A Novel, Nonpeptidic, Orally Active Bivalent Inhibitor of Human β -Tryptase

Sarah F. Giardina^a Douglas S. Werner^{b, c} Maneesh Pingle^{a–c}
Donald E. Bergstrom^d Lee D. Arnold^{b, e} Francis Barany^a

^aDepartment of Microbiology and Immunology, Joan and Sanford I. Weill Medical College of Cornell University, New York, NY, USA; ^bCoferon, Inc., 25 Health Sciences Drive, Stony Brook, NY, USA; ^cBlinkBio, Inc., The Scripps Research Institute, Jupiter, FL, USA; ^dDepartment of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, USA; ^eFount Therapeutics, LLC, Wilmington, DE, USA

Keywords

β -Tryptase · Bivalent inhibitor · X-ray analysis · Disiloxane · Tight-binding · Protease inhibitors

Abstract

β -Tryptase is released from mast cells upon degranulation in response to allergic and inflammatory stimuli. Human tryptase is a homotetrameric serine protease with 4 identical active sites directed toward a central pore. These active sites present an optimized scenario for the rational design of bivalent inhibitors, which bridge 2 adjacent active sites. Using (3-[1-acylpiperidin-4-yl]phenyl)methanamine as the pharmacophoric core and a disiloxane linker to span 2 active sites we have successfully produced a novel bivalent tryptase inhibitor, compound 1a, with a comparable profile to previously described inhibitors. Pharmacological properties of compound 1a were studied in a range of in vitro enzymic and cellular screening assays, and in vivo xenograft models. This non-peptide inhibitor of tryptase demonstrated superior activity (IC₅₀ at 100 pmol/L tryptase = 1.82 nmol/L) compared to monomeric modes of inhibition. X-ray crystallography

validated the dimeric mechanism of inhibition, and 1a demonstrated good oral bioavailability and efficacy in HMC-1 xenograft models. Furthermore, compound 1a demonstrated extremely slow off rates and high selectivity against related proteases. This highly potent, orally bioavailable and selective inhibitor of human tryptase will be an invaluable tool in future studies to explore the therapeutic potential of attenuating the activity of this elusive target. © 2018 S. Karger AG, Basel

Introduction

Mast cells, immune cells that play central roles in innate and adaptive immunity, can be activated by IgE-dependent and – independent mechanisms to release mediators such as tryptase, cytokines, and histamine [1–4]. The tryptase family of proteases are divided by their amino acid sequence into 4 types, α , β , δ and γ [5–8]. β -Tryptase, the most abundant protein in human mast cells, is stored at quantities as high as 15–30 pg/cell [9], and is hereafter referred to as tryptase. Tryptase is en-

zymatically active in a non-covalently linked tetramer form which is stabilized with negatively charged amino-glycans such as heparin and high ionic strength [5, 10]. The active sites of tryptase, while are similar to the structure of trypsin, are arranged in a tetrameric state that are directed toward a central pore, which possesses 2 narrow openings of $\sim 40 \text{ \AA} \times 15 \text{ \AA}$ [6, 10], restricting the access of substrates and inhibitors.

Direct evidence for a key role of tryptase proteolytic activity in inflammatory responses has been found in asthma [5, 11, 12], colitis [13], and arthritis [14, 15], where it plays a number of roles including the destruction of bronchodilators and peptides, propagation of mast cell degranulation, regulation of inflammatory cytokines, fibroblast and smooth muscle growth, extracellular matrix degradation and remodeling, recruitment of inflammatory cells, and activation of protease-activated receptor 2 on vasculature, eosinophils, keratinocytes, and neurons. Tryptase is believed to play important roles in the pathophysiology of smoke-induced emphysema, lung fibrosis and chronic obstructive pulmonary disease [16–18]. In experimental models of colitis and arthritis, the knockout of tryptase activity greatly reduces the severity of disease and the number of inflammatory infiltrates [13, 14] that contribute to hypersensitivity symptoms [19] and barrier dysfunction [20].

