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Structure Based Drug Design of Angiotensin-I Converting Enzyme Inhibitors

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Abstract: Cardiovascular disease (CVD) is responsible for ~27% of deaths worldwide, with 80% of these occurring in developing countries. Hypertension is one of the most important treatable factors in the prevention of CVD. Angiotensin-I converting enzyme (ACE) is a two-domain dipeptidylcarboxypeptidase that is a key regulator of blood pressure as a result of its critical role in the renin-angiotensin-aldosterone and kallikrein-kinin systems. Consequently, ACE is an important drug target in the treatment of CVD. ACE is primarily known for its ability to cleave angiotensin-I to the vasoactive octapeptide angiotensin-II, but is also able to cleave a number of other substrates including the vasodilator bradykinin and *N*-acetyl-seryl-aspartyl-lysyl-proline (acetyl-SDKP), a physiological modulator of hematopoiesis. Numerous ACE inhibitors are available clinically, and these are generally effective in treating hypertension. However some adverse effects are associated with ACE inhibition, such as the persistent dry cough and the potentially fatal angioedema. The solution of ACE crystal structures over the last decade has facilitated rational drug design which has contributed to the development of domain-selective ACE inhibitors, the most notable of which include RXP407 (N-domain) and RXPA380 (C-domain), which in principle may herald new therapeutic approaches for ACE inhibition. Additionally, dual inhibitors to ACE and other targets such as neprilysin, endothelin converting enzyme and chymase have been developed. The success of ACE inhibitors has also led to the search for novel inhibitors in food and natural products and the structure guided screening of such libraries may well reveal a number of new ACE inhibitors.

Keywords: Angiotensin-I converting enzyme, cardiovascular disease, crystal structure, domain selective inhibitors, hypertension, structure-based drug design, zinc metallopeptidase.

1. INTRODUCTION

Cardiovascular disease (CVD) due to hypertension, atherosclerosis and diabetes is the leading cause of death, accounting for 30% of deaths worldwide, with 80% of these deaths occurring in middle and low income countries [1,2]. The principal source of morbidity and mortality from CVD is due to end-organ damage, notably heart failure, nephropathy and retinopathy, due in large part to organ fibrosis.

Current treatment regimens for CVD and the underlying predisposing conditions are inadequate, and poorly controlled hypertension, atherosclerosis and diabetes are the norm rather than the exception. Consequently, progression of established heart failure, nephropathy and retinopathy can at best be slowed but not halted.

The importance of angiotensin-I converting enzyme (EC 3.4.15.1, ACE) and its critical role in the renin-angiotensin aldosterone system (RAAS) in relation to cardiovascular physiology and disease has been firmly established. This is mainly due to the fact that the drugs that block various components of this system are effective as treatments for hypertension, heart failure and prevention of vascular events caused by atherosclerosis (mainly heart attack and stroke), as well as slowing kidney disease caused by hypertension or diabetes. ACE inhibitors have been used clinically (Table 1), but there is still room for improvement given the side effects seen in patients on long-term treatment.

1.1. Angiotensin-I Converting Enzyme (ACE)

ACE is a zinc metallopeptidase that plays a critical role in blood pressure regulation by catalyzing the proteolysis of angiotensin-I to the vasopressor angiotensin-II [3-8]. Human ACE is a type I transmembrane protein of 1,306 amino acids and is processed to a 1277-residue mature form which is heavily glycosylated (Fig. 1). A sequence of 22 hydrophobic amino acids located near the carboxy terminus of the protein serves as a

transmembrane domain that anchors ACE to the cell surface. Thus, ACE contains a 28-residue cytosolic domain and a 1249-residue glycosylated extracellular domain. ACE is also a member of a large family of proteins that undergo cleavage, with release of their ectodomain as a soluble form of the protein [9].

There are two isoforms of human ACE: in somatic tissues, it exists as a glycoprotein composed of a mature single polypeptide chain of 1277-amino acids (sACE), with an active centre on both the N- and C-domains [10] (Fig. 1). In male germinal cells, it is synthesised as a lower molecular mass form and is thought to play a role in sperm maturation and the binding of sperm to the oviduct epithelium [11]. However the full nature of its role is still under investigation [12-14]. This isoform, testis ACE (tACE) is identical to the C-terminal half of somatic ACE, except for a unique 36-residue sequence constituting its amino terminus [15].

Despite sharing ~55% sequence identity with the C-domain, the N-domain of sACE has its own distinctive physicochemical and functional properties. It is thermally more stable than the C-domain [16], more resistant to proteolysis under denaturing conditions [17] and is less dependent on chloride activation as compared with the C-domain [18,19]. Both domains are heavily glycosylated (the N-domain has ten and the C-domain has seven *N*-linked glycosylation sites), a feature that has hampered the three-dimensional structure determination of the protein for a long time. The different glycan profile of the N-domain is likely to be responsible for the carbohydrate-mediated dimerisation of the somatic form which has been described under certain conditions [20]. Substrates such as the hemoregulatory and antifibrotic peptide *N*-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) [21], and the enkephalin precursor [22] Met⁵-Enk-Arg⁶-Phe⁷ are specific for the N-domain, whereas the physiological substrates bradykinin and angiotensin-I are hydrolysed with similar catalytic efficiency as the C-domain, *in vitro*. Interestingly, the N-domain preferentially hydrolyses the amyloid beta peptide of the amyloid precursor protein resulting in inhibition of amyloid beta aggregation and cytotoxicity in cell-based assays [23]; although this is not necessarily the case *in vivo* [24]. The N-domain may also have a role in modulating C-domain activity through a combination of inter-domain cooperativity and structural stabilisation.

