Charles University
Second Faculty of Medicine

Summary of the Ph.D. Thesis

Epigenetic and Cytotoxic Effects of Histone Deacetylase Inhibitors in Combination with Cytostatics on Neuroblastoma

Epigenetické a cytotoxické účinky inhibitorů histondeacetyláz v kombinaci s cytostatiky na buňky neuroblastomu

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Contents

1. Abstract

The enhanced expression of histone deacetylases (HDACs) in a variety of malignancies drew attention to investigate a new category of anti-cancer drugs that are based on the inhibition of those enzymes. Valproic acid (VPA) is a well-known antiepileptic drug that exhibits antitumor activities through inhibition of HDACs class I and IIa. Cancer stem cells (CSCs) have been recognized to drive the tumor growth and its progression hence; attention has been given to target the small subpopulation of CSCs rather than the whole bulk tumor cells. CD133 is considered to be a CSC marker in several tumors and its transcription is strongly influenced by epigenetic changes that will be altered upon administration of histone deacetylase inhibitors (HDACi) in cancer treatment. Therefore, we evaluated the cytotoxic and epigenetic effects of treatment with 1 mM VPA in combination with other chemotherapeutics and its influence on the expression of CD133 in human neuroblastoma (NB) cell lines.

Our results revealed that addition of VPA to DNA-damaging chemotherapeutics induced a synergistic anti-tumor effects including caspase-3 dependent induction of apoptosis in UKF-NB-4 cells. This synergism was related to the increase of the acetylation status of histones H3 and H4 and was only produced either by simultaneous treatment with both drugs or when the cells were pretreated with DNA-damaging chemotherapeutics before their exposure to VPA. On the other hand, we showed that VPA induced CD133 expression that was also dependent on increased acetylation of histones H3 and H4. On treatment with VPA and cytostatics, CD133+ cells were mainly detected in the proliferative phases of the cell cycle and they showed less activated caspase-3 compared to CD133− cells. UKF-NB-3 cells which express CD133 displayed higher colony and neurosphere formation capacities when treated with VPA, unlike IMR-32 cells which lack the CD133 protein. Induction of CD133 in UKF-NB-3 was associated with increased expression of phosphorylated Akt (p-Akt) and pluripotency transcription factors (Oct4, Sox2 and Nanog). VPA did not induce CD133 expression in cell lines with methylated P1 and and P3 promoters, unless they were pretreated with demethylating agent 5-aza-2'-deoxycytidine. In conclusion, VPA potentiates the cytotoxicity of DNA-damaging chemotherapeutics in NB that is conditioned by the sequence of drugs administrated. CD133 expression in NB can be regulated by histone acetylation and/or methylation of its CpG promoters hence influenced by VPA therapy. VPA can induce CD133+ cells which display high proliferation potential and low sensitivity to cytostatics in NB. Even though these results confirm the potentiating cytotoxic effect of VPA in cancer therapy; they give new insight into a possible limitation to use VPA in some types of cancer which require caution before its use in clinical application.

2. Souhrn

Zvýšená exprese histondeacetyláz (HDAC) u řady nádorů obrátila pozornost k možnosti využít jejich inhibici k protinádorové léčbě. Kyselina valproová (VPA) je nejen užívané antiepileptikum, ale vykazuje i protinádorové účinky podmíněné inhibicí HDAC třídy I a IIa. Předpokládá se, že nádorové kmenové buňky (CSC) jsou odpovědné za růst a progresi nádorů a proto je snaha zacílit léčbu na malou subpopulaci CSC spíše než na celý nádor. CD133 byl rozpoznán jako marker CSC u řady nádorů. Jeho exprese je významně ovlivněna epigenetickými změnami, mezi něž patří podání inhibitorů HDAC užívaných v léčbě nádorů. Proto jsme se zabývali epigenetickými a cytotoxickými účinky VPA v kombinaci s některými chemoterapeutiky a jejím vlivem na expresi antigenu CD133 u lidských neuroblastomových (NB) buněčných linií.

Naše výsledky ukázaly, že kombinace VPA s DNA poškozujícími cytostatiky má u buněk UKF-NB-4 synergistický účinek podmíněný indukcí apoptózy závislé na aktivaci caspázy-3. Tento synergizmus byl ve vztahu ke zvýšení acetylace histonů H3 a H4 a byl přítomen pouze při současné inkubaci s oběmi látkami nebo při inkubaci s DNA poškozujícím cytostatikem následované inkubací s VPA. Výsledky další studie ukazují, že VPA indukuje expresi CD133, která je závislá na zvýšení acetylace histonů H3 a H4. Při inkubaci s VPA a cytostatikem bylo více CD133+ buněk v proliferační fázi a tyto buňky vykazovaly nižší aktivaci caspázy-3 než buňky CD133-. UKF-NB-3 buňky exprimující CD133 vykazovaly vyšší tvorbu kolonií a neurosfér po ovlivnění VPA na rozdíl od buněk IMR-32, které CD133 neexprimovaly. Indukce CD133 u UKF-NB-3 byla provázena fosforylací Akt a expresí transkripčních faktorů typických pro kmenové buňky (Oct4, Sox2 a Nanog). VPA neindukoval expresi CD133 u buněčných linií s metylovanými promotory CD133 P1 a P3, pokud nebyly preinkubovány s demetylačním činidlem 5-aza-2’-deoxycytidinem.