Support for the use of tryptase inhibitors for the therapeutic benefit in allergic rhinitis [21], asthma [12, 22, 23], and colitis [24] has been produced in early clinical trials. In vitro, tryptase has been shown to cause mast cell degranulation [25], eosinophil and neutrophil migration [26], inactivation of fibrinogen [27], and amplification of histamine responses [28]. Recently, tryptase has been implicated in scar formation [29, 30], hepatic fibrosis [31], chronic pain associated with prostatitis [32], and irritable bowel syndrome [20]. Nafamostat, a potent, but poorly specific inhibitor of tryptase, has shown efficacy in reducing intestinal inflammation in preclinical models, and in colitis patients refractory to corticosteroids or sulfasalazine [33–35]. Inhibition of tryptase reduces protease-activated receptor 2 activation and decreases collagen content and subsequent hepatic fibrosis scores in rat models [31], suggestive of a possible therapeutic pathway for fibrotic disease. A correlation between mast cell density, VEGF mediated angiogenesis and tumor development has been reported [36–38]. In addition to using tryptase serum levels as a marker for surgery guidance in cancer patients, it has been suggested that tryptase inhibitors may be useful in adjuvant treatment in highly angiogenic tumors [37, 39]. Despite recent advances,

clear therapeutic roles for tryptase in these pathophysiological conditions are still somewhat speculative, in part due to the lack of potent, selective, and bioavailable pharmaceuticals.

Despite over 20 years of research, no viable tryptase inhibitor has been successfully approved for clinical use [5]. Inhibitors can be categorized into those that form covalent bonds with the catalytic serine [40], and those that contain a P1 group that forms H-bonding interactions with Asp189 in the S1 pocket [41–43]. Targeting the catalytic serine presents problems with selectivity, whereas targeting the P1 group requires a strong basic group and has typically led to molecules with poor bioavailability. Designing bivalent inhibitors for neutral P1 groups has failed to generate any binding to tryptase [44]. Some inhibitors have reached phase II clinical trials in allergy, asthma, and colitis. While the peptidomimetic inhibitor, APC366 lacked high potency and selectivity [45], and bivalent APC1390 did not have ideal drug-like properties, both were somewhat effective in treating asthma in clinical trials. APC366 did not progress in the clinic due to its slow acting nature, low efficacy, and lack of specificity [46]. The second generation, an injectable bivalent tryptase inhibitor, APC2059, had improved pharmacokinetics, reached phase II clinical trials in ulcerative colitis and psoriasis [24, 46, 47] but was not developed further due to limited success. Similar evidence supporting a key role for tryptase has emerged from clinical trials conducted in allergic rhinitis [21].

In this study, we introduced a novel bivalent tryptase inhibitor using a disiloxane linker moiety with oral bioavailability, high specificity, and extremely slow off rates. Our novel inhibitor is compared to a previously described bivalent tryptase inhibitor, compound 2 [44], referred to as compound 2a in the present manuscript, and the broad spectrum serine protease inhibitor, nafamostat [34, 48]. We anticipate that this compound will become an invaluable tool to help decipher the important roles of tryptase as a target for therapeutic interventions against inflammatory and allergic diseases.

Methods

Synthesis of Compound 1a and Compound 1b

To facilitate the design, exploration, and development of linker chemistries for inhibition of tryptase, we used (3-[1-(acylpiperidin-4-yl)phenyl]methanamine as the pharmacophoric core. Variants of this pharmacophore produce monovalent tryptase inhibitors with the N1-acyl moieties bound proximally in adjacent catalytic subunits and a range of potencies (10 nmol/L–1 μ mol/L) [44, 49].

Structures of compounds used in the current study are shown in online supplemental Figure S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000492078).

Scheme 1: Synthesis of 1a and 1b

Tert-Butyl N-([3-[1-[3-[hydroxy(dimethyl)silyl]benzofuran-5-carbonyl]-4-piperidyl]phenyl]methyl) carbamate (1): A stirred suspension of 2 (0.478 g, 0.93 mmol) in N-methylpyrrolidine (15 mL) was degassed with argon then charged with PdCl₂ (0.0164 g, 0.09 mmol), DIEA (0.721 mL, 5.5 mmol), 2-(di-tert-butylphosphino)biphenyl (0.055 g, 0.18 mmol), and 1,2-diethoxy-1,1,2,2-tetra-methyl silane (1.15 g, 5.5 mmol) and heated to 50°C for 14 h. The reaction mixture was charged with acetonitrile (20 mL), 1N aq. acetic acid (10 mL), and 2-(dimethylamino)ethanethiol hydrochloride (0.264 g, 1.8 mmol) and stirred at RT for an additional 2 h. The reaction mixture was diluted with H₂O (20 mL), filtered through a pad of celite, and the aqueous solution extracted with EtOAc (3 × 15 mL). The combined EtOAc extracts were dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo resulting in 1.1 g of crude product, which was further purified by chromatography on silica gel (230–400 mesh; eluting with 40% EtOAc in n-hexane), resulting in 0.390 g (35% yield) of 3 as a thick oil. MS (ES⁺): *m/z* = 558.9 [M+Na]⁺; HPLC: *t_R* = 2.97 min.

