Synthesis and Evaluation of Imidazole Acetic Acid Inhibitors of Activated Thrombin-Activatable Fibrinolysis Inhibitor as Novel Antithrombotics

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Abstract: Thrombin-activatable fibrinolysis inhibitor (TAFI) is an important regulator of fibrinolysis, and inhibitors of this enzyme have potential use in antithrombotic and thrombolytic therapy. Appropriately substituted imidazole acetic acids such as **10j** were found to be potent inhibitors of activated TAFI and selective versus the related carboxypeptidases CPA, CPN, and CPM but not CPB. Further, **10j** accelerated clot lysis in vitro and was shown to be efficacious in a primate model of thrombosis.

Thrombin-activatable fibrinolysis inhibitor (TAFI, EC 3.4.17.20)[1] was recently identified[2] as an important regulator of fibrinolysis.[3] It is present in plasma as a zymogen, which is activated by limited proteolysis primarily by thrombin/thrombomodulin.[4] The activated form, designated TAFIa, is a carboxypeptidase that serves to remove C-terminal arginine and lysine residues from protein and peptides. Initial degradation of fibrin by plasmin exposes new C-terminal lysine and arginine residues on the surface of fibrin. These then serve as binding sites for tissue plasminogen activator (tPA) and its substrate plasminogen, thereby bringing them in proximity and accelerating the production of plasmin.[5] The action of TAFIa serves to stabilize clots by removing these binding sites that allow for more rapid plasmin generation. Therefore, a TAFIa inhibitor should stimulate endogenous fibrinolysis and thereby exert an antithrombotic effect.

In the search for treatments of thrombotic disorders, much effort has been expended toward development of inhibitors of the coagulation cascade.[6] Since hemostasis results from a balance of procoagulant and fibrinolytic forces, an alternative approach would be agents that result in the enhancement of fibrinolysis. The availability of a natural peptide inhibitor of carboxypeptidases A, B, and TAFIa from potato tubers[7] (potato carboxypeptidase inhibitor or "PCI"), as well as TAFIa knock-out mice,[8] has allowed several groups to demonstrate that inhibition of TAFIa does indeed enhance fibrinolysis in vitro and in vivo.[9] Importantly, complete inhibition of TAFIa does not impart significant increases in bleeding,[8] suggesting that TAFIa inhibitors may have a wider therapeutic index than traditional antithrombotics. TAFIa inhibitors have also shown promise in animal models when used in combination with existing thrombolytic agents such as tPA[10] or with anticoagulants such as thrombin inhibitors.[11]

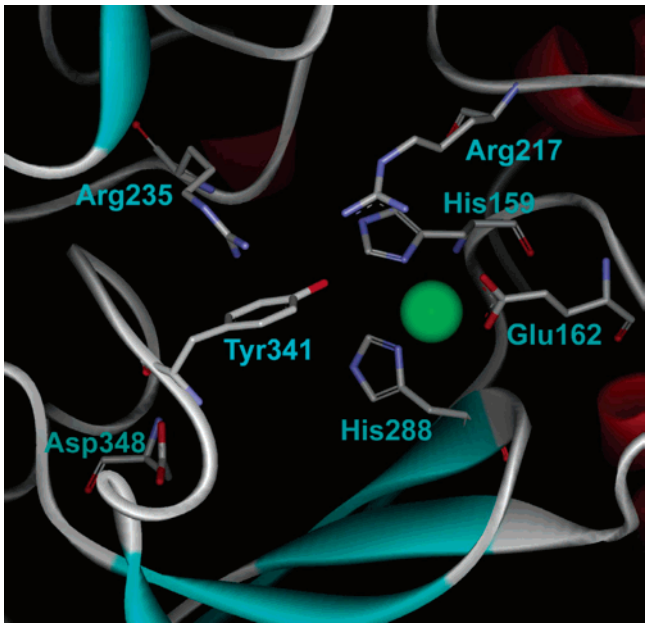

Figure 1. Homology model of the TAFI active site.

