Discovery of the Fibrinolysis Inhibitor AZD6564, Acting via Interference of a Protein−Protein Interaction

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S *Supporting Information*

ABSTRACT: A class of novel oral fibrinolysis inhibitors has been discovered, which are lysine mimetics containing an isoxazolone as a carboxylic acid isostere. As evidenced by X-ray crystallography the inhibitors bind to the lysine binding site in plasmin thus preventing plasmin from binding to fibrin, hence blocking the protein−protein interaction. Optimization of the series, focusing on potency in human buffer and plasma clotlysis assays, permeability, and GABAa selectivity, led to the discovery of AZD6564 (**19**) displaying an in vitro human plasma clot lysis IC_{50} of 0.44 μM, no detectable activity against GABAa, and with DMPK properties leading to a predicted dose of 340 mg twice a day oral dosing in humans.

KEYWORDS: Fibrinolysis, plasminogen, inhibitor, protein−protein interaction, isoxazolone, tranexamic acid
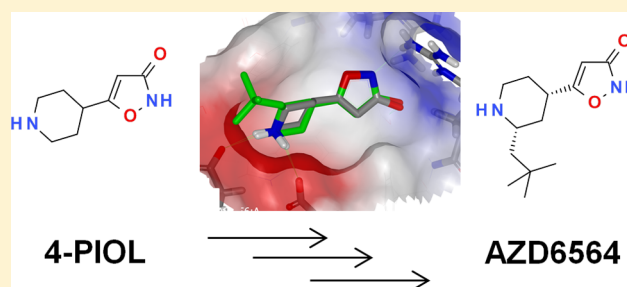
The physiological role of plasma coagulation is to prevent excessive blood loss from injured vessels, and the role of fibrinolysis is to dissolve fibrin clots after wound repair. Fibrinolysis is a cascade reaction with zymogene-to-enzyme conversion, feedback loops, and inhibitors.[1] The key protein in fibrinolysis is plasminogen, which is transformed into plasmin by the plasminogen activators (tPA and uPA). The localized activation of plasminogen and action of plasmin on the fibrin surface is facilitated by binding of C-terminal lysine residues on fibrin to lysine binding sites (LBS) in the kringle domains in plasminogen or plasmin, respectively.[2] Inhibition of fibrinolysis through blockade of LBS on plasminogen is an effective and safe treatment of bleeding conditions.[3] Two LBS inhibitors that are used clinically, ε amino caproic acid (EACA) and tranexamic acid (TXA), elicit their effects by preventing the protein−protein interaction between plasminogen and fibrin (Figure 1).[4]

The main use of EACA and TXA are in the treatment of heavy menstrual bleeding, although reduction of blood loss during surgery and trauma are other application areas.[4] In the recent CRASH-2 trial, it was demonstrated that TXA can be administered safely to a wide spectrum of patients with traumatic bleeding.[6] EACA and TXA have also been shown to have an effect as add-on treatment of mild hemophilia and in von Willebrands disease.[7]

TXA is more potent than EACA, but typical TXA efficacious doses in humans are 1−1.5 g given 3−4 times per day. Despite
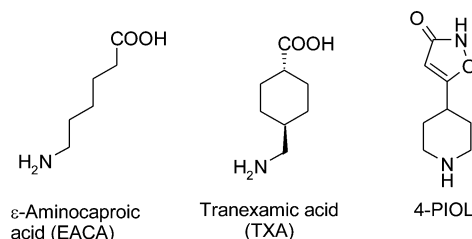

Figure 1. ε-Amino caproic acid (EACA), tranexamic acid (TXA), and 4-PIOL.[5]

attempts to reduce the dose by novel LBS inhibitors[8] or prodrugs of TXA,[9] no other LBS inhibitors have reached the market. Oral dosing of TXA is associated with dose-dependent gastrointestinal (GI) side effects, including nausea, vomiting, diarrhea, and cramping. This has limited the use and efficacious dosing in menorrhagia. There are also indications that treatment of menorrhagia in von Willebrands disease requires higher doses, and as a consequence, the dose-dependent side effects may become efficacy limiting.[10] In an attempt to prolong the exposure of TXA, it has been formulated as a modified release tablet, Lysteda (1.3 g three times daily), and this has recently received approval by the FDA.[11] Interestingly, GI side

effects in the treatment groups were not more frequent than in the placebo control group, indicating that the local amount of TXA released in the GI lumen may be causative for the GI side effects.[12]

Finally, there are rare reports of seizures during treatment with TXA, probably caused by a weak GABAa antagonistic activity.[12−14] It can be speculated that GI side effects associated with high dose TXA is also GABAa mediated. Thus, a novel oral fibrinolysis inhibitor, with the same mechanism of action as TXA but with more convenient dosing and higher selectivity over GABAa is hypothesized to be advantageous.