(4-[3-[Aminomethyl]phenyl]-1-piperidyl)-(3-[hydroxy(dimethyl)silyl]benzofuran-5-yl) methanone (Compound 1a)

A solution of 3 (0.39 g, 0.76 mmol) in DCM (20 mL) was charged with TFA (3.9 mL) and stirred at RT for 1 h. The mixture was concentrated in vacuo and the crude product was purified by preparative HPLC resulting in 20 mg (6.22% yield) of compound 1a as a white solid. ¹HnMR (400 MHz, DMSO-d₆, D₂O): δ = 8.00–7.92 (m, 1H), 7.69–7.60 (m, 2H), 7.42–7.23 (m, 4H), 4.00 (s, 2H), 2.93–2.71 (m, 4H), 2.81 (t, J = 59.7 Hz, 1H), 1.83–1.61 (m, 4H), 0.40 (s, 6H); ¹³CnMR (100 MHz, DMSO-d₆): δ = 169.0 (-CON-), 155.2, 152.3, 145.6, 131.2, 129.9, 128.4, 126.7, 126.0, 123.4, 120.5, 114.5 (Ar), 111.2 (-C-Si-), 43.1 (-CH₂NH₂), 41.7 (-CH₂N-), 40.1 (-C-CH₂-), 38.8 (-CH₂-C-), 1.3(-SiMe); MS (ES⁺): *m/z* = 408.95 [M+H]⁺ (monomer), 799.45 [M+H]⁺ (dimer); HPLC: *t_R* = 1.75 min (monomer), 2.07 min (dimer); HRMS (*m/z*): [M]⁺ calculated for C₄₆H₅₄N₄O₅Si₂, 799.3679; found, 799.3713.

(4-[3-[Aminomethyl]phenyl]-1-piperidyl)-(3-bromobenzofuran-5-yl)methanone (Compound 1b)

A solution of 2 (0.05 g, 0.09 mmol) in DCM (5 mL) was charged with TFA (0.5 mL) and stirred at RT for 1 h. The reaction mixture was concentrated in vacuo and the crude product was purified by preparative HPLC resulting in 15 mg (37.5% yield) of compound 1b as a brown oil. ¹HnMR (400 MHz, DMSO-d₆, D₂O): δ = 8.27 (s, 1H), 7.72 (d, J = 8.8 Hz, 1H), 7.55 (s, 1H), 7.46 (d, J = 8.8 Hz, 1H), 7.32–7.36 (m, 4H), 4.62 (bs, 1H), 3.97 (s, 2H), 2.66–3.17 (m, 4H), 1.53–1.82 (m, 4H); MS (ES⁺): *m/z* = 413.10 [M+H]⁺, 415.10 [M+H]²⁺; HPLC: *t_R* = 1.83 min, HRMS (*m/z*): [M]⁺ calculated for C₂₁H₂₁BrN₂O₂, 413.0865; found, 413.0831.

Tryptase Inhibition Screening in vitro

βII tryptase was incubated with compounds at RT for 3 h at 1.2 × their final concentration in assay buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 100 μmol/L EDTA, pH 7.4), with subsequent dilution into assay buffer containing a final concentration of

200 μmol/L (2 × K_m) N-tert-butoxycarbonyl-Gln-Ala-Arg-AMC (Enzo Life Sciences). A final concentration of 10 pmol/L – 1 nmol/L tryptase, calculated for the monomer from the average molecular weight, was used. The release of AMC was immediately measured every 30 sec for 15 min at Ex.: 367 nm, Em.: 468 nm on a Spectra-max M5 (Molecular Devices) microplate reader. The Softmax Pro (Molecular Devices) and GraphPad Prism 6 software were used to determine V_{Max} and IC₅₀ s, respectively.

Determination of Kinetic Curvature

Inhibitors were preincubated with 200 μmol/L substrate for approximately 5 min. The hydrolysis of AMC was immediately measured after the addition of tryptase every 5 sec for 30 min, as described above.

Tryptase Reversibility Studies

Bound inhibitors (10 μmol/L) to tryptase (100 nmol/L) were separated from unbound using 7kD cut-off Zeba desalting columns (Pierce), which had been equilibrated with 1 mol/L NaCl immediately prior to use. The subsequent eluant was diluted 1:100 in assay buffer and monitored over 216 h for tryptase activity as described above.

