УДК 616-006.004:618.19:615.373

COMBINED INFLUENCE OF TEICHOIC ACIDS FROM *STAPHYLOCOCCUS AUREUS* AND HETEROMETALLIK *Cu/Cd* ETHYLENEDIAMINE COMPLEX ON PERITONEAL MACROPHAGES AND TUMOR CELLS

V.V. NIKULINA [1], L.V. GARMANCHUK [1], N.V. SENCHYLO [1],
T.V. NIKOLAENKO [1], O.I. DZHUS [1], L.I. OSTAPCHENKO [1], N.M. KHRANOVSKA [2]

[1] Educational and scientific centre «Institute of biology» of Taras Shevchenko National University of Kyiv
[2] National Cancer Institute, Kyiv
E-mail: liudmylagarmanchuk@rambler.ru

We investigated the effects of teichoic acid (TA) from Staphylococcus aureus Wood 46 on tumor growth and metastasis of the experimental Lewis lung carcinoma (LLC) in mice. Intranasal administration of TA alone aggravated both tumor growth and metastasis, whereas combined administration of TA with a synthetic bimetallic (copper : cadmium) ethylene diamine complex PO244 resulted in pronounced antitumor and antimetastatic effects. The group of animals subjected to the combined treatment with TA and PO244 manifested the highest degree of lymphocyte infiltration into the tumor tissue, compared to the control group and those exposed to TA or PO244 alone. Moreover, the combined treatment negatively affected the adhesive properties of peritoneal macrophages in the LLC bearing mice. Co-cultivation of the isolated macrophages with primary LLC cultures revealed significant ($p < 0.05$) cytotoxic and cytostatic effects, detected as an increased level of apoptosis and a reduced fraction of replicating cells.

Key words: *teichoic acids, Lewis lung carcinoma, bimetallic complex, macrophages.*

Introduction. In order to overcome the adaptive mechanisms of tumor cells to form drug resistance there are a row of modern strategies of antitumor therapy, one of them is immunotherapy of tumor. On this base the application of immune modulators can promote tumor subclone formation. The bacterial cells components play a significant role as the immunomodulators. They are not antitumor drugs thereby such components modulate immune response and favour intensification of antitumor efficiency in combined use with classic chemotherapy [1].

The modern researches reveal anticarcinogenic influence one of the major bacterial cell wall components – teichoic acids (TA). It was shown that TAs of cell wall of some bacteria are responsible for enhancement of hypersensitivity reaction and suppresses antibody synthesis in big concentrations and is able to activate cell cytotoxicity. It is known that TA as a component of cell wall of Gram-positive bacteria as well as lipopolysaccharides of Gram-negative bacteria can stimulate TNF-α production. Bacterial substances which are responsible for tumor cell lysis are identified and used for enhancement of immunogenicity of antitumor vaccines. Indirect toxic influence of bacterial cells against tumors is realized by means of recruitment of immune system effect or sand cross-presentation of tumor antigens. A particular role in recognizing different bacterial structures and inducting the antitumor effects is dedicated to Toll-like receptors (TLRs) [2]. TLRs are known to be used very often in antitumor therapy [3].

TLR ligands are bacterial cell components, so-called pathogen-associated molecular patterns (PAMPs) [4]. In Gram-positive bacteria their role is fulfilled by teichoic acids.

Teichoic acids of Gram-positive bacteria are potential surface structures, which stimulate TNF-α production and induct IL-12 secretion in CD14-depended way by monocytes. It results cells the activation of natural killer cells, which leads to directly determined IFN-α production [5, 6]. Moreover, it has been shown that teichoic acids cause secretion of Th-1 type cytokines, which activate T-lymphocytes and natural killer cells, and are capable to change cytotoxic potential of lymphocytes with killer activity.

On the other hand, TA stimulates proliferative activity of both normal and immortalized lymphoid cells. It can cause progress of lymphoproliferative diseases [7]. In previous studies we have shown that TA modifies growth of primary tumor and level of metastatic dissemination of transplantable lung Lewis carcinoma dependently on term of administration.

