v některých případech využívají homologie ve struktuře β tubulinu a proteinu Bcl-2, vážou se přímo na Bcl-2, a tím snižují jeho antiapoptotickou aktivitu ***(Ferlini et al. 2009)***. Obvykle je ale úloha Bcl-2 proteinů v indukci buněčné smrti taxany poměrně komplikovaná, protože ztráta funkce jednotlivých Bcl-2 proteinů může být suplována zvýšenou aktivitou ostatních Bcl-2 proteinů s podobnou funkcí **(viz 3.2.2.2.2)**.

Zvýšení hladiny antiapoptotických proteinů bylo mnohokrát popsáno jako příčina rezistence nádorových buněk k taxanům ***(Ibrado et al. 1997, Fauzee et al. 2012, Watanebe et al. 2013)***. Nejčastěji došlo v nádorových buňkách k overexpresi proteinu Bcl-2 nebo Bcl-xL ***(Yoshino et al. 2006, Mhaidat et al. 2007)***, **(viz 3.2.2.2.2)**. V některých nádorových buňkách však může k taxanové rezistenci nepřímo přispívat též snížená hladina proteinu Bcl-2 ***(Calastretti et al. 2014)***.

Proteiny Bcl-2 a Bcl-xL byly v některých rakovinných buňkách po aplikaci taxanů fosforylovány ***(Basu a Haldar, 2003, Yoshino et al. 2006, Fabbri et al. 2008)***, což vedlo k inhibici jejich aktivity a k indukci apoptózy. Na fosforylaci Bcl-2 se zásadně podílely buněčné kinázy Raf (*rapidly accelerated fibrosarcoma*) a JNK ***(Shitashige et al. 2001, Chun a Lee 2004)***. Mutační analýzy fosforylačního místa navíc prokázaly, že blokace fosforylace proteinu Bcl-2 zvyšovala rezistenci nádorových buněk k taxanům ***(Blagosklonny et al. 1996, Haldar et al. 1997, Srivastava et al. 1999)***, avšak ne ve všech testovaných buňkách ***(Noguchi 2006)***. Po aplikaci taxanů na nádorové buňky byly totiž popsány i fosforylace Bcl-2, které vedly naopak k aktivaci tohoto proteinu ***(Brichese et al. 2002)***. Důležitou otázkou zůstává, jak jsou asociovány inhibiční a aktivační fosforylace Bcl-2 a Bcl-xL s rezistencí nádorových buněk k taxanům.

Na druhou stranu, snížení hladiny proapoptotických proteinů Bax a Bak, nebo inhibiční fosforylace těchto proteinů, např. PKB, vedly prokazatelně k rezistenci

nádorových buněk k taxanům ***(Strobel et al. 1996, Aoudjit a Vuori 2001)***. Zajímavé je, že v rakovinných i nerakovinných buňkách taxany snižovaly poměr proteinů Bcl2/Bax ***(Wang et al. 2004, Janssen et al. 2007, Sharifi et al. 2014)***, nebo rozrušovaly vazbu proteinů Bax a Bcl-xL ***(Flores et al. 2012)***. V obou případech tvořil volný protein Bax póry ve vnější mitochondriální membráně a spouštěl tak apoptózu **(viz 3.2.2.2.2 a 3.2.3.1)**.

Poněkud nejasná zůstává úloha BH3-only proteinů Bid, Bim a Bad **(viz 3.2.2.2.2)**. Byly popsány nádorové buňky se zvýšenou, ale i sníženou hladinou těchto proteinů, s různým výsledným vlivem na přežívání buněk po aplikaci taxanů ***(Mhaidat et al. 2007, Craik et al. 2010, Fauzee et al. 2012, Miller et al. 2013, Luo et al. 2015)***. V buňkách melanomu byla například apoptóza navozená taxany prakticky nezávislá na funkci proteinu Bid ***(Mhaidat et al. 2007)***. Přestože v některých dalších typech nádorových buněk dochází po aplikaci taxanů ke štěpení proteinu Bid, buňky bez funkčního proteinu Bid před účinky taxanu výrazně ochráněny nejsou ***(Janssen et al. 2007, Ho et al. 2008)***.

V některých případech dochází po aplikaci taxanů také k defosforylaci BH3-only proteinů ***(Janssen et al. 2007, Savry et al. 2013)*** a rozvolnění jejich interakcí s dalšími proteiny rodiny Bcl-2, např. proteinem Bak ***(Sunters et al. 2003, Kutuk and Letai, 2010)***, a tím k inhibici apoptózy.

3.5 Rezistence nádorových buněk k taxanům

Rezistenci nádorových buněk k účinkům paclitaxelu a docetaxelu můžeme rozdělit na rezistenci vrozenou, která je komplikací pro primární léčbu taxany a rezistenci získanou, která je vážnou komplikací opakované léčby taxany.

V nádorových buňkách bylo dosud popsáno několik mechanismů taxanové rezistence: Rezistence spojená s čerpáním taxanů z buněk, s metabolickou degradací taxanů, s vazbou taxanů k mikrotubulům, s defektní indukcí apoptózy, případně s neúčinnou indukcí dalších typů PCD (autofagie apod.), ***(Murray et al. 2012, Wang et al. 2015, Visconti et al. 2017)***.

3.5.1 Rezistence spojená se zvýšenou expresí ABC transportérů

Rodina ABC transportérů zahrnuje několik desítek transmembránových proteinů, které přenášejí hydrofobní molekuly přes plasmatickou membránu buňky za spotřeby energie. Dělí se na podrodiny ABCA (*ATP binding cassete subfamily A*) až ABCE (*ATP binding cassete subfamily E*), přičemž do některých podrodin se řadí až deset proteinů ***(Eckford a Sharom 2009)***.

Klinicky významný je zejména transportér ABCB1, neboli P-glykoprotein, který čerpá taxany z nádorových buněk, a tak buňky chrání před jejich účinkem ***(Fojo et al. 2005, Hansen et al. 2015)***. Intracelulární koncentrace látek se v přítomnosti ABCB1 transportéru na plazmatické membráně může lišit až dvacetkrát ***(Ehrlichová et al. 2005b)***. ABCB1 může mít *in vitro* afinitu pro více cytostatik zároveň, např. k doxorubucinu a zároveň k paclitaxelu ***(Chadderton et al. 2000)***. V některých nádorových buňkách P-glykoprotein transportoval také současně taxany z první a druhé generace. Taxany třetí generace jsou proto navrhovány tak, aby nebyly P-glykoproteinem rozeznávány a transportovány ***(Li et al. 2014, Ojima et al. 2016)***.

Složitost této problematiky ilustruje skutečnost, že některé nové taxany, např. cabazitaxel, které jsou účinné v indukci buněčné smrti nádorových buněk rezistentních k paclitaxelu, jsou proteinem ABCB1 rozeznávány a transportovány. Je zde tedy reálná a klinicky důležitá možnost navození rezistence k cabazitaxelu a příbuzným látkám ***(Duran et al. 2015)***.

Dlouhodobou otázkou zůstává snížení exprese ABCB1 transportéru v nádorových buňkách přímo v těle pacienta ***(Duan et al. 2004)*** z důvodu chybějícího výkonného a zároveň bezpečného transfekčního systému.

3.5.2 Rezistence spojená s metabolismem taxanů

Taxany jsou odbourávány v játrech v mikrozomech hepatocytů, nerozložené metabolity jsou poté transportovány do žluče a vyloučeny z těla. Ve studiích ***Václavíkové a kol. (2003, 2004, 2006)***, kde byl studován metabolismus paclitaxelu a docetaxelu v lidských a krysích mikrozomech, se ukázalo, že zatímco docetaxel se metabolizoval v obou typech mikrozomů podobně, odbourávání paclitaxelu bylo druhově specifické. V mikrozomech byly navíc do určité míry metabolizovány i nové taxany (SB-T-1216).

Na odbourávání taxanů se významně podílí v rámci redoxně-metabolických drah proteiny z rodiny cytochromů, např. cytochrom CYP3A4 (*cytochrome P450 3A4*),

(Václavíková et al. 2006). Předpokládá se, že změny exprese jednotlivých cytochromů mohou významně přispívat k taxanové rezistenci. Pokud se produkují ve vyšší míře cytochromy, které nejsou účinné v odbourávání taxanů, kumulují se taxany v buňkách a působí na ně déle ***(Miyoshi et al. 2002, Garcia-Martin et al. 2006)***.

3.5.3 Rezistence spojená s vazbou taxanů na mikrotubuly

Nádorové buňky rezistentní k taxanům se mohou lišit od senzitivních buněk bodovou mutací v genu kódujícím β tubulin, produkcí různých tříd β tubulinu, nebo změnou exprese proteinů regulujících aktivitu mikrotubulů **(viz 3.3.3)**, ***(shrnuto v Orr et al. 2003)***. V buňkách rezistentních k taxanům byly také popsány změny v expresi α tubulinu, nebo v proteinech asociovaných s mikrotubuly ***(Alli et al. 2002, Martello et al. 2003, Smoter et al. 2011, Sun et al. 2015)***. Jedná se však spíše o minoritní jevy.

Mutace v genu pro β tubulin můžeme rozdělit na dvě skupiny. První jsou mutace ve vazebném místě pro taxany, které snižují afinitu taxanů k β tubulinu, a tak chrání buňky před jejich účinkem ***(Giannakakou et al. 2000, Goncalves et al. 2001, Yin et al. 2010)***. Druhým typem jsou mutace v tzv. leucinovém klastru, které vedou ke snížení interakcí mezi β tubulinovými jednotkami. A ačkoliv jsou tyto mutace často neslučitelné s přežitím buňky, mohou za určitých podmínek zabránit stabilizaci mikrotubulů taxany ***(Hari et al. 2006, Wiesen et al. 2007)***.

V buňkách obratlovců bylo dosud popsáno šest tříd β tubulinu (I až VI), které se dále dělí do několika podtříd (IVa, IVb, apod.).

V mnoha typech nádorových buněk rezistentních k účinkům klasických taxanů byla opakovaně pozorována vyšší produkce β tubulinu III. třídy. β tubulin III. třídy má totiž nejvyšší dynamiku skládání a rozpadu tubulinových vláken a pro svoji účinnou polymeraci vyžaduje vysokou koncentraci jednotlivých tubulinových podjednotek ***(Kamath et al. 2005, Gan et al. 2007)***. Zvýšená exprese tohoto typu tubulinu tedy snižuje schopnost taxanů účinně polymerovat mikrotubuly v nádorových buňkách a může vést k navození taxanové rezistence.

Testování pacientek s nádorem prsu v řadě studií ukázalo, že zvýšená exprese β tubulinu III. třídy opravdu predikuje horší prognózu léčbou klasickými taxany ***(Paradiso et al. 2005, Séve et al. 2005)***.

Snížení exprese β tubulinu III. třídy v rezistentních nádorových buňkách pomocí antisense oligonukleotidů sensitivitu nádorových buněk k účinkům klasických taxanů částečně obnovilo. Předpokládá se, že aby byly zachovány životně důležité funkce mikrotubulů, byly v těchto nádorových buňkách produkovány ostatní třídy β tubulinu, které paclitaxelem polymerovány jsou ***(Noguchi 2006, Tommasi et al. 2007)***. Naopak, zvýšení exprese β tubulinu III. třídy vedlo k rezistenci nádorových buněk také k účinkům docetaxelu, taxanu druhé generace, ale také nového taxanového derivátu cabazitaxelu ***(Mhaidat et al. 2008a, Duran et al. 2015)***.

Řešení naznačují výsledky studie, ve které v buňkách rezistentních k paclitaxelu, které produkují více β tubulinu III. třídy, indukoval taxan nové generace IDN5390 apoptózu. A to zřejmě proto, že se na rozdíl od paclitaxelu k β tubulinu III. třídy účinně neváže ***(Ferlini et al. 2005)***.

3.5.4 Rezistence spojená se sníženou indukcí buněčné smrti

Rezistence k taxanům spojená se sníženou indukcí PCD může souviset např. se zvýšenou hladinou autofagie na úkor snížení indukce apoptózy, a tedy výsledným přežíváním buněk v důsledku inhibice proapoptotických mechanismů ***(Ajabnoor et al. 2012, Veldhoen et al. 2013)***. Obecně tedy rezistence může souviset s aktivací takových buněčných procesů, které brání indukci PCD. Nejčastější příčinou tohoto typu taxanové rezistence jsou však mutace v genech, které kódují proteiny apoptotického aparátu **(viz 3.4)**.

Jedná se zejména o absenci nebo sníženou aktivitu kaspáz **(viz 3.4.2)**, zvýšení hladiny antiapoptotických proteinů rodiny Bcl-2 nebo snížení hladiny proapoptotických proteinů Bcl-2 rodiny **(viz 3.4.3)**. Taxanovou rezistenci způsobuje také zvýšení exprese proteinů rodiny IAP1 **(viz 3.2.3.3)**, tj. survivinu, ***(Zaffaroni et al. 2002)***, XIAPu ***(Gagnon et al. 2008)***, IAP2 ***(Tian et al. 2014)*** nebo snížení hladiny proapoptotického proteinu Smac ***(Fandy 2008)***.

4. Komentář k publikacím

4.1 Molekulární mechanismy indukce apoptózy v buňkách nádorů prsu novými deriváty taxanů

V rámci studií zahrnutých do této disertační práce jsme se pokusili přispět k objasnění molekulárních mechanismů indukce apoptózy u buněk nádorů prsu klasickým taxanem paclitaxelem a některými novými taxanovými deriváty. Jedním z těchto nových derivátů byl taxan SB-T-1216, který se od paclitaxelu liší substituenty na uhlících C10, C3´a C3´N, a který překonává navozenou rezistenci nádorových buněk k paclitaxelu, ***(Kovář et al. 2009)***.

Je již dlouho známé, že zavedení určitých heteroatomů do molekuly taxanu může zvyšovat jeho cytotoxicitu, snižovat přirozenou rezistenci nádorových buněk nebo chránit taxany před metabolickou degradací, a tak prodlužovat dobu jejich působení. Proto byly připraveny nové deriváty taxanů, strukturou podobné SB-T-1216, které mají ve své struktuře atom fluoru: SB-T-12851, SB-T-12852, SB-T-12853 a SB-T-12854 ***(Pepe et al. 2009)***. V několika studiích byl testován účinek těchto taxanů na růst a přežívání senzitivních i rezistentních nádorových buněk ***(Ehrlichová et al. 2005a, Vobořilová et al. 2011, Jelínek et al. 2017)*** a ukázalo se, že přestože tyto fluorované taxany indukují apoptózu podobně účinně, taxan SB-T-12854 inhibuje proliferaci nádorových buněk nejefektněji.

Nejnižší koncentrace klasického taxanu paclitaxelu a nových taxanů, které v testovaných nádorových buňkách účinně indukují apoptózu (téměř žádné živé buňky na konci inkubace s taxany), jsme určili jako 100 až 600 nM, v závislosti na testované buněčné linii (buňky mléčné žlázy vs buňky duktu). Účinek všech testovaných taxanů na proliferaci a přežívání nádorových buněk byl velmi podobný, i když nové taxany SB-T-1216 a SB-T-12854 byly v indukci apoptózy mírně účinnější než paclitaxel ***(Jelínek et al. 2013, viz kap. 5, str. 54, Jelínek et al. 2017, viz kap. 5, str. 88)***.

Dále jsme zjistili, že se molekulární mechanismy indukce apoptózy paclitaxelem, SB-T-1216 a SB-T-12854 příliš neliší, zásadnější rozdíly v indukci apoptózy jsme však pozorovali mezi jednotlivými buněčnými liniemi **(viz níže)**.

4.1.1 Úloha kaspázy 2 v indukci apoptózy taxany

Nejprve jsme se věnovali mechanismu aktivace kaspázy 2 (což může napomoci objasnění její funkce v indukci apoptózy), a poté jsme studovali vlastní úlohu kaspázy 2 v indukci apoptózy taxany **(viz 3.4.2.2)** s důrazem na úlohu kaspázy 2 v aktivaci dalších kaspáz.

4.1.1.1 Aktivace kaspázy 2

Kaspáza 2 se obvykle aktivovala 24 a 36 hodin po aplikaci taxanů, což jsme prokázali mimo jiné detekcí štěpené aktivní formy kaspázy 2 a Golginu 160, ***(Mancini et al. 2000, Jelínek et al. 2013, viz kap. 5, str. 55, 56, Jelínek et al. 2017, viz kap. 5, str. 88)***. Po ověření faktu, že se v buňkách nádoru prsu kaspáza 2 skutečně aktivuje, jsme se zaměřili na mechanismus její aktivace.

Jelikož aktivovaný protein p53 zvyšuje hladinu proteinu PIDD, který může být zásadní pro aktivaci kaspázy 2 v komplexu PIDDosomu ***(Tinel et al. 2007)***, a kaspáza 2 může naopak stabilizovat protein p53 ***(Oliver et al. 2011)***, zjišťovali jsme, zda se v buňkách nádoru prsu protein p53 na aktivaci kaspázy 2 po aplikaci taxanů podílí.

Ukázalo se, že pokud měly testované nádorové buňky funkční protein p53, byl po aplikaci testovaných taxanů aktivován, což jsme prokázali detekcí exprese proteinu p21 ***(Jelínek et al. 2013, viz kap. 5, str. 59)***. Aktivace proteinu p53 ale pravděpodobně zásadní význam pro aktivaci kaspázy 2 neměla, neboť se kaspáza 2 aktivovala i v buňkách bez funkčního proteinu p53 ***(Jelínek et al. 2013)***. Nezávislost indukce apoptózy taxany na přítomnosti funkčního p53 byla již dříve mnohokrát popsána ***(Ehrlichová et al. 2005a, Baptiste-Okoh et al. 2008, Vobořilová et al. 2011)***. Naše výsledky navíc ukazují, že přestože se po aplikaci taxanů p53 aktivuje, neindukuje v buňkách expresi proteinu Bax ani proteinu PIDD. To jsou proteiny klíčové pro indukci apoptózy v důsledku aktivace p53 ***(Tinnel et al. 2007, Jelínek et al. 2013, viz kap. 5, str. 59, Jelínek et al. 2017, viz kap. 5, str. 90)***. Je tedy možné, že se p53 účastní pouze zastavení buněčného cyklu v G2/M fázi, nebo v následující G1 fázi, do které se buňky po aplikaci taxanů mohou dostat překonáním mitotického bloku ***(Castedo et al, 2004, Kovář et al. 2009).***

Dále jsme prokázali, že se kaspáza 2 váže na protein RAIDD i na protein PIDD, což by naznačovalo, že je aktivována v komplexu PIDDosomu ***(Tinnel et al. 2007,***

Jelínek et al. 2013, viz kap. 5, str. 60). Překvapivě, snížení exprese proteinu RAIDD specifickou siRNA, které zabránilo formování PIDDosomu, buňky před působením taxanů významně neochránilo ***(Jelínek et al. 2013, viz kap. 5, str. 61)***. Buď se tedy kaspáza 2 do komplexu PIDDosomu váže, ale ke kompletní aktivaci dochází jiným mechanismem, např. v apoptosomu nebo v komplexu DISC ***(Lavrik et al. 2006, Kim et al. 2009, Olsson et el. 2009)***, nebo je po zformování PIDDosomu a neúčinné aktivaci kaspázy 2 v tomto komplexu aktivována nějaká paralelní dráha vedoucí k aktivaci kaspázy 2 ***(Shin et al. 2005, Mhaidat et al. 2008b, Viana et al. 2010)***.
Určitou roli v aktivaci kaspázy 2 může hrát také translokace z jádra, kterou jsme pozorovali v testovaných nádorových buňkách ***(Jelínek et al. 2013, viz kap. 5, str. 58***). Otázkou zůstává, nakolik jde o aktivní proces a nakolik se jedná o víceméně mechanický přesun kaspázy 2 do cytosolu v důsledku rozpadu jádra během aberantní mitózy.

4.1.1.2 Vliv inhibice exprese kaspázy 2 na indukci apoptózy a na aktivaci ostatních kaspáz

Snížení exprese kaspázy 2 pomocí příslušné siRNA částečně ochránilo testované buněčné linie před účinkem taxanů po 48 i 96 hodinách inkubace v médiu s taxany. Některé typy nádorových buněk dokonce pokračovaly ve výrazně zpomalené proliferaci i v přítomnosti taxanů v takové koncentraci, která v senzitivních buňkách indukovala apoptózu ***(Jelínek et al. 2013, viz kap. 5, str. 57)***. Snížení exprese kaspázy 2 vedlo také k výraznému snížení aktivace kaspázy 9 a kaspázy 3, a do určité míry také aktivace kaspázy 8 a 7. Takto výrazný efekt inhibice exprese kaspázy 2 na aktivaci dalších kaspáz jasně naznačoval, že kaspáza 2 představuje v buňkách nádorů prsu po aplikaci taxanu apikální kaspázu ***(Jelínek et al. 2013, viz kap. 5, str. 58)***.
Kaspáza 2 byla již dříve popsána jako apikální kaspáza např. v buňkách melanomu po aplikaci docetaxelu a v buňkách nádoru prostaty po aplikací partikulí s docetaxelem ***(Mhaidat et al. 2007, Luo et al. 2010)***. Zde však docházelo později k aktivaci mitochondriální dráhy v důsledku štěpení proteinu Bid kaspázou 2 ***(Lavrik et al. 2006, Vakifahmetoglu-Norberg et al. 2013)***. My jsme ale v testovaných buňkách štepení proteinu Bid nepozorovali ***(Jelínek et al. 2017)***, a ani zde nepředpokládáme klíčovou úlohu mitochondriální dráhy indukce apoptózy **(viz**

4.1.2.2). Kaspáza 2 má tedy zřejmě ve výše zmíněných nádorových buňkách poněkud odlišnou funkci.

V každém případě se kaspáza 2 v buňkách nádoru prsu významně podílí na indukci apoptózy, a to pravděpodobně jako apikální kaspáza přímou aktivací kaspáz 9, 3 a 7 (**viz Obr. 5**).

4.1.2 Úloha iniciačních kaspáz 8 a 9 a exekučních kaspáz 3 a 7 v indukci apoptózy taxany

Ačkoliv jsme prokázali (alespoň v některých případech) zásadní význam kaspazy 2, v indukci apoptózy mají důležitou úlohu i ostatní kaspázy.

4.1.2.1 Aktivace iniciačních a exekučních kaspáz

V nádorových buňkách se 24 a 36 hodin po aplikaci taxanů aktivovaly iniciační kaspázy 8 a 9, což bylo potvrzeno mimo jiné aktivací exekučních kaspáz **(viz níže),** ***(Vobořilová et al. 2011, Jelínek et al. 2015, viz kap. 5, str. 70, 73)***.

Co se týká aktivace kaspázy 8, není příliš pravděpodobné, že byla aktivována v komplexu DISC, spíše sloužila jako substrát pro ostatní kaspázy v alternativních drahách vedoucích k exekutivním fázím apoptózy ***(van Haefen et al. 2003, Shin et al. 2005, Mhaidat et al. 2007)***.

Kaspáza 9 byla aktivována dvěma způsoby. V jedné testované linii došlo k poklesu MMP a uvolnění cytochromu c do cytosolu. Předpokládáme tedy, že se zde kaspáza 9 částečně aktivovala v apoptozómu, přestože ne vždy je translokace cytochromu c s aktivací kaspázy 9 spojena, ***(viz Samraj et al. 2007)*** a částečně přímou aktivací kaspázou 2. V dalších testovaných liniích ale k výraznému poklesu MMP nedošlo a cytochrom c se do cytosolu neuvolňoval. Dá se tedy předpokládat, že k formování apoptozómu nedochází a kaspáza 9 je aktivována převážně kaspázou 2 **(viz Obr. 5)**, ***(Ehrlichová et al. 2005a, Vobořilová et al. 2011, Jelínek et al. 2015, viz kap. 5, str. 76, 77)***.

Pokud byla v nádorových buňkách přítomna funkční kaspáza 3, 24 a zejména 36 hodin po aplikaci taxanů se aktivovala. Ve všech testovaných liniích jsme zároveň detekovali aktivní formy kaspáz 6 a 7, které byly již dříve detekovány v nádorových buňkách různých typů po aplikaci taxanů ***(Mao et al. 2007, Fabbri et al. 2008, Drago-Ferrante et al. 2008, Jelínek et al. 2015, viz kap. 5, str. 70, 71)***, a to i

v buňkách nádoru prsu po aplikaci různých proapoptotických faktorů ***(Mooney et al. 2002)***. Štěpení proteinu PARP prokázalo, že se v testovaných buňkách exekuční kaspázy opravdu aktivovaly ***(Jelínek et al. 2015, viz kap. 5, str. 70)***.

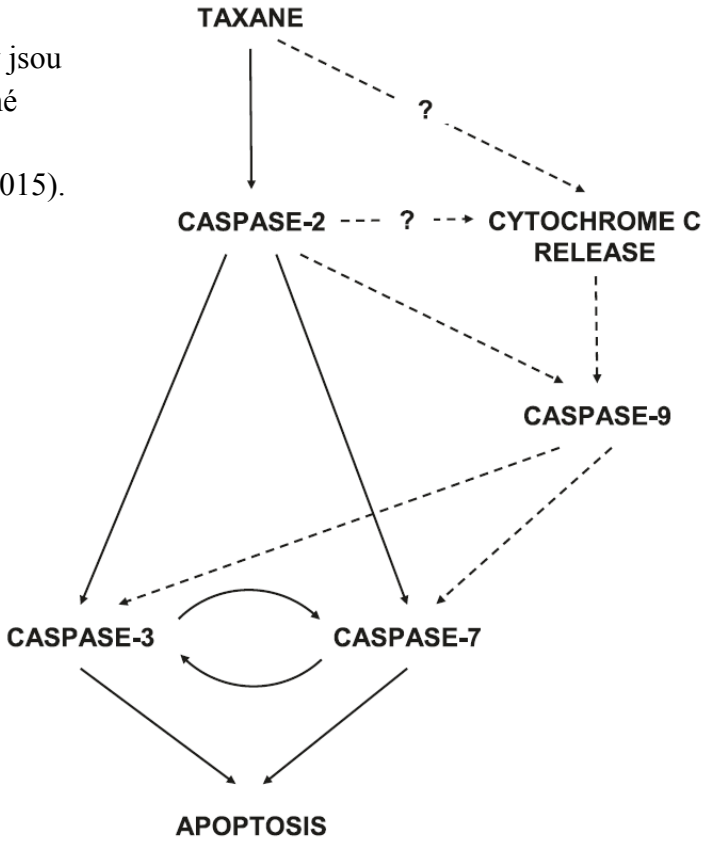
V buňkách duktálního původu k aktivaci kaspáz 2 a 9, a poněkud překvapivě ani k aktivaci hlavní exekuční kaspázy 3, nedocházelo ***(Jelínek et al. 2017, viz kap. 5, str. 89)***. Podobná buněčná smrt, probíhající bez aktivace kaspázy 3, byla popsána u buněk ovárií po aplikaci taxanů, kde ale nedocházelo k G2/M bloku ***(Kolfchoten et al. 2002)***. My jsme však G2/M blok pozorovali (naše nepublikovaná data). Pro detailní popis mechanismů indukce buněčné smrti v těchto buňkách bude tedy potřebné provést další experimenty.

4.1.2.2 Vliv inhibice exprese kaspázy 8, 9, 3 a 7 na indukci apoptózy a na aktivaci ostatních kaspáz

Snížení exprese kaspázy 9 ani kaspázy 8 nemělo významný vliv na indukci apoptózy taxany v testovaných buňkách. Naopak snížení exprese kaspázy 3 a kaspázy 7 buňky před apoptózou indukovanou taxany částečně ochránilo ***(Jelínek et al. 2015, viz kap. 5, str. 74)***. Žádná z kaspáz významně neštěpila prokaspázu 2, což potvrdilo apikální roli kaspázy 2 **(viz 4.1.1)**. Pokud byla v buňkách přítomna kaspáza 3, účastnila se aktivace kaspázy 7, kaspáza 9 aktivovala kaspázu 3 a 7 a konečně kaspáza 7 štěpila kaspázu 3 ***(Jelínek et al. 2015)***. V aktivaci kaspázy 8 jsme žádný signifikantní trend nenalezli a domníváme se, že v indukci apoptózy klíčovou roli nehraje ***(Janssen et al. 2007, Jelínek et al. 2015, viz kap. 5, str. 74, 75)***. Na základě těchto výsledků a výsledků inhibice exprese kaspázy 2 **(viz 4.1.1.2)** jsme navrhli model pravděpodobné vzájemné interakce kaspáz **(Obr. 5)**, ***(Jelínek et al. 2015)***.

Podle tohoto modelu taxany aktivují zatím ne zcela jasným mechanismem kaspázu 2, která následně aktivuje kaspázu 3 a 7. Paralelně se může uvolňovat cytochrom c z mitochondrií a v apoptozómu a/nebo pomocí kaspázy 2 aktivovat kaspáza 9. Kaspáza 9 se poté podílí na amplifikaci apoptotických signálů a (spolu s kaspázou 2) štěpí kaspázu 7 a 3. Kaspázy 3 a 7 se mohou navíc aktivovat vzájemným štěpením **(viz Obr. 5)**.

Obr. 5 Schéma vzájemné aktivace kaspáz. Plné čáry jsou potvrzené dráhy, čárkované čáry předpokládané dráhy (převzato z Jelínek et al. 2015).



Již dříve byly popsány některé dílčí dráhy z navrženého modelu ***(Ofir et al. 2002, Janssen et al. 2007, Ajabnoor et al. 2012)***. Podobný model byl také navržen pro aktivaci kaspázy 2 v důsledku poškození DNA ***(Janssens a Tinnel, 2012)***. V navrhovaných modelech indukce apoptózy však hrála aktivace mitochondriální dráhy klíčovou roli, nebo nebyla zcela jasná úloha kaspázy 2.

4.1.3 Úloha proteinů rodiny Bcl-2 v indukci apoptózy taxany

Apoptóza indukovaná taxany je obvykle spojena se změnou hladiny nebo aktivity jednoho nebo více proteinů rodiny Bcl-2.

My jsme v první řadě zjistili, že v buňkách s původem v mléčné žláze taxany zvyšovaly hladinu antiapoptotického proteinu Bcl-2 ***(Jelínek et al. 2017, viz kap. 5, str. 90)***. Zvýšení hladiny Bcl-2 proteinu je pravděpodobně nejběžnější forma obrany nádorových buněk před indukcí apoptózy ***(Janssen et al. 2007, Fauzee et al. 2012, Calastretti et al. 2014)***. V buňkách však zároveň docházelo k silné inhibiční fosforylaci Bcl-2 ***(Drago-Ferrante et al. 2008, Notte et al. 2013, Pan a Gollahon, 2013)***. Je tedy možné, že taxany nejprve zvyšují hladinu Bcl-2 proteinu,

nicméně poté je Bcl-2 fosforylován, inaktivován a není tedy dále schopen indukci apoptózy zabránit. Je zajímavé, že v jedné testované linii docházelo také k defosforylaci Bcl-2 ***(Jelínek et al. 2017, viz kap. 5, str. 90)***. Význam této defosforylace ale zůstává nejasný. Na druhou stranu, v buňkách s původem v duktu taxany mírně snižovaly hladinu proteinu Bcl-2 i bez výrazné fosforylace tohoto proteinu. Ke snížení hladiny Bcl-2 tedy může zřejmě docházet několika cestami.

Hladina proteinu Bax se výrazně neměnila v žádných z testovaných buněk nádoru prsu ***(Jelínek et al. 2017, viz kap. 5, str. 90)***. Naopak v buňkách retinoblastomu, melanomu a dalších typů nádorových buněk ke zvýšení hladiny proteinu Bax po aplikaci taxanů dochází ***(Mhaidat et al. 2007*, *Drago-Ferrante et al. 2008)***, což může být v souladu se zjištěním, že mitochondriální dráha je nezbytná pro indukci apoptózy ve výše zmíněných buňkách. Tato dráha ale nemá klíčovou úlohu u námi testovaných buněk nádoru prsu **(4.1.2.2 a Obr. 5)**. Tím, že se v buňkách neměnila hladina proteinu Bax a snížila se hladina Bcl-2, snížil se poměr hladin proteinů Bcl-2/Bax. To je významný indikátor indukce vnitřní dráhy apoptózy. Jestli má tento poměr důležitou úlohu v indukci apoptózy v buňkách nádoru prsu, nebylo zatím potvrzeno ***(Panno et al. 2006, Zheng et al. 2017)***. Apoptóza může být indukovaná taxany také v důsledku rozpadu vazby Bcl-xL/Bak ***(Flores et al. 2012)***. My jsme však žádné významné změny v hladině proteinu Bcl-xL nepozorovali ***(Jelínek et al. 2017, viz kap. 5, str. 90)***. Faktem je, že hladinu proteinu Bak jsme specificky nedetekovali, a proto tento mechanismus indukce apoptózy nemůžeme zcela vyloučit ***(Miller et al. 2013)***. Po aplikaci taxanů docházelo do určité míry také ke zvýšení hladiny proteinu Bok, jehož funkce v indukci apoptózy různými faktory zůstává poněkud nejasná. Protein Bok totiž indukoval apoptózu pouze v případě, že byl v buňce přítomen jeden z proteinů Bax/Bak. Jinak docházelo pouze k rozpadu ER a Golgiho apárátu, ale k indukci apoptózy nikoliv ***(Zhong et al. 2011, Echeverry et al. 2013)***.

Co se týká „BH3-only“ proteinů, ve všech liniích docházelo k signifikantnímu zvýšení hladiny proteinu Bad. Po delší inkubaci se však hladina proteinu Bad opětovně snížila. Protein Bad se může podílet na deaktivaci proteinu Bcl-2, pravděpodobně indukcí proteolytického štěpení Bcl-2 **(viz výše),** ***(Fauzee et al 2012, Craik et al. 2010)***. V buňkách nádoru prsu se protein Bad účastní PCD indukované taxany podle studie Craik a kol. **(2009)**. Nejednalo se ale přímo o apoptózu jako

v našem případě ale o jiný typ PCD. Objasnění přesné úlohy proteinu Bad v námi testovaných buňkách tedy vyžaduje další experimenty. V jedné buněčné linii se také dočasně zvýšila hladina proteinu Bim ***(Jelínek et al. 2017, viz kap. 5, str. 90)***. Bim je spojován v různých buněčných liniích nádorů prsu s rezistencí k taxanům. Literární údaje ale nejsou příliš konzistentní ***(Li et al. 2005, Czernick et al. 2009, Mac Fhearraigh et al. 2011, Ajabnoor et al. 2012, Savry et al. 2013, Miller et al. 2013, Jelínek et al. 2017)***. Jisté je, že se protein Bim aktivuje při poškození cytoskeletu, např. taxany, a zároveň jej aktivuje JNK kináza. JNK kináza může mimo jiné aktivovat také kaspázu 2 ***(Ley et al. 2005, Shin et al. 2005)***. Další experimenty zaměřené na úlohu JNK kinázy a proteinu Bim v indukci apoptózy taxany by tedy rozhodně mohly přinést zajímavé výsledky

Můžeme tedy shrnout, že se molekulární mechanismy indukce apoptózy výrazně liší více mezi buňkami jednoho typu nádoru s různým původem, než mezi testovanými taxany **(viz také 4.1.1, 4.1.2)**.

4.2 Rezistence buněk nádoru prsu k novým derivátům taxanů

V rámci této disertační práce jsme řešili úlohu vybraných funkčních skupin v molekule taxanů v navození a překonání rezistence nádorových buněk k taxanům, a to hlavně ve vztahu ke schopnosti P-glykoproteinu transportovat taxany z buněk, a tak buňky chránit před účinkem těchto taxanů.

4.2.1 Úloha substituentů v poloze C3´ a C3´N v rezistenci k taxanům

V naší laboratoři jsme postupnou adaptací etablovali sublinie z původně senzitivních linií buněk nádorů prsu, které jsou rezistentní k paclitaxelu, a také sublinie rezistentní k novému taxanu SB-T-0035 (podobně jako paclitaxel má v obou pozicích C3´ a C3´N fenylové zbytky). Rezistentní buňky přežívají a proliferují v takové koncentraci taxanů, která indukuje apoptózu v původních senzitivních buňkách ***(Němcová-Fűrstová et al. 2016, Jelínek et al. 2018, viz kap. 5, str. 105)***.

Dále jsme prokázali, že taxanovou rezistenci lze navodit právě proti takovým taxanům, které mají na obou pozicích C3´a C3´N fenylovou skupinu. Pokud je jedna

z fenylových skupin nahrazena jiným nearomatickým substituentem, nelze proti takovému taxanu rezistenci navodit a tyto taxany překonávají rezistenci nádorových buněk k paclitaxelu i SB-T-0035 ***(Ojima et al. 1996, Ojima et al. 2008, Jelínek et al. 2018, viz kap. 5, str. 102, 103, 104, 105)***.

4.2.2 Úloha P-glykoproteinu v rezistenci k taxanům s různými substituenty v polohách C3´a C3´N

ABC transportéry jsou proteiny, které transportují ven z buněk různé substráty, např. taxany, a tak je mohou chránit před jejich účinkem **(viz 3.5.1)**.