Protease Selectivity Studies

Selectivity for tryptase over trypsin, kallikrein, thrombin, plasmin, and factor XA was evaluated using 1 nmol/L of each enzyme and substrates at concentrations 2 × K_m. Kinetic reads were measured over 15 min with the following substrates and settings: Kallikrein, 150 μmol/L H-D-Val-Leu-Arg-AFC Ex: 400 nm Em: 505 nm; Thrombin, 100 μmol/L Benzoyl-FVR-AMC Ex: 367 nm Em: 468 nm; Plasmin, 700 μmol/L H-D-Val-Leu-Lys-pNA-2HCl absorbance 405 nm; trypsin, 150 μmol/L N-tert-butoxycarbonyl-Gln-Ala-Arg-AMC Ex: 367 nm Em: 468 nm; Factor XA, 700 μmol/L N-tert-butoxycarbonyl-Gln-Ala-Arg-AMC Ex: 367 nm Em: 468 nm.

Inhibition of Cellular Tryptase in HMC-1 Cells

HMC-1 cells were a gift from Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN, USA) [50]. Cultures of HMC-1 cells were grown at 37°C, 5% CO₂, in Iscove's modified Dulbecco's medium (Life Technologies), supplemented with 36 mmol/L sodium bicarbonate, 1.2 mmol/L monothioglycerol (Sigma-Aldrich), 10% Normal Calf Serum (Life Technologies), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were treated with inhibitors (10 nmol/L–100 μmol/L; 2 h) and subsequently washed in PBS. Degranulation was induced with 1 μmol/L A23187 in PBS. After 1 h the supernatant was assayed for tryptase activity as described above. Alternatively, treated cells were lysed in buffer (assay buffer containing 0.1% TX-100, with 1 mmol/L EDTA, 20 μg/mL heparin, 0.5 mg/mL soybean trypsin inhibitor, 100 nmol/L aprotinin, 0.5 μg/mL pepstatin, and 100 μmol/L N-Ethylmaleimide) and assayed for tryptase activity as described above.

Enzyme Histochemistry for in Situ Inhibition of Tryptase

For the evaluation of tryptase activity in situ, frozen lung tissue samples from patients with asthma (BioChain) were fixed in 4% paraformaldehyde to inhibit non-specific cleavage of the substrate and subsequently treated with compounds for 2 h at RT. Slides were washed in PBS and incubated with 0.25 mg/mL z-Ala-Ala-Lys-4-methoxy-2-naphthylamide (Enzyme System Products) in

0.1 mol/L phosphate buffer, pH 7.5, for 20 min at 37 °C, followed by a 5 min wash with 1% copper sulfate [51]. Slides were dehydrated, mounted, and were visualized using bright-field microscopy. Images are representative of $n = 10$ across 2–3 independent experiments.

Pharmacokinetics in Mice

Pharmacokinetics was determined in freely feeding male C57BL/6 mice following single intravenous doses (10 mg/kg dose, 2 mg/mL solution in 0.9% sterile saline) and oral (10 and 100 mg/kg at 1 and 10 mg/mL solutions in water, respectively). Blood samples were collected under isoflurane anesthesia and samples were obtained at pre-dose, 0.08, 0.25, 0.5, 1, 2, 4, 8 and 24 h (i.v.) and pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h (p.o.) using dipotassium EDTA as anticoagulant. Plasma samples were treated with 3 volumes of acetonitrile, vortexed, chilled on ice (1 h), and centrifuged. Drug levels in supernatant were analyzed by LC-MS/MS using a gradient of acetonitrile in water containing 0.1% formic acid at 1 mL/min over 5 min on an Agilent 1200 system equipped with an Eclipse XDB-C18 (4.6×150 mm) column at 40 °C with positive-ion MRM detection on an API4000-Q Trap (MDS Sciex, Applied Biosystems). Aliquots of dosing solutions were analyzed to determine actual concentrations (2.58, 0.86, and 8.17 mg/mL, for 10 mg/kg i.v., 10 and 100 mg/kg p.o., respectively). Pharmacokinetic parameters were calculated using the noncompartmental analysis tool of WinNonlin® Enterprise software (version 5.2).

HMC-1 Tumor Xenografts

HMC-1 cells (1×10^7) were resuspended in PBS (100 μ L) and implanted s.c. into the right and left flanks of female nu/nu CD-1 mice (Charles River), as previously described [52]. Tumors were established for 2–4 weeks and animals were randomized into treatment groups. Saline (0.5 mL; $n = 21$) or 1a (7.8 mg/kg; $n = 18$) dissolved in water (0.5 mL) was administered by oral gavage. Nafamostat Mesylate (10 mg/kg) was administered intravenously. Four hours post-dose animals were euthanized, plasma was collected, and excised tumors were flash frozen. Homogenized tumors were sonicated in lysis buffer and clarified by centrifugation. The supernatant was diluted 1:100 and trypsin activity was determined as described above. The concentration of compound 1a in plasma and tumor tissue homogenates was determined by LC/MS/MS.