Lze tedy shrnout, že VPA potencuje cytotoxické účinky DNA poškozujících cytostatik u NB, tato potenciace závisí na sekvenci těchto léků. Exprese CD133 u NB je regulována acetylací histonů a/nebo metylací jeho CpG promotorů, tedy může být ovlivněna terapií VPA. VPA u NB může indukovat CD133+ buňky, které mají velký proliferační potenciál a mají nízkou citlivost k cytostatikům. Přestože naše výsledky potvrzují potenciaci cytotoxického účinku některých cytostatik VPA, ukazují i nový pohled na možná omezení jeho použití u některých nádorů.

3. Introduction

Neuroblastoma (NB) is an embryonic tumor of the autonomic nervous system that arises from neural crest cells (NC). It is classified as the most commonly diagnosed tumor in infants and the most common extracranial solid tumor of childhood. Prognosis of high-risk NB tumors is poor and those tumors can be rapidly fatal because drug resistance arises in the majority of patients. To date, there are no salvage treatment regimens known to be curative and 50 to 60% of patients with high-risk NB develop a relapse. High doses of various chemotherapeutic combination that usually lead to toxic effect must be used during treatment of high-risk NB [1].

According to cancer stem cell theory, tumor is not a homogenous population but contains subpopulation of tumor initiating cells so called cancer stem cells (CSCs). This model presumes that CSCs are the main source of recurrence and metastasis due to their unique characters such as resistance to chemo- and radio-therapy. Thereby, targeting CSC can facilitate the mission of chemotherapy to eliminate all the tumor cells and thus decrease the possibility of recurrence [2].

Prominin-1, also known as CD133, is a pentaspan transmembrane glycoprotein that is expressed on neural precursor cells and several brain tumors. CD133+ cells display CSCs features such as self-renewal capacity, high clonogenicity, high proliferation potential *in vitro* and formation of neurospheres in serum free medium [3,4]. Moreover, sorted CD133+ cells displayed greater resistance to chemotherapeutic agents compared to CD133− cells [5]. Therefore, high expression of CD133 has been linked to a poor prognosis in several tumors including NB [6]. Furthermore, it has been recently clarified the functional association of CD133 molecule in the activation of PI3K/Akt pathway that promotes the tumorigenic capacity in glioma stem cells [7]. PI3K/Akt pathway is considered to be one of the most potent pro-survival signaling pathways that is activated in many types of cancer and associated with poor outcome in NB [8].CD133 transcription is regulated by epigenetic modifications such as histone acetylation and promoter methylation [9]. Thus, it is supposed that this correlation between CD133 expression and acetylation status of histones enables the histone deacetylase inhibitors to act as potent regulators of CD133 transcription.

The basic packaging unit of chromatin is the nucleosome which is formed of two copies of each histone H2A, H2B, H3 and H4. Acetylation of histone tail lessens itsoverall positive charges that disrupts the interaction with the negatively charged DNA, allowing access for transcription factors with consequent activation of many genomic regions [10]. Histone acetylation is tightly controlled by the balance between the opposing action of histone acetyl transferases (HATs) and histone deacetylases (HDACs). Enhanced expression of HDACs is linked to a variety of hematological and solid malignancies as well as their poor outcome; hence the rational for targeting HDACs in cancer therapy. Knockdown of selected HDACs in NB cancer cells resulted in cell cycle inhibition, induction of apoptosis and differentiation. Combining HDAC inhibitors (HDACi) with primary chemotherapeutic agents has shown promising results in preclinical research studies [11] as well as in clinical trials [12].

Despite of the fabulous effects of the HDACi in the preclinical studies that showed potent anticancer effects however, their clinical outcomes in solid tumors are disappointing when used as monotherapy [13]. It is not entirely clear this diversity in the response to HDACi that can be due to the lack of selectivity. Although, the broad spectrum inhibitors of HDACs may reactivate some tumor suppressors, they can also affect numerous other genes, for instance; induction of pluripotency in the cancer cells [14].

Valproic acid (VPA) is a branched short-chain fatty acid that has been successfully used for the treatment of different types of epilepsy. During the last few years, VPA has been under the spot light as a drug can be used in cancer therapy. This interest in VPA was based on its anti-cancer effect due to inhibition of HDACs class I and Class IIa with bearing in mind that it is already a registered drug and has been used safely for long term treatment of epilepsy [15]. In addition, it can cross the blood brain barrier and apply its action on brain tumors due to its lipid nature [16]. VPA as well as other HDACi have anti-proliferative effect and can induce apoptosis in cancer cells by different mechanisms such as upregulation of proapoptotic proteins as well as increase the molecules of the extrinsic apoptotic pathway. It also has anti-angiogenic effects and can induce tumor differentiation [17]. However, the exact anticancer mechanism of VPA is still unclear and it exhibits different effects in various tumors. For instance, it did not induce cell cycle inhibition in some NB cell lines such as SH-SY5Y and SK-N-BE [18]. Moreover, the expression of the pluripotency transcription factor *Fgf4* decreased in F9 embryonal carcinoma cell line after treatment with VPA while elevated in P19 cells [19]. Collectively, these remarks lead to suggest that the anticancer effect of VPA may be cancer type specific and dose dependent [20]. On the other hand, the growing assumption about the role of HDAC inhibitors as potential candidates for inducing the pluripotent stem cells has been confirmed in some studies. For example, the significant effect of VPA on amplification and maintenance of human hematopoietic stem cells [21], enhancement of the EMT of colorectal cancer cells [22] and induction of CD133 in human glioma [23] have been reported in different studies. These previous results raise a question whether treatment with VPA may amplify cancer cells with stem cell features such as CD133+ cells.

