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Pharmacokinetic Optimization of 3-Amino-6-chloropyrazinone Acetamide Thrombin Inhibitors. Implementation of P3 Pyridine N-Oxides to Deliver an Orally Bioavailable Series Containing P1 *N*-Benzylamides

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Abstract—In this manuscript we demonstrate that a modification principally directed toward the improvement of the aqueous solubility (i.e., introduction a P3 pyridine N-oxide) of the previous lead compound afforded a new series of potent orally bioavailable P1 *N*-benzylamide thrombin inhibitors. An expedited investigation of the P1 SAR with respect to oral bioavailability, plasma half-life, and human liver microsome stability revealed **5** as the best candidate for advanced evaluation.

Deep vein thrombosis, pulmonary embolism, and thromboembolic stroke represent a set of disorders that can arise from the formation of an occlusive vascular thrombus and are a major cause of mortality worldwide.[1] As a result of this medical need, an intensive search continues for antithrombotics that are safer and, at minimum, as efficacious as the current standard of care for the treatment of venous thrombosis and the prevention of cardiogenic thromboembolism (i.e., low molecular weight heparins and warfarin). An equally important requirement, particularly with regard to chronic therapy, is the development of orally bioavailable drugs with a convenient dosing regimen (e.g., once-daily dosing, no drug–drug interactions). Toward this end, recent approaches to anticoagulant therapy have been aimed at identifying direct small molecule inhibitors of specific coagulation cascade enzymes which possess the pharmacokinetic properties required for once daily oral administration.[2] Assuming predictable pharmacokinetics, the direct mechanism of action of these inhibitors should overcome the monitoring and safety liabilities associated with existing anticoagulation therapies. A prominent target to emerge from this effort is the trypsin-like serine protease thrombin (Factor IIa). Thrombin occupies a central role in hemostasis:[3] its primary procoagulant actions are the activation of

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platelets and cleavage of fibrinogen to fibrin, which together constitute the primary components of a vascular thrombus.

The majority of previously disclosed thrombin inhibitors retain a highly basic amidine that mimics the binding of the P1 Arg of natural peptide substrates to Asp-189 at the base of the S1 pocket. Although this salt-bridge interaction leads to a substantial amount of binding energy,[4] the incorporation of these extremely basic moieties is generally associated with low levels of oral bioavailability and high plasma clearance.[5] In an effort to produce orally bioavailable thrombin inhibitors with acceptable pharmacokinetics, our laboratories have focused on establishing compounds that incorporate less basic P1 binding elements (e.g., 2-aminopyridine).[6] Toward this end, a recent report detailed the metabolism directed optimization process which resulted in the identification of a series of potent, selective and orally bioavailable compounds containing weakly basic pyridine P1 moieties.[7] Although successful in producing a thrombin inhibitor **1** (Fig. 1) which showed oral bioavailability across three species and good stability in the presence of human hepatocyte preparations, an alternative approach to pharmacokinetic optimization was desired. These compounds exhibited poor solubility at physiologic pH (**1**: 11 μg/mL @ pH 7.4, logP = 1.50) and, most notably, the expedient production of analogues was hindered by the multi-step syntheses of the substituted P1 pyridines. In this paper, we demonstrate that a modification principally directed toward the improvement of the aqueous solubility additionally permitted a series of previously intractable P1 *N*-benzylamides thrombin inhibitors to show useful levels of oral bioavailability (Fig. 1). Due to the ready availability of the P1 benzylamines, this result allowed the rapid investigation of P1 SAR with respect to oral bioavailability, plasma half-life, and human liver microsome stability.

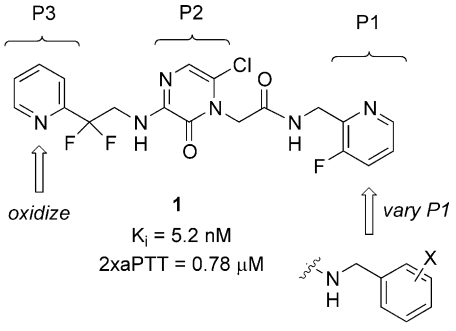


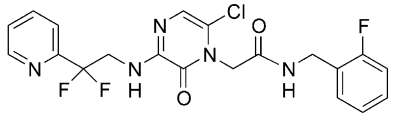
Figure 1. Modifications of thrombin inhibitor **1**.

