Valproic acid treatment response in vitro is determined by TP53 status in medulloblastoma

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Abstract
Purpose Histone deacetylase inhibitors (HDACi), as valproic acid (VA), have been reported to enhance efficacy and to prevent drug resistance in some tumors, including medulloblastoma (MB). In the present study, we investigated VA role, combined to cisplatin (CDDP) in cell viability and gene expression of MB cell lines.
Methods Dose-response curve determined IC₅₀ values for each treatment: (1) VA single, (2) CDDP single, and (3) VA and CDDP combined. Cytotoxicity and flow cytometry evaluated cell viability after exposure to treatments. Quantitative PCR evaluated gene expression levels of AKT, CTNNB1, GLI1, KDM6A, KDM6B, NOTCH2, PTCH1, and TERT, before and after treatment. Besides, we performed next-generation sequencing (NGS) for PTCH1, TERT, and TP53 genes.
Results The most effective treatment to reduce viability was combined for D283MED and ONS-76; and CDDP single for DAOY cells (p < 0.0001). TERT, GLI1, and AKT genes were overexpressed after treatments with VA. D283MED and ONS-76 cells presented variants in TERT and PTCH1, respectively and DAOY cell line presented a TP53 mutation.
Conclusions MB tumors belonging to SHH molecular subgroup, with TP53MUT, would be the ones that present high risk in relation to VA use during the treatment, while TP53WT MBs can benefit from VA therapy, both SHH and groups 3 and 4. Our study shows a new perspective about VA action in medulloblastoma cells, raising the possibility that VA may act in different patterns. According to the genetic background of MB cell, VA can stimulate cell cycle arrest and apoptosis or induce resistance to treatment via signaling pathways activation.

Keywords Medulloblastoma · HDACi · NGS · Gene expression · TP53

Introduction

Medulloblastoma (MB) is a primitive neuroepithelial tumor originating from the hindbrain and represents one of the leading causes of pediatric tumor-related death. MB is thought to arise from neural stem cell precursors in the granular cell layer of the cerebellum, and the incidence of MB in patients up to 19 years old ranges from 0.49 in girls to 0.75 in boys, per
100,000 patients-years, accounting for 16% of all pediatric brain tumors [1]. Despite current radical treatment, MB is associated to 30% of lethality. Survivors usually develop severe neurological side effects, underscoring the importance of developing alternative therapeutic strategies [2]. The 2007 WHO classification of CNS tumors recognized four MB histological subtypes: classic, desmoplastic/nodular, extensive nodularity (MBEN), and large cell anaplastic (LCA) [3]. In 2012, four molecular subgroups of MB were identified: WNT-activated, SHH-activated, and the numerically designated “group 3” and “group 4” [4]. A new risk classification was proposed in a consensus, in 2016, based in MB molecular subgroups and TP53 status [5].

Standard treatment for MB includes surgery, chemotherapy, and depending on the age of the patient, radiation therapy. Chemotherapy in addition to radiotherapy treatment has increased survival rates and allowed lowering of radiotherapy doses for standard risk patients [6, 7]. Cisplatin, vincristine, carboplatin, etoposide, and cyclophosphamide are used in various combinations, in most drug regimens for MB [8]. Also, synergistic drugs are used to enhance efficacy and to prevent drug resistance [9].

Histone deacetylase inhibitors (HDACi) are considered a promising cancer therapeutics, and are a key component of the epigenetic machinery, regulating gene expression though increased histone acetylation, and behaving as oncogenes in some cancer types. The first HDACi were initially characterized for their antitumor activity in vitro before the discovery that they were known to inhibit HDAC [10, 11]. Within HDACi, valproic acid (VA) inhibits both class I and II HDACs with resultant hyperacetylation of histone H3 and H4 [12]. VA is a short branched–chain carboxylic acid, FDA approved anti-seizure and antidepressant drug, a well-tolerated anticonvulsant, with several established properties, an extensively characterized toxicity profile and well-defined pharmacokinetic properties [12].

