AMINO-METHYL-CYCLOHEXANE-CARBOXYLIC ACID: AMCHA

A NEW POTENT INHIBITOR OF THE FIBRINOLYSIS

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Research Projects on Plasmin and Antiplasmin

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In 1953, S. Okamoto et al., co-operatively working with the chemist group leaded by Nagasawa, found that epsilon-amino-caproic acid (EACA) had a potent inhibitory effect on the fibrinolysis in vitro and in vivo(1). S. Okamoto(2) in 1959, and his co-worker, Yokoi(3), in 1960, described in detail the relations of chemical structures to the inhibitory effect on the basis of the results obtained from the examination of more than 300 kinds of chemical compounds. In particular, they mentioned that neither amino-group nor carboxylic group could be replaced without diminishing its original inhibitory effect, and that epsilon compounds had the most potent inhibitory effect of their close homologues(2,3).

Soon after the finding of EACA, a series of animal experiments were carried out by S. Okamoto et al., and the results obtained suggested that EACA would be applied to reverse the “hyperplasminic states” of patients(1,4). Then, S. Sato(5) and Itoga(6) demonstrated that the clinical signs due to accelerated fibrinolysis were successfully improved by the administration of EACA to patients. Many studies of clinical application of EACA have been conducted along this line by various workers and in various countries(7,8,9,10).

Thus it seems to be nearly accepted that EACA is available to reverse the hyperplasminic states of patients. However, clinical need remained us to search for more potent inhibitor than EACA. Therefore, 1-(aminomethyl)-cyclohexane-4-carboxylic acid (abbreviated as AMCHA) was remarked by S. Okamoto et al. under the close co-operation with the chemist group of Nagasawa(11). This paper deals with the first step of our studies in evaluating the antifibrinolytic effect of AMCHA in comparison with EACA. The more detailed results will be described by one of the authors and their colleagues in the following papers(12).

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MATERIALS

1-(aminomethyl)-cyclohexane-4-carboxylic acid which was abbreviated as AMCHA in this paper was synthesized in the central laboratories of Mitsubishi Kasei Kogyo Co. AMCHA is a colourless powder having a melting point of 237-238°C (decomposition) and being well soluble in water at room temperature\(^{11}\). EACA was synthesized by Daiichi-Seiyaku Co.

Commercial preparations of fibrinogen (Fraction I: Armour Laboratory), casein (Hammersten Co.) and thrombin (Mochida Co.) were used in the following experiments. Streptokinase preparation (Varidase) was offered from American Cynamid Co. A commercial preparation of Tosylarginine-methylester (Minophagen Co.) was used and abbreviated as TAMe. The standard human serum was prepared in our laboratory.

EXPERIMENTALS

The very preliminary examination was first made, and the spontaneously activated fibrinolytic system obtained from human serum was used in order to know whether AMCHA might be promising for clinical application. The measurement of the inhibitory effect of AMCHA was made by admixing a certain amount of the active ingredients with the fibrinolytic system which contained active euglobulin, fibrinogen, thrombin and an adequate amount of 1/20 M of phosphate buffer, and by measuring the time required for the complete dissolution of the formed fibrin clots incubated at 37°C, and comparing the said time with that of control experiments. Even though the inhibitory effect of AMCHA was varied by the difference of the system used, it was evidently observed that AMCHA added to the system caused the retardation of the lysis time and that such an effect was always much stronger than that of EACA. Results obtained from this preliminary experiment indicated that the detailed studies would be urgent.

(A) THE EFFECT OF AMCHA ON THE FIBRINOLYTIC SYSTEM CONTAINING HUMAN SERUM AND STREPTOKINASE

The effect of AMCHA was examined by adopting the plasminogen-streptokinase system with our expectation that AMCHA would most likely affect the activation process of plasminogen. A 0.1 ml of standard human serum, 0.4 ml of 1/20 M phosphate buffer saline solution and 0.1 ml saline solution containing 100 units of streptokinase were mixed together in a test tube at 0°C. Then 0.05 ml of a saline solution containing 5 units of thrombin and 0.3 ml of a 0.33% solution of bovine fibrinogen were added into the test tube. The mixture was incubated at 25°C and the time required for the complete lysis of the formed
clot was measured in seconds. The results obtained presented the control value\(^{(18)}\).

The inhibitory effect of AMCHA or EACA was examined by dissolving the agents in the phosphate buffer solution above mentioned; and the relations between the retardation of the lysis time and the concentration of AMCHA were inquired into and compared with EACA. The results were shown in Fig. 1.

