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Synthesis and Biological Evaluation of Menthol-Based Derivatives as Inhibitors of Plasminogen Activator Inhibitor-1 (PAI-1)

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Abstract—Compound **1** was identified by high throughput screening as a novel PAI-1 inhibitor. Optimization of the B and C-segments of **1** resulted in a series of structurally simplified compounds with improved potency. The synthesis and SAR data of these compounds are presented here.

Plasminogen activator inhibitor-1 (PAI-1) is a naturally occurring serine protease inhibitor, or serpin, that rapidly inhibits the activity of several proteases, including tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), by forming equimolar, irreversible complexes that are internalized and degraded.[1] PAI-1 plays a major role in preventing fibrinolysis by decreasing the activity of tPA and/or uPA, and consequently, the level of plasminogen converted to plasmin.[2] Plasmin is an enzyme critical to the lysis of fibrin clots and works by cleaving fibrin to small soluble products.[3] Clinically, PAI-1 is considered to be a thrombotic risk factor.[4] Elevated levels of PAI-1 have been described to correlate with an increased risk of deep vein thrombosis,[5] atherosclerosis,[6] unstable angina and myocardial infraction.[1,4] In addition, elevated levels of PAI-1 are also associated with a poor prognosis in cancer patients,[7] and PAI-1 is believed to play a role in angiogenesis,[8] cancer invasion,[8] and metastasis.[9] Thus, inhibition of PAI-1 would be expected to be of therapeutic benefit in a variety of cardiovascular and cancer diseases. To date, several PAI-1 inhibitors including antibodies,[10] peptides,[11] and small molecules[1,12] have been reported.

To develop small molecule PAI-1 inhibitors for the treatment of thrombosis, we established a protein-based PAI-1 assay.[13] High throughput screening of our compound library led to the identification of the lead compound **1** with an IC_{50} value of 1.4 μM. The lead **1** has a unique chemical structure, good potency, and moderate oral bioavailability. However, its complex chemical structure and poor aqueous solubility render it suboptimal as a drug candidate. Thus, an effort was made to simplify the structure and to improve the potency as well as the pharmacokinetic profile. Structurally, compound **1** can be divided into three segments designated as **A**, **B**, and **C** (Fig. 1). Optimization of the **B** and **C**-segments using a combined medicinal and combinatorial chemistry approach is detailed in this paper.

We started the optimization with the modification of the B-segment (Table 1). The synthesis of the series in Table 1 is exemplified by Scheme 1. Amidation of (−)-menthoxyacetyl chloride **2** with 4-nitrobenzylamine **3** under basic conditions afforded **4** in quantitative yield. Hydrogenation of **4**, followed by reductive-amination with commercially available Psoromic acid **6**, afforded compound **1** in 78% yield over two steps. The rest of analogues were prepared in a similar manner using different amines. Compared with **1**, shortening the B-seg-

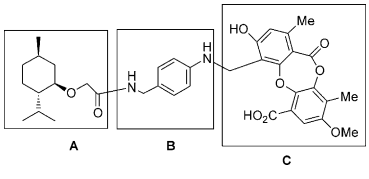

Figure 1. Lead compound **1** from library.

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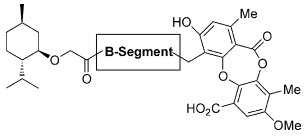
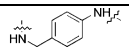
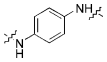
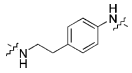
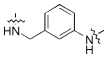
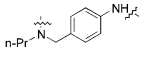
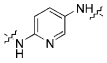
Table 1. Replacement of B-segment

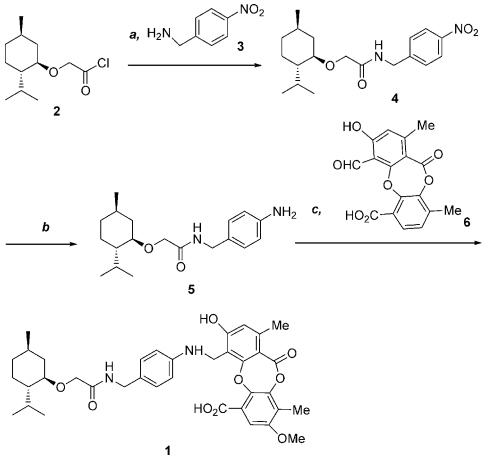


No.	Segment-B	IC_{50} (μM)[a]
1		1.4
7		1.1
8		3.9
9		1.4
10		5.0
11		0.53
12		>15

[a]IC_{50} values are averaged from multiple determinations ($n \geq 2$), and the standard deviations are <30% of the mean.

