EVALUATION OF MELANOGENSE IN A-375 MELANOMA CELLS TREATED WITH 5,7-DIMETHOXYCUMARIN AND VALPROIC ACID

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Abstract: Malignant melanoma (melanoma malignum) is one of the most dangerous types of tumor. It is very difficult to cure. In recent years, a lot of attention has been given to chemoprevention. This method uses natural and synthetic compounds to interfere with and inhibit the process of carcinogenesis. In this study, a new treatment strategy was proposed consisting of a combination of 5,7-dimethoxycoumarin (DMC), an activator of melanogenesis, and valproic acid (VPA), a well-known drug that is one of the histone deacetylase inhibitors (HDACis). In conjunction with 1 mM VPA, all of the tested concentrations of DMC (10-150 μM) significantly decreased the proliferation of A-375 cells. VPA and DMC also induced the synthesis of melanin and the formation of dendrite and star-shaped cells. Tyrosinase gene expression and tyrosinase activity significantly increased in response to VPA treatment. Pyrolysis with gas chromatography and mass spectrometry (Py-GC/MS) was used to investigate the structure of the isolated melanin. This showed that the quantitative and qualitative components of melanin degradation products are dependent on the...
type of applied melanogenesis inductor. Products derived from eumelanin were detected in the pyrolytic profile of melanin isolated from A-375 cells stimulated with DMC. Thermal degradation of melanin isolated from melanoma cells after exposure to VPA or a mixture of VPA and DMC revealed the additional presence of products derived from pheomelanin.

**Key words:** A-375 cell line, Malignant melanoma, Valproic acid, Tyrosinase, Gene expression, 5,7-dimethoxycoumarin, Melanin, Pyrolysis-gas chromatography/mass spectrometry

**INTRODUCTION**

Due to its rapid growth and early and extensive metastasis, malignant melanoma is one of the most malicious types of tumor. Prophylaxis and early detection are the most important factors in the fight against this malignancy. Standard therapy based on surgical intervention and chemo- or radiotherapy still does not give satisfactory results [1-4].

Agents that affect pathological epigenetic processes have given new hope for the effective therapy of many malignancies. Several epigenetic therapies are currently under investigation. One of them involves histone deacetylase inhibitors. Using both *in vitro* and *in vivo* approaches, it has been shown that histone deacetylase inhibitors (HDACis) restrain proliferation and promote the differentiation or apoptosis of various neoplastic cell types. These changes in cell phenotype are the result of altered patterns of gene expression caused by hyperacetylation of histone proteins [5-8]. HDACis belong to various chemical classes and are active against different HDAC classes [9, 10]. Valproic acid (Fig. 1A; VPA; a short-chain fatty acid) is a HDAC inhibitor [6, 11]. The selective initiation of apoptosis through a mitochondrial pathway and the activation of death receptors in tumor cells are responsible for the anticancer action of HDACis. Although the detailed mechanism of this process is not fully understood, it is assumed that HDACis can affect the activation of TNF receptors and suppress the overexpression of the genes BCL-X and BCL2, which code anti-apoptotic proteins [8, 12].

In recent years, increasing attention has been paid to cancer chemoprevention, which is defined as the use of natural or synthetic compounds to prevent the transformation of cells at early stages of tumorigenesis. Derivatives of coumarins activate the processes of cell differentiation and synthesis of melanin in malignant melanoma [13-15]. Coumarins are derivatives of α-pyrone and are widespread in plants [16]. They are known to be biologically active substances that can protect against reactive oxygen species-mediated damage and they show anti-mutagenic, anticancer and antibacterial properties [17-19]. They also cause vasodilation, have anti-thrombotic properties and suppress the activities of lipooxygenases and cyclooxygenases [13]. Coumarins and their synthetic derivatives have been used in clinical trials (in monotherapy or in conjunction...
Melanogenesis is the process of melanin biosynthesis taking place in specialized cells called melanocytes. These cells produce two different types of melanin: black and brown eumelanins, which are insoluble in alkaline solutions, and yellow or orange pheomelanin, which are soluble in hydroxides [23]. Tyrosine, the precursor of melanin, is hydroxylated to DOPA (3,4-dihydroxyphenylalanine) followed by oxidation to dopaquinone in a process catalyzed by tyrosinase. Dopaquinone is a highly reactive compound that is the key factor in the biosynthetic pathway of both eumelanin and pheomelanin. In the absence of sulfhydryl compounds, its cyclization leads to the formation of eumelanin, while in the presence of cysteine and glutathione, its cyclization leads to the formation of sulfhydryl conjugates that are precursors of pheomelanin [24].

