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Target-Directed Self-Assembly of Homodimeric Drugs Against β-Tryptase

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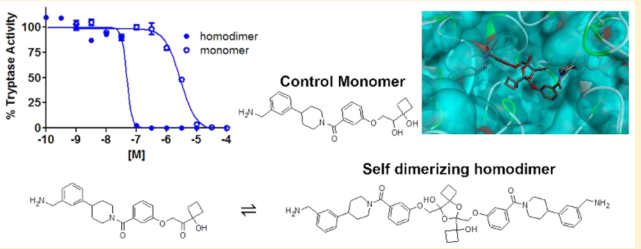
Supporting Information

ABSTRACT: Tryptase, a serine protease released from mast cells, is implicated in many allergic and inflammatory disorders. Human tryptase is a donut-shaped tetramer with the active sites facing inward forming a central pore. Bivalent ligands spanning two active sites potently inhibit this configuration, but these large compounds have poor drug-like properties. To overcome some of these challenges, we developed self-assembling molecules, called coferons, which deliver a larger compound in two parts. Using a pharmacophoric core and reversibly binding linkers to span two active sites, we have successfully produced three novel homodimeric tryptase inhibitors. Upon binding to tryptase, compounds reassembled into flexible homodimers, with significant improvements in IC_{50} (0.19 ± 0.08 μM) over controls (5.50 ± 0.09 μM), and demonstrate good activity in mast cell lines. These studies provide validation for this innovative technology that is especially well-suited for the delivery of dimeric drugs to modulate intracellular macromolecular targets.



KEYWORDS: *Coferon, small molecule, tryptase inhibitor, drug discovery, bivalent, homodimer*

One of the most formidable and fundamental challenges of small molecule drug design is to effectively and specifically target intracellular proteins.[1,2] Often small molecule ligands cannot achieve sufficiently high affinities to disrupt macromolecular interactions through localized binding sites, and extending their "reach" to exploit additional pharmacophoric contacts results in larger multivalent molecules with suboptimal drug properties.[3] To circumvent these limitations, we have developed coferons, reversibly self-assembling drug molecules that may be administered as a dimer, which dissociates into monomers *in vivo* for absorption, distribution, and permeation into tissues and cells. Upon binding to their macromolecular target, coferon monomers covalently reassociate to form a larger, more specific and higher-affinity dimeric assembly to effectively modulate interactions or activity. Each coferon monomer comprises an appropriate "pharmacophore", with an affinity for a binding site on the target, connected to a small linker moiety capable of a reversible, covalent reaction with a "partner" coferon binding at a proximal site on the target.[4] Thus, the macromolecular biological target serves as a template for the assembly of the higher affinity coferon dimer. The delivery of a larger dimeric drug molecule as two low molecular weight monomeric coferons affords greater flexibility in optimizing drug-like absorption, distribution, metabolism, and excretion (ADME) properties.

In conjoining two weaker pharmacophores into a higher affinity assembly, the coferon concept incorporates key attributes of multivalency[5−7] and fragment-based drug discovery (FBDD)[8,9] and, in using the biomolecular target as a template, also emulates protein-directed dynamic combinatorial chemistry (DCC).[10−13] Our efforts build upon the early precedents of weakly active drug fragments assembling covalently *in situ* (in cells) to form more active entities[14] and target directed self-assembly of higher affinity ligands and inhibitors.[12,15−17] Our approach is uniquely differentiated by the use of reversibly covalent, bioorthogonal linker moieties to enable drug delivery and subsequent *in situ* dimerization of pharmacophoric components upon the protein target inside cells.

