

## CHANGES IN EXPRESSION OF TLR-4, TGF- $\beta$ , INF- $\gamma$ , TNF- $\alpha$ IN CULTURED T24/83 CELLS OF INVASIVE BLADDER CANCER TREATED WITH CISPLATIN AND/OR POLYPHENOLIC ADJUVANT MELANIN

P.G. Yakovlev<sup>1,\*</sup>, O.I. Gorbach<sup>2</sup>, N.M. Khranovska<sup>2</sup>, A.V. Beliaeva<sup>3</sup>, O.V. Skachkova<sup>2</sup>, T.D. Skaterna<sup>4</sup>,  
L.G. Kalachniuk<sup>5</sup>, L.I. Ostapchenko<sup>3</sup>, L.V. Garmanchuk<sup>3,\*</sup>

<sup>1</sup>Clinical Hospital “Feofaniya”, Center of Urology and Oncourology, Kyiv 03143, Ukraine

<sup>2</sup>National Cancer Institute, Kyiv 03022, Ukraine

<sup>3</sup>Taras Shevchenko National University of Kyiv, Kyiv 01033, Ukraine

<sup>4</sup>Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, Kyiv 01054, Ukraine

<sup>5</sup>National University of Life and Environmental  
Sciences of Ukraine, Kyiv 03041, Ukraine

**Background:** Toll-like receptor 4 (TLR4) is known to be involved in carcinogenesis and cancer progression. Changes in TLR4 expression are associated with changes in the expression of key cellular cytokines (transforming growth factor- $\beta$  (TGF- $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (INF- $\gamma$ )), which affect cancer progression and metastasis. **Aim:** To study changes in the expression of *TLR4*, *TGF- $\beta$* , *TNF- $\alpha$* , *INF- $\gamma$*  genes, the level of apoptosis and cell cycle distribution in human invasive urothelial carcinoma T24/83 cells under the treatment with polyphenolic adjuvant compound of fungal origin melanin, cytotoxic drug cisplatin, and combination of both. **Materials and Methods:** T24/83 cells were incubated with cisplatin (0.05 mM), melanin (5  $\mu$ g/ml), or their combination. The expression level of *TLR-4*, *TGF- $\beta$* , *INF- $\gamma$* , *TNF- $\alpha$*  was evaluated by the real time polymerase chain reaction. The flow cytometry was used to study cell cycle distribution, proliferative activity and level of apoptosis. Morphological analysis of the T24/83 cells was performed as well. **Results:** Melanin, cisplatin, and their combination downregulate *TLR4* expression (2.67; 1.28; and 2.73-fold decrease, respectively) and *TNF- $\alpha$*  expression (6.5; 1.4; and 1.7-fold decrease, respectively). Melanin did not affect *TGF- $\beta$*  expression while cisplatin caused 13-fold downregulation of *TGF- $\beta$* . The combined use of cisplatin and melanin decreased *TGF- $\beta$*  expression by 6.5 times. The upregulation of *INF- $\gamma$*  by melanin, cisplatin, and their combination was demonstrated (4.3; 6.7; and 2-fold increase, respectively). All treatment modalities increased the level of apoptosis in T24/83 cells. Melanin treatment increased significantly the proportion of fibroblast-like cells in T24/83 culture with decreased cell adhesion to the substrate. **Conclusions:** Melanin, cisplatin, and combination of both agents affect significantly *TLR4*, *TNF- $\alpha$* , *TGF- $\beta$* , *INF- $\gamma$*  expression, cell cycle distribution and morphology in T24/83 cells suggesting their transition to less aggressive phenotype. **Key Words:** melanin, urothelial carcinoma, cell line T24/83, invasive bladder cancer, TLR4, TGF- $\beta$ , INF- $\gamma$ , TNF- $\alpha$ , apoptosis.

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Toll-like receptors (TLR) belong to the family of transmembrane sensors of cell death and tissue remodeling, which recognize the molecular fragments of bacterial and cellular origin, modify host immune response, affect the cell cycle, and promote cell proliferation [1]. The classical effects of TLR activation are enhancement and modification of the innate and acquired immune response depending on the tissue context [2–9].

Cancer immunotherapy has been the focus of intense research since the late 19<sup>th</sup> century when Coley observed that bacterial components could contribute to cancer regression by eliciting an antitumor immune response. Successful activation and maturation of tumor-specific immune cells is now known to be mediated by bacterial endotoxin, which activates TLR4 [10].

The TLR4 is the most studied of these pattern recognition receptors family members, present on the

immune and cancer cells. TLR4 recognizes pathogen-associated molecular patterns, such as Gram-negative bacterial lipopolysaccharide, endogenous damage-associated molecular patterns like fibronectin and hyaluronan, which are released during infectious and non-infectious inflammatory conditions [11]. Some chronic infections and inflammatory conditions are known to promote carcinogenesis and cancer progression through persistent activation of TLR4-induced inflammatory signaling [1, 4, 10, 12].

Recognition of non-self molecular patterns by pattern recognition receptors is a cornerstone of innate immunity. TLRs regulate a wide range of biological responses including inflammatory and immune responses during carcinogenesis. The high expression of TLRs by antigen-presenting cells, including dendritic cells, and their ability to induce antitumor mediators such as type I interferon has led to the efforts to utilize TLR agonists in cancer therapy in order to convert the tolerant immune response toward antitumor responses. However, TLRs are also recognized as regulators of tumor-promoting inflammation and promoters of tumor survival signals [1].

