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Antithrombotic Activity of an Indolinone Derivative – a Soluble Guanylate Cyclase Stimulator

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Abstract

Introduction. The article presents the results of studying the antithrombotic activity of a novel drug, a soluble guanylate cyclase stimulator (codename – GRS), in experimental models of arterial and venous thrombosis and thromboembolism.

Aim. Study the antithrombotic action of GRS compound in comparison with clopidogrel and rivaroxaban in the following models: arterial thrombosis induced by iron chloride application to carotid artery wall in rats; thromboembolism induced by intravenous administration of thrombin solution in mice; venous thrombosis induced by the ligature of inferior vena cava in rats.

Materials and methods. Arterial thrombosis was modelled in rats by applying a pad soaked in iron chloride ($FeCl_3$) to the carotid artery, GRS compound was administered orally in median effective dose of 10 mg/kg once 3 hours before pad application. Blood flow in carotid arteries and blood clot mass were registered. Thromboembolism was induced in mice by intravenous administration of thrombin solution, GRS in dose 10 mg/kg or the reference drug clopidogrel were administered once orally daily for 3 days. Animal mortality, survival time and blood clot size were registered. Venous thrombosis was induced in rats by the ligature of inferior vena cava below renal veins, GRS in dose 10 mg/kg, reference drug rivaroxaban in 5 mg/kg dose or their combination in these doses were administered once orally 1 hour before vein ligature. The mass of dry and wet blood clots was registered.

Results and discussion. In arterial thrombosis model GRS compound in 10 mg/kg, administered 3 hours before iron chloride application, increased the time to blood flow cessation in the carotid artery by 35 % and reduced the frequency of complete artery occlusion by 2 times compared to the control group ($p < 0.05$). 60 min after arterial thrombosis modelling complete occlusion was observed in 28 % of animals in GRS group and in 75 % of control animals, while after 24 hours the occlusion was observed in 14 % of animals in GRS group and in 50 % of control group animals ($p < 0.05$). In thrombin-induced thromboembolism model in mice GRS did not reduce the size of blood clots in pulmonary vessels, while clopidogrel reduced it by 48 %. In GRS group 80 % of animals died of thrombosis, compared to 30 % in clopidogrel group and 90 % in the control group. In ligature-induced venous thrombosis in rats GRS after single oral administration reduced the mass of dry blood clot, being as potent as rivaroxaban. Combined administration of GRS and rivaroxaban did not enhance their antithrombotic action.

Conclusion. GRS after single oral administration in 10 mg/kg dose had potent antithrombotic action in models of arterial and venous thrombosis in rats, while not preventing pulmonary vessel thrombosis in mice after thrombin administration. Therapeutic action of GRS in experimental venous thrombosis was as potent as that of rivaroxaban. Antithrombotic action of GRS compound results not only from its antiplatelet action, but also from the alleviation of endothelial dysfunction in arteries and veins.

Keywords: indolinone derivative, guanylate cyclase stimulator, rivaroxaban, clopidogrel, models of arterial and venous thrombosis and thromboembolism

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Vladimir V. Bykov has developed the concept and design of the manuscript. Arina V. Bykova, Vera I. Smolyakova, Galina A. Chernysheva, Oleg I. Alyev, Anna M. Anishenko and Anasrasia V. Sidekhmenova have performed the experiments. Aleksander I. Vengerovsky has edited the article. All authors participated in the discussion of the results.

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Антитромботическая активность производного индолинона – стимулятора растворимой гуанилатциклазы

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Резюме

Введение. В статье представлены результаты исследований антитромботической активности разрабатываемого лекарственного средства – стимулятора растворимой гуанилатциклазы (шифр – GRS) на экспериментальных моделях артериального и венозного тромбоза и тромбоэмболии.

Цель. Изучить в эксперименте способность соединения GRS в сравнении с действием клопидогрела и ривароксабана оказывать антитромботическое действие на моделях артериального тромбоза, вызванного у крыс аппликацией на стенку сонной артерии хлорида железа, при тромбоэмболии, вызванной у мышей внутривенным введением раствора тромбина, и венозном тромбозе, вызванном у крыс перевязкой нижней полой вены.

