RESEARCH ARTICLE

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

HEMOSTIMULATING PROPERTIES OF THE CONJUGATES OF GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR WITH ALENDRONIC ACID

G.G. Shimina, A.V. Bateneva, E.S. Tsyplenkova, S.G. Gamaley, T.I. Esina, E.A. Volosnikova, E.D. Danilenko

Institute of Medical Biotechnology of State Research Center of Virology and Biotechnology "VECTOR", Federal Service for Surveillance on Consumer Rights Protection and Human Well-being, 9, Khimzavodskaya St., Berdsk, Russia, 633004

E-mail: shimina_gg@vector.nsc.ru

Received 04 Aug 2022 After peer review 20 Oct 2022 Accepted 30 Oct 2022

The aim of the work is to evaluate the hemostimulating activity of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) conjugates with alendronic acid (ALN) in the model of cytostatic myelosuppression and the dynamics of rhGM-CSF accumulation as a part of the conjugate in the bone tissue and bone marrow of mice.

Materials and methods. The conjugates obtained by a solid-phase synthesis using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide or periodate oxidation, were used. A hemostimulating activity was evaluated in a model of a cytostatic myelosuppression induced by the administration of cyclophosphamide to CBA/Calac mice. RhGM-CSF preparations were injected subcutaneously for 4-5 days at the dose of 90 μg/kg. After the injections cycle had been completed, the total leukocyte and segmented neutrophil counts were carried out in the blood samples, and the total karyocyte count was carried out in the bone marrow samples.

The tissue distribution of rhGM-CSF preparations was assessed in outbred CD-1 mice after a single intravenous administration at the effective dose. The content of rhGM-CSF in blood, femoral tissue and bone marrow was determined by enzyme immunoassay.

Results. RhGM-CSF conjugates with ALN have been shown to retain the ability of the original protein to increase the number of leukocytes, segmented blood neutrophils, and bone marrow karyocytes under the action of conjugates. The stimulation of the neutrophil production used to be observed at earlier times than in the case of rhGM-CSF. The increase in the total number of bone marrow cells after the introduction of all three conjugates was more pronounced compared to the original protein (by 34%). The increased hemostimulatory effect of the AEG conjugate was accompanied by a more intense accumulation of rhGM-CSF in the bone tissue and bone marrow of mice. The rhGM-CSF introduced into the conjugate was detected in the bone tissue for 24 h and it circulated in the bloodstream for a longer time compared to the original protein.

Conclusion. The data obtained make it possible to conclude that further work on the development of effective hemostimulating drugs based on rhGM-CSF conjugates with ALN, is promising.

Keywords: recombinant human granulocyte-macrophage colony-stimulating factor; alendronic acid; conjugate; hemostimulating activity; accumulation in bone tissue and bone marrow.

Abbreviations: ALN – alendronic acid; GM-CSF – granulocyte-macrophage colony-stimulating factor; rhGM-CSF – recombinant human granulocyte-macrophage colony-stimulating factor; HAP – hydroxylapatite; EDC – 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide; CP – cyclophosphan; ELISA – electronic intelligence search and analysis; TKC – total karyocyte count.

For citation: G.G. Shimina, A.V. Bateneva, E.S. Tsyplenkova, S.G. Gamaley, T.I. Esina, E.A. Volosnikova, E.D. Danilenko. Hemostimulating properties of the conjugates of granulocyte-macrophage colony stimulating factor with alendronic acid. *Pharmacy & Pharmacology.* 2022;10(5): 472-482. **DOI:** 10.19163/2307-9266-2022-10-5-472-482

Для цитирования: Г.Г. Шимина, А.В. Батенева, Е.С. Цыпленкова, С.Г. Гамалей, Т.И. Есина, Е.А. Волосникова, Е.Д. Даниленко. Гемостимулирующие свойства конъюгатов гранулоцитарно-макрофагального колониестимулирующего фактора с алендроновой кислотой. *Фармация и фармакология.* 2022;10(5):472-482. **DOI:** 10.19163/2307-9266-2022-10-5-472-482

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОРИГИНАЛЬНАЯ СТАТЬЯ

DOI: 10.19163/2307-9266-2022-10-5-472-482

ГЕМОСТИМУЛИРУЮЩИЕ СВОЙСТВА КОНЪЮГАТОВ ГРАНУЛОЦИТАРНО-МАКРОФАГАЛЬНОГО КОЛОНИЕСТИМУЛИРУЮЩЕГО ФАКТОРА С АЛЕНДРОНОВОЙ КИСЛОТОЙ

Г.Г. Шимина, А.В. Батенева, Е.С. Цыпленкова, С.Г. Гамалей, Т.И. Есина, Е.А. Волосникова, Е.Д. Даниленко

Институт медицинской биотехнологии Федерального бюджетного учреждения науки «Государственный научный центр вирусологии и биотехнологии «Вектор» Федеральной службы по надзору в сфере защиты прав потребителей и благополучия человека, 633004, Россия, г. Бердск, ул. Химзаводская, д. 9

E-mail: shimina_gg@vector.nsc.ru

Получена 04.08.2022 После рецензирования 20.10.2022 Принята к печати 30.10.2022

Цель. Оценка гемостимулирующей активности конъюгатов рекомбинантного гранулоцитарно-макрофагального колониестимулирующего фактора человека (рчГМ-КСФ) с алендроновой кислотой (АЛН) на модели цитостатической миелосупрессии и динамики накопления рчГМ-КСФ в составе конъюгата в костной ткани и костном мозге мышей.

Материалы и методы. В работе использовали конъюгаты, полученные методом твердофазного синтеза с помощью 1-этил-3-[3-диметиламинопропил]карбодиимида или реакции периодатного окисления. Гемостимулирующую активность оценивали на модели цитостатической миелосупрессии, вызванной введением мышам CBA/Calac циклофосфана. Препараты рчГМ-КСФ вводили подкожно в течение 4–5 дней в дозе 90 мкг/кг. По окончании курса инъекций в образцах крови подсчитывали общее количество лейкоцитов, сегментоядерных нейтрофилов, в образцах костного мозга – общее число кариоцитов. Оценку распределения препаратов рчГМ-КСФ по тканям проводили на аутбредных мышах CD-1 после однократного внутривенного введения в эффективной дозе. Содержание рчГМ-КСФ в крови, ткани бедренной кости и костном мозге определяли иммуноферментным методом.

