Non-Clinical and Clinical Characterization of a Novel Acting Thrombolytic: Alfimeprase

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Key Words

Alfimeprase · Urokinase · Fibrinolysis · Thrombolysis · Thrombosis

Abstract

Alfimeprase (ALF) is a recombinant, truncated form of fibrolase, a directly fibrinolytic zinc metalloproteinase that was first isolated from the venom of the Southern copperhead snake (*Agkistrodon contortrix contortrix*). ALF has direct proteolytic activity against the fibrin(ogen) A α chain. ALF can be covalently bound and neutralized by serum α_2 -macroglobulin, a prevalent mammalian protease inhibitor. Preclinical pharmacology studies have shown that thrombolysis with ALF is up to 6-times more rapid than with select plasminogen activators. Additional studies suggest that intra-thrombus ALF has the potential to be a fast and effective thrombolytic without generation of a systemic lytic state. Investigations of phases 1 and 2 indicate that ALF is active and generally well tolerated. This paper reviews the biochemical characteristics of ALF and a review of the preliminary clinical experience in subjects with acute peripheral arterial occlusion and in those with central venous access device occlusion.

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Introduction

Alfimeprase (ALF) 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 metalloproteinases, family M12, subfamily B (the re-prolysins), a grouping of proteolytic enzymes that are comprised of many enzymes originally characterized from snake venoms [2]. The active site of the molecule has been identified in the zinc-binding region of the fibrolase molecule spanning amino acids 139–159 [3]. While similar in enzymatic activity, ALF contains 201 amino acids with an N-terminal sequence of SFPQR- in contrast to fibrolase, which contains 203 amino acids with an N-terminal sequence that begins with EQRF-PQR- [4].

Fibrinolytic Activity of Alfimeprase

Both fibrolase and ALF have been shown to be directly fibrinolytic. Fibrolase has documented 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 [5]. Venom fibrolase has demonstrated proteolytic activity against fibrinogen that is targeted at the Lys⁴¹³-Leu⁴¹⁴ site [6]. Dose-dependent, direct degradation of the fibrinogen A α chain can be dem-

onstrated in a gel-based method and confirmed in a quantitative fibrinogen assay [1]. In contrast, plasminogen activators like streptokinase, urokinase (UK), tissue-type plasminogen activator (tPA), and tPA variants indirectly promote thrombolysis by activation of the endogenous plasminogen (fibrinolytic) system. Hence, ALF can be distinguished from the plasminogen activators by its unique, direct mode of action.

Alpha₂-Macroglobulin

Alpha₂-macroglobulin is a prevalent protease inhibitor present in mammalian serum and one of the largest of the serum proteins (725 kDa) [7]. The tetrameric α_2 -macroglobulin molecule inhibits ALF by physical entrapment. Once entrapped, the ALF is sterically hindered and cannot gain access to macromolecular substrate. A covalent bond forms between α_2 -macroglobulin and ALF, such that the interaction between α_2 -macroglobulin and ALF is irreversible. The formation of this complex has been demonstrated, *in vitro*, to begin in seconds and be complete within minutes. Thus, within the general circulation, α_2 -macroglobulin has the potential to quickly and effectively neutralize ALF.

In vitro Clot Lysis with Human Blood

Using human whole blood, clots were formed by the addition of thrombin and excess calcium and then incubated at 37°C for 90 min. Retracted clots were packed into 5-cm sections of bypass graft material and placed in a closed circuit perfusion loop. Flow through the clotted graft segment was visually monitored while test compounds were administered. A 2-mg dosage of ALF successfully dissolved the clot in 2 of 5 cases. ALF dosages of 3 mg and higher were uniformly effective in achieving clot lysis. Lysis occurred more rapidly as the dose of ALF was increased to 5 mg (fig. 1). UK 30,000 U, a dose proportional to that used to treat peripheral arterial thromboses in Man, was also uniformly effective in achieving clot lysis, although not as rapid as ALF ($p < 0.05$ for the ALF 3- and 5-mg doses compared to the UK group by ANOVA and Fisher's PLSD testing).

Fig. 1. Time to clot lysis in an *in vitro* model of clot dissolution. Retracted human clot was placed in a parallel limb of a closed loop perfusion circuit and the system was pressurized to 60 mm Hg. Local administration of alteplase was used to dissolve thrombus and restore flow through the clotted segment. A 30,000 U dose UK had previously been established as the standard in this model and was used as a comparator. Increasing doses of alteplase resulted in progressively more rapid clot dissolution. The 5-mg dose of alteplase restores flow in half the time required to achieve the same results with the standard dose of UK.