Материалы и методы. Артериальный тромбоз моделировали аппликацией на сонную артерию крыс тампона, смоченного хлоридом железа ($FeCl_3$), соединение GRS в средней эффективной дозе 10 мг/кг вводили в желудок однократно за 3 ч до аппликации тампона. Регистрировали кровоток по общим сонным артериям и определяли массу тромбов. Тромбоэмболию вызывали внутривенным введением мышам раствора тромбина, соединение GRS в дозе 10 мг/кг или препарат сравнения клопидогрел вводили в желудок в течение 3 дней 1 раз в день. Регистрировали количество погибших животных, время до наступления их гибели и площадь тромбов. Для моделирования венозного тромбоза у крыс перевязывали нижнюю полую вену ниже почечных вен, соединение GRS в дозе 10 мг/кг или препарат сравнения ривароксабан в дозе 5 мг/кг, также оба вещества вместе в тех же дозах вводили в желудок однократно за 1 ч до перевязки вены. Определяли массу влажных и сырых тромбов.

Результаты и обсуждение. На модели артериального тромбоза соединение GRS в дозе 10 мг/кг при введении за 3 ч до аппликации хлорида железа на стенку сонной артерии на 35 % удлиняло время до остановки кровотока в сонной артерии и в 2 раза уменьшало частоту ее полной окклюзии по сравнению с показателями в контрольной группе ($p < 0,05$). Через 60 мин и 24 ч после моделирования артериального тромбоза полная окклюзия сосуда определялась у 28 и 14 % животных, получавших соединение GRS, и у 75 и 50 % контрольных животных ($p < 0,05$). На модели тромбоэмболии, вызванной внутривенным введением мышам тромбина, соединение GRS не уменьшало площадь тромбов в сосудах легких, клопидогрел уменьшал ее на 48 %. При введении соединения GRS от тромбоза погибало 80 % мышей, при введении клопидогрела – 30 %, в контроле – 90 %. На фоне экспериментального венозного тромбоза у крыс, вызванного перевязкой нижней полой вены, соединение GRS при однократном введении в желудок уменьшало массу высушенного тромба не слабее ривароксабана. При совместном введении соединения GRS и ривароксабана антитромботическое действие не усиливалось.

Заключение. Соединение GRS при однократном введении в желудок в дозе 10 мг/кг проявляло выраженное антитромботическое действие на моделях артериального и венозного тромбоза у крыс и не препятствовало тромбозу сосудов легких мышей при введении тромбина. Лечебное действие соединения GRS при экспериментальном венозном тромбозе не слабее, чем эффект ривароксабана. Антитромботическая активность соединения GRS обусловлена не только антиагрегантным влиянием, но и уменьшением дисфункции эндотелия артерий и вен.

Ключевые слова: производное индолинона, стимулятор гуанилатциклазы, ривароксабан, клопидогрел, модели артериального, венозного тромбоза и тромбоэмболии

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. В. В. Быков разработал концепцию и дизайн рукописи статьи. А. В. Быкова, В. И. Смольякова, Г. А. Чернышева, О. И. Алиев, А. М. Анищенко, А. В. Сидехменова выполнили эксперименты. А. И. Венгеровский редактировал статью. Все авторы принимали участие в обсуждении результатов.

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INTRODUCTION

Antiplatelet (also called antiplatelet) drugs inhibit the adhesion, activation and aggregation of platelets. Their mechanism of action is mainly based on blocking purine receptors $P2Y_{12}$ and glycoprotein receptors IIb/IIIa on platelet membrane, inhibiting the synthesis of thromboxane A_2 inside platelets, increasing the levels of adenosine and cyclic adenosine monophosphate. One of the dangerous adverse effects of antiplatelets is severe bleeding, arising in 1–2 % of patients receiving antiplatelet therapy [1].

This necessitates the development of effective and safe antiplatelet drugs, able to block different stages of hemostasis. The new antithrombotic drug, an indolinone derivative codenamed GRS, differs in its mechanism of action from the known antiplatelet drugs. Independently of nitric oxide it stimulates soluble guanylate cyclase (sGC) in the cytoplasm of platelets, endothelium and vascular smooth muscle and increases the synthesis of cyclic guanosine monophosphate (sGMP). This cyclic nucleotide activates calcium-dependent ATPase of platelet granules and sarcoplasmic reticulum with subsequent deposition

of calcium ions, it also participates in phosphorylation of vasodilator-stimulated phosphoprotein (VASP), reduces vascular tone and platelet cytoskeleton mobility [2].