Результаты. Показано, что конъюгаты рчГМ-КСФ с АЛН сохраняли присущую исходному белку способность повышать число лейкоцитов, сегментоядерных нейтрофилов крови и кариоцитов костного мозга. Стимуляция продукции нейтрофилов под действием конъюгатов наблюдалась в более ранние сроки, чем в случае рчГМ-КСФ. Увеличение общего числа клеток костного мозга после введения всех трех конъюгатов было более выраженным по сравнению с исходным белком (на 34%). Повышенный гемостимулирующий эффект конъюгата AEG сопровождался более интенсивным накоплением рчГМ-КСФ в костной ткани и костном мозге мышей. Введенный в состав конъюгата рчГМ-КСФ обнаруживался в костной ткани в течение 24 ч и более длительно циркулировал в кровеносном русле по сравнению с исходным белком.

Заключение. Полученные данные позволяют сделать вывод о перспективности дальнейших работ по созданию эффективных гемостимулирующих препаратов на основе конъюгатов рчГМ-КСФ с АЛН.

Ключевые слова: рекомбинантный гранулоцитарно-макрофагальный колониестимулирующий фактор человека; алендроновая кислота; конъюгат; гемостимулирующая активность; накопление в костной ткани и костном мозге

Список сокращений: АЛН – алендроновая кислота; ГМ-КСФ – гранулоцитарно-макрофагальный колониестимулирующий фактор; рчГМ-КСФ – рекомбинантный гранулоцитарно-макрофагальный колониестимулирующий фактор человека; ГАП – гидроксилапатит; EDC – 1-этил-3-[3-диметиламинопропил] карбодиимид; ЦФ – циклофосфан; ИФА – иммуноферментный анализ; ОКК – общее количество кариоцитов.

INTRODUCTION

The relevance of the search for new drugs and methods for the treatment of neutropenia of various etiologies, arising, in particular, as a result of chemotherapy and radiotherapy in cancer patients, is extremely high. Chemotherapy-induced disorders of hematopoiesis and, above all, suppression of leukocyte production, in some cases become the main indicators for interrupting the treatment, despite a distinct oncolytic therapy effect [1].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a natural cytokine protein that regulates the proliferation and differentiation of stem cells with the formation of colonies of neutrophilic, eosinophilic leukocytes, and macrophages [2–4]. The ability of GM-CSF to enhance hematopoiesis served as a basis for the development and introduction of drugs based on it, Leukine and Leucomax (USA), into clinical practice, to reduce the side effects of the antitumor therapy, increase resistance to infections, during bone marrow transplantation. However, despite a high level of GM-CSF preparations safety, their use leads to the development of adverse reactions such as fever, chills, lethargy, myalgia, bone pain, fever, body weight fluctuations,

generalized pruritus, redness and an aerythematous reaction around the site of the subcutaneous injection [5–8]. In this regard, the improvement of GM-CSF preparations in terms of increasing their affinity for the target tissues, reducing the therapeutic dose and, as a result, side treatment effects, is of undoubted interest.

Literature references describe examples of various vector molecules used for the targeted delivery of immunoregulatory ligands, in particular, cytokines [9–11]. As a means of delivering biologically active substances to the bone tissue and bone marrow, bisphosphonates which are characterized by a high affinity for calcium ions and the ability to rapidly accumulate in the bone, are used [12–17]. One of the methods for obtaining the targeted drugs based on bisphosphonates, is their conjugation with medicinal preparations [12, 13].

Taking into account all of the above, conjugates of a human tumor necrosis factor alpha with alendronic acid aminobisphosphonate (ALN), which demonstrated the ability to accumulate in the foci of bone metastasis of the tumor, thereby exhibiting an antitumor activity, was obtained [18, 19]. These data became a basis for the use of ALN as a vector molecule for a delivery of a recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) to bone marrow cells.

A technology for the production of rhGM-CSF in a prokaryotic expression system (recombinant strain E. coli SG20050/p280_2GM) [20], as well as methods for conjugating rhGM-CSF with ALN using different types of cross-linking agents, has been developed at the Institute of Medical Biotechnology of the State Research Center for Virology and Biotechnology “Vector”, Federal Service for Surveillance on Consumer Rights Protection and Human Well-being (IMBT FBRI SRC VB "Vector", Rospotrebnadzor) [21]. It has been shown that the resulting conjugates, compared to rhGM-CSF, have an increased affinity for hydroxylapatite, an analog of the mineral bone tissue matrix. The evaluation of the specific conjugates activity *in vitro* confirmed the preservation of the biological activity of rhGM-CSF in the composition [21].

THE AIM of the work is to evaluate the hemostimulatory activity of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) conjugates with alendronic acid (ALN) in the model of cytostatic myelosuppression and the dynamics of rhGM-CSF accumulation as a part of the conjugate in the bone tissue and bone marrow of mice.

MATERIALS AND METHODS

Study drugs

Experimental preparations were conjugates of rhGM-CSF with ALN obtained by the described methods [21]. The key point in the synthesis process was the choice of conditions that would minimize conformational changes in protein molecules in order to preserve their biological activity. For this, equimolar amounts of the rhGM-CSF protein and ALN were used. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) was chosen as the crosslinking agent; dextran with a molecular weight of 40,000 Da, activated with periodate, was used as a linker. The conjugation with EDC was carried out in two ways: a direct (rhGM-CSF →EDC → ALN) and reverse (ALN →EDC → rhGM-CSF) sequence of components application to the solid phase (hydroxyapatite sorbent, HAP).

For the direct sequence conjugation, the HAP chromatographic column was equilibrated with 2 mM potassium phosphate buffer, pH 7.0. A rhGM-CSF protein solution was applied to the balanced column, after the sorption of which the EDC solution was also fed to the column. The outlet of the solution from the column was blocked for 2 h to bind the components, then the column was washed with 2 mM potassium phosphate buffer (pH 7.0), and the ALN solution was applied. The column was repeatedly washed with phosphate buffer to remove the unbound components. The resulting conjugate was eluted from the column with 0.2 M potassium phosphate buffer, pH 7.0. The synthesized conjugate was transferred into a *physiological saline solution* by dialysis.

When dextran was used as a linker during the conjugation, it was added to the solution containing sodium periodate to form reactive aldehyde groups in the ratio of 1:40 (mol/mol), mixed, and incubated at 20°C for an hour. The activated dextran was separated by a gel filtration on the Sephadex G-25 column. The solutions of protein and ALN were added to the dextran solution in equimolar ratios: 1 mol of protein and 1 mol of ALN per 1 mol of dextran. The resulting mixture was incubated for 3 h at 20°C. To remove the unreacted components, the gel filtration was performed on the Sephadex G-25 column. The synthesized conjugate was transferred into the physiological saline by dialysis.

