Angiotensin-converting enzyme 2 (ACE2), a recently identified human homolog of ACE, is a novel metallocarboxypeptidase with specificity, tissue distribution, and function distinct from those of ACE. ACE2 may play a unique role in the renin-angiotensin system and mediate cardiovascular and renal function. Here we report the discovery of ACE2 peptide inhibitors through selection of constrained peptide libraries displayed on phage. Six constrained peptide libraries were constructed and selected against FLAG-tagged ACE2 target. ACE2 peptide binders were identified and classified into five groups, based on their effects on ACE2 activity. Peptides from the first three classes exhibited none, weak, or moderate inhibition on ACE2. Peptides from the fourth class exhibited strong inhibition, with equilibrium inhibition constants \( K_i \) values from 0.38 to 1.7 \( \mu M \). Peptides from the fifth class exhibited very strong inhibition, with \( K_i \) values <0.14 \( \mu M \). The most potent inhibitor, DX800, had a \( K_i \) of 2.8 nM.

Steady-state enzyme kinetic analysis showed that these potent ACE2 inhibitors exhibited a mixed competitive and non-competitive type of inhibition. They were not hydrolyzed by ACE2. Furthermore, they did not inhibit ACE activity, and thus were specific to ACE2. Finally, they also inhibited ACE2 activity toward its natural substrate angiotensin I, suggesting that they would be functional in vivo. As novel ACE2-specific peptide inhibitors, they should be useful in elucidation of ACE2 function, thus contributing to our better understanding of the biology of cardiovascular regulation. Our results also demonstrate that library selection by phage display technology can be a rapid and efficient way to discover potent and specific protease inhibitors.

One major control mechanism for blood pressure homeostasis is the renin-angiotensin system, in which angiotensin-converting enzyme (ACE)\(^1\) is a vital player. ACE, a zinc metallopeptidase, promotes blood pressure elevation at least in part by cleaving the inactive angiotensin I (Ang I) to the vasoconstrictor Ang II (1) and inactivating the vasodilator bradykinin by cleavage (2). Its role in regulating blood pressure and renal function is underscored by the effective clinical use of ACE inhibitors in the treatment of hypertension and other cardiovascular diseases.

ACE2 is a recently identified human homolog of ACE (3, 4). It contains a single zinc-binding catalytic domain, which is 42% identical to the human ACE active domain. Genomic structure comparison suggests that ACE2 and ACE genes arose by duplication of a common ancestor (3). Although both ACE2 and ACE are zinc metallopeptidases and angiotensin-converting enzymes with a membrane-associated and a secreted form, many differences exist between these two enzymes (for reviews, see Refs. 5 and 6). First, they are different in enzymatic activity; ACE2 is a carboxypeptidase, removing the C-terminal residue from the decapeptide Ang I to form angiotensin-(1–9) (Ang-(1–9)) (3, 4), whereas ACE is a dipeptidase, cleaving the C-terminal dipeptide from Ang I to form the octapeptide Ang II. Second, ACE2 and ACE have different substrate specificities; ACE2 cleaves Ang I, Ang II, apelin-13, apelin-36, dynorphin A-(1–13), and des-Arg bradykinin (3, 7); ACE cleaves Ang I, Ang-(1–9), bradykinin, and many other bioactive peptides such as substance P, neurotensin, and enkephalin (8). Another difference between these two enzymes is the inhibitor specificity; ACE2 cannot be inhibited by ACE inhibitors (3, 4). Finally, a difference in tissue expression has been observed; ACE2 is primarily expressed in the heart, kidney and testis, whereas ACE is more ubiquitously expressed in tissues including heart, lung, kidney, colon, small intestine, ovary, testis, prostate, liver, skeletal muscle, pancreas, and thyroid (3, 4).

