

Development of Domain-Selective Angiotensin I-Converting Enzyme Inhibitors

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ABSTRACT: Somatic angiotensin-converting enzyme (ACE) is an essential component of the renin-angiotensin system and consequently plays a key role in blood pressure and electrolyte homeostasis. Thus, ACE inhibitors are widely used in the treatment of cardiovascular disease, causing a decrease in the production of angiotensin II and an increase in the circulating vasodilator bradykinin. The ectodomain of ACE consists of two parts (N and C domains), each bearing an active site that differs in substrate and inhibitor specificity. Advances in the elucidation of the functional roles of these two domains and an expanded view of the renin-angiotensin system underscore the need for the next generation of domain-selective inhibitors with improved pharmacologic profiles. Moreover, recent breakthroughs in determining the crystal structure of testis ACE (identical to the C domain) and its homologue ACE2 provide new mechanistic insights into the interactions of ACE inhibitors and substrates with active site pockets. This review summarizes the structural basis and recent synthetic chemistry approaches to the development of novel domain-selective inhibitors.

KEYWORDS: angiotensin I-converting enzyme; inhibitors; domain selectivity; drug design

INTRODUCTION

Hypertension has emerged as one of the greatest public health challenges of the twenty-first century, affecting an estimated 26% of the world's adult population.¹ This figure is expected to increase to 29% by 2025, the greatest increase occurring in economically developing countries. The emergence of hypertension as a major risk factor in cardiovascular and kidney disease² has necessitated the development of novel therapeutic approaches that have centered upon the renin-angiotensin system.

Angiotensin-converting enzyme (ACE), a member of the M2 gluzincin family of metallopeptidases,^{3,4} occupies a central position in the renin-angiotensin system, where it is a key regulator of blood pressure, fluid, and electrolyte homeostasis.⁵ The

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larger somatic isoform of ACE (sACE) is a 1277 amino acid, 150–180-kDa⁶ type I transmembrane glycoprotein that is expressed in a variety of tissues including vascular endothelial cells, intestinal brush border cells, and renal proximal tubule epithelial cells.^{7–9} It is shed into the systemic circulation as a soluble ectodomain *via* cleavage at the Arg¹²⁰³–Ser¹²⁰⁴ bond of the juxtamembrane stalk by a zinc metalloproteinase.⁹ sACE comprises a C-terminal cytosolic tail, a hydrophobic membrane-anchoring domain, a juxtamembrane stalk, and an ectodomain consisting of two parts (C and N domains). The C and N domains, resulting from internal duplication of an ancestral gene, display a high level of homology.⁷ Both domains contain a characteristic HEMGH zinc-coordinating motif, crucial for the catalytic activity of sACE.^{10,11}

Alternate transcription from an internal promoter within the sACE gene has given rise to a smaller 701 amino acid, the 90–110-kDa testicular isoform of the C domain, which is restricted to male germinal cells. Testis ACE (tACE) lacks an N-terminal domain and consequently possesses only one active site per molecule.⁵ tACE is expressed in male spermatozoa under hormonal control¹² and plays an important role in fertilization.¹³ Recently, it was shown to cleave glycosylphosphatidylinositol (GPI)-anchored sperm proteins by a mechanism independent of its peptidyl dipeptidase activity, triggering sperm cell capacitation.¹⁴