Three types of conjugates were obtained:

1 – GEA – rhGM-CSF conjugate with ALN, obtained by a solid-phase synthesis through the carboxyl group of the protein using EDC by the method of a direct (protein→EDC→ALN) sequence of applying components to the sorbent, with a protein concentration of 1.09 mg/ml (Fig. 1, track 1);

2 – AEG – conjugate of rhGM-CSF with ALN, obtained by a solid-phase synthesis through the carboxyl group of the protein using EDC by the reverse (ALN→EDC→protein) sequence of applying components to the sorbent, with a protein concentration of 1.45 mg/ml (Fig. 1, track 2);

3 – DGA – conjugate of rhGM-CSF with ALN, obtained by a synthesis through the protein amino group *via* a dextran molecule as a linker using the Malaprade reaction [22], with a protein concentration of 0.86 mg/ml (Fig. 2, track 1).

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-5-472-482

Reference drug

The rhGM-CSF protein, obtained at the IMBT FBRI SRC VB "Vector", Rospotrebnadzor according to the described method [23], was used as a reference drug and to obtain conjugates with ALN.

The protein substance was characterized by quality indicators in accordance with the requirements of regulatory documentation[1]. The protein concentration in the used substance was 1.5 mg/ml, the homogeneity of the preparation was 99.2%.

Experimental animals

The study was carried out on 66 healthy male CBA/Calac mice weighing 19–23 g, obtained from the nursery of Goldberg Research Center of Pharmacology and Regenerative Medicine (Tomsk), and 45 female white outbred CD-1 (ICR) mice weighing 22–25 g from the nursery of the Federal Budgetary Institution of the State Research Center "Vector" of Rospotrebnadzor (Koltsovo, the Novosibirsk region). The age of the animals was 2.0–2.5 months. Before the start of the study, the animals had gone through a period of the adaptive quarantine. The mice were kept under standard vivarium conditions at the constant temperature and humidity; food and drink were available at any time of the day. The animals' keeping and experimental studies were carried out in accordance with the requirements of the International Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (Strasbourg, 1986), as well as in compliance with Directive 2010/63/EU of the European Parliament and Council of the European Union dated September 22, 2010, for the protection of the animals used for scientific purposes. The studies were approved by the Bioethical Commission of "Vector" (protocol No. 5 dated 01.10.2020).

Method for assessing hemostimulating activity of conjugates

The hemostimulating activity of the drugs was studied in a model of cytostatic myelosuppression induced by the administration of cyclophosphamide (CP, Sigma-Aldrich, USA) to CBA/Calac mice [24].

The animals were divided into 5 experimental groups (4 experimental and 1 control); there were 12 male mice in each one. All the experimental animals received a single intraperitoneal injection of a CP solution at the maximum tolerated dose (250 mg/kg) in the volume of 0.25 ml per 20 g of body weight.

24 hours after the CP injection, the mice of the experimental groups were injected subcutaneously for 4–5 days with one of the following drugs: 1) rhGM-CSF; 2) GEA conjugate; 3) AEG conjugate; 4) DGA conjugate. The administration dose, previously determined as an effective hemostimulating agent using the rhGM-CSF preparation [25], was 90 μg/kg; the volume of the administration was 0.2 ml per 20 g of the animal body weight. The mice of the control group received subcutaneous saline in the equivalent volume according to the similar scheme. Six intact animals were used as an additional control group (without the administration of rhGM-CSF and CF preparations). All manipulations with the animals were carried out at the same time (in the morning).

One day after the drug administration completion (on the 5th day after the CP administration), the blood samples from the tip of the tail were taken for the analysis from each group in half of the animals, and after the cervical dislocation of the cervical vertebrae, the bone marrow samples were taken. On that day, the second half of the animals received another injection; the biomaterial was taken for the analysis 24 hours after injection.

In the blood samples, the total leukocyte count was determined by a light microscopy (the blood was diluted 20 times with a 3% solution of acetic acid, the calculation was carried out in Gorjaev's chamber), the relative and absolute contents of neutrophils and other morphological forms of leukocytes were also detremined. In the bone marrow samples, the total karyocyte count (TKC) was carried out and the number of cells per femur was calculated. To obtain the bone marrow, the mouse femur was isolated, it was cleaned from the soft tissues of the femur, and the bone marrow canal was thoroughly washed with a 3% acetic acid solution in the volume of 1 ml. The TKC was carried out using Gorjaev's chamber.

Method for studying rhGM-CSF accumulation dynamics

To study the rhGM-CSF accumulation dynamics in bone tissue and bone marrow, CD-1 female mice were divided into 3 groups: a control group (5 individuals) and two experimental groups (20 individuals in each). On the eve of the experiment, at the end of the working day, the mice were transferred to clean cages without food. The animals were given food 2 hours after the drugs administration, there was water without restrictions.

The animals of the first experimental group received an intravenous rhGM-CSF injection; the mice of the second experimental group were injected with the AEG conjugate. The preparations were administered at the dose of 90 μg/kg of body weight, 0.2 ml per 20 g of animal weight. The intact mice served as control animals (the third group).

[1] GPM.1.7.1.0007.15 "Medicines obtained by recombinant DNA methods". Russian State Pharmacopeia ed 14. 2018;4: 2575-95. Available from: https://docs.rucml.ru/feml/pharma/v14/vol2/763/

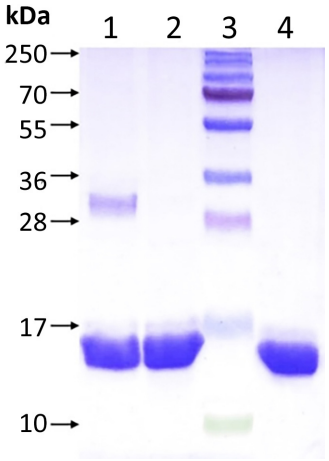

Figure 1 – Electrophoregram of conjugates obtained by direct sequence of applying components, GEA (1) and reverse sequence, AEG (2)

Note: Electrophoresis in 15% polyacrylamide gel under reducing conditions, R-250 Coomassie staining. Tracks: 3 – protein marker 10–250 kDa; 4 – rhGM-CSF protein, 20 µg.

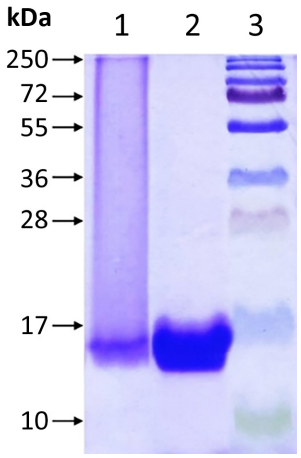

Figure 2 – Electropherogram of conjugate obtained using dextran (1)

Note: Electrophoresis in 15% polyacrylamide gel under reducing conditions, R-250 Coomassie staining. Tracks: 2 – rhGM-CSF protein, 20 µg; 3 – protein marker 10–250 kDa.

