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Thiol-based angiotensin-converting enzyme 2 inhibitors: P^1 modifications for the exploration of the S^1 subsite

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Abstract—Screening of a metalloprotease library led to the identification of a thiol-based dual ACE/NEP inhibitor as a potent ACE2 inhibitor. Modifications of the P^1 benzyl moiety led to improvements in ACE2 potency as well as to increased selectivity versus ACE and NEP.

Angiotensin-converting enzyme 2 (ACE2) is a recently identified clan MA, family M2 monocarboxypeptidase with highest homology to the dicarboxypeptidase angiotensin-converting enzyme (ACE, EC 3.4.15.1).[1,2] This membrane-associated and secreted metalloprotease is expressed in heart, kidney, testes, intestine, and lung, and has been implicated in cardiovascular disease, kidney disease, obesity, and lung disease.[3–5]

ACE2 processes angiotensin I and the AT_1 and AT_2 receptor agonist angiotensin II to produce angiotensin (1–9) and the *mas* receptor agonist angiotensin (1–7), respectively, but its exact role in the renin-angiotensin system (RAS) needs to be clarified. One group has reported that C57BL/6 ACE2 (−/−) mice exhibit a severe reduction in cardiac contractility, which is rescued in the ACE (−/−)/ACE2 (−/−) double knockout.[6] In contrast, another group has disclosed that ACE2 (−/−) mice of 129/SvEv, C57BL/6, and mixed background do not exhibit cardiac contractility defects, but have increased susceptibility to angiotensin II-induced hypertension.[7] While, a third group has revealed that male ACE (−/Y) mice are more susceptible to heart failure and death after transverse aortic constriction than their normal littermates.[8] Supporting a role for ACE2 in cardiac function, transgenic mice overexpressing ACE2 in the heart have lower mean arterial pressure with rare focal myocyte vacuolization, myofibril splaying, and nuclear enlargement. Many of these mice develop terminal ventricular fibrillation with lethal arrhythmias.[9]

Furthermore, male ACE2 (−/Y) mice, but not female ACE2 (−/−) mice, also accumulate fibrillar collagen in the renal glomerular mesangium, leading to development of glomerulosclerosis of the kidneys.[10] In addition, ACE2 (−/−) mice exhibit lower body weights than wild type mice with reduced fat mass.[11] Moreover, ACE2 is also utilized by the severe acute respiratory syndrome (SARS) coronavirus as the receptor for infection.[12] ACE2 (−/−) mice are resistant to SARS corona virus infection.[13] Finally, ACE2 (−/−) mice have enhanced vascular permeability, increased lung edema, and worsened lung function in several murine acute respiratory distress syndrome (ARDS) models.[14] With the many potential functions of ACE2, small molecule inhibitors of this enzyme could be utilized to help further define the physiological roles of this protease.

ACE2 belongs to the zinc metalloprotease family and it has been reported that classical ACE inhibitors such as captopril and lisinopril do not attenuate ACE2 enzyme activity. As part of a strategy to discover lead molecules for an ACE2 inhibitor program, a directed screen of ACE2 versus a set of metalloprotease inhibitors from

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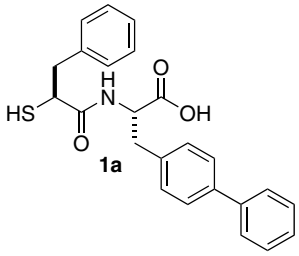
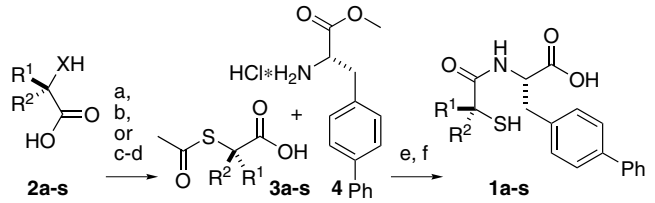
the GlaxoSmithKline compound collection was performed. Surprisingly, although confirming that classical ACE inhibitors like captopril were inactive in the screen, the thiol acid **1a** was identified as a potent ACE2 inhibitor ($K_{i\ App}$ = 86 nM).[15] This biphenyl analog **1a** is a known dual ACE and M13 metalloprotease neutral endopeptidase (neprilysin, NEP, EC 3.4.24.11) inhibitor (ACE $K_{i\ App}$ = 30 nM, NEP $K_{i\ App}$ = 1.1 nM).[16] It also inhibits the M14 metalloprotease carboxypeptidase A1 (CpA, EC 3.4.17.1, $K_{i\ App}$ = 1,200 nM). Speculating that the thiol functioned as a binding group for the active site zinc and that the carboxylic acid served as a recognition element for the enzyme's monocarboxypeptidase activity, the benzyl and methylene *p*-biphenyl moieties were surmised to be the P^1 and $P^{1'}$ substituents. With this premise, the structure activity relationships of the P^1 position of the lead inhibitor were explored with the goal of improving potency and reducing ACE and NEP inhibitory activity.



