EXPERIMENTAL STUDIES

CORRECTION OF SERUM PROONCOGENIC CYTOKINES AND METASTASES BY 5-HYDROXYPYRIMIDINE DERIVATIVES AND DOXORUBICIN AFTER REMOVAL OF A PRIMARY TUMOR NODE IN MICE WITH THE LEWIS LUNG EPIDERMOID CARCINOMA

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The effect of a single injection of doxorubicin, 8-day administration of two 5-hydroxypyrimidine derivatives, SNK-411 (2-Isobutyl-4,6-dimethyl-5-hydroxypyrimidine) and SNK-578 (hydrochloride of 2-isobutyl-4,6-dimethyl-5-hydroxypyrimidine), on metastases, lifespan and serum cytokines has been investigated in C57BL/6 mice after removal of a primary tumor node of Lewis lung carcinoma (LLC). LLC cells (1×10^6) were injected in the footpad of right hind feet of mice in control and experimental groups; after 14 days of tumor development the hind feet with the tumor were amputated at the ankle level. One hour before the amputation mice received a single injection of doxorubicin (4 mg/kg) and 8-day therapy with the 5-hydroxypyrimidine derivatives started. SNK-578 monotherapy was performed at a dose of 10 mg/kg administered intraperitoneally (i.p.). SNK-411 was administered *per os* at a dose of 25 mg/kg. In the case of combined therapy mice also received a single injection of doxorubicin (4 mg/kg; i.p.). The metastasis inhibition index in mice-treated with SNK-411 and SNK-578 were 53.3% as compared with control mice (with removed tumor). The mice-treated with SNK-411, doxorubicin, and the combination SNK-578 + doxorubicin had lifespan increased by 60.2%, 53.9%, and 42.9%, respectively. A single injection of doxorubicin, the course administration of the 5-hydroxypyrimidine derivative alone and in combination with single injection of doxorubicin completely decreased serum levels of the prooncogenic Th2 cytokines IL-4 and IL-6 and significantly decreased the level of the Th2 cytokine IL-5. Administration of doxorubicin, SNK-411 and SNK-578 did not influence serum concentration of Th1 cytokine interferon gamma (IFN-γ). These data confirm our previous findings that administration of the compounds studied decreased concentrations of prooncogenic IL-4 and IL-6 in tumor-bearing mice with LLC and had no effect on concentrations of the Th1 cytokine IFN-γ.

Key words: lung cancer model *in vivo*; doxorubicin; pyrimidines; metastasis; interleukins; survival

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INTRODUCTION

The antitumor therapy with doxorubicin causes increased formation of free radicals; this affects nucleic acids and intracellular protein-lipid structures and is one of the mechanisms of the cardiotoxic action of anthracycline antibiotics that cause cardiomyopathy. According to the literature data, the combined use of doxorubicin with drugs exhibiting antioxidant and anti-inflammatory effects increases antimetastatic activity and reduces the side effects of anthracycline antibiotics. For example, in the case of a double injection of doxorubicin at a dose of 4 mg/kg on day 7 after inoculation of Lewis lung carcinoma (LLC) cells (1×10^6) intramuscularly into the thigh of C57BI/6 mice, tumor growth inhibition (TGI) on day 22 of the experiment was 33.5%, while administration of doxorubicin accompanied by a 14-day administration of 50 mg/kg mexidol the TGI was 30.1%. In the case of doxorubicin administration alone, the metastasis inhibition index (MII) was lower (21.3%) than in the case of the combined administration of doxorubicin and mexidol (33.5%). Thus, the combined administration of mexidol with doxorubicin did not reduce TGI, but limited the process of spontaneous metastasis [1]. In other words, the parameters reflecting the antitumor and antimetastatic activity were higher during the combined use of doxorubicin and mexidol, than in the case of their separate administration to animals [2].

Recently, low-toxic derivatives of 5-hydroxypyrimidine, SNK-411 (2-isobutyl-4,6-dimethyl-5-hydroxypyrimidine) and highly water-soluble SNK-578 (2-isobutyl-4,6-dimethyl hydrochloride-5-hydroxypyrimidine), have been synthesized at the Department of Chemistry of Zakusov Research Institute of Pharmacology [3, 4].