The melanoma incidence rate for people with black skin is much lower than that for Caucasians [25]. This discrepancy is due to the better protection against UV radiation provided by melanin in the group with a higher level of constitutive pigmentation. However, melanin synthesis and its interaction with UV rays can result in ROS formation [26, 27]. Therefore, excessive melanin synthesis may be biologically harmful and may contribute to melanoma initiation. Melanoma cells are metabolically active and they are the source of numerous substances that are considered neoplastic markers, such as some cytokines, growth factors, neoplastic antigens, melanin precursors and metabolites, which are used in laboratory diagnostics [28, 29]. Pyrrole-2,3,5-tricarboxylic acid (PTCA, derived from eumelanin) and 4-amino-3-hydroxyphenylalanine (4-AHP, derived from pheomelanin) are usually analyzed as the markers of pigmented malignant melanoma [30, 31].

Melanogenesis is thought to be the main parameter of differentiation in both normal melanocytes and melanoma cells [32-34]. Neoplastic transformation is associated with aberrant cell differentiation. Metastatic melanoma cells possess
properties close to those of cells from the early stages of melanocyte development. Therefore, agents that can induce neoplastic cell differentiation can potentially be useful for tumor therapy (referred to as differentiation therapy) [35]. The aim of this study was to evaluate the proliferation rate and melanogenesis in A-375 cells that were treated with valproic acid and 5,7-dimethoxycoumarin. In addition, the pyrolytic profiles of melanin isolated from these cells were characterized.

MATERIALS AND METHODS

Tumor cells
The human malignant melanoma cell line A-375 was purchased from LGC Standards (Lomianki, Poland). The cell line was grown in a medium containing 90% Minimum Essential Medium Eagle (MEM, Sigma-Aldrich), 10% fetal bovine serum (PAA Laboratories, Pasching, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich) and 10 mM HEPES (Sigma-Aldrich). The cells were cultivated under standard conditions at 37°C in a humidified atmosphere containing 5% CO₂. To study the cell proliferation, melanoma cells were plated at an initial density of 10⁴ 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 test compounds (VPA at concentrations of 0.3, 1, 3, 10 mM and DMC at concentrations of 10, 50, 100, 150, 500 μM) for 72 h. They were then washed with PBS and fixed in 10% trichloroacetic acid. The proliferation of the cells was assessed using the In Vitro Toxicology Assay Kit, Sulforhodamine B Based (Sigma) according to the manufacturer’s instructions. Sulforhodamine B is a dye that binds to cellular proteins. After the liberation of the incorporated dye in tris base solution, the absorbance was measured at 570 nm and 690 nm (reference wavelength) using the MRX Revelation plate reader (Dynex Technologies).

Melanin isolation from A-375 melanoma cells
Melanoma cells (1 g) were mixed with 5 ml of 1% Triton X-100 (Sigma) and incubated for 1 h at room temperature [36]. Next, the sample was centrifuged (10,000 × g, 15 min) and the cell pellet was washed with phosphate buffer and once again centrifuged. The pellet was mixed with 5 ml of 5 mg/ml sodium dodecyl sulfate (SDS) in Tris-HCl buffer (50 mM, pH 7.4) with proteinase K (Sigma) to give a final concentration of 0.33 mg/ml. The mixture was incubated for 3 h at 37°C. After centrifugation (10,000 × g, 15 min), the melanin pigment was successively washed with 0.9% NaCl, methanol and hexane, and each time centrifuged (10,000 × g, 15 min). The melanin was dried at 37°C and stored in a glass desiccator over P₂O₅.

