Structure-Based Pharmacophore Design and Virtual Screening for Novel Angiotensin Converting Enzyme 2 Inhibitors

Monika Rella,[†] Christopher A. Rushworth,[†] Jodie L. Guy,[†] Anthony J. Turner,[†] Thierry Langer,[‡] and Richard M. Jackson*[,†]

Institute of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, U.K., and Department of Pharmaceutical Chemistry, Institute of Pharmacy, University of Innsbruck, Innrain 52c, A-6020 Innsbruck, Austria

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The metallopeptidase Angiotensin Converting Enzyme (ACE) is an important drug target for the treatment of hypertension, heart, kidney, and lung disease. Recently, a close and unique human ACE homologue termed ACE2 has been identified and found to be an interesting new cardiorenal disease target. With the recently resolved inhibitor-bound ACE2 crystal structure available, we have attempted a structure-based approach to identify novel potent and selective inhibitors. Computational approaches focus on pharmacophore-based virtual screening of large compound databases. Selectivity was ensured by initial screening for ACE inhibitors within an internal database and the Derwent World Drug Index, which could be reduced to zero false positives and 0.1% hit rate, respectively. An average hit reduction of 0.44% was achieved with a five feature hypothesis, searching ∼3.8 million compounds from various commercial databases. Seventeen compounds were selected based on high fit values as well as diverse structure and subjected to experimental validation in a bioassay. We show that all compounds displayed an inhibitory effect on ACE2 activity, the six most promising candidates exhibiting IC_{50} values in the range of 62−179 μM.

INTRODUCTION

Angiotensin Converting Enzyme 2 (ACE2) is a novel and unique human homologue of the Angiotensin Converting Enzyme (ACE).[1] ACE has long been known as a major regulator of the Renin Angiotensin System (RAS) and represents a well-established drug target for the treatment of hypertension and heart disease.[2] The discovery of ACE2 increases the complexity of the RAS—indicating a counter-regulatory role to ACE which makes it a promising new cardiorenal disease target. ACE2 has been associated with hypertension, heart, and kidney disease and more recently as the functional receptor for the SARS-coronavirus and its full physiological and pathophysiological roles are currently under intense investigation.[3] Like ACE, ACE2 is a member of the M2 metalloprotease family but functions as carboxypeptidase (as opposed to ACE which is a peptidyl dipeptidase) by removing the C-terminal residue from its peptide substrates. A screening study of 126 biological peptides identified 11 in vitro substrates including ACE substrates such as angiotensin I but not bradykinin.[4] Despite strong structural conservation of the overall fold and active site, ACE2 differs from ACE by a number of critical substitutions of active site residues. The key change involves the transition from a glutamate in ACE to Arg273 in ACE2, thereby abolishing the S2′ subsite. This provides the structural explanation for distinct catalytic activity and insensitivity to ACE inhibitors.[5] Recent mutagenesis data emphasizes the important role of Arg273 as essential for substrate binding and enzyme activity.[6]

In this study, a computational virtual screening approach is used to identify novel ACE2 inhibitors. Virtual screening has established itself as a valuable in silico technique alongside traditional high throughput screening for new active compounds in the pharmaceutical industry.[7,8] Several approaches exist to search small molecule structure databases for novel lead compounds depending on the presence or absence of an experimental protein structure.[9,10] Pharmacophore modeling is the approach widely used for a set of known active molecules which are analyzed for common functional groups responsible for specific drug-receptor interactions and accordingly aligned in three-dimensional space.[11] A pharmacophore is the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target structure and to trigger (or to block) its biological response.[12] The pharmacophore comprises chemical features such as hydrogen bonding, electrostatic and hydrophobic interactions among others and can be used as a query for database searching.

In cases where structural data is available, docking algorithms are most commonly employed, where small molecules are flexibly fitted into the active site of a receptor and scored for optimized positions.[9] Receptor-based pharmacophore models provide an efficient alternative to docking-based virtual screening of small molecules while still representing specific ligand−protein interactions. In the long history of the development and application of the pharmacophore concept, exploitation of available experimental protein structures is a new feature;[11] however, several studies

* Corresponding author phone: +44 (0)113 3432592; e-mail: r.m.jackson@leeds.ac.uk.
[†] University of Leeds.
[‡] University of Innsbruck.

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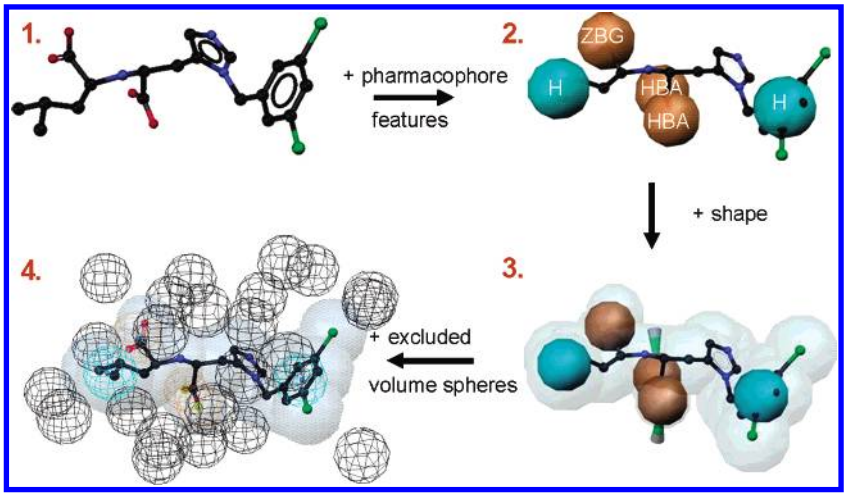


