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Short communication

In vitro PAI-1 inhibitory activity of oxalamide derivatives☆

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Abstract

A number of oxalamide derivatives have been synthesized and evaluated for PAI-1 inhibitory activity. *In vitro* PAI-1 inhibitory activities of oxalamide derivatives are evaluated by chromogenic assay. Few compounds have shown significant PAI-1 inhibitory activity.

Keywords: PAI-1; Oxalamide; Thrombosis; tPA; uPA

1. Introduction

The activity of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) is negatively regulated by a serine protease inhibitor (SERPIN) named plasminogen activator inhibitor-1 (PAI-1) [1]. Platelets contain high amount of PAI-1 [2,3] and release of PAI-1 from activated platelets may lead to its high local concentration, which may be responsible for higher incidence of tPA resistant thrombus formation [4–6]. PAI levels are reported to be elevated in various thrombotic disorders including deep vein thrombosis (DVT) [7,8] and other diseases like diabetes [9,10], obesity [11,12], and syndrome 'X' [11,13]. All of these disorders are associated with an increased risk of systemic thrombosis; therefore, inhibition of PAI-1 may represent a useful strategy for treating thrombotic diseases. This hypothesis is supported by studies suggesting that transgenic mice expressing high levels of PAI-1 develops spontaneous thrombosis [14,15], whereas PAI-1 knockout mice are resistant to venous or arterial thrombosis [16,17].

Recently, several classes of small molecule PAI-1 inhibitors have been reported [18], such as menthol based inhibitors **1** [19], piperazine analogues **2** [20], and indole oxoacetic acid **3** [21] (Fig. 1).

These inhibitors are found to show good in vitro PAI-1 inhibitory activity and are under different stages of preclinical/clinical development [20]. The process of finding a better inhibitor by high throughput screening of various compound libraries having carboxylic acid functionality culminated in the identification of oxalamide derivative **4** (Fig. 2) that showed an *in vitro* IC_{50} value of 96 μM in PAI-1 inhibitory assay.

Taking oxalamide derivative **4** as the suitable candidate for modification, several compounds **9** and **15** having carboxylic acid functionality were synthesized [22] and evaluated for their PAI-1 inhibitory activity in chromogenic assay [23] (Fig. 3). The PAI-1 protein and ligand interaction was also studied by Native PAGE (Poly Acrylamide Gel Electrophoresis) experiment.

2. Chemistry

The oxalamide derivatives **4**, **9** and **15** were prepared as shown in Schemes 1 and 2. The aminobiphenyl intermediate **6** was prepared by Suzuki coupling of 4-trifluoromethoxyphenyl boronic acid with 4-nitro-iodobenzene followed by reduction of $-NO_2$ group, which upon reductive amination with substituted aldehyde produced **7**. The reaction of **7** with methyl chlorooxoacetate furnished compound **8**. The conventional

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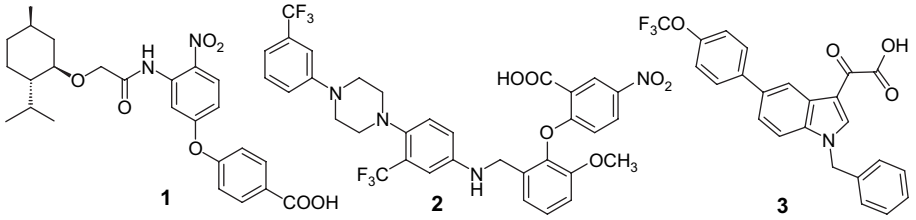


Fig. 1. PAI-1 inhibitors.

alkaline hydrolysis of compound **8** provided **9** (Scheme 1). Controlled reaction of substituted sulfonyl chloride **10** with *p*-phenylenediamine gave sulfonamide derivative **11**. The reductive amination of sulfonamide derivative **11** with 3,5-bistrifluoromethyl benzaldehyde gave **12**. The reaction of **12** with methyl chlorooxoacetate yielded oxalamide ester **13**. The alkylation of **13** with substituted alkyl halide provided compound **14** and subsequent alkaline hydrolysis of compound **14** gave **15** (Scheme 2).

