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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 15 (2005) 3514–3518

Synthesis and SAR of 2-carboxylic acid indoles as inhibitors of plasminogen activator inhibitor-1

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Received 21 April 2005; revised 19 May 2005; accepted 25 May 2005

Abstract—We synthesized and evaluated a novel series of 2-carboxylic acid indole-based inhibitors of plasminogen activator inhibitor-1 (PAI-1). Systematic modification of the N-1 position and the 5-position of the indole scaffold resulted in the identification of several compounds that showed good potency against PAI-1 in the spectrophotometric assay. This potency did not always translate to the antibody assay. Solubility and serum protein binding studies on selected analogs revealed that protein binding might be a factor in the poor correlation between the two assays.

Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor (serpin) gene family and is the principal inhibitor of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) in vivo.[1] These serine proteinases convert plasminogen, an inactive zymogen, to the active enzyme plasmin, which digests fibrin clots by degrading insoluble fibrin molecules to small soluble fragments.[2] In the acute setting, PAI-1 stored in platelet α-granules can be released upon platelet activation, resulting in significant local concentrations and the resistance of platelet-rich thrombi to lysis. In healthy individuals, PAI-1 expression is low, but it is elevated significantly in a number of diseases, including deep vein thrombosis,[3] atherosclerosis,[4] and type 2 diabetes.[5] Plasma PAI-1 is also elevated in postmenopausal women and has been proposed to contribute to the increased incidence of cardiovascular disease in this population.[6]

The importance of PAI-1, as a major regulatory protein in a variety of biological processes, has been supported by a number of studies in experimental animals.[7] PAI-1 null mice have normal coagulation, but unstable thrombi that lyse spontaneously. Furthermore, these null mice are viable and protected from the development of atherosclerosis, due in part to its role in regulating tissue proteolysis. Several patients with PAI-1 deficiency have been reported and observed to have normal coagulation.[8] Most abnormal bleedings were found only after trauma or surgery. No other physical anomalies were reported in individuals with complete PAI-1 deficiency. These results suggest that modulation of PAI-1 activity offers a beneficial therapy in treating a variety of cancers and cardiovascular diseases originating from tissue remodeling and fibrinolytic disorders.

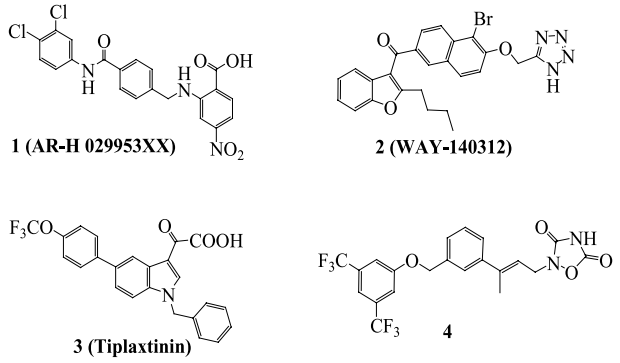

Figure 1. Structures of known PAI-1 small molecule inhibitors.

Keyword: PAI-1 inhibitors.
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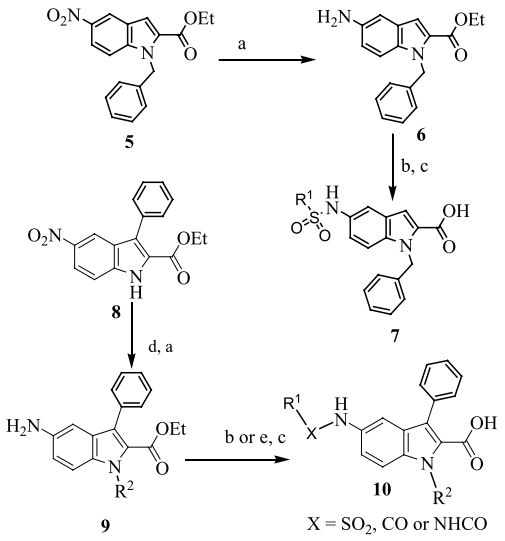
doi:10.1016/j.bmcl.2005.05.095

