

Structure-Based Discovery of a Novel Angiotensin-Converting Enzyme 2 Inhibitor

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Abstract—Angiotensin-converting enzyme 2 (ACE2) is considered an important therapeutic target for controlling cardiovascular diseases and severe acute respiratory syndrome (SARS) outbreaks. Recently solved high-resolution crystal structures of the apo-bound and inhibitor-bound forms of ACE2 have provided the basis for a novel molecular docking approach in an attempt to identify ACE2 inhibitors and compounds that block SARS coronavirus spike protein-mediated cell fusion. In this study, $\approx 140\,000$ small molecules were screened by *in silico* molecular docking. In this structure–activity relation study, the molecules with the highest predicted binding scores were identified and assayed for ACE2 enzymatic inhibitory activity and for their ability to inhibit SARS coronavirus spike protein-mediated cell fusion. This approach identified N-(2-aminoethyl)-1 aziridine-ethanamine as a novel ACE2 inhibitor that also is effective in blocking the SARS coronavirus spike protein-mediated cell fusion. Thus, the molecular docking approach resulting in the inhibitory capacity of N-(2-aminoethyl)-1 aziridine-ethanamine provides an attractive small molecule lead compound on which the development of more effective therapeutic agents could be developed to modulate hypertension and for controlling SARS infections. (*Hypertension*. 2004;44:903-906.)

Key Words: angiotensin-converting enzyme ■ cardiovascular diseases ■ hypertension

Angiotensin-converting enzyme 2 (ACE2) is a newly discovered membrane-bound aminopeptidase.^{1–3} This enzyme has been proven to be critical in impacting cardiovascular and immune systems by 2 distinct physiologically important mechanisms. ACE2 catalyzes the production of vasodilatory peptides, including angiotensin 1 to 7,^{4,5} and thus is responsible in counterbalancing the potent^{6–9} vasoconstrictor effects of angiotensin II.^{6–9} This counterbalancing property of ACE2 is proposed to be important for the development of novel pharmacotherapy against hypertension and related cardiovascular diseases.^{10–14} In addition to its critical role in regulation of hypertension, ACE2 has been demonstrated to be a functional receptor for the coronavirus that causes severe acute respiratory syndrome (SARS). The coronavirus, SARS-CoV, the primary cause of SARS, gains entry into pulmonary endothelial cells by membrane fusion on binding to this ectoenzyme. This interaction is mediated by the SARS-CoV spike protein.^{15–19} This conclusion is further supported by recent observations that pulmonary endothelial cells express high levels of ACE2.²⁰ These data provide the impetus to develop ACE2 modulators with anticipation that *in vivo* activation of ACE2 would lead to

protection and successful treatment for hypertension and other cardiovascular diseases. In contrast, ACE2 inhibitors would be expected to block ACE2/SARS-CoV spike protein interactions and inhibit SARS-CoV infection. In addition, ACE2 inhibitors could be potentially important in the mechanisms of hypertension. Despite this urgency, only a few studies have been attempted to develop potential inhibitors and activators of ACE2.^{4,21,22} Our objective in this study was to determine whether a rational structure-based approach can be used to identify molecules capable of inhibiting ACE2 activity and blocking SARS-CoV based on atomic motion deduced from ACE2 crystal structures.

The structure of human ACE2 was recently solved by x-ray crystallography in 2 forms: apo-bound and inhibitor bound.²³ A comparison of these structures revealed a striking conformational change in the active site that impacts a number of surrounding residues, including the ACE2 residues implicated in binding to the SARS-CoV spike protein.¹⁶ Based on these data, which associate ACE2 inhibition with conformational changes in the SARS-CoV binding site, we tested the hypothesis that novel ACE2 inhibitors could be developed with the use of molecular docking approach.