3. Results and discussion

The PAI-1 inhibitory activity of oxalamide derivatives **9a**–**9d** and **15a**–**15t** is summarized in Tables 1 and 2.

The compound **4** inhibited PAI-1 activity with an IC_{50} of 96 μM in chromogenic assay [23], hence compound **4** was selected for further modification in region 1 and region 2 (Fig. 2), which gave compounds **15a**–**15t** and **9a**–**9d** respectively (Fig. 3).

The first synthesized compounds *i.e.* 3-methanesulfonate phenyl derivative **9a** and pyridyl derivative **9b** did not show PAI-1 inhibitory activity in the chromogenic assay. Introduction of an electron-withdrawing and bulky trifluoromethyl group on phenyl ring **9c** showed considerable improvement in the activity with an IC_{50} of 23 μM. Additional increase in bulk by introducing trifluoromethyl group on **9c** gave 3,5-bistrifluoromethyl phenyl derivative **9d**, which displayed even better PAI-1 inhibitory activity with an IC_{50} 14.4 μM (Table 1). Based on these results, 3,5-bistrifluoromethyl-substituted phenyl ring in region 2 was finalized.

Furthermore, the modification of region 1, we introduced $-SO_2NR^2-$ as a spacer group between two phenyl rings (Table 2). Substitution of electron releasing groups at the *para* position of the phenyl ring (R^3 of region 1) such as chloro (**15a**) and methoxy (**15b**) produced compounds with low PAI-1 inhibitory activity. The compounds containing electron-withdrawing groups such as trifluoromethyl and trifluoromethoxy groups on phenyl ring demonstrated good PAI-1 inhibitory activity [20,21]. Taking clue from the literature, first trifluoromethoxy group was introduced on the phenyl ring, which resulted in compound **15d** and it inhibited PAI-1 activity with IC_{50} 9.3 μM. However, both positional isomers **15e** (*meta*) and **15f** (*ortho*) displayed inferior inhibitory activity than **15d** (*para*). Introduction of a less bulky electron-withdrawing fluoro substituent on phenyl ring produced compound **15c** with low PAI-1 inhibitory activity.

In order to see the effect of substituents at free H of sulfonamide group (R^2 of region 1), various compounds were synthesized in which H atom was replaced by methyl in **15g**, propyl in **15h**, pentyl in **15i** and allyl in **15j**. Results of PAI-1 assay indicate that bulky alkyl groups help in improving activity, as **15g** is less active than **15h**, which in turn is less active than **15i**. Compound **15j** showed similar activity to that of **15i**. However, benzyl substituted compound **15k** showed inferior activity. Introduction of 4-OCF_3 group on benzyl ring of **15k** produced compound **15l** with very good activity (IC_{50} 8.5 μM), which may be due to combined interaction of two trifluoromethoxy groups.

Next, compounds containing trifluoromethyl group on phenyl ring were synthesized. Introduction of 4-CF_3 group on phenyl ring (R^3 of region 1) produced very less potent compound **15m** with IC_{50} of 86 μM. Substitution of methyl group on sulfonamide linker of **15m** gave compound **15n** with marginally improved activity (IC_{50} 61 μM). Further changing the position of $-CF_3$ group from *para* to *meta*, **15o** showed dramatic improvement in potency (IC_{50} 4.5 μM). Substitution on sulfonamide linker of **15o** to get **15p** and **15q** also gave good compounds. The compound **15q** was found to be as

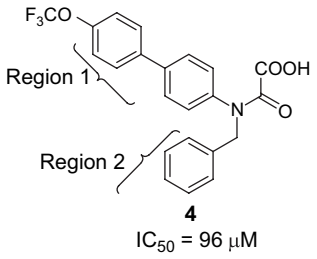


Fig. 2. Lead compound from library.

