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EPR STUDIES OF FREE RADICALS IN A-2058 HUMAN MELANOMA CELLS TREATED BY VALPROIC ACID AND 5,7-DIMETHOXYCOUMARIN

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Abstract: Free radicals in A-2058 human melanoma cells were studied by the use of electron paramagnetic resonance (EPR) spectroscopy. The aim of this work was to determine the changes in relative free radical concentrations in tumor A-2058 cells after treatment by valproic acid (VPA) and 5,7-dimethoxycoumarin (DMC). The influences of VPA and DMC on free radicals in A-2058 cells were compared with those for human *melanoma malignum* A-375 and G-361 cells, which were tested by us earlier. Human malignant melanoma A-2058 cells were exposed to interactions with VPA, DMC, and both VPA and DMC. The tumor cells A-2058 were purchased from LGC Standards (Lomianki, Poland), and they were grown in the standard conditions: at 37°C and in an atmosphere containing 95% air and 5% CO_2, in the Minimum Essential Medium Eagle (MEM, Sigma-Aldrich). The A-2058 cells were incubated with VPA (1 mM) and DMC (10 μM) for 4 days. The first-derivative EPR spectra of the control A-2058 cells, and the cells treated with VPA, DMC, and both VPA and DMC, were measured by the electron paramagnetic resonance spectrometer of Radiopan (Poznań, Poland) with microwaves from an X-band (9.3 GHz). The parameters of the EPR lines: amplitudes (A), integral intensities (I), line widths (ΔB_{pp}), and g-factors, were analyzed. The changes of amplitudes and line widths with microwave power increasing from 2.2 to 70 mW were drawn evaluated. o-Semiquinone free radicals of melanin biopolymer are mainly responsible for the EPR lines of A-2058 *melanoma malignum* cells. The amounts of free radicals in A-2058 cells treated with VPA, and both VPA and DMC, were lower than in the untreated control cells. Application of the tested substances (VPA, and both VPA and DMC) as the antitumor compounds was discussed. DMC without VPA did not decrease free radicals concentration in A-2058 cells. The studies confirmed that EPR spectroscopy may be used to examine interactions of free radicals with antitumor compounds.

Keywords: valproic acid, 5,7-dimethoxycoumarin, A-2058 human melanoma cells, free radicals, EPR spectroscopy

The pharmacologically active compounds may produce and interact with free radicals in cells (1–7). Free radicals are formed in tumor cells during pathological process (8–11). The modern antitumor compounds should decrease the amount of free radicals in cells to stop the toxic biochemical reactions during cancer transformation. Our earlier studies concentrate on changes in free radicals in melanin biopolymer isolated from A-375 and G-361 human *melanoma malignum* cells (12) after application of valproic acid (VPA), 5,7-dimethoxycoumarin (DMC), and free radicals in melanin isolated from A-375 cells exposed on valproic acid (VPA) and cisplatin (CPT) (13). Melanins are strongly paramagnetic and they contain the high amounts of o-semiquinone free radicals (12–21), so their free radicals are mainly responsible for polymer-drug interactions. The strong decrease of free radicals concentrations in melanin from A-375 and G-361 cells after treatment with both VPA and DMC was observed (12). Such effect was not obtained after A-375 cells treated with both VPA and CPT (13). Valproic acid, 5,7-dimethoxycoumarin and cisplatin were recommended as the antitumor compounds (12, 13). Their effect on free radicals in the whole tumor cells was not tested.

The aim of this work was to determine the changes in relative free radical concentrations in A-2058 human *melanoma malignum* cells after interactions with valproic acid (VPA) and 5,7-dimethoxycoumarin (DMC). The influences of VPA, DMC, and both VPA and DMC on free radicals in A-2058 cells were compared. The results were discussed relative to our earlier conclusions

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(12, 13) about the effectiveness of VPA and DMC as the antitumor compounds. Free radicals in melanin from tumor A-2058 cells were detected by the use of electron paramagnetic resonance (EPR) spectroscopy (12, 13). The same method of free radical examination in cells was used by now. EPR spectroscopy is the useful method in examination of drugs (22, 23) and the melanin complexes with drugs (18, 20, 21, 24, 25).