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\*Correspondence: E-mail: Dr.Yakovlev.Urology@gmail.com  
liudmyla\_garmanchuk@ukr.net

**Abbreviations used:** INF- $\gamma$  – interferon gamma; PCR – polymerase chain reaction; T24/83 – cell line of invasive urothelial carcinoma; TGF- $\beta$  – transforming growth factor beta; TLR – toll-like receptor; TNF- $\alpha$  – tumor necrosis factor alpha.

Activation of TLR4 can have opposing effects. While TLR4 activation can promote antitumor immunity, it can also result in increased tumor growth and immunosuppression. Nevertheless, TLR4 engagement by endotoxin as well as by endogenous ligands represents notable contribution to the outcome of different cancer treatments, such as radiation or chemotherapy. Further research of the role and mechanisms of TLR4 activation in cancer may provide novel antitumor vaccine adjuvants as well as TLR4 inhibitors that could prevent inflammation-induced carcinogenesis [10].

In the scenarios of established cancer, TLR4 facilitates an environment that is suitable for continued cancer cell proliferation. Pro-cancer mechanisms could include the evasion of cancer cells from immune surveillance [1, 6, 13, 14].

Immune response to any pathogenic stimuli includes activation of innate immunity, inflammation, and adaptive immunity. TLR4 signaling can eventually lead to a multitude of cellular effects, including the metastatic progression as the most deadly outcome of cancer [15]. Experimental evidence suggests that cancer cell migration and invasion are induced by triggering TLR4-NF- $\kappa$ B pathway under inflammatory conditions [16]. The blockade of TLR4 by siRNA and NF- $\kappa$ B inhibitors decreases the invasive ability of cancer cells [17]. Correspondingly, TLR4 silencing has been shown to decrease tumor burden in a murine model of colorectal metastasis and hepatic steatosis [18].

The persistence of tumor and chronic activation of the TLR4 can cause the production of pro-inflammatory cytokines, which may mimic the normal immune response. Change in the expression of the TLR4 is associated with the change in the expression of the key cellular cytokines (transforming growth factor- $\beta$  (TGF- $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ )), which affect progression of the cancer, enhance the mobility of cancer cells, and promote the metastatic spread. Activation of TLR4 upregulates TGF- $\beta$ 1 and IL-10, which leads to the active cellular migration, ensuing in the metastatic growth in Lewis lung carcinoma [19].

IFN- $\gamma$  can decrease tumor growth by acting not only directly on cancer cells, but also indirectly on endothelial cells causing ischemia and prompting immune cells in the tumor microenvironments [20].

TLR4 can be viewed as key player in the development of cancer. The drugs or adjuvants, which affect the expression or activity of the TLR4 deserve close attention and further clinical research.

It is known that polyphenolic compound melanin of herbal origin can downregulate the expression of TLR4 in colorectal carcinoma and its metastatic variant [21].

The high grade muscle invasive urothelial cancer belongs to one of the most aggressive diseases with poor prognosis due to the metastatic progression. It was observed that lower expression of TLR4 on the surface of bladder cancer cells correlates with less invasive potential of the bladder tumor [5]. We used invasive urothelial carcinoma cell line T24/83 as a screening model to study the effects and mechanisms of antitumor activity of polyphenolic adjuvant compound of fungal origin melanin in combination with the cytotoxic drug cisplatin based on the changes in the expression of TLR4, TNF- $\alpha$ , TGF- $\beta$ , IFN- $\gamma$ , cell morphology and apoptosis rate in cell culture. The aim of our study was to analyze changes in the expression of the TLR4, TGF- $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , level of apoptosis and cellular morphology in T24/83 cells of human invasive urothelial carcinoma under the treatment with melanin, cisplatin, and combination of both.

## MATERIALS AND METHODS

**Cell line and agents.** Cell line of invasive urothelial carcinoma T24/83, N85061107 (human bladder carcinoma) was obtained from Sigma, Inc. (USA). Cytotoxic drug cisplatin was obtained from Ebewe (Austria), polyphenolic compound melanin was obtained from Institute of Biology and Medicine of Taras Shevchenko Kyiv National University [22]. Concentration of cisplatin for experiment was 0.05 mM, concentration of melanin was 5  $\mu$ g/ml. T24/83 cells were incubated under standard conditions of 100% of humidity, 37 °C, and 5% of CO<sub>2</sub> in RPMI-1640 medium (Sigma, USA) supplemented with 10% FBS (Sigma, USA), 2 mM L-glutamine, and 40  $\mu$ g/mL gentamicin. Cisplatin and melanin at the indicated concentrations were added for 2 days.

