



ANTITROMBOTIC ACTIVITY OF A NEW BENZIMIDAZOLE DERIVATIVE WITH A SPATIALLY DIFFICULT PHENOLIC SUBSTITUTE IN ITS STRUCTURE

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The aim of the study was to investigate antithrombogenic properties of compound RU-1144 with previously identified pronounced antiplatelet and antioxidant activities. The thrombosis induced by *Ferric chloride* (FeCl_3) was carried out in rats' carotid artery, in comparison with the known antiaggregant drugs – acetylsalicylic acid (ASA) and clopidogrel, as well as with the antioxidant preparation – ethylmethylhydroxypyridine succinate (EMHPS).

Materials and methods. The antithrombotic activity of compound RU-1144 was studied on the model of the rats with carotid artery thrombosis, induced by the application of 50% *ferric chloride* (FeCl_3), and the Global Thrombosis Test model (the *Görög Thrombosis Test*). The evaluation of this type of activity was carried out by prolonging the time of a blood clot formation. The studies of the compound RU-1144 effect on the bleeding time parameter were performed in mice. Acetylsalicylic acid, clopidogrel and EMHPS were used as reference drugs.

Results. The antithrombotic effect of the RU-1144 substance revealed in the model of arterial thrombosis induced by the application of *ferric chloride* (FeCl_3), exceeded that of both acetylsalicylic acid and clopidogrel by 3.5 times and that of EMHPS by 2.9 times. In the model of the *in vitro* Global Thrombosis Test (the *Görög Thrombosis Test*), compound RU-1144 reduced the thrombogenic potential of the blood equally with acetylsalicylic acid and clopidogrel. The assessment of "the bleeding time", caused by the RU-1144 substance, showed that the prolongation of bleeding was twice as less pronounced than that caused by ASA and clopidogrel.

Conclusion. The performed studies demonstrated a pronounced antithrombotic activity of compound RU-1144, which exceeded that of acetylsalicylic acid, clopidogrel and EMHPS, while the ability to prolong the bleeding time was reliably lower than that of reference drugs.

Keywords: antithrombotic activity, thrombosis, benzimidazole, ASA, clopidogrel, ethylmethylhydroxypyridine succinate, the *Görög Thrombosis Test*, bleeding time

Abbreviations: EMHPS – ethylmethylhydroxypyridine succinate; ASA – acetylsalicylic acid.

АНТИТРОМБОТИЧЕСКАЯ АКТИВНОСТЬ НОВОГО ПРОИЗВОДНОГО БЕНЗИМИДАЗОЛА, ИМЕЮЩЕГО В СВОЕЙ СТРУКТУРЕ ПРОСТРАНСТВЕННО ЗАТРУДНЕННЫЙ ФЕНОЛЬНЫЙ ЗАМЕСТИТЕЛЬ

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Цель – изучение антитромбогенных свойств соединения РУ-1144 с ранее выявленной выраженной антиагрегантной и антиоксидантной активностью, на модели артериального тромбоза сонной артерии крыс, индуцированного хлоридом железа (III), в сравнении с известными антиагрегантными препаратами – ацетилсалициловой кислотой и клопидогрелом, а также антиоксидантным препаратом – этилметилгидроксипиридина сукцинат.

Материалы и методы. Антитромботическая активность соединения РУ-1144 была изучена на модели артериального тромбоза сонной артерии крыс, вызванного аппликацией 50% хлорида железа (III) и модели Global Thrombosis Test (по Горюгу). Оценку данного вида активности производили по удлинению времени образования тромба. Исследования влияния соединения РУ-1144 на параметр времени кровотечения проводили на мышах. В качестве препаратов сравнения использовали ацетилсалициловую кислоту, клопидогрел и ЭМГПС.

Результаты. Выявленное на модели артериального тромбоза, индуцированного аппликацией хлорида железа (III), антитромботическое действие субстанции РУ-1144, превосходило таковое как у ацетилсалициловой кислоты, так и у клопидогрела в 3,5 раза, и в 2,9 раза – у ЭМГПС. На модели Global Thrombosis Test (тест Горюга) *in vitro* соединение РУ-1144 снижало тромбогенный потенциал крови в равной степени с ацетилсалициловой кислотой и клопидогрелом. При оценивании «времени кровотечения» вещество РУ-1144 пролонгировало кровотечение в среднем в 2 раза менее выражено, чем АСК и клопидогрел.