Figure 1. Design strategy leading to a selective, structure-based hypothesis for retrieving novel ACE2 inhibitors. Relevant chemical features (zinc binding group: ZBG, hydrogen bond acceptor; HBA, hydrophobic feature: H) are mapped onto the bioactive conformation of a potent ACE2 inhibitor (1) and combined into a pharmacophore (2) which is enhanced by spatial constraints such as shape (3) and exclusion volume spheres mimicking the protein active site (4).

already indicate enhanced performance of a combined protein structure- and ligand-based approach in pharmacophore modeling.[13,14] The aim of this study was to generate a set of protein structure-based ACE2 pharmacophore models to apply as filters for virtual screening of several commercial databases. These screening hits will represent a promising source of candidate molecules for evaluation of the binding mode by docking and for further biological testing.

MATERIALS AND METHODS

Structure-Based Pharmacophore Modeling. LigandScout, a tool recently developed for the automatic construction and visualization of pharmacophore models derived from protein structures,[15,16] was used to study the active site of ACE2 in complex with the bound ACE2 inhibitor, MLN-4760.[5] The software captures and displays the main characteristics of the ligand−receptor interaction classified as hydrogen bonding, charge transfer, and hydrophobic regions. Multiple chemical features are detected and mapped onto the ligand functional groups, allowing the user to export all or specific pharmacophore patterns based on expert knowledge. Alternative hydrogen bond donor and/or acceptor sites are considered simultaneously on the protein within the limits of geometric constraints.

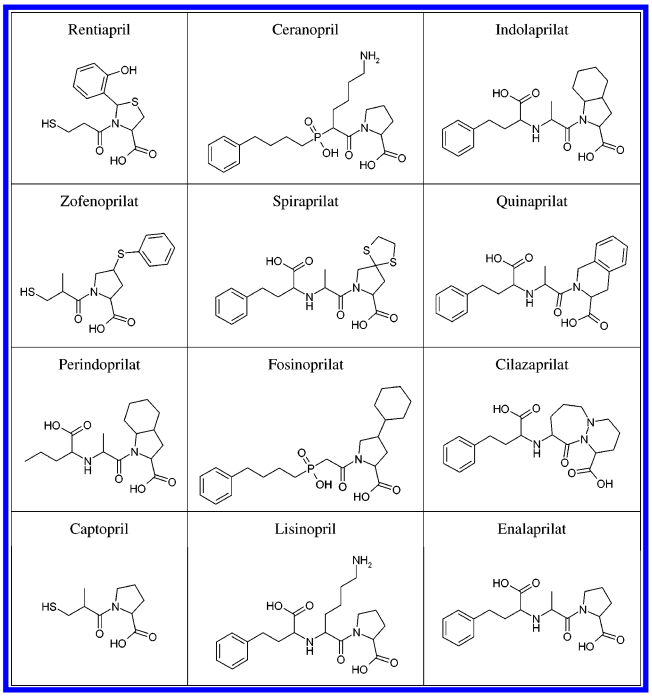
Exclusion volume spheres were added to the structure-based models onto coordinates defined by protein side chain atoms to characterize inaccessible areas for any potential ligand. Further model refinement such as addition of shape constraints was carried out using the Catalyst software package version 4.9[17] which also provided the screening platform hosting several commercial compound databases. All structures are stored as pregenerated, multiple low energy conformers which allows rapid screening by mapping each conformer against the query pharmacophore model.

Multiple hypotheses were built to overcome limitations in the feature definition and recognition within the Catalyst software, which only supports a single feature for each corresponding ligand functional group. If two features can be mapped to a group as in the case of the carboxyl group which can be described by both a hydrogen bond acceptor (HBA) and negative ionizable (negatively charged, ionizable group, NI), one has to be favored over the other for incorporation into the hypothesis. The same applies for the number of projection points and vectors. One projection point and vector are accepted per heavy atom. If two alternative, potential hydrogen bond donors (HBD) exist in the protein for a certain HBA in the ligand, only one can be selected as a projection point for the hypothesis. Projection points and vectors were omitted in all but one hypothesis to avoid a combinatorial explosion and increase the generality of the model.

Several features were selected and combined during ACE2 hypothesis generation. The C-terminal carboxylate moiety was represented by either 2 HBA or a NI, whereas the zinc binding carboxylate function was represented by a zinc binding group (ZBG) feature comprising known zinc ligands such as hydroxamic acid, phosphates, thiols, sulfides, carboxylic acid, carbazones, thiocarbazones, and azoles as well as by NI features. Additional hydrophobic features were also considered. A number of hypotheses were built, integrating different chemical feature combinations and with increasing complexity to enhance selectivity. The general design strategy is exemplified in Figure 1, starting with the bioactive conformation of the ACE2 inhibitor as geometric 3D template onto which chemical features are mapped.

