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3-Mercaptopropionic acids as efficacious inhibitors of activated thrombin activatable fibrinolysis inhibitor (TAFIa)

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Abstract—A novel series of cyclic potent, selective, small molecule, thiol-based inhibitors of activated thrombin activatable fibrinolysis inhibitor (TAFIa) and the crystal structures of TAFIa inhibitors bound to porcine pancreatic carboxypeptidase B are described. Three series of cyclic arginine and lysine mimetic inhibitors vary significantly in their selectivity against other human basic carboxypeptidases, carboxypeptidase N and carboxypeptidase B. (−)**2a** displays TAFIa IC_{50} = 3 nM and 600-fold selectivity against CPN. Inhibition of TAFIa with (rac)**2a** resulted in dose dependent acceleration of human plasma clot lysis in vitro and was efficacious as an adjunct to tPA in an in vivo rabbit jugular vein thrombolysis model.

Thrombin activatable fibrinolysis inhibitor (TAFI, plasma pro-carboxypeptidase B, carboxypeptidase R, carboxypeptidase U, EC 3.4.17.20) is a zymogen synthesized in the liver and circulating at a plasma concentration of about 50 nM.[1] The activated enzyme (TAFIa) is a zinc-based basic carboxypeptidase that suppresses fibrinolysis. Upon activation by the thrombin/thrombomodulin complex or plasmin, TAFIa inhibits the amplification of plasmin production by removing the newly exposed C-terminal lysine residues from partially degraded fibrin thus regulating fibrinolysis.[2] Thus, inhibition of TAFIa is a novel pro-fibrinolytic mechanism in which fibrinolysis is enhanced without directly affecting coagulation or platelet function. TAFIa inhibition is shown to be effective in enhancing tPA-induced fibrinolysis in several animal models using CPI[3a,b,c] (a selective peptidic TAFIa inhibitor isolated from potato) and small molecules.[3d,e] The safety of TAFIa inhibition has been demonstrated using TAFI-deficient mice.[4]

We[5] and others[3d,e,6] have recently reported data supporting small molecule TAFIa inhibitors as a treatment for anti-thrombotic indications. Here, we report a novel series of potent, selective TAFIa inhibitors that are efficacious as an *iv* adjunct to tPA in an in vivo model of thrombolysis.

Our starting point for optimization was the known carboxypeptidase inhibitor SQ-24,798 (Fig. 1).[7] However, SQ-24,798 is equipotent for porcine pancreatic carboxypeptidase B (CPB) and carboxypeptidase N (CPN).

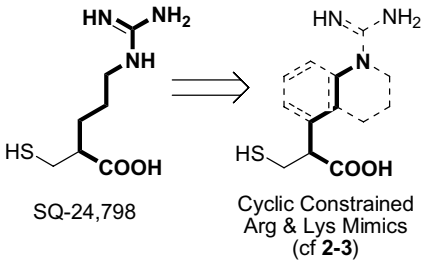

Figure 1. Inhibitor design scheme.

Keywords: TAFI; Fibrinolysis; Carboxypeptidase.
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CPN is a basic carboxypeptidase circulating in plasma that is critical in regulating the complement system.[8] Inhibition of CPN results in undesirable side effects. Therefore, it is critical to design inhibitors that are selective for TAFIa over CPN. Little risk is anticipated from transient inhibition of pancreatic CPB. Inhibition of CPB alone should not lead to intestinal malabsorption due to the presence of alternate active proteases available for digestion.

A crystal structure of human TAFIa would be an important aid for drug design. Unfortunately, TAFIa has not yet been crystallized due to its lack of stability.[1] CPB is an attractive alternative protein for structural studies. CPB has 47% sequence identity with human TAFIa, with >89% homology in the active site.

