

Valproic acid as a potent substance for increasing efficacy of topoisomerase I inhibitors

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ABSTRACT

Valproic acid (VPA) is a small molecule originally licensed as epilepsy drug, but then discovered as histone deacetylase inhibitor and anticancer drug. Topotecan is a chemotherapeutic drug, which functions as topoisomerase I inhibitor and has shown antiglioma efficacy in clinical trials. Here we tested the combination of these two agents in cultured glioma and medulloblastoma cells. 100µl of U87MG high grade glioma cells and MED 283 medulloblastoma cells were plated with 3000 cells/well in 96 well plates. After 24 hours drugs were added. VPA was used in concentration of 0.7, 1.4, 3.5 mM, topotecan was diluted 0.02 nM to 14.8 nM, with an incubation time of 72 and 120 hours. MTT-tests were used for measuring cell viability. VPA at concentration of 3.5 mM increased cell proliferation by 10 to 20%, 0.7 mM had no effect on cell proliferation, and higher concentrations reduced the cells numbers. The IC₅₀ of three days VPA was 13.9 mM. Topotecan incubation was cytotoxic. The IC₅₀ of topotecan (three days) was 0.9 nM. When both drugs were given together, the cytotoxicity was higher: with a constant concentration of dose of 0.7 mM VPA the IC₅₀ of topotecan was only 0.5 nM. The same effect was seen with 1.4 mM VPA. Incubation with 3.5 mM VPA over 72 h enhanced the reduction of cell viability, the IC₅₀ of topotecan and valproic acid was 0.2 mM. The effect became further enhanced, when increasing the incubation time. A combination treatment of the histone deacetylase inhibitor VPA and topotecan, enhanced the

antitumor efficacy of the topoisomerase-I-inhibitor. [Turk J Cancer 2009;39(3):104-109]

KEY WORDS: Valproic acid, topotecan, high-grade gliomas, medulloblastoma cell culture, MTT-test

INTRODUCTION

The poor outcome of children with malignant high-grade gliomas makes novel treatment approaches necessary.

Valproic acid (VPA) is a well known drug used for antiepileptic treatment (1, 2). More recently, antitumor effects were shown in several preclinical studies (3-7). Based upon those, the drug was also used in relapse high-grade glioma clinically, and first encouraging results confirmed the antitumor effects effect (8-10). The mechanism of action turned out to be histone deacetylase inhibition (11). Nucleosomal packing of the DNA is variable and implies different regulation steps of gene expression especially for those genes which play an important role in cellular proliferation and differentiation. Histone acetylation and deacetylation are important regulation mechanisms in this process. Therefore histone deacetylase inhibitors are currently evaluated as a novel class of anti-cancer agents (12).

Topotecan is a classical chemotherapeutic agent. Chemically it is a derivative of camptothecin, functionally it is a topoisomerase I inhibitor. Topoisomerase I is an enzyme active during the replication phase (S-phase) allowing DNA relaxation from torsion tension by cutting and resealing one of the DNA strands. Topoisomerase I inhibitors, such as topotecan, bind to the DNA-isomerase complex, inhibiting the resealing process and lead to reversible single strand breaks. The drug had shown efficacy in a high-grade glioma protocol within the HIT-GBM framework (13).

This cell culture study aimed to evaluate the effect of combining the two drugs in preparation of a possible future clinical study. Induction of differentiation is classically thought to reduce chemotherapy efficacy by reducing proliferation. However, histone acetylation is a general regulatory mechanism influencing many cellular pathways and histone deacetylase inhibition could therefore result in both, increased or decreased efficacy of the chemotherapeutic topotecan. Thus, before starting clinical testing we evaluated effect of combining VPA with the topoisomerase I inhibitor in preclinical experiments.

MATERIALS AND METHODS

Cell lines and reagents

The cell lines were originally purchased from the American Type Culture Collection (ATCC, Rockville, MD). Characterizations of the cell lines are readily available through ATCC. The U87MG (human glioblastoma cell line) had been maintained in the cell culture laboratory of the Department of Neurology at the University of Regensburg, Germany. MED283 (human medulloblastoma cell line) had been maintained in the cell culture laboratory of the Department of Neuropathology, University of Bonn, Germany.