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\(^3\) The abbreviations used are: ACE, angiotensin-converting enzyme; Ang I, angiotensin I; Ang II, angiotensin II; ACE2, angiotensin-converting enzyme homolog; Ang-(1–9), angiotensin-(1–9); Ang-(1–7), angiotensin-(1–7); varDNA, variegated DNA; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; Me2SO, dimethyl sulfoxide.
cleave bradykinin. However, ACE2 cleaves and inactivates des-Arg bradykinin, a local vasodilator functioning through binding to the B1 receptor expressed when inflammation or tissue damage occurs (15). In contrast, bradykinin, cleaved and inactivated by ACE, functions as a systemic vasodilator through binding to the B2 receptor (15). Based on the potential in vivo functions of Ang-(1–9) and des-Arg bradykinin, it is tempting to speculate that ACE2 plays a role in the regulation of vasomotor tone and blood pressure at least in part through cleavage of Ang I and des-Arg bradykinin. However, a recent knock-out mice study demonstrates that disruption of ACE2 in mice does not alter blood pressure and renal function but leads to increased levels of Ang II, up-regulation of hypoxia-induced genes, and decreased cardiac contractility that can be rescued by a second mutation causing ACE deficiency (16). Thus, ACE2 appears to be essential for regulating heart function in vivo. However, its role in blood pressure regulation remains unclear. Animal studies with specific ACE2 inhibitors should provide more information to our understanding of the physiological roles of ACE2 in cardiovascular regulation.

Here we described the discovery of novel ACE2 peptide inhibitors through selection of constrained peptides from libraries displayed on filamentous phage. We discovered very potent inhibitors through selection of constrained peptides from libraries and two linear libraries were selected using FLAG-ACE2 as bait.

**EXPERIMENTAL PROCEDURES**

*Materials—* Biotinylated anti-FLAG M2 monoclonal antibody, FLAG peptide, ACE, angiotensin I, NAD, resuzum, diaphorase, and captopril were purchased from Sigma. Horseradish peroxidase-conjugated anti-FLAG antibody-immobilized beads before selection on a Beckman Coulter Astra 20 (PBST) for 1 h and then incubated with biotinylated anti-FLAG antibody-immobilized beads before selection on the target. The depleted libraries were incubated with 6 μg of FLAG-ACE2 in 300 μl of phosphate-buffered saline (PBS) for 1 h and then incubated with biotinylated anti-FLAG antibody-immobilized beads for 1 h. The beads were washed 3 times with PBST, 0.1% Tween 20 (PBST) to remove unbound phage. The bound phage were then eluted with FLAG peptide (100 μg/ml) in Tris buffer (10 mM Tris-Cl, 150 mM NaCl, pH 7.5) for 30 min. Eluted phage were amplified and underwent two more similar rounds of selection and amplification. In round 1, the six constrained peptide libraries, TN6/6, TN7/4, TN8/9, TN9/4, TN10/9, and TN12/1, were selected separately. To accelerate

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the selection procedures, in the subsequent rounds of selection, these six libraries were combined into two pools: pool A composed of TN6/6, TN7/4, and TN8/9, and pool B composed of TN9/4, TN10/9, and TN12/1. The two linear peptide libraries, Ph.D.-7 and Ph.D.-12, were combined as Ph.D.-7/12, from the beginning of selection.

Screening for ACE2-binding by Phage ELISA

Sequence cluster analysis of ACE2 peptide binders. Peptide sequences from positive ELISA isolates were aligned to search for motif sequences. Shown here are peptide sequences comprising 10 motifs. Representative peptide sequences are listed in each motif. The motif sequences in each cluster are in boldface type. The template sequence is shown at the top of each cluster. The DX numbers at the right sides of the sequences are the names of the peptides synthesized, and the annotation within parentheses indicates the inhibitory activity of each peptide, same as described in Table II: --, no inhibition; +, weak inhibition; ++, moderate inhibition; ++++, strong inhibition; ++++, very strong inhibition.

Novel ACE2 Peptide Inhibitors
Peptide Library Peptide Sequence Inhibition IC50 Kd h_m h_e Kd

