

FULL PAPERS

EFFECT OF VALPROIC ACID ON THE PROLIFERATION AND APOPTOSIS
OF THE HUMAN MELANOMA G-361 CELL LINEEWA CHODUREK, ANNA KULCZYCKA, ARKADIUSZ ORCHEL,
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Abstract: Melanoma malignant is characterized by a high malignancy and low susceptibility to treatment. Due to these properties, there is a growing interest in compounds that would have the ability to inhibit proliferation, induce differentiation of tumor cells and initiate the apoptotic pathway. *In vitro* and *in vivo* research indicate that valproic acid (a histone deacetylase inhibitor) may have anti-cancer properties. In our study, the role of VPA on proliferation and apoptosis in G-361 human melanoma cell line was examined. Obtained results indicated that administration of VPA at concentrations above ≥ 1 mM led to significant inhibition of cell growth. Simultaneously, it was observed that VPA at higher concentrations (5 and 10 mM) caused an increase in caspase-3 activity.

Keywords: malignant melanoma, G-361 cell line, valproic acid, proliferation, caspase-3 activity

Malignant melanoma (*melanoma malignum*) is the most dangerous form of skin cancer. Melanoma that arises from melanocytes is an aggressive, immunogenic and malignant tumor that spreads early *via* the blood and the lymphatics. It is most commonly found in the skin or mucous membranes of the mouth and genitals. Melanoma can arise from clinically normal skin or pigmented nevi (mainly atypical nevi) (1, 2). It can also occur in the eye or meninges. Rapid growth, early and multiple metastases and low susceptibility to treatment determine the significant malignancy of the melanoma. The etiopathogenesis of malignant melanoma is associated with malignant transformation of pigment cells – melanocytes (3). Melanocyte during ontogeny can transform into pigmented cell, which lacks of cytoplasmic projections, has the ability to hyperplasia, could accumulate in the groups within the epidermis and instil into dermis. Nevus cells form benign pigmented lesions, called acquired nevi. Benign nevi can be converted into atypical moles (dysplastic). Atypical/dysplastic nevus syndrome (ANS/DNS) can be passed from generation to generation and is associated with a high risk of developing melanoma in a family. Clark et al. proposed a model which implies a gradual transformation of melanocytes

into dysplastic cells, and further into atypical cells with full malignant phenotype (1, 4, 5). More scientific papers point to the importance of tumor hypoxia in melanoma progression (6).

The acquisition of a malignant phenotype is caused by an imbalance between the action of two groups of genes – oncogenes and suppressors. Changes in these genes (during oncogenesis) result from the accumulation of mutations and epigenetic modifications. Extensive research in the field of melanoma etiopathology allowed identifying several of genes that mutation or inactivation contribute to the development of the disease (7).

In recent years, due to the low effectiveness of chemotherapy and radiotherapy in the anti-melanoma treatment, there is a growing interest in compounds that would have the ability to inhibit proliferation, induce differentiation of tumor cells and directing them to the apoptotic pathway. Scientific reports indicate a potential anti-cancer action of valproic acid (VPA), which belongs to the histone deacetylase inhibitors (HDACis) (8–11). It has been shown that VPA affects the activity of two of the four classes of HDAC, namely class I and II (subclass IIa). Exceptions in class IIa are HDAC 9, and HDAC 11, which are activated by VPA (12, 13).

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Cervoni and Szyf confirmed an indirect effect of VPA on the DNA demethylation, by increasing the availability of methylated nucleotides for DNA demethylases (14). VPA as a HDACi could form less condensed structure of chromatin, increase the DNA sensitivity to digestion with nucleases and increase the availability of DNA to intercalating agents. The compound also decreases the expression of proteins involved in the stabilization of chromatin, including SMC 1-6 (structural maintenance of chromosome), SMC binding proteins, DNA methyltransferase 1 (DNMT1), and heterochromatin protein 1 (HP1). Additionally, it may have an impact on transcription factors or other factors involved in important cellular processes, reduce the stability of the mRNA transcripts, and control proteasome-mediated protein degradation (15–18). All of these properties make probable the proapoptotic activity of this compound.

Different HDAC inhibitors, classified to short-chain fatty acids, benzamides, hydroxymates, cyclic tetrapeptides (19), may cause various changes at the molecular level (20, 21) resulting in various changes of the cellular phenotype. They may include e.g., downregulation of the antiapoptotic proteins, upregulation of the proapoptotic proteins, ROS generation or disruption of cell cycle checkpoints (22). One of the greatest challenge in developing the effective therapy is the precise evaluation of the effect of the compound on cell proliferation, differentiation and death.

The aim of the study was to investigate the influence of VPA on biology and function of human melanoma cell line (G-361).

EXPERIMENTAL

Cells

The malignant melanoma cell line (G-361) was purchased from ATCC-LGC Standards. To ensure proper proliferation, cells were grown in medium containing 90% McCoy's medium (Sigma-Aldrich), 10% fetal bovine serum (PAA), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich), and supplemented with 10 mM HEPES (Sigma-Aldrich). The culture flasks were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO₂.