**TLR-4, TGF- $\beta$ , INF- $\gamma$ , TNF- $\alpha$  expression levels** were evaluated by real-time polymerase chain reaction (PCR) on 7500 Real-Time PCR Systems (Applied Biosystems, USA) using specific primers and fluorochrome SYBR Green (Applied Biosystems, USA). GAPDH was used to normalize levels of mRNA for the relative quantification method of analysis. The sequences of TLR-4, TGF- $\beta$ , INF- $\gamma$  and TNF- $\alpha$  primers were constructed by Primer Express® Software v3.0 (Applied Biosystems, USA) (Table 1). Calculations were performed using the  $\Delta$ Ct relative quanti-

**Table 1.** Characteristics of the primers used in the experiment

| Subject of study | Sequence of primers                                         | Annealing temperature of primers (T <sub>m</sub> ), °C | Concentration of primer |
|------------------|-------------------------------------------------------------|--------------------------------------------------------|-------------------------|
| GADPH            | f-GCCAAGGTCATCCATGACAACTTTGG<br>r-GCCTGCTTCACACCTTCTTGATGTC | 60                                                     | 0.25                    |
| TLR-4            | f-AAATTTCGCTTCCTGGTCT<br>r-TCAGCCCATATGTTTCTGGA             | 60                                                     | 0.25                    |
| TGF- $\beta$     | f-GGACATCAACGGGTCACTA<br>r-CCGGTTCATGCCATGAATGG             | 60                                                     | 0.25                    |
| INF- $\gamma$    | f-CCAACGCAAAGCAATACATGA<br>r-TTTTCGCTTCCTGTTTAGCT           | 60                                                     | 0.25                    |
| TNF- $\alpha$    | f-CCCAGGCAGTCAGATCATCTTC<br>r-AGCTGCCCTCAGCTTGA             | 60                                                     | 0.25                    |

fication method. mRNA expression value was calculated by the formula:

$$x = 2^{-\Delta Ct}$$

where  $x$  — mRNA expression value,  
 $\Delta Ct = Ct(\text{GAPDH}) - Ct(\text{target gene})$ .

All tests were run in triplicate.

**Total RNA** was isolated by phenol–chloroform extraction using the Ribo-zol kit (AmpliSens). RNA concentration in all samples was measured by ThermoScientific NanoDrop-1000 (Thermo Fisher Scientific, USA) and samples were diluted to 200 ng/μL. cDNA was obtained from total RNA by RT-PCR using “High Capacity cDNA Reverse Transcription Kit” (Applied Biosystems, USA). The reverse transcription reaction was run under the following conditions: 25 °C — 10 min, 37 °C — 120 min and 85 °C — 5 s. DNA was diluted 2-fold with DNA buffer.

**Cell cycle distribution, and apoptotic level** were evaluated by flow cytometry.  $5 \cdot 10^5$  cells were used to prepare one sample. Cell suspension was pelleted by centrifugation at 400 g for 5 min and twice washed with phosphate buffered saline (PBS, pH 7.2). Cells were re-suspended in 200 μL PBS with next addition of 300 μL 0.1% Triton in citrate buffer (pH 6.8). In 1 min, 10 μL of ribonuclease and 10 μL of propidium iodide (Sigma, USA) were added to the samples. The samples were incubated for 10 min at 37 °C and 30 min at room temperature in the dark. Samples were centrifuged at 400 g for 10 min and supernatant was removed. The pellet was fixed by adding 400 μL of 0.4% formalin solution in PBS. Sample measurements were performed no later than in 3 days on flow cytometer FACS Calibur (Becton Dickinson, USA) with 488 nm argon laser and 582/42 nm narrowband filter in order to measure the fluorescence of PI. Flow cytometry data were analyzed using software Mod Fit LT 3.0 (BDIS, USA). The method of apoptosis level determination is based on DNA loss during programmed cell death due to its internucleosomal fragmentation. Flow cytometry evaluates the percentage of cells in the hypodiploid zone of the histogram, which consists of cells which lost DNA due to apoptosis [23].

**Morphological analysis.** Morphological analysis was performed by optical microscopy as we described earlier [24]. For this purpose, T24/83 cells were stained with Böhmer hematoxylin and May-Grünwald dyes (Alfarus, Ukraine).

**Statistical analysis.** Gaussian distribution of the group was checked with Shapiro-Wilk test. Statistical analysis included Mean  $\pm$  SE. To compare the data in four groups, we used One-way ANOVA with Tukey post-hoc test. Null-hypothesis of variables equality was rejected when  $p < 0.05$ . Statistical analysis was performed using Statistica 10.0 software package (Stasoft Inc., USA).

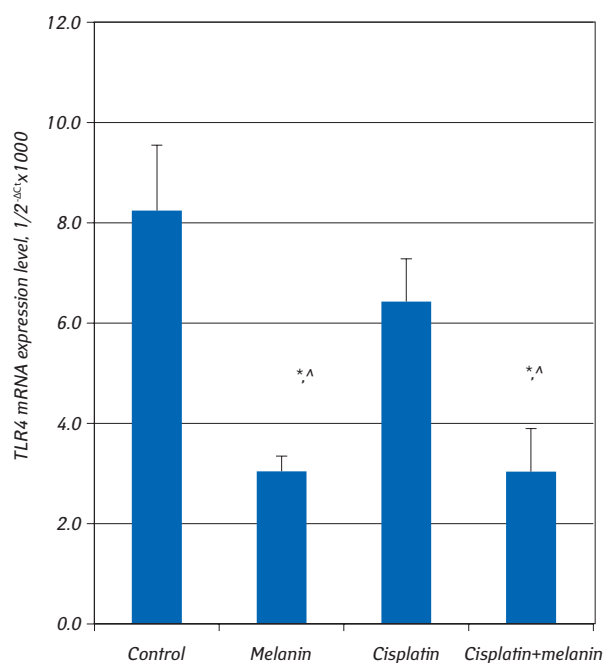
## RESULTS

TLR4 expression in T24/83 cells exposed to melanin, cisplatin or their combinations is presented in Fig. 1. Melanin and combination of melanin with cisplatin down-regulate TLR4 expression by 2.67 and 2.73 ( $p < 0.05$ )