Заключение. Проведенные исследования продемонстрировали у соединения РУ-1144 выраженную антитромботическую активность, превышающую таковую у ацетилсалициловой кислоты, клопидогрела и ЭМГПС, при этом способность удлинять время кровотечения была достоверно ниже, чем у препаратов сравнения.

Ключевые слова: антитромботическая активность, тромбоз, бензимидазол, АСК, клопидогрел, этилметилгидроксипиридина сукцинат, тромбоз по Горюгу, время кровотечения

Сокращения: ЭМГПС – этилметилгидроксипиридина сукцинат; АСК – ацетилсалициловая кислота.

INTRODUCTION

Cardiovascular diseases are currently the leading cause of global disability and mortality worldwide [1–3]. According to the World Health Organization, by 2030, more than 20 million deaths per year from the diseases associated with an increase in blood thrombogenic potential, will have been registered. Among them there is a coronary heart disease, a stroke, impaired peripheral circulation, complications of diabetes mellitus, therefore, antiplatelet therapy is an important component in various areas of clinical practice [4].

It is known that an atherosclerotic plaque causes narrowing of the vessel section and, as a result, when the bloodstream passes through this place, turbulent accelerations occur. In their turn, they affect blood corpuscles primarily erythrocytes and thrombocytes, increasing their aggregation ability. In addition to the formed elements, the vessel wall is exposed, which results in the endothelium damage. There is also the possibility of collagen fibers to come into contact with thrombocytes, followed by their adhesion to the damaged surface, activation, aggregation, and thrombus formation [5].

Thus, the activation of the platelet element of hemostasis, can become a cause of complications on behalf of the cardiovascular system, i. e. the formation of arterial thromboses [1]. Besides, an important role in the pathogenesis of thrombus formation is played by the activation of lipid peroxidation processes, the enhancement of which causes an increase in the thrombocyte aggregation and the coagulation element of hemostasis [6, 10]. This concept is the theoretical justification for the use of antioxidant agents as an additional pathogenetic therapy of arterial thromboses. Thus, timely and correct preventive measures aimed at inhibiting thrombocyte aggregation and lipid peroxidation, can prevent premature deaths, increase life expectancy, improve its quality, and reduce the economic costs of society for patients' treatment and rehabilitation [7–9].

In the course of the previous studies, among heterocyclic compounds, the substances exhibiting antiaggregant and antioxidant properties, had been identified [11–13]. The compound under code RU-1144 (1-(2,6-ditertbutyl-4-(1-hydroxyethyl)-phenyl-pyrimidobenzimidazole hydrochloride) can inhibit thrombocyte aggregation and lipid peroxidation, exceeding the reference drugs – acetylsalicylic acid, clopidogrel and ethylmethylhydroxypyridine succinate [31]. There are various methods for studying these types of activity [15, 16]. However, the most common is the study of antithrombotic properties in the models of arterial thromboses.

Therefore, the aim of the study was a comparative investigation of the antithrombotic activities of compound RU-1144 and antiaggregant drugs with a high evidence base – acetylsalicylic acid and clopidogrel, as well as the antioxidant agent EMGPS in the model of carotid arterial thromboses in rats, as well as in the model of the Global Thrombosis Test (the *Görög Thrombosis Test*) and their effect on “the bleeding time” parameters.

MATERIALS AND METHODS

Animals

The experiments were performed in 108 nonlinear white male rats weighing 250–300 g and 24 white mongrel male mice weighing 20–22 g, kept under vivarium conditions (the temperature of 22–24°C, the relative humidity of 40–50%) with natural light on a standard diet (GOST R 50258-92). All the animals were obtained from the nursery of the Research Center for Biomedical Technologies, OOO. The animals were kept under standard conditions in accordance with the Decree of the Chief State Sanitary Doctor of the Russian Federation No. 51 dated 29.08.2014, “On approval of SP 2.2.1.3218-14 “Sanitary and epidemiological requirements for the