Cell proliferation

To examine the proliferation activity, the *In Vitro* Toxicology Assay Kit based on reduction of the tetrazolium ring of XTT reagent (Sigma-Aldrich) was used. Melanocytes were cultured in 96-well plates (at initial density 8×10^3 cells/well) in 200 µL of medium for 24 h. Subsequently, the cells were treated with VPA (Sigma-Aldrich) in a wide range of concentrations: 0.1–10 mM. After 72 h of incubation, medium was removed and RPMI medium without phenol red (Sigma-Aldrich) with XTT reagent (20% of medium volume) was added. After 4 h, the absorbance was measured at 450 nm

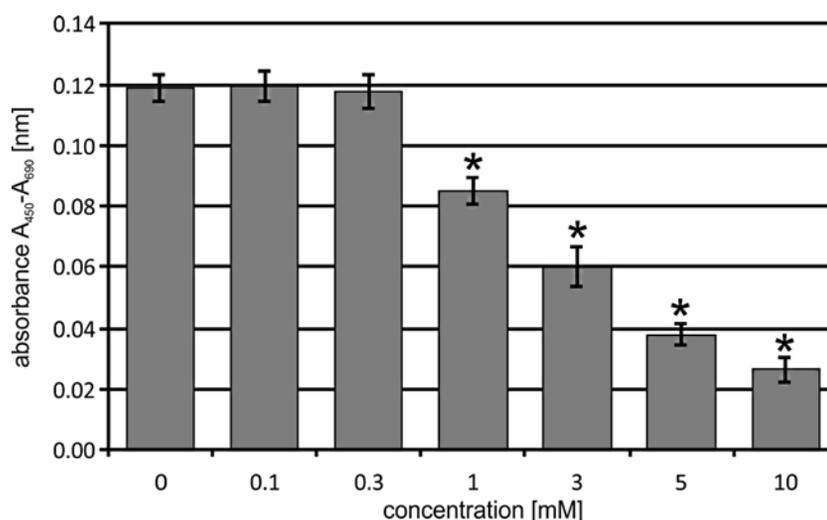


Figure 1. The effect of VPA on the proliferation of the human melanoma G-361 cell line. The cells were cultured for 72 h in the presence of various concentrations of VPA. Each bar represents the mean \pm SD; * $p < 0.05$ versus control cells (untreated)

and 690 (reference wavelength) using the MRX Revelation plate reader (Dynex Technologies).

Caspase-3 activity

To evaluate the proapoptotic caspase-3 activity, the Colorimetric Caspase-3 Assay Kit (Sigma-Aldrich) was used. Cells were seeded into tissue culture dishes (21.5 cm²) at the initial density of 2×10^6 cells/dish in 5 mL of culture medium. After 24 h cultivation, cells were incubated with VPA at 1, 3 and 10 mM for 2 days. Simultaneously, the control cells (untreated) and cells treated with 10 mM sodium butyrate (NaB) were cultivated. At the end of incubation, cells were lysed, centrifuged (16 000 \times g; 10 min) and stored at -80°C . Activity of the active caspase-3 was determined using Ac-DEVD-pNA as substrate. Enzyme samples were incubated with substrate at 37°C for 2 h. Preincubation of some samples with caspase-3 inhibitor (Ac-DEVD-CHO) was done to confirm the assay specificity. The absorbance of released p-nitroaniline (pNA) was measured at 405 nm using the MRX Revelation plate reader (Dynex Technologies). The enzyme activity was calculated relative to cellular protein content (assessed using Bradford's method).

RESULTS

Cell proliferation

In order to determine the influence of the tested compound on cell proliferation, melanoma cells

were incubated in the presence of a wide range of concentrations (0.1, 0.3, 1, 3, 5 and 10 mM) of VPA. The effect of 72 h of incubation is shown in Figure 1. The low concentrations of VPA (0.1 and 0.3 mM) did not exert a significant influence on the cell growth rate in a comparison to control group. At concentrations above 0.3 mM (1, 3, 5 and 10 mM), the concentration-dependent inhibition was seen. The higher the concentration the greater inhibition rate. The compound maximally inhibited cell proliferation at concentration of 10 mM.

Caspase-3 activity

In order to evaluate the influence of VPA on apoptosis in G-361 melanoma cell line, the activity of caspase-3 was tested. Cells were exposed to different concentrations of VPA (1, 5 and 10 mM) for 48 h. Preliminary studies with lower concentrations (0.1 and 0.3 mM) of VPA did not exert significant influence on cell apoptosis (data not shown). The effect of VPA on enzyme activity is presented in Figure 2. The increases in caspase-3 activity in cells treated with concentrations above 1 mM (compared to control cells) were statistically significant. Both concentrations of compound (5 and 10 mM) stimulated the enzyme activity at comparable level. At concentration of 1 mM of VPA, the activity of enzyme was slightly higher than in untreated control. To determine the apoptosis inducing power of VPA, the other histone deacetylase inhibitor –NaB, was used. Results showed that NaB exerted a

