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SYNTHESIS AND CHARACTERISATION OF NOVEL THROMBIN INHIBITORS BASED ON 4-AMIDINOPHENYLALANINE

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Thrombin inhibitors have been thought to play a pivotal role in myocardial infarct and stroke incidences and their aftermath. Quite some time ago potent synthetic thrombin inhibitors became known based on peptide derivatives D-Phe-Pro-Arg and benzamidine. One of them, fairly well characterised was β-naphthylsulphonylglycyl-*D,L*-4-amidino-phenylalanylpiperidide (NAPAP). NAPAP was prone to being administered intravenously due to its short plasma half life. Drawbacks to this compound such as effects on histamine release and blood pressure may have obstructed its clinical use. Long half life and oral bioavailability would be desirable for prophylactic treatment of thrombotic disorders.

We have used NAPAP as a template for new synthetic compounds to improve some characteristics of its profile. For screening purposes we have investigated fairly simple surrogate parameters, aspects that were considered to contribute to pharmacological effects. Potency was correlated to thrombin inhibition, side effects were addressed by specificity toward thrombin as well as reduction in basicity, and plasma half life was considered to be modulated by plasma stability of the compound. Oral bioavailability would be affected by instability during the passage through the gut wall. Chemical introduction of a carboxylic group and exchange of the naphthyl group for 4-methoxy-2,3,6-trimethylphenyl led to a compound that when compared to NAPAP, exhibited a 4-fold increase in thrombin inhibitory activity and a 3-fold increase in trypsin specificity. Plasma stability decreased to 22 h, however, sufficient enough not to play a major role in plasma half life. Gut homogenate stability of the compound has not changed. The potency increase did not translate into a reduction in IC_{50}-values for the coagulation assay aPTT and TT, in contrast to the IC_{50}-values for thrombin-induced platelet aggregation.

KEY WORDS: Thrombin inhibitors, Amidinophenylalanine, NAPAP

INTRODUCTION

Thromboembolic disorders leading for example to acute myocardial infarcts or stroke account nowadays for the most frequent incidences of mortality in the western world.

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Abbreviations: Adf, 4-amidinophenylalanine; CAMD, computer aided molecular design; Hepes, 4-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MD805, 2R,4R-4-methyl-[N^2-(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl-sulphonyl)-L-arginyl]-2-piperidinecarboxylic acid; Mtr, 4-methoxy-2,3,6-trimethylphenylsulfonyl; NAPAP, β-naphthylsulphonylglycyl-*D*,L-4-amidino-phenylalanylpiperidide; Pmc, 2,2,5,7,8-pentamethylchromane-6-sulfonyl, Tris, *tris*-(hydroxymethyl)-aminomethane.

During the acute phase as well as thereafter direct suppression of thrombin activity has been considered to prevent or to ameliorate the course of these disorders. Due to the great number of patients involved immense attention has been drawn to developing thrombin inhibitors for treatment and prevention of thrombosis.

Peptide derivatives based on D-Phe-Pro-Arg and benzamidine emanated successfully as compounds to reveal inhibitory activity toward thrombin.[1-7] Originated from benzamidine[8,9] in particular, highly potent thrombin inhibitors have been developed, with *β*-naphthylsulphonylglycyl-*D,L*-4-amidino-phenylalanylpiperidide (NAPAP) in the forefront. NAPAP displayed reasonable potency and specificity toward thrombin. However, associated with NAPAP were disadvantages like short plasma half life, side effects including reduction in blood pressure after intravenous injection and the lack of oral bioavailability.[10] This may have obstructed its clinical development.