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Table 1. N- and C- Domain Selectivity of Known (Current) ACE Inhibitors

Inhibitor	Inhibitor residues* -P ₂ -P ₁ -P ₁ ' -P ₂ '	Domain inhibition (nM) [‡]	
		N-domain	C-domain
BPPa	-Gln-Ile-Pro-Pro	100.0	1.0
BPP-10c	-Gln-Ile-Pro-Pro	200.0	0.5
BPPb	-Lys-Ile-Pro-Pro	10000.0	30.0
BPPc	-Pro-Ile-Pro-Pro	80.0	80.0
bBPP-12b	-Pro-Ile-Pro-Pro	5.0	150.0
Ang ₁₋₇	-Tyr-Ile-His-Pro	3400.0	130.0
RXPA380	Phe-Phe-Pro-Trp	10000.0	3.0
RXP407	Ac-Asp-Phe-Ala-Ala-NH ₂	2.0	2500.0
Captopril	Ala-Pro	8.9	14.0
Enalapril	Phe-Ala-Pro	26.0	6.3
Lisinopril	Phe-Lys-Pro	44.0	2.4
Keto-ACE	Bnz-Phe-Gly-Pro	1500.0	40.0
Trandolapril	Phe-Ala-Trp	3.1	0.3

*C-terminal amino-acid residues of peptide inhibitors (BPP peptides and Ang₁₋₇), or amino-acid analogs in the phosphinic peptides (RXPA380 and RXP407) and small-molecule ACE inhibitors (captopril, enalapril, lisinopril, keto-ACE, and trandolapril). [‡]Determined as K_i in every case except keto-ACE (IC₅₀), at 150 mM NaCl (keto-ACE and Ang₁₋₇ [22]), 200 mM NaCl (BPP and phosphinic peptides [69,96]), or 300 mM NaCl (small-molecule inhibitors [18]).

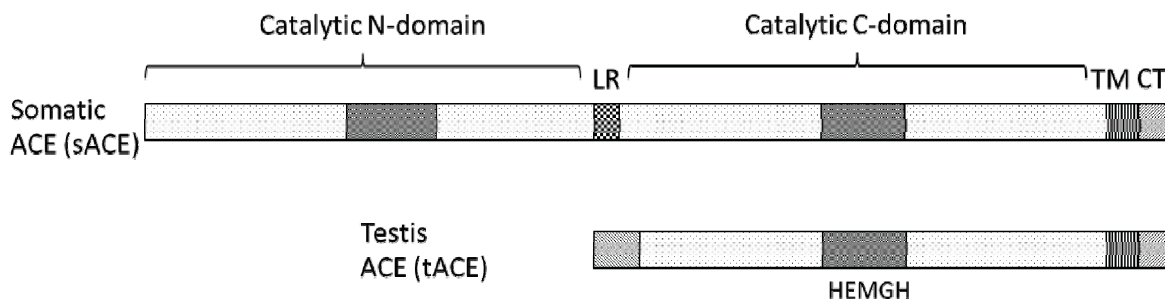


Fig. (1). Domain structure of somatic (sACE) and testis ACE (tACE or C-domain of ACE) indicating the catalytic domains, the inter-domain linker region (LR), the transmembrane domain (TM), and cytoplasmic domain (CT).

In 1977 Cushman *et al.* described the synthesis of a series of potent inhibitors of ACE designed on the basis of an assumed mechanistic homology with carboxypeptidase A [25]. This ushered in a generation of drugs for the management of hypertension, many of which are still used today. Remarkably, these inhibitors were developed without the benefit of any detailed chemical, kinetic or structural information on human ACE. Despite intensive efforts by several research groups since the 1970's, the ACE crystal structure could not be determined until 2003. This has largely been due to the inability to generate ACE proteins, from natural or recombinant sources, that could yield crystals suitable for structural study. These problems were eventually circumvented resulting in the solution of the first ACE structure [26]. This research showed that the structure of ACE (Fig. 2) is different from carboxypeptidase (used as a model for the design of well known and marketed ACE inhibitor captopril [25]) but similar to neurolysin, an unexpected similarity when comparing the amino acid sequences. The structure of tACE bound to the potent inhibitor lisinopril [18,27,28] showed that the inhibitor binds in a highly ordered, extended conformation [26] (Figs. 2A, B). The interactions of the inhibitor were found to be different from those for captopril, although they did encompass some features of what was envisaged in the early work. Thus, the research by Natesh *et al.* [26] not only represents an important scientific achievement in defining the structure of ACE for the first time, but also provided new and enlightening information.

Captopril differs from both lisinopril and enalaprilat in that its sulphhydryl group coordinates the zinc rather than a carboxyl group.

Its smaller size also results in less direct interactions with the protein, being held in place at only two positions, in addition to the zinc coordinating group. It was also found that the trend in the relative potencies of lisinopril, enalaprilat and captopril for the C-domain (L > E > C) [18], correlates with the number of interactions observed in the crystal structure complexes with tACE [29] and provided the details of the framework of residues important for drug binding.

Based on a similar strategy, batches of N-domain (sACE residues 1-629), expressed in Chinese hamster ovary (CHO) cells in the presence of alpha-glucosidase inhibitor *N*-butyldeoxy-nojirimycin (NB-DNJ), were subjected to extensive crystallisation trials, which eventually yielded crystals that diffracted to 2.9 Å resolution. The structure (both in the presence and absence of lisinopril) gave the first clues as to how these two domains differ in specificity and function. These structures of the highly homologous N- and C-domains provided a platform for the beginning of the design of next generation 'domain-specific' ACE inhibitors [30] (Fig. 2C).

2. CURRENT ACE INHIBITORS: ADVANTAGES AND LIMITATIONS

ACE inhibition is an approach for the treatment of hypertension and CVD that has been championed for over 30 years. Our understanding of the impact of ACE inhibitors in the early 80s could well be described as seeing through a glass, darkly compared

