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A conformationally constrained inhibitor with an enhanced potency for β-tryptase and stability against semicarbazide-sensitive amine oxidase (SSAO)

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ABSTRACT

A novel β-tryptase inhibitor with a basic benzylamine P1 group, a piperidine-amide linker, and a substituted indole P4 group was discovered. A substitution at 4-indole position was introduced to constrain the conformational flexibility of the inhibitor to the bioactive conformation exhibited by X-ray structures so that entropic penalty was decreased. More importantly, this constrained conformation limited the accessibility of this molecule to anti-targets, especially SSAO, so that an enhanced metabolic profile was achieved.

Mast cell stimulation leads to secretion of various types of proteolytic mediators among which is β-tryptase, a trypsin-like serine protease. Recent studies demonstrated a strong correlation between mast cell β-tryptase activity and a broad range of inflammatory and allergic reactions, which suggested that β-tryptase activity could be a key aspect of many inflammatory and allergic diseases, particularly asthma.[1] As a result, β-tryptase attracted wide-spread attention as a promising therapeutic target since the early 1990s.[2–5] Bode and coworkers successfully crystallized and resolved β-tryptase structures, which explained many of its biochemical properties and provided a strong foundation for structure-based drug design.[6] In the last two decades, a variety of β-tryptase inhibitors have been discovered including amidino derivatives,[2,3,7,8] di-basic inhibitors,[9,10] inhibitors with an azetidinone[11,12] or azepanone linker,[13] oxadiazole derivatives,[14,15] piperidine analogs,[16–20] and inhibitors with a benzylamine P1 group.

Comparing amidino-containing compounds, inhibitors with a benzylamine P1 group are preferable. While the best oral bioavailability achieved for benzamidine-like inhibitors without using a pro-drug strategy is ~5%, the one for benzylamine-containing compounds can reach 30% or higher. Benzylamine and benzamidine inhibitors with the same scaffold have very similar, if not identical, binding vectors to form the critical H-bonding interaction with D189. Benzylamine is an ideal bioisosteric replacement for benzamidine as a P1 group for inhibitors of trypsin-like serine proteases. When benzamidine is replaced by benzylamine without structural change to the rest of the inhibitor, the potency decreases more significantly for some trypsin-like serine proteases than for others. Fortunately, β-tryptase belongs to the later category and we were able to identify some highly potent inhibitors with a benzylamine P1 group. The liability of benzylamine, however, is that it is a known substrate for semicarbazide-sensitive amine oxidase (SSAO). Inhibitors with a benzylamine group are subject to oxidation under physiological condition, which renders them inactive towards β-tryptase. A major challenge was to develop benzylamine-containing β-tryptase inhibitors with a reasonable potency and metabolic profile, especially towards SSAO stability, which is the focus of the present study.

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As evidenced by both published X-ray structures[6,21,22] and our own in-house structural biology studies, the bioactive β-tryptase is a ring-like tetramer with four substrate binding pockets facing the central pore of the ring. Benzylamine inhibitors with a piperidine-amide linker exhibit conventional competitive binding modes in the β-tryptase substrate binding pocket. As shown in Figure 1, the P1 amino hydrogen atoms form H-bonding interactions with the backbone carbonyl oxygen of G219 and the side chain of D189, which represents the most recognizable interaction for trypsin-like serine proteases. The piperidine ring, *meta*-connected to the aminomethyl group, bypasses the catalytic triad which is formed by S195, D102, and H57, and projects the exiting binding vector directly into the S4 pocket located above W215. The carbonyl oxygen atom of the piperidine-amide has a direct H-bonding interaction with the backbone NH of G219 which is also a conserved interaction within this scaffold. Various aromatic P4 groups were explored and many of them exhibited promising activity against β-tryptase. In the present study, our effort was focused on various substituted indole rings as a P4 group which occupied the S4 pocket above the side chain of W215 and had a π-stacking interaction with the side chain of Q98.

Human SSAO is a dimeric membrane protein with a short N-terminal cytoplasmic tail, a membrane-spanning domain, and an extracellular catalytic domain.[23,24] The catalytic center of SSAO is featured by a cofactor, trihydroxyphenylalanine quinone (TPQ), and a copper ion coordinated by three conserved histidine residues. The catalytic center is deeply buried within the enzyme and is accessible only through a narrow channel with a diameter of about 4.5 Å. This channel is gated by the side chain of L469 which, along with the copper–TPQ coordination, controls the catalytic activity of SSAO. While specific interactions with residues lining the surface of the accessing channel are important for substrate specificity, the flexibility of substrates also plays an important role. As observed in molecular dynamics and induced docking studies, residues that form the accessing channel have reasonable flexibility to be able to reform the channel to various sizes and shapes to accommodate different substrates. However, such change in conformation increases the free energy of the system. The more rigid the substrate, the more significant the conformational change those residues must undergo to accommodate the substrate, which in turn further increases the free energy and decreases the probability of the substrate oxidation. Therefore, one possible strategy to enhance the SSAO stability of β-tryptase inhibitors is to constrain the conformation of the inhibitor in such a way that it is able to bind β-tryptase but not to pass through the accessing channel to the catalytic center of SSAO.