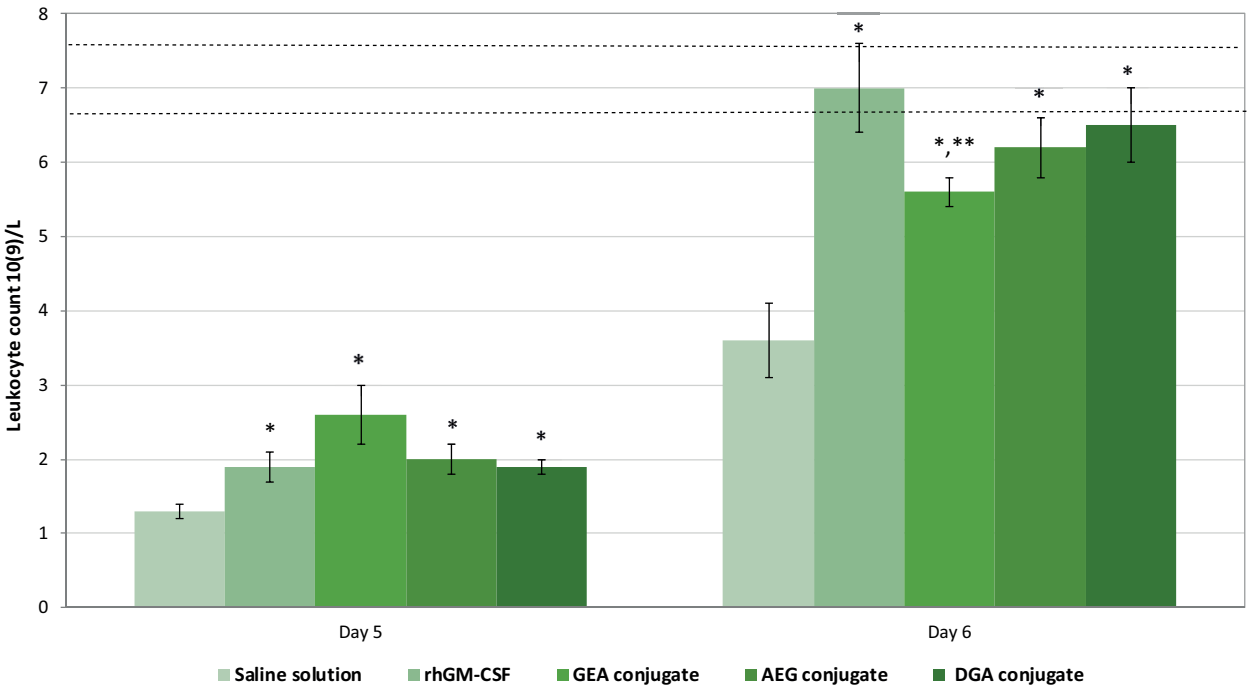

Figure 3 – Leukocyte count in peripheral CBA mice blood against the background of CP administration, rhGM-CSF drug and its conjugates with ALN

Note: abscissa shows study time (days); * – statistically significant difference in relation to the control (saline); ** – statistically significant difference in relation to rhGM-CSF at p ≤0.05. Area between dotted lines is confidence interval of indicator in intact mice.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-5-472-482

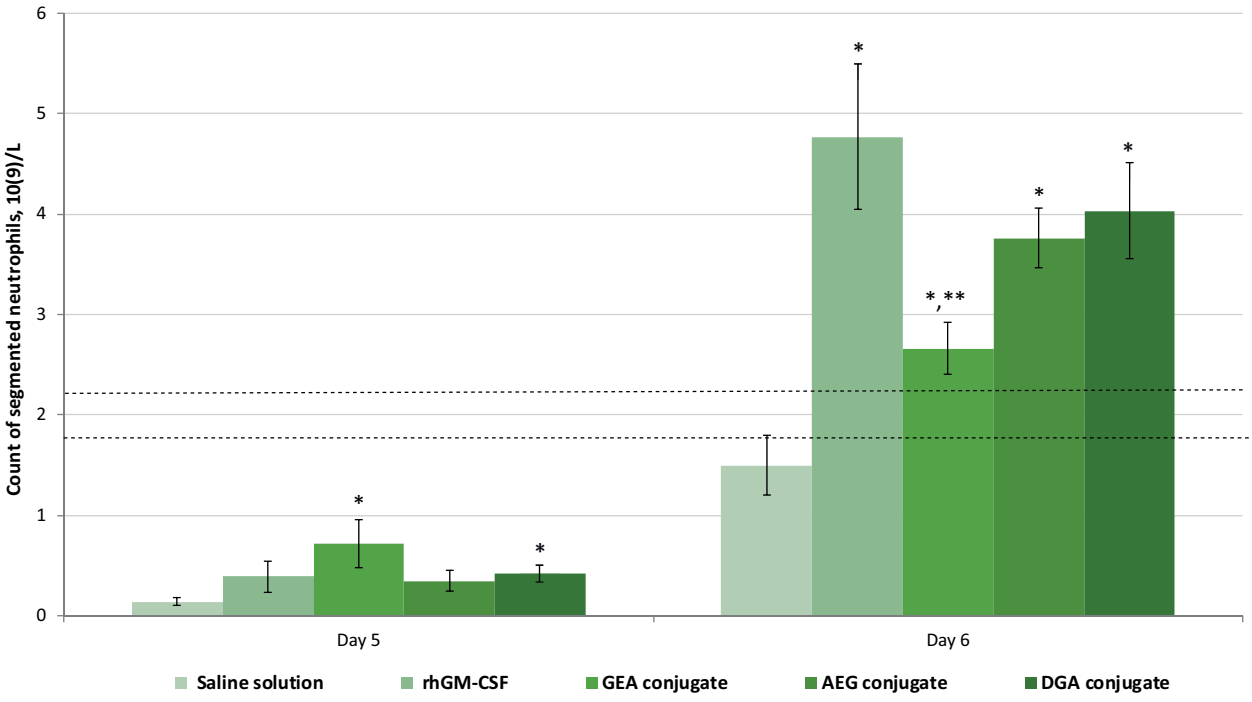

Figure 4 – Count of segmented neutrophils in peripheral CBA mice blood against the background of CP administration, rhGM-CSF drug and its conjugates with ALN

Note: abscissa shows study time (days); * – statistically significant difference in relation to the control (saline); ** – statistically significant difference in relation to rhGM-CSF at p ≤0.05. Area between dotted lines is confidence interval of indicator in intact mice.