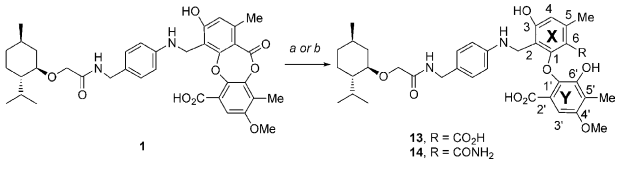
ment by one carbon retained potency (**7**), while lengthening by one carbon (**8**) decreased potency 3-fold. Changing the substitution pattern to *meta* (**9**) did not change potency, whereas blocking the hydrogen of the amide bond in **1** with *N*-propyl decreased potency 3-fold (**10**). The only modest improvement was found by inserting the heterocyclic spacer 2,5-pyridinediamine (**11**), which increased potency 2-fold. Non-aromatic spacers were also investigated, with most of the replacements decreasing potency. For this series, only one example (**12**) is listed, which is almost inactive.

We next explored the optimization of the C-segment. We initially did degradation studies on **1** to determine

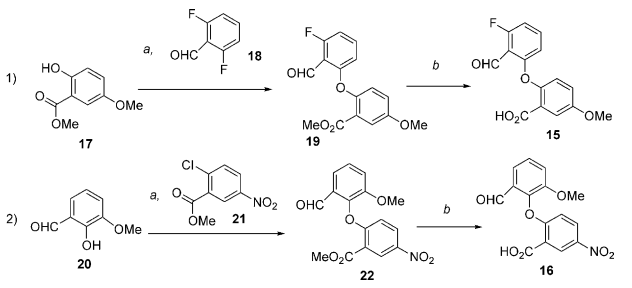


Scheme 1. Conditions: (a) Et_3N, CH_2Cl_2, **3**, 0 °C to rt, 2 h, 100%; (b) Pd/C, H_2, MeOH, rt, 2 h; (c) $NaBH_3CN$, MeOH, rt, 2 h, **6**, 78% in two steps.

which functional group of the Psoromic acid backbone is important to the inhibitory activity. Treatment of **1** with sodium methoxide and with ammonia afforded the ring-opened products, **13** and **14**, respectively (Scheme 2). The ring-opened products maintained similar potency to compound **1** (Table 2), indicating that diaryl ethers might be suitable C-segment replacements. For further elaboration of this series, we defined the two rings of the diaryl ether group as X and Y, and numbered the ring position as shown in Scheme 2. A series of simplified C-segment pieces (e.g., **15**–**16**) were designed and prepared (Scheme 3). Coupling of phenol **17** with difluorobenzaldehyde **18** under basic conditions afforded **19**, which was hydrolyzed to give **15**. Displacement of the chloride of **21** with phenol **20** under basic conditions, followed by saponification afforded **16**. The remaining analogues were prepared in a similar fashion. All the C-segment analogues were coupled with aniline **5** by reductive-amination to afford the compounds listed in Table 2. Most of these analogues (**24**–**31**) had potency similar to or better than **1**, except for the simple diphenyl ether analogue **23** which is devoid of a carboxylic group. Furthermore, the methyl or ethyl ester derivatives of the carboxylic acids (**24**–**31**) were inactive in the primary assay (data not shown), suggesting that an acidic group is essential for activity of this template. The need for an acid moiety is also consistent with the literature findings.[14] Compound **24** with a 2′-carboxylic acid and a 4′-methoxy on the Y-ring had the same potency as **1**, and introduction of fluoride at the 3-position of the X-ring (**25**) had no effect on potency. Compounds with a nitro group on the X- or Y-ring also had similar potency (**27**, **29**, and **30**). Improved potency was seen for the compounds with polar groups such as 6′-hydroxy (**26**) and 4′-amino group (**28**) on the Y-ring, and for a compound with a heterocycle as the Y-ring (**31**). Based on these results, we sought to test much simpler C-segments. Commercially available aromatic aldehydes were reacted with aniline **5** using solution phase parallel synthesis. The resulting library was tested without purification, and some of the compounds

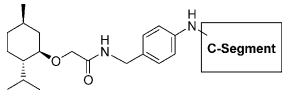


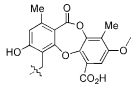
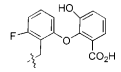
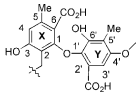
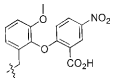
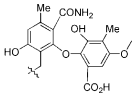
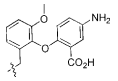
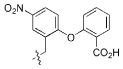
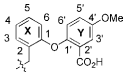
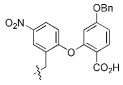
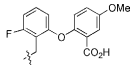
Scheme 2. Conditions: (a) NaOMe, MeOH, 0 °C to rt, 2 h, yielding **13** (57%); (b) $NH_3 \cdot H_2O$, THF, 50 °C, 1 h, yielding **14** (62%).