X-Ray Crystallography

The protein complexes with inhibitors were formed by the addition of 1 mmol/L compound to recombinant human β -Trypsin (1.95 mg/mL). The protein-compound mixtures were incubated on ice (30 min) and centrifuged to remove the precipitate. The co-crystallizations were setup using the vapor diffusion method. Equal volumes of the protein complex and reservoir solution (30% PEG 1500, 100 mmol/L sodium acetate pH 4.6 and 200 mmol/L ammonium sulfate) were mixed and subsequently incubated at 25 °C (3–5 days). Once formed, the monocrystal was soaked for 20 h in solution with 30% PEG 1500, 200 mmol/L ammonium sulfate, 100 mmol/L MES pH 5.5, and 1 mmol/L compound and flash-frozen in liquid nitrogen until x-ray diffraction.

Protein Data Bank: coordinates and structure factors for the co-crystal structure of the trypsin complex have been deposited with accession code 4MPW.

Materials

Dimeric β -tryptase inhibitor 2 (referred to as compound 2a in the current manuscript; online suppl. Figure S1) has been described [44]. Recombinant human β -tryptase from lung was purchased from Promega (Madison, WI, USA) and confirmed to be in a tetrameric state by non-denaturing Western blotting and x-ray crystallography. Trypsin was purchased from Calbiochem (San Diego, CA, USA). Kallikrein, plasmin, thrombin, H-D-Val-Leu-Arg-AFC, Benzoyl-FVR-AMC were purchased from EMD Millipore (Billerica, MA, USA); factor XA and N-*tert*-butoxycarbonyl-Gln-Ala-Arg-AMC from Enzo Life Sciences (Farmingdale, NY, USA). H-D-Val-Leu-Lys-pNA-2HCl was purchased from Oxford Biomedical Research (Oxford, MI, USA). Unless otherwise indicated, other reagents were from Sigma-Aldrich (St Louis, MO, USA) or Life Technologies (Grand Island, NY, USA).

Animal Welfare

All animal care and experimental investigations were in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the Institutional Animal Care and Use Committee at Stony Brook University.

Data Analysis and Statistical Procedures

Data are expressed as mean $\pm$ SEM. Differences between dose-response curves were analyzed by one-way ANOVA for repeated measurements, followed by unpaired Student *t* test. Xenograft studies were analyzed by one-way ANOVA and post hoc Tukey's multiple comparison test. Probability levels of $p < 0.05$ were considered statistically significant.

Results

Disiloxane -Based Dimers Afford Dramatic Improvements in Potency

The dimethylsilanol (-SiMe₂OH; DMS) linker was investigated, as it contributed nominal increases in molecular weight, H-bond donors/acceptors, rotatable bonds, polar surface area, and lipophilicity. Synthetic methodologies [53] and other conventional methods for installing an aliphatic DMS, producing trypsin inhibitors, which spontaneously dimerized upon lyophilization (compound 1a: 84.9–90.2% dimer at synthesis).

Compound 1a exhibited extraordinary improvements in apparent IC₅₀s over its corresponding monomeric analog, with IC₅₀s decreasing with trypsin concentration, as anticipated with tight-binding inhibitors (Fig. 1b). In comparison, the potency of the monomer analogs, Compound 1b and 2a were negligibly affected by the trypsin concentration. Compound 1a mediated trypsin inhibition was not significantly different from the previously described bivalent inhibitor, Compound 2a ($F[2,38] = 0.25$, $p = 0.86$; Fig. 1a). The disiloxane compound produced

Fig. 1. a Dose-response curves for the silanol bivalent compound, 1a, and its monomeric control, 1b, were compared to responses of the bivalent inhibitor, 2a, and its monomeric analog, 2b. **b** IC₅₀ s

and fold difference between nafamostat, the bivalent and control compounds are indicated for increasing concentrations of trypsinase.

more potent responses at each concentration of trypsinase examined and demonstrated a greater difference in potency over its monomeric analog (Fig. 1b). At 10 pmol/L trypsinase the apparent IC₅₀ of compound 1a approaches 300 pmol/L, corresponding to more than a 1,000-fold improvement relative to its monomeric control.

The detailed analysis of compound 1a with trypsinase provided a number of insights accounting for the marked potency. We confirmed the homodimeric mechanism of action via a co-crystal structure with trypsinase and found that the disiloxane bridged homodimer was in a fully relaxed pose, with Si-O-Si and C-Si-O bond angles of 145° and 108°, respectively (Fig. 2a; online suppl. Table S1) [54, 55].