design, equipment and maintenance of experimental biological clinics (vivariums)". All the animals (rats and mice) were quarantined for 14 days in separate vivarium boxes of the FSBEI HE of Volgograd State Medical University of the Ministry of Health of Russia. During the quarantine, the body weight of the animals was measured more than twice (on the 1st and the 14th days). The clinical condition was monitored in the groups every day by visual inspection. The animals with deviations found out during the examination, were excluded from the experimental groups. During the study, all the procedures with the animals were carried out in accordance with generally accepted ethical standards for the treatment of animals adopted by the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (1986) and considering the International Recommendations of the European Convention for the Protection of Vertebrate Animals used in experimental research (1997). All the procedures with the animals were performed in accordance with the standards, outlined in the eighth edition of the Guide for the Care and Use of Laboratory Animals and ARRIVE (Animal Research: Reporting of In Vivo Experiments). The experimental study was approved by the Regional Research Ethics Committee of the Volgograd Region, protocol No. 2083-2016, dated November 18, 2016. This study was carried out in accordance with the requirements of the "Guidelines for the Preclinical Studies of Medicines" [15].

Study design

An experimental study of the antithrombotic activity of the benzimidazole derivative under code RU-1144 (1-(2,6-ditretbutyl)-4-(1-hydroxyethyl)-phenyl-pyrimidobenzimidazole hydrochloride) (Scientific Research Institute of Physicochemical Physics of Southern Federal University), which has a spatially hindered phenolic substituent in its structure, has been carried out. Well known antiaggregant agents with a high evidence of activities – acetylsalicylic acid (Sigma, USA) and clopidogrel (Sanofi, France), and an antioxidant agent – ethylmethylhydroxypyridinesuccinate (EMGPS®, Pharmasoft LLC, Russia) [16] – had been chosen as reference drugs. Compound RU-1144 and reference drugs were administered intragastrically with the help of an intragastric tube.

As a solvent, purified distilled water was used. The antithrombotic activities of compound RU-1144 and reference drugs were studied in the model of rats' carotid arterial thromboses caused by the surface application of a 50% solution of *ferric chloride* (FeCl_3). The studied substances were administered to rats intragastrically once, 2 hours before the application of a thrombotic agent to the carotid artery of the animals [17]. 30 minutes before

the start of the experimental arterial thromboses, the rats were intraperitoneally anaesthetized with chloral hydrate (400 mg/kg). After the onset of anaesthesia, the skin and tissues were opened in layers, highlighting the carotid artery. A cotton pad moistened with a 50% solution of *ferric chloride* (FeCl_3) (0.025 ml) was placed on a small area of the carotid artery. The surrounding tissues were isolated with the help of a special "Parafilm" film. To record the changes in the blood flow, a Minimax-Doppler-K ultrasound dopplerograph (Minimax, St. Petersburg) was used. The ultrasound probe of the apparatus was installed at a small distance from the cotton pad, placed on the carotid artery. The blood flow was recorded till the complete occlusion of the vessel.

Compound RU-1144 and the reference drugs were studied at the doses of an equimolar dose of 19 mg/kg of acetylsalicylic acid (a pharmacologically active dose obtained in the model of ADP-induced rat aggregation in an *in vivo* test). For the test substance RU-1144, this dose was 48 mg/kg, and for the reference drugs of clopidogrel and EMGPS it was 32 and 28 mg/kg, respectively). In order to determine the ED_{50} depending on the manifested antithrombotic effect, (the dose at which the studied compounds increase the time of the onset of the complete vessel occlusion by a thrombus to the control by 50%), the studied doses of the substances and the reference drugs were either increased or decreased. Compound RU-1144 was also studied at the doses of 24 and 12 mg/kg, ASA – at 100 and 150 mg/kg, clopidogrel – at 60; 120 and 180 mg/kg, and the comparison drug EMGPS was studied at the doses of 150 and 100 mg/kg, respectively.

The antithrombotic activity of compound RU-1144 was investigated using the Global Thrombosis Test *in vitro* with the *ex vivo* study of the biological material after a single intragastric administration at the dose of 18.8 mg/kg (a pharmacologically active dose obtained in the model of ADP-induced thrombocyte aggregation in rats in the *in vivo* test) [18]. The reference drugs – acetylsalicylic acid and clopidogrel – were studied at the doses of 28.5 and 13.8 mg/kg, respectively. 2 hours after the administration of the test compounds, the blood was drawn from the abdominal aorta with a 5 ml syringe containing 20 μM of ADP thrombocyte aggregation inducer. The animals had been pre-anaesthetized with chloral hydrate (400 mg/kg, intraperitoneally). The resulting blood was immediately placed in a special *Görög* tube without any addition of stabilizers and preservatives. The main criteria for evaluating the antithrombotic effect of the test compound and reference drugs were indicators of the occlusion time and the lysis time, the analysis of which was carried out using the GTT Draw 2.3 software.