Functional studies have confirmed that HDACi and histone hyperacetylation are essential for VA antitumor activity in vitro before the discovery that they were known to inhibit HDAC [10, 11]. Within HDACi, valproic acid (VA) inhibits both class I and II HDACs with resultant hyperacetylation of histone H3 and H4 [12]. VA is a short branched–chain carboxylic acid, FDA approved anti-seizure and antidepressant drug, a well-tolerated anticonvulsant, with several established properties, an extensively characterized toxicity profile and well-defined pharmacokinetic properties [12].

Functional studies have confirmed that HDACi and histone hyperacetylation are essential for VA antitumor activity, once VA inhibits growth of human cancers [12–14], including pediatric solid tumors [15, 16]. Previous studies have reported that VA efficiently prevented MB growth, cell cycle arrest, apoptosis, senescence and differentiation, and suppressing colony forming [17–19]. Additionally, VA significantly inhibited the in vivo growth of DAOY and D283-MED cell lines [20], and presented radiosensitizing capacity, suggesting an adjuvant administration to improve MB therapy [21]. Previous studies have also provided evidences that VA sensitization works in conjunction with chemotherapy inducing apoptosis in MB cells [11].

Improving MB therapy would rely on a rational approach considering consecutive in vitro studies, followed by in vivo experiments [22]. In vitro studies, using long-established continuous cell lines have the advantages of being easy to expand and relatively uniform [23]. In the present study, we evaluated the role of VA, combined to cisplatin (CDDP), in cytotoxicity, viability, and gene expression profile in three well-established MB cell lines (DAOY, D283MED, and ONS-76). Additionally, we investigated PTCH1, TERT, and TP53 genes trough next-generation sequencing (NGS).

**Material and methods**

**Cell lines selection and culture**

For the present study, we used three well-characterized MB cell lines: DAOY (ATCC® HTB-186™), D283MED (ATCC® HTB-185™), and ONS-76 (JCRB® IFO50335). DAOY MB cell line was grown and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Cultilab®) with 10% fetal bovine serum (FBS, Cultilab®). D283MED cell line was grown in Minimum Essential Medium (MEM, GibcoTM, Thermo Fisher Scientific®), adding 2.2 g of NaHCO3 and 2.5 g of HEPES for each 1000 mL of medium. ONS-76 cells were grown in RPMI-1640 Medium (Cultilab®), containing 10% of FBS. Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO2 in air.

**DNA and RNA extraction, and cDNA synthesis**

DNA and RNA were extracted from untreated cell lines, and treated cells, submitted to the three groups of treatment: (1) VA single, (2) CDDP single, and (3) VA and CDDP combined, after 24, 48, and 72 h. For DNA and RNA extraction, we used NucleoSpin TriPrep Kit (Macherey-Nagel®). DNA was quantified in Qubit™ (ThermoFisher Scientific®), and RNA was quantified in NanoDrop™ (ThermoFischer Scientific®). cDNA was synthetized from 1 μg of RNA, using SuperScript Vilo™, following manufacture’s manual.

**Cell lines authentication**

Cell lines were authenticated using STR (short tandem repeat) profiling analysis, as published in ANSI/ATCC ASN-0002-2011 normative, which determines that eight loci are enough to authenticate a cell line, with correspondence > 80% [24]. For this genotyping, we utilized GlobalFiler® PCR Amplification Kit (Applied Biosystems™/Thermo Fisher Scientific®), in ABI PRISM 3500 Genetic Analyzer (Life Technologies™/Thermo Fisher Scientific®). Results were analyzed in GeneMapper ID-X software (Life TechnologiesTM/Thermo Fisher Scientific®). Correspondence index (CI) was calculated as suggested by Cosme et al. 2017 [25], and genotype profile was compared to ATCC STR database (www.atcc.org/str; last accessed February 15, 2017).
**Fluorescence in situ hybridization**

Fluorescence in situ hybridization (FISH) was performed in DAOY, D283MED, and ONS-76 methanol acetic acid-fixed cells using Vysis LSI N-MYC (2p24) SpectrumGreen/Vysis CEP 2 SpectrumOrange Probe (Abbott®). FISH procedure was performed according to manufacturer’s instructions. Slides were visualized by fluorescence microscopy, and images were captured using the ISIS software (Zeiss, Jena, Germany).

