Discovering Severe Acute Respiratory Syndrome Coronavirus 3CL Protease Inhibitors: Virtual Screening, Surface Plasmon Resonance, and Fluorescence Resonance Energy Transfer Assays

LILI CHEN,[1] SHUAI CHEN,[1] CHUNSHAN GUI,[1] JIANHUA SHEN,[1] XU SHEN,[1,2] and HUALIANG JIANG[1,2]

An integrated system has been developed for discovering potent inhibitors of severe acute respiratory syndrome coronavirus 3C–like protease (SARS-CoV 3CL[pro]) by virtual screening correlating with surface plasmon resonance (SPR) and fluorescence resonance energy transfer (FRET) technologies-based assays. The authors screened 81,287 small molecular compounds against SPECS database by virtual screening; 256 compounds were subsequently selected for biological evaluation. Through SPR technology-based assay, 52 from these 256 compounds were discovered to show binding to SARS-CoV 3CL[pro]. The enzymatic inhibition activities of these 52 SARS-CoV 3CL[pro] binders were further applied to FRET-based assay, and IC$_{50}$ values were determined. Based on this integrated assay platform, 8 new SARS-CoV 3CL[pro] inhibitors were discovered. The fact that the obtained IC$_{50}$ values for the inhibitors are in good accordance with the discovered dissociation equilibrium constants (K$_D$s) assayed by SPR implied the reliability of this platform. Our current work is hoped to supply a powerful approach in the discovery of potent SARS-CoV 3CL[pro] inhibitors, and the determined inhibitors could be used as possible lead compounds for further research. (*Journal of Biomolecular Screening* 2006:915-921)

Key words: SARS, SARS-CoV 3CL[pro], inhibitor, virtual screening, SPR, FRET

THE LAST OUTBREAK OF THE SEVERE ACUTE RESPIRATORY SYNDROME (SARS) epidemic in 2003 has led to thousands of lethally infected patients and hundreds of deaths,[1] and the serious aftereffect has caused major medical and economic concerns. It has been reported that SARS coronavirus (SARS-CoV) is responsible for SARS infection,[2,3] and SARS-CoV 3C–like proteinase (SARS-CoV 3CL[pro]) is an attractive target for the discovery of anti-SARS agents for its functional importance in the viral life cycle. To date, varied kinds of SARS-CoV 3CL[pro] inhibitors were discovered,[4-6] although no effective SARS-CoV 3CL[pro] inhibitor has yet been reported to treat SARS. Therefore, it is still a great challenge to explore new chemical classes of SARS-CoV 3CL[pro] inhibitors that can be possibly used in anti-SARS research.

As has been used in the current drug-discovery process, by using the high-performance computation technique to search the large chemical compound databases for the identification of possible drug candidates, the virtual screening approach is a technology that is based on the 3D structure of the target protein. It involves the rapid fitting of the chemical library members into the active sites of 3D protein structures and is critical to distinguish active from inactive substances at the primary screening stage. To date, there have been successful cases of virtual screening used in the discovery of lead compound candidates. For example, we recently reported that by using virtual screening technology with other methods, several new, small molecular specific cyclophilin A inhibitors were discovered[7]; through virtual screening with surface plasmon resonance (SPR) technology against a database containing structural information of more than 8000 available drugs, Cinanserin (SQ 10,643), a well-characterized serotonin antagonist that had undergone preliminary clinical testing in humans in the 1960s, was discovered to be the SARS-CoV 3CL[pro] inhibitor.[5]

Recently, SPR technology has been recognized as a powerful tool in monitoring receptor-ligand interactions with advantages of no-labeling, real-time, noninvasive measurements and low sample

consumption.[8] It has been widely used in hit determination,[7,9] and more and more successful cases have been published.[10,11] Fluorescence resonance energy transfer (FRET) technology–based assay belongs to another potent system applied in lead compound discovery. As a simple and sensitive approach, it has been effectively used in the detection of proteolytic activity and enzyme inhibitor evaluation.[4,6] Usually, FRET-based assay involves an intramolecularly quenched fluorogenic substrate that contains a donor and an acceptor chromophore. In our previous work, we constructed an efficient fluorogenic substrate-based platform for screening and evaluation of SARS-CoV 3CL[pro] inhibitors,[12] with which several potent SARS-CoV 3CL[pro] inhibitors were determined[5] and some related enzymatic features of SARS-CoV 3CL[pro] were studied.[13]

In the current work, considering the distinct advantages of these 3 above-mentioned methods, and according to the obtained successful experiences in enzyme inhibitor discovery,[5] we thus developed an integrated strategy for exploring novel SARS-CoV 3CL[pro] inhibitors. Our work is expected to supply a sound approach in the discovery of new potent SARS-CoV 3CL[pro] inhibitors, and the determined inhibitors could be used as possible lead compounds for further research.

