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Renin Inhibitor SC-51106 Complexed with Human Renin: Discovery of a New Binding Site Adjacent to P_3

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Abstract—SC-51106, a 'minimal-size' diol-based renin inhibitor lacking a P_4 residue, has been co-crystallized with human renin and the structure of the complex determined by X-ray crystallography. This study defines the mode of binding of this important class of renin inhibitor, and in conjuction with molecular modeling, has led to the discovery of a new binding site adjacent to S_3, which is termed the 'S_3aux(iliary)' subsite.

Introduction

The enzyme human renin is a critical part of a cascade known as the renin–angiotensin system which is involved in blood pressure homeostasis (Figure 1). For over a decade many groups have been interested in renin inhibitors[2,3] as potential antihypertensive agents. The structure of many inhibitors has taken the form of a 3-amino-1,2-diol ('diol') linked to a linear peptidomimetic fragment whose sequence is based on the renin natural substrate, angiotensinogen; the diol unit[4] represents a P_1–P_1'[5] angiotensinogen mimetic. A commerically viable renin inhibitor must be potent towards renin, bioavailable at the target organ and economical to synthesize. To achieve nanomolar potency, inhibitors must interact with a number of critical renin binding pockets; for example,[6] binding to the S_4–S_1' subsites as occurs with SC-53315 (Figure 2) is necessary and sufficient to produce IC_{50} values in the nano- or subnanomolar range. To optimize the likelihood of bioavailability at the target organ upon oral administration, an inhibitor must be well absorbed through the intestinal tract, stable to acidic and enzymatic degradation and poorly extracted by the liver and kidney.[7] The delicate balance needed to satisfy these conditions[8] has been attained[6] using a diol-based approach that employs a basic nitrogen at P_4 and a propargylglycine residue at P_2; for example, SC-53315 exhibits a bioavailability in the dog of *ca* 30 %. Lastly, to achieve favorable economics of production, the inhibitor should contain as few residues as possible, consonant with an acceptable cost of materials per daily treatment.

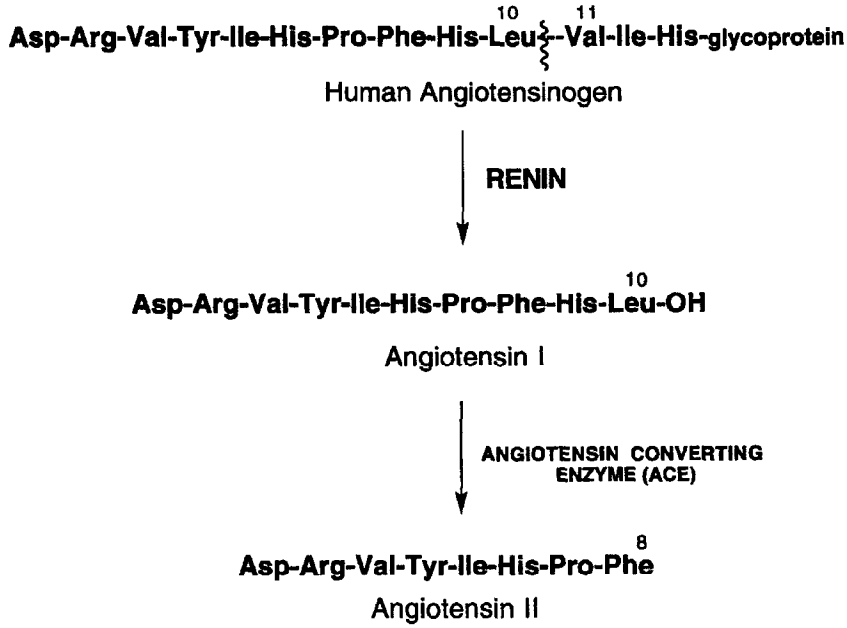
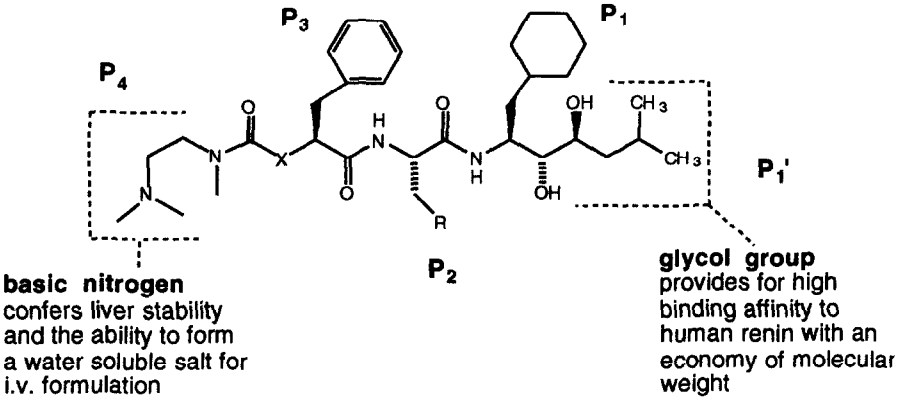
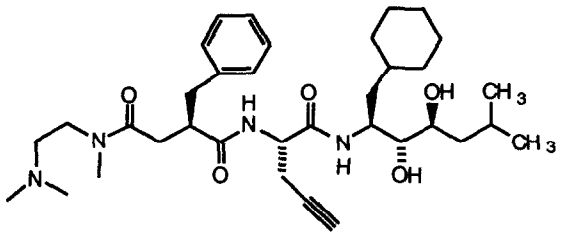
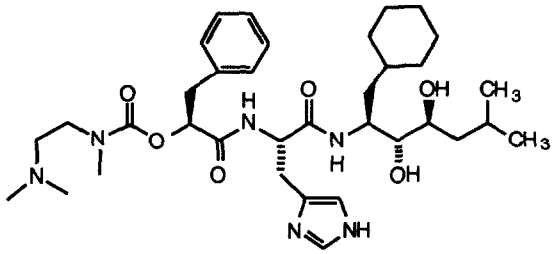


Figure 1. Renin-angiotensin system. The glycoprotein, angiotensinogen, is cleaved at the Leu^{10}-Val^{11} scissile bond by human renin to generate angiotensin I. This substance is then cleaved by angiotensin I coverting enzyme to the pressor substance angiotension II.