![Graph showing the inhibitory effect of AMCHA or EACA on the fibrinolytic system containing human serum and streptokinase.](image)

**Fig. 1** The inhibitory effect of AMCHA or EACA on the fibrinolytic system containing human serum and streptokinase.

The results in Fig. 1 indicated that the concentration of AMCHA required to double the control lysis time was about \(3 \times 10^{-7}\) g/ml, namely about \(2 \times 10^{-6}\) M, while that of EACA was about \(8 \times 10^{-5}\) g/ml. Therefore, it can be mentioned that the inhibitory action of AMCHA on the streptokinase-activation-process of the plasmin system of human serum in vitro is evidently very potent, that is, more than ten times of the action of EACA.

The inhibitory effect of AMCHA or EACA on the fibrinolysis caused by the partly purified plasmin preparation was, however, rather weak, \(10^{-6}\) g/ml of AMCHA or \(10^{-5}\) g/ml of EACA being required to double the control lysis time. This suggested that AMCHA might more strongly affect the activation process of plasminogen than the activity of plasmin per se. Results obtained from the experiments on the more purified plasmin preparation will be described in the following paper which appears, in this Journal, next to this paper\(^{(12)}\).
(B) THE EFFECT OF AMCHA ON THE FIBRINOGENOLYTIC SYSTEM CONTAINING HUMAN SERUM AND STREPTOKINASE

It was reported by U. Okamoto that fibrinogenolysis (fibrinogen splitting by the action of plasmin) was well estimated by the viscosity decreasing of the solution(13). In the following experiments, 2 ml of 5% fibrinogen saline solution, 0.2 ml of the standard human serum and 0.1 ml of saline solution containing a certain amount of AMCHA or EACA were mixed in a viscosimeter of Ostwald type which was placed in the 38°C (±0.005°C) water bath. After putting 0.1 ml of saline solution containing 1,000 units of streptokinase into the viscosimeter, the measurement of the viscosity was repeatedly made at a certain interval and the results obtained were plotted in Fig. 2. It seemed to indicate that the inhibitory effect of AMCHA on the mentioned fibrinogenolysis was more potent than that of EACA. It might be noteworthy that the concentrations of AMCHA or EACA requiring for the marked retardation of the viscosity-decreasing were rather high when compared with those concentrations in the fibrinolytic system above mentioned.

The similar results were also obtained by us in the caseinolysis so far as the inhibitory effect of AMCHA or EACA was concerned.

(C) THE EFFECT OF AMCHA ON THE TAMe ESTERASE ACTIVITY

It seems to be broadly accepted that TAMe can be used as a synthetic substrate of plasmin. In fact, it was reported by Sherry et al. that TAMe esterase activity was found in the very plasmin preparation which was purified.
by them to the great extent\(^{(14)}\). It is also known that TAMe esterase activity increases markedly in the human serum by adding streptokinase\(^{(15)}\). Therefore, the inhibitory effect of AMCHA on the activation process of TAMe esterase can be expected. Results obtained here, however, seem to contradict the current opinion mentioned above.

In our experiments, the assay of TAMe esterase activity was performed after the method described by Troll & Sherry in 1954\(^{(15)}\). Human euglobulin solution was prepared by diluting human serum with pure water, precipitating the fraction at pH 5.2 and resolving it in the tris-buffer solution. The reaction mixture was made by admixing 0.1 ml of euglobulin solution with 0.2 ml of 0.1 M TAMe solution, 0.5 ml of tris buffer (0.5 M, pH 9.0) and 0.1 ml of 1% AMCHA or EACA solution. The reaction mixture was incubated at 37°C. Results obtained were plotted in Fig. 3. It was indicated that AMCHA or EACA had no effect on the activity or activation process of TAMe esterase in the reaction mixture which contained serum euglobulin and streptokinase.

![Graph showing effect of AMCHA or EACA on TAMe esterase activity](image)

Fig. 3 Effect of AMCHA or EACA on the TAMe esterase activity. Ordinate indicates the amount of carboxylic acid freed from TAMe after incubation of various time at 37°C; abscissa indicates the time of incubation in min.

(D) THE EFFECT OF THE INTRAVENOUS ADMINISTRATION OF AMCHA TO RABBITS

The studies on the effect of AMCHA on the fibrinolytic system in vitro suggested that the administration of AMCHA to animals might be more effective that of EACA for suppressing the streptokinase activation test of fibrinolysis of the circulatory blood.

In the experiments shown in Table 1, 250 mg, 100 mg and 25 mg of AMCHA
or EACA were respectively given intravenously to rabbits weighing ca. 2.5 kg. Blood samples were drawn by venopuncture at the times mentioned in Table 1, and the actions of AMCHA or EACA were measured by the streptokinase activation test of the fibrinolysis. The results obtained are shown in Table 1 and summarized as follows.