Both somatic and testis ACE are heavily *N*-glycosylated with carbohydrates such as *N*-acetylglucosamine, fucose, mannose, glucose, and sialic acid, constituting 30% of their molecular weights.¹⁵ sACE contains 17 potential *N*-linked glycosylation sites,¹⁶ whereas tACE contains 7 such sites in addition to a unique 36-residue *N*-terminus, which is heavily *O*-glycosylated.¹⁷ sACE displays dual substrate specificity, acting both as an exo- and endopeptidase. For substrates like substance P and luteinizing hormone-releasing hormone (LHRH), where the C-termini are amidated, sACE acts not only as a dipeptidyl carboxypeptidase but also as an endopeptidase. Naqvi *et al.*¹⁸ recently characterized the molecular basis of the exopeptidase activity of the sACE C-domain. This involves interactions between the substrate C-terminal P₂' side-chain and the S₂' pocket of the C domain as well as carboxylate-docking interactions with residues Lys¹⁰⁸⁷ and Tyr¹⁰⁹⁶. These interactions are thought to stabilize the ground state, restricting the registration of substrates with a C-terminal carboxylate, limiting their processing to the cleavage of a C-terminal dipeptide. Other ACE substrates include acetyl-Ser-Asp-Lys-Pro and neurotensin.^{18,19} With respect to its role as a regulator of cardiovascular homeostasis, the principal physiologic substrates of ACE are angiotensin I and bradykinin. Acting as a dipeptidyl carboxypeptidase, ACE mediates the hydrolysis of the decapeptide angiotensin I to the active vasopressor octapeptide angiotensin II via removal of a C-terminal dipeptide, His-Leu. The vasodilator bradykinin is inactivated via sequential hydrolysis of two carboxy-terminal dipeptides at Pro⁷-Phe⁸ and Phe⁵-Ser⁶.^{15,20}

Both the N and C domains of membrane-bound sACE are responsible for the inactivating hydrolysis of bradykinin. However, in the soluble, circulating state, angiotensin I hydrolysis is also affected by the N domain, although it is the membrane-bound form of sACE that is primarily responsible for cardiovascular homeostasis.^{21–23}

The interaction of angiotensin II with its cognate endothelial angiotensin type-1 (AT₁) receptor results in vasoconstriction, aldosterone and vasopressin release, renal tubular sodium resorption, and decreased renal blood flow. The net effect, alongside

inactivation of the vasodilator bradykinin, is an elevation in blood pressure. Therefore, increased ACE activity is intimately linked to hypertension.

Despite the more than 60% sequence identity between the C- and N domains of sACE, each domain (both of which harbor an active site) demonstrates distinct substrate specificities. In addition to the differential hydrolysis of angiotensin I and bradykinin (discussed previously), the N domain preferentially hydrolyzes the hemoregulatory peptide *N*-acetyl-Ser-Asp-Lys-Pro (AcSDKP) as well as angiotensin 1-7.²⁴ Recently, the N domain was found to be responsible for the degradation of Alzheimer amyloid β -peptide, inhibiting its aggregation and cytotoxicity.²⁵ With respect to substrate hydrolysis, the C domain demonstrates a significantly greater chloride dependence than does the N domain, whereas the N domain is thermally more stable than the C domain.^{26,27}

EARLY DEVELOPMENT OF ACE INHIBITORS

In 1977, captopril (**1**, FIG. 1), the first nonpeptidic ACE inhibitor, was developed.²⁸⁻³⁰ Its development was initiated by the discovery of bradykinin-potentiating peptides isolated from the venom of the Brazilian snake *Bothrops jararaca*.³¹⁻³³ Some bradykinin-potentiating peptides have been shown to display domain-specific ACE inhibition.³⁴