GRS compound has successfully completed Phase I clinical trials in healthy volunteers and is currently in Phase II clinical trials in patients with ischemic heart disease, undergoing endovascular revascularization of coronary arteries.

Study goal – assess the antithrombotic action of GRS compound in comparison with clopidogrel and rivaroxaban in the following models: arterial thrombosis induced by iron chloride application to carotid artery wall in rats; thromboembolism induced by intravenous administration of thrombin solution in mice; venous thrombosis induced by the ligature of inferior vena cava in rats.

MATERIALS AND METHODS

Test article

GRS (figure 1) substance is (2-[2-[(5RS)-5-(hydroxymethyl)-3-methyl-1,3-oxazolidine-2-yliden]-2-cyanoethylidene]-1H-indole-3(2H)-one, lot 10320, assay 100.53 %, synthesized by J.S.C. "Organica" (Novokuznetsk, Russia). LD_{50} of GRS in rats is over 5000 mg/kg.

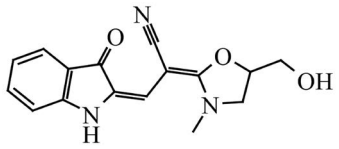

Figure 1. Structural formula of GRS

Animals

Studies were performed in the R&D center of IPHAR and Goldberg Research Institute of Pharmacology and Regenerative Medicine using 48 outbred male Sprague-Dawley rats with body mass 275–300 g and 35 male CD-1 mice with body mass 20-22 g. The animals were divided into 9 groups of 5–10 animals each, kept in plastic cages (4–5 animals per cage) at temperature 18–20 °C, relative humidity 45–65 %, air exchange 15 changes per hour and lighting mode 12:12 h.

The study was approved by institutional review boards of Goldberg Research Institute of Pharmacology and Regenerative Medicine (decision № 63012014 dated 30.01.2014), Siberian State Medical University (decision № 5378 dated 24.10.2016) and of IPHAR (decision № 59/2020 dated 14.08.2020) and is performed in accordance with the requirements of European Convention for the Protection of Vertebrate Animals (Directive 2010/63/EU) and the principles of Good Laboratory Practice (Eurasian Economic Commission decision N 81 dated 03.11.2016 and GOST 33044–2014). Surgery and manipulations were performed under diethyl ether anesthesia. The animals were sacrificed by diethyl ether overdose.

Arterial thrombosis model

The animals (rats) were divided into 2 groups of 8 animals. 3 hours before surgery the animals were administered GRS compound orally in median effective dose 10 mg/kg [2] as a suspension in 0.5 % aqueous solution of carboxymethyl cellulose (Ashland, USA, lot C181411). Control group rats received the same volume of carboxymethyl cellulose solution. Arterial thrombosis was modelled by applying a 1.5 mg cotton wool pad, soaked in 10 % iron chloride solution ($FeCl_3$, Sigma-Aldrich, USA, CAS 7705-08-0), to the carotid artery wall of rats [3]. The tissues surrounding the pad were covered by polyethylene film. After 15 minutes the pad was removed, the application site was washed with isotonic sodium chloride solution. After 60 min and 24 hours the blood flow in common carotid arteries was measured for 1 hours using electromagnetic blood flow meter MFV-1100 (Nihon Kohden, Japan) with graph registration using KSP-4 recorder (Teplopribor, Russia). The blood clot was then removed from the carotid artery, washed with isotonic sodium chloride solution, dried in a dry-air thermostat TS-1/20 SPU (Smolensk SCTB SPU, Russia) to constant mass at 60 °C for 24 hours and weighed on laboratory scales (Adventurer AR2140 Ohaus Corp., USA).

Thromboembolism model

The animals (mice) were divided into 4 groups of 10 animals. The animals were administered GRS compound in 10 mg/kg dose or clopidogrel (Plavix®, 75 mg film-coated tablets, Sanofi Winthrop Industry, France, lot AA686, expiration date – 01.04.2023) in median effective dose 10 mg/kg once orally for 3 days [4]. Control group rats received 0.5 % aqueous carboxymethyl cellulose solution. 3 hours after the last test article administration, the animals were infused 0.12 ml of thrombin solution (500 U/ml, Technology-Standard, Russia, lot E009031) in 60 U/animal dose [5]. Animal survival time and mortality were measured for 15 min. Mice still alive after 15 min were considered "survived" and were sacrificed. Animal lungs were histologically studied on dewaxed slices stained with hematoxylin (Biovitrum, Russia, lot 1436) and eosin (MiniMed, Russia, lot 70). Samples were examined using Carl Zeiss Axio Lab A1 microscope (Carl Zeiss, Germany) at ×100 magnifications. Clot area was measured in 10 fields of vision (from 10 angles).