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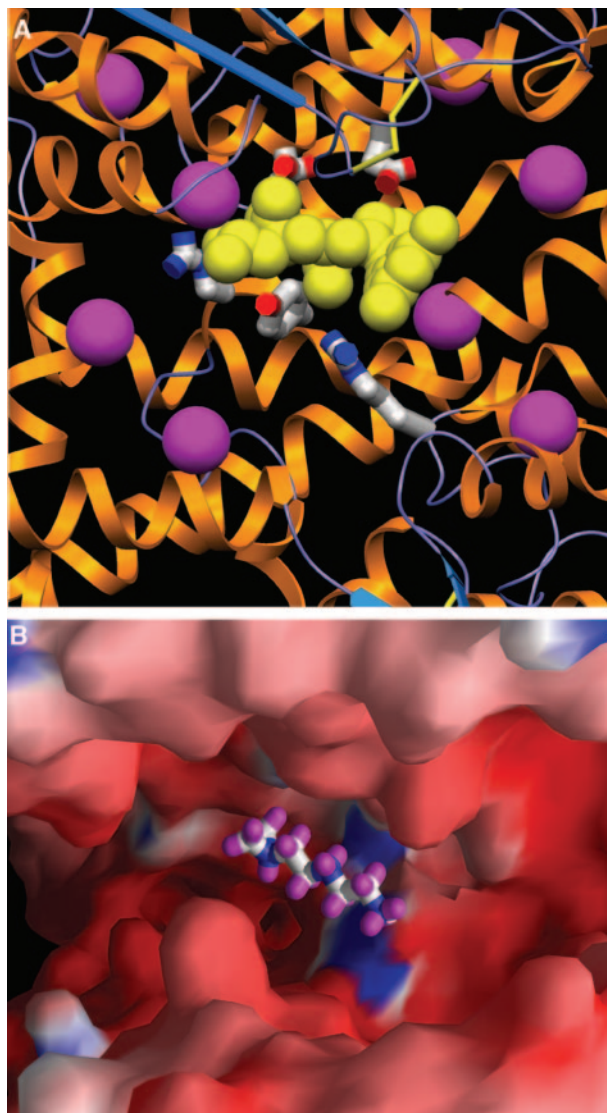


Figure 1. A novel ACE2 inhibitor identified by molecular docking. A, The active site residues of ACE2 are displayed. ACE2 β -strands are colored blue and α -helices are colored gold. Yellow spheres indicate the position of a known ACE2 inhibitor used as a guide for targeting small molecules. Magenta spheres mark the boundary of the scoring grid used to calculate energy scores for molecularly docked small molecules. B, The top scoring compound, N-(2-aminoethyl)-1 aziridine-ethanamine (NAAE), is shown in the ACE2 active site. ACE2 is colored to display the electrostatic molecular surface.

Methods

Database Preparation

The National Cancer Institute/Development Therapeutics Program (NCI/DTP) maintains a repository of $\approx 140\,000$ samples (the plated compound set) that are nonproprietary and offered to the extramural research community for the discovery and development of new agents for the treatment of cancer, AIDS, or opportunistic infections afflicting patients with cancer or AIDS.²⁴ The 3-dimensional coordinates for the NCI/DTP plated compound set was obtained in the MDL SD format and converted to the mol2 format by the DOCK utility program SDF2MOL2. Partial atomic charges, solvation energy, and van der Waals parameters for the ligands were calculated using SYDBD and added to the plated set mol2 file.

Molecular Docking

All docking calculations were performed with the October 15, 2002 development version of DOCK (v5.1.0).²⁴ The general features of DOCK include rigid orienting of ligands to receptor spheres, AMBER energy scoring, GB/SA solvation scoring, contact scoring, internal nonbonded energy scoring, ligand flexibility, and both rigid and torsional simplex minimization. Unlike previously distributed versions, this release incorporates automated matching, internal energy (used in flexible docking), scoring function hierarchy, and new minimizer termination criteria.