The inhibition constants (K_i) versus thrombin (IIa) and the concentration needed to double the activated partial thromboplastin time (2×aPTT) in human plasma were determined for each compound (Table 1).[8] As the principal goal of this study was the optimization of pharmacokinetics, compounds meeting our established potency criteria[9] were directly subjected to dog pharmacokinetic and human liver microsome stability experiments (Table 1).[10] The electron withdrawing effect of the β-difluoro substitution renders the P3 pyridine ring nitrogen of **1** relatively non-basic (pK_a's < 2.1), and in an effort to improve the low aqueous solubility of these compounds this pyridine was converted to its N-oxide. This modification not only improved the solubility (**2**: 44 μg/mL @ pH 7.4), but provided an improvement in enzyme binding potency (**2**: K_i = 3.1 nM).[11] This enhanced solubility translates into improved functional potency (2×aPTT = 0.54 μM) and excellent performance in the rat $FeCl_3$ arterial thrombosis model (0/6 occlusions, final plasma concentration = 1079 ± 69 nM).[12]

Upon oral dosing of **2** (1 mpk) to dogs, a 1.78 μM maximum plasma concentration (C_{max}) and a 2.5 h plasma half-life ($t_{1/2}$) were achieved (Table 1). The substantial decrease in plasma half-life observed upon the introduction of the P3 N-oxide into **1** can be attributed to the increased plasma clearance (Table 2). The excellent microsome stability (**2**, Table 1) indicated that hepatic clearance was low, therefore renal excretion was suspected to be the primary contributor to the higher overall clearance; indeed collection of urine from the dog pharmacokinetic experiment showed that 47% of **2** was excreted intact.[13]

In an attempt to increase the extremely low plasma protein binding (80% unbound), overall lipophilicity (log P = 0.19), and concomitantly reduce the renal clearance, the P1 3-fluoropyridine was replaced with a 3-chloropyridine (**3**, Table 1). This minimal substitution increased protein binding (50% unbound; log P = 0.75), effectively reduced the plasma clearance (3.0 mL/min/kg, Table 2) and accordingly improved the observed half-life ($t_{1/2}$ = 6.0 h).

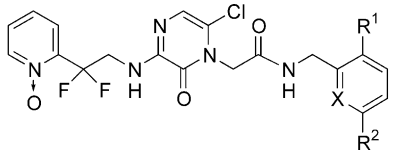
The incorporation of the P3 pyridine N-oxide successfully addressed the issue of low solubility, however the multistep syntheses of the 2-aminomethylpyridine P1s still prohibited a rapid SAR investigation. It was envisaged that the enhanced polarity acquired upon P3 N-oxide introduction could be exploited to improve the poor oral bioavailability of thrombin inhibitors containing benzylamine P1s;[14] for example, 2-fluorobenzylamine thrombin inhibitor **4** exhibited very low plasma levels in a dog oral pharmacokinetic experiment (Fig. 2).[15] Preparation of the N-oxide afforded **5**, in which the enzyme and anticoagulant potency were improved (Table 1); more significantly, this compound exhibited high plasma levels (C_{max} = 3.5 μM) and a good half-life ($t_{1/2}$ = 4.9 h) after oral dosing (F = 40%). To establish the general utility of this approach, the simple *N*-benzylamide analogue **6** was prepared and also



4
K_i = 4.8 nM
2xaPTT = 1.24 μM
C_{max} = 0.017 μM

Figure 2.