During subcutaneous inoculation of LLC and B-16 melanoma tumor cells (5×10^6) inducing formation of a primary tumor node in C57BL/6 mice, SNK-411 and SNK-578 showed antitumor and antimetastatic activity [5]. In the LLC model, on day 21 after tumor inoculation, MII in the lungs was 61.4% after 14 days of intraperitoneal (i.p.) administration of 50 mg/kg SNK-411 from day 2 to day 15 of the tumor development [5]. In the B-16 melanoma model, monotherapy with SNK-578 for 14 days

had a pronounced antimetastatic activity at doses of 10 mg/kg and 25 mg/kg (i.p.) (MII – 75.8-92.3%). Their course application together with a single injection of 4 mg/kg doxorubicin on day 2 of tumor development, this effect increased (MII – 98.9%) [6]. The study of anti-inflammatory properties of SNK-411 and SNK-578 in models of inflammation response to concanavalin A and carrageenan induced paw edema, revealed that 5-hydroxypyrimidine derivatives had a pronounced anti-inflammatory activity comparable to that of diclofenac [7].

SNK-411 (25 mg/kg) administered from day 2 to day 8 and from day 8 to day 15 of LLC development, reduced the content of pro-inflammatory interleukin 6 (IL-6) and pleiotropic IL-4 [8]. According to the existing literature data, these cytokines stimulate tumor formation and metastasis of many types of cancer through the STAT3 and STAT6 signaling pathways [9, 10].

However, characterization of the antimetastatic activity of the studied compounds requires data on significant inhibition of the development of metastases after removal of the primary tumor node because the removal of the primary tumor node may increase the metastatic processes.

The aim of this study was to evaluate the effect of a single injection of doxorubicin on the day 14 of the experiment, 8-day administration of the compounds SNK-411 and SNK-578 and their combinations with a single injection of doxorubicin to LLC bearing C57BL/6 mice on metastasis and the level of cytokines after removal of the primary tumor node.

MATERIALS AND METHODS

The work was performed on 120 male mice of the C57BL/6 line weighing 18-20 g (8 animals – intact control, 16 tumor-bearing animals in each group) obtained from the "Stolbovaya" nursery (Moscow Region). The animals were kept in the vivarium of the Zakusov Institute of Pharmacology with a 12-h light regime on a standard balanced briquetted feed with free access to food and water under natural light and an ambient temperature of 20-21°C.

The LLC model was used as an experimental model of malignant growth. The tumor cell strains were obtained from the Cell Culture Bank of the Research Institute for Experimental Diagnosis and Tumor Therapy (Blokhin Research Medical Center of Oncology, Russia). The LLC tumor cells were implanted in male C57BL/6 mice in the footpad of right hind foot at the maximum dose for this injection (1×10^6 LLC tumor cells). The day of transplantation of cells of the tumor strain was considered the zero day of the development of the primary tumor. On day 14 after the LLC inoculation, 1 h before amputation of the paw with the primary tumor node, doxorubicin was administered once at a dose of 4 mg/kg and the course 8-day administration of 5-hydroxypyrimidine derivatives was started. After i.p. administration of sodium thiopental at a dose of 25 mg/kg, the paws with the tumor were amputated at the level of the ankle joint with a tourniquet applied. The surgical wound was treated with medical glue in all groups (except animals of the intact control group no. 1 and the control group no. 2 with an unremoved tumor). Animals were divided into the following groups:

1) intact control (control no. 1);

2) control group with primary tumor node LLC/1% starch solution (control no. 2);

3) control group with removed primary tumor node LLC/1% starch solution (control no. 3);

4) LLC/single i.p. injection of doxorubicin at a dose of 4 mg/kg;

5) LLC/SNK-578 for 8 days i.p. at a dose of 10 mg/kg;

6) LLC/SNK-411 for 8 days orally at a dose of 25 mg/kg;

7) LLC/SNK-578 for 8 days i.p. at a dose of 10 mg/kg + doxorubicin once i.p. at a dose of 4 mg/kg;

8) LLC/SNK-411 for 8 days orally at a dose of 25 mg/kg + doxorubicin once intravenously at a dose of 4 mg/kg.