It is known that motogenic potential of normal as well as tumor cells is realized by system of adhe-

sive molecules. They are responsible for supporting tissue homeostasis, decreasing adhesive properties and abnormalities of intercellular contacts, that assist cell migration. Consequently, adhesive potential is inversely correlated with the ability to migrate.

By our research group it has been synthesized the complexes of copper and organic ligands in base for which bactericidal, fungicidal and antitumour activity have been revealed later. It has been found they permit to regulate a defensive reaction of organism on molecular and cell levels, promisinge therapeutic advances in cancer pathogenesis and infection diseases [8]. As a rule they cause both bactericidal and fungicidal effective influence, demonstrating antitumor effect and moderate resistance to microorganisms. Screening of bimetallic complexes has shown that the complex that consists of copper ions, cadmium and ethylenediamine – PO244 ($[Cu(en)_2][Cd_2(CH_3COO)_6]$) demonstrates the highest efficiency [9, 10].

The aim of this study was to investigate a combined influence teichoic acids from *Staphylococcus aureus* and heterometallik Cu/Cd ethylenediamine complex on peritoneal macrophages and tumor cells *in vitro* and *in vivo*.

Materials and methods. The investigation was carried out in C57Bl/6 female mice weighing 20–25 g aged 2 to 3 months from the vivarium of the Educational and scientific centre «Institute of biology» of Taras Shevchenko National University, in two groups of experiments (Table 1). All researches on animals were carried out according to Guide for the Care and Use of Laboratory Animals [11].

The strain of metastatic Lewis lung carcinoma (LLC) was kindly given by National Bank of Cell Lines and Tumor Strains of R.E. Kavetsky Institute of experimental pathology, oncology and radiobiology. The cell suspension of LLC (0.2 ml of 20 % cell suspension) was inoculated subcutaneously into the site of sacrum.

Administration of PO244 to the animals was carried out intraperitoneally at the total dose 0.8 mg/kg of body weight, whereas TA from *Staphylococcus aureus* Wood 46 was administrated intranasally in concentration 2 ng/kg of animal weight. Both TA and PO244 were administrated on 8th day after tumor cell inoculation. Antimetastatic and antitumor effects were defined according to the inhibition index of tumor weight, volume and number of metastases in lung as described [12].

The primary culture was obtained from transplantable Lewis lung carcinoma after 2–3 times trypsinization of tumor tissue in trypsin-EDTA solution with pH 7.0. Cell cultivation was conducted under standard and serum free conditions at 37 °C, 100 % humidity and 5 % CO_2.

Mononuclear phagocyte fraction of peritoneal exudate of mice was obtained by standard procedure of Pietrangeli [13]. Macrophages were incubated under standard conditions at 37 °C, 100 % humidity and 5 % CO_2 for 4 h and then their cultural medium was added to LLC culture.

The lymphocyte infiltration degree of tumor (1000 cells/1 g tumor tissue) was estimated after their fractionation in phycoll-verografin gradient with density 1,077 g/ml by centrifugation 40 min at 1.500 rpm. The adhesive capacity of macrophage and tumor cells was determined by the percent of cells which were attached to the substrate, after

Table 1. **The influence of heterobimetallic complex and teichoic acids on the growth and metastasis of transplantable Lewis lung carcinoma (27 days after tumor cell inoculation)**

Animal group	Control ($n_1 = 8$; $n_2 = 6$)	TA ** ($n_1 = 7$; $n_2 = 6$)	PO244 ($n_1 = 6$; $n_2 = 7$)	TA + PO244 ($n_1 = 8$; $n_2 = 6$)
Number of LLC bearing mice (%) ($n_t/n_1 + n_2$)	85,7 % (12/14)	100 %(14/14) *	69,2 % (9/13) *	64,3 % (8/14) *
Tumor weight (g)	5,3 ± 1,2	7,8 ± 1,4 *	4,1 ± 0,7 *	2,5 ± 0,6 *
WI		>47,2 %	<22,6 %	<52,8 %
Number of animals with metastases (%) ($n_m/n_1 + n_2$)	78,6 % (11/14)	100 % (14/14) *	63 % (8/13) *	57,2 % (8/14) *
Number of metastases in lungs	21,7 ± 3,2	37,3 ± 5,4 *	15,2 ± 3,6	9,3 ± 2,1 *
Metastatic index N_k-N(exp)/N_k · 100 (%)		>71,9,2 %		<57,3 %

* $p < 0,05$. **3 animals died before 27th day.

