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Substrate-Based Design of the First Class of Angiotensin-Converting Enzyme-Related Carboxypeptidase (ACE2) Inhibitors

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Recent advances in genetic technology have provided new tools to scientists for target identification. In concert with database mining and homology searching, we identified a new gene from 5′ sequencing of a cDNA library derived from the ventricle of a failed human heart. This gene encodes a protein that is highly expressed[1] in the following normal human tissues: heart, kidney, colon, small intestine, and testes. This protein has highest homology to the testes-specific isoform of angiotensin-converting enzyme (ACE) with 60% sequence homology and was designated angiotensin-converting enzyme-related carboxypeptidase, ACE2.[2] ACE2 is an 805 amino acid protein with a transmembrane domain and a single HEXXH zinc-binding consensus sequence (amino acid positions 374−378). The catalytic domain of ACE2 is 42% identical to that of ACE. A series of studies helped classify ACE2 as a metalloprotease that functions as a carboxypeptidase.[3] Potent and selective inhibitors of ACE2 may be useful pharmacological tools to help understand its biological relevance and ultimately its potential role in human disease. Herein we report the design and synthesis of the first potent and selective ACE2 inhibitors.

In lieu of conducting high throughput screening (HTS), we applied a rational design approach to develop ACE2 inhibitors on the basis of our enzyme characterization and substrate studies. Because ACE2 is a zinc metalloprotease that functions as a carboxypeptidase, we examined its ability to cleave the C-terminal residue of 130 known peptide hormones. Of these, ACE2 cleaved 11 peptides, including the endogenous ACE substrate, Ang I (1−10) to Ang 1−9.[4] However, the catalytic activity of ACE2 was not suppressed by known ACE inhibitors (enalaprilat, lisinopril, captopril), which may be due to the fact that ACE is a peptidyl dipeptidase and ACE2 is a carboxypeptidase.

To determine the minimal structural features required for binding to the ACE2 catalytic domain, we examined the substrate screening data and compared the P5−P1′ sequence alignment of the peptides that have moderate affinity for ACE2 (K_m < 10 uM).[3] Our analysis revealed two useful insights: (1) ACE2 preferentially cleaves Pro-X terminal peptides (X = hydrophobic amino acid) with high turnover rates, and (2) while Ang I, with a His−Leu cleavage site, binds to the enzyme with low micromolar affinity (K_m = 6.9 uM), it is proteolyzed slowly by ACE2 (K_{cat} = 0.034 s^{-1}). Additional experiments determined that Ang I inhibits ACE2 activity (K_i = 2.2 uM) and undergoes less than 2% proteolysis under the assay conditions. Collectively, these data offered clues to the structural requirements for ACE2 inhibitors, for example, the necessity for a His or Pro residue at P1 and maintaining a hydrophobic group at P1′.

For our small molecule design strategy, we conceived nonhydrolyzable His−Leu-like molecules bearing a C-terminal carboxylate and including a centrally located carboxylate to serve as a zinc coordinating element. We chose the carboxylate as the zinc binding fragment on the basis of precedence with a known carboxypeptidase A (CPDA) inhibitor, benzylsuccinic acid.[5]

The amino dicarboxylate core selected for evaluation is exemplified by **1a**, which inhibits ACE2 in the micromolar range, Figure 1. This molecule appears to be capable of occupying both S1 and S1′ and was amenable to further elaboration.

Prior to exploring substituent effects on the imidazole, we prepared the four stereoisomers of **1**. The *R*,*R* isomer (**1d**) is inactive (>50 uM); however, the other three isomers are equipotent (**1a**, *S*,*S* IC_{50} = 1.2 μM; **1b**, *R*,*S* IC_{50} = 1.4 μM; **1c**, *S*,*R* IC_{50} = 2.2 μM). Of these, the naturally derived *S*,*S* isomer (**1a**) appeared to be selective against ACE and CPDA (IC_{50}'s > 50 μM). With the goal of improving potency and maintaining selectivity, we alkylated the imidazole core of **1a** with groups that may be capable of occupying S2. A benzyl group at the N-1 position of the imidazole ring (**2**) decreased potency 10-fold. However, installing a benzyl substituent at the imidazole N-3 position[6] (**3**) greatly increased potency, providing a 24 nM inhibitor of ACE2, which maintained high selectivity (IC_{50}: ACE >100 uM; CPDA = 34 uM). Perhaps the N-3 substituent on the imidazole framework occupies the S2 pocket of ACE2, which could account for the observed potency enhancements.