On the next day after the course administration of the studied compounds the intact control animals and half of the animals of the other groups were decapitated using a guillotine (NPC "Open Science", Russia). Blood was collected, the number of metastases in the lungs was counted, using a Magnifier Lamp 8608 E-D XB ("Zhengte", China). Blood serum was obtained by centrifugation at 2500 rpm for 30 min in an Eppendorf Centrifuge 5804 R centrifuge ("Eppendorf", Germany). The effectiveness of the therapeutic effect was determined using the following parameters: the frequency of metastasis (%); the average number of metastases in the group; the magnitude of the difference in tumor metastasis between the control and experimental groups in terms of the metastasis inhibition index (MII), %: $MII = [(A_c \times B_c) - [(A \times B)] / (A_c \times B_c) \times 100\%$, where A_c and A are frequency of lung metastases in mice of control and experimental groups; B_c and B are the average number of lung metastases per animal in the control and experimental groups.

The effect of 8-day administration of 5-hydroxypyrimidine derivatives and a single administration of doxorubicin on the concentration of cytokines IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFN-γ, GM-CSF, TNF-α in the serum of mice with LLC were studied using a BD FACSCanto II flow cytometer ("BD Biosciences", USA) by the method of multiplex detection of fluorescent particles Mouse Th1/Th2 10plex Kit ("eBioscience", Austria), according

to manufacturer's protocol. The results were processed using the FlowCytoMix Pro 2.2.1 program and expressed in pg/ml.

Statistical processing of the experimental data was performed using the Statistica 10 program. All recorded animal characteristics are presented in table as the mean and standard error of the mean (Mean ± SEM). Normal distribution was tested using the Shapiro-Wilk test. To test the hypothesis of homogeneity of study groups with a normal distribution in the study population, testing for the absence of differences between groups was performed using the Student's t-test. In the case of a non-Gaussian distribution, the non-parametric Mann-Whitney test was used to compare the investigated parameters.

The dispersion homogeneity was assessed according to Levene. The significance of the influence of factors with homogeneous dispersion was determined using ANOVA analysis of variance, followed by the Dunnett method of multiple comparisons.

Survival analysis was performed using the Kaplan-Meier procedure, and the Cox F-test was used to assess the significance of differences between survival curves.

Differences were considered as statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Administration of LLC tumor cells (1×10^6) into the foot paw caused edema, which was maximally manifested on day 14 of the experiment (its volume increased to 280-320 mm^3) and was accompanied by the increase in volume of the hock joint.

On day 22 of the experiment, after LLC cell inoculation to animals followed removal of the primary tumor node on day 14 of the experiment, and 8 days of i.p. monotherapy with 10 mg/kg SNK-578 and oral administration of 25 mg/kg SNK-411, the average number of metastases in the experimental groups was significantly lower compared to the control group with non-removed (control no. 2, MII-50.9%) and with the removed tumor (control no. 3, MII-53.3%). After the course of administration of 10 mg/kg SNK-578 in combination with a single injection of 4 mg/kg doxorubicin at the average number of metastases in the group was significantly lower compared to control no. 2. A single intravenous injection of doxorubicin (4 mg/kg) on day 14 of tumor development, as well as its combination with oral administration of SNK-411 (25 mg/kg), insignificantly changed in the average number of metastases in these groups (Table 1). This differs from previously obtained data on doxorubicin administration on day 2 of the development of LLC and B-16 melanoma and increased the antimetastatic action when it was co-administered intravenously with SNK-411 and SNK-578 [5, 6].

The median survival according to the Kaplan-Meier method in control groups no. 2 and no. 3 was 15 days; in the case of SNK-411 administration it increased to 33 days, doxorubicin – 32 days, SNK-578 and doxorubicin – 30 days, SNK-411 and doxorubicin – 18 days (Fig. 1). In the group of animals treated with SNK-411, doxorubicin, or SNK-578 with doxorubicin, the life span expectancy (LSE) increased by 60.2%, 53.9%, and 42.9%, respectively.

The concentrations of the pleiotropic Th2 cytokine IL-4 and Th1 cytokine IFN-γ were determined in all three control groups, IFN-γ was determined in all control and experimental groups (Fig. 2).

The pro-inflammatory and pro-oncogenic Th2 cytokine IL-6 was detected in serum samples from control group no. 2 and at a higher concentration in control group no. 3; the Th2 cytokines IL-4 and IL-5 were also detected in control group no. 1. In all experimental groups, the concentrations of IL-4 and IL-6 cytokines in serum samples were below the determined minimum threshold value. This indicates a pronounced suppression of pro-oncogenic Th2 cytokines by doxorubicin and 5-hydroxypyrimidine derivatives.