Various approaches to constrain the piperidine-amide conformation were explored and the present study focused on the conformational effect of substitutions on the 4-indole position. Due to conjugation, the amide of the piperidine-amide linker largely remains planar, which is evidenced by both X-ray structures and in-silico models. However, the torsion angle between the amide and the indole ring is twisted by about 55° in its bound conformation within the β-tryptase binding pocket. This twisted torsion angle is not preferred by the conjugation between the amide and the indole ring. Even if the steric repulsion between the piperidine and the 4-position of the indole ring helps to stabilize this twisted conformation, significant conformational flexibility remains for this part of the molecule. Another major area of the conformational flexibility for the linker is the 4-position of the piperidine ring, which can adopt either a chair or a boat conformation. Substitution at the 4-indole position constrains the conformational flexibility of the linker for both of these areas. It strictly locks the torsion angle between the amide and the indole ring to the X-ray bound conformation and further increases the energy difference between the X-ray bound conformation (chair) and other possible conformations including the boat form.

The effect of the conformational constraint was further evidenced by a molecular dynamics (MD) simulation, as shown in Figure 2. The compound with or without the 4-indole substitution went through identical MD simulations, in which a 500 ps trajectory was collected following a heating period and a 100 ps equilibration period. The simulation was carried out using the CHARMm force field through Discovery Studio v2.1 from Accelrys. The time step for the simulation was 1 fs under 300 K. The 'Shake' option was turned on to scale down the movement of hydrogen atoms. Two exiting vectors of the piperidine-amide linker, the one between piperidine ring and the benzylamine ring (C1–C2) and the one between the amide and the indole ring (C3–C4), were monitored and the torsion angle between them was analyzed to reflect the conformational flexibility of the piperidine-amide linker.

As shown in Figure 2, the molecule without the 4-indole substitution (the top trajectory) was much more flexible than the one with the dimethylamide substitution at the 4-indole position (the bottom

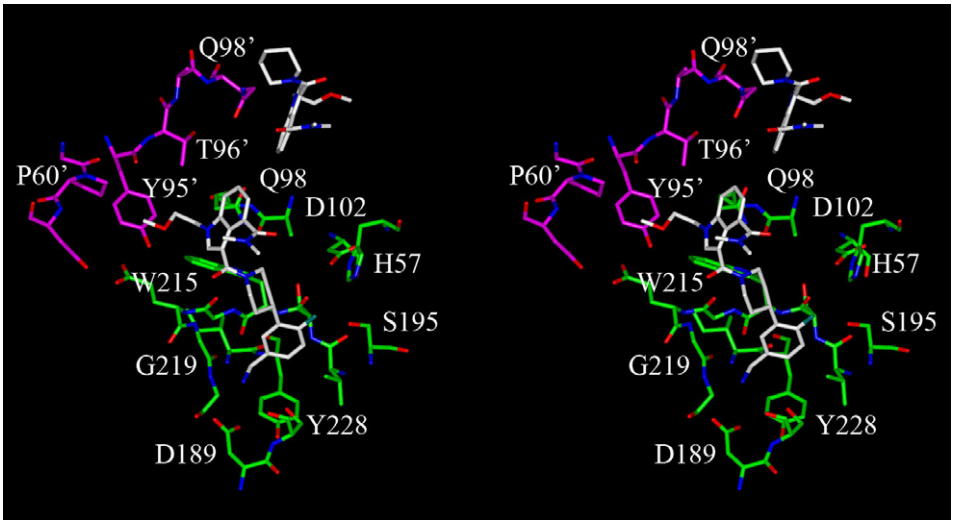


Figure 1. Stereo view of β-tryptase catalytic pocket. Carbon atoms of the inhibitors and two adjacent monomer of β-tryptase are colored white, green, and magenta, respectively. While the inhibitor in one substrate binding pocket is shown in whole, the one in the adjacent binding pocket is only shown in part to demonstrate the interface between the copies of the inhibitors.