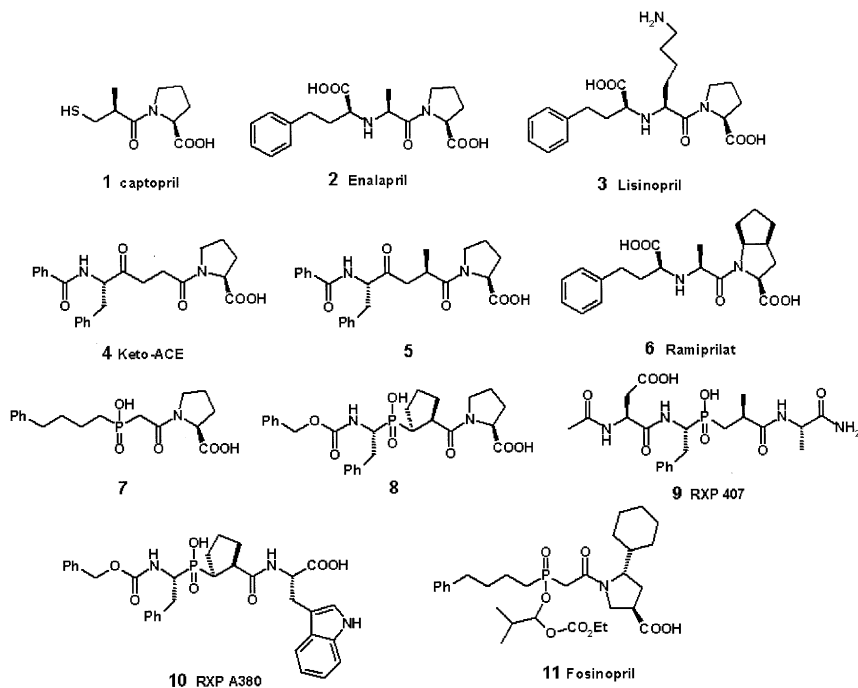


FIGURE 1. Some commonly known ACE inhibitors with different zinc-binding functionalities.

Captopril, together with other orally active ACE inhibitors such as enalapril and lisinopril (**2** and **3**, FIG. 1), are used extensively in the successful treatment and management of hypertension, congestive heart failure, myocardial infarction, and diabetic nephropathies.²⁴ However, these ACE inhibitors were designed without the knowledge of the three-dimensional structure of ACE.

The design of captopril was based on the structure of bovine pancreas carboxypeptidase A, a zinc-dependent carboxypeptidase thought to have similar catalytic mechanisms to those of ACE.^{28–30} Lisinopril and enalapril were developed based on their inhibition of thermolysin.^{35,36} These ACE inhibitors bind to somatic ACE at a 1:1 stoichiometry, indicating binding to only one of the two active sites.^{12,27}

The lack of domain specificity of these ACE inhibitors (lisinopril being only slightly more C-domain specific) may be the underlying cause of their adverse side effects. Because both the C and N domains are involved in bradykinin hydrolysis, nonspecific inhibition of both domains may be linked to the phenomena of persistent cough (5–20% of patients) and potentially life-threatening angioedema (0.1–0.5% of patients).^{37–40} This has been associated with systemic bradykinin accumulation as a result of the inappropriate suppression of N-domain bradykinin hydrolysis.

SYNTHESIS AND SELECTIVITY OF ACE INHIBITORS

Currently available ACE inhibitors include natural products and synthetic peptides belonging to the following classes of reactive compounds: sulfhydryls (captopril **1**); ketones (keto-ACE **4** and ketomethylene tripeptides **5**); carboxylates (lisinopril **3**); hydroxamic acids; and silanediols.

Although different approaches have been used in the synthesis of specific classes of inhibitor, they rely primarily on the interaction between a strong chelating group and the zinc-binding functionality of ACE. Because inhibitor binding is governed by the strength of this interaction, a drawback of this approach has been poor compound selectivity.⁴¹ This has necessitated the development of approaches that include interactions with specific ACE substrate-binding pocket residues.

The recently elucidated structure of a testis ACE-lisinopril complex⁴² as well as subsequent structures of ACE-inhibitor complexes^{43,44} has provided valuable insights into the molecular basis of the specific interactions between the ACE substrate-binding pockets and ACE inhibitors (FIG. 2).

Significant interactions with the inhibitor lisinopril **3** occur via residues occupying the S_1 , S_1' and S_2' pockets of the enzyme, highlighting the potential importance of these pockets in determining the domain selectivity of ACE inhibitors. The S_1' pocket of *t*ACE is shown to be very deep, and previous structure-activity relationship data have shown that the S_1' pocket can tolerate large hydrophobic P_1' side chains. This tendency is exhibited by most ACE inhibitors irrespective of their zinc-binding groups.

