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RESEARCH ARTICLE

Hemostatical activity of new benzylammonium salt 2-[3-methyl-1-n-propyl-7-(1,1-dioxotiethanyl-3)xantinyl-8-thio]acetic acid

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ABSTRACT

Background: Preventing and control of bleeding is important in various areas of clinical medicine in the light of increasing number of patients with hemorrhagic manifestations, extensive use of anticoagulants, increasing number of invasive diagnostic, and treatment methods. However, the drugs traditionally used in medical practice to control bleeding are often inefficient and unable to lead to effective reduction in blood loss. One of the ways to find new methods of bleeding prophylaxis is to develop means of pharmacological correction of hemostasis system. The results of the previous research show potentially high activity of some new nitrogen-containing heterocyclic derivatives relating to hemostasis system *in vitro* and *in vivo*. **Aims and Objectives:** To examine system hemostatic activity of first synthesized benzylammonium salt 2-[3-methyl-1-*n*-propyl-7-(1,1-dioxothietanil-3) xantinyl-8-thio]acetic acid (Compound I) under experimental conditions *in vitro* and *in vivo*. **Materials and Methods:** Experimental work *in vitro* is performed on the blood of healthy male donors, under conditions *in vivo* it is done on intraperitoneal injection of equimolar concentrations of the test substances. Thromboelastography (TEG) was carried out with apparatus TEG 5000. The analysis of the thromboelastograms enabled to define general tendency of coagulation, functional activity of platelets and fibrinogen, fibrinolysis activity, and physicomechanical properties of the formed clots. The influence of firstly synthesized xanthine derivative and ethamsylate on the functional activity of platelets *in vitro* and *in vivo* was studied using a laser platelet aggregation analyzer "Biola 230LA." The research evaluated the general nature of aggregation, value of maximum aggregation, maximum rate of aggregation, and average size of platelet aggregates. Experimental evaluation of the system specific hemostatic activity *in vivo* was carried out using the model of parenchymatous bleeding in immature male rats. **Results:** The interference came amid registration of bleeding stop time and extent of blood loss. Proagregate effect of Compound I is successfully realized into the system hemostatic activity under conditions of parenchymatous bleeding, exceeding the performance of the control group and the etamsylate group. **Conclusion:** The findings reveal potentially high systemic hemostatic activity of Compound I, convincing of the need to further study this compound and its analogues to create on their basis highly efficient, selective correctors of hemostasis system.

KEY WORDS: Xanthine Derivatives; Hemostasis System; Proaggragation Activity; Hemostatic Activity

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INTRODUCTION

Preventing and stopping the bleeding is essential in various areas of clinical medicine, especially in hematology,[1] surgery,[2] traumatology,[3] oncology,[4] and tocology,[5] in view of the increasing number of patients with hemorrhagic manifestations, extensive use

of anticoagulants (heparins and a number of coumarin drugs).[6]

Clinical experience shows that when planning surgical interventions with the integrity of the main arteries and veins, the capillary parenchymal hemorrhages are the primary sources of surgery blood loss.[7] To control them is achieved by local hypothermia using thermal and ultrasonic coagulation,[8] parenterally injecting fresh frozen plasma and its products, factors of blood clotting (isolated or in various combinations), aminocaproic acid, serine proteinase inhibitors (aprotinin), etamsylate, desmopressin, and several other drugs with different mechanisms of bleeding control.[9-13] Rationale of this research relates to the fact that the drugs traditionally used in medical practice to control bleeding are often inefficient and unable to lead to an effective reduction of blood loss which is dangerous due to the possible development of hemorrhagic shock, coagulopathy, ischemia, and the development of multiple organ failure. Overview of clinical recommendations show that among synthetic systemic hemostatic drugs the highly proved efficacy was confirmed only for tranexamic acid, aminocaproic acid, and etamsylate.[14] Most of hemostatic drugs are presented by fresh frozen plasma or recombinant factors,[15] the use of which is regulated by strict endeixes and is associated with high financial costs and the likelihood of complications and/or adverse effects.[16]

One of the ways to find new methods of bleeding prophylaxis is to develop means of pharmacological correction of hemostasis system. The results of the previous research show potentially high activity of some new nitrogen-containing heterocyclic derivatives regarding hemostasis system *in vitro* and *in vivo*.[17-19] This research is devoted to the study of hemostatic systemic activity of benzylammonium salt 2-[3-methyl-1-*N*-propyl-7-(1,1-dioxothietanil-3)xantinyl-8-thio]acetic acid (Figure 1) under experimental conditions.[20]