Table 1. Evaluation of the antimetastatic properties of compounds in the model of grafted Lewis epidermoid lung carcinoma

Groups; n=8	Control no. 2	Control no. 3	SNK-411, 25 mg/kg, *per os*	SNK-578, 10 mg/kg, i.p.	Doxorubicin, 4 mg/kg, i.p., single injection	SNK-411, 25 mg/kg + Dox., 4 mg/kg	SNK-578, 10 mg/kg + Dox., 4 mg/kg
Metastasis frequency, %	100	100	100	100	100	100	100
Average number of metastasis per one mouse	11.4±2.3	12.0±2.1	5.6±0.8*#	5.6±1.1*#	12.7±2.8	9.0±1.7	5.7±1.0#
MII, % compared to control no. 2	—	—	50.90%	50.90%	—	21.00%	50.00%
MII, % compared to control no. 3	—	—	53.30%	53.30%	—	25.00%	52.50%

* – $p<0.05$ compared to control no. 3 according to the Mann-Whitney test; # – $p<0.05$ compared to control no. 2 according to the Mann-Whitney test; n is the number of animals in the group.

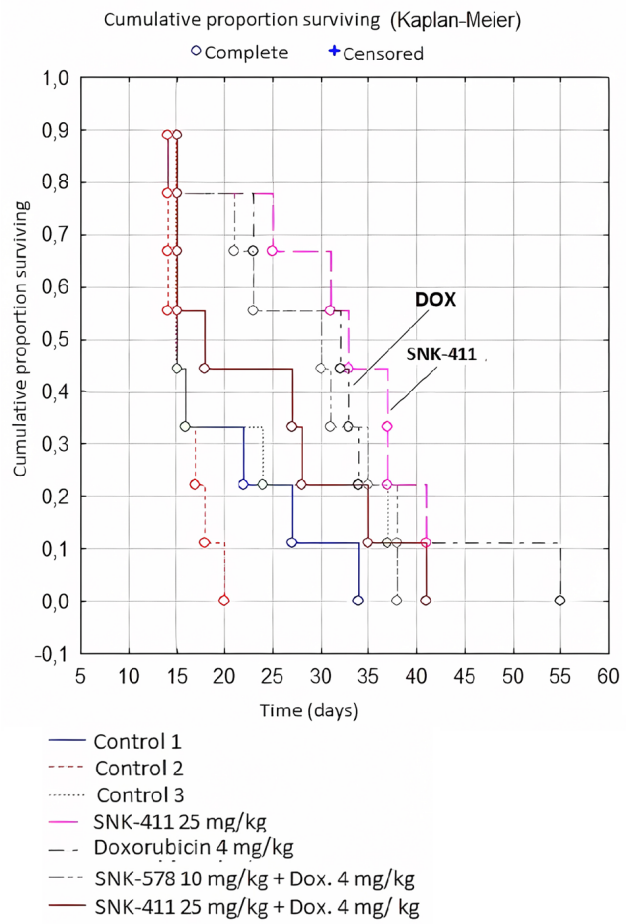

Figure 1. Evaluation of the survival of mice after removal of the primary tumor node LLC according to the Kaplan-Meier survival estimates, Cox's F-test.

Significant concentrations of the Th2 cytokine IL-5 were found in the serum samples of animals of all three control groups; in the serum samples of animals in other experimental groups it was below the determined minimum threshold value. This indicates a pronounced suppression of the hematopoietic Th2 cytokine IL-5, which stimulates tumor-associated eosinophilia, by 5-hydroxypyrimidine derivatives [11-13]. The remaining cytokines IL-1α, IL-2, IL-10, IL-17, GM-CSF, TNF-α were found in minimal single values in serum samples in each of the groups, and no statistically significant results were obtained.

Administration of SNK-411, SNK-578, and doxorubicin did not affect the concentration of the Th1 cytokine IFN-γ. This is consistent with previously obtained data on the suppression of the production of Th2 cytokines IL-6, IL-10, and IL-17A and the absence of a negative effect of 5-hydroxypyrimidine derivatives on IFN-γ, which has antiviral and antitumor properties, in groups of animals with cervical cancer treated with the studied compounds [14].