The minimal hypothesis was manually assembled from chemical functions and substructures and comprises five features: two HBA, two hydrophobic features (H), and a ZBG. The ZBG is an essential part of the pharmacophore and required for high affinity ligands due to ACE2's metallopeptidase activity. Another essential part of the pharmacophore is the C-terminal anchoring group, represented by two HBAs. A more selective hypothesis was created including spatial information by using the ACE2 inhibitor as template for a shape hypothesis and merging it with the five feature hypothesis. This defines shape similarity rather than shape containment, thus hits could have undesirable protrusions into the active site. To avoid such collision with protein residues, a set of exclusion volume spheres

Table 1. Examples of the ACEI Data Set Showing Potent ACE Inhibitors that Do Not Inhibit ACE2[19]



based on surrounding active site residues were added to the query.

Selectivity Evaluation: Validation Set and Model Creation. For validation of the generated, multiple ACE2 hypotheses, a set of known active ACE inhibitors including all marketed ACE drugs were extracted from the Derwent World Drug Index (WDI).[18] Large molecular weight compounds or those corresponding to peptides were removed as well as prodrugs or non-ACE inhibitors with false activity labels. The remaining 55 compounds are termed the ACE Inhibitor data set (ACEI), some of which are listed in Table 1 i.e., those that were confirmed by experiment not to inhibit ACE2.[19] This internal test set served to validate multiple ACE2 hypotheses by evaluating how well they distinguish between known ACE and potential ACE2 inhibitors by screening and reducing the hitlist. For a more widespread selectivity analysis and as a potential source for novel ACE2 inhibitors, the WDI version 2003, comprising 63 307 drugs and pharmacologically active compounds, including all marketed drugs, was searched with each ACE2 model.

ACE crystal structures were studied in complex with three commercial ACE drugs, lisinopril, enalaprilat, and captopril, to analyze and contrast inhibitor interaction with ACE2. Based on their bioactive conformations, a basic pharmacophore model for ACE was manually constructed to allow assessing the binding mode and essential requirements for ACE inhibitors by searching the ACEI. This model comprised a ZBG, 3 HBAs, and an H feature and was refined by adding shape information of enalaprilat. It was further used to validate hits resulting from database screening with ACE2 hypotheses for selectivity against ACE.

Database Searching. Catalyst[17] was used as the database search program and during hypothesis optimization to evaluate/fit individual compounds to a respective hypothesis. It handles conformational flexibility by pregenerating a representative set of diverse and low energy conformations with the poling algorithm[20] and storing those conformations in the database. This multiconformer database can be searched rigidly or flexibly, indicated by the fast or best search option. The FAST algorithm only considers existing conformers and interrupts a search as soon as a pharmacophore matching conformation is found, whereas the BEST algorithm additionally 'tweaks' bond distances, angles, and dihedral angles of pregenerated conformers on the fly to achieve the best matches. All database searches were conducted in the BEST search mode and only "all feature mappings" were considered during BEST fit calculations. Hit molecules can be ranked by their geometric fit values which indicate how well the chemical substructures were mapped onto the hypothesis feature location constraints and their distance deviation from the feature centers. High fit values indicate good matches with the maximum fit value set by the original ligand used to create the pharmacophore, which was MLN-4760 in its bioactive conformation.

Docking. Pharmacophore based database searches can generate many 'hits' of varying quality. To prioritize hits for purchase and biological testing, selected hits were docked into the binding site with eHiTS[21] and a new version of Q-fit[22] which allows flexible ligand docking[23] to a protein. Both tools use an empirical scoring function to rank ligand poses and discriminate between multiple ligands in a virtual screening experiment.

Biological Evaluation. Selected compounds were purchased and validated through a fluorogenic ACE2 activity assay. The assay utilizes a synthetic ACE2 substrate, Mca-APK(Dnp),[4] at a final concentration of 25 μM and following addition of substrate was carried out at room temperature. Each compound was incubated at 200 μM final concentration with cellular ACE2 protein for 20 min at 37 °C prior to administration of the substrate. Increase in fluorescence (excitation = 340 nm, emission = 430 nm) was observed upon substrate addition and hydrolysis in 3 min intervals up to 2 h using a Wallac Victor^2 fluorescence plate reader. For each compound, % inhibition was determined from the linear rate of fluorescence amplification.

RESULTS AND DISCUSSION

Structure-Based Pharmacophore Modeling. In the absence of small molecule ACE2 inhibitors that could be used for ligand-based pharmacophore modeling, a structure-based approach was applied based on the MLN-4760 inhibitor-bound ACE2 crystal structure complex. The active site was visualized and analyzed for protein−ligand interactions (Figure 2). The binding site is characterized by several directed interactions such as hydrogen bonding and zinc chelation in addition to unspecific hydrophobic interaction. The potent ligand, MLN-4760, acts as transition state analogue by mimicking a peptide substrate coordinating to the catalytic zinc with a carboxylate group and a C-terminal carboxylate moiety as anchoring group. Lipophilic side chains complement and reflect the hydrophobic nature of the active site. For an increased understanding of the ACE2 specificity and pharmacophore requirements of new ACE2 inhibitors, the structurally highly conserved ACE active site along with a set of known ACE inhibitors was also analyzed.

