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THE EFFECT OF NSC-631570 (UKRAIN) ALONE AND IN COMBINATION WITH PATHOGEN-ASSOCIATED MOLECULES ON CELL CYCLE DISTRIBUTION AND APOPTOSIS INDUCTION OF MOUSE MELANOMA CELLS WITH DIFFERENT BIOLOGICAL PROPERTIES

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Monotherapy and combined application of antitumor drug NSC-631570 (Ukrain) are successfully used for treatment of malignant melanoma since 1996. Melanoma cells of different origin have distinct susceptibility to components of Ukrain. *Aim:* To carry out comparative investigation of the effect of Ukrain used alone and in combination with pathogen associated molecules (PAM) on mitotic cycle and apoptosis induction in mouse melanoma cell lines with different biological properties. *Methods:* Two cell lines with different biological properties (rate of cell division, level of hematogenous metastasis, sensitivity to tumor necrosis factor (TNF)-induced apoptosis) established from B16 mouse melanoma cell line, were used. Apoptosis induction and cell viability were analyzed using trypan blue exclusion test, morphological criteria, DNA gel electrophoresis and flow cytometry. Cell cycle distribution of tumor cells was determined by flow cytometry. Transporters associated with antigen processing (TAP) genes expression was analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR) method. *Results:* The melanoma cells with different metastatic capabilities differed markedly by the growth rate, sensitivity to apoptosis inducers, and the character of TAP gene expression. Treatment of melanoma cells with Ukrain resulted in apoptosis induction in a dose dependent manner. Melanoma cells with high-metastatic properties were more sensitive to Ukrain than their low metastatic variants. However combined use of drug with PAM induced apoptosis more effectively in melanoma cells with low-metastatic potential. *Conclusion:* Sensitivity to Ukrain *in vitro* may depend on biological properties of melanoma cells and may be modified by combined treatment of cells with TLR ligands. The results can be useful to optimize the regimen of mono and combined treatment of melanoma with Ukrain.

Key Words: **antitumor drug NSC-631570 (Ukrain), melanoma cell lines, metastatic capabilities, TAP genes.**

Malignant melanoma incidence increases steadily all over the world. The number of patients with this pathology had been doubled in the last 20 years. At the early stages of melanoma development the patients are treated surgically, but the advanced disease is incurable [1, 2]. Melanoma research experts consider instability of melanoma cell biological properties among the primary reasons of aggressiveness of the tumor and its resistance to antitumor drugs. Melanoma cells with different metastatic ability can differ significantly in their sensitivity to anticancer agents [3–5].

NSC-631570 (Ukrain) is a semisynthetic product of thiosulfuric acid and celandine alkaloids (*Chelidonium majus L.)*. The drug has cytotoxic and cytostatic action on tumor cells *in vitro* and *in vivo* due to its ability to accumulate in tumor tissue selectively and cause cancer cell death but not normal cells death [6, 7]. For more than 20 years NSC-631570 had been used for benign and malignant tumor treatment [8]. Ukrain-based monotherapy, and also its usage in combination with vaccine based on self-restricted autologic dendritic cells demonstrated significant therapeutic effect in the treatment of experimental B-16 mouse melanoma [9, 10].

NSC-631570 monotherapy was successfully introduced into human melanoma therapy in 1996, and has been shown to be effective in combination with adjuvants [11, 12]. However, melanoma cells of different origin possess different sensitivity to Ukrain components [13].

The Toll-like receptor (TLR) agonists are among the most promising adjuvants used for more than 10 years in combined therapy of malignant melanoma. Pathogen associated molecules (PAM), such as peptidoglycane, lipopolysaccharide, flagellin, CpG-DNA etc. are the natural TLR agonists. It is known that TLR are expressed in a broad spectrum of cells and tissues including the effector cells of immune system. Stimulation of TLR-dependent immune cell mediates activation of inflammatory reactions and increases antitumor immunity [14]. TLR are also expressed on many tumor cells including melanoma cells [15, 16]. The use of natural (PAM) and synthetic TLR1, 2, 4, 7 and 9 agonists are shown to be effective for melanoma treatment [17]. In the present study, the extract of *S. aureus Wood 46* CPM was used as PAM. TLR6 in a complex with TLR1 and/or TLR2 is considered as a potential receptor for CPM [18].