We envisioned that a series of cyclic, constrained lysine and arginine mimics could improve potency and selectivity (Fig. 1). Our structure of SQ-24,798 complexed to CPB confirmed ample room in the active site surrounding the alkyl guanidine for the introduction of cyclic structures.[9] Since TAFIa substrates require a basic group to bind in the S_1' pocket, our design scheme covered a series of inhibitors **1**–**3** containing various basic groups substituted on phenyl or heterocyclic templates (Fig. 2).

The series of 2-piperidinyl 3-mercaptopropionic acids **1** was synthesized as follows (Scheme 1). 3 or 4-Piperidine **5a** or **5b** was generated from platinum oxide reduction of the appropriate 3 or 4-pyridine acetic acid esters **4**.

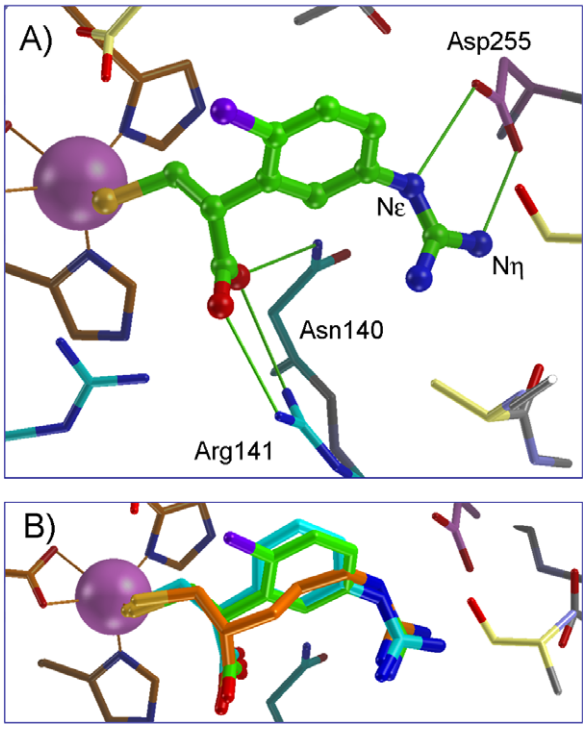


Figure 2. (A) The active site of CPB/**2d** complex showing the hydrogen bonds (pdb code 1ZG7). (B) The CPB/**2d** complex showing the superimposed structures of SQ-24,798 (orange, pdb code 1ZG9), (−)-**2a** (cyan, pdb code 1ZG8).

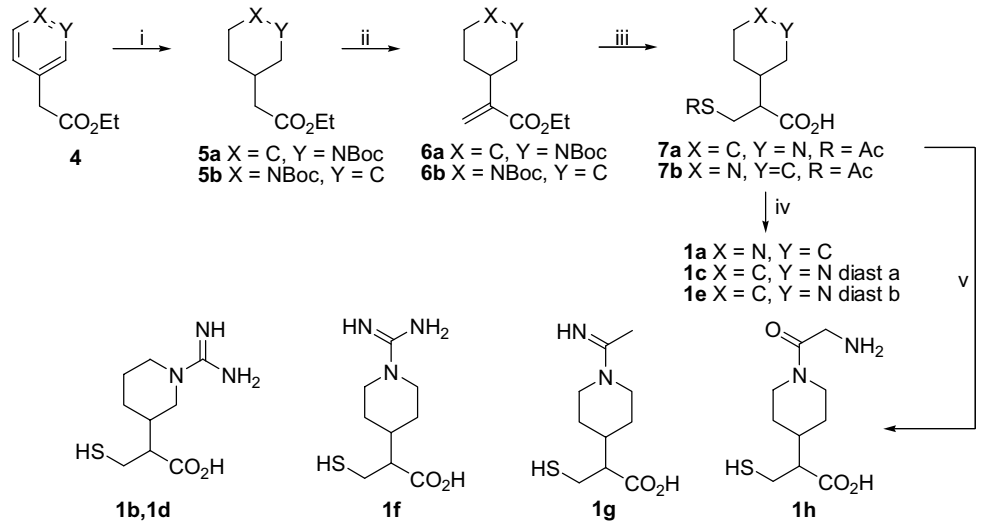
Boc protection followed by introduction of the methylene group by addition of formaldehyde to the lithium enolate produced **6**. Acid hydrolysis of both the ester and Boc group was followed by 1,4-addition of thioacetic acid to the double bond to provide **7**. Hydrolysis of the thioacetyl by ammonium hydroxide afforded the unsubstituted piperidinyl thiol inhibitors **1a**, **c**, and **e**. Further N-substituted piperidines **1b**, **1g**, **1f**–**h** were synthesized from **7**.