| Peptide | Library | Sequence | Inhibition | IC50 | Kd | h_m | h_e | Kd |
|---------|---------|----------|------------|------|----|-----|-----|-----|
| DX500   | TN6/6   | As-GSNRECHALFCDMFAPGEGGG-NH2 | +          | ND   | ND | ND  | ND  | ND  |
| DX501   | TN6/6   | As-GSSPCTRALFCDMFAPGEGGG-NH2 | +          | ND   | ND | ND  | ND  | ND  |
| DX504   | TN6/6   | As-GSSGMCALPCMFAPGEGGG-NH2 | +          | ND   | ND | ND  | ND  | ND  |
| DX507   | TN10/9  | As-GNDDYCTVFPTALCFLDFAPEGGG-NH2 | +          | ND   | ND | ND  | ND  | ND  |
| DX514   | TN10/9  | As-GSNQCGDIALFCDMFAPGEGGG-NH2 | +          | ND   | ND | ND  | ND  | ND  |
| DX508   | TN10/9  | As-GYDNCNLGLALNFCDFAPGEGGG-NH2 | +          | ND   | ND | ND  | ND  | ND  |
| DX510   | TN12/1  | As-GGDDDCHFAPGEGGG-NH2 | +          | ND   | ND | ND  | ND  | ND  |
| DX511   | TN12/1  | As-GDFPCEWDGPLTEGCGPGGG-GH2 | +          | ND   | ND | ND  | ND  | ND  |
| DX524   | TN6/6   | As-GRIGCDSRCWNPWAPGEGGG-NH2 | + + +      | 600  | 540 | ND  | ND  | ND  |
| DX525   | TN6/6   | As-GRGFCDRSSCPFPAPGEGGG-NH2 | + + + +     | 1.0 x 10^3 | 1.7 x 10^3 | ND  | ND  | ND  |
| DX527   | TN1/4   | As-AWWLFCPWEWDGDCDEKFGGG-NH2 | +          | ND   | ND | ND  | ND  | ND  |
| DX528   | TN1/4   | As-AGYWFCDFQDQEDMTEGCGGG-NH2 | -          | ND   | ND | ND  | ND  | ND  |
| DX529   | TN1/4   | As-AGYVECHWAPMMCKHGTEGCGGG-NH2 | + + +      | 400  | 380 | ND  | ND  | ND  |
| DX530   | TN1/4   | As-AQKKECGFPGYPCPLFWGCGG-GH2 | +          | 3.0 x 10^4 | ND  | ND  | ND  | ND  |
| DX531   | TN1/4   | As-AQGDCWCTWNCPFQFS | +          | 500  | 540 | ND  | ND  | ND  |
| DX512   | TN1/2   | As-GDRLHCKPFRQPWSMKDCHPDGGG-NH2 | + + +      | 60   | 139 | 1.4 x 10^4 | 1.4 x 10^-2 | 96  |
| DX513   | TN1/2   | As-GDLACVRPGDPWACTLDGPDGGG-NH2 | + + + +     | 90   | 126 | 2.4 x 10^4 | 4.0 x 10^-3 | 170 |
| DX509   | TN1/2   | As-DRYCLFLRQDKPWCKFWPDGGG-NH2 | + + + +     | 114  | 46.5 | 2.3 x 10^3 | 1.1 x 10^-2 | 48.6 |
| DX600   | TN1/2   | As-GDYSCHLPFWKCPYKPDGGG-NH2 | + + + +     | 151  | 56.8 | 4.5 x 10^-3 | 5.6 x 10^-3 | 56.3 |
| DX601   | TN1/2   | As-GGTCSPCRMPSWFWPFCRDLGPDGGG-NH2 | + + + +     | 967  | 1275 | 121.2 | 1.0 x 10^5 | 7.7 x 10^-3 | 74.4 |

* a Sequence; Ac- denotes N-terminal acetylation; -NH2 denotes C-terminal amidation.
* b Inhibition, +, no inhibition on ACE activity at concentrations up to 100 μM; +, weak inhibition (20-60% inhibition at 100 μM); ++, moderate inhibition (at least 80% inhibition at 100 μM, with IC50 values of about 30 μM); +++, strong inhibition (about 99% inhibition at 100 μM, with IC50 values of 0.4-1 μM); ++++, very strong inhibition (complete inhibition at 100 μM, with IC50 values <10 nM).
* c IC50 determined by inhibition assays with 20 nM ACE2, peptides ranging from 0 to 100 μM and 50 μM substrate M-2195.
* d M-2195 ranged from 14 to 50 μM. The inhibition assay of ACE was carried out essentially in the same way as that of ACE2 with the same substrate M-2195.

ACE2 Enzyme Assays Using Natural Substrate—ACE2 activity toward its natural substrate was measured by an assay based on a spectrophotometric assay system. ACE2 hydrolyzes Ang I (NH2-DRVYIHPFLHCOOH) to produce Ang-(1–9) (NH2-DVYIHPFH- COOH) and leucine. The released leucine can then be monitored by the activity of leucine dehydrogenase with concomitant conversion of NAD+ to NADH. The production of NADH is coupled to the diaphorase-catalyzed reduction of resazurin to resorufin, which can be monitored on a fluorescence reader.