Although a large number of potential protein interfaces could be considered for the validation of this technology, mast cell β-tryptase offered a number of advantages. Tryptase is a tetrameric trypsin family protease that is the most abundant protein in human mast cells,[18] and its release from granules has been linked

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to the pathology and progression in allergic and inflammatory disease.[19−24] Bivalent inhibitors spanning approximately 30 Å that engage pharmacophore binding sites of two adjacent subunits have demonstrated more than 1000-fold increase in selectivity and affinity and have shown efficacy in preclinical models and clinical studies.[25,26] However, the large size, double-charge and physicochemical properties of these molecules produced poor drug-like performance and precluded good bioavailability.[25,27−29]

Herein we demonstrate target-directed self-assembling of homodimeric inhibitors of human β-tryptase and achieve greatly enhanced affinity. Our preliminary evidence using α-hydroxyketo-based homodimeric linkers supports the concept, realizing the advantage of bivalent inhibitors and multivalency[7] without compromising cellular permeability.

Simulations suggested that micromolar affinity pharmacophores could combine to achieve subnanomolar inhibition, corresponding to potency enhancements in excess of several thousand-fold if certain reasonable criteria are met. We refer to this potency enhancement as the "coferon effect," calculated as the ratio of the IC_{50} of the control monomer and the apparent coferon IC_{50}. Improvements in apparent IC_{50}s are expected to be proportional to the root of the coferon dimerization and directly proportional to enhancements in monomer affinities until the tight-binding limit is approached. Interestingly, as expected for tight-binding inhibitors, the apparent IC_{50} for the coferons is predicted to decrease (with a corresponding increase in coferon effect) with decreasing target concentration.

To facilitate the design, exploration, and development of coferon linker chemistries to enable the self-assembly technology for inhibition of tetrameric β-tryptase, we selected the [3-(1-acylpiperidin-4-yl)phenyl]methanamine moiety (Figure 1a) as the pharmacophoric core. Relatively simple variants of

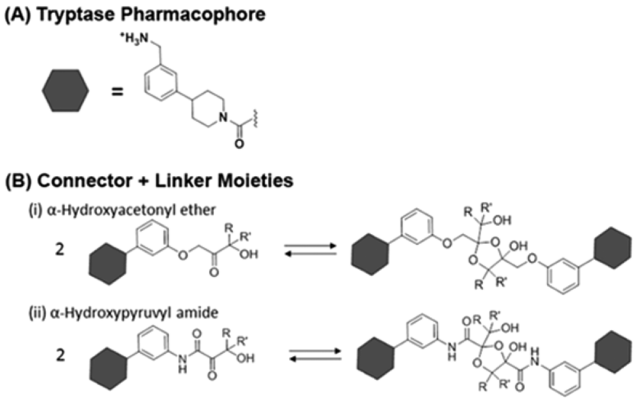

Figure 1. Designs for target-directed self-assembling dimeric drugs.(A) [3-(1-Acylpiperidin-4-yl)phenyl]methanamine was employed as the pharmacophoric core for the development of reversible homodimeric inhibitors of tetrameric human β-tryptase. One monomer binds each catalytically active subunit of tryptase, such that each homodimeric coferon inhibits an adjacent pair of tryptase subunits. (B) Examples of homodimerizing connector plus linker moieties explored in the current manuscript.

this pharmacophore produce monovalent tryptase inhibitors with potencies in the 10^{-6} to 10^{-8} M range with the *N*1-acyl moieties bound proximally in adjacent catalytic subunits.[26,30] Importantly, achieving suitable dimer dissociation constants (i.e., KDim < 10^{-2} M) with small linker moieties to produce significant coferon effects precluded the use of weak non-covalent bonding interactions[31] and compelled us to explore reversibly covalent α-hydroxyketo linker chemistries (Figure 1b).