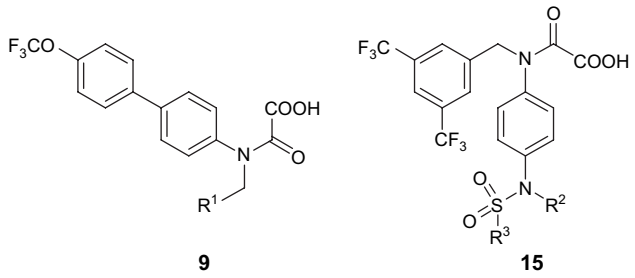
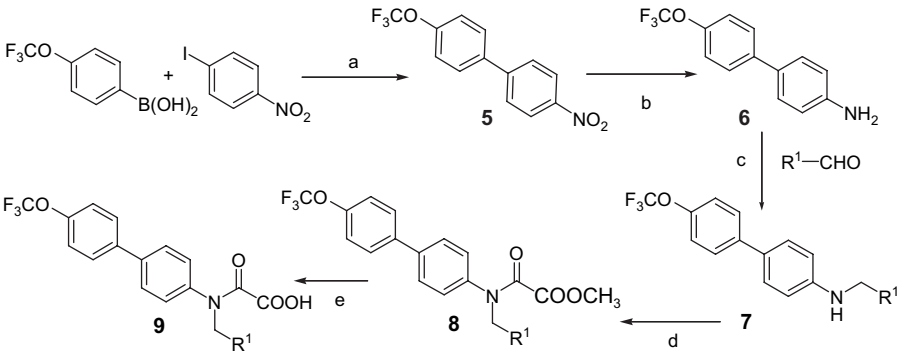
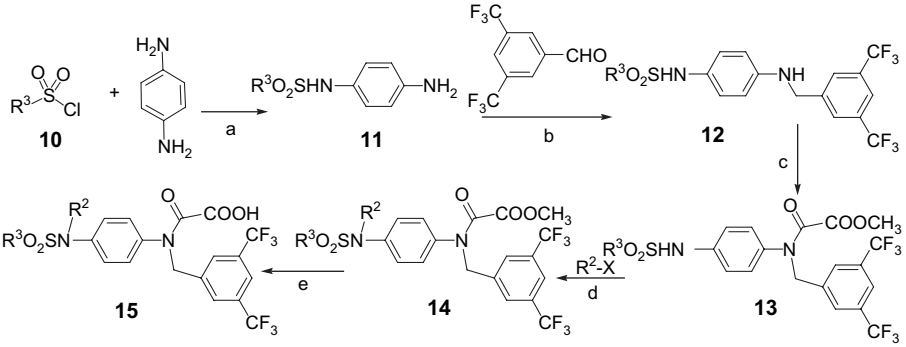


Fig. 3. General structure derived from **4**.



Scheme 1. Reagents and conditions: (a) $Pd(OAc)_2$, K_3PO_4, $(C_4H_9)_4N^+Br^-$, DMF, 45–50 °C, 16 h, 65%; (b) Pd–C, H_2 (60 psi), MeOH, 25–28 °C, 3 h, 90%; (c)$NaBH_4$, EtOH, 40–45 °C, 4–5 h, 80%; (d) ClCOCOOMe, pyridine, CH_2Cl_2, 5–10 °C, 3 h, 85% and (e) KOH, THF, H_2O, 25–28 °C, 80%.



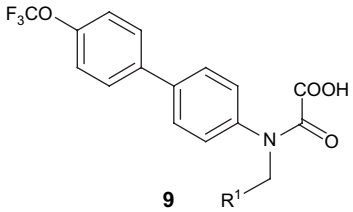
Scheme 2. Reagents and conditions: (a) DIPEA, CH_2Cl_2, 25–28 °C, 15–30 min, 75%; (b) $NaBH_4$, EtOH, 40–45 °C, 4–5 h, 80%; (c) ClCOCOOMe, pyridine, CH_2Cl_2, 5–10 °C, 3 h, 85%; (d) K_2CO_3, acetone, 25–28 °C, 2–10 h, 80%; (e) KOH, THF, H_2O, 25–28 °C, 80%.