**Genomic variants investigation**

We performed the investigation of MB molecular subgroups-related genes for DAOY, D283MED, and ONS-76 cell lines. For this investigation, we developed an AmpliSeq® custom panel, for Ion Torrent PGM® (ThermoScientific®) next-generation sequencer (NGS), comprising the complete genomic regions (CDS) of CTNNB1, PTCH1, DDX3X, and TP53 genes, and the CDS + UTR region for TERT gene. DNA from untreated cell lines was submitted to Ion PGM® library preparation. The resulting template was submitted to a micelles clonal amplification, using Ion PGM® Hi-Q® OT2 kit (Thermo Fisher Scientific®) in Ion OneTouch 2 machine (ThermoFisher Scientific®). Then, the template was enriched and applied to the 316 Ion Chip (Thermo Fisher Scientific®). The chip was inserted into the Ion Torrent PGM™ sequencer (Thermo Fisher Scientific®) to start sequencing.

**Dose-response curves**

Dose-response curve was used to determine the potential of VA and CDDP in promoting MB cells death. Treatments were distributed in three groups, during 24, 48, and 72 h: (1) VA single, (2) CDDP single, and (3) 24 h pretreatment with VA, and after 24 h, addition of CDDP (combined treatment). Untreated cells were the control for the assays. We plated 10^4 cells in a 96-wells plate. After 24 h, drugs were added individually or in combination at a serial dilution (0.078–200 mM). Every 24 h, cells were incubated for 2 h with 10 μl of the PrestoBlue™ cell viability reagent (Invitrogen, Carlsbad, CA) and then analyzed on the ELISA reader M3 SpectraMax (Molecular Devices). PrestoBlue™ reagent is reduced by metabolically active cells, providing a quantitative measure of cytotoxicity and cell viability, which allows the calculation of the inhibitory concentration (IC_{50}) of the cells. IC_{50} values were calculated using GraphPad Prism version 6.0 software (San Diego, CA). Values for concentrations were normalized, converted to log10 and plotted as a non-linear regression curve, following software manual. Once the IC_{50} values were determined for all treatments in each cell line, viability, migration, and invasion experiments were performed after the exposure to the treatments.

**Flow cytometry**

To evaluate cells viability after 48 h of exposure to the three groups of treatment, we selected DAOY and D283MED cells. We considered seven conditions for each cell line: (1) cells without treatment or DNA binding dye; (2) cells without treatment, marked with annexin; (3) cells without treatment, marked with 7-aminoactinomycin D (7-AAD); (4) death control (cells exposed to methanol); (5) cells treated for 48 h with VA; (6) cells treated for 48 h with CDDP; (7) cells treated for 24 h with VA, and after this, we added CDDP for 48 h. Following exposure of cells to drugs, after 48 h, cells were resuspended in 0.5 ml 1X PBS. Calibration of the markers was performed using controls 2 and 3. At least 10,000 events were considered for each study condition. Analysis was performed on the cell-flow analysis equipment BD LSRFortessa™ (BD Biosciences®). Results were analyzed using FlowJo® software (http://flowjo.com).

**Quantitative real-time PCR**

Quantitative real-time PCR (qPCR) evaluated expression levels of MB key genes AKT, CTNNB1, GLI1, KDM6A, KDM6B, NOTCH2, PTCH1, and TERT. Amplification was performed in triplicate, in a 96-well-plate, using 12-μl final volume of a reaction containing the following mixture: 6 μl of SYBR Green (ThermoFisher Scientific®), 3 μl of nuclease-free water and 1.5 μl of each qPCR primers. Primers were chosen with the assistance of Primer Express Software (ThermoFisher Scientific®). We conducted a BLAST search to confirm the total gene specificity of the nucleotide sequences chosen for primers. For all reactions, we used an ABI Prism 7500 thermal cycler (ThermoFisher Scientific®). To determine the relative quantification of gene expression, the comparative Ct (cycle threshold) method was used. Briefly, this method uses arithmetic formulas to determine relative quantification. To normalize the varying cDNA quantities, a gene housekeeping ACTB runned concurrently with the target gene. The Ct was determined by subtracting the ACTB Ct from the specific target gene Ct. The Ct of the control references was then subtracted from the ΔCt of the target gene, yielding the Ct and the relative quantitative value was expressed as 2^−ΔΔCt [19, 26].