Table 1. Effect of P1 substitution on thrombin inhibition (K_i), in vitro anticoagulant potency (2×aPTT), dog pharmacokinetic parameters, human liver microsome stability, and log P



Compd	X	R^1	R^2	K_i (nM)	2×aPTT (μM)	C_{max} (μM)[a]	$t_{1/2}$ (h)[b]	HLM[e]	Log P
2	N	F	H	3.1	0.54	1.78	2.5	6.5	0.19
3	N	Cl	H	2.9	0.47	1.09[c]	5.5	1.3	0.75
5	CH	F	H	2.3	0.40	3.50	4.9	1.1	1.39
6	CH	H	H	7.0	0.94	3.20	6.3	1.3	1.23
7	CH	Cl	H	2.5	0.54	0.70	2.9	0.15	1.89
8	CH	Me	H	6.3	0.77	1.76	3.6	0.29	1.74
9	CH	CF_3	H	3.6	0.93	1.43[c]	3.7	0.05	2.24
10	CH	OMe	H	3.0	0.56	2.48[c]	3.8	0.44	1.36
11	CH	OEt	H	5.2	0.87	0.77	1.2	0.08	1.91
12	CH	$OCHF_2$	H	3.0	0.69	2.03	2.8	0	1.63
13	CH	OCF_3	H	2.4	0.81	1.67	3.0	0	2.34
14	CH	OCH_2CF_3	H	1.8	0.58	2.29	2.5	0	1.93
15	CH	H	F	7.3	0.86	1.37[d]	4.1	0.71	NA
16	CH	H	Cl	0.26	0.23	1.91[c]	2.6	0	NA
17	CH	H	Br	0.19	0.28	1.01	3.6	0.14	2.11
18	CH	H	Me	0.8	0.30	2.19	1.1	0.58	1.81
19	CH	H	OMe	1.2	0.43	1.22	2.8	0.71	1.31
20	CH	H	$OCHF_2$	7.0	0.97	0.12	4.8	0.54	1.73
21	CH	F	F	2.4	0.43	2.29	3.9	0.05	NA
22	CF	F	H	0.85	0.33	0.21	6.2	3.4–0.61	1.23
23	CH	F	Cl	0.16	0.31	0.48	2.1	0	NA
24	CH	p-F	Cl	3.6	0.78	0.42	2.4	0	1.98
25	CH	Cl	Cl	0.087	0.39	0.11	2.1	1.04	2.61
26	CCl	H	Cl	2.0	0.88	0.29	2.5	0.39	2.14
27	CCF_3	F	H	1.3	0.88	0.07[c]	2.4	0	1.89
28	CF	F	F	1.1	0.31	1.51	3.5	0.04	1.42
29	CH	CF_3	F	1.7	0.63	0.99	2.8	0	1.99

[a]Dose = 1 mg/kg unless otherwise noted.
[b]po, Half-life.
[c]Dose = 0.7 mg/kg.
[d]Dose = 0.5 mg/kg.
[e]Human liver microsome stability versus an internal standard.[10]

Table 2. Comparison of dog pharmacokinetic parameters

Compd	PB (%free)		Cl_p (mL /min/kg)	V_d (L/kg)	iv $t_{1/2}$ (h)	F (%)
	Dog	Human				
1	33	14	2.2	0.62	4.5	66
2	80	70	4.91	1.13	2.9	83
3	50	41	3.03	1.17	6.0	80

shown to give high plasma levels and a good half-life upon oral administration (C_{max} = 3.2 μM, $t_{1/2}$ = 6.3 h). The better oral bioavailability is likely a result of improved oral absorption[14] due to the enhanced solubility of the N-oxides versus the non-basic pyridines.[16]

Having demonstrated that the P3 pyridine N-oxide confers bioavailability to inhibitors containing simple P1 *N*-benzylamides, an investigation was undertaken to explore the P1 SAR with respect to potency, pharmacokinetics, and human microsome stability (Table 1). The selection of P1s was biased towards simple, substituted benzylamines (e.g., R^1, R^2 = halogen, fluoroalkylether, Table 1) which contain no obvious metabolic 'soft-spots.' Substitution at the *ortho* position exhibited little effect on the thrombin inhibition with all prepared analogues satisfying our established potency criteria (**5**–**14**, Table 1). This class of compounds exhibited a range of C_{max} (0.70–3.50 μM), with a general trend toward lower log P compounds demonstrating higher plasma levels; the oral half-lives within this group ranged from 1.2–6.3 h. As anticipated,[17] substitution at the *meta* position of the P1 phenyl ring had a more pronounced effect on binding, with small lipophilic groups (e.g., Cl, Me) providing the best potency (**15**–**20**, Table 1). These compounds also exhibited good plasma levels and half-lives after oral dosing (C_{max} = 0.12–2.19 μM, $t_{1/2}$ = 1.1–4.8 h). Disubstituted P1s generally afforded compounds with lower plasma levels, possibly due to the higher log Ps observed within this group (**21**–**29**, Table 1). Overall, there emerged a trend, although not absolute, toward inhibitors with lower log P exhibiting better human microsome stability.