MATERIALS AND METHODS

Materials

The fluorogenic substrate Dabcyl-KNSTLQSGLRKE-Edans was synthesized by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd. (Shanghai, China). The chelating column and protein molecular weight marker for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) use were from Amersham Pharmacia Biosciences (Uppsala, Sweden). All other chemicals were of reagent grade or ultrapure quality and purchased from Sigma (St Louis, MO). Cinanserin was synthesized according to the published method.[5] The tested 256 compounds from virtual screening were purchased from SPECS Company (http://www.specs.net).

SARS-CoV 3CL[pro] preparation and activity assay

SARS-CoV 3CL[pro] was prepared according to the published procedure.[14] The enzymatic activity of SARS-CoV 3CL[pro] was measured by cleavage of the fluorogenic substrate Dabcyl-KNSTLQSGLRKE-Edans based on the reported protocols.[5,12] Briefly, the fluorescence intensity was monitored on a GENios microplate reader (Tecan, Mannedorf, Switzerland), and the enzyme activity was determined based on the increase in fluorescence upon continuous monitoring of the reactions in 96-well black microplates (BMG LABTECH, Offenburg, Germany) using wavelengths of 340 nm and 488 nm for excitation and emission, respectively.

Virtual screening

Virtual screening was performed on a 64-processor SGI Origin 3800 supercomputer. The virtual screening protocol adopted in this work was similar to that used in the previous publication.[5] Briefly, the 3D model of SARS-CoV 3CL[pro] was performed as the target for screening the SPECS molecular database containing 81,287 molecules by a docking approach[15] and the program DOCK 4.0.[16] Residues within a radius of 6 Å around the catalytic center (His41 and Cys145) were used to construct the scoring grids for the docking screen. During the docking calculations, Kollman-all-atom charges[17] and Geisterger-Hückel charges[18,19] were assigned to the protein and the small molecules, respectively. Conformational flexibility of the compounds from the database was considered in the docking search. In DOCK simulation, the ligand-receptor binding energy was approximated by the sum of the van der Waals and electrostatic interaction energies. After initial evaluation of orientation and scoring, a grid-based rigid body minimization was carried out for the ligand to locate the nearest local energy minimum within the receptor binding site. Position and conformation of each docked molecule were optimized using the single anchor search and torsion minimization method of DOCK 4.0. The top 2000 molecules with the highest DOCK score were further subjected to CScore[20] and Druglike[21] calculations. Finally, with visual inspection, 256 compounds were selected and purchased.

SPR technology–based binding assay and kinetic study

To inspect the possible active compounds with binding affinities to SARS-3CL[pro], SPR technology based Biacore 3000 biosensor was used (Biacore AB, Uppsala, Sweden). SPR assay was performed at 25 °C, and SARS-CoV 3CL[pro] purified at 3 mg/mL was diluted to a final concentration of 25 μg/mL in 10 mM sodium acetate buffer (pH 4.3) and immobilized to the CM5 sensor-chip by using the standard primary amine coupling method.[22] HBS-EP running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% [v/v] surfactant P$_{20}$, pH 7.4) was used as the running buffer during immobilization. The surface was activated by injecting a 1:1 mixture containing 0.2 M 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 50 mM N-hydroxysuccinimide (NHS) for 7 min at a flow rate of 20 μL/min. SARS-CoV 3CL[pro] was injected and the surface was then blocked by injecting 1 M ethanolamine at pH 8.5 for 7 min. The final immobilization level was about 4000 resonance units.

All the screening assays were performed over the unmodified dextran surface and SARS-CoV 3CL[pro] surface. During the SARS-CoV 3CL[pro] binder assay, the sample concentrations were set at 1 μM and 10 μM. For sample dilution, the pH and the concentration of both DMSO and buffer substances in samples and running buffer were carefully matched. Each sample

assay consisted of a 60-s buffer injection, a 60-s injection, and a 120-s undisturbed dissociation phase. The published SARS-CoV 3CL[pro] inhibitor Cinanserin[5] was used as a positive control to confirm the binding activity of SARS-CoV 3CL[pro], and the blank injection was used to check the carryover effects. The signal was adjusted for nonspecific binding of the samples to dextran matrix by subtracting the signal in the reference channel from the signal in the active channel. The binding hits could be directly determined by the signal difference between the 2 set concentrations of the samples.