In the inhibition assay, ACE2 was incubated with the peptide inhibitor or ACE inhibitor for 10 min at room temperature in reaction buffer consisting of 100 mM Tris, pH 8, 0.01% Tween, 4 mM NAD, 25 μM resazurin, 0.1 unit/ml leucine dehydrogenase, and 0.1 unit/ml diaphorase. The amount of MeSO was kept the same in each sample. The substrate M-2195 was added to achieve a final concentration of 50 μM, and the plates were read immediately on a SpectraMax Gemini fluorescence spectrophotometer at an excitation wavelength of 565 nm and an emission wavelength of 585 nm. Fluorescence was monitored at 1-min intervals for 2 h. The IC50 and Kd values were determined similarly as described for the assay with the synthetic substrate.
Novel ACE2 Peptide Inhibitors

**RESULTS**

Selection of ACE2 Peptide Binders—Six constrained loop peptide libraries, TN6/6, TN7/4, TN8/9, TN9/4, TN10/9, and TN12/1, and two linear peptide libraries Ph.D.-7 and Ph.D.-12, were used for selection against FLAG-ACE2 target. After incubation with libraries in solution, the target was immobilized to streptavidin-coated magnetic beads via biotinylated anti-FLAG antibody. The bound phage were eluted with FLAG peptide. After three rounds of selection, the fraction of input, which was calculated as the total amount of output phage divided by the total amount of input phage, increased from 6 to 10\$^{\text{a}}$ at the first round to 10\$^{\text{b}}$ to 10\$^{\text{c}}$ by the third round.

To identify positive phage binders, the eluted phage from the third round of selection were screened by ELISA. The ELISA positive isolates (n = 613) from constrained libraries were sequenced. The amino acid sequences of the encoded peptides were analyzed for shared motifs and, as shown in Fig. 1, 10 major motifs were found. Some clusters were found in multiple libraries, some were found exclusively in one library. For example, the ALFCVD(E)/F and RXXRXD-SRC motifs were found in both TN6/6 and TN10/9 libraries (Fig. 1, A and D); the (F/Y)(C/F/L/I)(D/E)/F motif, similar to the ALFCVD(E)/F motif, was found in TN8/9 and TN10/9 libraries (Fig. 1B); the DCTXTWXXPC motif was found in TN7/4 and TN8/9 libraries (Fig. 1F); and the CF(D/E)W(E/D) motif was identified in the TN7/4, TN8/9, and TN12/1 libraries (Fig. 1F). Whereas the (D/E)/C(E/D)WXX(F/W) and CXPXRXXPXXC motifs were found only in the TN12/1 library (Fig. 1, C and J), the CXTXDCV motif in the TN6/6 library (Fig. 1E) and the (Y/W)EXCH(W/Y)XP and KECFGXYYCLXW motifs were found in the TN8/9 library (Fig. 1, G and H). Based on these consensus motifs and the number of isolates occurring per sequence, 23 peptides representing these 10 motifs were synthesized.

Screening of Peptide Binders for ACE2 Inhibitors—The 23 peptides synthesized as ACE2 peptide binders were further screened for ACE2 inhibitors by assays using fluorogenic substrate M-2195. For initial screening of inhibitors, ACE2 (20 nM) was incubated with each peptide at 100 \text{ nM} prior to the addition of 50 \text{ nM} substrate. Based on their effects on ACE2 enzyme activity, the peptides were classified into 5 groups with none (–), weak (+), moderate (++), strong (+++), and very strong (++++) inhibition, respectively.
Novel ACE2 Peptide Inhibitors

FIG. 4. DX600 and DX512 peptides were stable ACE2 inhibitors. DX600 (A) and DX512 (B), at both low and high concentrations, were each incubated with ACE2 (20 nM) for 10 min or 20 h at room temperature prior to the addition of M-2195. The relative ACE2 activity was plotted against the peptide concentration. Open square, 10 min; filled square, 20 h.

FIG. 5. DX600 was a specific inhibitor to ACE2. A, effects of DX600 on ACE2 and ACE activity. Increasing concentrations of DX600 peptides were incubated with ACE2 (20 nM) or ACE (7.5 nM) prior to the addition of the substrate M-2195 (50 μM). B, effects of ACE peptide inhibitor teprotide on ACE2 and ACE activity. Increasing concentrations of teprotide were incubated with ACE2 (20 nM) or ACE (7.5 nM) prior to the addition of the substrate M-2195. The relative enzymatic activity was plotted against the peptide concentration. ACE2, filled circle; ACE, open circle.