The series of 2-phenyl 3-mercaptopropionic acids **2** was synthesized as follows (Scheme 2). Addition of formaldehyde to appropriately substituted phenylacetic esters **8** gave the key olefin intermediates **9**. Ester hydrolysis followed by 1,4-addition of thioacetic acid to the double bond afforded **11**. Para-substituted nitrile **11a** was converted to amidine **2f**. Racemic Boc-protected aniline **11b** was separated into pure enantiomers (+)**12** and (−)**12** by chiral HPLC. Deprotection of (+)**12**, (−)**12** and (rac)**12** afforded the chiral aniline intermediates and racemic aniline **2e**, respectively. Treatment of (+)**12**, and (−)**12** with diboc-trifluoromethanesulfonylguanidine followed by acid hydrolysis afforded both enantiomers of chiral guanidine (+)**2a** and (−)**2a**. (**rac**)**2a** was synthesized in a similar manner from **2e**.

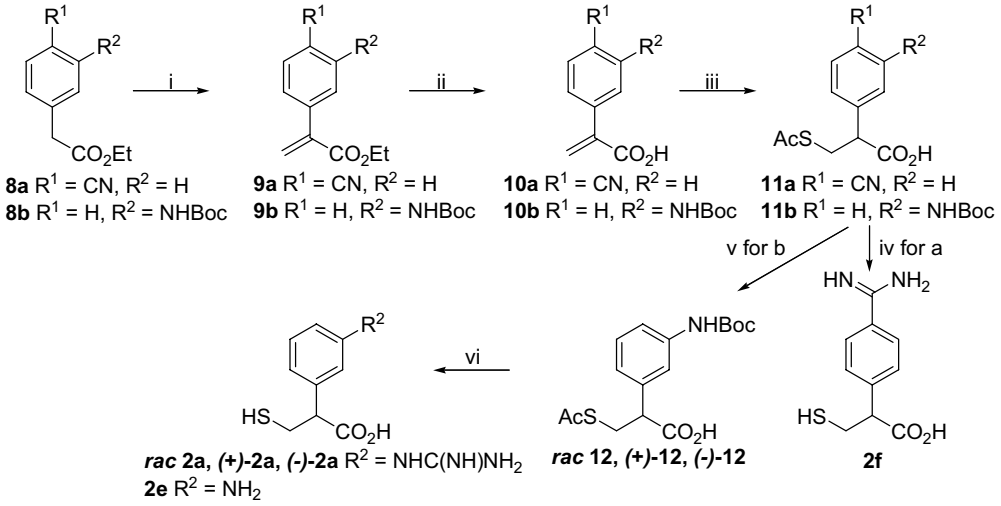
Chloro-substituted analog **2d** was synthesized as follows (Scheme 3). Reduction of the nitro group of **13** with iron, followed by Boc protection, and conversion of the alcohol via the mesylate gives benzylnitrile **14**. Nitrile hydrolysis to give the acid followed by Fischer esterification and Boc protection allowed for addition of formaldehyde to give the key double bond intermediate **15**. Ester hydrolysis, addition of thioacetic acid to the double bond, deprotection of the aniline, and generation of the Boc-protected guanidine gave **16**. Final deprotection with TFA followed by ammonium hydroxide afforded **2d**.