Recently, the generation of induced pluripotent stem cells (iPSCs) from differentiated adult cells was successfully achieved by the transduction of four basic transcription genes such as *Oct4, Sox2, Nanog*, and *Lin28* [24]. Currently, mouse iPSCs can be generated by reprogramming of a single gene such as Oct4 [25]. In fact, epigenetic changes including histone modification has been shown to play a valuable role in regulating both stem cell self-renewal and pluripotency. Surprisingly, among HDAC inhibitors and all other epigenetic modifiers, VPA exhibited the most potent effect on induction of iPSCs and enhanced the reprogramming efficiency more than 100-fold [26].

The generation of iPSC cannot be performed with the absence of the transcription factor Oct4 which has often been used as a marker of stemness and is highly expressed in side population cells of NB [27]. It is the core regulatory gene maintaining a multipotent state (self-renewal) of neural stem cells. Additionally, the differentiated cells show reduced or absence expression of this marker. It has been reported that over expression of *Oct4* is associated with poor prognoses in NB and promotes aggressiveness of *MYCN*-amplified NB cells. Furthermore, Oct4-positive NB cells has been shown to be resistant to conventional chemotherapy [28]

HDACis seem to be a promising group of anti-cancer drugs, particularly in combination with chemotherapy and/or radiotherapy. The important question is which will confer more efficient results in cancer treatment, using the pan-HDACi or the selective HDACis. Furthermore, assumptions about the role of some HDAC inhibitors particularly VPA as cancer stem cells inducers [29] or about the phenomenon that HDAC inhibition may enhance the EMT of cancer cells need to be further explored.

4. Aim of the study

The main aim of the presented thesis is to analyze the synergistic effect as well as the possible limitations of treatment high risk NB cells with HDACi along with commonly used cytostatics. In this work, we could significantly explore and discuss the following issues:

- Cytotoxic effect of low dose of VPA on NB cell lines.
- Cytotoxic effect of low dose of VPA in combination with different cytostatics on NB cell lines.
- Effect of epigenetic alterations on expression of CD133
- Effect of VPA on the expression of CD133
- Effect of CD133 induction on activation of Akt pathway.
- Effect of induction of VPA on the pluripotent transcription factors
- Distribution of the CD133 in the cell.

5. Materials and methods

A variety of experimental methods were used in the thesis work. These methods are described in chapter materials and methods, page 22, in the original thesis. The majority of experiments were carried out utilizing the flow cytometry and western blot analysis. NB cell lines were used as model to study cytotoxic and epigenetic effects of VPA combined with different anticancer drugs. Here, some of the basic methods and difficulties are further explained.

5.1 **Measurement of apoptosis:** Different methods were used to assess apoptosis depending on the experiment design. Annexin V / propidium iodide (PI) labeling and detection of active caspase-3 were basically used for measuring apoptosis by flow cytometry. PI was not suitable to detect apoptosis in CD133 population because both PI and anti-human CD133/2 PE-conjugated primary antibody that we used have very close emmition values and their co-staining would interfere with the integrity of the results. So, we used cleaved caspase-3 Alexa Fluor® 647 conjugated antibodies as a indicator for apoptosisin CD133− and CD133+ populations.

5.2 Measurement of cell cycle: PI is a fluorescent DNA stain that can't cross the intact plasma membrane. Cell cycle was measured by permeabilization of cell membrane followed by nuclei staining with PI. 4',6-diamidino-2-phenylindole (DAPI) stain is also a fluorescent DNA intercalating agent that we used in combination with CD133 antibodies (PI couldn't be used for the previously mentioned reason) to assess the cell cycle of CD133− and CD133+ populations.

5.3 Measurement of CD133: We detected both surface CD133 by using the flow cytometry and total CD133 protein by western blotting. Immunofluorescence staining of CD133 was done for visualization of the surface CD133 while confocal microscopy was used for visualization of the intracytoplasmic CD133 deposition.

5.4 Isolation of histones: Histones are among the most basic proteins in the nucleus that allowing them to be extracted in ice-cold sulfuric acid. We used the methods described by Shechter*et al* [30] that is based on acid extraction followed by precipitation of histones using trichloroacetic acid. This method is simple and provides high purity for the extracted histones.

5.5 Real-time monitoring of cell viability by xCELLigence system: A recent technique is used to monitor the proliferation of the cells. The presence of adherent cells at the electrode-solution interface impedes electrons flow across gold microelectrodes fused to the bottom surface of a micro-plate well. The electrical impedance represents the readout and expressed as cell index.

5.5 Methylation sensitive-high resolution melting analysis: Detection of methylation status of CD133 promoters was performed on two steps. First is *"bisulfite conversion"* where whole genomic DNA was treated with sodium bisulfite using an Epitect Bisulfite Kit *(Qiagen)* to convert unmethylated cytosine to uracil. Second step is *"methylation-sensitive high resolution melting (MS-HRM)"* where the melt profiles of experimental samples are compared to profiles from DNA with known methylation levels using real time PCR.

5.6 Assessment of cologenecity and neurosphere formation capacity: Are used to calculate the frequency of progenitors or stem cells. The ability of single cell to make a colony after using specific agents, whether cells grow attached to culture plate forming colonies or floating in serum free culture forming neurospheres is an indicator of the self-renewal activity of CSCs.

