Virtual Screening of Novel Noncovalent Inhibitors for SARS-CoV 3C-like Proteinase

Zhenming Liu,[†,‡] Changkang Huang,[†] Keqiang Fan,[†] Ping Wei,[†] Hao Chen,[†,‡] Shiyong Liu,[†,‡] Jianfeng Pei,[†,‡] Lei Shi,[†] Bo Li,[†] Kun Yang,[†,‡] Ying Liu,*[,†] and Luhua Lai*[,†,‡]

State Key Laboratory for Structural Chemistry of Unstable and Stable Species, College of Chemistry and Molecular Engineering, and Center for Theoretical Biology, Peking University, Beijing 100871, China

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The SARS coronavirus 3C-like proteinase is considered as a potential drug design target for the treatment of severe acute respiratory syndrome (SARS). Owing to the lack of available drugs for the treatment of SARS, the discovery of inhibitors for SARS coronavirus 3C-like proteinase that can potentially be optimized as drugs appears to be highly desirable. We have built a "flexible" three-dimensional model for SARS 3C-like proteinase by homology modeling and multicanonical molecular dynamics method and used the model for virtual screening of chemical databases. After Dock procedures, strategies including pharmocophore model, consensus scoring, and "drug-like" filters were applied in order to accelerate the process and improve the success rate of virtual docking screening hit lists. Forty compounds were purchased and tested by HPLC and colorimetric assay against SARS 3C-like proteinase. Three of them including calmidazolium, a well-known antagonist of calmodulin, were found to inhibit the enzyme with an apparent K_i from 61 to 178 μM. These active compounds and their binding modes provide useful information for understanding the binding sites and for further selective drug design against SARS and other coronavirus.

INTRODUCTION

Severe acute respiratory syndrome (SARS) is a respiratory illness that had an ever dramatic outbreak and was spread in Asia, North America, and Europe in early 2003.[1,2] Many evidences indicate that a previously unrecognized coronavirus exists, which is called SARS coronavirus, and is the leading hypothesis for the cause of SARS.[3−5] Many therapies including different drugs have been tested and used for SARS.[6−8] For example, ribavirin, a nucleoside analogue with broad antiviral activity has been used in combination with interferon $\alpha 2\beta$ to treat this disease and decrease the death rate.[9,10] However, there is no evidence that they are valid and reliable measures.[11−13] Threatened by such a severe disease, it is a significant challenge for scientists in all areas to try and find reliable countermeasures.

It was known that the cleavage process of the SARS-CoV polyproteins by a special proteinase, the so-called SARS coronavirus 3C-like proteinase (CoV Mpro), is a key step for the replication of SARS-CoV.[14] Homology modeling for the SARS 3C-like proteinase has been performed by various groups,[15−17] and the conformational flexibility of the substrate-binding site has been studied.[16] Virtual screening of chemical compounds libraries has predicted possible inhibitors.[17,18] An 8mer peptide has been docked on the model of SARS 3C-like proteinase to study the possible interactions of the protein and the substrate.[19] Two crystal structures of coronavirus 3C-like proteinase from transmissible gastroenteritis virus (TGEV)[20] and human coronavirus (hCoV) 229E[15] have been solved. The SARS coronavirus 3C-like proteinase crystal structure has also been solved by two groups recently with the coordinates deposited in the Protein Data Bank (http://www.rcsb.org). One of the groups (Z. H. Rao's) also reported a complex structure of SARS 3C-like proteinase with a covalent bonded substrate analogue at the same time.[21]

The 3C-like proteinase and similar proteinases have been found in many viruses including rhinovirus and hepatitis A virus.[22,23] It is known to be an attractive target enzyme against the common cold and hepatitis. Unlike other thiol proteases such as papain, it is a very specific cysteine protease, which has an overall backbone fold similar to trypsin-like serine proteases, and is responsible primarily for the catalytic cleavage of glutamine-glycine (serine) peptide bonds.[24,25] Covalent-bonded peptide and non-peptide inhibitors have been designed and discovered to inhibit 3C proteinases and therefore to treat related diseases.[26,27] These inhibitors can be used as a guidance to design drugs for SARS.[18,28] Active compounds have been obtained by covalent bonding with the cysteine at catalytic site. However, side effects and toxicity can often be caused by covalent-bonding.[29,30] It somehow prevents the inhibitors to be developed into drugs and used for clinic therapy. Thus, for SARS treatment, it is more attractive to design and discover noncovalent inhibitors.