The U87MG cell line was maintained in DMEM, the MED 283 in RPMI medium. Fetal calf serum (10%), 1% penicillin/streptomycine and 1% L-glutamine were added. The cells were cultured in a standard humidified incubator at 37° in a 5% CO₂ 95% air atmosphere. Cell culture reagents were purchased from Sigma. VPA and topotecan, licensed for clinical use, were purchased from the hospital's pharmacy.

Cell growth and viability assay

100 µl of U87MG and MED283 were plated with a concentration of 3000 cells/well (30 cells/ µl) in 96 well plates. After 24 hours drugs were added. VPA was dissolved in complete medium to reach final concentrations in the experimental wells of 0.7, 1.4 and 3.5 mM. This was done by preparing double concentrations and then adding 100 µl of double concentrated drug solutions to 100 µl cell culture medium in the wells. Topotecan was diluted to final concentrations of 0.02 nM to 14.8 nM in complete medium. After incubation times of 72 and 120 hours the cell viability measuring using MTT-tests (14). Experiments were performed quadruplicate and replicated with similar results.

MTT-measurement and statistical analysis

The MTT test was measured by Dynex-Photometer at a wave length of 570 nm. IC₅₀ was determined by the program Revelation (Version 4.22). Plots were drawn by the Excel program by exporting the measured data to the Excel program. The significance of differences was evaluated by Wilcoxon test. For the analyses the statistical package for social studies (SPSS® Inc, San Francisco, Ca, USA, version 12.0) was used.

RESULTS

VPA and topotecan incubation

Cellular proliferation after incubation with VPA was measured in the MED283 and U87MG cell line (Figure 1) after 1, 3, 6 and 7 days of incubation. A concentration less than 0.7 mM VPA did not change cellular proliferation, independent of the incubation time. At a concentration of 3.5 mM VPA, an increased cellular proliferation of 20% was noted in the glioblastoma cell line, but not in the medulloblastoma cell line. In higher concentrations, the drug became toxic for both cell lines. Topotecan was toxic in much lower concentrations. For an incubation time of three days, the IC₅₀ of topotecan was 0.9 nM for U87MG, and 0.014 nM for the MED283 (Table 1).

VPA-Topotecan co-incubation for three and six days

In the glioma cell line, three-day incubation with 0.7 mM of VPA slightly decreased cell viability of the U87MG

Table 1

INCUBATION	U87MG IC ₅₀	MED283 IC ₅₀
VPA, 3-day-incubation (3d)	13.9 mM	0.6 mM
Topotecan, 3-day-incubation	0.9 nM	0.014 nM
Topotecan + VPA 0.7 mM, 3 d	0.5 nM	0.014 nM
Topotecan + VPA 1.5 mM, 3d	0.9 nM	0.005 nM
Topotecan + VPA 3.5 mM, 3 d	0.2 nM	No cells alive
VPA, 6-day-incubation (6 d)	13.9 μM	
Topotecan, 6-day-incubation	0.5 nM	
Topotecan + VPA 0.7 mM, 6 d	0.3 nM	
Topotecan + VPA 1.5 mM, 6 d	0.3 nM	
Topotecan + VPA 3.5 mM, 6 d	0.18 nM	

$X^1=0.0001$ Doxo (100% IC)+(VPA 29% IC), $X^2=0.0001$ Doxo (100% IC)+(VPA 3.8% IC)

compared to incubation with topotecan alone (Figure 2A). When repeating the experiments with higher VPA concentration (3.5 mM), the additional toxicity became more prominent ($p=0.012$, Figure 2A). The difference between the cell viability curve of 0.7 and 3.5 mM VPA was statistically different ($p=0.012$). Six-day incubation did not further enhance the toxic effect of valproic acid (Figure 2B). The difference between the concentration of 0.7 and 3.5 mM VPA remained significant ($p=0.036$).