Assay for tyrosinase activity
The assay for the DOPA oxidase activity of tyrosinase was carried out according to the modified method of Slominski et al. [37] using a Hewlett Packard 8452A
spectrophotometer. A-375 cells were seeded in 100-mm culture dishes at a density of $1 \times 10^6$ cells/dish in 12 ml of the above-mentioned medium. The cells were allowed to attach and grow for 24 h. The culture medium was changed and the cells were treated with 1 mM VPA, 10 $\mu$M DMC or their combination for 3 and 7 days. At the end of the incubation periods, cells were washed with PBS and collected by trypsinization. Detached cells were centrifuged at 4,000 × g for 5 min. Subsequently, the cell pellet was lysed in 0.1 M phosphate buffer (pH 6.8) containing 1% Triton X-100 (Sigma) for 30 min. The cell lysate was incubated with an equal volume of DOPA (3 mg/ml in 0.1 M phosphate buffer, pH 6.8) for 3 h at 37ºC and the absorbance was measured at 490 nm. The tyrosinase activity in cells treated with VPA and DMC was expressed as an N-fold increase with respect to the vehicle-treated control cells.

**Tyrosinase expression**

A-375 cells were seeded and treated as described above. The mRNA expression of the tyrosinase gene was determined using a real-time reverse transcriptase-PCR technique. Total RNA was extracted from A-375 cells using a NucleoSpin RNA II Kit (Macherey-Nagel) according to the manufacturer’s instructions. All RNA samples were treated on-column with RNase-free DNase I (Macherey-Nagel). The RNA concentration was determined using a Quant-iT RiboGreen RNA Assay Kit (Invitrogen) according to the manufacturer’s instructions. The primers for PCR amplification of the tyrosinase transcript were designed using Primer Express 2.0 software on a sequence obtained from GenBank (ref. No. U01873). The primer sequences were: TF 5’-CTTCGATTGGAGTGCCCC AGA-3’; TR 5’-CCAAGCAGTGCATCCATTGAC-3’. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was used as an endogenous control. The GAPDH primer pair was: GF 5’-GAAGGTGAAGGTCGGAGTC-3’; GR 5’-GAAGATGGTGATGGGATTTC-3’ [38].

The reverse transcription and amplification reactions were performed using the Power SYBR Green RNA-to-CT 1-Step Kit (Invitrogen) in a 20 μl reaction volume containing 50 ng RNA and 0.2 μM primers. The cycle parameters were as follows: 30 min at 48ºC and 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 30 s at 54ºC, and 30 s at 72ºC. Under these reaction conditions, the amplification efficiencies were between 95 and 100%. The specificity of the PCR reaction was confirmed by melting curve analysis and by electrophoresis on 2% agarose gels stained with ethidium bromide. The threshold cycle ($C_T$) values were used to determine the relative expression ratios between the controlled and treated cells. Real-time RT-PCR was run in triplicate for both genes in each sample.

**Py-GC/MS of melanin**

Pyrolysis was performed using Pyrojector II (SGE Analytical Science) coupled directly to an Agilent Technologies 7890A gas chromatograph interfaced with an Agilent Technologies 7000 GC/MS Triple Quad instrument. The pyrolysis temperature was 500ºC and GC separations of pyrolysis products were
performed on an Rtx-5MS (Restek, Bellefonte, PA) fused-silica capillary column (5% diphenyl, 95% dimethyl polysiloxane, 60 m × 0.32 mm i.d., × 0.5 μm film thickness). Helium was used as the carrier gas at a flow rate of 2.4 ml/min. The inlet temperature was constant at 250ºC, while the GC oven was held at 35ºC for 5 min, then heated at 5ºC/min to 100ºC and next at 10ºC/min to 260ºC. The final temperature was maintained for 16 min. The temperature of the MS ion source was 230ºC and that of the quadrupole was 150ºC. The electron impact ionization energy was 70 eV. Mass spectra were recorded at m/z 45-400. MassHunter GC/MS Acquisition B.05.00.412 and MassHunter Workstation Software B.03.01 (Agilent Technologies) software were used. Wiley Registry of Mass Spectral Data 8th Edition software was used for data collection and mass spectra processing.