4.2.2.1 ABC transportéry a rezistence k taxanům

V řadě buněčných linií rezistentních k paclitaxelu byla popsána zvýšená hladina ABCB1 (P-glykoproteinu), ***(Reed et al. 2010, Ajabnoor et al. 2012, Shi et al. 2014, Li et al. 2014, Kathawala et al. 2015, Aldonza et al. 2016)***. V některých případech se navíc zvyšovala i hladina transportéru ABCC3 ***(Němcová-Fűrstová et al. 2016)***. P-glykoprotein byl ve zvýšené míře produkován i v buňkách rezistentních k účinkům docetaxelu, cabazitaxelu ***(Ojima et al. 1998, Duran et al. 2015, Li et al. 2014)***, a také taxanu SB-T-0035 ***(Jelínek et al. 2018, viz kap. 5, str. 105)***. V naší laboratoři snížení exprese P-glykoproteinu pomocí specifické siRNA částečně obnovilo senzitivitu testovaných buněk k paclitaxelu i novému taxanu SB-T-0035. Předpokládáme tedy, že je P-glykoprotein významně zapojen do rezistence k taxanům ***(Němcová-Fűrstová et al. 2016, Jelínek et al. 2018, viz kap. 5, str. 105)***.

4.2.2.2 Interakce taxanových derivátů s P-glykoproteinem

Jelikož je transport molekul P-glykoproteinem spojen s ATPázovou aktivitou tohoto transportéru, lze z míry ATPázové aktivity nepřímo určit transportní aktivitu tohoto proteinu ***(Ojima et al. 1996, Ferlini et al. 2000, Marchetti et al. 2014)***. Ukázalo se, že ATPázovou aktivitu P-glykoproteinu aktivovaly (v koncentraci indukující apoptózu) všechny testované taxany. Pozorované rozdíly v aktivaci P-glykoproteinu paclitaxelem a novými taxanovými deriváty nebyly sice statisticky významné, přesto však ATPázovou aktivitu P-glykoproteinu nejvíce aktivovaly taxany s dvěma fenyly na uhlících C3´ a C3´N ***(Jelínek et al. 2018, viz kap. 5, str. 106)***.

Dále jsme pomocí metody molekulárního modelování predikovali afinitu testovaných taxanových derivátů a vazebného místa P-glykoproteinu. Nejvyšší afinitu k P-glykoproteinu měly taxany s dvěma fenyly, poněkud slabší byla afinita taxanů s jedním fenylem na uhlíku C3´ nebo C3´N. Taxany, které neměly na těchto pozicích fenyl, měly k P-glykoproteinu afinitu nejnižší ***(Jelínek et al. 2018, viz kap. 5, str. 107)***.

Detailní molekulárně-modelovací studie vazebného místa ukázaly, že vazebné místo pro taxany tvoří hydrofobní kapsa obsahující fenyly, do které se svými fenylovými skupinami vážou taxany ***(Ojima et 1998, Liu et al. 2013, Jelínek et al. 2018, viz kap. 5, str. 107)***. Důležitý pro vazbu taxanů je také fenyl 339, který tvoří první mechanickou zábranu přístupu do hydrofobní kapsy P-glykoproteinu ***(Zhang et al. 2015)***.

Zdá se tedy velice pravděpodobné, že se dva fenyly na uhlících C3´ a C3´N v molekule taxanu vážou s vysokou afinitou do vazebného místa P-glykoproteinu, aktivují jeho ATPázovou doménu a zprostředkovávají transport tohoto taxanu ven z buňky. Pokud je jeden z fenylů nahrazen nearomatickou skupinou, taxan se neváže s tak vysokou afinitou k P-glykoproteinu, není účinně transportován ven z (rezistentní) nádorové buňky a může v ní tedy následně indukovat apoptózu.

5.1 Publikace věnované molekulárním mechanismům indukce apoptózy v buňkách nádorů prsu novými deriváty taxanů:

5.1.1 Jelínek M, Balušíková K, Kopperová D, Němcová-Fürstová V, Šrámek J, Fidlerová J. Zanardi I, Ojima I, Kovář J: Caspase-2 is involved in cell death induction by taxanes in breast cancer cells. Cancer Cell Int. 13(1): 42, 2013.

5.1.2 Jelínek M, Balušíková K, Schmiedlová M, Němcová-Fürstová V, Šrámek J, Stančíková J, Zanardi I, Ojima I, Kovář J: The role of individual caspases in cell death induction by taxanes in breast cancer cells. Cancer Cell Int. 15(1): 8, 2015.

5.1.3 Jelínek M, Kábelová A, Šrámek J, Seitz J, Ojima I, Kovář J: Differing mechanisms of death induction by fluorinated taxane SB-T-12854 in breast cancer cells. Anticancer Res. 37(4): 1581-1590, 2017.

5.2 Publikace věnované rezistenci buněk nádorů prsu k novým derivátům taxanů:

5.2.1 Jelínek M, Balušíková K, Daniel P, Němcová-Fürstová V, Kirubakaran P, Jaček M, Wei L, Wang X, Vondrášek J, Ojima I, Kovář J: Substituents at the C3' and C3'N positions are critical for taxanes to overcome acquired resistance of cancer cells to paclitaxel. Toxicol. Appl. Pharmacol. 347: 79-91, 2018.

5.1 Publikace věnované molekulárním mechanismům indukce apoptózy v buňkách nádorů prsu novými deriváty taxanů:

5.1.1 Jelínek M, Balušíková K, Kopperová D, Němcová-Fürstová V, Šrámek J, FidlerováJ, Zanardi I, Ojima I, Kovář J: Caspase-2 is involved in cell death induction by taxanes in breast cancer cells. Cancer Cell Int. 13(1): 42, 2013.

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Caspase-2 is involved in cell death induction by taxanes in breast cancer cells

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Abstract

Background: We studied the role of caspase-2 in apoptosis induction by taxanes (paclitaxel, novel taxane SB-T-1216) in breast cancer cells using SK-BR-3 (nonfunctional p53, functional caspase-3) and MCF-7 (functional p53, nonfunctional caspase-3) cell lines.

Results: Both taxanes induced apoptosis in SK-BR-3 as well as MCF-7 cells. Caspase-2 activity in SK-BR-3 cells increased approximately 15-fold within 48 h after the application of both taxanes at the death-inducing concentration (100 nM). In MCF-7 cells, caspase-2 activity increased approximately 11-fold within 60 h after the application of taxanes (300 nM). Caspase-2 activation was confirmed by decreasing levels of procaspase-2, increasing levels of cleaved caspase-2 and the cleavage of caspase-2 substrate golgin-160. The inhibition of caspase-2 expression using siRNA increased the number of surviving cells more than 2-fold in MCF-7 cells, and at least 4-fold in SK-BR-3 cells, 96 h after the application of death-inducing concentration of taxanes. The inhibition of caspase-2 expression also resulted in decreased cleavage of initiator caspases (caspase-8, caspase-9) as well as executioner caspases (caspase-3, caspase-7) in both cell lines after the application of taxanes. In control cells, caspase-2 seemed to be mainly localized in the nucleus. After the application of taxanes, it was released from the nucleus to the cytosol, due to the long-term disintegration of the nuclear envelope, in both cell lines. Taxane application led to some formation of PIDDosome complex in both cell lines within 24 h after the application. After taxane application, $p21^{WAF1/CIP1}$ expression was only induced in MCF-7 cells with functional p53. However, taxane application did not result in a significant increase of PIDD expression in either SK-BR-3 or MCF-7 cells. The inhibition of RAIDD expression using siRNA did not affect the number of surviving SK-BR-3 and MCF-7 cells after taxane application at all.

Conclusion: Caspase-2 is required, at least partially, for apoptosis induction by taxanes in tested breast cancer cells. We suggest that caspase-2 plays the role of an apical caspase in these cells. Caspase-2 seems to be activated via other mechanism than PIDDosome formation. It follows the release of caspase-2 from the nucleus to the cytosol.

Keywords: Caspase-2, Cell death, Taxanes, Breast cancer cells

Background

Taxanes represent a well-known but relatively new group of anticancer drugs. There are two established (classical) taxanes, paclitaxel (Taxol®) and docetaxel (Taxotere®), currently used for treatment of breast and ovarian cancer as well as head and neck, lung and prostate cancer [1,2]. In addition to the aforementioned classical taxanes, novel taxanes have been developed. They represent a new generation of taxoids (taxane analogs). They are not yet used in clinical practice but they are substantially more effective in resistant cancer cells *in vitro* and *in vivo* [3-7].

Taxanes are mitotic poisons. They bind to the β subunit of the tubulin heterodimer, thereby stabilizing microtubules and inhibiting their depolymerization [8-10]. In this way, taxanes are thought to block progression through the M-phase of the cell cycle [9,11]. However, the relationship between mitotic arrest and the induction of cell death by taxanes remains unclear [12-14].

The molecular mechanism of cell death induction by taxanes is not fully understood either. It has been

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previously shown that apoptosis induced by taxanes seems to be p53 independent [4,15]. On the other hand, several findings concerning cytochrome c release, caspase-9 activation and caspase-3 activation strongly indicate, that at least in some cases, a mitochondrial pathway is involved in apoptosis induction by taxanes [4,16,17]. However, alternative nonmitochondrial pathways could also be involved [4,7,18], including caspase-8 activation [7,14,19]. Recently, the role of caspase-2 in apoptosis induction by taxanes has come under consideration [7,14,20,21].

Caspase-2 is ubiquitously expressed and represents an evolutionarily highly conserved mammalian caspase. However, its precise physiological function has not been identified. Several lines of evidence point to caspase-2 as a major player in apoptosis induction [22,23]. Procaspase-2 interacts with other proteins, such as CARD-containing RAIDD protein, via its caspase recruitment domain (CARD). RAIDD interacts with another death domain containing protein PIDD *via* its death domain [24]. The complex of procaspase-2, RAIDD and PIDD, known as PIDDosome, facilitates caspase-2 activation. PIDD is a p53-inducible protein [23,25]. In some cases, PIDD seems to function as a regulator of caspase-2 activity [26]. However, caspase-2 activation independent of p53, as well as RAIDD and PIDD, has also been reported, e.g. in cases of cell death via a mitotic catastrophe [27-30]. Caspase-2 has been found in the cytosol, Golgi complex and mitochondria. It is also present in the nucleus. Active caspase-2 specifically cleaves golgin-160 which is present in the Golgi complex [31].

It has been suggested that caspase-2 functions as the most apical caspase when apoptosis is induced by DNA damage and cytotoxic stress [32,33]. The involvement of caspase-2 activation in apoptosis of breast cancer cells, induced by various stimuli, has also been found [27,34-36]. Several other studies have also demonstrated caspase-2 activation in various types of cancer cells following apoptosis induction by taxanes [21,37,38].

We have previously found that caspase-2 is significantly activated in breast cancer cells (together with the activation of caspase-3, caspase-9 and caspase-8) following apoptosis induction by taxanes [7,14]. We have also shown that the mitochondrial pathway is not, at least in some cases, the predominant pathway of apoptosis induction by taxanes in breast cancer cells, and that caspase-2 may be a major player in this process [7]. In our present study, we investigated the role of caspase-2 in apoptosis induction by taxanes in breast cancer cells. We used breast cancer cells SK-BR-3 (nonfunctional p53, functional caspase-3) and MCF-7 (functional p53, nonfunctional caspase-3) as an experimental model and tested both classical (paclitaxel) and novel (SB-T-1216) taxanes. We demonstrated that caspase-2 is required for apoptosis induction by taxanes in the tested breast cancer cells, probably as an apical caspase. Caspase-2 is activated via other mechanism than PIDDosome formation.

Results

Effect of taxanes on growth and survival

The effects of paclitaxel and SB-T-1216 on growth and survival of SK-BR-3 cells were tested over a wide range of concentrations (0.3-1000 nM). Paclitaxel and SB-T-1216 both induced death of SK-BR-3 cells within 96 h of incubation at a concentration of 30 nM and higher concentrations. The C_{50} values (concentration of taxanes resulting in 50% living cells compared to controls after 96 h of incubation) were 15 nM and 3 nM for paclitaxel and SB-T-1216, respectively (Figure 1).

In the case of MCF-7, the effects of taxanes were also tested over a wide range of concentrations (0.3-3000 nM). Both paclitaxel and SB-T-1216 induced the death of MCF-7 cells within 96 h of incubation at a concentration of 100 nM and higher concentrations. The C_{50} values of paclitaxel and SB-T-1216 were similar, 5 nM and 8 nM, respectively (Figure 1).

The data showed that MCF-7 cells were more resistant to cell death induction by both taxanes at higher concentrations (30 nM and higher concentrations) than SK-BR-3 cells. In MCF-7 cells, paclitaxel and SB-T-1216 exerted similar effects. However, in SK-BR-3 cells, SB-T-1216 seemed to be more efficient than paclitaxel (Figure 1).

On the basis of our data, we selected 100 nM and 300 nM as the cell death-inducing concentrations, i.e. the lowest concentration having complete death-inducing effect, of tested taxanes for SK-BR-3 cells and MCF-7 cells, respectively. These concentrations were used in subsequent experiments.

Effect of taxanes on caspase-2 activity

Employing a commercial kit and flow cytometry (see "Materials and Methods"), we tested the time course of caspase-2 activation in both SK-BR-3 and MCF-7 cells after taxane application.

Caspase-2 activity in SK-BR-3 cells increased within 48 h after the application of paclitaxel and SB-T-1216 at death-inducing concentrations (100 nM) approximately 16-fold and 14-fold, respectively. A substantial increase (approximately 10-fold for both taxanes) was seen 36 h after taxane application as well as a noticeable increase (about 3-fold) 24 h after application (Figure 2).

In the case of MCF-7 cells, caspase-2 activity increased within 60 h after the application of both taxanes at death-inducing concentrations (300 nM) approximately 11-fold. A significant increase (approximately 4-fold for both taxanes) was seen 48 h after taxane application and a noticeable increase (about 2-fold) 36 h after application (Figure 2).

In order to confirm caspase-2 activation, we assessed the cleavage of procaspase-2 by measuring the level of

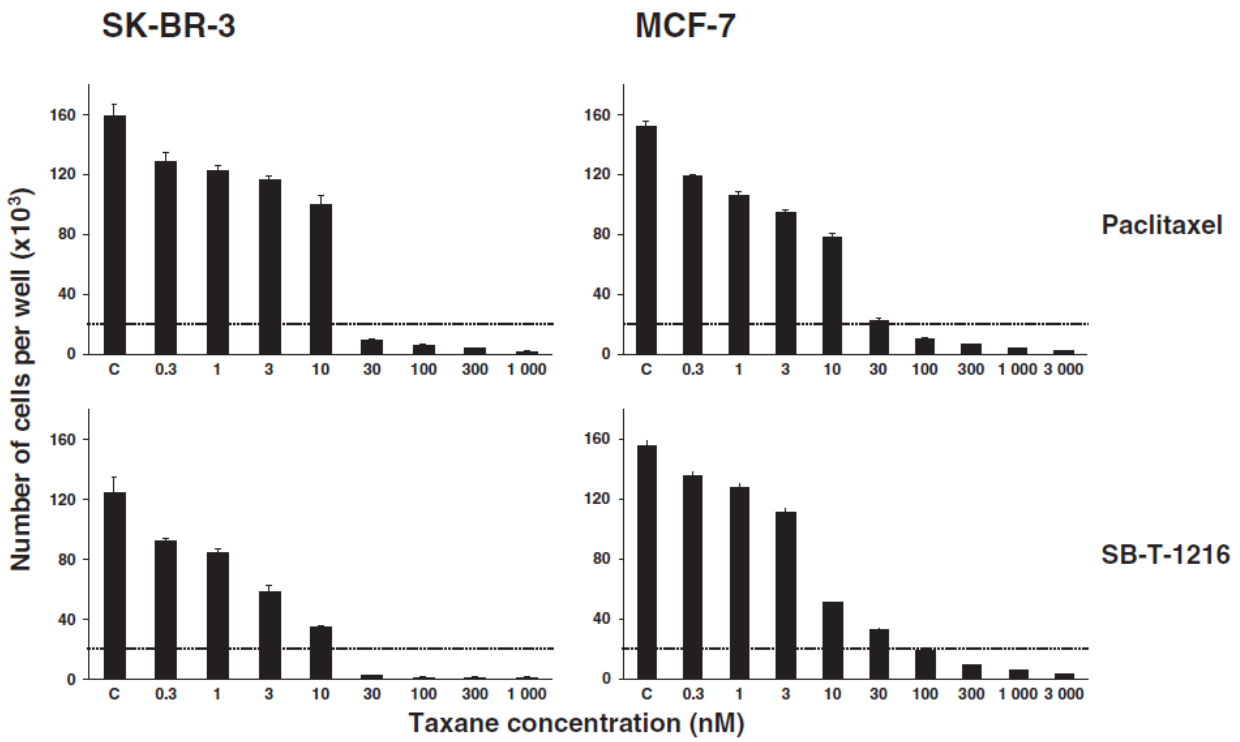

Figure 1 Effect of paclitaxel and SB-T-1216 (0.3-3000 nM) on the growth and survival of SK-BR-3 and MCF-7 cells. Control cells (C) were incubated without taxane. The cells were seeded at 20×103 cells/100 μl of medium per well. The number of cells of the inoculum is shown as a dotted line. The number of living cells was determined after 96 h of incubation (see "Materials and Methods"). Each column represents the mean of 8 separate cultures ± SEM.

procaspase-2 using western blot analysis. Procaspase-2 level decreased significantly 36 h after the application of both taxanes in SK-BR-3 cells. After 48 h, the level of procaspase-2 in SK-BR-3 cells was very low (Figure 3A). Concerning MCF-7 cells, some decrease in procaspase-2 level was seen 24 h after taxane application, but a more significant decrease was seen 36 h and 48 h after application. After 60 h, the level of procaspase-2 in MCF-7 cells was very low (Figure 3A).

Decreasing procaspase-2 level points at procaspase-2 cleavage resulting from formation of active caspase-2. The decrease of procaspase-2 level in SK-BR-3 cells correlated with increased level of the cleaved form 36 h after taxane application. However, 48 h after taxane application cleaved caspase-2 disappeared (Figure 3A). Similarly, the decrease of procaspase-2 level in MCF-7 cells correlated with increased level of the cleaved form 36 h and 48 h after taxane application. Again, 60 h after taxane application we can see decreasing level of cleaved caspase-2 (Figure 3A).

The levels of caspase-2 substrate golgin-160 using western blot analysis were also assessed. A significant decrease in golgin-160 level corresponded with decreased procaspase-2 level in both SK-BR-3 and MCF-7 cells after application of both taxanes (Figure 3A,B).

Effect of the inhibition of caspase-2 expression on taxane induced cell death

Employing RNA interference (see "Materials and Methods"), we assessed the effect of specific inhibition of caspase-2 expression on cell death induction after taxane application.

First, the efficiency of the RNA interference was tested. The inhibition of caspase-2 expression was about 80% compared to control (Figure 4A) in SK-BR-3 cells and more than 80% in MCF-7 cells (Figure 4A). Moreover, nonsense siRNA or specific caspase-2 siRNA did not significantly affect cell growth or survival in either cell line. However, cells transfected with siRNAs seemed to grow slightly slowlier in comparison with control (Figure 4B).

After 48 h of incubation with taxanes at death-inducing concentrations (100 nM), the inhibition of caspase-2 expression resulted in an approximately 2-fold increase in the number of surviving SK-BR-3 cells. It represents a statistically significant increase from 26% to 60% of the original number of cells for paclitaxel and from 33% to 64% for SB-T-1216. After 96 h, the effect was even more pronounced. In the case of paclitaxel, the number of surviving SK-BR-3 cells increased from 4% to 30% and from 7% to 26% for SB-T-1216 (Figure 4C).

As for MCF-7 cells, the inhibition of caspase-2 expression, after the application of both paclitaxel and SB-T-1216

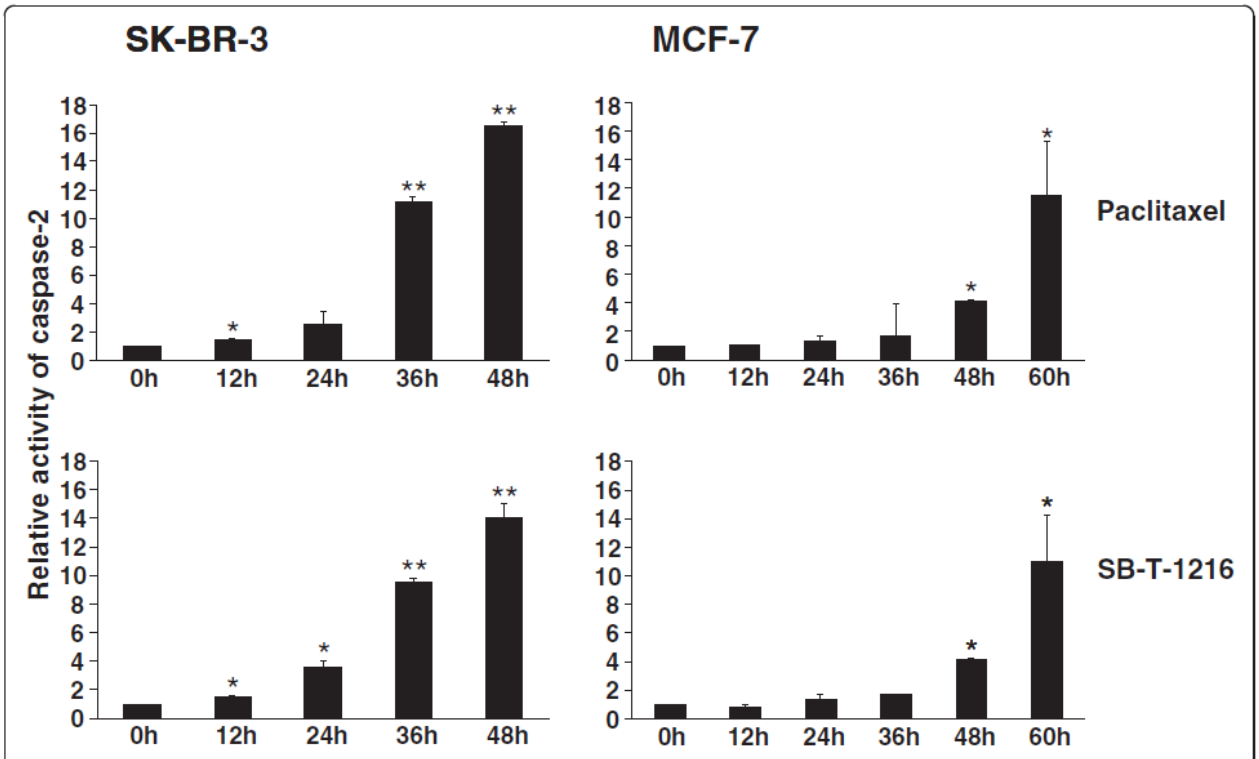

Figure 2 Effect of paclitaxel and SB-T-1216 on the activity of caspase-2 in SK-BR-3 and MCF-7 cells. After 0, 12, 24, 36, 48 and 60 h of incubation with paclitaxel or SB-T-1216 (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), the activity of caspase-2 was measured by flow cytometry employing CaspGLOWTM Active Caspase Staining Kit (see "Materials and Methods"). Mean fluorescence at 0 h was 23.9 for SK-BR-3 cells and 28.5 for MCF-7 cells. Each column represents the mean of 2 experimental values ± SEM. *P < 0.05, **P < 0.01 when comparing the effect with that at 0 h.

at death-inducing concentrations (300 nM), increased the number of surviving cells roughly 2-fold after 48 h and 96 h of incubation. After 48 h of incubation, there was a statistically significant increase from 96% to 203% of the original number of cells for paclitaxel and from 78% to 147% for SB-T-1216. After 96 h, the number of surviving cells increased from 42% to 112% and from 50% to 121% for paclitaxel and SB-T-1216, respectively (Figure 4C). It was also a statistically significant increase. These data demonstrate that MCF-7 cells grew even after application of both taxanes at death-inducing concentrations when caspase-2 expression was inhibited (Figure 4C).

Effect of the inhibition of caspase-2 expression on taxane induced activation of caspase-8, -9, -3 and -7

Using siRNA technique (see "Materials and Methods"), we also assessed the effect of specific inhibition of caspase-2 expression on the activation of caspase-8, -9,-3 and -7 after taxane application.

SK-BR-3 cells with suppressed expression of caspase-2 (for the efficiency see previous section) were incubated with paclitaxel or SB-T-1216 (100nM) for 24 h. Subsequent western blot analysis showed significantly decreased cleavage of caspase-9 and caspase-3 due to inhibition of caspase-2 expression. No considerable change in the level of cleaved caspase-8 was observed. Surprisingly, the level of cleaved caspase-7 significantly increased (Figure 5).

MCF-7 cells (without functional caspase-3) with inhibited expression of caspase-2 were incubated with tested taxanes (300nM) for 48 h. Western blot analysis showed significantly decreased levels of cleaved caspase-8 and cleaved caspase-9. Decreased level of cleaved caspase-7 was not as pronounced (Figure 5).

Effect of taxanes on cellular distribution of caspase-2

The effect of tested taxanes on cellular distribution of caspase-2 was assessed using confocal microscopy (see "Materials and Methods").

Confocal microscopy showed that caspase-2 (detected by two different antibodies) seemed to be mainly found in the nucleus of control SK-BR-3 cells. However, caspase-2 did not colocalize with DNA, as we demonstrated when comparing interphase and mitotic control cells. After 36 h of incubation with paclitaxel or SB-T-1216 at death-inducing concentrations (100 nM), caspase-2 was released from the nucleus to the cytosol. This was probably due to the mitotic block following taxane application which is associated with the disintegration of the nuclear envelope

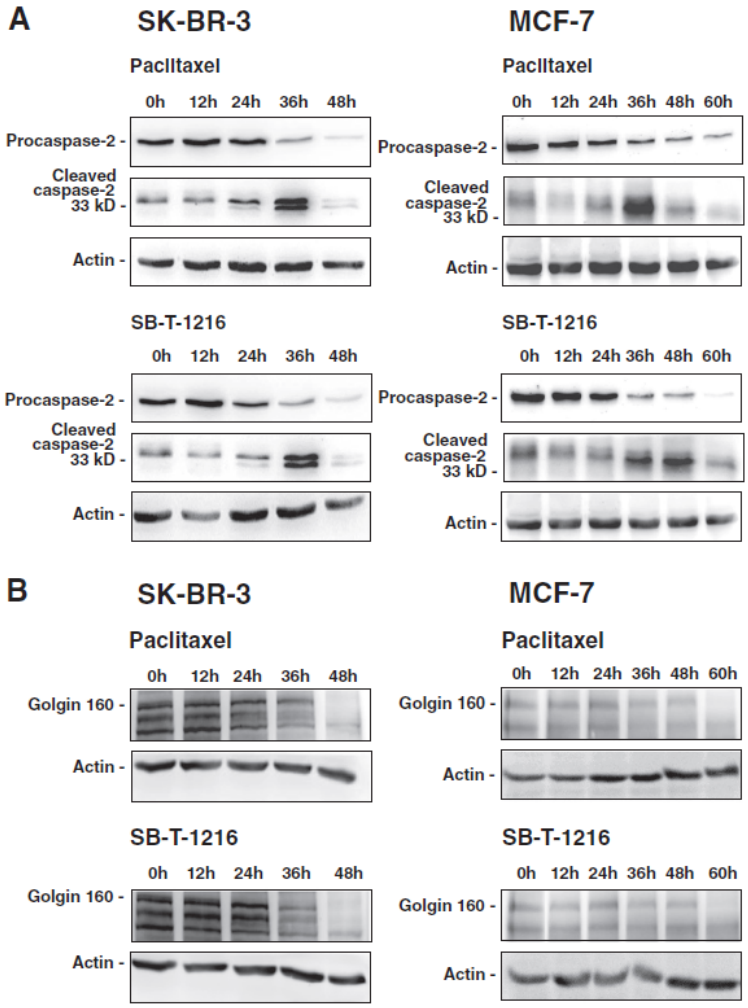

Figure 3 Effect of paclitaxel and SB-T-1216 on (A) the level of procaspase-2, cleaved caspase-2, and (B) the level of golgin-160 in SK-BR-3 and MCF-7 cells. After 0, 12, 24, 36, 48 and 60 h of incubation with paclitaxel or SB-T-1216 (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), levels of procaspase-2, cleaved caspase-2 and levels of golgin-160 were determined using western blot analysis and relevant antibodies (see "Materials and Methods"). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of three (procaspase-2, cleaved caspase-2) or two (golgin 160) independent experiments. MCF-7 data demonstrating the level of procaspase-2 and the level of cleaved caspase-2 are from different experiments.

(Figure 6). However, caspase-2 was not redistributed into mitochondria (data not shown). Similar data were obtained with MCF-7 cells (data not shown).

Effect of taxanes on p53 activation

We assessed the activation of p53 by induction of $p21^{WAF1/CIP1}$ expression employing real-time PCR (mRNA level) and western blot analysis (protein level).

Within 36-h incubation of SK-BR-3 cells (nonfunctional p53) with paclitaxel at the death-inducing concentration (100 nM), the level of $p21^{WAF1/CIP1}$ mRNA decreased to 40% of the original value (statistically significant decrease). Western blot analysis did not detect any $p21^{WAF1/CIP1}$ protein in these cells during 36 h of incubation with the taxane (Figure 7). Similar data were obtained with SB-T-1216 in SK-BR-3 cells (data not shown).

With regard to MCF-7 cells (functional p53), $p21^{WAF1/CIP1}$ mRNA level increased approximately 9-fold during 36 h of incubation with paclitaxel at the death-inducing concentration (300 nM). This pronounced and statistically significant increase of $p21^{WAF1/CIP1}$ mRNA level corresponded to a pronounced increase in $p21^{WAF1/CIP1}$ protein level (Figure 7). Again, similar data were obtained with SB-T-1216 in these cells (data not shown).

Effect of taxanes on PIDD expression

Effect on PIDD expression was assessed using real-time PCR (mRNA level) and western blot analysis (protein level).

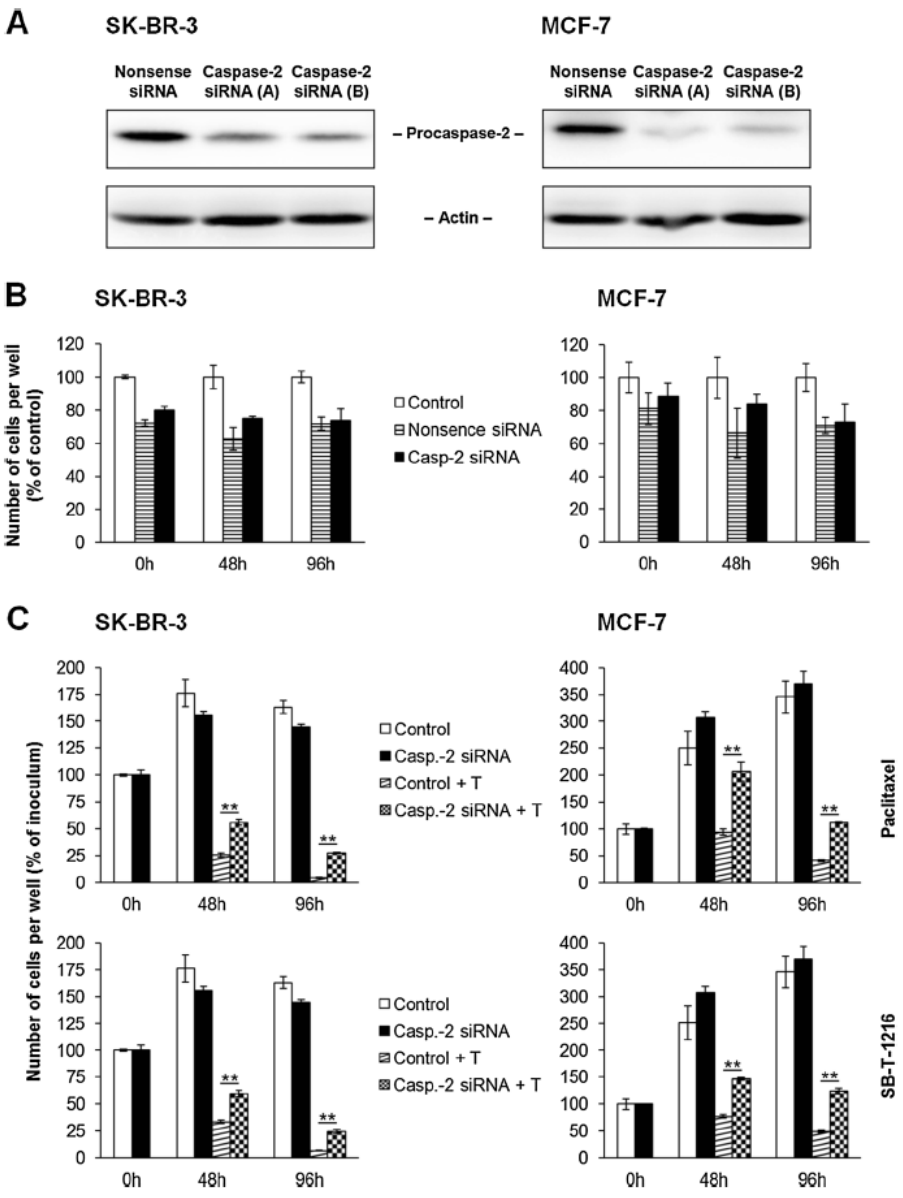

Figure 4 Effect of the inhibition of caspase-2 expression on the growth and survival of SK-BR-3 and MCF-7 cells after paclitaxel and SB-T-1216 treatment. (**A**) Efficiency of caspase-2 suppression by two employed specific siRNAs, i.e. **A** and **B**, in SK-BR-3 and MCF-7 cells is shown. Levels of procaspase-2 were determined using western blot analysis and relevant antibodies (see "Materials and Methods"). Actin levels were used to confirm equal protein loading. (**B**) Effect of nonsense siRNA and specific caspase-2 siRNA on the growth and survival of SK-BR-3 and MCF-7 cells without taxane treatment is also shown. (**C**) The effect of specific caspase-2 siRNA on the growth and survival of SK-BR-3 and MCF-7 cells after taxane (T) treatment (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells) is presented. The cells were seeded at 7×10^3 cells/200 μl of medium per well and prepared as described (see "Materials and Methods"). After 0, 48 and 96 h of incubation, the number of living cells was determined (see "Materials and Methods"). Each column represents the mean of 4 separate cultures ± SEM. *$P < 0.05$, **$P < 0.01$ when comparing the effect.

The level of PIDD mRNA showed a decrease (to 60% of the original value) during 36-h incubation of SK-BR-3 cells with paclitaxel at the death-inducing concentration (100 nM). PIDD protein level also seemed to decrease slightly after 36 h of incubation with paclitaxel (Figure 8). Similar data were obtained with SB-T-1216 (data not shown).

PIDD mRNA in MCF-7 cells showed similar levels during 36-h incubation with paclitaxel at the death-inducing concentration (300 nM). No significant change was found for PIDD protein level (Figure 8). As was the case for SK-BR-3 cells, similar data were obtained with SB-T-1216 (data not shown).

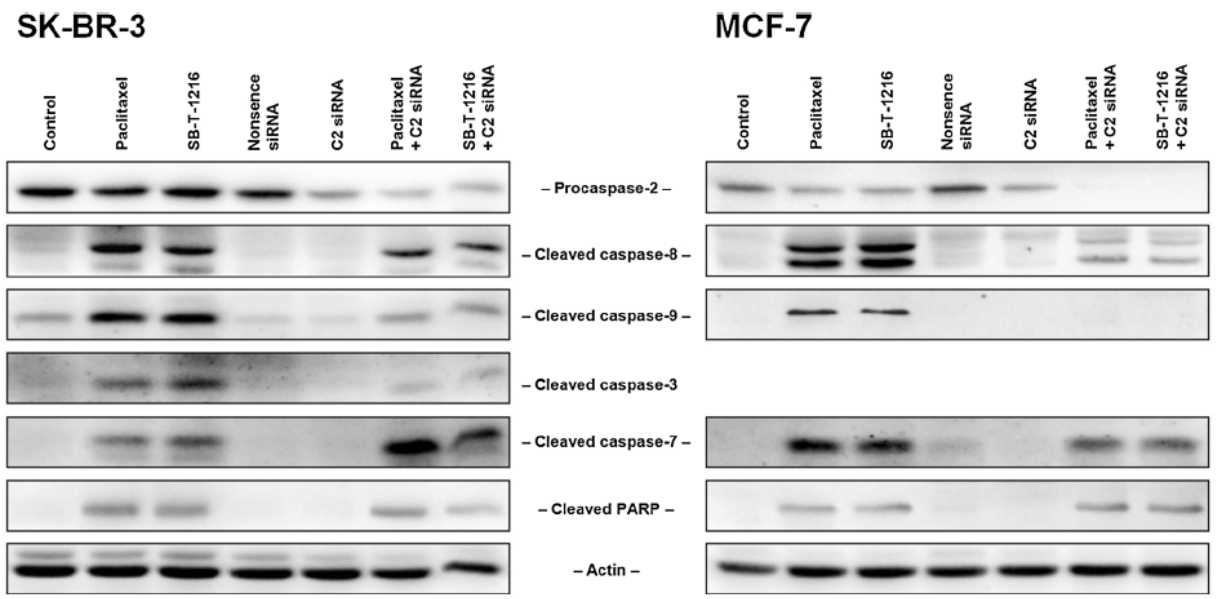

Figure 5 Effect of the inhibition of caspase-2 expression on the activation of caspase-8, -9, -3 and -7 in SK-BR-3 and MCF-7 cells after paclitaxel and SB-T-1216 treatment. A specific caspase-2 siRNA (C2 siRNA) was used. Control cells were incubated without taxane and siRNAs. Effects of nonsense siRNA as well as caspase-2 siRNA without taxane treatment are also shown. After 24 h (SK-BR-3) or 48 h (MCF-7) of incubation with tested taxane (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), levels of cleaved caspases were determined using western blot analysis and relevant antibodies (see "Materials and Methods"). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments.

