Structure-Based Identification of Small-Molecule Angiotensin-Converting Enzyme 2 Activators as Novel Antihypertensive Agents

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Abstract—Angiotensin-converting enzyme 2 (ACE2) is a key renin-angiotensin system enzyme involved in balancing the adverse effects of angiotensin II on the cardiovascular system, and its overexpression by gene transfer is beneficial in cardiovascular disease. Therefore, our objectives were 2-fold: to identify compounds that enhance ACE2 activity using a novel conformation-based rational drug discovery strategy and to evaluate whether such compounds reverse hypertension-induced pathophysiology. We used a unique virtual screening approach. In vitro assays revealed 2 compounds (a xanthenone and resorcinolnaphthalene) that enhanced ACE2 activity in a dose-dependent manner. Acute in vivo administration of the xanthenone resulted in a dose-dependent transient and robust decrease in blood pressure (at 10 mg/kg, spontaneously hypertensive rats decreased 71±9 mm Hg and Wistar-Kyoto rats decreased 21±8 mm Hg; \( P<0.05 \)). Chronic infusion of the xanthenone (120 \( \mu \)g/day) resulted in a modest decrease in the spontaneously hypertensive rat blood pressure (17 mm Hg; 2-way ANOVA; \( P<0.05 \)), whereas it had no effect in Wistar-Kyoto rats. Strikingly, the decrease in blood pressure was also associated with improvements in cardiac function and reversal of myocardial, perivascular, and renal fibrosis in the spontaneously hypertensive rats. We conclude that structure-based screening can help identify compounds that activate ACE2, decrease blood pressure, and reverse tissue remodeling. Administration of ACE2 activators may be a valid strategy for antihypertensive therapy. (Hypertension. 2008; 51:1312-1317.)

Key Words: structure-based drug design ◼ angiotensin-converting enzyme 2 ◼ virtual screening ◼ molecular docking ◼ cardiovascular disease ◼ angiotensin (1-7)

The recent discovery of angiotensin-converting enzyme (ACE) 21,2 and its role in the control of renin-angiotensin system activity is relevant as a potentially valuable target for antihypertensive therapies. ACE2 is a zinc-dependent monooxygenase that plays a central role in balancing vasoconstrictor and proliferative actions of angiotensin (Ang) II with the vasodilatory and antiproliferative effects of Ang-(1-7).3 Altered expression of this enzyme is associated with cardiac, vascular, and renal dysfunctions.4,5 In addition, blocking the synthesis of Ang II by Ang-converting enzyme inhibitors or its actions by Ang II receptor blockers has been shown to increase cardiac ACE2 expression.6,7 Furthermore, overexpression of ACE2 by gene transfer8 protects the heart from hypertension-induced cardiac remodeling. It was demonstrated that ACE2 is an effective enzyme in attenuating fibrosis and structural remodeling.8 Based on these observations, we hypothesize that pharmacological enhancement of ACE2 activity would have beneficial effects on the cardiovascular system and would protect against hypertension-induced pathophysiology. Therefore, our objectives in this study were 2-fold: to identify compounds that enhance ACE2 activity and to determine the effects of ACE2 enhancers on hypertension and associated pathophysiology.

Materials and Methods
Synthesis of xanthenone, measurement of ACE2 activity, histological analysis, and statistical analysis are described in the supplemental information (available online at http://hyper.ahajournals.org).