We optimized the inhibitor potency and selectivity of **3** by exploring the putative P2, P1, and P1′ sites. Modifications of the P1′ hydrophobic residue decreased potency (cf. **4** and **5**) and appeared to diminish selectivity versus CPDA. In the P1 binding pocket, various substituted aromatic groups were explored,[7] and the imidazole-based scaffold provided superior potency and synthetic flexibility for substituent studies. To explore the presumed interaction at S2, a variety of substituted benzyl groups were installed at the imidazole N-3 position. We replaced the N-3 benzyl group with a cyclohexylmethyl group (**6**), which reduced ACE2 inhibition by an order of magnitude, while the homologue (**7**) yielded a 10 nM inhibitor of ACE2. Many substituents[8] were tolerated, and no significant electronic or steric effects (cf. **8**−**11**) were observed, providing many low nanomolar inhibitors (Table 1) that retained selectivity (>100 fold) against the related enzymes tested (ACE and CPDA). A systematic study of regioisomers revealed a profound preference for meta-substitution (cf. **11**−**13**). While maintaining a 3-substituent on the phenyl group, a series of disubstituted derivatives was prepared, and a significant preference for the 3,5-isomer exists. For example, a second methyl group at the 4-position of **13** decreased potency (**14**), but when the additional

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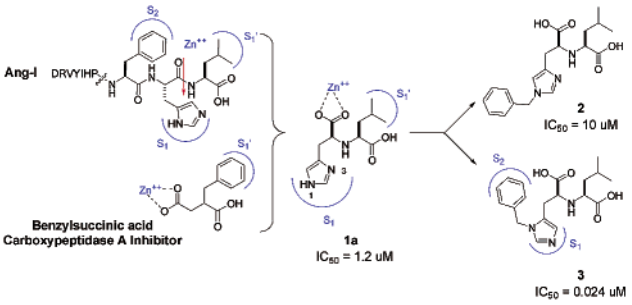
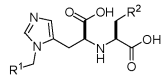


Figure 1. Design of ACE2 inhibitors.

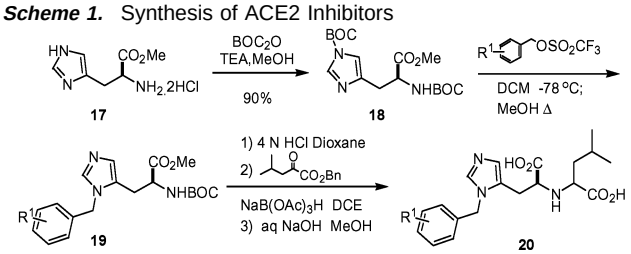
Table 1. ACE2 Inhibitors[a]



			IC$_{50}$[b] (μM)		
compound	R^1	R^2	ACE2	ACE	CPDA
4	Ph	Me	0.30		2.8
5	Ph	Ph	0.34		
6	cyclohexyl CH_2	$CH(CH_3)_2$	0.27		
7	cyclohexyl $(CH_3)_2$	$CH(CH_3)_2$	0.010	>10	12
8	4-NO_2Ph	$CH(CH_3)_2$	0.076		
9	4-ClPh	$CH(CH_3)_2$	0.021		>10
10	4-CF_3OPh	$CH(CH_3)_2$	0.052		
11	4-MePh	$CH(CH_3)_2$	0.032	>10	>10
12	2-MePh	$CH(CH_3)_2$	0.29		
13	3-MePh	$CH(CH_3)_2$	0.0042	>10	>10
14	3,4-diMePh	$CH(CH_3)_2$	0.010	>10	>10
15	3,5-diMePh	$CH(CH_3)_2$	0.0014	>10	>10
16	3,5-diClPh	$CH(CH_3)_2$	0.00044	>100	27

[a] $n \geq 2$, SEM within 50% of IC$_{50}$ values. [b] Assay conditions: soluble human ACE2, porcine, or human testicular ACE, Bovine CPDA.

Scheme 1. Synthesis of ACE2 Inhibitors



methyl group was installed at the 5-position, the potency modestly increased (**15**). The 3,5-dichloro analogue (**16**) inhibits ACE2 in the picomolar range and has excellent selectivity (>5000-fold) versus related enzymes.[9] All four stereoisomers of **16** were prepared, and the greatest potency remained within the *S*,*S* isomer.[10]

The preparation of the N-3 substituted imidazole-containing inhibitors is outlined in Scheme 1. Treatment of (*S*)-histidine methyl ester (**17**) with Boc_2O affords the fully protected histidine derivative **18**.[11] Selective alkylation of the N-3 imidazole nitrogen using the triflate of a substituted benzyl alcohol (or alkyl alcohol derivative) provides the N-3 alkylated histidine derivative **19**.[12,13] Following Boc deprotection, reductive amination between the histidine derivative **19** and the β-keto ester furnishes the diester amine.[14] Hydrolysis of this diester yields the desired amino dicarboxylate ACE2 inhibitor **20** as a mixture of diastereomers.[15] The diastereomers are separated and purified using HPLC and crystallization.

ACE2 is a recently identified zinc metalloprotease with carboxypeptidase activity that was discovered using our genomics platform. As a strategy to circumvent the caveats associated with HTS (i.e., expense, time commitment, and low probability for success for this type of target), we implemented a rational design approach to identify potent and selective ACE2 inhibitors. To this end, we successfully designed and synthesized picomolar inhibitors of ACE2 using our understanding of the enzymatic mechanism, the substrate data, and related protease literature. These inhibitors have low molecular weights, are synthetically accessible from commercially available chiral building blocks, and display excellent selectivity versus related enzymes. Currently, these inhibitors are proving useful in exploring the physiological relevance of ACE2 in vivo.

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Supporting Information Available: Synthetic procedures and assays protocols (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) Site of alkylation was confirmed using ROESY NMR.
(7) Data not shown.
(8) Representative data shown.
(9) Configuration confirmed by X-ray crystallography.
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