Virtual screen by molecular docking of chemical databases is one of the most powerful approaches to discover noncovalent inhibitors.[31,32] We report here the database screening, design, biological evaluation, and structure modification of reversible, noncovalent inhibitors of SARS 3C-like proteinase (See details in Figure 1.). An improved virtual docking strategy considering pharmacophore model, consensus scoring, and "drug-like" filters was applied to improve the success rate of VDS hit lists. Forty compounds were purchased and experimentally tested by HPLC and colorimetric assays for SARS 3C-like proteinase. Three of them were found to inhibit SARS 3C-like protease with apparent

* Corresponding authors phone: 86-10-62757486; fax: 86-10-62751725; e-mail: lhlai@pku.edu.cn.
† College of Chemistry and Molecular Engineering.
‡ Center for Theoretical Biology.

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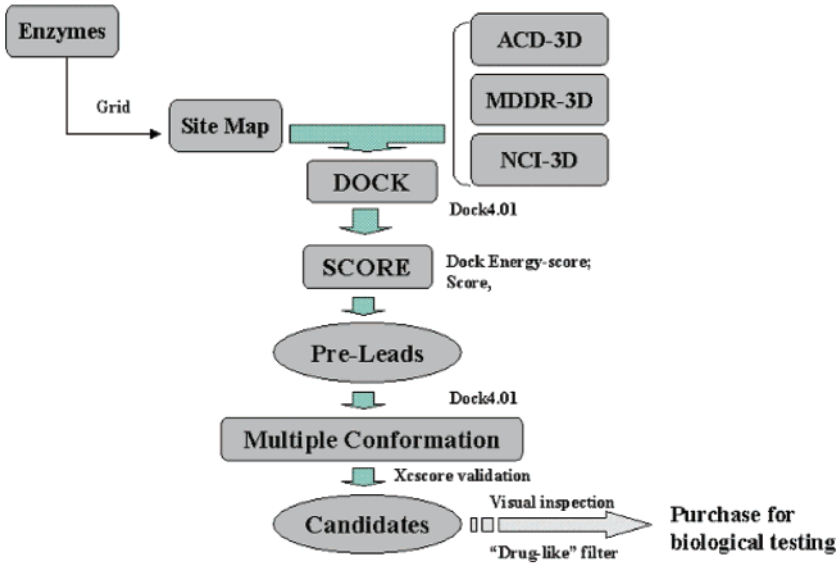


Figure 1. Docking strategy for data generation and validation. Consensus scores were used here to rerank and validate the virtual screening result. "Drug-like" filter considering the enzyme properties makes the selection more likely to inhibit the target.

K_i from 61 to 178 μM which can be used for further optimization.

MATERIALS AND METHODS

Generation of the "Flexible Model" of SARS 3C-like Proteinase for Docking. An accurate three-dimensional (3D) structure of the target is required for virtual screening by docking. The crystal structure of SARS 3C-like proteinase was not publicly available at the time of this work. A 3D homology model of SARS 3C-like proteinase based on the structure of transmissible-gastroenteritis-virus coronavirus 3C-like proteinase was built by using MODELLER6.0 program.[16] When the coordinates of SARS coronavirus 3C-like proteinase or SARS-CoV Mpro were recently released, we compared our model to the crystal structure determined by Rao Z's group.[21] The comparison result shows good conservation, especially for the catalytic domain. The crystal structure was finally used to validate the hit list and to find the possible binding conformations.

Considering the case that the binding site conformation may change when binding with a ligand,[33] the conformational flexibility of the active site was simulated by molecular dynamics combined with multicanonical sampling for a flexible target.[16] Molecular dynamics simulations coupled with the multicanonical sampling method were carried out with an implicit solvation model for 10 ns. Conformational clustering gave two major groups of structures for further docking studies. The active site loops were found to have two typical conformations, which may be related to the conformational movement associated with the enzymatic reaction. One of the conformations from molecular dynamics simulation is similar to the original model, which has a smaller binding site with a close loop conformation; the other one has a larger pocket caused by the open loop conformation. As the pocket in the first conformation seems to be small for VDS, the structure with a larger pocket was selected for further docking study.