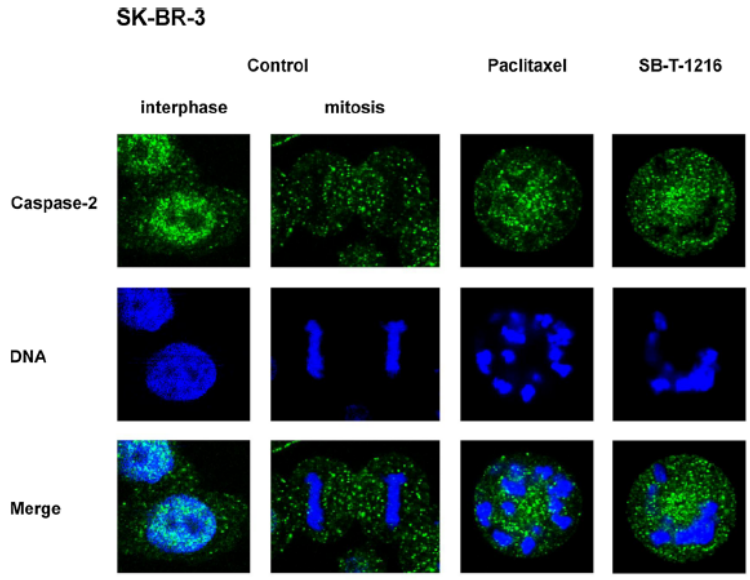

Figure 6 Effect of paclitaxel and SB-T-1216 on cellular distribution of caspase-2 in SK-BR-3 cells. Control cells were incubated without taxane. After 36 h of incubation with tested taxane (100 nM), the localization of caspase-2 and DNA position were detected using confocal microscopy, following staining with DAPI (DNA) and indirect immunofluorescence staining (caspase-2) with antibody against human caspase-2 (see "Materials and Methods"). The localization of caspase-2 (green), of DNA (blue) and the merge of caspase-2 and DNA within the cell are shown. The data shown were obtained in one representative experiment of two independent experiments.

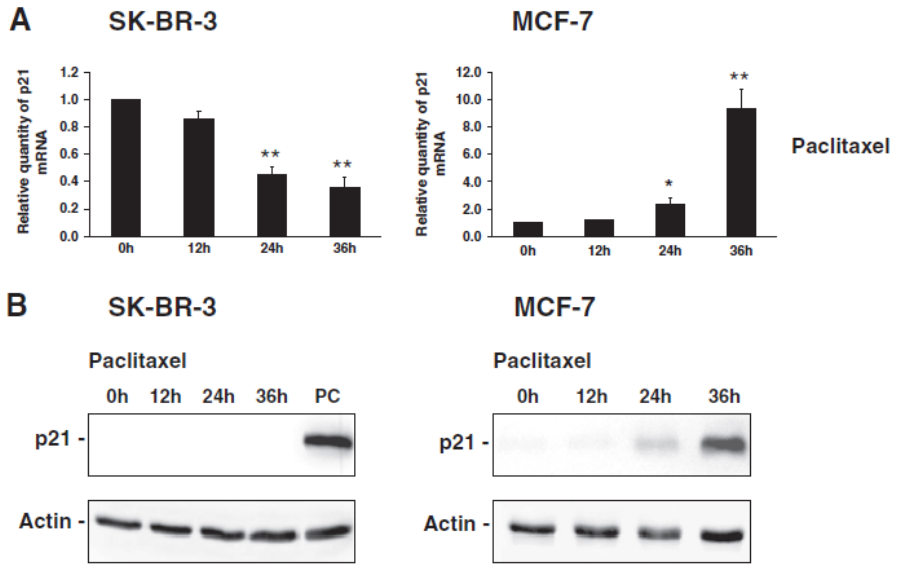

Figure 7 Effect of paclitaxel on the level of (A) $p21^{WAF1/CIP1}$ mRNA and (B) $p21^{WAF1/CIP1}$ protein in SK-BR-3 and MCF-7 cells. After 0, 12, 24 and 36 h of incubation with paclitaxel (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), the level of mRNA was determined using RT-PCR and relevant primers and the level of protein was determined using western blot analysis and relevant antibodies (see "Materials and Methods"). Each column of mRNA data represents the mean of 4 experimental values ± SEM. *P < 0.05, **P < 0.01 when comparing the effect with that at 0 h. Actin levels of protein data were used to confirm equal protein loading. In the case of SK-BR-3 cells, MCF-7 cells after 36 h of incubation with paclitaxel were used as a positive control (PC). The data shown were obtained in one representative experiment of two independent experiments.

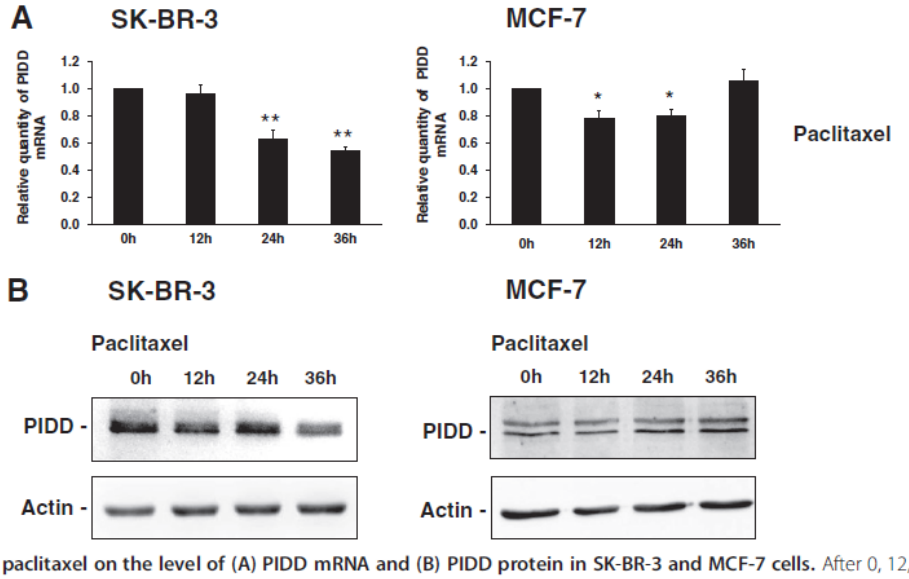

Figure 8 Effect of paclitaxel on the level of (A) PIDD mRNA and (B) PIDD protein in SK-BR-3 and MCF-7 cells. After 0, 12, 24 and 36 h of incubation with paclitaxel (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), the level of mRNA was determined using RT-PCR and relevant primers and the level of protein was determined using western blot analysis and relevant antibodies (see "Materials and Methods"). Each column of mRNA data represents the mean of 4 experimental values ± SEM. *P < 0.05, **P < 0.01 when comparing the effect with that at 0 h. Actin levels of protein data were used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments.

Similar data were obtained, when PIDD protein levels were assessed using flow cytometric analysis after staining with relevant primary and secondary antibodies, for both cell lines (SK-BR-3 and MCF-7) and both taxanes (paclitaxel and SB-T-1216) (data not shown).

Effect of taxanes on the coimmunoprecipitation of caspase-2 and PIDD with RAIDD

Coimmunoprecipitation (see "Materials and Methods") of RAIDD protein with other components of PIDDosome, i.e. PIDD protein and caspase-2, was assessed using subsequent western blot anylysis.

Both caspase-2 and PIDD protein (detected by antibody against C-form) were coimmunoprecipitated with RAIDD protein after 24-h incubation of SK-BR-3 cells with paclitaxel at the death-inducing concentration (100nM) (Figure 9).

Similarly, caspase-2 and PIDD protein were detected in coimmunoprecipitated complex with RAIDD protein in MCF-7 cells after 24 h incubation with paclitaxel at the death-inducing concentration (300 nM). Thus it seems that PIDDosome is formed in both SK-BR-3 and MCF-7 cells when taxane is applied at death-inducing concentrations.

Effect of the inhibiton of RAIDD expression on taxane induced cell death

Employing RNA interference (see "Materials and Methods"), we assessed the effect of specific inhibition of RAIDD expression on cell death induction after taxane application.

The inhibition of RAIDD expression by RNA interference was found to be highly efficient for both SK-BR-3 and MCF-7 cells (Figure 10A). Specific RAIDD siRNA did not significantly affect cell growth or survival in ether cell line (data not shown).

After 48 h as well as 96 h of incubation with paclitaxel at death-inducing concentration (100 nM), the inhibition of RAIDD expression did not result in any significant change in the number of surviving SK-BR-3 cells (Figure 10B).

Similarly for MCF-7 cells, the inhibition of RAIDD expression did not result in any significant change in the number of surviving cells after 48 h and 96 h of incubation with paclitaxel at death-inducing concentration (300 nM) (Figure 10B).

Discussion

In our previous studies [7,14], we demonstrated that caspase-2 was significantly activated (up to 20-fold) along with other caspases (caspase-3, caspase-9 and caspase-8) during apoptosis induction by taxanes in some cancer cells. We have also shown that, at least in some cases, the mitochondrial pathway does not represent the main pathway of apoptosis induction by taxanes. Recently, we found that apoptosis was induced by taxane application in SK-BR-3 and MCF-7 breast cancer cells and that caspase-2 was also significantly activated in these cells. Furthermore, MCF-7 cells are without functional caspase-3, as we confirmed (data not shown). Thus it is reasonable to assume that caspase-2 could play an important role in apoptosis induction by taxanes in breast cancer cells.

In order to study the role of caspase-2 in apoptosis induction by taxanes in breast cancer cells, we employed a convenient model using SK-BR-3 and MCF-7 cells. SK-BR-3 cells have nonfunctional p53 and functional caspase-3 [39]. On the other hand, MCF-7 cells have functional p53, but they do not have functional caspase-3 [40]. The

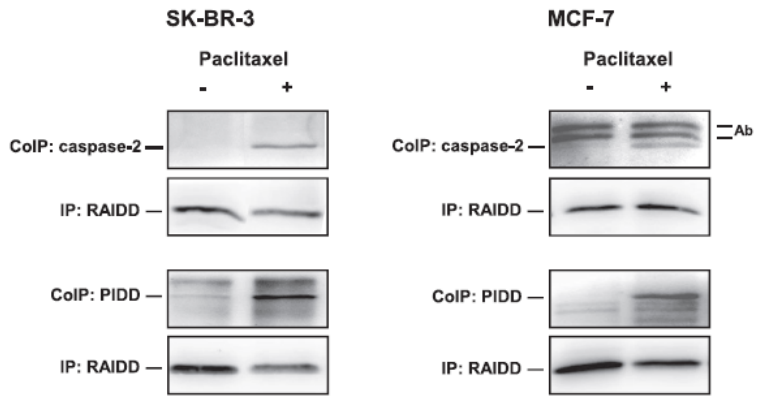

Figure 9 Effect of paclitaxel on coimmunoprecipitation of caspase-2 and PIDD with RAIDD in SK-BR-3 and MCF-7 cells. After 24 h of incubation with paclitaxel (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), the level of precipitated RAIDD and coimmunoprecipitated caspase-2 as well as PIDD (detected by antibody against C-form) were determined using western blot analysis and relevant antibodies (see "Materials and Methods"). The heavy chain of the antibody used for RAIDD precipitation (Ab) is seen. The data shown were obtained in two independent experiments.

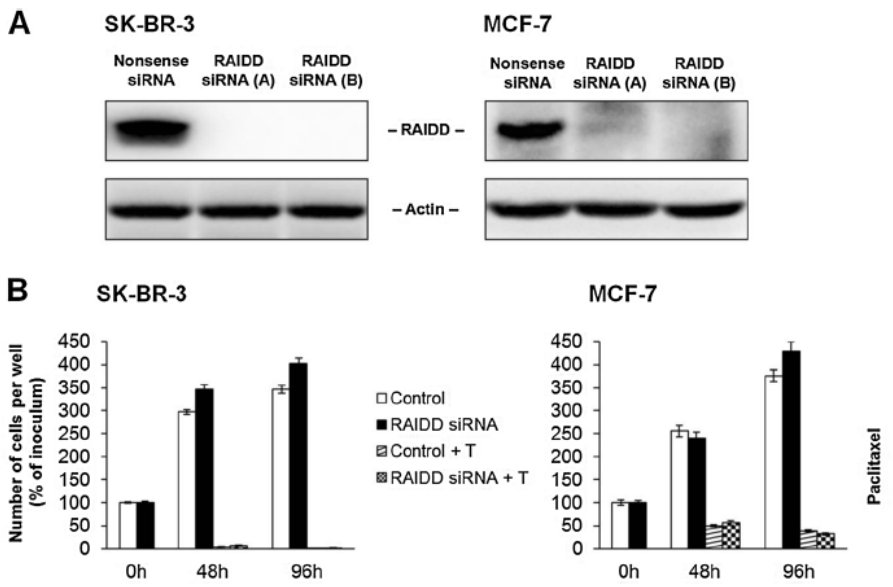

Figure 10 Effect of the inhibition of RAIDD expression on the growth and survival of SK-BR-3 and MCF-7 cells after paclitaxel treatment. (**A**) Efficiency of RAIDD suppression by two employed specific siRNA, i.e. **A** and **B**, in SK-BR-3 and MCF-7 cells is shown. Levels of RAIDD were determined using western blot analysis and relevant antibodies (see "Materials and Methods"). Actin levels were used to confirm equal protein loading. (**B**) The effect of specific RAIDD siRNA on the growth and survival of SK-BR-3 and MCF-7 cells after paclitaxel (T) treatment (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells) is presented. The cells were seeded at 7×10^3 cells/200 μl of medium per well and prepared as described (see "Materials and Methods"). After 0, 48 and 96 h of incubation, the number of living cells was determined (see "Materials and Methods"). Each column represents the mean of 2 separate cultures ± SEM.

use of these two cell lines, with their opposite properties, could help to elucidate the role of caspase-2 in apoptosis induction by taxanes. It could particularly contribute to our knowledge concerning the relationship between caspase-2 activation and the activation of other caspases as well as the mechanism of caspase-2 activation itself.

We tested both a classical taxane paclitaxel and a novel (second-generation) taxane SB-T-1216. In our previous study with MDA-MB-435 and NCI/ADR-RES cell lines [14], SB-T-1216 was shown to be more effective than paclitaxel, particularly in NCI/ADR-RES cells resistant to paclitaxel. However, SB-T-1216 and paclitaxel seemed to use the same or similar mechanism of cell death induction [7,14]. In this study, MCF-7 cells were shown to be a slightly more resistant to taxanes than SK-BR-3 cells but both cell lines showed similar sensitivity to paclitaxel and SB-T-1216 (see Figure 1). Furthermore, it seems that paclitaxel and SB-T-1216 use the same mechanism of apoptosis induction (see Figures 2, 3, 4, 5, 6 and "Results").

Significant activation of caspase-2 in SK-BR-3 and MCF-7 cells after taxane application at death inducing concentrations (see Figure 2) was confirmed using several different methods (see Figure 3). It is known that the measurement of caspase-2 activity can be affected by the activity of caspase-3 [41]. Thus, the measured activity of caspase-2 (see Figure 2) can be in fact a sum of caspase-2 and caspase-3 activities. It can explain the discrepancy in timing of measured caspase-2 activity (see Figure 2) and detected caspase-2 activation (see Figure 3). In MCF-7 cells, we only detected cleavage of procaspase-2 (see Figure 3A) under different conditions than procaspase-2 was detected. However, the problem of detecting the cleaved form of caspase-2 in MCF-7 cells could be a specific feature of these cells as described previously [42]. With regard to other cell types, several studies have demonstrated caspase-2 activation in various types of cancer cells after apoptosis induction by taxanes [37,38,43].

Several studies with human prostate cancer cells, human melanoma cells and mouse embryonic fibroblasts have shown that caspase-2 is required for apoptosis induction by taxanes. These studies employed various techniques using siRNA, caspase-$2^{-/-}$ cells and specific caspase-2 inhibitors [21,23,44]. We also demonstrated that caspase-2 is required, at least partially, for apoptosis induction by taxanes in both studied breast cancer cell lines. The inhibition of caspase-2 expression using siRNA technique resulted in a significantly increased number of surviving cells following application of taxanes at death-inducing concentrations. MCF-7 cells, with inhibited caspase-2 expression, grew even after application of death-inducing concentrations of taxanes (see Figure 4). This effect could

not be attributed to the stimulatory effect of siRNA application on cell proliferation (see Figure 4B).

In spite of the fact that several studies have shown the involvement of caspase 2 in apoptosis induction by various stimuli in breast cancer cells [27,34,35], the mechanism of caspase-2 involvement in apoptosis induction is not fully understood. Caspase-2 has been described as an apical caspase [21,23] as well as a possible executioner caspase [45], in various types of cancer cells, together with its functions which are independent of apoptosis [46,47]. In this study with SK-BR-3 and MCF-7 cells we demonstrated that, together with caspase-2, other caspases (caspase-8, caspase-9, caspase-3, caspase-7) were activated after application of taxanes.

The activation of caspase-9, -3 and -7 was significantly affected in SK-BR-3 cells with inhibited caspase-2 expression after cell death induction by taxane application. While the cleavage of caspase-9 and caspase-3 significantly decreased, caspase-7 cleavage increased. In MCF-7 cells (without functional caspase-3) the cleavage of caspase-9 was nearly blocked and the cleavage of caspase-7 was significantly decreased. Cleavage of caspase-8 decreased slightly in SK-BR-3 cells and significantly in MCF-7 cells (see Figure 5). The substantial inhibitory effect of the inhibition of caspase-2 expression on the activation of caspase-8 and caspase-9, as well as the activation of executioner caspase-3 in SK-BR-3 cells and executioner caspase-7 in MCF-7 cells, supports a suggestion that caspase-2 functions as an apical caspase. The increase of caspase-7 activation together with the decrease of caspase-3 activation, due to the inhibition of caspase-2 expression, in SK-BR-3 cells seems rather obscure (see Figure 5). Some kind of compensatory mechanism could be involved.

Additional information concerning caspase-2 function could come from testing the cleavage of caspase-2 substrates. However, there are only a few known specific substrates of caspase-2 such as golgin-160 [31,46]. In this study, we demonstrated golgin-160 cleavage after application of death-inducing taxane concentrations in both studied cell lines (see Figure 3B). Unfortunately, it was not very helpful in elucidating caspase-2 function without data connecting golgin-160 cleavage to other relevant events.

Caspase-2 has been found in the cytosol, Golgi complex, mitochondria and also in the nucleus of cells [46]. Regarding SK-BR-3 and MCF-7 cells, we showed that caspase-2 seemed to be primary localized in the nucleus. However, caspase-2 did not colocalize with DNA, as demonstrated with mitotic cells. Treating the cells with death-inducing taxane concentrations seemed to lead to a redistribution of caspase-2 from the nucleus to the cytosol (see Figure 6). Caspase-2 was probably released from the nucleus because of the long-term disintegration of the nuclear envelope associated with the mitotic block after application of taxanes. The question, whether caspase-2 is activated within the nucleus or in the cytoplasm, has not been answered yet [47]. In our case, long-term exposure of most of the cellular caspase-2 to the cytoplasmic environment, which can comprise caspase-2-activating capacity, could lead to caspase-2 activation. Thus, there could be a very simple explanation for caspase-2 activation in breast cancer cells after taxane application.

Caspase-2 activation is usually connected with PIDDosome formation and PIDD protein upregulation via the induction of expression by activated p53 [23-25]. We found significant p53 activation assessed by the induction of $p21^{WAF1/CIP1}$ expression, after the application of death-inducing taxane concentration in MCF-7 cells with functional p53 [48]. On the other hand, in SK-BR-3 cells without functional p53 [39] we confirmed that there was no p53 activity (see Figure 7). In the next step, we assessed the effect of taxanes on PIDD expression. No significant effect of taxanes on PIDD upregulation was found in either SK-BR-3 or MCF-7 cells (see Figure 8). This means that p53 activation, and the subsequent PIDD upregulation, is not involved in caspase-2 activation.

Nevertheless, we detected some coimmunoprecipitation of RAIDD protein with both PIDD protein and caspase-2 in both cell lines 24 h after taxane application at the death-inducing concentrations. However, suprisingly the inhibition of RAIDD expression using siRNA technique did not affect the number of surviving SK-BR-3 as well as MCF-7 cells after taxane application at all. Therefore, we suggest that PIDDosome formation [49] does not represent the main platform for caspase-2 activation in breast cancer cells when apoptosis is induced by taxanes. Recently, other pathways of caspase-2 activation which circumvent PIDDosome formation have been reported [50], e.g. the activation of caspase-2 in DISC complex [51]. Therefore other pathways of caspase-2 activation in cells treated with taxanes should be considered.

Conclusions

We can summarize that significant caspase-2 activation is associated with apoptosis induction by taxanes in tested breast cancer cells and that caspase-2 is required, at least partially, for the induction as well. Caspase-2 could be activated due its release from the nucleus and subsequent long-term exposure to the cytoplasmic environment after taxane application resulting in long-term disintegration of the nuclear envelope. Concerning the mechanism of caspase-2 activation, caspase-2 seems to be activated via other mechanism than PIDDosome formation. The activation of both initiator and executioner caspases after taxane application depends on caspase-2 expression. Thus we can suggest that caspase-2 functions as an apical caspase in apoptosis induction by taxanes.

Materials and methods

Materials

Paclitaxel was obtained from Sigma-Aldrich (St. Louis, MO, USA). SB-T-1216 [13] was synthesized at the Institute of Chemical Biology and Drug Discovery (Stony Brook, NY, USA). Taxanes were dissolved in DMSO (tissue culture quality) to obtain a 1 mM stock solution.

For western blot analysis, the following primary antibodies were used: mouse monoclonal antibody against caspase-3 (#9668), rabbit antibody against cleaved caspase-3 (#9661), rabbit antibody against cleaved caspase-7 (#9491), mouse monoclonal antibody against caspase-8 (#9746), and rabbit antibody against cleaved caspase-9 (#9505) from Cell Signaling Technology (Danvers, MA, USA), rat monoclonal antibody against caspase-2 (ALX-804-355-C100) and mouse monoclonal antibody against PIDD (ALX-804-837) from Enzo Life Science (Farmingdale, NY, USA), rabbit monoclonal antibody against caspase-2 (ab32021), rabbit polyclonal antibody against caspase-2 (ab 18737), goat polyclonal antibody against golgin-160 (anti-GOLGA3, ab40837), and rabbit monoclonal antibody against RAIDD (Ab52621) from Abcam (Cambridge, UK), rabbit polyclonal antibody against caspase-2 (H-145, sc-15379), mouse monoclonal antibody against p21 (F-5, sc-6246), goat polyclonal antibody against PIDD (S-17, sc-32161), and rabbit polyclonal antibody against PIDD (H-300, sc-2354) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and mouse monoclonal antibody against actin (AC-40, A3853) from Sigma-Aldrich.

For precipitation, Protein A/G PLUS- agarose beads (sc-2003) from Santa Cruz Biotechnology were used.

Cells and culture conditions

Human breast carcinoma cell lines SK-BR-3 and MCF-7 were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA) and National Cancer Institute (Frederick, MD, USA). The cells were maintained in a culture medium at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium consisted of basic medium supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany). The basic medium was RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), HEPES (15 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml), as previously described [52].

Assessment of cell growth and survival

Cells were harvested and seeded at 20×10^3 cells/100 μl of culture medium into the wells of a 96-well plastic plate. After 24-h preincubation period allowing cells to attach, the culture medium was replaced by either the culture medium without taxane (control) or with one of tested taxanes (paclitaxel or SB-T-1216) at desired concentrations. Cell growth and survival were evaluated after 96 h of incubation. The number of living cells was determined using a hemocytometer after staining with trypan blue [53].

Measurement of caspase-2 activation

A commercial CaspGLOW™ Active Caspase Staining Kit (Biovision, Mountain View, CA, USA) was used to detect the active form of caspase-2, as previously described [54]. Cells (approximately 3×10^5 cells per sample) were seeded and after a 24-h preincubation period allowing cells to attach, the culture medium was replaced by either taxane-free culture medium (control) or with medium containing taxane (paclitaxel or SB-T-1216) at desired concentrations. After the required incubation period, the cells were harvested using low-speed centrifugation and staining was performed according to the manufacturer's instructions. Fluorescence was measured using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Real-time PCR

Cells were harvested and seeded at 1.2×10^6 cells/6 ml of the culture medium into Petri dishes. After a 24-h preincubation period, the culture medium was either replaced by taxane-free culture medium (control) or with medium containing taxane (paclitaxel or SB-T-1216) at desired concentrations. Total RNA was isolated from SK-BR-3 and MCF-7 cells using a RNAeasy MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions after the required incubation period.

Prepared RNA was reverse transcribed using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA) with random primers, in accordance with the manufacturer's instructions. Transcribed cDNA was subjected to real-time quantitative PCR in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using a commercially available TaqMan Gene Expression Master Mix kit (Applied Biosystems) with TaqMan Gene Expression Assays (Applied Biosystems) for CDKN1A (cyclin-dependent kinase inhibitor 1A, p21), PIDD (p53-induced death domain protein), CASP2 (caspase-2) and for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as the control gene. All data were normalized relative to the amount of GAPDH cDNA in the sample and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in genes expression using ABI Prism 7000 SDS Software Version 1.1 (Applied Biosystems).

Western blot analysis

Cells (approximately 1×10^7 cells per sample) were seeded and taxanes were applied after 24-h preincubation. Cells were harvested after the incubation period by low-speed centrifugation, washed in PBS and centrifuged. Cell pellets

were stored at -80°C. Frozen pelets were resuspended in RIPA buffer (Sigma Aldrich, St. Louis, MO, USA) containing a mixture of protease inhibitors (Sigma Aldrich). The protein lysate was centrifuged (14,000 rpm, 20 min, 4°C) and the supernatant was stored at -20°C.

Western blot was carried out with some modifications as has been previously described in detail [4]. Proteins separated by SDS-PAGE were blotted onto 0.2 μm nitrocellulose membrane PROTRAN BA 83, (Whatman-Schleicher and Schuell, Maidstone, UK) for 3 h at 0.25 A, using a MiniProtean II blotting apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat milk or 5% BSA in TBS for 15 min. TBS containing Tween-20 (0.1%) was used for washing. The washed membrane was incubated with the primary antibody. Following incubation (overnight, 4°C), the membrane was washed (three times) and then incubated for 1–2 h with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterward, the membrane was washed three times and the chemiluminescence signal was detected using a Supersignal West Pico Chemiluminescence Substrate from Pierce (Thermo Fisher Scientific Inc., Rockford, IL, USA) and and the KODAK Gel Logic 1500 Imaging System (Eastman Kodak Company, Rochester, NY, USA).

RNA interference

In order to optimize the RNA interference procedure, two independent siRNAs targeting the caspase-2 mRNA sequence, i.e. MISSION® esiRNA human CASP2 (Sigma Aldrich, St. Louis, MO, USA) and CASP2 siRNA (s2412, Applied Biosystems, Foster City, CA, USA), and two independent siRNA targeting the RAIDD mRNA sequence, i.e. two CRADD (RAIDD) Silencer® Select siRNAs (s16654 and s225028, Applied Biosystems, Foster City, CA, USA), were tested. Two different transfection agents, i. e. INTERFERin™ (Polyplus transfection™, Illkirch, France) and siPORT™ NeoFX™ Transfection Agent (Applied Biosystems) were also tested. We used GAPDH siRNA (Applied Biosystems) as a positive transfection control and Silencer® Negative Control siRNA (Applied Biosystems) as the non-targeting siRNA. The efficiency of caspase-2 and RAIDD inhibition was tested after 48-h incubation of cells with a medium containing transfection mixture for mRNA levels (real-time PCR, data not shown) and after 72-h incubation for protein levels (western blot analysis, Figure 4A). The efficiency of caspase-2 and RAIDD inhibition reached similar levels using any combination of the two tested transfection agents and the two siRNAs. For experiments, INTERFERin™ transfection agent (Polyplus transfection™), CASP2 siRNA (Applied Biosystems) and CRADD (RAIDD) siRNA s225028 (Applied Biosystems) were used.

Based on the manufacturer's instructions (INTERFERin™ in vitro siRNA Transfection Protocol, Polyplus transfection™) we performed RNA interference in SK-BR-3 and MCF-7 cells. The cells were seeded at 7×10^3 cells/200 μl of culture medium into a 96-well plate or at 7×10^4 cells/2 ml of culture medium into a12-well plate for 24-h preincubation. The siRNA was diluted in OPTI-MEM® I Reduced Serum Medium (Gibco, Invitrogen™ Life Technologies, Carlsbad, CA, USA) for a final concentration of 5 nM siRNA. INTERFERin™ transfection agent (0.75 μl per 96-well and 4 μl per 12-well) was added. The mixture was incubated for 10 min at room temperature to form the transfection complex. Preincubation medium in wells was replaced by fresh culture medium (150 μl in 96-well plates and 1 ml in 12-well plates). Prepared transfection complex was added to fresh culture medium in cultivation wells and gently mixed. Cells were incubated with the medium containing transfection complex for 72 h. After incubation, the medium containing transfection complex was replaced with the culture medium containing tested taxane at the death inducing concentration for further analyses.

Confocal microscopy

Cells were seeded onto coverslips (approximately 2×10^5 cells per coverslip) and taxanes were applied after 24 h of preincubation as described above (see "Measurement of caspase-2 activation"). After 36 h of incubation for SK-BR-3 and 60 h of incubation for MCF-7, cells were fixed with 4% paraformaldehyde for 15 min at 37°C and permeabilized with 0.1% Triton X-100 in 4% paraformaldehyde for 15 min. After washing with PBS, cells were blocked with Image-iT™ FX signal enhancer (Molecular Probes, Invitrogen, Eugene, OR, USA) for 30 min. Next, cells were washed with PBS and stained with 30 μl of the primary antibody against caspase-2 (H-145, Santa Cruz Biotechnology, Santa Cruz, CA, USA or ab18737, Abcam, Cambridge, UK), diluted 1:50 in PBS, at 4°C overnight. Cells were than washed with PBS and incubated with 30 μl of Alexa Fluor® 488 goat anti-rabbit secondary antibody (Molecular Probes), diluted 1:200 in PBS, for 1 hour in a dark at room temperature. Finally, cells were washed again with PBS. Stained cells on coverslips were transferred onto a droplet of Vectashield® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and sealed. Samples were analyzed using a Leica TCS SP5 confocal microscope (Bannockburn, IL, USA) using a 63× oil objective at relevant excitation and emission wavelengths.

Immunoprecipitation

First, 25 μl of agarose beads coated with bacterial proteins A and G (sc-2003) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were washed (resuspended in 0.5 ml of lysis buffer and centrifuged at 5000 rpm,

1 min) four times and finally resuspended in 0.5 ml of lysis buffer. 3.3 μl of anti-RAIDD (see "Materials") antibody was added to the beads and mixture was incubated for 4 h in a refrigerator. Beads with bound antibody were washed as described and supernatant was removed. Beads with bound antibody were stored at 4°C.

Cell were harvested (see "Western blot analysis") and lysed by non-denaturing lysis buffer (1% NP-40, 20 mM TRIS pH 7.4, 1 mM EDTA, 5% glycerol, 250 mM NaCl). Cell lysates were incubated for 20 min on ice and then centrifuged (14, 000 rpm, 15 min.) at 4°C. Supernatants containing cell proteins were stored on ice and protein concentrations were assessed.

Beads with bound antibody were diluted (to final volume of 100 μl) in lysate buffer containing 300 μg of cell proteins. The mixture was incubated overnight while agitated. After incubation, the mixture was centrifuged (6500 rpm, 7 min) and supernatant was discarded. Beads with bound immunocomplexes were washed twice as described with lysis buffer and twice with 50 mM Tris HCl pH 7.5. Washed beads with bound immunocomplexes were finally resuspended in 40 μl of sample buffer (see "Western blot analysis") and heated for 15 min at 75°C to disintegrate the beads and release the immunocomplexes. 15 μl of the samples were loaded on 15% polyacrylamide gel for Western blot analysis (see "Western blot analysis").

Statistical analysis

Statistical significance of difference was determined using the Student's *t*-test. $P < 0.05$ and $P < 0.01$ were considered statistically significant at the 5% and 1% levels, respectively.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MJ carried out western blot experiments and worked on the manuscript, KB carried out real-time PCR experiments and siRNA techniques, DK participated in western blot experiments and in coimmunoprecipitation, VNF carried out confocal microscopy, JŠ participated in coimmunoprecipitation, JF carried out flow cytometry analysis of caspases-2 activity, IZ and IO participated in the preparation of SB-T-1216 taxane, JK coordinated the experiments and helped to complete the manuscript. All authors read and approved the final manuscript.

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The role of individual caspases in cell death induction by taxanes in breast cancer cells

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Abstract

Background: In previous study we showed that caspase-2 plays the role of an apical caspase in cell death induction by taxanes in breast cancer cells. This study deals with the role of other caspases. We tested breast cancer cell lines SK-BR-3 (functional caspase-3) and MCF-7 (nonfunctional caspase-3).

Methods and results: Using western blot analysis we demonstrated the activation of initiator caspase-8 and -9 as well as executioner caspase-6 and -7 in both tested cell lines after application of taxanes (paclitaxel, SB-T-1216) at death-inducing concentrations. Caspase-3 activation was also found in SK-BR-3 cells. Employing specific siRNAs after taxane application, suppression of caspase-3 expression significantly increased the number of surviving SK-BR-3 cells. Inhibition of caspase-7 expression also increased the number of surviving SK-BR-3 and MCF-7 cells. On the other hand, suppression of caspase-8 and caspase-9 expression had no significant effect on cell survival. However, caspase-9 seemed to be involved in the activation of caspase-3 and caspase-7. Caspase-3 and caspase-7 appeared to activate mutually. Furthermore, we observed a significant decrease in mitochondrial membrane potential (flow cytometric analysis) and cytochrome c release (confocal microscopy, western blot after cell fractionation) from mitochondria in SK-BR-3 cells. No such changes were observed in MCF-7 cells after taxane treatment.

Conclusion: We conclude that the activation of apical caspase-2 results in the activation of caspase-3 and -7 without the involvement of mitochondria. Caspase-9 can be activated directly via caspase-2 or alternatively after cytochrome c release from mitochondria. Subsequently, caspase-9 activation can also lead to caspase-3 and -7 activations. Caspase-3 and caspase-7 activate mutually. It seems that there is also a parallel pathway involving mitochondria that can cooperate in taxane-induced cell death in breast cancer cells.

Keywords: Taxanes, Breast cancer, Caspases, Cell death

Background

Taxanes are known mitotic poisons. There are two taxanes currently used in cancer therapy, paclitaxel (Taxol®) of natural origin and semi synthetic docetaxel (Taxotere®). They are routinely used in chemotherapy of solid tumors, e.g. breast cancer, ovary cancer, lung cancer and prostate cancer [1]. Unfortunately, resistance of cancer cells to clinically used taxanes (classical taxanes) became a problem. Novel taxanes have been developed in order to overcome resistance of cancer cells [2-4]. Some of these novel taxanes are significantly more effective in resistant cancer cells [5,6].

Taxanes bind to the β subunit of the tubulin heterodimer and prevent depolymerization of microtubules. The stabilization of microtubules blocks progression through the M phase of the cell cycle [7,8]. This state of mitotic arrest normally results in cell death and it is supposedly associated with mitotic catastrophe, which has been observed by many authors in taxanes-treated cells [9-12]. Although there are numerous studies concerning taxane-induced cell death in cancer cells, the molecular mechanism remains elusive [12-14].

It is well known, that functional caspases are required for completing apoptosis after various stimuli. Initiator caspase-9, -8, -10, -2 are involved in apoptosis induction and executioner caspase-3, -6 and -7 are involved in apoptosis execution. The activation of various caspases has been observed after taxane application in many types

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of cancer cells. The activation of initiator caspase-8, often associated with the death receptor signaling pathway, has been found in cells treated with taxanes [15,16]. In contrast, the role of caspase-8, apart from its involvement in certain amplification loops, has been seriously questioned, particularly in regard to melanoma cancer cells [13,15]. The activity of caspase-10, which, together with caspase-8, is involved in the extrinsic apoptotic pathway, has been observed in human leukemia cells after taxane application. However it was not associated with the activation of death receptors [17].

Caspase-2 is a highly conservative protease and it is known to be involved in cell death induction by several different stimuli, e.g. heat shock, growth factors withdrawal or cytoskeleton damage [18]. It is often activated within a cytoplasmic complex, containing in addition PIDD protein and RAIDD protein, referred to as a PIDDosome [19]. Recently, several laboratories, including ours have reported that caspase-2 appears to play a pivotal role in taxane-induced cell death [13,20,21].

Initiator caspase-9 is involved in the mitochondrial pathway of apoptosis induction and its activity has been found in several cancer cell lines [14,16,22] and also in non-cancer cells [23] after taxane application. It indicates that mitochondria can play an important role in the taxane-induced apoptosis at least in certain cancer cell lines [9,24]. Taxanes have also been found to induce the release of cytochrome c from isolated mitochondria [25] as well as from mitochondria in cancer cells [26,27] or embryonic cells [21]. The release of cytochrome c is a hallmark of apoptosis induction via the intrinsic apoptotic pathway. Another significant feature is decreasing mitochondrial membrane potential ($\Delta\psi_m$). Some studies in melanoma and prostate cancer cells have observed decreasing $\Delta\psi_m$ after taxane treatment [13,28].

The activation of the key executive caspase-3 and/or cleavage of its substrate PARP have been observed in many cancer cell types after taxane application [12,14,16]. On the other hand, the role of caspase-6 and -7 in cell death induction remains somewhat unclear [17,29]. However, activation of caspase-7 has been detected in breast cancer cells after taxane exposure [30] as well as after combination treatments [31].