To further quantitatively investigate the kinetic binding features of the determined SARS-CoV 3CL[pro] inhibitors by FRET-based assay, SPR technology–based Biacore 3000 was also used. During the assay, samples were diluted to 8 different concentrations and injected at a constant flow of 30 μL/min at 25 °C. Sensorgrams were processed by automatically subtracting for nonspecific bulk refractive index effects. The kinetic parameters were then analyzed using a global data analysis program (BIAevaluation 3.1 software, Biacore AB).

SARS-CoV 3CL[pro] inhibition assay

SARS-CoV 3CL[pro] inhibition assay was performed according to the previously described method.[5] The inhibition of SARS-CoV 3CL[pro] activity was assayed by preincubating the enzyme (1 μM) with the test compounds (50 μM) in 100 mM NaCl, 20 mM phosphate buffer (pH 7.4) at 4 °C for 2 hours. The reaction was initiated by the addition of 10 μM of fluorescent substrate (Dabcyl-KNSTLQSGLRKE-Edans), and the change of the fluorescent intensity was monitored on a GENios microplate reader (Tecan) using an excitation wavelength of 340 nm and an emission wavelength of 488 nm. Control reactions were carried out using the same reaction mixture without the test compound or the enzyme. Concentration of inhibitor producing 50% inhibition (IC_{50}) calculation was carried out in the absence or presence of varying concentrations of the inhibitors by using Origin 7.0 software (OriginLab Corp., Northampton, MA).

RESULTS AND DISCUSSION

Virtual screening and docking results

The DOCK program was employed as the first step in preliminarily screening the potential binding molecules against SARS-CoV 3CL[pro] based on the substrate-binding pocket formed by residues within a radius of 6 Å around the catalytic center (His41 and Cys145) of the model structure.[23] As a result, 256 compounds from the SPECS database (containing 81,287 compounds) were thus selected for subsequent biological assays according to their DOCK score, Cscore, Druglike score, and finally with visual inspection. The results obviously implied that the performed virtual screening has supplied potent compound
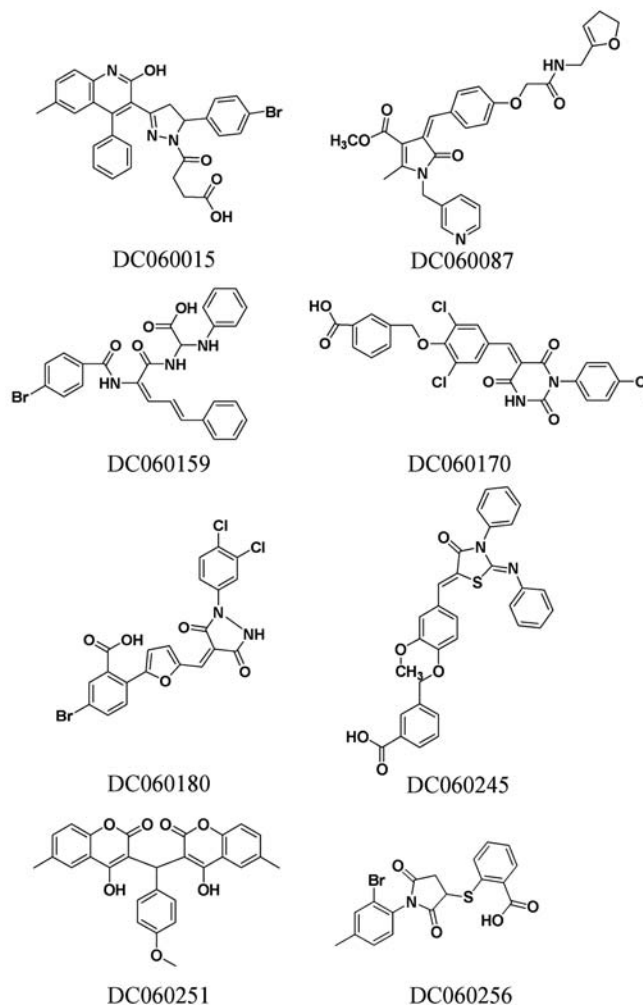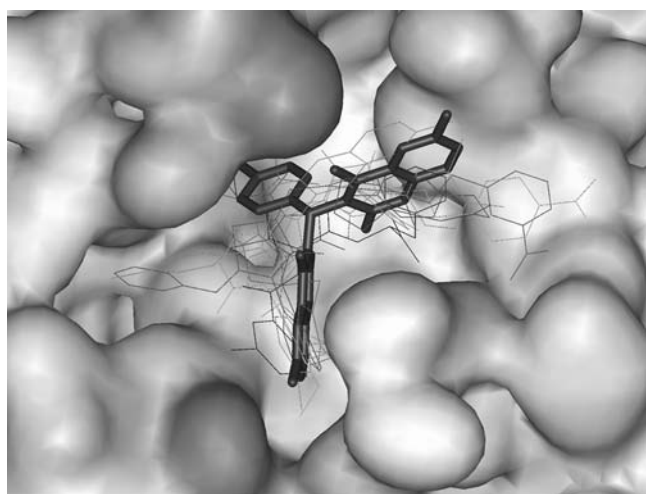

