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Research Article

STUDY OF THE ANTICOAGULANT ACTIVITY OF ALKALOID AKUZUN

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ABSTRACT

It is known that the search for effective and safe agents that have a complex effect on vascular-platelet hemostasis remains an urgent task of experimental biology. The purpose of this work is to study the alkaloid Akuzun isolated from H. acutifolium plants on the hemostasis system. The effect of anticoagulants on human plasma coagulation in vitro was assessed using generally accepted tests: activated partial platelet time, prothrombin time, thrombin time, Reaklot-Heparin; fibrinogen was used to study the effect on fibrinogen polymerization. All coagulological tests (with human plasma) were performed on a single-channel coagulometer. The indicators that integrally reflect the total blood plasma coagulation activity (recalcification time), the rate of interaction of thrombin with fibrinogen (thrombin time) and monomeric fibrin autopolymerization (fibrin self-assembly time in plasma).

KEYWORDS

Akuzun, anticoagulants, plasma, coagulation activated partial platelet time, prothrombin time, thrombin time, Reaklot-Heparin; fibrinogen.

INTRODUCTION

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It is known that anticoagulants are used to prevent and treat thrombosis in humans. [1-3]. There are anticoagulants of indirect and direct type of action. [4]. The mechanism of action of direct-acting anticoagulants is associated either with direct inhibition of the activity of thrombin or factor Xa, or with the activation of their plasma inhibitor antithrombin [5].

Currently used anticoagulant drugs do not fully satisfy the needs of practical medicine. With undoubted effectiveness, indirect anticoagulants and heparin preparations have limitations. One of the most common side effects of all modern anticoagulants is bleeding [6-9]. Along with heparin preparations and anticoagulants of indirect action, bleeding is also provoked by modern thrombin / factor Xa inhibitors for oral and parenteral use [10-14]. Therefore, the development of new anticoagulant compounds with different chemical structures and less hemorrhagic activity is relevant.

It is known that platelets play a key role in the development of heart attacks, strokes and complications after bypass surgery and angioplasty. Platelets are involved in the formation of blood clots, the regulation of inflammation and immune processes. By acting on platelets, it is possible to delay the formation of blood clots and the development of coronary heart disease.

It is now known that taking antiplatelet drugs based on plant alkaloids reduces the risk of developing acute vascular complications, including ischemic diseases [15,16].

For this reason, the search for effective and safe agents that have a complex effect on vascular-platelet hemostasis remains an urgent task of experimental biology [17-19].

Thus, the search for both new chemical compounds for the subsequent creation of drugs with anticoagulant activity and the search for a possible antidote to them are relevant. In in vitro experiments it is possible to determine the specific activity of the compounds, to select an antidote, in in vivo experiments to evaluate the pharmacodynamics, to determine the dose of anticoagulant and antidote in experimental animals.

Experimental research and analysis of new compounds with different mechanisms of anticoagulant action is relevant for the search and creation of potential pharmacological drugs.

The aim of the study is to study the Akuzun alkaloid isolated from H. acutifolium plants on the hemostasis system.

Materials and methods.

The effect of anticoagulants on human plasma coagulation in vitro was assessed using generally accepted tests: activated partial platelet time (APTT) [20], prothrombin time (PT) [21], thrombin time (TT) [22], set of REAC from - Heparin (NPO " Renam" Russia, Moscow) [23]; fibrinogen (thrombin and buffer. Cypress Diagnostics. Belgium) was used to study the effect on fibrinogen polymerization [24]. All

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coagulological tests (with human plasma) were performed on a single-channel coagulometer (CYANCoag, Belgium.CY003, SN:5400439). To assess the anticoagulant potential of the obtained alkaloid, the effective concentrations of APTT, PT, TT, Reaklot were graphically determined, which were found by the abscissa of the points located on the curves of dependence of the concentration of the anticoagulant - effect; ordinates of points - 2-fold increase in plasma clotting time, in comparison with control, that is, without the addition of anticoagulants.