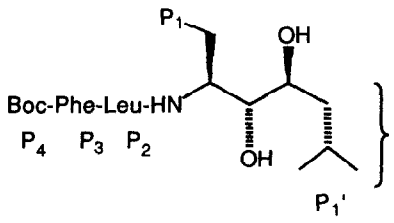
SC-53315



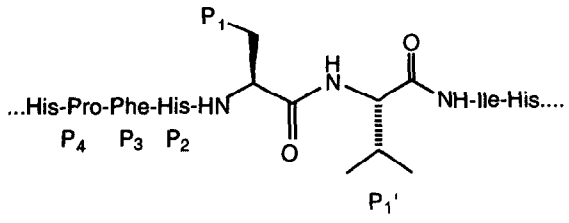
SC-48473

Figure 2. Structure of SC-53315 (R = ethynyl, X = CH_2, IC_{50} = 1 nM) and SC-48473 (R = 4-imidazolyl, X = O, IC_{50} = 1 nM), showing the P subsites corresponding to angiotensinogen and key structural features.

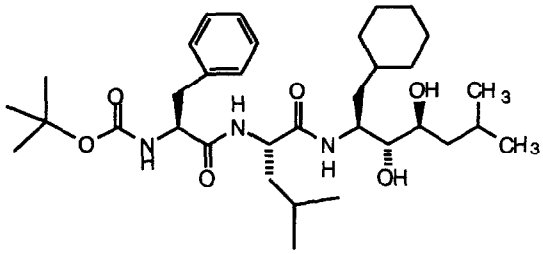
Following the independent discovery of renin inhibitory dipeptide diols by Matsueda[9] and Hanson,[10] the diol motif was elaborated by the introduction[11,12] of an isobutyl group as a P_1' mimic of Val[11] in angiotensinogen. This alkylation increased potency[4] from 190 to 5 nM (Figure 3). Subsequent modifications including the introduction of a basic nitrogen at P_4 and an ethynyl group at P_2 led to the highly bioavailable[6] SC-53315 (Figure 2, MW = 612). With the discovery of this series of highly potent and bioavailable renin inhibitors with molecular weights in the 600–700 Da range, emphasis shifted to the discovery of new analogues of lower molecular weight.



Dipeptide Glycols



Human Angiotensinogen



SC-46507

Figure 3. P subsite correspondence between diol-based inhibitors and angiotensinogen. Bracket shows the introduction aof an isobutyl group in the dipeptide glycol series (SC-46507, IC_{50} = 5 nM) to mimic the Val[11] side chain in angiotensinogen. In the original series, where the isobutyl group is replaced by hydrogen, IC_{50} = 190 nM.

In order to pursue the design of smaller, economical inhibitors of high potency, it was necessary to have detailed information at the molecular level of enzyme–inhibitor interactions. To this end, the X-ray crystal structure of human renin and SC-48473 (Figure 2) was obtained.[13] This structure showed that the P_4 chain is solvent exposed; from this followed a proposal that excision of this appendage would produce smaller 'truncated' compounds that would retain potency by leaving intact the residues proximal to the active site, P_3–P_1'. In this report we present an analysis of the X-ray crystal structure of such a truncated compound, SC-51106, complexed to human renin. We present for the first time experimental data that define the mode of binding of diol-based inhibitors and that demonstrate[14] the existence of a new binding site adjacent to S_3, designated as S_3aux(iliary), or referred to as the inhibitor residue, P_3aux. The existence of P_3aux had been hypothesized through SAR and enzyme–inhibitor modeling studies based on similar compounds not containing a P_3aux group.[14]

Results and Discussion

Removal of the P_4 side chain in SC-48473 gave rise to the hydrocinnamide SC-47921 (Figure 4) which showed a 70-fold decrease in *in vitro* potency (IC_{50} = 70 nM[15]), demonstrating the importance of P_4, not just as a solubilizing group, but as a contributor to potency; in addition, SC-47921 was not orally active in the marmoset monkey.[16] Thus a stratagem was needed to increase enzyme affinity and oral activity. We chose to introduce an (*S*)-methyl group at the β-carbon of the hydrocinnamoyl moiety of SC-47921 to increase the steric bulk surrounding the proximal amide carbonyl group in order to shield this linkage from proteolytic attack. In the event, the analogue[14,17] SC-51106 displayed a seven-fold increase in inhibitory potency towards human renin, giving a therapeutically acceptable IC_{50} of 11 nM (Figure 4). The (*S*) stereochemistry is important: analogues with the (*R*) configuration are essentially inactive.[14]

To illuminate the molecular interactions responsible for this potency increase, the crystal structure of SC-51106 complexed with human renin was determined to 3 Å resolution (Figure 5). The hydrogen bonds that SC-51106 makes to the enzyme residues are shown in Figure 6, and a stereoview of the bioactive conformation is given in Figure 7. Table 1 lists the protein residues within 4.5 Å of the inhibitor. SC-51106, like other inhibitors complexed to renin,[13] binds in an extended conformation with the P_2 (imidazole) and P_1' (isobutyl) side chains adjacent to one another on one side of the molecule, and with adjacent P_3 (phenyl) and P_1 (cyclohexyl) groups on the other. These side chain associations serve to conformationally restrict this molecule by the principle of 'hydrophobic collapse'.[18] The polar functional groups capable of hydrogen bonding are pointed perpendicular to the P side chains; these groups form hydrogen bonds to the enzyme in roughly the manner of an anti-parallel β-pleated sheet. Although the overall shape is extended, the diol unit introduces a deviation from an idealized extended β-sheet; the diol conformation likely approximates that of the scissile bond region of angiotensinogen as it is activated for hydrolysis by renin.