Moreover, the lysyl amine forms a salt bridge with D³⁷⁷ of *t*ACE, which is replaced by a glutamine in the N domain (FIG. 2B), and an E162D substitution also occurs in the S_1' subsite. Thus, extension of P_1' substituents into the S_1' pocket of ACE may provide a means of developing ACE inhibitors with specific C and N domain selectivities. The stereochemistry of the P_1' substituents is very important in the determination of ACE inhibitory potency. The P_1' substituents with *S*-stereo-

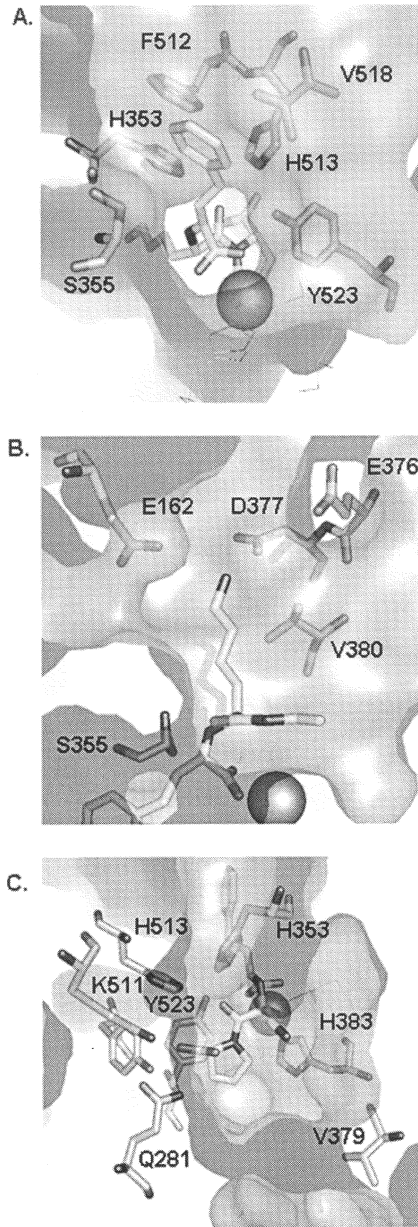


FIGURE 2. S_1 (A), S_1' (B) and S_2' (C) subsites of tACE and their interactions with lisinopril. The catalytic zinc atom is shown.

chemistry have been shown to possess greater potency than their *R* counterparts. The P_2' group of lisinopril fits into a relatively large S_2' pocket (FIG. 2C). This interaction with S_2' subsite residues such as K^{511} and Q^{281} via the hydrophobic prolyl ring⁴⁵ increases the ACE potency by at least 25-fold. Most available ACE inhibitors incorporate a proline or a tryptophan moiety at the P_2' position. The C-domain selective nature of a bulky P_2' residue may arise from the hydrophobic interactions with Val^{379} , which is replaced by a serine in the N domain.

Based on interactions with the zinc-binding functionality and surrounding subsite residues, two classes of compounds have been designed and developed: (1) those in which the zinc-binding group is flanked on both sides by amino acid residues (Type I and II inhibitors), and (2) those in which the amino acid residues are present on the right-hand side of the zinc-binding groups (Type III inhibitors) (FIGS. 3 and 4). A variety of different ACE inhibitors have been synthesized using this approach, incorporating a range of different zinc-binding functionalities.⁴⁶

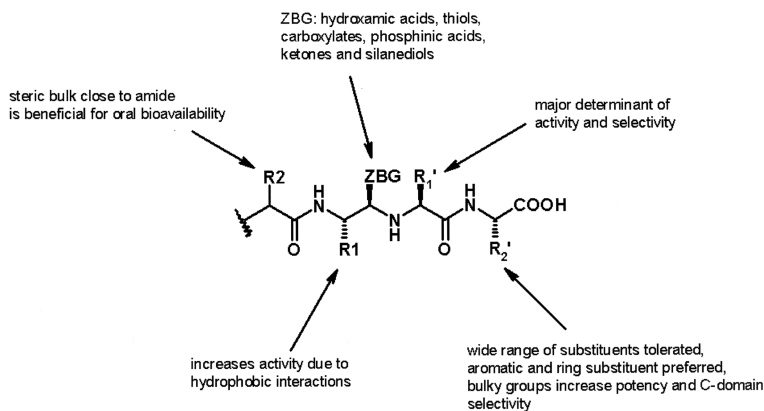


FIGURE 3. Structure-activity relationships for the left- and right-hand side of ACE inhibitors.

