Alfimeprase: Pharmacology of a Novel Fibrinolytic Metalloproteinase for Thrombolysis

Christopher F. Toombs

Product Development, Amgen Inc., Thousand Oaks, Calif., USA

Key Words

Fibrolase · Alfimeprase · Thrombosis · Fibrinolysis · Metalloprotease · Thrombolysis

Abstract

Alfimeprase is a recombinantly produced, truncated form of fibrolase, a known directly fibrinolytic zinc metalloproteinase that was first isolated from the venom of the southern copperhead snake (*Agkistrodon contortrix contortrix*). Both fibrolase and alfimeprase have been shown to have direct proteolytic activity against the fibrinogen A α chain. In vivo pharmacology studies have shown that thrombolysis with alfimeprase is up to 6 times more rapid than with plasminogen activators. Alfimeprase can be bound and neutralized by serum α_2 -macroglobulin, a prevalent mammalian protease inhibitor which is capable of forming a macromolecular complex with alfimeprase. As a result, systemic

bleeding complications have been greatly reduced due to the inhibitory effects of α_2 -macroglobulin. This article reviews the biochemical in vitro and in vivo characteristics of this novel acting thrombolytic.

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Introduction

Alfimeprase is a recombinantly produced, truncated form of fibrolase, a known fibrinolytic zinc metalloproteinase that was first isolated from the venom of *Agkistrodon contortrix contortrix*, the southern copperhead snake [1]. Fibrolase is a member of clan MB of metallopeptidases, family M12, subfamily B (the repolysins), a grouping of proteolytic enzymes that are comprised of many enzymes originally characterized from snake venoms.

The alfimeprase molecule differs slightly from fibrolase at the amino terminus where alfimeprase contains 201 amino acids with an

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Fax +41 61 306 12 34
E-Mail karger@karger.ch
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Christopher F. Toombs, PhD
Product Development/Mailstop 27-5-A, Amgen Inc.
1, Amgen Center Drive, Thousand Oaks, CA 91320 (USA)
Tel. +1 805 447 8121, Fax +1 805 375 6524
E-Mail ctoombs@amgen.com

Fig. 1. Coomassie-based visualization of proteolytic cleavage of fibrinogen A α chain. Samples of human fibrinogen (741 μ g) were incubated with 1 μ g alfineprase (+ lane) or buffer (– lane) at 37°C for 30 min. The microgram quantity of fibrinogen was selected such that alfineprase was combined with each substrate in a 1:50 molar ratio (alfimeprase:substrate). Separation of the reaction mixture by SDS-PAGE and visualization of proteins with Coomassie staining reveals proteolytic degradation of the fibrinogen A α chain in the presence of alfineprase (dashed box).

N-terminal sequence of SFPQR---, in contrast to the N-terminal sequence of fibrolase, which begins with EQRFPQR--- and is 203 amino acids in length [2]. While alfineprase is a modest 2-amino-acid truncation of fibrolase, the two enzymes are similar with respect to enzymatic activity and the ability to be bound and inhibited by α_2 -macroglobulin. This is consistent with the data of Manning [3] indi-

cating that the active site of the fibrolase molecule spans amino acids 139–159, and its location, in three-dimensional space, is distant from the amino terminus where the truncation was made.

Fibrinolytic Activity of Alfineprase

Both fibrolase and alfineprase have been shown to be fibrinolytic, and fibrolase has been previously documented to have proteolytic activity against the fibrinogen A α chain, with reduced proteolytic cleavage of the B β chain and no activity against the γ chain of fibrinogen [4]. In contrast to the plasminogen activator class of thrombolytic drugs, fibrolase and alfineprase do not rely on the endogenous fibrinolytic system (conversion of plasminogen to plasmin). Hence, fibrolase and alfineprase can be distinguished from the plasminogen activators by their unique mode of action and are defined as directly fibrinolytic agents.

Substrates of Alfineprase and Fibrolase

Published literature on venom fibrolase has demonstrated the proteolytic activity against fibrinogen at the Lys⁴¹³-Leu⁴¹⁴ site [5] and against the oxidized β -chain of insulin at the Ala¹⁴-Leu¹⁵ site [6, 7]. Alfineprase has also been determined to have proteolytic activity on these substrates. The degradation of the fibrinogen A α chain can easily be demonstrated in a gel-based method, as first described with fibrolase by Retzios and Markland [8] and replicated in figure 1 using alfineprase.

α_2 -Macroglobulin

α_2 -Macroglobulin is a prevalent protease inhibitor present in mammalian serum and is one of the largest of the serum proteins (725 kD). The properties of α_2 -macroglobulin that are relevant to alfineprase are described in this section, and for a more thorough review, see Barrett [9]. The specificity of α_2 -macroglobulin for proteases is broad, encompassing serine, cysteine, aspartic and metalloprotease classes. The α_2 -macroglobulin molecule is a tetramer of identical subunits that are disulfide bonded in pairs with a noncovalent association of the half molecules. Thus, under reducing conditions, α_2 -macroglobulin can be dissociated into its four monomeric subunits.