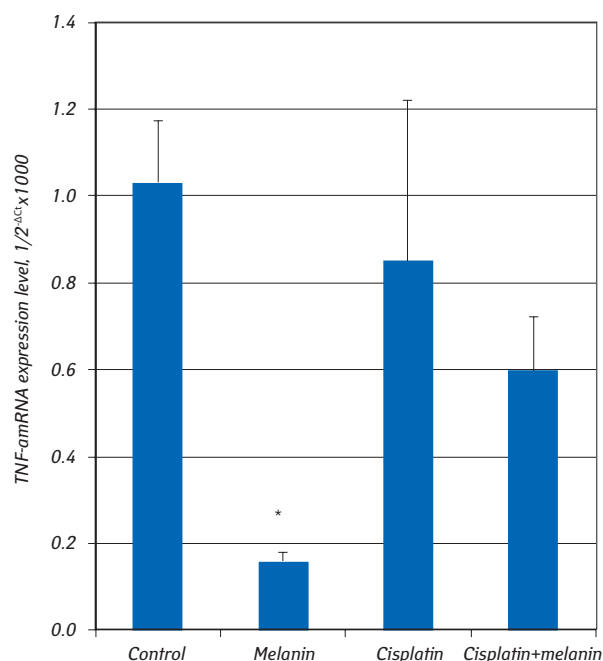
times, respectively, compared to control, and by 2.13 times compared to cisplatin ( $p < 0.05$ ). The application of cisplatin decreases TLR4 expression by 1.28 times ( $p < 0.05$ )

Fig. 2 demonstrate 6.5-fold suppression of TNF- $\alpha$  expression in the cell culture under the treatment with melanin ( $p < 0.05$ ), while combination of both compounds or cisplatin only led to reduction by 1.7 and 1.4 times, respectively ( $p < 0.05$ )

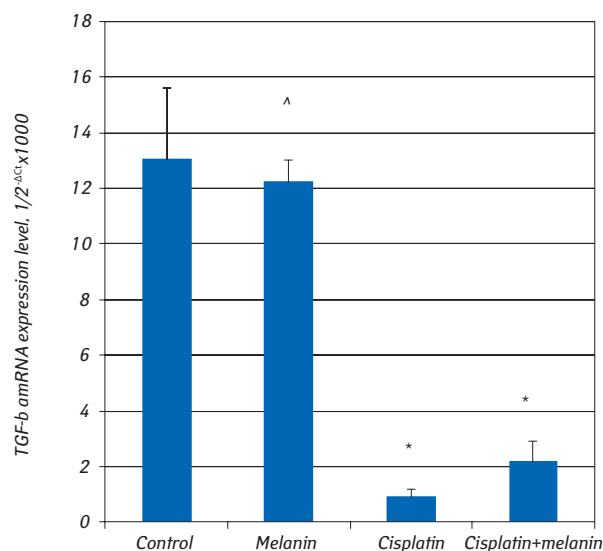
Fig. 3 demonstrates statistically significant (13-fold) downregulation of TGF- $\beta$  in T24/83 under the treatment with cisplatin, and by 6.5 times under



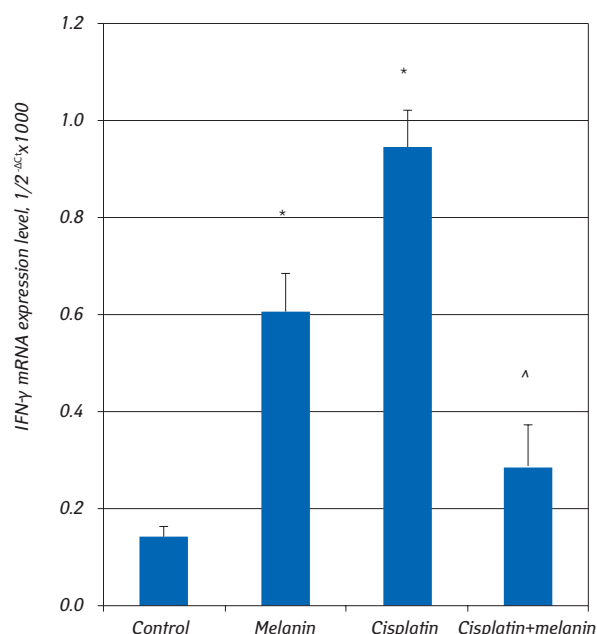
**Fig. 1.** Expression of TLR4 in the urothelial cancer cell line T24/83 under the treatment with melanin, cisplatin and combination of both compounds. \* $p < 0.05$  vs control; ^ $p < 0.05$  vs cisplatin



**Fig. 2.** Expression of TNF- $\alpha$  in the urothelial cancer cell lines T24/83 under the treatment with melanin, cisplatin and combination of both compounds. \* $p < 0.05$  vs control



**Fig. 3.** Expression of TGF-β in the urothelial cancer cell line T24/83 under the treatment with melanin, cisplatin and combination of both compounds. \* $p < 0.05$  vs control; ^ $p < 0.05$  vs cisplatin