Compound 1a Exhibits High Affinity for Trypsinase and a Very Slow Off-Rate

A negative curvature gradually leading to a steady-state constant slope in the enzyme kinetics was observed for compound 1a at concentrations in the vicinity of the IC₅₀ (Fig. 2b) suggestive of slow, albeit tight, binding. The high stability of trypsinase's proteolytic activity at room temperature enabled reversibility studies of compounds to be conducted over an extended period of time. After the removal of excess unbound inhibitor from trypsinase by a gel filtration spin-column, we monitored the recovery of enzymic activity. Monomeric inhibitors were readily dissociated under these conditions to immediately restore full trypsinase activity, while less than 25% of activity was recovered after 9 days with compound 1a (Fig. 2c).

Bivalent Inhibitor 1a is Highly Specific for Trypsinase

High specificity for trypsinase among trypsin and related proteases in the coagulation cascade is essential for a potential therapeutic agent but has been challenging to

achieve with monovalent inhibitors [21, 56]. Consistent with the tailored fit of compound 1a and bivalent inhibition of trypsinase, outstanding specificity was revealed by >2,000-fold selectivity against a panel of related trypsin-family proteases (online suppl. Figure S2) with improvements over the monomeric analogs diminishing in parallel. Indeed, Nafamostat was found to potently inhibit other related proteases, as previously described [49], whereas the bivalent compounds were found to be only marginally more potent than their monomeric counterparts (online suppl. Figure S2).

Compound 1a Retains Potency in the Presence of Plasma and its Components

As evidence of specificity and bioorthogonal behavior, the potency of compound 1a against trypsinase was negligibly altered in the presence of plasma components including albumin (5%), L-Glutathione (10 mmol/L), α-1-acid glycoprotein (0.1%), glucose (8 mmol/L), and marginally affected (<8×) by the addition of 10–20% human or mouse plasma (online suppl. Table S2). Furthermore, LC/MS/MS studies demonstrated excellent stability of compound 1a, with no evidence of decomposition after 72 h in plasma, or over 25 days at pH 2.1, 4.7 or 7.4.

Compound 1a Permeates Cells to Effectively Pre-Inhibit Trypsinase Prior to its Release

Trypsinase is stored at high concentrations in mast cell granules and from our in vitro studies and co-crystallization efforts, the potent bivalent inhibitor retained its high affinity for trypsinase even under the more acidic conditions (pH 5.5–6) encountered in the storage granules. The very slow off-rate of compound 1a allowed us to treat HMC-1 cells with inhibitors to pre-inhibit the

Fig. 2. **a** Co-crystal structures of 1a confirmed a bivalent mechanism of inhibition, providing insights into the relaxation of the disiloxane angular deformation to 145°. Compound 1a is depicted in stick format and the protein as a surface colored by pKa. **b** Curvature was observed with 1a in a continuous fluorimetric enzyme assay at 1 nmol/L tryptase, particularly in the vicinity of the IC₅₀, consistent with tight binding. RFU, relative fluorescence units. **c** Extremely slow off-rates

of the bivalent silanol were observed in reversibility studies. Tryptase (100 nmol/L) was treated with 1a or monomer control 1b (10 μmol/L) and unbound inhibitor was removed by gel filtration spin column. The molar ratio of 1a to tryptase subunits was determined to be 1.3 (±0.3):1 by LC-MS/MS. The subsequent solutions were diluted to 1 nmol/L tryptase and assayed daily for tryptase activity. There was no recovery in activity without removal of excess inhibitor.

target, wash, and then demonstrate a concentration-dependent inhibition of the tryptase activity (Fig. 3a). Control compounds demonstrated some inhibition in vitro, albeit more than 130 fold less potent than the bivalent compounds (Fig. 3b). Further supporting the inhibition of tryptase in mast cell granules, we observed the effective inhibition of the tryptase proteolytic activity in histochemical studies of formaldehyde-fixed sections of human asthmatic lung tissues after treatment with 100 μmol/L 1a but not with its monomeric analog, 4b (Fig. 3c–e).

Compound 1a is Orally Bioavailable and Inhibits Human Tryptase Activity In Vivo

Studies of the intravenous and oral pharmacokinetics of 1a at 10 mg/kg in C57BL/6 male mice revealed high oral bioavailability (F = 96%), with peak plasma levels of