Each subunit of α_2 -macroglobulin possesses a region that is very susceptible to proteolytic cleavage (the 'bait' region). Proteolysis of the bait region induces a conformational change in α_2 -macroglobulin, which entraps the protease within the α_2 -macroglobulin molecule. This process is described in the literature as a 'Venus-flytrap' interaction. Once the protease is entrapped, the protease is sterically hindered and therefore cannot access macromolecular substrate.

In addition, a covalent bond can form between α_2 -macroglobulin and many of the proteases that it entraps. Thus, within the general circulation, α_2 -macroglobulin can effectively neutralize a variety of proteases by entrapment of the protease which, in some instances, also becomes covalently bonded to α_2 -macroglobulin.

Interaction of Alfineprase with Purified Human α_2 -Macroglobulin

As previously mentioned, α_2 -macroglobulin is a prevalent protease inhibitor present in mammalian serum, which is capable of form-

Fig. 2. Rapidity of alfineprase: α_2 -macroglobulin complex formation. Visualization of the rate at which alfineprase can form complex with purified human α_2 -macroglobulin. Reaction products were detected using a SDS-PAGE separation and Western blotting with a polyclonal antibody that recognizes alfineprase. Alfineprase and purified human α_2 -macroglobulin are incubated together at 37°C with α_2 -macroglobulin present in a 3-fold molar excess of alfineprase. At various time points, the formation of complex is stopped by the addition of a buffer containing the reducing agent dithiothreitol (DTT). If α_2 -macroglobulin is treated with DTT prior to the addition of alfineprase (DTT lane), formation of the complex is completely prevented, indicating that DTT is an effective stopping agent. Upon the addition of alfineprase, complex formation begins as early as 5 s (5s lane) and is nearly complete within 1.5 min (1.5m lane). Purified alfineprase appears alone in the last lane.

ing a macromolecular complex with alfineprase. Unlike some proteases that can form a dissociable complex with α_2 -macroglobulin, alfineprase forms a complex and cannot be dissociated from α_2 -macroglobulin under physiologic conditions.

As shown in figure 2, alfineprase remains bound to α_2 -macroglobulin even under the harsh laboratory conditions used to process samples for Western blotting (reducing agent, detergent, and boiling), confirming the non-

dissociable nature of the alfineprase- α_2 -macroglobulin complex.

α_2 -Macroglobulin Binding in Human Plasma Is Saturable

Although α_2 -macroglobulin is one of the major plasma proteins, there is nonetheless a finite quantity of α_2 -macroglobulin available to bind and neutralize alfineprase, and therefore the α_2 -macroglobulin binding capacity is saturable. Using Western blotting techniques a titration experiment can be performed where a range of alfineprase concentrations is added to a plasma sample from an individual human donor. When low concentrations of alfineprase are added to plasma, only the high molecular weight alfineprase- α_2 -macroglobulin complex form is detectable. At concentrations that approach and then slightly exceed the α_2 -macroglobulin binding capacity, the unbound alfineprase begins to become detectable. Once the α_2 -macroglobulin binding capacity has been exceeded, the concentration of unbound alfineprase rises proportionally as additional alfineprase is added to the sample. Using this methodology, the alfineprase binding capacity of human plasma was estimated to be $\sim 40 \mu\text{g/ml}$ plasma (data not shown).

In vitro Lysis of Clotted Human Whole Blood

The activity of alfineprase has been demonstrated in an in vitro model of clot lysis. Human blood can be clotted by the addition of a high calcium concentration. Once formed, these clots can be solubilized with thrombolytic agents. The rate of clot lysis can be assessed by visual inspection of the clot mass.

As can be visually observed in the data of figure 3, when a fixed amount of alfineprase is used to degrade clots of varying sizes, the time required for clot dissolution increases proportionally with the mass of the clot.

Studies Which Demonstrate Biologic Activity in Animals: Rat Carotid

Pharmacology studies were conducted in carotid arteries that have been acutely thrombosed by locally injuring the vessel with anodal current. Once an occlusive thrombus forms, the thrombosed carotid artery is observed for a period of 30 min to assure that the carotid occlusion is stable. Heparin and aspirin are then administered intravenously to prevent further propagation of the thrombus. In these studies, a small needle (30 gauge) that was introduced into the artery in the vicinity of the occlusive thrombus and then cemented into place such that test agents could be administered locally.

Thrombolysis was attempted by intra-arterial infusion of test agents in a blinded fashion using numbered, coded syringes. While study drugs were being administered, flow through the carotid artery was monitored with a perivascular flow probe to determine the time at which reflow occurs. The time to lysis was recorded and group means were calculated for those experiments where clot lysis was successful. Blood pressure and heart rate were observed during the study to monitor any untoward effects of the test agents. As a measure of the hemorrhagic potential of the test agents, any blood that was shed from the surgical site was collected with gauze swabs. The swabs were placed in a detergent solution to solubilize red blood cells and release hemoglobin, which was then quantified spectrophotometrically. Shed hemoglobin was mathematically converted into a volume of blood

Fig. 3. In vitro time to clot lysis with alfimeprase is directly proportional to clot mass. Scatter plot of individual clot lysis experiments. Retracted whole human blood clots were formed from citrated whole blood by the addition of excessive calcium and incubation for 90 min. The clotted blood was cut into various sizes, weighed and transferred to new tubes containing 2 mg of alfimeprase (0.4 ml of a 5 mg/ml solution). The tubes were inspected visually over a period of 5 h and time to clot lysis was determined as the time point at which no visible clot mass remained.

loss by using the hemoglobin concentration of whole blood (in g/dl), which was measured for each animal.

