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ENHANCING EFFECT OF NEW BIOLOGICAL RESPONSE MODIFIER SULFOETHYLATED (1→3)-β-D-GLUCAN ON ANTITUMOR ACTIVITY OF CYCLOPHOSPHAMIDE IN THE TREATMENT OF EXPERIMENTAL MURINE LEUKOSES

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Aim: **One of the advanced methodologies of the tumor therapy is the application of the so-called biological response modifiers used for activation of the endogenous antitumor mechanisms and combined with classical cytotoxic agents. The aim of this work was the investigation of the effect of sulfoethylated (1→3)-β-D-glucan (SEG) in the treatment of experimental murine leukoses in combination with cyclophosphamide (CPA) and its ability to modulate the activity of lysosomal enzymes in tumor tissues.** ***Materials and Methods:*** **The solid forms of inoculated murine leukoses P388 and L1210/1 were transplantated to male DBA/2 mice. The therapy was performed by treating animals with CPA (Biokhimik, Saransk, Russia) alone or in combination with SEG (Institute of Chemistry, Slovak Academy of Sciences, Slovakia). CPA was administered in saline as a single intraperitoneal (ip) injection on the 10th day after tumor transplantation; SEG was administered to mice ip 3 days after tumor transplantation with the intervals in 3 days. The therapy effect was estimated by measuring of solid tumor volume. Activity of the cysteine proteases — cathepsins B and L — was measured fluorometrically using fluorescent substrates Z-Arg-Arg-MCA and Z-Phe-Arg-MCA (Sigma, USA), respectively. The apoptosis was estimated evaluating the number of cells with fragmented nuclei using optical microscope.** ***Results:*** **It has been demonstrated that application SEG leads to inhibition of tumor growth and potentiates therapeutic action of CPA, especially at repeated administrations during the whole treatment/observation At addition of SEG, therapeutic effect of a one-half reduced dose of CPA is equal or higher than that of the full dose. Therapeutic action of CPA and SEG on the studied tumors is realized predominantly through induction of apoptosis and is accompanied by a substantial increase of the activity of cysteine proteases cathepsins B and L in tumor tissues. The highest cathepsin B and cathepsin L activity in tumor tissue accompanied with the strongest inhibition of tumor growth. It is suggested that this phenomenon is due to the infiltration of the macrophages rich in the named enzymes into the tumor, where they phagocytize the apoptotic cells and tissue debris.** ***Conclusion:*** **Utilization of this polysaccharide BRM, sulfoethylated (1→3)-β-D-glucan, might potentially enhance efficiency of antitumor therapy with standard cytostatics without a need of substantial increase of their dosage and hence avoiding their toxic side-effects.**

Key Words: **murine leukosis, cathepsins B, L and D, yeast glucan, cyclophosphamide.**

Increased incidence of oncological diseases has led to the search of the new efficient therapies for malignant neoplasia. One of the advanced methodologies of the tumor therapy is development of such approaches that involve not only a direct cytotoxic effect on the tumor cells, but also consider activation of the endogenous antitumor mechanisms [1]. One of the most efficient applications was the use of the so-called biological response modifiers (BRMs), such as the culture *Corinebacterium parvum*, anti-tuberculosis vaccine BCG [2], as well as the biologically active compounds mainly of the polysaccharidic nature derived from bacteria and fungi, such as prodigiosan, mannan, glucan, muramyldipeptide, etc [3]. In the last decade, much attention has been given to the investigation of the antitumor activity of the natural non-specific immunomodulators — fungal (1→3)-β-D-glucans [4–7]. Glucans are structurally distinct polysaccharides consisting of D-glucose units linked by (1→3)-β and (1→6)-β glycosidic linkages. Many experimental data corroborate the ability of (1→3)-β-D-glucans to non-specifically stimulate cellular and humoral components of the immune system [8]. One of the (1→3,1→6)-β-D-glucans, lentinan, isolated from the oriental edible mushroom shiitake or *Lentinus edodes*, was admitted in Japan for application in clinical antitumor therapy [9–11]. Another fungal glucan, (1→3,1→6)-β-D-glucan isolated from the cell wall of baker's yeast *Saccharomyces cerevisiae* has attracted attention of many researchers due to its ability to enhance the functional status of macrophages and neutrophils [12], modify immunosuppression [13], increase resistance to infections by Gram-negative bacteria [14], as well as exert antitumor activity [15, 16]. Since (1→3)-β-D-glucans consist solely of glucose, they are not toxic to animals and humans, however insolubility in water hinders their application in animal models and humans. In order to circumvent this obstacle, we have synthesized several water-soluble derivatives of yeast β-D-glucan including carboxymethylated and sulfoethylated ones [17, 18]. In a