Received December 14, 2007; first decision December 24, 2007; revision accepted March 4, 2008.
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Hypertension is available at http://hyper.ahajournals.org
DOI: 10.1161/HYPERTENSIONAHA.107.108944
Virtual Screening
The DOCKv5.2 package was used for in silico screening of ~140,000 compounds available from the National Cancer Institute Developmental Therapeutics Program. This computer database was prepared with DOCK accessory software (SF2MOL2, University of California San Francisco) and Sybyl (Tripos, Inc.). Each compound was docked as a rigid body in 100 different orientations. The orientations were filtered by default bump filter parameters to exclude compounds with pronounced steric clashes. The grid-based scoring system was used for scoring with a nonbonded force field energy function implemented in DOCK. A standard 6 to 12 Lennard-Jones potential was used to evaluate van der Waals contacts. Spheres used by DOCK during matching algorithms were generated by SPHGEN.10

Sites for molecular docking were identified by structural analysis in which the differences between the molecular surfaces of ACE2 in the open and closed conformation were calculated with DSSP.11 Three different molecular surface pockets, remote to the active site of ACE2, were selected with SPHGEN to dock and rank the compounds of the National Cancer Institute database. Two sites were selected in the inhibitor-bound form of the enzyme (sites 2 and 3, PDBID 1R4L), and a single site was selected in the open conformation of ACE2 (site 1, PDBID 1R42). Structural analysis indicates that these surface sites are unique to only 1 of the 2 conformations.

After ranking with DOCK, the top-scoring compounds for each site were tested in vitro with human recombinant ACE2 (R&D Systems). Active compounds were modeled bound to ACE2 with DOCK and were docked in ≥3000 orientations, energy minimized, and refined with flexible bond parameters enabled. Other parameters such as the number of minimization steps and number of conformations were also exhausted until the score for each compound converged and did not improve further.

Animals
All of the animal procedures were performed in compliance with approved International Animal Care and Use Convention protocols and University of Florida regulations. Male Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) of 14 to 16 weeks of age (300- to 325-g body weight) were purchased from Charles River Laboratories (Wilmington, Mass).

Acute Hemodynamic Measurements
Mean arterial pressure and heart rate (HR) were continuously monitored in SHRs and WKY animals (n = 3 to 9) fitted with both a jugular and carotid cannula. Briefly, animals were anesthetized with a mixture of ketamine, xylazine, and acepromazine (30, 6, and 1 mg/kg, respectively). A polyethylene cannula (PE-50, Clay Adams) was introduced into the carotid artery for direct blood pressure (BP) measurements, whereas a silicone elastomer cannula (Helix Medical) was introduced into the descending jugular vein for acute intravenous injections of the drug. Both cannulae were filled with heparin saline (40 U/mL, Sigma), and sealed with stylets. Dose-response curves were obtained in awake, freely moving animals after a 24- to 48-hour recovery period. Doses of xanthenone (XNT) (0.5, 1.0, 5.0, and 10.0 mg/kg) were applied as a bolus administration via the jugular cannula, and BP and HR data were recorded and interfaced to a PowerLab (ADInstruments) signal transduction unit. Data were analyzed using the Chart program supplied with the PowerLab system.

Chronic Hemodynamic Measurements
Osmotic minipumps (Alzet, model 2004) containing either 10 mg/mL of XNT (60 μg/d; 28 days; n = 9) or vehicle (saline; pH 2.0 to 2.5) were implanted SC after allowing them to equilibrate in sterile saline at 37°C for 24 hours. XNT was delivered at an infusion rate of 260 ng/kg per minute. BP was measured indirectly by the tail-cuff method in conscious animals every week for 4 weeks. After 28 days of saline or XNT infusion, animals were cannulated as described above, and acute hemodynamic responses to Ang II (5, 10, 20, 40, 80, and 160 ng/kg), bradykinin (BK; 0.06, 0.60, 6.00, 14.00, and 28.00 ng/kg), and losartan (0.25 mg/kg) were measured in both WKY rats and SHRs.

