Clinical Medical Research Award

Design of angiotensin converting enzyme inhibitors

DAVID W. CUSHMAN &
MIGUEL A. ONDETTI

Specific inhibitors of angiotensin-converting enzyme, known to the lay public as ACE inhibitors, have now been embraced by the medical community as first-line therapy for hypertensive disease and congestive heart failure. However, it is important for historical and scientific perspective to note that in the late 1960s, the so-called renin–angiotensin system, of which ACE is a component, was a poorly understood enzyme system that was not widely accepted as being important for blood pressure regulation. Renin was discovered in the late 19th century as a hypertensive substance in blood, and was shown in 1934 to be an enzyme that was elevated in the blood of renal hypertensive rats[1]. The potent blood pressure-elevating octapeptide angiotensin II, produced in blood by the action of renin, was isolated in the late 1930s (ref. 2). But ACE, which cleaves a His–Leu dipeptide from the inactive decapeptide renin product angiotensin I to form angiotensin II, was not identified until 1954 (ref. 3).

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu **Angiotensin I**
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe **Angiotensin II**

DAVID W. CUSHMAN

In 1967, John Vane, a consultant at The Squibb Institute for Medical Research suggested that someone at the Institute should become interested in studying ACE and in searching for inhibitors of its action. I was a young enzymologist looking for a new project, and immediately volunteered for this one, which involved a peptidase of apparently unique mechanism and an enzyme product of potentially great physiological and medical significance. Although most of the medical cognoscenti had reservations concerning the role of the renin–angiotensin system in human hypertension, I, along with Vane and a few other key individuals at Squibb, felt that inhibitors of this unique enzyme might allow us to determine once and for all the biological significance of angiotensin II.

In 1967, however, ACE, found in high concentration in the vascular beds of lung[4], was still a poorly characterized peptidase. It was not yet clear whether it was an endopeptidase or a unique type of exopeptidase that cleaved dipeptide residues from the carboxy-terminal end of substrates such as angiotensin I. Its enzymatic activity was usually assayed by measuring the smooth-muscle-contracting activity of its product, angiotensin II, a laborious and nonquantitative procedure. What was needed was a simple quantitative assay for this enzyme. With this in mind, I developed a spectrophotometric assay to purify rabbit lung ACE (ref. 5) and to study its enzymatic properties, and soon thereafter, Miguel Ondetti and I began a fruitful collaborative effort to discover or design potent and specific inhibitors of ACE.

The modern student of biochemistry and molecular biology might need to be reminded how primitive our understanding of enzymes was in the late 1960s, before the internet or personal computers and before modern developments in nucleic acid and protein sequencing. Only a very few protein structures had been determined by X-ray crystallography; fortunately, one of these was that of carboxypeptidase A (ref. 6), a zinc-containing exopeptidase that cleaves a single amino acid from the carboxy-terminal end of peptide substrates. Our studies of the properties of ACE suggested that it might be a very similar kind of exopeptidase, albeit one whose catalytic center had evolved to bring about cleavage of the penultimate peptide bond of its substrates, thus releasing a dipeptide product. We had found that ACE, in analogy to carboxypeptidase A, was inhibited by EDTA and other metal-chelating agents, was reactivated to similar degrees by the metal ions manganese, zinc and cobalt, and would not cleave substrate analogs with terminal carboxamide or dicarboxylic amino acids. Thus, long before we were able to develop useful specific inhibitors of ACE, Miguel Ondetti and I had formulated a hypothetical working model of ACE as a zinc metallopeptidase with a catalytic center similar to that of carboxypeptidase A (Fig. 1).

The first major breakthrough leading to truly specific ACE inhibitors came again through our consultant John Vane, who suggested that we collaborate with his colleague Sergio Ferreira, who had shown that the venom of a Brazilian pit viper contained a factor that greatly enhanced the smooth-muscle-relaxing action of the nonapeptide bradykinin. Vane's lab had evidence that the

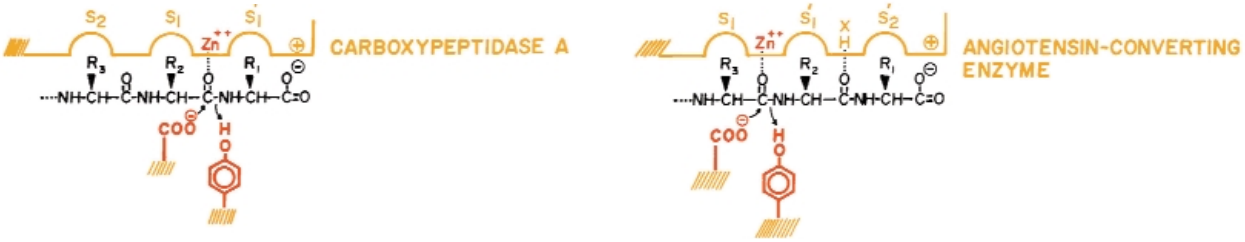


Fig. 1 The known active site of carboxypeptidase A and a hypothetical model of the active site of ACE. Subsites S_1, S_2 and so on are areas or pockets in the structure of each enzyme that interact with adjacent side-chains of amino-acid residues of an enzyme-bound peptide substrate. Functional groups (red) participate in catalysis of peptide bond cleavage. X–H is a postulated hydrogen bond donor. In the known structure of the active site of carboxypeptidase A, the carboxylate, phenolic and positively charged groups are Glu_{270}, Tyr_{248} and Arg_{145}, respectively.