FIG. 1. Structures of the 8 compounds screened in this study.

information, although it might suffer from false positives in view of some factors like inaccurate scoring functions, crude conformational statement of target protein and some solvent-related terms,[15] or false negatives due to the neglect of allosteric effect as carried out in our assay. Shown in **Figure 1** are the structures of the 8 FRET-determined SARS-CoV 3CL[pro] inhibitors from virtual screening. These compounds show good druglikeness according to our in-house druglike filter.[21] Furthermore, 5 of the 8 compounds pass the rule of 5 based on the results shown in **Table 1**.

The docking results (**Fig. 2**) demonstrated that the discovered inhibitors can fit well to the active site of SARS-CoV 3CL[pro]. **Figure 3** shows the detailed binding information of DC060251 to SARS-CoV 3CL[pro] as a typical example. Moreover, by using DC060251 as a query structure to search MDL Drug Data Report (MDDR), Comprehensive Medicinal Chemistry (CMC), and

| Compound	No. Molecular Weight	No. Hydrogen Bond Donors	No. Hydrogen Bond Donors	CLogP	Rule of 5 (Pass or Fail)
DC060015	558.4	2	7	6.5484	Fail
DC060087	487.5	1	9	2.9486	Pass
DC060159	519.4	4	7	5.6938	Fail
DC060170	541.3	2	9	4.551	Pass
DC060180	522.1	2	7	4.507	Pass
DC060245	536.6	1	7	7.056	Fail
DC060251	470.5	2	7	5.7126	Pass
DC060256	420.3	1	5	3.2525	Pass

FIG. 3. Schematic representation of SARS-CoV 3CL^pro interaction with DC060251. DC060251 and protein residues are rendered in ball-and-stick and sticks, respectively.

FIG. 2. The binding model of the 8 inhibitors against SARS-CoV 3CL^pro in the active site. DC060251 is rendered in sticks, whereas others are shown in lines.

China Natural Products Database (CNPD), several known drugs or preclinical compounds were also identified (**Fig. 4**), whose structures share 90% similarity to that of DC060251. For example, compound A (3,3',3'',3'''-*p*-Xylene-α,α,α',α'-tetrayltetrakis(4-hydroxy-2H-1-benzopyran-2-one)) from MDDR was a known HIV-1 protease inhibitor with IC_{50} of 1.7 µM and a mean EC_{50} of 11.5 µM in an XTT-based anti-HIV assay.[24] Compound B (Ethyl Biscoumacetate) from CMC was found in 1943 with anticoagulant effect,[25] and compound C (Gerberinol 1) from CNPD was the main component extracted from a kind of plant gerbera with many biological activities.[26] These results thus implied that compound DC060251 might be of great interest for further study.

SPR technology–based binder identification

As a 2nd key process, SARS-CoV 3CL^pro binder identification from the virtual screening results was based on SPR

technology–based Biacore 3000 biosensor (Biacore AB). SPR technology has been widely used to investigate protein-macromolecule[27,28] and protein–small molecular compound[5,7] interactions for its obvious advantages such as label-free, sensitive, real-time, noninvasive measurements, low sample consumption, and high throughput. Recently, this technology has also been applied in the discovery of inhibitors against varied enzymes, such as HIV-1 protease,[9] human cyclophilin A,[7] and human 5-lipoxygenase.[10] In our previous reports, we ever successfully used SPR technology for discovering SARS-CoV 3CL^pro small molecular inhibitors[5] and performing SARS-CoV 3CL^pro–related research.[13]

In the current work, the SPR-based results showed that of the selected 256 compounds from the virtual screening, 52 compounds were identified as SARS-CoV 3CL^pro binders. SPR-based technique has supplied a rapid approach in the discovery of SARS-CoV 3CL^pro binder. However, it is noticed that in some cases some hits obtained by SPR assay are probably nonspecific binding molecules, which show no inhibitory activities. To solve this problem, the screened hits should be further tested by enzymatic inhibitory assays.