2-Piperidinylmethyl 3-mercaptopropionic acid analogs **3** were synthesized as follows (Scheme 4). Addition of benzyl methyl malonate to 3- or 4-pyridine aldehyde followed by platinum oxide reduction and Boc protection of the piperidine gave **17**. Mannich reaction followed by ester hydrolysis and Boc deprotection gave key double bond intermediates **18**. Addition of thioacetic acid to the double bond gave **19**. Hydrolysis with ammonium hydroxide provided thiol inhibitors **3a**–**c**.

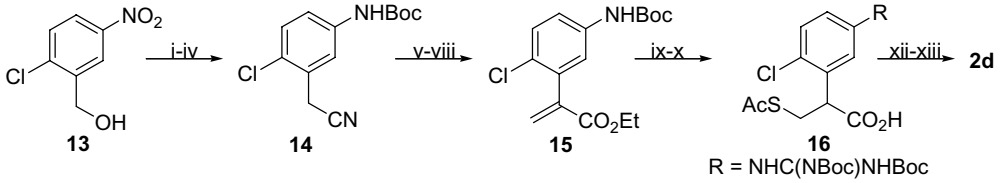
The 2-piperidine 3-mercaptopropionic acid series of TAFIa inhibitors **1** yielded potent and selective compounds against CPN and CPB (Table 1). The unsubstituted 4-piperidine analog **1a** was the most potent in the series (TAFIa IC_{50} = 37 nM), but showed little selectivity over CPN or CPB. To further probe binding affinity and selectivity within the S_1' pocket, substitution of the 4-piperidine nitrogen of **1a** with various other basic groups afforded less potent inhibitors (guanidine **1f**, amidine **1g**, amine **1h**; TAFIa IC_{50} = 1070, 5300, 7350 nM, respectively). The two unsubstituted diastereomeric 3-piperidine analogs **1c** and **1e** provided potent compounds (TAFIa IC_{50} = 68 and 445 nM, respectively). Compound **1c** showed the highest



Scheme 1. Reagents and conditions: (i) H_2, PtO_2, CH_3COOH; (ii) LDA, CH_2O, THF, −78 °C; (iii) a—6 N HCl, dioxane, 100 °C; b—AcSH; (iv) NH_4OH; (v) for **1b**, **1d**, **1f**: a—*N*,*N*′diBocN″trifluoromethanesulfonylguanidine, DIEA, $CHCl_3$; b—4 N HCl, dioxane; c—NH_4OH for **1g**: a—$CH_3C(=NH)OC_2H_5$ HCl; b—NH_4OH for 1 h: a—bocglycine *N*-hydroxysuccinimide ester, dioxane; b—TFA; c—NH_4OH.



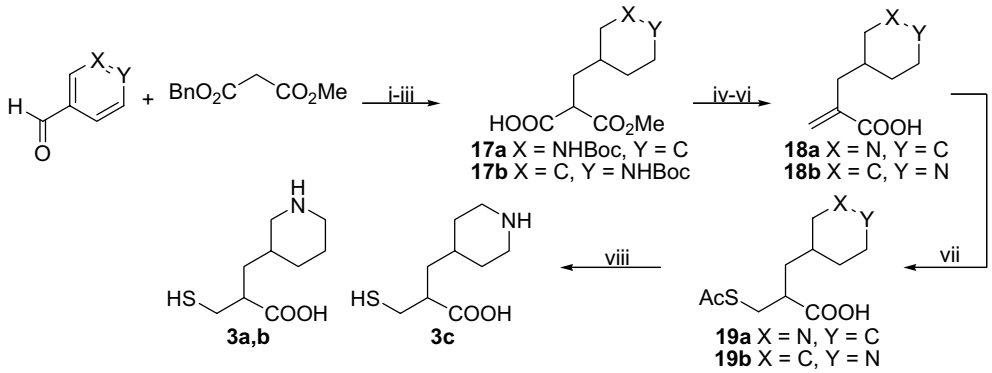
Scheme 2. Reagents and conditions: (i) paraformaldehyde, K_2CO_3, Bu_4NI, toluene, 100 °C, 4 h; (ii) NaOH, water–MeOH; (iii) AcSH, $CHCl_3$; (iv) HCl(g)–EtOH then NH_3(g)–EtOH; (v) HPLC; (vi) a—TFA, CH_2Cl_2; for **2a**, *N*,*N*′diBocN″trifluoromethanesulfonylguanidine, DIEA, $CHCl_3$ then HCl(g)–EtOH.