TAFIa is a zinc metalloprotease with significant amino acid sequence homology (approximately 50%) to the digestive carboxypeptidases A and B.[12] CPA recognizes and cleaves C-terminal hydrophobic residues such as phenylalanine, while CPB, like TAFIa, prefers C-terminal basic residues (lysine and arginine). An interesting difference between TAFIa and other carboxypeptidases is that it is conformationally unstable (thus the alternative nomenclature[2a,3] "CPU"), losing half of its activity in about 10 min at 37 °C.[13]

Greatly assisting the inhibitor design process is the presence of high-resolution X-ray crystallographic structures of CPA and CPB with and without inhibitors bound.[14] A TAFI homology model was created[15] from these structures, and the active site is depicted in Figure 1. The C-terminal carboxylate of the substrate or inhibitor is bound by salt bridges from two arginine residues (Arg235 and Arg217)[16] as well as by hydrogen bonds from Asn234 (not shown) and a mobile tyrosine (Tyr341) that covers the active site after binding. The catalytic zinc atom (green sphere) is coordinated by two histidines, a glutamate, and a displaceable water molecule. CPA features an isoleucine residue located at the bottom of the S1′ specificity pocket to help recognize hydrophobic residues, while in CPB and TAFIa this residue is an aspartic acid (Asp348) that binds to basic side chains. The S1 region is bordered in part by the

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Table 1. In Vitro Inhibition of Carboxypeptidases[a]

compd	config	R	TAFIa[b] IC_{50} (μM)	CPB[b] IC_{50} (μM)	CPA[c] IC_{50} (μM)	CPN[c] IC_{50} (μM)	CPM[c] IC_{50} (μM)	CLT_{50} (μM)
PCI			0.0021 ± 0.0005	0.001 ± 0.0002	0.0009 ± 0.00016	>50	>50	0.015 ± 0.005
1	±		>50	1.6 ± 0.4	0.4±0.1	>50	>50	>50
2	±		>100	>100	1.7±0.3	>50	>50	>50
3	±		0.2 ± 0.02	0.0082 ± 0.0009	>50	0.017 ± 0.004	0.013 ± 0.003	3.5
10a	±	H	0.08 ± 0.003	0.067 ± 0.002	>50	>50	>50	0.97
10b	+	H	1.8 ± 0.15	1.0 ± 0.2	>50	>50	>50	>10
10c	−	H	0.04 ± 0.006	0.035 ± 0.01	>50	>50	>50	0.5
10d	±	Me	0.042 ± 0.008	0.05 ± 0.01	NT	>50	>50	0.42
10e	±	Et	0.048 ± 0.002	0.036 ± 0.01	NT	>50	>50	0.65
10f	±	iPr	0.069 ± 0.006	0.052 ± 0.009	6.4 ± 0.3	>50	>50	1.06
10g	±	Bu	0.008 ± 0.001	0.006 ± 0.002	NT	>50	>50	0.15
10h	±	Bn	0.028 ± 0.0007	0.021 ± 0.001	NT	>50	>50	0.74
10i	±	iPent	0.005 ± 0.001	0.004 ± 0.0004	2.2 ± 0.3	>50	>50	0.12
10j	−	iPent	0.002 ± 0.0005	0.0027 ± 0.0006	1.4 ± 0.4	>50	>50	0.056 ± 0.021

[a] Values represent the average of two runs or the mean ± standard error of the mean. [b] Tested in ECL format; see ref 21. [c] Tested in spectrophotometric format; see ref 22.

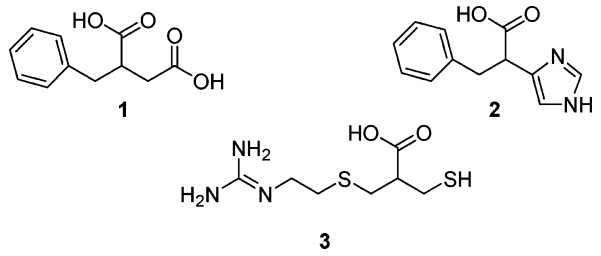
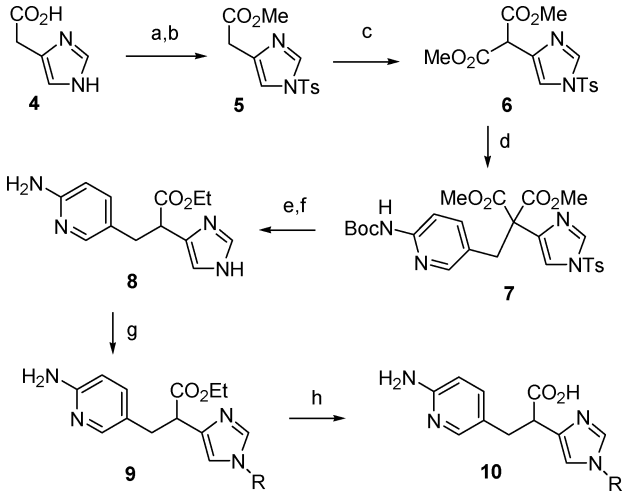