The detected ACE2 pharmacophore was compared with the structurally similar ACE active site in complex with three

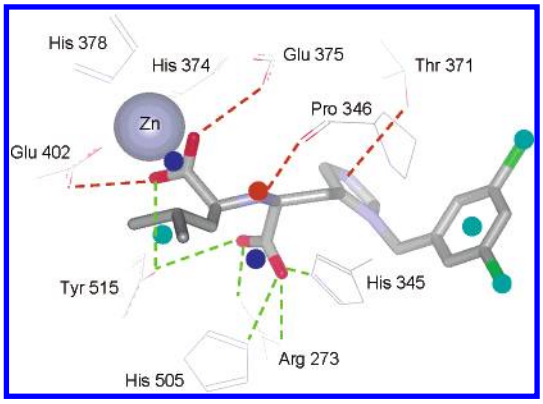


Figure 2. Protein−ligand interactions in the ACE2 active site with MLN-4760.[5] Important residues and the zinc (Zn) cofactor are displayed. Feasible hydrogen bonds are shown as green, less likely occurring ones as red dotted line. Hydrophobic regions are visualized as cyan spheres, negative and positive ionizable features as blue and red spheres, respectively.

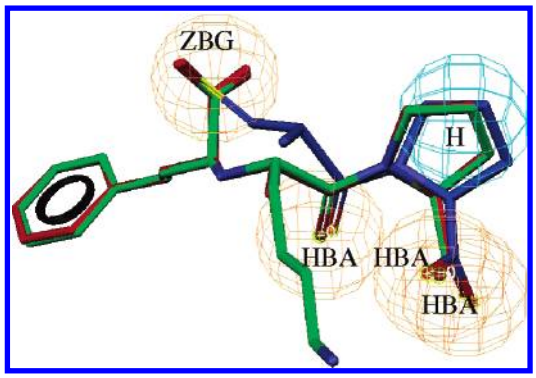


Figure 3. Three ACE inhibitors (lisinopril in green, enalaprilat in red, and captopril in blue) in their bioactive conformation, aligned in the corresponding ACE pharmacophore model. The model consists of the ZBG, one H feature and three HBA features.

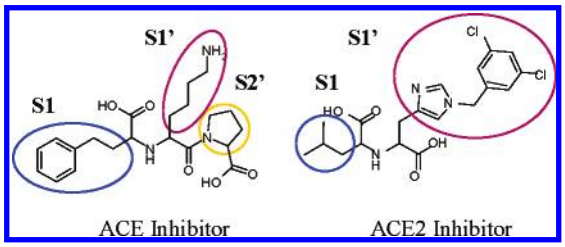


Figure 4. Schematic view of ACE versus ACE2 inhibitor binding in the corresponding active site subpockets (S1, S1′, and S2′).

different ACE inhibitors. Common features were deduced from the bound ACE inhibitors and a pharmacophore hypothesis constructed comprising a C-terminal hydrophobic group, three HBAs representing the carbonyl group and C-terminal carboxyl group, and a customized zinc ligand feature (Figure 3).

That ZBG covered a number of functional groups derived from other metalloprotease inhibitors known to chelate zinc. The first class of ACE2 inhibitors does not give clues about alternative zinc binding groups and C-termini.[24] Thus, known ACE inhibitors were analyzed for occurrence/variability of respective ZBG and C-termini using the generated pharmacophore model. This pharmacophore model also allowed studying the overall structure of ACE inhibitors and their relationship to ACE2 and its inhibitors (Figure 4). The model was able to retrieve all 55 ACE inhibitors demonstrating sufficient generality for the purpose of this analysis.

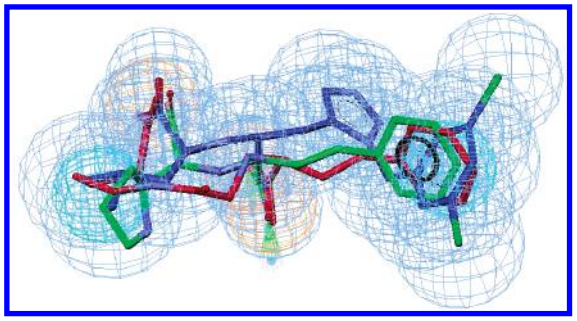


Figure 5. Overlay of ACE2 inhibitor with lisinopril derivates that lack the lysine side chain as potential ACE2 inhibitors. ACE2 inhibitor (blue), lisinopril derivates A (green), and B (red).

Several zinc ligands were found, mainly comprising free thiols and carboxyl groups as well as phosphate groups that constitute the three known ZBG classes (sulfuryl, phosphinyl, carboxyl) of marketed ACE drugs. Additionally, the analysis suggests that ZBGs not observed in ACEI such as ringlike structures are unlikely to act as ZBG, a conclusion that is further supported by the narrow bottleneck nature of the zinc binding region in both the ACE and ACE2 active sites. Sulfides are not observed, and S-methylation is known from ACEI design to diminish binding affinity dramatically.[25]

A free carboxyl is always found as the C-terminal group and never any other HBA. In this sense the inhibitors are typical peptidomimetics, copying the C-terminal carboxyl in peptides. The question arises whether other functional groups (that are negative ionizable and able to accept a hydrogen bond) would be able to replace the carboxyl group (isosteric replacement).