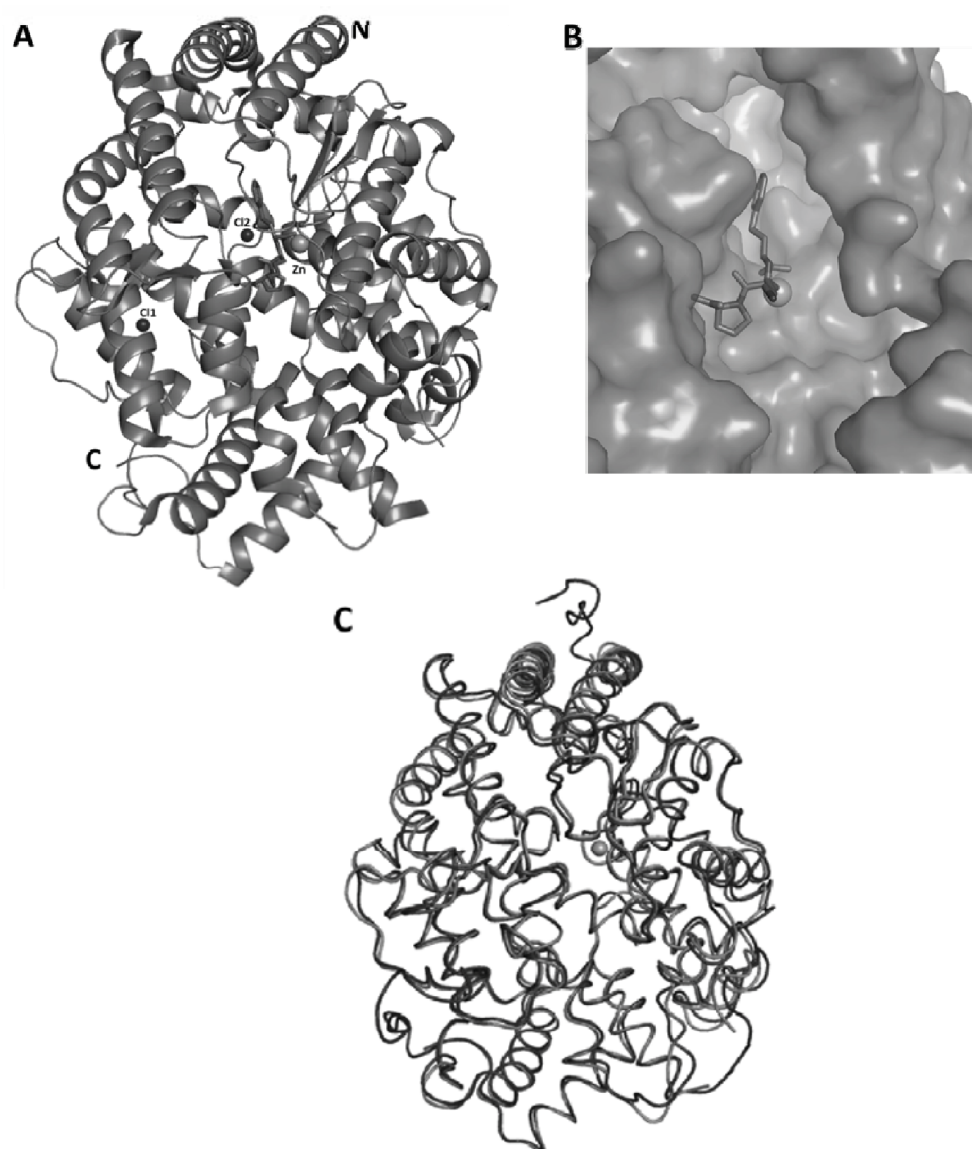


Fig. (2). Structure of tACE [26]. **A.** Cartoon representation with zinc ion (large sphere), chloride ions in small spheres, and the ACE inhibitor lisinopril (black) bound in the active site. **B.** Surface representation of tACE active site cavity with bound lisinopril (black). **C.** Comparison with the N domain (thin line), the zinc ion is shown in large sphere [30].

to our current knowledge of all the angiotensin-related peptides and their cognate receptors; the interactions and relative contributions of the different blood pressure regulating cascades; and the cell signalling mechanisms. The fact that they have withstood rigorous testing in large clinical studies and are still so widely used today, bears testimony to their success.

ACE inhibitors are one of the front-line therapies for essential hypertension. In both hypertensive and salt-depleted normotensive subjects they show efficacy in lowering mean systolic and diastolic pressures [31,32]. One of their hallmarks is that they decrease peripheral vascular resistance without altering baroreceptor activity [33] or causing an increase in heart rate [34-36], while also inhibiting the tonic effect of angiotensin-II on the sympathetic nervous system [37]. The advantages of ACE inhibitors in the treatment of atherosclerosis, congestive heart failure, myocardial infarction, diabetic nephropathy, plaque stabilization and cancer have been reviewed previously [38].

ACE hydrolyses a diverse range of substrates including AcSDKP, gonadotropin-releasing hormone (GnRH) [also known as

luteinizing-hormone-releasing hormone (LHRH)], substance P, neurotensin, dynorphin and enkephalin and thus ACE inhibition results in a variety of effects independent of blood pressure reduction [39]. Hence, ACE inhibitors augmented the effects of bradykinin in blood vessels that had negligible levels of ACE and increased the effect of ACE-resistant B₂-kinin receptor agonist [40]. These data support the notion that ACE inhibitors selectively potentiate the vascular effects of bradykinin mediated by the B₂-receptor, independently of their ACE inhibiting properties. Furthermore, ACE has been shown to dimerise with the B₂-kinin receptor resulting in the protection of high-affinity receptors, blocking receptor desensitization, and decreasing internalisation thereby potentiating bradykinin beyond simply preventing its hydrolysis [41,42].

Kohlstedt *et al.* showed that ACE inhibitors are able to induce phosphorylation of Ser1270 located in the cytoplasmic domain of ACE resulting in outside-in signalling that increases expression of cyclooxygenase-2 and ACE [43]. The ACE inhibitor effect on cyclooxygenase-2 is caused by the transcription factor activator

protein and this results in an increased release of prostacyclin and prostaglandin E2 by endothelial cells [44].

The most common adverse effect of ACE inhibitors is a persistent cough which can affect as many as one in five subjects in certain populations. Angioedema, on the other hand, is a much rarer side effect, but potentially life-threatening. It is widely accepted that these effects are caused by elevated levels of bradykinin or substance P and stimulation of vagal C fibers [45]. Furthermore, ACE inhibitors lead to lower levels of aldosterone which can cause hyperkalemia, especially in patients with poor kidney function or patients on potassium supplements [46-48].

Sulphydryl-containing ACE inhibitors have been associated with skin rash, neutropenia and nephrotic syndrome and these side effect are often associated with concomitant end organ disease (especially renal insufficiency) [49]. Initially captopril was administered at doses that are now recognised to be far in excess of those necessary for drug action and this likely aggravated the adverse effects. Moreover, dosages were given without considering deficiencies of renal function, now known to be the main excretory route of captopril. In contrast to these adverse effects caused by other sulphydryl ACE inhibitors, long-term treatment with zofenopril may slow the progression of carotid atherosclerosis in addition to its blood pressure lowering effect [50].

Chronic use of ACE inhibitors can lead to ACE inhibitor escape, where levels of angiotensin-II are not reduced to normal despite substantial ACE inhibition. One of the mechanisms for ACE inhibitor escape is the hydrolysis of angiotensin-I by the serine protease chymase, found in cardiac interstitial space and cardiac endothelial cells [51]. Recently, Wei *et al.* showed that chronic ACE inhibition resulted in a marked increase in left ventricle chymase activity mediated by bradykinin/B₂ receptor in mice [52]. Moreover, combination of ACE and chymase inhibition improved left ventricle function, decreased adverse cardiac remodelling and improved survival post myocardial infarction.

