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Discovery of inhibitors of plasminogen activator inhibitor-1: Structure–activity study of 5-nitro-2-phenoxybenzoic acid derivatives ☆

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ABSTRACT

Two novel series of 5-nitro-2-phenoxybenzoic acid derivatives are designed as potent PAI-1 inhibitors using hybridization and conformational restriction strategy in the tiplaxtinin and piperazine chemo types. The lead compounds **5a**, **6c**, and **6e** exhibited potent PAI-1 inhibitory activity and favorable oral bioavailability in the rodents.

Plasminogen activator inhibitor-1 (PAI-1), a member of serpin (serine protease inhibitor) superfamily, prevents the formation of plasmin by inhibiting the activity of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), the key enzymes

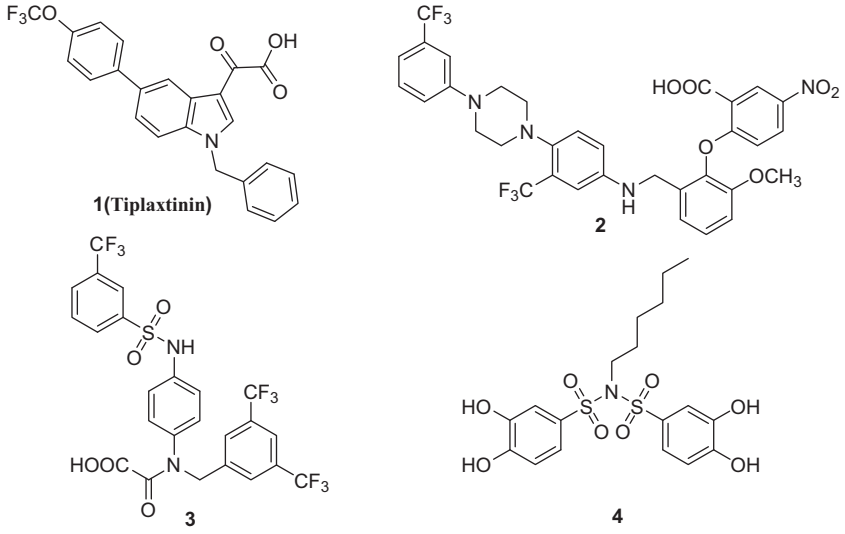

Figure 1. Structures of known PAI-1 inhibitors.