EXPERIMENTAL

Tumor cells

The human malignant melanoma cell line A-2058 was purchased from LGC Standards (Łomianki, Poland). The cell line was grown in the medium containing the following composition: 90% Minimum Essential Medium Eagle (MEM, Sigma-Aldrich), 10% fetal bovine serum (FBS, PAA), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich) and 10 mM HEPES (Sigma-Aldrich). The cells were cultivated in standard conditions (at 37°C, in a humidified atmosphere containing 5% CO_2).

Tested compounds

Synthetic valproic acid (VPA) and 5,7-dimethoxycoumarin (DMC) were purchased from Sigma-Aldrich.

Cells A-2058 were incubated with test compounds (1 mM VPA, 10 mM DMC or their combination) for 4 days.

EPR measurements

Free radicals in A-2058 tumor cells were examined by the use of an X-band (9.3 GHz) electron paramagnetic resonance (EPR) spectrometer with magnetic modulation of 100 kHz produced by Radiopan (Poznań, Poland). The EPR spectra were measured for the cell samples located in thin walled glass tubes with the external diameter of 1 mm. The empty tubes were free of paramagnetic impurities, and they did not reveal EPR signals. The EPR spectra as the first derivative of absorption curves were recorded by the Rapid Scan Unit from Jagmar (Kraków, Poland). The numerical acquisition (100) of EPR spectra for each sample was done. The total microwave power (M_o) produced by klystron of the EPR spectrometer was 70 mW. The microwave power was changed by different attenuation in the range from 2.2 to 70 mW. Additionally, the influence of microwave power on the parameters of the EPR spectra was drawn.

Spectroscopic programs of Jagmar (Kraków, Poland) and LabVIEW 8.5 by National Instruments were used to measure and to analyze the EPR spectra. g-Factors, line widths (ΔB_{pp}), amplitudes (A), and integral intensities (I) of the examined EPR spectra were obtained. g-Factors were calculated according to the formula (26, 27):

$$g = h\nu/\mu_B B_r$$

where: h – Planck constant, ν – microwave frequency, μ_B – Bohr magneton, B_r – induction of resonance magnetic field. Microwave frequency (ν) was directly measured by MCM101 recorder of EPRAD (Poznań, Poland). The induction of resonance magnetic field (B_r) was determined from the EPR line. ΔB_{pp} depend on magnetic interactions in the samples (1, 26).

Amplitudes (A) and integral intensities (I) increase with increasing amount of free radicals in the sample (26). Free radical concentration is proportional to integral intensity of the EPR line, which is defined as the area under the absorption curve (26). Integral intensities were calculated by double integration of the first derivative EPR spectra. To compare the relative concentrations of free radicals in the tested samples, the values of integral intensities were divided by the volume of the cells in the glass tube, and the integral intensities were done in the arbitrary units. Integral intensities reflect the concentrations in the cells. The integral intensities of the analyzed samples were compared with those for the free radical reference. Ultramarine was the reference which was used. The second reference permanently placed in a resonance cavity – a ruby crystal (Al_2O_3: Cr^3) was used to rise the accuracy of the measurements.

The effect of microwave power (2.2–70 mW) on amplitudes (A) and line widths of the EPR spectra of the cell samples was examined. Type of broadening (homogeneous or inhomogeneous) of EPR lines was determined from the correlations between microwave powers and both amplitudes (A) and line widths. The spin-lattice relaxation processes were evaluated from the changes of amplitudes (A) with microwave power. The faster spin-relaxation processes give EPR lines, which saturate at the higher microwave powers (26).

RESULTS

In the present study, we have assessed the morphology of human malignant melanoma cells A-2058 incubated in the presence of 1 mM VPA, 10 μM DMC or their combination. In the control culture (Fig. 1a) large aggregates of spindle-shaped cells were visible, which were readily adhere to the surface of the culture vessel. At low concentrations

of VPA (0.1 and 0.3 mM) there was no difference in the appearance of cells in comparison to the control. In contrast, in cultures exposed to 1 mM VPA (Fig. 1b), the number of cells was decreased. The cells were most often found separate from one another and their shape was irregular. Part of the cells was taken a round shape, which indicates their poor adhesion to the surface of the culture vessel. A similar effect was observed in cultures exposed to 10 μM DMC (Fig. 1c) and mixture of 1 mM VPA and 10 μM DMC (Fig. 1d).

