Antiplatelet Activity of Riamilovir under Conditions of Lipopolysaccharide Intoxication

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We studied the effect of antiviral agent riamilovir on ADP-induced platelet aggregation in the absence and presence of LPS. Unlike acetylsalicylic acid (reference drug), riamilovir did not exhibit antiplatelet effect *in vitro*. However, it markedly suppressed platelet reactivity in LPS-treated blood samples and was 2.2-fold superior to acetylsalicylic acid in terms of IC_{50} value. In *in vivo* experiments, riamilovir under conditions of hypercytokinemia blocked platelet aggregation in rats by 64%.

Key Words: *riamilovir; platelet aggregation; LPS; hypercytokinemia*

In addition to their traditional role in thrombosis and hemostasis, platelets mediate the key aspects of inflammatory and immune processes [3]. SARS-CoV-2 is known to penetrate into endothelial cells, and the resulting endothelial damage can cause platelet migration to sites of infection. Activated platelets express P-selectin and CD40L on the cell membrane, interact with neutrophils, and can release α-granules and complement component C3 as well as various cytokines such as CCL2, CCL3, CCL7, IL-1β, IL-7, IL-8, *etc.* [8]. In addition to the release of cytokines, another important factor in COVID-19 is the recruitment of neutrophils into the vascular network, which plays a crucial role in triggering immunothrombosis [10]. Binding of activated platelets to neutrophils facilitates platelet migration into the lumen of the alveoli and contributes to pulmonary edema, which causes further platelet activation [14].

Hence, therapeutic approaches to COVID-19 can include drugs targeting platelet activity in addition to drugs targeting other sources of inflammation.

Riamilovir (Triazaverin) is an original Russian antiviral drug (against RNA-containing viruses, including influenza types A and B viruses); it is used for the treatment of influenza in outpatient and inpatient conditions. According to provisional guidelines, this drug is used to treat and prevent COVID-2019: as monotherapy for mild subclinical forms and in combination with other etiotropic agents for moderate forms [4]. Preliminary data suggest that the drug is effective and produces no adverse reactions. Nevertheless, current additional data on the efficacy and new properties of the drug remain relevant. A significant advantage of riamilovir is its ability to protect against hemorrhagic pneumonia [1].

As a specific feature of thrombosis in COVID-19 is poorly controlled inflammation and hyperactivation of the clotting system, which allows considering severe coronary infection as a pathogenetic mechanism of thrombosis, a thrombotic storm. Thus, platelets play a key role in the pathogenesis of sepsis and thrombosis and can be a potential target for the prevention of these complications. The role of exogenous LPS in the development of endotoxemia leading to a proinflammatory and procoagulant state and contributing to platelet activation, interaction with neutrophils, and fibrinogen binding has been shown in the experiment [11]. Here we studied the effect of the antiviral drug riamilovir on platelet activation without and after macrophage stimulation with LPS.

MATERIALS AND METHODS

The experiments were performed on 6 Chinchilla rabbits and 24 white outbred male rats weighing 250-270 g obtained from the Scientific Center of Bio-

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medical Technology of the Federal Medical-Biological Agency of Russia and kept in a vivarium conditions (22-24°C, relative humidity 40-50%, and natural light conditions); the animals received standard diet (GOST R 50258-92). The experiments were performed in accordance with the Principles of Good Laboratory Practice for Preclinical Studies in the Russian Federation (GOST R 51000.3-96 and 1000.4-96) and the Directive 2010/63/EU of the European Parliament and of the Council (September 22, 2010; On the Protection of Animals Used for Scientific Purposes).

This study was performed in accordance with the requirements of the current Manual for Preclinical Studies of New Pharmacological Substance [2]. Riamilovir, an antiviral agent (Medsintez), was studied as an object of study. In *in vitro* experiments, the antiplatelet agent acetylsalicylic acid (Sigma) served as a reference drug. The choice of this medicine is based on the fact that acetylsalicylic acid is widely used as an antiplatelet agent with a high level of evidence. Riamilovir was dissolved in saline; the reference drug was dissolved in 30 μl DMSO and then in saline to the required volume. The effect of the drugs on platelet aggregation was studied using Chrono-Log-700 (Chronolog) dual-channel lumi-aggregometer using the impedance detection method.