6. Results and discussion

The results found in this study demonstrate that VPA used at the clinically relevant dose (1mM) has synergistic effect on the cytotoxicity of DNA-damaging drugs cisplatin (CDDP) and etoposide with activation of caspase-3-mediated induction of apoptosis. However, no sensitizing effect of VPA was produced in compination with the mitotic inhibitor vincristine (VCR). These results indicate that HDAC inhibitor-mediated capability of increasing cytotoxic efficiency of anticancer drugs may be connected with the chemotherapeutics that target cellular DNA [31]. In the second section of the thesis, we studied the effect of VPA on expression of the stem cell marker "CD133" in NB cell lines. We also gave overview about the variability of CD133 protein expression in relation to the acetylation status of histones and methylation status of CD133 promoters.

6.1 <u>Cytotoxic effect of 1mM VPA on NB cell lines.</u>

The cytotoxic effect of 1 mM VPA on NB cell lines was confined to cell cycle arrest with minimal induction of apoptosis that did not exceed 5% at maximum (i.e. IMR-32). Even though VPA noticeably decreased the proliferation index of NB cell lines, it shows irrelevant effect on the proliferation index of SH-SY5Y cell line which highly expresses CD133.

Table 1: IC_{50}, apoptosis, and proliferation index of NB cell lines after treatment with 1 mM VPA for 72 hrs.

Cell line	IC_{50}VPA mM	Apoptosis Annexin V / PI labeling		Proliferation index (G_2M + S) / (G_0G_1 + S + G_2M)	
		Control (%)	VPA (%)	Control	VPA
UKF-NB-3	1.27 ± 0.46	2.27 ± 0.49	4.86 ± 1.74**	0.50 ± 0.05	0.43 ± 0.02*
IMR-32	1.30 ± 0.69	2.08 ± 0.26	7.02 ± 0.88**	0.55 ± 0.01	0.43 ± 0.01**
UKF-NB-4	2.00 ± 0.60	0.61 ± 0.15	4.72 ± 0.48**	0.64 ± 0.02	0.58 ± 0.01**
SH-SY5Y	9.00 ± 1.44	3.84 ± 1.02	7.38 ± 0.78**	0.35 ± 0.04	0.34 ± 0.03^{Ns}

Ns- Non-significant
* $p < 0.05$
** $p < 0.01$
paired t-test

6.2 <u>Cytotoxic effect of 1 mM VPA in combination with DNA-damaging conventional chemotherapy</u>

On the other hand, we found that addition of the same concentration of VPA (1mM) significantly potentiats the cytotoxicity of DNA damaging chemotherapeutics (CDDP and etoposide but not VCR) on UKF-NB-4 human NB cells whether those drugs were used in high or low concentrations. This potentiating effect was associated with increased the acetylation status of histones H3 and H4. It was also conditioned by the sequence of drugs administrated. Our results showed that the optimal regimens for combination of cisplatin with HDACi (VPA) were administeration of both drugs simultaneously or treating cells with DNA damaging chemotherapeutics prior to VPA. These findings suggest that DNA damage is crucial for the synergistic effect of VPA. Recently, our group has shown that co-treatment of cells with ellipticine and VPA increased the formation of ellipticine-derived DNA adducts, which indicates an easier accessibility of ellipticine to DNA when co-treated with VPA [32]. Similarly, we suggest that changes in the structure of DNA caused by CDDP or etoposide (i.e., formation of DNA adducts or DNA cross-links by cisplatin and intercalation of etoposide into DNA) [34–35] can increase accessibility of nucleosomal core histones for acetylation by VPA, which additionally determines transcription of genes involved in DNA repair or apoptosis. Surprisingly, evaluation of etoposide-mediated H2AX phosphorylation showed no further enhancement of its expression after addition of VPA. This indicates that other mechanisms can be responsible for this synergistic effect of VPA on induction of cell death in UKF-NB-4 rather than enhancement of double-strand DNA breaks caused by etoposide (due to inhibition of topoisomerase-II activity). This suggestion however needs to be further investigated. In figure 1, combination of cisplatin with VPA led to a higher elimination of UKF-NB-4 cells in contrast to cells cultured with CDDP alone.

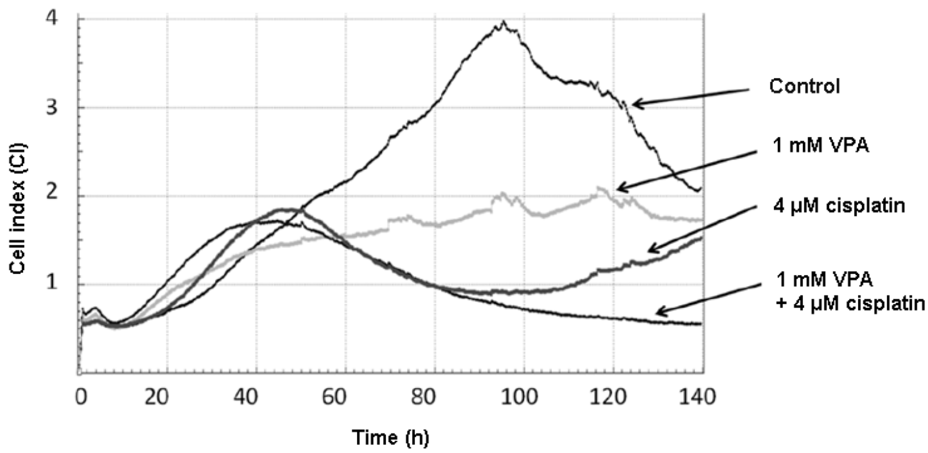

Figure 1. Viability of neuroblastoma UKF-NB-4 cells treated with 1 mM VPA, 4 μM cisplatin, or with both drugs. Viability was measured by the xCELLigence system for real-time label-free monitoring of cells and expressed as cell index. Representative data from one of three independent experiments are shown. During cell cultivation, cell index is changing, combined cultivation of cells with VPA and CDDP decreased cell index. A similar trend was observed by cultivation of cells with CDDP alone, but after approximately 100 h of cultivation, cell index started to rise again.