For all the tested A-2058 tumor cells: the control cells and the cells treated with valproic acid and 5,7-dimethoxycoumarin, EPR spectra were obtained (Fig. 2). The spectra without microwave saturation effect were observed. In Figure 2, the EPR spectra measured with microwave power of 62 mW are compared.

Free radicals exist in all the tested cell samples, but their amounts and their EPR parameters differed for the cells treated with the individual compounds.

g-Factors characteristic for o-semiquinone free radicals were obtained for the examined A-2058 human *melanoma malignum* cells (Table 1). The broad EPR spectra with line widths in the range: 1.86-1.91 mT were measured (Table 1). Integral intensities indicated that VPA, and VPA together with DMC decrease free radical concentrations in the cells. The lower values of integral intensities, so the lower free radical concentrations, revealed A-2058 cells treated with VPA, and VPA and DMC together (Table 1). The lowest integral intensities were measured for the tested cells treated with both VPA and DMC. Integral intensity of the EPR spectrum of A-2058 cells interacting with VPA is lower than integral intensity of the spectrum for these cells treated with DMC. Integral intensity of the EPR spectrum of A-2058 cells interacting only with DMC revealed similar integral intensity as the control A-2058 cells (Table 1), so DMC did not quenched free radicals in the tested tumor cells.

The spectral amplitudes and line widths depend on microwave power. The changes of the parameters of the EPR spectra of A-2058 cells are shown in Figures 3 and 4. The influence of microwave power (M/M_o) on linewidths of EPR spectra of the control A-2058, and the cells treated with VPA, DMC, and both VPA and DMC, shows the broadening of all the spectra with an increase of microwave power (Fig. 3). This correlation indicates homogeneous broadening of EPR lines of the examined tumor cells.

Amplitudes (A) of EPR lines of all the studied A-2058 human malignant melanoma cells increase with increasing microwave power, and these lines are not saturated (Fig. 4). Only for A-2058 cells treated with both VPA and DMC at the beginning of

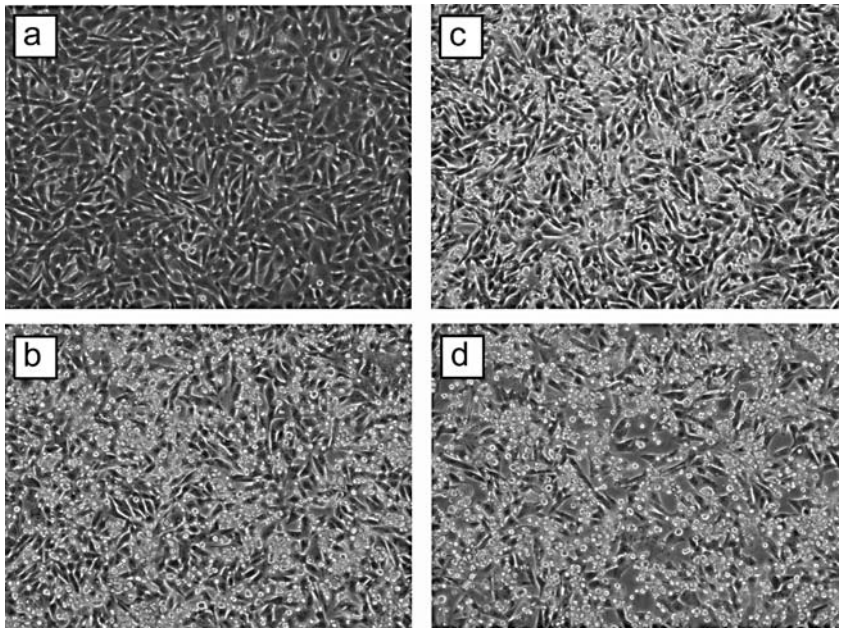

Figure 1. Morphology of malignant melanoma A-2058 cells: control (a), cells treated with 1 mM VPA (b), cells treated with 10 mM DMC (c), cells treated with 1 mM VPA and 10 mM DMC (d)