As shown in table 1, clot lysis in the 2-mg alfimeprase group was more rapid than in all other groups. In addition, urokinase (at 250 U/min) was associated with a significant amount of blood loss from the surgical site. While mean blood loss in the 2-mg alfimeprase and 25 U/min urokinase groups was higher than in the saline group; these changes did not reach statistical significance. While

some changes in mean arterial pressure reached statistical significance in the saline and urokinase 250 U/min groups, no significant changes were noted in the alfimeprase treatment group.

These results indicate that alfimeprase has the potential to be more effective than a reference plasminogen activator in terms of both efficacy (speed of lysis) and safety (hemorrhagic complication).

Table 1. Selected parameters from a comparison of alteplase and urokinase on thrombolysis in acutely thrombosed rat carotid arteries

	Incidence of lysis %	Time to lysis min	Carotid flow ml/min		Blood volume loss at 90', ml	Mean arterial pressure, mm Hg		Heart rate min ⁻¹	
			base	90' Rx		base	15' Rx	base	15' Rx
Saline (n = 6)	0 (0 of 6)	N/A	4.3 ± 1.5	0.0 ± 0.0	0.10 ± 0.10	85.2 ± 3.6	76.5 ± 3.2 ¹	315 ± 23	311 ± 20
Urokinase 25 U/min (n = 15)	47% (7 of 15)	55.3 ± 4.3	3.2 ± 0.4	0.7 ± 0.3	1.06 ± 0.41	87.7 ± 3.6	87.1 ± 3.4	306 ± 9	313 ± 8
Urokinase 250 U/min (n = 15)	87% (13 of 15)	33.5 ± 4.1	4.1 ± 0.7	1.8 ± 0.5	1.43 ± 0.38 ²	89.8 ± 5.6	82.5 ± 4.1 ¹	320 ± 8	317 ± 8
Alteplase 2 mg (n = 14)	71% (10 of 14)	6.3 ± 1.6 ³	3.8 ± 0.5	2.1 ± 0.7	0.97 ± 0.21	87.1 ± 4.6	84.5 ± 3.6	305 ± 9	310 ± 9

All data are presented as mean ± standard error.

Base: baseline time point following surgical instrumentation, but prior to the formation of thrombus. 15' Rx and 90' Rx, time points at 15 and 90 min following the administration of test agents.

¹ Significant difference (p < 0.05) from baseline time point by paired t test.

² Significant difference (p < 0.05) from saline group by ANOVA.

³ Significant difference (p < 0.05) vs. all other groups by Kaplan Meier estimates.

Effects of Alteplase on Fibrinogen and Platelet Aggregation in vitro

Alteplase has been evaluated for potential effects on human plasma fibrinogen and platelet aggregation using citrated whole blood.

In general, we found that plasma fibrinogen was not affected by incubation with alteplase at concentrations of 10 and 20 µg/ml. Presumably, the alteplase that was added to the sample was completely inhibited by the α₂-macroglobulin that was also present in the sample. In contrast, incubation of plasma with alteplase at 100 µg/ml resulted in complete degradation of fibrinogen in all plasma samples within the first 30 min of the incubation (data not shown), suggesting that at an alteplase concentration of 100 µg/ml, the capacity for α₂-macroglobulin to bind and neutralize alteplase has been exceeded.

Incubation of platelet-rich plasma with alteplase up to and including 40 µg/ml did

not affect platelet aggregation. When alteplase was incubated with platelet-rich plasma at 100 µg/ml, a concentration which completely degraded plasma fibrinogen in all subjects studied (as noted above), a platelet aggregation response was still present, although it was diminished compared to untreated controls.

The retention of a platelet aggregation response when alteplase is incubated at 100 µg/ml appears to be consistent with the ability of activated platelets to release the fibrinogen that is stored in platelet α-granules. This release of fibrinogen appears sufficient to support some, but not fully normal, platelet aggregation.

Conclusion

In conclusion, the pharmacology of alteplase appears to demonstrate a high degree of novelty in relation to the plasminogen activa-

tor class of thrombolytic agents. Specifically, the animal data thus far indicate the speed of lysis is greatly accelerated and the potential risk of hemorrhagic complications greatly diminished. Despite the potential drawback in the requirement for local delivery of alfineprase, the advantage gained in local delivery

is more rapid thrombolysis combined with a reduced risk of bleeding. If these attributes are maintained as clinical safety and efficacy are assessed, alfineprase will represent the first of a new therapeutic class (fibrinolytic metalloproteases) for use in thrombolysis.

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