6.3 <u>Expression of CSC marker CD133 is regulated by epigenetic changes in neuroblastoma</u>

a. **CD133 expression is regulated by acetylation of histones H3 and H4:** Our results offer evidence for induction and maintenance of CD133+ cells in NB cell lines through inhibition of HDAC by VPA. We relate this effect to its action on increasing the acetylation of histones which leads to chromatin accessibility and consequent transcription of many genes including CD133. The following results are confirming this suggestion:

- **Effect of VPA on the expression of CD133:** VPA increased the expression of CD133 protein as detected by western blot and the number of CD133+ cells detected by flow cytometry. The increase of CD133 expression was only seen in UKF-NB-3 and SH-SY5Y while CD133 protein was not detected in UKF-NB-4 or in IMR-32 cell lines where CD133 promoters are highly methylated.
- **Effect of various histone deacetylase inhibitors on expression of CD133:** Different classes of HDAC inhibitors increased the expression of CD133 in variable degrees that were in positive correlation with the status of H3 and H4 acetylation. The maximum acetylation we noticed was induced by entinostat and was associated with highest expression of CD133 (Figure 2). Similarly, the low dose of TSA that we tested did not seem to affect the acetylation of histones H3 and H4 and was not associated with increased expression of CD133. This suggests the concomitant relation between CD133 expression and acetylation of histones H3 and H4 induced by HDAC inhibitors.

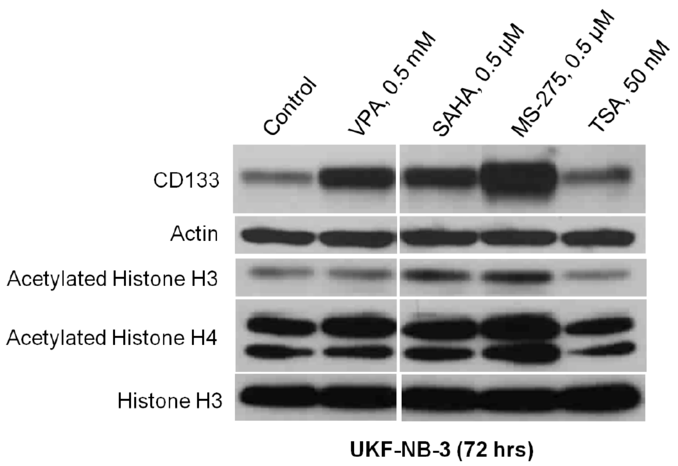

Figure 2: Changes of CD133 expression and histones H3 and H4 acetylation in UKF-NB-3 after cultivation with different classes of HDAC inhibitors. CD133 expression correlated with the changes of histones H3 and H4 acetylation when cultured with different HDAC inhibitors.

- **Effect of valpromide (VPM) on expression of CD133:** Treatment of UKF-NB-3 cells with different concentrations of VPM (1 mM, 2 mM and 3 mM) for 72 hrs showed no effect on the expression of CD133 as well as on the acetylation status of histones H3 and H4 at any of the tested concentrations (Figure 3). VPM is VPA analogue that doesn't affect acetylation of histones.
- **Effect of high dose of cytostatics on CD133 expression:** Interestingly, we noticed down-regulation of CD133 when treated with high doses of VCR or CDDP that was associated with low acetylation of histones H3 and H4 (Figure 3). The low expression of CD133 should not be interpreted to mean that CD133 permanently disappeared, because the same cells were able to significantly express CD133 when combined with VPA. The point to highlight here is that VPA had the potential to turn cells that were detected as CD133− using flow cytometry into CD133+. This variability in CD133 expression is under control of the epigenetic modification which could explain why sorted CD133− cells can acquire the CD133 surface marker or CD133+ cells can lose it in some tumors [36].

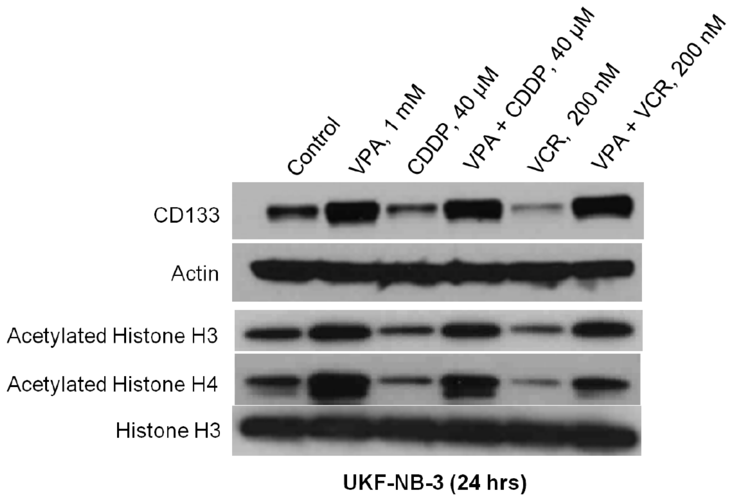

Figure 3: Changes of CD133 expression and histones H3 and H4 acetylation in UKF-NB-3 after cultivation with high doses of cytostatics. CD133 expression correlated with the changes of histones H3 and H4 acetylation when cultured with high doses of CDDP or VCR and in combination with VPA for 24 hrs.

b. **CD133 expression is regulated by methylation of promoter P1 and P3:** We also showed that CD133 could be controlled by methylation of its CpG promoters that can silence the expression of CD133 protein.