650 nmol/L after oral dosing, and an i.v. elimination half-life of 5.4 h (Fig. 4a). A higher oral dose of 100 mg/kg safely achieved peak plasma levels of ~10 μmol/L and sustained levels of ~1 μmol/L for 24 h with no apparent adverse effects, suggesting good toleration and oral efficacy. To demonstrate in vivo efficacy of these novel inhibitors of human tryptase, bilateral human mast cell tumor (HMC-1) xenografts were grown in nude mice [52], and the TFA salt of 1a was administered orally at 7.8 mg/kg. Tumors were excised 4 h post-dose, assayed for tryptase activity, and the associated levels of 1a were determined by LC/MS/MS. 1a significantly reduced the tryptase proteolytic activity within the tumors of treated mice by 61% (±5.8; F[2, 44] = 5.160, *p* = 0.0097), with associated mean plasma and tumor tissue levels of 10 and 13 nmol/L respectively (Fig. 4b). The magnitude of the inhibition at this concentration is comparable to that of the potent, nonspecific, irreversible pro-

Fig. 3. a Tryptase activity is potently inhibited by treatment of HMC-1 cells with 1a ($IC_{50} = 58 \text{ nmol/L} \pm 0.05$) and 2a ($IC_{50} = 44 \text{ nmol/L} \pm 0.05$), followed by washing, and induction of degranulation with a calcium ionophore. Monomeric controls were more than 130 less potent than the bivalent inhibitors. **b** Inhibition of mast cell associated tryptase activity in formaldehyde-fixed human lung tissue from asthmatic patients. Fixed sections were incubated with DMSO (**c**), 100 $\mu\text{mol/L}$ 1a (**d**), or 1b (**e**) for 1 h prior to histochemical staining with a colorimetric tryptase substrate. Staining of mast cells (arrows) was completely inhibited by 1a but not its monomeric analog 1b.

tease inhibitor, nafamostat [34, 48]. Based upon protease activity and ELISA determinations in the tumor homogenates we estimated the intratumoral tryptase concentrations to be 3–75 nmol/L, suggesting we are essentially titrating the enzyme in the tumor tissue.

Discussion

We sought bioorthogonal, single-bond linkers with the flexibility to conform to the demands of linking pharmacophoric sites while maintaining good drug-like properties and evading metabolism and conjugation. In earlier studies, Tacke et al. [57] installed silanol moieties in place

of carbinols of known drugs, and demonstrated the retention of biological activity in vitro and in vivo, good stability, toleration, and pharmacokinetics, with resistance to dehydration and metabolism, and no detectable glucuronide conjugates. In several instances, disiloxane dimers of these silanol drug analogs were noted in the solid state and concentrated solutions, but dimers were not exploited and did not appear to contribute to the pharmacology [58]. Water-soluble disiloxanes were explored by Molnar et al. [59] as modulators of multidrug resistance and potential anticancer agents. Compound 1a demonstrated enhanced inhibition and ideal drug-like properties and was therefore investigated further. Disiloxane inhibitors were initially designed to act as monomers and dimerize upon the

Fig. 4. a The pharmacokinetics of 1a as its formate salt were determined in freely feeding male C57BL/6 mice following single intravenous (10 mg/kg in 0.9% sterile saline) and oral (10 and 100 mg/kg in water) doses. At 10 mg/kg p.o. the oral bioavailability was 96 %, with peak plasma levels of 653 nmol/L (± 265). Only monomeric silanol was detected in all bioanalytical samples. **b** Nude mice bearing bilateral HMC1 xenograft tumors were treated orally with 1a (7.8

mg/kg as TFA salt in water) or intravenously with nafamostat (10 mg/kg i.v.). After 4 h, tumors were excised, homogenized, and assayed for tryptase activity. 1a significantly reduced tryptase activity ($p = 0.0057$) by 61% (± 5.8), with mean plasma and tumor tissue levels of 4.1 ng/mL (± 1.2) and 5.2 ng/mg (± 2.2) respectively. * $p < 0.05$, ** $p < 0.01$.

tryptase target [60]. However, monomerized compounds seemed unable to reform dimers under the current enzymic screening conditions in vitro and improved potencies to levels of the initial mixed homodimer solution were not observed for the duration of tryptase activity at RT (several days, data not shown). Likewise, stock solutions of compound 1a diluted into assay buffer did not spontaneously monomerize and the dimer concentration was consistent with that at the time of synthesis (89.4–90.2%). The silanol dimer demonstrated remarkable oral bioavailability and efficacy, providing all the advantages of bivalent inhibition with the added benefit of oral efficacy. Analysis of the silanol compound from animals treated both by oral and intravenous administration only identified monomeric form in plasma. We found that the equilibrium shifted progressively at pH 4.7 and 2.1 to significantly favor monomer, with rapid hydrolysis of the dimeric disiloxanes at pH 2.1. While possibly the orally administered dimer monomerized under the acidic conditions of the stomach, we cannot rule out the contributions of sample preparation when we consider findings from intravenous samples. Indeed, 1a existed primarily as dimer in the 2 mmol/L aqueous oral dosing solution and it is highly unlikely that such a magnitude of inhibition found in the xenograft studies occurred from monomeric compound. The poor thermostability of tryptase at 37°C hindered further investigations and efforts to assess the dimer equilibrium and pharmacokinetics are beyond the scope of the current studies.