MATERIALS AND METHODS

Design of the Research

All research work has been carried out in two phases;[21] the first phase examined the impact of Compound I on the hemostasis system *in vitro*. Then, the research rated systemic hemostatic activity of Compound I under bleeding conditions on intraperitoneal injection to rats. Under *in vitro* conditions, the study of the effect on the functional activity of platelets began

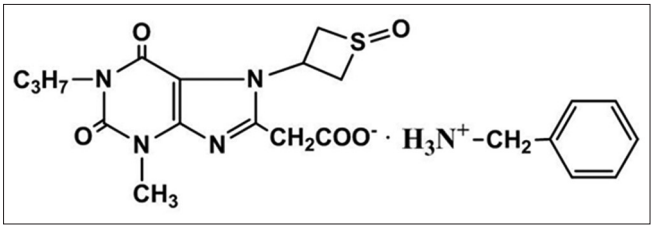

Figure 1: The structure of Compound I

with concentration of 2×10^{-3} mol/L, coagulation hemostasis component - 10^{-3} g/L, gradually reducing the concentration. Under *in vivo* conditions, rats were intraperitoneally injected with the test substances in equimolar concentration which for etamsylate was 38.1 mg/kg, for the Compound I is 73.8 mg/kg.

Experimental work *in vitro* is performed on blood of 54 male donors aged 18-24. The study was approved by Ethics Committee of State-Financed Educational Institution of Higher Professional Education "Bashkir State Medical University" of Health Ministry of Russia (No. 2 Dated from 17.10.2012). An informed consent was obtained from all research participants before blood sampling.

Experimental research *in vivo* is performed on 50 lab rats in compliance with the International recommendations of the European Convention for protection of vertebrate animals in experimental animals, laboratory practice regulations on conducting preclinical studies in Russia (GOST 51000.3-96 3 and 51000.4-96, GOSTR 50258-92) and the order of the Ministry of Health and Social development of Russia No. 708 n dated from 23/08/2010 "on approval of the rules for laboratory practice" (good laboratory practice). The animals were kept in standard conditions of animal quarters with natural lighting, air temperature of 20 ± 2°C and humidity of 55-60% in plastic cages with bedding from sawdust. 24 h before the research the feeding was stopped without limiting the access to water.

Blood Collection and Centrifugation

Blood sampling from donor volunteers was carried out from cubital vein using the systems of vacuum blood sampling BD Vacutainer® blood collection (Dickinson and Company, United States). The stage of working with laboratory animals included their anesthesia with diethylether, fixation on a peeling block and blood sampling from the jugular vein into siliconized tubes. Venous blood was stabilized by 3.8% sodium citrate solution in a ratio of 9:1.

All tests were carried out on enriched and platelet depleted dry blood. Samples of platelet-rich plasma were obtained by centrifuging the citrated blood with 100 g during 10 min, plateletless plasma - with 300 g during 15 min. The work included centrifuge OPN-3.02 (OJSC TNC "DASTAN", Kyrgyzstan).

Platelet Aggregation

Research on the influence of Compound I and comparators on platelets aggregation was carried out with a laser analyzer of platelet aggregation "Biola 230LA" (LLC "Biola", Russia). For aggregation inductor was used adenosine diphosphate (ADP) with a concentration of 20 μg/ml and collagen - 5 mg/ml produced by "Technology-Standard" (Russia). Aggregatogram analysis was conducted using the AGGR software, taking into

account the following indicators: General nature of aggregation (single-wave, two-wave; complete, partially reversible, irreversible), value of maximum aggregation (MA), maximum speed of aggregation (tg α), average size of platelet aggregates in relative units (mean radius aggregate).

Coagulation Component of Hemostasis

Study of influence of Compound I and comparator on coagulation component of hemostasis was conducted by widely accepted clotting tests on turbodimetric hemocoagulometer Solar CGL 2110 (CJSC "SOLAR", Belarus). The research included the indicators of activated partial thromboplastin time, thrombin time, prothrombin time, and A. Clauss fibrinogen concentration. Procoagulation activity of the substances under study *in vitro* was defined in concentration of 10^{-3} g/ml. The research applied reagents produced by "Technology-Standard" (Russia).