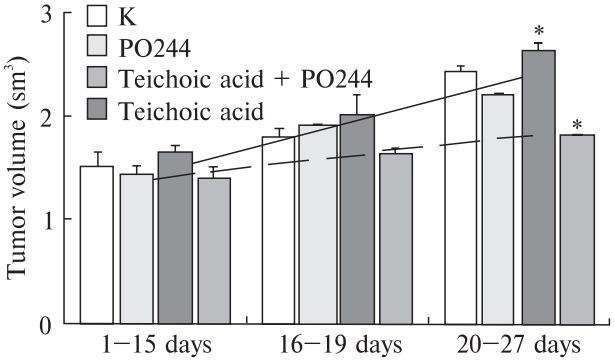

Fig.1. The influence of teichoic acids and PO244 (mono and combined therapy) on growth of primary tumors. Here and Fig. 3, 4 *P < 0.05 *vs* control

crystal violet staining by measuring absorbance at 570 nm using a multiwell spectrophotometer, Synergie Biotec (USA).

Cytotoxic/cytostatic effect, necrotic/apoptotic index and proliferative activity after the influence of PO244 ($[Cu(en)_2][Cd_2(CH_3COO)_6]$) and TA with respect to cells of primary culture of LLC were assessed by cytofluorimetry, counting cells stained with trypan blue and MTT-test assay, cells were incubated with TA and PO244 for 24 and 48 h under normal conditions in 96 well plates. The initial cell concentration was approximately $5 \cdot 10^4$ cells/ml in the sample volume of 100 μl. It has been used a culture media RPMI («Sigma», USA) with 10 % FBS («Sigma», USA), 2 mM L-glutamine, and 40 μg/ml gentamicin. Different concentrations ($1 \cdot 10^{-6}$–$1{,}25 \cdot 10^{-4}$ M) of PO244 and TA (1 ng/ml–1 μg/ml) were added to cell cultures in 100 μl of media after the period of cell adaptation under standard conditions (5 % CO_2, 100 humidity, 37 °C) during 4 h. The number of living cells was determined in wells using MTT-colorimetric assay and cell counting were performed using a tripan blue dye after 48 h incubation with test-agents [14]. The cytotoxic effect was evaluated as a percent of live cells relative to control and characterized by IC_{50} index [15] and mitogenic effect was evaluated as percent increase number alive cells relative to control.

The isolated peritoneal macrophages from tumor bearing mice after TA and PO244 treatment have been co-cultivated with primary LLC culture during 48 h. Apoptotic level and distribution of cells in phases of cell cycle were assessed by cytofluorimetry [16]. For this purpose the samples were stained with PI, which selectively joins with intercalating places in DNA. Cytofluorymetry was carried out on the instrument FACS Calibur («Becton Dickinson», USA). Special mathematical program Mod Fit LT 2.0 (BDIS, USA) for Macintosh computers was used for acquisition and data analysis. Narrowband filter 585/42 nm was used in order to measure the fluorescence of PI. It has been performed the histological mounts of tumor samples after their exposure to TA, PO244, and their combination by standard methods. Coloring of Lewis carcinoma tumor sections was performed by the standard method of hematoxylin-eosin staining [17].

Results and conclusions. For the first time, it has been shown antitumor and antimetastatic activity of heterometallic coordinational composition on the model of transplantable Lewis lung carcinoma. Under the condition of PO244 administration tumors were found out in 69,2 % of experimental animals (Table 1), the administration of TA to LLC-bearing mice resulted in 100 % tumor outcome, in comparison with 85,7 % in control group, whereas combined application of PO244 with teichoic acids led to 64,3 % tumor outcome

The tumors in animals with certain therapy differed in the rate of growth and weight from each other. The administration of TA increased the tumor weight by 42,7 % compared to control, whilst PO244 injection resulted in the decrease of tumor weight by 22,6 %, whereas LLC-bearing mice that received PO244 + TA combined treatment had 52,8 % smaller tumor weight. Moreover, this pattern remained in determining the tumor weight at different stages of growth. From the dynamics of tumor growth the growth rate of the tumor was seen to be reduced in the application of TA with PO244 compared to the control group (Fig. 1).