When assessing changes in the coagulation and aggregation activity of platelets, platelet-rich plasma obtained from the blood of human donors was used. Platelets were isolated by centrifugation at 1150 rpm for 5 min to precipitate erythrocytes. Platelet-rich plasma was centrifuged again for 10 min at 3,000 rpm. The platelet pellet was suspended in 5 ml of medium containing 150 mM NaCl, 2.7 mMKCl, 0.37 mM NaH_2PO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES-NaOH, pH 6.55, 50 units/ml heparin, 0.35% serum albumin and 0.15 mg/ml apyrase. All operations were carried out in plastic containers at room temperature. To assess coagulative activity, the tests APTT, prothrombin time were used. All coagulation tests (with human plasma) were performed on a single channel coagulometer (CYANCoag, Belgium.CY003, SN:5400439). Platelet aggregation was recorded according to the Born method [25] on a Biola ALAT-2 aggregometer (No. FSR2007/01301, Russia). ADP (5-10 μM), epinephrine (5 μM) and collagen and ristomycin (0.5 U/ml) (Sigma) were used as inducers of platelet aggregation. The process of formation of aggregates and the degree of platelet aggregation were expressed as a percentage of the maximum level of light transmission (T%, max). Obtaining information in the form of aggregation curves with automatic calculation of indicators was carried out using a computer interfaced with an aggregometer.

Statistical data processing and illustrations were performed using the Origin 6.1 computer program (Microsoft, USA).

Results and discussion.

In the course of the study, the effect of the Akuzun alkaloid on the hemocoagulation of laboratory animals was studied, as well as the comparison of their anticoagulant effect with heparin.

The influence of the studied compounds on the plasma clotting time in the test activated partial thromboplastin time, thrombin, prothrombin time and Reaklot-heparin (NPO Renam).

To assess the effectiveness of anticoagulants in practice, the clinic uses the indicator of twice the clotting time of plasma or human blood in some coagulological tests, in comparison with the indications before the administration of the drug [26,27].

In experiments, when samples are added to human citrate plasma in vitro, the time for the appearance of a fibrin clot in the APTT, TT, PT and ReaClot-heparin

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tests increases (Fig.1). Akuzun antithrombin activity reached 7.6 ± 0.6 μg / ml, respectively, and effective concentrations in the TT test did not significantly differ from those for unfractionated heparin and were equal to 5.2 ± 0.1 and 7.6 ± 0 .5 μg/ml, respectively (Table 1).

Table 1. Effective concentrations of antithrombin activity of Akuzun

№	MM, кDa	CC	PT, mcg / ml plasma	TT, mcg / ml plasma	APTT, mcg / ml plasma	ReAclot, mcg / ml plasma
Heparin	15	1,8	14,6±1,3	5,2±0,1	0,7±0,07	2±0,01
Akuzun	358	2,01	86,4±4,1	7,6±0,5	7,6±0,6	44,4±2,4

Note: Effective concentrations (PT, TT) - concentrations of Akuzun, at which the plasma clotting time increases by 2 times, in comparison with control (without anticoagulant); M±n; n= 6.

In addition, it was noted that the activity against factor Xa in the most promising compounds is several times less than the antithrombin activity, when, like in heparin, these activities are the same [28].

Above, we showed that the more architecturally complex the clotting test, the more Akuzun is required to achieve the same braking efficiency. Therefore, the possibility of Akuzun's action at the stages preceding the coagulation transformation of fibrinogen, at which part of the alkaloids are consumed, is not excluded. Otherwise, their concentrations in various tests would be equal, or at least comparable. This doubt is reinforced by the fact that, according to electrocoagulograms, Akuzun lengthens the period before the formation of, a fibrin clot.

To resolve this issue, we used a technique that allows us to isolate the process of coagulation conversion of fibrinogen from the general cascade of coagulation reactions. The essence of this technique is that the pooled donor plasma is released from fibrinogen by mild thermal denaturation (56°C, 3 min). If fibrinogen, Akuzun are added to such defibrinated plasma and then coagulation is provoked, then Akuzun can affect any of the stages of plasma coagulation. If, however, in defibrinated plasma, the activation of the coagulation cascade is first initiated, and after the formation of thrombin, fibrinogen and Akuzun are added, then the latter can only affect the coagulation transformation of fibrinogen.