Thus, some of the 'limitations' of ACE inhibitors involving ACE's substrate promiscuity and other enzymes ability to hydrolyse its primary physiological substrate have heralded possibilities for fine tuning the RAAS using combination therapies and dual peptidase (vasopeptidase) inhibitors. It is likely that these advances in our understanding of ACE and its blockade will result in further additions to the arsenal of drugs for the treatment of hypertension and CVD and hopefully a more precise approach towards personalised medicine.

3. MINIMUM GLYCOSYLATION REQUIREMENTS FOR THE EXPRESSION AND CRYSTALLISATION OF ACE

The use of protein crystal structures solved in complex with specific inhibitors is a valuable tool for rational drug design. Given the importance of ACE inhibitors in the treatment of cardiovascular disease, numerous efforts have been made to solve the X-ray crystal structure of this protein. Attempts to crystallise the full length sACE enzyme have as yet been unsuccessful. This is most likely due to a high level of surface glycosylation and the flexibility of the linker region, joining the two domains. However, the complex problem of sACE crystallisation has been solved in part by attempting to crystallise the N- and C-domains separately.

While researchers have been able to crystallise the individual N- and C-domains, this was not a trivial matter. Initial attempts to crystallise tACE (equivalent to the C-domain of ACE) were unsuccessful. Thus a truncated form was developed by the removal of the transmembrane region at the C-terminus, and a heavily O-glycosylated region at the N-terminus (not present in the C-domain of sACE). This shortened, soluble form of the enzyme, expressed in the presence of a glucosidase inhibitor NB-DNJ, and treated with endoglycosidase-H (which leaves just a single N-acetylglucosamine

residue attached to the Asn of the glycosylation sequon), was able to form diffracting crystals and led to the solution of the first X-ray crystal structure for human tACE [26]. The solution of the N-domain structure was also hampered by the high degree of surface glycosylation and once again, expression in the presence of the glucosidase inhibitor, NB-DNJ, was required to produce a crystallisable form of the N-domain [30]. Although the use of NB-DNJ allowed for the solution of the crystal structure for the N- and C-domains, in both cases crystallisation was not reproducible, and combined with the high cost of NB-DNJ, this approach was deemed unfeasible for routine use.

Since N-glycosylation is required for the folding of ACE into its active conformation [53,54], attempts were made to determine the minimum number of N-glycosylation sites required for the expression of functional N- and C-domains of ACE. Producing forms of the N- and C-domains which contained the fewest possible number of intact glycosylation sites would reduce glycan complexity and avoid the need for expensive glycosylation inhibitors.

Mass spectrometry based studies on tACE revealed that although glycosylation was found at six of the seven potential N-glycosylation sites on the C-domain, only the three N-terminal sites were consistently found in the glycosylated state, with the three C-terminal sites being found in both the glycosylated and unglycosylated forms [55]. These data seemed to imply that the N-terminal sites were of particular importance for the C-domain. Therefore, extensive site-directed mutagenesis of the various glycosylation sites was carried out, disrupting the glycosylation sites from the C-terminus. Results from this study revealed that glycosylation of only one or two of the N-terminal sites was required for expression of an active glycoform of the C-domain [54] (Fig. 3A). Using one of these hypoglycosylated forms of tACE, tACEg1,3 (tACE containing glycosylation at only sites one and three), reproducible and diffraction-quality crystal formation was achieved [56]. An alignment of the two C-domain crystal structures, shows that they are essentially identical, with a root-mean square deviation (RMSD) of 0.51 Å for all atoms [57], indicating that mutation of the glycan sequons did not affect the structure of the enzyme.

Mass spectrometry studies revealed that virtually all of the ten N-glycosylation sites on the N-domain were glycosylated, with only one site found to be unglycosylated, while one site was not detected [58]. Therefore, no inference about the relative importance of the different glycosylation sites could be made. However, given the high degree of structural similarity between the N- and C-domains (RMSD of 0.72 Å for all atoms) it was expected that the generation of a similarly hypoglycosylated form of the N-domain would be suitable for high throughput inhibitor-enzyme crystallisation studies. Thus, glycosylation sites were sequentially disrupted from the N-terminus. However, in contrast to the C-domain where N-terminal glycosylation was important, it was found that C-terminal glycosylation was required for the expression of functional N-domain [58]. Further investigation revealed that three C-terminal glycosylation sites (sites seven, eight and nine) were sufficient for the expression of functional N-domain, although an N-terminal site was able to compensate for the absence of a C-terminal site, such that Ndom389 (N-domain containing glycosylation at sites three, eight and nine) was expressed as an active N-domain glycoform [58] (Fig. 3B). Recent crystallization trials with this variant have shown that it is able to crystallize reproducibly, the best of which diffracted to 2.0 Å resolution [58]. It is often a concern that introducing mutations may affect the structure of the protein, which would be problematic for this approach to crystal structure determination. However, aligning the structures of wild type N-domain and Ndom389 revealed an RMSD of 0.70 Å for all atoms [57].