**Data and statistical analysis**

After removing adaptor sequences, we evaluated the quality of raw reads obtained through NGS. Following a laboratory-developed pipeline, we selected amplicons through the coverage data analysis with the ratio between forward and reverse strands as ≥0.6 and ≤1.4, and a sequencing coverage of 200X reads was used as a minimum requirement. After processing the reads to meet a certain quality standard, they were aligned against human references (NCBI build 37, human genome 19).
We performed gene expression statistical analyses using GraphPad Prism version 5.0 software (California). The efficacy of the treatments was compared using the “Extra-sum-of-squares F test”, which assesses whether or not the null hypothesis (in this case, the fact that the treatments are equally effective) should be rejected. Comparisons between the median values of gene expression in the treated and untreated cell lines (control) were evaluated using the Wilcoxon rank test. Continuous data on gene and protein expression were evaluated and compared using non-parametric tests: Mann-Whitney, Wilcoxon, Kruskal-Wallis, correlation coefficient (R), Spearman and Friedman, followed by Dunns post-test for multiple comparisons and two-way ANOVA. Results were considered statistically significant when \( p < 0.005 \).

### Results

#### Cell lines authentication

DAOY, D283MED, and ONS-76 cell lines were authenticated, with > 90% of CI. Genotype profile and CI are described in Table 1.

#### Fluorescence in situ hybridization

We did not observe \( MYCN \) gene amplification in DAOY, D283MED, and ONS-76 cell lines.

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Table 1  Cell lines authentication results

<table>
<thead>
<tr>
<th>Locus</th>
<th>DAOY (ATCC® HTB186™)</th>
<th>D283MED (ATCC® HTB-185™)</th>
<th>ONS-76 (JCRB No. IFO50355)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>Observed</td>
<td>Reference</td>
<td>Observed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWA</td>
<td>14, 20</td>
<td>16, 18</td>
<td>14, 16</td>
</tr>
<tr>
<td>D16S539</td>
<td>10</td>
<td>11</td>
<td>9, 10</td>
</tr>
<tr>
<td>CSF1PO</td>
<td>11</td>
<td>9, 12</td>
<td>10, 12</td>
</tr>
<tr>
<td>TPOX</td>
<td>8, 10</td>
<td>8, 11</td>
<td>8</td>
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<td>AMEL</td>
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<td>( X )</td>
<td>( X ) ( Y )</td>
</tr>
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<td>TH01</td>
<td>9</td>
<td>7, 7</td>
<td>9, 9</td>
</tr>
<tr>
<td>D5S818</td>
<td>11, 13</td>
<td>11</td>
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<td>D13S317</td>
<td>13, 14</td>
<td>8, 10</td>
<td>8, 13</td>
</tr>
<tr>
<td>D7S820</td>
<td>8, 10</td>
<td>10</td>
<td>11, 12</td>
</tr>
<tr>
<td>Correspondence index (CI)</td>
<td>ATCC®</td>
<td>ATCC®</td>
<td>ATCC®</td>
</tr>
<tr>
<td></td>
<td>Observed</td>
<td>Observed</td>
<td>Observed</td>
</tr>
</tbody>
</table>
|ღ N.A, not available

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Fig. 1 MB cell lines characteristics, somatic variants, pathogenic predictors (\( S = \) SIFT, \( P = \) PolyPhen, \( MT = \) mutation taster) and human genetic variation (HGV; \( 1G = \) 1000 genomes, \( Ex = \) ExAC) obtained through NGS sequencing, considering exonic variants with coverage \( > 200\times \), for our cohort.
Genomic variants investigation

DAOY cell line presented six variants. Of these, three exonic variants: one missense variant in exon 12 of TERT gene (c.2915G > A:p.Arg972His), and two missense variants in TP53 gene, both in exon 3: c.215C > G:p.Pro72Arg variant, also known as codon 72 polymorphism of TP53 gene, and c.329G > T:p.Cys110Phe variant. D283MED cell line presented 10 variants. One of these variants is c.215C > G:p.Pro72Arg variant, of TP53 gene, and the other is the synonymous variant in exon 13 of TERT gene (c.2850C > T:p.His950=). ONS76 cell line presented 15 variants. Of these, we observed two synonymous variants in TERT gene, one in exon 2 (c.915G > A:p.Ala305=), and another in exon 14 (c.2850C > T:p.His950=). Also, we observed one missense variant in exon 22 of PTCH1 gene: c.3583A > G:p.Thr1195Ser. The relation of variants observed is described in Fig. 1.