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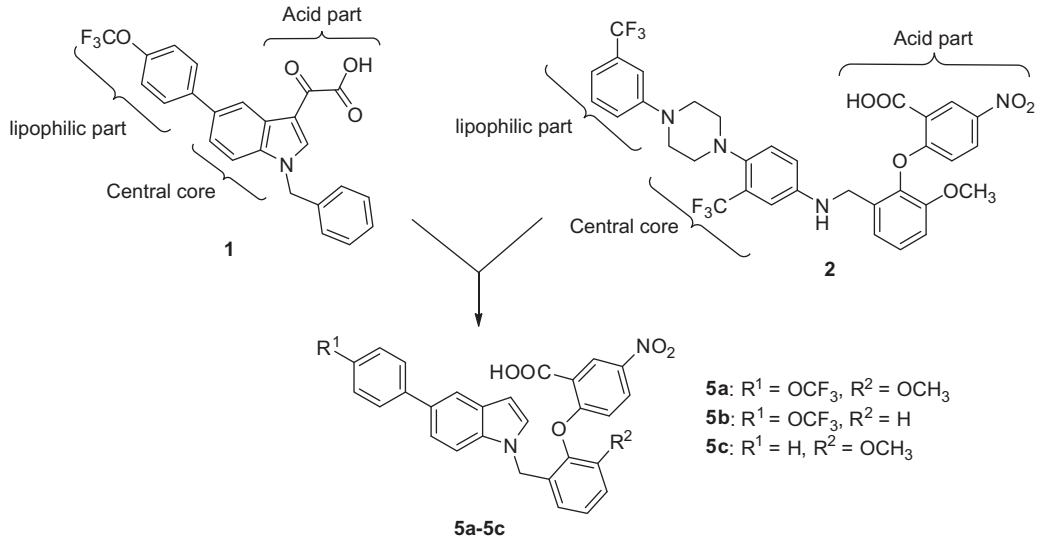
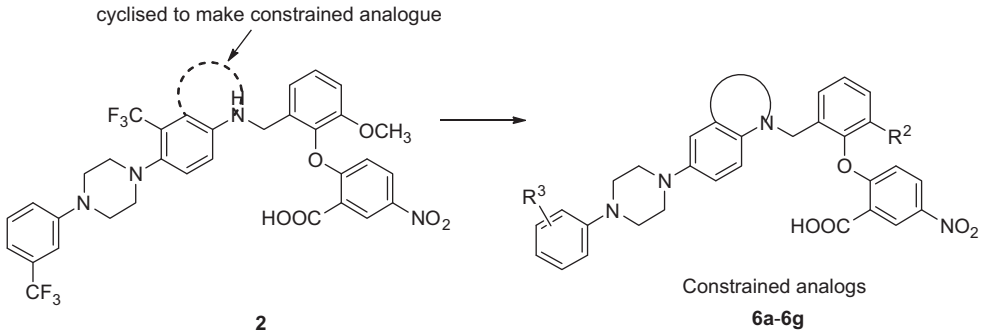
whose proteolytic action converts plasminogen to plasmin.[1] Plasmin dissolves fibrin clots by degrading insoluble fibrin molecules to small soluble fragments. Deficiency of PAI-1 in humans results in a hyperfibrinolytic state suggesting its important role in the fibrosis.[2–4] PAI-1 has been reported to have its implications in various patho-physiological processes, such as cancer,[5] diabetic nephropathy,[6] obesity,[7] metabolic syndrome,[8] venous thrombosis,[9] and atherosclerosis.[10,11] The therapeutic potential of PAI-1 inhibitors has been reviewed recently.[12] Several small molecules have been identified using the concept of the high throughput screening or virtual screening.[13] The most studied PAI-1 inhibitor tiplaxtinin **1** could reach up to Phase I.[14] Consistent research efforts resulted in the development of inhibitors with improved potency over tiplaxtinin exemplified by piperazine derivative **2**,[15] and aryl sulfonamide derivative **4**[16] (Fig. 1). There are few PAI-1 inhibitors which, demonstrated good antithrombotic efficacy in the various preclinical models however, none of them could advance to clinic.[17–26]

As a part of our research endeavor to develop viable therapies for the treatment of thrombotic diseases, we have previously reported the structure-activity relationship of a series of oxalamide derivatives **3**[27] (Fig. 1) identified by highthroughput screening of our compound library. However, none of those oxalamide derivatives could be further evaluated due to their poor oral bioavailibility. In continuation of our efforts to identify potent and orally bioavailable PAI-1 inhibitors, we further opted hybridization and conformational restriction strategies using known chemotypes. In order to achieve this objective, we selected tiplaxtinin **1**(IC_{50} = 2.7 μM, Lit. value),[14] and piperazine derivative **2**, with potent PAI-1 inhibitory activity (IC_{50} = 0.5 μM, Lit. value).[15] The known PAI-1 inhibitors reported mostly possess a carboxylic acid

Strategy 1



Strategy 2



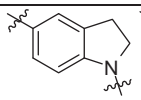
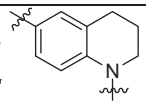
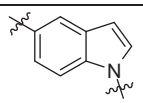
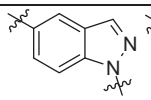
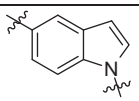
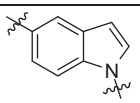
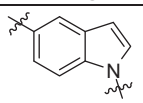
	6a	6b	6c	6d	6e	6f	6g
Central ring							
R^2	H	H	H	H	OCH_3	OCH_3	H
R^3	*m*-CF_3	*m*-CF_3	*m*-CF_3	*m*-CF_3	*m*-CF_3	*p*-CF_3	H