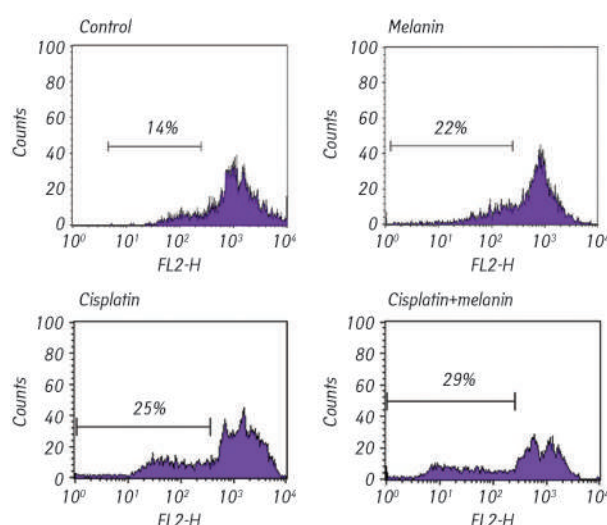


**Fig. 4.** Expression of IFN-γ in the urothelial cancer cell line T24/83 under the treatment with melanin, cisplatin and combination of both compounds. \* $p < 0.05$  vs control; ^ $p < 0.05$  vs cisplatin

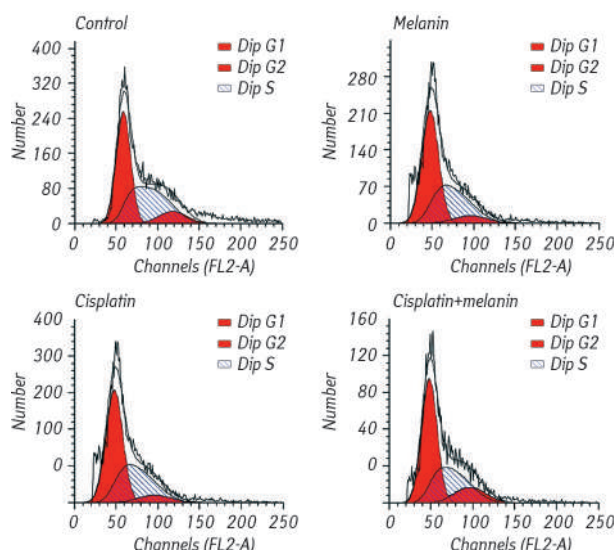
combination of melanin with cisplatin compared to control ( $p < 0.05$ ). Melanin did not affect the TGF-β expression.

Expression of IFN-γ in the cell culture of invasive bladder carcinoma T24/83 under the treatment with melanin, cisplatin and combination of both is presented in Fig. 4. Presented data demonstrate significant upregulation of IFN-γ in the cell line under the treatment with both melanin (by 4.3 times,  $p < 0.05$ ) and cisplatin (by 6.7 times,  $p < 0.05$ ). Combination of melanin with cisplatin only doubled the expression of IFN-γ in the cellular culture ( $p < 0.05$ ).

The fraction of apoptotic cells is represented in Fig. 5 and Table 2. We found that highest and sta-



**Fig. 5.** Changes in the level of apoptosis estimated as a percentage of hypodiploid cells from T24/83 urothelial cancer cells under the treatment with cisplatin, melanin and combination of both compounds.  $p < 0.05$  vs control



**Fig. 6.** Histogram of cell cycle in the urothelial cancer cell line T24/83 under the treatment with melanin, cisplatin and combination of both compounds

tistically significant level of apoptosis was achieved with combined application of melanin with cisplatin (by 2.1 times), and cisplatin (by 1.8 times) ( $p < 0.05$ ).

The results of cell cycle distribution analysis are given in Fig. 6 and Table 2. Melanin demonstrates the suppression of mitosis (by 1.5 times,  $p < 0.05$ ), while cisplatin increased the number of cells in mitosis (by 2.6 times,  $p < 0.05$ ). Cisplatin exerts the most suppressive effect at the phase of DNA synthesis (by 2.4 times), while the number of cells in G<sub>0</sub>/G<sub>1</sub> increases [1, 4, 10, 25–27]. The combination of both studied compounds demonstrates moderate effect on the cell cycle distribution.

The morphology of T24/83 cells treated with melanin, cisplatin, or combination of both is depicted in Fig. 7. Intact cells have typical for this line mixed epithelial-fibroblast-like morphology (Fig. 7, a) [28], demonstrating a polymorphic set of cells with protrusions, among which there are both spread polygonal

**Table 2.** The level of cell apoptosis and distribution per phases of the cell cycle in the urothelial cancer cell line T24/83 under the treatment with melanin), cisplatin and combination of both compounds)

| Test agent          | Apoptotic cells (%) | Cell cycle phases (%)          |                           |                            |
|---------------------|---------------------|--------------------------------|---------------------------|----------------------------|
|                     |                     | G <sub>0</sub> /G <sub>1</sub> | G <sub>2</sub> /M         | S                          |
| Control             | 14.43 ± 0.91        | 40.45 ± 0.87                   | 5.36 ± 0.34               | 39.73 ± 1.43               |
| Melanin             | 23.72 ± 5.00*       | 39.86 ± 1.30* <sup>^</sup>     | 3.16 ± 1.03* <sup>^</sup> | 33.97 ± 0.17* <sup>^</sup> |
| Cisplatin           | 25.12 ± 4.70*       | 48.01 ± 0.65*                  | 12.29 ± 2.07*             | 14.71 ± 0.46*              |
| Cisplatin + melanin | 28.9 ± 3.21*        | 41.14 ± 0.87* <sup>^</sup>     | 6.75 ± 0.63* <sup>^</sup> | 24.10 ± 2.21* <sup>^</sup> |