The results of such an experiment showed that the braking efficiency in both cases described above is practically equal to each other when used as Akuzun. Therefore, the initial assumption that Akuzun is realized at the level of coagulation transformation of fibrinogen turned out to be correct, and differences in the mechanism of Akuzun's influence on coagulation are observed precisely at the stage of fibrinogen transformation.

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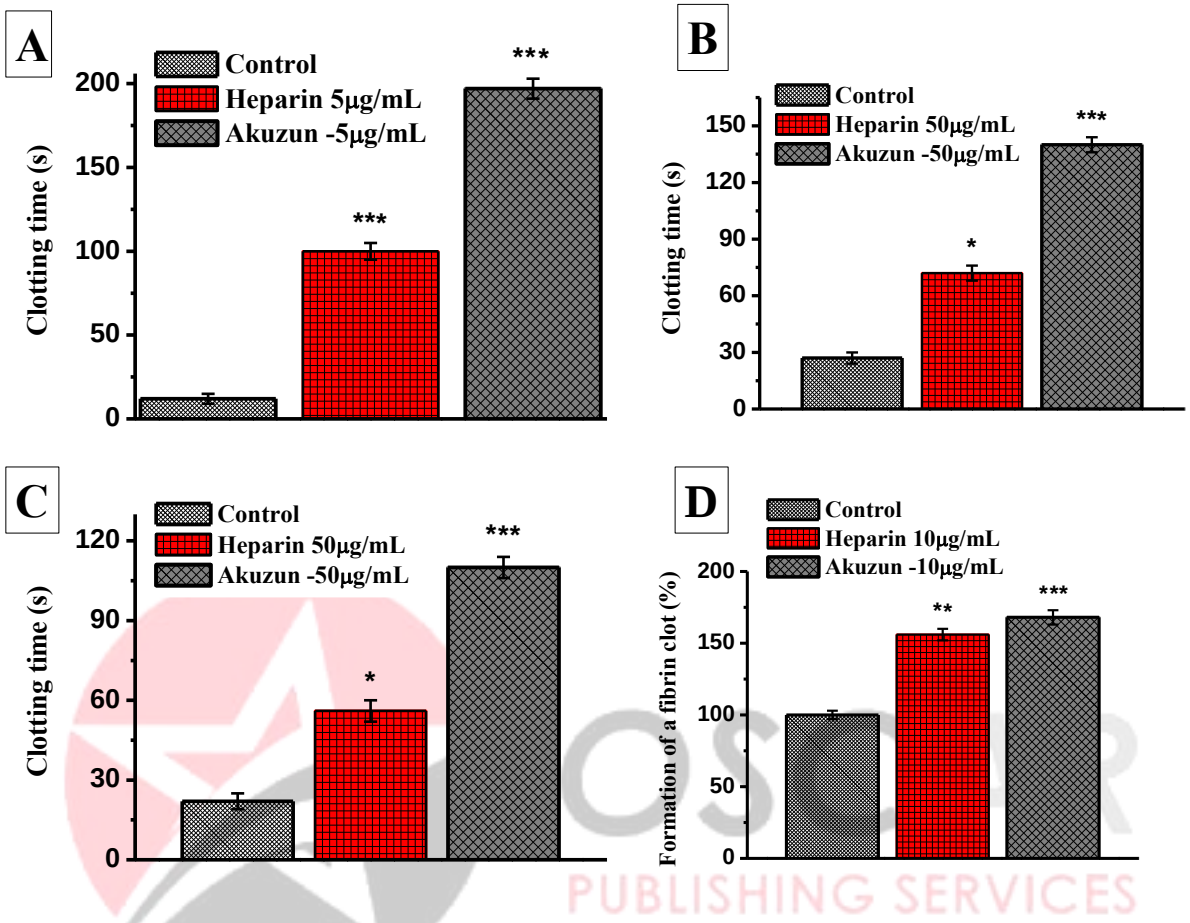