Selectivity Evaluation. The ACE2 active site is highly conserved relative to ACE and the main difference is the absence of the S2′ pocket in ACE2. This pocket is exploited by ACE inhibitors verified by three ACE inhibitor complexes (scheme shown in Figure 4), and its absence in ACE2 prevents binding for a range of ACE inhibitors including those crystallized (captopril, lisinopril, enalaprilat) as well as others such as cilazaprilat and spiraprilat.[19]

For further selectivity evaluation, conformational models were generated for the three ACE inhibitors, lisinopril, enalaprilat, and captopril, in addition to the bioactive conformations. Conformers of each ACE inhibitor were mapped against several ACE2 models. It was shown that the basic five feature model was not able to distinguish between ACE and ACE2 due to adoption of a reversed binding mode with the C-terminal carboxylate mapping the ZBG and the real zinc binding carboxylate fitting to the two C-terminal HBA as shown in the example of lisinopril with a fit value close to MLN-4760. This is possible due to internal symmetry of the scaffold but does not reflect the experimental binding mode due to clashes of the lysine residue of lisinopril with the protein. Introduction of additional spatial constraints in the form of exclusion volume spheres and shape can overcome this effect and prevent lisinopril from mapping the ACE2 model. The binding mode suggests that lisinopril may be a reasonable inhibitor of ACE2 without the lysine residue. Removal of lysine from lisinopril indeed enabled mapping (Figure 5) even in the presence of shape and exclusion volume spheres with a fit value close to MLN-4760 but prevented mapping when the other side chain proline was removed. Two derivates were

Table 2. Selectivity Analysis of Alternative Hypotheses against WDI and ACEI Databases

model	description[a]	# feature	# hits Derwent	% hits Derwent	# hits ACEI	% hits ACEI
HypA	H−NI-2HBA-H-shape	5	145	0.23	2	3.6
HypB	H-2NI−H-shape	4	12	0.02	2.	3.6
HypBM	2NI−Har-shape	3	7	0.01	1	1.8
HypC	H-ZBG-2HBA-H-shape-exVol	5	77	0.12	0	0
HypD	H-2HBA-HBD-HBA-2HBA-H−H−H	10	1	0.002	0	0
HypE	H−PI−HBA5P−H-shape	8	24	0.04	0	0
HypH	Hal-Har-ZBG-NI-shape	4	11	0.02	2	3.6

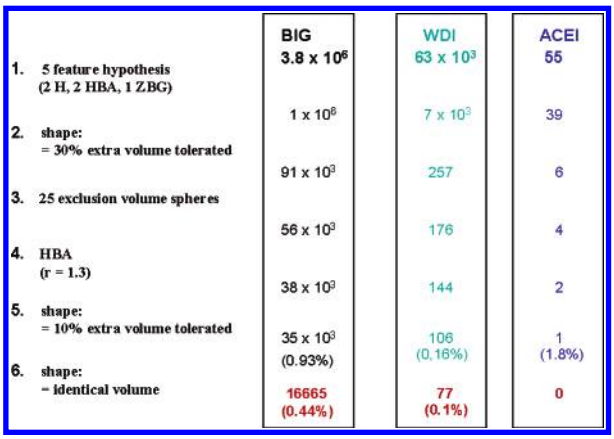
[a] Hypothesis features described in sequence N→C terminus. Hydrophobic (H), hydrophobic aromatic (Har), hydrophobic aliphatic (Hal), hydrogen bond donor (HBD), hydrogen bond acceptor (HBA), projected points only (P), negative ionizable (NI), positive ionizable (PI), zinc binding group (ZBG), exclusion volumes (exVol).

fitted into the model, one mimicking an N-terminal proline in a peptide with a phenylethyl C-terminus (derivate A), the other simply without the lysine side chain keeping the phenylethyl group at the N- and proline at the C-terminus (derivate B). The latter displayed a high fit value but demonstrated an unrealistic, reversed binding mode with the carboxylate C-terminus of proline acting as a conformationally restricted ZBG (attached to the ring instead of the aliphatic chain). It is hypothesised that a truncated lisinopril comprising proline at the N-terminus and phenylpropanyl at the C-terminus could yield a reasonable ACE2 inhibitor, especially since proline was found to be the most favorable residue in biological peptide screening assays.[4]

Selectivity Analysis and Virtual Screening. The standard approach to validation and quality evaluation of a generated hypothesis is by assembling a test set of known active compounds and assessment of the retrieval rate when screening it with the hypothesis. If satisfactory enrichment is achieved, the hypothesis can be applied to search for new compounds by matching the functional and spatial requirements. High fit values based on this geometric scoring function indicate candidate molecules for further evaluation. However fit values must not be interpreted as estimated activity since this information can only be gained from a ligand-based hypothesis designed from a set of active compounds. Due to the lack of small molecule ACE2 inhibitors it was not possible to assess the enrichment quality of the generated models for active compounds. Emphasis is placed on model validation in terms of selectivity against ACE and retrieval of a restricted hitlist from the WDI. ACE selectivity was assessed twice, prior and posterior to virtual screening: First, by searching the ACEI data set with each generated ACE2 pharmacophore model with the aim to retrieve a restricted hitlist based on experimental evidence that ACE inhibitors do not inhibit ACE2. Second, promising hits from ACE2 virtual screening experiments were counter-screened against a pharmacophore model of the structurally close ACE.

