Chapter 15
Structure-Based Design of Domain-Selective Angiotensin-Converting Enzyme Inhibitors

Ross G. Douglas and Edward D. Sturrock

Abbreviations

ACE	Angiotensin-converting enzyme
AcSDKP	*N*-acetyl-serylaspartyllysylproline
ADE	Adverse drug event
AngI	Angiotensin I
AngII	Angiotensin II
Ang1-7	Angiotensin 1-7
AT_1R	Angiotensin type 1 receptor
AT_2R	Angiotensin type 2 receptor
BK	Bradykinin
BPF	Bradykinin-potentiating factor
CPA	Carboxypeptidase A
CVD	Cardiovascular disease
kAF	keto-ACE Phe inhibitor
kAW	keto-ACE Trp
LisW	Lisinopril Trp
PDB	Protein Data Bank (http://www.rcsb.org/pdb)
RAAS	Renin-angiotensin-aldosterone system
tACE	Testis angiotensin-converting enzyme
WHO	World Health Organisation
ZMRG	Zinc Metalloprotease Research Group

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15.1 Introduction

Cardiovascular disease (CVD) accounts for a large number of deaths and is a major cause for morbidity and disability worldwide. While the occurrence of CVD is global, statistics indicate that an alarming 80% of cases occur in developing nations [1]. The prevalence of CVD in such countries warrants a combined effort in the establishment of hygiene education, primary health-care facilities, improved monitoring systems and the development of improved and cost-effective treatments for those afflicted with CVD.

Hypertension is a major risk factor in the development of CVD, as illustrated by a decrease in incidence of adverse cardiovascular events (including stoke and myocardial infarction) when blood pressure is controlled. As already stated, hypertension affects predominantly developing nations, with Africa being no exception. In 1998, approximately 21% of the South African adult population was estimated to have hypertension [2], with general sub-Saharan data estimates at approximately 27% of the adult population [1]. Ten years later, even with the increased knowledge of vasoaction, CVD and antihypertensive drug design, the World Health Organisation (WHO) estimates that approximately 37% of adult South Africans have raised blood pressure (SBP $\geq$ 140 or DBP $\geq$ 90) [3].

Given the prevalence of hypertension in sub-Saharan Africa, and given the diversity of populations present, African researchers have a distinct responsibility in the development of suitable technologies and appropriate strategies to combat this dangerous risk factor. Certainly, the employment of early warning diagnosis, education and reputable primary health-care facilities are indispensable to prevention and early intervention; however, the use of necessary therapies is vital in the continuing efforts to minimise this threat. The use of angiotensin-converting enzyme (ACE) inhibitors has made a substantial contribution in the control of blood pressure [4]. However, the prevalence of adverse drug events (ADEs) associated with current ACE inhibitor treatment has necessitated the need to develop second-generation ACE inhibitors. Interestingly, for factors currently not fully understood, individuals of African origin can tend to have increased risk of occurrence of these drug events including life-threatening angioedema and persistent cough [5, 6]. Also, second-generation ACE inhibitors could be efficacious in the treatment of conditions not limited to blood pressure control [7]. Thus, the Zinc Metalloprotease Research Group (ZMRG) at the University of Cape Town, South Africa, has been investigating a single piece of the cardiovascular puzzle: the structure–function relationship of ACE with emphasis on the design of domain-selective ACE inhibitor drug candidates.

15.2 The Renin-Angiotensin-Aldosterone System: A Key System in Blood Pressure Regulation

The renin-angiotensin-aldosterone system (RAAS) is one of the central pathways in the regulation of blood pressure [8]. Angiotensinogen is a 55-kDa protein expressed in the liver, and an N-terminal decapeptide of this protein is released through

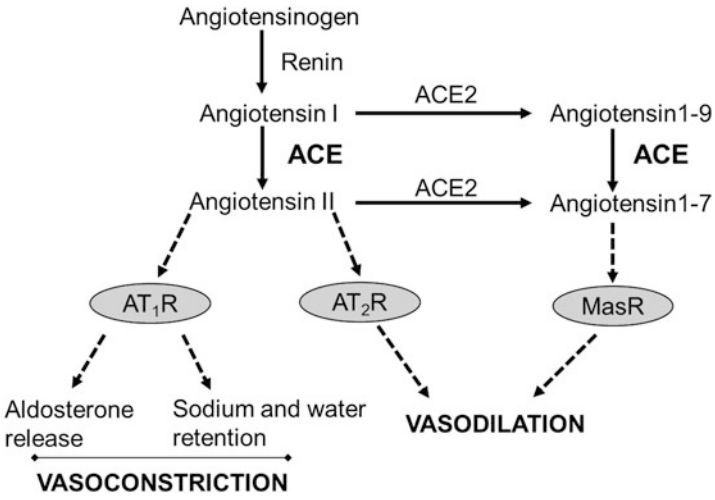

Fig. 15.1 A schematic representation of the renin-angiotensin-aldosterone system (RAAS). Angiotensin I is produced through the proteolytic action of the enzyme renin, and angiotensin I, in the classic pathway, is subsequently converted to angiotensin II (AngII) by ACE (indicated in *bold font*). AngII mediates its vasconstrictive effects through the AT type 1 receptor (AT_1R) due to the predominant tissue distribution of this receptor over the type 2 isoform. Recently added elements of the RAAS include the production of angiotensin1–9 and angiotensin1–7 (Ang1–7) by the action of ACE homologue ACE2. Ang1–7 mediates vasodilatory effects through the Mas receptor and thus serves as a counter-regulatory arm of this system

the action of the highly specific aspartyl protease renin [9]. This N-terminal decapeptide, referred to as angiotensin I (AngI), is an inactive form of the angiotensin hormone and is cleaved at the penultimate C-terminal peptide bond by ACE to yield the vasoactive octapeptide angiotensin II (AngII) [10, 11]. AngII binds and mediates its vasoconstrictive action predominantly through the angiotensin type 1 receptor (AT_1R). Downstream signalling from this receptor interaction promotes aldosterone release and subsequent water and sodium retention, thus resulting in vasoconstrictive events [4, 12]. While a type 2 receptor also exists that mediates vasodilatory events, the predominant tissue expression and distribution of AT_1R in the adult cardiovascular system accounts for a net increase of systemic blood pressure upon AngII production [13] (Fig. 15.1).