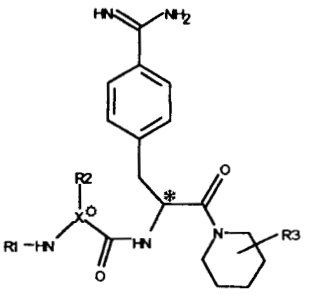
Therefore, the goal of our investigation was to design compounds that were not associated with the above mentioned disadvantages. An ideal inhibitor of thrombin was thought to combine a reasonably low inhibition constant, high specificity toward thrombin, longer half time, reduced effects on blood pressure compared to NAPAP, stability in physiological environments with the potential of being administered orally.[11] To undertake this task we modified chemically the structure of NAPAP by combining the information of crystal structure data of thrombin and known inhibitors like 2R,4R-methyl-[N^2-(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl-sulphonyl)-L-arginyl]-2-piperidinecarboxylic acid (MD-805).[12] To reduce the effect on blood pressure one hypothesis was, to introduce a hydrophilic group into the NAPAP molecule, preferentially a carboxyl group thereby neutralising the charge of the amidino group. Another approach was to introduce a less bulky hydroxyl group. With this in mind additional modifications of the NAPAP structure were carried out, leaving in most cases the 4-amidinophenyl moiety untouched.

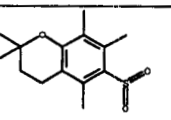
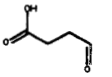
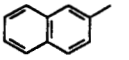
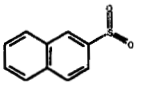
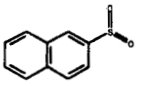
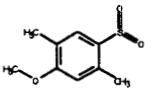
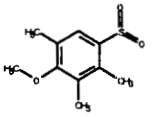
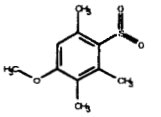
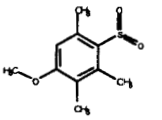
MATERIALS AND METHODS

Modelling

Molecular modelling was performed employing a workstation (Silicon-Graphics Model W4D25TG) equipped with software Insight™ and Discover™(BIOSYM). Prior to chemical synthesis inhibitors depicted in scheme 1 were positioned manually into the active cleft of thrombin biased on its crystal structure data with known inhibitors, preferentially that of D-Phe-Pro-Arg-CH_2-Cl.[13] All 4-amidino-phenylalanine based compounds have been calculated using the D-form. During minimisation and dynamisation calculations the coordinates of thrombin were kept constant. The binding quality of each tested inhibitor was judged following a sequence of calculations: after positioning of each inhibitor in the active site cleft of thrombin minimisation was performed for 100 fs using conjugate gradients. Subsequently dynamisation calculation was initiated for 5 ps at 50 K followed by minimisation for 1 ps using conjugate gradients. These steps were repeated up to 250 K in increments of 50 K. A criterion for a reasonable fit of an inhibitor was considered to be that the inhibitor would at least not be positioned outside the catalytic pocket.

Scheme 1



Com. No.	R1	R2	X	R3	O	*
1		H	N	-	-	D
2			CH	-	D	D
3		H	CH	3-OH	-	D,L
4			CH	-	L	D,L
5			CH	-	L	D,L
6			CH	-	L	D,L
7			CH	-	L	D,L
8			CH	-	L	D,L

Chemical synthesis

Compounds depicted in Scheme 1 were synthesised according to commonly known procedures and have been published elsewhere.[14]

Determination of inhibition constants

All measurements were carried out on a COBAS BIO instrument (Hoffman LaRoche) at 37°C. After 10 min of preincubation of an enzyme with inhibitor, assays were initiated by adding the appropriate substrate. All substrates were *p*-nitroaniline-based substrates. Concentrations of the product *p*-nitroaniline, formed after hydrolysis by an enzyme, were determined from the extinction coefficient at 405 nm of 9920 M^{-1} cm^{-1}. Inhibitors were used within the concentration range of 0–400 μM. Inhibition constants were calculated employing tight-binding algorithm.[15,16,17]

Thrombin

Human α-thrombin was prepared by Behringwerke AG. Ampoules of lyophilised powder consisted of 4.7 μg thrombin in the presence of the stabilisers bovine serum albumin (10 mg), sodium glutaminate (10 mg), and 1 μl kathon DP™. Final concentration of thrombin was based on active site titration with methylumbelliferyl *p*-guanidinobenzoate and was found to be 4.1 μg per ampoule.[18] Thrombin concentration in the assay was 100 pM. Substrate was S2238 (Kabi) at a concentration of 100 μM. Buffer consisted of 50 mM Tris and 75 mM NaCl, pH 7.8. The Michaelis-Menten constant was determined to be 8.2 μM.