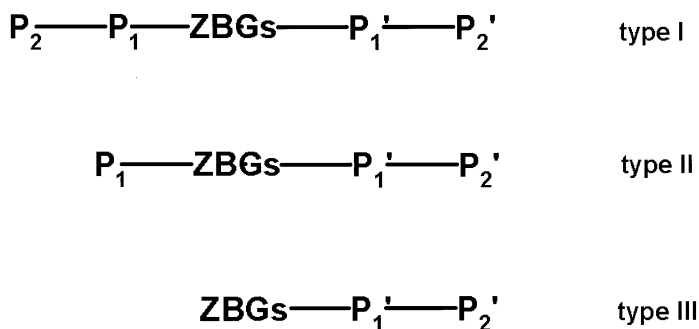


FIGURE 4. Schematic representation of the three major types of ACE inhibitors.

Synthesis of left- or right-hand side inhibitors such as hydroxamates (to be discussed) and thiolates has permitted probing of either the primed or the unprimed side of the active site. Here, potency is primarily reliant upon the strength of the zinc-chelating group, but selectivity is compromised. By contrast, compounds that mimic the peptide substrate sequence to the left- and right-hand sides of the scissile bond and incorporate a weaker zinc-binding group, such as a phosphinate, exhibit more domain-selective inhibition.⁴¹

Phosphinic Acid Derivatives

Phosphinic peptide chemistry has been used to develop inhibitors that interact with both primed and unprimed sides of the active site. One of the early phosphinic acid ACE inhibitors developed in the 1980s was [hydroxyl-(4-phenylbutyl)phosphinyl]acetyl-*L*-proline (**7**).⁴⁷ This inhibitor had an IC₅₀ value of 180 nM. The insertion of a methylene spacer between the phosphinic acid zinc-binding group and the carbon bearing the P₁' substituents results in increased potency (compound **8**) (FIG. 1). Furthermore, despite weaker phosphinic acid zinc-binding functionality, C- and N-domain selectivity has been greatly enhanced by various modifications of the P₁ and P₁' inhibitor residues. Using this approach, Dive and co-workers^{41,48,49} recently reported the synthesis of two domain-specific phosphinic peptide ACE inhibitors, RXP 407 (**9**) and RXP A380 (**10**) (FIG. 1). RXP 407 is more selective for the N domain ($K_i = 12$ nM), whereas RXP A380 is approximately 1,000-fold more C-domain selective ($K_i = 3$ nM). RXP 407, characterized by the unusual incorporation of an aspartate residue in the P₂ position, was obtained using a solid-phase peptide synthesis approach, whereas the synthesis of RXP A380 was a classical synthetic chemistry approach similar to the right-hand to left-hand methodology as for type I ACE inhibitors. The N-domain selectivity of RXP407 has been attributed to an interaction between its acidic P₂ aspartate and an S₂ subsite arginine as well as its C-terminal carboxamido group. In the C domain, this S₂ subsite residue is replaced by a glutamate. The C-domain selectivity of RXPA380 can be attributed to the trans-amide geometry of the P₁'-P₂' residues required for an effective hydrogen bonding interaction between enzyme and inhibitor. Docking of RXP A380 at the active site showed that the pseudo-proline in the S₁' pocket imposes a particular orientation to the P₂' tryptophan residue, thus improving the C-domain selectivity. Given their *in vivo* stability, these domain-selective phosphinic acid derivatives have been useful in probing the functional roles of the N and C domains, confirming the role of both domains in bradykinin hydrolysis.^{21,50,51} Thus, domain-specific inhibition of the C domain may be necessary and sufficient for the treatment of hypertension and phosphinic acid derivatives, potential lead compounds in the development of therapeutic drugs. Fosinopril (**11**, FIG. 1), belonging to the same class of inhibitors, is currently in clinical use for the treatment of hypertension.⁴¹