In order to determine the undesirable effect of the

antiaggregant drugs, “the bleeding time in mice” model was used [19]. To reproduce this model, the animals were preliminarily anaesthetized using chloral hydrate at the dose of 400 mg/kg. After that 5 mm of the tip of the tail was cut off, then placed in a test tube with physiological saline in a water bath (37°C).

To evaluate the effect, the time expressed in seconds, from the moment of cutting off the tip of the tail to the moment the bleeding stopped completely, was recorded. According to the effect on this parameter, compound RU-1144 was studied at the dose of 18.8 mg/kg and the reference drugs ASA and clopidogrel – at the doses of 28.5 and 13.8 mg/kg, respectively. The administration of the studied compounds was carried out 2 hours before the start of the experiment.

The groups of the control animals were given purified distilled water as a single dose in the equivalent volume intragastrically.

Statistical processing of results

Statistical processing of the experimental data was carried out using the Mann-Whitney criterion, the one-way ANOVA criterion with Bonferroni correction using the GraphPad Prism 5.0 statistical software package (“GraphPad”, USA) and Microsoft Excel 2007 (Microsoft, USA).

RESEARCH RESULTS

In the course of the arterial thrombosis study, the data indicating the presence of antithrombotic properties of the test substance and reference drugs, were obtained.

The average time of the carotid artery occlusion of the control group animals, was 19.4 ± 1.5 min. (Table 1), which is consistent with the published data [23, 26].

Compound RU-1144 at the dose of 48 mg/kg, reliably prolonged the time of the carotid artery complete occlusion to 31.4 minutes, which was 61.1% reliably higher than this indicator in the control group animals. With a further decrease of the test compound dose to 24 mg/kg, the time of thrombus formation also reliably decreased and amounted to 27 minutes. A further dose reduction to 12 mg/kg, reliably prolonged the onset of the carotid artery complete occlusion by 14.1% (Table 1).

At the dose of 19 mg/kg, ASA unreliably with respect to the control, prolonged the time of thrombus formation by 6.4%. Therefore, in the further study, the doses of acetylsalicylic acid were increased to 100 and 150 mg/kg. At the same time, at the dose of 100 mg/kg, the reference drug increased the time of the onset of the carotid artery complete occlusion by 29.5%, and at the dose of 150 mg/kg – by 58.5%. Thus, an increase

of the dose of acetylsalicylic acid increased the studied parameter by 58.5% (Table 1).

At the dose of 32 mg/kg, clopidogrel reliably extended the thrombosis time by 9.0% compared with the control group animals. A further increase of the dose to 60 mg/kg and then to 120 and 180 mg/kg, led to an increase in time till the complete occlusion of the carotid artery by 21.8, 34.6 and 65.4%, respectively (Table 1).

At the dose of 28 mg/kg, EMHPS increased the onset time of the rats' carotid artery complete occlusion by 8.11%. The increase of the drug doses to 100 and 150 mg/kg, led to the prolongation of this indicator by 41.03 and 75.21%, respectively.

Based on the obtained data, ED_{50} antithrombotic activities of compound RU-1144 and reference drugs were calculated. So, for the tested RU-1144 sample, this value was 37.8 mg/kg, for acetylsalicylic acid – 133.0 mg/kg, and for clopidogrel and EMHPS – 132.0 and 108.4 mg/kg, respectively. Thus, in terms of ED_{50} antithrombotic activities, RU-1144 compound exceeded the antiaggregant drugs – acetylsalicylic acid and clopidogrel – by 3.52 and 3.49 times, respectively, and the antioxidant agent EMHPS – by 2.87 times.

At the next stage, the antithrombotic activities of compound RU-1144 and the reference drugs of ASA and clopidogrel, were studied in the Global Thrombosis Test model (the *Görög Thrombosis Test*). When performing this experiment in the control group of the animals, the onset of the complete occlusion in the test system was 95.2 seconds (Table 2). The study of the biological material of the animals administered intragastrically with compound RU-1144, showed a statistically reliable increase in the time of the complete occlusion onset by 37% compared with the values obtained in the control group, and amounted to 130.5 seconds. At the same time, the test compound unreliably increased the lysis time relative to the control (Table 2).