The following discussion of the crystallographic results will deal with the diol (P_1–P_1') and hydrocinnamide moieties (P_3aux) of SC-51106 as well as the flap region of the enzyme.

The diol unit

The structure–function of the diol moiety is revealed by the crystallographic data. The overall conformation is similar to that obtained in our earlier modeling studies[19] based on a model aspartyl protease, endothiapepsin; this conformation is also similar to that of a diol-based inhibitor bound to pepsin.[20] The 3(*R*) hydroxyl, antiperiplanar to the 4(*S*) hydroxyl, points towards the catalytic aspartates (Asp^{37} and Asp^{225}) in a direction parallel to the other carbonyls, and is within favorable hydrogen bonding distance to these aspartates. This 3(*R*) hydroxyl displaces a bound water molecule, present in the crystal structure of the native enzyme,[13] which is presumably delivered to the carbonyl of Leu^{10} in angiotensinogen.

Table 1. Specificity pockets for human renin complexed with SC-51106

S_3aux	Phe123 (sc), Gly227, Ser229 (sc)
S_3	Gln18 (sc), Thr84 (sc), Pro117 (bb,sc), Phe118 (bb), Leu120 (bb), Ala121 (bb,sc), Phe123 (sc), Ser229 (sc), Water3
S_2	Ser83 (sc), Thr84 (sc), Ala228 (sc), Ser232 (sc), His300(sc), Met302 (sc), Ile304 (sc), 313Ala (sc), Water151
S_1	Val35 (sc), Asp37 (sc), Gly39, Tyr82 (sc), Thr84 (bb,sc), Phe118 (sc), Phe123 (sc), Val126 (sc), Asp225 (sc), Gly227
S_1'	Gly39, Tyr82 (bb,sc), Ser83 (bb,sc), Leu223 (sc), Asp225 (sc), Ile304 (sc), Water130, Water142

Inhibitor–protein–solvent contacts within 4.5 Å in the crystallographically refined complex of renin with SC-51106 are listed. (bb) Denotes contact with a backbone {N,CA,C,O} atom and (sc) denotes contact with a side chain atom. Only the inhibitor side chain atoms are considered to contact the S subsites.

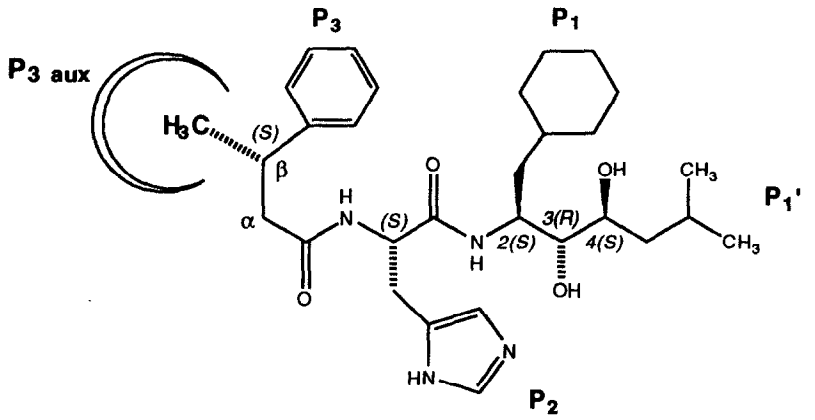


Figure 4. The structure and stereochemical configuration of SC-51106, IC_{50} = 11 nM. The P_3aux *des* -methyl analogue SC-47921 exhibits an IC_{50} = 70 nM.