In addition to the above classical description of the RAAS, other angiotensin peptides have been discovered to also play a regulatory role within this system. Of perhaps greatest relevance is Ang1–7, a peptide produced either by the carboxypeptidase action of ACE homologue ACE2 on AngII or by the initial cleavage of AngI by ACE2 with subsequent cleavage by ACE [14] (Fig. 15.1). Ang1–7 mediates its effects through the Mas receptor [15] and promotes vasodilatory actions, thereby serving as a counter-regulatory arm of the RAAS [16].

The use of gene knockout technology in the production of mice lacking certain aspects of the RAAS has provided vital information regarding the importance of

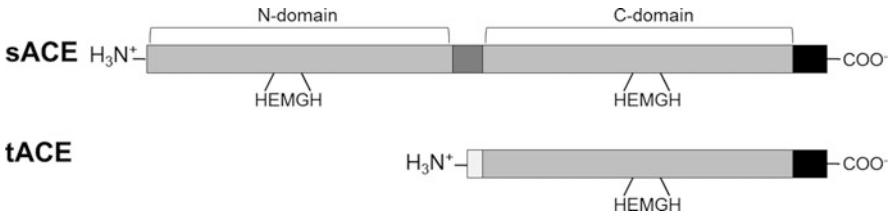

Fig. 15.2 The domain assembly of angiotensin-converting enzyme (ACE) isoforms. Each domain is presented in *light grey* with the zinc coordinating motif crucial for catalytic function (HEMGH) indicated. In somatic ACE (sACE), the linker region between the two domains is shown in *dark grey* and the C-terminal transmembrane and cytoplasmic tail in *black*. Testis ACE (tACE) is identical to the C-domain of sACE with the exception of a unique 36 amino acid sequence on the N-terminus (*white*)

the RAAS elements in the regulation of blood pressure and general physiological function. Mice containing increasing numbers of angiotensinogen gene copies tended to display increased blood pressure [17]. Mice homozygous for renin knockout showed significantly reduced blood pressure compared to controls [18], as did mice lacking AT_1R expression [19]. Similarly, homozygous ACE knockout mice displayed significantly reduced blood pressure, renal defects and fertility compared to wild-type mice [20, 21]. Thus, while there are other systems contributing to blood pressure regulation, such models emphasise the central importance of the RAAS in the systemic regulation of blood pressure.

15.3 Angiotensin-Converting Enzyme: A Central Figure in the RAAS

15.3.1 General Background

ACE belongs to the gluzincin family (MA clan) of metallopeptidases and contains a characteristic HEXXH zinc-binding motif critical for the catalytic mechanism of substrate cleavage [22]. Furthermore, it is a zinc dipeptidyl carboxypeptidase and, while best known and named for its cleavage of AngI, possesses the ability to cleave a diverse set of substrates and even displays endopeptidase activity *in vitro* [23–26]. ACE is heavily glycosylated and such a posttranslational modification is important for correct intracellular folding prior to export, thermal stability and homodimerisation [27–30]. Somatic ACE is 1,277 amino acids in length and expressed in many human tissues, especially vascular endothelial cells [31]. Interestingly, somatic ACE possesses two homologous domains (designated N- and C-domains based on their location in the polypeptide chain), each of which contains a functionally active catalytic site (Fig. 15.2) [31, 32]. Comparison of overall sequence identity between the N- and C-domains reveals approximately 60% similarity. In the domain active sites regions, the sequence identity is even more

conserved with 90% sequence similarity. With this being said and perhaps surprisingly, despite high-sequence identity and similar structural topology between the domains, differences are noticed in domain-specific substrate processing and inhibitor-binding profiles (see Sects. 15.3.2, 15.6.2 and 15.6.4). An appreciation of the differential functioning of each domain has prompted efforts to develop domain-selective inhibitors that could be useful in distinct disease states.

15.3.2 Pertinent Substrates of ACE

15.3.2.1 Vasoactive Peptides: Angiotensin I and Bradykinin

ACE was first isolated and characterised on the basis of the enzyme's ability to convert AngI (DRVYIHPFHL) to produce the potent vasoconstrictive octapeptide AngII (DRVYIHPF) [10, 11]. While vasoconstriction is one response from AngII production, the downstream effects of AT_1R activation also result in increased cell proliferation, hypertrophy and fibrosis, thus making ACE a promising drug target for the treatment of disorders relating to vascular function [33].

Bradykinin (RPPGFSPFR, BK) is a nonapeptide member of the kallikrein-kinin system and is currently the best physiological substrate of ACE, with a tenfold higher catalytic efficiency than AngI conversion [34]. Contrasting AngII action, BK mediates nitric oxide release and prostaglandin synthesis that promotes vasodilation [35]. ACE is able to abolish the vasodilatory ability of this peptide through its sequential dipeptidase activity. Thus, to put it simply, ACE activity serves to result in a net increase in blood pressure through the introduction of vasoconstrictive and removal of vasodilatory peptides.

Gene modification technologies that allow for the generation of mice that contain only one functional domain of the somatic ACE molecule have provided important in vivo understanding of the domain contribution in substrate processing. BK has been shown to be cleaved with approximately similar catalytic efficiencies by both domains [36, 37]. In contrast, the C-domain has been shown to be the primary site for the conversion of AngI [37].

15.3.2.2 *N*-acetyl-Ser-Asp-Lys-Pro: Another Important ACE Substrate

While AngI and BK are certainly the most celebrated substrates of ACE, others exist that could be important in disease progression or prevention thereof. These include gonadotropin-releasing hormone and amyloid β-peptide, although the *in vivo* role of ACE cleavage of these peptides remains controversial [24, 38]. *N*-acetyl-Ser-Asp-Lys-Pro (AcSDKP) is a negative regulator of haematopoiesis and a potent anti-fibrotic agent. ACE is the principal enzyme involved in the cleavage of AcSDKP [39]. Numerous mouse models of hypertension have provided strong support for the role of AcSDKP in minimising collagen deposition in the heart

and kidney through peptide infusion or ACE inhibition and thus suggest an important role of this peptide in tissue injury [40–47].

Cleavage of AcSDKP is highly N-domain specific both *in vitro* and *in vivo* [48, 49]. Mice lacking a functional N-domain catalytic site displayed increased plasma concentration of AcSDKP, as did mice treated with an N-domain-selective inhibitor [49, 50]. N-domain active site knockout mice also showed increased protection against bleomycin-induced lung fibrosis, with this protection being linked to increased AcSDKP levels [51].