Number of animals with metastases in control group was 78,6 %, where as number of metastases in lungs was 21,7 ± 3,2, TA treatment resulted in the increase metastatic level to 100 % and 37,3 ± 5,4 correspondently, PO244 utilization led to decrease metastasis to 63 % animals with metastases and 15,2 ± 3,6 metastases in lungs, after combined use PO244 and TA antimetastatic effect was the highest: 52,7 % of animals with metastases had 9,3 ± ± 2,1 metastases in lungs.

On experimental model of metastatic Lewis lung carcinoma hyperactivation of primary tumor growth simultaneously with metastasis was as certained in intranasal administration of TA on the stage of primary node formation. During the application

in combined therapy TA with synthetic bimetallic complex (PO244), which demonstrated both antitumor and antimetastatic influence, intensifying of antitumor effect of PO244 was shown (Fig. 1).

Our results argue that considerable depression of tumor growth occurs to all phases of tumor formation with combined use PO244 and TA. It is known that recognition of bacterial structures including LTA and TA passes with involvement of toll-like receptors [18]. This receptors (including TLR2 and TLR4, there ligands are respectively TA and LTA) are expressed on surface of different cell types including different types of tumors [19]. The interaction TA and LTA with specific receptors results in activation of cytokine production. Moreover, in depending on ligand doze the activation both pro-inflammatory and anti-inflammatory cytokines are possible [20].

For the purpose to define the mechanisms of combined TA and PO244 influence, cytotoxic/cytostatic screening of these substances was carried out on primary LLC culture. However, the intensification of cytotoxic effect of PO244 and TA on tumor cells of primary culture was not detected in such conditions (Table 2). IC_{50} index was $2.20 \pm \pm 0.04 \cdot 10^{-5}$ M under the influence PO244, addition of TA didn't impact on the index $3.03 \pm 0.06 \cdot 10^{-5}$ M, whereas monotherapy by TA stimulated of primary LLC culture proliferation insignificantly.

Table 2. **The influence of PO244 and TA on LLC cells with their separated and combined application in vitro. Incubation of cells with above-mentioned agents was carried out 48 h**

Cells	PO244	TA+PO244
	M·10⁻⁵	
IC_{50}	2.20 ± 0.04	3.03 ± 0.06
$IC_{50}/10$	0.22 ± 0.00	0.30 ± 0.01
Viability ($IC_{50}/10$,%)	71.6 ± 7.3	79.4 ± 3.1
Apoptotic level ($IC_{50}/10$,%)	24.4 ± 4.3	18.1 ± 0.4
Cells	Control	TA*
Mitogenic index		1.12
Viability (%)	92.4 ± 3.7	94.3 ± 5.6
Apoptotic level (%)	11.7 ± 2.7	9.1 ± 0.5

*Apoptotic level, viability and mitogenic index were assessed after cells incubation with TA (concentration of TA was 0.25 ng/ml).

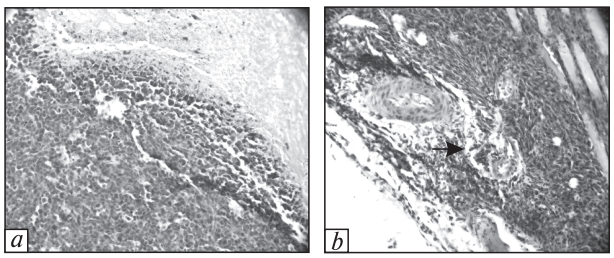

Fig. 2. Slide mount of mouse lung demonstrates level of lymphocyte infiltration in control (*a*) and under the influence of teichoic acid and PO244 (infiltrative lymphocyte is marked) (*b*)