FRET technology–based IC_{50} determination

As demonstrated in the SPR-based SARS-CoV 3CL^pro binder assay, 52 SARS-CoV 3CL^pro binders were discovered. To further
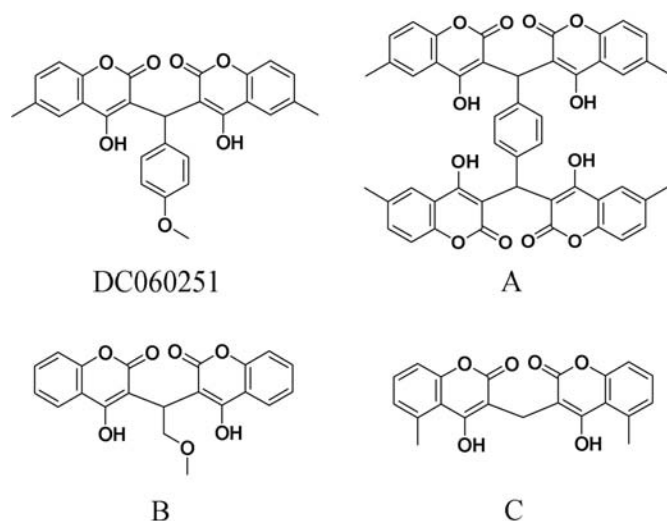
FIG. 4. Structures of 3 compounds (A, B, C) from MDL Drug Data Report (MDDR), Comprehensive Medicinal Chemistry (CMC), and China Natural Products Database (CNPD) databases. DC060251 shows 90% structural similarity to them.

investigate their possible inhibitory activities against SARS-CoV 3CL[pro], the inhibitory effects of the selected 52 binders were tested by the FRET method. For this assay, the internally quenched fluorogenic substrate Dabcyl-KNSTLQSGLRKE-Edans was applied, whose Michaelis constant (Km) against SARS-CoV 3CL[pro] is 49.38 ± 5.80 µM (data not shown). This value is comparable to those of other recently published fluorogenic peptides.[6,29]

Table 2 lists the yielded IC$_{50}$ values of the 8 screened inhibitors of SARS-CoV 3CL[pro], and **Figure 5** shows the dose-response curves for 3 typical inhibitors DC060170 (**A**), DC060180 (**B**), and DC060251 (**C**). The IC$_{50}$ values of these inhibitors are in the range of 6.86 to 80.46 µM.