The goal of the study was comparative assessment of the effect of NSC-631570 (Ukrain) used alone and in combination with PAM, on the mitotic cycle and

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Abbreviations used: CPM – cytoplasmic membrane extract; MHC – major histocompatibility complex; PAM – pathogen associated molecules; PCR – polymerase chain reaction; RT – reverse transcriptase; TLR – toll-like receptor; TNF – tumor necrosis factor.

apoptosis of melanoma cells which possess various biological properties.

MATERIALS AND METHODS

Cell cultivation, treatment and cell analysis. Mouse melanoma MM-4 and MM-4M2 cell lines were obtained from Bank of Cell Lines of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine. The cells were cultured *in vitro* in Dulbecco modified Eagle medium (DMEM, Sigma, USA), supplemented by 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µl/mL) at 37 °C in 5% CO_2 atmosphere.

To study cell growth kinetics, melanoma cells were seeded on 24-well plates at a density of 1 x 10^4 cells per well in 1.5 ml of medium containing 10% FBS and incubated at standard conditions for 6 days. The number of viable cells was determined daily with Trypan Blue 0.4% staining as described in [19–21].

To test the cytotoxicity of drugs, cells were treated at logarithmic phase of growth with Ukrain (Nowicky Pharma, Austria) at the concentrations of 20 µg/ml and 250 µg/ml for 24–48 h. *Staphylococcus aureus* cytoplasmic membrane extract (CPM), which was kindly granted by prof V.K. Pozur (T. Shevchenko Kyiv National University), was used as PAM and was added in culture medium at the concentrations of 2 µg/ml and 25 µg/ml for 24–48 h. Ukrain (20 µg/ml) + PAM (2 µg/ml) and Ukrain (250 µg/ml) + PAM (25 µg/ml) were used for combined treatment. Solvent was added to the controls.

For a morphologic investigation, cytospin specimens of cells were dried, Pappenheim-stained and analyzed using Axiostar Plus microscope (Carl Zeiss, Germany) at x 400–1000 magnification.

DNA extraction, gel electrophoresis and flow cytofluorimetry were performed as it was described earlier [19–21].

RNA isolation and RT-PCR analysis of TAP1 and TAP2 mRNA expression. Total RNA was isolated from homogenized cells using the "RiboSorb" KIT (AmpliSens, Russia). Extracted RNA was transcribed into cDNA using M-MuLV Reverse Transcriptase (Fermentas, Lithuania) following recommendations of the manufacturer. The cDNA concentration was measured and 2 µl of each cDNA samples were taken for semiquantitative analysis. To circumvent false negative results, primers detecting the GAPDH gene were used in the same reaction tube. Each reaction mixture for PCR contained: Taq-buffer with $(NH_4)_2SO_4$, 2.5 µM Mg^{2+}, 0.2 µM each dNTP, 1U Taq-polimerase (Fermentas, Lithuania), 50 µM of each primer and 2 µl of cDNA. Following primers were used: TAP1 primers — forward 5'GGACTGTCAGCAGCGGCAACC-3' and reverse 5'CAAGGCCTTTCATGTTTGAGGG-3'; TAP2 primers — forward 5'CAGGATGCAGTGGCCAGGGCG-3' and reverse 5'TAGATACACGTCTTTTTCCAGG-3'; GAPDH primers — forward 5'GCCAAGGTCATCCATGACAACTTTGG-3' and reverse 5'GCCTGCTTCACCACCTTCTTGATGTC-3'. PCR program: initial denaturation step at 94 °C, 4 min, and 30 cycles (93 °C 35 s, 60 °C 35 s, 72 °C 35 s). PCR products were visualized by electrophoresis in 2% agarose gels with ethidium bromide using UV-transilluminator. Semiquantitative analysis of the expression level was performed in TotalLab TL120 program (Great Britain).

