EVALUATION OF BIOLOGICAL ACTIVITY OF THE CONJUGATES OF GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR WITH ALENDRONIC ACID

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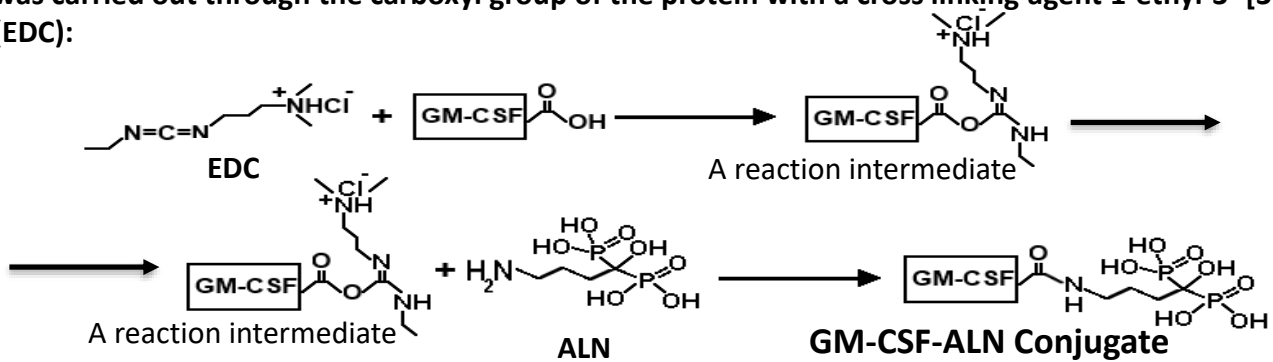
Introduction Recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) is used as a hematopoietic stimulant in patients with various diseases accompanied by neutropenia. Targeted drug delivery to the bone marrow can enhance the growth and differentiation of hematopoietic cells due to GM-CSF localization at the site of hematopoiesis.

Aim Study of biodistribution and biological (hemostimulating) activity of the conjugates of GM-CSF with alendronic acid (ALN).

Materials and methods In this study we used reagents from AppliChem (Germany), Sigma-Aldrich (USA). For the synthesis of conjugates we used the preparation of GM-CSF (substance), with protein concentration of 1.15 mg/ml and specific activity of 0.28 ng/ml, manufactured by IMBT FBRI SRC VB "Vector". The molecular weight of the conjugates was determined by use of vertical gel electrophoresis in 15% polyacrylamide gel under denaturing conditions, with Coomassie R-250 staining. The study of the conjugates` ability to accumulate in bone tissue was performed in vitro in a bone matrix model by use of hydroxyapatite (HAP) chromatography. The in vitro biological activity of GM-CSF within the conjugates was evaluated by the stimulation level of proliferation of cytokine-dependent human erythroleukemia cells (TF-1). The in vivo hemostimulating activity was determined by use of a model of cytostatic myelosuppression induced by cyclophosphamide (CP) administration into mice.

Results

Alendronic acid, which has an affinity for the bone tissue, was used as a vector molecule. The conjugation of GM-CSF with ALN was carried out through the carboxyl group of the protein with a cross linking agent 1-ethyl-3- [3-dimethylaminopropyl]carbodiimide (EDC):



Synthesis on solid phase

Results

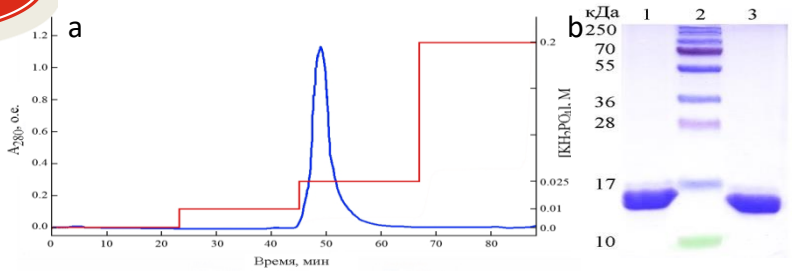


Fig. 2 The elution profile of GM-CSF-ALN conjugate, on a column with HAP (a), on the ordinate axis: optical absorption of the solution at 280 nm, AU (left), the molarity of the elution buffer, M (right); on the abscissa axis - elution time, min. The electrophoregram for the conjugate sample, in 15% PAAG under reducing conditions, Coomassie R-250 staining (b). GM-CSF-ALN conjugate, 20 µg / well (1); protein ladder 10-250 kDa (2); control, GM-CSF, 20 µg / well (3).