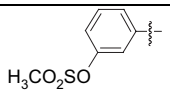
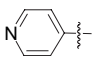
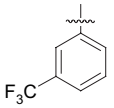
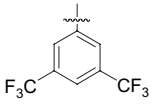
potent as **15o** in PAI-1 inhibitory activity. Compound **15r** with 3,5-bistrifluoromethyl-substituted ring (R^2 of region 1) showed a very promising PAI-1 inhibitory activity with an IC_{50} of 5 μM. However, changing methyl group from sulfonamide linker (R^2 of region 1) of **15r** with bulky groups allyl (**15t**) and propyl (**15s**) lead to compounds with mediocre *in vitro* activity. This is in contrast to the observation in compounds **15g–15l**, which may be due to crowding of alkyl groups with *meta*-CF_3 group. In Native PAGE (Poly Acrylamide Gel Electrophoresis) experiment, we observed that PAI-1-ligand complex stays above the free PAI-1 (gel picture is not given). The intensity of the PAI-1-ligand complex in Native PAGE and unavailability of free PAI-1 after ligand interaction clearly demonstrates that high concentration of the ligand does not induce any PAI-1 inactivation.

4. Conclusion

In summary, we have explored the structure–activity relationships around the PAI-1 inhibitor **4** by modification of the regions 1 and 2. It was observed that regions 1 and 2 accommodate electron-withdrawing bulky groups on phenyl ring with enhancement of potency, but electron releasing groups in region 1 and polar groups in region 2 were not tolerated. Introduction of sulfonamide spacer retained its inhibitory

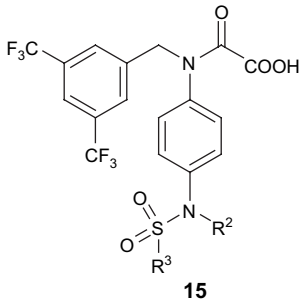
Table 1
PAI-1 inhibitory activity of oxalamide derivatives **9a–9d**



Compound	R^1	IC_{50} (μM)[a]
9a		No inhibition
9b		No inhibition
9c		23
9d		14.4
3 Tiplaxtinin	–	10

[a] Values determined using *in vitro* chromogenic assay.

Table 2
PAI-1 inhibitory activity of oxalamide derivatives **15a–15t** with sulfonamide spacer



15

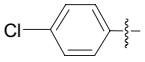
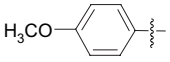
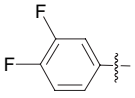
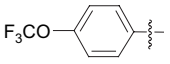
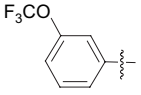
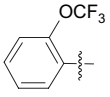
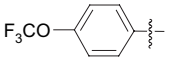
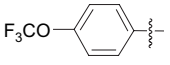
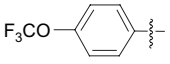
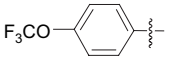
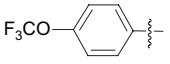
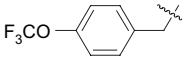
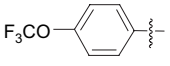
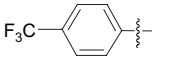
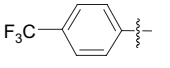
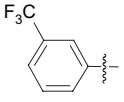
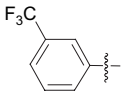
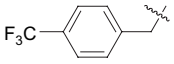
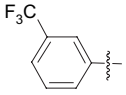
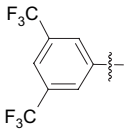
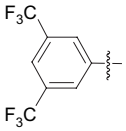
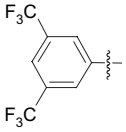
Compound	R^2	R^3	IC_{50} (μM)[a]
15a	H		75
15b	H		>80
15c	H		29.9
15d	H		9.3
15e	H		15.4
15f	H		114
15g	CH_3		25
15h	C_3H_7		21
15i	C_5H_{11}		14.9
15j			13.3
15k	Bn		43
15l			8.5
15m	H		86
15n	CH_3		61
15o	H		4.5

Table 2 (*continued*)

Compound	R^2	R^3	IC_{50} (μM)[a]
15p			11
15q			5.4
15r	CH_3		5
15s	C_3H_7		8.4
15t			12.8
3 Tiplaxtinin	–	–	10

[a] Values determined using *in vitro* chromogenic assay.

activity. Substitution at sulfonamide group with bulky alkyl groups and benzyl group substituted with electron-withdrawing groups resulted some good compounds **15l**, **15o**, **15q**, **15r** and **15s** with good potency.

