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New Fibrinolytic Agents: Benzothiophene Derivatives as Inhibitors of the t-PA–PAI-1 Complex Formation

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Abstract—The synthesis and activity of novel benzothiophene derivatives are described. In the t-Pa-induced fibrin clot lysis assay, several compounds inhibit the formation of the tPa–PAI-1 complex with submicromolar IC_{50}. This class of compounds potentially represents a new generation of antithrombotic-fibrinolytic agents.

The conversion of plasminogen to plasmin through the proteolytic cleavage of the Plasminogen Activators (Tissue type Plasminogen Activator, t-PA, and Urinary Plasminogen Activator, or urokinase, u-PA) is the main event occurring in the fibrinolytic pathway, ultimately leading to the dissolution of the clot by plasmin. The proteolytic activity of t-PA is regulated by Plasminogen Activator Inhibitor-1 (PAI-1). The molecular mechanism of inhibition of t-PA by PAI-1 is common to all the members of the serpin superfamily, and is characterised by the formation of a stable 1:1 stoechiometric complex.[1]

When released from endothelial cells into the circulation, a fraction of PAI-1 is spontaneously inactivated into a latent form; a second fraction of circulating PAI-1 gives a 1:1 complex with vitronectin. This stabilized complex allows PAI-1 to act as a pseudo-substrate for t-PA by mimicking plasminogen sequence, using a 'bait' dipeptidic residue Arg 346-Met 347, located on the reactive center loop. Cleavage of this peptidic bond by Ser 478 of t-PA leads to the formation of the t-PA/PAI-1 1:1 complex. Deactivation into the latent form as well as cleavage of the bait residue give rise to the internalization of the reactive center loop into β-sheet A.[2]

Numerous animal studies have highlighted the central role of PAI-1 in the development of thrombotic events, among which those obtained using transgenic mice: animals overexpressing human PAI-1 displayed venous occlusions, while deficient mice are virtually protected against venous thrombosis.[3] In human, elevated PAI-1 levels have been shown to be correlated with several thrombotic disease states, including venous thromboembolism, coronary artery disease, acute myocardial infarction, obesity, diabetes, sepsis, surgery and trauma.[4]

Beside this important role in the fibrinolytic pathway, PAI-1 has also been implicated in several other crucial biological processes including tumor invasion, neovascularization, inflammation and wound healing.[5]

Several hypothesis have been put forward in order to decrease PAI-1 activity and to restore fibrinolysis: for example, it has been shown that the inhibition of the formation of the stoechiometric t-PA/PAI-1 complex by anti-PAI-1 monoclonal antibodies such as MAI-12[6] could be of value in order to decrease prothrombotic PAI-1 activity. More recently, a tetradecapeptide based upon the peptidic sequence of the reactive center loop was shown to decrease PAI-1 activity in an in vitro clot lysis model.[7] In 1996, Xenova reported the first low molecular weight inhibitor of the t-PA/PAI-1 interaction: XR 334 **1** was found to enhance fibrinolysis ex vivo and reduce the formation of the thrombus in the rat electrically stimulated carotid artery model.[8]

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Optimization of this very insoluble lead gave rise to compounds with improved activity and solubility, such as XR 5082 **2** and XR 5118 **3**.[9] Meanwhile, the optimization of a family of 2,3-disubstituted benzothiophenes selected from our corporate library giving submicromolar activity in the t-Pa-induced fibrin clot lysis assay has been reported, yielding compounds exemplified here by **4**.[10] More recently, Xenova has disclosed the structures of novel inhibitory templates.[11]