Figure 2. Carboxypeptidase inhibitors.

Scheme 1. Synthesis of Inhibitors



[a] (a) MeOH, HCl; (b) TsCl, Et_3N, CH_2Cl_2; (c) LHMDS, MeO_2CCN, THF; (d) NaH, 5-bromomethyl-2-(boc-amino)pyridine, DMF, 0 °C; (e) 6 N HCl, 100 °C; (f) EtOH, HCl; (g) NaH, R−Br, DMF; (h) 6 N HCl, 90 °C, 2 h.

β-sheet at the "front" of Figure 1.[17] At the initiation of this work, several inhibitors of carboxypeptidases A and B such as **1** (CPA),[18] **2** (CPA),[19] and **3** (CPB)[20] (Figure 2) were known and were attractive starting points because of their small size and high water solubility.

Inhibitor **2** was chosen for further modification because imidazole was considered to be the most attractive zinc ligand and offered several points for exploration of S1. We began by converting the lipophilic phenyl of CPA inhibitor **2** into the basic aminopyridine group as shown in Scheme 1. Direct alkylation of **5** was somewhat problematic; however, after conversion to malonate derivative **6** (LHMDS, MeO_2CCN), alkylation proceeded smoothly to give adduct **7** in good yield. Heating in aqueous 6 N HCl removed the tosyl and carbamate protecting groups, hydrolyzed the esters, and induced decarboxylation. Reesterification (HCl, EtOH) afforded key intermediate **8**, which could be selectively alkylated on the least hindered imidazole nitrogen (3:1 ratio) when treated with NaH and an appropriate alkylating agent. Hydrolysis of the ester afforded the products shown in Table 1. Enantioenriched **10b**, **10c**, and **10j** were produced by resolution (chiral preparative HPLC) of **8** or **9** followed by hydrolysis. This hydrolysis did not cause any decarboxylation, and racemization was minimal (≤5%) with short reaction times.

Compounds were assayed for carboxypeptidase activity using ECL[21] or spectrophotometric[22] assays and are reported in Table 1 as IC_{50} values. As expected, conversion from the lipophilic phenyl P1 group present in **2** to the basic aminopyridine **10a** decreased CPA activity and improved TAFIa and CPB activity presumably by interaction with Asp348 at the bottom of the S1′ pocket (Figure 1). Although the absolute stereochemistry of the resolved enantiomers **10b** and **10c** has not been established, activity resides predominately with the (−)-isomer **10c**. This small compound (MW = 232) is remarkably potent (40 nM) and selective versus other carboxypeptidases, save CPB, and serves as a core structure that can be decorated with other potency and selectivity enhancing features. The imidazole N1 position proved to be a good site for further substitution and was explored further. As seen in **10d**, **10e**, and **10g**, longer alkyl chains gradually improved potency; however, bigger chains were not as beneficial as seen by the modest improvements of isopropyl (**10f**) and benzyl (**10h**). Branching further from the imidazole with an isopentyl chain (**10i**) was beneficial, and the (−)-isomer **10j** proved to be a low nanomolar inhibitor of TAFIa suitable for further study. The inhibitory activity of these compounds can be rationalized by examining **10j** in the TAFIa homology model (Figure 3). The imidazole acetic acid anchors the inhibitor between the zinc and the carboxylate-binding arginines because the aminopyridine reaches down into S1′, forming a salt bridge with Asp348. The N1 imidazole nitrogen then orients the isopentyl group toward S1.