The statistical significance of the differences between mean values was assessed by the Student's *t*-test.

RESULTS AND DISCUSSION

MM-4 cell line and MM-4M2 subline, which were used as the models in the experiments, were established earlier from transplantable B-16 melanoma of C57BL/6 mice [1]. MM-4 cells have relatively low metastatic ability: the cells rarely metastasize spontaneously when transplanted intramuscularly or subcutaneously, and upon intravenous application of 2 x 10^5 cells at average 10–20 pigmented metastases will be formed in the lungs of 70–100% of animals.

MM-4M2 highly metastatic subline was obtained by double *in vivo* passage of MM-4 cells in C57BL/6 mice: the cells were administered intravenously with further *in vitro* recultivation of the largest lung metastases that developed after 21 days. After the preparative cultivation of metastatic cells, they were intravenously administered to mice, and then the cells from large metastases were again recultivated. The cells of newly established line possessed much higher metastatic potential than the initial cells: the metastases are formed in 100% of animals, their average number reached 206.6 ± 56 (ranging from 68 to 360). MM-4M2 cells don't differ significantly from the initial cell line by morphology and pigmentation, although they have a clear epithelioid morphology and higher adhesion (data not presented). MM-4 and MM-4M2 cell lines dramatically differ by growth rate: doubling time at logarithmic phase of growth for MM-4 cells is 19.3 h, and for MM-4M2 line — 22.7 h; maximal density of MM-4 cell growth reaches 5.7 x 10^5/cm^2, and of MM-4M2 cells — only 2.78 x 10^5/cm^2 (Fig. 1).

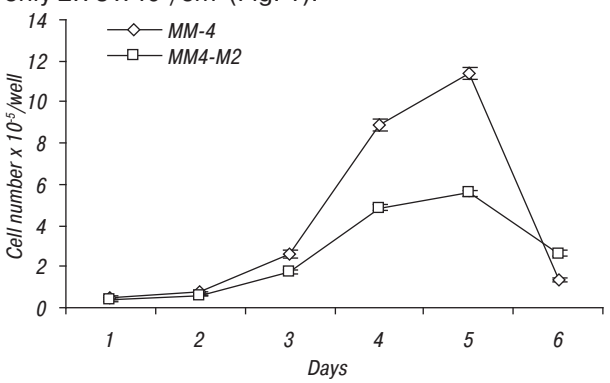

Fig. 1. Cell growth kinetics of MM-4 and MM-4M2 melanoma cell lines

The MM-4 and MM-4M2 cell lines are sensitive to various apoptosis inducers, particularly to tumor necrosis factor (TNF) (Fig. 2, *a*, *b*).

Interestingly, the sensitivity to growth inhibition and apoptosis induction caused by TNF is much higher in MM-4M2 highly metastatic subline than in MM-4 cells. The difference between cell lines is especially expressed at TNF concentrations < 8–10 000 IU/ml, but at higher TNF concentrations

the cells of both lines become equally sensitive to TNF (Fig. 2, *b*; Fig. 3).

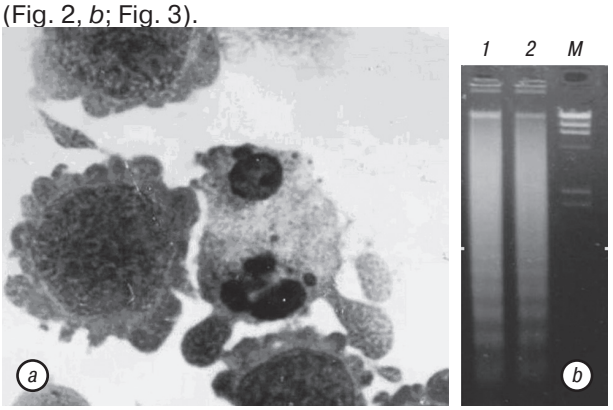

Fig. 2. Apoptosis in MM-4 (*a*) and MM-4M2 (*a*, *b*) cells treated with TNF: *a*, morphology of apoptotic MM4 cells treated with TNF; *b*, DNA laddering of MM4-M2 and MM4 cells treated with TNF