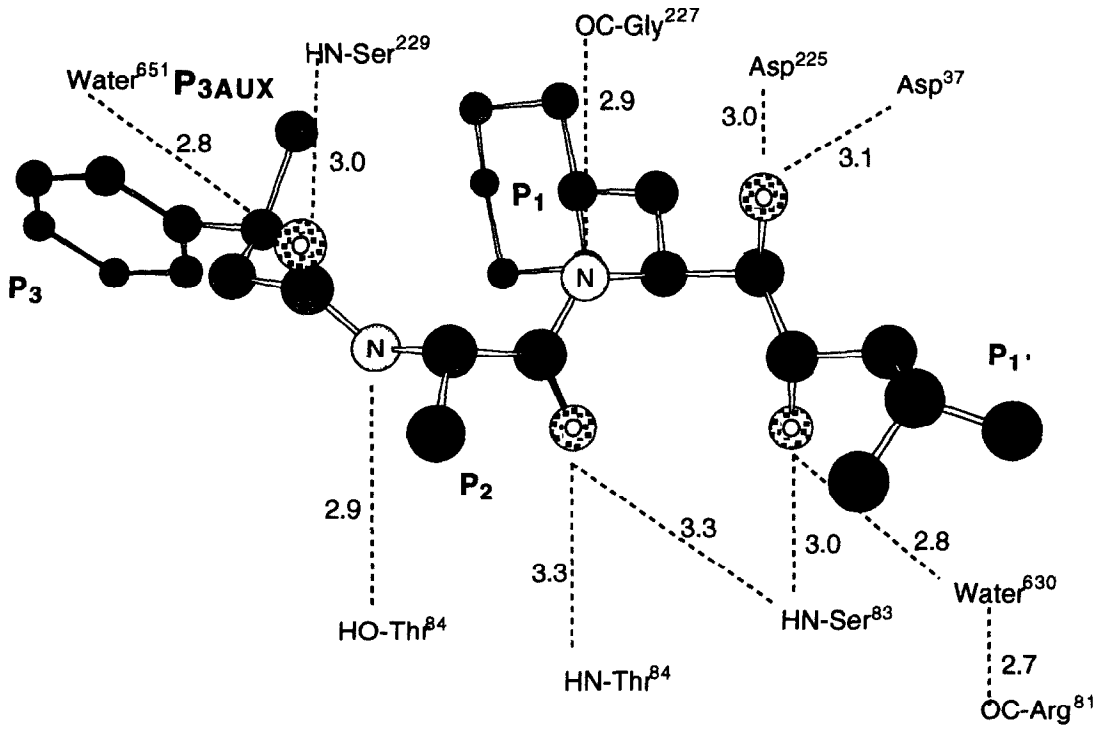


Figure 6. The hydrogen bonding patterns of SC-51106 complexed with human renin. (The P_2 imidazole is hidden to show the P_2 carbonyl.) Hydrogen bond distances (in Å units) are from Donor (heavy atom) to Acceptor (heavy atom) according to this definition: Donor--H--Acceptor--Acceptor Antecedent. At the resolution of this structure (3 Å), the H-bond distances listed here are not significantly different. The associated bond angles are consistent with good H-bonds.

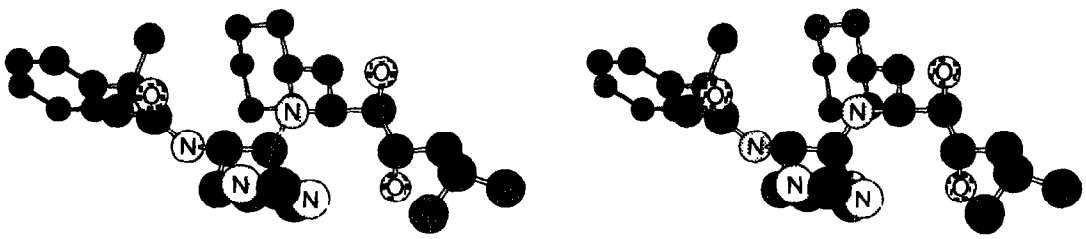


Figure 7. Stereoview of bioactive conformation of SC-51106.

An expanded view of the diol group in its bioactive conformation interacting with key 'flap' residues is shown in Figure 8. The 4(*S*) hydroxyl points away from the catalytic aspartates, hydrogen bonded to the NH of the flap residue Ser^{83}. This result resolves the question of how the 4(*S*) hydroxyl binds to renin, a subject investigated in a variety of modeling studies.[21] This 4(*S*) hydroxyl–flap interaction is analogous to that found in a rhizopuspepsin–inhibitor complex,[22] in which the P_1' carbonyl of a substrate-like inhibitor makes a hydrogen bond with the

corresponding flap residue; an analogous inhibitor–flap hydrogen bond was also noted for the P_1' carbonyl of a hydroxyethylene isostere binding to the Ser flap residue in a complex of CGP 38,560 and human renin.[23] Thus we propose that the 4(*S*) hydroxyl in the peptidomimetic SC-51106 binds in a mode similar to the P_1' backbone carbonyl in the substrate angiotensinogen. Notably, the 4(*S*) hydroxyl is also hydrogen bonded to a water molecule, which is in turn hydrogen bonded to the carbonyl of flap residue Arg^{81}; modeling of an angiotensinogen–human renin complex places the P_3' NH within hydrogen bonding distance of the carbonyl of Arg^{81}. Thus, through the agency of a water molecule, the 4(*S*) diol hydroxyl does double duty: it likely mimics the P_1' carbonyl and P_3' amide NH of angiotensinogen.

The isobutyl group of SC-51106, which corresponds to the P_1' residue Val^{11} in angiotensinogen, completely fills its specificity pocket (Figure 9). The function of this pocket, may be to 'pull' the P_1' side chain towards the enzyme, thereby setting the stage for a conformational change in the Leu^{10}–Val^{11} scissile carbonyl; this change would force this carbonyl out of a β-sheet extended conformation into a position roughly parallel to the P_1 side chain, allowing for the axial delivery of a water molecule in the enzymatic hydrolysis step. The renin inhibiting activity of a different series of compounds that incorporate a Phe^{10}-Phe^{11} (Burton's RIP inhibitors[24]) as a scissile bond replacement can be explained by the idea that the larger phenyl side chain of Phe^{11}, to be accomodated by the P_1' pocket that normally binds the much smaller Val^{11} side chain, abuts against the enzyme, restricting the flexibility of the inhibitor main chain; bulky Phe^{11} would thereby prevent the scissile bond carbonyl from rotating into the necessary position parallel to the P_1 side chain. Thus, the carbonyl group in RIP inhibitors would be locked into pointing towards the catalytic aspartates, possibly displacing the active site bound water molecule, but in any case hindering the deployment of the catalytic hydrolysis machinery.

P_3aux and the hydrocinnamide moiety

SC-51106 possesses a β-(*S*)-methyl hydrocinnamoyl group as its P_3 unit. The phenyl ring fits into the S_3 specificity pocket that is defined by the enzyme residues shown in Table 1. Adjacent to this pocket, bound on one side by the phenyl ring of Phe^{123}, is a previously unreported binding site, that we have named S_3aux, which binds the P_3aux β-(*S*)-methyl side chain (Figure 10). This binding pocket contains in the native enzyme two water molecules, one of which is displaced by the β-methyl substituent of SC-51106 (see discussion below); the remaining water forms one of the boundaries of S_3aux. The β-(*S*)-methyl group is well accommodated by this pocket.