With an understanding of the centrality of ACE substrate cleavage in the regulation of vascular function, it is not surprising that ACE was identified as a promising antihypertensive drug target.

15.4 First-Generation ACE Inhibitors

The discovery of the first orally active ACE inhibitors in the late 1970s is one of the classic examples of structure-based rational drug design that appears in most medicinal chemistry textbooks. However, remarkably, these inhibitors were successfully developed with no knowledge of the enzyme's three-dimensional structure and instead were based on an assumed mechanistic homology to carboxypeptidase A (CPA). There are several excellent reviews of the discovery and development of the first-generation ACE inhibitors captopril, lisinopril and enalaprilat that have been published over the last 30 years (Fig. 15.3) [4, 52–57]. Thus, the intention of this chapter is not to provide a comprehensive review of this fascinating story, but to highlight some of the key events and insights in the context of the structural data that has been elucidated more recently in our laboratory and those of our collaborators.

The key discovery of Kevin Ng and John Vane that AngI is converted to AngII in the lung [58] and the speculation that the enzyme responsible was a carboxypeptidase sparked a research programme at the pharmaceutical company Squibb to find an inhibitor of the candidate enzyme with potential application in hypertension. Ferreira et al. convincingly showed that snake venom contained inhibitors of the degradation of both bradykinin and AngI [59]. At Squibb, Miguel Ondetti and David Cushman built on these findings and were the first to synthesise the potent snake venom inhibitor SQ20881—the same as the nonapeptide bradykinin-potentiating factor (BPF) teprotide. This peptide was rapidly progressed into clinical trials and shown to decrease blood pressure in hypertensive patients [60]. A key insight derived from work on the BPFs was that the C-terminal sequence Phe-Ala-Pro and its acylated derivatives provided optimal enzyme inhibition. The next challenge was to synthesise orally active, non-peptide analogues of BPF, and the programme at Squibb was temporarily halted in 1973 due to failure to achieve this objective.

One of the key breakthroughs in the development of the first antihypertensive drug captopril was the hypothesis that ACE is structurally and mechanistically related to CPA. This led Ondetti and Cushman to propose a model of the ACE

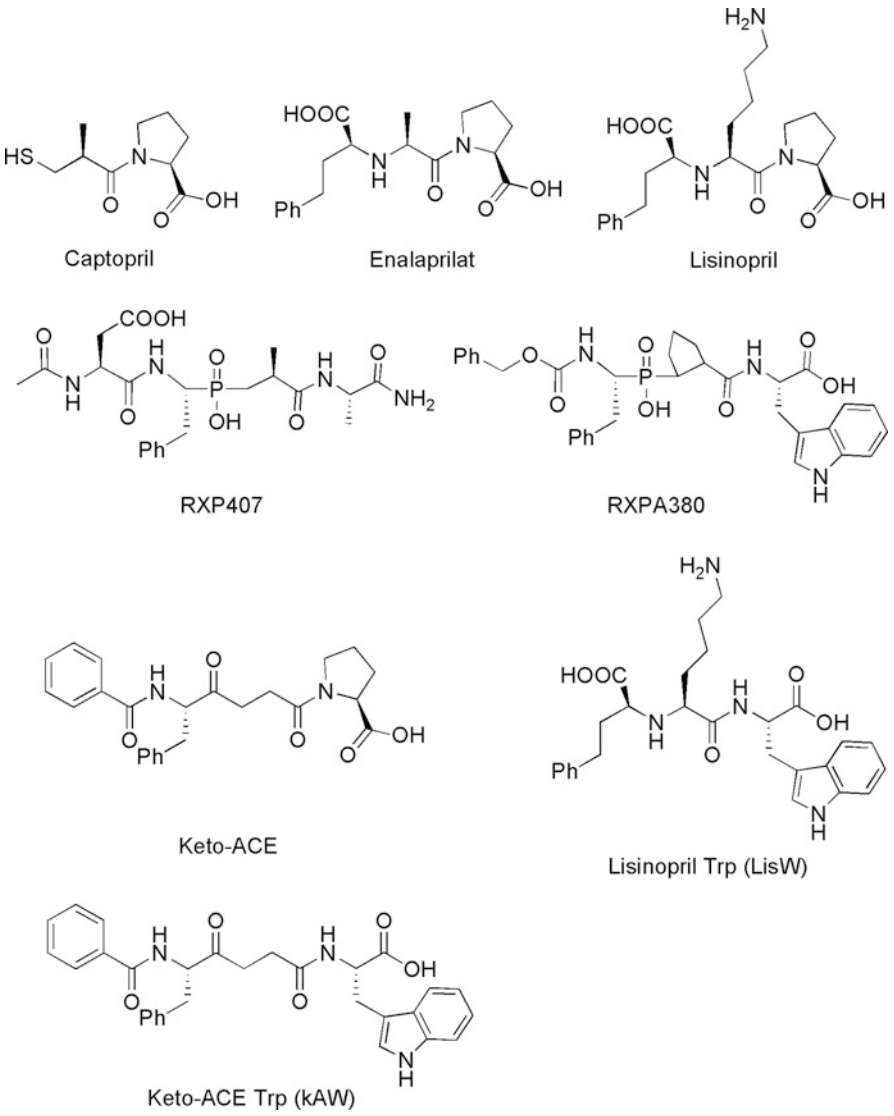

Fig. 15.3 Chemical structures of a selection of ACE inhibitors

active site with the critical zinc ion positioned to activate the scissile carbonyl of peptidic substrates. This key concept, together with work by Byers and Wolfenden showing that D-2-benzylsuccinic acid was a potent by-product inhibitor of CPA [61], prompted the design of similar succinyl amino acid derivatives with analogous structures to the dipeptide product of ACE hydrolysis. On the basis of the BPF Phe-Ala-Pro sequence, Ondetti synthesised methylsuccinyl Pro which was a specific inhibitor of ACE with an IC_{50} in the low micromolar range. By replacing the