Studies Which Demonstrate Thrombolysis in Animals

Pharmacology studies were conducted in carotid arteries of rats, piglets and dogs that had been acutely thrombosed following injury to the vessel from locally applied anodal current. Heparin and aspirin were administered following arterial thrombosis to prevent propagation. Thrombolysis was attempted by intra-arterial infusion of ALF or UK. While study drugs were being administered, flow through the carotid artery was monitored with a perivascular flow probe to determine the time at which restoration of flow occurred. In all studies, observations were continued for 90 min postinitiation of the thrombolytic treatment regimen. As shown in figure 2, time to clot lysis in the preclinical studies with ALF has been very rapid in comparison to UK. Regardless of animal species tested, ALF successfully lysed stable, occlusive arterial thrombus much faster than UK.

A recombinant preparation of fibrolase (r-fibrolase), the compound upon which ALF was derived, has also been evaluated in comparison to tPA in the piglet model

Fig. 2. Comparison of time to lysis for ALF or UK in rat, piglet and dog models of acute carotid thrombosis. In each study, the administration of alfineprase produced more rapid clot lysis than a reference dosage of UK. Rat study: ALF = 2 mg and UK = 250 U/min $\times$ 60 min. Piglet study: ALF = 3 mg and UK = 2,000 U/min $\times$ 60 min. Dog study: ALF = 2 mg and UK = 4,500 U/min. Data are expressed as mean $\pm$ standard error.

of acute carotid thrombosis as described above. A fixed dose of 5 mg r-fibrolase achieved a 100% incidence of clot lysis at 4.4 min after drug delivery compared to a 70% incidence at 17.8 min with 2 mg/kg of tPA infused over 90 min. Average blood loss in this animal study was 1.7 ml in the r-fibrolase group, considerably lower than the 17.1 ml average blood loss in the tPA group (Nuvelo, Inc. data on file).

Summary of Non-Clinical Results

Collectively, the preclinical efficacy of ALF has been demonstrated in human clotted blood *in vitro* and in rat, piglet and canine models of arterial thrombosis, clearly establishing the biologic activity of ALF across a broad range of species [8]. The pharmacology of ALF appears to demonstrate a high degree of novelty in relation to the plasminogen activator class of thrombolytic agents. Specifically, the animal data thus far indicate that the speed of lysis with ALF appears greatly accelerated. In addition, the rapid neutralization of ALF by α_2 -macroglobulin provides a mechanism whereby a systemic lytic state may be avoided, potentially

lowering the risk of hemorrhagic complications, an adverse effect widely observed with the plasminogen activators.

Clinical Use of Thrombolytic Agents

Clinical limitations of currently available thrombolytic agents, such as streptokinase, UK, tPA, reteplase, and TNK-tPA include the need for an adequate plasminogen supply to generate lytic activity, inactivation by endothelial cell and platelet-derived plasminogen activator inhibitor-1, and the generation of a systemic lytic state even during local intra-vascular drug delivery [9]. These limitations of the plasminogen activators may contribute to the need for extended infusion durations, platelet-rich arterial thromboses being recalcitrant to thrombolysis, and unacceptable rates of major bleeding and intra-cranial hemorrhage. The observed bleeding complications likely reflect the fact that plasminogen activators can generate increased circulating levels of plasmin that result in a systemic 'lytic state' that does not distinguish between physiologic and pathologic thrombosis. Based on the described non-clinical studies, ALF has the potential to provide rapid and effective direct thrombolysis of arterial and venous thrombosis with an improved safety profile compared to plasminogen activators.

Potential Clinical Indications for Alfineprase

Alfineprase has the potential to effectively, rapidly, and with an improved safety profile restore vascular patency in a broad range of thrombotic conditions. Arterial thrombotic diseases including acute coronary syndromes, stroke, and acute peripheral arterial occlusion (PAO) as well as venous thrombotic diseases such as pulmonary embolism, extremity deep venous thrombosis, and central venous access device (CVAD) thrombosis are potential targets for ALF thrombolysis. The initial clinical development of ALF includes programs in subjects with acute PAO and those with CVAD occlusion.

In acute PAO, also known as acute limb ischemia, native arteries or arterial bypass grafts become occluded secondary to thrombosis or thromboembolism. The resulting obstruction to arterial blood flow can result in significant pain, impairment of activities of daily living, and limb loss. Catheter directed thrombolysis with plasminogen activators was first explored in acute PAO over two

decades ago [10]. More recently, the use of plasminogen activators, compared with surgery, was shown to be associated with higher risk of bleeding of all types (5–15%), including intra-cranial hemorrhage (1–2%) [11]. Given the safety drawbacks, the role and relative benefits of thrombolysis with plasminogen activators in acute PAO are still being debated.