Scheme 3. Reagents and conditions: (i) Fe, HCl, EtOH; (ii) (*t*-BuCO)$_2$O; (iii) MsCl, DIEA, CH_2Cl_2; (iv) KCN, KI, 18-Crown-6, EtOH; (v) 10% H_2SO_4, MeOH; (vi) HCl(g)–EtOH; (vii) (*t*-BuCO)$_2$O, THF, 50 °C; (viii) paraformaldehyde, K_2CO_3, Bu_4NF, toluene; (ix) NaOH; (x) AcSH; (xi) HCl; (xii) *N*,*N*′diBocN″trifluoromethanesulfonylguanidine, DIEA, $CHCl_3$; (xiii) a—TFA, CH_2Cl_2; b—NH_4OH.

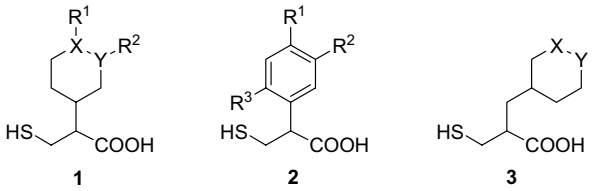
selectivity against both CPB (345-fold) and CPN (470-fold) of the entire thiol series **1**–**3**. Both 3-piperidine N-substituted guanidine diastereomers **1b** and **1d** displayed good potency (IC_{50} = 45 and 165, respectively) in contrast to 4-piperidine N-substituted guanidine **1f** (IC_{50} = 1070 nm).

The 2-piperidinylmethyl 3-mercaptopropionic acid series of inhibitors (**3a**–**c**) showed slightly less potency than the one carbon shorter 2-piperidine analogs **1a**, **c**, **e**. 4-Piperidine **3c**[6c] was more potent for CPN and CPB than TAFIa. Pure diastereomer 3-piperidine **3b** showed significant selectivity against CPB (250-fold). However,



Scheme 4. Reagents and conditions: (i) piperidine, benzene, Dean–Stark reflux; (ii) H_2, PtO_2, AcOH; (iii) $(t\text{-BuCO})_2O$, $NaHCO_3$, dioxane–water; (iv) NEt_2, formalin, THF; (v) NaOH, dioxane–water; (vi) 6 N HCl, 80 °C; (vii) AcSH, *i*-PrOH; (viii) NH_4OH.