carboxyl zinc-binding group with a sulfhydryl, Cushman and Ondetti were able to improve the potency of the inhibitor and thus the competitive inhibitor captopril with a K_i of 1.7 nM was launched (Fig. 15.3) [62, 63]. Its proposed contacts with the active site S_1' and S_2' pockets and the interaction of the prolyl carboxylate with a positively charged residue are remarkably similar to those observed in the captopril-ACE crystal structure almost 30 years later [64]. Captopril was approved for clinical use in 1981 and widely used for the treatment of hypertension and congestive heart failure. However, various ADEs caused by the sulfhydryl group led to renewed efforts by Patchett and associates at Merck to design tripeptide mimetics with a carboxyl zinc-binding group [65]. Like captopril, their efforts were based on the work of Byers and Wolfenden, and they reasoned that it should be possible to raise the potency of their early lead carboxylate compounds by making them more similar to the C-terminal peptides of BPF. To achieve this, an amine group was introduced into the backbone of the molecule affording the *N*-carboxymethyl derivative of Ala-Pro. Disappointingly, this compound had a similar inhibition constant to its CH_2 analogue. The realisation that compensating hydrophobic effects likely influenced these results prompted the addition of a P_1 methyl followed by a P_1 Phe that had vastly improved IC_{50} values of 92 nM and 3.8 nM, respectively [65]. It was thought that these residues made contact with a hydrophobic surface in the S_1 pocket, and once maximal interactions had been made, changes to affect lipophilicity are tolerated because these groups do not interact with the active site. The Phe-Ala-Pro compound (enalaprilat, Fig. 15.3) had poor oral activity [66]; however, conversion of the zinc-binding group to an ethyl ester (enalapril) altered the lipophilicity of the compound and dramatically improved oral availability. The approach of synthesising a prodrug that required hydrolysis of the ester in the liver to yield the active free acid was subsequently used with a number of ACE inhibitors that are presently in clinical use. Substitution of a P_1' Lys for the Ala in enalaprilat yielding the Phe-Lys-Pro analogue (lisinopril, Fig. 15.3) also improved the oral activity resulting in longer duration of action and no requirement for metabolic activation.

With the benefit of the three-dimensional crystal structures of enalaprilat-ACE and lisinopril-ACE [64, 67], it is now clear that the postulated hydrophobic interactions of the amino-terminal Phe of the inhibitors, made by the Merck group, are validated by contacts with Phe^{512} and Val^{518} in the S_1 subsite. In the lisinopril-ACE complex, the P_1' lysyl amine, which occupies the deep S_1' pocket, interacts ionically with Glu^{162} and with Asp^{377} via water-mediated interactions. Interestingly, Glu^{162} is replaced by an Asp in the N-domain, and this amino acid substitution is likely responsible for lisinopril's modestly increased affinity for the C-domain active site [68] (see Sect. 15.6.3 on C-selective inhibitors). Enalaprilat and captopril, on the other hand, have a methyl in the P_1' position which does not make significant contact with any of the S_1' residues. The interaction of the C-terminal carboxylate with an arginine in the case of carboxypeptidase was shown to be with Lys^{511} in the C-domain, consistent with the notion that this interaction was important for the correct positioning of the scissile bond carbonyl with respect to the catalytic zinc. Finally, the hypothesis that large conformational changes of the Ala-Pro in captopril and enalaprilat occurred on binding to the active

site of ACE led to the design and synthesis of numerous conformationally restricted analogues [69]. These compounds were created by bridging a carbon chain between the P_1' methyl and the prolyl C-5. Medium-sized lactams were predicted to be good mimics of the low-energy conformations of Ala-Pro. These bulky conformationally restricted analogues were well accommodated by the prime side of the ACE active site, and this is in agreement with the architecture of the cavernous S_1'/S_2' subsite revealed in the crystal structure.

The successful development of these first-generation ACE inhibitors was achieved prior to the discovery of ACE containing two catalytically active and differentially functioning domains [31, 32, 37, 49]. These inhibitors, while they interact slightly differently with each domain, lack any significant domain selectivity [68]. The high-affinity binding of inhibitors to both domain active sites is thought to be a contributor of the observed ADEs associated with treatment. While the mechanisms of ACE inhibitor associated ADEs are complex, the elevation of BK due to dual domain blockade has been implicated as a key contributor to these events [70–72]. As presented above, BK is cleaved at approximately the same rate by both domains. Thus, the selective inhibition of a single domain would allow the other active site to be involved in BK clearance and potentially lower ADE incidence. This would allow for inhibitors that could have efficacious effects in treatment of hypertension (C-domain-selective inhibitors) or pulmonary fibrosis (without affecting blood pressure, N-domain-selective inhibitors) with reduced side effect profiles [4, 7]. The development of such inhibitors has been a strong research focus in our laboratory and stands on a strong tradition of ACE research in South Africa.

15.5 Historical African Contributions

One of the first South African studies on ACE started at almost the same time as the first clinical ACE inhibitors became available. The high incidence of tuberculosis and sarcoidosis in the Western Cape prompted the evaluation of serum ACE levels in disease activity in relation to remission and steroid therapy [73]. The serum ACE assay was shown to provide a good monitor of disease activity and found to be useful in the management of patients with sarcoidosis. Serum ACE is today still used as a marker for sarcoidosis; however, ACE levels probably reflect the total granulomatous burden rather than the degree of lung involvement by sarcoidosis [74].

This work was followed by a detailed biochemical investigation of the somatic form of ACE which was also carried out in the Department of Medicine at the University of Cape Town. The paradigm of collaborating with the Department of Chemistry in an effort to solve ACE-related biological chemistry problems was initiated by Mario Ehlers who synthesised a novel N-carboxyalkyl dipeptide CA-Gly-Gly that was used for the rapid affinity purification of human lung and kidney ACE [75]. The weak competitive ACE inhibitor CA-Gly-Gly had a terminal amino group available for the covalent attachment to Sepharose. This approach, together with the use of CA-Phe-Gly [76], revolutionised the previously tedious and

time-consuming purification of ACE. In addition, the CA-Gly-Gly ligand could be prepared by a relatively simple and efficient 4-step synthetic procedure [77]. CA-Gly-Gly was one of the weakest inhibitors in the N-carboxyalkyl dipeptide class, and thus ACE could be easily eluted from a CA-Gly-Gly affinity resin under mild conditions without denaturing the enzyme. Today, this purification method has been adapted and the N-carboxyalkyl dipeptide replaced with the highly specific ACE inhibitor lisinopril which is coupled to Sepharose via its lysyl amine [78, 79]. Ehlers went on to kinetically characterise the unique chloride activation of human ACE [80]. The effect of chloride on ACE-catalysed hydrolysis of AngI was shown to be complex and dependent on both chloride concentration and pH. In addition, at high chloride concentrations, the enzyme was inhibited by chloride by a complex mechanism which may involve more than one low-affinity inhibitory binding site on free enzyme, enzyme–substrate complex and/or enzyme–product complex. This *in vitro* study suggested that ACE is probably maximally activated in the vascular endothelium and renal proximal tubular epithelium, since at these sites, pH and chloride concentration are optimal and do not fluctuate. However, in the intestinal microvilli that contain large amounts of ACE, the enzyme's activity could be further controlled by ion fluxes.