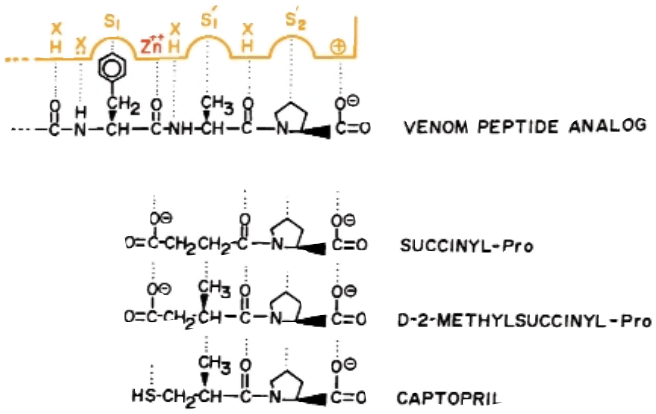


Fig. 2 Proposed binding to the active site of ACE by a substrate or venom peptide inhibitor with terminal sequence Phe–Ala–Pro, by succinylamino acids, and by captopril.

venom also inhibited ACE, although it was not clear at the time whether or not the two activities were related. As it turned out the proposed collaboration never occurred; Ferreira's group isolated bradykinin-potentiating peptides from the venom[8], using bioassay methods, and we independently isolated ACE-inhibitory peptides using the spectrophotometric assay[9,10]. All of the peptides were shown to be potent and specific inhibitors of ACE, which turned out to be an important bradykinin-inactivating enzyme. The venom peptide with the best duration of ACE-inhibitory activity *in vivo*, teprotide, was also the first ACE inhibitor to be studied in hypertensive patients and to show useful blood-pressure-lowering activity. Only its lack of oral activity precluded its general therapeutic use. Structure–activity studies with synthetic venom peptide analogs, however, improved our understanding of the active site of ACE, and indicated that the optimal carboxy-terminal amino-acid sequence of inhibitors or substrates for binding to the enzyme was Phe–Ala–Pro. The side-chains of these three amino-acid residues were assumed to specifically interact with subsites, or pockets, at the active site of ACE, called S_1, S'_1 and S'_2, respectively (Figs. 1 and 2).

Another observation related to carboxypeptidase A led to design of a new class of structurally simple ACE inhibitors with none of the drawbacks of the venom peptides. On 13 March 1974, as documented in a handwritten memo, Miguel Ondetti and I discussed a paper describing a potent new inhibitor of carboxypeptidase A (ref. 11). The authors called this inhibitor, benzylsuccinic acid, a 'biproduct' analog inhibitor, by which they meant that it combined in one molecule active site interactions characteristic of an aromatic amino-acid product of the enzyme's action and that of the terminal carboxyl group of the other peptide product. In our discussion of this paper, we made two conceptual breakthroughs. We agreed that the inhibitor was probably bound to the active site of carboxypeptidase A much like an amino acid product, but speculated that the succinyl carboxyl group would most likely bind to the catalytically important zinc ion of the enzyme. We further speculated that a similar type of compound, a succinylamino acid derivative, might specifically inhibit ACE, its structure being analogous to a dipeptide product with a zinc-binding succinyl carboxyl function (Fig. 2). The first compounds synthesized to test this simple hypothesis were not exceptionally potent inhibitors of ACE, but were specific ACE inhibitors as judged by a variety of *in vitro* and *in vivo* test systems, and one of them (an analog of Ala–Pro) was potent enough to demonstrate oral activity in hypertensive rats[12]. We postulated five specific interactions between this type of inhibitor and the active site of ACE (Fig. 2). All of these proposed interactions were tested and confirmed by measuring inhibitory potency of structural analogs with no amide bond, no terminal carboxyl, and so on[13]. Extensive structure–activity studies of this type showed that the simple structure of the Ala–Pro analog (D-2-methylsuccinyl-L-proline; Fig. 2) was optimal for binding to ACE in all but one respect: its zinc-binding carboxyl group. Replacement of this carboxyl by a sulfhydryl group led to a 1,000-fold increase in inhibitory potency, and the resulting compound, captopril (Fig. 2), proved to be one of the most potent competitive inhibitors known and the first truly useful antihypertensive drug designed to bind to the active site of ACE.