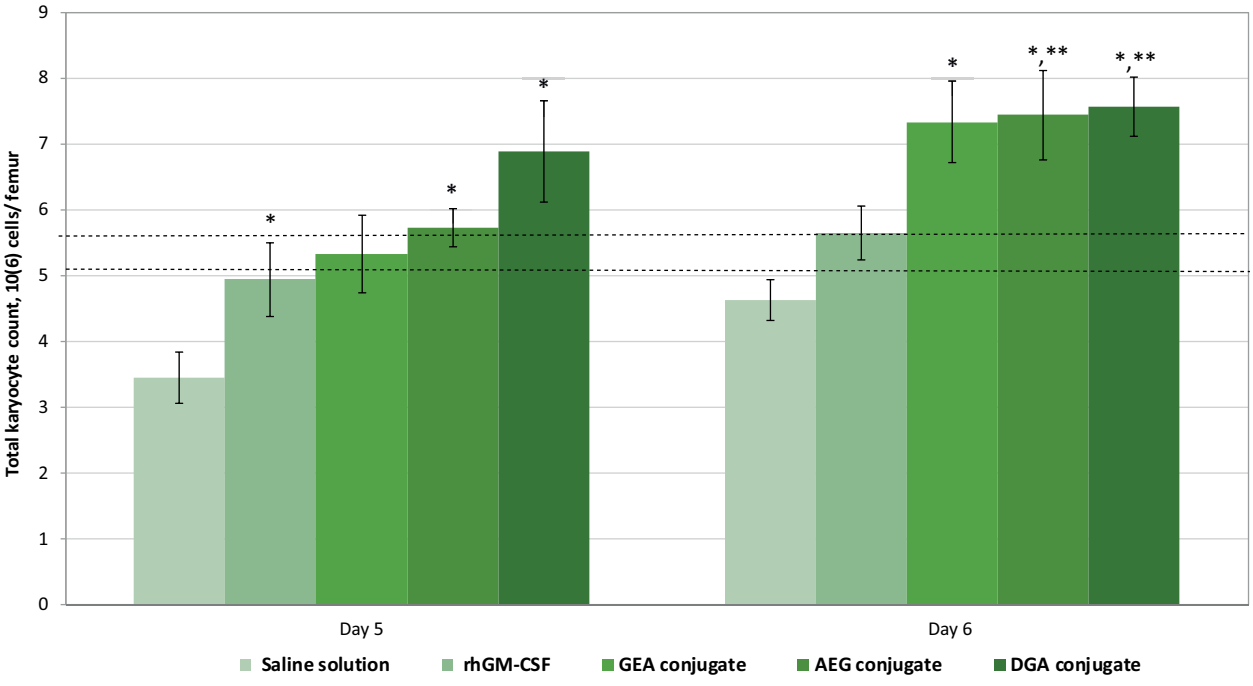

Figure 5 – Karyocyte counts in CBA mice bone marrow against the background of CP, rhGM-CSF its conjugates with ALN administration

Note: abscissa shows study time (days); * – statistically significant difference in relation to the control (saline); ** – statistically significant difference in relation to rhGM-CSF at p ≤0.05. Area between dotted lines is confidence interval of indicator in intact mice.

Table 1 – Dynamics of changes in the level of rhGM-CSF in the blood, bone tissue and bone marrow of mice after a single intravenous administration of rhGM-CSF preparations

Drug	Concentration of rhGM-CSF in samples			
	Blood serum, pg/ml			
	3 minutes	1 hour	4 hours	24 hours
rhGM-CSF	151 715±32 571	3 138±214	24.5±2.3	0.174±0.148
AEG conjugate	406 468±54 586**	8 500±2 539**	48.8±9.0**	0.676±0.676
	Femur, pg/g			
Control	184±139			
rhGM-CSF	3 869±458*	322±151	436±52	137±105
AEG conjugate	11 154±1 613*,**	1 108±387	1 706±374*,**	1 652±449
	Bone marrow of femur, pg/femur			
Control	3.52±2.03			
rhGM-CSF	237±36*	9.84±7.47	0±0	1.20±1.20
AEG conjugate	567±127*	20.3±12.8	5.64±5.64	15.7±9.7

Note: experimental data are presented as arithmetic mean and standard error (M±m); * – differences are statistically significant compared to control; ** – differences are statistically significant compared to mice that were injected with rhGM-CSF ($p<0.05$ for blood; $p<0.017$ for tissues).

3 minutes later, 1, 4 and 24 hours after the drugs administration, the blood and one femur was taken from 5 animals from each experimental group after euthanasia. On the first day of the experiment, a similar material was taken from the mice in the control group. The serum was obtained from the blood; the bone marrow was extracted from the femur by washing the bone marrow canal with a 0.9% sodium chloride solution in the volume of 1 ml. The bone marrow cells were resuspended with a dispenser until a homogeneous suspension. After the bone marrow removal, the femur was weighed and a 10% homogenate was prepared in 0.1 M potassium phosphate buffer (pH 7.2) using a GlasCol homogenizer (USA). The resulting biomaterial was stored at the temperature not exceeding –20°C. On the day of the analysis, the bone marrow and femur homogenates were thawed, centrifuged (5810R centrifuge, Eppendorf, Germany) at 5000 g and the temperature of 2–8°C for 5 min, and the supernatants were collected.

In the blood serum and supernatants of femur homogenatesthe content of rhGM-CSF was determined by electronic intelligence search and analysis (ELISA) using reagent kits for the determination of human GM-CSF in serum, blood plasma, supernatants of cell cultures and organ homogenates "Human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) ELISA Kit", "CUSABIO", China. The range of the standard sample determined concentrations included in the kit, was 15.6–1000 pg/ml, the sensitivity was less than 3.9 pg/ml.

Statistical processing of obtained results

The results obtained were processed using the "Statgraphics, Version 5.0" software package ("Statistical Graphics Corp.", USA). Due to the small sample sizes, non-parametric tests were used to assess the significance of intergroup differences – the two-sample Mann-Whitney U-test and the Kruskal-Wallis H-test of multiple comparisons with a critical level of statistical significance (p) equal to 0.05. When statistically significant differences were found in the H-test, post-hoc comparisons were performed using the U-test, while the adjusted critical significance level (p) for three pairwise comparisons was taken equal to 0.0170 [26, 27]. The experimental data are presented as the arithmetic mean and standard error (M±m). The figures are constructed using Microsoft Excel.

RESULTS AND DISCUSSION

The study showed that a single CP administration to the mice at the dose of 250 mg/kg led to the regular development of myelosuppression. The total karyocyte count of the bone marrow in the control group mice on the 5th day after the CP administration decreased by 1.5 times, the blood total leukocyte count- by 5.5 times, the count of segmented neutrophils – by 13.9 times.