The reference drug ASA, studied at the dose of 28.5 mg/kg, also reliably led to the prolongation of the complete occlusion time in the test system, while this indicator was 1.2 times higher than that of the control group animals, though not affecting the lysis clot time.

In the group of the animals treated with clopidogrel, the occlusion time was 57.6% longer than in the control group, but the lysis time was comparable to the values obtained in the control group animals (Table 2).

Thus, the results of the study obtained in this model, showed that the greatest antithrombotic effect was demonstrated by the reference drug clopidogrel, which, in the studied dose, increased the occlusion time of the test system by 1.7 times, unreliably exceeding compound RU-1144 and reliably exceeding acetylsalicylic acid by 1, 3 times. The studied compounds have shown no effect on the rate of lysis either.

Table 1 – The effect of compound RU-1144, ASA, clopidogrel and EMHPS on the complete occlusion time of rats' carotid artery in the model of arterial thromboses induced by the application of ferric chloride (FeCl₃) (M ± m) (n = 6)

No. in sequence	Tested samples	Dose, mg/kg	Thrombus formation time, min	Δ% of prolonging thrombus formation time	ED ₅₀ , mg/kg
1	Control		19.4±1.5		
2	RU-1144	12	22.3±0.7*	14.1±3.6*	37.8
		24	27.0±0.6*	38.5±2.8*	
		48	31.4±1.0*	61.1±5.4*	
3	ASA	19	20.8±0.3*	6.4±1.6*	133.0
		100	25.3±0.5*	29.5±2.5*	
		150	30.9±0.3*	58.5±1.4*	
4	Clopidogrel	32	21.3±0.3*	9.0±1.3*	132.0
		60	23.8±0.3*	21.8±1.6*	
		120	25.8±0.4*	34.6±1.3*	
		180	32.3±0.4*	65.4±2.2*	
5	EMHPS	28	21.1±0.3*	8.1±1.6*	108.4
		100	27.5±0.6*	41.0±2.9*	
		150	34.2±0.8*	75.2±4.3*	

Notes:

n – the number of the animals in the group;

* – the data relative to the control (the Mann-Whitney test, p≤0.05).

Table 2 – Antithrombotic activities of compound RU-1144 and reference drugs – ASA and clopidogrel – in the model of the *ex vivo* Global Thrombosis Test (the Görög Thrombosis Test) (M ± m, n = 6)

No. in sequence	Tested samples	Dose, mg/kg	Occlusion time, sec	Lysis time, sec
1	Control	–	95.2 ± 1.4	635.2 ± 29.0
2	RU-1144	18.8	130.5 ± 7.8*	711.2 ± 39.4
3	ASA	28.5	117.5 ± 4.1*	629.3 ± 15.7
4	Clopidogrel	13.8	150.0 ± 4.0*	631.5 ± 17.1

Notes:

n – the number of the animals in the group;

* – the data reliable relative to the control (the Mann-Whitney test, p<0.05).

Table 3 – The effects of compound RU-1144 and reference drugs on the time of bleeding from the mice's tail veins, at the doses of ED₅₀ antiaggregant activity *in vivo* (M ± m) (n = 6)

No. in sequence	Tested samples	Dose, mg/kg	Bleeding time, sec	Δ% of prolonging thrombus formation time
1	Control		349.3 ± 7.2	
2	RU-1144	18.8	445.5 ± 10.5*#	27.5 ± 3.0*#
3	ASA	28.5	583.9 ± 9.1*	67.2 ± 2.6*
4	Clopidogrel	13.8	566.0 ± 10.0*	62.0 ± 2.9*

Notes:

n – the number of the animals in the group;

the data reliable relative to the control, one-way ANOVA criterion with Bonferroni correction (p < 0.05);

– the data reliable relative to the reference drugs, one-way ANOVA criterion with Bonferroni correction (p < 0.05).