Phe-Ala-Pro and Ketomethylene Analogues

Phe-Ala-Pro and ketomethylene analogues (compounds **2–6**, FIG. 1) are type I and II ACE inhibitors, respectively (FIG. 4). The synthesis of type II inhibitors proceeds by synthesizing the building block P₁-ZBGs (the P₁ residues incorporating the zinc-binding group) followed by successive coupling of the P₁' and P₂' residues. A

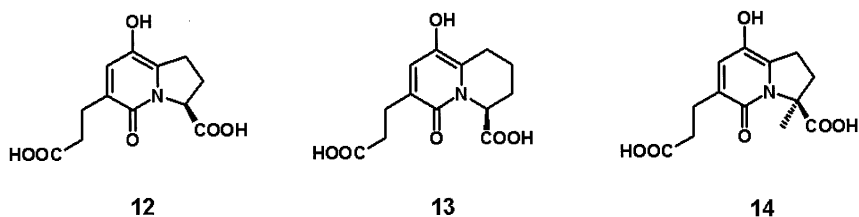


FIGURE 5. Nonpeptidic bicyclic ACE inhibitors.

similar approach is followed in the synthesis of type I ketomethylene analogues such as keto ACE, except that the P_2 residue is coupled with the P_1 residue. Exploiting their P_1' and P_2' residues, Phe-Ala-Pro analogues, such as enalaprilat (**2**) and ramiprilat (**6**), were some of the first commercially available ACE inhibitors for the treatment of hypertension. These inhibitors show IC_{50} values in a similar nanomolar range as those of lisinopril and captopril.

In general, peptides and peptidic compounds are usually unsuitable as drug candidates, possessing undesirable physical properties such as poor solubility, susceptibility to degradative enzymes, and poor oral bioavailability. To reduce the peptidic nature of ketomethylene inhibitors the P_1' and P_2' substituents may be cyclized to form a lactam, where there is a correlation between the inhibitory potency and the ring size.^{52–54} Thus, the bicyclic dicarboxylic acids (**12** and **13**) and the nonpimerizable 3-methyl analogue (**14**, FIG. 5) were found to inhibit ACE in the low nanomolar range, similar to those of the commercially available inhibitors captopril and enalapril.

The synthetic chemistry of these ACE inhibitors was reviewed by De Lima.⁴⁶ Keto-ACE (**4**, FIG. 1), originally described in 1980, has emerged as a potential lead compound for C-domain-specific ACE inhibitors, with a 40–50-fold greater specificity for this domain compared with the N domain.^{55,56} Keto-ACE and its analogues, which were found to inhibit ACE in the nanomolar range, contain a ketomethylene isostere replacement at the scissile bond that is believed to mimic the tetrahedral transition state of the proteolytic reaction.⁵⁷

Keto-ACE, a tripeptide analogue of Phe-Gly-Pro, contains a bulky P_1 group and a P_2 benzyl ring that might confer C-domain selectivity.⁵⁸ With the availability of the three-dimensional C-domain (tACE) structure, the interaction of keto-ACE with the active site pockets of ACE was investigated. In this respect, synthesis of keto-ACE analogues with Trp or Phe at the P_2' position led to a marked increase in C-domain selectivity (unpublished data), highlighting the importance of the C-domain S_2' pocket. Inhibitory potency may further be enhanced by the incorporation of hydrophobic substituents such as a phenyl group at the P_1' position. In this instance, the stereochemistry of P_1' substituents is important. P_1' substituents with *S*-stereochemistry have been shown to possess greater inhibitory potency than have their *R* counterparts.⁵⁹

Iterative docking experiments performed on a keto-ACE analogue synthesized in our research group (unpublished work) illustrated that the inhibitor makes protein-ligand contacts with the S_1 , S_2 , S_1' and S_2' residues of the ACE active site. The ori-

entation of its benzyl ring permits a stacking interaction with the aromatic side chain of F³⁹¹. In the N domain, this residue is replaced by a less favorable Y³⁶⁹ at the S₂ subsite, explaining in part the C-domain selectivity of keto-ACE. Additional contacts include those between the ketone group and the catalytic zinc atom, an interaction believed to be one of the most significant forces holding the complex together. Moreover, the peptide backbone of the inhibitor makes hydrogen bonding contacts with the main chain of the protein.