Table 1. Inhibition of human TAFIa, human CPN, and porcine pancreatic CPB



Compound	X	Y	R^1	R^2	R^3	TAFIa IC_{50}[a] (nM)	CPN IC_{50}[a] (nM)	CPB IC_{50}[a] (nM)
SQ-24,798	—	—	—	—	—	8 ± 6	10 ± 7	2 ± 1
1a	N	C	H	H_2	—	37 ± 5	195 ± 21	60 ± 17
1b	C	N	H_2	$C(NH)NH_2$	—	45 ± 18	38 ± 14	38 ± 0
1c	C	N	H_2	H	—	68 ± 10	>30,000	23,500 ± 4950
1d	C	N	H_2	$C(NH)NH_2$	—	165 ± 21	3 ± 2	58 ± 6
1e	C	N	H_2	H	—	445 ± 106	19,000 ± 5660	1750 ± 778
1f	N	C	$C(NH)NH_2$	H	—	1070 ± 325	45 ± 6	3100 ± 707
1g	N	C	$C(NH)CH_3$	H	—	5300 ± 266	1040 ± 14	29,000 ± 4240
1h	N	C	$C(O)CH_2NH_2$	H	—	7350 ± 3040	>30,000	4950 ± 2470
(−)**2a**	—	—	H	$NHC(NH)NH_2$	H	3 ± 1	1720 ± 239	6 ± 4
(rac)**2a**	—	—	H	$NHC(NH)NH_2$	H	4 ± 1	137 ± 15	3 ± 0.2
(+)**2a**	—	—	H	$NHC(NH)NH_2$	H	5 ± 1	36 ± 5	8 ± 2
2d	—	—	H	$NHC(NH)NH_2$	Cl	11 ± 3	4650 ± 81	7 ± 1
2e	—	—	H	NH_2	H	785 ± 353	>30,000	357 ± 110
2f	—	—	$C(NH)NH_2$	H	H	905 ± 695	845 ± 219	1000 ± 7070
3a	NH	CH_2	—	—	—	92 ± 33	4150 ± 354	420 ± 99
3b	NH	CH_2	—	—	—	129 ± 72	1950 ± 778	>30,000
3c[6c]	CH_2	NH	—	—	—	139 ± 52	29 ± 12	61 ± 25

[a] Values are mean of more than 2 experiments ± standard deviation. For assay descriptions see Ref. 11.

pure diastereomer 3-piperidine **3a** showed 45-fold selectivity against CPN.

A key discovery was replacing the guanidinopropyl chain of SQ-24,798 by an aryl guanidine (**2**). This 2-phenyl 3-mercaptopropionic acid series of inhibitors **2** yielded the most potent and CPN-selective TAFIa inhibitors; however, the series is equipotent against CPB. Both enantiomers of aryl guanidine **2a** show nanomolar potency against TAFIa with (−)**2a** being slightly more potent (TAFIa IC_{50} = 3 nM). (−)**2a** is about 270-fold more potent than aniline **2e** and 300-fold more potent than amidine **2f**. Compound (−)**2a** shows increased selectivity of nearly 600-fold over CPN, compared to (+)**2a** (7-fold). Introduction of a chloro substituent to aryl guanidine **2a** affords **2d**, a potent and very selective inhibitor against CPN (TAFIa IC_{50} = 11 nM, 420-fold selective against CPN).

X-ray structures of SQ-24,798, (−)**2a**, and **2d** bound to porcine pancreatic CPB reveal a common binding mode.[9] (Fig. 2) The thiol chelates the zinc, and the carboxylic acid and guanidine group form salt bridges to Arg145 and Asp255, respectively. These inhibitors are close mimics of arginine and probably bind in a manner similar to the natural substrates.

The high selectivity of (−)**2a** and **2d** for TAFIa over CPN could be explained based upon comparisons of our structures[9] to the published structure of carboxy-

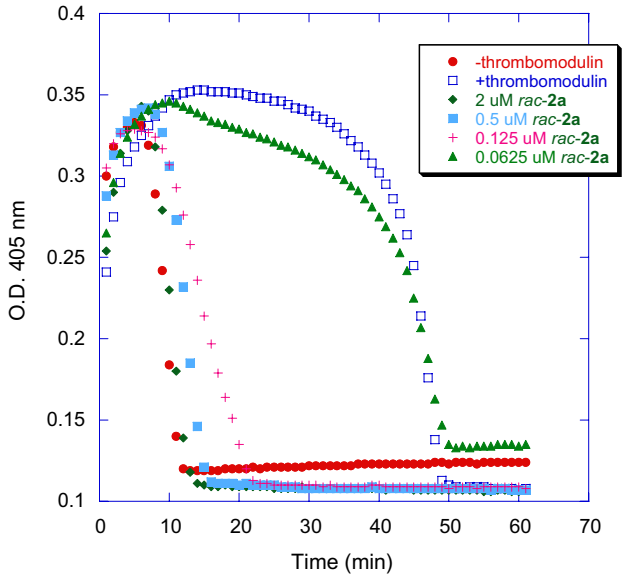


Figure 3. Clot lysis time exhibits dose dependent acceleration by (rac)**2a**.