Note: \* $p < 0.05$  vs control; <sup>^</sup> $p < 0.05$  vs cisplatin

cells (epithelioid morphology, about 60% of cells in the field of view) (Fig. 7, a) and elongated spindle cells (fibroblast-like, about 40% of cells) (Fig. 7, b). All cells show a high degree of adhesion to the substrate. The cells have a well-defined large nucleus with a nuclear-cytoplasmic ratio of 1:1 — 1:2 and 2–4 well-defined nucleoli, which indicates a high functional activity of the nuclei. In the field of view, up to 10 cells are in the state of apoptosis (with a pyknotic nucleus) (Fig. 7, c), and 5–7 binuclear cells (Fig. 7, a) are observed.

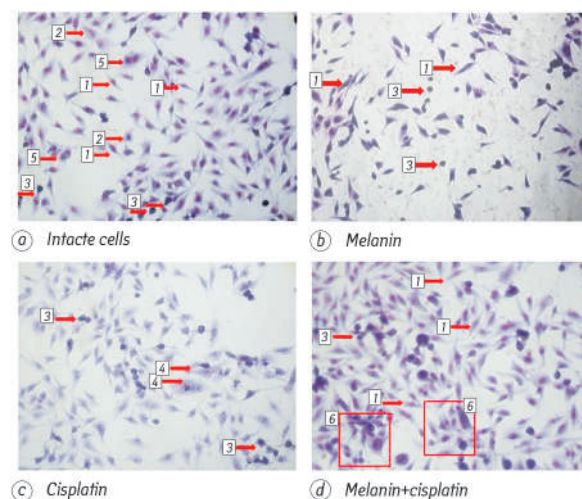
The treatment with melanin significantly increases the proportion of fibroblast-like cells (up to 80%), and decreases the number of cell protrusions, the degree of cell stratification and adhesion to the substrate. Almost all cells have a compact nucleus with high hyperchromia, which indicates their functional deactivation. The number of cells in the state of apoptosis in the field of view increases to 10–15, i.e. is 2–3 times higher than in the intact cells. In cells treated with cisplatin, the morphology of the nucleus (in particular, its color, the distribution of heterochromatin and the number of nucleoli) is maintained at a level close to control cells. The ratio of epithelioid and fibroblast-type cells is preferably preserved. However, cell size variability increases (anisocytosis), and individual giant cells appear. The number of cells with morphological signs of apoptosis in comparison with intact cells also increases by 30–50% in different fields of view.

In general, morphological changes in cells treated with both compounds are closer to the effects of cisplatin (Fig. 7, d). The proportion of fibroblast-like cells is 70–80%. The degree of stratification and adhesion of cells to the substrate is reduced. The number of cells in the state of apoptosis is 15–20 in the field of view. There is a decrease in the number of cell protrusions and pronounced cellular anisocytosis (significant differences in cell size). Nuclear-cytoplasmic ratio is 1:1 — 1:2, which coincides with the control values. The control indicators also correspond to the general morphological features of the nucleus — the degree of hyperchromia and basophilia of the nuclei, the visibility and number of nucleoli.

Summarizing the morphological changes in T24/83 cells under the treatment of the studied compounds, their effects can be ranged as follows: Intact cells < cisplatin ≤ cisplatin + melanin < melanin.

## DISCUSSION

The carcinogenesis and progression of established cancer are strictly controlled by the host immune system, which employs multiple cytokines and their receptors [1, 4, 10, 26, 27]. The tumor and its microenvironment produce cytokines themselves in the process called immune mimicry enabling cancer cells to evade



**Fig. 7.** Morphological changes in the urothelial cancer cell lines T24/83 in control (7, a) and under the application of melanin (7, b), cisplatin (7, c) and combination of both compounds (7, d); 1 — fibroblast-like cells; 2 — spread epithelioid cells; 3 — cells with morphological signs of apoptosis; 4 — giant cells; 5 — binuclear cells; 6 — cells with pronounced anisocytosis

the immune surveillance [29–31]. Understanding the interplay at the different stages of cancer growth between key receptors and downstream cytokines regulating the cell cycle in the tumor and its microenvironment will help better manage cancer and its metastatic potential. One of the major challenges in cancer biology is a search for agents and their combinations, which can abrogate the growth of the cancer cell in the long run, downregulate the expression of pro-proliferative molecular stimuli, prevent the migration and ensuing metastatic dissemination of cancer cells.

In this study, we estimated changes in the expression of the panel of cytokines and key cellular receptor TLR4, engaged in immune control of tumor growth, in T24/83 cells of invasive urothelial cancer upon exposure to cytotoxic chemotherapeutic drug cisplatin, and polyphenolic adjuvant compound melanin originating from the fungus *Nadsodniela nigra sp.*, which has capacity to modify cell growth. We found that the application of cisplatin, melanin and combination of both agents affects substantially the expression of TLR4, TNF- $\alpha$ , TGF- $\beta$ , and INF- $\gamma$ , redistribution of cells through the phases of cell cycle, and the level of apoptosis.