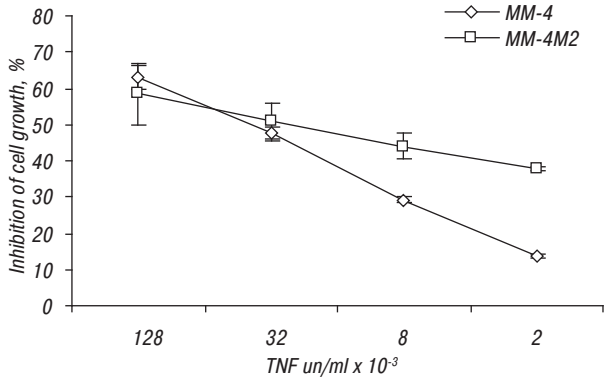

Fig. 3. Inhibition of cell growth of MM-4 and MM-4M2 melanoma cells

Expression of TAP1 and TAP2 in low- and high-metastatic melanoma B16 cells. Level of metastasis is dependent on antigenic characteristics of tumor cells, and an enhanced or decreased antigenicity of disseminated tumor cells will lead to their immune recognition and rejection or, conversely, enhancement of metastasis. TAP proteins (transporters associated with antigen processing) take part in the transport of oligopeptides from proteasomes into endoplasmic reticulum. In the endoplasmic reticulum these oligopeptides are bound to MHC class I molecules and transported to the cell surface. TAP proteins consist of two subunits: TAP1 and TAP2. It has been previously shown that TAP protein expression can be decreased in malignant cells, followed by reduced protein expression or complete lack of MHC class I antigens on the cell surface [11, 12]. Thus, character of TAP proteins expression may be closely linked to metastatic ability of melanoma cells.

RT-PCR analysis revealed a total inhibition of mRNA transcription of the peptide transporter TAP1 in high-metastatic MM-4M2 cells compared to low-metastatic MM-4 cells. mRNA levels of TAP2 had no significant differences in these melanoma sublines (Fig. 4).

TAP1 and TAP2 down-regulation in primary melanoma lesions is associated with the decrease of tumor immunogenicity and significantly correlated with tumor progression and development of metastases [13].

Thus, MM-4M2 cell subline is a useful model which allows to reproduce post-invasive stage of hematogenous metastasis, and also to examine the mechanisms of melanoma cell sensitivity to various programmed cell death inducers.

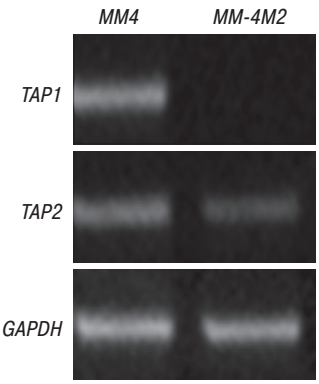

Fig. 4. RT-PCR analysis of TAP mRNA expression in mouse melanoma cells with different metastatic potential

It should be noted that some characteristics of high metastatic MM-4M2 cells, such as a reduced rate of cell proliferation and increased sensitivity to TNF-induced apoptosis contradict, in part, to known characteristics of some metastatic models. It is considered that resistance to TNF is accompanied by an increase of the metastatic ability. However, in this case the sensitivity to TNF did not affect the selection of *in vivo* highly metastatic cells.

In the first part of the study we have examined the effect of Ukrain alone or in combination with CPM on the growth of MM-4 and MM-4M2 cell sublines [18].

The results have shown that the effect of Ukrain used alone or in combination with PAM differs for MM-4 and MM-4M2 cells (at least for 48 h treatment period). Ukrain used alone at low concentration (20 μg/ml) caused significant (treatment time independent) growth inhibition of MM-4 cells at average by 65% ($p < 0.001$) (Fig. 5, *a*). In MM-4M2 cells, the progressive inhibitory action of Ukrain has been observed: the number of viable cells decreases by 64.6% in 24 h and by 79.4% in 48 h ($p < 0.001$) (Fig. 5, *b*). In this case, the sensitivity of MM-4M2 cells to Ukrain was significantly higher ($p < 0.01$) than that of MM-4 cells. At high concentration (250 μg/ml) the drug caused death of nearly 100% cells of both sublines after 24–48 h of incubation.