In our experiments, administration of SNK-411 and SNK-578 to albino guinea pigs inhibited systemic anaphylactic reaction to ovalbumin [7]. Allergy and autoimmune diseases are associated with an excessive Th2- and Th17-mediated immune response, chronic infections and oncological diseases are accompanied by an insufficient Th1-mediated type of immune response. In this regard, the need for drugs that would inhibit the excessive activity of the Th2 pro-oncogenic and pro-allergenic immune response requires the search

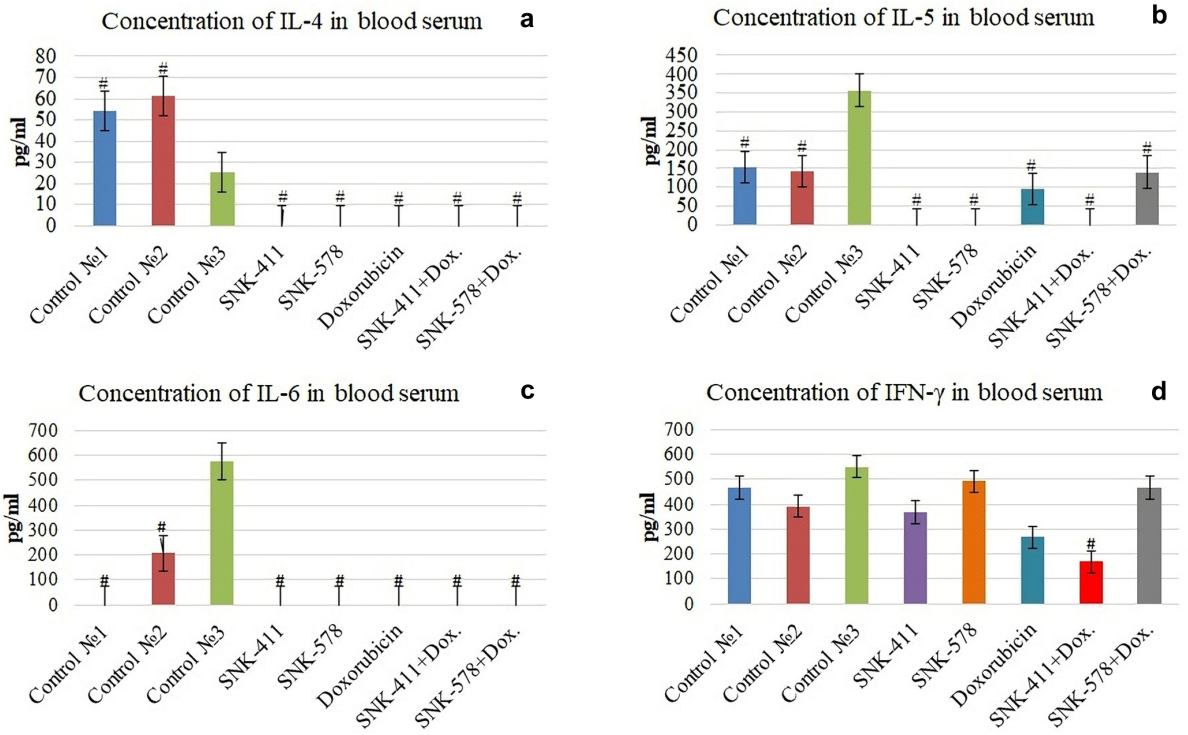

Figure 2. The content of cytokines: **a**) IL-4, **b**) IL-5, **c**) IL-6, **d**) IFN-γ in the blood sera of mice with an LLC tumor against the background of removal of the primary tumor node. # – compared to control no. 3 by Dunnett's test.

for compounds aimed at correcting the balance of Th1/Th2 helper lymphocytes and cytokines [15-18]. According to the latest literature data, the question whether antitumor therapy with anti-PD-1/PD-L1 monoclonal antibodies is effective for some patients is actively discussed, while in others it rarely accelerates tumor development. In the Th1-mediated type of immune-inflammatory response in cancer patients, the prognosis of treatment with checkpoint inhibitors is the most favorable, while in the Th2-mediated type of immune-inflammatory response with a certain set of Th2 cytokines in the tumor microenvironment, no effect or even hyperprogression of the disease can be expected [19-25].

Thus, doxorubicin and 5-hydroxypyrimidine derivatives decreased the serum levels of pro-oncogenic Th2 cytokines IL-4, IL-5, and IL-6 without reducing the level of Th1 cytokine IFN-γ and increased survival of animals. The results obtained in this study confirm the antimetastatic activity in CNK-411 and CNK-578 in the LLC model after removal of the primary tumor node.