Figure 1. Anticoagulant activity of Akuzun (A) APTT, (B) TT, (C) PT and (D) formation of a fibrin clot plasma of human blood versus the heparin

***- P<0.05; **- P<0.01; ***- P<0.001. (n=6).**

To confirm this conclusion, we determined the effectiveness of inhibition of the reaction of interaction of thrombin with fibrinogen and Akuzuns separately and their sum, while comparing the expected effect and actually obtained. The expected (theoretical) effect was significantly lower (on average by 70%), which indicates the synergy of Akuzun. At the same time, these data also indicate that the mechanism of influence of the studied Akuzun on the coagulation transformation of fibrinogen is different: in the case of the same mechanism, we would observe the summation of effects with a lack of Akuzun in the system, or antagonism - with an oversaturation of the system with Akuzuns.

We received even more objective information about the difference in the mechanism of action of Akuzun by observing the process of coagulation transformation

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of fibrinogen using a nephelometer with automatic registration of the stages of this process.

Akuzun, compared with control, on average only 9.7% delays the formation of oligomers and 81% increases the time required for the formation of a fibrin clot. Since the aggregation of protofibrils is almost an instantaneous process, Akuzun mainly inhibits the autopolymerization of sufficiently mature oligomers. However, at the same time, the time of formation of a fibrin clot increases on average by 70% in relation to the control.

To study the effect of Akuzun on the anticoagulant activity of the plasma of rabbits, the test compound was administered at various doses intravenously into the marginal ear vein. In the rabbit plasma obtained at different time intervals after the administration of Akuzun, the clotting time was determined in the APTT / ReaClot-Heparin tests and the aXa of plasma activity was calculated when compared with heparin.

It is known that to control heparin therapy, blood clotting time, plasma clotting time in the APTT test, activated blood clotting time, antithrombin and aXa plasma activities are determined. APTT is the most widely used test to determine the degree of AK action after the administration of heparin at therapeutic doses [29-33].

The work determined a significant increase in rabbit plasma coagulation in the APTT and Reaclot-Heparin tests with an increase in the dose of Akuzun (3, 5 and 7 mg/kg) or heparin (0.75 and 1 mg/kg) when administered intravenously. The time of action (according to APTT and Reaklot-Heparin) with the introduction of heparin at doses of 0.75 and 1 mg/kg reached 72 minutes and 113 minutes, respectively. The long duration of the anticoagulant effect of Akuzun, in comparison with heparins, is associated with large doses. To achieve the same effect in terms of plasma clotting time in the APTT test (15 minutes after administration), Akuzun needed 7 times more.

We noted the maximum aXa plasma activity at 5 minutes after the introduction of Akuzun and heparin into the blood, which coincides with the literature data for intravenous administration of the direct-acting anticoagulant heparin [34-35]. The complete disappearance of aXa activities in the plasma of rabbits was observed with the introduction of only heparin at doses of 0.75 and 1 mg/kg after 170 and 110 minutes, which coincides with the data on the plasma clotting time in the APTT and Reaclot-Heparin tests.

We observed consistently greater antithrombin activities of rabbit plasma, in comparison with aXa activities after administration of Akuzun in different doses. This is understandable, since the specific aII activity of the Akuzun alkaloid is greater than the aXa activity. To neutralize the anticoagulant effect of heparin in clinical practice, the administration of protamine sulfate is used [36-37]. Sequential administration of protamine sulfate after unfractionated heparin (in the same doses) led to a decrease in rabbit plasma clotting time in the APTT /

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ReaClot-Heparin tests and to a decrease in plasma aXa activity. So, 15 minutes after the administration of Akuzuns and protamine sulfate (SP), the plasma clotting time in the APTT test, depending on the dose, was 2.3–4.4 times less than with the administration of Akuzuns alone; for heparin and SP, this difference for 15 minutes was 6 and 9 times, depending on the dose. When analyzing plasma 15 minutes after the administration of Akuzun and SP using the Reaklot-Heparin test, the clotting time was lower, depending on the dose, by 1.5 - 2.0 times; for heparin and SP, this difference for 15 minutes was 1.9 and 3.4 times, depending on the dose. This effect of SP on heparin has long been known [38].

