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# PROLIFERATION AND CELLULAR DEATH OF A375 CELL LINE IN THE PRESENCE OF HDACs INHIBITORS

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Dynamic increase of a skin malignant melanoma (*melanoma malignum*) morbidity rate is observed in the world for several years. Though, this type of carcinoma comprises just 5% of all malignant skin cancers, it is responsible for a 75–80% of deaths caused by these tumors (1).

*Melanoma malignum* is a cancer formed as a result of malignant transformation of melanin synthesizing cells – melanocytes. It can develop from moles or *de novo* in a normal skin. Over 90% of all melanoma cases occur in a skin, whereas in an eyeball, brain meninges, mucous membrane of oral cavity and genitalia it is more rarely observed (about 10%). For 50 years, progressive increase in melanoma incidence in the Western world is observed. The high grade of malignancy of that neoplasm is associated with rapid proliferation, numerous early metastases and high resistance to conventional treatment. Therefore, there is a demand for development of alternative antimelanoma therapies (2–4).

Inhibitors of histone deacetylases (HDACs) are a group of compounds displaying clear anticancer activity (5, 6). They have been shown to inhibit proliferation, induce apoptosis and augment differentiation in a variety of tumor cells *in vitro*. Valproic acid (VPA, 2-propylpentanoic acid), a well known antiepileptic drug, has been shown to inhibit HDACs in some transformed cell lines. Several studies confirmed the influence of VPA on proliferation, apoptosis and differentiation processes in malignant cells. Besides, its antitumor properties include the inhibition of angiogenesis and metastasis. In melanoma cells, VPA induced the cell cycle arrest in G1 phase as well as apoptosis. This effect was associated with up-regulation of P16 protein – a cell cycle inhibitor. Generally, VPA is considered as a candidate drug useful both in the chemotherapy of advanced neoplasias and chemoprevention or control of residual minimal disease. Clinical trials included phase I/II study of the therapy with VPA combined standard chemoimmunotherapy of patients suffering from advanced stage melanoma (7, 8).

Butyric acid, a four-carbon fatty acid, is a well known inhibitor of histone deacetylases. It is formed in the human colon as a result of anaerobic bacterial fermentation of dietary fiber. It is believed that butyrate plays an important chemopreventive role in colorectal carcinogenesis (9).

The aim of our study was to compare the influence of sodium valproate (NaVP) and sodium butyrate (NaB) on morphology, growth rate and apoptosis in human melanoma cell line A375.

## EXPERIMENTAL

A375 cell line was purchased from LGC Promochem (Łomianki, Poland). The cells were grown in minimum essential medium (MEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES. The cell cultures were maintained at 37°C in an atmosphere of 5% CO2/95% air.

To study the cell proliferation, melanocytes were plated at an initial density of $10^3$ cells per well in 200 μL of culture medium in 96-well plates. Cells were allowed to attach and grow for 24 h prior to exposure to test reagents. Cells were incubated with HDACs inhibitors for 72 h. Subsequently, they were

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washed with PBS and fixed in 10% trichloroacetic acid. Proliferation of the cells was quantitated using "In Vitro Toxicology Assay Kit, Sulforhodamine B Based" (Sigma-Aldrich, Poznań, Poland) according to the manufacturer's protocol. The sulforhodamine B is a dye staining cellular proteins. After the liberation of the incorporated dye absorbance was measured at $\lambda = 570$ nm and $\lambda = 690$ nm (reference wavelength) using the MRX Revelation plate reader (Dynex Technologies).

Caspase-3 activity was determined using the "Colorimetric Caspase-3 Assay Kit" (Sigma-Aldrich, Poznań, Poland) according to the manufacturer's protocol. Cells were plated into tissue culture dishes (56 $cm^2$) at an initial density of $2 \times 10^6$ cells/dish and cultured for 24 h. Subsequently, the cells were treated with HDACs inhibitors for 24 h followed by the cell scraping from surface of the dish and lysis. Cell lysates were cleared by centrifugation and frozen at –80°C. Enzyme activity was measured at 37°C using Ac-DEVD-pNA as a substrate. The amount of *p*-nitroaniline liberated was determined spectrophotometrically at $\lambda = 405$ nm using the MRX Revelation plate reader (Dynex Technologies). The results are expressed relative to cellular protein content, which was determined spectrophotometrically using Bradford's method (10).