- **Methylation status of CD133 promoters P1 and P3 was correlated with CD133 expression:** Cell lines with highly methylated promoters (UKF-NB-4 and IMR-32) did not express CD133 protein, while those with low methylation (UKF-NB-3 and SH-SY5Y) expressed CD133 as detected by western blot and flow cytometry.

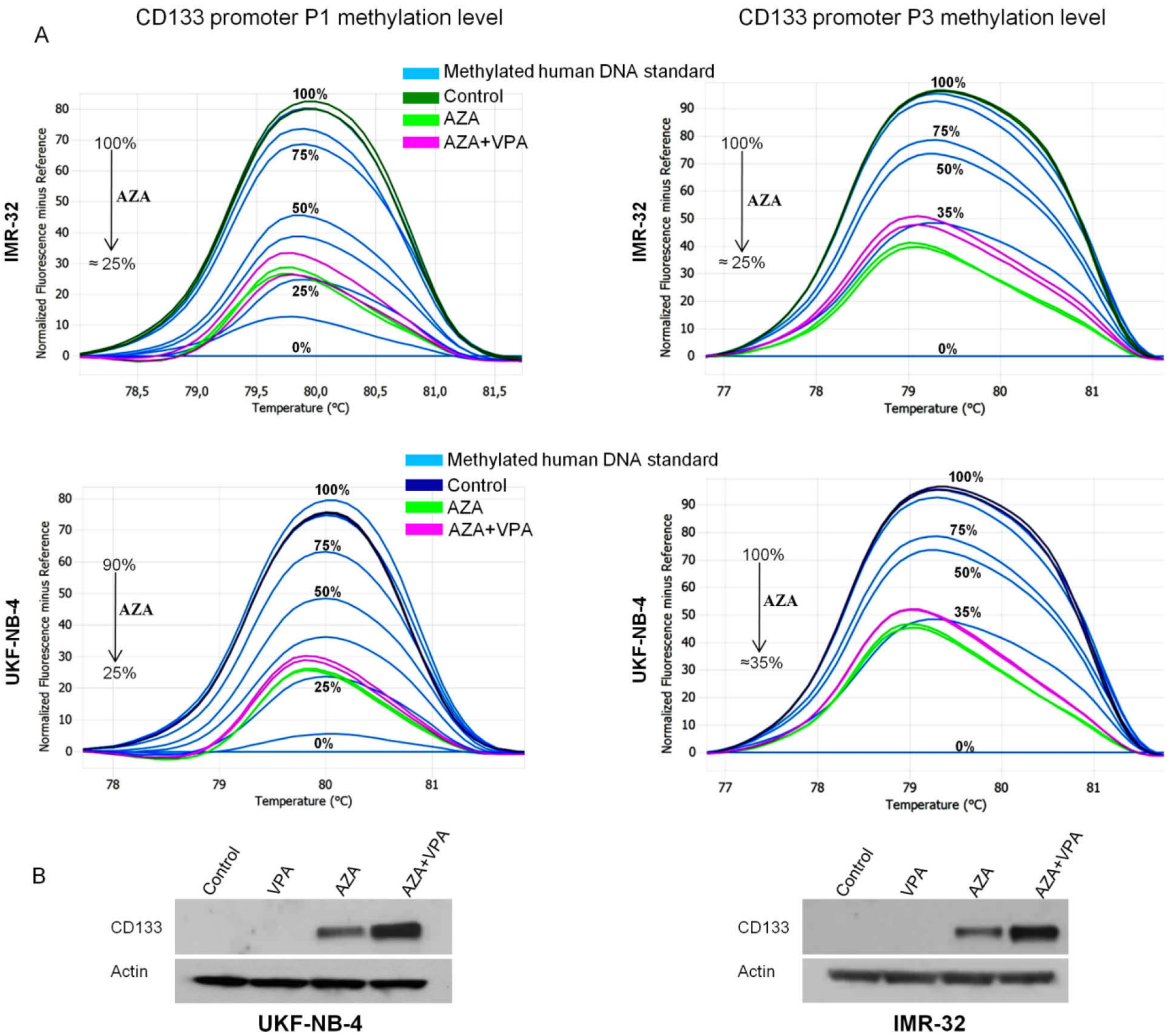

Figure 4: Normalized melt curves showing methylation status of promoters P1 and P3 in UKF-NB-4 and IMR-32 before and after using AZA and in combination with VPA. Western blot is showing the effect of 1mM VPA and AZA on CD133 expression in methylated cell lines. (A) Treatment with AZA decreased the methylation of both promoters P1 and P3 in UKF-NB-4 (P1 from 90% to 25% and P3 from 100%.to 35%) and IMR-32 (P1 from 100% to 25% and P3 from 100%.to < 35%). **(B)** CD133 was not detected in cell lines with methylated promoters (UKF-NB-4, IMR-32). Re-expression of CD133 was seen in a small amount after applying AZA and in high amount when AZA was combined with VPA. Treatment with VPA alone did not induce CD133 protein in UKF-NB-4 and IMR-32.