Statistical analysis
Cell proliferation analysis. The data obtained from 3 independent series of experiments were expressed as mean values ± standard deviations. Differences in cell proliferation were analyzed for statistical significance using analysis of variance (ANOVA) and the Kruskal-Wallis test. A P-value of < 0.05 was considered significant. Analysis was performed using Statistica 8 PL software for Windows (StatSoft, Poland).

Tyrosinase activity. The results were analyzed using a one-way ANOVA, followed by a Tukey post hoc test. Analysis was performed using Statistica 8 PL software for Windows (StatSoft, Poland).

Tyrosinase expression analysis. The relative expression calculations and statistical analyses were performed using the REST 2009 software. REST 2009 software was developed by Pfaffl et al. [39] and uses randomization and bootstrapping methods to test the statistical significance.

RESULTS
We investigated the cytotoxic effects of valproic acid, 5,7-dimethoxycoumarin and a combination of the two compounds on human melanoma cell line A-375. The effect was measured using a colorimetric test after the cells had been treated for 72 h with different concentrations of the tested compounds (Fig. 2). It was observed that VPA and DMC inhibited the proliferation of A-375 cells in a concentration-dependent manner. Only in the case of 0.3 mM VPA the number of cells did not considerably differ from the control. In cultures exposed to 1 mM VPA or 10 μM DMC, a significant decrease in proliferative activity was observed. Much stronger inhibition of proliferation was observed when cells were treated with a mixture of 1 mM VPA and 10 μM DMC. As the DMC concentration in a medium with 1 mM VPA increased, the proliferative activity of the cells decreased. In the presence of higher DMC concentrations, the cells took an irregular shape and grew in small clusters or separately, and an
increasing number became detached. This proves that the combination of 1 mM VPA and 100 or 150 µM DMC is cytotoxic for A-375 cells. In the case of cells that were exposed to just one of the compounds, significant cytotoxic effects were observed only for 500 µM DMC and for 3 mM and 10 mM VPA.

Fig. 2. Growth of A-375 cells cultured in the presence of various concentrations of VPA and DMC and in the presence of a combination of 1 mM VPA with different concentrations of DMC. Each bar represents the mean ± SD; *P < 0.05 versus control.

Fig. 3. Morphology of A-375 cells. A – control culture, B – culture treated with 10 mM VPA, C – culture treated with 500 µM DMC (magnification ×100).

Morphological changes in A-375 human melanoma cells after treatment with VPA and DMC are shown in Fig. 3. A-375 cells are adherent, flattened cells growing as a monolayer (Fig. 3A). In cultures supplemented with valproic acid (Fig. 3B) or 5,7-dimethoxycoumarin (Fig. 3C), morphological changes were observed, including the growth of dendrites and formation of star-shaped cells. The effect on tyrosinase activity of cell exposure to 10 µM DMC and 1 mM VPA (added individually or together) for 3 or 7 days is shown in Fig. 4. After 3 days, increased DOPA oxidase activity was detected relative to the control,
1.11-fold with exposure to 10 µM DMC alone, 1.32-fold with 1 mM VPA alone, and 1.78-fold with the combination of the two. However, the increase in enzyme activity was only statistically significant in cells treated simultaneously with VPA and DMC. When the incubation period was prolonged to 7 days, the tyrosinase activity increased 1.26-, 2.18- and 2.57-fold relative to the control level, respectively. Statistically significant increases were observed in cells that were incubated with VPA. The highest tyrosinase activity in A-375 cells was observed when the combination of 10 µM DMC and 1 mM VPA was used, both after 3 and 7 days of culture.