Selectivity screening results for each hypothesis against ACE and WDI are summarized in Table 2. The search for ACE2 inhibitors was conducted in a composite database comprising of largely commercial and some in-house databases with ~3.8 million compounds of which ~2.5 million are unique. All data sets were restricted in a stepwise fashion by using increasingly selective models at each stage which is summarized in Table 3. At each step the resulting hitlist was screened with a more restrictive hypothesis introducing additional spatial constraints thereby further reducing the number of hits. The final, most selective hypothesis C was able to restrict the composite database to 0.44%, exhibiting best fit values for 127 hits >4, 1898 hits >3, and 5598 hits >2 with the best achievable fit being 5 (Table 4). The WDI and ACEI were reduced to 0.1% and 0%, respectively, showing best fit values for 2 hits >4, 12 hits > 3, and 34 hits >2 for the former. The majority of WDI hits are likely to be false positives based on visual inspection that revealed unlikely binding modes; however, we cannot exclude some of the well fitting compounds as actives. Conclusions can only be drawn once subjected to biological evaluation in an ACE2 assay. Database screening of the WDI may help to elucidate unknown activity profiles of known therapeutic compounds (reverse screening) e.g. natural compounds and was successfully used as a source for discovering nanomolar inhibitors of ERG2, EBP, and the sigma1 receptor.[26] Some compounds from the WDI carried the activity label vasodilators or hypotensives, of which several cimicifugic acid derivatives were among the top exhibiting high fit values. An example of such a cimicifugic acid hit mapped to the ACE2 hypothesis with a best fit value of 4.2 (the maximum fit value was 5) is shown in Figure 6.

Table 3. Step by Step Virtual Screening, Refinement, and Validation of a Five Feature Pharmacophore Hypothesis with Increasing Selectivity through Spatial Parameter Settings (Shape, Exclusion Volume Spheres, Tolerance Radius *r*)*



* Number of retrieved molecules stated after each screening round for the composite database BIG, WDI, and ACEI. Reduction of database given in % for most selective hypotheses.

Analysis of Virtual Screening Hits. With hypothesis C, many compounds and diverse functional groups are classified as satisfying the C-terminus HBA query. This includes a carbonyl and adjacent hydroxyl group that are unlikely to interact efficiently with the guanidinium group of the

Table 4. Reduction of Structural Databases by Pharmacophore-Based Virtual Screening Using Five Feature Hypothesis C with Shape and Exclusion Volume Spheres

DB name	DB entries	hits total	hits %	hits best fit[a] >2	>3	>4
Analyticon	7046	21	0.30	13	6	0
Arkive	29794	74	0.25	31	12	1
Asinex_Gold	224371	904	0.40	310	91	9
Asinex_Platinum	114652	699	0.61	171	47	4
Aurora	29272	202	0.69	96	51	4
Bionet/Keyorganics	43142	229	0.53	71	20	1
ChemBridge	422648	1655	0.39	491	249	12
ChemDiverse_DC	505649	2519	0.50	994	283	14
ChemDiverse _NC	35747	145	0.41	50	19	0
Chemstar	60153	180	0.30	49	20	0
Comgenex	181422	183	0.10	29	2	0
Derwent	63307	77	0.12	34	12	2
Enamine	359492	3155	0.88	1134	430	30
IFLab/Life Chemicals	126645	816	0.64	278	60	5
Interbioscreen_Syn	344706	1515	0.44	425	111	0
Interbioscreen_Nat	40158	177	0.44	96	50	6
Maybridge 2004	59652	420	0.70	161	55	9
MDPI	10571	7	0.07	3	0	0
Microsource	1971	2	0.10	0	0	0
MedchemLabs_Starfish	72305	285	0.39	95	16	1
MedchemLabs_Shark	103208	284	0.28	61	24	1
Nanosyn_Pharma	46668	84	0.18	30	8	2
Nanosyn_Explore	18597	38	0.20	13	9	0
NCI	123219	138	0.11	60	26	2
Pharmecs	105175	530	0.50	201	77	2
Specs	216823	750	0.35	199	74	4
Strasbourg	4793	2	0.04	1	0	0
Timtec	164493	695	0.42	253	68	12
TosLab_Collection	21990	39	0.18	10	5	0
TosLab_UGI	4317	0	0.00	0	0	0
Vitas-M_Stock	187819	660	0.35	189	43	4
Vitas-M _Tulip	24663	20	0.08	8	3	1
Worldmolecules	33198	160	0.48	42	27	1
total	3787666	16665	0.44	5598	1898	127

[a] Maximum best fit value = 5.