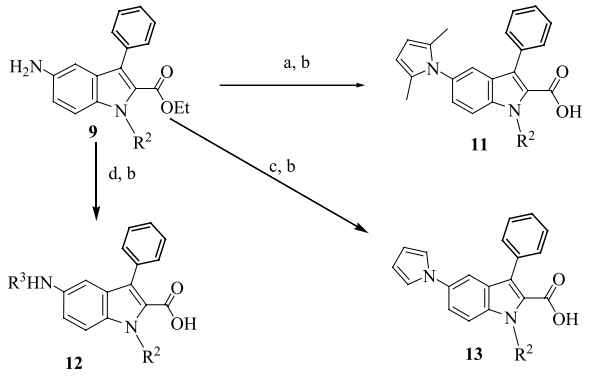
To date, several PAI-1 inhibitors including antibodies, peptides, and small molecules have been reported.[1b,9] Some of the recent small molecule inhibitors (Fig. 1) are flufenamic acid-derived AR-H029953XX (**1**),[10] the diketopiperazine analogues,[11] benzothiophene derivatives,[12] menthol,[13] and piperazine analogues.[14] In addition, Wyeth has previously reported a number of potent PAI-1 inhibitors,[15] such as the naphthyl benzofuran **2**,[15a] the 3-indole oxoacetic acid **3** (Tiplaxtinin),[15b] and oxadiazolidinediones **4**.[15c] The key structural features present in all inhibitors reported so far include either a carboxylic acid or an acid bioisostere attached to a lipophilic aromatic ring scaffold. As part of our ongoing research to find additional novel, orally active PAI-1 inhibitors, we designed a group of 2-indole carboxylic acid-based compounds. In our design, the oxoacetic acid of Tiplaxtinin was replaced by a carboxylic acid and the position of the carboxylic acid was switched from 3-indole to 2-indole. The relocation of the acid moiety allowed easier access to indole substituent variation, which could provide an avenue for the exploration of additional chemical space.

2-Carboxylic acid indoles were conveniently prepared by following synthetic Schemes 1 and 2. 5-Nitro indole **5**[16] was first reduced to amine **6** upon treatment with Raney® nickel in a mixture of hydrazine and ethanol (Scheme 1). Sulfonylation of the aniline with sulfonyl chlorides, followed by basic hydrolysis of ethyl esters in LiOH, gave the desired carboxylic acids **7**. The 1*H*-indole **8** was alkylated with either iodomethane or benzyl bromides in the presence of Cs_2CO_3. The resulting nitro intermediate was then converted to acid **10** upon Raney® nickel reduction, sulfonylation/acylation, and LiOH hydrolysis.

Condensation of aniline **9** with excess hexane-2,5-dione utilizing a Dean-Stark trap for azeotropic water removal led to a 2,5-dimethylpyrrolylindole, which was converted to **11** upon saponification (Scheme 2). Similarly,



Scheme 1. Reagents: (a) Raney® nickel/EtOH; (b) R^1SO_2Cl/NEt_3; (c) LiOH; (d) R^2Br/Cs_2CO_3; (e) R^1COCl or R^1-NCO.



Scheme 2. Reagents: (a) hexane-2,5-dione, THF, reflux; (b) LiOH; (c) 2,5-dimethoxytetrahydrofuran, THF, reflux; (d) $R^3B(OH)_2$/2,6-lutidine/$Cu(OAc)_2$.

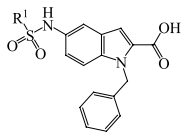
reaction of aniline **9** with a large excess of 2,5-dimethoxytetrahydrofuran, followed by a basic hydrolysis, gave pyrrole **13**. The aniline **9** can also be converted to a diaryl amine with a boronic acid in the presence of $Cu(OAc)_2$[17] and the final acid **12** was obtained via basic hydrolysis.

The inhibition[18] of PAI-1 by 2-carboxylic acid indoles is presented in Tables 1–3, together with comparative data for AR-H029953XX (**1**) and Tiplaxtinin (**3**).[15b] As an initial in vitro screen, preincubation of recombinant human PAI-1 with tPA resulted in complete inhibition of the enzymatic action of tPA with a chromogenic substrate, as determined spectrophotometrically. Addition of 2-carboxylic acid indoles to human PAI-1 restored the proteolytic activity of tPA, indicating successful inhibition of PAI-1. For the most potent inhibitors, the IC_{50} values were generated using a secondary assay that quantified residual active PAI-1 by antibody binding following incubation with various concentrations of the compound.

We began our SAR studies with the modification of *N*-benzyl-C_3-H indoles (Table 1). In general, lipophilic sulfonamides were preferred. Thus, the presence of a bisphenyl (**16**) or 3,4-dichlorophenyl (**17**) increased PAI-1 % inhibition at an initial screening concentration (100 μM) over *n*-butyl- (**14**) or phenyl (**15**)-substituted compounds. The bisphenyl sulfonamide **16** also showed 49% inhibition at 25 μM; however, it was found to be a weak PAI-1 inhibitor, with an IC_{50} value of 63.1 μM in the secondary antibody assay.