PAM treatment of MM-4 and MM-4M2 cells influenced their growth only during first 24 h of incubation, but not later. PAM at the low concentration (2 μg/ml) significantly ($p < 0.01$) inhibited cell growth in MM-4 cell line. However, at the high concentration (25 μg/ml) led to the increase of the number of viable cells (see Fig. 5*a*). In MM-4M2 cells, by contrast, increased concentrations of PAM (from 2 to 25 ug/ml) was accompanied by a significant decrease in the number of living cells ($p < 0.01$). The PAM, regardless of concentration after short-time exposure, did not change the sensitivity of both cell lines to the cytotoxic action of Ukrain (Fig. 5, *a*, *b*). Apoptosis inducing effect after prolonged treatment of MM-4 cells with Ukrain (48 h) in combination with PAM was more expressed than that after treatment with Ukraine alone.

It is known that Ukrain attenuates tumor cell growth by inducing arrest of the cells which are at G2/M phase (or G0/G1 phase *in vitro*) with further apoptosis induction [22, 23]. Analysis of cell cycle of MM-4 and MM-4M2 cells

treated by Ukrain *in vitro* has distinctly shown the ability of the drug to modulate the cell cycle in a dose-dependent way differently for the cells with various biological properties (Table). NSC-631570 did not affect apoptosis rate of MM-4 cells at concentration of 20 μg/ml after 24 h of incubation. However, the number of G0/G1 cells increased significantly (by 56% compared to control cells), the number of cells in G2/M phase was 1.4 times lower, and S-phase cells — two times lower, what can point on cell cycle arrest in G0/G1. 24-hour incubation of MM-4 cells with Ukrain at 250 μg/ml concentration resulted in apoptosis of 98.4% cells.

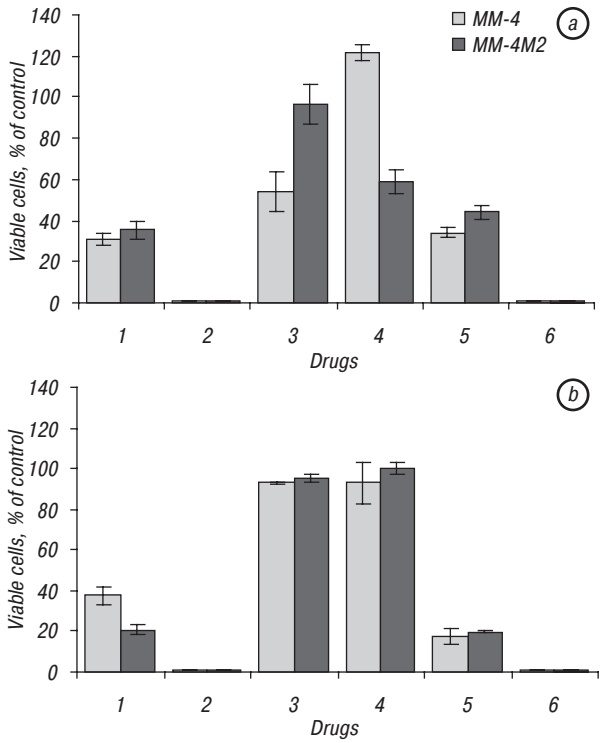

Fig. 5. The effect of Ukrain and PAM on the growth of mouse melanoma cells with different biological properties: 24 h (*a*) and 48 h (*b*): 1 — Ukrain 20 μg/ml; 2 — 250 μg/ml; 3 — PAM 2 μg/ml; 4 — PAM 25 μg/ml; 5 — Ukrain 20 μg/ml + PAM 2 μg/ml; 6 — Ukrain 250 μg/ml + PAM 25 μg/ml

The MM-4M2 cells appear to be more sensitive to NSC-631570 at the concentration of 20 μg/ml: the number of apoptotic cells was 26.67% (4 times higher than in MM-4 cells). Moreover, the number of cells in G0/G1 phase has increased while the number of G2/M and S-phase cells has dropped. Incubation of MM-4M2 cells with Ukrain for 24 h at 250 μg/ml concentration resulted in apoptosis of 97.2% of cells.