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Abbreviations used: CPA – cyclophosphamide; SEG – sulfoethylated (1→3)-β-D-glucan; BRMs – biological response modifiers; Z-Arg-Arg-MCA – Z-arginine-arginine-methylcumarilamide; Z-Phe-Arg-MCA – Z-phenyl-arginine-methylcumarilamide.

previous paper, we have demonstrated the possibility of enhancement of antitumor activity of cyclophosphamide by means of addition of the carboxymethylated (1→3)-β-D-glucan in the Lewis lung carcinoma model [1]. In this work, we have investigated the effect of another soluble (1→3)-β-D-glucan derivative, sulfoethylated (1→3)-β-D-glucan (SEG), in the treatment of experimental murine leukoses, its synergistic action with cyclophosphamide, as well as its ability to modulate the activity of lysosomal enzymes in tumor tissues in the process of therapy of leukoses.

MATERIALS AND METHODS

Preparation of SEG. The water-insoluble (1→3)-β-D-glucan was isolated from the commercial baker's yeast biomass purchased from Slovlik (Trenčín, Slovakia). Yeast cells were treated with 6% NaOH at 60 °C followed by 4% phosphoric acid extraction at room temperature as previously described [19]. After the removal of all soluble material, β-D-glucan was left as the insoluble residue. Sulfoethylation of the insoluble β-D-glucan was performed according to Chorvatovičová et al. [20].

Animals and tumors. Male DBA/2 mice, 3–4 months of age used in the experiments were obtained from Research Institute of Pharmacology Siberian Branch of RAMS (Tomsk, Russia). The animals were kept in plastic cages in groups of 8–10 at natural illumination and had free access to a standard pellet diet (Laboratorsnab, Moscow, Russia) and water. Two experimental tumor models were used: transplantable murine leukoses P-388 and L1210/1. The tumors were received from the experimental animal laboratory of the Institute of Cytology and Genetics Siberian Branch of RAS (Novosibirsk, Russia). Leukosis L1210/1 is a version of the leukosis L1210 obtained after a series of *in vitro* passages of initial tumor and characterized by rather benign behavior and absence of visible macroscopic signs of generalization [21]. The work with animals was approved by Ethic committee.

Tumor transplantation and animal treatment. The cryoconserved suspensions of tumor cells were thawed and implanted into abdominal cavity of DBA/2 mice and the developed ascites were used for transplantation to the experimental animals. The ascites were diluted with 20 volumes of physiological saline and 1 ml of a suspension ($1.5–1.7 \times 10^6$ tumor cells) was inoculated intramuscularly (im) into animal's right thigh. In each experiment, shortly after tumor transplantation the animals were divided into four groups, one of which (group 1) was a control group and three other groups (2 to 4) were subjected to therapy. The therapy was performed by treating animals with cyclophosphamide (CPA, Biokhimik, Saransk, Russia) alone (groups 2 and 3) or in combination with SEG (group 4). CPA was administered in saline as a single intraperitoneal (ip) injection on the 10th day after tumor transplantation at the doses of 20 and 40 mg/kg in one experiment and 25 and 50 mg/kg in other experiments. SEG was dissolved in saline and administered to mice ip 3 days after tumor transplantation three times in one experiment and 5–7 times in other experiments. Each single dose of SEG was 25 mg/kg and the intervals between the individual injections were 3 days.

Estimation of tumor growth. Three perpendicular diameters of the tumor were measured with a caliper and tumor volume was calculated by multiplication of their values. When the mice were sacrificed, the excised tumor nodules were homogenized in buffered 0.1% Triton X-100 solution for subsequent determination of proteases activity.

Cysteine protease assay. Activity of the cysteine proteases — cathepsins B and L — was measured fluorometrically as previously described by Svechnikova et al. [22]. Shortly activity of cathepsins B and L were assessed in the tumor tissue using fluorescent substrates Z-Arg-Arg-MCA and Z-Phe-Arg-MCA (Sigma, USA), respectively. At assay of cathepsin L a selective inhibitor of cathepsin B — CA-074 (kind gift of Prof. N. Katunuma, Japan) was added into incubation mixture. Fluorescence was measured at 355 nm (excitation) and 460 nm (emission) using a fluorescent spectrophotometer Perkin-Elmer 650–10S (Japan). The results were expressed as nmol of methylcoumarylamide (MCA)/ min per mg protein. The measurements were carried out in the treated animals on the 3rd day after CPA administration, i. e. 13 days after tumor implantation.