Modeling of a β-n-propyl hydrocinnamide analogue

Prior to the solution of the SC-51106–human renin complex (Figure 5), the existence of S_3aux was predicted based on a detailed examination of the potential energy surfaces of SC-48473 (Figure 2) complexed with human renin.[25] β-(*S*)-Methyl, ethyl and *n*-propyl substituents were added to the hydrocinnamoyl moiety of SC-48473 in renin–inhibitor modeling experiments; these appendages fit nicely into the S_3aux channel (Figure 11). Each of the modeled P_3aux (*S*)-side chains point back into the binding cleft. The β-ethyl case exemplifies the side chain disposition. All ethyl conformations, except where the dihedral angle given by Cα–Cβ–Cγ(ethyl)–Cδ(ethyl) = *ca* 70 °, are strongly prohibited by the superposition of the ethyl group with the protein or with the cyclohexyl P_1 side chain (Figure 12). Upon initial docking, near 70 ° the ethyl Cδ not only experiences large VDW repulsions from Ser^{229} Oγ and Hγ, but is superimposed on a crystallographically determined water position. However, the P_3aux conformation is allowed with the following changes to the protein: (1) the side chain of Ser^{229} changes from a *gauche*(–) to a *gauche*(+) conformation. Ser^{229} is found in a X_1 = [–50 °,–40 °] conformation in several crystallographically determined, renin-inhibitor complex

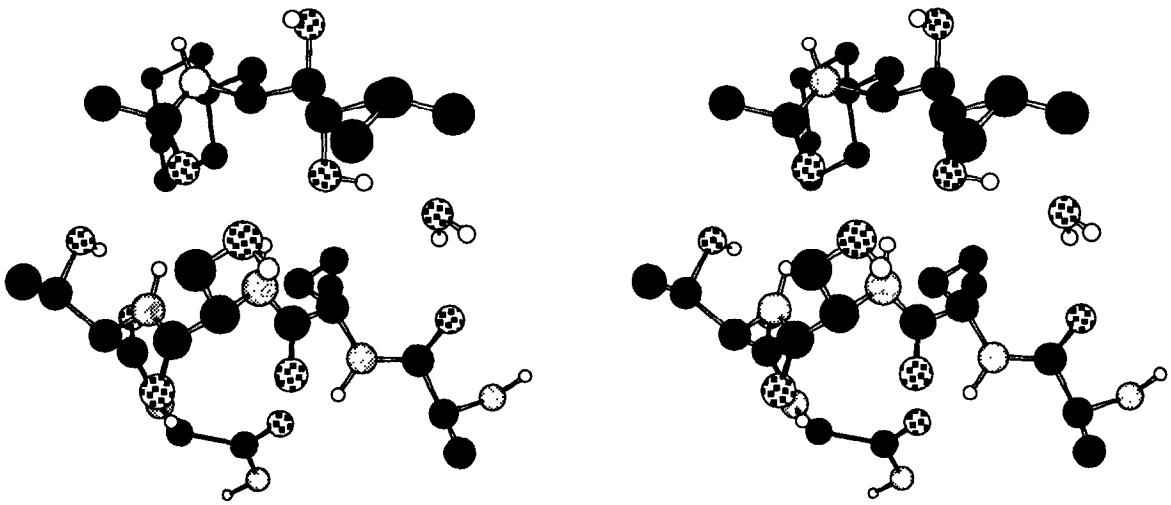


Figure 8. Stereoview of the SC-51106-human renin complex (partial structure). Top molecule is the P_1-P_1' diol portion (including the P_2 carbonyl on the left) of SC-51106 complexed with human renin. Bottom structure is a cutaway of the flap region binding to the diol. The NH of the Ser^{83} (i+1) residue (coming out of the plane of the page) of the fap region β-turn forms an hydrogen bond to the 4(*S*) hydroxyl (nearest to the viewer). On the right is a bound water molecule that bridges via hydrogen bonds the 4(*S*) hydroxyl with the carbonyl of $Arg.^{81}$

structures, including the SC-48473 complex.[13] The accompanying B factors are small, implying little conformational flexibility for the sidechain. However, using potential energy surfaces analysis algorithms previously developed,[25] a second minimum near X_1 = [35 °,50 °] was detected. The qualitative fingerprint for local configurational entropy is good for each conformation and the transition barrier appears to be quite low (readily accessible at physiological temperature). All directional interactions are satisfied for Ser^{229} in the new conformation; (2) one water ($Water^5$ in the SC-48473 complex) is displaced. Its only strong interactions were to the side chain of Ser^{229} and $Water^6$ (in the SC-48473–renin complex) prior to its conformational change. Once that change is made, only a small amount of energy should be necessary to displace it from the P_3aux site; (3) a second nearby water molecule ($Water^6$ in the SC-48473 complex) reorients and drifts (less than 1 Å) to re-establish a hydrogen bond to a solvent-inaccessible protein backbone atom which was lost when Ser^{229} was rotated. The relative dispositions of these two molecules upon binding at the S_3aux site was also subsequently confirmed crystallographically. Even with the larger propyl substituent, the $Water^6$ remains at the pocket boundary (Figure 11).

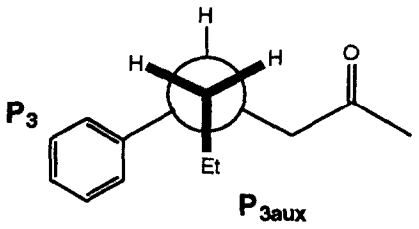


Figure 12. Newman projection of the hydrocinnamide moiety of SC-51106 with an *n*-propyl group appended at P_3aux, in the most favorable conformation.