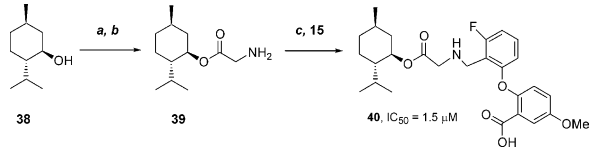
Scheme 3. Conditions: (a) DMSO, NaH, rt, 10 h, **18** or **21**; (b) LiOH, THF/H_2O, rt, 6 h.

Table 2. Simplified C-segment: diphenyl ether



No.	Segment-C	IC_{50} (μM)[a]	No.	Segment-C	IC_{50} (μM)[a]	No.	Segment-C	IC_{50} (μM)[a]
1		1.4	**26**		0.71	**32**		>15
13		1.8	**27**		1.2	**33**		>15
14		0.6	**28**		0.7	**34**		>15
23		>15	**29**		0.71	**35**		7.8
24		1.4	**30**		2.6	**36**		3.2
25		1.4	**31**		0.5	**37**		2.7

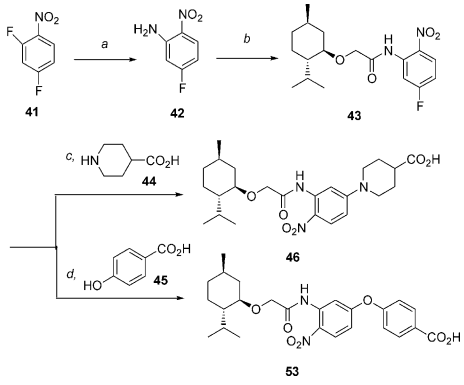
[a]IC_{50} values are averaged from multiple determinations ($n \geq 2$), and the standard deviations are $\leq 30\%$ of the mean.



Scheme 4. Conditions: (a) EDC, DMAP, *N*-Boc-glycine, CH_2Cl_2, rt, overnight; (b) TFA, CH_2Cl_2, rt, 2 h; (c) $NaBH(OAc)_3$, **15**, $ClCH_2CH_2Cl$, rt, 3 h.

showed moderate inhibitory activity. Table 2 lists some typical examples (**32**–**37**) which were re-synthesized, purified, and re-tested. Basically, neutral (**32**), basic (**33**) and strong acidic (**34**) analogues were detrimental to potency. Acid (**35** and **36**) and phenol (**37**) substituents reduced potency 2- to 5-fold.

After finding that the C-segment could be simplified without a significant loss in potency, we looked at simplifying the B and C fragments together. Reductive-amination of aldehyde **15** with amine **39** prepared from (−)-menthol **38** (Scheme 4) afforded compound **40** with an IC_{50} value of 1.5 μM, which has equal potency as compound **25**. Although the structure of compound **40** did not look like an exact deletion of the B-segment (4-amino-benzylamine) of compound **25**, it appeared that the B-segment may not be necessary for the potency. Therefore, intermediate **43** was prepared (Scheme 5), because a large number of analogues can be prepared by simple displacement of the 5-fluoride with different nucleophiles. Since the carboxylic acid group is essential for the potency of this template, only necleophiles containing acid were selected. Most of the amino-acid

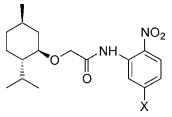


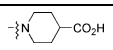
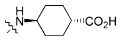
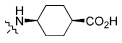
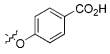
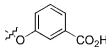
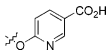
Scheme 5. Conditions: (a) NH_3, −78 °C to rt, 12 h; (b) DIEA, DMAP, CH_2Cl_2, **2**, 0 °C to rt, 2 days, 44%; (c) CH_3CN, **44**, reflux, 4 h, K_2CO_3, 80%; (d) NaH, DMSO, **45**, 80 °C, 2 h, 78%.

adducts (**46**–**52**) maintained potency (Table 3). Compounds **49**, **50**, and **51** were a mixture of diastereomers since they were prepared from racemic amino-acids. Additionally, 3- and 4-hydroxybenzoic acid, and 6-hydroxy-3-pyridinecarboxylic acid were investigated (**53**–**55**) because the corresponding final products contain a diphenyl ether acid moiety, which was thought to be an important functional group for potency. Interestingly, compared with **1**, compounds **53** and **54** have 4-fold higher potency, while **55** is about 2-fold less potent. In contrast to the case of **31**, changing from benzene (**53**) to pyridine (**55**) caused a decrease in potency.