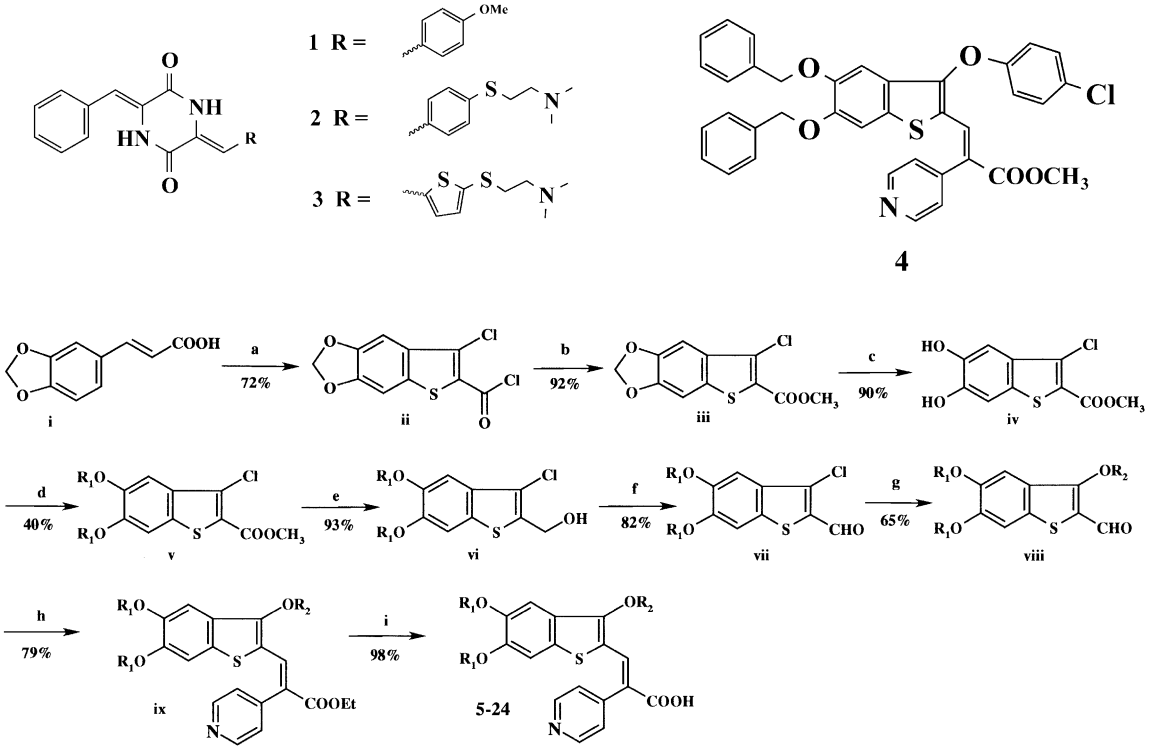
The compounds described herein were prepared according to Scheme 1. Starting from 3,4-methylenedioxy-cinnamic acid **i**, Higa-type cyclization[12] gave the acid chloride of 3-chloro-5,6-methylenedioxy-benzo[*b*]thiophene-2-carboxylic acid **ii** with 75% yield. Methanolic hydrolysis and removal of the methylenedioxy group afforded the key dihydroxy intermediate **iv**. Alkylation with the appropriate halide yielded the corresponding 5,6-disubstituted benzothiophene derivative **v**. Reduction of the ester function gave alcohol **vi**, which was further oxidized to aldehyde **vii**. Functionalisation at position 3 on the benzothiophene ring was then realized by substitution of the chlorine atom with a properly substituted phenol; finally, Knoevenagel reaction followed by saponification gave the final carboxylic acid derivatives **5–24**.

Biological evaluation of this new series of compounds was realized by measuring the inhibitory potency of the new compounds against the formation of the t-PA–PAI-1 complex in a t-PA-induced fibrin clot lysis assay, according to our previously published protocol.[13,14]

Xenova compounds **1**, **2** and **3** proved to be potent inhibitors of the t-PA–PAI-1 complex formation giving IC_{50} of 59.4 ± 5.7, 51 ± 3 and 106.7 ± 1.6 μM, respectively. In our previous report, we had already shown that **4** was significantly more active with an IC_{50} of 0.47 ± 0.15 μM (Table 1).[10]

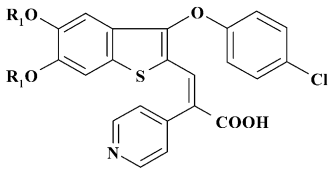
Variously substituted biphenyl systems were prepared: the biphenyl-4-yl methyloxy moiety (**7**) was preferred to its 2- (**5**) and 3-yl (**6**) counterparts. Activity was maintained with a 4′-methoxy substitution in **8**. Finally, introduction of a spacer between the two phenyl rings in **9–12** proved to be advantageous for activity, culminating with **11** which came out as the most active tPa–PAI-1 complex formation inhibitor of this chemical series with an IC_{50} of 39 nM. Having established the biphenyl-4-yl methyloxy moiety as one of the optimal substitutions at positions 5 and 6, modifications were effected at position 3 (Table 2): the non-substituted derivative **13** proved to be as potent as starting compound **7**, while introduction of a fluorine atom at position 3 on the 4-chloro substituted phenyl ring gave a sharp drop in activity (**14**). One methyl group at position 3 was acceptable (**15**), while 3,5-dimethyl substitution found in **16** was 2 times less potent; 3-fluoro (**17**) and 3,4-difluoro (**18**) substitutions restored interesting sub-micromolar activities. Finally, introducing a pyridine ring (**19–21**) as well as substitution with larger polycyclic ring systems such as in **22–24**, led to an overall decrease in activity.