In our previous study, we described the activation of caspase-8, -9 and -3 in sensitive and resistant breast cancer cells after apoptosis induction by paclitaxel, the novel taxane SB-T-1216 as well as certain novel fluorinated taxanes. We observed that cytochrome c was released from the mitochondria in one of the tested cancer cell lines. We also showed that caspase-2 was significantly involved in taxane-induced cell death in breast cancer cells [5,12]. Caspase-2 affected the activation of caspase-3, -7, -8 and -9. Thus caspase-2 seemed to play the role of an apical caspase [20].

In the present study we tested the role of individual caspases in taxane-induced cell death in breast cancer cells. We tested two breast cancer cell lines, SK-BR-3 and MCF-7. It is known that functional p53 is absent in SK-BR-3 cells but present in MCF-7 cells. On the other hand, SK-BR-3 cells are known to have functional caspase-3 while MCF-7 cells are deficient in functional caspase-3 [32,33]. Such differences in proteins involved in apoptosis facilitated elucidation of the role of individual caspases in taxane-induced cell death. We found that the activation of apical caspase-2 led to the activation of executioner caspase-3 and −7 without the mitochondria involvement. Although a parallel pathway involving cytochrome c release and caspase-9 activation may also be involved.

Results

Effect of taxanes on caspase-3 activation

In order to assess caspase-3 activation in SK-BR-3 cells after taxane application (MCF-7 cells have no functional caspase-3), we tested the time course of procaspase-3 cleavage using western blot analysis.

Procaspase-3 levels decreased significantly 36 h after application of both taxanes at death-inducing concentration (100 nM in SK-BR-3 cells, see "Materials and methods"). After 48 h, the level of procaspase-3 in SK-BR-3 cells was extremely low (data not shown). The decrease of procaspase-3 levels correlated with dramatically increased levels of the cleaved form of caspase-3 which occurred 36 h after taxane application. However, low levels of cleaved caspase-3 were detectable 24 h after taxanes application (Figure 1A). The time course of caspase-3 activation is in agreement with our finding that most of SK-BR-3 cells are dead before 48 h of taxane treatment (our unpublished data).

Using western blot analysis, the time course of the levels of caspase-3 substrate PARP was also assessed. A significant decrease in PARP levels after 36 h as well as significant increase in cleaved PARP levels after 24 h and 36 h corresponded with caspase-3 activation after taxane application (Figure 1B). In MCF-7 cells, the cleavage of PARP was also detected 36 h after taxane application (data not shown).

Effect of taxanes on caspase-6 and -7 activations

In order to assess the activation of other executioner caspases (caspase-6 and -7) in both studied cell lines after taxane application, we tested the time course of procaspase-6 and -7 cleavage using western blot analysis.

Procaspase-6 levels decreased somewhat 36 h after the application of taxanes at death-inducing concentration (100 nM) in SK-BR-3 cells. Some decrease was also seen 24 h after the application. Concerning MCF-7 cells, similar effect was seen at least 60 h after taxane

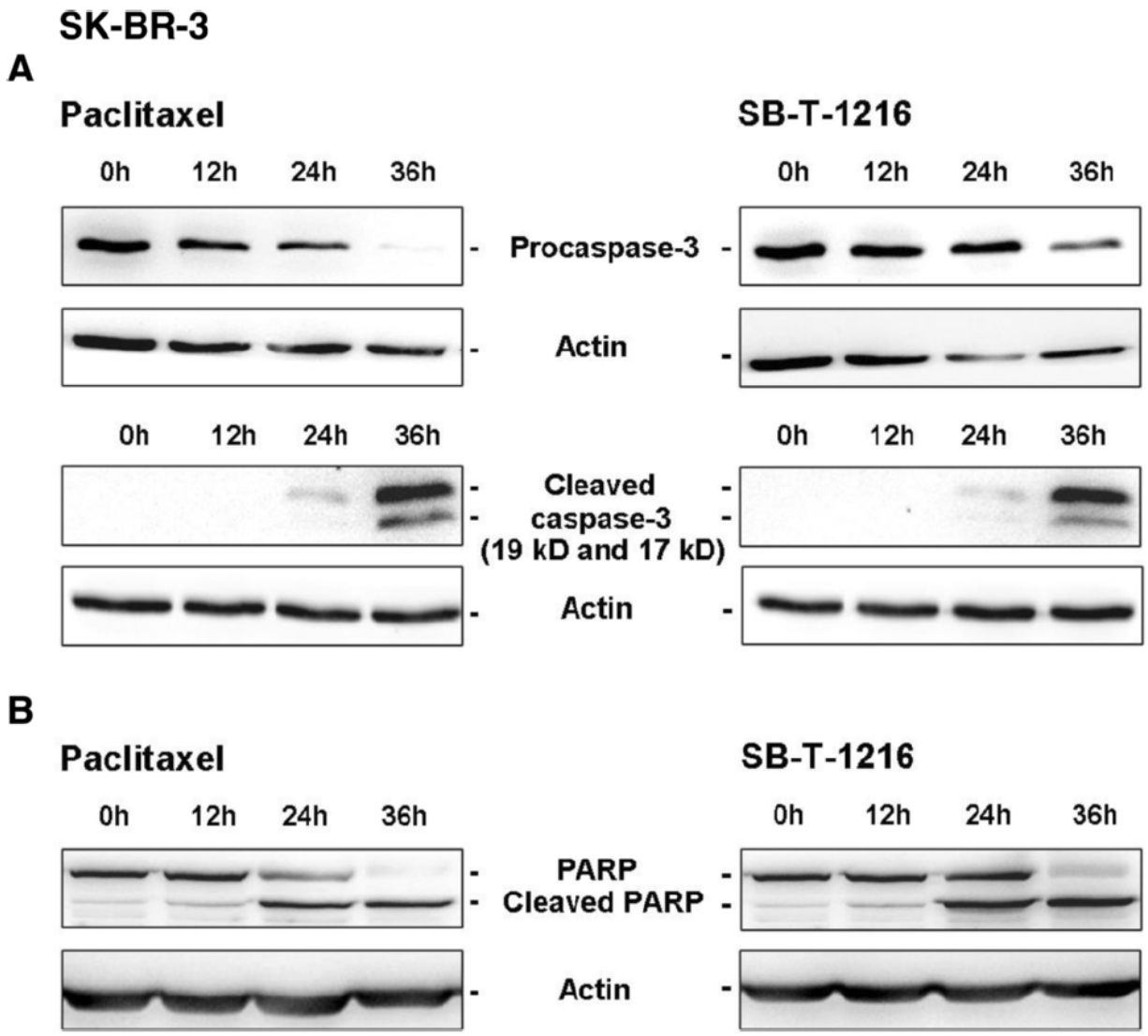

Figure 1 Effect of paclitaxel and SB-T-1216 on the activation and activity of caspase-3 in SK-BR-3 cells. After 0, 12, 24 and 36 h of incubation with paclitaxel or SB-T-1216 (100 nM), levels of **(A)** procaspase-3 and cleaved caspase-3 as well as **(B)** levels of PARP were determined using western blot analysis and relevant antibodies (see "Materials an methods"). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments.

application at death inducing concentration (300 nM in MCF-7 cells, see "Materials and methods"). The decrease of procaspase-6 levels in SK-BR-3 cells correlated roughly with increased levels of the cleaved form of caspase-6 24 h and 36 h after taxanes application. In MCF-7 cells, cleaved caspase-6 was seen from 36 h to 60 h after application (Figure 2A).

Procaspase-7 levels decreased slightly 36 h after taxane application in SK-BR-3 cells. In MCF-7 cells, there was also certain decrease in procaspase-7 level 60 h after taxane application. Increased levels of the cleaved form of caspase-7 were detected 24 h and particularly 36 h after taxane application in SK-BR-3 cells and from 24 h to 48 h or even 60 h in MCF-7 cells (Figure 2B). The time course of caspase-6 and -7 activations is also in agreement with our finding that most of cells are dead before 48 h of taxane treatment (our unpublished data).

Effect of taxanes on caspase-8 and -9 activations

Using western blot analysis, we assessed the activation of initiator caspase-8 and -9 after taxane application by testing the time course of procaspase-8 and -9 cleavages.

Procaspase-8 levels decreased 36 h after the application of both taxanes at death-inducing concentration (100 nM) in SK-BR-3 cells. Concerning MCF-7 cells, a decrease in procaspase-8 levels after the application of

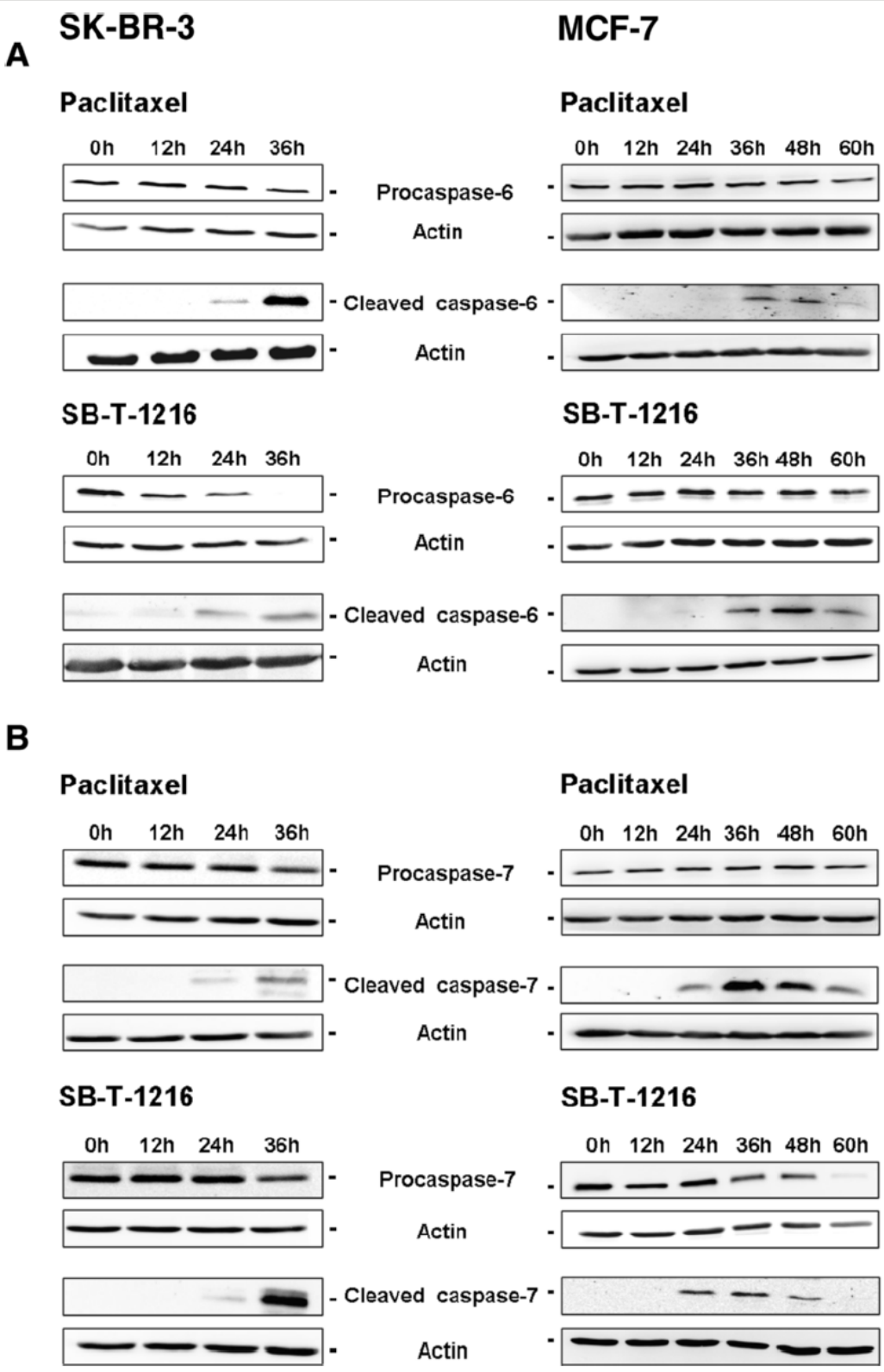

Figure 2 (See legend on next page.)

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Figure 2 Effect of paclitaxel and SB-T-1216 on (A) the activation of caspase-6 and (B) the activation of caspase-7 in SK-BR-3 and MCF-7 cells. After 0, 12, 24, 36, 48 and 60 h of incubation with paclitaxel or SB-T-1216 (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), levels of procaspases and cleaved caspases were determined using western blot analysis and relevant antibodies (see "Materials and methods"). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments.

taxanes at death-inducing concentration (300 nM) was not detected. The decrease of procaspase-8 levels in SK-BR-3 cells correlated with increased levels of the cleaved form of caspase-8 36 h after taxanes application. Cleaved caspase-8 was seen in MCF-7 cells from 24 h to 60 h after taxane application, in spite of the fact that the cleavage of procaspase-8 was not detected here (Figure 3A).

Regarding caspase-9, the decrease of procaspase-9 levels after taxane application was not clearly detected in SK-BR-3 or MCF-7 cells. Increased levels of the cleaved form of caspase-9 appeared 24 h after taxanes application in SK-BR-3 cells. The increase was more pronounced 36 h after the application. Cleaved caspase-9 levels were also seen from 24 h to 60 h after taxane application in MCF-7 cells (Figure 3B). Similarly like in the case of caspase-3, -6 and -7, the time course of caspase-8 and -9 activations is in agreement with our finding that cells are principally dead before 48 h of taxane treatment (our unpublished data).

Effect of the inhibition of caspase-3, -7, -8 and -9 expression on taxane induced cell death

Employing RNA interference, we assessed the effect of specific inhibition of caspase-3, -7, -8 and -9 expressions on cell death induction by taxane application.

First, the efficiency of the RNA interference was tested. It revealed that inhibition of the expression of individual caspases was efficient enough in both SK-BR-3 and MCF-7 cells (Figure 4A). Furthermore, nonsense siRNA or specific caspase siRNAs did not significantly affect cell growth or survival in either cell line. Cell transfected with siRNAs seemed to grow slightly slower when compared with control cells (data not shown). In spite of this fact, in the case of caspase-3 and -7 we were able to detect significant increase of cell growth and survival after the inhibition of caspase expression by siRNA application when cells were incubated with taxanes (see below).

After 48 h of incubation with taxanes at death-inducing concentrations (100 nM), inhibition of caspase-3 expression (81%) resulted in an approximately 6-fold increase in the number of surviving SK-BR-3 cells. For both taxanes, it represents a statistically significant increase from about 2% to 12-14% of the number of cells cultured without taxane. The inhibition of caspase-7 expression (81%) resulted in an approximately 4-fold increase in the number of surviving SK-BR-3 cells after 48 h of incubation. It again represents a statistically significant increase (from about 2% to 7-8%). After 96 h, the effect of the inhibition of caspase-3 and caspase-7 expression was similar or even more pronounced. On the other hand, we did not detect any significant effect of caspase-8 (94%) or caspase-9 (70%) suppression on cell death induction by taxanes in SK-BR-3 cells (Figure 4B).

Concerning MCF-7 cells, inhibition of caspase-7 expression (81%) increased the number of surviving cells by approximately 2-fold after 48-h incubation with taxanes at death-inducing concentration (300 nM). It was a statistically significant increase from about 20% to about 40% of the number of cells cultured without taxane. After 96 h, the effect of the inhibition of caspase-7 expression was very similar. We did not detect a significant effect of caspase-8 (89%) and caspase-9 (61%) suppression on taxane-induced cell death in MCF-7 cells. Perhaps there was a slight, although insignificant, increase of the number of surviving cells linked to the inhibition of caspase-9 expression (Figure 4B).

Effect of the inhibition of caspase-8, -9, -3 and -7 expression on taxane induced activation of caspase-8, -9, -3, -7

Using the siRNA technique, we assessed the effect of specific inhibition of caspase-3, -7, -8 and -9 expression on the activation of caspase-8, -9, -3, and -7 after taxane application. Inhibition efficiency of individual caspases is mentioned above (or see "Materials and methods"). To confirm caspase-2 role as an apical caspase [20], the effect of specific inhibition of the expression of tested caspases on procaspase-2 cleavage was also assessed after taxane application. No significant effect was found (Figure 5).

After 36-h incubation of SK-BR-3 cells with taxanes at death inducing concentration (100 nM), subsequent western blot analysis showed significantly decreased cleavage of caspase-8, -9 and -7 due to inhibition of caspase-3 expression. Decreased cleavage of caspase-3 was observed in response to inhibition of caspase-7 expression. Decreased cleavage of caspase-9 was seen in response to inhibition of caspase-8 expression and decreased cleavage of caspase-3 and -7 linked to inhibition of caspase-9 expression (Figure 5A).

Concerning MCF-7 cells after 36 h of incubation with taxanes at death-inducing concentration (300 nM), inhibition of caspase-7 expression did not significantly affect the cleavage of caspase-9 but it slightly affected the cleavage of caspase-8. Inhibition of caspase-8 expression did not affect the cleavage of caspase-9 and -7.

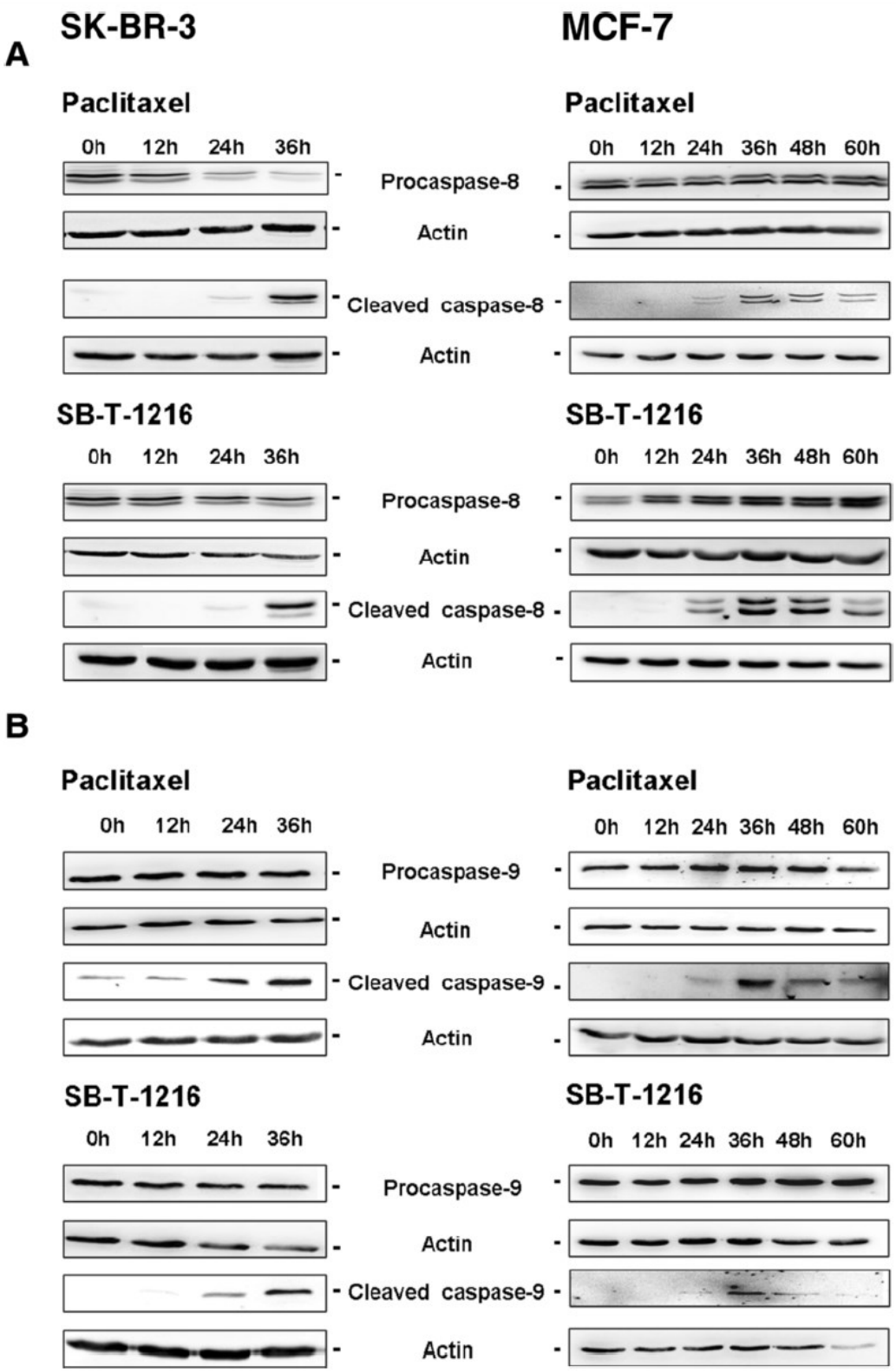

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Figure 3 Effect of paclitaxel and SB-T-1216 on (A) the activation of caspase-8 and (B) the activation of caspase-9 in SK-BR-3 and MCF-7 cells. After 0, 12, 24, 36, 48 and 60 h of incubation with paclitaxel or SB-T-1216 (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), levels of procaspases and cleaved caspases were determined using western blot analysis and relevant antibodies (see "Materials and methods"). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments.

Inhibition of caspase-9 expression did not affect the cleavage of caspase-8, but it significantly decreased the cleavage of caspase-7 (Figure 5B).

Effect of taxanes on mitochondrial membrane potential ($\Delta\psi_m$)

We employed [DiOC6(3)] staining and subsequent flow cytometry to assess the effect of taxanes on mitochondrial membrane potential ($\Delta\psi_m$).

In SK-BR-3 cells, within 36 h of incubation with taxanes at death-inducing concentration (100 nM), $\Delta\psi_m$ decreased significantly close to a total collapse. On the other hand, $\Delta\psi_m$ did not show any significant decrease in MCF-7 during 60 h after taxane application. It clearly demonstrated different responses of mitochondrial membrane potential to taxane application in SK-BR-3 and MCF-7 cells (Figure 6).

Effect of taxanes on cytochrome c release

The effect of tested taxanes on cytochrome c release from mitochondria was assessed using confocal microscopy and cell fractionation followed by western blot analysis.

Confocal microscopy showed that cytochrome c was mainly localized in the mitochondria of control SK-BR-3 as well as MCF-7 cells. After 36 h of incubation of SK-BR-3 cells with taxanes at death-inducing concentration (100 nM), cytochrome c was released from the mitochondria into the cytosol. However, in MCF-7 cells, cytochrome c was still found within mitochondria 36 h after taxane application (300 nM) (Figure 7A).

These results were confirmed using western blot analysis after cell fractionation. Cytochrome c was shown to be mainly localized in the mitochondrial cell fraction in both SK-BR-3 and MCF-7 cells before taxane application. In SK-BR-3 cells, cytochrome c levels increased rapidly in the cytosolic fraction 24 h and particularly 36 h after taxane application. In MCF-7 cells, cytochrome c stayed mainly in the mitochondrial fraction after taxane application. Thus, after taxane application, cytochrome c is released from mitochondria only in SK-BR-3 cells, but not in MCF-7 cells (Figure 7B).

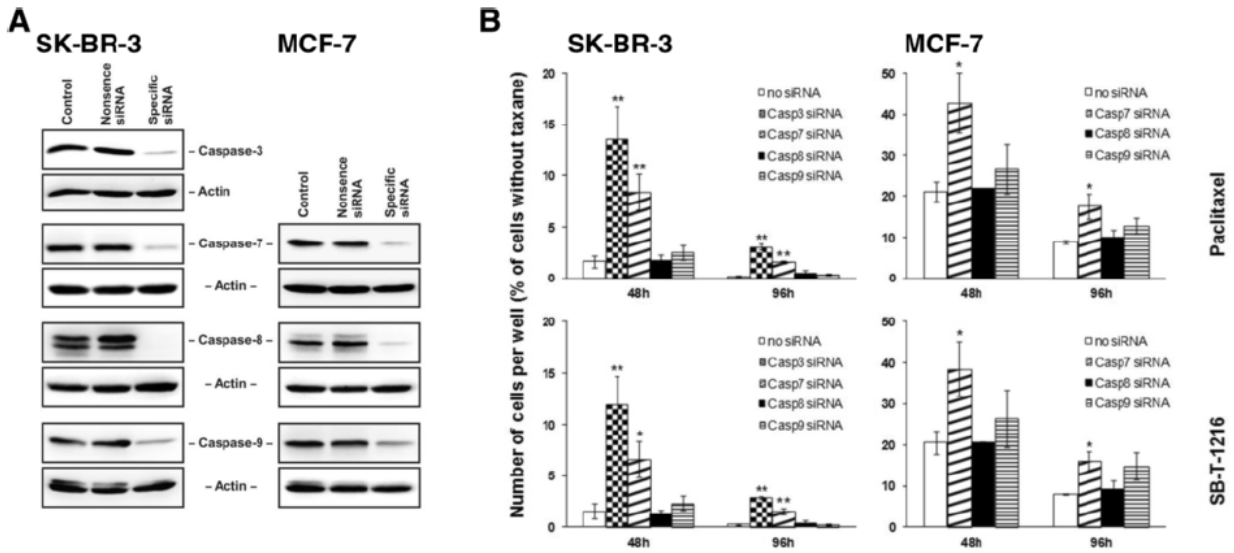

Figure 4 Effect of inhibition of caspase-3, -7, -8 and -9 expression on cell growth and survival of SK-BR-3 and MCF-7 cells after paclitaxel and SB-T-1216 application. (A) Efficiency of caspase suppression based on specific siRNAs in SK-BR-3 and MCF-7 cells is shown. Levels of caspases were determined using western blot analysis and relevant antibodies (see "Materials and methods"). Actin levels were used to confirm equal protein loading. **(B)** The effect of specific caspase siRNAs on the growth and survival of SK-BR-3 and MCF-7 cells after taxane treatment (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells) is presented. The cells were seeded at 20×10^3 cells/100 μl of medium per well and prepared as described (see "Materials and methods"). After 48 and 96 h of incubation, the number of living cells was determined (see "Materials and methods"). Each column represents the mean of 3 or 4 separate cultures ± SEM. *$P < 0.05$, **$P < 0.01$ when comparing the number of living cells in cultures with individual specific siRNAs and culture with no siRNA.

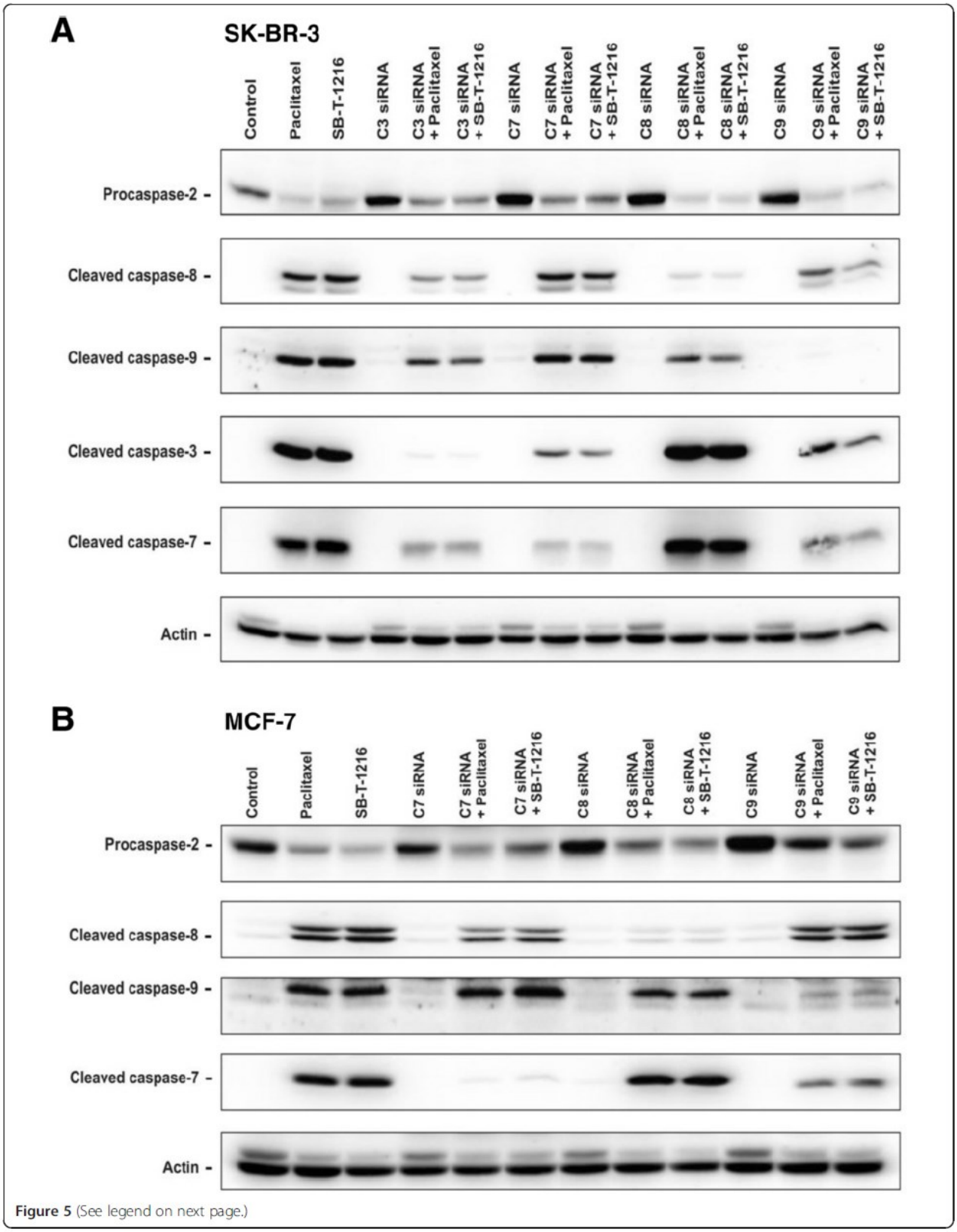

Figure 5 (See legend on next page.)

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Figure 5 Effect of the inhibition of caspase-3, -7, -8 and -9 expression on the activation of caspase-3, -7, -8 and -9 in (A) SK-BR-3 and (B) MCF-7 cells after paclitaxel and SB-T-1216 application. Specific caspase-3 siRNA, caspase-7 siRNA, caspase-8 siRNA and caspase-9 siRNA were used. Control cells were incubated without taxane and siRNA. After 36 h of incubation with the tested taxane (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells) and relevant specific siRNA, levels of cleaved caspases were determined using western blot analysis and relevant antibodies (see "Materials and methods"). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments.

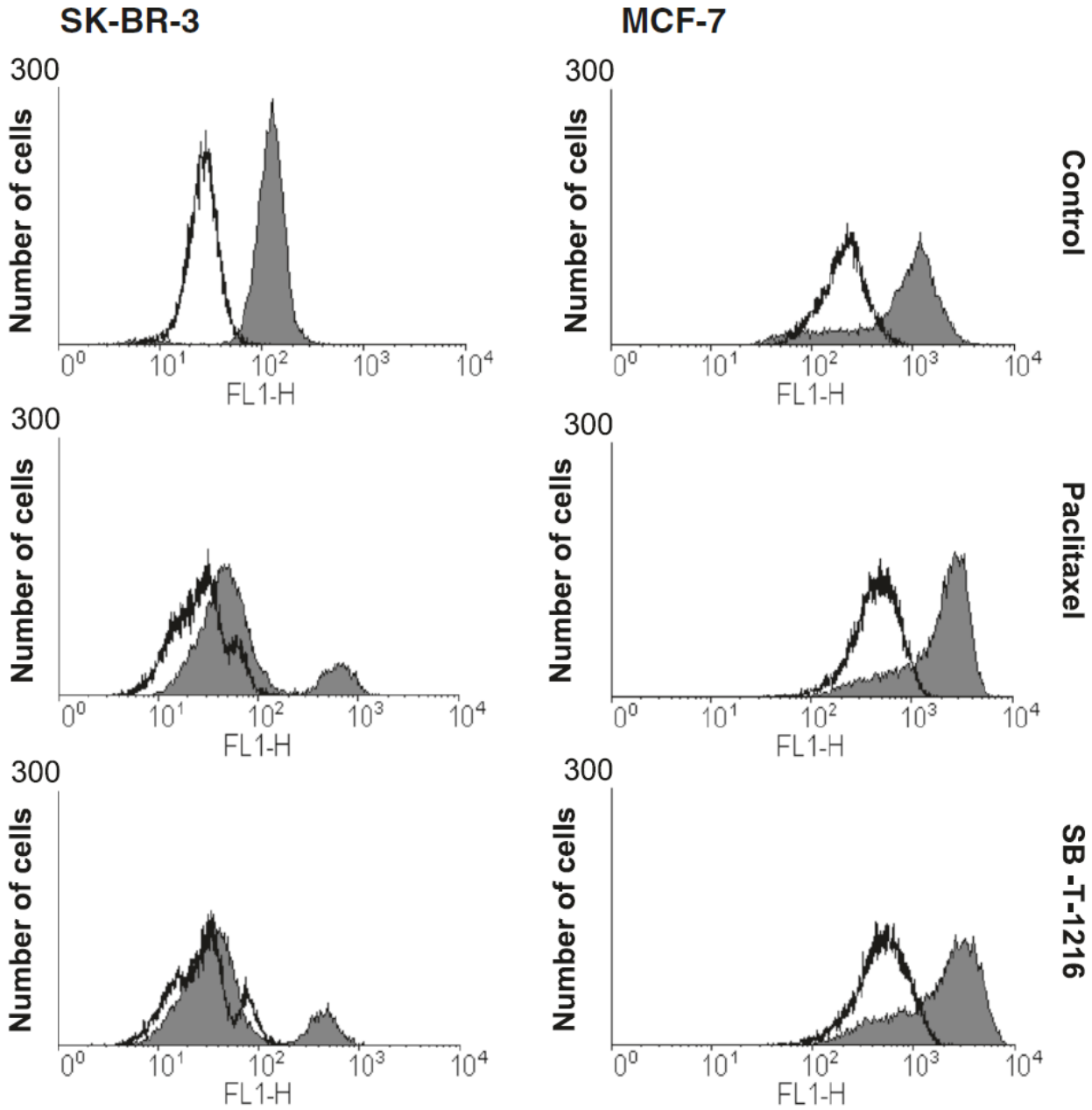

Figure 6 Effect of paclitaxel and SB-T-1216 on the mitochondrial membrane potential ($\Delta\psi_m$) in SK-BR-3 and MCF-7 cells. Control cells were incubated without taxane. After 36 h (SK-BR-3) or 60 h (MCF-7) of incubation with tested taxane (100 nM for SK-BR-3 and 300 nM for MCF-7) the mitochondrial membrane potential was assessed using flow cytometry analysis of cells after staining with [DiOC6(3)] (see "Materials and methods"). The collapse of $\Delta\psi_m$ caused by the application of CCCP is shown (blank areas). The data shown were obtained in one representative experiment of three independent experiments.

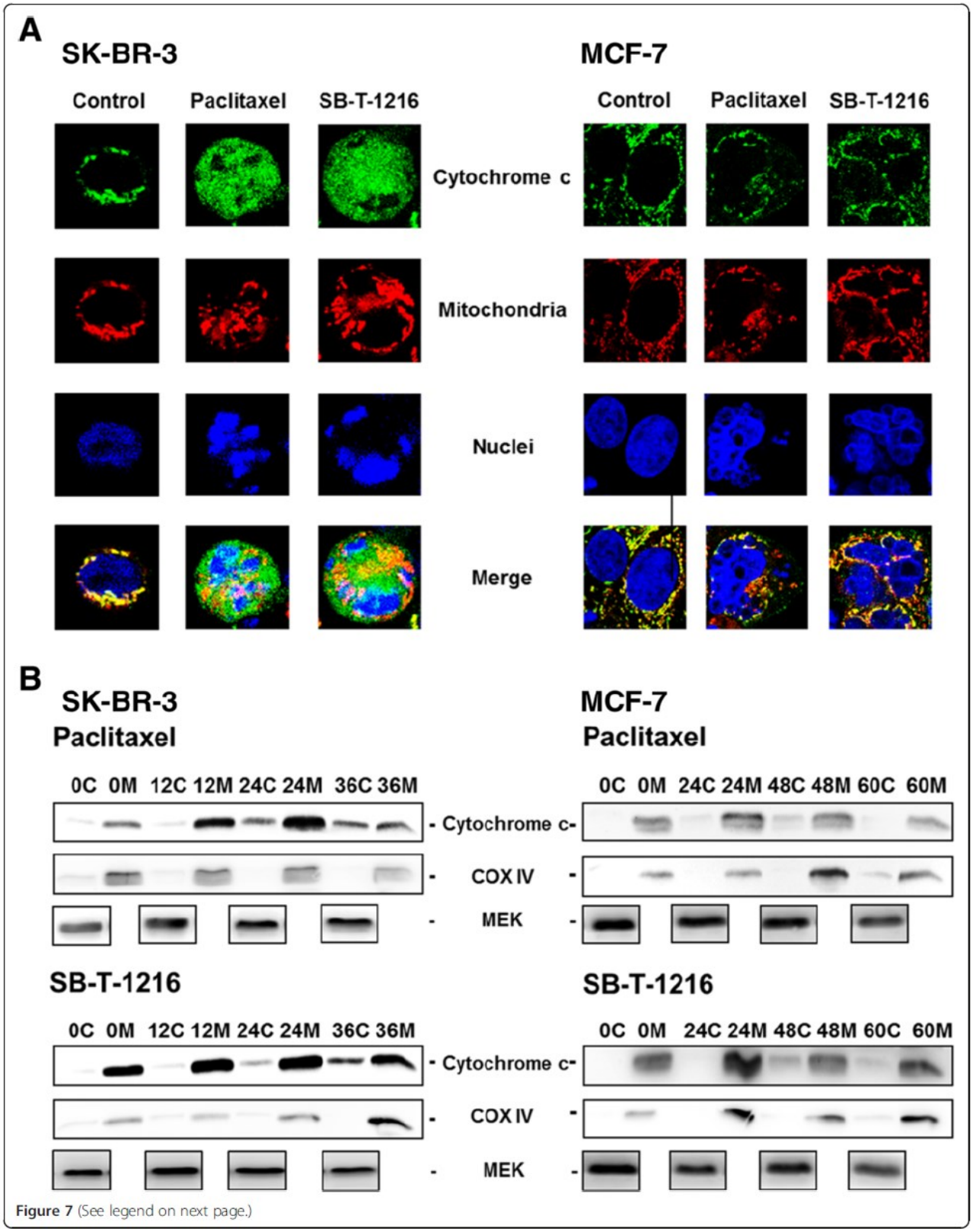

Figure 7 (See legend on next page.)