Venous thrombosis model

The animals (rats) were divided into 4 groups of 8 animals. The animals were administered GRS compound in 10 mg/kg dose or rivaroxaban (Xarelto®, 15 mg film-coated tablets, Bayer, Germany, batch BXJB9R1, lot 35457, expiration date 01.05.2022) in median effective dose 5 mg/kg [6]. The third group received the combination of GRS and rivaroxaban in the same doses. Control group rats received carboxymethyl cellulose solution.

Venous thrombosis was induced 1 hour after test article administration by ligating the inferior vena cava below renal veins, also ligating all visible side branches [7]. After 24 hours the clot was extracted from the inferior vena cava, weighed, dried in a dry-air thermostat TS-1/20 SPU (Smolensk SCTB SPU, Russia) to constant mass at 60 °C for 24 hours and then weighed again on laboratory scales (Pioneer 213C Ohaus Corp., USA) to calculate the clot's density.

Data processing

Experimental data was statistically processed using Statistica 8.0 software. The results were presented as means and mean standard deviation (M ± m). Statistical significance level between groups ($p < 0.05$) was calculated using Mann–Whitney U-test as one most acceptable for small sample size [8] and Fisher test.

RESULTS AND DISCUSSION

In arterial thrombosis model control group rats (without treatment) had carotid artery thrombosis in 75 % cases. Carotid artery occlusion was observed in 75 % of animals 60 minutes after modelling and in 50 % of animals after 24 hours. GRS compound, administered orally in 10 mg/kg dose 3 hours before iron chloride administration, has increased mean time until blood flow cessation in the carotid artery by 35 % ($p < 0.05$). Thrombosis in GRS group developed in 28 % of animals, complete carotid artery occlusion in 28 % of animals after 60 min and in 14 % of animals after 24 hours ($p < 0.05$). Blood clot mass did not statistically differ between groups (table 1).

Therefore, the indolinone derivative GRS has considerably reduced the frequency of thrombosis in experimental arterial thrombosis, but did not considerably affect blood clot mass. High efficacy of GRS at the initial thrombosis stage may be associated with its stimulation of soluble guanylate cyclase in endothelial and smooth muscle cells, which prevented endothelial dysfunction, developed a resistance to blood clot formation and induced vasodilation.

Intravenous thrombin administration lead to generalized thrombosis after 1–3 min with thrombosis of mainly pulmonary arteries, killing 9 animals out of 10, which conforms to literature data [9]. After 3 days of oral administration of GRS in 10 mg/kg dose once daily, mortality in the test was 8 animals out of 10, while clopidogrel administration in 10 mg/kg dose has reduced mortality to 3 animals out of 10 ($p < 0.05$). Clopidogrel, as a classic antiplatelet drug and an inhibitor of platelet $P2Y_{12}$ receptors, had potent antithrombotic action. Blood clot area in the lungs in clopidogrel group was 48 % smaller than in the control group ($p < 0.05$).

Time to animal mortality did not differ between GRS and clopidogrel groups ($p > 0.05$) (table 2), likely because this model involves immediate (in several minutes) pulmonary embolism.

In a model of venous thrombosis in rats (table 3), induced by the ligature of inferior vena cava, GRS compound in 10 mg/kg dose has reduced wet blood clot mass by 66 %, while rivaroxaban in 5 mg/kg dose reduced it by 81 % ($p < 0.05$). Dried blood clot mass did not statistically differ between GRS and rivaroxaban group ($p > 0.05$). High efficacy of GRS compound in this deep vein thrombosis model is associated not only with its antiplatelet effects, since it's well known that in antiplatelets are less effective in preventing and treating deep vein thrombosis and pulmonary embolism than anticoagulants [10], but also the anti-inflammatory and anti-atherosclerotic effects of GRS, and with an increase of sGC sensitivity to endogenous nitric oxide [11].