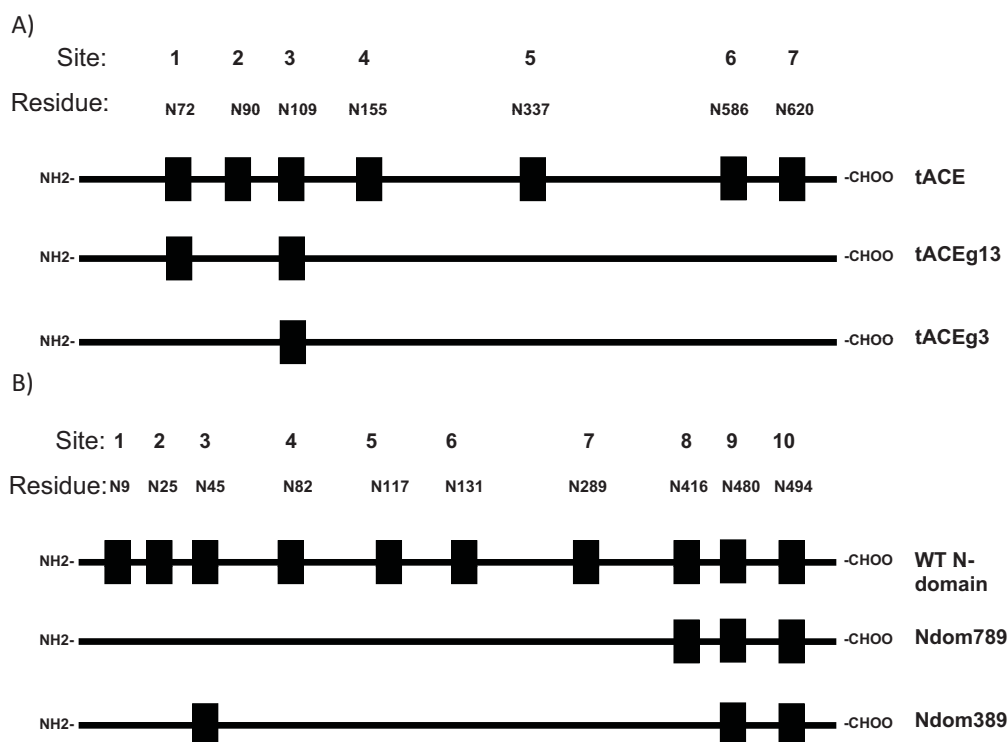


Fig. (3). Schematic representation of the glycosylation sites on the N- and C-domains of ACE showing the minimum number of glycosylation sites required for expression and crystallisation of the N- and C-domains. Black boxes indicate intact glycosylation sites.

4. DOMAIN SELECTIVE ACE INHIBITORS

4.1. N-Domain Inhibitors

The N-domain has been shown to be the main site involved in the cleavage of Angiotensin (1-7) [Ang1-7], amyloid beta peptide (A-beta42), GnRH and AcSDKP [21,22,59], although others have shown the hydrolysis of Ang1-7 is not domain-selective [60]. Perhaps the most important of these is AcSDKP. This substrate was first identified as a regulatory peptide involved in hematopoietic stem cell proliferation [61], but has also been shown to have a number of beneficial effects on the cardiac system. These include preventing or reversing cardiac, vascular and renal inflammation and fibrosis [62]. AcSDKP mediates these effects, in part, by inhibiting macrophage differentiation, activation, migration and cytokine release. AcSDKP has also been shown to prevent end-organ damage resulting from hypertension [63], as well as protecting against diabetic cardiomyopathy [64]. Additionally, increased levels of AcSDKP, following ACE inhibition, are thought to contribute to the beneficial effects of ACE inhibitors by a novel mechanism whereby AcSDKP inhibits collagen deposition in the left ventricle of the heart following vascular injury [65]. N-domain knock-out mice, and mice undergoing chronic ACE inhibitor treatment, have AcSDKP levels six to seven times that of wild-type (WT) mice [66-68], illustrating the importance of the N-domain in the regulation of AcSDKP levels.

Not surprisingly, these findings highlight the value of developing inhibitors selective for the N-domain of ACE, which may prove useful in the treatment of certain diseases such as renal and pulmonary fibrosis, where inhibition of the N-domain could be beneficial, while the C-domain would remain active to allow for normal regulation of blood pressure.

The most notable N-domain selective inhibitor is RXP407, which displays roughly 2000-fold selectivity for the N-domain (Table 1) [69]. The S₂ pocket residues Tyr369 and Arg381 have been implicated in conferring the N-domain selectivity of this

inhibitor (Fig. 4D), positioning RXP407 in an optimal conformation for the N-domain active site [70]. These findings further confirm those established during the synthesis of RXP407, where it was found that varying the P₂ group had a notable effect on the selectivity of the inhibitor [69]. Furthermore, Dive *et al.* [69] found that the presence of a Glu, Asn or Asp at the P₂ position promoted N-selectivity, while the presence of an Arg at the P₂' position reversed the N-selectivity of the inhibitor. The importance of the S₂ pocket in conferring the selectivity of RXP407 was further highlighted in the crystal structure of the N-domain in complex with this inhibitor, which showed that both Tyr369 and Arg381 make close contacts with the P₂ group of RXP407 (Figs. 4C and D) [58].

4.2. C-Domain Inhibitors

Recent findings have shown that the major site of angiotensin-I cleavage is at the C-domain active site [68,71], while both domains cleave bradykinin with equal efficiency [19]. These findings have given rise to the pursuit of C-domain selective inhibitors which, it is hoped, will provide control of blood pressure, while leaving the N-domain active to prevent bradykinin accumulation and the associated side effects [72].

Keto-ACE, a ketomethylene analogue of the tripeptide Phe-Gly-Pro, is about 50-fold more selective for the C-domain of ACE and was the first ACE inhibitor to display notable domain-selectivity (Table 1) [22]. The domain-selectivity of this inhibitor has subsequently been improved by adding a bulky hydrophobic group at the P₂' position [73]. For example, changing the P₂' Pro to a Trp or Phe causes the domain selectivity to increase to about 250-fold in favor of the C-domain. The selectivity of the keto-ACE derivatives is likely due to interactions between the aromatic P₂' group and the S₂' residues Glu367 and Val380 in the C-domain [74]. These interactions are lost in the N-domain where the residues in question are replaced by the polar residues Asp354 and Thr358, respectively. It has been suggested that hydrophobic interactions