Full length plasminogen (Glu-plasminogen) may exist in a compact closed conformation and as an open extended confirmation.[15] There are five kringle domains in plasmin-(ogen), and each has an LBS. The amino acid sequence identity between the kringle domains are around 70%. The X-ray crystal structure of the full length plasminogen (pdb code: 4A5T) revealed that only kringle-1 (K1) is exposed on the surface of the closed complex, as such available for ligand binding. The respective LBS from K2, K4, and K5 are engaged in interdomain interactions contributing to the formation of the closed conformation, whereas the LBS on K3, being less exposed, does not engage any lysine from the neighboring domain. However, in the N-terminal truncated Lys-plasmi-nogen, having an open conformation, all LBS should be available for binding. Full length Glu-plasminogen with the closed conformation has one strong affinity binding site for TXA (K_d = 1.1 μM, K1) and several low affinity binding sites (K_d = 750 μM), while elongated Lys-plasminogen has two high affinity binding sites (K_d = 2.2 μM, K1, and K_d = 36 μM, probably K4).[16,17] TXA binds to plasmin(ogen) noncovalently by a charge interaction (pdb code: 1CEB). The carboxylic acid of TXA interacts with two arginines, ARG34 and ARG70, and the basic piperidine binds to two aspartate residues, ASP54 and ASP56, in the small and shallow pocket.[18] Thus, a novel ligand would require both positive and negative charges, i.e., to be a zwitterion, to effectively bind to K1. Although other kringles may be important in the binding and activation of plasminogen and subsequent fibrinolysis, this report focuses on the K1 ligand−protein interactions.

In the search for new chemical leads as plasmin(ogen) inhibitors, we previously reported that 4-PIOL (Figure 1) was identified via a small-scale virtual screening campaign using an electrostatic similarity approach using TXA as a seed molecule (Figure 2).[19] 4-PIOL was also found to bind to the shallow but very polar pocket of the K1 domain of plasmin. This interaction occurs in a similar way to TXA, with ionic interactions to the basic nitrogens in the two aspartates (ASP54 and ASP56) and acidic interactions to the two arginine residues (ARG34 and ARG70).

4-PIOL is a very interesting lead compound as it displays approximately 4-fold improvement of potency in both human buffer and plasma clot lysis assays compared with TXA (Table 1). However, 4-PIOL has also been reported to be a GABAa partial agonist.[20] In an in-house GABAa binding assay, measuring displacement of radiolabeled muscimol from rat brain vesicles, the IC$_{50}$ was 35 μM. This can be compared to the IC$_{50}$ of TXA in the same assay of 1600 μM. Although 4-PIOL serves as a promising lead from a potency perspective, it has poor permeability properties as measured in a Caco-2 cell permeability assay (P_{app} A to B < 0.01 × 10^{-6} cm s^{-1}) and development of a new oral pharmaceutical compound will require not only optimization of potency but also increased
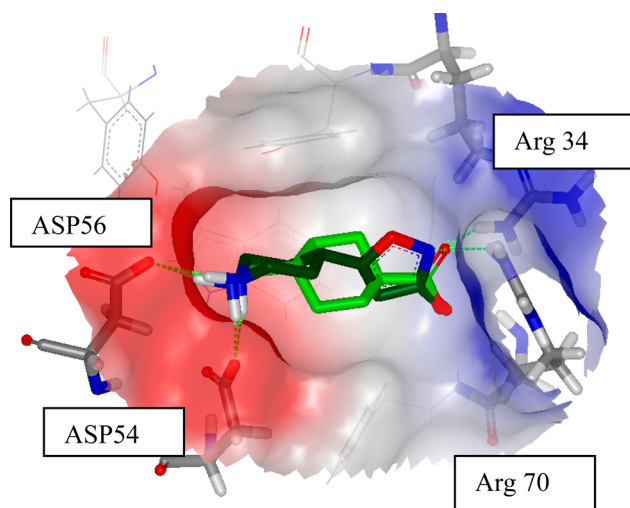

Figure 2. Overlay of TXA (light green) and 4-PIOL (dark green) in the binding site of plasmin(ogen).[19,21] The position of 4-PIOL is generated by docking into the binding pocket using Glide from Schrödinger.