1) When 250 mg of AMCHA or EACA solved in saline solution was given intravenously to rabbits, a very marked retardation of the lysis time of the streptokinase activation test of fibrinolysis was observed one hour to six hours after the injection of these two agents.

2) The action of AMCHA, however, was different from that of EACA when 100 mg or 25 mg of these agents were given to rabbits. The intravenous injection of 100 mg or 25 mg of AMCHA produced a marked retardation of lysis time, while that of EACA was obviously weaker than of AMCHA.

3) Then in giving rabbits, such a small amount of the active agents as 5 mg, the action of AMCHA was compared with that of EACA. Results obtained are shown in Table 2.

Table 1.
The effect of the intravenous administration of AMCHA or EACA to rabbits

<table>
<thead>
<tr>
<th>Chemical substance</th>
<th>Dosage (mg)</th>
<th>Lysis time (sec.)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Before experiment</td>
</tr>
<tr>
<td>AMCHA</td>
<td>250</td>
<td>1,320</td>
</tr>
<tr>
<td>EACA</td>
<td>250</td>
<td>1,200</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>EACA</td>
<td>25</td>
<td>1,992</td>
</tr>
</tbody>
</table>

Table 2.
The effect of the intravenous administration of a smaller amount of AMCHA or EACA to rabbits

<table>
<thead>
<tr>
<th>Chemical substance</th>
<th>Dosage (mg)</th>
<th>Lysis time (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before experiment</td>
</tr>
<tr>
<td>AMCHA</td>
<td>5</td>
<td>465</td>
</tr>
<tr>
<td>AMCHA</td>
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<td>690</td>
</tr>
<tr>
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<tr>
<td>EACA</td>
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</tr>
<tr>
<td>EACA</td>
<td>5</td>
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AMINO-METHYL-CYCLOHEXANE-CARBOXYLIC ACID

In three rabbits to which 5 mg of AMCHA was given intravenously, the retardation of lysis time was obviously confirmed in each blood sample which was taken 30 minutes to six hours after the injection. In two rabbits to which 5 mg of EACA was given intravenously any retardation of lysis time was hardly confirmed.

The mentioned results indicated that some relatively small amount of AMCHA could reveal its potent antifibrinolytic effect to the circulatory blood though the same amount of EACA was little effective.

Fig. 4 The antifibrinolytic effect of the intravenous administration of 2 mg per kg of AMCHA or EACA to rabbits. Ordinate indicates percentage of the retardation of the lysis time estimated by the streptokinase activation test.

Fig. 5 The effect of the oral administration of AMCHA or EACA to rabbits.

(E) THE EFFECT OF AMCHA GIVEN PER OS TO RABBITS

In order to present the basis of the clinical application of AMCHA and compare the effect with that of EACA, oral administration was performed in rabbits. Results obtained indicated that the antifibrinolytic effect of AMCHA was well demonstrated when a small amount of AMCHA was given per os to rabbits.

It could be said that a certain amount of the oral administration of AMCHA showed the more potent antifibrinolytic action in its rate and duration. Clinical application of AMCHA per os to patients were thus suggested to be promising.
REVERSAL BY AMCHA OF LYTIC SYSTEM IN BLOOD STREAM PRODUCED IN RABBITS

It has been reported by the authors et al. or Miller et al. that the intravenous administration of EACA was effective to reverse the activated fibrinolysis of the circulatory blood in animal experiments. The same effect was examined in the following experiments using AMCHA instead of EACA.

In order to activate the lytic system in the circulatory blood of rabbits, 5 ml of human serum and 30,000 units of streptokinase were given intravenously to rabbits. The activation of the lytic system in blood was easily demonstrated by estimating the time required for the complete lysis of the formed clots of fresh blood samples (natural clot lysis). Results shown in Fig. 6a indicated that the very activated state of lytic system in blood lasted for 70 minutes or more after the injection of streptokinase.

Results shown in Fig. 6b, however, indicated that, even though the natural clot lysis time was shortened to 30 min. by the activation procedure above mentioned, it was turned normal by the injection of 5 ml of 5% AMCHA solution.
to the rabbit. It was also indicated that the administration of AMCHA prior to the activation procedure prevented the appearance of the rapid clot lysis in circulatory blood which would otherwise occur (Fig. 6c).