Apoptosis assay. In order to evaluate apoptosis, mice were euthanized 3 days after administration of CPA, tumors were extracted and minced with scissors in physiological solution. The resulting suspension was filtered through nylon sieve. Upon 3 min centrifugation, sedimented cells were fixed in Carnois fluid, dyed with acetocarmine in 40% acetic acid and the number (percentage) of cells with fragmented nuclei (cells in the preterminal apoptosis phase) was evaluated using optical microscope. It should be however pointed out that this method yields underestimated data since it does not take into account cells totally disintegrated to apoptotic corpuscles [23].

Statistical analysis. Statistical analysis was performed using Wilcoxon — Mann — Whitney non-parametrical criteria.

RESULTS

Fig. 1 illustrates the results of the first experiment with leukosis P-388. As can be seen, on the 10th day after tumor transplantation its size was smaller in mice treated with SEG in comparison with those, which did not receive the polysaccharide. During 3–4 days upon administration of CPA at all modes of treatment applied, the tumor size has decreased, however it started to grow again, and this growth was especially pronounced in mice of the second group, which were administered CPA alone in a low dose (25 mg/kg). Mice from the fourth group, which received the same dose of CPA in combination with SEG revealed smaller size of tumor up to the 14th day of the experiment than the mice from the third group treated with higher dose of CPA alone (50 mg/kg), while on the 14th day the size of tumor was similar in mice of these two groups. How-

ever, afterwards tumors in mice of the fourth group grew faster than in the animals of group 3 (see Fig. 1). Thus, application of low dose of CPA after three administrations of SEG exerted better effect than higher dose of CPA only during the early phase of the experiment, while later this advantage disappeared.

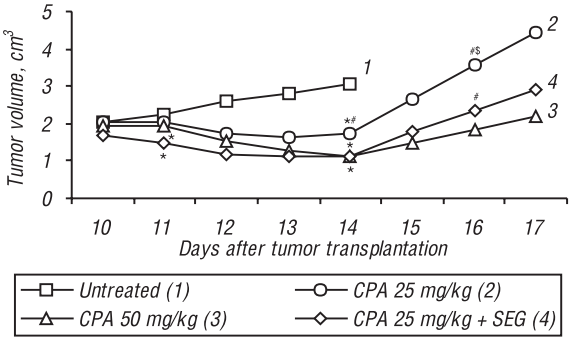

Fig. 1. Effect of cyclophosphamide and sulfoethylated (1→3)-β-D glucan on tumor volume in mice with leukosis P-388
*$P < 0.05$ compared to the untreated mice. # $P < 0,05$ compared to the mice treated with CPA 50 mg/kg. $ $P < 0.05$ compared to the mice treated with CPA 25 mg/kg + SEG. 7–10 mice in each group. CPA was administered to mice on the 10th day after tumor transplantation. SEG was administered to mice on the 3rd, 6th, and 9th days after tumor transplantation (25 mg/kg). Tumor volume was calculated as the product of multiplication of 3 perpendicular diameters of tumor node in murine thigh.

Taking this into account, in the following experiment SEG was applied both before (on the 3rd, 6th, and 9th days), as well as after (on the 12th and 15th days) administration of CPA, which was similarly to the first experiment injected in a dose of 25 mg/kg on the 10th day after transplantation of leukosis P-388. At this scheme of treatment, therapeutic effect of the combined application of low dose CPA and SEG was similar to that of the treatment using higher dose of CPA throughout whole duration of the experiment. Tumor volume at the end of the monitoring (on the 17th day) was 0.42 ± 0.125 cm^3 (combined CPA and SEG application) and 0.48 ± 0.090 cm^3 (application of 50 mg/kg CPA alone), while in mice that received 25 mg/kg CPA alone tumor volume reached 1.61 ± 0.223 cm^3 at the end of the experiment and all control (untreated) animals did not survive till the 17th day.

Similar results have been obtained in the experiments involving leukosis L1210/1. Using this tumor model, CPA in doses of 20 mg/kg and 40 mg/kg was administered once on the 10th day after tumor transplantation, while taking into account results of the preceding experiments, SEG was applied to the animals, which received 40 mg/kg CPA, seven times during the whole monitoring — on the 3rd, 6th, 9th, 12th, 15th, 18th, and 21st days after tumor implantation.