Rabbit blood for *in vitro* studies was sampled from the marginal ear vein by free drop method and stabilized with 3.8% sodium citrate (9:1). From the obtained sample, a constant blood volume of 450 μl was used for the study. The preparations in a concentration of 100 μM were added directly to a cuvette containing whole blood. ADP (Sigma) in a concentration of 5 μM was used as an inducer of platelet aggregation.

In case of high antiplatelet activity, to calculate the IC_{50} value (concentration at which platelet aggregation is blocked by 50%), the tested samples were additionally studied in concentrations of 10 and 1 μM. Antiplatelet activity was also analyzed under conditions of hypercytokinemia. To this end, LPS solution (*E. coli* O111:B4, Sigma) in a final concentration of 20 μM [5] was added simultaneously with the test drug to a cuvette with the whole blood sample and after 5-min incubation, platelet aggregation inductor was added.

In vivo experiments were performed on rats divided into 4 groups (6 animals per group): two groups without LPS administration (intact rats and rats intragastrically treated with riamilovir) and two groups with LPS intoxication (control animals received intravenous injection of LPS and experimental rats received LPS intravenously and riamilovir intragastrically).

Riamilovir in a dose of 20 mg/kg (a dose equivalent to human dose calculated using interspecies conversion factor) was administered once intragastrically using an atraumatic gastric probe 1 h before blood sampling (the corresponding to maximum concentration in the blood). The rats were anaesthetized with chloral hydrate (400 mg/kg intraperitoneally) and the biomaterial for the study was taken from the abdominal aorta (blood stabilizer indicated above). Hypercytokinemia was modeled by intravenous injection of 2 mg/kg LPS [6] into the caudal vein. Riamilovir was administered orally once 1 h before LPS administration, the blood was taken in 4 h after LPS administration. Controls intragastrically received an equivalent volume of distilled water.

The results were processed statistically using GraphPad Prism 8.0 software (one-way ANOVA with Bonferroni correction, $p < 0.05$). IC_{50}, the mean and standard deviation in each group were calculated using Microsoft Excel 2020 built-in functions.

RESULTS

At the first stage, we studied the effects of riamilovir and acetylsalicylic acid on platelet aggregation *in vitro*. The control impedance of rabbit whole blood induced by ADP was 8.3 Ω. The reference drug in a concentration of 100 μM reduced the amplitude of platelet aggregation to 3.4 Ω, which corresponded to a significant inhibition of platelet functional activity relative to control by 59.8% (Table 1). Reducing acetylsalicylic acid concentration to 10 and 1 μM was followed by a decreased in the amplitude of platelet aggregation to 5.5 and 6.6 Ω, respectively. Thus, at the above concentrations, acetylsalicylic acid blocked platelet aggregation by 23.7 and 11.6%, respectively. IC_{50} of the reference drug was 57.5 μM. Riamilovir in a concentration of 100 μM reduced the amplitude of ADP-induced platelet aggregation to 6.9 Ω, *i.e.* inhibited this process by 17.1% (Table 1).

During incubation of the whole blood with LPS, the amplitude of platelet aggregation significantly increased from 8.3 to 11.9 Ω, which indicated higher activation of the platelet hemostasis in response to macrophage activation (Table 2). Antiplatelet activity of the studied drugs was evaluated in relation to the range of the difference between the level of ADP-induced aggregation of intact platelets and platelets treated with LPS. Acetylsalicylic acid in a concentration of 100 μM significantly reduced the amplitude of platelet aggregation to 8.91 Ω, *i.e.* inhibited this process by 83.2% (Table 2, Fig. 1). In concentration of 10 and 1 μM, acetylsalicylic acid reduced activity by 52.4 and 3.2%, respectively, and amplitude of platelet aggregation to 10.02 and 11.8 Ω, respectively. The IC_{50} value of acetylsalicylic acid was 12.4 μM (Table 2). Thus, after macrophage stimulation with LPS, activity of the reference drug increased by 4.6 times in comparison with that in intact blood.