1a

The thiol analogs **1a**–**1s** were prepared as depicted in Scheme 1. The acids **3a**–**3s** were activated in situ via the carbodiimide, converted into the activated esters with the aza-hydroxybenzotriazole, and then coupled to the amine hydrochloride **4** to produce the fully protected amides. Subsequent hydrolysis of the methyl ester, as well as the thioacetate, with lithium hydroxide afforded the thiol acids **1a**–**1s**.[17]

The thiol acids **3a**–**3s** were produced by three different routes. Acids **3c** and **3e** were prepared by reaction of the commercially available thiols **2c** and **2e** (X=S) with acetyl chloride. In contrast, the acids **3k**, **3m**, and **3n** were produced from the alcohols **2k**, **2m**, and **2n** (X=O) via the Mitsunobu reaction with thioacetic acid.[18] Alternatively, the acids **3a**–**3b**, **3d**, **3f**–**3j**, **3l**, and **3o**–**3s** were synthesized from the commercially available



2a-s **3a-s** **4** **1a-s**

Scheme 1. Reagents and conditions: (a) **2c** and **2e**, X=S, AcCl, NEt_3, dioxane, 0 °C to rt, 15–37%; (b) **2k**, **2m**, and **2n**, X=O, AcSH, DIAD, PPh_3, THF, 0 °C to rt, 24–67%; (c) **2a**–**2b**, **2d**, **2f**–**2j**, **2l**, **2o**–**2s**, X=NH, HBr, $NaNO_2$, H_2O, 0 °C, 25–80%; (d) AcS^-K^+, DMF, 0 °C to rt, 11–72%; (e) EDC, HOAt, *i*-Pr_2NEt, CH_2Cl_2, 48–96%; (f) LiOH·H_2O, THF, H_2O, 30–94%.

amino acids **2a**–**2b**, **2d**, **2f**–**2j**, **2l**, and **2o**–**2s** (X=NH), by diazotization of the amines to afford the bromides, then subsequent displacement of the bromides, by potassium thioacetate, with stereochemical inversion. Some stereochemical erosion, presumably resulting from formation of the α-lactone before nucleophilic trapping, resulted during the Mitsunobu and diazotization reaction steps, but after coupling with the amine **4**, the minor diastereomer could be removed via chromatography.

The structure/activity relationships of P^1 analogs are depicted in Table 1. The (R) isomer **1b** derived from L-phenylalanine displays the P^1 benzyl substituent in the unnatural configuration. This orientation of the P^1 substituent was detrimental to the ACE2 inhibitory activity of diastereomer **1b** ($K_{i\ App}$ = 1400 nM) as well as that of ACE ($K_{i\ App}$ = 520 nM). This loss in inhibition is not surprising, since nature has evolved proteases to recognize the natural configuration of physiological peptide substrates. In contrast, NEP ($K_{i\ App}$ = 1.3 nM) accommodated the alternate configuration with no loss in potency.

Complete removal of the P^1 moiety caused a modest loss in inhibitory potency versus ACE2 (H, $K_{i\ App}$ = 320 nM). This decrease in potency could arise from reduced van der Waals interactions due to the lack of a P^1 substituent, the increased entropic cost for the inhibitor to bind to the protease given the inhibitor's increased rotational freedom, or a combination of these factors. In contrast to the D-isomer **1b**, the ACE activity ($K_{i\ App}$ = 16 nM) of the glycine-like analog **1c** was not affected by elimination of the P^1 element, while NEP potency ($K_{i\ App}$ = 13 nM) of **1c** decreased by over an order of magnitude.

The alanine-derived analog **1d** (Me, $K_{i\ App}$ = 6.9 nM) decreases rotational freedom and was an even more potent ACE2 inhibitor than the starting lead **1a**, while increasing selectivity versus both ACE ($K_{i\ App}$ = 21 nM) and NEP ($K_{i\ App}$ = 23 nM). In contrast, the geminal dimethyl analog **1e** (ACE2 $K_{i\ App}$ = 2300 nM) decreased inhibitory potency versus not only ACE2, but also ACE ($K_{i\ App}$ = 3,400 nM) and NEP ($K_{i\ App}$ = 730 nM). Similar to the methyl analog **1d**, the linear P^1 ethyl and *n*-butyl analogs **1f** (Et, $K_{i\ App}$ = 1.4 nM) and **1g** (*n*-Bu, $K_{i\ App}$ = 1.8 nM) were also potent ACE2 inhibitors, but these extensions in P^1 also increased activity versus ACE (**1f** $K_{i\ App}$ = 8.6 nM, **1g** $K_{i\ App}$ = 9.3 nM) and even more so versus NEP (**1f** $K_{i\ App}$ = 0.80 nM, **1g** $K_{i\ App}$ = 1.2 nM).

With the hope of improving the selectivity of these thiol-based inhibitors versus ACE and NEP, the effect of branching along the P^1 side chain of this inhibitor class was explored. Branching along the P^1 chain was well tolerated in the S^1 subsite of ACE2. The α-branched *iso*-propyl **1h** (*i*-Pr, $K_{i\ App}$ = 1.5 nM), (R)-*sec*-butyl **1i** ((R)-*s*-Bu, $K_{i\ App}$ = 1.5 nM), (S)-*sec*-butyl **1j** ((S)-*s*-Bu, $K_{i\ App}$ = 1.6 nM), cyclobutyl **1k** (Cyb, $K_{i\ App}$ = 2.4 nM), and cyclopentyl **1l** (Cyp, $K_{i\ App}$ = 1.8 nM) analogs and the β-branched *iso*-butyl **1o** (*i*-Bu, $K_{i\ App}$ = 1.4 nM)