Table 2  IC<sub>50</sub> values for each treatment, in each cell line, in 24, 48, and 72 h of exposure

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>VA (mM)</th>
<th>CDDP (mM)</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAOY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>3 97</td>
<td>3 mM AV + 12.9 mM CDDP</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>2.7 6</td>
<td>3 mM AV + 4.7 mM CDDP</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>2.5 2</td>
<td>3 mM AV + 2.6 mM CDDP</td>
<td></td>
</tr>
<tr>
<td>D283MED</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>5 9.2</td>
<td>0.5 mM AV + 5.7 mM CDDP</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>2.7 6</td>
<td>0.5 mM AV + 4.7 mM CDDP</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>2.5 2</td>
<td>0.5 mM AV + 2.6 mM CDDP</td>
<td></td>
</tr>
<tr>
<td>ONS-76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>18,598 6356</td>
<td>18,598 mM AV + 9261 mM CDDP</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>8116 4044</td>
<td>8116 mM AV + 5564 mM CDDP</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>6277 4739</td>
<td>6277 mM AV + 4421 mM CDDP</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2  Graphics comparing viability rates for DAOY cell line after exposure to single VA and CDDP, and VA and CDDP combined after 24 h (a), 48 h (b), and 72 h (c)
Dose-response curves

VA and CDDP were used single and in combination. We obtained the IC\textsubscript{50} value for each MB cell lines, in each group of treatment (single VA and CDDP, and VA and CDDP combined), in each time (24, 48, and 72 h) (Table 2). The best time for dose-effect curve and IC\textsubscript{50} was achieved after 48 h. For DAOY cells, we observed that single CDDP treatment significantly reduced cells viability, when compared to single VA and combined treatments. This result was observed in 24 (\(p=0.0418\)), 48, and 72 h (\(p<0.001\)) (Fig. 2). For D283MED cells, we observed that combined treatment significantly reduced cells viability, when compared to single VA and CDDP treatments. This result was observed when treatments were compared in 24 (\(p=0.0154\)) and 48 h (\(P<0.0001\)) (Fig. 3). ONS-76 cells presented significant reduction of viability in the presence of VA treatment (single or combined) (\(p<0.0001\)) (Fig. 4).

Flow cytometry

For DAOY cell line, we observed that the highest percentage of viable cells was present in single VA treatment (33%), while the highest percentage of cell death was present in single CDDP treatment (92%). For combined treatment, we observed 9\% of viability and 81\% of cell death. For D283MED cell line, we observed that single VA treatment resulted in 42\% of viable cells. However, we observed that combined treatment presented the lowest percentage of viable cells (11\%), less than half of the percentage of viable cells observed in single CDDP treatment (26\%). Also, we observed that combined treatment presented 50\% of cell death, double that observed in single VA (26\%) and CDDP (25\%) treatments. Results are demonstrated in Fig. 5.

Quantitative real-time PCR

We observed an important gene expression modulation in relation to treatments with combined and single VA.
Cell lines isolated analysis demonstrated that \(KDM6A\), \(KDM6B\), \(CTNNB1\), \(TERT\), \(GLI1\), \(AKT\), \(PTCH1\), and \(NOTCH2\) genes expression, when submitted to the three groups of treatment proposed, varied significantly for D283MED (\(p < 0.0001\)) and ONS-76 cells (\(p < 0.0001\)) (Fig. 6).

For D283MED cells, we observed that \(TERT\) gene expression was significantly increased in groups of treatment with VA single and combined, in 48 h, compared to control (Fig. 6a). For ONS-76 cells, we observed that \(GLI1\) gene expression was significantly increased in groups of treatment with VA single and combined (Fig. 6b). When comparing gene expression and treatments, individually, we observed that in single VA treatment, \(TERT\) gene was significantly overexpressed in D283MED cells (\(p = 0.05\)) (Fig. 7a). In addition, for single VA treatment, \(AKT\) gene was overexpressed in ONS-76 cells (\(p = 0.0286\)) when compared to control (Fig. 7b). \(GLI1\) gene presented significant overexpression in group of treatments with VA (single or combined), when comparing the three cell lines (\(p = 0.0176\)) (Fig. 7c), and after 48 h (Fig. 7d).