The high specificity of 1a for human tryptase was also reflected in the $\sim 20 \times$ lower potency and more modest 21-fold effect relative to the monomeric 1b observed with the mouse ortholog of human β -tryptase, despite the $>90\%$ sequence homology in the inhibitor binding sites (73 nmol/L IC_{50} at 2 nmol/L mMCP-6; online suppl. Figure S2). The most significant difference in the binding site region between human and murine tryptase is the Q117E substitution, placing glutamate sidechains of each subunit proximal to the disiloxane linkage. The effects of the acidic substitution upon the formation and stability of the bivalent inhibitor are uncertain, but one could speculate that it could accelerate the hydrolysis of bound dimer and enhance the off-rate. Therefore, to demonstrate in vivo efficacy of 1a against the target for which it was designed, human tryptase, bilateral human mast cell tumor (HMC-1) xenografts were used [52]. These studies revealed that 1a was able to inhibit human β -tryptase after oral administration to levels comparable to the potent, yet non-specific, Nafamostat (Fig. 4; online suppl. Figure S2).

The relevance of mast cells and their secretory products to human pathology has been the focus of significant research and has given rise to considerable efforts to develop antagonists to many of the mast cell mediators, including tryptase. Clinical trials with non-ideal candidates resulted in the discontinuance of programs, despite some early success, largely due to unwanted side effects, off target responses and lack of oral bioavailability. These fail-

ures have also hindered progress in defining clear roles of tryptase in the many pathophysiological conditions in which it has been implicated. McNeil (2008) developed the mMCP6 knockout model and has demonstrated a direct role for this protease in arthritis [14], colitis [13], abdominal aortic aneurysms [18], and chronic obstructive pulmonary disease [16]. Indeed, tryptase might act as a novel target for the adjuvant treatment of tumors through the selective inhibition of angiogenesis [39], and the treatment of fibrotic diseases [31, 61, 62], where current treatment options are limited. The benefits of tryptase inhibition for asthma have been demonstrated, where tryptase is thought to play a role beyond the acute airway hyper-responsiveness. Tryptase's actions as a chemoattractant for neutrophils [13, 14, 16, 63], a mitogen for fibroblasts [61, 64, 65], and its role in tissue remodeling and repair [22, 66] are all points of potential therapeutic invention.

Polyvalent binding increases the affinity and specificity of interactions between molecules [67], as confirmed in the current study. Bivalent inhibitors of tryptase that engage 2 pharmacophoric binding sites spanning approximately 34 Å have demonstrated more than a 1,000-fold increase in selectivity and affinity and have shown efficacy in early clinical studies [41, 68, 69]. However, these molecules possess high molecular weights, are double-charged, and the physicochemical properties of these molecules produce poor drug-like performances [24, 69]. The current study successfully developed a bivalent compound containing a disiloxane linker, which demonstrates good bioavailability after oral administration, and exhibits high selectivity and specificity.

Like previously reported dimeric inhibitors of tryptase [24, 41, 44, 47, 69], compound 1a, which bridges 2 active sites, has a higher affinity for tryptase than monomeric controls [23]. Consistent with bivalent binding, we demonstrated extremely slow off rates and superior selectivity for tryptase [23, 70], with remarkable potencies, never seen before in orally available inhibitors of tryptase.

The prospect of selective inhibition of tryptase is of considerable pharmacological significance and therapeutic potential for allergic and inflammatory diseases. While considerable effort has been made to design selective and potent inhibitors of tryptase, to date no candidate has advanced into stage 3 clinical trials. Small molecule inhibitors have been unsuccessful due to the lack of selectivity and poor efficacy, and while larger molecules demonstrate improved potencies and selectivity but they do not have ideal drug-like properties. The current study successfully developed a bivalent inhibitor with good oral bioavailability, selectivity, and specificity for tryptase. We

predict that compound 1a will be a useful pharmacological tool to help delineate the roles for tryptase in inflammatory disorders.

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Disclosure Statement

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Author Contributions

S.F.G. and L.D.A. contributed to study concept and design. F.B., D.E.B., M.P., and L.D.A. developed the Coferon concept and linker chemistries. D.S.W. performed and oversaw syntheses. Acquisition of data: S.F.G. Analysis and interpretation of data: S.F.G., L.D.A., D.W.S. S.F.G. and D.S.W. wrote the manuscript. All authors approved of the final version.

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