Table 1. Initial SAR Development around 4-PIOL

compd	clotlysis IC$_{50}$ (μM)[a] buffer/plasma	GABAa IC$_{50}$ (μM)[b]	Caco-2 P_{app} A to B (10^{-6} cm s^{-1})
EACA	105/40		
TXA	12/3.1	1600	
4-PIOL	2.8/0.8	35	<0.01
1	100/100		1.0
2	31/40		0.4
3	5.7/2.0	51	
4	146/−		0.4
5[c]	6.3/2.1	>2000	0.6
6	12.2/−	267	0.5

[a]Values are means of three experiments. [b]Top concentration tested was 2000 μM. [c]Compound **5** is the racemic cis-isomer. − = not measured.

GABAa selectivity and increased permeability. These parameters became the important key factors in our optimization program described below.

First, a methyl scanning strategy was performed to evaluate structure and activity/property relationships (Table 1, synthetic details for compounds **1−6** can be found in the Supporting Information).

N-Substitution of 4-PIOL either at the piperidine (**1**) or isoxazolone (**2**) resulted in a drastic decrease in potency suggesting a more optimal geometry and zwitterionic character for 4-PIOL as compared to **1** and **2**. Further substitution on the isoxazolone ring, as in **3**, was tolerated from a potency perspective, but no improvement on the GABAa selectivity could be seen. Methyl substitution in the 4-position of the piperidine ring (**4**) decreased potency. However, methyl substitution in the 2-position of the piperidine ring as in **5**

gave a potency comparable with 4-PIOL, and most importantly, compound **5** dramatically improved the selectivity over GABAa with no detectable activity at a concentration of 2000 μM, hence revealing the 2-position as a site for further exploration. Finally, the regioisomeric isoxazolone **6** also lost potency compared with 4-PIOL.

Encouraged by the improved GABAa selectivity and equivalent potency of **5** as compared with 4-PIOL, further substituents in the 2-position of the piperidine ring were investigated. In Scheme 1, the synthetic route to the benzyl substituted compounds **14−17** is depicted.

Scheme 1. General Synthesis for 14−17[a]
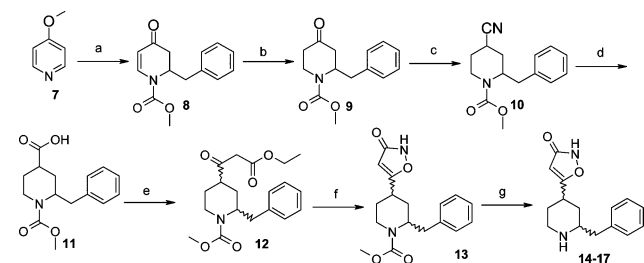
[a]Reagents and conditions: (a) (i) BnMgCl, THF, (ii) methyl chloroformate, (iii) HCl; (b) Pd/C, EtOAc; (c) TOSMIC, KtBuO; (d) conc. HCl; (e) (i) CDI, THF, (ii) MgCl$_2$, potassium ethylmalonate, (iii) diastereoisomer separation; (f) (i) NaOH, MeOH, H$_2$O, −20 °C, (ii) NH$_2$OH, −20 °C, (iii) 6 N HCl, 80 °C; (g) (i) chiral HPLC, (ii) 33% HBr in HOAc.

The addition of benzyl magnesiumchloride to 4-methoxypyridine **7** via the in situ formed 1-acyl pyridinium salt gave, after acidic workup, the 2-substituted dihydropyridone **8** in a method analogous to the one pioneered by Comins et al.[22] Hydrogenation over Pd/C gave the corresponding piperidone **9** that could be converted to the nitrile **10** by treatment with TOSMIC.[23] Hydrolyis of the nitrile in the presence of the methylcarbamate could be achieved by conc. HCl to yield the carboxylic acid **11**. The 3-isoxazolone moiety could be prepared in analogy to the method reported by Krogsgaard-Larsen et al.[24] via formation of the β-keto-ester **12**, and subsequent treatment of **12** with NaOH and then cyclization at −20 °C using hydroxylamine for the formation of **13**. The cis- and trans-diastereomers were conveniently separated by chromatography on the stage of the β-keto-esters **12**, whereas separation of the enantiomers by chiral HPLC was best undertaken with intermediates **13** before final removal of the methyl carbamate with HBr in acetic acid to give the benzyl substituted isoxazolone derivatives **14−17**.