## RESULTS AND DISCUSSION

Melanoma in an advanced stage is resistant to all conventional methods of treatment. Therefore, the development of a new, alternative therapy is justified. Melanin accumulation in a melanoma cell is a phenomenon efficiently hindering therapy. However, intense melanogenesis is a specific marker of melanoma cells differentiation. Chemical compounds such as sodium butyrate, valproic acid, 5,7-dimethoxycoumarin or dimethyl sulfoxide seems to influence the differentiation of cancer cell *in vitro* effectively, therefore, inducing the process opposite to carcinogenesis (11).

A375 cell line was used as a model of skin *melanoma malignum* cells. Cells were cultured in the presence of various concentrations (0.1, 0.3, 1.0, 3.0, 10 mM) of sodium butyrate (NaB) and sodium valproate (NaVP). Both tested compounds at low concentrations (0.1 and 0.3 mM) did not exerted any influence on the growth rate of the cells as compared to control culture (Fig. 1, Fig. 2). Significant decrease of the cell proliferative activity was observed in cultures exposed to 1 mM NaB and NaVP. The impact of tested compounds on the melanocyte growth was markedly enhanced when their concentrations were increased to 3 mM and 10 mM. NaVP and NaB, used at the highest concentrations, caused not only strong inhibition of cell proliferation but also cell detachment from the tissue culture plastic due to the induction of cellular death. NaB has been shown to inhibit proliferation and induce apoptosis in a variety of tumor cells *in vitro* (12–15). However, clinical efficacy of butyrate is hampered by its rapid metabolism and clearance rate resulting in the inability to sustain biologically relevant *in vivo* drug concentrations (16). In contrast to

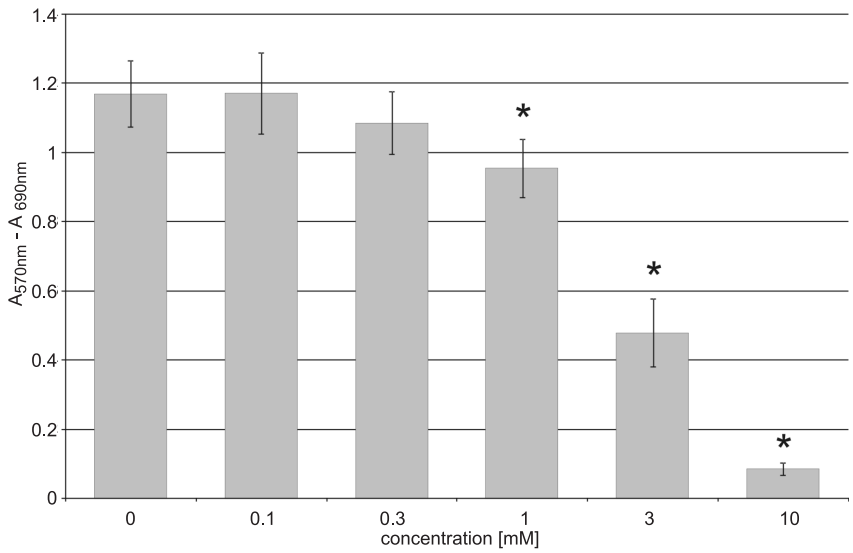


Figure 1. Growth of A375 cells cultured in the presence of various concentrations of NaB. Each bar represents the mean ± SD; *$p < 0.05$