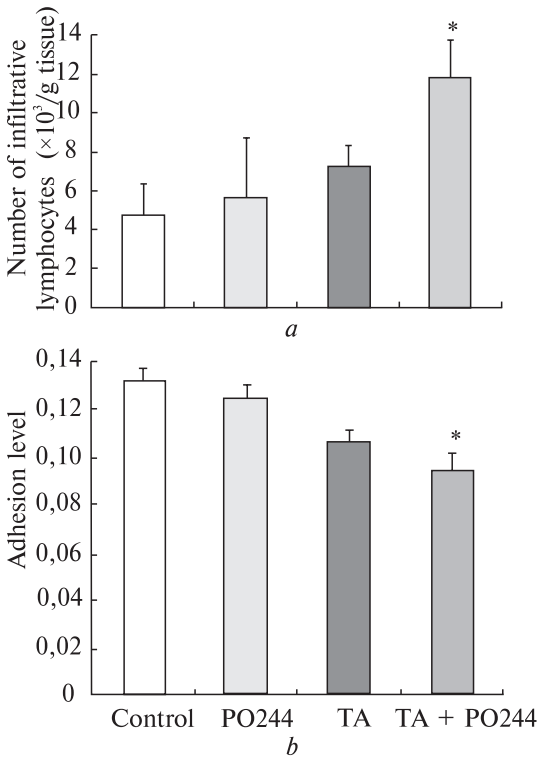

Fig. 3. The influence of agents (TA, PO244 and TA+ + PO244) degree of infiltrative lymphocytes in tumor tissue (*a*). The adhesion parameter of peritoneal macrophages of LLC bearing (*b*)

The PO244 substance showed distinct cytotoxic effect whereas TA in certain concentrations activated cell proliferation, as we can see from mentioned data. The survival curve under combine influence of these substances practically doesn't differ from the curve under the influence of PO244.

The modern researchers consider the degree of lymphocytes infiltration of tumor as diagnostic and prognostic marker of clinical course [20, 21].

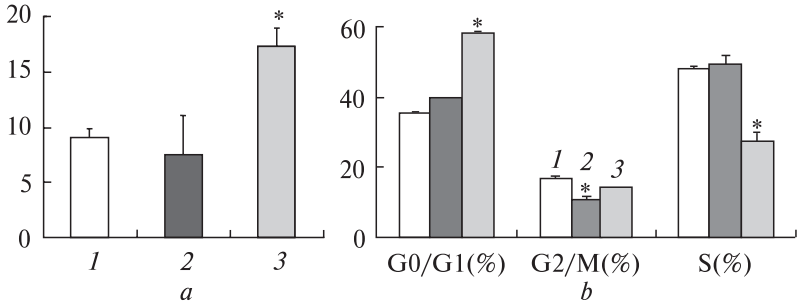

Fig. 4. Apoptotic level, % (*a*) of LLC cells under condition of contactless cocultivation with macrophages, which were obtained from LLC bearing mice, LLC bearing mice after effective combine therapy by teichoic acid and PO244 and distribution of LLC cells in phases of cell cycle, % (*b*); *1* – LLC (control), *2* – LLC(Mph_tumor), *3* – LLC(Mph_tumor+TA+PO244)

During the experiment of primary tumor therapy we registered the increase of lymphocyte infiltration in animal groups with application of bimetallic complex PO244 or TA (Fig. 2, *a*, *b*).

The highest degree of lymphocyte infiltration in tumor was detected in animal group which was treated by mixture of TA and PO244. It should be noted that the increase of rate of lymphocyte infiltration in tumor tissue after mono or combined therapy is one probable mechanism of inhibition of primary tumor (Fig. 3, *a*) [22–24].

The TLR-mediated effect can be modifying of cell-cell interaction and cell interaction with molecules of extracellular matrix. We have tested the TA influence on adhesive potential of peritoneal macrophages of LLC bearing mice or intact animals.

It was shown that application of synthetic bimetallic complex PO244 in therapeutic concentration ($1.1 \pm 0.01 \cdot 10^{-5}$M/g weigh animal) didn't result in a cytotoxic impact on peritoneal macrophages, whereas the modifying effect on adhesive behavior of peritoneal macrophages was observed (Fig. 3, *b*).

Since, adhesive potential inversely correlates with cell ability to migrating, the *in vitro* findings indicate that migration and tumor infiltration can be activate when tumor growing *in vivo*. We have shown it in combined therapeutic scheme application of TA and PO244 on transplantable Lewis lung carcinoma (Fig. 1). Monotherapy by TA stimulates tumor infiltration by lymphocytes insignificantly, whereas in combined therapy with PO244 this parameter is increased 2.4 times ($p < 0.05$).