Fig. 4. The effect of VPA and DMC on tyrosinase activity in A-375 melanoma cells after 3 or 7 days in culture. Each bar represents the mean ± SD; C-control. *P < 0.05 versus control.

Examination of the influence of VPA and DMC on the expression of the tyrosinase gene revealed that an increase in transcriptional activity preceded the accumulation of the enzyme protein. Treatment of the cells with 1 mM VPA (alone or in combination with 10 µM DMC) for 3 days resulted in a statistically
significant increase in tyrosinase gene expression (Fig. 5). When the incubation period was prolonged to 7 days, the levels of tyrosinase transcripts decreased and fell below the control value (data not shown).

Melanin accumulation within cells was observed in all of the cultures exposed to VPA and DMC. Melanin was not isolated from the control (A-375 cell culture devoid of melanogenesis stimulators; Tab. 1).

Tab. 1. The influence of melanogenesis stimulants on the synthesis of melanin by human malignant melanoma A-375 cells.

| Culture                        | The quantity of melanin/1 g of cells [g] |
|--------------------------------|-----------------------------------------|
| A-375 cells                    | Not detected                            |
| A-375 + 1 mM VPA               | 0.0016                                  |
| A-375 + 10 μM DMC              | 0.0037                                  |
| A-375 + 10 μM DMC + 1 mM VPA   | 0.0018                                  |

Py-GC/MS analysis (Fig. 6) showed that there were qualitative and quantitative differences between products obtained after the pyrolysis of melanin isolated from A-375 cells after 7 days culture in the presence of 10 μM DMC, 1 mM VPA, or a combination of the two.

Styrene (16) and α-methylstyrene (19) were predominant among the products of thermal degradation of the melanin isolated from cells exposed to 10 μM DMC (Fig. 6A). Toluene, methylethylbenzene and small amounts of benzene, pyridine, pyrrole, phenol, indole and their methyl derivatives were also detected. All these compounds are products of thermal degradation of the eumelanin biopolymer.

Benzene, pyridine, pyrrole, toluene, styrene, 4-methylphenol and indole were detected after Py-GC/MS analysis of the melanin isolated from the investigated cells exposed to 1 mM VPA (Fig. 6B). These products indicate the presence of an eumelanin component in the analyzed biopolymer. However, in contrast with the DMC-melanin pyrolysate, benzothiazole (26 in Fig. 6), which is a sulfur-containing heterocyclic compound, was identified in the VPA-melanin pyrolysate. This compound is one of the characteristic products of pheomelanin pyrolysis.

Many sulfur-containing products such as thiophene (2 in Fig. 6) and thiazole (3 in Fig. 6), which are characteristic for the pyrolysis of pheomelanin, were identified after the pyrolysis of melanin isolated from A-375 cells that had been treated with a combination of 1 mM VPA and 10 μM DMC (Fig. 6C). The products of eumelanin thermal degradation were also detected, including pyrrole and its alkyllic derivatives, methyl derivatives of pyridine, styrene and α-methylstyrene.

Products of thermal degradation of lipids (alkenes and alkanes) and proteins (amino acids) were also identified among the pyrolysis products. This proved that the method used for melanin isolation did not completely separate the pigment from the protein and lipid components.
Fig. 6. Chromatogram of the products formed during the pyrolysis of melanin isolated from the human A-375 cell line, which had been treated with 10 µM DMC (A), 1 mM VPA (B), or 1 mM VPA and 10 µM DMC together (C). Peak designation: (1) benzene, (2) thiophene, (3) thiazole, (4) pyridine, (5) pyrrole, (6) toluene, (7) L-alanine, (8) 1-decene, (9) decane, (10) 2-methylpyridine (11) 2-methylpyrrole (12) 3-methylpyrrole (13) ethylbenzene, (14) 4-methylpyridine (15) not identified, (16) styrene, (17) not identified, (18) methylethenylbenzene, (19) α-methylstyrene, (20) not identified, (21) phenol, (22) not identified, (23) 4-methylphenol (24) indene, (25) benzyl nitrile, (26) benzothiazole, (27) not identified, (28) indole.