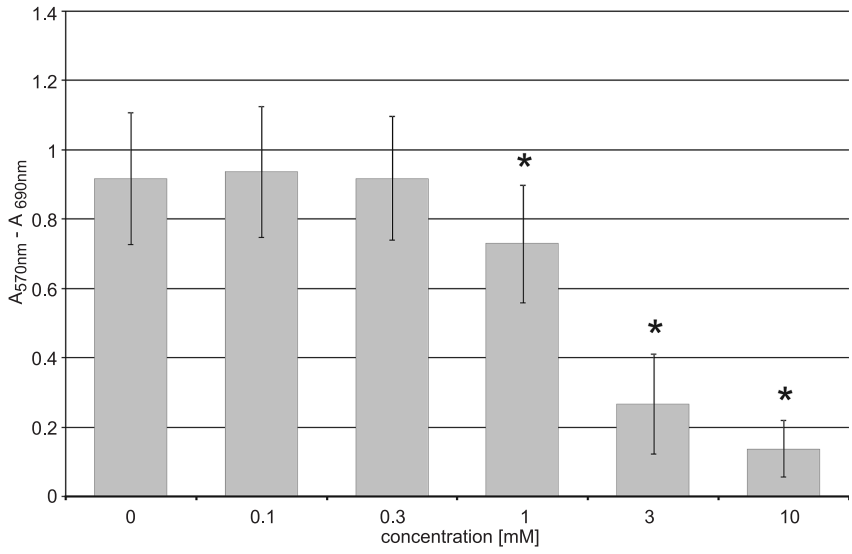


Figure 2. Growth of A375 cells cultured in the presence of various concentrations of NaVP. Each bar represents the mean ± SD; *p < 0.05

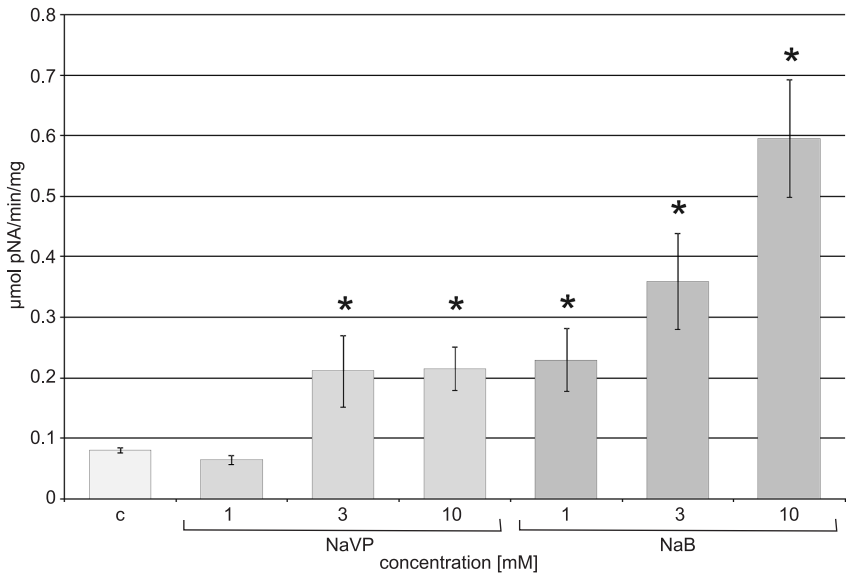


Figure 3. Effect of NaVP and NaB on caspase-3 activity in A375 cells. Each bar represents the mean ± SD; *p < 0.05; c – control

NaB, NaVP is relatively slowly metabolized and its therapeutic concentrations in blood are readily achieved.

Apoptosis was characterized morphologically by acridine orange staining of detached cells to visualize the condensed chromatin and fragmented nuclei of apoptotic cells. Moreover, caspase-3 activity was determined for reliable quantitative evaluation of apoptosis.

Caspase-3, belonging to cystein proteases ICE (*interleukin-1β converting enzyme*), is considered as an enzyme involved in the apoptosis (17, 18). Findings showed that NaVP influenced caspase-3 activity in A-375 cells less than NaB (Fig. 3).

Generally, the results of our study indicate that NaVP and NaB inhibited proliferation of melanoma cells with a similar potency. Therefore, valproic acid should be considered as potential alternative for standard cytotoxic tumor chemotherapy and additional studies on its mechanisms of action are needed.

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