Melanoma (melanoma malignum) is a malignancy originating from melanocytes, the cells synthesizing pigment called melanin. This cancer develops predominantly in the skin but also in mucous membrane or the uvea. Melanoma is a highly aggressive tumor of a dangerous high incidence and high metastatic potential. Its occurrence and mortality rates are constantly growing worldwide (1, 2). Malignant melanoma is responsible for 65% of skin malignancy-related deaths and long-term survival rate for patients with metastatic melanoma is only 5% (3).

Due to its significant unresponsiveness to conventional treatments, especially at advanced stages of disease, prophylaxis and early detection seems to be crucial in fight against melanoma. Recently, a several chemopreventive strategies for treatment of this tumor have been proposed (4–7). Cancer chemoprevention involve the use of natural or synthetic substances to reverse, suppress, or prevent the development of cancer. One of such strategies is to use therapeutic agent that is capable of inducing differentiation of tumor cells and their conversion to normal melanocytes (8, 9).

Valproic acid (VPA) (Fig. 1a), currently used as an anti-convulsant and anti-depressant, is one of the histone deacetylase inhibitors (HDACis). HDACis alter acetylation of chromatin influencing gene expression in such a way that tumor suppressive genes are activated, whereas oncogenes expression is suppressed (10–12). VPA, by inhibition of HDAC1 and HDAC2, elongates the G1 phase of cell cycle and influences many cell growth and replication processes. As a result, it exhibits antiproliferative, proapoptotic and differentiation-stimulating properties (12–14). Moreover, it seems that VPA sensitizes certain cancers to the standard chemotherapeutic (15).

5,7-Dimethoxycoumarin (DMC) (Fig. 1b) is another compound with chemopreventive potential. This natural product, also called citropten, is found in the essential oils of lime, lemon and bergamot. DMC influences phosphorylation of Mek1/2/ERK1/2 proteins, which are components of mitogen activated protein kinase (MAPK) signalling pathway. Consequently, cell cycle is blocked in the G0/G1 phase, leading to tumor cells growth reduction and differentiation (16).

The aim of the study was to investigate the influence of VPA and DMC on proliferation of A2058 human melanoma cells. It was also examined whether these compounds have a synergistic antiproliferative activity against the investigated cells.

**ANTIPROLIFERATIVE EFFECT OF VALPROIC ACID AND 5,7-DIMETHOXYCOUMARIN AGAINST A2058 HUMAN MELANOMA CELLS**

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**Abstract:** Melanoma is one of the most malignant tumors of a dangerous high incidence and high metastatic potential. It grows quickly and in an advanced stage is resistant to radio-, chemo- and immuno-therapy, which makes it difficult to cure. Therefore, research efforts are focused on the development of new therapeutics or chemopreventive strategies. The aim of the study was to investigate whether the valproic acid and 5,7-dimethoxycoumarin have an antiproliferative activity against A2058 human melanoma cell line. Investigated compounds inhibited the proliferation of cells, however, no synergistic effect of their co-administration was observed.

**Keywords:** melanoma, A2058 human melanoma cells, valproic acid, 5,7-dimethoxycoumarin

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EXPERIMENTAL

Cell cultures
Human malignant melanoma cell line A2058 were obtained from American Type Culture Collection. The cells were cultured in minimum essential medium (MEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 100 IU/mL penicillin G, 100 µg/mL streptomycin and 10 mM HEPES (Gibco). The cell were cultured at standard condition (37°C, humidified atmosphere of 95% air and 5% CO₂).

Proliferation assays
The XTT (In Vitro Toxicology Assay Kit, XTT Based, TOX-2, Sigma-Aldrich) and sulforhodamine B (In Vitro Toxicology Assay Kit, Sulforhodamine B Based, TOX-6, Sigma-Aldrich) assays were used to assess cell proliferation in the presence of tested compounds.

Tetrazolium ring of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) is cleaved by mitochondrial dehydrogenases of viable cells and soluble in water formazan crystals are formed. Cell were seeded in 96-well plates at initial density of 8 × 10³ per well and cultured in MEM for 24 h. Next medium was exchanged for medium containing VPA (concentration range: 0.1–10 mM), DMC (10–500 mM) and mixture of 1 mM VPA and 10, 50, 100 and 150 mM DMC. After 72 h of incubation cells were washed three times with RPMI (without phenol red dye) then 100 µL XTT solution was added into each well for 4 h. The absorbance was measured at 450 nm (reference 690 nm) using a MRX Revelation plate reader (Dynex) and was directly proportional to amount of the living cells.