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Figure 7 Effect of paclitaxel and SB-T-1216 on cytochrome c release from mitochondria in SK-BR-3 and MCF-7 cells. (A) After 36 h of incubation with tested taxane (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), the localization of cytochrome c within the cells was detected using confocal microscopy (see "Materials and methods"). Control cells were incubated without taxane. The localization of cytochrome c (green), mitochondria (red), nuclei (blue) and the merge of cytochrome c, mitochondria and nuclei are shown. The data shown were obtained in one representative experiment of three independent experiments. **(B)** After 0, 12, 24, 36, 48 and 60 h of incubation with tested taxane (100 nM for SK-BR-3 cells and 300 nM for MCF-7 cells), levels of cytochrome c in mitochondrial (M) and cytosolic (C) fractions were determined using western blot analysis and relevant antibodies (see "Materials and methods"). COX IV (integral mitochondrial protein) level was used to confirm proper fractionation. MEK levels were used to confirm equal protein loading of cytosolic fraction. The data shown were obtained in one representative experiment of three independent experiments.

Discussion

In our previous paper [20] we studied the role of caspase-2 in taxane-induced cell death in breast cancer cells. It was suggested that caspase-2 plays the role of an apical caspase. This study deals with the role of other caspases, i.e. initiator caspase-8 and -9 as well as executioner caspase-3 and -7 in taxane-induced cell death in breast cancer cells. The relationship between activation of individual caspases was of particular interest. We used two breast cancer cell lines, SK-BR-3 (nonfunctional p53, functional caspase-3) and MCF-7 (functional p53, nonfunctional caspase-3) as an experimental model. We previously reported that p53 is activated in MCF-7 cells after taxane application. However, we did not observed p53-induced increased expression of PIDD protein or any other function of p53 in activation of caspase-2 that plays important role in cell death induction [20]. The role of p53 in apoptosis induction after taxane application is also questioned because we did not find the activation of mitochondrial death pathway to have a decisive role in cell death induction here. Importantly, apoptosis was induced in both tested cell lines even SK-BR-3 have no functional p53 [20]. Taken together, we do not suppose p53 to play an important role in cell death induction after taxane application in tested breast cancer cells.

We tested two taxanes, classical (clinically used) paclitaxel and novel taxane SB-T-1216. The effect of both taxanes was found to be very similar.

We found that caspase-8 was activated after taxane application at death-inducing concentrations in both tested cell lines (see Figure 3). However, the suppression of caspase-8 expression did not affect cell survival after taxane treatment at all (see Figure 4). Activation of other caspases, especially the executioner caspases, was not significantly affected in cells where caspase-8 expression had been inhibited (see Figure 5). The role of caspase-8 in various types of cancer cells after taxane treatment has been previously discussed in several reports [12,15]. Interestingly, caspase-8 activation was usually observed only with the simultaneous activation of other initiator caspases (caspase-2 and caspase-9) after taxane application [12,16,22]. The role of caspase-8 in taxane-induced cell death was questioned by Park et al. [17]. They did not find any significant role of caspase-8 in taxane-induced cell death in lymphoid cells. Similarly, we suggest that caspase-8 does not play any significant role in cell death induction by taxanes in breast cancer cells.

On the contrary, caspase-2 seems to play a key role in cell death induction by taxanes [20]. We and others have shown that cancer as well as non-cancer cells without functional caspase-2 were more resistant to taxanes [13,20,21]. We showed that the inhibition of caspase-2 expression resulted in decreased activation of caspase-9, -3 and -7 after taxane application in breast cancer cells which implies that caspase-2 plays the role of an apical caspase in these cells [20] (see Figure 8).

Taxanes have been shown to induce the mitochondrial pathway of apoptosis induction by several authors [5,9,23]. Also in this study, we observed significant decreases of $\Delta\psi_m$ and cytochrome c release from mitochondria in SK-BR-3 cells after taxane application. However, we did not detect any change in $\Delta\psi_m$ or cytochrome c release in taxane-treated MCF-7 cells (see Figures 6 and 7). Previous reports have shown that cytochrome c was released from mitochondria and $\Delta\psi_m$ decreased in certain types of cancer cells treated with taxane [5,27]. On the other hand, other types of cancer cells underwent cell death without cytochrome c release from mitochondria [5]. Recently, the role of cytochrome c in taxane-induced cell death was also assessed by Li et al. [34]. They observed cytochrome c release from mitochondria but cell death was induced without cytochrome c involvement. Thus, it seems that the mitochondrial pathway does not play an essential role in apoptosis induction by taxanes, at least in certain types of cancer cells (see Figure 8).

Caspase-9 was activated in SK-BR-3 cells and a relatively low degree of activation was also found in MCF-7 cells (see Figure 3). Activation of caspase-9 has been observed by many authors in various types of cancer cells after taxane treatment and it usually appeared together with caspase-3 activation [12,16,35]. Concerning the mechanism of caspase-9 activation following taxane treatment, we suggest that there are at least two pathways involved. Caspase-9 can be activated in the cytosol via the classical pathway after cytochrome c release from mitochondria or it can be activated via caspase-2

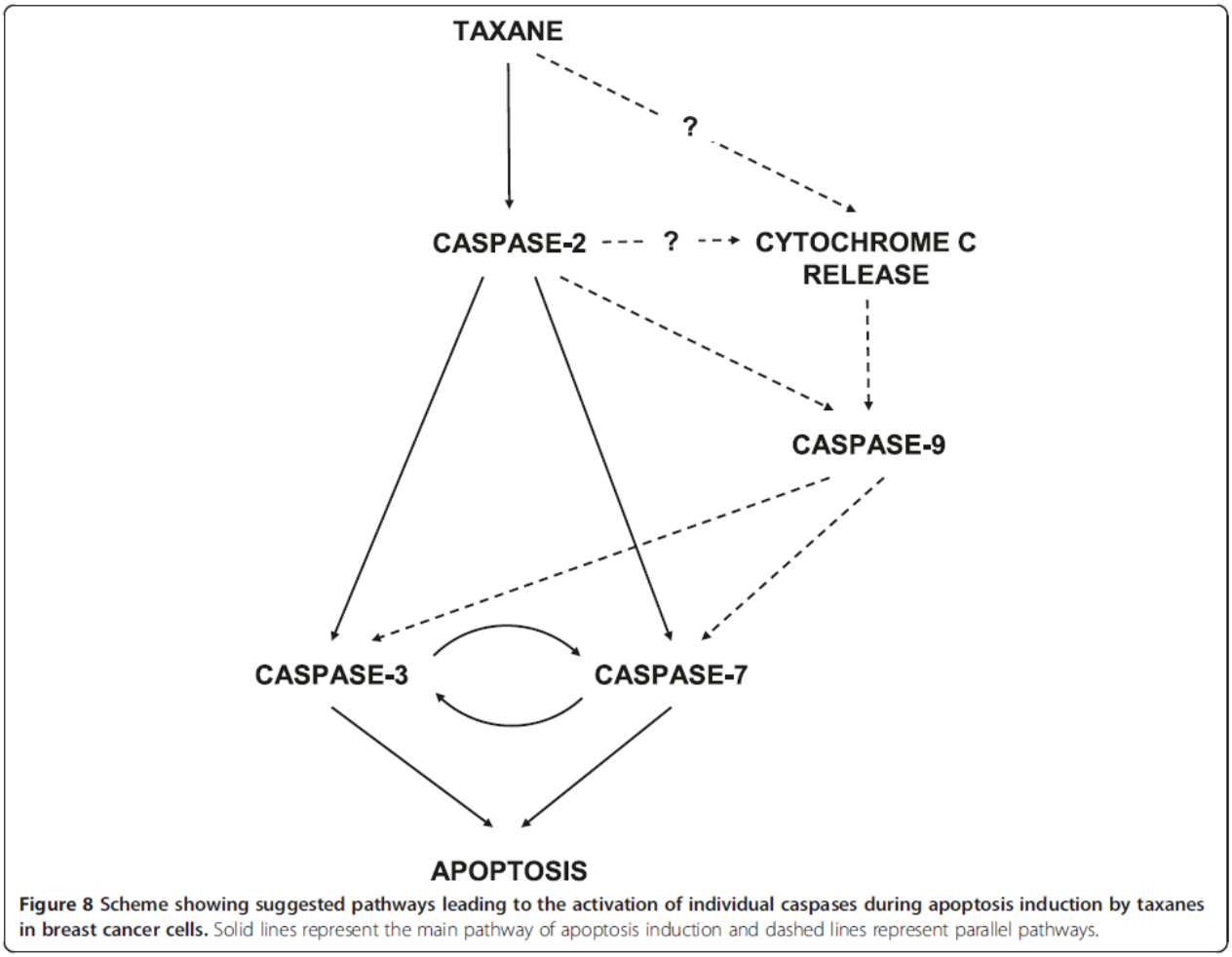

Figure 8 Scheme showing suggested pathways leading to the activation of individual caspases during apoptosis induction by taxanes in breast cancer cells. Solid lines represent the main pathway of apoptosis induction and dashed lines represent parallel pathways.

activation without the involvement of cytochrome c (see Figure 8). Although cells were slightly protected against cell death induction by the inhibition of caspase-9 expression, the effect on cell survival after taxane application was not significant in both tested cell lines (see Figure 4). Independence of cell death induction by taxanes in MCF-7 cells on caspase-9 activity was described previously [36]. Using caspase-9 siRNA, we also found some decreased activation of executioner caspase-3 and -7 after taxane application (see Figure 5). Finally, we suggest that caspase-9 could play a role in the parallel pathway of executioner caspase-3 and -7 activations after taxane application (see Figure 8).

Caspase-3 was activated and its substrate PARP was cleaved in SK-BR-3 cells after taxane application (see Figure 1). Caspase-3 was also found to be activated in other cancer cell types after exposure to paclitaxel [16,24,37] or docetaxel [38]. Inhibition of caspase-3 expression increased the number of surviving SK-BR-3 cells significantly (see Figure 4). Concerning MCF-7 cells, there are previous reports demonstrating that overexpression of caspase-3, after transfection of the caspase-3 gene, increased the sensitivity of cells to paclitaxel [32]. On the other hand, caspase-3-independent cell death has been also reported to occur in MCF-7 cells transfected by the caspase-3 gene after paclitaxel application [36]. We also demonstrated that the suppression of caspase-3 expression decreased the activation of caspase-7 (see Figure 5). It confirms that caspase-7 is a caspase-3-activated caspase. Thus, caspase-3 seems to be a universally important executioner caspase for taxane-induced cell death in a variety of cancer cell types.

Executioner caspase-7 and caspase-6 were activated in tested cell lines after taxane application (see Figure 2). Caspase-7 activation was also observed during cell death induction by zoledronic acid in breast cancer cells [39] or in other types of cancer cells after paclitaxel application [24,30]. We showed that the suppression of caspase-7 expression significantly increased the number of surviving SK-BR-3 as well as MCF-7 cells (see Figure 4). MCF-7 cells without functional caspase-7 have been recently described to be more resistant to paclitaxel

[40]. We also demonstrated that the inhibition of caspase-7 expression led to a decrease of caspase-3 activation in SK-BR-3 cells (see Figure 5). Thus it seems that caspase-3 and -7 cooperate in the later phases of apoptosis induction by taxanes via their mutual activation (see Figure 8). Cooperation of caspase-3 and caspase-7 during cell death induction was recently described by Brentnall et al. [41].

Conclusion

We can summarize that caspase-2 plays the key role of an apical caspase in main death-inducing pathway after taxane application in tested breast cancer cells. This pathway leads from activated caspase-2 to the activation of executioner caspase-3 and -7 without the involvement of mitochondria. There is mutual activation of caspase-3 and -7. Furthermore, taxanes appear to be able to induce cytochrome c release from mitochondria in some breast cancer cells. In these cases caspase-9 is activated. However, caspase-9 can also be activated here without the mitochondrial involvement, probably via caspase-2 activation. This parallel pathway represents another possibility for caspase-3 and caspase-7 activation. It suggests that both pathways can cooperate in cell death induction by taxanes in at least some types of breast cancer cells.

Materials and methods

Materials

Paclitaxel was obtained from Sigma-Aldrich (St. Louis, MO, USA). SB-T-1216 was synthesized at the Institute of Chemical Biology and Drug Discovery (Stony Brook, NY, USA). Taxanes were dissolved in DMSO (tissue culture quality) to obtain a 1 mM stock solution.

For western blot analysis, the following primary antibodies were used: mouse monoclonal antibody against caspase-3 (#9668), rabbit polyclonal antibody against cleaved caspase-3 (#9661), rabbit polyclonal antibody against caspase-6 (#9762), rabbit polyclonal antibody against cleaved caspase-6 (#9761), rabbit polyclonal antibody against caspase-7 (#9492), rabbit polyclonal antibody against cleaved caspase-7 (#9491), mouse monoclonal antibody against caspase-8 (#9746), rabbit monoclonal antibody against cleaved caspase-8 (#9496), rabbit polyclonal antibody against caspase-9 (#9502), rabbit polyclonal antibody against cleaved caspase-9 (#9505), rabbit polyclonal antibody against COX IV (#4844), rabbit polyclonal antibody against cytochrome c (#4272), rabbit polyclonal antibody against PARP (#9542) from Cell Signaling Technology (Danvers, MA, USA), and mouse monoclonal antibody against actin (AC-40, A3853) from Sigma-Aldrich.

Casp3 Silencer® Select Validated siRNA 4427038, Casp7 Silencer® Select Validated siRNA 4427038, Casp8 Silencer® Select Validated siRNA 4427038, and Casp9 Silencer® Select Validated siRNA 4427037 were from Life technologies (Carlsbad, CA, USA).

Cells and culture conditions

Human breast carcinoma cell lines SK-BR-3 and MCF-7 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and the National Cancer Institute (Frederick, MD, USA), respectively. The cells were maintained in a culture medium at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium consisted of basic medium supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany). The basic medium was RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), HEPES (15 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) [42]. For experiments, paclitaxel and SB-T-1216 were diluted in culture medium to produce a final concentration of 100 nM (SK-BR-3) and 300 nM (MCF-7). These cell death inducing concentrations are the lowest taxane concentrations with nearly maximum effect on tested cells. The concentrations were derived from dose response experiments described in detail previously [20]. DMSO was used as a dissolvent agent for taxanes. DMSO itself at used concentrations was found without any effect on tested cells.

Assessment of cell growth and survival

Cells were harvested and seeded at 20×10^3 cells/100 μl of culture medium into the wells of a 96-well plastic plate. After a 24-h preincubation period allowing cells to attach, the culture medium was replaced by either culture medium without taxane (control) or medium with one of tested taxanes (paclitaxel or SB-T-1216) at desired concentrations. Cell growth and survival were evaluated after 48 h and 96 h of incubation. The number of living cells was determined using a hemocytometer after staining with trypan blue.

Preparation of cell lysates

Cells at desired concentrations were seeded into wells of a plastic plate, Petri dishes or culture flasks and taxanes were applied after a 24-h preincubation. After the incubation period, cells were harvested by low-speed centrifugation (2000 rpm, 9 min, 4°C), washed in PBS and centrifuged. Cell pellets were stored at -80°C. Frozen pellets were resuspended in RIPA buffer (Sigma Aldrich) containing a 1% mixture of protease inhibitors P8340 (AEBSF 104 mM, Aprotinin 80 μM, Bestatin 4 mM, E-64 1.4 mM, Leupeptin 2 mM, Pepstain A 1.5 mM, Sigma Aldrich). Protein lysates were centrifuged (14,000 rpm, 20 min, 4°C) and the supernatants containing proteins were stored at -80°C. Protein lysates were than analyzed using western blot.

Cell fractionation

Cells (approximately 3.6×10^6 cells per sample) were seeded into Petri dishes or culture flasks and taxanes were applied after a 24-h preincubation. After the incubation period, cells were harvested by low-speed centrifugation (2000 rpm, 9 min, 4°C), washed in PBS and centrifuged again. Cell pellets were resuspended in a specific lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose and 1% mixture of protease inhibitors P8340 from Sigma Aldrich) containing 0.635 mM digitonin D141 (Sigma-Aldrich) and vortexed for 30 s. Lysates were than centrifuged (14,000 rpm, 1 min, 4°C) and supernatants (cytosolic fractions) were removed and stored at -80°C. The specific lysis buffer described above containing 6.35 mM digitonin D141 (Sigma-Aldrich) was added to the pellets and suspensions were vortexed for 30 s and centrifuged (14,000 rpm, 1 min, 4°C). After centrifugation, supernatants (mitochondrial fractions) were removed and stored at −80°C. Cell fractions were analyzed using western blot.

Western blot analysis

First, the concentration of proteins in cell lysates was assessed using BCA Protein Assay Reagent from Pierce (Thermo Fisher Scientific, Rockford, IL, USA).

Depending on protein concentration, cell lysates were diluted in RIPA buffer to the gel-loading concentration of proteins (2.5 μg/μl), mixed with equal volumes of sample buffer (0.125 M Tris/HCl pH 6.8, 10% glycerol, 4% SDS, 0.25 M DTT) and heated for 5–7 min at 110°C. Protein samples were separated using a protein electrophoresis (Bio-Rad, Hercules, CA). Proteins separated by SDS-PAGE were blotted onto 0.2 μm nitrocellulose membrane PROTRAN BA 83 (Whatman-Schleicher and Schuell, Maidstone, UK) for 3 h at 0.25 A, using a Mini-Protean II blotting apparatus (Bio-Rad). The membrane was blocked with 5% non-fat dry milk or 5% BSA in TBS for 15–20 min and incubated with the primary antibody at 4°C overnight. After the incubation, the membrane was washed three times (5–10 min) with TBS containing 0.1% Tween-20. Then it was incubated for 1–2 h with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterward, the membrane was washed (as described above) and the chemiluminescence signal was detected using the Supersignal reagents from Pierce (Thermo Fisher Scientific) and a CCD device (Kodak).

RNA interference

Based on the manufacturer's instructions (INTERFERin™ in vitro siRNA Transfection Protocol, Polyplus transfection™), RNA interference in SK-BR-3 and MCF-7 cells was completed. The cells were seeded at 2.1×10^5 cells/6 ml of culture medium into a Petri dish for a 24-h preincubation. The siRNAs (see "Materials") were diluted in 400 μl OPTI-MEM® I Reduced Serum Medium (Gibco, Invitrogen™ Life Technologies, Carlsbad, CA, USA) to a final concentration of 5 nM and the INTERFERin™ transfection agent (18 μl per reaction mixture) was added. The mixture was intensively vortexed and incubated for 10 min at room temperature to form a transfection complex. The preincubation medium in Petri dish was replaced by 4 ml of fresh culture medium. Transfection mixture was added and gently mixed. Cells were incubated in the presence of transfection complexes for 72 h. After incubation, cells were harvested into fresh culture medium and seeded at 2×10^5 cells/ml for further analysis. The efficiency of gene silencing using RNA interference was confirmed using western blot analysis followed by densitometry: in the case of SK-BR-3 cells 81% for caspase-3, 81% for caspase-7, 94% for caspase-8 and 70% for caspase-9, and in the case of MCF-7 cells 81% for caspase-7, 89% for caspase-8 and 61% for caspase-9.

Flow cytometric analysis of the mitochondrial membrane potential ($\Delta\psi_m$)

Cells (approximately 5×10^5 cells per sample) were seeded into Petri dishes and taxanes were applied after a 24-h preincubation. After the incubation period, cells were harvested by low-speed centrifugation (2000 rpm, 9 min, 4°C) and resuspended in PBS. To assess $\Delta\psi_m$, cells were incubated with 20 nM 3, 3′-dihexyloxacarbocyanine iodide [DiOC6(3)] from Invitrogen (Grand Island, USA) at 37°C for 20 min. As a negative control, the cells were pre-incubated with 100 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, St. Louis, USA), a protonophore causing a complete disruption of the $\Delta\psi_m$, at 37°C for 20 min. After incubation, samples were kept on ice. The fluorescence of cells was measured using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Confocal microscopy

Confocal microscopy was described previously [43]. Briefly, cells were seeded onto coverslips (approximately 2×10^5 cells per coverslip) and taxanes were applied after 24 h of preincubation. After 36 h of incubation, cells were stained with Mitotracker Red 480 (Molecular Probes, Grand Island, USA), fixed with 4% paraformaldehyde at 37°C for 15 min and permeabilized with 0.1% Triton X-100 in 4% paraformaldehyde for the next 15 min. After washing with PBS, cells were blocked with Image-iT™ FX signal enhancer (Molecular Probes, Invitrogen, Eugene, OR, USA) for 30 min. Next, cells were stained with the corresponding primary antibody at 4°C

overnight. Cells were than washed with PBS and incubated with the corresponding secondary antibody for 1 hour in the dark at room temperature. Finally, cells were washed again with PBS. Stained cells on coverslips were transferred onto a droplet of Vectashield® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and sealed. Samples were analyzed using a Leica TCS SP5 confocal microscope (Bannockburn, IL, USA) with relevant excitation and emission wavelengths.

Statistical analysis

Statistical significance of differences was determined using the Student's t-test. $P < 0.05$ and $P < 0.01$ were considered statistically significant at the 5% and 1% levels, respectively.

Competing interests

The authors declare that they have no competing of interests.

Author's contribution

MJ carried out western blot experiments and worked on the manuscript, KB carried out siRNA techniques and western blot experiments, MS carried out measuring of mitochondrial membrane potential, VNF carried out confocal microscopy and cell fractionation, JŠ participated in cell fractionation, JS participated in western blot experiments, IZ and IO participated in the preparation of SB-T-1216 taxane, JK coordinated the experiments and helped to complete the manuscript. All authors read and approved the final manuscript.

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Differing Mechanisms of Death Induction by Fluorinated Taxane SB-T-12854 in Breast Cancer Cells

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Abstract. *Background/Aim: Classical taxanes are routinely used in cancer therapy. In this study, mechanisms involved in death induction by the novel fluorine-containing taxane SB-T-12854 were investigated. Materials and Methods: We employed breast cancer SK-BR-3, MCF-7 and T47D cell lines to assess activation of individual caspases, changes in the expression of proteins of the Bcl-2 family, and the release of pro-apoptotic factors from mitochondria into the cytosol after SB-T-12854 treatment. Results: Caspase-2, -8, and -9 were activated in SK-BR-3 and MCF-7 cells. Only caspase-8 was activated in T47D cells. Caspase-7 and -6 were activated in all tested cells while caspase-3 was activated only in SK-BR-3 cells. Pro-apoptotic Bad protein seems to be important for cell death induction in all tested cells. Anti-apoptotic Bcl-2 and pro-apoptotic Bim, Bok, Bid and Bik seem to be also associated with cell death induction in some of the tested cells. The mitochondrial apoptotic pathway was significantly activated in association with the release of cytochrome c and Smac from mitochondria, but only in SK-BR-3 cells, not in MCF-7 and T47D cells. Conclusion: Cell death induced by SB-T-12854, in the tested breast cancer cells, differs regarding activation of caspases, changes in levels of pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family and activation of the mitochondrial apoptotic pathway.*

Taxanes are well known mitotic poisons used in the treatment of solid tumors. Currently two taxanes, *i.e.* paclitaxel (Taxol®) and docetaxel (Taxotere®) are routinely used in chemotherapy of breast, ovary, lung, and other cancers (1-3). Taxanes bind tubulin dimers and block de-polymerization of microtubules (4). The hyper-polymerization of microtubules blocks the cell cycle in the G_2/M interphase and leads to mitotic arrest (5, 6). Subsequently, mitotic arrest usually results in apoptosis induction. However, other alternative pathways leading to cell death can be induced in cells treated with taxanes (7-10).

Pro-apoptotic Bax, Bak and Bok proteins of the Bcl-2 family form channels in the outer mitochondrial membrane and thus enable the release of cytochrome *c* into the cytosol (11). The level of Bax was found to increase, and the level of Bak was observed to decrease as well as to increase after taxane application (11-13). The role of the Bok protein remains somewhat unclear. Similarly, the levels of anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2 and Bcl-xL (they prevent formation of Bax/Bak channels in mitochondrial membrane), have been reported to be increased as well as decreased after taxane application (11, 12, 14, 15). In addition, apoptosis induced by taxanes is usually associated with Bcl-2 phosphorylation (16). Pro-apoptotic BH3-only proteins of the Bcl-2 family, that suppress the activity of anti-apoptotic Bcl-2 proteins, can effectively induce the intrinsic mitochondrial pathway of apoptosis induction (17-19). There are reports showing Bim protein to be directly involved in cell death induction by taxanes (20-21). Furthermore, some roles for Bad, Bik and Puma in cell death induction by taxanes in breast cancer cells have been reported (11, 17, 22).

Cytochrome *c* and Smac protein release from mitochondria are fundamentally important for the activation of the mitochondrial apoptotic pathway and activation of caspases. The release of cytochrome *c* and/or Smac occurrs *in vitro* and *in vivo* in cancer cells treated with taxanes (23-25). On the other hand, cell death induced by taxanes in cancer cells can also be realized without release of cytochrome *c* (24).

Concerning caspases, the activation of caspase-8, the main initiator caspase of the extrinsic pathway of apoptosis induction, and caspase-9, the main initiator caspase of the intrinsic mitochondrial pathway of apoptosis induction, have been observed in various cell types after taxane treatment (7, 15, 26, 27). Recently, several groups including ours have

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reported that caspase-2, a highly conservative protease involved in cell death induction by different stimuli (28), appears to play an important role in taxane-induced cell death (29-31). Executioner caspase-3, -7 and -6 have also been activated after taxane application in various types of cancer cells (7, 12, 32). Caspase-3 probably plays the most important role here, while caspase-6 and -7 seem to have minor roles (23, 32).

There is an abundance of data concerning cell death induction by novel taxanes (6, 7, 24, 31, 32). However, the precise roles of some components of the apoptosis-inducing pathways (Bcl-family proteins, mitochondria, and caspases) have not yet been elucidated.

Because innate or acquired resistance of cancer cells to clinically used taxanes remains a problem in chemotherapy (33), novel taxanes have been developed to overcome resistance (34-37). Some of these novel taxanes have proven to be significantly more effective than the classical taxanes in cell death induction in resistant cancer cells (7, 38).

We previously reported that novel fluorine-containing taxanes are effective in cell death induction in cancer cells as well as in cancer cells resistant to paclitaxel (7). In the present study, we showed that the novel fluorine-containing taxane SB-T-12854 (39) is effective in cell death induction in tested breast cancer cell lines SK-BR-3, MCF-7 and T47D. However, mechanisms of cell death induction seem to differ in these cells based on the observed changes in the expression of the Bcl-2 family proteins, the release of pro-apoptotic factors from mitochondria, and activation of caspases.

Materials and Methods

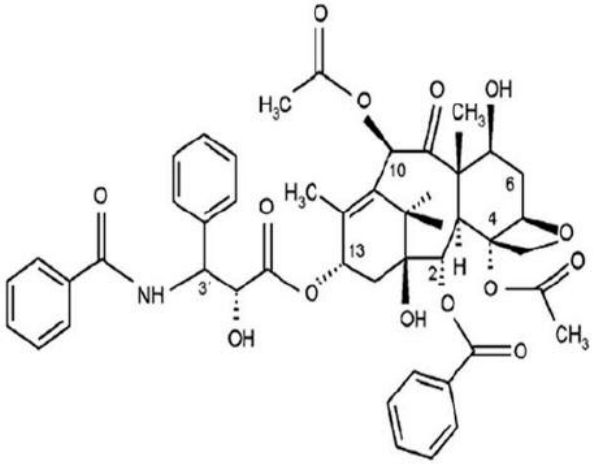
Materials. SB-T-12854 (39) was synthesized in the laboratory of Professor Iwao Ojima at the Institute of Chemical Biology and Drug Discovery (Stony Brook, NY, USA). For the structures of SB-T-12854 and classical paclitaxel see Figure 1. The taxane was dissolved in DMSO (tissue culture quality) to obtain a 1 mM stock solution. For western blot analysis, the following primary antibodies were used: rabbit monoclonal antibody against Bid (ab32060) and Bok (ab186745) from Abcam (Cambridge, UK), rabbit polyclonal antibody against Bad (#9292), Bax (#5023), Bik (#4592), and Bok (#4521), rabbit monoclonal antibody against Bcl-xL (#2764) and Bim (#2933), rabbit polyclonal antibody against cleaved caspase-3 (#9661), cleaved caspase-6 (#9761) and cleaved caspase-7 (#9491), rabbit monoclonal antibody against cleaved caspase-8 (#9496), rabbit polyclonal antibody against cleaved caspase-9 (#9505), rabbit polyclonal antibody against COX IV (#4844) and cytochrome *c* (#4272) and mouse monoclonal antibody against Smac/Diablo (#2954) from Cell Signaling Technology (Danvers, MA, USA), mouse monoclonal antibody against Bax (sc-7480) and Bcl-2 (sc-7382) from Santa Cruz Biotechnology, INC. (Dallas, TE, USA), rat monoclonal antibody against caspase-2 (ALX-804-356-C100) from Enzo Life Sciences (Farmingdale, NY, USA), and mouse monoclonal antibody against actin (AC-40, A3853) from Sigma-Aldrich.

Cells and culture conditions. Human breast carcinoma cell lines SK-BR-3, MCF-7, and T47D were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), the National Cancer Institute (Frederick, MD, USA), and the European Collection of Cell Cultures (ECCC, Porton Down, Salisbury, UK), respectively. SK-BR-3 adenoma cells are without functional p53, with functional caspase-3, and they overexpress Her2/Neu receptor. MCF-7 adenoma cells are with functional p53, without functional caspase-3 (40), and they carry progesterone and estrogen receptors. T47D ductal cells are without functional p53, with functional caspase-3, and they carry progesterone and estrogen receptors. These cell lines represent cells with differing status of key molecules and thus it helps elucidate various mechanisms involved in cell death induction. The basic medium was RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), HEPES (15 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (41). The culture medium consisted of basic medium supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany). The cells were maintained in a culture medium at 37˚C in a humidified atmosphere of 5% CO_2 in air. For experiments, taxanes were diluted in culture medium to achieve the required concentrations. Culture medium without taxane was used as controls.

Assessment of cell growth and survival. Cells were harvested and seeded at 20×10^3 cells/100 μl of culture medium into wells of 96-well plastic plates. After a 24-h pre-incubation period, allowing cells to attach, the culture medium was replaced by either culture medium without taxane (control) or medium with tested taxane at the desired concentrations. Cell growth and survival were evaluated 96 h after taxane application. The number of living cells was determined using a hemocytometer after staining with trypan blue.

Preparation of cell lysates. Cells at desired concentrations were seeded in Petri dishes or culture flasks and taxane was added after a 24-h pre-incubation period. After the incubation period, cells were harvested by low-speed centrifugation (2,000 rpm, 9 min, 4˚C), washed in PBS and centrifuged again. Cell pellets were stored at –80˚C. Next, pellets were re-suspended in RIPA buffer (Sigma Aldrich, St. Louis, MO, USA) containing a 1% mixture of protease inhibitors (P8340, Sigma Aldrich). Protein lysates were centrifuged (14,000 rpm, 20 min, 4˚C) and the supernatants containing proteins were stored at –80˚C (24).

Cell fractionation. Cells (approximately 3.6×10^6 cells per sample) were seeded into Petri dishes or culture flasks and taxanes were added after a 24-h pre-incubation. After the incubation period, cells were harvested by low-speed centrifugation (2,000 rpm, 9 min, 4˚C), washed in PBS and centrifuged again. Cell pellets were re-suspended in a specific lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, and 1% mixture of protease inhibitors P8340 from Sigma Aldrich, St. Louis, MO, USA) containing 0.635 mM digitonin D141 (Sigma-Aldrich) and vortexed for 60 s. Lysates were than centrifuged (14,000 rpm, 1 min, 4˚C) and supernatants (containing cytosolic fractions) were removed and stored at –80˚C. The specific lysis buffer described above, but containing 6.35 mM digitonin D141 (Sigma-Aldrich), was added to the pellets and suspensions were mixed for 60 s and centrifuged (14,000 rpm, 1 min, 4˚C). After centrifugation, supernatants (containing mitochondrial fractions) were stored at –80˚C. Cell fractions were analyzed using western blot.



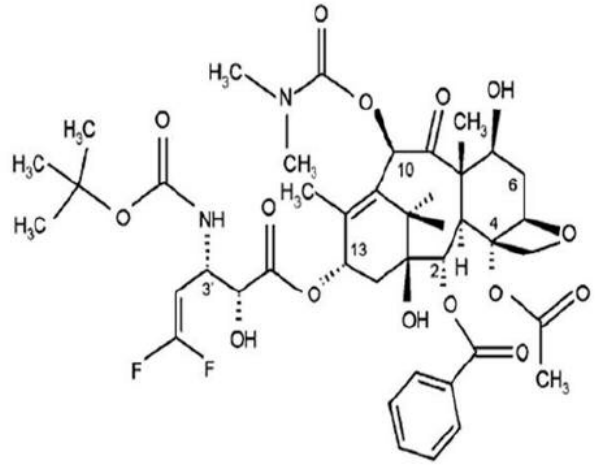


Figure 1. *Chemical structures of paclitaxel and SB-T-12854.*

Western blot analysis. Concentration of proteins in cell lysates and cell fractions were assessed using the BCA Protein Assay Reagent from Pierce (Thermo Fisher Scientific, Rockford, IL, USA). Depending on protein concentrations, cell lysates and cell fractions were diluted in RIPA buffer to the gel loading concentration of proteins (2 μg/μl), mixed with equal volumes of sample buffer (0.125 M Tris/HCl pH 6.8, 10% glycerol, 4% SDS, 0.25 M DTT) and heated for 5-7 min at 110˚C. Protein samples were separated using protein electrophoresis (Bio-Rad, Hercules, CA, USA). Proteins separated by SDS-PAGE were blotted onto a 0.2 μm PROTRAN BA 83 nitrocellulose membrane (Whatman-Schleicher and Schuell, Maidstone, UK) for 3 h at 0.25 A, using a MiniProtean II blotting apparatus (Bio-Rad). The membrane was blocked with 5% non-fat dry milk or 5% BSA in TBS for 15-20 min and incubated with the primary antibody at 4˚C overnight. After the incubation, the membrane was washed three times (5-10 min) with TBS containing 0.1% Tween-20. Then it was incubated for 1-2 h with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterward, the membrane was washed (as described above) and the chemiluminescence signal was detected using Supersignal reagents from Pierce (Thermo Fisher Scientific) and a CCD device (Carestream).

Results

Effect of fluorinated taxane SB-T-12854 on growth and survival. We assessed the effect of fluorine-containing taxane SB-T-12854 (Figure 1) on cell growth and survival in breast cancer SK-BR-3, MCF-7 and T47D cells (see Materials and Method section) at wide range of concentrations (0.1-3,000 nM). In SK-BR-3 cells, SB-T-12854 induced cell death at a concentration of 30 nM and higher concentrations. C_{50} (concentration of taxane resulting in 50% of living cells relative to controls after 96 h of incubation) for SK-BR-3 cells was determined to be approximately 4 nM. In MCF-7 cells, the taxane also induced cell death at a concentration of 30 nM and higher concentrations. C_{50} for MCF-7 cells was also about 4 nM. Similar data were obtained with T47D cells. SB-T-12854 induced cell death at a concentration ≥30 nM and the C_{50} was approximately 5 nM (Figure 2).

In further experiments, we used the death inducing concentration, *i.e.* the lowest concentration with near maximum death-inducing effect, 100 nM of SB-T-12854 for SK-BR-3 cells and 600 nM for MCF-7 cells. For T47D cells, we used 300 nM because concentrations with a near maximum death-inducing effect were extremely high (Figure 2).