To understand the stereoisomeric significance of substitution in the 2-position of the piperidine ring, the four stereoisomers of the benzyl substituted piperidines (**14−17**) were studied in more detail (Table 2). It appears that an (*S*)-configuration at the 4-position is critical for potency as highlighted with **15** and **17**, while the 2-position is more open for both configurations with **17** (2*R*,4*S*), having a cis-conformation, being somewhat more potent than the corresponding *trans*-analogue **15**. Introduction of a larger group such as benzyl into the 2-position resulted in the desired selectivity over GABAa, which is in line with what was seen for methyl-substituted **5**, but also gave an increased permeability as shown for **14−17**.

A crystal structure was obtained for **17** binding to K$_1$ of plasminogen (pdb code: 4CIK; Figure 3).

Table 2. Stereoisomeric Evaluation of 2,4-Disubstituted Piperidines 14−17

compd	clotlysis IC$_{50}$, (μM)[a] buffer/plasma	GABAa IC$_{50}$ (μM)[b]	Caco-2 P_{app} A to B (10^{-6} cm s^{-1})
14 (2S,4R)	150/−	1350	4.1
15 (2S,4S)	2.9/0.58	>2000	1.2
16 (2R,4R)	169/−	>2000	1.0
17 (2R,4S)	0.95/0.28	>2000	1.9

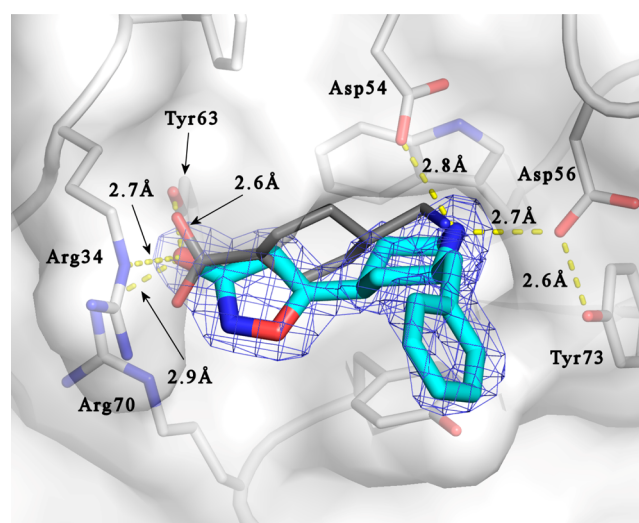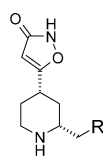[a]Values are means of three experiments. [b]Top concentration tested was 2000 μM.

Figure 3. X-ray crystal structure of **17** (blue) binding to plasminogen (pdb code: 4CIK). The structure was overlaid with the kringle 1/TXA complex (pdb code: 1CEB). The position of TXA (gray) is shown for clarity.

The structure shows that **17** binds in the same binding pocket as TXA, interacting with the same key amino acids. The isoxazolone carbonyl overlays well with the carboxylic acid moiety of TXA and is interacting with Arg34, Tyr63, and Arg70. The piperidine nitrogen of **17** takes a similar position as the amino group of TXA (about 1 Å apart between the respective nitrogen atoms), both are within H-bond distance to Asp54 (2.8 Å) and Asp56 (2.7 Å). Evidently, the benzyl substituent does not make any specific interactions with the K1 kringle, which is in line with the observed small difference in potency between the two stereoisomeric compounds **15** and **17**. Nevertheless, as indicated by the lower GABAa affinity for 2-substituted 4-PIOL analogues as **14−17**, it suggests that the benzyl groups populate an unfavorable region in the GABAa binding site. Similar substitution effects on the GABAa affinity has been observed for a related structural series indicating the generality of this finding.[25]

In order to assess the scope of the 2-substituted 4-PIOL derivatives, we further evaluated a range of analogues focusing on the 2*R*,4*S* piperidine configuration (Table 3, synthetic details for compounds **18−25** can be found in the Supporting Information). Introduction of larger aliphatic groups in the 2-