Sulforhodamine B is a dye capable of binding to basic amino acid residues of cell proteins. A2058 cell were cultured in 96-well plates (initial density of 10³ per well) for 24 h. Subsequently, cells were treated with VPA, DMC and their mixture at the same concentration as in XTT assay. Following 72 h of incubation, cells were washed with PBS (Sigma-Aldrich), fixed with 10% trichloroacetic acid (Sigma-Aldrich) and incubated with sulforhodamine B for 30 min. Next, unbound dye was washed off using 1% acetic acid (Sigma-Aldrich). Protein-bounded sulforhodamine B was quantitatively liberated with 10 mM Tris base solution and absorbance was measured at 565 nm (reference 690 nm).

Statistics
To evaluate the influence of VPA and DMC on the A2058 cells proliferation the arithmetic mean as a measure of the average and standard deviation as a measure of dispersion were used. Differences in cells proliferation were analyzed for statistical significance using analysis of variance (ANOVA) followed by Tukey test. Normality was verified by Shapiro-Wilk test, and homogeneity of variance by Brown-Forsythe test. The p-value of less than 0.05 was considered significant. Analysis was performed using Statistica 10.0 software (StatSoft).

RESULTS AND DISCUSSION

Melanoma is a cancer difficult to treat. The prognosis for patient at the late-stage of disease are very poor. Therefore, it is crucial to develop new therapies. Histone deacetylase inhibitors (HDACis) are very promising new antitumor agents. HDACis restore the equilibrium between the acetylation and deacetylation of histones, resulting in selective activation of the genes responsible for the inhibition of proliferation and the induction of tumor cells differentiation (17).
Cytotoxic effect of valproic acid, 5,7-dimethoxycoumarin and a combination of these two compounds on A2058 human melanoma cell line was investigated. The effect was measured using two colorimetric test after the cells had been treated for 72 h with various concentration of investigated compounds. Both XTT and sulforhodamine B based test revealed that VPA, DMC and their mixture inhibited the proliferation of A2058 cells in a concentration depended manner (Fig. 2). This findings are consistent with results of Chodurek et al. (18, 19) concerning the influence of VPA and DMC on A-375 and G-361 human melanoma cell lines. The statistically significant inhibition of cells proliferation was observed even at the lowest concentrations. After supplementation of investigated cells with VPA, DMC and their mixture, the growth of dendrites and formation of star-shape cells were observed. These morphological changes may be a result of melanoma cell differentiation to normal melanocytes.

There are evidences that VPA makes tumor cells more sensitive to standard chemotherapy. Hubaux et al. (20) investigated the effect of VPA on small cell lung carcinoma (SCLC) cells in combination with cisplatin and etoposide. The 1 mM VPA significantly increased apoptosis induced by 10 µM cisplatin or 10 µM etoposide. Valentini et al. (21) also observed similar effect on M14 melanoma cells when VPA was co-administrated with standard chemotherapeutics. They observed that 1 mM VPA combined with 2.5 µM cisplatin or 0.5 µM etoposide inhibited the proliferation of cells by 50% in comparison to cells treated by these compounds alone. Finding of Chodurek et al. (18, 19) revealed that valproic acid increased the apoptosis of G-361 and A-375 human melanoma cells caused by 5,7-dimethoxycoumarin. As observed in Figure 2, although VPA and DMC caused the inhibition of A2058 melanoma cell proliferation, their co-administration did not increase this effect.

Figure 2. The effect of VPA and DMC on the proliferation of the human melanoma A2058 cell line evaluated by sulforhodamine B (A) and XTT assay (B). Each bar represents the mean ± SD; * p < 0.05 versus untreated cells
CONCLUSION

The results of our study showed that valproic acid and 5,7-dimethoxycoumarin inhibited the proliferation of A2058 human melanoma cells. However, no synergistic effect of combined compounds have been observed.

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REFERENCES