- **Silenced CD133 was re-expressed after treatment with demethylating agent 5-aza-2'-deoxycytidine:** Cell lines that showed no expression of CD133, even after incubation with VPA (UKF-NB-4 and IMR-32), were treated with the demethylating agent AZA. We found that CD133 protein was restored again in a small amount after AZA treatment and in a larger amount when AZA was combined with VPA. The re-expression of CD133 was associated with a drop in promoters P1 and P3 methylation levels (Figure 4). Thus, our work is in agreement with Shmelkov et al., who stated that promoter methylation may play a key role in CD133 regulation [37].

6.4 <u>CD133+ neuroblastoma cells induced by VPA were sharing features with CSCs:</u>

We used the cytometry to examine the cell cycle and apoptosis-sensitive cells of both CD133+ and CD133− populations within the pool of the cultured cells

a. **Resistance to chemotherapeutic agents:** Caspase-3 is the effector caspase that cleaves some cellular proteins including caspase-activated DNAase, which causes DNA fragmentation that is characteristic for apoptosis [38]. Therefore, low level of cleaved caspase-3 we detected in CD133+ compared to CD133− cells in UKF-NB-3 cell line when treated with VPA and cytostatics, indicates resistance to apoptosis (Figure 5). Interestingly, we detected a high level of p-Akt (active form) in UKF-NB-3 and SH-SY5Y in contrast to IMR-32 and UKF-NB-4 which lack for CD133 protein and showed low level of activated Akt (Figure 6). The level of activated Akt in UKF-NB-3 was enhanced by VPA therapy and repressed by high dose of VCR that goes along with the changes in the CD133 expression in both conditions. Our results are supported by previous studies revealed that the phosphorylated Y828 residue in CD133 cytoplasmic tail binds to PI3K regulatory subunit p85 which results in the activation of PI3K/Akt pathway, subsequently promoting the self-renewal and tumor formation of glioma stem cells [39]. In addition, the activation of Akt is proposed to trigger pro-survival signaling via Akt mediated cell cycle arrest and anti-apoptotic mechanisms leading to chemotherapeutic resistance [40]. Altogether, we supposed that cancer cells expressing CD133 may get benefits from activation of Akt signaling during VPA therapy such as resisting apoptosis which manifested by low activated caspase-3 in our study.

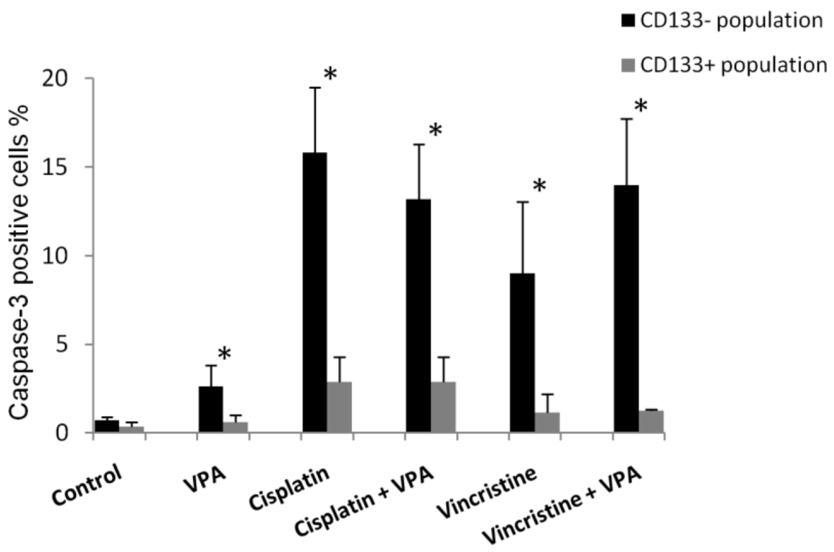

Figure 6: Activated caspase-3 in CD133– versus CD133+ populations after treatment with VPA combined therapy for 72 hrs. The graph shows that mean percentage of cells with cleaved caspase-3 was significantly higher in CD133− than in CD133+ cells in all tested samples. Hence, activated caspase-3 was higher in CD133− cells compared to CD133+ cells. *$p < 0.05$— paired t-test.

b. **High proliferation rate:** We demonstrated that CD133+ cells are mainly present in the S/ G_2M phases, while CD133− cells largely reside in G_0/G_1 either in the control or in samples treated with VPA or cytostatics which agree with other published results [41]. Collectively, we suggest that CD133+ cells form a characteristic population in tumor and represent a significant proliferative fraction in NB cell lines.

c. **Increase the colony formation and neurosphere capacities of tumor cells after VPA treatment:** Pretreatment cells with VPA significantly enhanced colony formation capacity in the N-type NB cell line UKF-NB-3 but not IMR-32 which lacks the CD133 protein. We suggest that pretreatment with a low dose of VPA can enhance colony formation capacity of susceptible tumors, which may be related to the increase in CD133+ cells. Additionally, it has been reported the role of CD133 in cell adhesion [42] which greatly can affect the colony formation in N-type with low attachment characters rather than the S-type with high attachment ability. Therefore, we examined the capacity of neurospheres formation which can be more reliable test for the self-renewal activity *in vitro*. We found that VPA significantly increased the number of formed neurospheres in UKF-NB-3 but not in IMR-32, whereas the S-type neuroblastoma consistently formed adherent colonies.