SPR-based quantitative inhibitor binding affinity determination

It has been known that although the IC$_{50}$ value represents the inhibitory activity of an inhibitor to an enzyme, this value is subject to the experimental conditions, for example, the structure of the substrate and the total concentrations of both enzyme and substrate. If any of the experimental conditions change, the IC$_{50}$ value of a given compound will be altered, making IC$_{50}$ from different literatures hard to be compared.[30] In comparison with IC$_{50}$, the equilibrium dissociation constant (K$_D$) for the enzyme-inhibitor complex is independent of the experimental conditions. SPR-based assay can directly measure K$_D$ values between the inhibitors and SARS-CoV 3CL[pro]. To further investigate the K$_D$ values of the inhibitors to SARS-CoV 3CL[pro], an SPR technology–based Biacore 3000 instrument was also used. K$_D$ values of the 8 discovered inhibitors were calculated using
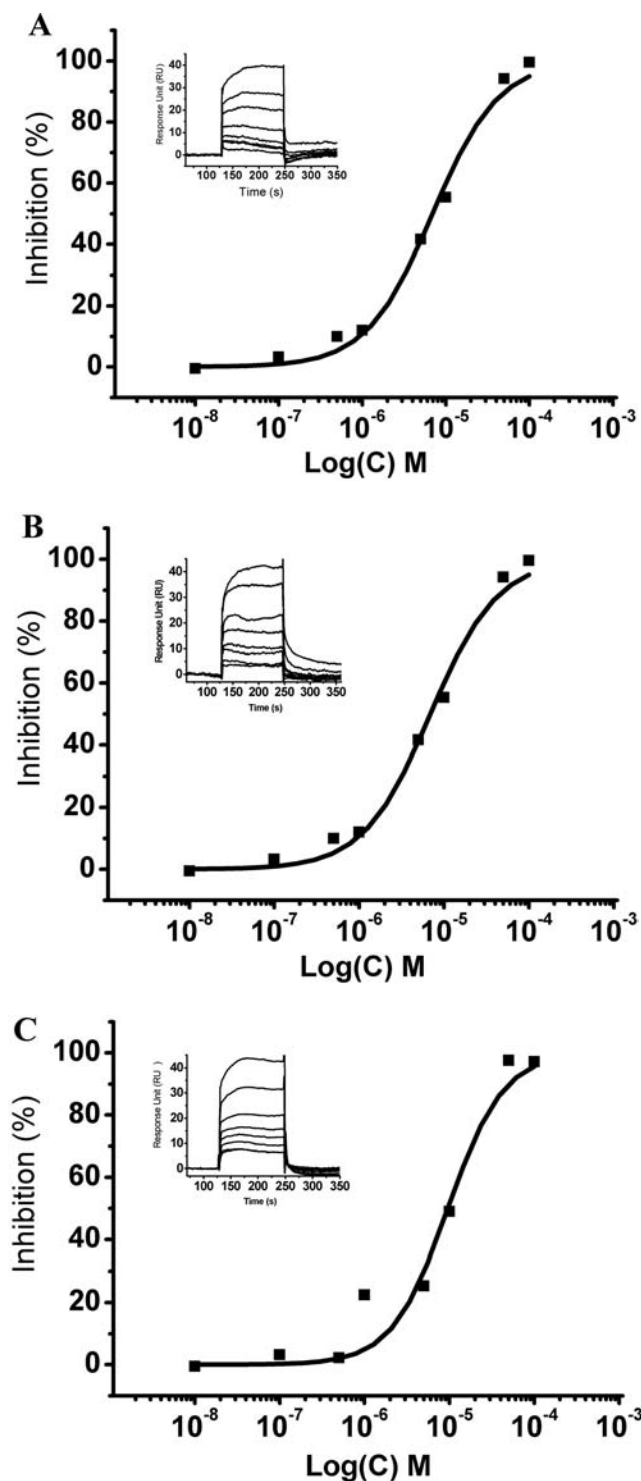
Table 2. Summary of Concentration of Inhibitor Producing 50% Inhibition (IC$_{50}$) Values and Equilibrium Dissociation Constants (K$_D$s) for SARS-CoV 3CL[pro] Inhibitors

| Compound	Molecular Weight (Da)	IC$_{50}$ (µM)	K$_D$ (µM)
DC060015	558.4	49.96 ± 4.62	18.60 ± 1.18
DC060087	487.5	80.46 ± 2.07	42.50 ± 2.10
DC060159	519.4	42.19 ± 3.02	19.14 ± 2.03
DC060170	541.3	6.86 ± 0.91	4.23 ± 0.81
DC060180	522.1	9.19 ± 2.34	6.50 ± 0.23
DC060245	536.6	21.49 ± 2.97	23.91 ± 5.63
DC060251	470.5	9.56 ± 1.75	4.78 ± 0.29
DC060256	420.3	56.24 ± 3.48	49.91 ± 3.98

the 1:1 Langmuir binding model or the steady-state affinity model (BIAevaluation 3.1 software, Biacore AB). **Figure 5** (inset) shows the binding dose-response curves for the 3 compounds, DC060170 (**A**), DC060180 (**B**), and DC060251 (**C**). As indicated in **Table 2**, the K$_D$ values of the compounds range from 4.23 to 49.91 µM. As demonstrated in **Figure 6,** the K$_D$ values are well correlated with IC$_{50}$ values by the correlation coefficient (R) of 0.92.

CONCLUSIONS

In this work, we developed an integrated approach for discovering novel SARS-CoV 3CL[pro] inhibitors. Virtual screening method based on the 3D structure of SARS-CoV 3CL[pro] can reliably narrow down the number of potential candidates before experimental testing. Although virtual screening might suffer from false positives, SPR technology was thus used to determine the binders of SARS-CoV 3CL[pro] and further reduce the screening scale and time. Because the hits obtained by SPR-based assay may be nonspecific binding compounds, FRET method is applied for SARS-CoV 3CL[pro] inhibitor identification. Our current strategy for discovering SARS-CoV 3CL[pro] inhibitor has integrated virtual screening and SPR- and FRET-based assays, and with this integrated platform, we have successfully identified 8 novel potent SARS-CoV 3CL[pro] inhibitors. Although the hit enrichment might not always be higher than a random selection, the screened active compounds from virtual screening could involve more structural information for virtual screening based on the compound database that comprises a large number of chemical structures, whereas for random screening, the obtained active compounds might contain lower structural information because of the possible limitation of the compound quantity. The obtained results thereby suggested that the inhibitor screening is more effective compared with the traditional screening when integrated strategy is applied. It is believed that such an integrated approach may help speed up the