Inhibitor with a Ki of 2.8 nM (Table II and Fig. 2). Kinetic analyses of DX600 by Dixon plots (Fig. 2) indicated a mixed inhibition pattern consisting of competitive and non-competitive components, with the maximum velocity (Vmax) reduced, and the apparent Michaelis constant (Km) increased. The other very strong and strong inhibitors also showed a similar inhibition pattern (data not shown).

Binding Affinity of ACE2 Inhibitors—The binding affinity of the very strong peptide inhibitors was measured by BIAcore as described under “Experimental Procedures.” Sensorgrams were analyzed using the simultaneous association and dissociation 1:1 Langmuir fitting model. For all measurements, the χ value, the standard statistical measure of the closeness of the fit, is less than 0.4 (data not shown), indicating a close fit. Representative sensorgrams of DX600 and DX512 are shown in Fig. 3. The on-rates (k_on), off-rates (k_off), and Kd values of all six very strong inhibitors (DX512, DX513, DX599, DX600, DX601, and DX602) are listed in Table II. The k_on values of all six inhibitors were in the order of $10^4$ to $10^5$ M$^{-1}$ s$^{-1}$; the k_off values were in the order of $10^{-2}$ to $10^{-3}$ s$^{-1}$, and the Kd values (Kd = k_on/k_off) ranged from 10.8 to 170 nM (Table II). DX600 had the slowest off-rate (4.6 × 10$^{-4}$) and the lowest Kd (10.8 nM), which was consistent with it being the most potent inhibitor with a Ki of 2.8 nM. The Kd values of the other inhibitors were close to their respective Ki values (Table II).

Stability of ACE2 Inhibitors—To test the stability of ACE2 peptide inhibitors, DX600 and DX512 peptides were individually incubated with ACE2 (20 nM) for 10 min or 20 h at room temperature prior to the addition of M-2195. The results showed that incubation of ACE2 with DX600 or DX512 for up to 20 h did not affect the inhibitory activities of these peptides.
Novel ACE2 Peptide Inhibitors

Inhibition on ACE2 Activity toward Its Natural Substrate Ang I—The inhibitory activities of identified peptide inhibitors were determined based on ACE2 assays using synthetic fluorogenic substrate. Such inhibition on ACE2 activity toward the synthetic substrate may not necessarily correspond to the inhibition on ACE2 toward its natural substrate. Thus, to determine whether the identified peptide inhibitors also inhibit ACE2 toward its natural substrate Ang I, we developed an ACE2 assay using Ang I as the substrate based on a spectrofluorometric enzyme-coupled system, which is superior to high pressure liquid chromatography-based assays that are discontinuous and time consuming. This assay is based on the cleavage of the C-terminal leucine from Ang I substrate after ACE2 catalysis. The leucine formation is then monitored by the activity of leucine dehydrogenase with concomitant conversion of NAD$^+$ to NADH. The production of NADH is coupled to the diaphorase-catalyzed reduction of resazurine to resorufin, which can be monitored on a fluorescence reader. Before its use in ACE2 inhibition studies, experiments were undertaken to verify that the observed inhibition was not due to the other two enzymes (leucine dehydrogenase and diaphorase) in the assay (data not shown).

Determination of DX600 Peptide Using ACE2 Assays with the Natural Substrate Ang I—DX600, at concentrations ranging from 0 to 12.5 nM, was preincubated with 7 nM ACE2. The substrate Ang I was added at concentrations ranging from 40 to 160 μM. A, Dixon plot. Filled squares, 40 μM substrate (Ang I); open squares, 60 μM; filled triangles, 80 μM; ×, 100 μM; open triangles, 120 μM; and filled circles, 160 μM. B, Dixon secondary plot. The slope at each substrate concentration in A was plotted against the reciprocal substrate concentration. Data were fitted to a linear regression ($y = mx + b$, where $m = K_i/(K_i \times V_{max}) = 23.041$, $b = -0.039$). $K_i$ (145.52 μM) and $V_{max}$ (2.26 farads/s) were obtained by a fit of the data in the absence of inhibitor to the Michaelis-Menten equation by nonlinear regression analysis. $K_i$ was calculated to be 2.8 nM from the equation $K_i = K_i(V_{max} \times m)/17$.