d. VPA induced pluripotent transcriptional factors Oct4 and Sox2 in cell lines expressing CD133: This action of VPA can be related to the effect of HDAC inhibitors in cell reprogramming and induction of pluripotent cells [20]. In fact, we detected variable expression of pluripotent transcriptional factors Oct4, Sox2 and Nanog in CD133 expressing cell lines and they were obviously induced by VPA treatment. Although Oct4 and Nanog were also expressed in NB cell lines with MYCN amplification (IMR-32 and UKF-NB-4), these cell lines did not show evidence of Sox2 protein expression which was only detected in CD133 expressing cell lines. A previous reports have demonstrated that Oct4 and Sox2 orchestrate together to maintain the self-renewal and pluripotency of embryonic and neural stem cells [43]. Based on previous remarks, combined induction of these transcription factors together with CD133 expression after VPA treatment might suggest induction of pluripotency.

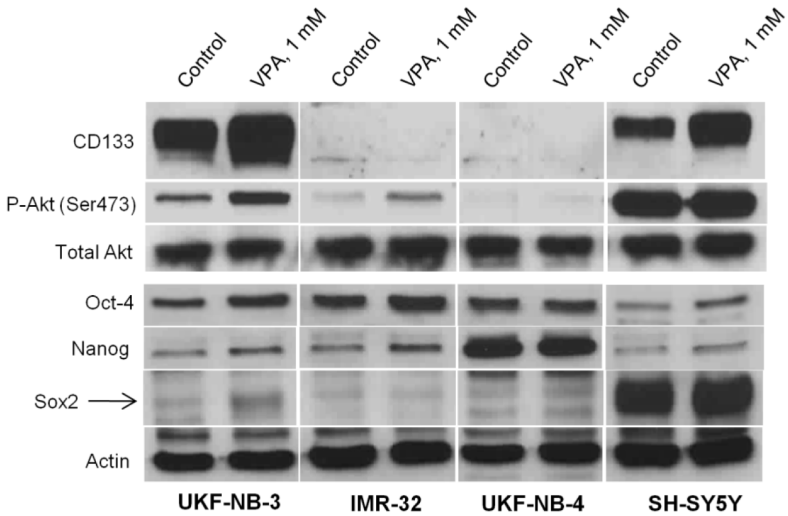

Figure 6: Expression of CD133, p-Akt, Oct4, Nanog and Sox2 in NB cell lines after treatment with 1 mM VPA. 72 hrs cultivation with VPA induced CD133 overexpression only in UKF-NB-3 and SH-SY5Y cell lines. p-Akt was highly expressed in CD133-expressing cell lines. Phophorylation of Akt was increased with VPA treatment in UKF-NB-3, whereas its expression was extremely high in SH-SY5Y whether in the control or in VPA treated sample. Oct4 was mainly expressed in MYCN amplified cell lines (UKF-NB-3, IMR-32 and UKF-NB-4) and was enhanced after treatment with VPA in UKF-NB-3, IMR-32 and SH-SY5Y. Nanog expression was detected in low amount that was enhanced by VPA in UKF-NB-3, IMR-32 and SH-SY5Y, while it was highly expressed in UKF-NB-4. Sox2 was slightly expressed in UKF-NB-3 and was induced by VPA while it was over expressed in SH-SY5Y independent of VPA effect.

7 Conclusion:

In our study, we found that addition of VPA to cancer chemotherapeutics could synergize the cytotoxicity of DNA-damaging chemotherapeutics effectively in UKF-NB4 cells. This synergism was related to increase the acetylation status of histones H3 and H4 and was significant either by simultaneous treatment with both drugs or by pretreatment of cells with DNA-damaging chemotherapeutics before their exposure to VPA.

On the other hand, we found that CD133 expression in NB can be regulated by histones acetylation and/or methylation of its CpG promoters. Thus, we showed that the HDAC inhibitors can increase CD133 significantly in NB cell lines that show low methylated CpG promoters.

We also found that VPA treatment can increase the number of CD133+ cells that show higher resistance to cytostatics than CD133– cells. VPA treatment enhanced the ability of CD133 expressing cell line UKF-NB-3 to generate more colonies and neurospheres, induce Akt phosphorylation, induce expression of the pluripotency transcriptional factors which collectively may lead to induce chemoresistance and preserve tumor growth. Amplification of cancer stem like cells might be unwanted action of VPA during cancer treatment as it antagonizes the idea of cancer stem cell theory that gives attention to target the CSCs rather than the more differentiated tumor cells.

No doubt that addition of high doses of VPA to conventional chemotherapy has revealed a synergistic effect on cell cycle inhibition and apoptosis induction in tumor cells but we have to keep in mind that VPA dose is limited by its toxicity and has different anti-cancer effects depending on tumor cell biology. Therefore, it is of great value to determine the type of tumors that can be beneficial from VPA therapy.

Finally, further investigations are needed for evaluation of epigenetic therapy (HDAC inhibitors and demethylating agents) on induction of CSCs in tumors prior to their use in clinical medicine. In addition, Selective HDAC inhibitors should be designed to overcome the unwanted effects of pan HDAC inhibitors to be efficient in different types of cancers.

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Publications related to thesis:

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