FIG. 5. Dose-response inhibition and kinetic assay curves for the 3 compounds from the screening procedure. IC$_{50}$ values were extracted from the curves using nonlinear regression analysis, and dissociation equilibrium constant (K$_D$) values were analyzed using a global data analysis program of Biacore 3000. **A**, **B**, **C** represent the dose-response inhibition curves (Inset: the responding binding curve) of the compounds (DC060170, DC060180, DC060251), respectively.
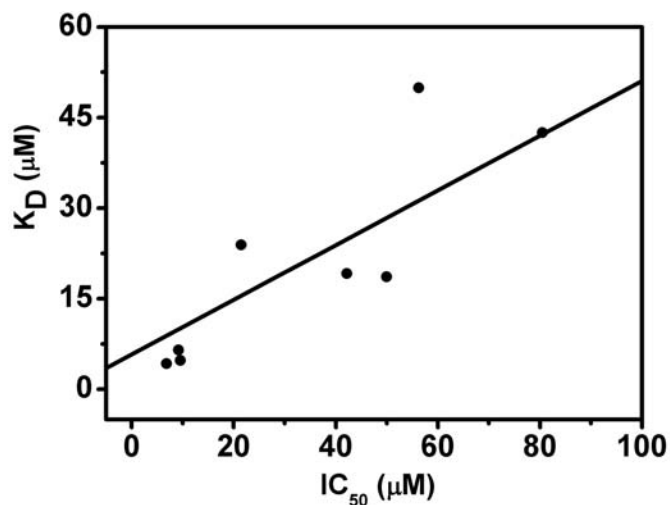
FIG. 6. Correlation between the concentration of inhibitor producing 50% inhibition (IC$_{50}$) and equilibrium dissociation constants (K$_D$) for SARS-CoV 3CL^{pro} inhibitors. IC$_{50}$ values were obtained from the fluorescence resonance energy transfer (FRET)–based assay, and K$_D$ values from biosensor measurements. The symbol (●) represents the compounds with correlation.

drug-discovery process. In addition, it is expected that the determined inhibitors could be used as possible lead compounds for further research.

ACKNOWLEDGMENTS

REFERENCES

1. von Grotthuss M, Wyrwicz LS, Rychlewski L: mRNA cap-1 methyltransferase in the SARS genome. *Cell* 2003;113:701-702.
2. Drosten C, Preiser W, Gunther S, Schmitz H, Doerr HW: Severe acute respiratory syndrome: identification of the etiological agent. *Trends Mol Med* 2003;9:325-327.
3. Fouchier RA, Kuiken T, Schutten M, van Amerongen G, van Doornum GJ, van den Hoogen BG, et al: Koch's postulates fulfilled for SARS virus. *Nature* 2003;423:240.
4. Bacha U, Barrila J, Velazquez-Campoy A, Leavitt SA, Freire E: Identification of novel inhibitors of the SARS coronavirus main protease 3CLpro. *Biochemistry* 2004;43:4906-4912.
5. Chen L, Gui C, Luo X, Yang Q, Gunther S, Scandella E, et al: Cinanserin is an inhibitor of the 3C-like proteinase of severe acute respiratory syndrome coronavirus and strongly reduces virus replication in vitro. *J Virol* 2005;79:7095-7103.

6. Blanchard JE, Elowe NH, Huitema C, Fortin PD, Cechetto JD, Eltis LD, et al: High-throughput screening identifies inhibitors of the SARS coronavirus main proteinase. *Chem Biol* 2004;11:1445-1453.

7. Li J, Chen J, Gui C, Zhang L, Qin Y, Xu Q, et al: Discovering novel chemical inhibitors of human cyclophilin A: virtual screening, synthesis, and bioassay. *Bioorg Med Chem* 2006;14:2209-2224.

8. Cooper MA: Optical biosensors in drug discovery. *Nat Rev Drug Discov* 2002;1:515-528.

9. Markgren PO, Hamalainen M, Danielson UH: Screening of compounds interacting with HIV-1 proteinase using optical biosensor technology. *Anal Biochem* 1998;265:340-350.

10. Du L, Zhang Z, Luo X, Chen K, Shen X, Jiang H: Binding investigation of human 5-lipoxygenase with its inhibitors by SPR technology correlating with molecular docking simulation. *J Biochem (Tokyo)* 2006;139:715-723.