Virtual Docking Screening Combined with Pharmacophore Model, Consensus Scoring, and "Drug-like" Filters. Our goal was to screen chemical databases including

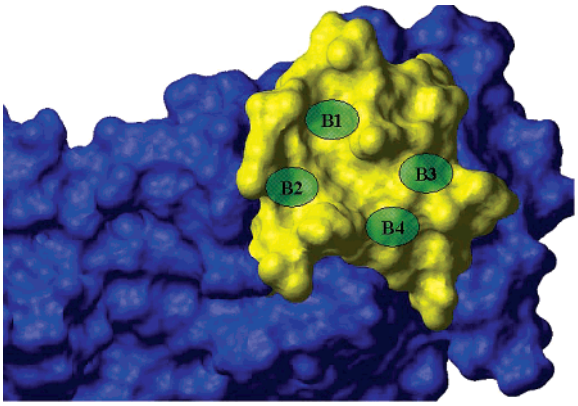


Figure 2. Top view of the large active site of SARS 3C-like proteinase and a rectangle box that defines the mass center of binding pocket. Three important binding residues including His41, Cys145, and Glu166 were at the positions B3, B4, and B2

The National Cancer Institute Diversity Set (~230 000 compounds total), ACD-3D (Available Chemical Database, Release: ACD 3D 2002.2, ~280 000 compounds in total), and MDDR-3D (MDL Drug Database Report, Release: MDDR 3D 2002.2, 120 000 compounds in total). The Dock program, developed by Kuntz and co-workers,[33] has been applied successfully in many screening studies. In this work by using the MPI Edition Dock4.01 program modified in our lab, a million compounds could be screened and scored on Linux cluster within a reasonable period of time.

The first step for docking is to define the possible ligand binding sites (Figure 2.). The binding cleft of SARS 3C-like proteinase is located between domain 1 and domain 2. His41 and Cys145, known as two catalytic residues, were included when considering the possible inhibitors binding. The TGEV coronavirus 3C-like proteinase (3C-likepro) structure, determined by Anand et al.,[15] and the structure of rhinovirus 3C proteinase (PDB code 1CQQ)[23] were used to determine the possible pocket. The former structure contains an eight residues peptide substrate analogue, which takes up the S3, S2, and S1 position. The latter structure is a 3C protease from rhinovirus, with a binding pocket similar to SARS 3C-like proteinase. The covalent inhibitor AG7088

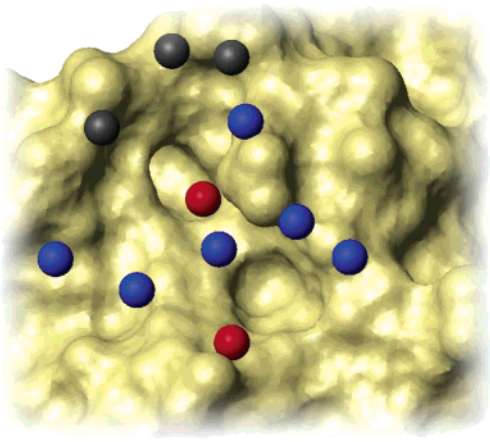


Figure 3. Pharmacophore model generated by the POCKET module from LigBuilder from protein model based on pocket residues: **red ball,** hydrogen-bond acceptor; **blue ball,** hydrogen-bond donor; and **black ball,** hydrophobic sites.

Table 1. Residues Chosen To Limit the Binding Conformation Based on Pharmocophore Model

type	residues
donor	HIS41, THR25
acceptor	GLU166
hydrophobic site	LYS141, MET165

locates in the binding cleft and takes up most of the volume. A pharmocophore model (Figure 3) was generated by the POCKET module from LigBuilder.[34] A few residues including His41 and Glu166 were chosen as the key residues for site-directed docking (Table 1).

Virtual screening with docking was performed on a Linux Cluster Platform which contains 128 CPU (Inter P−IV 2.6GHz) on 64 computing nodes. For each single database docking/score result, those compounds with both dock energy-score and contact-score ranked top 10% were chosen as the primary selection list. For example, about 28 000 compounds were selected by docking scores from the pool of 280 000 compounds in ACD database.

The second step, following the consensus scoring approach, the Score program developed by Wang et al.[35] was applied to reevaluate the top-ranked molecules. Molecules with calculated pK_i higher than 4.0 were retained in the hit list. Almost 22 000 molecules were removed after this filter. The remaining 8962 compounds were then redocked by using Dock4.0 program to generate 500 conformations per molecule for further scoring.[36]

Xcscore Program developed by Wang et al.[37] combines three empirical functions to calculate protein−ligand binding affinity. The calculated pK_i can somewhat remove the score bias caused by single empirical function and was considered more accurate and possible to reflect the actual binding ability. The multiple conformations of 8962 compounds were then calculated and reevaluated by Xcscore to generate the final "hit lists". All compounds with calculated pK_i higher than 4.0 and ranked in the top 2000 for both Score and Xcscore results were kept in the hit lists. Finally, 984 compounds were selected after docking/scoring filter evaluation.