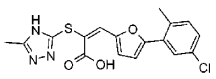
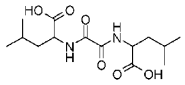
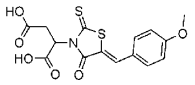
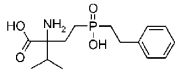
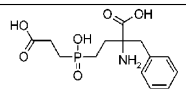
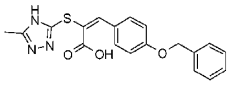
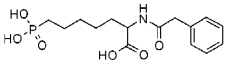
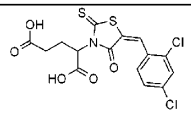
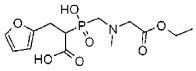
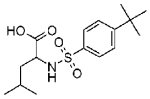
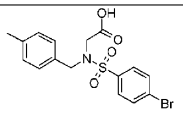
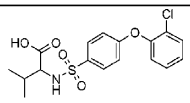
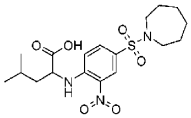
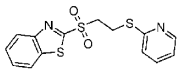
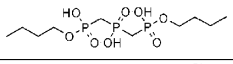
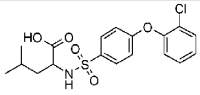
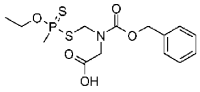
Figure 6. Example of top scoring hit from the Derwent database. Cimicifugic acid C is mapped into the five-feature ACE2 hypothesis C with shape.

substrate binding residue, Arg273 in ACE2. Nevertheless, hypothesis C proved to be the richest source of interesting hits. For each retrieved hitlist, a geometric best-fit value was calculated, and only top scoring hits were subjected to further visual inspection. In many cases retrieved compounds were highly similar to one another both within a database as well as across different databases. Compounds were not filtered for duplicates initially to identify all vendors offering a certain hit compound. Care was taken to ensure structural diversity in terms of constituents such as the zinc binding group, C-terminal HBA, and hydrophobic side chains. H features were usually mapped by five- or six-membered heterocycles with various substituents but overall little diversity. The majority are considered to be too large for the S1 pocket, following experimental evidence of peptide substrate screening where neither tyrosine nor phenylalanine is accepted at the P1 position.[4] Therefore this hydrophobic feature was removed in hypothesis BM. Compounds without that H feature were assumed to have reduced but sufficient binding affinity. Scaffolds were limited to aliphatic chainlike structures due to the restrictive nature of the ACE2 active site and the fact that potent inhibitors are likely to be peptidomimetics. Screening with any pharmacophores that included the ZBG feature resulted in the majority of hits bearing heteroaromatic rings or sulfide groups (as the ZBG feature). However, compounds containing either feature are unlikely to exhibit high affinity. Exchanging the ZBG in hypothesis C for a NI creates hypothesis A, which exclusively yielded carboxylates as the zinc ligand in 200 top scoring hit compounds. Specification of a second NI at the C-terminus in hypothesis B yielded 72 hits and only 17 with shape constraints, again mostly yielding carboxylates at the C-terminus.

No compound was found with ideal spacer distance between ZBG and C-terminal HBA and/or combinations of functional groups expected to yield highly potent inhibitors such as the original ACE2 inhibitor. However, to evaluate the potential of the most promising, high scoring hits in a biochemical assay, 25 compounds were selected, originating from various hypotheses but mainly hypothesis C. Each was screened against the ACE model, and all but one compound passed this selectivity test. The identified compound (1N-08795) was previously considered to be an unlikely ACE2 inhibitor but included for selection to evaluate specific aspects of its structure and binding capability. It may also be a candidate for ACE inhibition.

Biological Validation. Availability and vendor issues guided the selection of 17 of the 25 compounds for purchase. Preliminary results from ACE2 competition assays showed that all selected compounds were found to be active with various degrees of inhibition at 200 μM compound concentration with up to 93% for the most promising candidates considered for IC_{50} analysis (Table 5). This corresponds to a hit rate of 41%, with a minimal cut off at 70% inhibition and can be interpreted as good success. Six primary hits were further subjected to IC_{50} studies, and all revealed novel micromolar ACE2 inhibitors. The dose−response curve for one of the three candidate lead compounds with IC_{50} values of <100 μmol/L is shown in Figure 7. All six novel ACE2 inhibitors—comprising synthetic and natural compounds—were purchased from Interbioscreen,[27] which proved to be the richest source of compounds with ACE2 modulating activity. The best three are visualized in their respective pharmacophore model (Figure 8). An interesting revelation is the high affinity of compound 1N-08795, which was solely included in the biological analysis to evaluate its C-terminal hydrogen bonding capabilities, presumably by contribution of the carbonyl and carboxyl group. At the same time it fulfills the ACE pharmacophore, by matching perfectly its carboxyalkyl ZBG, the carbonyl HBA, and the C-terminal carboxylate including correct spacing. The only difference is found in the C-terminal hydrophobic part which is made up by a leucine rather than a hydrophobic ring, However replacement of the aromatic ring by a methyl has been observed in the dual ACE/NEP inhibitors alatrioprilat and

Table 5. Compounds Selected and Purchased for Biological Validation: ACE2 Inhibition at 200 μM Concentration and IC_{50} of Selected Compounds

Name	2D Structure	% Inhibition [200 μM]	IC_{50}* [μM]
4S-16659		76	62
1N-08795		90	96
1S-91206		75	84
1N-28616		93	116
1N-26923		93	134
4S-14713		70	179
1N-27714		89	ND
T0507-4963		41	ND
3S-95223		40	ND
7857351		27	ND
7490938		20	ND
7850455		20	ND
T0515-3007		13	ND
7870029		11	ND
1S-90995		11	ND
T0513-5544		4	ND
5115980		1	ND

* ND indicates not determined.