Our homodimeric coferon designs explored simple α-hydroxyketo (α-HK) moieties as linkers.[32] The idea was supported by the knowledge that dihydroxyacetone exists as a dimer in concentrated solutions and in the solid state[33] and that α-HK minerolo- and gluco-corticoid progestagens form dimers in aqueous solutions.[34] Of the regioisomeric α-HK dimer assemblies that could be produced, several diastereomeric five-membered spiroketal (1,3-dioxolan-4-ol) linkages (Figure 1) are predicted to be thermodynamically favored over cyclic six-membered bis-hemiketals (i.e., 1,4-dioxane-2,5-diol). Our initial design based upon α-hydroxyacetonyl ether (R,R′ = H in Figure 1b); IC_{50} = 49 nM at 1 nM tryptase) displayed a marginal 5-fold improvement in activity compared to its nondimerizable diol analog; however, the linker moiety proved to be unstable in aqueous buffers and plasma due to tautomerization to the enediol and hydrolytic fragmentation.[35] This issue was circumvented through the geminal dialkyl substitution of the α-hydroxymethyl moiety to block the tautomerization, discourage oxidation, and inhibit metabolic conjugation.

The α-hydroxyacetonyl 1a (Figure 1b, *R*,*R*′ = $-(CH_2)_3-$) and the α-hydroxypyruvylamido 2a (Figure 1b, *R*,*R*′ = $-(CH_2)_3-$) derivatives exhibited improved stability under aqueous conditions, and significant improvements in IC_{50} *in vitro* of over 200-fold of their nondimerizable racemic vicinal diol analogs (1b, 2b, and 3b, respectively) (Figures 2a and S1b). As predicted from simulations, the *in vitro* IC_{50}s of these coferons improved with decreasing tryptase concentrations, such that the IC_{50} for 3a was 10.9 nM at 10 pM tryptase, while the potency of the monomeric diols was affected minimally (Table 1). The impressive potency enhancement of 3a was also reflected in tryptase cellular degranulation assays (IC_{50} = 52 nM) and cell lysates from cultured HMC1 human mast cells (IC_{50} = 113 nM; Table 2). X-ray cocrystallographic studies supported the proposed homodimeric mechanism of tryptase inhibition for 1a, 2a, and 3a, with contiguous electron density bridging two proximal pharmacophore binding sites (Table S1). For the most potent analog, 3a, the observed electron density could be accounted for by a single spiroketal diastereomer bound in both directions (Figures 2b and S1a), but for 1a and 2a, contributions from multiple diastereomeric spiroketal assemblies were evident (Figure 2c). This illustrates how the biomolecular target may promote the formation of one or more homodimeric assemblies with similar energies. Protein Data Bank (PDB): coordinates and structure factors for the cocrystal structures of the tryptase complexes with 2a and 3a have been deposited with accession codes 4MPU and 4MPV, respectively.

The high stability of tryptase'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 tryptase 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 tryptase activity, while less than 25% of activity was recovered after 9 days with compound 1a (Figure S1c).

Through the use of computer simulations based upon basic equilibrium models and relatively simple homodimeric linker chemistries, we have established the foundation for the delivery of bivalent drugs, based on the target-directed and reversible