The final stage was the unavoidable visual inspection of the remaining top ranked candidates. It always means a cost of time, especially when the hit list still contains thousands of molecules. In our case, we applied our calculated pharmacophore model to reduce the working load. Those molecules that were a better match with the pharmocophore were kept. Others that cannot match enough pharmacophore elements or have more than double of the elements were removed from the candidate lists. This method was also applied to cluster the final hits within the same binding mode and prepare a molecule list for purchase.

To make the hit lists more reliable and possible to be used in animals and humans, some "drug-like" rules were applied which include the following:

1. Molecular weight < 1000. Considering the binding site volume of the target, this is necessary for possible large inhibitors.

2. Drug-like molecule. All the hit structures were searched in the MDDR database. Chemical structures similar to the original drug framework were considered for priority.

3. Anti-viral consideration. Molecules in the candidates list were similar structure fragments to former antiviral compounds (for example nucleotide and analogs), and proteinase inhibitors (for example HIV-1 proteinase inhibitors and inhibitors of rhinovirus and hepatitis A virus 3C protease) were paid more attention and kept in the final hit list.

Enzyme Assays. *HPLC Assay.* The SARS 3C-like proteinase HPLC assay was done as reported previously.[14] The inhibitors were solved in DMSO and mixed with the substrate peptide S01 (TSAVLQSGFRK) aqueous solution. The SARS 3C-like proteinase was incubated with the mixture in 40 mM Tris-HCl buffer, pH 8, at room temperature, with a final concentration of 1.8 μM enzyme, 1.6 mM substrate, 0.1 mM inhibitor, 17 mM DTT, and 10% DMSO. The cleavage reaction was stopped after 20 min by adding of 0.1% TFA aqueous solution and analyzed by RP-HPLC (LabPrep System, Gilson) on a Zorbax C18 analytic column (4.6 by 250 mm, Agilent). Cleavage products were resolved using a 15-min, 0−50% linear gradient of acetonitrile in 0.1% TFA. Peak areas were calculated by integration. The inhibiting percentage was calculated by comparing the peak area of the two cleavage products with those without any inhibitor and averaged.