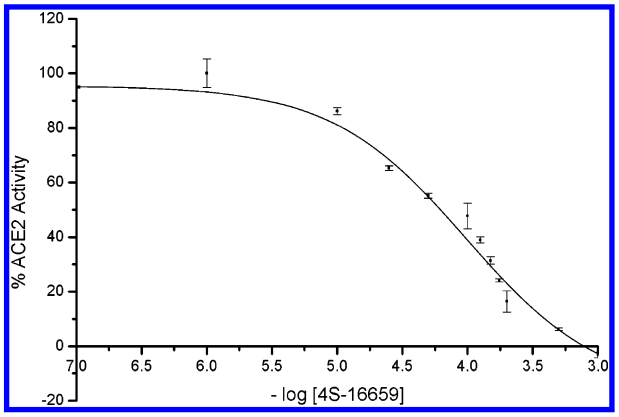


Figure 7. Dose−response curve of ACE2 inhibition by compound 4S-16659.

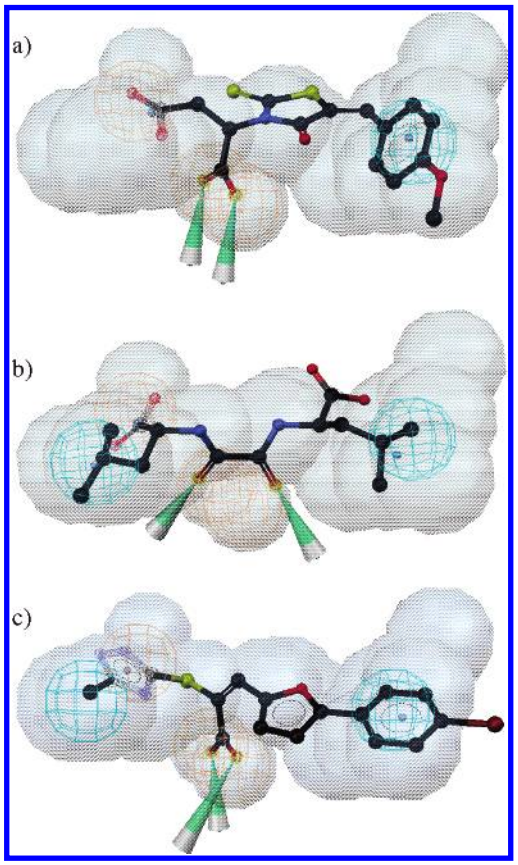


Figure 8. Novel ACE2 inhibitors mapped to four and five feature pharmacophore hypotheses and integrated shape: 1S-91206 (a), 1N-08795 (b), and 4S-16659 (c).

RB-105. The compound 1N-08795 deviates from typical ACE inhibitors by the presence of a carbonyl group at the S1′ side chain position. This is usually taken up by a methyl or lysine. Evaluation in an ACE bioassay would clarify its potential to inhibit ACE.

Docking Analysis. All selected compounds were also docked into the active site to confirm the correct binding mode and ensure geometric fit. Docking proved to be difficult for this target. This was expected due to the low resolution of the crystal structure and the metalloprotease character which is not handled well by most docking programs.[28] For successful docking it is essential to recreate the correct zinc coordination geometry, proving docking programs that use geometrical models superior over those that model metal−ligand interactions simply with electrostatic and van der Waals interactions.[28] Compound 4S-16659 is visualized in the active site by one of its low energy docking poses next to the bioactive conformation of the potent ACE2 inhibitor MLN-4760 (Figure 9). The pose indicates a novel zinc binding group, a 1,2,4-triazole heterocycle, not found in any marketed ACE drugs. This conformation of 4S-16659 aligns well with MLN-4760; however, the true binding mode will only be revealed by experimental structure elucidation.

Figure 9. Novel ACE2 inhibitor docked in the active site of ACE2. The potent inhibitor MLN-4760 is shown for comparison in its bioactive conformation (blue) next to compound 4S-16659 (green). ACE2 is visualized as molecular surface sliced through to display the catalytic center and bound ligands along with the zinc ion (gold sphere).

CONCLUSION

A successful virtual screening application was presented revealing novel, micromolar ACE2 inhibitors. The approach was based on structure-based pharmacophore modeling and screening a large database comprising products of various commercial compound vendors. The software packages LigandScout and Catalyst were used for identification and visualization of protein−ligand interaction sites, pharmacophore model generation, and database searching, respectively. Chemical feature-based pharmacophore models were generated based on the ACE2 X-ray crystal structure in complex with a potent inhibitor. Requirements for ACE2 inhibition were established through in depth analysis of a set of known ACE inhibitors and known ACE2 substrates. The models were assessed for selectivity by retrieving a restricted hitlist searching the internal ACE Inhibitor database and the WDI. Resulting hitlists were further visually analyzed for structural and functional ACE2 inhibitor requirements and structural diversity of functional group constituents. A subset of 25 compounds was proposed for biological testing, 17 of which were purchased and subjected to an ACE2 competition assay at 200 μM. All compounds showed some level of inhibitory activity at this stage. The best six compounds were selected for IC_{50} studies, all yielding novel ACE2 inhibitors, three with $IC_{50} < 100$ μmol/L, the rest with $IC_{50} < 180$ μmol/L. Their binding mode and interactions were further analyzed via docking and revealed close similarity to the complexed ACE2 inhibitor.

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