**Discussion**

The current challenge in MB treatment is to identify which of the conventional chemotherapy agents and which new-targeted drugs might be expected to work best in each of the MB molecular subtypes. In this way, it is important to ascertain the subtype each cell line belongs to in order to link preclinical data to tumor type of the patients. For the present study, we selected three MB cell lines: DAOY and ONS-76, both classified as SHH subtype, carrying mutated (\(TP53\text{MUT}\)), and wild-type \(TP53\) (\(TP53\text{WT}\)), respectively, and D283MED classified as either group 4 or group 3, with \(TP53\text{WT}\) [23]. To validate our findings, first we authenticated the cell lines using STR profiling analysis. As previously reported, we did not observe \(MYCN\) amplification in DAOY, ONS-76, and D283MED cells [27]. Also, corroborating to literature, D283MED and ONS-76 cell lines did not present \(TP53\) mutations, and DAOY cell line, in turn, has a pathogenic mutation in \(TP53\) gene (c.329G>T:p.Cys110Phe).

It has been proposed that VA leads to a specific DNA damage [28], the double strands breakage (DSB), which can result in altered gene expression and induction of apoptosis.
The histone hyperacetylation induced by VA causes structural alterations in chromatin, exposing portions of DNA to DNA-damaging agents, that are normally protected by heterochromatin [30, 31]. HDACi, as VA, function by increasing histone acetylation, but also by enhancing acetylation of nonhistone proteins as TP53 [32]. The outcome of an anticancer chemotherapy can depend on the TP53 status of the tumor [33]. The exact mechanisms of HDACi anti-tumoral action under influence of the status of TP53 are not yet fully understood [34–36].

In our study, MB cell lines investigated presented different response to VA treatment. DAOY has TP53MUT while ONS-76 and D283MED have TP53WT. This difference can be explained by the fact that DAOY and ONS-76 belongs to SHH molecular medulloblastoma subgroup, and D283MED belongs to molecular subgroup 3/4 [23]. SHH subgroup is characterized by activation of the SHH pathway, and tumors commonly harbor mutations in components of the SHH pathway, specifically PTCH, SMO, and SUFU [37]. A proportion of SHH tumors exhibits amplification of MYCN and GLI2, and mutations in TP53, frequently associated with anaplastic morphology [38]. In addition to belonging to the subgroup SHH, the DAOY cell presents TP53MUT. It was recognized that group 3 is more related to group 4 than WNT and SHH, with some overlapping features, and therefore these two groups were introduced as provisional entities within non-SHH/non-WNT medulloblastomas, where the subgrouping is unequivocal [38].

In addition to the genetic background, another striking difference between the MB cells investigated is the site of origin. DAOY and ONS-76 cell lines originated from a primary tumor site, and D283MED cell line originated from a metastatic tumor site. Recent studies described that medulloblastoma metastasis are different from primary tumor, showing only around 10% of molecular targets presented at diagnosis [15]. Probably, due to cell lines origins and molecular subgroups, it might presents different genetic backgrounds, and so, other confounding genes related to different responses to VA treatment, as observed in our study [39].

ONS-76 cells has a more aggressive behavior, presenting higher proliferation and migration capability, greater division rate, and is more immature (undifferentiated) than other medulloblastoma cell lines [13, 14, 23, 26]. Fujiki et al. (2013) demonstrated proapoptotic and antiproliferative effects of VA on neural progenitor cells (NPCs) in the embryonic...
development. Also, the authors reported that cells apoptosis after VA exposure was exerted transiently only during early embryonic brain development [40]. Catalano et al. (2005) reported that VA strongly inhibits cell growth through induction of apoptosis and cell cycle arrest in poorly differentiated cancer cells, using the intrinsic pathway of apoptosis, activating caspase 9, and leading to recruitment of caspase 3. The authors concluded that the intrinsic pathway is a selective pathway of VA-induced apoptosis, and that the effect on cells apoptosis is VA dose-dependent [41].