Thromboelastography (TEG)

TEG was carried out with apparatus TEG 5000 (Haemoscope Corporation, United States). The analysis of the thromboelastograms defined general tendency of coagulation (R), functional activity of platelets and fibrinogen (MA, Angle), activity of fibrinolysis clot lysis time (CLT), and the physicomechanical properties of the formed clots (G). For TEG activator was used 0.2 M $CaCl_2$ ('Technology-Standard', Russia).

The Model of Parenhimatosis Hemorrhage in Rats

The experimental evaluation of the specific systemic hemostatic activity *in vivo* was carried out on viripotent male rats weighing 190-220 g. The drugs were injected intraperitoneally 1 h before parenhimatosis bleeding simulation. Then was midline laparotomy under ether anesthesia, delivering the front surface of the liver. The liver was resected using a special stopper (for the same volume, shape and size). The wound made had an elliptic shape with an area of 2.5 cm^2 and depth of 0.3 cm. The wound surface was tightly mopped with a gauze wad before the final hemostasis. The interference came amid registration of bleeding stop time and extent of blood loss. The amount of blood loss was determined by the gravimetric method, weighing the blood-soaked gauze material on electronic scales.[21]

Statistical Processing

The findings are processed using the statistical package Statistica 10.0 (StatSoft Inc., USA). The normality of the distribution of actual data was checked by using the criterion of Shapiro-Wilka. The groups were described using the median and interquartile interval. Variance analysis was performed using the criterion of Kraskel-Wallis test (for independent observations) and Friedman (for repeated observations). Critical level of *P* significance for statistical criteria was taken equal to 0.05.

RESULTS

Results of Studies *In Vitro*

The influence of Compound I and etamsylate on the hemostasis system *in vitro* was studied starting with TEG as a method that enables to evaluate the hemostatic system taken together on the main key units. The findings determined that the Compound I shows hemostatic properties exceeding level wise the values of etamsylate (Table 1). MA indicator, which characterizes the functional activity of platelets, in the presence of Compound I has been increased by 26.4% ($P = 0.001$) and by 13.8% ($P = 0.001$) compared to the control and etamsylate, respectively.

This leads to a statistically significant increase of the total coagulation potential toward hypercoagulation - the

Table 1: The indicators of TEG under the impact of Compound I and etamsylate *in vitro* (*n*=7)

Indicator	Control	Etamsylate	Compound I	P_2
R, min	11.6 (9.7-13.2)	8.7 (6.4-9.3)$^{\alpha}$	9.6 (8.9-10.3)$^{\alpha}$	0.03
Angle, deg	43.7 (42.4-44.3)	48.3 (44.5-49.1)$^{\beta}$	57.4 (50.3-62.7)$^{\beta}$	0.005
MA, mm	55.9 (51.2-57.8)	60.1 (58.3-64.2)$^{\alpha}$	68.4 (64.1-74.7)$^{\beta}$	0.001
TMA, min	35.4 (32.4-38.5)	28.7 (24.1-30.5)$^{\beta}$	23.6 (22.7-27.9)$^{\beta}$	0.005
G, dyne/cm^2	6.3 (5.7-6.4)	6.9 (6.2-7.3)	8.9 (8.1-9.4)$^{\beta}$	0.006
E, dyne/cm^2	127.3 (115.4-142.3)	130.5 (121.7-134.3)	140.1 (137.2-143.5)$^{\beta}$	0.001
TPI/s	14.1 (13.2-15.1)	16.2 (15.7-17.1)$^{\alpha}$	18.4 (16.3-19.2)$^{\beta}$	0.001
CL30,%	97.5 (91.3-99.5)	95.3 (93.1-98.6)	96.5 (94.8-99.4)	0.6
LY30,%	0.6 (0.2-0.9)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.4
CLT, min	36.9 (32.4-38.3)	35.9 (34.6-37.3)	34.7 (33.7-37.5)	0.5
CI	0.6 (0.3-1.3)	1.6 (1.4-1.8)$^{\beta}$	2.5 (2.1-3.4)$^{\beta}$	0.001

$^{\alpha}P<0.05$; $^{\beta}P<0.001$ - Etamsylate or Compound I versus control, P_2 - Etamsylate versus Compound I, MA: Maximum aggregation, TEG: Thromboelastography, CI: Confidence interval, CLT: Clot lysis time

trimethylamine indicator reduced by 33.3% (P = 0.001) and 17.8% (P = 0.005) and triose-phosphate isomerase increased by 30.5% (P = 0.001) and 14.2% (P = 0.001) in comparison with the control and etamsylate. The values of the clot flowage increase - the E and G indexes increase in the group of Compound I by 1.3 times compared to the control. Etamsylate had no effect on indicators of the clot strength. Indicators that are responsible for fibrinolysis system (CLT, CL30, LY30), remain at the level of the stated values.