Isolated Heart Preparation
After analysis of the BP responses to Ang II, BK, and losartan, animals were allowed to recover for 24 hours. An IP injection of heparin (400 IU) was administered to each animal. Ten to 15 minutes later, the hearts were dissected and perfused according to the Langendorff technique. Briefly, hearts were perfused through an aortic stump with Krebs Ringer solution containing 118.4 mM/L of NaCl, 4.7 mM/L of KCl, 1.2 mM/L of KH2PO4, 1.2 mM/L of MgSO4·7H2O, 2.5 mM/L of CaCl2·2H2O, 11.7 mM/L of glucose, and 26.5 mM/L of NaHCO3. The perfusion flow was maintained constant (8 to 10 mL/min) at 37±1°C along with constant oxygenation (5% CO2 and 95% O2). Intraventricular pressure and coronary perfusion pressure were continuously recorded using a PowerLab signal transduction unit (ADInstruments). After 20 to 30 minutes of stabilization, functional parameters were recorded for an additional period of 30 minutes. Data from vehicle or XNT-treated animals were analyzed electronically with Chart software.

Results
Identification of Small Molecules That Enhance ACE2 Activity
To identify ACE2 activators, small drug-like molecules were screened in silico at molecular surface sites remote from the active site. We targeted 3 separate structural pockets based on the crystal structures of ACE2 in the substrate-free (PDBID 1R42) and inhibitor-bound form (1R4L; Figure 1A). One pocket (site 1) was selected on the open (substrate-free) form of ACE2 at the hinge region between subdomains 1 and 2 (Figure 1B).13 Two additional pockets were selected on the closed (inhibitor-bound) form of the enzyme. Site 2 is located distal from the active site, whereas site 3 is located on the interface of subdomains 1 and 2 at the active site entrance (Figure 1B). All of the pockets were selected in both conformations to maximize the likelihood of identifying conformation-specific compounds capable of enhancing ACE2 activity. We used a rapid molecular docking approach to computationally screen a large chemical library. Validity of molecular docking approaches has been established in a large number of studies, primarily to identify enzyme inhibitors.14,15 In this study, we applied the high-throughput molecular docking technique to a significant and challenging problem: identification of compounds that enhance, rather than inhibit, enzyme activity.

Approximately 140,000 small molecules were each docked (DOCK 5.2) into the 3 selected sites in 100 different orientations and ranked by energy score. The top 10 scoring compounds for each of 3 sites are listed in Table S1. The compounds directed at site 1 share physicochemical characteristics with Food and Drug Administration–approved orally available drugs. These predominantly uncharged compounds are consistent with drug-likeness criteria (molecular weight: <500; octanol/water partition coefficients: <5; H-bond donors: <5; H-bond acceptors: <10).16 In contrast to site 1, compounds molecularly docked into sites 2 and 3 included charged compounds of larger molecular weight or with those other unfavorable characteristics for drug absorption and permeability. The shared characteristics of these molecularly...
docked compounds likely reflect the properties of the structural pockets selected for virtual screening, and the results suggest that site 1 is a better fit for the ligation of a drug-like molecule.

The highest scoring compounds directed at ACE2 structural pockets in the open and closed conformations were tested in vitro for their ability to modulate ACE2 activity. Gene transfer experiments leading to increased ACE2 mRNA levels by 2- to 3-fold\(^1\) and studies on clinically available Ang-converting enzyme inhibitors\(^1\) that report a similar increase in ACE2 mRNA and plasma Ang-(1-7) show improvements in the pathophysiology of cardiovascular diseases (CVDs). Based on this evidence and the emerging trend that these beneficial effects are mediated by ACE2 activity and Ang-(1-7), compounds capable of enhancing ACE2 activity by 2- to 3-fold are expected to have a physiological effect in vivo. We found 2 compounds that enhanced ACE2 activity by 1.8- to 2.2-fold of control levels (Figure 1G), XNT and resorcinolnaphthalein, respectively. C and E show ACE2 in a similar orientation. Compounds are colored according to the elements: green for carbon, red for oxygen, orange for sulfur, and blue for nitrogen. Likely hydrogen bonding interactions are labeled with dashed yellow lines. D and F, Chemical structures of XNT and resorcinolnaphthalein, respectively. G, ACE2-specific enhancement by XNT and resorcinolnaphthalein (100 \(\mu\)mol/L), in red. ACE2 activators have no effect on ACE activity in the same conditions, in blue. *\(P<0.001\).