Next, we examined various *N*-benzyl-C_3-phenyl-substituted indoles (Table 2). The sulfonyl chlorides chosen for use in the synthesis of sulfonamides **18**–**26** were selected based on an earlier SAR study using a different scaffold.[19] Since the C_3–phenyl sulfonamides are much more potent than the C_3–H sulfonamides, only the inhibitions at lower concentrations (10 and 25 μM) are included in Table 2. Looking across the subseries (R^1 = Bn and Me), the less lipophilic 8-quinoline substituent at the R^2 position (**19** and **24**) was the least potent, and highly lipophilic R^2 groups [e.g., 4-^{t}Bu phenyl (**20** and **25**), 4-phenyl-phenyl (**21** and **26**)] were the most

Table 1. PAI-1 inhibitory activity of C_3–H indoles



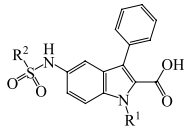
Compound	R^1	% Inhibition[a] at 100 μM	% Inhibition[a] at 25 μM	IC_{50} (μM)[b]
1		74	51	17.8
3		83	47	2.7
14	*n*Bu	14		
15	Ph	32		
16	4-Ph-Ph	64	49	63.1
17	3,4-diCl-Ph	62		

[a] Initial spectrophotometric assay.
[b] Antibody assay.

potent. Comparing *N*-methyl with *N*-benzyl pairs (**18** and **23**, **19** and **24**, **20** and **25**, and **21** and **26**), the *N*-benzyl analogs always showed greater potency at a concentration of 10 μM. Several *N*-benzyl-3-phenyl indoles exhibited high inhibition (>25%) at a lower concentration (10 μM) in the initial spectrophotometric assay. For example, the 4-phenyl benzyl sulfonamide **22** showed 79% inhibition. However, this high percent inhibition did not get transferred to our antibody assay (IC_{50} = 28 μM for **22**, vide infra). The most potent sulfonamide in the antibody assay was found to be the 4-CF_3O-phenyl sulfonamide **18** with an IC_{50} value of 8.3 μM, which is 2-fold more potent than the literature standard AR-H029953XX (**1**).

To examine the effect of sulfonamide pharmacophore on the inhibition of PAI-1, the sulfonamide functionality was replaced by a number of other groups, as shown in Table 3. Compared to the sulfonamides (**18**–**21**), an amide derivative (**27**) and a urea analog (**28**) showed similar potency. To explore a non-amide lipophilic piece, a series of biphenyl anilines (**29**–**32**) was synthesized and all anilines showed similar potency as well. For example, an *N*-(4-phenyl)benzyl aniline analog

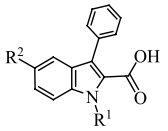
Table 2. PAI-1 inhibitory activity of C_3–Ph indoles



Compound	R^1	R^2	% Inhibition[a] at 25 μM	% Inhibition[a] at 10 μM	IC_{50} (μM)[b]
1				3	
3				0	
18	Bn	4-CF_3O-Ph	81	33	8.3
19	Bn	8-Quinoline	43	8	
20	Bn	(4-^{t}Bu)-Ph	82	41	28.7
21	Bn	4-Ph-Ph	61	44	23.0
22	4-Ph-Bn	Ph	87	79	28.0
23	Me	4-CF_3O-Ph	19	0	
24	Me	8-Quinoline	1	0	
25	Me	(4-^{t}Bu)-Ph	46	5	
26	Me	4-Ph-Ph	68	11	

[a] Initial spectrophotometric assay.
[b] Antibody assay.

Table 3. PAI-1 inhibitory activity of non-sulfonamide-based indoles



Compound	R^1	R^2	% Inhibition[a] at 25 μM	% Inhibition[a] at 10 μM	IC_{50} (μM)[b]
27	Bn	(2,4-diF)Ph-CONH	43	24	
28	Bn	(2,4-diF)Ph-NHCONH	61	27	
29	Bn	3-MePh-NH	69	24	
30	Bn	(4-^{t}Bu)-Ph-NH	73	45	
31	Bn	4-Ph-Ph-NH	71	36	
32	4-Ph-Bn	(4-^{t}Bu)-Ph-NH	80	52	12.0
33	Bn	1-Pyrrole	69	12	
34	Bn	(2,5-diMe)-1-Pyrrole	83	23	

[a] Initial spectrophotometric assay.
[b] Antibody assay.

(**32**) showed 52% inhibition at 10 μM and had an IC_{50} value of 12.0 μM in the antibody assay. Similarly, 5-pyrrole derivatives **33** and **34** showed essentially the same potency in the initial in vitro screen. This preliminary SAR information suggested that the 5-position of the indole ring was flexible and could accommodate a variety of substitutions, although no additional increase in the PAI-1 inhibitory activity was achieved compared to **18**.