Figure 2. Strategies for the synthesis of PAI-1 inhibitors.

or an acid equivalent group attached to a lipophilic aromatic ring as a key structural feature. Tiplaxtinin **1** contains indole oxoacetic acid scaffold and published SAR[14] suggests importance of 4-triflorometho xyphenyl group (lipophilic part) and also its position on indole. Piperazine derivative **2** contain 5-nitro-2-phenoxybenzoic acid part as an acid group, which was found to be optimum after employing various acid groups.[15] We thus proceeded and designed the compounds by incorporating the acid part of **2** in the **1** as a probable replacement of oxoacetic acid group of **1,** to get the hybridized molecules **5a**–**5c** (Fig. 2, strategy 1). Further, rational has been derived from docking study[28] of **5a** and tiplaxtinin, which reveled that both compounds possess similar orientation in the ligand binding pocket of PAI-1. The H bond interaction of carboxylic group of **5a** with Arg118 was found to be the key interaction (Fig. 3). As an alternative strategy, we intended to make the constrained analogues of **2** and subsequently few analogue **6a**–**6g** (Fig. 2, strategy 2) were synthesized. The compounds **5a**–**5c** and **6a**–**6g** were evaluated for their PAI-1 inhibitory activity (Tables 1 and 2). The pharmacokinetics parameters[29] of the potent compounds **5a**, **6c**, and **6e** were studied in the male wistar rats (Table 3).

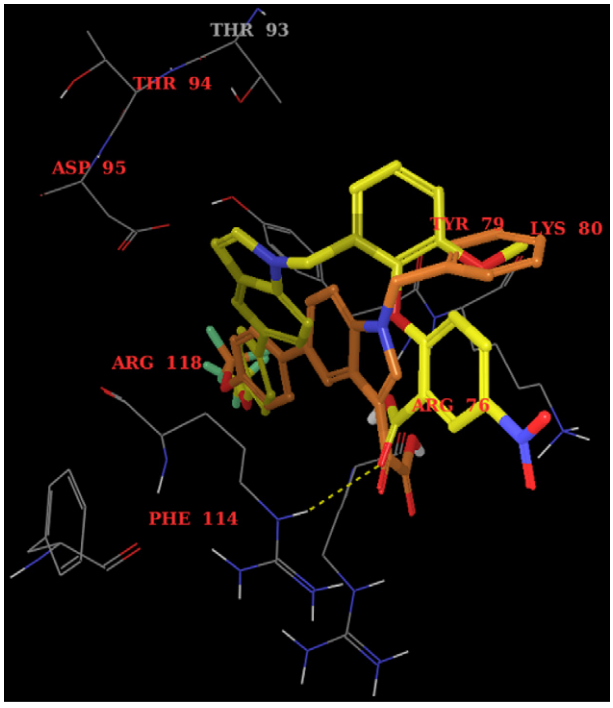
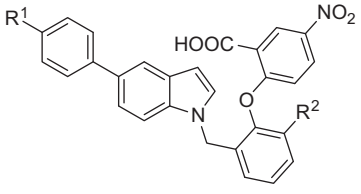


Figure 3. Overlay of docking images of tiplaxtinin (orange) and **5a** (yellow) into active site of PAI-1: H bond interaction of **5a** with Arg118 is shown in dashed line.

Table 1
PAI-1 inhibitory activity of compounds **5a**–**5c**



Compound	R^1	R^2	IC_{50}[a] (μM)
5a	OCF_3	OCH_3	3.4
5b	OCF_3	H	4.9
5c	H	OCH_3	98
Tiplaxtinin (**1**)			14.8

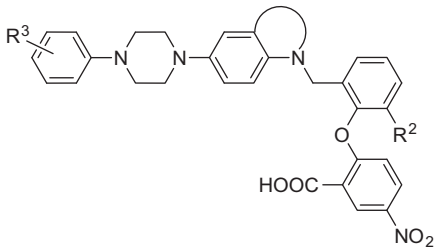
[a] Values determined using in vitro chromogenic assay.