Prolonged (for 48 h) MM-4 cells incubation with Ukrain at the concentration of 20 μg/ml has resulted in the increase of apoptotic cell number (from 1% of control to 25%). Apoptosis rate in MM-4M2 after 48-hour incubation with NSC-631570 at concentration of 20 μg/ml was lower than that after short-term (24-hour) exposure, though 15 times higher than that in the control cells. Ukrain caused stable apoptotic effect in both cell lines at the concentration of 250 μg/ml after 48 h of incubation.

Thus, melanoma cells with different metastatic potential are characterized by different sensitivity to NSC-631570. Short-term drug treatment at low concentrations had caused the apoptosis of significant number of MM-4M2 cells only. The response of MM-4 cells to Ukrain had developed more slowly (after 48-hour of incubation). The results suggest that sensitivity of melanoma cells to apoptosis induced by TNF may coincide with sensitivity to Ukrain and possibly may have some common mechanisms. These biological patterns of the cells may be useful for the development of therapeutic schedule of NSC-631570 drug usage.

Table. Cell cycle distribution in low- (MM-4) and high-metastatic (MM-4M2) cell line after their treatment with Ukrain and PAM *in vitro*

Cells and treatment	Cell number (%) in cell cycle phases			
	Apoptosis	G0/G1	S	G2/M
	Incubation for 24 h			
Control MM-4	4.73 ± 0.14	44.06 ± 2.12	30.68 ± 0.99	25.26 ± 1.94
Ukrain 20 μg/ml	5.66 ± 0.18	68.74 ± 1.60	13.39 ± 1.07*	17.87 ± 0.98*
Ukrain 250 μg/ml	98.4 + 0.06 *		Apoptosis	
PAM 2 μg/ml	1.13 ±0.02*	43.55 ± 1.83	32.68 ± 0.88	23.76 ± 2.00
PAM 25 μg/ml	1.66 ±0.02*	49.10 ± 1.99	32.49 ± 0.76	18.41 ± 1.34
Ukrain 20 μg/ml + PAM 2 μg/ml	29.2 ± 1.94*	70.15 ± 2.09*	17.16 ± 0.99*	12.69 ± 1.10*
Ukrain 250 μg/ml + PAM 25 μg/ml	94.46 ± 0.09*		Apoptosis	
Control MM-4M2	5.61 ±0.11	44.64 ± 2.17	33.64 ± 2.01	21.72 ± 0.21
Ukrain 20 μg/ml	26.67 ± 0.79*	67.7 ± 1.60*	16.20 ± 0.98*	16.10 ± 1.04*
Ukrain 250 μg/ml	97.18 ± 0.09		Apoptosis	
PAM 2 μg/ml	1.96 ± 0.07*	48.06 ± 2.98	18.49 ± 1.00	33.44 ± 1.01
PAM 25 μg/ml	6.45 ± 0.09	44.45 ± 1.06	21.20±0.86	34.34 ± 1.07
Ukrain 20 μg/ml + PAM 2 μg/ml	9.22 ± 0.09*	74.62 ± 1.35*	13.02 + 1.00	12.36 ± 1.01
Ukrain 250 μg/ml + PAM 25 μg/ml	96.82 ± 1.00*		Apoptosis	
	Incubation for 48 h			
Control MM-4	1.06 ± 0.08	72.43 ± 3.08	15.54 ± 1.16	12.03 ± 1.10
Ukrain 20 μg/ml	24.98 ± 1.94*	66.38 ± 2.96	16.76 ± 1.12	16.86 ± 1.10*
Ukrain 250 μg/ml	97.74 ± 1.04*		Apoptosis	
PAM 2 μg/ml	0.61 ± 0.02*	77.20 ± 1.98	8.01 ± 1.31*	14.79 ± 1.20
PAM 25 μg/ml	0.61 ± 0.05*	78.32 ± 3.11	7.11 ± 1.12*	14.57 ± 1.22
Ukrain 20 μg/ml + PAM 2 μg/ml	18.73 ± 1.00*	63.95 ± 2.99	24.97 ± 0.85 *	11.08 ± 1.02
Ukrain 250 μg/ml + PAM 25 μg/ml	92.51 ± 1.00*		Apoptosis	
Control MM-4M2	0.58 ± 0.03	64.29 + 2.08	22.72 ± 1.55	13.00 ± 1.01
Ukrain 20 μg/ml	8.83 ± 0.99*	68.81 ±2.08	15.85 ± 1.48	15.34 ± 1.11
Ukrain 250μg/ml	83.80 ± 3.06*	63.88 + 1.77	23.41 ± 2.00	12.71 ± 3.16
PAM 2 μg/ml	0.57 ± 0.08	69.10 ± 2.68	16.06 ± 1.90	14.85 ± 1.05
PAM 25 μg/ml	0.64 ± 0.02	69.65 ± 2.41	17.53 ± 1.10	12.92 ± 1.10
Ukrain 20 μg/ml + PAM 2 μg/ml	14.28 ± 1.07*	72.59 ± 1.36*	14.70 ± 0.96*	12.64 ± 2.13
Ukrain 250 μg/ml + PAM 25 μg/ml	78.95 ± 3.03*	68.03 ± 1.42	17.54 ± 1.29	14.43 ± 1.16