Figure 1. Structure-based identification of an ACE2 activator. A, Free (open) and inhibitor bound (closed) ACE2 structures (PDBID: 1R42 and 1R4L respectively\(^1\)). Secondary structure is shown in red for helices, gray for loops, and gold for strands. B, Sphere clusters targeting 3 sites on ACE2. Spheres are in yellow and secondary structure is shown as in A. B shows the structure of the inhibitor-bound conformation of ACE2 (inhibitor not shown). The cluster for site 1 was generated based on the structure of the open form of the enzyme, but it is shown superposed on the closed form to show its relative position to the other clusters. The view of the structure is rotated 90° around the horizontal axis. C and E, Molecular docking models of ACE2 activators XNT and resorcinolnaphthalein, respectively. C and E show ACE2 in a similar orientation. Compounds are colored according to the elements: green for carbon, red for oxygen, orange for sulfur, and blue for nitrogen. Likely hydrogen bonding interactions are labeled with dashed yellow lines. D and F, Chemical structures of XNT and resorcinolnaphthalein, respectively. G, ACE2-specific enhancement by XNT and resorcinolnaphthalein (100 \(\mu\)mol/L), in red. ACE2 activators have no effect on ACE activity in the same conditions, in blue. *\(P<0.001\).

Effects of an ACE2 Activator on BP, Heart Function, and Tissue Remodeling

Acute intravenous injections of XNT resulted in a rapid and transient decrease in BP (Figure 2A and 2B). It caused a significant decrease in BP in the SHRs with a dose as low as 1 mg/kg. A maximal decrease of 71±9 mm Hg on BP was observed with 10 mg/kg (Figure 2B). Decreases in BP were accompanied by decreases in HR (Figure 2C and 2D). In contrast to SHRs, XNT had no significant effect on WKY rat BP with 1 mg/kg and showed only modest decreases in BP with 5 and 10 mg/kg. Thus, the antihypertensive effect of XNT was significantly more pronounced in the SHRs compared with WKY rats (Figure 2A and 2B). Compared with the 71±9 mm Hg decrease observed in the SHRs, only a 21±8 mm Hg decrease was observed in WKY rats with a dose of 10 mg/kg (\(P<0.05\)). Sprague-Dawley rats showed a response to XNT that was similar to WKY rats (data not shown). In addition, vehicle alone did not show any significant effects on BP or HR in either strain of rats. Chronic infusion of XNT produced a significant reduction in the BP of SHRs but not in WKY rats. The decrease in BP during XNT infusion was gradual, and it achieved the maximal effect (17 mm Hg; 2-way ANOVA; \(P<0.05\)) by the third week (Figure S1). However, chronic treatment of SHRs with XNT did not cause any significant changes in either HRs (363±19 bpm, \(n=8\) in controls versus 366±24 bpm, \(n=7\) in XNT-treated rats) or body weights (321±8 g, \(n=8\) in controls versus 332±12 g, \(n=9\) in XNT-treated rats).

Because ACE2 is involved in the metabolism of Ang peptides and kallikrein-kinin system peptides, the BP re-
responses to acute administration of BK and Ang II and to the Ang II type 1 receptor antagonist losartan were evaluated in WKY rats and SHRs after 4 weeks of XNT infusion. BK-induced decreases in BP were more pronounced in XNT-treated WKY rats and SHRs (Supplemental Figure S1). Also, the potentiation of this BK hypotensive effect in XNT-treated rats was significantly greater in the SHRs compared with the WKY rats (43±12 mm Hg versus 28±8 mm Hg; P<0.05; Figure S1). However, no significant differences in Ang II–induced increase or losartan-induced decrease in BP were observed between saline and XNT-treated WKY rats and SHRs (data not shown).

In addition, XNT effects on cardiac function were analyzed using the Langendorff preparation. Chronic infusion of XNT resulted in an increase in +dP/dt and −dP/dt in SHRs (Figure S1). No significant changes were observed in left ventricular systolic pressure, left ventricular end diastolic pressure, perfusion pressure, or HR (data not shown).