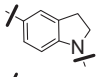
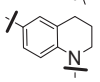
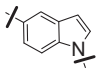
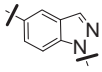
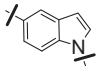
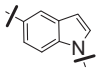
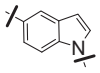
The compounds **5a–5c** were prepared as shown in the Scheme 1. The salicylaldehyde derivative **8** was reacted with methyl 2-chloro-5-nitrobenzoate **7** using NaH as a base to give diphenyl ether derivative **9**, which upon reduction with sodium borohydride followed by bromination with PBr_3 afforded the bromo derivative **10**. The BOC protected 5-bromoindole **11** was reacted with the appropriate boronic acids **12** under Suzuki coupling[30] reaction conditions to afford the indole derivative **13** after the BOC deprotection using the TFA. The coupling of indole derivative **13** with the **10** in presence of *t*-BuOK provided the ester derivative **14**, which upon basic hydrolysis with KOH afforded the target compounds **5a**–**5c**.

The compounds **6a**–**6g** were prepared as depicted in the Scheme 2. The piperazine derivative **15** was coupled with the BOC protected heterocycle **16** using $Pd(OAc)_2$ as a catalyst and BINAP as a ligand to give compound **17**,[31] which was subsequently deprotected using either TFA or concd sulfuric acid to furnish **18**. The coupling of **18** with the halogen derivative **10** in presence of *t*-BuOK or K_2CO_3 as a base provided the ester derivative **19**. The basic hydrolysis of derivative **19** with KOH afforded the compounds **6a**–**6g**.

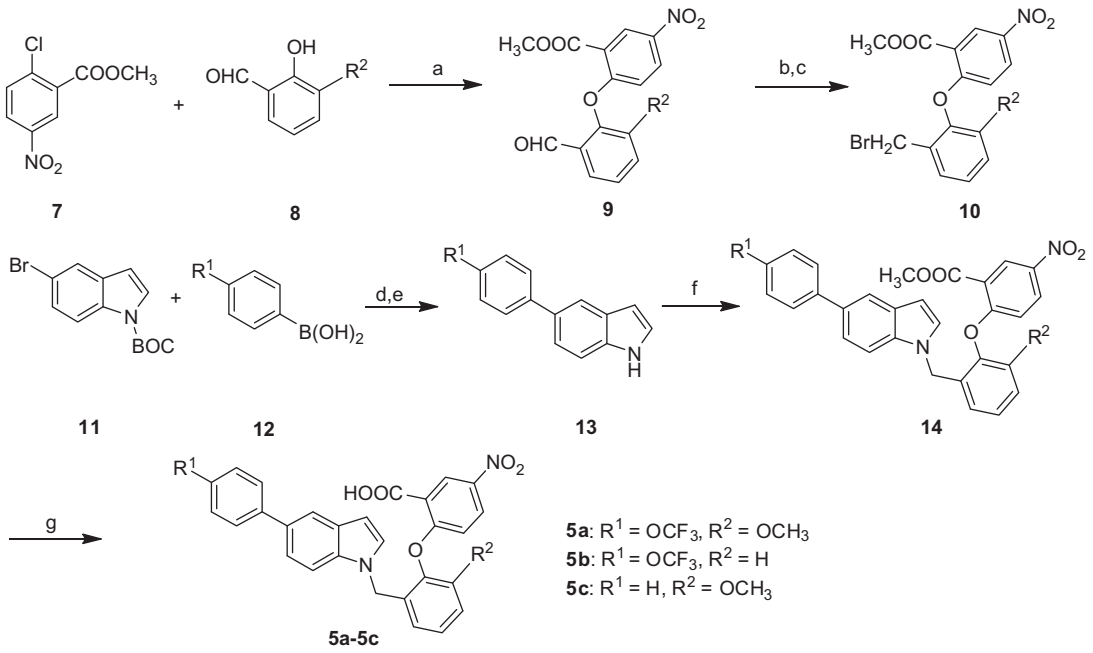
All the compounds **5a**–**5c** and **6a**–**6g** synthesized[32] were evaluated for their in vitro PAI-1 inhibitory activities[33] (Tables 1 and 2). The hybridized derivative **5a** synthesized as a part of strategy 1,