Central venous access devices are inserted to provide durable and reliable venous access in order to facilitate frequent blood sampling and the infusion of parenteral therapy including chemotherapy, antibiotics, analgesics, and blood products. Catheter dysfunction most commonly develops as a result of thrombotic occlusion. Rapid restoration of catheter function is necessary to insure timely delivery of vital therapy. Cathflo® Activase® is a plasminogen activator that is approved in the United States for the restoration of function in occluded CVADs. Cathflo® Activase® in a dose of 2 mg has been shown to restore function in 74% of occluded CVADs at 2 h after dosing and up to 87% of occluded CVADs after 2 doses and 4 h of treatment [12]. A 2–4-hour course of thrombolytic therapy, though, may delay crucial infusional therapies and blood tests.

First in Human Experience

An open-label phase 1 study to evaluate the safety, pharmacokinetics, and thrombolytic activity of ALF was conducted in subjects with chronic PAO. Twenty subjects with worsening symptoms of lower extremity ischemia within 6 months of study enrollment were treated with ALF in 5 escalating dose cohorts (0.025, 0.05, 0.1, 0.3, and 0.5 mg/kg) by means of intra-arterial and/or intra-thrombus injections. Safety was assessed for up to 3 months. Pharmacokinetic parameters were evaluated by an assay that measures both free and α_2 -macroglobulin-bound (total) ALF.

No local or systemic bleeding was noticed, and plasminogen and fibrinogen concentrations were not substantially altered by treatment. No intra-cranial hemorrhage was reported. Two transient drug-related adverse events were reported; a skin rash and one incidence of headache. Both adverse events were graded as mild in severity. No other systemic adverse events, peripheral embolism, anaphylactic shock, tissue damage or electrocardiogram changes from baseline were observed. The half-life for total ALF ranged from 11 to 54 min. The serum α_2 -macroglobulin concentrations were transiently decreased by ALF treatment in a dose-response-like manner. Angio-

graphic evidence in 8 (40%) cases showed signs of improved limb perfusion following treatment with ALF. This was an unexpected observation because of the chronic nature of the arterial disease.

Phase 2: Acute PAO Experience

An open-label phase 2 study to evaluate the safety and activity of ALF in patients with acute PAO was conducted in multiple sites in the US, Europe, and South Africa. The primary objective of the study was to evaluate the safety including major hemorrhagic events (including intra-cranial hemorrhage), embolic events, and all other serious and non-serious adverse event rates at a range of ALF doses. The secondary objectives of the study included determination of the activity of ALF by restoration of arterial flow rate, determination of the open surgery free survival rates at 30 days, and assessment of the severity of interventions after ALF treatment compared with baseline planned interventions. Serum α_2 -macroglobulin levels and ALF immunogenicity were also assessed.

Subjects received ALF 0.1, 0.3, or 0.6 mg/kg as divided dose (2/3 and 1/3 of the total dose 2 h apart) via a slit-hole catheter positioned within the clot via multiple manual 1-ml pulses of ALF given every minute. Adults (≥ 18 years of age) with acute PAO of the lower limb, onset of symptoms within 14 days of enrollment, and acute limb ischemia severity of Rutherford Class I or IIA were eligible for enrollment. Safety and activity were evaluated during the 2 h following each ALF administration and at 18–24 h and 3, 7, and 30 days following administration.

At study completion, 113 subjects had received the study drug and completed the evaluation period. Five of 48 subjects (10.4%) in the 0.6-mg/kg dose group, 2 of 49 subjects (4.1%) in the 0.3-mg/kg dose group, and no subjects (0%) in the 0.1-mg/kg dose group were reported to have major bleeding events. Most major bleeding events involved hematoma formation at arterial puncture sites. Eleven subjects (23%) in the 0.6-mg/kg dose group, 4 subjects (8%) in the 0.3-mg/kg dose group, and 1 subject (6%) in the 0.1-mg/kg dose group experienced at least one hypotensive event. The study investigator attributed the hypotensive events to ALF in 8 cases (50%), all but one of which involved the 0.6 mg/kg dose level. The mean percent decline of α_2 -macroglobulin from baseline at 18–24 h was 38, 53, and 63% in the 0.1-, 0.3-, and 0.6-mg/kg dose groups, respectively. No subjects recorded a 100% decline. Most α_2 -macroglobu-