TABLE 1. Antiplatelet Activity of Antiviral Agent Riamilovir and Acetylsalicylic Acid in Model of ADP-Induced (5 µM) Whole Blood Platelet Aggregation in Rabbits *In Vitro* (*n*=6; *M*±*m*)

Experimental conditions	Concentration, µM	Amplitude of platelet aggregation, Ω	Δ% of inhibition of platelet aggregation	IC$_{50}$, µM
Control (intact)	—	8.3±0.2		
Acetylsalicylic acid	100	3.4±0.1*	59.8±4.0*	57.5
	10	5.5±0.2	23.7±8.0	
	1	6.6±0.2	11.6±7.0	
Riamilovir	100	6.9±0.2*	17.1±6.7	>100
	10	0±0	0±0	
	1	0±0	0±0	

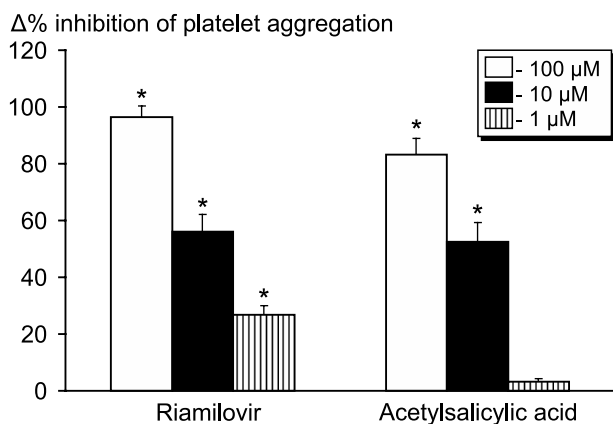**Note.** *p<0.05 in comparison with the control (one-way ANOVA).

Fig. 1. Antiplatelet activity of acetylsalicylic acid and antiviral agent riamilovir in concentrations of 100, 10, and 1 µM on ADP-induced (5 µM) platelet aggregation in the presence of LPS *in vitro*. *p<0.05 in comparison with the control (100%).

Riamilovir showed high antiplatelet activity in the presence of LPS: in a concentration of 100 µM, it inhibited platelet aggregation by 96.3% (Fig. 1) and reduced the amplitude of this process to 8.43 Ω (Table 2). When the concentration of riamilovir was reduced to 10 and 1 µM, the amplitude of platelet aggregation decreased to 9.9 and 10.9 Ω, respectively, *i.e.* platelet aggregation was inhibited by 56.1 and 26.8%, respectively (Fig. 1). The IC$_{50}$ for riamilovir was 5.2 µM.

Thus, *in vitro* experiments showed that under conditions of LPS stimulation of macrophages, the IC$_{50}$ value for riamilovir was 2.4 times higher than for acetylsalicylic acid.

The second step was to find out whether riamilovir would have this effect in *in vivo* experiments. The amplitude of ADP-induced platelet aggregation in control rats was 7.9 Ω. The antiviral drug riamilovir in a dose of 20 mg/kg reduced the amplitude of platelet aggregation to 5.57 Ω, functional activity of platelets was inhibited by 29.4% (Table 3). Thus, riamilovir produced an antiplatelet effect *in vivo*.

The amplitude of ADP-induced platelet aggregation in rats injected intravenously with LPS increased significantly relative to that in intact controls (to 10.9 Ω), which attested to platelet activation under the influence of hypercytokinemia. Riamilovir reduced the amplitude of platelet aggregation to 8.98 Ω. Thus, antiplatelet activity under conditions of cytokine intoxication increased by 2.2 times in comparison with that in intact animals.