Table 2
PAI-1 inhibitory activity of compounds **6a**–**6g**

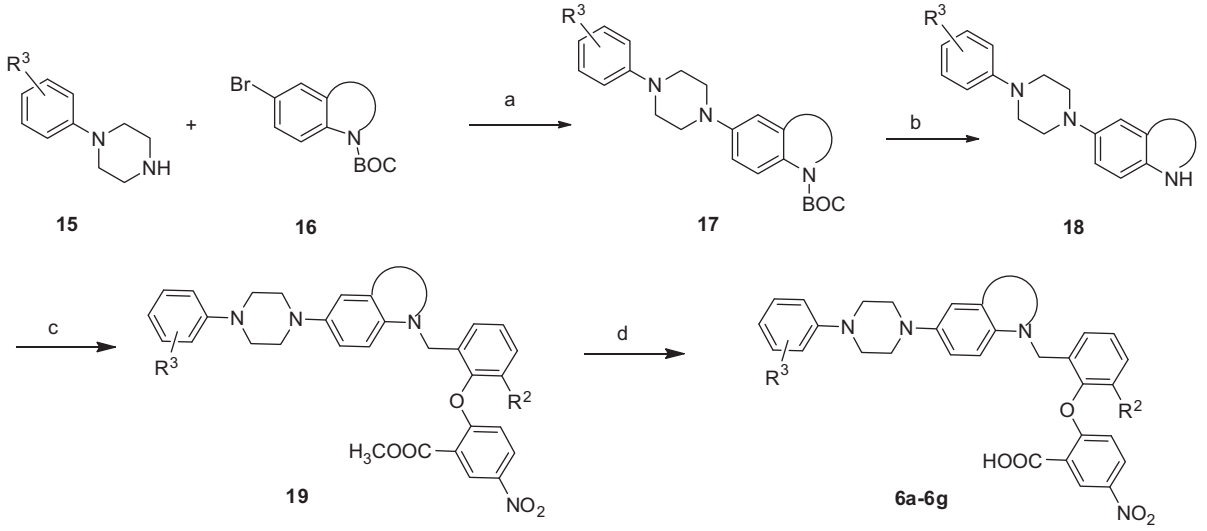


Compound	R^2	R^3	Central ring	IC_{50}[a] (μM)
6a	H	*m*-CF_3		29
6b	H	*m*-CF_3		63.8
6c	H	*m*-CF_3		3.2
6d	H	*m*-CF_3		14.6
6e	OCH_3	*m*-CF_3		2.4
6f	OCH_3	*p*-CF_3		22
6g	H	H		No inhibition
Tiplaxtinin (**1**)	–	–	–	14.8

[a] Values determined using in vitro chromogenic assay.



Scheme 1. Reagents and conditions: (a) NaH, DMSO, 0–25 °C, 60–70%; (b) $NaBH_4$, MeOH, 10–25 °C, 90–95%; (c) PBr_3, CH_2Cl_2, 10–25 °C, 75–80%; (d) $Pd(OAc)_2$, MeOH, reflux, 70–80%; (e) TFA, CH_2Cl_2, 25 °C, 80–90%; (f) **10**, *t*-BuOK, DMF, 10–25 °C, 60–75%; (g) KOH, MeOH, H_2O, 25 °C, 85–95%.



Scheme 2. Reagents and conditions: (a) $Pd(OAc)_2$, BINAP, K_3PO_4, DME, reflux, 20–50%; (b)TFA, CH_2Cl_2 or neat H_2SO_4, 0–25 °C, 80–90%; (c) **10**, *t*-BuOK, DMF or K_2CO_3, CH_3CN, 10–25 °C, 70–80%; (g) KOH, MeOH, H_2O, 25 °C, 85–95%.