In our study, we used higher VA concentrations in ONS-76 cells than in the other two cell lines investigated to reach cytotoxic levels (Table 2). Fujiki et al. (2013) reported that VA had dose-dependent effects both on apoptosis and hyperacetylation of histone H3 in NPCs [40], Yagi et al. (2010) described that inhibition of VA in gastric cancer cells was dependent on the dose and incubation time [42], and Jung et al. (2015) reported that the rate of cell death was found to increase in a dose-dependent manner in undifferentiated human cells [43]. We suggest that the accentuated VA cytotoxicity observed in ONS-76 cell line compared to CDDP is due to its ability to induce apoptosis and cell cycle arrest in poorly differentiated cancer cells, like ONS-76, possibly by caspase 9 activation and histone hyperacetylation in a dependent dose-response mechanism.

Some studies point to a largely p53-independent action of HDACis [3, 34], while other studies, however, suggest an essential role of TP53 in the response of tumor cells to HDACi treatment [44, 45]. In our study, as observed by other authors, the use of VA as a single agent did not decreased
DAOY cell viability [32], and DAOY cells treated with single CDDP had a large drop in the viability rates. We suggest that treatment response observed in combined VA and CDDP was not similar to single CDDP due to TP53 status. DAOY cells presents TP53MUT, making us suggest a possible selection and treatment resistance for CDDP, after previous exposition to VA.

Altered expression of multiple genes has been reported in cells exposed to VA treatment [46]. Indeed, excessive histone acetylation at transcribed regions could destabilize chromatin, thus leading to an increase of transcription at erroneous starting sites [10, 31]. In our study, we observed that TERT, GLI1, and AKT genes were, four to ten times, overexpressed after VA treatment (p < 0.0001). GLI1 and PTCH1 belongs to SHH signaling pathway, and while PTCH1 is a suppressor of this pathway, GLI1 is an important transcription factor and final product of SHH pathway [47]. GLI1 overexpression observed in ONS-76 cells, after treatment with single and combined VA, might be related to the presence of c.3583A > T.p.Thr1195Ser missense PTCH1 gene variant, observed in heterozygosis. Once this variant is related to PTCH1 loss of function [48], SHH pathway may become activated, and this might be the reason of GLI1 overexpression in ONS-76 cell line.

Another hypothesis for GLI1 overexpression after treatment with VA, observed in ONS-76 cell line, might be through AKT overexpression observed in these cells. Studies have shown that the chronic use of VA is associated with reactivation of the AKT pathway, which may be involved with mechanisms of treatment resistance. Furthermore, AKT signaling pathway activates SHH pathway independently [49]. In transformed cells, as tumor cells, epigenetic regulators are required to maintain the expression of a few key target genes, and a slight tip in this regulation balance is sufficient to result in a cell catastrophe [50]. This result suggests an activation of GLI1 by AKT, and this activation may trigger a treatment resistance mechanism.

Our study shows a new perspective about VA action in medulloblastoma cells, raising the possibility that VA may act in different patterns. According to the genetic background of MB cell, VA can stimulate cell cycle arrest and apoptosis or induce resistance to treatment via signaling pathways.
activation. VA may help in treatment, acting as a sensitizer to conventional therapies, however, this is a tiny balance and we believe that VA chronic use may select resistant cells through the high expression levels of genes related to proliferation and undifferentiation. Mechanisms that regulate gene expression and resistance after exposition to HDACIs, including VA, are really complex and little understood. Inhibition of HDACs show many chances of interfering on cells fundamental functions, and should be expected positive and negative effects upon tumorigenic and oncosupression processes [16].

Based on our results, the main take home message about MB cells and VA is about MB tumors belonging to SHH molecular subgroup, with TP53MUT, that would be the ones presenting high risk in relation to VA use during the treatment. When TP53MUT cells were exposed previously to VA, CDDP effect was not as efficient as expected in the treatment with CDDP single agent. On the other hand, TP53WT MBs can benefit from VA therapy, both SHH and groups 3 and 4. Some additional studies are necessary to understand the precise VA mechanism of action upon dose-dependent proapoptotic effects and resistant-selected cells, to establish VA safety when prescribed to MB patients.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

References