As can be seen in Fig. 2, implants of L1210/1 tumor continued to grow during the first three days after injection of CPA, while within the following three days tumor size decreased to one half (at 20 mg/kg dose) or to one fourth of the initial size (at 40 mg/kg) and the tumor began to grow again after 4 or 7 days, respectively, with practically similar rate as can be judged upon the slope of the curves. In contrast to that, in mice, which were administered CPA and SEG together, tumors started to diminish immediately upon application of CPA and at the end of the experiment, when in the group of mice treated with CPA alone tumor size doubled in two days, at a combined administration of CPA and SEG tumor grew only gradually and the size increase was insignificant (see Fig. 2).

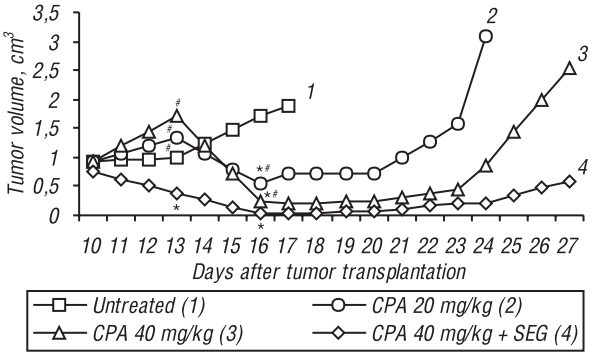

Fig. 2. Effect of CPA and SEG on tumor volume in mice with leukosis L1210/1
*$P < 0,05$ compared with the untreated mice.#$P < 0,05$ compared with the mice treated with CPA 40 mg/kg + SEG. 7–10 mice in each group. CPA was administered to mice on the 10th day after tumor transplantation. SEG was administered to mice on the 3rd, 6th, 9th, 12th, 15th, 18th, and 21rd days after tumor transplantation (25 mg/kg). Tumor volume was calculated as the product of multiplication of 3 perpendicular diameters of tumor node in murine thigh.

The results of the investigation of the activity of the cysteine proteases — cathepsins B and L — in the tumor tissue of mice with implanted leukoses P-388 and L1210/1 on the 3rd day after CPA injection are presented in the Table. As can be seen, untreated animals with either of the tumors revealed practically equal activities of the individual cathepsins and after the onset of treatment activity of cathepsins increased in all experimental groups, while the observed increase was more pronounced with P-388 tumor in comparison with the L1210/1. The highest cathepsin B and cathepsin L activity in tumor tissue accompanied with the strongest inhibition of tumor growth was detected in the animals treated with CPA and SEG (Table).

Table. Tumor weight and cysteine proteases activity in tumor transplants of leukosis P388 and L1210/1 (M ± S.D.)

Group of animals	Tumor weight (g)	Cathepsin B (nmol MCA/min per mg of protein)	Cathepsin L (nmol MCA/min per mg of protein)
Leukosis P388			
1. Untreated animals	1.6 ± 0.11 (100%)	0.16 ± 0.040 (100%)	0.04 ± 0.004 (100%)
2. CPA, 50 mg/kg × 1	1.0 ± 0.13*** (62.5%)	0.51 ± 0.159 (319%)	0.11 ± 0.025* (275%)
3. CPA, 25 mg/kg × 1 + SEG, 25 mg/kg × 4	0.6 ± 0.14*** (37.5 %)	0.74 ± 0.060*** (463%)	0.13 ± 0.009*** (325%)
Leukosis 1210/1			
1. Untreated animals	4.7 ± 0.31 (100%)	0.18 ± 0.017 (100%)	0.04 ± 0.004 (100%)
2. CPA, 40 mg/kg × 1	3.1 ± 0.25** (66.0%)	0.35 ± 0.041** (194%)	0.06 ± 0.004*** (150%)
3. CPA, 40 mg/kg × 1 + SEG, 25 mg/kg × 4	1.8 ± 0.19***## (38.3%)	0.46 ± 0.022***# (256%)	0.07 ± 0.002***# (175%)

Notes: CP was administered to mice on the 10th day after tumor transplantation; SEG was administered on the 3rd, 6th, 9th, and 12th days after tumor transplantation. In parentheses: percentage relatively to the untreated group. The number of animals in each group was 7–10. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to the untreated group, #$P < 0.05$, ##$P < 0.01$ compared to the CPA-treated group.