With the introduction of heparin and the subsequent neutralization of its effect at 15 minutes after administration, we noted a decrease in antithrombin activity by 8 times. Sequential administration of protamine sulfate led to a decrease in the aXa activity of the plasma of rabbits after the administration of Akuzun by an average of 2 times, compared with the results without the administration of the antidote. The introduction of an antidote after the administration of Akuzun or heparin led to a decrease in the aXa activity of rabbit plasma. So, at the 6th minute of the aXa activity of the plasma with the introduction of Akuzun and SP at doses of 3, 5, 7 mg/kg, on average, it decreased by almost 3 times, in comparison with the introduction of only Akuzun with the disappearance of aXa plasma activity after 160 minutes. With the introduction of SP for heparin aXa, plasma activity decreased by 13 times.

In experiments in vitro, we determined that in order to neutralize the anticoagulant effect of Akuzun, depending on the concentration, the addition of protamine sulfate may be required in weight ratios to the anticoagulant from 1 to 10. In experiments on experimental animals, we have shown that the use of protamine sulfate and an anticoagulant in the same doses may be sufficient to neutralize the anticoagulant activity of Akuzun.

We have studied the effect of Akuzun (at equal concentrations of 10-100 μM) on the aggregation function of platelets. ADP (2.5 and 10 μg/ml) and adrenaline (Tonogen solution) were used as inducers, since the results of the aggregation intensity values caused by the above inducers are the most satisfactory, and the intensity of aggregation caused by ADP correlates well with the intensity aggregation caused by adrenaline [39].

Under these conditions, the studied alkaloid Akuzun inhibits platelet aggregation, but its mechanism of action, as well as the effect on the process of coagulation transformation of fibrinogen, differs significantly.

Thus, when using ADP at a concentration of 2.5 μg/ml, Akuzun at 10-100 μM practically does not change the first wave of aggregation, which characterizes the aggregation of platelets under the influence of an inductor, but significantly inhibits the second wave,

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which characterizes the release of internal mediators of aggregation (Fig.2). The maximum values of the second wave of aggregation for Akuzun practically do not differ from each other and are less than the control value by 31 and 38%, respectively. Akuzun shorten the time required to reach the maximum value of the second wave, but with different intensity - by 62.5 and 37.5%,
respectively. In addition, Akuzun activates the process of disaggregation.

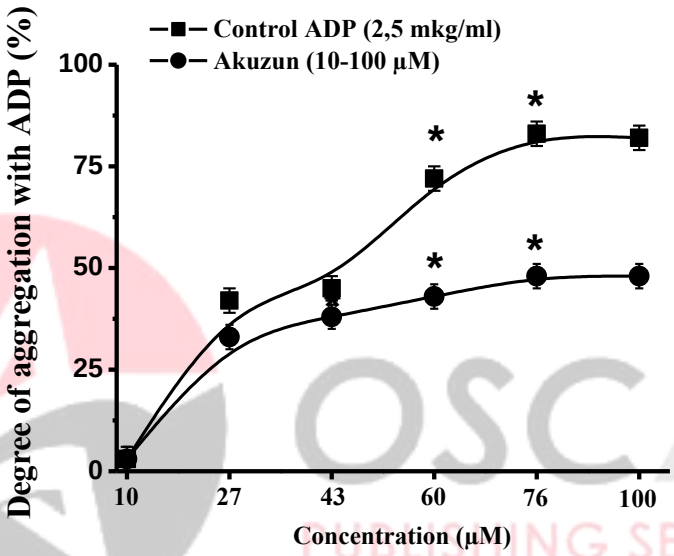

Figure 2. The effect of Akuzun on platelet aggregation with ADP 2.5 µg/ml. *- P<0.05; **- P<0.01; *- P<0.001. (n=6).**

Significant differences in the mechanism of antiaggregation action are observed with an increase in the concentration of the inducer to 10 µg / ml. In this case, no aggregation is observed in the control of the first wave. The first wave of aggregation appears in the presence of Akuzun.