Table 1. Antithrombotic action of an indolinone derivative GRS (10 mg/kg, oral) after single administration in a rat model of arterial thrombosis, induced by iron chloride application (M ± m)

Group ($n = 8$)	Time to blood flow cessation, min	Percentage of rats with complete occlusion		Percentage of rats with blood clot after 24 h	Blood clot mass after 24 h, mg
		60 min	24 h		
Animals with carotid artery thrombosis (control)	20.6 ± 1.4	6 (75)	4 (50)	6 (75)	0.28 ± 0.05
Animals receiving GRS	31.5 ± 1.0*	2 (28)*	1 (14)*	2 (28)*	0.25 ± 0.05

Note. * Statistically significant difference from control animals (Mann–Whitney test), $p < 0.05$.

Table 2. Antithrombotic action of an indolinone derivative GRS (10 mg/kg, oral) after 3-fold administration in a mice model of thromboembolism, induced by intravenous thrombin administration (M ± m)

Parameters	Experimental conditions ($n = 10$)		
	Thrombosis (control)	GRS 10.0 mg/kg	Clopidogrel 10.0 mg/kg
Mortality, %	90	80	30*
Time to death onset, min	2.35 ± 0.27	2.83 ± 0.58	2.72 ± 0.40
Blood clot area, mcm^2	2542 ± 494	2168 ± 260	1225 ± 275#

Note. * Statistically significant difference in mortality (Fisher test), $p < 0.05$; # – statistically significant difference from control animals (Mann–Whitney test), $p < 0.05$.

Table 3. Antithrombotic action of GRS compound (10 mg/kg, oral), rivaroxaban (5 mg/kg, oral) and their combination after single administration in a rat model of venous thrombosis (M ± m)

Experimental conditions	Wet blood clot mass, mg	Dry blood clot mass, mg
Venous thrombosis (control, $n = 5$)	51.3 ± 6.8	13.1 ± 2.1
GRS, 10 mg/kg ($n = 10$)	17.1 ± 3.3*	4.3 ± 0.9*
Rivaroxaban, 5 mg/kg ($n = 10$)	9.6 ± 3.4*#	2.55 ± 0.8*
Combination of GRS and rivaroxaban ($n = 10$)	12.5 ± 3.5*	3.6 ± 0.9*

Note. * Statistically significant difference from control animals (Mann–Whitney test), $p < 0.05$; # – statistically significant difference from GRS group (Mann–Whitney test), $p < 0.05$.

High efficacy of GRS compound in this deep vein thrombosis model is associated not only with its antiplatelet effects, since it's well known that in antiplatelets are less effective in preventing and treating deep vein thrombosis and pulmonary embolism than anticoagulants [10], but also the anti-inflammatory and anti-atherosclerotic effects of GRS, and with an increase of sGC sensitivity to endogenous nitric oxide [11].

Combined administration of 10 mg/kg GRS and 5 mg/kg rivaroxaban did not increase the antithrombotic effect in this model, since GRS as a sGC stimulator inhibits the development of platelet thrombus, while rivaroxaban as a direct Xa factor inhibitor blocks the final stage of coagulation hemostasis.

The obtained data demonstrate the high antithrombotic activity of a new soluble guanylate cyclase stimulator GRS in models of arterial thrombosis, induced by iron chloride application to the carotid artery, and venous thrombosis, induced by the ligature of inferior vena cava. GRS compound had no significant antithrombotic effect in a model of acute thromboembolism induced by intravenous thrombin administration. Thrombin increases platelet and coagulation hemostasis, increasing platelet aggregation and activation with active participation of PAR-1 receptors and, being a proteolytic enzyme, catalyzes fibrin synthesis. Considering the fast and potent effect of thrombin, P2Y12 platelet receptor inhibitor clopidogrel has more potent antithrombotic effects in this model than sGC stimulator GRS.

According to prior studies, antiplatelet action of GRS compound is more pronounced towards increased platelet aggregation in cardiovascular diseases. In addition, GRS improves the function of vascular endothelium. Unlike GRS, clopidogrel irreversibly blocks platelet aggregation and increases the risk of bleeding.

Absence of antithrombotic activity of GRS compound in a model of thrombin-induced acute pulmonary embolism shows that GRS can't serve as a first-line drug in generalized thrombosis or high risk of thromboembolism.

CONCLUSION

The new indolinone derivative GRS has potent antithrombotic action in models of arterial and venous thrombosis in rats, while not preventing pulmonary vessel thrombosis in mice after thrombin administration. Being a stimulator of soluble guanylate cyclase, GRS compound not only inhibits platelet aggregation, but also improves the function of endothelium in cardiovascular pathologies.

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