Shape of the curves in Fig. 2 (increase of tumor volume in the first 2–3 days after CPA injection and the subsequent regression) may suggest that the therapeutic effect in the used model is to a significant extent mediated through induction of apoptosis of the tumor cells [23]. Upon performing the analysis of cell suspensions obtained from the implanted L1210/1 tumor on the 3rd day after beginning of the treatment, it has been found that indeed the predominant portion of the cells contains picnotic and fragmented nuclei, i.e. are in apoptotic state. Number of such cells in the tumors of the treated mice was 3–4 times higher than in the control animals, and in mice treated with CPA after three SEG administrations this number was significantly higher than in those that received CPA alone (Fig. 3, *a*). As can be seen in Fig. 3, *b*, leukosis P388 is also susceptible to CPA-induced apoptosis (effect of a combined administration of CPA and SEG was not studied in this case).

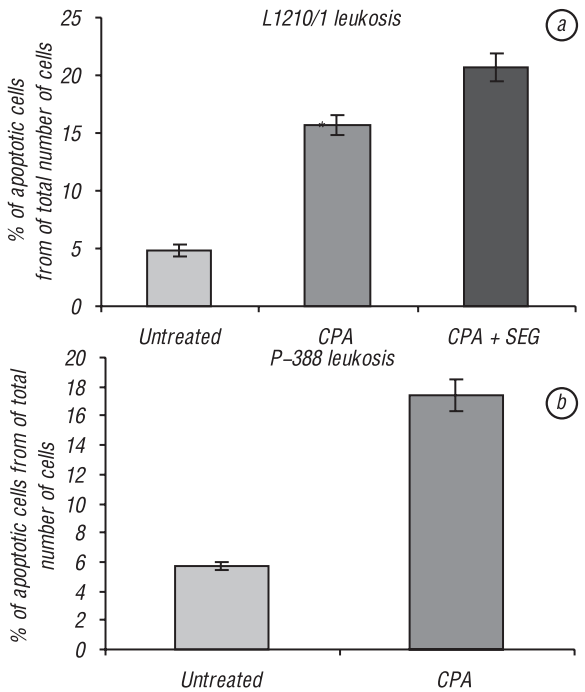

Fig. 3. Apoptosis of the tumor cells in mice with L1210/1 and P-388 leukoses 3 days after CPA administration: *a*) L1210/1 leukosis, CPA 40 mg/kg; *b*) P-388 leukosis, CPA 50 mg/kg

DISCUSSION

The results presented in the study indicate that the prepared sulfoethylated derivative of yeast cell wall (1→3)-β-D-glucan possesses an ability to significantly augment the therapeutic action of CPA in the murine lymphoid tumors. Multiple applications of SEG revealed enhanced effect in comparison to its single administration simultaneously with CPA (data not shown) or its three-time application prior to injection of CPA. Since protective activity of the glucans is known to be mediated via the stimulation of the immune system of the host rather than through direct interaction of the polysaccharide with the infective agent [4, 5, 8] it was not anticipated that application of SEG alone could affect tumor growth and therefore there was no experimental group of animals that received SEG alone. Nevertheless, it was observed that besides potentiating the therapeutic action of CPA, SEG itself was capable of exerting inhibitory effect on tumor growth: upon its three-time administration a certain retardation of the implanted tumor growth was observed already prior to the onset of cytostatics treatment (Fig. 1 and 2). The biological effects of (1→3)-β-D-glucans are initiated through their recognition and binding to the specific cell surface receptors: CR3 [24] lactosylceramide [25] scavenger receptors [26] and dectin-1 [27]. Besides monocytes and macrophages, presence of β-D-glucan receptors has been established also on the neutrophiles [25, 28], NK-cells [29–31], fibroblasts [32] and some tumor cells [33]. Previously we demonstrated that SEG alone suppressed growth of susceptible to CPA murine lymphosarcoma [18].