Table 3. Optimization of the Substituent in the 2-Position of the Piperidine

compd	R	clotlysis IC$_{50}$ (μM)^a plasma	GABAa IC$_{50}$ (μM)^b	Caco-2 A to B (10^{-6} cm s^{-1})
17	Ph	0.37	>2000	1.9
18	iPr	1.02	>2000	1.0
19	tBu	0.44	>2000	2.6
20	−CH$_2$C(CH$_3$)$_3$	0.77	>2000	4.5
21	−C$_6$H$_{11}$−	1.06	>2000	6.2
22	−CH$_2$Ph	0.39	>2000	2.6
23	2-F−Ph	1.26	>2000	1.8
24	3-F−Ph	0.36	>2000	3.1
25	4-F−Ph	0.45	1090	3.7

^{a}Values are means of three experiments. ^{b}Top concentration tested was 2000 μM.

position resulted in an increase in potency compared with methyl-substituted **5** as seen for the isobutyl **18** and neopentyl **19** derivatives. Further elongation with an additional methylene linker (**20**) gave a slight decrease in potency indicating a limitation in length and volume of the side chain. Introduction of an aliphatic ring as in **21** reduced potency. In contrast to **20**, elongation of the benzyl substituent to a phenethyl group was tolerated as shown for **22** displaying excellent potency as well as GABAa selectivity and permeability. Fluorine atoms in the phenyl ring (**23**−**25**) were also tolerated, but no significant improvement in the desired properties was achieved.

The analogues in Table 3 all show a narrow span in potency. Despite varying the side chain from aliphatic to aromatic, varying chain length, and introducing fluorine atoms, the potency varies less than 5-fold. This is in agreement with the conclusions from the crystal structure of 4-PIOL and **17** (Figure 3) that the substituent in the 2-position does not make any specific interactions to the protein. However, some steric limitations could be seen, for example, **20**. The small variation noted could possibly be attributed to differences in solvation and entropic effects.

Substitution in the 2-position of the piperidine ring led to more permeable compounds compared to 4-PIOL (Table 3). Introduction of the 2-substituents led to an increase in lipophilicity, which has been shown to increase membrane permeability, especially in the low lipophilicity range of these derivatives.[26] Systematic modification on the substitution pattern and stereochemistry revealed how the zwitterionic nature of the compounds contribute to the permeability profile. This is seen by stereoisomers **14** and **15** in Table 2, where the experimentally determined pK_a difference between the basic and acidic centers falls from 4.9 for compound **15** to 4.4 for compound **14**, while P_{app} increases from 1.2 to 4.1 × 10^{-6} cm s^{-1}, respectively. For compounds with similar lipophilicity, the compound with a lower difference between the acid and base pK_a values had the higher P_{app} value. Although not evident with classical molecular descriptors related to permeability such as logD or number of H-bond donors, which are identical for the stereoisomers, the permeability is also influenced by the pK_a difference between the acid and base within the molecule, and

permeability increases with reducing zwitterionic character. This effect has also been showed for other molecular series.[27] For more details on the relationship between pK_a and permeability for several compounds in this article, please see the Supporting Information.

Compounds in this series, including **17**, **19**, and **22**, showed very high metabolic stability in human hepatocytes (<1 μL/min/10^6 cells) and liver microsomes (<5 μL/min/mg), as well as displaying no notable inhibition of CYP450s and no inhibition of hERG. Biotransformation experiments in human hepatocytes supported the observed low metabolism. The only observed metabolism of these compounds, if any could be seen, was trace amounts of glucuronidation on the isoxazolone ring.

Compounds **17**, **19**, and **22** were selected for further exploration in vivo based on the balanced potency, permeability, and GABAa selectivity properties. The pharmacokinetic profiles of **17**, **19**, and **22** were studied in rat and dog after i.v. and oral dosing (Table 4).

Table 4. Pharmacokinetic Profiles of Selected Compounds

compd	CL_{tot}/CL_{renal} rat (mL/min/kg)^a	F% rat^a	CL_{tot}/CL_{renal} dog (mL/min/kg)^b	F% dog^b
17	46/11	33	2.9/0.2	73
19	13/6	39	1.1/0.8	53
22	24/6	19	1.8/0.12	59

^{a}Doses used: i.v. 5 μmol/kg and oral 20 μmol/kg. ^{b}Doses used: i.v. 3 μmol/kg and oral 6 μmol/kg.