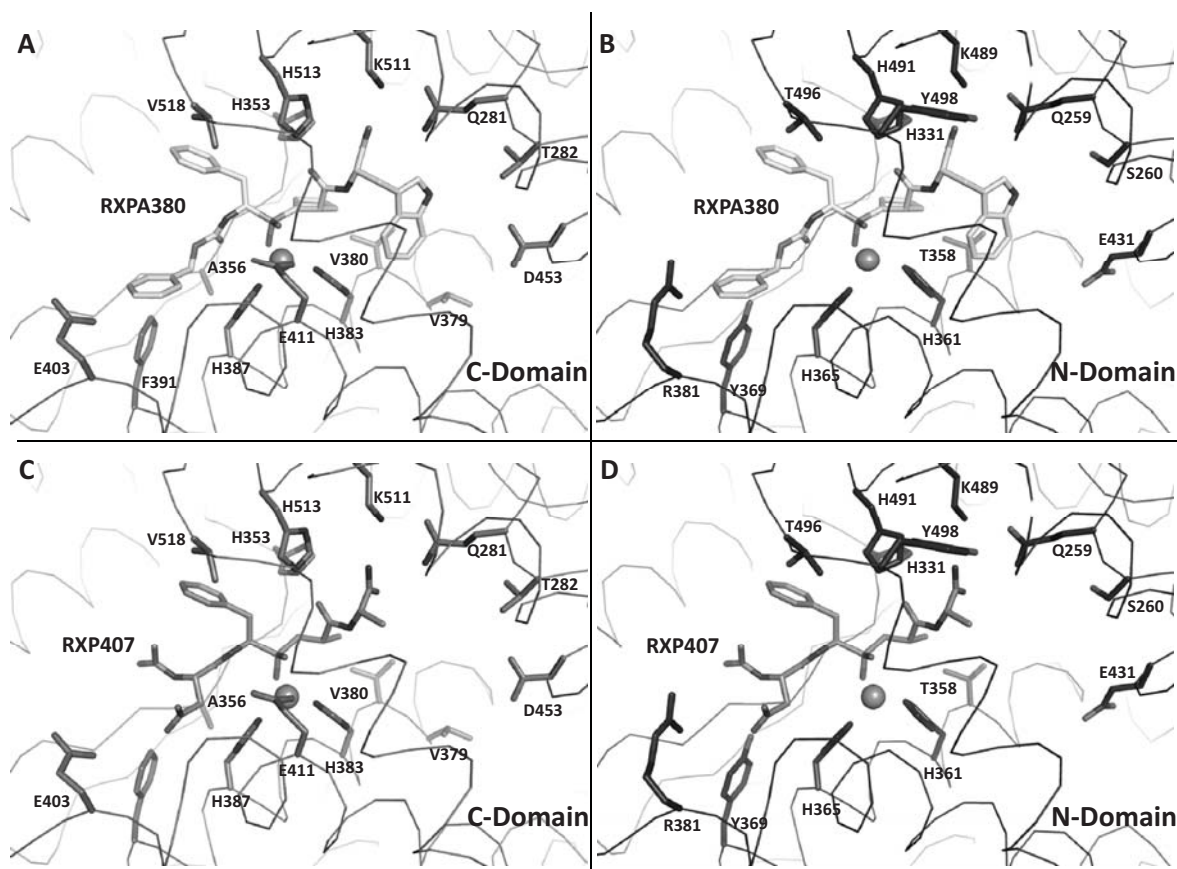


Fig. (4). Stick representation of the active site residues involved in inhibitor selectivity for the C-domain (phosphinic peptide RXPA380) [77] and N-domain (phosphinic peptide RXP407) [58] of ACE. **A)** The C-domain specific inhibitor RXPA380 is shown; **B)** structure-based superposition of RXPA380 molecule in the active site of N-domain; **C)** structure-based superposition of RXP407 molecule in the active site of C-domain and **D)** The N-domain specific inhibitor RXP407 is shown.

mediated by C-domain residues Phe391 (S_2) and Val518 (P_1) also contribute to C-domain selectivity of the ketoACE derivatives, as these residues are replaced by more polar amino acids in the N-domain (Tyr369 and Thr496 respectively) [74].

The bradykinin potentiating peptide pGlu-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro (BPPb) also shows about 260-fold C-domain selectivity (Table 1) [75]. The selectivity of the BPPs' is thought to be mediated by the P_2 residue. Thus, the presence of the P_2 Lys in BPPb, as opposed to the Gln, Pro and Pro in BPPa, BPP2 and BPPc respectively, drives the C-domain selectivity of this peptide.

The most potent C-domain selective inhibitor to date is the phosphinic peptide analogue RXPA380, which is approximately 2000-fold more C-domain selective (Table 1) [76]. Based on the crystal structure of C-domain in complex with RXPA380 inhibitor [77] and mutagenesis studies, the potency of this inhibitor has been attributed largely to hydrophobic interactions between the P_2 group and Phe391, which is replaced by the more polar Tyr369 in the N-domain may present a steric clash with the P_2 Phe of RXPA380 binding (Figs. 4A and B) [70]. Additionally, the C-domain S_2 pocket residue Glu403 is replaced by Arg381 in the N-domain, and may also cause steric clash with the P_2 group, further contributing to the C-domain selectivity of this inhibitor. Interestingly, Asp354 and Glu403, which were implicated in the selectivity of the keto-ACE derivatives, did not appear to affect domain selectivity of RXPA380 [70].

However, the future of C-domain selective inhibitors has been coloured by recent murine studies which have shown that genetic

inactivation of the C-domain did not result in a decrease in blood pressure, due to a compensatory mechanism involving the upregulation of renin [71]. This upregulation of renin allows for the maintenance of regular blood pressure, despite the absence of the C-domain activity [71,78]. Thus, it is possible that the use of C-domain selective ACE inhibitors for the treatment of chronic hypertension may not be as effective as initially thought. The development of dual C-domain/endothelin converting enzyme inhibitors, however, may be one possible solution to this problem.

5. DUAL ACE-ECE INHIBITORS

Apart from ACE, neutral endopeptidase (neprilysin, NEP, EC 3.4.24.11) and endothelin converting enzyme-1 (ECE-1, EC 3.4.24.71) also exert vasoconstrictive and vasodilatory activities. Hence both NEP and ECE-1 have proven to be suitable targets for the treatment of cardiovascular disease [7,79-83]. However, considerable side-effects have been documented, most likely due to elevated levels of bradykinin [45,84]. Moreover, it is now known that ACE inhibition can induce an increase in chymase activity in cardiac interstitial fluid and hence provides a different mechanism for the accumulation of angiotensin-II [52]. Hence, blood pressure control remains sub-optimal in a significant proportion of patients on this therapy [85], providing a compelling case for the development of new improved strategies. To address this concern, potent vasopeptidase inhibitors able to target ACE and NEP were developed [86,87], followed by dual NEP/ECE-1 inhibitors and ultimately triple inhibitors blocking ACE/NEP/ECE-1 simultaneously [88]. Even though the dual ACE/NEP inhibitor

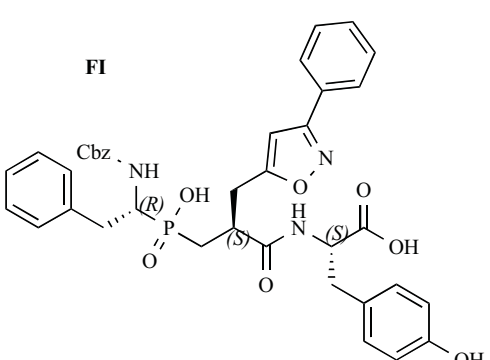
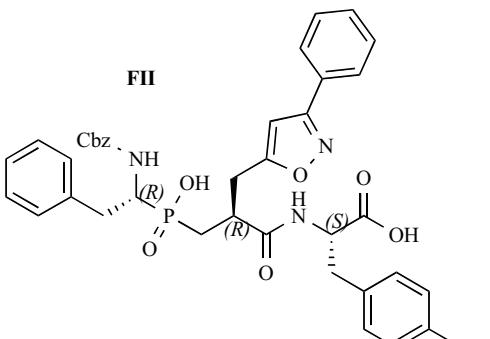
Inhibitor	Apparent K_i (nM)			
	ACE C-domain	ACE N-domain	NEP	ECE-1
 <p>FI</p>	0.41±0.03	180±25	172±24	275±60
 <p>FII</p>	0.65±0.03	150±20	6700±300	14±2