The findings on how Compound I and etamsylate influence functional activity of platelets *in vitro* are presented in Table 2. The research showed that the injection of 2×10^{-3} mol/l of Compound I into the aggregometer cuvet during 5 min of inductors makes platelet aggregation increase on average by 9.7% (P = 0.006) for ADP and 8.3% (P = 0.005) for collagen that two times exceeds etamsylate indicators. Analysis on "dose-effect" relationship shows that at a concentration of 5×10^{-4} mol/L of Compound I the platelet aggregation grows at an average by 2.3% for both inductors. Etamsylate in this concentration already shows no activity.

The findings on how Compound I and comparator influences plasma hemostasis component (Table 3) show that new benzylammonium salt and etamsylate do not change indicators of the coagulogram at concentrations of 10^{-3} g/ml.

Results of Studies *In Vivo*

The next stage examined the impact of Compound I and etamsylate on functional activity of platelets at intraperitoneal injection into rats (Table 4). Maximal platelet aggregation on injecting Compound I exceeded indicators of the control by more than 20%, and the values of etamsylate - by an average of 10%; platelet aggregation rate increased by 25.0% in respect to the control and by 15.5% in comparison with the etamsylate for both inductors of aggregation. The average radius of platelet aggregates in the presence of Compound I is higher the similar index in the control group by 40.0% and in the group of etamsylate - by 28.5%.

The findings of systemic hemostatic activity on the model of parenchymatous hemorrhage in rats are presented in Table 5. Table 5 data show that on intraperitoneal injection into rats the etamsylate reduced bleeding time by 26.5% (P = 0.0016) in comparison with the control group without significant impact on the total amount of blood loss. Compound I reduces the bleeding time by 37.4% (P = 0.003) and by 14.7% (P = 0.002) compared to the control and etamsylate, respectively. Wherein the blood loss volume reduced efficiently by 40.5% compared to the control (P = 0.002) and the group of etamsylate (P = 0.001).

DISCUSSION

The findings of the experimental work determined that the new benzylammonium salt shows proagregative activity that

Table 2: Indicators of ADP-and collagen-induced platelet aggregation under the influence of etamsylate and Compound I *in vitro* in blood of healthy donors (n=7)

Code number	Concentration mol/L	Spontaneous aggregation of platelets (% to the control)	Enhancement of ADP-induced platelet aggregation (% to the control)	Enhancement of collagen-induced platelet aggregation (% to the control)
Ethamsylate	2×10^{-3}	22.1 (20.7-25.2)	3.7 (1.5-5.9)	4.3 (2.2-6.9)
	10^{-3}	10.2 (7.4-13.5)	4.2 (2.1-6.3)	5.3 (3.1-8.6)
	0.5×10^{-3}	2.9 (1.2-5.7)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Compound I	2×10^{-3}	0.0 (0.0-0.0)	9.7 (7.4-12.3) P=0.006	8.3 (7.1-10.3) P=0.005
	10^{-3}	0.0 (0.0-0.0)	7.2 (5.6-9.3) P=0.002	6.7 (5.4-8.1) P=0.003
	0.5×10^{-3}	0.0 (0.0-0.0)	3.4 (2.7-4.2) P=0.002	3.9 (2.9-4.4) P=0.001

P - Level of statistical significance of differences of etamsylate groups versus Compound I, ADP: Adenosine diphosphate

Table 3: Influence of Compound I and etamsylate on koagulogram *in vitro* (n=7)

Indicator	Control	Etamsylate	P_1	Compound I	P_2	P_3
APTT, s	23.1 (21.6-24.7)	23.4 (22.7-24.8)	0.7	21.9 (20.3-24.2)	0.6	0.7
TT, s	27.2 (26.4-28.9)	28.3 (27.5-29.6)	0.2	27.8 (26.3-28.9)	0.8	0.3
PT, s	12.4 (11.5-13.9)	12.9 (11.5-14.3)	0.3	13.2 (11.5-14.7)	0.4	0. 3
Fibrinogen, s	24.3 (22.5-26.7)	25.9 (24.7-26.3)	0.2	23.8 (22.3-24.8)	0.8	0.5