In our previous work, we have demonstrated cytostatic and cytotoxic effects of melanin on estrogen-dependent breast cancer cells MCF-7 and cervical carcinoma cells HeLa with accompanying increase of the adhesive capacity of these cells hinting at possible diminishment of their migratory and invasive properties [32].

Cisplatin is the basic chemotherapeutic drug in modern oncological practice. However, because of drug resistance and numerous undesirable side effects the use of cisplatin might have been restricted and combination with other agents have been searched for [33]. Previously, we have shown that melanin enhanced dose dependently the growth inhibitory effects of cisplatin on T24/83 cells [34].

Cancer cells and their microenvironment produce wide range of cytokines, which mimic the normal immune response, which allow the tumor evade the host immune surveillance through the suppression of the host immune response, in particular through the suppression of functional activity of immune cells and their number in the peripheral blood. We demonstrated progressive decline of absolute and relative lymphocyte count in peripheral blood with progression of urological cancer from stage I to stage IV as reflection of deterioration of immune defense in cancer patients [35]. The TLR4 is one of key receptors engaged in regulating immune response of the host. Domenis *et al.* [31] showed that higher level of TLR4 expression was typical for high grade, high stage invasive carcinoma of the bladder, higher capability of cancer cells to evade the immune surveillance, and acquiring the higher motility. Low levels of TLR4 expression were associated with low grade carcinoma and more benign course of the disease [36].

In current study, we demonstrated that application of melanin, cisplatin and combination of both compounds to T24/83 cells led to the statistically significant suppression of TLR4 and TNF- $\alpha$  expression. TNF- $\alpha$  is a pro-inflammatory cytokine, produced by macrophages that can induce cellular growth, death, and regeneration [37]. Cancer tissue is infiltrated with monocytes, T cells, and other cells capable of producing TNF- $\alpha$ ; cells in the tumor microenvironment produce soluble TNF- $\alpha$  receptors. Selective extracorporeal removal of soluble TNF- $\alpha$  receptors can cause local enhancement of endogenous TNF- $\alpha$  activity and provide enhanced tumor cell death without associated systemic toxicities [38]. In the tissue of superficial bladder cancer, the TNF- $\alpha$  is considered a main mediator of curative effect of BCG intravesical therapy due to its direct antitumor activity [39], while in the tissue of invasive tumor its effect is less clear. Systemic TNF- $\alpha$  administration results in an unacceptable level of toxicities, in contrast, localized administration of TNF- $\alpha$  yields excellent results, for example, in soft tissue sarcomas [38]. Wu *et al.* [40] demonstrated that pro-inflammatory activity of TNF- $\alpha$  has been suppressed by TLR4 antagonist Ibudilast (AV4II), which indicated potential direct stimulatory relations between TLR4 and TNF- $\alpha$ . Considering importance of the inflammatory state for cancer progression, the suppression of pro-inflammatory cytokine TNF- $\alpha$  expression could contribute to diminishing stimulatory tumor microenvironment.

TGF- $\beta$  is a key cytokine regulating cell migration, which plays an important role in epithelial-to-mesenchymal transition and formation of metastases in carcinogenesis [41–44]. In our study, application of melanin exerted no effect on TGF- $\beta$  expression. The application

of cisplatin and combination of both compounds led to 13- and 6.5-fold suppression of TGF- $\beta$  expression, respectively. This finding is worthy of further research.

INF- $\gamma$  is a pro-inflammatory cytokine, which plays dual role in cancer [20]. INF- $\gamma$  may suppress tumor growth by acting not only directly on cancer cells, but also indirectly on endothelial cell inducing ischemia in the tumor, and on immune cell in the tumor microenvironment. On the other hand, IFN- $\gamma$  contributes to the subsequent cancer evasion by promoting tumorigenesis and angiogenesis eliciting the expression of tolerant molecules and inducing homeostasis program [20]. In our study, we observed that application of melanin, cisplatin and combination of both compounds led to 4-, 6.7- and 2-fold rise in the expression of IFN- $\gamma$  in the cell culture, respectively. Considering dual role of the IFN- $\gamma$  in the carcinogenesis, the significance of these results needs to be further analyzed.

In current study, we observed significant increase in the number of apoptotic cells in the cell culture treated with melanin, cisplatin and combination of both agents. Cancer cells are more resistant to apoptotic cell death, allowing them to bypass critical biological checkpoints that normally maintain cell turnover in healthy tissues. Specifically, checkpoints can fail following an introduction of mutations in apoptotic genes such as p53, or DNA-repair genes like *BRCA1/2* [45]. Administration of high dose of chemotherapeutic drugs with aims of achieving apoptosis may cause inadvertent effects on healthy tissues. That is why it is of critical importance to find a combination of commonly used cytotoxic drugs with adjuvant agents, which could reduce the dosage of drug without compromising the efficiency of systemic treatment.

Redistribution of cells per phases of cell cycles under the treatment of studied compounds was modest. Under melanin treatment, we observed decrease of cells in phase G<sub>2</sub>/M and S, and increase in phase G<sub>0</sub>/G<sub>1</sub>. Cisplatin caused more pronounced redistribution than that of melanin, namely, significant increase of cells in phases G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M, decrease in phase S. Combined application of compounds shifted more cells in phases G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M, and less in phase S.