Compound **17** had higher clearance in rat and dog compared with **19** and **22**. Compound **19** demonstrated good bioavailability of 39% and 53% in rat and dog, respectively, whereas compound **22** showed 19% and 59%, respectively. Investigation of the elimination pathways revealed that between 25% (**17** and **22**) and 45% (**19**) of the dose was eliminated renally in the rat and that between 7% (**17**) and 70% (**19**) was eliminated renally in the dog. To confirm reduction in bleeding in vivo, compounds **17**, **19**, and **22** were tested in a rat bleeding model (please refer to Supporting Information for details). It could be demonstrated that inhibition of fibrinolysis measured as the prolongation of the human plasma clot lysis time in vitro translated to a shortening of tPA-prolonged bleeding time in vivo (Table 5). For example, compound **19** reduced bleeding

Table 5. In Vivo Fibrinolysis Inhibition EC$_{50}$ and ED$_{50}$ Values in a Rat Bleeding Model^a

compd	rat in vivo EC$_{50}$ (μM)	rat in vivo ED$_{50}$ (μmol/kg·min)
TXA	19.2	15.9
17	0.79	0.65
19	1.62	0.84
22	1.92	1.44

^{a}Compounds were infused at four different doses in 6 individual animals per dose. The ED$_{50}$ and EC$_{50}$ were calculated as the infused dose and plasma concentration giving 50% reduction in bleeding time, respectively.

times from an incision in the tail by 50% at a 19- and 12-fold lower dose and plasma concentration, respectively, as compared to TXA. The corresponding values for **17** and **22** were 25- and 11-fold reductions in dose and 24- and 10-fold decrease in plasma concentration, respectively.

From clinical experience, it is known that a total TXA plasma concentration of at least 30 μM is necessary to achieve the desired clinical effect suggesting that a 95% receptor occupancy is required.[28] For compound **19**, the plasma and blood clot lysis IC$_{95}$ estimates fall in the range of 1.5 and 6.6 μM. These results are used as potency estimates in the dose prediction in man. If, as observed in rat and dog studies, hepatic metabolism and glomerular filtration are the routes of elimination the total human clearance is estimated to be 17.5 L h^{-1}, a composite of metabolism and renal filtration. Considering the 95% coverage of LBS and the absorption and clearance properties of **19** gives a human predicted dose estimate of twice daily dosing of 340 mg.[29]

In summary, we have described the discovery of a novel series of fibrinolysis inhibitors acting via interference of a protein−protein interaction. On the basis of the improved potency over TXA, excellent GABAa selectivity, no inhibition of CYP450 isozymes or hERG[30,31] (the human ether-à-go-go-related gene), good pharmacokinetic properties with both hepatic and renal routes of elimination, and a much improved predicted daily dose to man, compound **19** was selected as the drug candidate AZD6564 for development. Further profiling and testing will be the subject of future publications.

ASSOCIATED CONTENT

S Supporting Information

Synthetic procedures and experimental details (**1−25**). This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Project leader L.C.; Lead author D.P.; Medicinal chemists P.S., D.P., B.O., M.K., S.P., M.J., A.P., and T.F.; Computational chemists E.E. and J.B.; Bioscientists A.T., G.W., and D.G.; DMPK C.H., W.L., and L.L.; X-ray Y.X.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

TXA, tranexamic acid; 4-PIOL, 5-(4-piperidyl)-3-isoxazolone; EACA, epsilon aminocaproic acid; tPA, tissue-plasminogen activator; PLG, plasminogen; LBS, lysine binding site; DMPK, drug metabolism and pharmacokinetics; GABA, γ-aminobutyric acid; TBSCl, *tert*-butyl-dimethyl silyl chloride; DMF, dimethyl formamide; DCM, dichloromethane; THF, tetrahydrofuran; TBAF, *tert*-butyl ammonium fluoride; CDI, carbonyldiimidazole; HPLC, high performance liquid chromatography

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(29) Human dose was predicted from the equation

$$\text{dose} = C_{\text{uss}} \frac{Cl_{\text{u}}}{F} \tau$$

where C_{uss} is the unbound plasma concentration, Cl_{u} is the unbound clearance, τ is the dosing interval, and F is the bioavailability. Inserting a C_{uss} of 4 μM, Cl_{u} of 50 L h^{-1}, F of 0.65, and τ of 12 h gives a predicted dose of 340 mg BID.

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