Silanedioles

One approach to protease inhibition that has proven very successful is the incorporation of a nonhydrolyzable isostere of the tetrahedral intermediate of amide hydrolysis (structure **15**, FIG. 6). Ketone or aldehyde hydrates (**16**) are examples that effectively mimic the tetrahedral intermediate, but such structures are often reactive and form undesirable covalent bonds with other nucleophilic species *in vivo*.^{60,61} Other molecular structures that mimic the “geminal diol motif,” such as **17** or **18**, have also been incorporated into peptide derivatives and shown to serve as effective isosteres. The phosphorous geminal diol (**17**) is very similar to the phosphinic acid zinc-binding group functionality present in the phosphinic acid class of ACE inhibitors.^{47,48} Silicon forms a dialkylsilanediol compound (structure **18**) that is a stable isostere, providing the diol component that is sufficiently hindered to prevent the formation of a siloxane polymer. In fact, it has been demonstrated that silanediol-based dipeptide analogues are potent inhibitors of metalloproteases and aspartic proteases.

Synthesis of the first silanediol analogues of type I ACE inhibitors (FIG. 7) was reported by Sieburth and co-workers.^{59,62,63} The synthetic approaches to these silanedioles used classical synthetic chemistry methodology resembling the right- to left-hand side coupling approach described for the ketomethylene tripeptide analogues. Because silanedioles are more stable than carbon diols, they are likely to exhibit a longer half-life. The silanediol (**19**) was found to be approximately fourfold

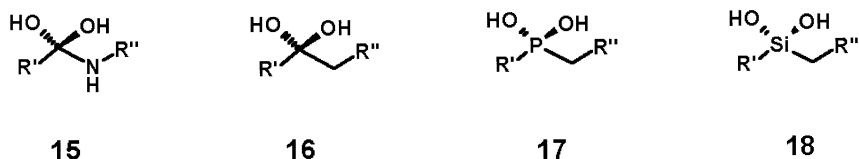


FIGURE 6. Schematic representation of peptide bond hydrolysis isosteres.

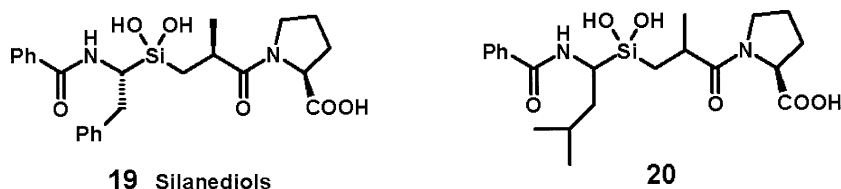


FIGURE 7. Silanediol ACE inhibitors with different P₁ substituents.

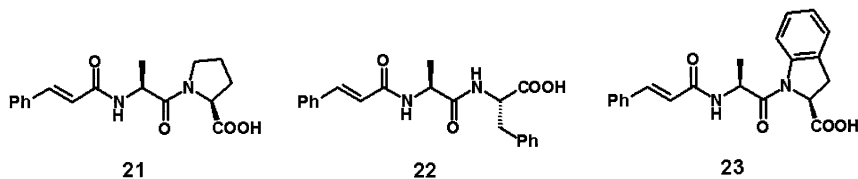


FIGURE 8. Irreversible ACE inhibitors comprising α,β -unsaturated carbonyls.

less potent than the ketone analogue (**5**)⁵⁹ due to the weaker zinc chelation of silanediols as compared with ketones.