Specificity of ACE2 Inhibitors—To determine whether the identified peptide inhibitors were specific to ACE2, we analyzed the effect of DX600 on the enzyme activity of the other ACE. In this assay, increasing concentrations of DX600 peptide were incubated with ACE2 (20 nM) or ACE (7.5 nM) prior to the addition of the substrate M-2195. DX600, which greatly inhibited ACE2 activity with a $K_i$ of 2.8 nM, did not inhibit ACE activity at concentrations up to 100 μM (Fig. 5A). Similarly, other ACE2 inhibitors including DX512 and DX513 did not inhibit ACE (data not shown). These results indicated that DX600 and other peptide inhibitors were specific ACE2 inhibitors.

Likewise, the effect of an ACE inhibitor on ACE2 activity was also tested. As shown in Fig. 5B, teprotide, the ACE peptide inhibitor, inhibits the activity of ACE at 7 nM with an IC$_{50}$ of about 38 nM but did not inhibit ACE2 activity at concentrations up to 100 μM. Captopril, another ACE inhibitor, also showed no inhibition on ACE2 activity (data not shown), which was consistent with reports from others (3).

Kinetic Analysis of DX600 showed a $K_i$ of 2.8 nM and a mixed inhibition pattern consisting of competitive and non-competitive components as demonstrated by Dixon plot (Fig. 6), which were similar to the results from ACE2 assays using the syn-
thetic substrate. These results indicated that DX600 inhibited ACE2 activity toward its natural substrate with the same potency as inhibition on ACE2 toward the synthetic substrate. Another peptide inhibitor, DX512, was also tested by this assay. As for DX600, the $K_e$ and inhibition pattern for DX512 were similar to those when using the synthetic substrate (data not shown).

Because ACE2 and ACE share the same natural substrate Ang I, we wanted to know if ACE2 inhibitors may also inhibit ACE2 activity in this assay. Two ACE inhibitors, teprotide and captopril, were tested for inhibition on ACE2 activity toward Ang I. As shown in Fig. 7, the $IC_{50}$ of DX600 was $\approx 8$ nM, whereas the $IC_{50}$ of the ACE peptide inhibitor teprotide was over 700 $\mu$M, and the $IC_{50}$ of the small compound inhibitor captopril was over 1 $\mu$m. This further indicated that DX600 was a specific ACE2 inhibitor.

**DISCUSSION**

Here we report novel ACE2 specific peptide inhibitors discovered through selection of peptides from libraries displayed on M13 filamentous phage. Six constrained peptide libraries were constructed and used for selection against FLAG-ACE2 target. In parallel, two commercially available linear peptide libraries were also used for selection. Surprisingly, no ACE2-binding phage were identified from the linear libraries; all ACE2 binders were obtained from the constrained loop libraries. Sequence analysis of positive phage isolates identified 10 motifs. The 23 representative peptides derived from these motifs showed a range of inhibitory properties from none to very potent. Peptides derived from half of the motifs exhibited either no or weak inhibition, whereas peptides from the other half of the motifs exhibited moderate, strong, or very strong inhibition, showing that selection of peptide libraries for binders to enzymes by phage display technology is a rapid and efficient way to discover enzyme inhibitors.

Interestingly, the most abundantly isolated motifs such as \textit{ALFCV(D/E)F, (F/Y)C(F/L/I)(D/E)F, and CF(D/E)W(E/D)} were very poor inhibitors. In contrast, the highly inhibitory sequences such as those from the \textit{CXPRLXXPWXXC} motif were each seen as unique sequences. Peptides derived from the same motif are likely to bind ACE2 at the same site and thus share similar binding and inhibitory properties. However, due to the slight sequence variations between consensus or non-consensus residues, peptides from the same motif could show either no or weak inhibition. Such examples included peptides derived from the \textit{ALFCV(D/E)F, (F/Y)C(F/L/I)(D/E)F, and (D/E)C(E/D)WXX(F/W)} motifs. Similarly, the extent of inhibition among peptides from the same inhibitory motif could vary significantly. This is well illustrated by the peptides from the \textit{CXPRLXXPWXXC} motif. Although all of the peptides derived from this motif showed very strong inhibition, the inhibitory potency varied, with $K_e$ values ranging from 2.8 to 139 nM. Thus, in order to find the best inhibitor, it is necessary to synthesize and test many peptides from an inhibitory motif.