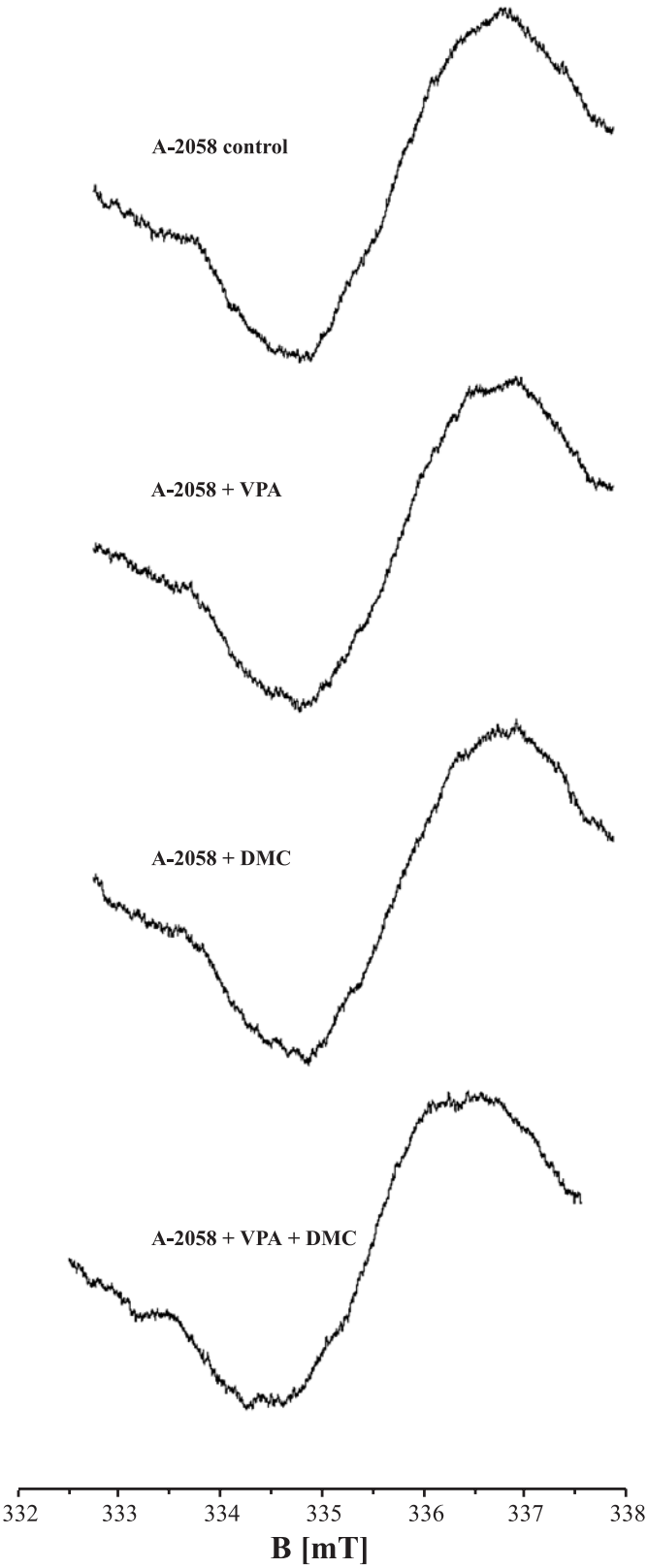

Figure 2. EPR spectra of control A-2058 human *melanoma malignum* cells, and the cells treated with VPA, DMC, and both VPA and DMC. The spectra were measured with microwave power of 62 mW. B – magnetic induction