This solid biochemical foundation paved the way for the cloning studies of ACE carried out by Ehlers in collaboration with James Riordan at Harvard. A race between the Harvard group and Pierre Corvol's group in Paris to clone the enzyme ensued. The cloning of somatic ACE revealed the striking feature that the large ectoprotein region was comprised of two homologous domains that had likely resulted from a gene duplication [31]. Moreover, each domain contained short sequences of the critical residues identical to those of the active sites of other metalloproteases, such as thermolysin and neutral endopeptidase, suggesting that each contained a putative active site. Molecular cloning of the human testis ACE (tACE) isoform showed that it was virtually identical to the C-terminal half of somatic ACE also bearing a functionally active catalytic site (Fig. 15.2) [81]. At that stage, scientists could only speculate on the significance of the curious finding that there was a unique testis isoform localised only in sperm cells and that this isozyme was a simpler version of the ubiquitously expressed somatic ACE. These early studies provided important biochemical and biophysical insights into the mechanism of action of ACE and paved the way for the engineering of soluble constructs that would be suitable for three-dimensional structure determination.

15.6 Current Activities: The Structure-Based Design of Second-Generation ACE Inhibitors

15.6.1 Crystal Structures of ACE

The three-dimensional structure of drug targets has played a pivotal role in drug design and development. However, the crystal structure of ACE has eluded scientists for almost half a century since its discovery, and this has been largely

due to the "glycosylation problem", namely, the heterogeneity of the surface glycans prevent crystal lattice formation and the growth of diffraction-quality crystals. Herculean efforts by a number of groups in the late 1980s and early 1990s to express large quantities of somatic ACE and to crystallise the enzyme were unsuccessful due to the high level of surface glycosylation and the flexibility of the linker region joining the two domains. However, the complex problem of somatic ACE crystallisation has been solved in part by expressing the N- and C-domains separately and solving their crystal structures (Fig. 15.4). In the mid-1990s, we started investigating the glycan occupancy of tACE (the equivalent of the C-domain) and the effect of glycosylation on the functional integrity of the enzyme [82]. This study was followed by mutagenesis of the Asn-Xxx-Ser/Thr glycosylation sites of tACE in order to produce minimally glycosylated forms of the enzyme [83]. Two of the seven glycans at the N-terminus of tACE were found to be necessary and sufficient for the expression of correctly folded and enzymatically active enzyme. Because of the homology between the two domains, we assumed that the N-terminal preference for a minimally glycosylated C-domain would be similar for the N-domain. Surprisingly, this was not the case, and the N-domain showed an additional requirement for C-terminal glycosylation [28]. Thus, a construct with glycans in positions Asn^{45}, Asn^{416} and Asn^{480} was fully active and gave reproducible crystallisation results yielding diffraction-quality crystals. These studies blazed the trail for the high-resolution crystal structures of the individual N- and C-domains.

A truncated form of tACE that lacked the N-terminal glycosylated region and the transmembrane domain was expressed in the presence of a glucosidase inhibitor N-butyldeoxynojirimycin, and the N-linked glycans were removed with endo-glycosidase-H (which leaves just a single *N*-acetylglucosamine residue attached to the Asn). This deglycosylated construct formed diffractable crystals and led to the solution of the first X-ray crystal structure for human tACE [67]. The hypoglycosylated form of tACE that had only two intact glycosylation sites at Asn^{72} and Asn^{109} (tACE numbering) was also used to solve the structure of native tACE and tACE in complex with different inhibitors [84–86]. The overall structures of tACE [67] and the N-domain [87] are very similar. Both molecules are ellipsoid in shape and comprised mainly of α-helices with a few short regions of β-strand. They are divided by a deep channel in which the active site zinc ion is located, and this channel is shuttered by the N-terminal two α-helices or lid helices. Normal mode analysis of tACE suggests that a hinge movement opens the active site to allow substrate and inhibitor access [84]. The lid helices α1 and α2 swivel to one side, and the channel opens up more on one end than the other with a similar observation having been seen in a N-domain structure [28]. Helices α13 (tACE) and α 15 (N-domain) contain the HEXXH zinc-binding motif with its two zinc-coordinating histidines. A glutamate on α14 (α17 for the N-domain) and an acetate ion (from the crystallisation medium) or a water molecule complete the tetrahedral coordination of the zinc ion. The N- and C-domain crystal structures revealed differences in chloride binding: two buried chloride ions were observed in tACE