The study of the accumulation of GM-CSF-ALN conjugates in a bone matrix model (HAP) in vitro was performed by HAP chromatography. The binding of the obtained conjugates is not less than 1 mg / ml of resin, the conjugate is eluted with 0.025 M potassium phosphate and does not contain intact GM-CSF (Fig. 3)

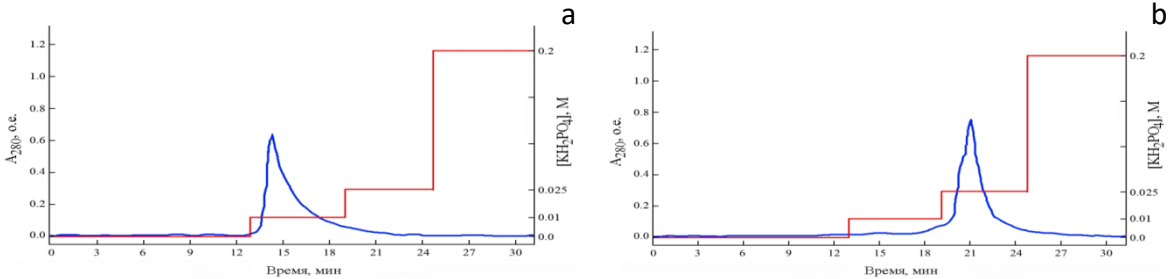


Fig. 3 The HAP desorption curve of free GM-CSF (a), and GM-CSF within the conjugate obtained by use of EDC (b). On the ordinate axis: optical absorption of the solution at 280 nm, AU (left), the molarity of the elution buffer, M (right); on the abscissa axis - elution time, min.

Fig. 3 shows an increased affinity for hydroxyapatite in comparison with an intact protein.

The analysis of **proliferative activity** of the cytokine-dependent human erythroleukemia cells TF-1 confirmed the **safety of biological properties of GM-CSF within the conjugates**. The stimulation of TF-1 cells proliferation was calculated as a percentage in relation to the control. The number of living cells in the negative control was taken as 100%. Estimated ED50 values (concentration at which a 2-fold proliferative effect is achieved) for the preparations: GM-CSF substance - 0.28 ng / ml, GM-CSF conjugate - 0.28 ng / ml.

Preparation	Activity of GM-CSF, ng/ml	Homogeneity,%	Molecular weight, Da	Affinity for hydroxyapatite (concentration of elution buffer, mol / l)
Intact GM-CSF	0.28 ± 0.01	98.2	15 400 ± 300	0.010 ± 0.002
Conjugated GM-CSF	0.28 ± 0.01	98.1	15 400 ± 300	0.025 ± 0.002

Results

On the **cytostatic myelosuppression** model, it was shown that the GM-CSF conjugate, as well as the intact protein, accelerated the restoration of bone marrow cellularity and number of segmentoneuclear neutrophils in the peripheral blood, while the stimulating effect of the conjugate on the **total number of karyocytes was more pronounced**.