Trypsin

Bovine pancreatic trypsin (Sigma) was used with the substrate Chromozym TRY (Boehringer) at assay concentrations of 5 nM and 500 μM, respectively. The buffer consisted of 100 mM triethylamine, 10 mM $CaCl_2$, pH 7.8 at 25°C. The Michaelis-Menten constant was determined to be 122 μM.

Factor Xa

Human Factor Xa (Boehringer) was used with the substrate Chromozym X (Boehringer) at assay concentrations of 3 nM and 900 μM, respectively. The buffer consisted of 20 mM Hepes, 140 mM NaCl, 4 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.2. The Michaelis-Menten constant was determined to be 390 μM.

Plasmin

Human plasmin (Behringwerke AG) was used with the substrate D-norvalyl-cyclohexylalanyl-lysyl-*p*-nitronilide (Behringwerke AG) at assay concentrations of 4 nM and 600 μM, respectively. The buffer consisted of 10 mM KH_2PO_4, 75 mM NaCl, 11% (w/v) glycerol, pH 7.5. The Michaelis-Menten constant of substrate was determined to be 49 μM.

Coagulation parameters aPTT and TT

Activated partial thromboplastin time (aPTT) was carried out using Standard Human Plasma (SHP, Behringwerke AG) and an aPTT-reagent kit (Behringwerke AG). Inhibitors were preincubated with plasma for 5 min at 37°C. Coagulation was initiated

by pathromtin and $CaCl_2$ (Behringwerke AG) employing a coagulometer (Schnitger and Gross). Thrombin time (TT) was determined using a reagent kit and SHP (Behringwerke AG). Plasma was incubated with inhibitor for 1 min and the clot formation was initiated with thrombin concentrations of inhibitors that double aPTT- and TT-times are listed as IC_{50}-values.

Thrombin induced platelet aggregation

Platelet-rich plasma (PRP) was prepared from human blood of healthy volunteers. Blood was anticoagulated with 0.38% trisodium citrate (ASID) and centrifuged for 20 min at 150 g at room temperature. Platelet containing supernatant ($1–5\times10^8$/ml, PRP) was removed and stored in Falcon test tubes (Greiner) at 0° prior to the use in the aggregation assay. To four volume equivalents of PRP were consecutively added inhibitor (0–400 nM, final) and after 1 min of preincubation human α-thrombin (2.5 nM, final) totalling one volume equivalent. Aggregation was monitored in a APACT aggregometer (LAbor). Calibration was performed using PRP (0%) and platelet poor plasma (100%) that was obtained by centrifugation of PRP at 14 000 X g. IC_{50}-values of compounds were calculated by interpolation to the point of 50% aggregation.

Stability in plasma and gut homogenate

Standard Human Plasma was provided by Behringwerke AG. Gut homogenate was prepared from the entire rat intestine. The intestine was minced and homogenised in cold physiological NaCl solution. After centrifugation at 10,000 × g at 4°C for 30 min the liquid between sediment and floating fatty layer was removed and recentrifuged at 15,000 × g for 10 min. The supernatant was use for further stability experiments. Compounds (500 μM) to be tested in plasma or gut homogenate were incubated at 37°C. Aliquot samples were taken at several time intervals within 24 h. Acetonitrile was added to samples (4:1) and subsequently centrifuged at 15,000 × g. The supernatant was diluted with water (1:3) and injected into an HPLC. Analysis was carried out on a Nucleosil 100, 3 μm, C18 column (Bischoff) with a phosphate-based (pH 2.5) solvent gradient altering the acetonitrile content from 20 to 75% using a UV detector at 205 nm. Pseudo-first order degradation kinetics were assumed.