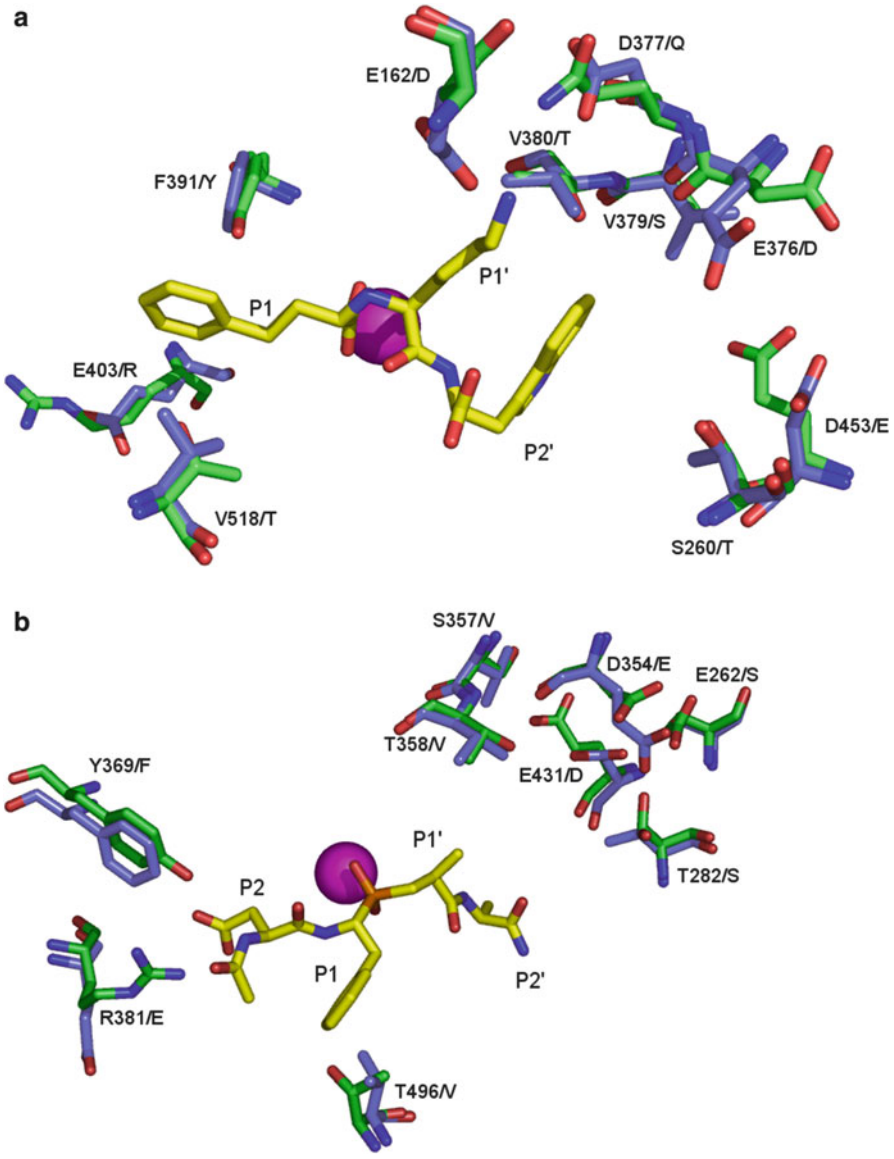

Fig. 15.4 Three-dimensional crystal structures stick representation of ACE with inhibitors. (**a**) Stick representation of lisinopril-Trp (lisW-S, *yellow*) within the tACE (C-domain) active site (PDB accession code 3L3N). Unique residues of interest in the C- and N-domains are shown in *blue* and *green sticks* respectively and residues labelled with the C-domain residue number followed by the corresponding N-domain counterpart. The catalytic zinc ion is shown in *magenta*. (**b**) Stick representation of RXP407 (*yellow*) within the N-domain active site (PDB accession code 3NXQ). Unique residues of interest in the C- and N-domains are shown in *blue* and *green sticks*, respectively, and residues labelled with the N-domain residue number followed by the corresponding C-domain counterpart. Domains were aligned using the programme *ALIGN* [103] and figures generated with PyMOL software (v 0.99, DeLano Scientific)

(chloride I and II), whereas in the N-domain structure, there was a single chloride ion corresponding to chloride II, located distally to the zinc ion.

When comparing the superimposed N- and C-domain structures, the most clearly observable difference is the extra length of the N-domain at the N- and C-termini, the latter of which includes the linker region. The well-defined nature of this region in the electron density map allowed us to propose a model for the two-domain somatic ACE. The loop between helices 19 and 20 (residues 409–417) that was not visible in the tACE structure is well defined in the N-domain. Furthermore, three other flexible loops, between helices 3 and 4, strands 1 and 2 and strand 6 and helix 23, show small differences between the two domains. Despite the structural homology between the two domains, there are some important differences between the N- and C-active sites that are responsible for the domain-selectivity of certain substrates and inhibitors. These residue substitutions in the obligatory binding site have been instrumental in our design of novel domain-selective ACE inhibitors. These active site differences will be discussed in more detail under the sections below (see Sects. 15.6.3 and 15.6.5).

15.6.2 C-Domain-Selective ACE Inhibitors

The first highly selective C-domain inhibitor, the phosphinic peptide RXPA380, effectively blocked the hydrolysis of AngI in a mouse model [36]. In contrast, bradykinin protection from ACE cleavage required the inhibition of both N- and C-active sites. A subsequent study of several RXPA380 analogues investigating the contribution of each residue towards its selectivity revealed that the P_1' pseudoproline and the P_2' tryptophan of RXPA380 were the key determinants of its C-domain specificity [88].

In our design and preparation of C-domain inhibitors, initial synthetic efforts focussed on analogues of the moderately C-selective ketomethylene derivative 5-S-5-Benzamido-4-oxo-6-phenylhexanoyl-L-proline (keto-ACE) [89]. The keto-methylene scaffold has been used extensively in the design of protease inhibitors, and it facilitated the design of non-peptidic small molecules. Keto-ACE has a proline in the P_2' position and inspection of the tACE-lisinopril structure [67] revealed that the P_2' proline of lisinopril did not make contact with any S_2' residues that are unique to the C-domain. Thus, the objective was to introduce bulky hydrophobic P_2' residues that were more likely to interact with the Val^{379} and Val^{380} that are replaced by a Ser and Thr in the N-domain, respectively. A simple five-step synthetic approach yielded a carboxylic acid which could be coupled with different *O*-protected amino acids to provide the corresponding esters in excellent yields [90]. These were hydrolysed at room temperature to afford the desired compounds in quantitative yields. Compounds with a P_2' Trp (kAW) or Phe (kAF) were 1,300-fold and at least 600-fold more selective for the C-domain using the substrate Abz-FRK(Dnp)P-OH and had K_i values of 0.83 μM and 0.68 μM, respectively [85]. Moreover, the co-crystal structures of these compounds

with the C-domain confirmed our hypothesis that the nonpolar P_2' moieties of the keto-ACE derivatives would make close contacts with residues in the cavern-like S_1'/S_2' pocket, e.g. Val^{379}, Val^{380}, His^{387}, Phe^{457}, Phe^{512}, Val^{518} and Phe^{527} and contribute favourably to the binding entropy by the hydrophobic effect [85].