DISCUSSION

Late-stage malignant melanoma is a cancer with a very poor prognosis. The average lifetime of an advanced melanoma patient usually does not exceed one year and, in some cases (≤ 5% of patients), can only be extended to about five years with chemo- or immunotherapy. Therefore, new therapies have to be developed [9, 11, 40]. Histone deacetylase inhibitors (HDACis) are the most promising new antitumor agents. HDACis are considered to selectively activate the genes responsible for the inhibition of proliferation and the induction of differentiation of tumor cells.
The function of HDACis is mainly associated with restoring the equilibrium between the acetylation and deacetylation of histones [41]. Many HDACis, among them VPA, are currently undergoing phase I or II clinical trials [11, 41]. Daud et al. [42] used VPA (30-90 mg/kg/24 h) and the topoisomerase I inhibitor Karenitecin (KNT at 0.8 and 1 mg/m²/24 h) and achieved stabilization of the disease in 13 out of 33 (39%) patients with the diagnosed melanoma. The phase I and II clinical trials conducted by Rocca et al. [11] revealed that combined treatment with VPA, decarbazine and INF-γ did not give desirable effects. The application of some natural compounds, such as coumarin derivatives that activate the processes of cell differentiation, correlated with the increased level of melanin synthesis and was proposed as another strategy for melanoma therapy [16, 22].

In this study, we investigated the influence of valproic acid and 5,7-dimethoxycoumarin on the proliferation and differentiation of human malignant melanoma A-375 cells. It is proposed that melanoma resistance to chemotherapy is correlated with silencing of some specific genes caused by their excessive deacetylation. Valentini et al. [43] investigated the effect of VPA on M14 melanoma cells treated by cisplatin and etoposide. The co-administration of VPA with standard chemotherapeutics gave a synergistic effect of the combined treatment. 1 mM VPA together with 2.5 μM cisplatin inhibited the proliferation of cells by 50% in comparison to cells treated with cisplatin alone. A similar result was obtained in cultures simultaneously stimulated with 1 mM VPA and 0.5 μM etoposide; therefore, VPA made M14 cells more sensitive to standard chemotherapy. This suggests that some of the HDACis may be useful in the combined therapy of malignant melanoma [42]. It is noteworthy that the results presented in Fig. 2 showed a similar synergistic effect in the case of A-375 cells exposed to VPA and DMC. A significant inhibition of cell proliferation was observed after their co-treatment with 1 mM VPA and 10 μM DMC.

Melanogenesis is considered to be the main parameter of differentiation both in normal melanocytes and melanoma cells. Differentiating melanocytes accumulate melanosomes, which store tyrosinase, an enzyme responsible for cell pigmentation. Therefore, melanin synthesis, tyrosinase activity, and the expression of the tyrosinase gene are widely accepted as the main markers of melanocyte differentiation under in vitro conditions [32-34]. Melanin itself seems to be a two edged sword: its production can be considered both photoprotective and photosensitizing. Melanin synthesis and its interaction with UV radiation can result in ROS production. Overproduction of melanin may be biologically harmful and may contribute to melanoma initiation [44, 45]. On the other hand, induction of melanoma cell differentiation (evaluated using the above-described markers) was usually linked to inhibition of cell proliferation [22, 33, 34].

Alesiani et al. [22] investigated the influence of DMC on the B16 mouse melanoma and A-375 human melanoma cell lines. They observed increased melanogenesis for both cell lines. At concentrations of 100 μM, 250 μM and
500 μM, DMC increased the melanogenesis in A-375 cells over the control values 1.7-, 2.9- and 5.2-fold, respectively. The observed augmentation of melanin content was a result of moderate increases in tyrosinase activity (1.13-, 1.20- and 1.23-fold compared to the control).