RESULTS AND DISCUSSION

Efficacy and tolerability of thrombin inhibitors were linked and reduced to surrogate criteria like inhibition constants, *in vitro* coagulation parameters and specificity. When positions of inhibitors in the active cleft of thrombin were calculated using CAMD the resulting enthalpy values did not correlate well with inhibition constants for thrombin (data not shown) CAMD was still a very useful tool for visualisation of inhibitor-thrombin complexes. To address oral bioavailability we focused on the aspects of stability in plasma and gut homogenates. Stability is considered among other factors such as absorption by the gut wall or first pass elimination in liver, an essential factor for systemic plasma level after oral administration of a compound.

TABLE 1
Inhibition constants of thrombin inhibitors

Compound	K_i[nM]			
	thrombin	factor Xa	trypsin	plasmin
NAPAP	11	63 400	499	39 000
1	2.4	31 400	2035	7 500
2	73	n.d.	1404	n.d.
3	91	40 400	478	12 500
4	20	73 500	505	19 600
5	279	226 100	582	28 200
6	103	5349	618	150 000
7	3.1	172 000[a]	670	21 100[a]
8	33	71 100	2952	3 088

[a] K_i-value for stereoisomer **7a**

Our template for chemical alterations was the molecule NAPAP. The inhibition constant toward thrombin was determined to be 11 nM at assay conditions being pH 7.8 at 37°C (Table 1). Reports indicate that the introduction of a carboxyl group at the piperidine constituent of NAPAP would implement improved pharmacokinetic properties and enhanced tolerability. The most potent compound was *β*-naphthylsulfonylylcyl-*D,L*-4-amidinophenylalanyl-L-proline associated, however, wih an unacceptably low affinity toward thrombin (K_i = 510 nM).[19] The loss of affinity was also in agreement with our results of modelling compounds containing an hydroxyl group in the piperidine ring. It suggested that even fairly small alterations at R3 position will disturb optimal occupation of the hydrophobic pocket. In this case the K_i-value increased by roughly one order of magnitude (Table 1) compared to NAPAP.

TABLE 2
Effect of stereoisomers of thrombin inhibitors on inhibition constants and coagulation parameters

Compound	stereoisomer	K_i [nM]	IC_{50} [nM]	
		thrombin	aPTT	TT
7a	L-Asp, D-Adf	2.5	337	35
7b	D-Asp, L-Adf	1864	> 1000	> 1000
7c	D-Asp, D-Adf	288	> 1000	> 1000
7d	L-Asp L-Adf	788	> 1000	> 1000

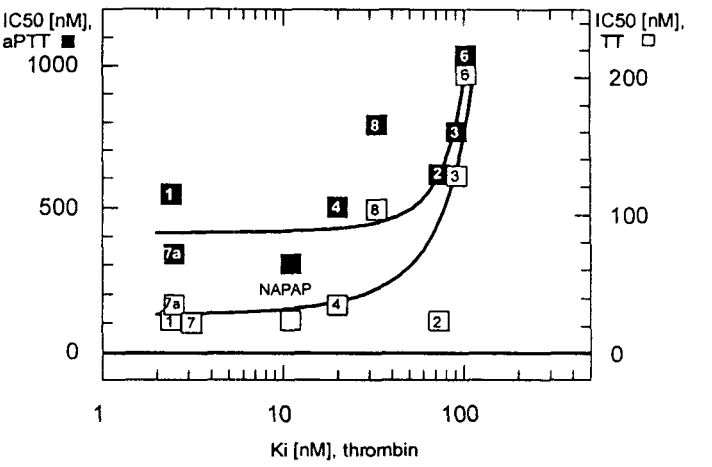


FIGURE 1 Influence of inhibition constants toward thrombin on IC_{50}-values of aPTT and TT. Inhibitor concentrations that were needed to double clotting times in aPTT (■) and TT (□) assays depended to a greater extent on their K_i-value when greater than 20 nM, 35 nM for aPTT, TT, respectively. At lower K_i-values the influence on IC_{50}-values became less pronounced. The exception was **8**.