Table 3
Pharmacokinetic parameters for compounds[a] **5a**, **6c**, **6e**

Compound	C_{max} (μg/mL)	T_{max}(h)	$T_{1/2}$ (h)	AUC(0–24) (h μg/mL)
5a	2.4	1	3.27	10.38
6c	6.8	6	9.86	80.18
6e	1.78	4	6.88	5.39

[a] Compounds were dosed in fasted male wistar rats at 30 mpk po formulated with a Tween-80: PEG: CMC(5:5: 90% v/v).

inhibited PAI-1 with an IC_{50} of 3.4 μM in the chromogenic assay. The removal of methoxy group from **5a** gave **5b**, which exhibited slightly lower potency (IC_{50} = 4.9 μM) than **5a**. The removal of triflorometh-oxy group is found to be detrimental for activity as evident from the compound **5c** (IC_{50} = 98 μM). Further more the compounds **6a**–**6g** were synthesized (Table 2) as part of strategy 2 (Fig. 2). The conformational restriction of **2** with two carbon atoms produced indoline derivative **6a**, which showed less potency (IC_{50} = 29 μM) compared to the tiplaxtinin (IC_{50} = 14.8 μM). The ring expansion in the **6a** to get tetrahydroquinoline derivative **6b** found to be detrimental to PAI-1 inhibitory activity (IC_{50} = 63.8 μM), probably due to conformational misfit of the molecule (six-membered ring of **6b** vs five-membered ring of **6a**). The introduction of the double bond in the indoline derivative **6a** to get indole derivative **6c** inhibited the PAI-1 activity with impressive IC_{50} of 3.2 μM, (Table 2). The incorporation of an extra N atom in the ring gave indazole derivative **6d**, which showed inferior potency with an IC_{50} of 14.6 μM. Further, a methoxy group was introduced in the most potent compound **6c** to get the compound **6e**, interestingly the compound **6e** exhibited slightly

higher potency (IC_{50} = 2.4 μM) compared to **6c** (IC_{50} = 3.2 μM). The translocation of *m*-CF_3 group of **6e** (IC_{50} = 2.4 μM) at para position was found to be detrimental in terms of potency as witnessed from IC_{50} value of **6f**, (IC_{50} = 22 μM). The removal of CF_3 group from **6c** to get the compound **6g** resulted in the deterioration of the PAI-1 inhibition (Table 2), which further supported the importance of the of the CF_3 group. The compounds with potent PAI-1 inhibitory activity, **5a**, **6c**, and **6e** were evaluated for their pharmacokinetic parameters in rats (Table 3).

The compound **5a** showed good plasma levels (C_{max} = 2.4 μg/mL) and a half life ($T_{1/2}$ = 3.27 h) when dosed orally at 30 mg/kg in wistar rats (Table 3). The compound **6c** showed impressive plasma levels (C_{max} = 6.8 μg/mL) and a long ($T_{1/2}$ = 9.86 h), which is favorable for this class of compounds. However, plasma concentration of the methoxy derivative **6e** was found to be modest when compared to **6c**. The significant plasma concentration and long half life of the compounds **5a**, **6c**, and **6e** prompted us to study the compounds for their in vivo efficacy in rats using $FeCl_3$ induced arterial thrombosis model using Clopidogrel, a well known antiplatelet agent as a positive control.[34] However, compound **6c** exhibited moderate antithrombotic efficacy while compounds **5a** and **6e** failed to show any in vivo efficacy, inspite of their impressive in vitro PAI-1 inhibitory activity and favorable pharmacokinetic parameters (Fig. 4). The further optimization efforts for this class of compounds to get the appropriate pharamacodynamics and pharmacokinetics correlation are in progress.

In summary, the novel 5-nitro-2-phenoxybenzoic acid derivatives derived using hybridization and conformational restriction strategies display potent PAI-1 inhibitory activity and favorable pharmacokinetic parameters. Oxoacetic acid part of Tiplaxtinin **1** has been effectively replaced with 5-nitro-2-phenoxybenzoic acid part of **2** producing potent PAI inhibitor **5a**. The docking study confirmed the similar orientation of **5a** and tiplaxtinin in PAI-1 ligand binding site. Conformational restriction of **2** with indole as a central core (**6c**) showed potent PAI-1 inhibitory activity and excellent pharmacokinetic profile with moderate efficacy in rats using $FeCl_3$ induced arterial thrombosis model. These findings provided the impetus for further studies on the refinement of these templates which will be reported in due course.