Table 4. Antiaggregant activities of compound RU-1144 and reference drugs in *in vitro* (IC₅₀) and *in vivo* (ED₅₀) studies

No. in sequence	Tested samples	IC ₅₀ , μM	ED ₅₀ , mg/kg
1	RU-1144	5,5	18,8
2	ASA	120,0	28,5
3	Clopidogrel	—*	13,8

Note:

* – in view of being a prodrug, clopidogrel cannot be used in *in vitro* tests.

Thus, compound RU-1144 led to a reliable increase in the bleeding time by 27.5% relative to the control mouse group, while ASA and clopidogrel prolonged the bleeding time twice as active – by 67.2 and 62.0%, respectively (Table 3). Thus, compound RU-1144 had a weaker effect on this parameter than the reference drugs.

DISCUSSION

Complex mediator interactions between thrombocytes with the involvement of various aggregation factors, play a significant role in initiating the processes of the arterial thrombosis development. Preliminary studies of the antiaggregant activity of compound RU-1144 in comparison with clopidogrel and acetylsalicylic acid, both *in vitro* and *in vivo*, made it possible to determine the inhibitory concentrations (IC_{50}) and effective doses (ED_{50}) of these compounds (Table 4).

As Table 4 shows, in the *in vitro* studies, compound RU-1144 predominates over the ASA activity by 21.8 times, *in vivo* – by 1.5 times, while it is 1.3 times weaker than clopidogrel. According to the published data, by application of 50% *ferric chloride* ($FeCl_3$), in the thrombotic lesion of the vascular wall Fe directly interacts with hydrogen peroxide, causing the formation of hydroxyl anions and a change of thrombocyte membranes in the phospholipid composition. The above-mentioned processes increase the functional activity of thrombocytes [21–23, 29].

Besides, oxidized fibrinogen accumulates in blood, activating the process of thrombus formation. Lipid peroxidation in endotheliocyte membranes leads to systemic endothelial dysfunctions and increases its penetration. This model of thrombosis allows us to study the effect of the compounds on the formation rate of an arterial (white) thrombus, which mainly consists of thrombocytes. That is why this model was chosen to study compound RU-1144, combining two types of activity: antiaggregant and antioxidant.

Based on the data obtained, it can be concluded that the compound RU-1144 exhibits a pronounced antithrombotic activity which exceeds that of the reference drugs due to its ability to inhibit thrombocyte aggregation and lipid peroxidation, and thereby prevent

the occurrence of arterial thrombosis in the rats' carotid artery.

The Global Thrombosis Test makes it possible to research not only the antithrombotic but also the thrombolytic activities of the compounds. Under the conditions of increased turbulence of blood flow in the *Görög Thrombosis Test*, compound RU-1144 showed the ability to increase the time of thrombus formation compared to the values in the control group animals and the unreliable predominancy of the activity compared to acetylsalicylic acid, however, it was unreliably weaker than clopidogrel. The lysis time under the action of the test sample did not change, which makes it possible to conclude that RU-1144 compound does not have any fibrinolytic activity.

It is known that during a prolonged therapy with antiaggregant drugs, a side effect such as bleeding, is observed. Bleedings in the gastrointestinal tract are most often, and intracranial hemorrhages resulting in increased risks of ischemic events, are not uncommon either [25, 27, 28].

The study showed that the compound RU-1144 increases the bleeding time from the tail vein of mice, however, in contrast to ASA and clopidogrel, this effect is less pronounced. That makes it possible to suppose a low probability of a side effect in the form of bleeding.

CONCLUSION

In the model of rats' carotid arterial thrombosis induced by a 50% solution of *ferric chloride* ($FeCl_3$), the compound under the laboratory code RU-1144 exerts a pronounced antithrombotic effect, exceeding that of the antiaggregant drugs – acetylsalicylic acid and clopidogrel – by 3.52 and 3.49 times, respectively, and the antioxidant agent EMHPS by 2.87 times. Under the conditions of increased turbulence of blood flow in the *Görög Thrombosis Test*, the tested sample of RU-1144 showed the ability to increase the time of thrombus formation, comparable to acetylsalicylic acid and clopidogrel, without affecting the time of its lysis. When studying compound RU-1144 in the test "bleeding time", the ability of the test sample to prolong this indicator was shown. This factor is typical of the group of antiplatelet agents, but at the same time, in comparison with acetylsalicylic acid and clopidogrel, it can prolong this time to a lesser extent.

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AUTHORS' CONTRIBUTION

All authors equally contributed to the research work.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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