We subsequently kept the amidinophenylalanine-piperidide structure constant and concentrated on modifying the N-terminal site. Substituting the R1-moiety by pentamethylchromanesulfonyl (Pmc) and the X-moiety by aza (compound **1**) an almost 5-fold increase in inhibitory activity was observed. Surprisingly the higher affinity did not translate into lower IC_{50}-values in *in vitro* coagulation assays aPTT and TT (Table 3 and Figure 1). The aPTT IC_{50}-value almost doubled compared to NAPAP.

TABLE 3
Potency of thrombin inhibitors in coagulation assays

Compound	IC_{50} [nM]		
	aPTT	TT	platelet-aggregation
NAPAP	304	24	31
1	546	24	140
2	615	24	n.d.
3	767	128	214
4	502	35	85
5	n.d.	n.d.	51
6	1035	202	115
7	337[a]	22	9[a]
8	792	104	32

[a] IC_{50}-value for stereoisomer **7a**. n.d. not determined

However this could, at least in part, be explained by its stability pattern in plasma. Compound **1** deteriorated in human plasma (Table 4) and therefore during the time course of preincubation of the assay the effective inhibitor concentration diminished. When exposure to plasma was minimised as was done in the *in vitro* TT-assay, **1** exhibited TT-IC_{50}-values that were within the expected range of the intact inhibitor (Table 3). IC_{50}-value for thrombin-induced platelet aggregation showed lower potency by a factor of 5 (Table 3).

Placing the naphthyl group in the R2 position and a succinyl group in the R1 position (compound **2**) caused also a significant fall in affinity (K_i = 73 nM) and a similar pattern of potency in the coagulation assays was noticed as had also been seen with **1** (Table 3). This suggested that the R1 substituent as well as the position of an acidic group play a pivotal role in instilling potency into amidinophenyl based inhibitors.

Introducing a carboxyl group in R2 position (aspartyl, **4**) caused only a decrease in affinity by a factor of 2. Exchanging the R1 subtituent in compound **4** by a 4-methoxy-2,3,6-trimethylphenylsulfonyl (Mtr) group (**7**), increased affinity toward thrombin and the potency in the coagulation assays. In attempts to optimise the substituent pattern at the phenyl ring it became more obvious that the occupation of the hydrophobic pocket is crucial with respect to loss or gain of potency. To dwell further on this aspect we investigated compounds with slight changes of the substituent pattern at the phenyl ring (**5**, **6**, **7**). Removing the methyl group of Mtr (**7**) in the 2-position yielded **5** or shifting the methyl group of Mtr from position 6 to position 5 yielded **6**. In both cases inhibitory activities decreased, either 25-fold or two orders of magnitudes, respectively (Table 1). Increases in coagulation parameters paralleled the changing affinities. CAMD studies revealed that indeed the Mtr group occupied the hydrophobic pocket almost perfectly. The methyl group in position 6 had enough space while a methyl group in position 5 collided with the hydroxyl group of Tyr60A of thrombin. The methoxy group in the 4 position also was located in a favoured position in comparison to an ethoxy group which led to a decrease in K_i by a factor of 10 (data not shown).

As mentioned above introducing an additional carboxyl group was thought to counteract the negative influence of basicity. Compounds **4–8** all contained an additional carboxyl group in form of aspartyl substituting glycyl (R2) with the exception of compound **8** that contained a glutamyl moiety. Expanding the carboxyl group by one methylene group caused a reduction in thrombin affinity (K_i = 33 nM). CAMD studies could provide a possible molecular explanation: the expanded side chain collided with Trp 148. In addition the interaction of the β-carbonyl group with Gly 219 NH was hampered due to its unfavourable distance.

These findings prompted us to investigate the influence of stereoisomers of **7** (Table 2). The L-Asp, D-Adf (4-amidinophenylalanine) isomeric configuration (**7a**) proved to be the most effective one, displaying a K_i-value of 2.5 nM while the D-Asp, D-Adf isomer (**7b**) proved to be poorly effective with an almost 3 orders of magnitude higher K_i-value.