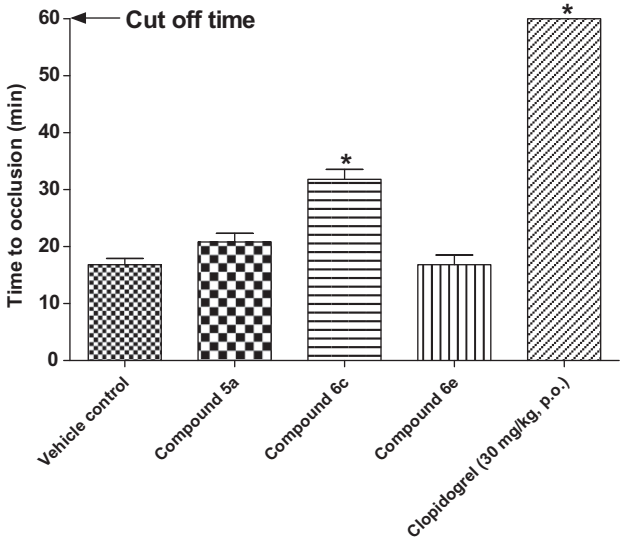


Figure 4. Effects of the compound **5a** and **6c** and **6e** on time to thrombus formation in $FeCl_3$-induced arterial thrombosis in rats at 30 mpk. Each value represents mean ± SEM (n = 6). * indicates $p < 0.05$ versus vehicle control. Clopidogrel was used as positive control and administered orally 2 h before application of $FeCl_3$ paper on the carotid artery.