Based upon thrombin and in vitro anticoagulant potency, dog oral bioavailability and human liver microsome stability, the best compound to emerge from this survey is **5**. This inhibitor is extremely selective for thrombin, exhibiting minimal (>49 μM) activity versus trypsin, t-PA, plasmin, factor Xa, plasma kallikrein, activated protein C, urokinase and chymotrypsin. Further in vivo characterization (Table 3), revealed that this inhibitor exhibited good bioavailability in dog (F=40%), rat (F=52%) and rhesus monkey (F=21%). Clearance is low in dogs (1.55 mL/min/kg) and, based on in vitro metabolic scaling studies, is also predicted to be low in man.[13]

Table 3. Pharmacokinetic parameters of **5** in dog, rat and rhesus monkey[a]

Species	C_{max} (μM)	Cl_p (mL/min/kg)	V_d (L/kg)	iv $t_{1/2}$ (h)	F (%)
Dog	3.28	1.55	0.49	6.3	40
Rat	1.94±0.79	43±7	0.93±0.12	0.43±0.05	52
Rhesus	1.05	19	1.5	1.1	21

[a]Dose = 1 mg/kg (dog), 10 mg/kg (rat), 5 mg/kg (rhesus).

This compound demonstrated dose-dependent efficacy in the rat $FeCl_3$ arterial thrombosis model upon iv dosing (Table 4). At the maximum dose of 10 μg/kg/min (solubility limit in vehicle) near complete efficacy (2/12 occlusions, 1RF) was observed at plasma levels that minimally elevated the aPTT (ca. 1.5-fold elevation); a dose dependent effect on the thrombin time (TT) was observed.

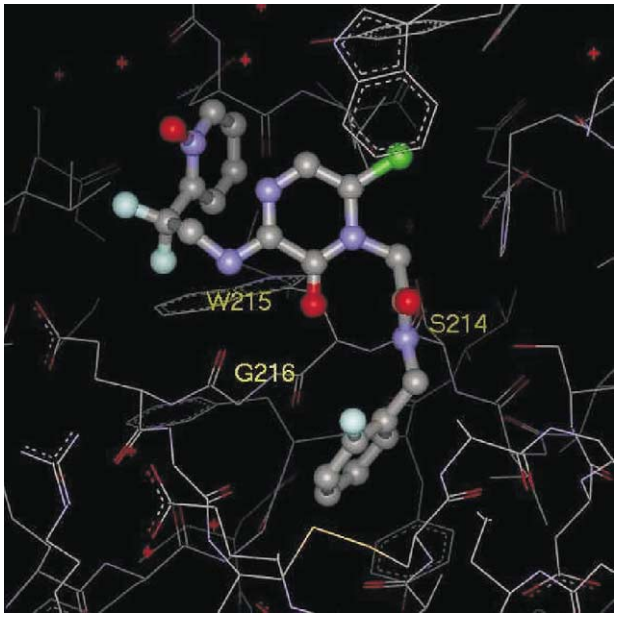
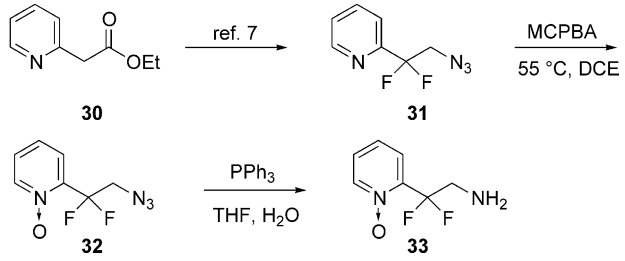


Figure 3. X-ray crystal structure of **5** bound in the thrombin active site.