In an effort to increase the binding strength of the C-selective keto-ACE inhibitors and improve the drug-like characteristics of the molecule, we used the insights from the inhibitor RXPA380 [88] and kAW to design a lisinopril analogue with a carboxylic acid zinc-binding group and a P_2' tryptophan (lisW) [91]. Synthesis of lisW was achieved by reductive amination of ethyl 2-oxo-4-phenyl butyrate 1 and N-ε-(*tert*-butoxycarbonyl)-L-lysine to give a key intermediate that was coupled with the L-tryptophan methyl ester affording the Boc-protected ester as a mixture of diastereoisomers. Saponification of the ester with LiOH followed by semi-preparative HPLC afforded the two lisW diastereomers**.** The K_i value of the S enantiomer of lisW is 6.6 nM, 600-fold lower than the keto-ACE equivalent [86]. However, the R configuration bound considerably more weakly with a K_i three orders of magnitude greater than that of the lisW-S diastereomer. This decreased affinity is probably due to a steric clash between the β-carbon of the P_1 lysine and the hydroxyl group of the conserved Tyr^{523} if the R diastereomer is modelled into the active site. In the lisW-S-tACE crystal structure, there are nine amino acids that differ between the N- and C-domains and are thus likely to contribute to its C-selective binding (Fig. 15.4). In the S_1' pocket, Glu^{162} is substituted with Asp^{140} in the N-domain and Asp^{377} with Gln^{355}. Both these C-domain residues are close enough to the amine of the P_1' lysine to make ionic interactions or weak hydrogen bonds. LisW-S makes interactions with unique C-domain residues Glu^{376} and Val^{380} in the S_2' pocket and Val^{518} in the S_1 subsite, similar to the key contacts in the crystal structures of kAW and RXPA380. Interestingly, the P_2' tryptophan of lisW-S takes a different conformation from that observed in the co-crystal structures of tACE with kAW [85] and RXPA380 [92]. This conformation is likely favoured because of the hydrophobic interactions between the P_2' tryptophan, the P_1' lysine and Val^{380} that is positioned between the two. The two different conformations of the P_2' tryptophan emphasises the large volume of the S_2' pocket, which allows different orientations of this side chain during binding.

Surprisingly, trandopril which is also a potent ACE inhibitor lacks the domain selectivity seen in lisW-S and RXPA380 despite the fact that all three compounds have bulky hydrophobic P_2' groups. A possible explanation for the lack of selectivity of trandolapril is that its P_2' tryptophan lacks an indole nitrogen, which would prevent it from hydrogen bonding with Asp^{415}, as seen in tACE- lisW-S structure [86].

15.6.3 Site-Directed Mutagenesis: An Assessment of C-Domain Residue Contribution

The structural modification of selective inhibitors is an important and useful strategy in the assessment of functional group contribution to selective binding.

However, further information regarding the relative contribution of unique amino acids in the domain active sites provides vital information in the drug discovery process, allowing for a further basis for improved inhibitor contacts. In our laboratory, site-directed mutagenesis was carried out, whereby C-domain residues implicated for selectivity in several crystal structures were converted to their corresponding N-domain counterparts. Binding affinities of these mutants with C-selective inhibitors were then assessed and revealed that contributions of amino acids across several subsites were important for the observed C-selectivity.

Assessment of RXPA380-binding affinity with these mutants suggested an important hydrophobic interaction between Phe^{391} of the C-domain and the carboxyphenyl moiety of the inhibitor [93]. In addition, S_2' residues Thr^{282}, Glu^{376}, Val^{380} and Asp^{453} have been shown to contribute to C-domain inhibitor selectivity [85, 86, 93]. Variations in the magnitude of the effect of the mutations on different inhibitors can be attributed both to the different degrees to which individual interactions contribute to the overall affinity of each inhibitor and to the flexibility of the P_2' side chain. Interestingly, the mutation of Val^{380} to Thr had little effect on lisW-S affinity in contrast to the decrease in affinity observed for kAW, kAF and RXPA380. This emphasises the unpredictable effects of flexibility in this position. Such an approach was useful in fully clarifying the contribution of novel C-selective ACE inhibitors that were based on nominally selective inhibitor templates keto-ACE and lisinopril. Observations from co-crystal structures with different domain-selective inhibitors such as kAW and lisW-S (see above) are important as this information has allowed for identification of a consistent set of unique binding residues (Fig. 15.5 and Sect. 15.6.2). This mutagenic approach has thus allowed for a further refinement of our understanding of the active site residue contributions and provides a solid foundation for the development of next-generation inhibitors. In addition, inhibitors kAW and lisW-S provide important leads in the drug discovery process that are currently under investigation in animal studies.

15.6.4 N-Domain-Selective Inhibitors

Original efforts focussed on the development of selective inhibitors that could be used in the treatment of CVD by reduced AngII production (C-selective ACE inhibitors). However, the increasing number of publications supporting the prominent role of AcSDKP in fibrotic tissue injury (presented in Sect. 15.3.2.2) has identified the N-domain of ACE as another important drug target for the treatment of diseases relating to collagen deposition. This includes the use of an N-selective inhibitor as a co-treatment with bleomycin [51], a potent anti-neoplastic agent that results in discontinuation of treatment in many patients due to excessive lung fibrosis. Moreover, N-domain inhibitors could also be used for the treatment of idiopathic pulmonary fibrosis, for which there are no currently no approved

effective drugs [94]. Thus, N-selective inhibitors could be used as a treatment in these diseases without affecting blood pressure.

RXP407, like RXPA380, is a phosphinic peptidomimetic inhibitor that has provided an important starting point in the design of N-selective ACE inhibitors (Fig. 15.3) [95]. A combinatorial chemistry approach was employed to synthesise such an inhibitor and provides important guidance in functional group contribution to N-selectivity. Keeping the P_1 and P_1' moieties in a consistent R-Pheψ-(PO_2-CH_2)-Ala-R′ form, amino acids were modified in the P_2 and P_2' positions and such products were assessed for their N-selective inhibitory ability. This resulted in the inhibitor Ac-Asp-Pheψ-(PO_2-CH_2)-Ala-Ala-NH_2, a compound with two unusual structural characteristics compared with previous ACE inhibitors: a C-terminal amide (traditionally a free carboxylate) and a protected P_2 group. Synthesis of structural analogues lacking these groups indicated the importance of these unique features in allowing for N-selective binding [95].

Several models have been proposed previously to account for the unique contacts of RXP407 in the N-domain active site, with differences in precise inhibitor functional group positioning [87, 96–98]. The recently resolved crystal structure of the N-domain in complex with RXP407 provides helpful information and is a very important step in the design of novel N-selective ACE inhibitors (Fig. 15.4) [28]. In this structure, RXP407 makes 12 direct hydrogen bonds with the N-domain active site. Of these interactions, Tyr^{369} and Arg^{381}, residues present in the S_2 subsite, are the only residues that differ to their corresponding C-domain counterparts and appear to have a prominent interaction with the P_2 Asp of RXP407. Thus, the crystal structure provides a structural basis for the importance of the Asp in N-selectivity. Interestingly, the C-terminal amide has no unique contacts with the S_2' subsite. Further mechanistic insight as to the residue contribution in the dynamic active site, as stated in a previous section, provides information that is useful in the actual contribution of these residues.