In summary, optimization of a family of benzothiophene derivatives as inhibitors of the t-Pa/PAI-1 complex formation led to novel structures with sub-micromolar IC_{50} values. Further structural modification as well as in vivo evaluation of the new inhibitors will be reported in due course.



Scheme 1. Reagents: (a) $SOCl_2$, pyridine, chlorobenzene; (b) methanol, toluene; (c) BBr_3, methylene chloride; (d) R_1-Cl, K_2CO_3, DMF; (e) $LiAlH_4$, THF; (f) MnO_2, toluene; (g) R_1-substituted phenol, NaH, DMF; (h) ethyl aryl acetate, Ac_2O; (i) NaOH, EtOH.

Table 1. tPa/PAI-1 complex formation inhibition data for compounds **5–12**



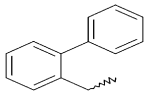
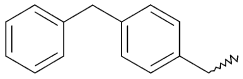
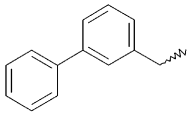
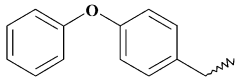
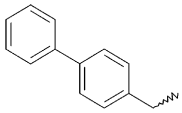
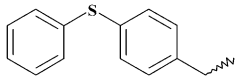
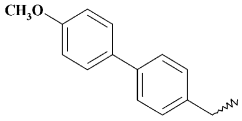
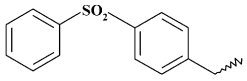
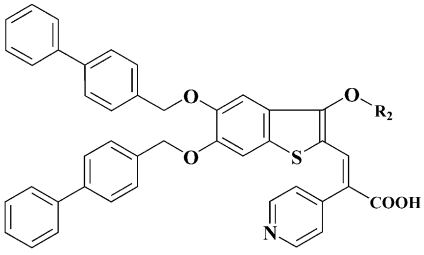
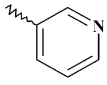
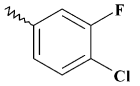
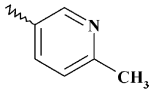
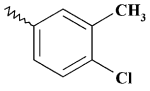
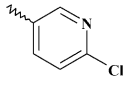
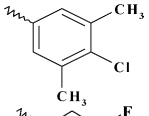
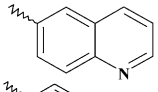
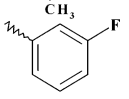
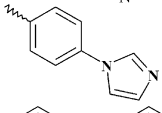
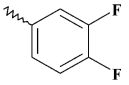
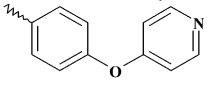
Compd	R_1	IC_{50} (μM)	Compd	R_1	IC_{50} (μM)
5		10.8±0.1	**9**		0.27±0.01
6		0.22±0.05	**10**		0.10±0.08
7		0.13±0.06	**11**		0.039±0.01
8		0.14±0.04	**12**		0.77±0.1

Table 2. tPa/PAI-1 complex formation inhibition data for compounds **13–24**



Compd	R_2	IC_{50} (μM)	Compd	R_2	IC_{50} (μM)
13		0.10±0.04	**19**		2.40±0.87
14		2.28±0.72	**20**		2.48
15		0.48±0.15	**21**		2.07
16		1.09±0.29	**22**		6.06
17		0.24±0.04	**23**		2.91±1.5
18		0.59±0.04	**24**		10.7±2

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14. tPa (0.42 nM) was mixed with plasminogen (800 nM), fibrinogen (7.5 mg/mL), and human recombinant active PAI-1 (Molecular Innovations, 2 nM) in the presence or not of the tested compound; thrombin (14 nM) was then added and absorbance was measured each 10 min for 12 h at 37 °C to determine lysis times. A calibration curve (lysis times *vs* active PAI-1 concentrations) was constructed to calculate the active PAI-1 concentration inhibited by each compound concentration; the concentration of compound inhibiting 50% of active PAI-1 (IC_{50}) was then determined.