The flap region

The flap region is a β-pleated sheet containing a β-turn. Upon binding of an inhibitor, the flap region undergoes a conformational change, moving closer to the main body of the enzyme and makes important hydrogen bonding (Figure 6) and hydrophobic interactions with the diol-based inhibitor. The turn (Figure 13) is a non-standard type I β-turn that includes the following residues making contact with SC-51106: Arg^{81}, Tyr^{82} (i), Ser^{83} (i+1), and Thr^{84} (i+2). (The designation 'nonstandard' is used because the side chain of the i+2 residue Thr^{84} is oriented equatorially.[26]) The most interesting hydrophobic interaction is afforded by Tyr^{82}, whose phenyl side chain defines one side of the P_1 pocket, making hydrophobic contact with one face of the P_1 cyclohexyl ring.

Conclusion

The complex of SC-51106 and human renin reveals the nature of the interactions at the molecular level. This structure lays the basis for rational efforts directed at the design of potent, minimal size renin inhibitors, based either on a linear peptide theme or *de novo* nonpeptide structures such as fused heterocycles. The new binding site S_3aux provides an avenue of exploration in the search for the 'minimal renin inhibitor'.[2]

Experimental Section

Crystal structure determination of the SC-51106 complex with human renin

SC-51106 was co-crystallized with deglycosylated human renin at pH 4.5 in the presence of 13 % (w/v) polyethylene glycol 3350 and 0.4 M NaCl using vapor phase

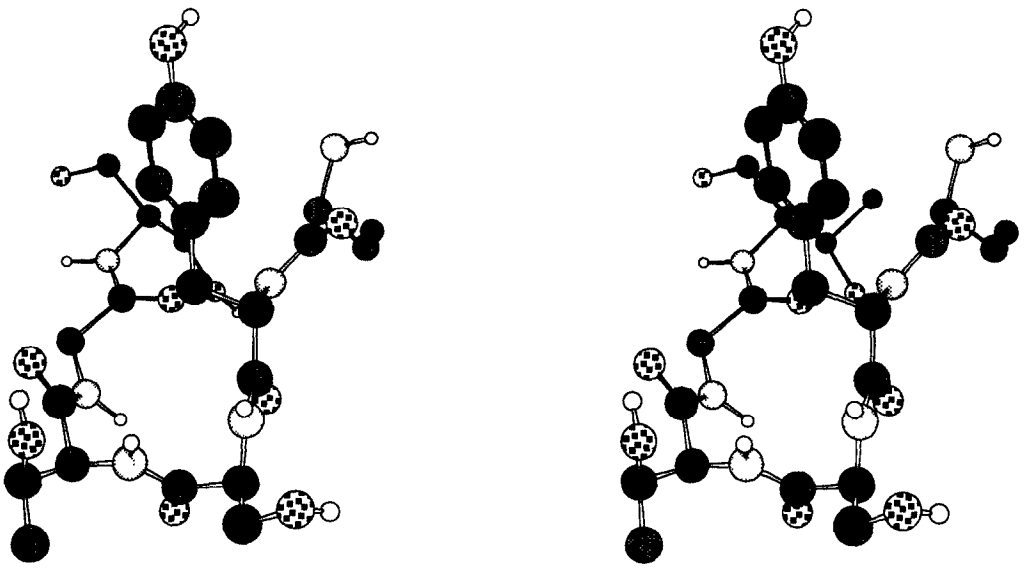


Figure 13. Stereoview of the β-turn of the flap. The corner residues, Ser^{83} and Thr^{84} have equatorially disposed side chains. The Tyr^{82} residue bounds the S_1 specificity pocket.