15.6.5 Site-Directed Mutagenesis: An Assessment of N-Domain Residue Contribution

Owing to the implicated importance of the S_2 and the S_2' subsites in providing interactions with RXP407 and the importance of assessing residue contribution in the drug development process, our laboratory sought to generate mutations in these pockets and analyse the effects of these amino acid substitutions on inhibitor binding. Consistent with that observed in the crystal structure, conversions to C-domain residues in the S_2' subsite had little effect on the binding ability of RXP407 [93]. Individual mutations in the S_2 subsite, namely, Y369F and R381E, had modest effects on RXP407 inhibitor binding. However, production of a mutant active site with both Y369F and R381E mutations resulted in a decrease in inhibitor-binding affinity of more than 100-fold compared to the wild-type

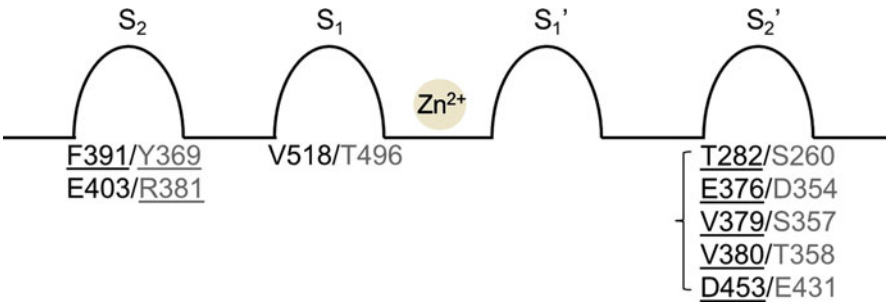

Fig. 15.5 Schechter and Berger representation [104] of subsites indicating a selection of unique residues in the N- (*grey*) and C-domain (*black*) active sites. Residues that were shown to be important contributors to selective binding are underlined. The *bracket* indicates the combined role of all the C-domain S_2' residues to facilitate C-selective binding

N-domain, suggesting crucial interactions with RXP407. Thus, the mutational analysis is in agreement with the crystal structure while the exact role of the C-terminal amide in contributing to RXP407 N-selectivity remains unclear (Fig. 15.5). In further support of the lack of importance of the C-terminal amide, it has been noted that keto-ACE analogues containing a C-terminal amide are poor inhibitors and not significantly more N-selective than the keto-ACE parent compound (unpublished observations).

Such information provides a solid basis for the design of novel N-selective ACE inhibitors. Current work involves the addition of P_2 functionalities that would have optimal interactions with residues Tyr^{369} and Arg^{381} in a keto-ACE template. This will provide an important "proof of concept molecule" that could be a promising lead in the development of N-selective inhibitors with clinical relevance.

15.7 Conclusions and Future Directions

Over the past 15 years, great strides have been made in the development of second-generation domain-selective ACE inhibitors that possess similar efficacy to that of original inhibitors but with reduced side effect profiles. In addition, increased understanding of the *in vivo* role of each domain has allowed for the initiation of the design of inhibitors that perhaps 10 years ago would not have been identified as potentially useful (e.g. AcSDKP and its role in lung fibrotic injury).

Several technologies have been indispensable in the process of ACE drug design and refinement. Firstly, the resolution of the crystal structures of each ACE domain has been invaluable in providing a structural basis in the rational design of inhibitors. This has further allowed for an identification and implication of unique residues present in the active sites that could play a role in selective binding. Another technology that has been important in allowing for a functional assessment of residue contribution towards binding has been the invaluable technique of site-directed

mutagenesis for studying protein structure–function relationships. This has allowed for the prioritisation of particular "contact points" with the inhibitor and therefore assisted in the design process. These, coupled with appropriate synthetic chemistry strategies, have allowed for additional steps to be taken in the design of domain-selective ACE inhibitors. Future drug discovery work certainly involves the incorporation of other new technologies. For example, isothermal titration calorimetry is a rapid technique that will provide detailed binding analyses that will fuel the design process. Such a technique has been used for ACE inhibitors previously [99] and is currently being optimised for use in our research group.

The novel C-selective compounds LisW-S and kAW have displayed good *in vitro* selectivity (presented above). The next logical step in the drug discovery pipeline has been the investigation of the *in vivo* potential of such compounds. The efficacy of these inhibitors in hypertensive rat models is currently under investigation and will provide further indications of the druggability of these candidates.

The development of N-selective ACE inhibitors is a more recent and exciting addition to the possible therapeutic benefits of domain-selective inhibition. The identification of the important contribution of the S_2 residues provides a good starting point in terms of inhibitor design. Such compounds are the focus of current work and could provide important early lead compounds. Following this, compound refinement in terms of ADMET properties will probably be required in the move towards an appropriate drug candidate.

An interesting development in the field of CVD treatment has been the development of vasopeptidase inhibitors: compounds that are capable of inhibiting two or more enzymes involved in vasoaction (reviewed in [100]). Most work has indicated that these inhibitors tended to be associated with increased risk for ADEs, as was the case for dual ACE/neprilysin inhibitor omapatrilat [100, 101]. In an effort to lower risks associated with this form of treatment, interest has now developed in trying to produce molecules that are selective within enzymes while still inhibiting two or more enzymes. Such molecules include compound 8 F_2, an inhibitor that is C-selective for ACE (to free the N-domain for BK clearance) and also inhibits endothelin-converting enzyme (another enzyme that produces a peptide in vasoaction), which has proved efficacious in a rat model [102]. With increasing knowledge of the structure–function relationships of these important enzymes, the possibility of achieving success in these developments seems possible.

In closing, our research has employed technologies that have provided a strong impetus in the drug development process of ACE inhibitor design. While there remains a significant amount of work to be done prior to the possibility of approval, the information generated during the last 15 years could contribute to such inhibitors being produced. With the need of better outcomes of CVD in the African context and the need for treatment of lung fibrosis, it is hoped that our and others' pursuit of the development of novel domain-selective ACE inhibitors will be of lasting benefit to the people of Africa and beyond.

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