Compound **20** is a weaker ACE inhibitor than compound **19**, presumably due to the *i*-butyl group of compound **20** compared to the benzyl group. Moreover, the introduction of a hydrophobic methyl phenyl provides a modest increase in potency over an analogue with a *tert*-butyl group at P_1 , suggesting that there is improved recognition with the S_1 pocket.

Irreversible ACE Inhibitors

In principle, irreversible inhibitors should be devoid of the inherent drawbacks associated with the classical reactive warhead groups, that is, lack of specificity, excessive reactivity, and instability. Park Choo *et al.*⁶⁴ reported a novel class of ACE inhibitors comprised of α,β -unsaturated carbonyls (FIG. 8, compounds **21–23**). These compounds had IC_{50} values of 0.23, 2.0, and 3.19 mM, respectively.

Although the IC_{50} values of these compounds were significantly higher than those reported for most ACE inhibitors, the rationale was that the α,β -unsaturated moiety might react with a catalytic nucleophile at the active site to afford a Michael-type adduct, rendering the enzyme inactive. Because of this covalent bond formation between the enzyme and the inhibitor, this class of inhibitors has the advantages of increased half-life, oral bioavailability, and increased resistance to hydrolytic degradation. The tenfold difference in inhibitory potential between compounds **22** and **23**, on the one hand, and **21**, on the other, might be due to the extra π - π interaction between the electron-rich phenyl or benzene ring and the active site S_2' residues that reduce the covalent strength of the Michael-type adduct, thereby rendering the enzyme more active.

Captopril Analogues

Because the S_2' subsites of both the C and N domains are relatively large and can accommodate various linear and cyclic side chains, the selectivity of ACE inhibitors might depend on interactions with the S_2' pockets.^{48,49,65} Before the solution of the ACE crystal structure,^{42,43} many ACE inhibitors were designed with bulky P_2' residues. Captopril (**1**) is one of the simplest ACE inhibitors, comprising a P_1' and P_2' residue and a thiol zinc-binding group. Hanessian and co-workers⁶⁶ reported the synthesis of modified captopril analogues (**24–26**) in which the P_2' proline residue of captopril had been modified by a conformationally constrained heterocycle (FIG. 9). These analogues showed increased ACE inhibition. Synthesis of these con-

and N domains display about 90% identity at their active site, targeting specific residues that are not conserved in both domains, might aid in the discovery of potent and domain-selective ACE inhibitors. This review discusses new insights to consider during the design of domain-selective ACE inhibitors.

The compounds might serve as attractive chemical entities for drug discovery research in the field hypertension and heart-related diseases. However, caution is needed when using structural information in a predictive manner in the design of ACE inhibitors, because conformational changes may have occurred in the active site upon binding of the inhibitor in the ACE crystal structure.

The clinical benefits of ACE inhibitors are undeniable, and their use in the treatment of conditions extending beyond that of hypertension is growing. In this regard, ACE inhibitors have been shown to be beneficial in treating hypertension accompanied by the cardiometabolic syndrome and type II diabetes mellitus.⁶⁹ ACE inhibitors have been shown to retard myocardial remodeling and contractile dysfunction leading to heart failure,⁷⁰ improve endothelial function,⁷¹ and reduce atherogenesis.⁷² Recently, the role of ACE as a signal transduction molecule was investigated. Here the ACE inhibitors ramiprilat and perindoprilat together with bradykinin were shown to enhance phosphorylation of the ACE cytoplasmic tail in endothelial cells. This “outside-in signaling” pathway results in an increase in ACE expression.⁷³ Furthermore, ACE inhibitors are associated with increased expression of cyclooxygenase-2 (COX-2), resulting in increased production of prostaglandin E2 and prostacyclin.⁷⁴

The burgeoning use of ACE inhibitors, especially in the treatment of conditions comorbid with hypertension, underscores the importance of understanding at a structural level the nature of their interactions with the ACE active site. Moreover, in improving the efficacy of ACE inhibitors and reducing the prevalence of adverse side effects, the need exists for the development of drugs that can specifically distinguish between the physiologically diverse C and N domains of this enzyme.

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