The coordinates for the crystal structure of a fragment of human ACE2,²⁶ PDB code 1RL4, was used in the molecular docking calculations. To prepare the site for docking, all water molecules were removed. Protonation of receptor residues was performed in Sybyl (Tripos, St. Louis, Mo). The structure was explored using sets of spheres to describe potential binding pockets. The number of orientations per molecule was 100. Intermolecular AMBER energy scoring (van der Waals + columbic), contact scoring, and bump filtering were implemented in DOCK5.1.0. SETOR and GRASP were used to generate molecular graphic images.

ACE2 Enzyme Activity Assay

Human recombinant ACE2 (R&D Systems, Minneapolis, Minn) was used to measure the effect of test compounds on ACE2 activity, essentially as described previously.^{22,25} The assay is based on the use of the fluorogenic peptide substrate VI [7Mca-Y-V-A-D-A-P-K(Knp)-OH; R&D Systems]. ACE2 removes the c-terminal dinitrophenyl moiety that quenches the inherent fluorescence of the 7-methoxycoumain group, resulting in an increase in fluorescence in the presence of ACE2 activity at excitation and emission spectra of 328 nm and 392 nm, respectively. Samples containing ACE2 (1 nM) and varying concentrations of test compounds were incubated with 50 $\mu\text{mol/L}$ the fluorogenic peptide substrate VI in a final volume of 100 $\mu\text{mol/L}$ with reaction buffer (1 mol/L NaCl, 75 mmol/L Tris, 0.5 $\mu\text{mol/L}$ ZnCl₂, pH 7.4). Samples were read in triplicate every 36 seconds for 10 minutes immediately after the addition of fluorogenic peptide substrate VI using a Spectra Max Gemini EM Fluorescence Reader (Molecular Devices).

SARS Fusion Assay

A fusion assay was used to measure the ability of compounds to inhibit SARS-ACE2 interaction as described previously.¹⁸ Fusion inhibitors were tested by β -galactosidase (β -gal) reporter gene-based cell-cell fusion assay; 293T cells (7×10^6) were plated in T75 flasks 1 day before transfection. The next day, cells were transfected with pSectag2B-S and pCDNA3-ACE2 using the Polyfect transfection kit (Qiagen). Four hours after transfection, cells transfected with S constructs were infected with T7 polymerase-expressing vaccinia virus VTF7.3, and cells transfected with ACE2 constructs were infected with β -gal-encoding vaccinia virus (VCB21R). Two hours after infection, cells were supplemented with fresh medium and transferred to 30°C for overnight incubation. The next day, S glycoprotein-expressing cells and ACE2-expressing cells were collected by brief trypsin digestion. ACE2-expressing cells were pre-incubated with ACE2-binding small molecules at the indicated concentration for 30 minutes at room temperature, and then mixed in 1:1 ratio with S glycoprotein-expressing cells and incubated at 37°C. Additionally, a positive control peptide (N-terminus-GDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELG-C terminus) directed against one of the heptad repeats in the SARS-CoV S pre-incubated with an equivalent batch of ACE2-expressing cells. Three hours later, cells were lysed by adding NP-40 to a final concentration of 0.5%. Cell lysates (50 μL) were mixed with equal volume of chlorophenol red- β -D galactopyranoside (CPRG) substrate (Roche, Mannheim, Germany), and OD595 was measured 1 hour later.

Results

We used a novel, rapid, and economical structure-based approach in which molecular docking is combined with

Top Compounds Identified by Molecular Docking and Their Capacity to Inhibit ACE2 Activity

Compound Name/Chemical Formula	DOCK Score (kcal/mol)	IC ₅₀	K _i
N-(2-Aminoethyl)-1-aziridineethanamine			
C ₆ H ₁₅ N ₃	-23.7	57 ± 7 μmol/L	459 μmol/L
C ₈ H ₁₀ N ₂ O ₂	-19.6	2.5 mmol/L	6.3 mmol/L
C ₁₁ H ₁₄ ClN	-19.2	5 mmol/L	ND
3-Phenylhydrazonopentane-2,4-dione			
C ₁₁ H ₁₂ N ₂ O ₂	-18.4	1 mmol/L	ND
C ₁₁ H ₁₃ N ₅ O ₃ S	-15.9	4.5 mmol/L	ND
5-Methyl-8-quinolinol, Tiliquinol			
C ₁₀ H ₉ NO	-15.2	1.99 mmol/L	4.5 mmol/L
1-Chloro-2-ethylbenzene			
C ₈ H ₉ Cl	-14.3	3 mmol/L	ND
C ₁₀ H ₉ NO	-13.4	2.5 mmol/L	ND
C ₁₂ H ₁₅ ClO ₃	-13.3	3 mmol/L	ND