The change in the counts of leukocytes, segmented neutrophils, and bone marrow cells in the mice after the drug administration was evaluated in comparison with the indicators of the control group (physiological saline), the values of which were taken as 100%. The administration of both rhGM-CSF and its conjugates with ALN to the animals resulted in a faster statistically significant ($p \leq 0.05$) increase in the blood leukocyte count compared to the control values (Fig. 3). On the 5th day after the CP administration, the blood leukocyte count in the mice treated with rhGM-CSF preparations exceeded the control value by 46–100%, on the 6th day – by 56–94%. Significant differences in the level of the leukopoiesis stimulation in the groups that had been injected with different types of conjugates or rhGM-CSF, were not observed.

On the 5th day after the CP administration, in the mice groups that had been injected with GEA and

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-5-472-482

DGA conjugates, a statistically significant increase in the number of segmented neutrophils compared with the control ones, was notified by 414 and 200%, respectively, p≤0.05). On the 6th day, with the conjugates administration, the increased values of the indicator were registered in all the two groups (by 77 and 169%). A significant stimulating effect of the initial rhGM-CSF was observed only on the 6th day, the count of the segmented neutrophils in the blood during this period increased by 218% compared with the index of the animals treated with saline (Fig. 4).

The data presented in Fig. 5, shows that 5 days later, the rhGM-CSF administration led to a statistically significant increase in the total karyocytes count, by 43% after the CP administration compared with the control values. Similar, but more pronounced, changes were notified in the groups of mice that had been injected with the conjugates. At the same time, the DGA effect (an increase of 100% compared to the control) was maximal. Six days after the cytostatic administration, the karyocytes count in the bone marrow of the mice that had been injected with conjugates, was 58–63% higher than the control level, and 30–34% higher than the level recorded after the rhGM-CSF administration.

Thus, the conjugation of rhGM-CSF with ALN did not lead to a decrease in the hemostimulatory protein activity. No significant differences were found in the level of the leukostimulating activity of the studied preparations. The GEA and DGA conjugates accelerated the recovery of the murine blood neutrophil count. The AEG and DGA conjugates had a more pronounced stimulating effect on the production of bone marrow cells compared to GM-CSF, which was manifested in a more intense and earlier increase in the total bone marrow cellularity after the exposure to the cytostatic.

The enhancement of the stimulating rhGM-CSF effect in the conjugate on the production of karyocytes can obviously be associated with an increased affinity of the protein to the bone tissue cells.

To confirm the targeted rhGM-CSF delivery in the composition of the ALN conjugate to the bone tissue, a comparative study of the drug accumulation in the bone tissue and bone marrow after the intravenous rhGM-CSF and its AEG conjugate administration, was carried out.

The dynamics in protein concentration changes in the blood, the bone tissue and bone marrow of mice after a single intravenous rhGM-CSF and its conjugate administration at the effective hemostimulating dose (90 μg/kg), is presented in Table 1.

The obtained data indicate that the highest rhGM-CSF blood values in the mice were registered 3 min after the drug administration and amounted to 19.2% of the administered dose in the mice treated with AEG conjugate, and to 7.2% in the mice after the rhGM-CSF administration. (Table 1). The protein was retained in the blood serum of mice of the both experimental groups for 4 hours of the observation. 1 hour after the drugs administration, the values of the indicator decreased by 48 times compared to the “3 min” point, and in the subsequent periods, their further decrease to the background level was notified. The rhGM-CSF concentration in the blood of mice that had been administrated with the conjugate, exceeded the corresponding indicator after the rhGM-CSF administration by 2.7 times after 3 minutes and 1 hour, and twice after 4 hours after the administration (the differences are statistically significant, p ≤0, 05). 24 hours after the injection, rhGM-CSF was detected in small amounts in the blood of two out of five animals after the rhGM-CSF administration, and in one out of five mice that had been administrated with the AEG conjugate. In the blood serum of the control animals, the values of the indicator did not differ from the zero in any of the observation periods.

The highest rhGM-CSF level in the mice femoral bone homogenates was detected 3 min after the drug administration. At the same time, the protein content in the bone tissue of the mice treated with the conjugate at this point was 60 times higher than that of the animals administrated with rhGM-CSF. In the subsequent periods, the protein concentration in all the mice femurs that had been administrated with rhGM-CSF, decreased to the control level (1 hour after the administration, Table 1). In the bone tissue samples of the mice that had been administrated with AEG conjugate, the concentration of rhGM-CSF 1 hour after the injection was 6 times higher than the control level (the differences are not statistically significant, p ≤0.017), in the subsequent periods (4 and 24 hours) – by 9 times (the statistial differences were observed at the 4-hour point). 24 hours after injection, only two out of five mice that had been administrated with rhGM-CSF, had an increased rhGM-CSF content in the bone tissue (541 and 146 pg/g of the tissue), while a higher level of rhGM-CSF than in the control CSF (from 1408 to 2460 pg/g) after the administration of the AEG conjugate was observed in four out of five animals.

The maximum rhGM-CSF concentration in the bone marrow of the mice from the both experimental groups, as well as in the blood and femoral bone homogenates, was recorded at the first point, 3 minutes after the administration (Table 1). The values of the indicator in the mice that had been administrated with the AEG conjugate, were 161 times higher than the control values, and 2.4 times higher than the indicator of the mice that had been administrated with rhGM-CSF. In the subsequent periods, the protein content in both experimental groups decreased. However, it should be notified that 4 and 24 hours after the rhGM-CSF administration of all five mice, the protein in the bone marrow was not detected, while in the group that had been administrated with the AEG conjugate, the trace amounts of rhGM-CSF were registered in 2 mice (from 28.2 to 42.2 pg/femur).

Thus, the introduction of alendronic acid into the

composition of rhGM-CSF contributed to an increase in the accumulation and distribution of rhGM-CSF in the bone tissue and bone marrow of the mice, which is consistent with the available literature data on the accumulation of alendronic acid preparations in the bone tissue, their strong binding and retention by the bone matrix [28-34]. The concentration of the conjugated protein in the blood in the first hours after the administration was higher than that of the free rhGM-CSF, which may be due to an increase in the protein resistance to proteolytic enzymes as a result of modification and, as a result, the appearance of its ability to circulate in the bloodstream for a longer time.

CONCLUSION

It has been established that rhGM-CSF conjugates with ALN have a hemostimulating activity comparable to the activity of the original protein. In the composition of conjugates, the effect of rhGM-CSF on the bone marrow cells was more pronounced and prolonged, which is apparently due to the longer presence of the protein in the conjugate in the blood and its increased accumulation in the bone tissue and bone marrow. The data obtained make it possible for us to conclude that further work on the development of hemostimulating drugs based on rhGM-CSF conjugates with alendronic acid, is promising.