Fig. (5). Chemical structures of ACE/ECE-1 dual inhibitors FI and FII and their potency. Compound FI adopts *S* configuration and compound FII adopts *R* configuration [90].

omapatrilat was more clinically efficacious than a single ACE inhibitor, the higher incidence of angioedema observed in patients treated with omapatrilat halted the development of omapatrilat and raised concerns about the risk/benefit ratio of dual ACE/NEP inhibitors for therapeutic applications [89]. While the level of angioedema associated with triple ACE/NEP/ECE-1 inhibitor treatment is yet to be evaluated, it has been suggested that NEP inhibition (in the context of either dual or triple inhibitor treatment) might be responsible for the increase in adverse effects [88]. Indeed it has been established that inhibition of NEP results in increased levels of bradykinin and endothelin-I (ET-I). These data initiated the development of novel inhibitors that interact with the C-domain of ACE and ECE-1 [90]. This led to the discovery of the first potent and selective dual C-domain ACE/ECE-1 inhibitors and revealed that the configuration of the P1' residue was a key factor in the control of inhibitor selectivity (Fig. 5). Instead of the classical *S* configuration of the inhibitor's P1' group (corresponding to an L-amino acid, as observed in all ACE inhibitors known to date) (compound FI in Fig. 5), it was discovered that an *R* configuration in compounds (compound FII in Fig. 5) containing long and bulky P1' side chains was well accommodated by ACE, as well as by ECE-1, but much less so by NEP [90]. Furthermore, in a spontaneous hypertensive rat (SHR) model, an intravenous administration of C-domain ACE/ECE-1 dual inhibitor (FII) (10 mg/kg) lowered the mean arterial blood pressure by 24 ± 2 mm/Hg as compared with controls [90]. Thus, targeting both ACE and ECE-1 lowers plasma concentrations of angiotensin-II and endothelin-I, the two most potent vasoconstrictive peptides without increasing the bradykinin levels.

A structural basis for the binding of a highly potent and selective dual ACE/ECE-1 phosphinic inhibitor was provided by the first structures in complex with the C- (at 1.97 Å) and N-domain of ACE (at 2.15 Å) [57]. The structural determinants revealed the

unique features of the binding of two molecules of the dual inhibitor in the active site of the C-domain of ACE (Figs. 6A and C) and one molecule in the active site of the N-domain of ACE (Figs. 6B and D), respectively. In both structures, the first molecule was positioned in the obligatory binding site and has a bulky bicyclic P1' residue with the unusual *R* configuration which, surprisingly, was accommodated by the large S2' pocket (Fig. 6). In the C-domain complex, the isoxazole phenyl group of the second molecule makes strong *pi-pi* stacking interactions with the amino benzoyl group of the first molecule locking them in a 'hand-shake' conformation. These features for the first time highlighted the unusual architecture and flexibility of the active site of the C-domain of ACE, which could be further utilized for structure-based design of new C-domain or vasopeptidase inhibitors.

6. LESSONS IN ACE INHIBITOR DESIGN FROM NATURAL PRODUCTS

6.1. ACE Inhibitors that Bind to the Non-Prime Binding Site

The naturally occurring phosphonotripeptide K-26 was initially discovered by fractionation of extracts of a soil dwelling prokaryote, "actinomycete strain K-26". Although its target among soil bacterium is unknown, K-26 is a potent ACE inhibitor (comparable to captopril) containing an unusual α -amino phosphonic acid analogue of tyrosine [91,92]. Recently, it has also been determined that phosphoryl substitution is a critical determinant of activity, resulting in a 1500-fold increase in ACE inhibition when compared to its carboxyl analogues [93]. In addition, the absolute configuration of the terminal α -amino phosphonate and *N*-acetylation were found to be important for modulation of ACE inhibitory activity. Recently, a structural basis of K-26 binding was provided through a detailed 3D structure of