An apoptotic index increase and reduction of proliferative cells quantity in non-contact coculture with primary culture LLC were observed as a result of adding the peritoneal macrophages from intact animals, the animals bearing the tumors with and without combined influence of TA and PO244 (Fig. 4, *a, b*).

In order to determine possible mechanism of TA impact on the tumor through the immune cells we studied macrophages that were received from different groups of animals at the last stage of carcinogenesis. Macrophages from animals after therapy and control group in vivo were added and cocultivated with primary LLC culture during 48 h; we founded cytotoxic/cytostatic influence which was expressed as increasing of apoptotic level and decreasing of cell population of proliferative pool (Fig. 4). Thereby, the screening of potential antitumor agent PO 244 (mono and combined application with TA) was carried out. The results of the study of influence of teichoic acids on transplantable Lewis lung carcinoma results strongly suggest about implication this bacteria cell wall component of *Staphylococcus aureus* Wood 46 in antitumor and antimetastatic response *in vivo* and modification influence *in vitro*.

КОМБИНИРОВАННОЕ ВЛИЯНИЕ ТЕЙХОЕВОЙ КИСЛОТЫ ИЗ *STAPHYLOCOCCUS AUREUS* И ГЕТЕРОМЕТАЛЛИЧЕСКОГО *Cu/Cd* ЭТИЛЕН ДИАМИНОВОГО КОМПЛЕКСА НА ПЕРИТОНЕАЛЬНЫЕ МАКРОФАГИ И ОПУХОЛЕВЫЕ КЛЕТКИ

В.В. Никулина, Л.В. Гарманчук, Н.В. Сенчило, Т.В. Николаенко, Е.И. Джус, Л.И. Остапченко, Н.Н. Храновская

Исследовали модифицирующее влияние тейхоевой кислоты (ТК) на рост и метастазирование перевиваемой карциномы легких Льюис у мышей. Выявлена гиперактивация роста и метастазирования первичной опухоли при интраназальном введении животным ТК, в то время как при комбинированном влиянии с РО244 наблюдали усиление противоопухолевого эффекта. Самый высокий уровень инфильтрации опухолевой ткани лимфоцитами зафиксировали в группе животных, которым проводили комбинированную терапию ТК с РО244. Показано снижение адгезивных свойств перитонеальных макрофагов под влиянием биметаллического комплекса.

После сокультивирования макрофагов от животных, которым проводили комбинированную терапию с первичной культурой LLC, выявлено значительное ($p < 0.05$) цитотоксическое/цитостатическое влияние, что проявлялось в увеличении уровня апоптоза и уменьшении популяции клеток пролиферативного пула.