ND indicates not determined.

functional testing using a large library of small molecules (molecular weight <500) available by request through NCI/DTP. Approximately 140 000 compounds with known 3-dimensional structures were positioned into the active site of ACE2 in the closed conformation *in silico* (Figure 1A). This approach combined resources available through the NCI/DTP (atomic coordinates and small molecules) with improved molecular docking and scoring algorithms imposed in DOCK.²⁶ Each compound was positioned in the active site of ACE2 in 100 orientations, and predicted binding energies of interaction between each compound and ACE2 were calculated. Compounds with the highest overall energy scores were obtained and assayed for their ability to inhibit ACE2 enzyme activity. Table lists top scoring compounds as predicted by the molecular docking program DOCK 5.1.0. The compound with the highest energy score (vdw + electrostatic potential) based on molecular docking is N-(2-aminoethyl)-1-aziridine-ethanamine (NAAE) (Figures 1B and 2A and Table), with an energy score of -23.7 kcal per mol. Each of these top-scoring compounds was tested in ACE2 enzyme assays to determine their inhibitory capacities. NAAE, which showed the highest DOCK score, also demonstrated highest potency in enzyme inhibition. A dose-dependent inhibition of ACE2 activity was observed (Figure 2B) with an IC₅₀ of 57 ± 7 μmol/L and a K_i of 459 μmol/L. Because lead compounds identified by high throughput screening and molecular docking approaches are typically selected if the compounds inhibit activity with IC₅₀ values of <100 μmol/L, NAAE represents a novel lead compound for ACE2 modulation. These data support the structure-based strategy in which the closed conformation of ACE2 provided the basis for the development of novel small molecule ACE2 modulators.

Next, we tested the hypothesis that ACE2 inhibitors block SARS-CoV infection by modulating SARS-CoV S-glycoprotein-mediated membrane fusion. We determined the ability of NAAE to inhibit SARS-CoV S-glycoprotein interactions and compared it with a well-characterized peptide-based

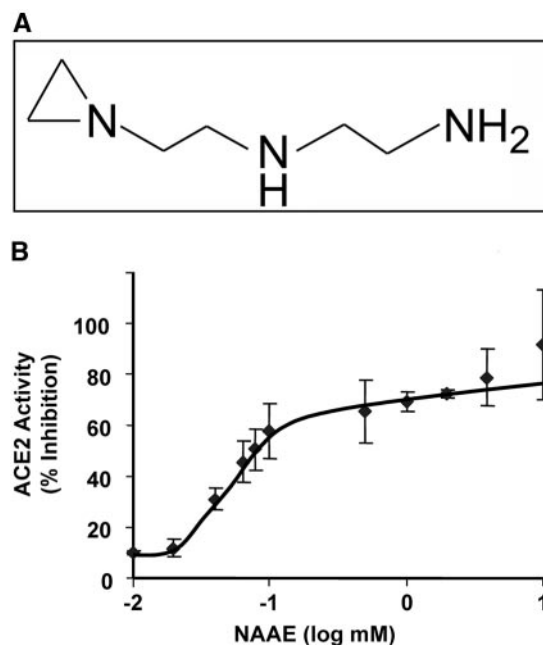


Figure 2. Inhibition of catalytic function of ACE2 by N-(2-Aminoethyl)-1-aziridineethanamine (NAAE). A, The 2-dimensional structure of NAAE is shown. B, Dose-response of ACE2 inhibition by NAAE is shown. ACE2 activity was measured at indicated concentrations of NAAE as described in the Methods.