Importantly for in vivo studies, **10a**−**j** were inactive versus the critical regulatory enzymes[23] CPN and CPM in part because of the low sequence identity between TAFIa and CPN & M (<15%). Unfortunately, no differences between TAFIa and CPB were revealed in the P1 area because all the compounds prepared have similar

Figure 3. **10j** docked in the TAFI homology model.

Table 2. Pharmacokinetic Profile of **10j**

species	% *F*	$T_{1/2}$ (h)	Cl (mL/(min·kg))	Vd_{ss} (L/kg)
rat[a]	47	1.1 ± 0.2	32 ± 5.7	1.36 ± 0.06
dog[b]	38	0.6	6.1	0.28
rhesus[c]	12	0.8	6.1	0.26

[a] 2 mg/kg iv (n = 3); 14 mg/kg po (n = 3) noncrossover. [b] 0.55 mg/kg iv (n = 2); 0.9 mg/kg po (n = 2) crossover. [c] 1 mg/kg iv (n = 2); 2 mg/kg po (n = 2) crossover.

activity against the two enzymes. The significance of inhibiting CPB (thought to only be present in the gut) remains to be determined, but the presence of carboxypeptidase inhibitors in potatoes and tomatoes[24] suggests that some CPB activity might be manageable.

Compound **10j** was extensively counterscreened versus other coagulation enzymes, Zn metalloenzymes, and others (e.g., thrombin, factor Xa, plasmin, uPA, tPA, trypsin, chymotrypsin, MMP-1, NEP, and ACE) and found to be inactive (>50 μM). The potency and specificity for TAFIa make **10j** an excellent tool for further studies and prompted further evaluation in vivo.

Compound **10j** was first examined for ADMET properties and has an excellent profile. Even though **10j** is very polar (log P = −0.9), it is bioavailable in rat and dog (Table 2), although the terminal half-life is somewhat short. Microsomal incubations show no metabolites, and 80% of an iv dose was recovered unchanged in rats. Further, **10j** was found not to be an inhibitor of the cytochrome P450 enzyme isoforms CYP3A4, 2D6, or 2C9, despite containing an imidazole moiety.

A clot lysis assay was developed to measure functional activity of TAFIa inhibitors in pooled human plasma. Clot formation and lysis were triggered by the addition of thrombin, $CaCl_2$, and tPA and were detected by turbidity changes. As shown in Figure 4, clots form rapidly (increased turbidity and thus absorbance at 405 nM) and lyse after 2 h with no TAFIa inhibitor present (curve A). Increasing concentration of inhibitor **10j** (curves B−I) decreases the time required for clot lysis;

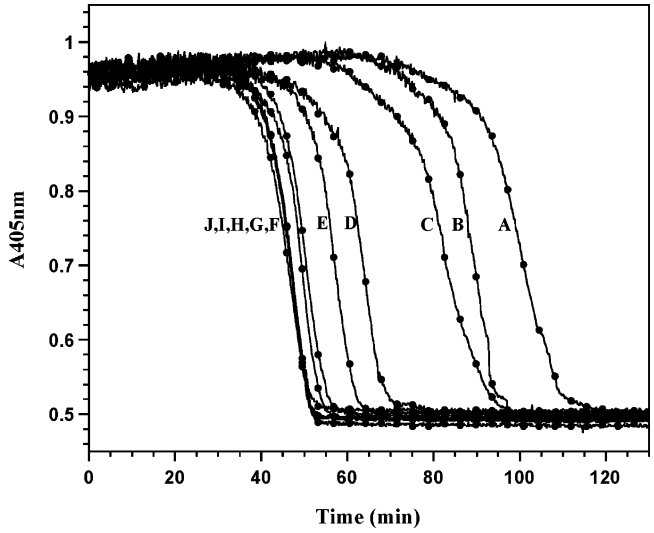


Figure 4. **10j** accelerates human plasma clot lysis in a dose-dependent manner. Human plasma (125 μL) was mixed with thrombin (25 nM), $CaCl_2$ (25 mM), and tPA (0.065 μg/mL) in 100 μL of buffer and varying concentrations of **10j** (μM): A, 0; B, 0.008; C, 0.016; D, 0.063; E, 0.125; F, 0.250; G,0.500; H, 1.0; I, 2.0; J, PCI (1 μM) as a control for maximal acceleration of clot lysis.

however, the effect is saturable and lysis faster than 50 min does not occur under these conditions even at extremely high doses of inhibitor. The CLT_{50} value represents the concentration of inhibitor that gives 50% of the maximum acceleration of lysis, and as seen in Table 1, activity in this functional assay tracks well with the IC_{50} for enzyme inhibition.