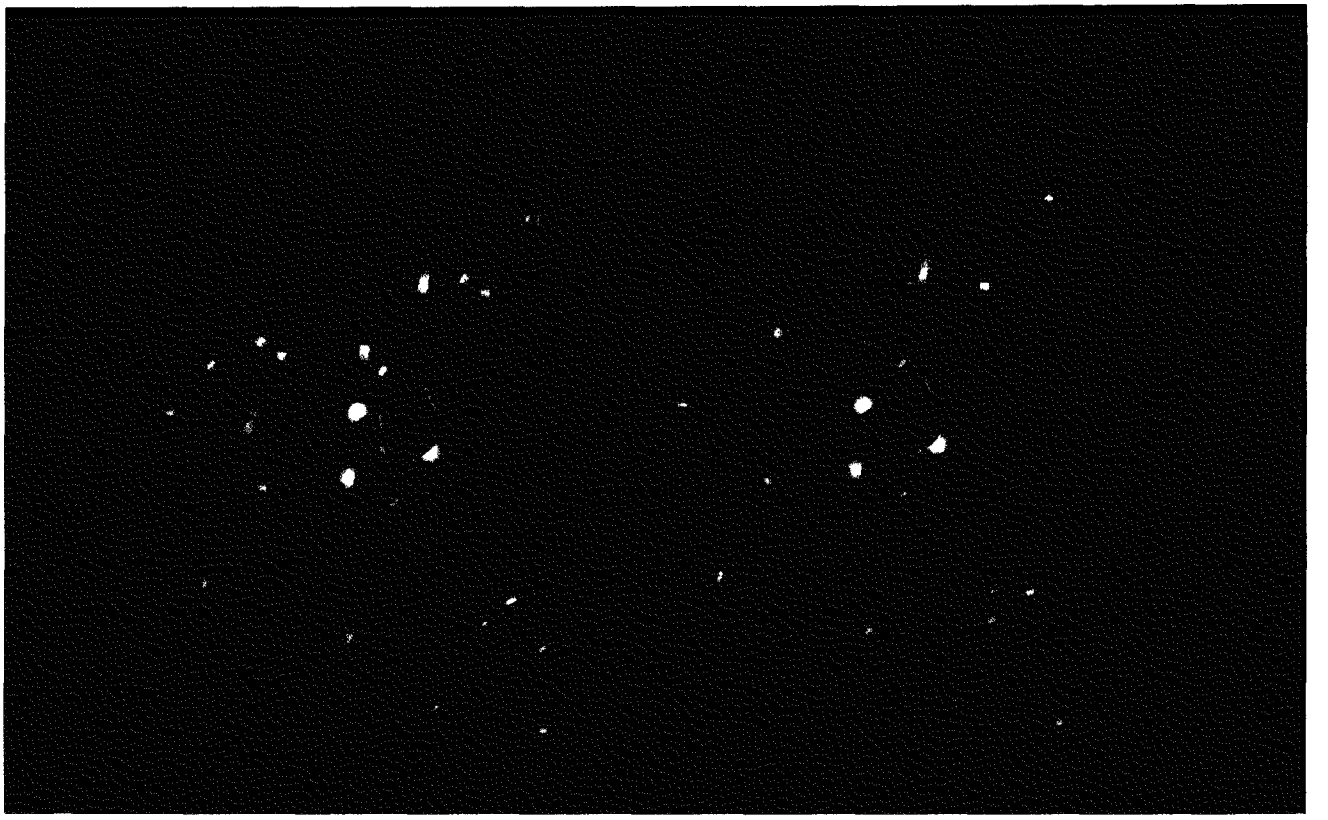


Figure 5. Global view of the SC-51106 (CPK model)-human renin (ribbon) complex.

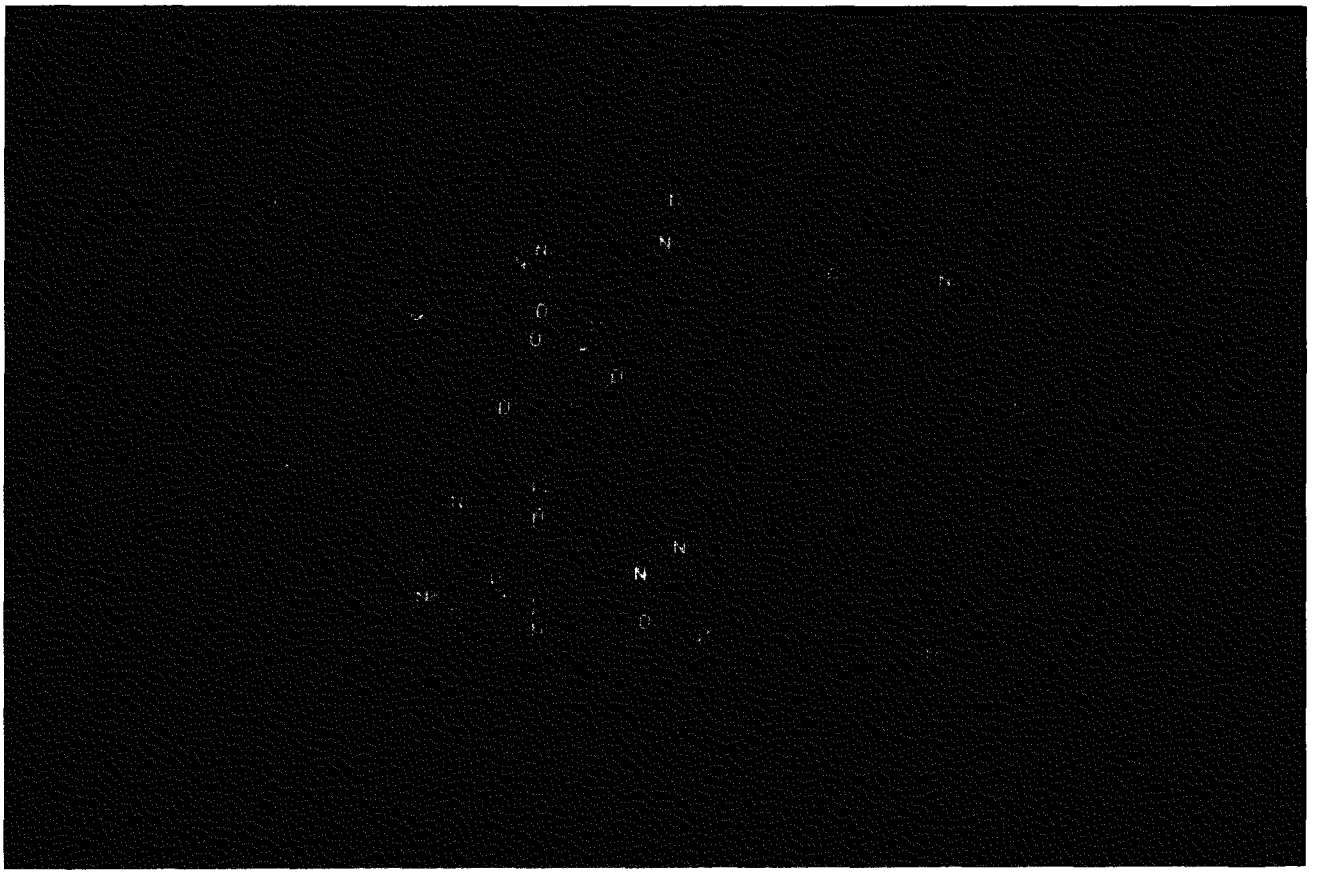


Figure 9. The P_1' isobutyl group completely fills its specificity pocket. The dot surface shows the VDW surface of the isobutyl methyl groups (with added hydrogens) plus 1.4 Å.