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[22] Characterization data: **9d**. 96.5% purity by HPLC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.04 (s, 1H), 7.9 (s, 2H), 7.78 (d, $J = 8.74$ Hz, 2H), 7.73 (d, $J = 8.49$ Hz, 2H), 7.44 (d, $J = 8.19$ Hz, 2H), 7.33 (d, $J = 8.43$ Hz, 2H), 5.19 (s, 2H); **15l**. 98% purity by HPLC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.91 (bs, 3H), 7.64 (d, $J = 8.82$ Hz, 2H), 7.50 (d, $J = 8.34$ Hz, 2H), 7.31 (d, $J = 8.18$ Hz, 2H), 7.1–7.18 (m, 4H), 6.99 (d, $J = 8.72$ Hz, 2H), 5.0 (s, 2H), 5.0 (s, 2H); **15r**. 98.8% purity by HPLC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.49 (s, 1H), 7.92 (bs, 3H), 7.74 (bs, 2H), 7.24 (bs, 2H), 7.06 (d, 2H), 5.07 (s, 2H), 3.1 (s, 3H). The product formation was supported by ESI-MS: 695.2 (M − H). **15o**. 98% purity by HPLC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.58 (bs, 1H), 7.94 (m, 4H), 7.75 (m, 3H), 7.11 (d, 2H), 6.98 (d, 2H), 5.0 (s, 2H). The product formation was supported by ESI-MS: 613.1 (M − H). **15q**. 98.5% purity by HPLC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.0 (bs, 2H), 7.8 (m, 4H), 7.6 (m, 3H), 7.48 (d, 2H), 7.14 (m, 4H), 5.06 (s, 2H), 4.87 (s, 2H). The product formation was supported by ESI-MS: 771.3 (M − H).
[23] Chromogenic assay: *in vitro* PAI-1 inhibitory activity of compounds was determined using chromogenic assay that was based upon the interaction between tPA and active PAI-1. tPA coated assay plates obtained from Trinity Biotech., NY, USA were kept at 4 °C. Required quantity of phosphate buffer containing EDTA and tween 20 was added in each well and incubated for 2 min at 27–28 °C with gentle shaking in order to dissolve tPA. Oxalamide derivatives were dissolved in DMSO and diluted to a range of concentration between 1 and100 μM. Varying concentrations oxalamide derivatives were then incubated with human PAI-1 (50 nM, Molecular Innovations, MI, USA) for 30 min at 25 °C. An aliquot of this solution along with a monoclonal antibody against human PAI-1 conjugated with HRP (Trinity Biotech., NY, USA) was added to the t-PA-coated plate. The Plate was then incubated for 30 min at 27–28 °C with gentle shaking. The solution was aspirated from the plate, which was then washed thrice with a buffer consisting of 0.05% tween 20 and 0.1% BSA in PBS. Aliquot of 100 μL of HRP substrate solution was added and incubated for 5 min at 25 °C. Reaction was terminated with the addition of 50 μL of 1.6 M H_2SO_4 followed by the determination of absorbance at 490 nm. This assay detects only active inhibitory PAI-1 (not latent or substrate) bound to the plate. The quantitation of residual active PAI-1 bound to t-PA at varying concentrations of oxalamide derivative was used to determine the IC_{50} by fitting the results to a logistic dose-response program (Graphpad Prism, CA, USA). IC_{50} was defined as the concentration of compound required to achieve 50% inhibition of PAI-1 activity. The assay sensitivity was 5 ng/mL of human PAI-1 as determined from a standard curve ranging from 0–100 ng/mL of human PAI-1.