It is worth to note that the highest increase in number of cells in M phase was observed under the application of cisplatin that explains why cisplatin is widely used as radiosensitizer. To the contrary, the least amount of cells in M phase was observed under the application of melanin, which signified the cytostatic effect of melanin.

To sum up, the application of melanin, cisplatin, and combination of both agents to T24/83 cells of the invasive urothelial carcinoma demonstrates the evident effect on the expression of *TLR4*, *TNF- $\alpha$* , *TGF- $\beta$* , *INF- $\gamma$* , redistribution of cells per phases of cell cycle, cell morphology, and rising apoptosis rate, which may contribute to the change in phenotype towards less aggressive one.

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### ЗМІНИ ЕКСПРЕСІЇ TLR-4, TGF- $\beta$ , INF- $\gamma$ , TNF- $\alpha$ В КУЛЬТУРІ КЛІТИН T24/83 ІНВАЗИВНОГО РАКУ СЕЧОВОГО МІХУРА ПІД ДІЄЮ ЦИСПЛАТИНУ ТА/АБО ПОЛІФЕНОЛЬНОГО АД'ЮВАНТУ МЕЛАНІНУ

П.Г. Яковлев<sup>1,\*</sup>, О.І. Горбач<sup>2</sup>, Н.М. Храновська<sup>2</sup>, А.В. Беляєва<sup>3</sup>, О.В. Скачкова<sup>2</sup>, Т.Д. Скатерна<sup>4</sup>, Л.Г. Калачнюк<sup>5</sup>, Л.І. Остапченко<sup>3</sup>, Л.В. Гарманчук<sup>3,\*</sup>

<sup>1</sup>Клінічна лікарня «Феофанія», Центр урології та онкоурології, Київ 03143, Україна

<sup>2</sup>Національний інститут раку, Київ 03022, Україна

<sup>3</sup>Київський національний університет ім. Тараса Шевченка, Київ 01033, Україна

<sup>4</sup>Інститут біохімії ім. О.В. Палладіна НАН України, Київ 01054, Україна

<sup>5</sup>National Національний університет біоресурсів та природокористування, Київ 03041, Україна

**Стан питання:** Зміни експресії Toll-подібного рецептора 4 (TLR4) асоційовані зі змінами в експресії ключових цитокінів (трансформуючого фактора росту- $\beta$  (TGF- $\beta$ ), фак-

тора некрозу пухлини- $\alpha$  (TNF- $\alpha$ ), інтерферону- $\gamma$  (IFN- $\gamma$ ), що мають вплив на прогресування злоякісних пухлин та метастазування. **Мета:** Дослідити зміни експресії генів *TLR4*, *TGF- $\beta$* , *TNF- $\alpha$* , *IFN- $\gamma$* , рівень апоптозу та розподіл за фазами клітинного циклу в клітинах T24/83 інвазивної уротеліальної карциноми людини під дією поліфенольної ад'ювантної сполуки меланіну, виділеної з грибів, та цитотоксичного хіміопрепарату цисплатину, а також поєднаної дії обох цих сполук. **Матеріали та методи:** Клітини T24/83 інкубували з цисплатином (0.05 мМ), меланіном (5 мкг/мл), або з обома цими сполуками. Рівень експресії *TLR-4*, *TGF- $\beta$* , *INF- $\gamma$* , *TNF- $\alpha$*  визначали за допомогою полімеразної ланцюгової реакції в режимі реального часу. Розподіл за стадіями клітинного циклу, проліферативну активність та рівень апоптозу визначали за допомогою проточної цитометрії. Досліджували також морфологію клітин під дією зазначених сполук. **Результати:** Меланін, цисплатин та їх комбінація знижують експресію *TLR4* (у 2.67; 1.28 та 2.73 раза відповідно) та *TNF- $\alpha$*  (у 6.5; 1.4 та 1.7 раза відповідно). Меланін не впливає на експресію *TGF- $\beta$* , у той час як цисплатин знижує його експресію в 13 разів. У разі поєднаної дії цисплатину та меланіну експресія *TGF- $\beta$*  знижується в 6.5 раза. Експресія *INF- $\gamma$*  за дії меланіну, цисплатину або поєднаної дії цих сполук збільшується у 4.3; 6.7 та 2 рази відповідно. Як меланін або цисплатин, так і поєднана дія цих сполук спричинює підвищення рівня апоптозу в клітинах T24/83. Меланін суттєво збільшує частку фібробластоподібних клітин в культурі T24/83 зі зменшенням адгезії клітин до субстрату. **Висновки:** Меланін, цисплатин, у разі окремого або комбінованого застосування суттєво впливають на експресію *TLR4*, *TNF- $\alpha$* , *TGF- $\beta$* , *INF- $\gamma$* , розподіл за клітинним циклом та морфологію клітин T24/83, що дозволяє припустити набуття цими клітинами менш агресивного фенотипу.

**Ключові слова:** меланін, уротеліальна карцинома, клітинна лінія T24/83, інвазивний рак сечового міхура, *TLR4*, *TGF- $\beta$* , *INF- $\gamma$* , *TNF- $\alpha$* , апоптоз.