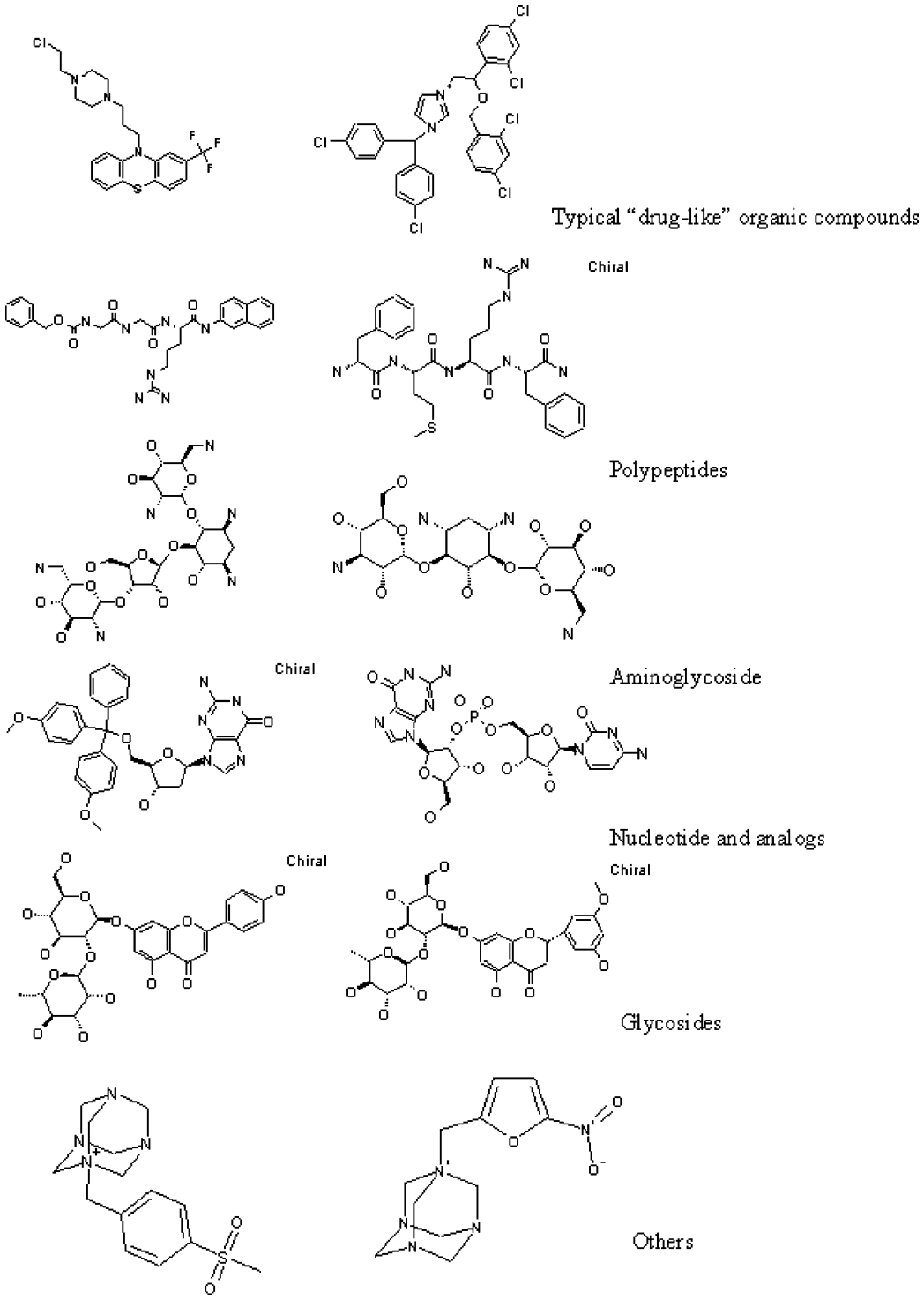
Continuous Colorimetric Assay. We have developed a continuous colorimetric bioassay for SARS 3C-like proteinase.[38] The experiment was performed with a multiwell ultraviolet spectrometer (SPECTRA MAX 190, Molecular Device). The substrate, Thr-Ser-Ala-Val-Leu-Gln-pNA, which is cleaved at the Gln-pNA bond was purchased from GL Biochemistry Ltd., China. All inhibitors were solved in dimethyl sulfoxide (DMSO). One hundred forty-five microliters of buffer (20 mM Tris-HCl, 1 mM DTT, pH 7.63), 20 μL of SARS 3C-like proteinase stock solution (1.5 mg/mL buffer solution), and 10 μL of inhibitor stock solution were mixed at 37 °C for about 5 min. The absorbance of the solution was recorded after 25 μL of substrate stock solution (2 mM in water) was added for 20 min. v_0, the velocity of absorbance change without inhibitors, can be denoted as

$$v_0 = (k_c/K_m)[ES]/(1 + [S]/K_m) \quad (1)$$

and v, the velocity under the inhibitor concentration I:

$$v = (k_c/K_m)[ES]/(1 + [S]/K_m + [I]/K_i) \quad (2)$$

Chart 1. Structures of Candidates after Virtual Docking Screening



The inhibitory percentage under the concentration [I] was calculated as

$$1 - v/v_0 \tag{3}$$

A series of velocity for different inhibitor concentrations was were measured to get the dissociation constant:

$$v_0/v = 1 + [\mathrm{I}]/(1 + [\mathrm{S}]/K_\mathrm{m})K_\mathrm{i} \tag{4}$$

$$v_0/v \text{ is linear to [I], and } K_\mathrm{i} = 1/\text{slope } (1+[\mathrm{S}]/K_\mathrm{m}) \tag{5}$$

As the concentration of the substrate [S] (~250 μM) is much less than K_m (~6 mM), K_i can be calculated approximately as

$$K_\mathrm{i} = 1/\text{slope} \tag{6}$$

RESULTS AND DISCUSSION

After the above procedure, a total of 126 molecules with more than 10 types of chemical structures were selected for further study. Some of them were listed below in Chart 1. In the final hit lists, there are 56 typical "drug-like" organic compounds, 50 polypeptides and analogues, 7 aminoglycoside compounds, 2 nucleotide and analogues, 5 glycosides, and 2 other compounds considered with antiviral activity.