We evaluated most of these compounds in a functional clot lysis assay,[15] and explored their pharmacokinetic

Table 3. Simplified analogues



No.	X	IC_{50} (μM)[a]
46		0.94
47		1.1
48		0.9
49		0.89
50		2.9
51		2.2
52		2.9
53		0.38
54		0.40
55		2.6

[a]IC_{50} values are averaged from multiple determinations ($n \geq 2$), and the standard deviations are $<30\%$ of the mean.

properties in rat. Table 4 shows the data for the selected compounds. Compound **27** has equal potency as **1** in both the primary and functional assays, but its oral bioavailability (F%) in rat was improved from 23 to 94%. Compounds **46** and **53** are 10-fold more potent than **1** in the functional assay, and **53** has the same level of oral bioavailability as **1**. It is interesting to note that **54** is 10-fold less potent than **53** in the functional assay even though they are equally potent in the primary assay. The reason for this discrepancy remains unclear. Compounds **46** and **54** both showed low plasma level when dosed orally.

In summary, we have explored structure–activity relationships around the novel PAI-1 inhibitor **1** by modification of the B- and C-segments. The B-segment could be replaced with a variety of aromatic diamines without loss of potency, but aliphatic diamines were not tolerated. Efforts to optimize the C-segment resulted in the identification of a series of novel diphenyl ether carboxylic acid derivatives having improved potency, moderate to good functional activity, and oral bioavailability. Further simplification led to the discovery of the most potent inhibitor **53** in both the primary and functional assays. Optimization of the A-segment will be reported in due course.

Table 4. Selected data for PK and different assay

No.	IC_{50} (μM)[a]		F% (rat)[b]
	Primary	Functional	
1	1.4	0.1	23
27	1.2	0.1	94
46	0.94	0.01	<10[c]
53	0.38	0.01	24
54	0.40	0.1	<10[c]

[a]IC_{50} values are averaged from multiple determinations ($n \geq 2$), and the standard deviations are $<30\%$ of the mean.
[b]Compounds were dosed by IV/PO in cannulated conscious rats with a dose amount of 2 mg/kg. 94% PEG/2% 0.1HCl/4% EtOH solution was used as a vehicle.
[c]Below limit of detection.

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13. **PAI-1 primary assay**: PAI-1 activity was determined by its inhibition on the activity of uPA. Assays were performed in 96-wells microtiter plates at room temperature in a total volume of 200 μL. Human recombinant PAI-1 (6 nM, Molecular Innovation) was incubated with compounds for 2 min in assay buffer containing 50 mM TrisHCl, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% polyethylene glycol 6000, pH 7.5. Human kidney urokinase (0.0175 units/mL; Sigma) was then added and incubated for another 2 min. Reactions started by addition of a chromogenic peptide substrate, S2444 (32 μM; Diapharma). Reaction rates were determined by measuring the rate of the absorbance change at 405 nm in a ThermoMax microplate reader (Molecular Devices). Controls without compounds and with uPA only or with uPA plus PAI-1 were assayed in the same plate. IC_{50} values for the inhibitors were determined from the dose response curve by fitting the data to the Hill equation with an automated analysis method using a computer spreadsheet.

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15. **Plasma clot lysis assay**: A plasma clot lysis assay was also used to evaluate the potency of small molecule PAI-1 inhibitors. In this assay, pooled human plasma was diluted (1:3) in a buffer containing 150 mM NaCl, 2 mM $CaCl_2$, 20 mM Hepes, pH 7.4. Fibrin clot formation was initiated by addition of human thrombin (30 nM). The newly formed fibrin clot remained stable at 37 °C for at least 2 h. If exogenous human tPA (4 nM) was included in the assay, the fibrin clot will be lysed within 20 min at 37 °C, which was monitored by optical absorbance at 405 nm. In the presence of recombinant human PAI-1 (4.3 nM), the activity of tPA was reduced and clot lysis time was delayed. When both PAI-1 and PAI-1 inhibitors were added, the PAI-1 activity was significantly inhibited, as demonstrated by the shortened clot lysis times.