In order to get an idea of the desired potency of compounds with respect to their thrombin affinities, K_i-values were correlated to *in vitro* coagulation parameters. K_i-values plotted versus molar IC_{50}-values of aPTT and TT assay suggested that compounds with K_i-values down to 20 to 30 nM corresponded well with an increase

in potency. However, at lower K_i-values the increased affinity to thrombin did not translate into reduced molar IC_{50}-values (Figure 1). This suggested that in plasma, inhibiors with K_i-values below 20 nM existed almost completely in the form of their thrombin-inhibitor complexes. In platelet rich plasma a somewhat higher binding power was needed (Table 3). That could be explained by taking into account the competing thrombin receptor of platelets.

Specificity of inhibition

Oligo-specific binding or inhibition of other enzymes by thrombin inhibitors could be thought of as acting synergistically as well as counteractively. In the case of inhibiting, i.e. factor Xa, thrombin generation would be reduced whereas inhibiting plasmin, endogenous lysis of a formed thrombus would be prevented. When rational drug design has been directed to oral absorption of thrombin inhibitors, trypsin binding is undesired. Trypsin binding in particular must also be considered as a crucial parameter for specificity, based on the similarity of its catalytic site to thrombin. Therefore, it was not surprising that trypsin specificities were found to be lower than specificities for plasmin or factor Xa (Figure 2). Enzyme specificity was defined by the ratio of K_i(enzyme) over K_i(thrombin). Depending on the K_i-value of thrombin for a particular compound specificities ranged for trypsin, plasmin and factor Xa from 2–850, 100–8500 to 50–13100, respectively. Analysis of the specificity data revealed that the K_i-value of thrombin (denominator) set the tone of the specificity magnitude where the binding affinity to the other three enzymes (numerator) generally has been kept constant. Exceptions to these generalised findings were compound for trypsin (Figure 2), **6** and **8** for plasmin (Figure 4) and **6** for factor Xa (Figure 3).

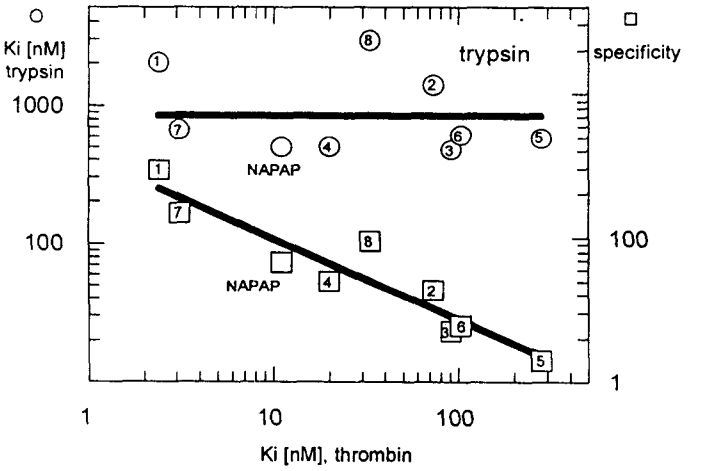


FIGURE 2 Inhibition constants toward thrombin and trypsin and specificity for thrombin versus trypsin. All chemical modifications affected the K_i-value of thrombin while the K_i of trypsin (○) was less sensitive with the exception of compounds **1,8**. Therefore the improvement of specificity over trypsin (□) is due to higher inhibitory potency toward thrombin.