Determination of the X-ray crystal structure of **5** complexed to thrombin revealed the same general binding mode as observed with **1**:[7] the fluorobenzene ring occupies the S1 specificity pocket, the chlorine of the pyrazinone fills the insertion loop, and the P3 aryl group binds in the distal hydrophobic pocket (Fig. 3). The antiparallel β-sheet hydrogen bonding motif between the aminopyrazinone and Gly-216 is maintained (d=2.63 and 2.94 Å); similarly the Ser-214 H-bond to the inhibitor amide is conserved (d=3.10 Å). In S3, the N-oxide projects toward solvent and appears not to be involved in any specific interaction; the improved potency gained upon incorporation of the π-deficient P3 pyridine N-oxide may be due to reinforcement of the edge-to-face σ–π interaction between the P3 aryl group and the π-rich Trp-215.[18]

Synthesis of the 2,2-difluoro-2-(2-pyridyl-*N*-oxide)ethylamine **33**[19] commenced with azide **31**, prepared by our previously disclosed route.[7] Oxidation of the electron deficient pyridine of **31** could be effected by treatment with MCPBA at elevated temperature in the presence of Kishi's radical inhibitor (Scheme 1).[20] Staudinger reduction afforded the 2,2-difluoro-2-(2-pyridyl-*N*-oxide)ethylamine **33** as a low-melting solid.



Scheme 1.

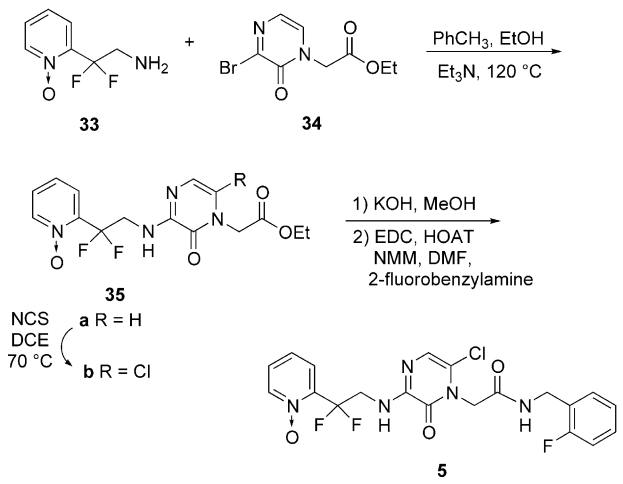
Coupling of the P3 amine **33** to 3-bromopyrazinone **34** required heating in a sealed tube at 120 °C for 24 h (Scheme 2). Chlorination of **35a** with 1 equivalent of NCS in DCE occurred with complete regioselectivity to afford the 6-chloropyrazinone **35b**. Hydrolysis of ethyl ester afforded the corresponding acids, which underwent EDC-mediated amide coupling with the P1 amines to afford the final products (e.g., **5**).

In this paper, we have demonstrated that a modification principally directed toward the improvement of the aqueous solubility (i.e., introduction a P3 pyridine N-oxide) of the previous lead compound afforded a new series of potent orally bioavailable P1 *N*-benzylamide

Table 4. Effects of intravenous administration of **5** in the rat model of FeCl3-induced carotid thrombosis[a]

Dose	Incidence of Occlusion	TTO	TT (s)	APTT (s)	Plasma level (μM)
—[b]	19/22 (2RF)	15±1	14.4±0.2	17.2±0.4	—
10	2/12 (1RF)	26±5	64.7±5.4	25.4±0.9	788±51
5	3/6 (1RF)	15±1	59.3±6.5	22.6±0.9	402±30
3	5/6	19±1	31.6±1.9	24.1±1.1	219±20

[a]Dose @ μg/kg/min infusion; TTO = time to occlusion; TT = thrombin time (ref 12).
[b]Vehicle = 0.3% DMSO.



Scheme 2.

thrombin inhibitors. This finding expedited an investigation of the P1 SAR with respect to oral bioavailability, plasma half-life, and human liver microsome stability which revealed **5** as the best candidate for advanced evaluation. Further studies are required to evaluate the safety, tolerability, pharmacokinetics, and, ultimately, the long-term utility of these thrombin inhibitors as anticoagulant therapeutics.

Acknowledgements

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