The maximum values of the second wave of aggregation for Akuzun are less than the control value by 44 and 32%, respectively. Akuzun does not significantly change the time of the onset of the maximum value of the second wave (Fig. 3).

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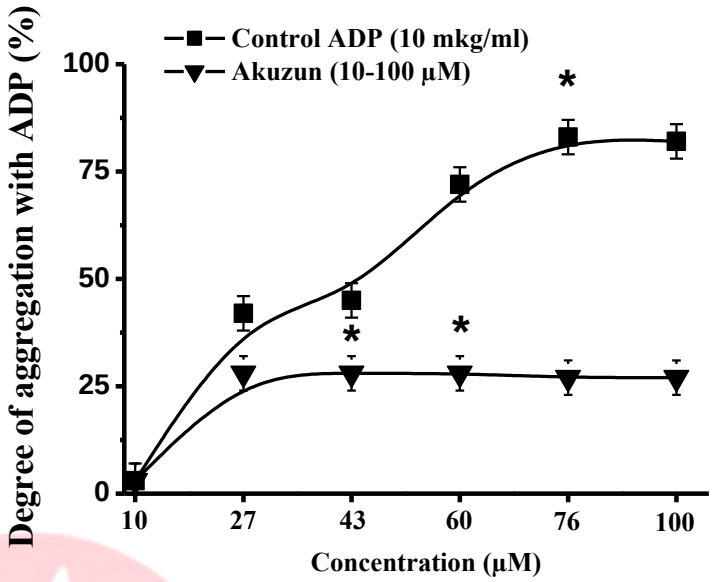

Figure 3. The effect of Akuzun on platelet aggregation with ADP 10 µg/ml.

***- P<0.05; **- P<0.01; ***- P<0.001. (n=6).**

Akuzun, without changing the value of the maximum of the first wave of aggregation, doubles the time required to reach it, reduces the maximum value of the second by 50.4%, and lengthens the time to reach its maximum by 20.6%.

When deciphering the mechanism for limiting the coagulation activity of blood plasma, first of all, with the help of an objective method (registration of the plasma coagulation process in the dynamics of the CYANCOag coagulogram), we were convinced that Akuzun has a pronounced anticoagulant activity. In this case, pooled donor plasma was used as a substrate, and Akuzun was used jointly and separately as an anticoagulant. At the same time, some differences were revealed in the effect of carriers on blood plasma recalcification, especially reflected in the change in time before the onset of coagulation, as well as in the rate of retraction and fibrinolysis.

Differences were also revealed in the study of the effect of inhibitors on the process of coagulation transformation of fibrinogen by another objective method - using the USB-2000 spectrometer: Akuzun has a pronounced inhibitory effect on the early stages of polymerization. With its joint effect on fibrinogen coagulation, a synergism of effects is observed, which confirms the aforementioned difference in the mechanisms of action.

Thus, the main point of application of the studied anticoagulant activity can be considered the final

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phase of coagulation with the predominant effect of Akuzun on the self-assembly of monomeric fibrin.

We determined only those indicators that integrally reflect the total coagulation activity of blood plasma (time of recalcification), the rate of interaction of thrombin with fibrinogen (thrombin time) and autopolymerization of monomeric fibrin (time of fibrin self-assembly). brine in plasma).

These properties of these components are of particular interest and require further detailed study of the physicochemical characteristics and mechanisms of their action, which ultimately will allow them to be used as an anticoagulant drug.

CONCLUSIONS

1. The action of Akuzun on the recalcification of blood plasma was revealed, especially reflected in the change in time before the onset of coagulation, as well as in the rate of retraction and fibrinolysis.
2. Direct acting anticoagulant Akuzun is active by interacting with antithrombin. The maximum antithrombin activity of Akuzun was 7.6±0.6 μg/ml.
3. The indicators that integrally reflect the total blood plasma coagulation activity (recalcification time), the rate of interaction of thrombin with fibrinogen (thrombin time) and monomeric fibrin autopolymerization (fibrin self-assembly time in plasma).

Akuzun does not independently cause aggregation of human platelets and does not potentiate the aggregation of human platelets induced by adenosine diphosphoric acid.

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