The most potent ACE2 inhibitor was derived from the \textit{CXPRLXXPWXXC} motif. Interestingly, a recent study of ACE2 binding properties of peptides from an inhibitory motif \textit{CXPRLXXPWXXC} shows resemblance to the substrate consensus sequence, three key differences exist. First, there is a conserved basic amino acid arginine lying between the two prolines in the inhibitory motif but not in the substrate consensus. Second, the space between two prolines is different: four residues in the inhibitory motif and one to three residues in the substrate consensus. Third, the inhibitory sequence is constrained by disulfides whereas the substrate is not. Probably because of these differences, peptides from this inhibitory motif were stable inhibitors and not hydrolyzed by ACE2, indicating that they were not better ACE2 substrates than the assay substrate M-2195 but true inhibitors.

As expected, these peptides also inhibited ACE2 enzymatic activity toward its natural substrate, Ang I. For DX600 and DX512, the $K_e$ values determined by using Ang I were similar to those determined by using M-2195. Thus, these peptides were functional in inhibiting ACE2 toward its natural substrate. However, they were not inhibitory on ACE, which shares great sequence homology and the same natural substrate Ang I with ACE2. Likewise, the ACE inhibitors such as the peptide inhibitor teprotide and the $\alpha$-benzylsuccinic acid derivative captopril specifically inhibited ACE but not ACE2. Thus, although ACE2 and ACE share a homologous catalytic domain, they are structurally distinct.

Kinetic analyses of the strong and very strong ACE2 inhibitors by Dixon plots showed that these inhibitors exhibited a mixed competitive and non-competitive type of inhibition, with $V_{\text{max}}$ reduced and apparent $K_e$ increased. These data suggest that the inhibitors bind to a site adjacent to the active site in a manner that interferes with substrate binding. Interestingly, ACE inhibitors such as captopril also exhibit a mixed competitive and non-competitive type of inhibition toward ACE (18, 19).

The most potent ACE2 inhibitor (DX600) identified had a $K_e$ of 2.8 nM. It is remarkable that a peptide inhibitor with a $K_e$ in the low single digit nanomolar range can be extracted from the selection of first generation peptide libraries, demonstrating the utility of such well constrained peptide libraries for the rapid identification of high affinity enzyme inhibitors. If more potent inhibitors with $K_e$ values at subnanomolar concentrations are needed, affinity maturation by peptide optimization through soft randomization can be conducted (20).

Although small molecule ACE2 inhibitors have been recently synthesized (21), the peptides discovered here are the first ACE2-specific peptide inhibitors. These inhibitors should be useful for \textit{in vivo} studies to elucidate ACE2 function. In fact, one of the inhibitors, DX512, which is the first one synthesized and tested to be a very strong inhibitor, has been studied in spontaneously hypertensive rats. Upon intravenous bolus injection in the awake rats, the peptide inhibitor DX512, but not the control peptide DX510 (with no inhibitory effect on ACE2), caused a dose-dependent depressor response characterized by an initial transient fall in mean arterial pressure lasting about 1–2 min at the lower doses and about 6 min in duration at the 3 mg/kg dose level, with the maximal average depressor response at 70.5 ± 4.6 mm Hg from an average mean arterial pressure of 155 ± 10 mm Hg. The depressor response was also accompanied by transient tachycardia. The \textit{in vivo} demonstration of the antihypertensive effect of the ACE2 inhibitor is not consistent with the recent findings from the knock-out mice study showing that disruption of ACE2 in mice does not alter blood pressure and renal function (16). Further investigation including inhibition studies with ACE2-specific inhibitors will be needed to elucidate the physiological roles of ACE2 in blood pressure mediation.

In summary, potent ACE2 peptide inhibitors with the lowest $K_e$ in the low single digit nanomolar range were discovered by selecting constrained peptide libraries. These inhibitors exhibited a mixed competitive and non-competitive type of inhibition. They were stable inhibitors not hydrolyzed by ACE2 and were specific to ACE2. These specific ACE2 inhibitors can be
used in \textit{in vivo} studies to elucidate the physiological functions of ACE2 in cardiovascular regulation.

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