Effect of SB-T-12854 on activation of caspases. We used western blot analysis in order to assess the activation of caspase-2, -8, -9, -3, -7, and -6 after application of SB-T-12854 at the death-inducing concentration in tested cell lines. Caspase activation was assessed by detecting the cleaved forms of caspases (see Materials and Methods section). Cleaved caspase-2, cleaved caspase-8 and cleaved caspase-9 appeared in significant amounts 36 h after taxane treatment in SK-BR-3 cells. Lower levels of cleaved caspase-8 and -9 were observed after only 24 h. As for executioner caspases, we detected high levels of cleaved caspase-3 and -7 after 36 h. Lower levels of cleaved caspase-3 and -7 were also observed after 24 h. Similarly, cleaved caspase-6 was detected 36 h after taxane treatment (Figure 3). In MCF-7 cells, the level of cleaved caspase-2 significantly increased 36 h after SB-T-12854 application. However, after 48 h the level substantially decreased. Cleaved caspase-8 was present after 36 as well as 48 h. Cleaved caspase-9 was detected between 24 h and 48 h after taxane application. Since there is not functional caspase-3 in MCF-7 cells (40), cleaved caspase-3 was not detected. Low levels of cleaved caspase-

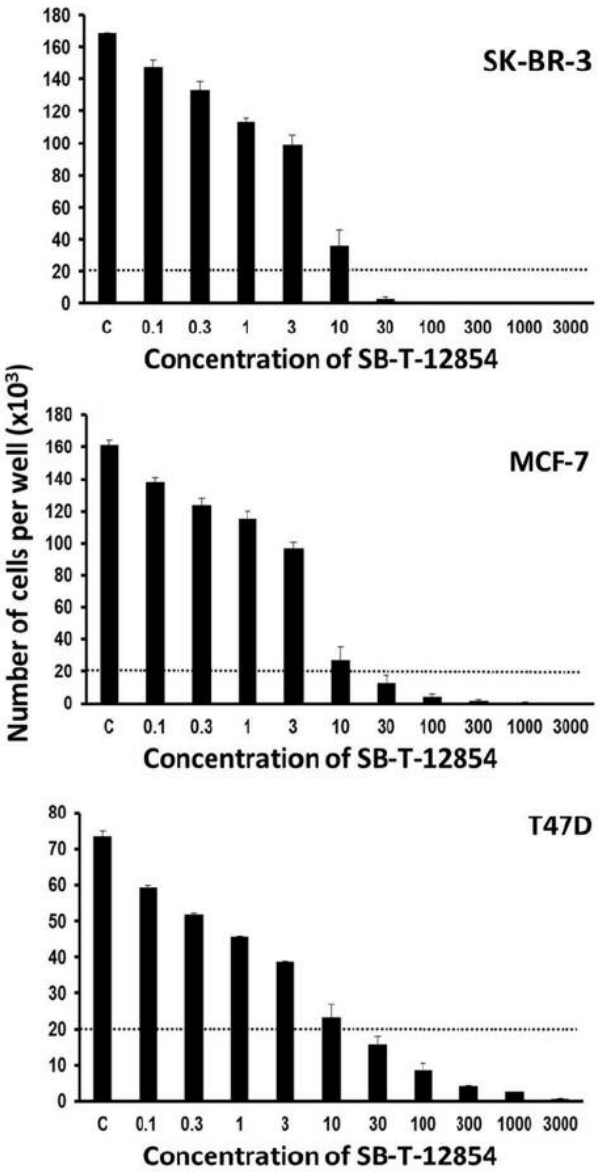


Figure 2. *Effect of SB-T-12854 (0.1-3,000 nM) on growth and survival of SK-BR-3, MCF-7, and T47D cells. Control cells (C) were incubated without SB-T-12854. The cells were seeded at 20×10³ cells/100 μl of medium per well (dotted line). The number of living cells was determined after 96 h of incubation (see Materials and Methods section). Each column represents the mean of two separate cultures±SEM.*

7 were seen after 24 h with a significant increase after 36 h. However, the level of cleaved caspase-7 decreased 48 h after taxane application. Cleaved caspase-6 was detected 36 h after taxane treatment with its subsequent decrease in cleaved caspase-6 level (Figure 3). A significant increase of the level of cleaved caspase-2 was not detected in T47D cells after SB-T-12854 application. Concerning caspase-8, the level of cleaved caspase-8 increased between 72 h and 120 h after taxane application. Nearly undetectable levels of cleaved caspase-9 were observed between 48 h and 120 h after taxane treatment. Interestingly, only low levels of cleaved caspase-3 were detected after the treatment. Concerning other executioner caspases, levels of cleaved caspase-6 as well as caspase-7 were significantly increased 120 h after SB-T-12854 application (Figure 3).

To summarize, all tested initiator and executioner caspases were activated in SK-BR-3 cells within 36 h. The same was true for MCF-7 cells with the exception of the non-functional caspase-3. In T47D cells, only the activation of caspase-8, -7 and -6 was observed.

Effect of SB-T-12854 on levels of proteins of the Bcl-2 family. We measured changes in levels of proteins of the Bcl-2 family in cells after taxane treatment (see Materials and Methods section). The level of pro-apoptotic (channel forming) Bax protein did not change due to SB-T-12854 application, while the level of Bok protein significantly decreased 36 h after taxane application in SK-BR-3 cells (Figure 4A). The level of anti-apoptotic Bcl-2 protein significantly increased 12 h after taxane application. Most of proteins seemed to be phosphorylated (phosphorylated form is presented by the upper band of the double band observed). On the other hand, the level of anti-apoptotic Bcl-xL protein did not change significantly (Figure 4B). Concerning the BH3-only pro-apoptotic proteins of the Bcl-2 family, the level of Bad protein started to increase 12 h and peaked 24 h after taxane application. After 36 h a decrease was seen. The level of Bid protein decreased significantly 36 h after taxane treatment. The level of Bik seemed to increase 12 h after taxane treatment and then decreased 24 h after treatment. Bim level decreased significantly 24 h after treating with taxane (Figure 4C). In MCF-7 cells, SB-T-12854 did not significantly affect the level of Bax protein. However, the level of Bok protein increased significantly 36 h after taxane application with a subsequent decrease (Figure 4A). The level of anti-apoptotic Bcl-2 started to increase 12 h after taxane application. At the same time some of Bcl-2 was phosphorylated. Interestingly, Bcl-2 became significantly dephosphorylated 36 h after application. The level of Bcl-xL did not change significantly after taxane treatment (Figure 4B). As to the BH3-only proteins, the level of Bad seemed to be slightly increased 24 h after treatment. We did not observe any change in the level of Bid. Concerning Bik level, it appeared to increase 12 h after treatment. The level of Bim protein increased 12-24 h and decreased 36 h after taxane treatment (Figure 4C). There were no significant changes in the level of Bax and Bok proteins in T47D cells after SB-T-12854 application (Figure 4A). The level of anti-apoptotic Bcl-2 was found to decrease from 72 h to 96 h after taxane application. Phosphorylation of

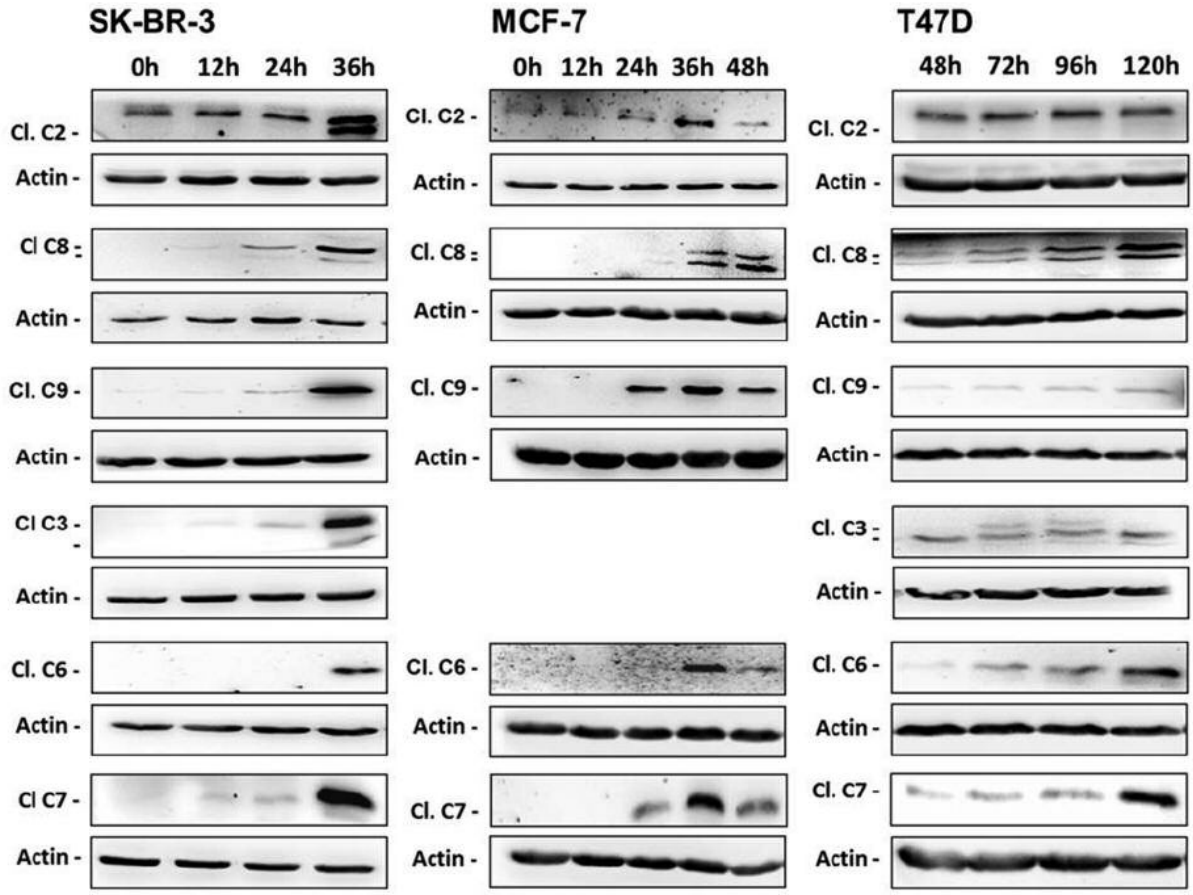


Figure 3. *Effect of SB-T-12854 on the activation of caspase-2, -8, -9, -3, -6 and -7 in SK-BR-3, MCF-7, and T47D cells. After incubation period with the death-inducing concentration of SB-T-12854 (0-36 h and 100 nM for SK-BR-3 cells, 0-48 h and 600 nM for MCF-7 cells, 48-120 h and 300 nM for T47D cells), levels of cleaved forms of caspases were determined using western blot analysis and relevant antibodies (see Materials and Methods section). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments.*

Bcl-2, which was detected in SK-BR-3 and MCF-7 cells, was not observed in T47D cells. The level of Bcl-xL did not seem to be significantly affected by taxane treatment (Figure 4B). Concerning the BH3-only proteins, the level of Bad protein started to increase 48 h after taxane application. The level of Bid protein seemed to decrease 72 h after taxane application. There was nearly no effect of the taxane on the level of Bik protein. The level of Bim protein was found to increase 48 h after SB-T-12854 treatment (Figure 4C).

In summary, higher levels of Bcl-2 together with its phosphorylation were detected in SK-BR-3 and MCF-7 after SB-T-12854 application. However, there was no up-regulation and phosphorylation of Bcl-2 in T47D cells. The level of Bad increased in all cell lines after application. The most conspicuous effect was observed in SK-BR-3 cells. Finally, the levels of Bok and Bim seemed to change significantly, but the changes differed in the individual tested cell lines.

Effect of SB-T-12854 on cytochrome c and Smac release from mitochondria. To clarify the involvement of mitochondria in apoptosis induction by SB-T-12854 in the tested cell lines, the release of cytochrome *c* and Smac protein from mitochondria into the cytosol was assessed using western blot analysis after cell fractionation (see Materials and Methods section). As expected, cytochrome *c* and Smac were detected in mitochondrial fractions in control SK-BR-3, MCF-7 as well as T47D cells. For SK-BR-3 cells, cytochrome *c* and Smac, in significant amounts, were detected in the cytosolic fraction 36 h after SB-T-12854 treatment at death-inducing concentration. We observed small amounts of cytochrome *c* in the cytosolic fraction of MCF-7 cells 36-48 h after taxane treatment, albeit it was not a significant release. We did not detect any release of cytochrome *c* or Smac into the cytosol of T47D cells after taxane treatment (Figure 5).

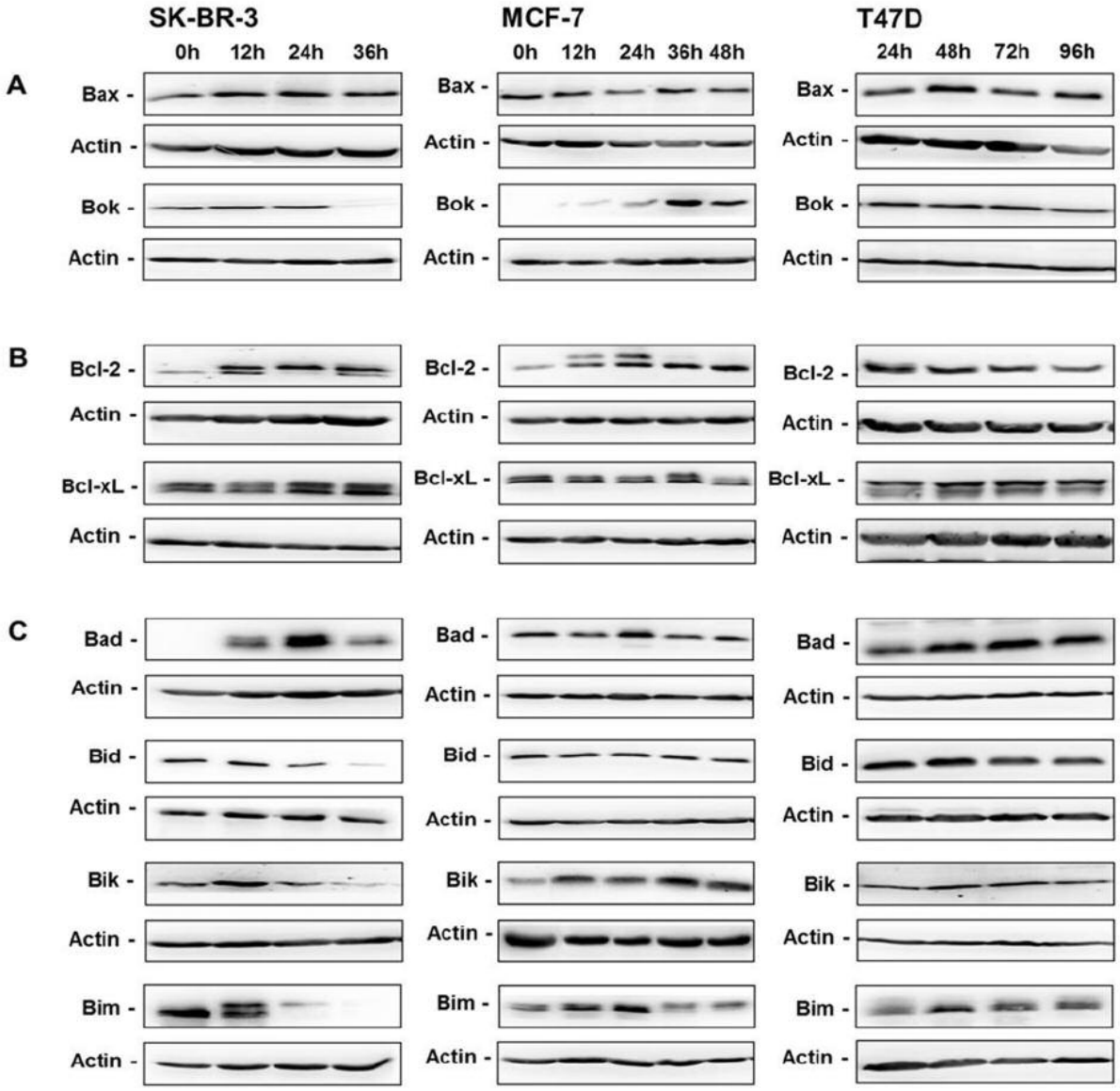


Figure 4. *Effect of SB-T-12854 on the level of proteins of the Bcl-2 family in SK-BR-3, MCF-7, and T47D cells, i.e. (A) proapoptotic proteins of the Bax subfamily, (B) antiapoptotic proteins of the Bcl-2 subfamily and (C) proapoptotic proteins of the BH3-only subfamily. After the incubation period with the death-inducing concentration of SB-T-12854 (0-36 h and 100 nM for SK-BR-3 cells, 0-48 h and 600 nM for MCF-7 cells, 24-96 h and 300 nM for T47D), levels of the proteins were determined using western blot analysis and relevant antibodies (see Materials and Methods section). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments.*

Thus, the mitochondrial pathway seems to play an important role in apoptosis induction in SK-BR-3 cells, but not in MCF-7 and T47D cells.

Discussion

In our previous studies on the mechanisms of cell death induction by taxanes in breast cancer cells, we reported that during cell death induction by taxanes caspase-2 was involved as the apical caspase and its activation results in the mutual activation of caspase-3 and -7 (31, 32). In the present study we investigated molecular mechanisms of cell death induction by novel fluorinated taxane SB-T-12854 (39) in breast cancer cells. We used SK-BR-3 and T47D cells (without functional p53) and MCF-7 cells (without functional caspase-3) (see Materials and Methods section).

SB-T-12854 induced cells death in breast cancer cell lines examined (Figure 2). The taxane has also been shown to be effective in cell death induction in other cancer cell lines (7, 42). The effect of SB-T-12854 on the growth and survival of breast cancer cells was similar to the effect of previously studied novel non-fluorinated taxane SB-T-1216 (31, 32). Compared to SB-T-1216, the fluorine atoms in the structure of SB-T-12854 are supposed to increase the resistance of the taxane molecule to metabolization (42). Interestingly, both taxoids SB-T-12854 and SB-T-1216 were able to overcome the acquired resistance of breast cancer cells to paclitaxel (7, 43). Thus, SB-T-12854 seems to be a promising agent for induction of cell death in sensitive as well as resistant breast cancer cells.

Initiator and executioner caspases have been found to be activated in many types of cancer cell lines after the application of classical as well as novel taxanes (7, 12, 27, 31, 44). Activation of individual caspases seems to be cell type and also applied taxane specific. Therefore, we focused on the activation of all initiator and executioner caspases, except of caspase-10. In this study, we found that SB-T-12854 activated initiator caspase-8, caspase-9 and caspase-2 in SK-BR-3 and MCF-7 cells (Figure 3). Previously we have reported the activation of caspase-2 in breast cancer cells after taxane application, but this activation was not associated with PIDDosome formation (31). We detected a significant release of cytochrome *c* into the cytosol only in SK-BR-3 cells, but not in MCF-7 cells after SB-T-12854 treatment. Therefore, we suggest that activation of caspase-9 in MCF-7 cells could result from direct activation by caspase-2 (32). Interestingly, caspase-8 was the only initiator caspase activated significantly in T47D cells (see Figure 3). Concerning the mechanism of activation of caspase-8, it seems different from the previously described non-receptor activation of caspase-8 by caspase-3 (26) or by caspase-2 (32) since we did not observe significant activation of caspase-3 or caspase-2 in T47D cells. It seems that the role of caspase-8 in apoptosis induction after taxane treatment in T47D cells is more important than that in SK-BR-3 and MCF-7 cells as discussed previously (32).

Caspase-3 was significantly activated only in SK-BR-3 cells, taking into account the fact that there is not functional caspase-3 in MCF-7 cells. On the other hand, caspase-7 and caspase-6 were activated in all tested cells. The activation of executioner caspases has obviously been described after taxane application previously (12, 26, 32, 44) and caspase-3 was usually the most important cell death inducer. Caspase-3 is known as the executioner caspase functioning upstream of caspase-7 and caspase-6. This fact led to a question. How are caspase-7 and caspase-6 activated in MCF-7 and T47D cells? It is possible that initiator caspases, such as caspase-2 or caspase-8, are somehow involved. It seems that the activation of caspases induced by SB-T-12854 in SK-BR-3 and MCF-7 cells follows

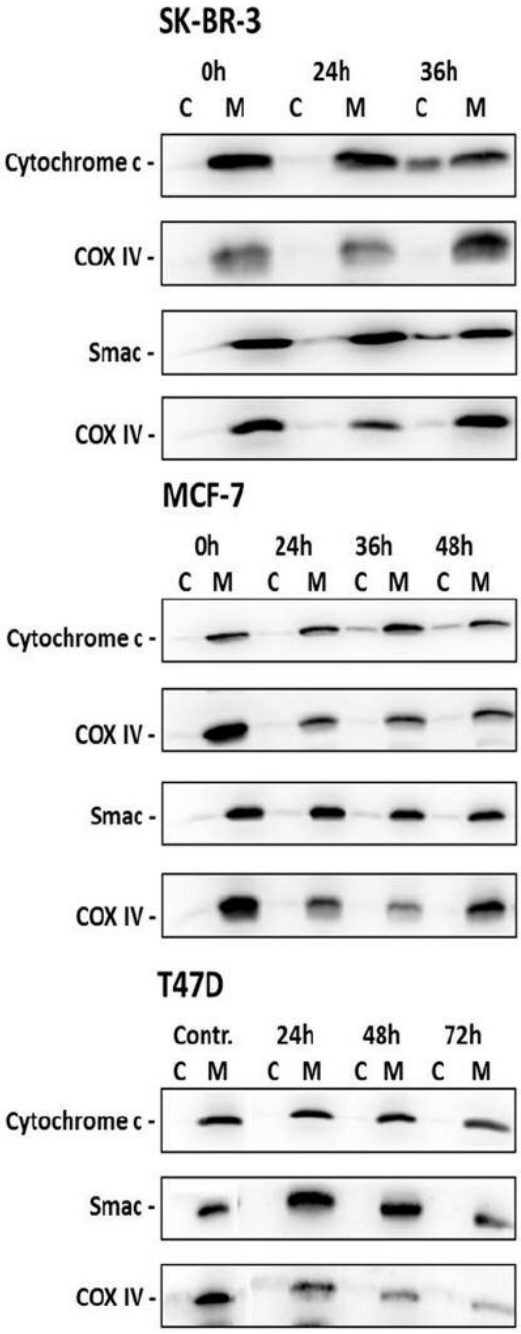


Figure 5. *Effect of SB-T-12854 on the release of cytochrome c and Smac protein from mitochondria in SK-BR-3, MCF-7, and T47D cells. After incubation period with the death-inducing concentration of SB-T-12854 (0-36 h and 100 nM for SK-BR-3 cells, 0-48 h and 600 nM for MCF-7 cells, 24-72 h and 300 nM for T47D cells), levels of cytochrome c and Smac were determined in mitochondrial (M) and cytosolic (C) fractions using western blot analysis and relevant antibodies (see Materials and Methods section). Cells incubated in taxane-free medium for 24 h (Control) were used as control for T47D cells. COX IV (integral mitochondrial protein) level was used to confirm proper fractionation. The data shown were obtained in one representative experiment of two independent experiments.*

a previously proposed scheme (32). The activation of caspases seems to differ in T47D cells. In particular, caspase-8 may play a more important role here (see above).

Proteins of the Bcl-2 family are known to play an important role in taxane-induced cell death in many types of cancer cells (12, 21, 22, 45). Concerning proteins of the Bcl-2 family, we decided to test channel-forming Bax protein and less studied Bok protein. Anti-apoptotic Bcl-2 and Bcl-xL proteins were selected according to our preliminary data. Selected Bad, Bid, Bik and Bim proteins from the BH3-only subfamily had been previously reported to have potential functions in cell death induction by taxanes. As for the channel forming pro-apoptotic proteins of this family, Bok expression increased after SB-T-12854 treatment in MCF-7 cells, showing a possible role in cell death induction in these cells (Figure 4A). As far as we know, there are no data in the literature demonstrating the role of Bok in taxane-induced cell death in breast cancer cells. Interestingly, Bok has been reported to be important in cell death induction after blocking the cell cycle (46), which results from taxane treatment. However, there was no significant increase in Bok levels in SK-BR-3 and T47D cells and thus Bok does not seem to play a key role in cell death induction by SB-T-12854 in breast cancer cells.

Considering anti-apoptotic proteins of the Bcl-2 family, we observed increased levels of Bcl-2 in SK-BR-3 and MCF-7 cells after SB-T-12854 application. In addition, Bcl-2 was phosphorylated in both cell lines after taxane application. In T47D cells, the level of Bcl-2 decreased after SB-T-12854 treatment and there was no significant phosphorylation of Bcl-2 (Figure 4B). Increased level of Bcl-2 has been previously shown to be protective cells against the effect of taxanes (12). However, some contra-indicatory data exist concerning the role of Bcl-2 in taxane effect (14). We suggest that increased levels of Bcl-2 followed by its phosphorylation could represent some type of protective mechanism of SK-BR-3 and MCF-7 cells against taxane effect. The situation seems to be different in T47D cells, where decreasing levels of Bcl-2 after taxane application correlated with cell death induction (Figure 4B).

As for the BH3-only proteins of the Bcl-2 family, the level of Bad was found to be more or less transiently increased in all tested cells (Figure 4C). Apoptosis induction by taxanes has been shown to be associated with the pro-apoptotic activity of Bad protein (12). Furthermore, the involvement of Bad in the regulation of cell-cycle progress associated with cell death induction has also been demonstrated (22). We assume that Bad is, directly or indirectly, involved in cell death induction by SB-T-12854. The level of Bim was transiently higher in MCF-7 and T47D cells after SB-T-12854 application (Figure 4C). Bim protein can induce cell death by affecting Bcl-2 protein during cytoskeleton stress, however, recently other indirect mechanisms of cell death induced by Bim that were associated with mitotic aberrations have been described (21, 45, 47). This could also be the case of tested MCF-7 and T47D cells. However, Bim levels were significantly decreased after taxane application in SK-BR-3 cells (Figure 4C). Similarly, a decrease of Bik and Bid levels was also found in SK-BR-3 cells after SB-T-12854 application. The relationship between the decrease and cell death induction remains unclear.

Previously we and other groups have reported that cell death dependent on as being well as independent of cytochrome *c* release from mitochondria into the cytosol. It means dependence on or independence from mitochondrial the apoptotic pathway (24, 44). Thus, we tested activation of this crucial pathway of apoptosis induction. Significant release of cytochrome *c* and Smac into the cytosol after SB-T-12854 application was only observed in SK-BR-3 cells (Figure 5). Thus, it is likely that caspase-9 is activated by an alternative mechanism unrelated to the classical mitochondrial apoptotic pathway in MCF-7 cells. We suggest that the mitochondrial apoptotic pathway is activated only in SK-BR-3 cells after taxane application and it is not required for cell death induction in MCF-7 and T47D cells.

We can conclude that the novel taxane SB-T-12854 induces cell death effectively in SK-BR-3, MCF-7 and T47D breast cancer cells. The activation of initiator caspase-2, -8, -9, in SK-BR-3 and MCF-7 cells and caspase-8 in T47D cells, as well as activation of executioner caspase-3 in SK-BR-3 cells and caspase-7, -6 in all tested cell lines were found to be associated with SB-T-12854 application. The pro-apoptotic Bad protein of the Bcl-2 family seems to be important for cell death induction by taxane SB-T-12854 in the tested cells. The anti-apoptotic Bcl-2 protein also seems to be associated with apoptosis induction in SK-BR-3 and MCF-7 cells and the pro-apoptotic Bim in MCF-7 and T47D cells. Similarly, the pro-apoptotic Bok in MCF-7 cells as well as the pro-apoptotic Bid and Bik in SK-BR-3 cells could be involved in apoptosis induction by SB-T-12854. The mitochondrial apoptotic pathway, after SB-T12854 application, was significantly activated in association with the release of cytochrome *c* and Smac from mitochondria, but only in SK-BR-3 cells, and not in MCF-7 and T47D cells.

In summary, cell death induced by SB-T-12854 in the tested breast cancer cells differs with regard to the activation of caspases, changes in levels of pro-apoptotic as well as anti-apoptotic proteins of the Bcl-2 family, and release of pro-apoptotic factors (cytochrome *c*, Smac) from mitochondria into the cytosol.

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5.2 Publikace věnované rezistenci buněk nádoru prsu k novým derivátům taxanů

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Substituents at the C3′ and C3′N positions are critical for taxanes to overcome acquired resistance of cancer cells to paclitaxel

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ABSTRACT

We tested the role of substituents at the C3′ and C3′N positions of the taxane molecule to identify taxane derivatives capable of overcoming acquired resistance to paclitaxel. Paclitaxel-resistant sublines SK-BR-3/PacR and MCF-7/PacR as well as the original paclitaxel-sensitive breast cancer cell lines SK-BR-3 and MCF-7 were used for testing. Increased expression of the ABCB1 transporter was found to be involved in the acquired resistance. We tested three groups of taxane derivatives: (1) phenyl group at both C3′ and C3′N positions, (2) one phenyl at one of the C3′ and C3′N positions and a non-aromatic group at the second position, (3) a non-aromatic group at both C3′ and C3′N positions. We found that the presence of phenyl groups at both C3′ and C3′N positions is associated with low capability of overcoming acquired paclitaxel resistance compared to taxanes containing at least one non-aromatic substituent at the C3′ and C3′N positions. The increase in the ATPase activity of ABCB1 transporter after the application of taxanes from the first group was found to be somewhat higher than after the application of taxanes from the third group. Molecular docking studies demonstrated that the docking score was the lowest, i.e. the highest binding affinity, for taxanes from the first group. It was intermediate for taxanes from the second group, and the highest for taxanes from the third group. We conclude that at least one non-aromatic group at the C3′ and C3′N positions of the taxane structure, resulting in reduced affinity to the ABCB1 transporter, brings about high capability of taxane to overcome acquired resistance of breast cancer cells to paclitaxel, due to less efficient transport of the taxane compound out of the cancer cells.

1. Introduction

Paclitaxel (Taxol®), a natural product from the bark of *Taxus brevifolia*, and the semisynthetic analog docetaxel (Taxotere®) from the needles of *Taxus baccata*, are taxanes used for the treatment of many solid cancers including breast, lung, prostate, ovarian and head and neck cancers (Chu et al., 2005; Markman, 2008; Nabholtz and Gligorov, 2005; Yared and Tkaczuk, 2012). A third taxane in clinical use, cabazitaxel (Jevtana ®), was approved by the Food and Drug Administration for treatment of metastatic castration-resistant prostate cancer in 2010 (Paller and Antonarakis, 2011).

The antitumor activity of taxanes is associated with their ability to bind to the β-tubulin subunit on the luminal side of the assembled microtubule (Parness and Horwitz, 1981; Rao et al., 1995; Rao et al., 1999; Snyder et al., 2001). Taxane treatment causes a mitotic arrest due to the binding of taxanes to the mitotic spindle. After prolonged exposure to taxane, the mitochondrial pathway of apoptosis induction is often initiated (Jordan et al., 1996; Woods et al., 1995).

The effectiveness of taxane treatment is influenced by innate and/or acquired resistance of tumor cells. Several mechanisms of taxane resistance have been proposed (Murray et al., 2012). Taxane resistance involves drug efflux mediated by transporters of the ATP-binding

Abbreviations: ABC, ATP-binding casette; ABCB1/MRP1, ATP-binding cassette B1/multidrug resistance protein 1; ABCC3/MRP3, ATP-binding cassette C3/multidrug resistance protein 3; MAPs, microtubule associated proteins; Pgp, P-glycoprotein

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casette transporter (ABC) family that can be responsible for poor bioavailability of classical taxanes (Fojo and Menefee, 2005). Mutations in the taxol-binding site of β-tubulin (Orr et al., 2003), alterations in β-tubulin isotype expression, especially β-III tubulin (Kavallaris et al., 1997), and the effect of microtubule associated proteins (MAPs), which can either affect tubulin dynamics or bind to the taxol-binding site, have all been observed in cell line models (Alli et al., 2007; Smoter et al., 2011; Sun et al., 2015).

To overcome resistance to classical taxanes, modified taxanes such as Cabazitaxel (Jevtana ®) (de Bono et al., 2010) and Ortataxel (SB-T-101131, *IDN*-5109, BAY 59-8862) have been developed with the goal of overcoming P-glycoprotein-associated resistance (Geney et al., 2005).

Classical taxanes containing a baccatin III core (paclitaxel) or 10-deacetylbaccatin (docetaxel) are esterified at the C13 position and thus they have a specific side chain at this position. The side chain contains two phenyl groups at the C3′ and C3′N positions in paclitaxel or a phenyl group at C3′ position and *tert*-butoxycarbonyl at the C3′N position in docetaxel. A series of second generation taxanes modified at the C2, C3′, C3′N, C10 and C13 positions, have been synthetized and their potency to overcome resistance to taxanes mediated by P-glycoprotein (Pgp) or mutations in β-tubulin has been assessed in some cancer cell lines or in mouse models (Matesanz et al., 2014; Ojima et al., 1994; Ojima et al., 1996; Ojima et al., 2008). These structure-activity relationship studies have revealed that novel taxanes are extremely efficient in overcoming some mechanisms of the acquired resistance described above.

We have established paclitaxel-resistant breast cancer cell sublines, SK-BR-3/PacR and MCF-7/PacR, capable of long-term proliferation in a medium containing concentrations of paclitaxel that are death-inducing for original sensitive cell lines SK-BR-3 and MCF-7 (100 nM and 300 nM, respectively). Both resistant sublines overexpress multi-drug resistance transporters ABCB1 and ABCC3 (Němcová-Fürstová et al., 2016). Moreover, both sublines also express other proteins which may be involved in resistance to paclitaxel (Pavlíková et al., 2014; Pavlíková et al., 2015). Our previous results have showed that novel taxanes with modified structures, especially at the C′3 and C3′N positions (Jelínek et al., 2013; Kovář et al., 2009; Vobořilová et al., 2011), were able to overcome paclitaxel resistance and induced cell death in tested paclitaxel-resistant breast cancer sublines (Němcová-Fürstová et al., 2016).

In the present study we tested the role of substituents at the C3′ and C3′N positions of the taxane structure to assess the capability of taxanes to overcome acquired resistance to paclitaxel in SK-BR-3 and MCF-7 cancer cells. We found that taxanes with phenyl groups at the C3′ and at C3′N positions are able to induce apoptosis in paclitaxel-resistant cells only at very high concentrations. However, taxanes with a nonaromatic group instead of a phenyl group at least at either the C3′or C3′N position are able to overcome acquired resistance to paclitaxel. These taxanes induce apoptosis at significantly lower concentrations. These acquired resistance to paclitaxel results from a dramatically increased expression of the ABCB1 (Pgp) transporter. Mechanism of the capability of taxanes to overcome this acquired resistance is based on lower affinity to ABCB1 transporter and thus lower export from resistant cells.

2. Materials and methods

2.1. Materials

The following primary and secondary antibodies were used for the detection of proteins: *anti*-ABCB1 (#12683, dilution 1:1000) from Cell Signaling Technology (Danvers, MA, USA), *anti*-actin (AC-40, A3853, dilution 1:1000) from Sigma-Aldrich, HRP-linked goat *anti*-mouse (sc-2005, dilution 1:6000) and HRP-linked goat *anti*-rabbit (sc-2004, dilution 1:6000) antibody from Santa Cruz (Santa Cruz, CA, USA).

Paclitaxel and docetaxel were purchased from Sigma-Aldrich (St. Louis, MO, USA), deacetyl paclitaxel from Santa Cruz (Santa Cruz, CA, USA). Acetyl docetaxel, SB-T-0035, SB-T-1102, SB-T-1211, SB-T-1212N1, SB-T-1214 and SB-T-1216 were synthesized at the Institute of Chemical Biology and Drug Discovery (Stony Brook, NY, USA), (Ojima et al., 1996; Ojima et al., 1997). Taxanes were dissolved in DMSO (tissue culture quality) to obtain 10 mM stock solution.

2.2. Cells and culture conditions

Human breast carcinoma cell lines MCF-7 and SK-BR-3 were obtained from the National Cancer Institute (Frederick, MD, USA) and the American Type Culture Collection (ATCC) (Manassas, VA, USA), respectively. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air in RMPI-1640 based culture medium containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), HEPES (15 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml), and supplemented with 10% heat-inactivated fetal bovine serum.

Taxane-resistant cell sublines were maintained as long-term culture in a taxane-containing medium. Concentrations were as follows: 100 nM paclitaxel for the SK-BR-3/PacR cell subline, 300 nM paclitaxel for the MCF7/PacR cell subline, 300 nM SB-T-0035 for the MCF7/SB-T-0035R cell subline.

2.3. Assessment of cell growth and survival

Cells were harvested and seeded into 96-well plate at a density of 2×10^4 cells per well in 100 μl of culture medium. After 24 h pre-incubation period allowing cells to attach, the culture medium was replaced by a culture medium without taxane (control) or with a medium containing tested taxane at desired concentrations. The number of living cells was determined after 96 h of incubation using a hemocytometer after staining with trypan blue.

2.4. Establishment of MCF7 subline resistant to SB-T-0035

The MCF7 cell subline resistant to the effect of SB-T-0035, referred to as MCF7/SB-T-0035R, was established in a similar way as the paclitaxel-resistant SK-BR-3/PacR and MCF7/PacR sublines (Němcová-Fürstová et al., 2016). The MCF7/SB-T-0035R subline was established by gradual adaptation of the original cell line to increasing concentrations of SB-T-0035. The starting concentration of SB-T-0035 was 1 nM. SB-T-0035 concentration then was increased as follows: 1 nM → 3 nM → 5 nM → 10 nM → 20 nM → 30 nM → 50 nM → 70 nM → 100 nM → 300 nM. Cells were maintained at a particular SB-T-0035 concentration for approximately 10 passages or until they displayed, more or less, standard growth and survival. The final concentration of SB-T-0035 achieved was 300 nM. Long-term growth and survival of MCF7/SB-T-0035R in 300 nM SB-T-0035 was similar to cells without SB-T-0035. On the other hand, most of the original MCF-7 cells exposed to 300 nM SB-T-0035 died within 96 h.

2.5. Preparation of cell lysates

Cells were harvested and seeded to 60 mm Petri dish at the density 1.5×10^6 cells in 5 ml of culture medium. After a 24 h pre-incubation period allowing cells to attach, the culture medium was replaced with taxane-free medium or taxane-containing medium. After each particular period of incubation, cells were harvested by trypsinization, washed three-times with ice-cold PBS and centrifuged at 500*g* for 10 min. Cell pellets were frozen at− 80 °C for 1 h and then dissolved in the RIPA lysis buffer containing protease and phosphatase inhibitors (described in detail in Jelínek et al., 2015). Protein concentrations were determined using the BCA method (Pierce BCA Protein Assay Kit, Thermofisher Scientific, MA, USA).