Table 2. Compound Types and Their Inhibition Activity

compound types	purchased	activity >10%	%age (%)	activity >40%	%age (%)
organic compounds	21	6	33.33	1	4.76
polypeptides analogues	10	4	40.00	2	20.00
aminoglycoside	3	1	33.33	0	0.00
nucleotide analogues	2	1	50.00	0	0.00
glycosides	3	2	66.67	0	0.00
others	1	0	0.00	0	0.00
total	40	14	35.00	3	7.50

As the target protein is a proteinase from a viral source, in addition to drug-like structure framework, two types of molecules were paid more attention: polypeptide analogues and molecules with potential antiviral activity. Polypeptides and analogues were investigated carefully to find possible inhibitors that bind the enzyme similar to substrate binding mode. Aminoglycoside was another larger series of compounds in our hit list. Considering both the structure flexibility and their potential antiviral activity, three of them were selected and purchased for biological testing.

Enzyme Inhibition. Following the above process, 40 compounds from the hit list were selected and ordered from the SIGMA and ICN chemical archives and then tested qualitatively by HPLC and quantitatively by a continuous colorimetric assay developed in our laboratory.[38] Among those compounds from the hit lists, there are 21 typical "drug-like" organic compounds, 10 polypeptides and analogues, 3 aminoglycoside compounds, 2 nucleotides and analogues, 3 glycosides, and 1 other compound. In our experiments, inhibition rate at the inhibitor concentration 250 μmol/L for each compound was tested. Fourteen compounds were found to inhibit SARS-coronavirus 3C-like proteinase by more than 10% (Table 3 and Figure 4). Among them, three of the selected compounds turned out to inhibit more than 40% of the enzymatic activity of SARS coronavirus 3C-like proteinase. The K_i values for these three compounds were experimentally determined to be 61 ± 6 μmol/L, 84 ± 2, and 178 ± 9 μmol/L, respectively. They are all noncovalent inhibitors of SARS coronavirus 3C-like proteinase. This is the first time that potent noncovalent inhibitors of SARS coronavirus 3C-like proteinase have been experimentally verified. Although several groups have reported potential active compounds by docking studies,[17,39,40] no enzyme inhibition experiments were reported.

Binding Mode of Calmidazolium. It is interesting that the compound with the strongest inhibition ability is a well-known calmodulin antagonist: C3930 or calmidazolium. In body, it takes part in and modulates many physiological processes including signal transduction and cell trafficking, ion channel modulators, and steroid genesis. It is also a regular tool compound used as an inhibitor of brain calmodulin-dependent phosphodiesterase and voltage-gated Ca^{2+}channels in many biological experiments. All those advantages make it possible to be used as a low toxic compound directly in animals and human.

The binding pose found by Dock4.0 for C3930 is at the center of the cleft and makes contact with residues His41, Glu166, and Met 165. As we can see from Figure 5, the ring 1,3,4 of C3930 occupies approximately the S2, S1, and S1′ position in the binding site. Volume occupation of the pocket with hydrophobic interactions was considered a basic requirement for binding and biologic activity. The binding mode of C3930 shows that the S1′ position is quite large and can be used to develop potent inhibitors for SARS 3C-like proteinase. Strong electrostatic interaction between ring 1 and Asp48 was found to increase binding affinity. Only one typical hydrogen bond was found between ring 1 and His41. However, considering that His41 is one of the catalytic residues, the aromatic ring stacking between ring 1 of C3930 and His41 may also contribute to the binding. Further experimental analysis is necessary to prove this interaction.

Model Structure Versus Crystal Structure. The flexible model of SARS 3C-like proteinase used in a virtual screen turns out to be successful for producing an active compound list. We would like to know if there is any difference between the docking/score result from the model structure used here and the crystal structure.

Two crystal structures published by Z. H. Rao's group were used in the redocking calculation. The structure with PDB entry 1UK4 has an octapeptide analogue as the covalent inhibitor in the binding site. The other structure (PDB entry 1UK3) does not have a small molecule in the pocket. The compounds in the hit lists were docked again by using the Dock program against the two crystal structures. Only the best energy-score conformation of each molecule was kept and used for analysis. Figure 6 shows the correlation of docking energies by using the crystal structures and our model.