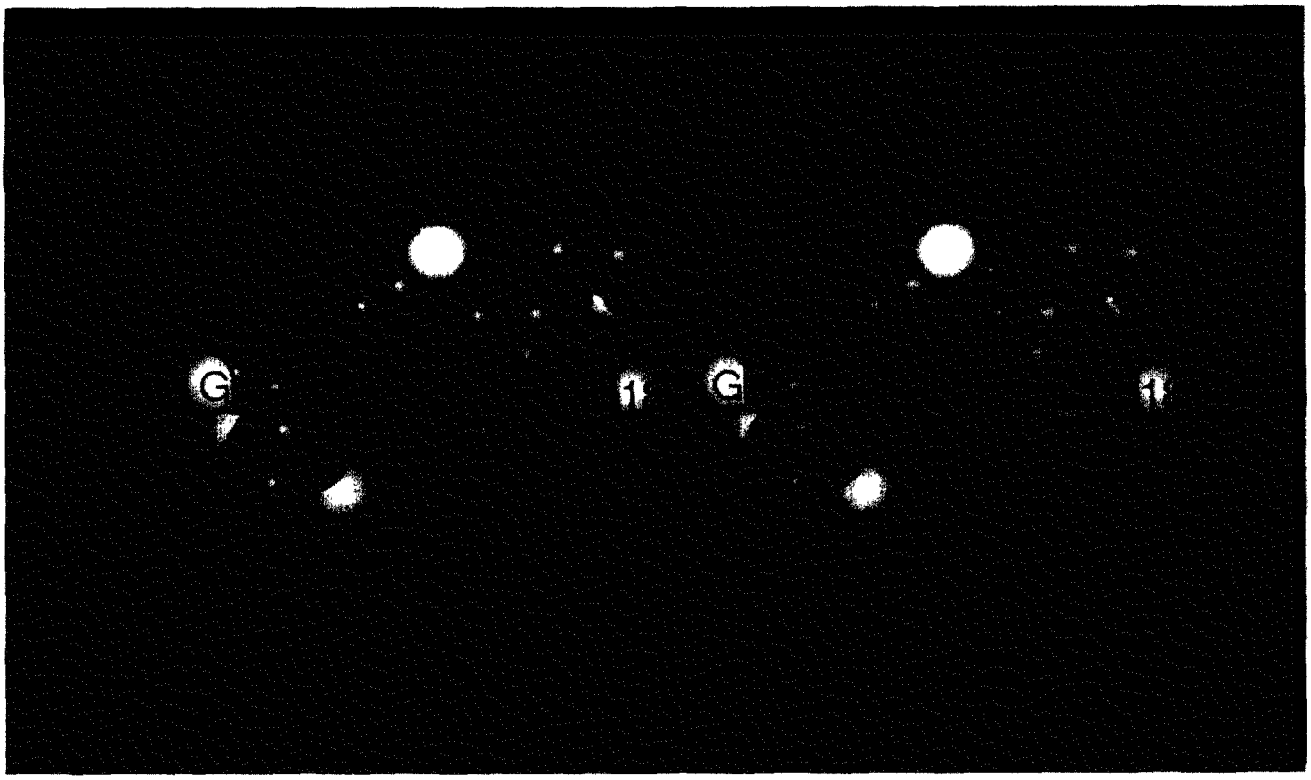


Figure 10. Stereoview of S_3aux. The β -carbon of the hydrocinnamide moiety is substituted with an (*S*)- *n*-propyl group to clearly delineate the S_3aux pocket.

Figure 11. The S_3aux pocket in the human renin/SC-51106 complex with P_3aux depicted by dot surfaces. The blue dots correspond to the VDW surface (plus 1.4 Å) of the β-methyl group of SC-51106; the cyan dots correspond to the volume of a modified SC-51106 in which a β-*n*-propyl group replaces the β-methyl.

equilibration and the hanging drop method.[27] The inhibitor was solubilized in dimethyl sulfoxide (DMSO) with the final DMSO concentration in the crystallization droplet less than 1 %. Lattice parameters are: $a = 67.6$, $b = 97.7$, $c = 117.5$ Å in space group $P2_12_12_1$ with 8 renin–inhibitor complexes in the unit cell. V_M is 2.3 Å^3/Dalton. The data were collected using a dual-chamber Xuong–Hamlin multiwire area detector;[28] R_{sym} is 9.0 % based on 132,715 observations of 15,391 unique reflection intensities measured to 3.0 Å resolution. Protein atoms and 304 solvent molecules from the structure of the isomorphous renin complex with SC-48473, determined at 2.4 Å resolution with a crystallographic residual of 18.7 %, provided the starting point for refinement using the positional refinement algorithms implemented in XPLOR.[29] After the initial refinement against the X-ray data from the SC-51106 complex, residual electron density from difference Fourier maps calculated in the vicinity of the active site could be interpreted with a 3-dimensional model of the new inhibitor. Refinement, including parameters of SC-51106, was continued in XPLOR with iterative rounds of rebuilding and examination of the active site solvent structure. The final crystallographic residual is 21.0 % for all 14,641 structure factors between 3.0 and 8.0 Å resolution; the R factor is 16.0 % based on 10,592 structure factors greater than $\sigma(F)$. Rms deviations from ideal bond lengths and valence angles are 0.017 Å and 3.7 °, respectively. The final model includes 303 solvent molecules. Several residues located at the N-termini of the renin chains are not visible in electron density maps and have not been included in the structure factor calculations.

Computational studies

Many methods to dock small molecules to proteins have appeared in the literature.[21] The principle difference between them lies in the algorithms and procedures which are used to separate structures which might be physically observable from those which are not. We have used an extension of the algorithms[25] for differentiating between well-folded and poorly-folded protein structures to make the distinction. The process relies upon the assumption that from the protein's point of view, any tightly bound small molecule is indistinguishable from any other well-folded piece of itself. Hence, any complex formation in which (1) the small (peptidyl or nonpeptidyl) molecule can be docked in a low energy conformation and (2) all of the energetic fingerprints of well-folded proteins can be satisfied for the protein in the complex, is considered to be possible. To date, in practice most complexes which can be generated numerically or through manual modeling, fail to satisfy both criteria. All calculations were performed using the program CHARMM[30] and parameter sets detailed elsewhere.[30,31]

Acknowledgement

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