REFERENCES

1. *Bingle L., Brown N.J., Lewis C.E.* The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies // J. Pathol. – 2002. – **196**. – P. 254–265.
2. *Shcheblyakov D., Logunov D., Tukhvatulin A. et al.* Toll-Like Receptors: The role in tumor progression // Acta Naturae. – 2010. – **2**, № 3. – P. 21–29.
3. *So E.Y., Ouchi T.* The application of Toll like receptors for cancer therapy // Int. J. Biol. Sci. – 2010. – **6**, № 7. – P. 675–681.
4. *Сківка Л.М., Позур В.В.* Реакції за участю Toll like рецепторів у протективному імунітеті та за патологічних станів // Укр. біохім. журн. – 2008. – **80**, № 3. – С. 5–20.
5. *Senchylo N.V., Olishevskyy S.V., Pozur V.K.* The influence of teichoic acid of *Staphylococcus aureus* Wood 46 on cylolytic activity of natural killer cells // Biopolym. Cell. – 2003. – **19**, № 3. – P. 238–241.
6. *Senchylo N.V., Garmanchuk L.V., Yavorska N.V. et al.* Teichoic acid from *Staphylococcus aureus* increases antitumour and antimetastatic effects of the heterometallik Cu/Cd complex // Cell/Tissue Injury and cytoprotection/Organoprotection : 5th Int. Symp. Физиол. журн. – 2009. – **55**, № 1. – С. 101.
7. *Сківка М.П., Рудик В.В., Позур Н.В. та ін.* Ефекти тейхоєвої кислоти *Staphylococcus aureus* при пухлинному рості // Biopolym. Cell. – 2008. – **24**, № 3. – С. 212–217.
8. *Тутельян А.В.* Иммунорегуляторная оценка естественных и синтетических препаратов в системе прайминга лейкоцитов // Эпидемиология и вакцинопрофилактика. – 2003. – № **5**. – P. 55–57.
9. *Пат.* № 36261 Україна. Застосування ацетатного комплексу міді та кадмію з етилендіаміном як протипухлинного та антиметастатичного засобу / Федорчук О.Г., Перепеліцина О.М., Кокозей В.М. та ін. Опубл. 27.10.08. Бюл. № 20.
10. *Яворская Н.В., Гарманчук Л.В., Перепелицына Е.М. и др.* Бактерицидное, фунгицидное и цитотоксическое действие гетеробиметаллических комплексов // Биологически активные вещества : Тез. докл. – Новый Свет, 2009. – С. 451–452.
11. *Guide* for the care and use of laboratory animals. – Washington : Acad. press, 1996. – 140 p.
12. *Pyaskovskaya O.N., Dasyukevich O.I., GarmanchukL.V. et al.* Changes in VEGF level and tumor growth characteristics during Lewis lung carcinoma progression towards cis-DDP resistance// Exp. Oncol. – 2007. – **29**, № 3. – P. 197–202.
13. *Pietrangeli C.E., Edelson P., Congshavn P.* Measurement of 5'-nucleotidase in mouse peritoneal macrophages in listeriosts // Inf. Immun. – 2001. – **32**, № 3. – P. 1206–1210.
14. *Mosmann T.* Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assayas // J. Immun. Meth. – 1983. – **65**, № 1/2. – P. 55–63.
15. *Alley M.C., Scudiero D.A., Monks P.A. et al.* Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay // Cancer Res. – 1988. – **48**. – P. 589–601.
16. *Nicoletti I., Migliorati G., Pagliacci M.C.* A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry // J. Immun. Meth. – 1991. – **139**, № 2. – P. 271–280.
17. *Горальський Л.П., Хомич В.Т., Кононський О.І.* Основи гістологічної техніки і морфофункціональні методи досліджень у нормі та при патології. – Житомир : Полісся, 2005. – 288 с.
18. *Jassens S., Beyaert R.* Role of toll-like receptors in pathogen recognition // Clin. Microbiol. Rev. – 2003. – **16**, № 4. – P. 637–646.
19. *Zarember K.A., Godowski P.J.* Tissue expression of human toll-like receptors and differential regulation of toll-like receptors mRNAs in leucocytes in to microbes, their products, and cytokines // J. Immun. – 2002. – **168**. – P. 554–561.
20. *Netea M.G., van der Graaf C., van der Meer J.W., Kullberg B.J.* Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system // J. Leuc. Biol. – 2004. – **75**, № 5. – P. 749–755.
21. *Nikolaenko T., Senchylo N., Garmanchuk L. et al.* Teichoic acid modulates the adhesive properties of MCF-7 tumor cells // Primary therapy of early breast cancer : 13th Int. conf. St. Gallen // Breast. – 2013. – **22**, Suppl. 1.
22. *Funada Y., Noguchi T., Kikuchi R. et al.* Prognostic significance of CD8+ T cell and macrophage peritumoral infiltration in colorectal cancer // Oncol. Rep. – 2003. – **10**. – P. 309–313.
23. *Drescher K.M., Sharma P., Watson P. et al.* Lymphocyte recruitment into the tumor site is altered in patients with MSI-H colon cancer // Farm. Cancer. – 2009. – **8**. – P. 231–239.
24. *Macchetti L.H., Cosiski Marana H.R., Silvall J.S. et al.* Tumor-infiltrating CD4+ T lymphocytes in early breast cancer reflect lymph node involvement // Clinics. – 2006. – **61**, № 1. – P. 203–208.

Received 28.12.12