In the medulloblastoma cell-line MED283 a three-day incubation with topotecan and additional 0.7 mM of VPA did not decrease the cell viability as compared to incubation with topotecan alone. However, higher VPA concentrations (1.5 mM) revealed the same effect as in the glioma cells: The cellular proliferation was significantly decreased compared to incubation with topotecan alone ($p=0.012$). It was also significantly decreased as compared to incubation with topotecan with or without 0.7 mM VPA ($p=0.012$). Coincubation of topotecan with 3.5 mM VPA was toxic to almost all of the cells (Figure 3). In those higher concentrations, the additive effect of VPA was significantly more enhanced in the medulloblastoma cell line as compared to the glioma cell line ($p=0.012$ at 0.7, 1.5 and 3.5 mM VPA).

DISCUSSION

High-grade malignant gliomas and diffuse intrinsic pontine glioma remain a challenge as the dismal outcome calls for novel therapeutically approaches (15). Several pieces of evidence indicate that progress has been made recently. A large multicenter trial in adults with glioblastoma multiforme showed significant benefit from temozolomide, a chemotherapeutic agent (16). In children, multiagent approaches have shown high survival rates at least when treatment could be started with gross total resection, but the toxicity of intense multiagent chemotherapy reached a level, which might prohibit further intensification (17). Understanding the intracellular mechanisms

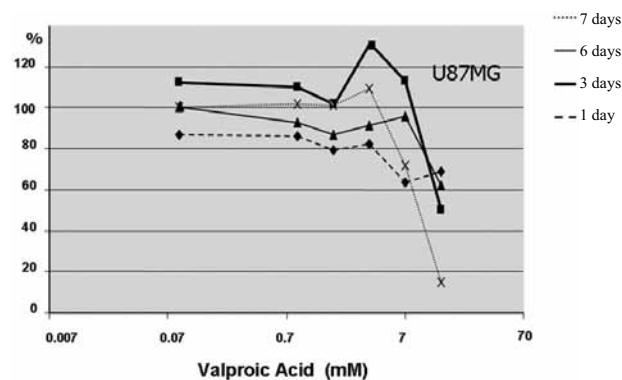


Fig 1. Valproic acid incubation over several days in different concentration in high grade glioma cells