P_1 - Etamsylate versus control, P_2 - Compound I versus control, P_3 - Etamsylate versus Compound I, APTT: Activated partial thromboplastin time

Table 4: Indicators of ADP-and collagen-induced platelet aggregation in rats following intraperitoneal injection of etamsylate and Compound I into rats, *n*=7

Indicator	Control	Etamsylate	Compound I	P_2
Collagen, mm	55.9 (53.8-58.1)	67.9 (64.2-69.3) P_1=0.006	74.3 (70.2-77.9) P_1=0.003	0.001
MRA (collagen)., r.u.	6.5 (6.2-6.7)	9.1 (7.8-11.4) P_1=0.001	12.3 (11.7-13.5) P_1=0.0005	0.03
tg α.(collagen)	36.5 (34.3-38.2)	37.2 (35.7-38.6) P_1=0.02	44.2 (41.7-49.2) P_1=0.001	0.004
ADP, mm	54.1 (50.6-57.4)	67.1 (64.9-68.6) P_1=0.002	77.6 (74.2-79.3) P_1=0.0001	0.004
MRA (ADP), r.u.	6.3 (6.2-7.1)	8.9 (7.2-10.3) P_1=0.002	11.4 (8.1-9.3) P_1=0.003	0.001
tg α (ADP)	42.7 (40.2-44.9)	46.4 (45.9-47.1) P_1=0.001	53.4 (51.2-56.3) P_1=0.0001	0.006

P_1 - etamsylate or Compound I versus control, P_2 - etamsylate versus Compound I, ADP: Adenosine diphosphate

Table 5: Indicators of hemostatic activity of etamsylate and Compound I following intraperitoneal injection into rats (*n*=7)

Medicament	Control	Etamsylate	Compound I
Bleeding time, s	97.9 (91.2-99.1)	71.9 (70.6-73.4) P_1=0.0016	61.3 (59.8-64.6) P_1=0.003 P_2=0.002
Δ weight of drapes, g	7.6 (7.4-8.1)	6.9 (5.9-7.5) P_1=0.23	4.1 (3.8-4.3) P_1=0.002 P_2=0.001

P_1 - etamsylate or Compound I versus control, P_2 - etamsylate versus Compound I

exceeds the values of etamsylate both *in vitro* and *in vivo*. There was no registered case of impact on coagulation component of hemostasis *in vitro* of the etamsylate and Compound I neither through the standard clotting tests nor through TEG. The Compound I activity data *in vivo* regarding spectrum-wise proagregate activity fully corresponds to the data obtained at the stage *in vitro*. The Compound I showed proagregate activity upon intraperitoneal injection to laboratory rats, being more effective than etamsylate, reducing the amount of blood loss and bleeding time.

Etamsylate (2.5-dihydroxibenzolsulphonatediethylamm onium salt) was developed in 1959 Esteve et al. and has been used as a hemostatic agent since1964.[22] The main domain of usage is to reduce menorrhagia and prevent/treat periventricular hemorrhage in children with low birth weight, as well as to control surgical or postsurgical capillary bleeding. In the year 1980, Vinazzer found that the etamsylate affects mechanisms of platelets adhesion and reduces capillary bleeding.[23] The research by Sack and Dujovne shows that etamsylate provokes agregation of platelets in platelet-enriched plasma, but such platelet aggregation is minor and reversible.[24] The study of biochemical prerequisites of pro-agregation effect of etamsylate helped to determine that it enhances platelet aggregation and ATP release induced by arachidonic acid, thromboxane A2, collagen and calcium ionophore A23187 but not ADP and/or adrenaline.[25] Based on the research findings, it was determined that injection of 2×10^{-3} mol/l of etamsylate *in vitro* induced spontaneous platelet aggregation, reaching an average of 22.1% relating to the control. Thus, the main effect of etamsylate falls on intact platelets, thereby potentially increasing the risk of blood clot formation, on the one hand, and remaining ineffective during non-capillary bleeding, on the other.[26-29] It should be noted that the Compound I did not cause spontaneous aggregation. This fact suggests that at the moment of induced aggregation the Compound I has a more selective effect on activated platelets and increases the overall hemostatic potential.

CONCLUSION

The findings reveal potentially high systemic hemostatic activity of benzylammonium salt 2-[3-methyl-1-*N*-propyl-7-(1,1-dioxothietanil-3) xantinyl-8-thio] acetic acid and convince of the need to further study this compound and its analogues to create on their basis highly efficient systemic selective hemostatic agents.

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