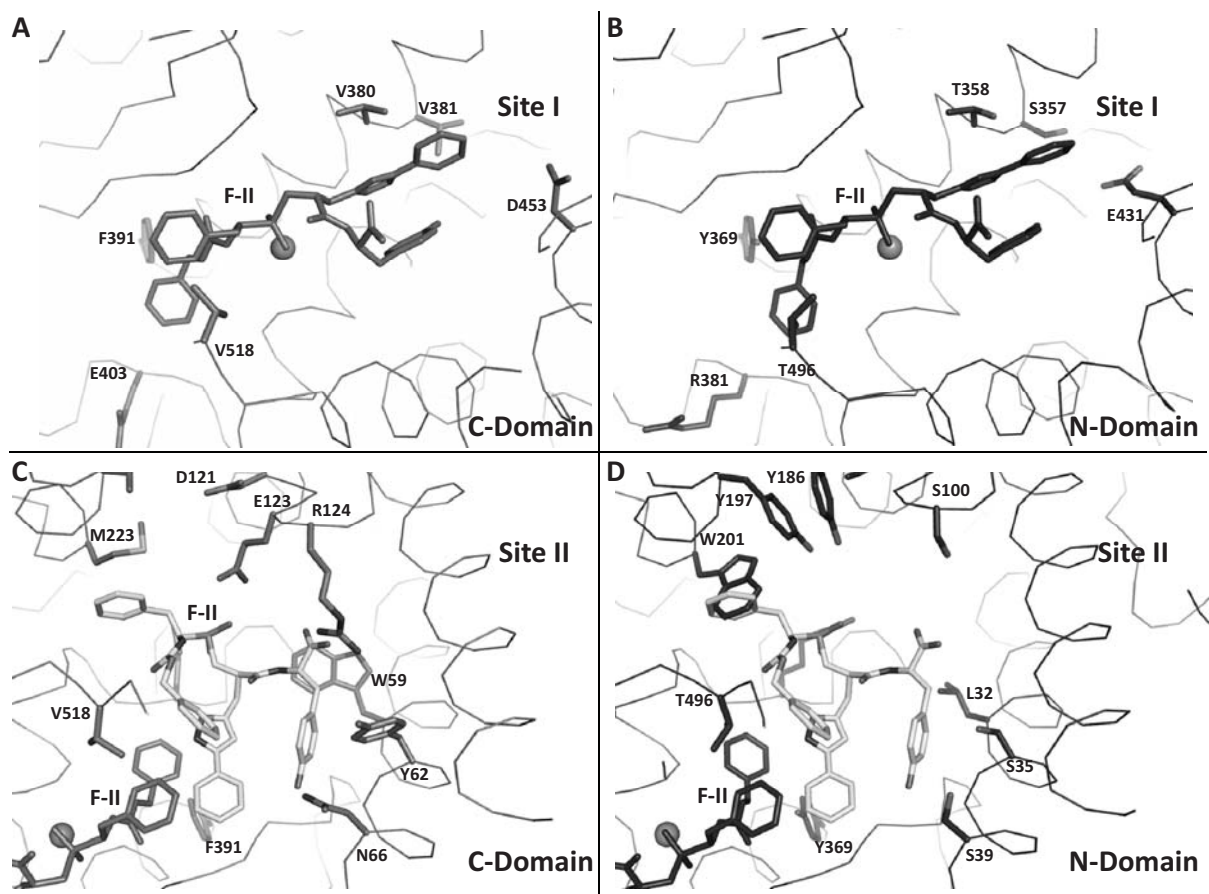


Fig. (6). Details of FII binding in C- and N-domain ACE active sites [57]. **A)** Interaction for C-domain with the dual inhibitor at the primary site FII (site I). **B)** Interaction for N-domain with the dual inhibitor at the primary site FII (site I). **C).** Interaction of C-domain with dual inhibitor at the secondary binding site FII (site II) and **D)** Comparison of residues at the N-domain active site which precludes the binding of FII at site II.

AnCE (a homologue of ACE from *Drosophila melanogaster* with a single enzymatic domain) with K-26 at 1.96 Å resolution [94]. The structure revealed that the inhibitor K-26 only occupies the S₂ and S₁ pockets (i.e., non-prime pockets of the active site). Based on this complex it was possible to predict how K-26 might interact with the N- and C-domain active sites in human somatic ACE. Thus the new structure provides useful information for further exploration of ACE inhibitor pharmacophores involving phosphonic acids and other K-26 related natural products and analogues.

6.2. Vitamin B₁₂ Derivatives as Possible ACE Inhibitors

While captopril is not used as widely as other ACE inhibitors due to its side effect profile, sulfhydryl containing ACE inhibitors are advantageous in terms of their ability to stimulate nitric oxide activity and reduce oxidative stress in hypertensive patients. Mukherjee *et al.* [95] recently reported that the synthesis of captopril-cobalamin (a derivative of vitamin B₁₂ in which captopril is bound *via* its thiol group at the β-axial site of cobalamin) could improve captopril in terms of absorption, cellular uptake and tissue penetration. Moreover, it is likely that the compound would have less adverse reactions arising from its sulfhydryl group. Structural characterisation of this molecule by X-ray diffraction and nuclear magnetic resonance (NMR) spectroscopy showed the formation of two isomers of the inhibitor which differ in the stereochemistry of captopril – the *trans* isomer is formed preferentially in the solid state. This study provides an additional avenue for the exploration of non-conventional vitamin B₁₂ derivative/s as potential ACE inhibitor/s.

7. CONCLUSIONS

ACE inhibitors have long been established as a frontline therapy in the treatment of hypertension and look set to remain a standard treatment for this disease in the foreseeable future. Recent advances have led to the development of domain-selective ACE inhibitors, which may provide new therapeutic avenues for ACE inhibition. These include C-domain selective inhibitors, which may offer improved side-effect profiles as compared with conventional ACE inhibitors, as well as N-domain selective inhibitors, which may prove useful in the treatment and prevention of fibrosis.

The development of dual inhibitors is an approach to inhibitor design that has gained a lot of attraction in the drug discovery industry. While clinical studies showed ACE/NEP dual inhibitors were more efficacious than single ACE inhibitor treatment, the increase occurrence of adverse effects have halted the progression of lead compounds to the market. However an ACE/ECE-1 dual inhibitor (selective for the C-domain of ACE) has shown promising results in short-term SHR experiments. In addition, treatment with both ACE and chymase inhibitors showed improved efficacy compared to ACE inhibitor monotherapy in an animal model, indicating that there is potential for an ACE-chymase dual inhibitor. Thus, dual ACE inhibitors may well usher in a new line of hypertensive treatments, further extending the scope of ACE inhibiting drugs used in the treatment of cardiovascular and other disease. Undoubtedly, the use of the 3D structures of these enzymes will be a vital aid in the successful design of such dual ACE inhibitors.

The development of easily crystallisable forms of both ACE domains has opened the way for more high throughput analysis of enzyme-inhibitor complexes, which will hopefully allow for increased development of rationally designed ACE inhibitors, particularly with respect to domain selective and dual ACE inhibitors.

Additionally, the identification of ACE inhibiting compounds in natural products may help develop novel lead compounds and shed light on the evolution of natural product biosynthetic gene clusters. The analysis of natural product libraries may be particularly insightful when combined with a structure-based screening process, potentially helping to identify a range of novel ACE inhibitors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ACE	=	Angiotensin-I converting enzyme
tACE	=	testis angiotensin-I converting enzyme
sACE	=	somatic angiotensin-I converting enzyme
Ang 1-7	=	angiotensin (1-7)
(A-beta42)	=	amyloid beta peptide
CHO	=	chinese hamster ovary
ECE-1	=	endothelin converting enzyme-1
GnRH	=	gonadotropin-releasing hormone (GnRH)
LHRH	=	luteinizing-hormone-releasing hormone
NB-DNJ	=	N-butyldeoxynojirimycin
NEP	=	neprilysin
NMR	=	nuclear magnetic resonance
CVD	=	cardiovascular disease
RAAS	=	renin-angiotensin aldosterone system
AcSDKP	=	N-acetyl-seryl-aspartyl-lysyl-proline
RXPA380	=	(Cbz-PheΨ[P(O)(OH)CH]Pro-Trp-OH)
RXP407	=	(Ac-Asp-(L)Phe(PO ₂ CH ₂)(L)Ala-Ala-NH ₂)
RMSD	=	root mean square deviation
SHR	=	spontaneous hypertensive rat

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