CONCLUSIONS

The most important components of inflammation, signaling pathways, cytokines, tumor-associated macrophages (TAMs) associated with cancer are involved in a coordinated system that affects tumor development; the study of this system may shed light on the development of new potential antitumor therapies [26].

Under conditions of removal of the primary tumor node LLC, a pronounced suppression of pro-oncogenic Th2 cytokines by 5-hydroxypyrimidine derivatives and doxorubicin without changes in the level of Th1 cytokine IFN-γ was confirmed. The antimetastatic effect of SNK-411 and SNK-578 compounds, the positive effect of SNK-411, doxorubicin, and co-administration of SNK-578 (i.p.) with doxorubicin on survival indicate the prospects for further study of the antitumor properties of 5-hydroxypyrimidine derivatives. It can be assumed that the antitumor effect is enhanced by the combined use of SNK-411 and SNK-578 with cytostatics and high-molecular targeted antitumor drugs that cause hypersensitization, which requires further research.

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COMPLIANCE WITH ETHICAL STANDARDS

All experiments with animals were carried out in accordance with international rules (Directive 2010/63/EU of the European Parliament and the Council of the European Union of September 22, 2010 on the protection of animals used for scientific purposes) and the rules for working with animals approved by the Ethical Commission of the Zakusov Research Institute of Pharmacology.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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КОРРЕКЦИЯ УРОВНЯ СЫВОРОТОЧНЫХ ПРООНКОГЕННЫХ ЦИТОКИНОВ И МЕТАСТАЗИРОВАНИЯ ПРОИЗВОДНЫМИ 5-ОКСИПИРИМИДИНА И ДОКСОРУБИЦИНОМ ПОСЛЕ УДАЛЕНИЯ ПЕРВИЧНОГО ОПУХОЛЕВОГО УЗЛА У МЫШЕЙ С МЕТАСТАЗИРУЮЩИМ РАКОМ ЛЁГКОГО LLC

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Изучено влияние на метастазирование, продолжительность жизни и концентрацию цитокинов в сыворотках крови мышей после удаления первичного опухолевого узла карциномы лёгкого Lewis (LLC), однократного введения мышам линии C57BL/6 доксорубицина, 8-дневного введения производных 5-оксипиримидина СНК-411 (2-изобутил-4,6-диметил-5-оксипиримидин) и СНК-578 (хлоргидрат 2-изобутил-4,6-диметил-5-оксипиримидина), раздельно, а также в сочетании с однократным введением доксорубицина. Клетки LLC в концентрации 1×10^6 вводили в подушечку правой задней стопы животным контрольных и опытных групп, по истечении 14 дней лапки с опухолью ампутировали по голеностопному суставу. За 1 ч до ампутации лапки с первичным опухолевым узлом однократно вводили доксорубицин в дозе 4 мг/кг и начинали 8-дневную монотерапию СНК-578 в дозе 10 мг/кг внутрибрюшинно (в/бр), СНК-411 в дозе 25 мг/кг перорально и в сочетании с однократным в/бр введением доксорубицина в дозе 4 мг/кг. При введении СНК-411 и СНК-578 индекс ингибирования метастазирования составил по 53,3% по сравнению с контролем с удалённой опухолью. В группе животных, которым вводили СНК-411, отмечено увеличение продолжительности жизни на 60,2%, доксорубицин — на 53,9%, СНК-578 с доксорубицином — на 42,9%. Однократное введение доксорубицина, 8-дневное введение производных 5-оксипиримидина в сочетании с однократным введением доксорубицина полностью снижало содержание проонкогенных Th2 цитокинов IL-4 и IL-6 в образцах сыворотки крови животных опытных групп и значимо уменьшало содержание Th2 цитокина IL-5. Введение доксорубицина, СНК-411 или СНК-578 не снижало концентрацию Th1 цитокина IFN-γ, что подтверждает раннее полученные нами данные об уменьшении концентрации Th2 цитокинов IL-4 и IL-6 у мышей с LLC, и об отсутствии негативного влияния на Th1 цитокин IFN-γ в группах животных, получавших исследуемые соединения.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: модель рака лёгкого *in vivo*; доксорубицин; пиримидины; метастазирование; интерлейкины; выживаемость

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