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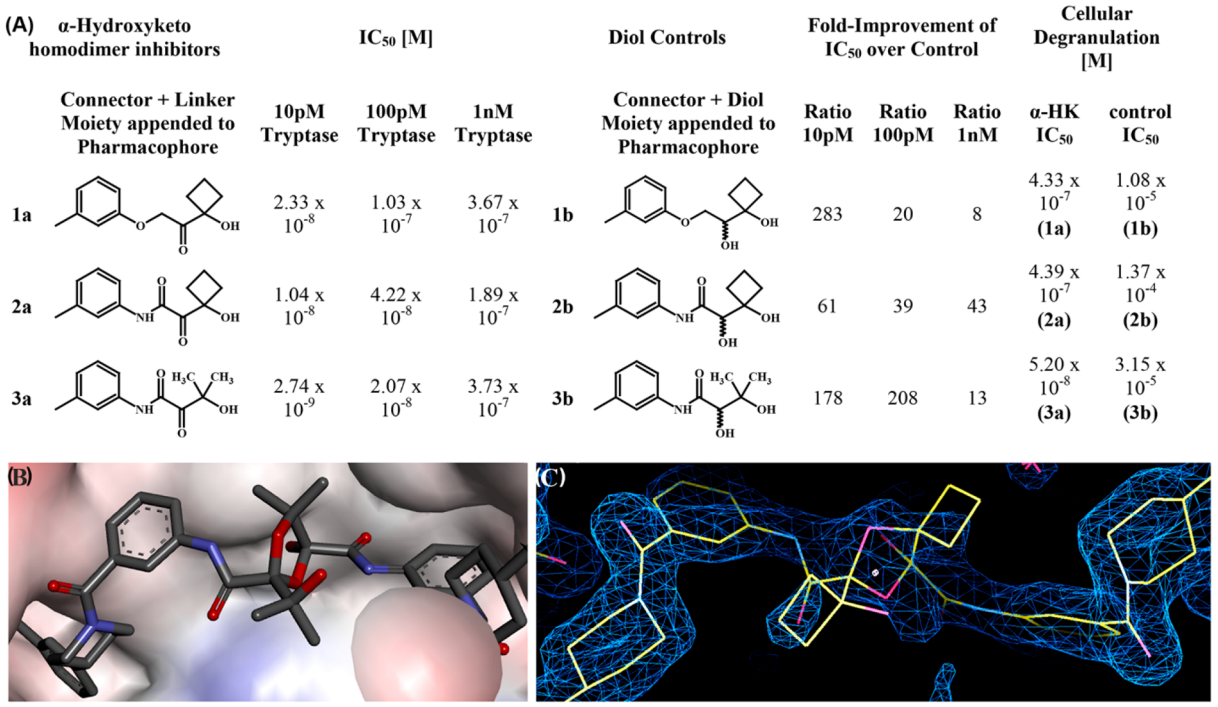

Figure 2. Tryptase promotes the assembly of α-hydroxyketo-based coferon dimers resulting in improved potency. (A) α-Hydroxyketo coferons 1a, 2a, and 3a display significant *in vitro* and cellular potency improvements (ratios) over their nondimerizable racemic vicinal diol analogs (1b, 2b, and 3b, respectively). (B) Cocrystal structure of 3a at 2.3 Å resolution with human β-tryptase indicates that pharmacophore binding sites in adjacent subunits are bridged by a dimeric spiroketal assembly. The (2*R*,4*S*)-4-hydroxy-2-(1-hydroxy-1-methyl-ethyl)-1,3-dioxolane diastereomer in left-to-right and right-to-left configuration (depicted displayed on protein surface colored by electrostatic character) were best suited in fitting the bridging density. (C) Contiguous electron density was also observed in the cocrystal structure of 2a (1.65 Å resolution), consistent with occupancy by dimeric spiroketal assemblies. While an R,S-diastereomer, analogous to that of 3a, contributes to the observed 1σ density (depicted), no single spiroketal configuration could account for all aspects (Table S1).

Table 1. IC_{50}s for the α-Hydroxyketo Homodimers[a]

	IC_{50} [M] ± SEM						IC_{50} Improvement over control (Coferon Effect)		
	a-HK #			Diol Control #					
	1a	2a	3a	1b	2b	3b	1a/1b	2a/2b	3a/3b
10 pM	$2.3 \times 10^{-8} \pm 0.06$	$1.0 \times 10^{-8} \pm 0.07$	$2.7 \times 10^{-9} \pm 0.11$	$6.6 \times 10^{-6} \pm 0.26$	$6.4 \times 10^{-7} \pm 0.14$	$4.9 \times 10^{-7} \pm 0.15$	282.8	61.7	177.8
100 pM	$1.0 \times 10^{-7} \pm 0.15$	$4.2 \times 10^{-8} \pm 0.13$	$2.1 \times 10^{-8} \pm 0.05$	$2.0 \times 10^{-6} \pm 0.15$	$1.7 \times 10^{-6} \pm 0.09$	$4.3 \times 10^{-6} \pm 0.14$	19.6	39.1	208.3
1 nM	$3.7 \times 10^{-7} \pm 0.04$	$1.9 \times 10^{-7} \pm 0.04$	$3.7 \times 10^{-7} \pm 0.05$	$3.1 \times 10^{-6} \pm 0.04$	$8.1 \times 10^{-6} \pm 0.05$	$4.9 \times 10^{-6} \pm 0.06$	8.5	43.1	13.1