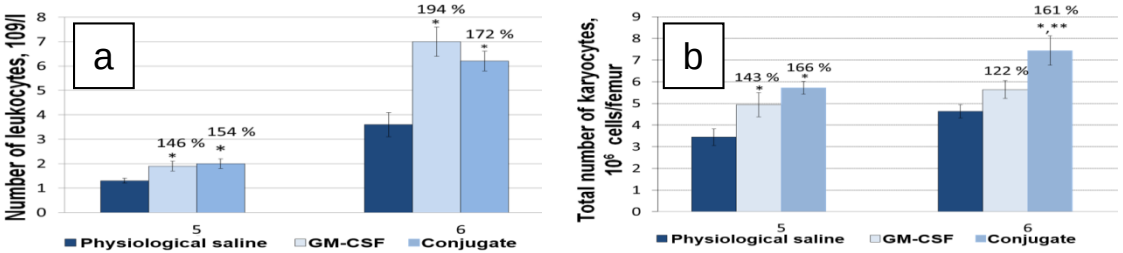


Fig. 4 The number of leukocytes (a), karyocytes (b) in the bone marrow of CBA mice exposed to CP. The abscissa indicates the time of study (day).
% - the number of blood cells or bone marrow cells of the experimental animals in relation to the corresponding indicator of the control animals, expressed as a percentage.

A study of distribution and accumulation of GM-CSF conjugates in the organs and tissues of mice confirmed their increased ability to penetrate into the bone tissue and bone marrow. Thus, the protein content in the femur during 4 hours after the conjugate administration was 2.9-3.9 times higher than the values recorded after the administration of GM-CSF substance (Fig. 5a). The values of the indicator in this group remained higher compared to the control level (intact animals) until the end of the first day. The level of GM-CSF in bone marrow cells 3 minutes after the conjugate administration significantly exceeded the control indicator by 161 times, while after the substance administration, only 67 times (Fig. 5b). The conjugated GM-CSF circulated longer in the peripheral blood of mice. Thus, 3 minutes post administration, the protein content in the blood was 19.2% of the administered dose for the conjugate and 7.2% for the substance; statistically significant differences between the groups persisted for at least 4 hours (Fig. 5c). It is important to note that GM-CSF conjugate was not detected in the blood by the end of the first day after the administration.

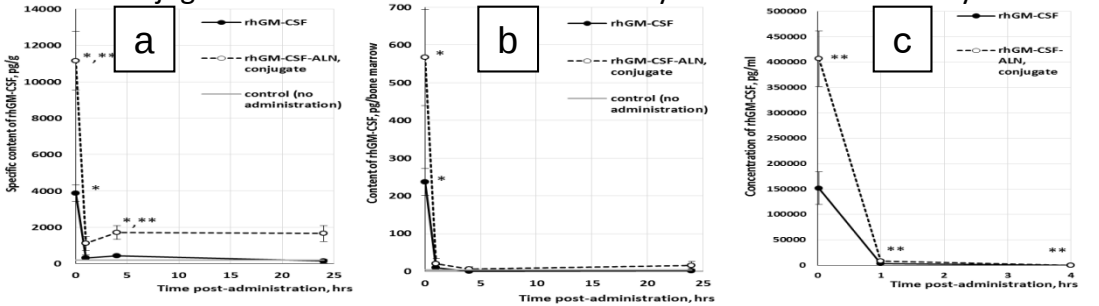


Fig. 5. Dynamics of changes in the content of GM-CSF in the femurs (a), bone marrow (b) and blood serum (c) of mice after a single intravenous administration of GM-CSF preparations.

The significance of intergroup differences was assessed using the nonparametric Mann-Whitney U-test at a significance level of $p < 0.0170$ for tissues, $p < 0.05$ for blood; * - differences are statistically significant compared with the control; ** - differences are statistically significant compared with GM-CSF substance.

Conclusions

The data obtained indicate that the conjugation of GM-CSF with ALN has enhanced the accumulation of GM-CSF in the bone marrow and increased its level of hemostimulating activity. Therefore, the developed approach is a promising platform for further development of drugs with increased tropism to the bone marrow and hemostimulant effect.

Thank you for your attention!

This work was financially supported by the Russian Foundation for Basic Research **(RFBR),** a project in the framework of the contest "My First Grant", application **No. 18-315-00236 \ 19.**