While the saturable effect on clot lysis represents a potential safety feature, whether a potent TAFIa inhibitor will display useful antithrombotic efficacy is a more difficult question to answer. Of concern is the report that TAFI knock-out mice responded no differently than wild-type to several acute models of thrombosis.[8] It is difficult to accurately model human disease, including deep-vein thrombosis. However, a model of electrolytic vascular injury in the African green monkey (AGM)[25] wherein an occlusive thrombus forms gradually over ∼90 min is suitable to evaluate the antithrombotic effect of a TAFIa inhibitor. This model has been validated with novel and standard-of-care clinical agents such as antiplatelet (aspirin and glycoprotein IIb/IIIa inhibitors) and anticoagulant (direct and indirect thrombin inhibitors) compounds. In preparation for this model, **10j** was assayed for activity versus AGM TAFIa (IC_{50} = 18 nM) and in AGM plasma (CLT_{50} = 35 nM). Although intrinsic potency was slightly diminished, functional activity was improved possibly because of decreased protein binding in AGM plasma (40% bound) versus human (87% bound). As was seen for PCI,[25] **10j** did indeed prolong time-to-occlusion (TTO) in the jugular vein in a dose-dependent manner without a physiologically meaningful difference in template bleeding time (Table 3). Further, there was no effect on APTT, platelet aggregation, hemoglobin, platelet count, or hemodynamics at the initial high dose (0.3 mg/kg bolus + infusion of 0.1 mg/(kg·min)). The success of **10j** in this model suggests that TAFIa inhibitors should be considered as novel, stand-alone antithrombotic agents.

Table 3. AGM in Vivo Data for **10j**

n	dose[a] (mg/kg + mg/(kg·min))	vein TTO (min)	artery TTO (min)	bleeding time (min)	concn[b] (μM)
10	saline	96 ± 6	51 ± 6	2.0 ± 0.1	0
6	0.3 + 0.1	216 ± 22[c]	85 ± 14[c]	2.9 ± 0.2	26.1 ± 2
8	0.1 + 0.03	171 ± 24[c]	52 ± 7	2.3 ± 0.2	9.6 ± 1
8	0.05 + 0.02	147 ± 17[d]	62 ± 5	2.2 ± 0.1	5.7 ± 0.7
8	0.03 + 0.01	128 ± 18	61 ± 10	2.0 ± 0.1	2.4 ± 0.1

[a] iv bolus followed by continuous infusion for 300 min. [b] Steady-state plasma concentration measured by LC/MS. [c] $p < 0.01$ vs vehicle. [d] $p < 0.05$ vs vehicle.

These studies demonstrate that the imidazole acetic acid framework **10** serves as a good scaffold for constructing potent TAFIa inhibitors with good selectivity versus most other enzymes. Further, **10j** accelerates clot lysis in vitro and is efficacious in a primate model of thrombosis. Studies aimed at improving CPB selectivity and terminal half-life are ongoing and will be reported in due course.

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Supporting Information Available: Experimental procedures and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) The homology model of TAFIa was created from the crystal structure of CPA (PDB code 2CTC) using MOE software (Chemical Computing Group, Inc., Montreal). Ten intermediate models were created, and the best-scoring model was minimized to a root-mean-squared gradient of 1 kcal/(mol·Å). The positions of the Zn, Zn-binding residues (His159, Glu162, and His288), and Asp348 were altered to those found in the crystal structure of procarboxypeptidase B (PDB code 1NSA). These residues, along with the residues flanking them, were allowed to relax in the context of the entire protein using the AMBER force field and the GB/SA solvation model as implemented in BatchMin (Schrödinger, Inc., Portland, OR) with the Zn−ligand distances constrained to those found in 1NSA.

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