Platelets play a key role in the pathogenesis of sepsis and thrombosis [9]. Endothelial damage typical of hypercytokinemia induced the release of platelet agonists and leads to their hyperactivation and activation of the internal coagulation system, and then together with tissue factors from damaged endothelial cells in the vascular network activates the external coagulation system, which can contribute to immunothrombosis in viral infections [13]. Hence, disease progression can be prevented or delayed by affecting platelets via blockade of P-selectin expression on the cell surface, interaction with neutrophils, and cytokine release. In addition, recently published studies showed that production of tissue factor, the key element in the immunocoagulation process, can be blocked by acting on platelets [7,12].

Riamilovir, in contrast to acetylsalicylic acid, produced no antiplatelet effect *in vitro*. However, in LPS-treated blood samples, *i.e.* under conditions of hypercytokinaemia, this drug pronouncedly inhibited platelet reactivity and was superior to acetylsalicylic acid by its IC$_{50}$. However, *in vitro* and *in vivo* antiplatelet activity of the drugs can differ. Therefore, activity of riamilovir was studied after its single peroral administration to rats in a dose of 20 mg/kg. Antiplatelet activity of riamilovir under conditions of hypercytokinemia increased by 2.2 times. Therefore, the studied antiviral agent exhibited antiplatelet effect *in vitro* only after macrophage activation with LPS, while in *in vivo* experiments, it suppressed platelet aggregation without and under conditions of

TABLE 2. Antiplatelet Activity of Antiviral Agent Riamilovir and Acetylsalicylic Acid on Model of ADP-Induced (5 µM) Platelet Aggregation of Rabbit Whole Blood in the Presence of LPS *In Vitro* ($n=6$; $M\pm m$)

Experimental conditions	Concentration, µM	Amplitude of platelet aggregation, Ω	IC_{50}, µM
Control (intact)	—	8.3±0.2	
Control+LPS	—	11.9±0.1*	
Acetylsalicylic acid	100	8.9±0.1°	12.4
	10	10.0±0.2°	
	1	11.8±0.0	
Riamilovir	100	8.4±0.0°	5.2
	10	9.9±0.1°	
	1	10.9±0.1°	

Note. $p<0.05$ in comparison with *control, °control+LPS (one-way ANOVA).

TABLE 3. Antiplatelet Activity of Riamilovir on a Model of ADP-Induced (5 µM) Platelet Aggregation in Intact Rats after Single Intragastric Administration in a Dose of 20 mg/kg without and in the Presence of LPS ($n=6$; $M\pm m$)

Group	Dose, mg/kg*	Amplitude of platelet aggregation, Ω	Δ% of inhibition of platelet aggregation
Control (intact)	—	7.9±0.0	
Control+LPS	—	10.9±0.1⁺	
Riamilovir	20.0	5.6±0.1	29.4±2.2⁺
Riamilovir+LPS	20.0	9.0±0.0°	64.0±6.5°

Note. *Riamilovir dose equivalent to the dose used for humans in clinical practice calculated using the interspecies conversion factor. $p<0.05$ in comparison with ⁺control, °control+LPS (one-way ANOVA).

hypercytokinemia. Hence, it can be concluded that antiplatelet therapy can have a beneficial effect in viral infection. However, there are many questions about the use and usefulness of antiplatelet agents in COVID-19. It is not clear at which phase of the disease the treatment should be administered, which antiaggregant is optimal, and at what dose it can be effectively used for minimizing the risk of bleeding. The use of riamilovir in COVID-19 is mainly aimed at its interaction with the S-protein/ADP2 complex, in order to block the binding and entry of the virus into the host cells. However, its ability to stabilize hemostatic processes and in particular its antiplatelet activity during macrophage activation suggests its positive contribution to reducing the risk of COVID-19-associated thrombosis. However, this assumption can be definitively confirmed only in clinical trials.

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