Table 1. HA003 summary of activity results

Cumulative patency restoration rate, %	T = 0	Dose 1				Dose 2			
		5 min	15 min	30 min	120 min	5 min	15 min	30 min	120 min
ALF 0.3 mg (n = 16)	0	13	13	25	38	38	38	38	44
ALF 1.0 mg (n = 16)	0	13	44	44	56	56	56	56	56
ALF 3.0 mg (n = 10)	0	40	50	60	60	60	60	60	80
CathfloActivase 2.0 mg (n = 13)	0	0	0	23	46	54	62	62	62

Fig. 3. Comparison of activity assessments for ALF 0.1, 0.3, and 0.6 mg/kg in subjects with acute PAO. ABI = Ankle-brachial index, a non-invasive indicator of limb perfusion.

lin levels had returned to baseline levels after 14 days. There were no subject deaths and no cases of intra-cranial hemorrhage observed during the 30 days after ALF dosing.

Key activity results are summarized in figure 3. In the intent-to-treat population, restoration of arterial flow, based on blinded angiographic core lab evaluation, was observed in 60% of subjects in the 0.6-mg/kg dose group, 55% in the 0.3-mg/kg dose group, and 31% in the 0.1-mg/kg dose group ($p = 0.025$ by Kruskal-Wallis test for ordered categorical data). Thrombolysis was observed in 73% of subjects in the 0.6-mg/kg dose group, 76% in the 0.3-mg/kg dose group, and 50% in the 0.1-mg/kg dose group ($p = 0.025$ by Kruskal-Wallis test for ordered categorical data). Overall, 58% were open surgery free at 30 days. The aggregate activity results support an ALF dose response in acute PAO.

Phase 2: CVAD Occlusion Experience

A double-blind phase 2 study to compare the safety and efficacy of one or two instillations of three intraluminal doses of ALF in patients with occluded CVADs was conducted. In this trial an active control arm utilized the clinically proven and FDA approved 2.0 mg dose of CathfloActivase [12].

Eligible subjects were clinically stable and had a dysfunctional indwelling CVAD defined as the inability to withdraw 3.0 ml of blood from the catheter. Peripherally inserted central catheters, catheters with valves, and implanted ports were allowed. Catheters inserted for the purposes of hemodialysis or therapeutic pheresis were excluded. One or two instillations (final volume of 2 ml) of one of three intraluminal doses of ALF (0.3, 1.0, or 3.0 mg) or CathfloActivase 2.0 mg were used. Patency was assessed at 5, 15, 30, and 120 min after each dose of study drug. Restoration of patency was defined as the ability to withdraw 3 ml of blood and infuse 5 ml of saline through the treated catheter lumen. Adverse events were assessed for 30 days.

Fifty-five subjects with CVAD withdrawal occlusion were enrolled and randomized to treatment. No systemic bleeding, intracranial hemorrhages, embolic events, hypotensive events, or study drug-related deaths were reported in any treated subject. One subject (2%) was diagnosed with a catheter-related infection during the 30 day follow-up period.

Table 1 summarizes the activity results observed in this study. The ALF 3.0-mg dose produced the highest patency rate at 120 min after the first and second doses. All three ALF doses were more active than CathfloActivase during the first 30 min of treatment. The ALF 1.0- and 3.0-mg doses resulted in 44 and 50% patency restoration rates at 15 min, respectively, compared to 0% for CathfloActivase ($p = 0.0084$ and 0.0075 , respectively).

Clinical Summary and Future Directions

The low major hemorrhagic event rates and the absence of intracranial hemorrhage in the phase 2 studies, are consistent with the non-clinical study-based prediction that ALF could be a safe thrombolytic agent. Infrequent, transient bouts of hypotension were almost exclusively seen in the 0.6-mg/kg dose group in the phase 2 acute PAO study. All other studied dose groups in that trial were essentially free from hypotension related to ALF. In the CVAD occlusion phase 2 trial, ALF in total doses up to 6 mg was well tolerated without any episodes of hypotension or major bleeding. Anti-ALF antibodies have not been detected in any of the ALF-treated subjects to date.

Data from the phase 2 studies also support the following ALF efficacy-related statements. ALF facilitates the

restoration of arterial patency in less than 4 h in the majority of treated acute PAO subjects and thus may facilitate the performance of endovascular therapy and avoidance of open vascular surgery. ALF is capable of restoring patency to occluded central venous catheters and may be able to do so in under an hour in the majority of cases. Phase 3 studies in both acute PAO and CVAD occlusion are scheduled to begin in the near future.

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