peptidase D (CPD) bound with the related arginine mimic GEMSA.[10] CPD has 50% sequence homology with CPN and the homology is >87% in the active site. GEMSA binds Asp192 in CPD via terminal (N^{η}) guanidine nitrogen atoms. SQ-24,798, (−)**2a**, and **2d** bind Asp255 in CPB via internal (N^{ε})and terminal (N^{η}) guanidine nitrogen atoms. Since replacement of the alkyl chain with an aromatic ring eliminates the possibility of a terminal guanidine nitrogen binding conformation, the selectivity of our inhibitors may stem from the hydrogen bonds formed by the guanidine moiety.[9]

TAFIa inhibition by aryl guanidine (rac)**2a** was tested in an in vitro human plasma clot lysis assay to show that the inhibition of activated TAFI observed in the enzyme inhibition assay translated to an effect in human plasma.[3b] Addition of thrombin, thrombomodulin, and calcium to human plasma leads to activation of TAFI and subsequent prolongation of clot lysis time. Clot lysis was monitored by measuring turbidity at 405 nm. Lysis time is accelerated by (rac)**2a** in a dose dependent manner (Fig. 3).

An in vivo rabbit jugular vein thrombolysis model was used to test the hypothesis that TAFIa inhibition with (rac)**2a** in combination with tPA would enhance fibrinolysis.[3b] A segment of the jugular vein was isolated with ligatures and injected with a mixture of autologous citrated blood and thromboplastin to induce thrombus formation. Doses of tPA, CPI, (rac)**2a** or phosphate-buffered saline (PBS) vehicle were administered as *iv* bolus injections, followed by a constant infusion for 90 min via the right marginal ear vein. Fibrinolysis was assessed by measuring clot weight at the end of the study. An inefficacious sub-threshold dose of tPA was combined with either the TAFIa inhibitor (rac)**2a** or the peptidic TAFIa inhibitor CPI. CPI was used as a positive control to confirm the effect of TAFIa inhibition in this model.

Table 2. Efficacy of (rac)**2a** in rabbit jugular vein thrombolysis model

Dose[a] (mg/kg + mg/kg h)	Thrombus weight (mg)	SEM[b]	% reduction in thrombus wt
PBS	141	15	—
tPA	145	19	—
tPA + CPI	82	21	42[c]
tPA + (rac)**2a**	97	11	31[c]

[a] Dose: tPA = 0.01 mg/kg + 0.07 mg/kg h; CPI = 0.5 mg/kg + 0.3 mg/kg h; (rac)**2a** = 15 mg/kg + 15 mg/kg h; n = 8–9/group.
[b] Standard error of the mean.
[c] $p < 0.05$ Fisher's PLSD compared to vehicle and tPA.

When either CPI or (rac)**2a** was given together with the inefficacious sub-threshold dose of tPA, the effect of tPA was enhanced with a significant reduction in thrombus weights being observed when compared to the vehicle or tPA treated group (Table 2). The assessment of bleeding risk was evaluated by measuring the weight of blood loss from toe nail clips performed prior to thrombolytic treatment, and at the end of the study. No significant differences in bleeding were observed in any of the treatment groups. Thus, TAFIa inhibition with (rac)**2a** in combination with tPA enhances fibrinolysis in vivo.

In conclusion, the data shown describe the discovery of a series of cyclic, constrained arginine and lysine mimetics as potent and selective TAFIa inhibitors. Several inhibitors show significant selectivity against CPN and CPB. (rac)**2a** dose dependently accelerates clot lysis in human plasma in vitro and is efficacious in an in vivo thrombolysis model as an *iv* adjunct to tPA. These data provide further support for TAFIa inhibitors as a treatment for managing thrombosis in the clinic.

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