[a]α-Hydroxyketo coferons demonstrate a concentration-dependent increase in potency over their vicinal diol analogs in assays with purified enzyme. IC_{50}s for the homodimers decreased with decreasing target concentration and corresponded to an increase in fold improvements. Fold difference was determined from the monomeric diol analogs. Intensity of red boxes indicates the degree of fold difference. IC_{50}s were determined from nonlinear regression, with no constraints on Hill slope using Graphpad prism.

Table 2. α-Hydroxyketo Coferons Demonstrate Good Potency in Degranulation and Whole Lysate Assays in HMC1 Cells[a]

	IC_{50} in HMC1 cellular degranulation [M]				IC_{50} in HMC1 cellular lysates [M]			
a-HK #	IC_{50} [M]	SEM	diol control	SEM	IC_{50} [M]	SEM	diol control	SEM
1a	4.33×10^{-7}	0.4	1.08×10^{-5}	0.07	4.54×10^{-7}	0.12	8.40×10^{-6}	0.07
2a	4.39×10^{-7}	0.19	1.37×10^{-4}	0.41	3.14×10^{-7}	0.06	2.18×10^{-5}	0.11
3a	5.20×10^{-8}	0.43	3.15×10^{-5}	0.1	1.13×10^{-7}	0.05	1.33×10^{-5}	0.1

[a]Cells treated with inhibitors (10 nM to 100 μM; 2 h) were degranulated in the presence of 1 μM A23187 in PBS. After 1 h, the supernatant was assayed for tryptase activity. Alternatively, IC_{50}s were determined in cell lysates. IC_{50}s and SEM were determined from nonlinear regression, with no constraints on Hill slope using Graphpad prism.

covalent assembly of monomeric coferons. Our approach allows significant flexibility for optimizing monomers for improved permeability, metabolic stability, and pharmacokinetics while delivering the superior potency of the larger dimeric payload.

The current study uses a novel chemistry platform to establish self-assembling homodimeric inhibitors of tryptase. Our efforts have demonstrated the attributes of synthetic ease of introduction, effectiveness, specificity, kinetic facility, biological

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inertness, and physiologic compatibility of the linkers, while minimally perturbing the affinity and drug-like behavior of the pharmacophoric moiety to which it is appended. Thus, we anticipate that this technology will provide a versatile, broadly applicable linker, deployable in diverse structural arrangements for drug discovery and delivery of bivalent molecules with improved cellular permeability. Our coferon dimers are sufficiently stable to be chromatographed, crystallized, and stored without decomposition, making them suitable as active pharmaceutical constituents. Thus, we anticipate that our coferon platform will provide a versatile and broadly applicable coferon linker for drug discovery and delivery efforts, and amenable to tuning and engineering properties through entropic, electronic, and steric effects.[4]

EXPERIMENTAL PROCEDURES

For details regarding the synthesis of the compounds and experimental procedures see the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00204.

Synthesis of compounds (PDF)

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Author Contributions

Study concept and design: S.F.G. and L.D.A. M.P., F.B., and D.E.B. developed the Coferon concept, and L.D.A., D.E.B., and M.P. developed the 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., and D.W.S., K.W.F. S.F.G., L.D.A., and D.S.W. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare the following competing financial interest(s): S.F.G., L.D.A., M.P., D.E.B., and F.B. are Blinkbio Inc. shareholders. D.S.W. and M.P. are employed by Blinkbio Inc. S.F.G., L.D.A., D.S.W., M.P., D.E.B., and F.B. hold patents on the Coferon technology.

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ABBREVIATIONS

α-HK, α-hydroxyketo; FBDD, fragment-based drug discovery; AMC, 7-amino-4-methylcoumarin

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