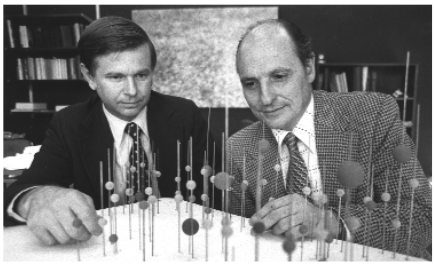
MIGUEL A. ONDETTI

In 1968, the research management of The Squibb Institute for Medical Research proposed that the peptide synthesis group under my supervision join David Cushman in the study of the ACE inhibitors present in the venom of *Bothrops jararaca*. Until then, our drug discovery efforts had concentrated on the synthesis of gastrointestinal peptide hormones[14]: first secretin and then cholecystokinin. Despite this somewhat drastic change in research direction, the new project was very appealing because it allowed me to return to a field that had been the subject of my research experience in Argentina: the isolation and structural elucidation of natural products as potential drugs. Peptide isolation and sequence determination was a new field for my group of synthetic peptide chemists, and we had to learn quickly many new techniques as we isolated and characterized these new enzyme inhibitors. However, our synthetic expertise was an invaluable tool in allowing us to compensate for our isolation/structure elucidation inexperience. We finally succeeded in characterizing and synthesizing large amounts of the most active of these venom peptide inhibitors, which, with the generic name teprotide, was introduced to clinical studies in the very early 1970s.

In the hands of clinical investigators like John Laragh[15], teprotide was essential in demonstrating the potential value of an ACE inhibitor in the treatment of hypertension . However, in terms of drug development, it was a disappointment, as it brought back to us all the shortcomings of 'peptides-as-drugs' that we had experienced in our work on gastrointestinal hormones. The large molecular size needed to achieve substantial intrinsic activity and the metabolic instability of the multiple peptide bonds conspired to eliminate all hopes of oral activity, essential for a cardiovascular drug. The extensive structural modifications of teprotide explored during the early 1970s had little effect in improving its shortcomings, but provided key, albeit indirect, information on the architecture of the active site of ACE.

As usual in drug research, the lack of results and the pressing needs of other projects dictated that we put aside our research on ACE inhibitors. David Cushman has described what brought us back. The observations of Wolfenden on carboxypeptidase A (ref. 11) and our understanding of ACE made a compelling argument for a new attempt at designing small molecular weight inhibitors. We had at our disposal not only an enzyme assay but also a highly discriminatory combination of isolated smooth muscle assays developed by Bernard Rubin, which could tell us

if a new compound was or was not a specific inhibitor of ACE. Using these tools we were excited to confirm that the first 'designed' inhibitor, succinyl -L-proline, seemed to be a specific inhibitor of ACE although of very low potency. Subsequent structural modifications of this early lead convinced us that we were indeed on the right track.



David Cushman and Miguel Ondetti, 1977.

It is important to point out that from our very first reading of Wolfenden's paper we were firmly convinced that one of the carboxyl groups of benzylsuccinic acid functioned as a ligand for the zinc atom present at the active site. And, being a carboxypeptidase, the other carboxyl was bound to an arginine moiety like the substrate carboxyl group. Therefore, in our consideration of structural modifications of succinyl proline, we gave special attention to the replacement of the carboxyl group of the succinyl moiety with other atoms or group of atoms that could also function as zinc ligands. Among those tested early on were the hydroxamic acid group and the sulfhydryl group, which eventually gave us captopril.

All the structural modifications that were studied with captopril confirmed that this molecule was interacting very specifically with the active site of ACE. Practically every functional group in captopril is important in the overall inhibitory activity by providing strong and specific interactions with the different 'pockets' of this active site[13]. Because of its sulfhydryl group, captopril can show inhibitory activity towards other zinc metalloproteases, but these activities are several orders of magnitude weaker than that seen with ACE. The assumption that the sulfhydryl group functions as a ligand of the active site zinc has been shown to be correct by studying the three-dimensional structure of a specially designed thiol-containing inhibitor with the metalloprotease thermolysin using X-ray crystallography[16].

Of course, all this potency and specificity would have been of little value without good oral absorption. Fortunately, captopril has one-fifth of the molecular size of teprotide and, strictly speaking, has no peptide bonds. It was therefore no surprise that its antihypertensive activity was equal or better by the oral route than by the parenteral route.

Conclusions

The active site model that we described in our original studies[12] used simple chemical concepts guided by a hypothetical 'paper-and-pencil' model of substrate and inhibitor binding to the enzyme. This rational design approach has led to a class of structurally simple compounds that can inhibit the action of the enzyme with great potency and specificity, properties that translate *in vivo* into effective antihypertensive activity with a remarkably low level of unwanted side effects or toxicity. It has also led to new generations of ACE inhibitors with additional interactions at the active site[17]. Also, the chemical, biological and clinical methods used to develop ACE inhibitors have inspired and aided attempts to develop angiotensin II receptor antagonists as antihypertensive drugs[18]. The concept of zinc ligand motif has been more recently exploited in the design of other metalloproteinase inhibitors of clinical significance[19,20].

Clinical research and drug discovery have always been closely associated. Not simply because a large amount of clinical research is done with new drugs, but, more importantly, because clinical research often indicates new areas in which drug discovery could be profitable. The motivation for the search for ACE inhibitors was the controversy centered on the physiological and clinical role of the renin–angiotensin system. The clinical studies with ACE inhibitors not only confirmed the important role postulated by the advocates of the renin–angiotensin system, but brought into focus clinical applications that were not even apparent to the clinical researchers and/or drug discoverers at the beginning of this venture.

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