FUNDING

The study was carried out as a part of the work on the state task implementation of the of State Research Center of Virology and Biotechnology "VECTOR", Rospotrebnadzor, subject GZ-39/21.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

GGSh – conducting experimental studies, statistical data processing, diagramming, writing the article text;
AVB – literature search, experimental research, statistical data processing, writing the article text;
EST – conducting experimental studies; SGG – conducting a literature search, forming the purpose and objectives of the study, developing the design of the study, finalizing the article text;
TIE – obtaining drugs for research; EAV – obtaining drugs for research; EDD – approval of the pilot study plan, editing and revision of the article text content, approval of the final article text version.

REFERENCES

1. Dygai AM, Zhdanov VV, Miroshnichenko LA, Zyuz'kov GN, Udut EV, Simanina EV, Stavrova LA, Khrichkova TY, Agafonov VI. Comparison of Specifi c Activity of Granulocytopoiesis Stimulators after Treatment with Cytostatics with Different Mechanisms of Action. Bull Exp Biol Med. 2013;55:631–5. DOI: 10.1007/s10517-013-2212-3
2. Hamilton JA, Anderson GP. GM-CSF Biology. Growth Factors. 2004 Dec;22(4):225–31. DOI: 10.1080/08977190412331279881
3. Bhattacharya P, Budnick I, Singh M, Thiruppathi M, Alharshawi K, Elshabrawy H, Holterman MJ, Prabhakar BS. Dual Role of GM-CSF as a Pro-Inflammatory and a Regulatory Cytokine: Implications for Immune Therapy. J Interferon Cytokine Res. 2015 Aug;35(8):585–99. DOI: 10.1089/jir.2014.0149
4. van de Laar L, Coffer PJ, Woltman AM. Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy. Blood. 2012 Apr 12;119(15):3383–93. DOI: 10.1182/blood-2011-11-370130
5. Hübel K, Dale DC, Liles WC. Therapeutic use of cytokines to modulate phagocyte function for the treatment of infectious diseases: current status of granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and interferon-gamma. J Infect Dis. 2002 May 15;185(10):1490–501. DOI: 10.1086/340221
6. Steward WP, Scarffe JH, Austin R, Bonnem E, Thatcher N, Morgenstern G, Crowther D. Recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) given as daily short infusions--a phase I dose-toxicity study. Br J Cancer. 1989 Jan;59(1):142–5. DOI: 10.1038/bjc.1989.28
7. Morita M, Lamkhioued B, Soussi Gounni A, Aldebert D, Delaporte E, Capron A, Capron M. Induction by interferons of human eosinophil apoptosis and regulation by interleukin-3, granulocyte/macrophage-colony stimulating factor and interleukin-5. Eur Cytokine Netw. 1996 Dec;7(4):725–32.
8. Lieschke GJ, Burgess AW. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (1). N Engl J Med. 1992 Jul 2;327(1):28–35. DOI: 10.1056/NEJM199207023270106
9. Lee JH, Nan A. Combination drug delivery approaches in metastatic breast cancer. J Drug Deliv. 2012;2012:915375. DOI: 10.1155/2012/915375
10. Mu CF, Shen J, Liang J, Zheng HS, Xiong Y, Wei YH, Li F. Targeted drug delivery for tumor therapy inside the bone marrow. Biomaterials. 2018 Feb;155:191–202. DOI: 10.1016/j.biomaterials.2017.11.029
11. Kamada H, Tsutsumi Y, Yamamoto Y, Kihira T, Kaneda Y, Mu Y, Kodaira H, Tsunoda SI, Nakagawa S, Mayumi T. Antitumor activity of tumor necrosis factor-alpha conjugated with polyvinylpyrrolidone on solid tumors in mice. Cancer Res. 2000 Nov 15;60(22):6416–20.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2022-10-5-472-482