The comparison indicates that the docking/score result using our model correlates well with that from the complex

Table 3. Docking/Score, Experimental Testing, and Inhibition Rate for Candidates

compound	corank (dock and score)	docking score (KJ/mol)	score	Xcscore	inhibition rate % (250 μmol/L)	Ki (μM)
153706	20	−47.60	5.96	4.79	$15 \pm 5.$	
F102	133	−44.65	5.23	5.37	12 ± 3	
D7154	294	−42.28	5.03	5.28	27 ± 4	
P3847	18	−44.81	5.96	5.26	11 ± 1	
C9521	1526	−49.85	4.29	4.81	40 ± 3	178 ± 9
A8906	11	−43.53	7.05	5.64	25 ± 1	
A8750	544	−39.81	4.90	5.15	12 ± 9	
A3261	688	−57.51	4.68	4.88	12 ± 2	
S78, 784-1	59	−44.40	5.48	5.14	13 ± 2	
P4170	242	−44.71	5.04	6.09	$12 \pm 4.$	
195623	1964	−43.88	4.35	4.84	75 ± 1	84 ± 2
41548-5000	13	−43.72	6.50	7.77	22 ± 3	
13241-33-3	8	−47.05	8.09	5.88	12 ± 2	
C3930	115	−47.95	5.33	5.73	inhibits 43% at 40 μmol/L	61 ± 6

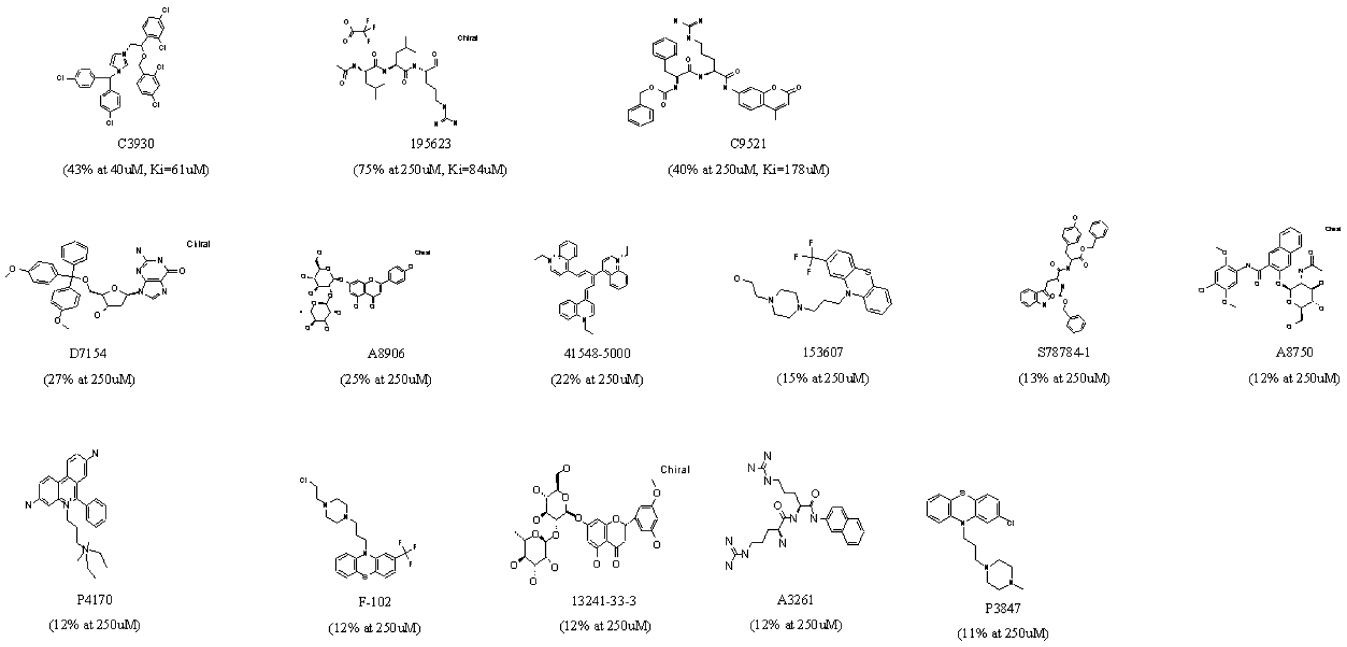


Figure 4. Chemical structures of active compounds identified by virtual docking screening.