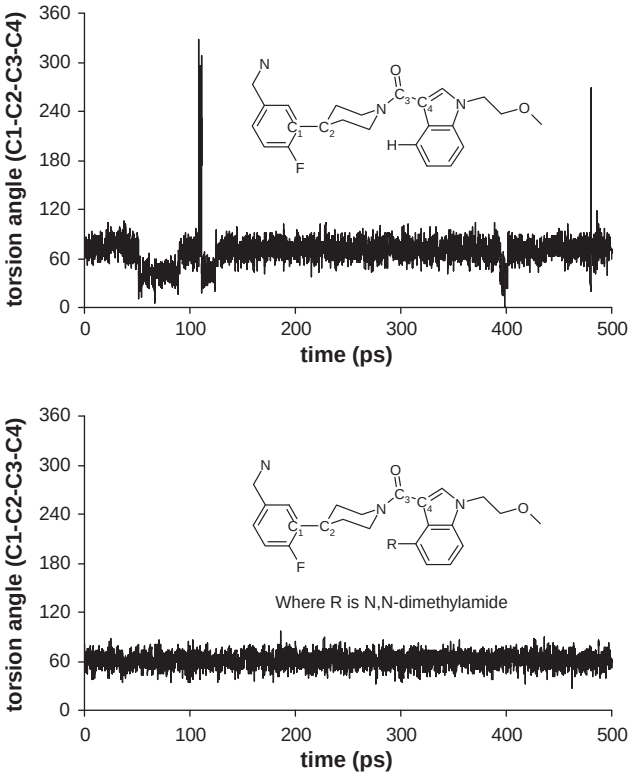
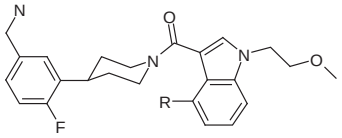


Figure 2. Torsion angle between the two exiting vectors of the piperidine-amide scaffold during a course of 500 picosecond (ps) molecular dynamics (MD) simulation. The bottom figure represented the trajectory of a molecule with a dimethylamide substitution at 4-indole position and the top represented the one without such substitution.

trajectory). The molecule without the 4-indole substitution experienced nine major conformational transformations for the linker within 500 ps while the molecule with a 4-indole substation stayed at the X-ray bound conformation through the entire simulation. Even during the timeframe in which neither molecule experienced any major conformational change (from 150 ps to 350 ps), the average linker flexibility of the molecule without the 4-indole substitution was higher than the one with the substitution. The standard deviation of the torsion angle between the two exiting vectors for the substituted molecule was 8.5° and the one for the unsubstituted molecule was 10.1°. Based on this MD simulation, the amide substitution at the 4-indole position properly constrained the conformation to the one exhibited in the X-ray structures and significantly reduced the flexibility of the molecule.

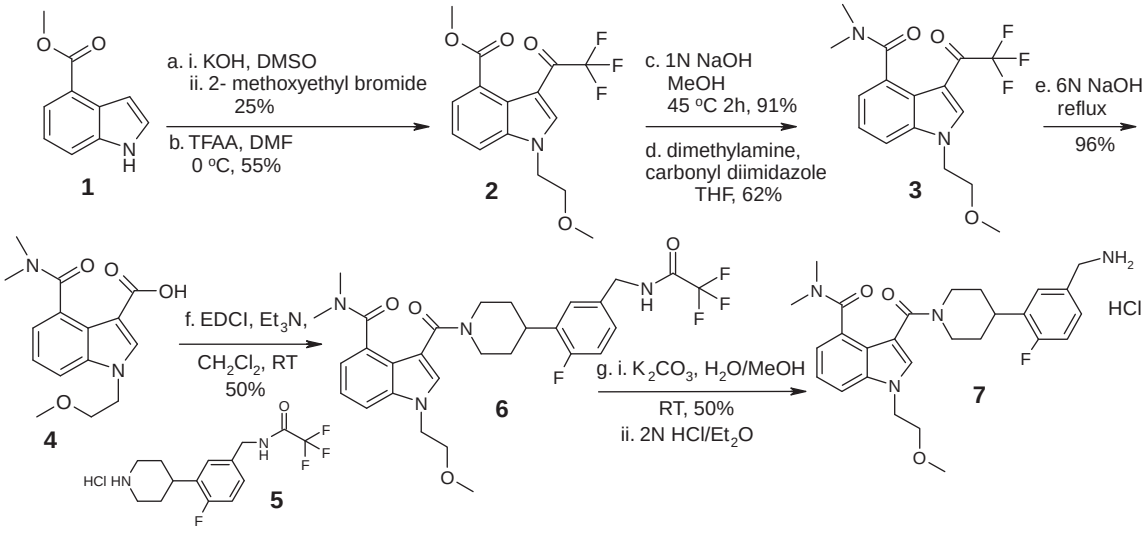
Table 1
β-Tryptase activity and selective DMPK profiling data for of compound **7** where R is *N*,*N*-dimethylamide