Finally, previous studies have demonstrated that ACE2 overexpression by gene transfer prevents cardiac fibrosis. This observation led us to test the possibility that XNT activation of ACE2 would reverse cardiac fibrosis induced by hypertension. Thus, the effect of chronic XNT infusion on cardiac and renal fibrosis was examined. Chronic XNT treatment caused a significant reversal of both myocardial and perivascular fibrosis in the SHR heart (Figure 3A and 3B). Similarly, a significant reversal in renal interstitial fibrosis was observed in SHRs chronically treated with XNT (Figure 3C). Representative images demonstrating the effect of XNT on fibrosis are presented as Figure S2.

**Discussion**

The most significant observation in this study is that XNT, a compound that enhances ACE2 activity, causes considerable reductions in BP and a striking reversal of cardiac and renal fibrosis in the SHR model of hypertension. This observation is remarkable, because rational drug design is traditionally directed at the discovery of enzyme inhibitors or receptor blockers that compete with the natural ligand. Here we present for the first time a structure-based drug-discovery approach to enable rational development of enzyme activators. In addition, we identified a compound that, for the first time, results in a beneficial outcome on both BP and tissue remodeling in the heart and kidney. The clinical ramifications of this study are directly significant for CVD and diseases associated with hypertension, such as obesity and diabetes. Moreover, we define a novel rational drug design strategy to address new challenges in the prevention and treatment of human diseases.

In this study, we began to test the hypothesis that targeting a specific enzyme conformation with small drug-like molecules will enhance enzymatic activity by shifting the conformational equilibrium of the enzyme favorably for its activity. This hypothesis is based on recent enzyme structure, dynamic and kinetic data demonstrating that conformational changes involved in the binding or release of ligands may be rate limiting. Importantly, the monovalent anion-dependent...
enhancement of activity observed for our model enzyme, ACE2, has been suggested to occur by this mechanism. For hinge-bending enzymes, such as ACE2, the large conformational change that opens and closes their active site allows for a unique opportunity to measure the effects of targeting specific enzyme conformations in a key protein involved in regulating BP and CVD. In this study, we attempted to understand how drug-like molecules can be developed to probe protein dynamics and enhance enzyme activity.

With these considerations in mind, >140,000 small molecules were molecularly docked into structural pockets present in crystal structures of ACE2 in the open and closed conformations (Table S1). Selected compounds were tested in vitro and allowed us to identify 2 active compounds directed at a structural pocket present in the open conformation of ACE2: XNT and resorcinolnaphthalene. Both compounds enhanced ACE2 activity in a dose-dependent manner and were ACE2 specific, because they did not significantly affect ACE activity. These data demonstrate the selective strength of this novel approach in pinpointing specific structural pockets and conformations, because the ACE2 and ACE catalytic domains share 42% sequence identity.

The observation that 20% of the compounds directed at site 1 function in enhancing ACE2 activity, whereas no compounds directed at sites 2 and 3 enhance enzyme activity, suggests that the structural pocket defined by site 1 in the open conformation may be a valid target for therapeutic development. Structural analysis shows that both conformations of ACE2 have 10 to 15 surface pockets with adequate solvent accessible volumes (DSSP11 and castP23), but only a few of these sites are unique to 1 specific conformation. This structure-based approach is distinctly different from those used in previous efforts, because multiple specific enzyme conformations were targeted distal to the active site with the goal of enhancing enzyme activity. Molecular docking, using the parameters described in this study, has been shown previously in inhibitor studies to yield hit rates significantly higher (20% to 80%) than by random screening (0.2 to 2.4). Our results suggest that virtual screening methods may also become rapid and economical tools for the development of enzyme activators.