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28. *Protocol for docking study*: (a) X-ray crystal structure of PAI-1 (PDB code: 3Q03) was obtained from PDB database. Protein crystal structure of PAI-1 was prepared using the Schrödinger's protein preparation wizard module. The binding site of the protein structure was identified using the sitemap module of the Schrödinger software. The probable site according to the literature was used as the centroid to generate the grid files for the docking. Docking study was carried out by using the induced fit docking; (b) Schrödinger Suite 2010 Induced Fit Docking protocol; Glide version 5.6, Schrödinger, LLC, New York, NY, 2010; Prime version 2.2, Schrödinger, LLC, New York, NY, 2010; (c) Docking study of Tiplaxtinin using the Glide software in PAI-1 protein crystal structure (PDB code: 1B3K) obtained from PDB did not gave a reported binding mode,[22] hence PAI-1 crystal structure recently reported by Jensen et al. was used for docking study of compounds along with tiplaxtinin; (d) Jensen, J. K.; Thompson, L. C.; Bucci, J. C.; Nissen, P.; Gettins, P. G. W.; Peterson, C. B.; Andreason, P. A.; Morth, J. P. *J. Biol. Chem.* **2011**, in press. doi:10.1074/jbc.M111.236554.
29. *Pharmacokinetic study*: Compounds were formulated with a Tween-80:PEG:CMC (5:5:90% v/v), A graduated dose volume (5 ml/kg) of suspension was administered to fasted male Wistar rats at 30 mg/kg po. The animals were anesthetized for blood sample collection from retro-orbital plexus. Serial blood samples were collected into heparinised containers at various time points and blood centrifuged to yield plasma. Plasma concentration was determined by using LC–MS/MS method.
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32. *Spectroscopic characterization of selected compounds*: **5a**: 97.8% by HPLC; mp 225–228 °C; ^{1}H NMR (400 MHz, DMSO-d_6): 8.5 (d, J = 2.8 Hz, 1H), 8.08 (dd, J = 2.8 and 9.4 Hz, 1H), 7.7 (m, 3H), 7.48 (d, J = 8.8 Hz, 1H), 7.44 (d, J = 3.2 Hz, 1H), 7.40 (d, J = 8 Hz, 2H), 7.33 (dd, J = 1.6 and 8.8 Hz, 1H), 7.26 (m, 1H), 7.16 (dd, J = 1.2 and 8.4 Hz, 1H), 6.83 (d, J = 7.2 Hz, 1H), 6.49 (d, J = 9.2 Hz, 1H), 6.41 (d, J = 2.8 Hz, 1H), 5.34 (s, 2H), 3.68 (s, 3H); ESI-MS: 578.9 $(M+H)^+$, **6c**: 97% by HPLC; mp 180–185 °C; ^{1}H NMR (300 MHz, DMSO-d_6): 8.56 (d, J = 3.2 Hz, 1H), 8.23 (dd, J = 2.9 and 9.1 Hz, 1H), 7.42 (m, 2H), 7.33 (m, 3H), 7.21 (m, 2H), 7.21 (m, 2H), 7.12 (m, 3H), 7.0 (m, 2H), 6.78 (d, J = 9.1 Hz, 1H), 6.3 (d, J = 3 Hz, 1H), 5.34 (s, 2H), 3.37 (t, J = 4.2 Hz, 4H), 3.16 (t, J = 4.4 Hz, 4H); ESI-MS: 617.2 $(M+H)^+$; **6e**: 97.5% by HPLC; mp 120–125 °C; ^{1}H NMR (400 MHz, DMSO-d_6): 8.51 (d, J = 2.8 Hz, 1H), 8.07 (dd, J = 2.8 and 9.2 Hz, 1H), 7.44 (m, 1H), 7.28 (m, 3H), 7.24 (m, 2H), 7.18 (dd, J = 0.8 and 8 Hz, 1H), 7.10 (d, J = 7.6 Hz, 1H), 6.95 (d, J = 2 Hz, 1H), 6.87 (dd, J = 2 and 8.8 Hz, 1H), 6.80 (d, J = 7.6 Hz, 1H), 6.45 (d, J = 9.2 Hz, 1H), 6.24 (d, J = 3.2 Hz, 1H), 5.26 (s, 2H), 3.69 (s, 3H), 3.38 (t, J = 4 Hz, 4H), 3.14 (t, J = 4.8 Hz, 4H); ESI-MS: 647 $(M+H)^+$.
33. *Chromogenic assay*: The chromogenic assay 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 overnight. phenoxybenzoic acid derivatives were dissolved in DMSO and diluted to a range of concentration between 1 and100 μM. Varying concentrations of phenoxybenzoic acid 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 room temperature 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. This assay detects only active inhibitory PAI-1 (not latent or substrate) bound to the plate. 100 μl aliquot 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. The quantization of residual active PAI-1 bound to t-PA at varying concentrations of phenoxybenzoic acid 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.
34. *Protocol for in vivo study*: In this study $FeCl_3$ induced chemical injury was used as a model of arterial thrombosis in rat model. Rats (n = 6) were anaesthetized with urethane (1.25 g/kg, ip) and secured in supine position. A midline cervical incision was made on the ventral side of the neck, and left carotid artery was isolated by blunt dissection. Compound **5a**, **6c** and **6e** (each 30 mg/kg) were formulated in polyethylene glycol (PEG) and 0.5% sodium carboxymethyl cellulose (1:10) and administered by oral gavage. Exactly after 2 h of administration, a 2 × 3 mm strip of Whatman # 1 filter paper saturated with 35% (w/v) $FeCl_3$ was placed on the carotid artery for 5 min. A temperature probe (Thermalert-TH8, Physitemp Instruments Inc., Clifton, N.J., USA) was placed distal to filter paper to monitor the temperature of carotid artery. A sudden fall in temperature (about 1–2 °C) was taken as an indication of cessation of blood flow as a result of thrombus formation. Time to occlusion (TTO) was defined as the time from $FeCl_3$ application to time of thrombus formation (indicated by sudden fall in carotid temperature). In case if no thrombus formation was seen in drug-treated animals, a cutoff time was fixed at 1 h.