We next focused on a preliminary investigation of the potency correlation discrepancy between the initial spectrophotometric assay and the antibody assay. Since these compounds are highly lipophilic, we initially suspected that the lower than expected potency of newly synthesized PAI-1 compounds (**18**, **20**, **22**, and **32**) in the antibody assay compared to **3** may be due to solubility. The concentrations of the five compounds (**3**, **18**, **20**, **22**, and **32**) were determined in the two assay buffers at a target concentration of 50 μM by LC-MS.[20] All the compounds were soluble (>25 μM) in the antibody assay buffer and in the initial spectrophotometric assay buffer. Thus, solubility was probably not a factor for the discrepancy between the two assays.

Since the test compounds were run in the presence of 10 mg/L bovine serum albumin (BSA) in the antibody assay, and serum proteins were not present in the spectrophotometer assay, the antibody assay faced an additional hurdle of competition of the test compounds between BSA and PAI-1. Binding characteristics of the five compounds (**3**, **18**, **20**, **22**, and **32**) with BSA were then evaluated by equilibrium dialysis[21] and Biacore assays.[22] It was found that all five compounds were highly bound to BSA with an unbound fraction below the detection limit (<0.05%) in the equilibrium dialysis experiment. Biacore analysis showed a 1:1 stoichiometric binding and comparable binding constants ($K_d \sim 10$ μM) for **3** and **18**, while other three compounds were rather non-specific (stoichiometry: 2.5–3.7 at 10 μM). Thus, the presence of BSA decreased the concentration of free drugs for **20**, **22**, and **32** in the antibody assay much more than it did for compounds **3** and **18**. It may thus be concluded that the magnitude of drug–BSA protein interaction influenced the biological activity and this may partially account for the potency correlation discrepancy between the two assays.

In summary, we have explored the structure–activity relationships for the novel 2-carboxylic acid indole-based PAI-1 inhibitors. Introduction of a lipophilic phenyl group at the C_3 position of the indole ring significantly increased PAI-1 potency. Solubility study indicated that the selected compounds were soluble in the initial assay buffer and in the antibody assay buffer. The interaction of BSA with the selected compounds was investigated by equilibrium dialysis and Biacore assays, and the initial results have suggested that the drug–protein interaction might account for the biological activity correlation discrepancy between the initial screening assay and the antibody assay. This study has shown that comparative selective and nonselective protein binding analysis can be used in further understanding of the SAR of small molecule PAI-1 inhibitors.

Acknowledgments

The authors thank Drs. Thomas J. Commons and Eugene J. Trybulski for leading this project in chemistry. We also thank the Discovery Analytical Chemistry group at Wyeth Research, Collegeville, PA, for spectral data.

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20. Initial spectrophotometric assay buffer (pH 6.6): Na_2HPO_4 50 mM, NaCl 200 mM, and EDTA 1 mM. Antibody assay buffer (pH 7.5): Tris 50 mM, NaCl 150 mM, BSA 10 mg/L, and 0.01% Tween 80.
21. Equilibrium dialysis: stock solutions at concentrations of 400 μM of compounds were prepared in 100% DMSO, 4% BSA in phosphate buffer (67 mM, pH 7.4) was spiked with an appropriate compound stock solution to obtain 20 μM final drug concentration. Four percent (w/v) dextran 70,000 was used in dialysis buffer to maintain similar osmolarity in reciprocal dialysis chambers minimizing the volume shift phenomena. DMSO concentration in all dialysis units was 5%. Buffer samples spiked with the test compound were used to verify the ability of the compounds to permeate across the dialysis membrane. Samples were dialyzed for 18 h in a 37 °C water bath. Following dialysis, samples were collected from all dialysis compartments and combined with an equal volume of acetonitrile. After 12 min of centrifugation at 12,000 rpm, the supernatant of each sample was collected and analyzed by HPLC. Based on HPLC data, percentage drug free was calculated for the compound.
22. Biacore assay: BSA was immobilized on the flow cell of a CM-5 sensor chip. Stock solutions (0.8 mM) of compounds were prepared in DMSO. Test solutions of compounds were prepared from the stock solutions and diluted to their final concentration in phosphate buffer (67 mM, pH 7.4). Samples of all test compounds in the concentration range of 0–10 μM were injected over the biosensor chip surface. Dilutions for all prepared samples were made in such a way that the final DMSO concentration was 5% in samples and running buffer. Matrix binding effect from blank runs was subtracted from dose–response curve data points.