What mechanism could be involved in its inhibitory activity on tumor growth? The antitumor action of SEG could be ascribed to the ability of yeast β-D-glucan to stimulate release of tumor necrosis factor TNF-α from monocytes/macrophages, as we have previously demonstrated [34]. Some indications of the additional mechanism are provided by the results of the current investigation of the mechanism of death of the tumor cells and of the activities of cathepsins B and L in tumor tissues of the treated animals. Increased content of apoptotic cells in the tumors subjected to the treatment by a combination of CPA and SEG in comparison with those treated with CPA alone implies that SEG is able to induce apoptosis in susceptible cells. Augmented activity of the lysosomal proteases in tumor tissues is justified if these enzymes take part in the induction or realization of apoptosis. However as a more reasonable explanation appears to be a suggestion that the observed post-treatment enhancement of activity of cathepsins B and L in tumor tissues results not from their activation in tumor cells, but rather is due to the increased number of macrophages infiltrating the tumor cells to resorb dying apoptotic tumor cells. We are currently attempting to verify this hypothesis.

In conclusion, combined application of CPA and SEG resulted in the enhanced apoptosis of the leukemic cells and was accompanied by a substantial increase of the activity of cysteine proteases cathepsins B and L in tumor tissues.The results obtained in the present work demonstrate that at addition of SEG, therapeutic effect of a one-half reduced dose of CPA is equal or higher than that of the full dose (Fig. 1 and 2). Thus, utilization of this polysaccharide BRM might potentially enhance efficiency of antitumor therapy with standard cytostatics without a need of substantial increase of their dosage and hence avoiding their toxic side-effects. These results together with the previously published data on the beneficial effect of administration of the derivatives of yeast β-D-glucan in the antitumor therapy of various types of cancer indicate possible utilization of these preparations especially at a combined application with classical anticancer agents.

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ПОТЕНЦИРУЮЩИЙ ЭФФЕКТ СУЛЬФОЭТИЛИРОВАННОГО (1→3)-β-D-ГЛИКАНА НА ПРОТИВООПУХОЛЕВУЮ АКТИВНОСТЬ ЦИКЛОФОСФАНА ПРИ ЭКСПЕРИМЕНТАЛЬНЫХ ЛЕЙКОЗАХ МЫШЕЙ

Цель: **одним из перспективных методов противоопухолевой терапии является использование так называемых модификаторов биологического ответа, применяемых для активации эндогенных противоопухолевых механизмов и комбинируемых с классическими цитотоксическими препаратами. Цель данной работы — исследование эффекта сульфоэтилированного (1→3)-β-D-гликана (SEG) при лечении экспериментальных лейкозов мышей в комбинации с циклофосфаном (CPA) и его способность модулировать активность лизосомных ферментов в опухолевой ткани.** ***Материалы и методы:*** **солидные формы перевиваемых лейкозов мышей P388 и L1210/1 трансплантировали мышам-самцам DBA/2. Для лечения использовали CPA (Биохимик, Саранск, Россия) и его комбинацию с SEG (Институт химии Словацкой Академии Наук, Братислава, Словакия). CPA вводили внутрибрюшинно на 10 сут после перевивки опухолей; SEG вводили внутрибрюшинно начиная с 3 сут после трансплантации лейкозов с интервалом в 3 дня. Терапевтический эффект оценивали путем измерения объема солидной опухоли. Активность цистеиновых протеаз — катепсинов B и L — определяли флюориметрическим методом, используя флюоресцентные субстраты Z-Arg-Arg-MCA и Z-Phe-Arg-MCA (Sigma, США). Апоптоз оценивали по результатам подсчета клеток с фрагментированными ядрами в световом микроскопе.** ***Результаты:*** **в работе показано, что использование SEG приводит к торможению опухолевого роста и потенцирует терапевтический эффект CPA, особенно при повторном введении в течение всего лечения. В сочетании с SEG терапевтический эффект половинной дозы CPA равнозначен или превышает действие полной дозы цитостатика. Воздействие CPA и SEG на использованные в исследовании опухоли реализуется в основном через индукцию апоптоза и сопровождается существенным повышением активности цистеиновых протеаз катепсинов B и L в опухолевой ткани. Наиболее высокая активность катепсинов B и L сопровождается максимальным подавлением опухолевого роста. Предположительно это обусловлено инфильтрацией опухолевой ткани макрофагами с высоким содержанием вышеназванных ферментов, где они фагоцитируют клетки в апоптозе.** ***Выводы:*** **использование сульфоэтилированного (1→3)-β-D-гликана, нового модификатора биологического ответа дает возможность существенно повысить эффективность противоопухолевой терапии стандартными цитостатиками без повышения их дозы, что позволяет избежать побочных эффектов данных препаратов.**

Ключевые слова: **лейкозы мышей, катепсины, дрожжевые гликаны, циклофосфан.**