inhibitor of the ACE2/SARS-CoV S-glycoprotein interactions.¹⁸ As shown in Figure 3, NAAE blocks membrane fusion with an IC₅₀ in the micromolar concentration range. This degree of inhibition was comparable to the ability of NAAE to inhibit ACE2 enzyme activity. Because a large hinge-bending motion is presumed to accompany enzyme inhibition to transform the open conformation into the closed conformation, a mechanism for inhibition of SARS infection by NAAE is inferred from structural and functional data. These data suggest that the SARS-CoV S-glycoprotein binding residues of ACE2 are shifted on NAAE interaction to a sufficient degree that ACE2 binding to SARS-CoV S-glycoprotein is inhibited.

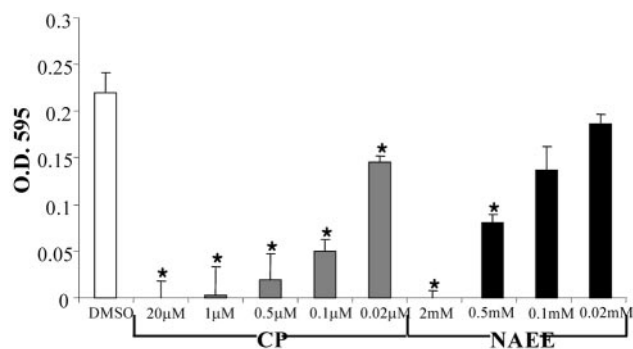


Figure 3. NAAE inhibits SARS S-glycoprotein-mediated cell fusion. 293T cells expressing ACE2 were incubated with NAAE at the indicated concentrations or with a control peptide directed against one of the heptad repeats in the SARS-CoV glycoprotein. Thirty minutes later, these cells were mixed with SARS S1-expressing cells. β-galactosidase activity was used to monitor fusion. DMSO (the solvent for NAAE) and a peptide-based inhibitor were assayed as negative control and positive controls. *P < 0.05 vs DMSO.

Discussion

In this study, we used a structure-based approach to identify a novel inhibitor of human ACE2, NAAE. The ability of NAAE to modulate ACE2 activity and prevent SARS spike protein-mediated cell fusion suggests that it serves as a potentially valuable lead compound for optimization into therapeutic modulators of hypertension and SARS-CoV infectivity. A significant component of this study is the successful use of a novel, rapid, and economical technique to identify lead compounds. This approach builds on well-established techniques in molecular docking by using the atomic coordinates of $\approx 140\,000$ small molecules (all of which represent unique structural diversity) that are available through the NCI/DTP. This UNIX-based molecular docking approach, combined with the availability of this large well-characterized database of small molecules, allowed for a rapid turnaround time to conduct molecular docking and to obtain and test the top scoring compounds to identify NAAE as an ACE2 inhibitor capable of blocking SARS-CoV spike protein-mediated cell fusion. Because high-resolution structural information is becoming available for a large number of targets through structural genomics and other efforts, this study shows that this rapid structure-based method may be a tractable approach to lead development in other biological systems.

This study is significant in the field of hypertension because it provides a novel small molecule, NAAE, capable of modulating ACE2. Because there is a reputable paradigm for optimization of small molecule lead compounds into therapeutic agents, variants of NAAE with chemical groups placed at sites that interact with specific ACE2 residues are expected to act as ACE2 antagonists and agonists. For example, we are currently considering a hypothesis that a compound could lodge into the active site and may force the ACE2 substrate to be more accessible for catalysis. Thus, the strategy could be used for screening and development of ACE2 agonists that would have beneficial use in controlling hypertension.

Perspectives

This study demonstrates that UNIX-based molecular docking approach in combination with a well-characterized database is potent technology to screen and identify ACE2 inhibitors, and activators in particular, and other compounds in general with therapeutic potential for other diseases. This will lead to the development of highly effective drugs for the management of hypertension and SARS infection.

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