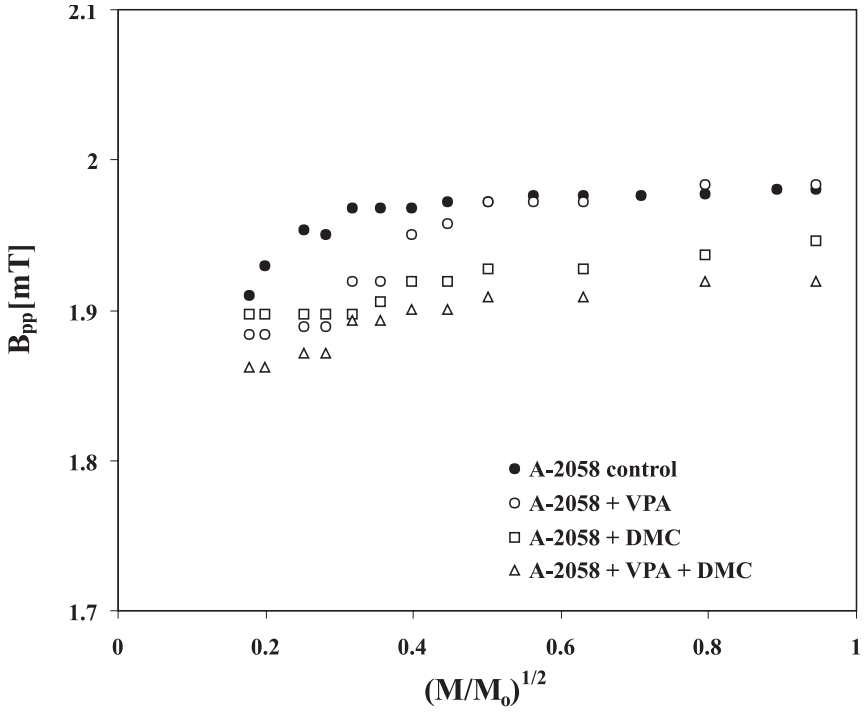

Figure 3. Influence of microwave power (M/M_o) on line widths (ΔB_{pp}) of EPR spectra of A-2058 control cells (●), and the cells treated with VPA (○), DMC (□), and both VPA and DMC (△). M, M_o – the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively

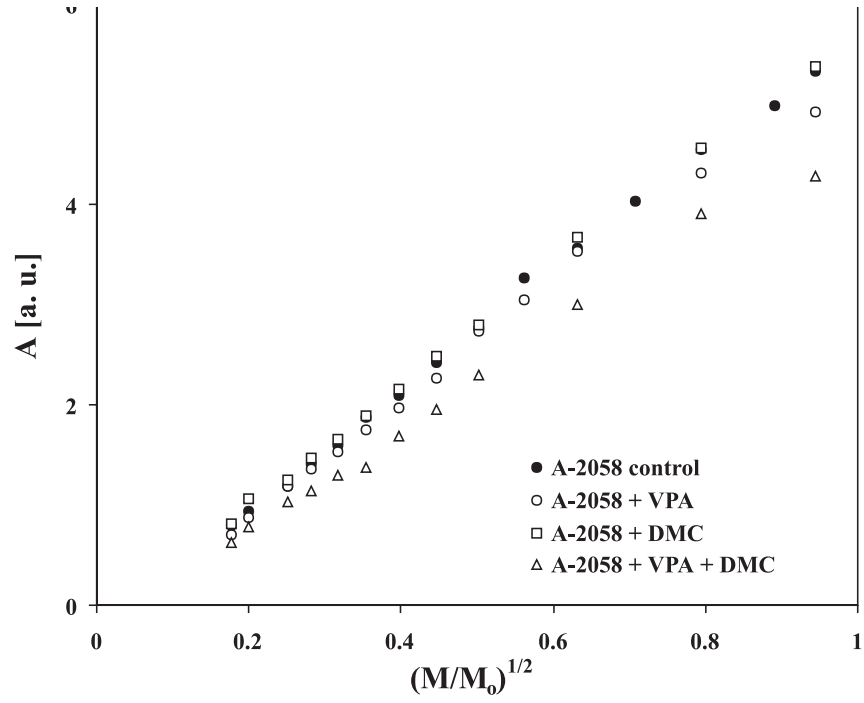

Figure 4. Influence of microwave power (M/M_o) on amplitudes (A) of EPR spectra of A-2058 control cells (●), and the cells treated with VPA (○), DMC (□), and both VPA and DMC (△). M, M_o – the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively

Table 1. Integral intensities (I), g-factors, and line widths (ΔB_{pp}) of EPR spectra of the studied A-2058 human *melanoma malignum* cells. Free radicals concentrations in the cells are proportional to the integral intensities (I). The data for the control cells and cells treated with VPA, DMC, and both VPA and DMC, are shown. The EPR spectra were measured with microwave power of 2.2 mW.