12. Danilenko ED, Belkina AO. The use of bisphosphonates as a means of drug delivery. Russian Journal of Biopharmaceuticals. 2014;6(6):44-53. Russian
13. Farrell KB, Karpeisky A, Thamm DH, Zinnen S. Bisphosphonate conjugation for bone specific drug targeting. Bone Rep. 2018 Jul 3;9:47-60. DOI: 10.1016/j.bonr.2018.06.007
14. Sedghizadeh PP, Sun S, Junka AF, Richard E, Sadrerafi K, Mahabady S, Bakhshalian N, Tjokro N, Bartoszewicz M, Oleksy M, Szymczyk P, Lundy MW, Neighbors JD, Russell RG, McKenna CE, Ebetino FH. Design, Synthesis, and Antimicrobial Evaluation of a Novel Bone-Targeting Bisphosphonate-Ciprofloxacin Conjugate for the Treatment of Osteomyelitis Biofilms. J Med Chem. 2017 Mar 23;60(6):2326–43. DOI: 10.1021/acs.jmedchem.6b01615
15. Kamble S, Varamini P, Müllner M, Pelras T, Rohanizadeh R. Bisphosphonate-functionalized micelles for targeted delivery of curcumin to metastatic bone cancer. Pharm Dev Technol. 2020 Nov;25(9):1118–26. DOI: 10.1080/10837450.2020.1798458
16. Yamashita S, Katsumi H, Sakane T, Yamamoto A. Bone-targeting dendrimer for the delivery of methotrexate and treatment of bone metastasis. J Drug Target. 2018 Nov;26(9):818–28. DOI:10.1080/1061186X.2018.1434659
17. Xie Z, Liu G, Tang P, Sun X, Chen S, Qin A, Zhu P, Zhang J, Fan S. Bone-targeted methotrexate-alendronate conjugate inhibits osteoclastogenesis in vitro and prevents bone loss and inflammation of collagen-induced arthritis in vivo. Drug Deliv. 2018 Nov;25(1):187–97. DOI: 10.1080/10717544.2017.1422295
18. Volosnikova EA, Demin IF, Levagina GM, Lebedev LR, Zakabunin AI, Danilenko ED. The Synthesis of Conjugates of TNF-Alpha and Alendronic Acid. Russian Journal of Bioorganic Chemistry. 2016;42(6):704–11. DOI: 10.7868/S0132342316060154
19. Zakabunin AI, Danilenko ED, Volosnikova EA, Levagina GM, Demin IF. Antitumor agent. Russian Federation Patent No. 2609871 C. 2017 Feb 06. Russian
20. Gileva IP, Esina TI, Volosnikova EA, Gogina YaS, Lebedev LR, Danilenko ED. Recombinant plasmid DNA p280_2GM coding polypeptide with properties of granulocyte-macrophage colony-stimulating factor of human, strain Escherichia coli SG 20050/p280_2GM – producer of polypeptide with properties of granulocyte-macrophage colony-stimulating factor of human and method of obtaining of said polypeptide. Russian Federation Patent No. 2708556. 20198 Dec 09. Russian
21. Esina TI, Volosnikova EA, Lebedev LR, Kochneva GV, Grazhdantseva. A. A. Study on the Methods for Synthesis of GM-CSF Conjugates with Alendronic Acid. Russian Journal of Bioorganic Chemistry. 2020;46(3):1–8. DOI: 10.31857/S0132342320030112. Russian
22. Mamaeva SA, Kruppa IS, Dyatlov VA, Rustamov IR, Gumnikova VI. Influence of periodate oxidation on fractional inhomogeniety and molecular weight of polysacharides. Uspekhi v himii i himicheskoj tekhnologii=Success in Chemistry and Chemical Technology. 2014;28(3): 45–8. Russian
23. Esina TI, Lebedev LR, Volosnikova EA, Gileva IP, Gogina YaS, Tereshchenko TA, Kochneva GV, Grazhdantseva AA, Danilenko ED. Method for obtaining recombinant human granulocyte-macrophage colony-stimulating factor. Biotechnology in Russia. 2019;35(3): 68–73. DOI: 10.21519/0234-2758-2019-35-3-68-73
24. Goldberg ED, Dygai AM, Zyuzkov GN, Zhdanov VV, Simanina EV, Guryantseva LA, Khrichkova TYu, Udut EV, Stavrova LA, Sotnikova NV. Method of granulocytopenia-stimulating effect evaluation for pharmacological substances. Russian Federation Patent No. 2332667. 2008 Aug 27. Russian
25. Shimina GG, Bateneva AV, Gamaley SG, Esina TI, Tereshchenko TG, Danilenko ED. Study on Hemostimulating Properties of Granulocyte-Macrophage Colony Stimulating Factor. BIOpreparations. Prevention, Diagnosis, Treatment. 2020;20(4):268–76. DOI: 10.30895/2221-996X-2020-20-4-268-276. Russian
26. Grzhibovsky AM. Analysis of quantitative data for two independent groups. Human Ecology. 2008;(2):54-61. Russian
27. Grzhibovsky AM. Analysis of three or more independent groups of quantitative data. Human Ecology. 2008;(3):50-8. Russian
28. Nguyen TD, Pitchaimani A, Aryal S. Engineered Nanomedicine with Alendronic Acid Corona Improves Targeting to Osteosarcoma. Sci Rep. 2016 Nov 8;6:36707. DOI: 10.1038/srep36707
29. Pan H, Sima M, Kopecková P, Wu K, Gao S, Liu J, Wang D, Miller SC, Kopecek J. Biodistribution and pharmacokinetic studies of bone-targeting N-(2-hydroxypropyl)methacrylamide copolymer-alendronate conjugates. Mol Pharm. 2008 Jul-Aug;5(4):548–58. DOI: 10.1021/mp800003u
30. Miller K, Eldar-Boock A, Polyak D, Segal E, Benayoun L, Shaked Y, Satchi-Fainaro R. Antiangiogenic antitumor activity of HPMA copolymer-paclitaxel-alendronate conjugate on breast cancer bone metastasis mouse model. Mol Pharm. 2011 Aug 1;8(4):1052-62. DOI: 10.1021/mp200083n
31. Chen H, Li G, Chi H, Wang D, Tu C, Pan L, Zhu L, Qiu F, Guo F, Zhu X. Alendronate-conjugated amphiphilic hyperbranched polymer based on Boltorn H40 and poly(ethylene glycol) for bone-targeted drug delivery. Bioconjug Chem. 2012 Sep 19;23(9):1915–24. DOI: 10.1021/bc3003088'
32. Akyol UK, Sipal S, Demirci E, Gungormus M. The influence of low-level laser therapy with alendronate irrigation on healing of bone defects in rats. Lasers Med Sci. 2015 Apr;30(3):1141–6. DOI: 10.1007/s10103-015-1720-y
33. Lin JH, Duggan DE, Chen IW, Ellsworth RL. Physiological disposition of alendronate, a potent anti-osteolytic bisphosphonate, in laboratory animals. Drug Metab Dispos. 1991 Sep-Oct;19(5):926–32.
34. Lin JH, Chen IW, Duggan DE. Effects of dose, sex, and age on the disposition of alendronate, a potent antiosteolytic bisphosphonate, in rats. Drug Metab Dispos. 1992 Jul–Aug;20(4):473–8.

AUTHORS

Galina G. Shimina – Researcher of the Department of Biological Research, Institute of Medical Biotechnology of State Research Center of Virology and Biotechnology "VECTOR", Rospotrebnadzor. ORCID ID: 0000-0002-1078-7033. E-mail: shimina_gg@vector.nsc.ru

Alena V. Bateneva – Researcher of the Department of Biological Research, Institute of Medical Biotechnology of State Research Center of Virology and Biotechnology "VECTOR", Rospotrebnadzor. ORCID ID: 0000-0002-3761-7798. E-mail: bateneva_av@vector.nsc.ru

Elena S. Tsyplenkova – Junior Researcher, Department of Biological Research, Institute of Medical Biotechnology of State Research Center of Virology and Biotechnology "VECTOR", Rospotrebnadzor. ORCID ID: 0000-0002-1277-6258. E-mail: tsyplenkova_es@vector.nsc.ru

Svetlana G. Gamaley – Head of the Department of Biological Research Institute of Medical Biotechnology of State Research Center of Virology and Biotechnology "VECTOR", Rospotrebnadzor. ORCID ID: 0000-0002-7441-333X. E-mail: gamaley_sg@vector.nsc.ru

Tatyana I. Esina – Junior Researcher at the Department of Technology Development and Pilot Production of Biological Products, Institute of Medical Biotechnology of State Research Center of Virology and Biotechnology "VECTOR", Rospotrebnadzor. ORCID ID: 0000-0001-9006-8313. E-mail: esina_ti@vector.nsc.ru

Ekaterina A. Volosnikova – Candidate of Sciences (Biology), Head of the Laboratory for Obtaining and Analyzing Biosubstances of the Department for Technology Development and Pilot Production of Biological Products, Institute of Medical Biotechnology of State Research Center of Virology and Biotechnology "VECTOR", Rospotrebnadzor. ORCID ID: 0000-0001-5028-5647. E-mail: volosnikova_ea@vector.nsc.ru

Elena D. Danilenko – Candidate of Sciences (Biology), Director of the Institute of Medical Biotechnology of State Research Center of Virology and Biotechnology "VECTOR", Rospotrebnadzor. ORCID ID: 0000-0001-5026-1602. E-mail: danilenko_ed@vector.nsc.ru