β-Tryptase (K_i)	20.0 nM
Compound remaining (SSAO, 24 h)	100%
Metabolic stability (human)	67%
Metabolic stability (guinea pig)	69%
Metabolic stability (rat)	92%
hERG (percent inhibition at 10 μM)	16%
CYP inhibition (3A4)	>30 μM

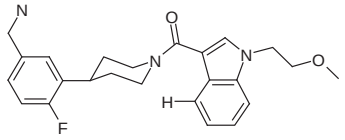
Synthesis of the dimethylamide substituted indole, inhibitor **7**, is described in Scheme 1. 4-Dimethylcarbamoyl-1-(2-methoxyethyl)-1H-indole-3-carboxylic acid **4** was prepared via N-alkylation of 1H-indole-4-carboxylic acid methyl ester **1** with 2-methoxyethyl bromide using powdered KOH followed by trifluoroacetylation to provide fluoroform **2**. Selective base hydrolysis of the ester **2** then subsequent treatment with the coupling agent carbonyl diimidazole and dimethylamine gave the corresponding amide **3**. Hydrolysis of fluoroform **3** under refluxing 6 N NaOH solution provided the key carboxylic acid intermediate **4** in high yield. Acid **4** was coupled with 2,2,2-trifluoro-*N*-(4-fluoro-3-piperidin-4-yl-benzyl)-acetamide hydrochloride **5** (see reference for synthesis) in the presence of EDCI to afford the penultimate intermediate **6**. Removal of the trifluoroacetyl protecting group of **6** with aqueous potassium carbonate followed by 2 N HCl in Et_2O provided the desired product **7**.

The inhibition of human β-tryptase was assayed with a chromogenic substrate S-2366 (Diapharma), L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroaniline hydrochloride. For determining IC_{50}, eight



Scheme 1. The reaction scheme for synthesizing compound **7**.

Table 2
β-Tryptase activity and SSAO stability of the reference compound



β-Tryptase (K_i)	54.2 nM
Compound remaining (SSAO, 24 h)	67%

concentrations of the compound were used: 0.0046, 0.0137, 0.04, 0.12, 0.37, 1.11, 3.3, and 10 μM. Briefly, the compound was mixed with 50 ng/mL β-tryptase, followed by addition of 500 μM substrate S-2366. The reaction mixture was incubated at room temperature for 30 min. The absorbance of released product, *p*-nitroaniline, was measured at a wavelength of 405 nm with Wallac Victor2 V spectrophotometer. The IC_{50} value was determined by fitting the dose–response data into a sigmoid curve and the K_i value was calculated from a competitive inhibition equation: $K_i = IC50/(1 + [S]/K_m)$. The K_i of compound **7** was determined to be 20 nM, as shown in Table 1, which represented a reasonable enhancement as compared to the reference compound without the dimethylamide substitution at the 4-indole position, as shown in Table 2.

For stability determination in the presence of SSAO, 0.5 μM of compound was incubated with 56 μg/mL of recombinant human full-length SSAO over-expressed in CHO cells for 0 and 24 h. The mixture was then extracted with acetonitrile containing an internal standard. After centrifugation, 50 μl of the acetonitrile fraction was subjected to LC/MS analysis with a PE Sciex API 3000 mass spectrometer. The percentage remaining of the compound was determined by the ratio of the integrated area at 24 h to the integrated area at 0 h. While significant metabolism was observed for many similar compounds without the dimethylamide substitution, compound **7** was found to be 100% stable after 24-h incubation with SSAO, which represented a significant improvement over the reference compound, as shown in Table 2.

An in vitro liver microsome stability assay with appropriate co-factors was used for further metabolic stability evaluation. Compound **7** was incubated with liver microsomes for 20 min, before the reaction was stopped and the amount of compound remaining was measured. As shown in Table 1, the metabolic stability of compound **7** for human, guinea pig, and rat are 67%, 69%, and 92%, respectively. The inhibition against hERG and CYP (3A4) was also measured and found to be satisfactory, as shown in Table 1.

In conclusion, a substitution of *N*,*N*-dimethylamide at the 4-indole position significantly decreases the flexibility of the piperidine-amide linker, which constrained the conformation of the inhibitor to the X-ray bound conformation. Such a constraint decreases the entropic penalty of binding, which leads to enhanced potency against β-tryptase. More importantly, the reduced flexibility decreases the chance for the inhibitor to bind with anti-targets, especially SSAO, which is critically important for further development of this series of compounds as therapeutic agents.

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