To validate our strategy, we selected XNT for in vivo studies because of its more favorable solubility properties for administration. Bolus injection of XNT caused a dose-dependent decrease in BP and HR, which was significantly more pronounced in the SHRs compared with WKY and Sprague-Dawley rats. The effect on HR could be the consequence of a direct action of XNT in the heart, direct change in the autonomic activity (increase vagal or decrease sympathetic tonus), or changes in the set point of the baroreflex at the central nervous system. An effect on the central nervous system is consistent with observations after overexpression of ACE2 in the rostral ventrolateral medulla, which resulted in a marked decrease of BP and HR in SHRs. It is important to note that XNT did not elicit any changes in the HR of isolated hearts. However, we cannot exclude a direct effect of XNT on HR, because isolated heart perfusion was performed after 4 weeks of systemic XNT infusion and not directly with a solution containing XNT. More importantly, chronic infusion of XNT also induced a reduction in the BP of SHRs but did not alter HR. The unaffected HR in this protocol was probably because of the different approaches used (acute versus chronic administration) and the final effective plasma concentration of XNT after acute and chronic treatment. Finally, we observed a modest decrease in high BP in the SHRs by chronic infusion of XNT in contrast to its robust effect when this compound was administered acutely. This difference in the efficacy could be because of half-life, biodegradability, solubility, and/or pharmacokinetics of this lead compound in vivo under chronic conditions.

Consistent with the beneficial effects of ACE2 activation on BP, we found that cardiac function was improved in isolated hearts after chronic infusion of XNT in the SHRs. The mechanism of this improvement remains to be elucidated; however, an indirect effect as a result of the decrease in BP is a possibility. Because XNT-treated SHRs also presented a reversal in myocardial and perivascular fibrosis, the improvement in heart function is more likely because of the marked reduction in collagen deposition in cardiac tissue. In fact, if after ACE2 activation there is an increased Ang-(1-7) production with concomitant degradation of Ang II, this hypothesis is plausible, because Ang II is a profibrotic peptide and Ang-(1-7) possesses antifibrotic actions. The antifibrotic effect of XNT was not limited to the heart, because it also reversed interstitial fibrosis in kidneys of SHRs.

As anticipated, the hypotensive effect of BK is more pronounced in SHRs than in WKY rats. Furthermore, we observed that XNT infusion potentiates the BK response in WKY rats and SHRs. Again, these data suggest that the XNT actions may be, at least partially, mediated by an increased Ang-(1-7) production, because it has been demonstrated that Ang-(1-7) potentiates the hypotensive effect of BK in previous preparations.

This study is of broad relevance for 2 reasons. First, evidence suggests that development of a new class of antihypertensive drugs (specific for ACE2) may serve as a complementary strategy in the treatment of CVD. Second, we describe a rational structure-based approach for increasing specific enzymatic activities. This novel approach is suited to a variety of challenging clinical goals, such as the prevention and treatment of protein conformational disorders (eg, retinitis pigmentosa, α1 antitrypsin deficiency, and Alzheimer's disease).

**Perspectives**

Despite recent successes in the treatment and control of hypertension and its associated cardiovascular pathophysiology, the prevalence of CVD has risen steadily in the last several decades. Thus, innovative strategies are needed, and new targets must be developed to overcome this debilitating disease. In this study, we present evidence that a novel and rational structure-based approach can be used to discover activators of ACE2 with effective antihypertensive properties. It also demonstrates that the activator is effective in the reversal of cardiac and renal fibrosis induced by hypertension. Our observations may also have a broader relevance in that the structure-based approach of increasing enzyme ac-
tivity may be well suited to a variety of other challenging clinical goals involving protein conformational disorders, such as Alzheimer’s disease and α-1 antitrypsin deficiency.

Acknowledgment
We thank Edwin A. Homan for his contributions to the synthesis of the xanthenone.

Source of Funding
This work was supported by the National Institutes of Health grant HL56921.

Disclosures
None.

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_Hypertension._ 2008;51:1312-1317; originally published online April 7, 2008;
doi: 10.1161/HYPERTENSIONAHA.107.108944

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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