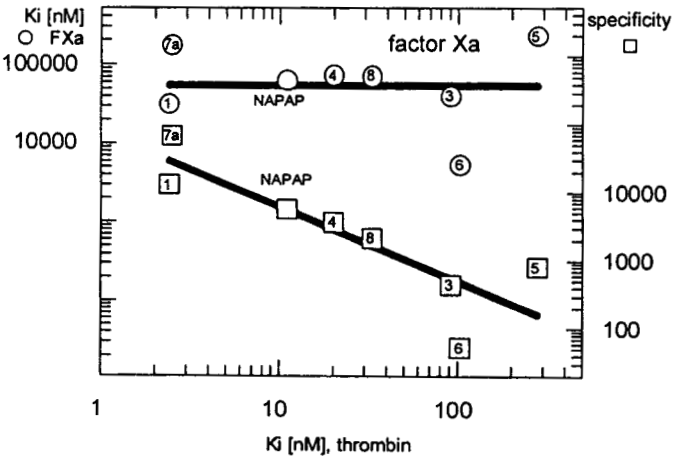


FIGURE 3 Inhibition constants toward thrombin and factor Xa and specificity for thrombin versus factor Xa. All chemical modifications affected the K_i-value of thrombin while the K_i of factor Xa (○) was less sensitive with the exception of compound **6**. Therefore the improvement of specificity over factor Xa (□) is due to higher inhibitory potency toward thrombin.

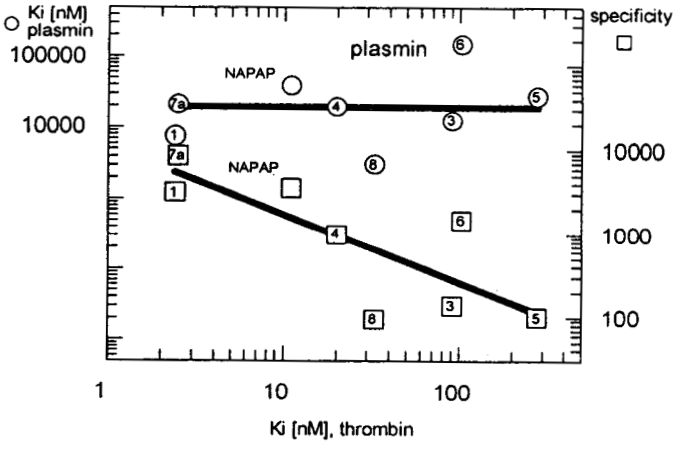


FIGURE 4 Inhibition constants towards thrombin and plasmin and specificity for thrombin versus plasmin. All chemical modifications affected the K_i-value of thrombin while the K_i of plasmin (○) was less sensitive with the exception of compounds **6** and **8**. Therefore the improvement of specificity over plasmin (□) is due to higher inhibitory potency toward thrombin.

TABLE 4
Stability of thrombin inhibitors in plasma and gut homogenate at 37°C with the assumption of pseudo-first order degradation kinetic

Compound	plasma		gut homogenate	
	$k[h^{-1}]$	$t_{90}[h]$	$k[h^{-1}]$	$t_{90}[h]$
NAPAP	0.00033	320	< 0.00036	> 290
1	0.78	0.1	0.0063	17
4	< 0.00036	> 290	< 0.00036	> 290
7a	0.0049	22	< 0.00036	> 290

In summary thrombin inhibitors based on the Adf-piperidide moiety that produced the maximal effect in coagulation assays (K_i < 20 nM) displayed a specificity of 3 to 5 orders of magnitudes, likely to be sufficient not to affect the thrombin inhibitor efficacy in preventing thrombin mediated clot formation.

Stability of the inhibitor

Stability in plasma environment on one hand must be thought of as a contributor to the half life of the pharmacokinetic effect. On the other hand aiming at an orally available compound a certain stability in gastro-intestinal (GI) tract would be desired. For stability studies four compounds (NAPAP, **1,4,7a**) were selected each representing a step change in the molecular design (Table 4). All compounds appeared to be rather stable in gut homogenate. Assuming pseudo-first order rate degradation kinetics for the most labile compound (**1**) t_{90} stability was calculated to be 17 h. Also in plasma **1** was the most labile compound with a t_{90} stability of 0.1 h. In summary it could be said that for these compounds stability in the GI tract would not be the rate limiting step for systemic plasma levels after oral administration.

Compound **7a** combined the features of good thrombin inhibitory activity, sufficient potency in the coagulation assay (Table 2) as well as reduced basicity, reducing the disadvantage of causing histamine release related fall in blood pressure (data not shown). Furthermore, due to its high stability in gut homogenate and therefore presumably in the GI tract, compound **7a** could be a good candidate for reasonable absorption by the gut wall.

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