Sample	I [a. u.] ± 0.2	g ± 0.0002	ΔB_{pp} [mT] ± 0.02
A-2058 control	18.0	2.0060	1.91
A-2058 + VPA	15.3	2.0060	1.88
A-2058 + DMC	17.9	2.0058	1.90
A-2058 + VPA + DMC	13.3	2.0060	1.86

EPR line the saturation effect is observed. These correlations (Fig. 4) are characteristic for fast spin-lattice relaxation processes in the samples.

DISCUSSION AND CONCLUSIONS

Electron paramagnetic resonance (EPR) measurements indicated that o-semiquinone free radicals exist in both untreated and treated with VPA and DMC A-2058 human *melanoma malignum* cells. Microwaves were absorbed for all the samples located in magnetic field, and EPR spectra were detected (Fig. 2). The characteristic values of g-factors (Table 1) for such type of free radicals (14, 18–21, 24–26) were obtained.

Free radicals in A-2058 tumor cells are located near each others, because their EPR lines are very broad (Table 1). Such located o-semiquinone free radicals strongly dipolar interact, what is responsible for the measured line broadening (26). Strong dipolar interactions were also found in the melanin biopolymers (14–21). EPR lines of the A-2085 cells are homogeneously broadened (Figs. 3 and 4), similar to EPR lines of melanin biopolymers (14, 18–21, 24, 25). Spin-lattice relaxation processes in A-2058 tumor cells were different than those in melanin biopolymers of A-375 and G-361 human *melanoma malignum* cells (12). Spin-lattice interactions in A-2058 cells were fast (Fig. 4), and slow spin-lattice relaxation processes were detected in melanin of A-375 and G-361 cell (12). Probably, these differences results from the magnetic interactions of o-semiquinone free radicals of melanin with the whole structures in A-2085 cells.

Free radicals have unpaired electrons, which are responsible for their biochemical activity (1). Effective drugs should quench free radicals content in cells to avoid pathological transformation. Such positive interactions were observed for the tested VPA (Table 1). Because of the lowest intensities of EPR lines of A-2058 cells after VPA used together with DMC (Table 1), it seems better to use them both. VPA and DMC used together strongly decrease the amount of o-semiquinone free radicals in melanin biopolymers from A-375 and G-361 cells (12). In this study, we obtained the next proof of the effectiveness of VPA, and VPA used together with DMC as the antitumor compounds by the use of electron paramagnetic resonance.

Reports in the literature indicate that DMC and VPA can be a new strategy in the treatment of melanoma (28, 29). 5,7-Dimethoxycoumarin, which is a natural substance widely distributed in the plant kingdom, has a wide spectrum of biological activities. It is a natural chemopreventive agent (28). Furthermore, valproic acid, applied in the treatment of epilepsy, can be used in the chemoprevention and cancer chemotherapy (29).

Taking into account our spectroscopic results for free radicals, it can be concluded that VPA, and VPA applied with DMC together may be used as the antitumor compounds in A-2058 human malignant melanoma cells therapy, because they decrease free radical concentrations in these cells. It is expected that VPA is more effective than DMC, because it strongly quenches free radicals in A-2058 tumor cells, while such effect was not observed for DMC. The lower amounts of free radicals exist in A-2058 cells after treatment with VPA, and VPA applied with DMC together, so the optimal for antitumor therapy is to use VPA or VPA and DMC together. The similar conclusions were obtained by us earlier (12) for VPA, and VPA and DMC combination applied in therapy of G-361 human *melanoma malignum* cells. The usefulness of DMC without the other substance was not confirmed in the case of A-2058 human malignant melanoma cells.

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