The results of our study indicate that treatment of A-375 cells with VPA increased tyrosinase activity in a concentration-dependent manner, but the highest enzymatic activity was determined after simultaneous application of 1 mM VPA and 10 μM DMC. Moreover, the increased enzyme activity resulted from an enhanced transcription of the tyrosinase gene. Elevated levels of tyrosinase transcripts were detected in cells treated with VPA for 3 days. After extending the incubation period to 7 days, the levels of tyrosinase mRNA transcripts decreased below the control value. We found an identical pattern of tyrosinase expression changes in our previous study of melanocytes treated with dimethyl sulfoxide (DMSO) [46]. We suggest that the decreased tyrosinase gene expression accompanying the accumulation of enzyme protein was a result of negative feedback regulation.

Our observations indicate that DMC induces morphological changes in A-375 melanoma cells because treatment of these cells with DMC caused the formation of star-shaped structures. Similar morphological changes in both mouse and human melanoma cells treated with DMC were observed by Alesiani et al. [21]. These changes in the cell structure did not disappear after DMC removal from the culture medium. Therefore, they could be regarded as a sign of differentiation induced by DMC, because star-shaped morphology is one of characteristics of normal melanocytes.

At high concentrations, VPA induced morphological changes in A-375 cells that were similar to those caused by DMC. Star-shaped cells appeared in cultures treated with 3 mM VPA. Takahashi et al. studied the impact of another two HDACis on differentiation in human melanoma cells: sodium butyrate (NaB) and DMSO [47]. They showed that NaB caused the formation of star-shaped cells, whereas DMSO did not affect the cell morphology. The latter was also confirmed in our recent study [46].

In this paper, melanogenesis was evaluated on the basis of the amount of melanin isolated from A-375 cells. The highest isolation efficiency was achieved when cells were treated with DMC alone or in combination with VPA. Py-GC/MS analysis (Fig. 6) showed that the pyrolytic profile of melanin isolated from A-375 cells is influenced by the melanogenesis inducer used. Visible qualitative and quantitative differences were found for all studied melanin types. In the pyrolytic profile of melanin isolated from cells exposed to DMC, there are compounds characteristic of thermally degraded eumelanin [36, 48] whereas pyrolysis products of pheomelanin are absent [49]. The pyrogram of melanin isolated from cells treated with VPA is distinguished by the presence of the sulfur-containing heterocyclic compound, benzothiazole, alongside the eumelanin component. The predominant pyrolysis products of melanin from cells treated with both compounds were sulfur-containing markers of
pheomelanin, such as thiazole and thiophene [49, 50]. It is worth noting that natural types of melanin are tightly bound to a lipoprotein component that is difficult to remove from the melanin polymer [36], so thermal degradation products of proteins and lipids were also detected in pyrolysates of studied melanin.

Rosso et al. [51] determined the type of melanin as a risk indicator for malignant melanoma and assessed the eumelanin content in patients’ hair samples. The amount of eumelanin was estimated using an indirect method based on the assay of 2,3,5-pyrroletricarboxylic acid released as a result of the chemical degradation of eumelanin. It is considered one of the main markers of these biopolymers. Nevertheless, a Py-GC/MS approach may be used to establish more useful criteria for identifying persons at risk of developing melanoma by providing a more detailed picture reflecting structural features of melanin isolated from neoplastic cells.

In summary, the results reported here show that valproic acid and 5,7-dimethoxycoumarin are able to inhibit melanoma cell proliferation and the maximal inhibition of cell growth was achieved as a result of the combined treatment with these compounds. Moreover, both VPA and DMC enhanced the synthesis of melanin in melanoma cells in vitro. Generally, the compounds show promise as potential chemopreventive and therapeutic agents for patients with malignant melanoma.

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