*Difference is significant compared to the control ($p < 0.05$).

Nowadays information concerning TLR agonists effect on cell cycle distribution is quite scanty and controversial. It is known that TLR activation by natural ligands causes increase of malignant cell proliferation under certain conditions [24, 25]. It is recently shown that TLR agonists can activate apoptosis of tumor cells [26]. Our experiments have shown that in almost all cases and regardless of the dose, PAM causes suppression of basal level of apoptosis in MM-4 cells. The PAM response pattern of MM-4M2 subline differed from that of MM-4 cells. Exposure of MM-4 cells to 2 μg/ml PAM for 24 h had led to decrease of the apoptosis level by 65%. Usage of high PAM concentration (25 μg/ml) did not cause any reliable influence on mitotic cycle of MM-4 cells. Reliable influence on cell cycle was also absent when the exposure was prolonged.

Thus, B16 melanoma cell sublines which possess different biological properties, had different response to TLR agonists. We suggest that it could be considered upon its usage in antitumor adjuvant immunotherapy, especially in the case of low metastatic tumor variant.

However, PAM treatment causes different effect on cell cycle in melanoma cell sublines if it used in combination with Ukrain.

Ukrain at the concentration of 20 μg/ml combined with PAM at the concentration of 2 μg/ml caused increased MM-4 tumor cell death rate. The number of apoptotic cells in some samples was 6 times higher in comparison to the control, 5 times higher compared to Ukrain-treated samples and 22 times higher than that in PAM-treated samples (see Table).

Effect of Ukrain and PAM combination (20 μg/ml and 2 μg/ml correspondingly) on highly metastatic MM-4M2 cells was weaker than that of Ukrain used alone after shot-term exposure. However, when the exposure was prolonged, apoptosis inducing effect of combined treatment was more expressed than that after treatment of MM-4M2 cells with Ukrain alone.

Total apoptosis was observed in the cells of both cell sublines treated by combination of Ukrain and PAM at high concentrations (250 μg/ml and 25 μg/ml correspondingly), as well as in the samples treated with Ukrain at the same concentration. These data have demonstrated that the PAM does not reduce the apoptotic effect of Ukrain.

Thus, melanoma cells with different metastatic potential had different response pattern to NSC-631570 (Ukrain) used alone or in combination with PAM. 24-hour treatment with drug at the low concentration resulted in considerable death rate of MM-4M2 cells only. MM-4 cells were more sensitive to combined treatment at low concentrations. It is possible that such effect could be caused by different TLR expression level in these cells.

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