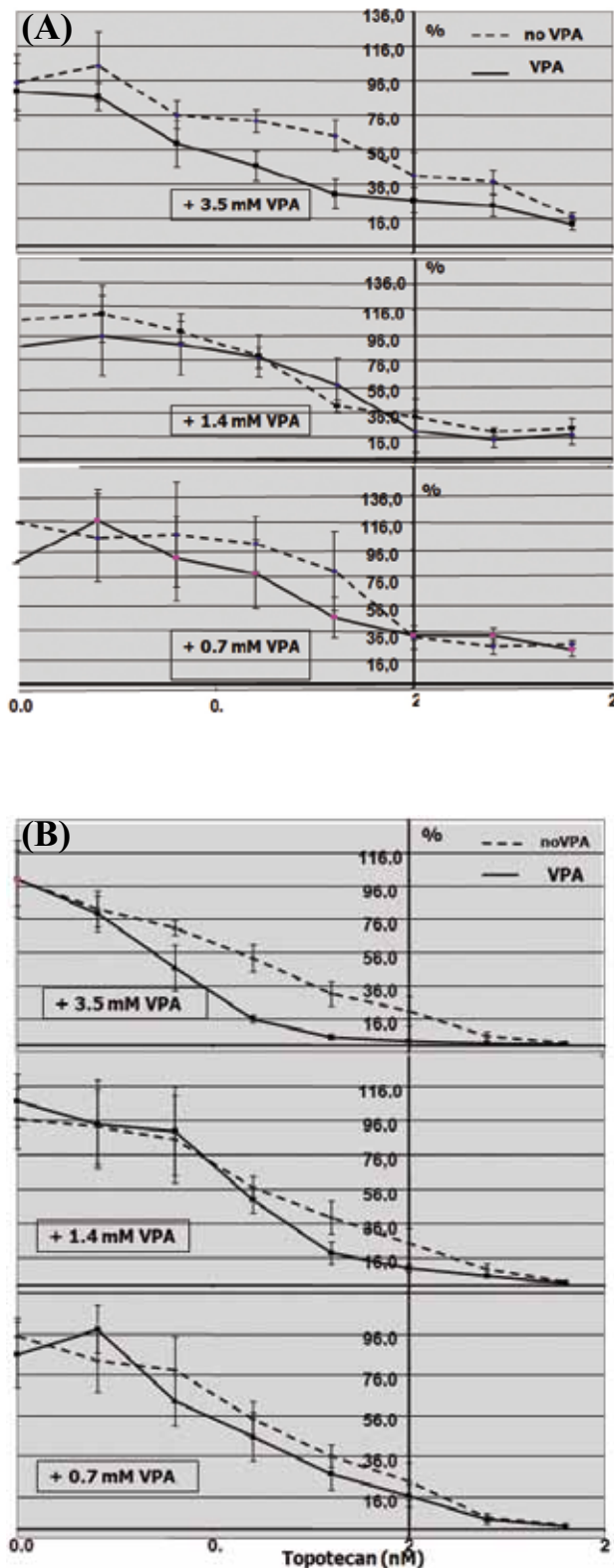


Fig 2 (A&B). (A): Three- and (B): six-day incubation of a glioblastoma cell line (U87MG) with valproic acid and topotecan. The Y axis represents cell survival in comparison to solvent treated controls. Error bars represent standard deviations, of three identical experiments

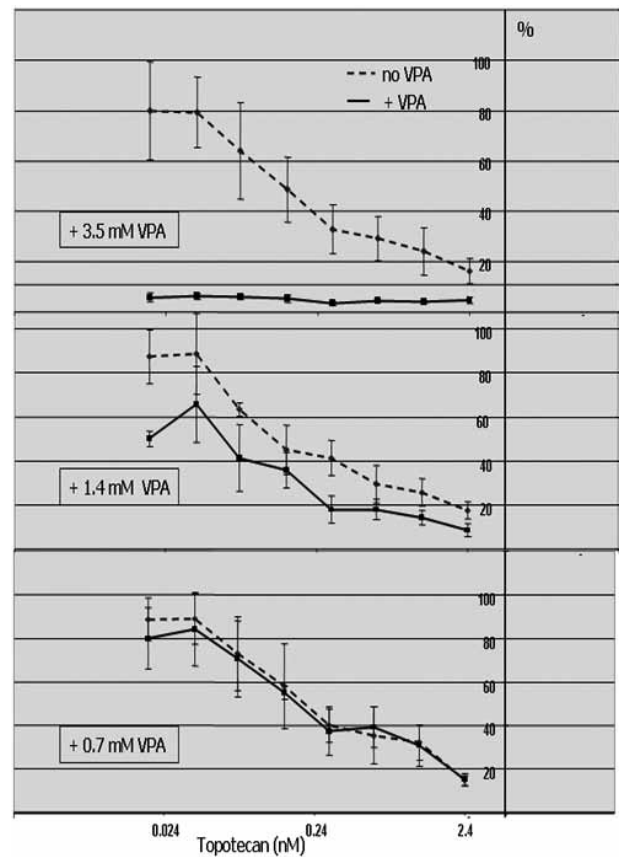


Fig 3. Three-day-incubation of a medulloblastoma cell line (MED283) with valproic acid and topotecan. The Y axis represents cell survival in comparison to solvent treated controls. Error bars represent standard deviations, of three identical experiments

of individual agents and modifying biochemical pathways in glioma cells has a higher likelihood to further improve clinical treatment protocols.