**DISCUSSION**

Since the discovery of a fetal hemorrhage with accelerated fibrinolysis by Soulier et al. in 1952(19), attention was paid by several workers to the evidence that the usual treatments for haemostasis were not effective. Some attempts, however, had been started to search for antifibrinolytic agent by Jobling et al. in 1915(20), and by Rosenmann in 1938(21). The systematic studies by S. Okamoto et al. had been also started in 1948, and the potent inhibitory effect of EACA on fibrinolysis was reported by them in 1953(1). Kaula et al. in 1953 examined the antifibrinolytic effect of antibiotics(22). In 1958 Mounter reported the inhibition of plasmin by phosphorus compounds, but they were toxic(24). Sarker examined the antifibrinolytic effect of the close homologues of EACA in 1958, and confirmed that EACA was most effective among those compounds(23).

In recent years, EACA has been rather broadly applied to ameliorate the fibrinolytic hemorrhage or the allied syndromes. A very low toxicity of EACA and its highly specific action to the fibrinolytic system have been generally accepted. However, it was also noticed by the authors that some workers administered to patients such a large amount of EACA as 30 g per day in order to suppress the very extensively accelerated fibrinolysis of blood. This was the reason why a more potent inhibitor than EACA was inquired into, even though EACA is yet regarded by the authors as one of the most ideal inhibitors for the clinical purpose.

1-(aminomethyl)-cyclohexane-4-carboxylic acid which was abbreviated as AMCHA in this paper was synthetized by Nagasawa, Takagi, Yokoi and Mangyo in the central laboratories of Mitsubishi Kasei Kogyo Co.(2).

Studies of the mode of action of AMCHA will involve many a problems, so results described in this first paper of AMCHA will be limited to the approach of evaluating the inhibitory effect of AMCHA and comparing it with that of EACA.

As to the in vitro test of the inhibitory effect of these agents, fibrin, fibrinogen, casein and TAME were used as substrates, and the clot lysis time for fibrin, viscosity decreasing for fibrinogen, ultraviolet absorbance for casein and acid liberation for TAME were respectively estimated. The reaction mixture was made by admixing the one of the mentioned substrates with the human standard serum and streptokinase.
Results obtained from the fibrinolysis test (Fig. 1) indicated that the inhibitory effect of AMCHA was far more potent than that of EACA. Calculation made from the lowest concentrations of AMCHA or EACA for doubling the lysis time of the control clots indicated that the action of AMCHA was ca. 27 times of that of EACA.

Action of AMCHA given to rabbits intravenously or orally was examined by administering various amounts of AMCHA or EACA and estimating the streptokinase activation test of blood samples taken from the rabbits at various intervals. Results obtained (Tables 1, 2, Figs. 4, 5 and 6) indicated that the more potent effect of AMCHA than EACA was clearly demonstrated when some small amounts of the agents were given to the animals. Results shown in Table 2, for instance, indicated that the inhibition of the streptokinase activation test was markedly observed in the blood samples taken from rabbits 6 hours after the intravenous administration of 5 mg of AMCHA; while the inhibition was not observed at all even in the blood samples taken 30 min. to one hour after the intravenous administration of the same amount of EACA. Results obtained from the oral administration (Fig. 5) indicated that the inhibition by AMCHA was also more potent than EACA in its grade and duration. Results shown in Fig. 6 demonstrated reversal or inhibition by AMCHA of lytic system in blood stream produced in rabbits by streptokinase.

Thus, basing upon the mentioned results, the authors should like to suggest that it is urgent to extend the investigation of AMCHA towards the clinical application. It is also known that toxicity of AMCHA is very weak when examined by acute and chronic tests in animals (unpublished). The authors expect that AMCHA will be effectively applied, either intravenously or orally, to suppress the extensively accelerated fibrinolysis in blood of patients which otherwise may require the administration of a very large amount of EACA.

The remained problem is to discuss the results obtained from examination on TAMe esterase activity. However, one of the authors, U. Okamoto is now studying this subject. The other aspect of the mode of action of AMCHA will be described in the following papers.

**SUMMARY**

1) A new potent inhibitor of the fibrinolytic system was found. Its chemical name was 1-(aminomethyl)-cyclohexane-4-carboxylic acid and abbreviated as AMCHA in our laboratories.

2) The inhibitory effect of AMCHA in vitro was far more potent than that of EACA when examined by the fibrinolytic system.
3) The inhibitory effect of AMCHA given to rabbits intravenously or orally was also more potent than that of EACA when the effect was examined by the streptokinase activation test of blood samples taken at various intervals.

4) Reversal by AMCHA of the accelerated fibrinolysis in circulatory blood produced in rabbits by streptokinase was demonstrated.

REFERENCES