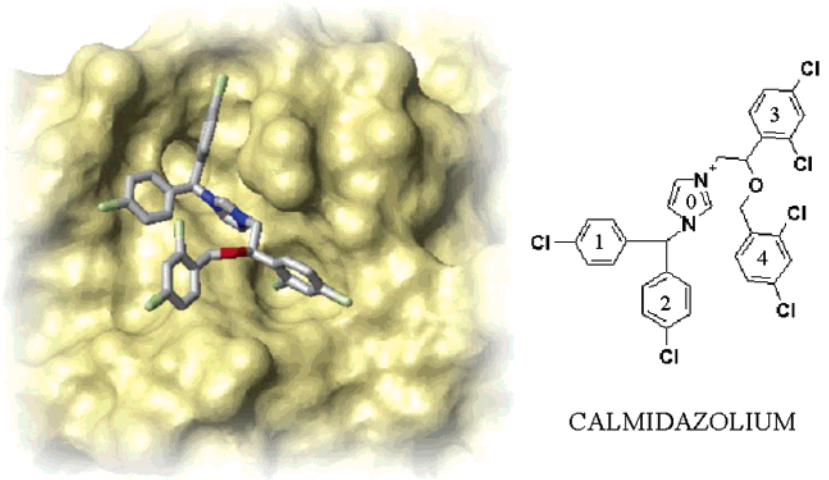


Figure 5. Binding mode of C3930 with SARS coronavirus 3C-like proteinase.

structure but worse with that from the free enzyme. The binding site of the free enzyme structure 1UK3 is smaller than 1UK4 and our model structure due to closed loops. This indicates that the flexibility of binding sites especially the loop flexibility was important for SARS coronavirus 3C-like proteinase ligand screening.

CONCLUSION

This research led to the discovery of novel noncovalent inhibitors of SARS coronavirus 3C-like proteinase. After virtual docking screen and post-dock scoring filters, 40 potential compounds were selected and purchased for biological testing. Three of them were found to inhibit SARS coronavirus 3C-like proteinase in the micromolar range. The compound calmidazolium, a well-known calmodulin antagonist, was found to inhibit the enzymatic activity of SARS coronavirus 3C-like proteinase with a K_i value of 61 μM. These active compounds serve as the basis for developing drugs against SARS. The binding conformation of active compounds also provides useful clues for finding and optimizing 3C-like proteinase inhibitors.

Overall, our results further demonstrate the effectiveness of docking based virtual screening for the de novo discovery of inhibitors and their subsequent optimization. In this context, refinement of the enzyme with molecular dynamics and using a "flexible model" for docking screening turn out to be important aspects of the design.

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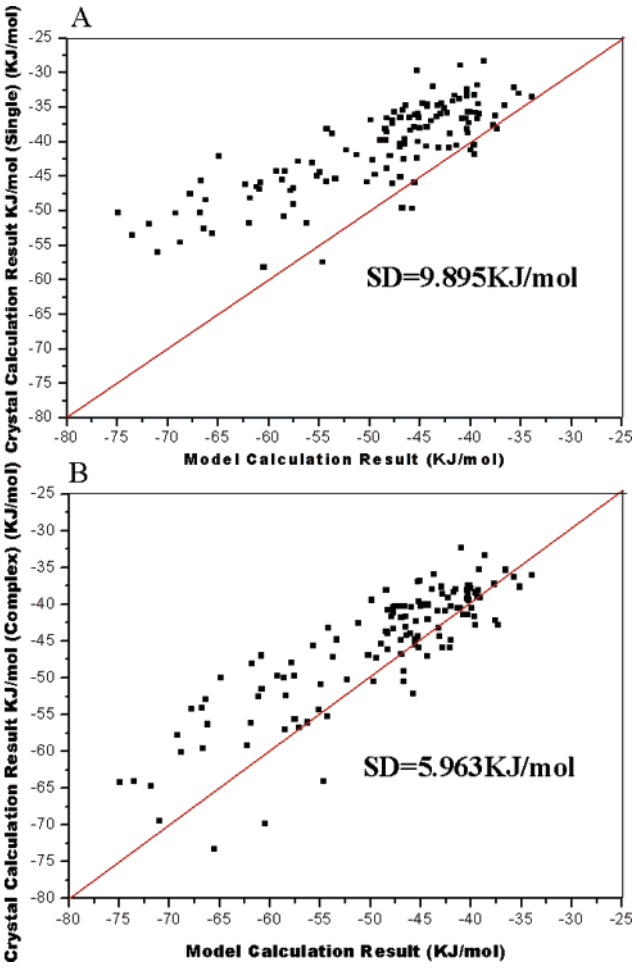

Figure 6. Comparison of docking/score energies using model structure and crystal structures. (A) SARS 3C-like proteinase crystal structure of the free enzyme and the model structure. (B) SARS 3C-like proteinase crystal structure of the complex and the model structure.

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