2.6. Western blot analysis

Protein samples (20 μg) were mixed with sample buffer (0.125 M

Tris/HCl pH 6.8, 10% glycerol, 4% SDS, 0.25 M DTT) and heated for 5 min at 100 °C. For detection of the ABCB1 transporter, samples were heated for 5 min at 37 °C. Samples were separated in 10% acrylamide gels using protein electrophoresis (Bio-Rad, Hercules, CA). Proteins separated by SDS-PAGE were blotted onto 0.2 μm nitrocellulose membrane PROTRAN BA 83 (Whatman-Schleicher and Schuell, Maidstone, UK) for 3 h at 0.25 A, using a MiniProtean II blotting apparatus (Bio-Rad). The membrane was blocked with 5% BSA in TBS for 20 min and incubated with the primary antibody at 4 °C overnight. After incubation, the membrane was washed three times (5–10 min) with TBS containing 0.1% Tween-20. Then the membrane was incubated for 1–2 h with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterwards, the membrane was washed (as described above) and the chemiluminescence signal was detected using the Supersignal reagents from Pierce (Thermo Fisher Scientific, MA, USA) and a CCD camera (Carestream).

2.7. Silencing by siRNA

ABCB1 knock-down was performed similarly to a protocol already reported (Němcová-Fürstová et al., 2016). The following siRNA were used: ABCB1 specific siRNA (catalog no: 4427037, ID: s10419, Life Technologies) and nonspecific siRNA (catalog no.: AM4635, Life Technologies) as a negative control.

The siRNA transfection mixture was prepared using INTERFERin (PolyPlus-Transfection, Illkirch, France) according to manufacturer` instructions. In the transfection mixture, ABCB1 or nonspecific siRNAs were diluted with the Opti-MEM® Reduced Serum Medium to a final concentration of 5 nM of siRNA in the culture medium together with INTERFERin transfection reagent at a 1:250 dilution.

2.8. Assessment of ABC transporter ATPase activity

The effect of tested taxanes on P-glycoprotein ATPase activity was assessed using the Pgp-Glo™ Assay System with a P-glycoprotein kit (Promega, CA, USA) according to the manufacturer's protocol. Briefly, membranes (25 μg) containing human recombinant P-glycoprotein were incubated with verapamil (positive control), sodium orthovanadate (inhibitor of ATPase activity), tested compound (paclitaxel, SB-T-1216, SB-T-0035) or Pgp-Glo buffer (as a control) with non-limiting concentration of ATP in Nunc™ F96 MicroWell™ (Thermofisher Scientific, MA, USA) white plate for 120 min at 37 °C. Afterwards, ATP-detection reagent, containing recombinant Ultra-Glo™ Luciferase and luciferin substrate, was added to the samples and luciferase activity signal was determined using a TECAN Infinite M200 Pro luminometer (TECAN, Männedorf, Switzerland).

2.9. Assesment of intracellular taxane level

Cells were harvested and lysed by buffer containing 8 μg/μl digitonin (Sigma Aldrich) (see Jelínek et al., 2015).

Samples were prepared by mixing 200 μl of acetonitrile (ACN) with 100 μl of cell lysate. After centrifugation, 50 μl of the supernatant was injected onto HPLC system equipped with a column Ascentis Express C18 (150 × 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA). Our method was based on principles of previously published work (Kim et al., 2005). As a mobile phase, mixture of deionized water and ACN was used. Flow rate was 1 ml/min, maintaining first 5 min ACN concentration of 34% and increasing by linear gradient to ACN concentration of 61% by 23rd min. It was followed by purifying gradient with ACN (99%) from 24th min to 28th min and finished by the decrease of ACN concentration back to 34%.

For UV detection, the wave length $\lambda = 228$ nm was used, retention time was 17.82 min for paclitaxel, 17.87 min for SB-T-0035 and 20.85 min for SB-T-1216. The areas of peaks of individual taxanes were finally compared for tested cell lines to determine relative taxane level.

2.10. Molecular modeling studies

The human ABCB1 sequence was retrieved from the UniProt database with the accession ID: P08183. An ABCB1 homology model was constructed using the Swiss-Model automated homology model server (Biasini et al., 2014). The sequence identity of the target and template protein was 87.36%. This value indicated that the P-glycoprotein (PDB: 4Q9I) was a good model for use as a template (Szewczyk et al., 2015). The homology model of ABCB1 was further utilized for the docking studies.

The protein was prepared using the Protein Preparation Vizard in Schrodinger following a standard protocol described elsewhere (Sastry et al., 2013). Hydrogen atoms were added and minimization was done using OPLS_2005 force field. This ABCB1-homology model was further used to predict the potential small-molecule binding site using the SiteMap (SiteMap, version 3.3, Schrodinger, LCC, New York, 2014). This module in Schrodinger employs three stages of calculation-finding of appropriate sites, mapping the sites and evaluating the sites for possible binding region of ligand molecules. The receptor grid was generated around the predicted binding site with a scaling factor of 1 Å and a partial cutoff charge of 0.25 Å. Ligand docking was confined to the enclosing box of 20 Å to the selected binding site. The compounds were prepared using the LigPrep module (LigPrep, version 3.2, Schrodinger, LCC, New York, 2014) and possible ionization states were generated for neutral pH. The docking calculations were carried out using the Glide Extra Precision (Glide XP) mode with default parameters (Glide, version 6.5, Schrodinger, LCC, New York, 2014), followed by post-docking minimization to maintain the accuracy of the binding complex. The 10 most favorable docking poses for each ligand were selected for structural analysis. The mean docking scores, and hydrogen bond amino acids were provided for each protein-ligand complex.

2.11. Statistical analysis

Statistical significance of differences was determined using the Student's *t*-test. $P < 0.05$ and $P < 0.01$ were considered statistically significant at the 5% and 1% levels, respectively.

3. Results

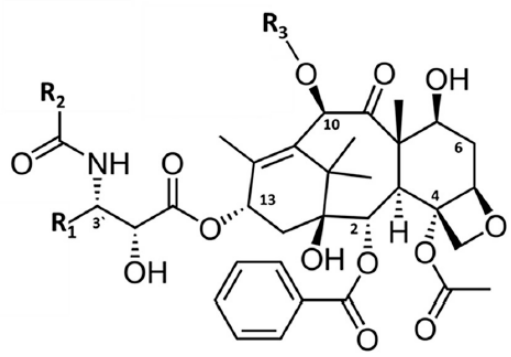
3.1. Three groups of tested taxanes

In this study we tested three groups of taxanes. The first group, including classical paclitaxel, is represented by taxanes containing phenyl groups at both the C3′(R_1) and C3′N (R_2) positions, i.e. deacetyl paclitaxel, paclitaxel, and SB-T-0035. Taxanes from this group have different chemical groups at the C10 (R_3) position (Fig. 1).

The second group contains taxanes with a phenyl at either the C3′(R_1) or C3′N (R_2) positions and with a non-aromatic group at the second position, i.e. *tert*-butoxycarbonyl at the C3′N position or 2-methylpropenyl at the C3′ position. This group of taxanes includes classical docetaxel, acetyl docetaxel, and SB-T-1212N1. The analogs of this group have two different types of substituents at the C10 (R_3) position (Fig. 1).

The third group is represented by taxanes bearing a non-aromatic 2-methylpropenyl group at the C3′ (R_1) position and a non-aromatic *tert*-butoxycarbonyl group at the C3′N (R_2) position. This group of taxanes is represented by SB-T-1211, SB-T-1102, SB-T-1216, and SB-T-1214. Taxanes of this group have different substituents at the C10 (R_3) position (Fig. 1).

A



B

GROUP OF TAXANES	TAXANE	C3′ (R_1)	C3′N (R_2)	C10 (R_3)
1	DEACETYL PACLITAXEL	phenyl	phenyl	- H
	PACLITAXEL	phenyl	phenyl	- CO-CH_3
	SB-T-0035	phenyl	phenyl	- CO-N(CH_3)$_2$
2	DOCETAXEL	phenyl	- O-C(CH_3)$_3$	- H
	ACETYL DOCETAXEL	phenyl	- O-C(CH_3)$_3$	- CO-CH_3
	SB-T-1212N1	- CH=C(CH_3)$_2$	phenyl	- CO-CH_3
3	SB-T-1211	- CH=C(CH_3)$_2$	- O-C(CH_3)$_3$	- H
	SB-T-1102	- CH-CH(CH_3)$_2$	- O-C(CH_3)$_3$	- CO-CH_3
	SB-T-1216	- CH=C(CH_3)$_2$	- O-C(CH_3)$_3$	- CO-N(CH_3)$_2$
	SB-T-1214	- CH=C(CH_3)$_2$	- O-C(CH_3)$_3$	- CO-◁

- CH=C(CH_3)$_2$: 2-methylpropenyl, -CH-CH(CH_3)$_2$: 2-methylpropyl, -O-C(CH_3)$_3$: *tert*-butoxycarbonyl, -CO-CH_3: acetyl, -CO-N(CH_3)$_2$: dimethylcarbamoyl, -CO-◁ : cyclopropanecarboxyl

Fig. 1. A) Chemical structure of taxanes. R_1, R_2 and R_3 represent substituents at the C3′, C3′N and C10 positions, respectively. B) Groups of tested taxanes. Substituents at the positions C3′ (substituent R1), C3′N (substituent R_2) and C10 (substituent R_3) are described.

3.2. Characterization of paclitaxel-sensitive and paclitaxel-resistant counterpart cells

We established, from original paclitaxel-sensitive SK-BR-3 and MCF-7 cell lines, paclitaxel-resistant counterpart cell sublines SK-BR-3/PacR and MCF-7/PacR *via* adaptation to gradually increasing concentrations of paclitaxel. The established paclitaxel-resistant SK-BR-3/PacR and MCF-7/PacR cells display long-term survival and proliferation in a culture medium with 100 nM paclitaxel (SK-BR-3/PacR) or 300 nM paclitaxel (MCF-7/PacR). Application of these concentrations of paclitaxel results in cell death in most cells of the original lines within 36 h (Němcová-Fürstová et al., 2016).

Activation of executioner caspase-3 and caspase-7 was not detected in SK-BR-3/PacR cells after 100 nM paclitaxel application, while activation of these caspases in original sensitive SK-BR-3 was clearly seen. Similar data were obtained for caspase-7 in MCF-7/PacR versus original MCF-7 cells. There is no functional caspase-3 in MCF-7 cells (Němcová-Fürstová et al., 2016).

We found that the expression of ABCB1 (PgP) and ABCC3/MRP3 transporters are significantly upregulated in both resistant sublines SK-BR-3/PacR and MCF-7/PacR (Fig. 2A). Employing ABCB1 silencing by a specific siRNA, we tested whether the overexpression of ABCB1 was responsible for developed resistance to paclitaxel. Silencing of the ABCB1 expression to nearly undetectable level resulted in a significant decrease in the number of surviving cells 96 h after paclitaxel application in both resistant sublines. It was a decrease to about 70% of the number of control cells (without paclitaxel) for SK-BR-3/PacR cells and about 20% for MCF-7/PacR cells (Fig. 2B). Such differing effects of ABCB1 silencing on the number of surviving SK-BR-3/PacR cells and MCF-7/PacR cells after paclitaxel application could simply reflect differing dependence of the resistance of these sublines on ABCB1 transporter.

3.3. Effect of tested taxanes on growth and survival of paclitaxel-sensitive and paclitaxel-resistant cells

We assessed the effect of tested taxanes on growth and survival of paclitaxel-sensitive and corresponding paclitaxel-resistant cells. Taxane concentrations 10–300 nM for SK-BR-3 cells and 3–3000 nM for MCF-7 cells were used.

Data for the first group (phenyl groups at both C3′ and C3′N positions) of tested taxanes are shown in Fig. 3. Data for the second group (phenyl at either C3′or C3′N position and a non-aromatic substituent at the other position) of taxanes are shown in Fig. 4. Data for the third group (non-aromatic substituents at both C3′ and C3′N positions) are in Fig. 5.

The results are summarized in Table 1. The C_0 values (taxane concentration for which there is no increase or decrease in the number of cells of the inoculum after 96-hour incubation) concerning individual tested taxanes for sensitive and resistant SK-BR-3 as well as MCF-7 cells are presented. Furthermore, fold increase of the C_0 value for resistant cells compared with the C_0 value for sensitive cells is also presented. In the first group of taxanes, the increase in the C_0 value for resistant cells was between 8.6× and > 13.6× in the case of SK-BR-3 cells and between 26× and 132× in the case of MCF-7 cells. In the second group, the increase in the C_0 value was between 0.5× and 4.5× for SK-BR-3 cells and between 8.0× and 14.1× for MCF-7 cells. As to the third group, the increase in the C_0 value was between 1.6× and 3.5× for SK-BR-3 cells and between 2.8× and 5.9× for MCF-7 cells. We can see that the C_0 increase in the first group of taxanes was noticeably higher when compared with the C_0 increase in the second and third group of taxanes for both SK-BR-3 and MCF-7 cells.

3.4. Establishment of MCF-7 subline resistant to SB-T-0035

Like in the case of established paclitaxel-resistant counterpart cell sublines, we established SB-T-0035-resistant cell subline MCF-7/SB-T-0035R, from the original SB-T-0035-sensitive MCF-7 cell line, by adaptation to gradually increasing concentrations of SB-T-0035.

Cells of the established SB-T-0035-resistant subline MCF-7/SB-T-0035R display long-term survival and proliferation in a medium containing 300 nM SB-T-0035. Cells of the original sensitive MCF-7 line cultured with such SB-T-0035 concentration die nearly completely within 96 h (see Fig. 7).

3.5. Characterization of SB-T-0035-resistant cells

As with the paclitaxel-resistant sublines, we found that the expression of ABCB1 (PgP) transporter is significantly upregulated in the SB-T-0035-resistant subline of MCF-7 cells (Fig. 6A).

Again, we tested whether the overexpression of ABCB1 was responsible for the developed resistance using ABCB1 silencing. Silencing of ABCB1 expression by a specific siRNA to suitable level led to a significant decrease in the number of surviving cells 96 h after SB-T-0035 application in SB-T-0035-resistant cells. It was a decrease to about 30% of the number of control cells (without SB-T-0035) (Fig. 6B).

3.6. Effect of tested taxanes on growth and survival of SB-T-0035-resistant cells

We also assessed the effect of some of the tested taxanes on growth and survival of SB-T-0035-sensitive and SB-T-0035-resistant MCF-7 cells (see previous two sections) over a range of concentrations

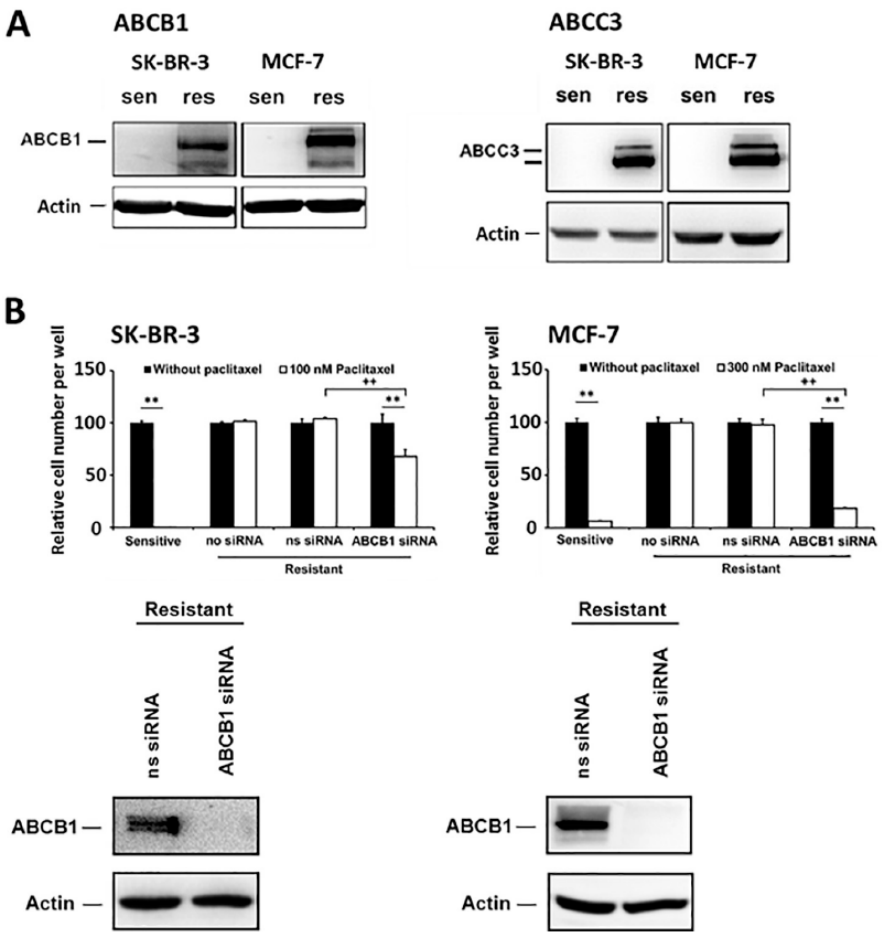


Fig. 2. (A) The level of ABCB1 and ABCC3 transporters in paclitaxel-sensitive (sen) and paclitaxel-resistant (res) SK-BR-3 and MCF-7 cells. (B) The effect of ABCB1 silencing on the growth and survival of paclitaxel-resistant SK-BR-3 and MCF-7 cells after paclitaxel treatment. (A) After 24 h of incubation with paclitaxel (100 nM for SK-BR-3 and 300 nM for MCF-7) the levels of ABC transporters were determined using western blot analysis and relevant antibodies (see "Materials and methods"). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments. Western blot quantification by densitometry is shown. Data are presented as the mean of relative density ± SEM. *$P < 0.05$ when comparing the density in sensitive and resistant cells. (B) The cells were prepared as described in "Materials and methods" and seeded at 20×10^3 cells/100 µl of medium per well. The relative number of living sensitive cells, resistant cells (no siRNA), resistant cells treated with non-specific siRNA (ns siRNA) and resistant cells treated with an ABCB1 specific siRNA (ABCB1 siRNA) was determined after 96 h of incubation without paclitaxel (control cells) or with paclitaxel (100 nM for SK-BR-3 and 300 nM for MCF-7). Each column represents the mean of 4 separate culture ± SEM. **$P < 0.01$ when comparing the effect in cells without paclitaxel and treated with paclitaxel. $^{++}P < 0.01$ when comparing the effect in ns siRNA-treated and ABCB1 siRNA-treated cells after paclitaxel application. The data shown were obtained in one representative experiment of three independent experiments. The effect of non-specific siRNA (ns siRNA) and specific siRNA (ABCB1 siRNA) on ABCB1 expression in paclitaxel-resistant SK-BR-3 and MCF-7 cells is also shown. Actin levels were used to confirm equal protein loading.

(3–3000 nM).

Data for paclitaxel and SB-T-0035 (the first group of taxanes with phenyl groups at both C3′ and C3′N positions) and SB-T-1216 (the third group of taxanes with non-aromatic substituents at both C3′ and C3′N positions) are in Fig. 7. The results are summarized in Table 2. The C_0 values concerning individual tested taxanes for sensitive and resistant MCF-7 cells are presented. Fold increase of the C_0 value for resistant cells compared with the C_0 value for sensitive cells is also shown. The increase in the C_0 value for resistant MCF-7 cells was 95× and 133× for paclitaxel and SB-T-0035 (the first group of taxane derivatives), respectively, and 2.0× for SB-T-1216 (the third group of taxanes). Again, we can see that the C_0 increase in the first group of taxanes was significantly higher compared with the C_0 increase in the third group of taxanes.

3.7. Effect of tested taxanes on ATPase activity of ABCB1 transporter

We assessed the effect of some of the tested taxanes (300 nM) on the ATPase activity of the ABCB1 (PgP) transporter employing a non-cellular system, i.e. membranes containing human recombinant ABCB1 (see "Materials and methods").

We found that all three tested taxanes (paclitaxel, SB-T-0035, and SB-T-1216) increased basal activity of the ATPase. However, the increase in the ATPase activity was 1.7–2.1 times higher for taxanes from the first group (paclitaxel, SB-T-0035) than for the taxane from the third group (SB-T-1216). The difference was not found to be statistically significant. When comparing the increase in the basal ATPase activity after the application of tested taxanes and the application of verapamil (plus control), the increase after the application of taxanes was 2.8–5.8 times lower than after verapamil application (Fig. 8).

3.8. Intracellular level of tested taxanes after their application in paclitaxel-resistant and SB-T-0035-resistant cells

Using a HPLC system (see "Materials and methods"), we assessed intracellular level of some of tested taxanes after their application (300 nM) in sensitive, paclitaxel-resistant and SB-T-0035-resistant MCF-7 cells. We supposed that intracellular level of particular taxane after the incubation of the cells with this taxane reflects the efficacy of taxane transport out of the cells.

We found that both taxanes from the first group (paclitaxel, SB-T-0035) were transported out of paclitaxel-resistant as well as SB-T-0035-resistant MCF-7 cell very efficiently. Intracellular level of both taxanes in resistant cells represented only about 5% or less of the level in control sensitive MCF-7 cells. However, intracellular level of the taxane from the third group (SB-T-1216) in resistant cells represented about 40–50% of the level in sensitive cells. It showed that transport of SB-T-1216 out of both types of resistant cells was much less efficient (Fig. 9).

3.9. Molecular docking of tested taxanes to the ABCB1 transporter

We performed molecular docking of some of tested taxanes to the human ABCB1 (PgP) transporter (Fig. 10) using Schrodinger docking suite (see "Materials and methods").

To investigate the binding affinities computationally, deacetyl paclitaxel, paclitaxel, SB-T-0035, SB-T-1212N1, acetyl docetaxel and SB-T-1216 were docked into the active site of the homology model of the ABCB1 protein. The obtained docking scores were divided into three

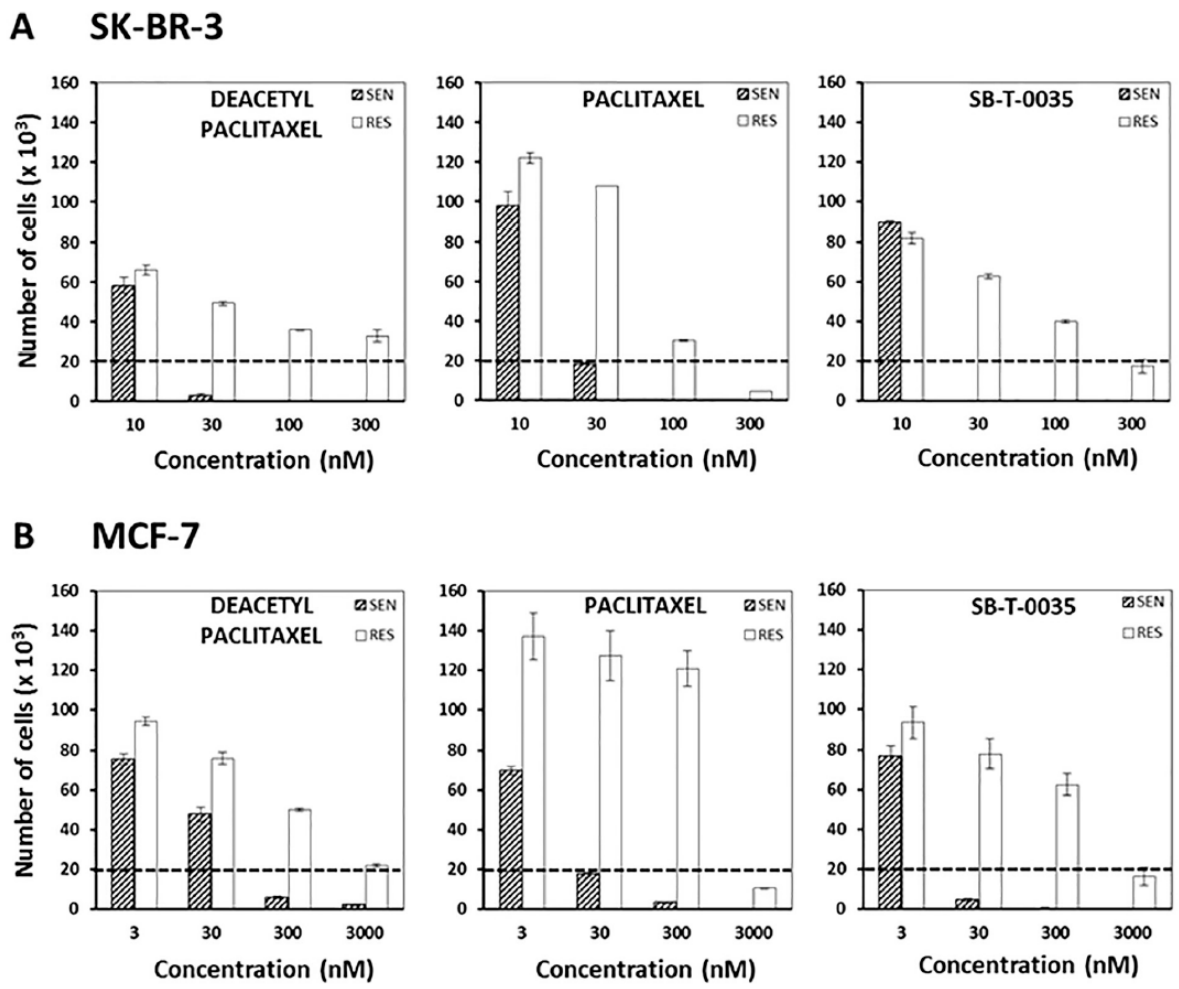


Fig. 3. Effect of deacetyl paclitaxel, paclitaxel and SB-T-0035 on the growth and survival of paclitaxel-sensitive (sen) and paclitaxel-resistant (res) (A) SK-BR-3 cells (10–300 nM taxane) and (B) MCF-7 cells (3–3000 nM taxane). The number of cells of the inoculum (20×10^3 cells/100 µl of medium per well) is shown as a dotted line. The number of living cells was determined after 96 h of incubation (see "Materials and methods"). Each column represents the mean of three separate experiments ± SEM.

groups based on the respective substitution of the side chains in the ligand molecules. The first group of taxanes included deacetyl paclitaxel (−10.77 ± 0.21), paclitaxel (−10.12 ± 0.19) and SB-T-0035 (−10.02 ± 0.15) and exhibited the lowest docking scores, i.e. highest predicted binding affinity. This probably reflects the importance of the two phenyl groups at the C3′ and C3′N positions in their structures. The second group contained SB-T-1212N1 (−9.89 ± 0.07) and acetyl docetaxel (−9.49 ± 0.18). These taxanes, lacking one phenyl group at either C′3 or C3′N position in their structures, had worse docking scores compared to taxanes of the first group. The last taxane SB-T-1216 (8.15 ± 0.25), from the third group, bearing no phenyl group at the C′3 and C3′N positions, had a docking score reflecting the lowest predicted binding affinity compared to the other taxanes. The docking predictions are summarized in Table 3.

These results demonstrated that the docking energy score reflecting the predicted free energies of individual taxanes to the ABCB1 transporter, was the lowest (the highest predicted free energy) for taxanes from the first group (deacetyl paclitaxel, paclitaxel, SB-T-0035), i.e. from −10.02 to −10.77. The taxanes from the second group (acetyl docetaxel, SB-T-1212N1) had lower predicted free energy compared to taxanes from the first group, i.e. from −9.49 to −9.89. SB-T-1216 from the third group had the lowest predicted free energy to ABCB1, i.e. −8.15 (Table 3).

Schemes showing molecular interactions of individual taxanes with the ABCB1 transporter at their binding sites are shown in Fig. 11.

4. Discussion

Previously we have established paclitaxel-resistant sublines of the original sensitive breast cancer cell lines SK-BR-3 and MCF-7 by adaptation to gradually increasing concentrations of paclitaxel (Němcová-Fürstová et al., 2016). Interestingly, we found that both paclitaxel-resistant sublines maintained their sensitivity to taxane SB-T-1216 (Ojima et al., 1996) that was similar to the sensitivity of the original sensitive lines. Furthermore, despite repeated efforts, we were unable to establish variants of the original cell lines resistant to SB-T-1216 (Němcová-Fürstová et al., 2016). These findings together with results of subsequent pilot experiment with other taxanes led us to hypothesize that substituents at the C3′and C3′N positions of the taxane structure are critical with regard to capability of overcoming resistance to paclitaxel.

We have decided to test this hypothesis. As an experimental model, we used our previously established paclitaxel-resistant sublines SK-BR-3/PacR and MCF-7/PacR versus original paclitaxel-sensitive lines SK-BR-3 and MCF-7. Resistant sublines are capable of long-term survival and near-normal proliferation in a medium with such concentrations of paclitaxel (100 nM for SK-BR-3/PacR and 300 nM for MCF-7/PacR) in which most cells of the original sensitive lines die within 96 h (Němcová-Fürstová et al., 2016). Later, within the framework of this

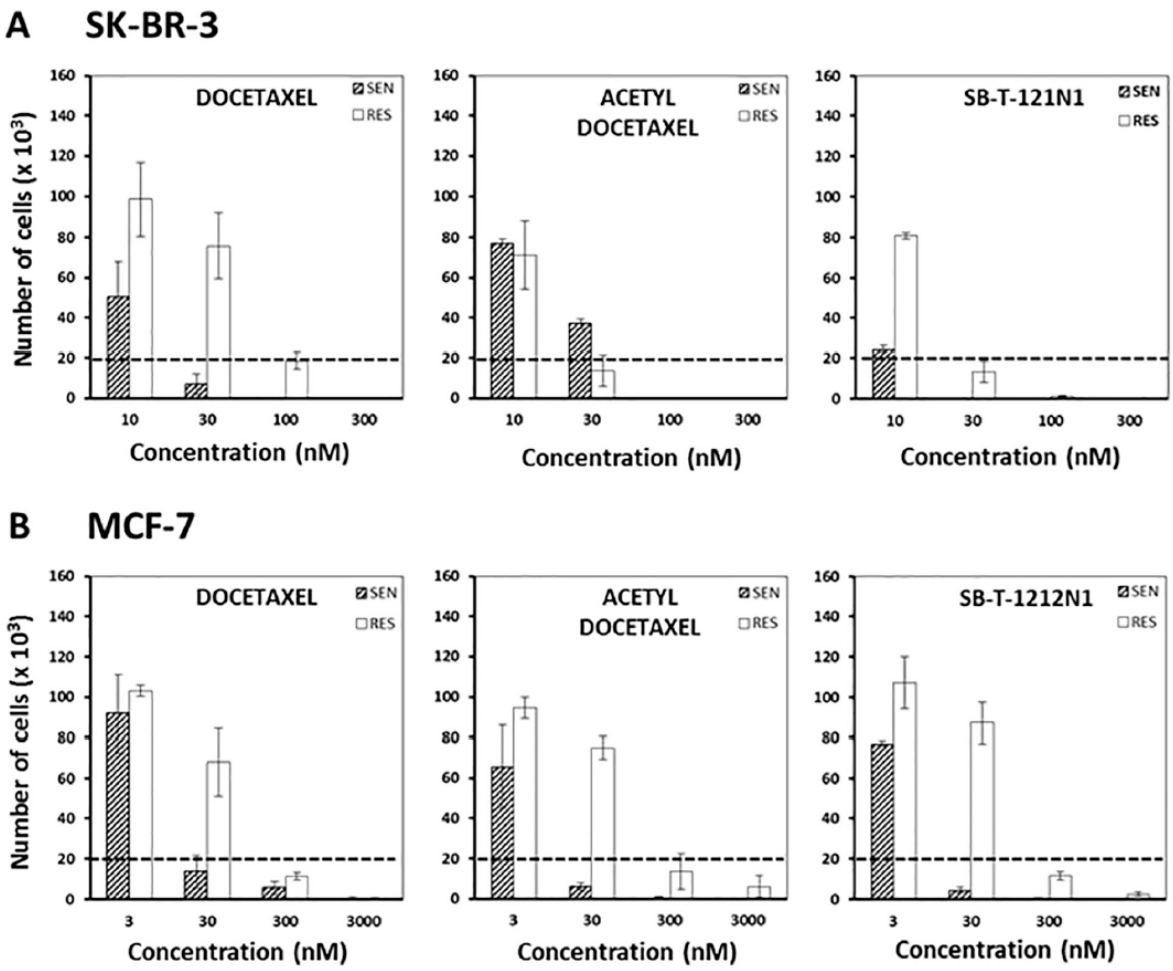


Fig. 4. Effect of docetaxel, acetyl docetaxel and SB-T-1212 N1 on the growth and survival of paclitaxel-sensitive (sen) and paclitaxel-resistant (res) (A) SK-BR-3 cells (10–300 nM taxane) and (B) MCF-7 cells (3–3000 nM taxane). The number of cells of the inoculum (20×10^3 cells/100 μl of medium per well) is shown as a dotted line. The number of living cells was determined after 96 h of incubation (see "Materials and methods"). Each column represents the mean of three separate experiments ± SEM.

study, we established SB-T-0035-resistant subline from the original sensitive MCF-7 cell line using similar approach as used for paclitaxel-resistant sublines (see "Results"). Taxane SB-T-0035 has the same substituents at the C3′and C3′N positions as paclitaxel. We used the model of established SB-T-0035-resistant MCF-7 cells versus the original sensitive MCF-7 cells in our experiments in order to confirm results regarding paclitaxel-resistant versus paclitaxel-sensitive cells.

Previously, we also found that both paclitaxel-resistant sublines significantly upregulated the expression of ABCB1 (PgP) or ABCC3 (MRP3) transporters which was detected by western blot analysis. Furthermore, effective silencing of ABCB1 expression employing a specific siRNA significantly decreased resistance to paclitaxel in the paclitaxel-resistant cells. It suggests the important role for ABCB1 in mechanisms of paclitaxel resistance (Němcová-Fürstová et al., 2016). In this study, we also found that ABCB1 expression in SB-T-0035-resistant sublines of MCF-7 cells is significantly upregulated. Together with this, we confirmed that effective silencing of ABCB1 expression by a specific siRNA results in a significant decrease in resistance to SB-T-0035 in SB-T-0035-resistant cells (see Fig. 6). Upregulation of ABCB1 and ABCC3 expression in taxane-resistant variants of breast cancer cells has been demonstrated by several authors (Ajabnoor et al., 2012; Hembruff et al., 2008; O'Brien et al., 2008; Reed et al., 2010; Shi et al., 2014; Wang et al., 2014). However, there are papers indicating that ABCB1 upregulation is not required for the development of paclitaxel resistance in breast cancer cells (Kars et al., 2006; Kenicer et al., 2014).

Data from our pilot experiments pointed to the possibility that only the presence of phenyl groups at both C3′ and C3′N positions are responsible for low capability of taxane to overcome acquired resistance to paclitaxel as well as a high probability of developing resistance to this taxane. When there is a non-aromatic group at one or both C3′ and C3′N positions, such taxane has higher capability of overcoming resistance to paclitaxel and there is low probability of developing resistance to this taxane. In further studies we tested this hypothesis. In order to do that we compared three groups of taxanes. Taxanes of the first group have phenyl groups at both C3′ and C3′N positions. Taxanes of the second group have a phenyl group at either C3′ or C3′N position and a non-aromatic group at the other position. The third group involves taxanes with non-aromatic groups at both C3′and C3′N positions (see Fig. 1). The role of substituents at the C3′and C3′N positions of the taxane structure in resistance of tumor cells has been previously studied (Ojima et al., 1994; Ojima et al., 1996; Ojima et al., 1997; Ojima et al., 2000). However, these studies were not focused on acquired resistance to paclitaxel.

The effect of individual taxanes from the three mentioned groups on tested cells was assessed by the C_0 value (see "Results"). Then the degree of resistance of paclitaxel-resistant cells to individual taxanes was assessed by the increase of the C_0 value of resistant cells when compared with the C_0 value of corresponding sensitive cells (see Table 1). In

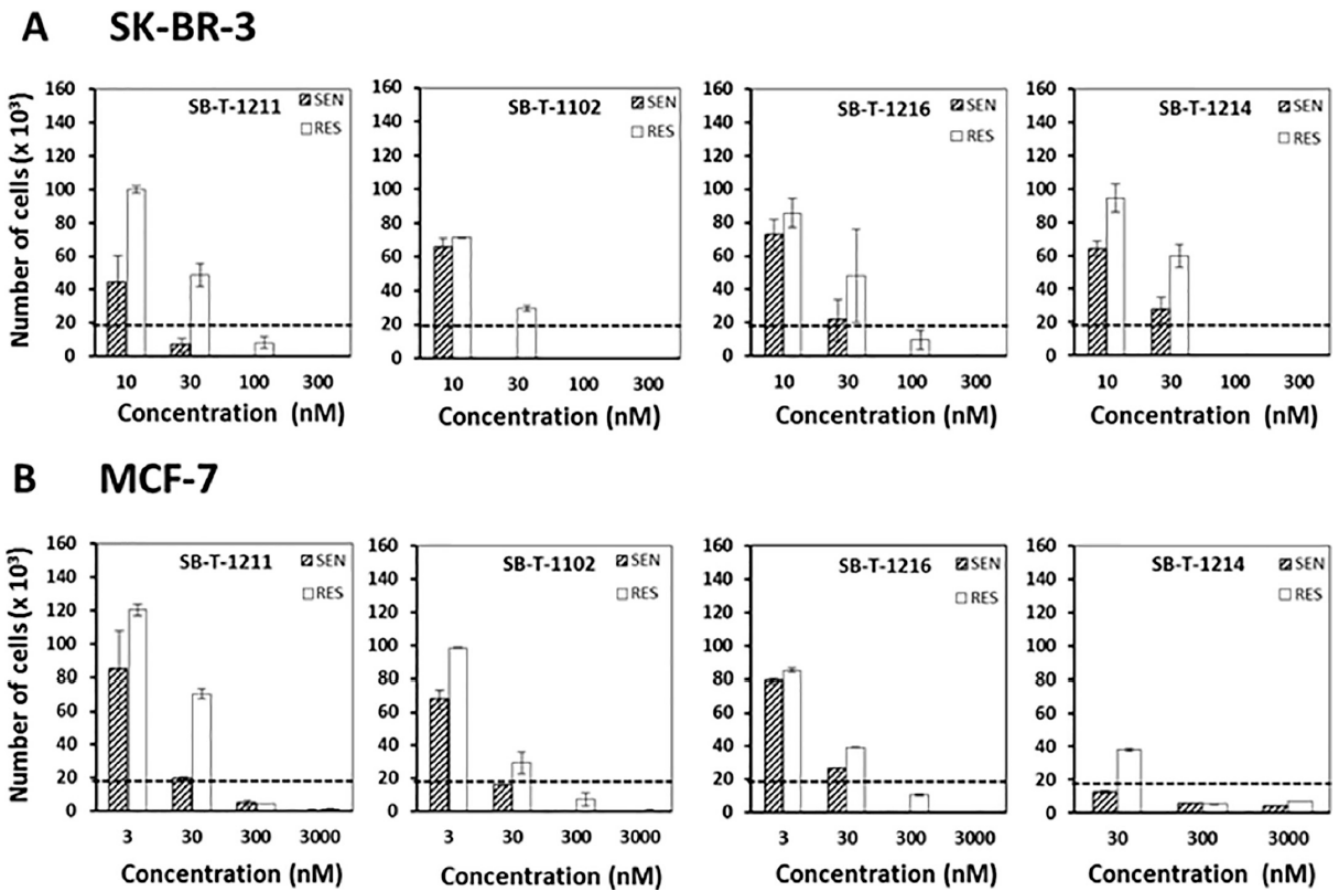


Fig. 5. Effect of SB-T-1211, SB-T-1102, SB-T-1216 and SB-T-1214 on the growth and survival of paclitaxel-sensitive (sen) and paclitaxel-resistant (res) (A) SK-BR-3 cells (10–300 nM taxane) and (B) MCF-7 cells (3–3000 nM taxane). The number of cells of the inoculum (20×10^3 cells/100 µl of medium per well) is shown as a dotted line. The number of living cells was determined after 96 h of incubation (see "Materials and methods"). Each column represents the mean of three separate experiments ± SEM.