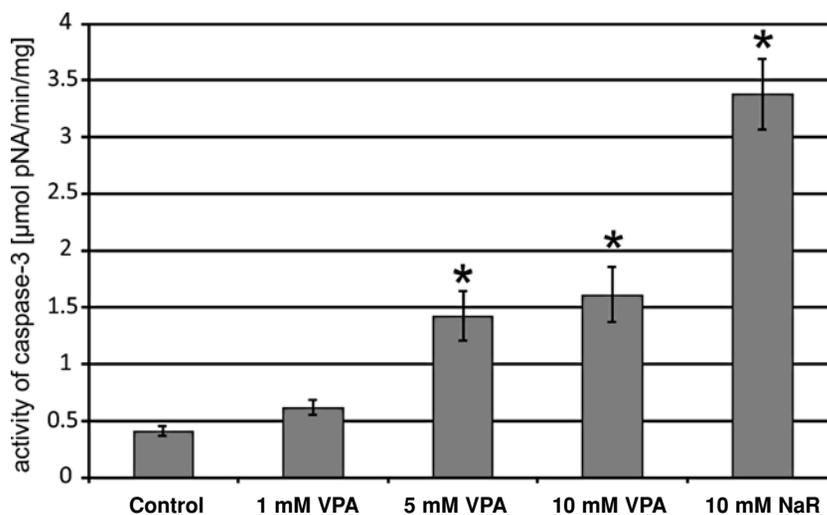


Figure 2. Effect of VPA and NaB on caspase-3 activity in G-361 cells. Each bar represents the mean \pm SD; * $p < 0.05$

stronger effect on caspase-3 activity in G-361 than VPA (both compounds at 10 mM).

DISCUSSION

The malignant melanoma causes up to 80% of death from skin cancer (5). An increased proliferation, capacity to metastasize and broad spectrum of associated genetic and epigenetic changes contribute to resistance development of melanoma to conventional therapy. Lack of effectiveness of anti-melanoma therapies make it necessary to search for new drugs that improve or replace the standard chemotherapy. The new promising strategy is the administration of a histone deacetylase inhibitor (HDACi) – valproic acid (11, 13). The mechanism of anti-melanoma effect of VPA is based on induction of apoptosis in cancer cells by different mechanisms. The first utilizes the fact that VPA, as a histone deacetylase inhibitor (HDACi), leads to inhibition of histone deacetylases involved in formation of heterochromatin. Inhibition of histone deacetylation results in increased level of histone acetylation, euchromatin formation and re-expression of genes associated with cell cycle inhibition and apoptosis like *CDKN1A* encoding the p21 protein or *CDKN2A* encoding the p16 protein (8, 12, 23). HDAC inhibitors *via* both extrinsic and intrinsic apoptosis pathways contribute to the activation of the caspase cascade that degrades the cell structures and causes the cell death. Histone deacetylase inhibitors increase the expression of death receptors and their ligands in cancer cells and inhibit the antiapoptotic gene expression e.g., cFLIP, Bcl-2, Bcl-XL. In consequence, an imbalance in the level of proapoptotic and antiapoptotic genes leads to apoptosis (18, 24, 25).

To evaluate the inhibition of cell proliferation and proapoptotic properties of VPA, the XTT assay and caspase-3 activity test were performed. The results of XTT assay showed that VPA, at higher concentrations (1, 3, 5 and 10 mM) caused a statistically significant cell growth inhibition in the studied G-361 cell line. Similar results were obtained previously by determining the cytotoxicity (using *In Vitro* Toxicology Assay Kit, Sulforhodamine B based) mediated by valproic acid in G-361 and A-375 melanoma cell lines (10, 11, 23). Previous studies also showed that conjunction of 1 mM VPA with 5,7-dimethoxycoumarin significantly decreased the proliferation in A-375 melanoma cell line and could be useful in chemoprevention (10). In our studies, lower concentrations of VPA (0.1 and 0.3 mM) did not cause a significant reduction of cell growth.

Subsequently, the proapoptotic effect of VPA was examined. This assessment bases on measurement of released p-nitroaniline as a result of caspase-3 action. Caspase-3 is an effector caspase that cleaves cellular proteins and leads to cell death so its activity could be a good apoptosis marker. Incubation with higher concentrations of VPA – 5 and 10 mM caused an increase in enzyme activity (statistically significant). These results were compared with results obtained after incubation with another histone deacetylase inhibitor – sodium butyrate. In our studies, both substances stimulated the enzyme activity but NaB was much powerful inducer than VPA at the same concentration. The same findings were observed in A-375 line (26).

Summarizing, obtained results suggest that VPA seems to be a potent anti-melanoma agent, reducing proliferation of human melanoma G-361 cell line in a concentration-dependent manner. Currently, the possibility of synergistic action of VPA with known anti-cancer drugs should be tested.

Acknowledgments

This work was supported by SUM grant KNW-1-002/M/4/0 and KNW-2-023/D/3/K.

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