Valproic acid, a well-tolerated and well-known anti-epileptic drug also has antitumor effects. In cell culture VPA resulted in reduced cellular proliferation, matured cellular morphology, increased CD56 and decreased CD44 expression in various malignant glioma cell lines (5,8). Those effects were summarized originally as “differentiating” activity mimicking similar effects shown in cultured neuroblastoma cells, and in vivo in erythropoiesis (3,4,18). The in vitro observations prompted the use in clinical studies of high-grade glioma in patients within the HIT-GBM-framework of front-line protocols and in relapse patients. Surprisingly not only prolongation of survival but also tumor shrinkage was observed, which is inconsistent with the concept of tumor differentiation (10). The solution to this puzzle might come from the re-

cent discovery of VPA as a histone deacetylase inhibitor, which brought attention of a broad spectrum of scientists and will likely cause fast data production in the near future (11). Side effects of valproic acid have a different organ spectrum than most chemotherapeutic agents and are generally less severe. This adds further rationale to test combined treatments. The hypothesis is that the surviving cells, perhaps the tumor stem cells, could be affected by continuous inhibition of histone deacetylase inhibition increasing apoptosis or reducing mechanisms of chemoresistance to classical chemotherapeutic agents. Nevertheless, before starting combination treatment, the effect of the differentiation drug VPA in addition to other chemotherapeutic drug has to be elucidated.

For drug combination in our experiments we have chosen topotecan, a topoisomerase I inhibitor, since it had demonstrated activity in a relapse protocol (13). The mechanisms of topoisomerase I inhibitors include the inhibition of progression from the G2-phase to the M-phase, unbalanced growth due to a continuation of RNA-synthesis and protein in the absence of DNA-synthesis and polyploidy (19-21). In these experiments, we found that the toxicity of topotecan in a glioblastoma cell line (U87MG) was significantly increased when adding VPA in concentration of 0.7-1.5 mM. Those concentrations can be achieved in clinical settings in serum. A longer incubation period increased the toxic effect of the drug combination. One of the mechanisms could be that when inhibiting normal gene regulatory steps, a misbalance of gene function and DNA-replication and transcription takes place. Acetylation of core histones has been shown to weaken histone-DNA interactions and consequently increase DNA accessibility for DNA-toxic drugs. When using an unusual high concentration of 3.5 mM VPA almost all the cells were killed even with topotecan concentration, which in monoculture only reduced cell viability to 30% of the total cell amount. This confirmed the results of experiments of Phiel et al. (22), who described that VPA only acted as HDAC inhibitor at relative high concentrations.

Our results appear to be opposite to the results of Kim et al. (23). In these experiments, there was no effect of other HDAC inhibitors SAHA and TSA in combination with the topoisomerase-I inhibitor camptothecin, but a large effect in combination with topoisomerase II-inhibitors. An explanation for the differences could be that VPA might have a different mechanism or subtype specificity concerning HDAC inhibition than TSA or SAHA. Marks et al. (12) described that VPA contributed to the short-chain fatty acids class of HDAC while TSA / SAHA contributed to the hydroxamic acids class followed by other histone DNA and DNA protein interaction. Results of one HDAC inhibition could not be transferred to other HDACs type concerning efficacy and toxicity in clinical trials. Therefore each HDAC has to be evaluated separately. The HDAC with the best additive or supra-additive effect to conventional chemotherapeutics drugs and with the lowest toxicity should be chosen for further clinical evaluation. VPA might not be the most effective HDAC but with the given clinical information it has to be considered the safest at present. Our experiments did not address topoisomerase II inhibitors yet, those have to be done in the next future.

In summary, we found that the histone deacetylase inhibitor valproic acid potentiates the cyto-toxicity of topoisomerase-I inhibition in glioma and medulloblastoma cell lines. Acetylation of the core histone seemed to reduce the histone-DNA interactions and increase therefore DNA accessibility (24,25) for topoisomerase-inhibition. The medulloblastoma cell line was more sensitive than the glioma cell line for both, the treatment with topotecan only, and the combination treatment with valproic acid and topotecan.

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