Table 1
Comparison of the effect of tested taxanes on the growth and survival of paclitaxel-sensitive (SEN) and paclitaxel-resistant (RES) SK-BR-3 and MCF-7 cells.

TAXANE	SK-BR-3			MCF-7		
	C_0/SEN (nM)	C_0/RES (nM)	INCREASE (x)	C_0/SEN (nM)	C_0/RES (nM)	INCREASE (x)
DEACETYL PAC.	22	> 300	> 13.6	138	3600	26.1
PACLITAXEL	29	250	8.6	27	2500	92.6
SB-T-0035	23	260	11.3	19	2500	132
DOCETAXEL	22	98	4.5	25	200	8.0
ACETYL DOC.	52	26	0.5	17	240	14.1
SB-T-1212N1	12	27	2.2	18	230	12.8
SB-T-1211	20	70	3.5	29	170	5.9
SB-T-1102	22	45	2.0	25	84	3.4
SB-T-1216	32	72	2.2	51	141	2.8
SB-T-1214	41	67	1.6	< 30	114	> 3.8

C_0 represents taxane concentration (nM) for which there is not any increase or any decrease of the number of cells of the inoculum (20×10^3 cells/100 µl of medium per well) after 96 h of incubation. Fold increase (x) of C_0 value for resistant cells in comparison with C_0 value for sensitive cells is shown.

the third group of taxanes with non-aromatic groups at both C3′and C3′N positions, the increase in the C_0 value of resistant cells was 1.6–3.5 times for SK-BR-3 cells and 2.8–5.9 times for MCF-7 cells. As to the second group of taxanes with one phenyl and one non-aromatic group at the C3′and C3′N positions respectively, the increase in the C_0 value for resistant cells was similar or somewhat higher. It was 0.5–4.5 times for SK-BR-3 cells and 8.0–14.1 times for MCF-7 cells. Presented unexpected increase (only 0.5 times) in the C_0 value for acetyl docetaxel and resistant SK-BR-3 cells (see Table 1) may represent just a stochastic fluctuation. However, in the first group of taxanes with phenyl groups at both C3′and C3′N positions, the increase in the C_0 value was noticeable. It was 8.6–13.6 times for SK-BR-3 cells and 26–132 times for MCF-7 cells. The data show that the increase in the C_0 value for resistant cells is significantly higher for taxanes of the first group compared with the increase in the C_0 value for taxanes of the second and third group. Thus, these results confirm our hypothesis that phenyl group at both C3′and C3′N positions of taxane is associated with low capability of taxane to overcome acquired paclitaxel resistance compared with taxanes containing at least one non-aromatic substituent at the C3′and C3′N positions. To the best of our knowledge, this is the first direct confirmation of such fact (see Ojima et al., 1994; Ojima et al., 1996; Ojima et al., 1998; Ojima et al., 2008). Our finding is strongly supported by the data obtained with SB-T-0035-resistant cells (see Table 2). Similarly to paclitaxel, SB-T-0035 has phenyl groups at both C3′and C3′N positions.

As with paclitaxel-resistant SK-BR-3 and MCF-7 cells (Němcová-Fürstová et al., 2016), we detected significant upregulation of the expression of ABCB1 (PgP) transporter in SB-T-0035-resistant MCF-7 cells (see Fig. 6A). It points at the possibility that upregulation of ABCB1 transporter can play an important role in acquired resistance to paclitaxel, as it was shown by several groups previously (Aldonza et al., 2016; Kathawala et al., 2015; Reed et al., 2010), and also to SB-T-0035.

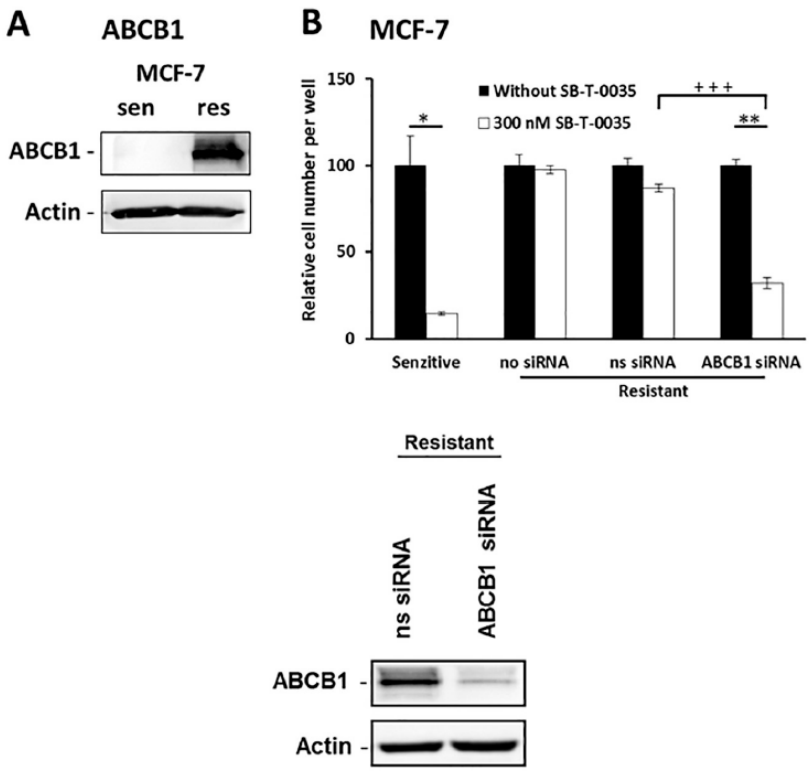


Fig. 6. (A) The level of ABCB1 transporter in SB-T-0035-sensitive (sen) and SB-T-0035-resistant (res) MCF-7 cells. (B) The effect of ABCB1 silencing on the growth and survival of SB-T-0035-resistant MCF-7 cells after SB-T-0035 treatment. (A) After 24 h of incubation with 300 nM SB-T-0035 the level of ABCB1 transporter was determined using western blot analysis and relevant antibody (see "Materials and methods"). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments. (B) The cells were prepared as described in "Materials and Methods" and seeded at 20×10^3 cells/100 µl of medium per well. The relative number of living sensitive cells, resistant cells (no siRNA), resistant cells treated with nonspecific siRNA (ns siRNA) and resistant cells treated with an ABCB1 specific siRNA (ABCB1 siRNA) was determined after 96 h of incubation without SB-T-0035 (control cells) or with 300 nM SB-T-0035. Each column represents the mean of 4 separate culture ± SEM. *$P < 0.05$, **$P < 0.01$ when comparing the effect in cells without SB-T-0035 and treated with SB-T-0035. $^{+++}P < 0.001$ when comparing the effect in ns siRNA-treated and ABCB1 siRNA-treated cells after SB-T-0035 application. The data shown were obtained in one representative experiment of three independent experiments. The effect of non-specific siRNA (ns siRNA) and specific siRNA (ABCB1 siRNA) on ABCB1 expression in SB-T-0035-resistant MCF-7 cells is also shown. Actin levels were used to confirm equal protein loading.

The possibility was confirmed by our experiments with paclitaxel-resistant and SB-T-0035-resistant cells by inhibiting ABCB1 expression using a specific siRNAs (Němcová-Fürstová et al., 2016, see Fig. 6B).

Increased level of ABCB1 in cells resistant to docetaxel and cabazitaxel (taxanes belonging to the second group of taxanes) (Duran et al., 2015, Li et al., 2014, Ojima et al., 1996, Ojima et al., 1998) and transport of novel taxane BMS-275,183 (taxane belonging to the third group of taxanes) by ABCB1 were described previously (Marchetti et al., 2014). The ability of taxanes SB-T-1212 and SB-T-1213 (with non-aromatic groups at C3′and C3′N positions) to overcome established P-glycoprotein-related resistance was also described (Ferlini et al., 2000; Ojima et al., 1996). Thus the hydrophobicity of molecules, which was highest for taxanes from the first group of taxanes, seems to be a key feature of molecules to be transported by ABCB1 (Liu et al., 2013;

Table 2
Comparison of the effect of tested taxanes on the growth and survival of SB-T-0035-sensitive (SEN) and SB-T-0035-resistant (RES) MCF-7 cells.

TAXANE	MCF-7		
	C_0/SEN (nM)	C_0/RES (nM)	INCREASE (x)
PACLITAXEL	20	1900	95
SB-T-0035	18	2400	133
SB-T-1216	66	135	2.0

C_0 represents taxane concentration (nM) for which there is not any increase or any decrease of the number of cells of the inoculum (20×10^3 cells/100 µl of medium per well) after 96 h of incubation. Fold increase (x) of C_0 value for resistant cells in comparison with C_0 value for sensitive cells is shown.

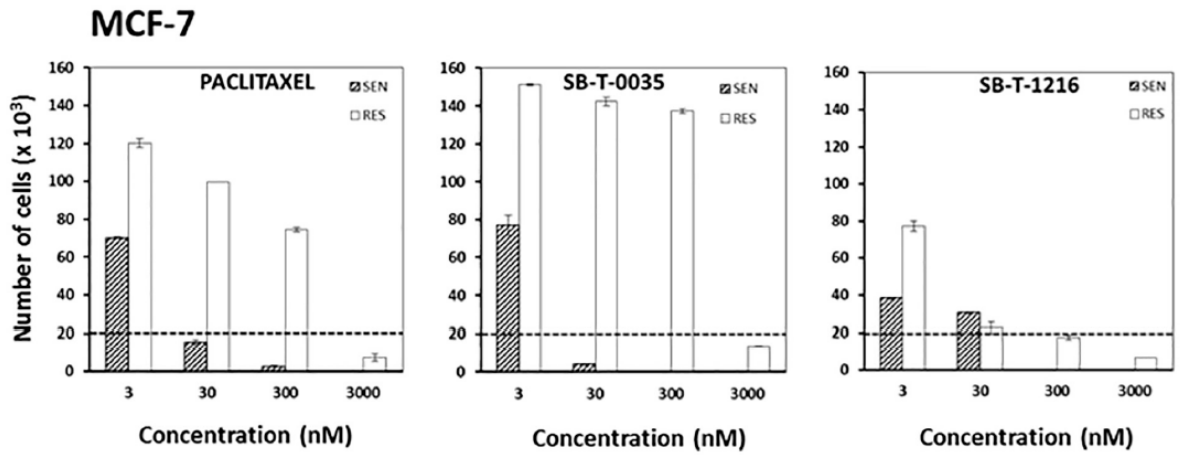


Fig. 7. Effect of paclitaxel, SB-T-0035 and SB-T-1216 (3–3000 nM) on the growth and survival of SB-T-0035-sensitive (sen) and SB-T-0035-resistant (res) MCF-7 cells. The number of cells of the inoculum (20×10^3 cells/100 µl of medium per well) is shown as a dotted line. The number of living cells was determined after 96 h of incubation (see "Materials and methods"). Each column represents the mean of three separate experiments ± SEM.

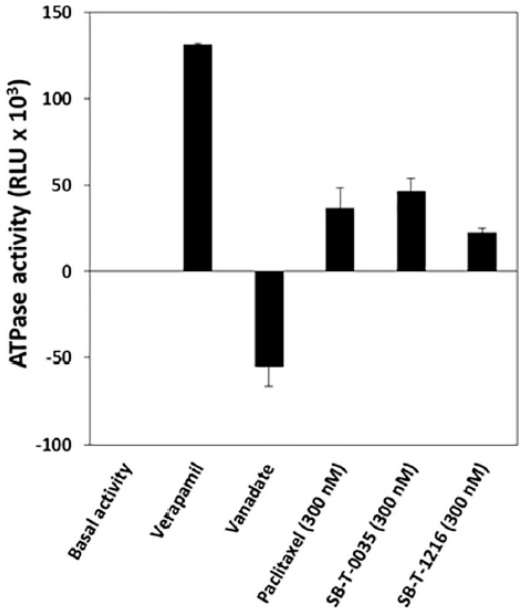


Fig. 8. Effect of paclitaxel, SB-T-0035 and SB-T-1216 (300 nM) on the ATPase activity of ABCB1 transporter. To assess the ATPase activity of ABCB1 (PgP) transporter, commercial Pgp-Glo™ Assay System with P-glycoprotein kit (Promega) using membranes with human recombinant P-glycoprotein was used (see "Materials and methods"). The ATPase activity is expressed as relative light units (RLU). The ATPase activity after verapamil application was used as a positive control and after sodium orthovanadate application as a negative control. Basal ATPase activity of ABCB1 transporter (without taxane, verapamil or vanadate application) is shown. Each column represents the mean of two independent experiments ± SEM.

Ojima et al., 1998).

The visual inspection of the docking poses of deacetyl paclitaxel, paclitaxel and SB-T-0035, from the first group of taxanes, acetyl docetaxel and SB-T-1212N1, from the second group of taxanes, and SB-T-1216 from the third group of taxanes, provided almost identical interactions with few exceptions in the binding conformation/orientation of the compounds to the active site of the ABCB1 protein (see Fig. 11). The 5 Å surrounding region of the bound compound was mostly occupied by hydrophobic amino acid residues. The two phenyl groups at the C3′and C3′N positions are buried into the hydrophobic cleft of the ABCB1 protein, indicating a tight binding of deacetyl paclitaxel, paclitaxel, SB-T-0035, acetyl docetaxel and SB-T-1212 N1. Docking energy scores of

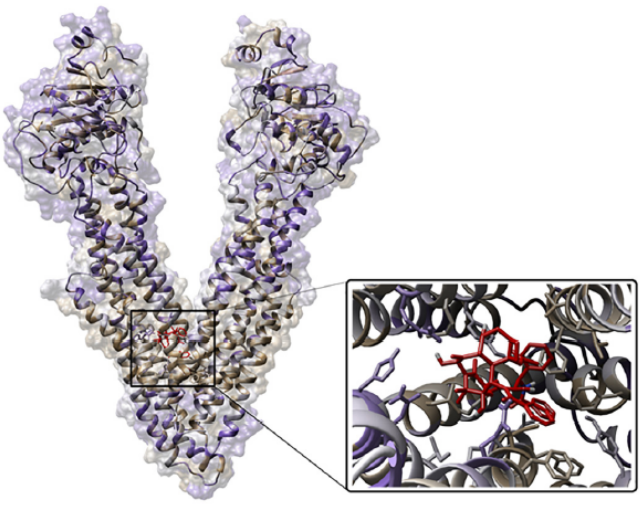


Fig. 10. Structure of the human ABCB1 transporter with bound deacetyl paclitaxel.

taxanes of all three taxane groups reflect the importance of substitutions at the C3′and C3′N positions of the C13 side chain of taxanes. The importance of hydrophobic interactions was documented previously. Phe 339 of ABCB1 molecule was found to form a gate for entry of paclitaxel into its binding site, while several other Phe residues (Phe71, Phe332, Phe728) helped paclitaxel to be stabilized in the binding site of ABCB1 (Zhang et al., 2015).

Further, hydrogen bonding interaction was predicted between the protein-ligand complexes. The hydrogen bond involving the polar carboxyl group of Ser337 is maintained by the oxygen atom (=O) near the NH of all reported taxanes and it helps to keep the same orientation and position of the phenyl group in deacetyl paclitaxel, paclitaxel, SB-T-0035 and SB-T-1212 N1. On the other hand, acetyl docetaxel and SB-T-1216 lack the phenyl group near the oxygen atom (=O), which results in the slight change in the orientation of the binding pose. Moreover, the interaction of Gln347 was observed with the carbonyl oxygen atom, which is connected to the phenyl group at the C2 position of deacetyl paclitaxel, SB-T-0035, acetyl docetaxel and SB-T-1212 N1. For so far unknown reason, this interaction is not present in complexes with paclitaxel and SB-T-1216. Additionally, the interaction of side chain of the Gln946 was predicted in the baccatin core of all tested taxanes.

Docking scores reflecting predicted free energy of taxanes to ABCB1 transporter correspond to the three groups of tested taxanes (see

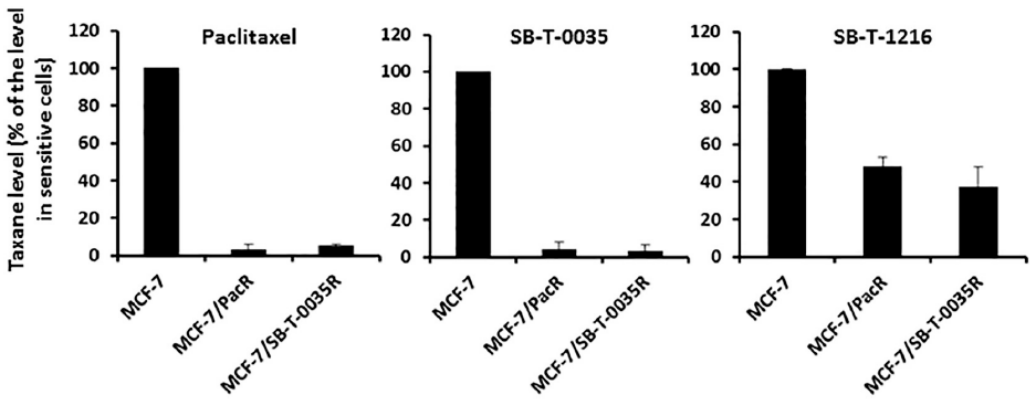


Fig. 9. Level of paclitaxel, SB-T-0035 and SB-T-1216 after their application in sensitive MCF-7 cells, paclitaxel-resistant MCF-7/PacR cells and SB-T-0035-resistant MCF-7/SB-T-0035R cells. After 24 h of incubation with 300 nM concentration of tested taxane relative taxane levels were assessed using a HPLC system (see "Materials and methods"). Levels of individual taxanes in sensitive MCF-7 cells were used as controls (100%). Each column represents mean of two independent experiments ± SEM.

Table 3
The docking score and hydrogen bond interactions of the ABCB1 transporter with tested taxanes. The values are based on the average score of top ten conformations.

Taxane	Docking conformations	Score (kcal/mol)	Hydrogen bond
DEACETYL PACLITAXEL	10	−10.77 ± 0.21	S 337, Q 347, Q 946
PACLITAXEL	10	−10.12 ± 0.19	S 337, Q 946
SB-T-0035	10	−10.02 ± 0.15	S 337, Q 347, Q 946
SB-T-1212 N1	10	−9.89 ± 0.07	S 337, Q 347, Q 946
ACETYL DOCETAXEL	10	−9.49 ± 0.18	S 337, Q 347, Q 946
SB-T-1216	10	−8.15 ± 0.25	S 337, Q 946

Table 3). Taxanes from the first group (two phenyl groups at the C3′and C3′N positions) have the highest predicted free energy, taxanes from the second group (one phenyl and one non-aromatic substituent at the C3′and C3′N positions) have intermediate predicted free energy, and taxane from the third group (two non-aromatic substituents at the C3′and C3′N positions) has the lowest predicted free energy. Lower predicted free energy means less effective transport out of cancer cell and *vice versa*. These data are in agreement with our data concerning the efficacy of taxane transport out of cells (see Fig. 9). The higher capability of taxanes, with at least one non-aromatic group at the C3′and C3′N positions, to overcome acquired resistance to paclitaxel as well as reduced probability of developing resistance is very likely based on their less effective transport out of cancer cell. The affinity of taxane to the ABCB1 transporter was reported to be strongly affected by the substituent at the position C10 previously (Ferlini et al., 2000). However, authors did not use the human ABCB1 transporter in their

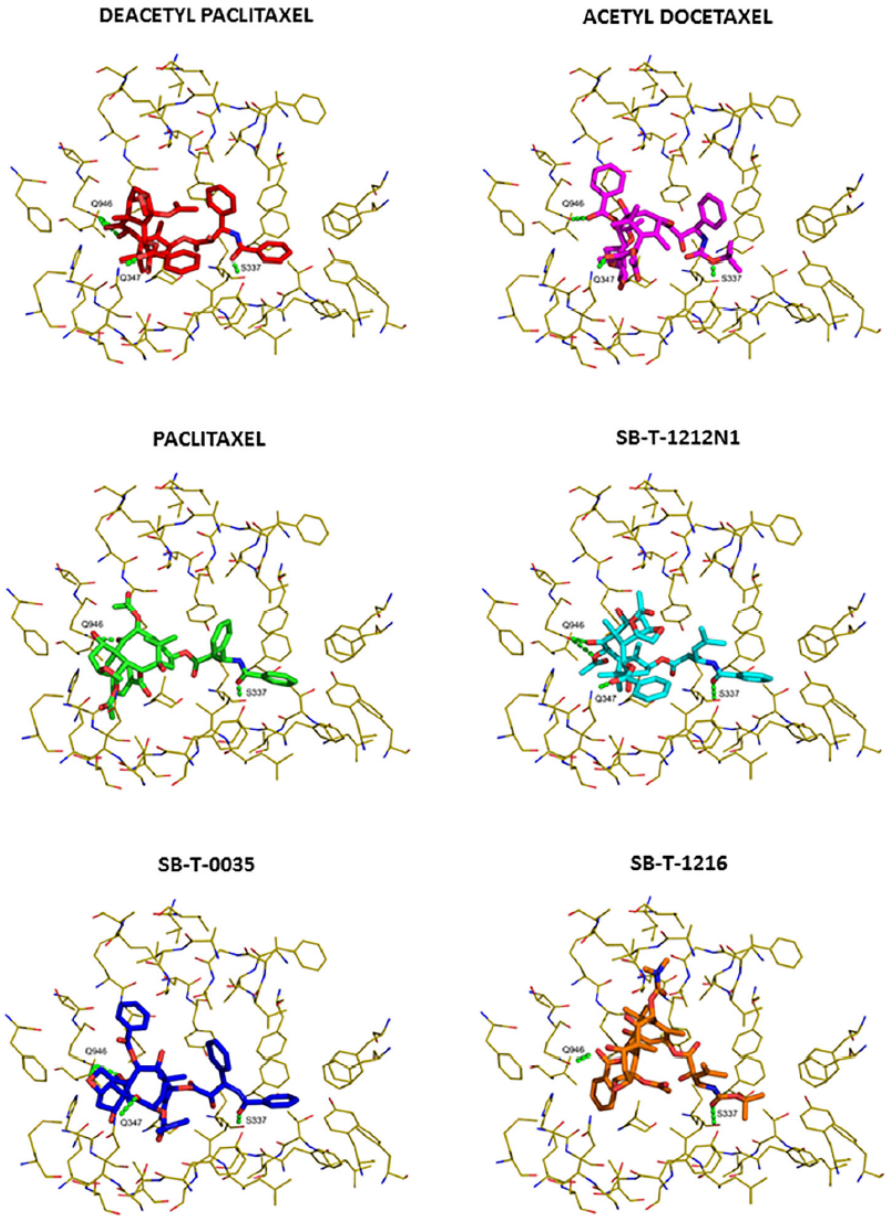


Fig. 11. Visualization of representative docking poses of tested taxanes (deacetyl paclitaxel, paclitaxel, SB-T-0035, acetyl docetaxel, SBT-1212N1, and SB-T-1216) into the ABCB1 binding site. The hydrogen bonding interactions are shown as green dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecular docking study. We did not confirm that finding in our study when comparing taxanes from the first group.

We can summarize that the high capability of a taxane to overcome acquired resistance of breast cancer cells to paclitaxel and the low probability of developing resistance to this taxane correspond to the type of substituents at the C3′ and C3′N positions of the taxane molecule. At least one non-aromatic group at these positions means high capability to overcome acquired resistance and low probability to develop resistance to the taxane. On the other hand, the presence of phenyl groups at both C3′and C3′N positions leads to low capability of overcoming acquired resistance and high probability of developing resistance. These findings are related to the function of the ABCB1 transporter which plays a crucial role in acquired resistance to taxanes.

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Author's contributions

MJ carried out some western blot analyses, analyzed dose responses and significantly contributed to manuscript preparation, KB performed siRNAs experiments, PD carried out ATPase assay and helped with manuscript preparation, VNF carried out some western blot analyses, PK realized molecular docking studies, MJ carried out HPLC method, LW and XW were involved in the preparation of taxane derivatives, JV designed molecular docking studies, IO designed taxane derivatives and contributed to manuscript preparation and JK was main coordinator of the research and manuscript preparation. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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6. Nepublikované výsledky a plány další práce

6.1 Vliv taxanů na buněčný cyklus a jeho regulaci v buňkách nádorů prsu

V rámci testování účinku taxanů na zastavení buněčného cyklu jsme ověřili, že taxany u buněk nádoru prsu s původem v mléčné žláze 12 hodin po aplikaci buněčný cyklus kompletně zastavují. Podrobnější průběh dalších procesů jsme určovali detekcí hladin polo-like kinázy 1 (*Plk1*) a Aurora kináz A a B. Hladiny všech testovaných kináz po aplikaci taxanů v důsledku zastavení buněčného cyklu výrazně vzrostly. Je zajímavé, že 36 hodin po aplikaci taxanů prudce klesla hladina Aurory A, a po 48 hodinách i hladiny Aurory B a Plk1. To může naznačovat, že je buněčný cyklus těchto buněk zastaven na více než 20 hodin, a teprve poté se spouští apoptóza ***(I. Bartoňová: nepublikované výsledky)***.

Dále jsme ověřovali, jestli jsou buňky zastaveny opravdu v G2/M fázi buněčného cyklu. Jelikož docházelo 36 hodin po aplikaci taxanů k fosforylaci Chk1 (*check kinase 1*) na serinu 345 a zároveň k mírnému zvýšení hladiny CDC25 (*cell division cycle protein 25*), což jsou dva procesy typické pro buňky v mitóze, je velmi pravděpodobné, že jsou buňky zastavené opravdu na rozhraní G2/M fáze, respektive v nějaké formě aberantní mitózy **(viz 3.2.1)**, ***(I. Bartoňová: nepublikované výsledky)***.

6.2 Další výsledky týkající se molekulárních mechanismů indukce apoptózy v buňkách nádorů prsu taxany

Již dříve bylo popsáno, že taxany indukují stres endoplasmatického retikula a aktivují i další buněčné signální dráhy. My jsme v buňkách nádoru prsu ale aktivaci stresu ER nepozorovali, rovněž sledování aktivace a deaktivace různých kináz po aplikaci taxanů nevedlo k odhalení nějakého trendu v aktivaci/deaktivaci, nebo dokonce úlohy jednotlivých kináz v přežívání buněk nebo v indukci buněčné smrti ***(D. Kopperová a J. Šrámek: nepublikované výsledky)***.

Naše pilotní experimenty však ukázaly, že se taxany v mohou v buňkách účastnit zastavení translace, přesný molekulární mechanismus ale zůstává nejasný. Za zmínku stojí, že aplikace taxanů vedla k mírné aktivaci JNK kinázy ***(D. Kopperová a J. Šrámek: nepublikované výsledky)***, která může podle literatury fosforylovat kaspázu

2, a tak ji aktivovat **(viz 3.2.3.2)**. Další experimenty zaměřené na interakci JNK kinázy a kaspázy 2 by tedy mohly přinést zajímavé výsledky.

6.3 Testování účinnosti taxanových derivátů s různými substituenty v poloze C2

Prokázali jsme, že substituenty na pozicích C3´a C3´N jsou klíčové pro překonání rezistence k taxanům. Pro účinnost taxanů mohou být důležité i substituenty na dalších uhlících, např. na uhlíku C2 **(viz 3.3.4)**. Testovali jsme proto účinek nových taxanových derivátů s modifikacemi právě na uhlíku 2: SB-T-121402, SB-T-121405, SB-T-121406 a další příbuzné deriváty. Tyto deriváty mají k benzenovému jádru na uhlíku 2 přípojenou methylovou skupinu bez fluorů nebo s dvěma či třemi fluory. Naše pilotní studie ukazují, že tyto nové taxanové deriváty překonávají rezistenci k paclitaxelu (jak jsme očekávali), ale že jsou navíc v indukci buněčné smrti účinnější než taxanové deriváty, které modifikace na uhlíku 2 nemají. Poměrně důležitou úlohu v indukci buněčné smrti těmito novými taxanovými deriváty mají fluory připojené k výše zmíněné methylové skupině, v naší další experimentální práci se chceme věnovat objasnění úlohy právě těchto atomů fluoru.

6.4 Úloha autofagie a produkce různých tříd β tubulinu v rezistenci buněk nádorů prsu k taxanům

Kromě výše popsané úlohy ABCB1 transportéru **(viz 4.2)** jsme dále řešili úlohu autofagie a β tubulinu II. a III. třídy **(viz 3.5.3)** v rezistenci nádorových buněk k paclitaxelu.

Aplikace taxanů snižovala v buňkách s původem v mléčné žláze míru autofagie, pravděpodobně v důsledku indukce apoptózy. Naopak v buňkách s původem v duktu docházelo k aktivaci autofagie a dá se předpokládat, že buňky používaly autofagii jako obranný proces k indukci apoptózy taxany (**viz 3.2.1**). Poněkud překvapivě, aplikace induktorů a inhibitorů autofagie nevedla k výrazným změnám rezistence nádorových buněk k paclitaxelu, přesná úloha autofagie v rezistenci k paclitaxelu tedy zůstává dále nejasná ***(A. Kábelová: diplomová práce, 2015)***. Použití aktivátorů a inhibitorů autofagie, zvláště se současnou aplikací taxanů, může mít na buňky značně pleiotropní účinky, případně i řadu vedlejších efektů. V dalších experimentech by bylo zajímavé snížit nebo zvýšit hladinu určitých autofagických

proteinů pomocí specifických siRNA nebo transfekcí plazmidů nesoucích geny pro příslušné proteiny.

U senzitivní buněčné linie s původem v duktu docházelo po aplikaci taxanů ke zvýšení hladiny βII a βIII tubulinu, zatím jsme však neurčili, která z těchto tříd tubulinu je důležitější pro případnou rezistenci k paclitaxelu. Významnou úlohu rozdílné exprese těchto tříd β tubulinu ovšem zpochybňuje fakt, že bez ohledu na hladinu βIII a βII tubulinu není možné v těchto buňkách navodit rezistenci k paclitaxelu ***(A. Kábelová: diplomová práce, 2015, nepublikované výsledky)***. V každém případě by bylo zajímavé pozorovat případné změny rezistence k paclitaxelu po snížení nebo zvýšení hladiny β tubulinu II. a III. třídy.

Aplikace taxanů na buňky s původem v mléčné žláze nezvyšovala hladinu ani jedné testované třídy β tubulinu. Na druhou stranu jsme pozorovali významné rozdíly v produkci, zejména β tubulinu III. třídy, mezi testovanými senzitivními a rezistentními buňkami. Jelikož se nám ale nepodařilo najít žádnou signifikantní korelaci mezi hladinou β tubulinu III. třídy a rezistencí k paclitaxelu, je pravděpodobné, že tento typ β tubulinu v námi testovaných buňkách zásadní úlohu nehraje ***(A. Kábelová: diplomová práce, 2015)***.

7. Závěr

Znalost molekulárních mechanismů indukce apoptózy taxany a resistence nádorových buněk k taxanům je velmi důležitá pro účinnou chemoterapii taxany. V této disertační práci jsme se tedy pokusili přispět k objasnění těchto dvou důležitých otázek.

1) Molekulární mechanismy indukce apoptózy taxany v buňkách lidských nádorů prsu

- Zjistili jsme, že pokud se v buňkách nádoru prsu kaspáza 2 po aplikaci taxanů aktivovala, hrála v indukci apoptózy významnou roli. Na aktivaci kaspázy 2 se v testovaných buňkách nepodílel protein p53, K aktivaci kaspázy 2 také nedocházelo v komplexu PIDDosomu. Je možné, že k aktivaci kaspázy 2 přispívala nepřirozeně dlouhá přítomnost prokaspázy 2 v cytosolu v důsledku rozpadu jaderné obálky během mitotického bloku.

- Po aplikaci taxanů se v buňkách nádorů prsu s původem v mléčné žláze aktivovala kaspáza 2, iniciační kaspázy 8 a 9 i exekuční kaspázy 3 a 7. Kaspáza 8 se však indukce apoptózy přímo neúčastnila a kaspáza 9 se aktivovala pravděpodobně pouze v rámci apoptotické dráhy s nižším významem vedoucí k aktivaci kaspáz 3 a 7. Poněkud nejasná zůstává úloha mitochondriální dráhy indukce apoptózy. Nezdá se však, že by byla nezbytná pro indukci apoptózy taxany. Dle námi navrženého modelu je tedy hlavní dráha indukce apoptózy v buňkách původem ve žláze zahájena aktivací kaspázy 2, která poté aktivuje kaspázu 3 a 7. Současně zde probíhá aktivace kaspázy 9 kaspázou 2 a vzájemná aktivace kaspáz 3 a 7.
- V buňkách s původem v duktu se však významně aktivovala pouze iniciační kaspáza 8 a exekuční kaspázy 7 a 6. Apoptóza je tedy v těchto buňkách indukována nezávisle na kaspázách 2, 3 a 9.

- Pokud jde o proteiny rodiny Bcl-2, tak nejdůležitější úlohu v indukci apoptózy po aplikaci taxanů má pravděpodobně proapoptotický „BH3-only“ protein Bad. Jeho zvýšenou hladinu jsme pozorovali ve všech testovaných liniích. Dále jsme pozorovali relativně časté snížení hladiny antiapoptotického proteinu Bcl-2 a zvýšení hladiny proapoptotického proteinu Bim. U těchto proteinů určitou úlohu v indukci apoptózy předpokládáme. Otázkou zůstává úloha proteinu Bok, jehož přesná fukce v indukci apoptózy není obecně známá. Ostatní proteiny rodiny Bcl-2 se indukce apoptózy u buněk nádorů prsu taxany zřejmě významnějším způsobem neúčastní.

2) Molekulární mechanismy navozené rezistence buněk lidských nádorů prsu k taxanům

- Ukázali jsme, že u buněk nádorů prsu lze navodit rezistenci pouze proti takovým taxanům, které mají v obou pozicích C3´ a C3´N fenylovou skupinu. Pokud je alespoň jeden z fenylů ve zmíněných pozicích nahrazen nearomatickou funkční skupinou, nelze proti takovému taxanu rezistenci navodit. Tyto taxanové deriváty překonávají navozenou rezistenci nádorových buněk k taxanům s dvěma fenyly v pozicích C3´a C3´N.

- Dále jsme zjistili, že navozená rezistence buněk nádoru prsu k taxanům s dvěma fenyly na uhlících C3´a C3´N je úzce spojena s vysokou afinitou těchto derivátů k ABCB1 transportéru (P-glykoproteinu). Taxanové deriváty s vysokou afinitou k ABCB1 jsou totiž účinně transportovány z buněk, což je zřejmě zásadní příčinou rezistence k těmto taxanům. Pokud je jeden ze substituentů ve výše zmíněných pozicích nahrazen nearomatickou skupinou, mají tyto taxanové deriváty nižší afinitu k ABCB1 transportéru, nejsou jím trasportovány ven z buněk a rezistenci k nim tedy v podstatě navodit nelze.

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Diplomové práce:

KÁBELOVÁ, Adéla: Úloha autofagie a vybraných izotypů beta-tubulinu v rezistenci k taxanům u nádorových linií prsu. Praha **2015**. **Diplomová práce.** Universita Karlova. Přírodovědecká fakulta. Katedra buněčné biologie.

9. Publikace nesouvisející s tématem disertační práce

Šrámek J, Němcová-Fürstová V, Balušíková K, Daniel P, Jelínek M, James RF, Kovář J: p38 MAPK is activated but does not play a key role during apoptosis induction by saturated fatty acid in human pancreatic β-cells. Int. J. Mol. Sci. 17(2): 159, 2016.