11. Ye F, Zhang ZS, Luo HB, Shen JH, Chen KX, Shen X, et al: The dipeptide H-Trp-Glu-OH shows highly antagonistic activity against PPARgamma: bioassay with molecular modeling simulation. *Chembiochem* 2006;7: 74-82.

12. Chen S, Chen LL, Luo HB, Sun T, Chen J, Ye F, et al: Enzymatic activity characterization of SARS coronavirus 3C-like protease by fluorescence resonance energy transfer technique. *Acta Pharmacol Sin* 2005;26:99-106.

13. Chen S, Chen L, Tan J, Chen J, Du L, Sun T, et al: Severe acute respiratory syndrome coronavirus 3C-like proteinase N terminus is indispensable for proteolytic activity but not for enzyme dimerization. Biochemical and thermodynamic investigation in conjunction with molecular dynamics simulations. *J Biol Chem* 2005;280:164-173.

14. Sun H, Luo H, Yu C, Sun T, Chen J, Peng S, et al: Molecular cloning, expression, purification, and mass spectrometric characterization of 3C-like protease of SARS coronavirus. *Protein Expr Purif* 2003;32:302-308.

15. Doman TN, McGovern SL, Witherbee BJ, Kasten TP, Kurumbail R, Stallings WC, et al: Molecular docking and high-throughput screening for novel inhibitors of protein tyrosine phosphatase-1B. *J Med Chem* 2002; 45:2213-2221.

16. Ewing TJ, Makino S, Skillman AG, Kuntz ID: DOCK 4.0: search strategies for automated molecular docking of flexible molecule databases. *J Comput Aided Mol Des* 2001;15:411-428.

17. Cornell WD, Cieplak D, Bayly CI, Gould IR, Merz KM, Ferguson DM, et al: A Second generation force field for the simulation of proteins, nucleic acids, and organic molecules. *J Am Chem SOC* 1995;117:5179-5197.

18. Gasteiger J, Marsili M: Iterative partial equalization of orbital electronegativity-A rapid access to atomic charges. *Tetrahedron* 1980;36: 3219-3228.

19. Purcell WP, Singer JA: Brief review and table of semiempirical parameters used in the Hückel molecular orbital method. *J Chem Eng Data* 1967;12: 235-246.

20. Clark R, Strizhev A, Leonard J, Blake J, Matthew J: Consensus scoring for ligand/protein interactions. *J Mol Graph Model* 2002;20:281-295.

21. Zheng S, Luo X, Chen G, Zhu W, Shen J, Chen K, et al: A new rapid and effective chemistry space filter in recognizing a druglike database. *J Chem Inf Model* 2005;45:856-862.

22. Johnsson B, Lofas S, Lindquist G: Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal Biochem* 1991;198: 268-277.

23. Xiong B, Gui CS, Xu XY, Luo C, Chen J, Luo HB, et al: A 3D model of SARS_CoV 3CL proteinase and its inhibitors design by virtual screening. *Acta Pharmacol Sin* 2003;24:497-504.

24. Wang S, Milne GW, Yan X, Posey IJ, Nicklaus MC, Graham L, et al: Discovery of novel, non-peptide HIV-1 protease inhibitors by pharmacophore searching. *J Med Chem* 1996;39:2047-2054.

25. Stahmann MA, Wolff I: Studies on 4-hydroxycoumarins: I. The synthesis of 4-hydroxycoumarins. *J Am Chem Soc* 1943;65:2285-2287.

26. Sengupta P, Sen M, Karuri P: Structure of gerberinol novel dimethyl-dicoumarol from gerbera lanuginosa benth. *J Indian Chem Soc* 1985;62: 916-919.

27. Luo H, Wu D, Shen C, Chen K, Shen X, Jiang H: Severe acute respiratory syndrome coronavirus membrane protein interacts with nucleocapsid protein mostly through their carboxyl termini by electrostatic attraction. *Int J Biochem Cell Biol* 2006;38:589-599.

28. Luo H, Chen Q, Chen J, Chen K, Shen X, Jiang H: The nucleocapsid protein of SARS coronavirus has a high binding affinity to the human cellular heterogeneous nuclear ribonucleoprotein A1. *FEBS Lett* 2005; 579:2623-2628.

29. Kao RY, To AP, Ng LW, Tsui WH, Lee TS, Tsoi HW, et al: Characterization of SARS-CoV main protease and identification of biologically active small molecule inhibitors using a continuous fluorescence-based assay. *FEBS Lett* 2004;576:325-330.

30. Gao Z, Luo H, Chen L, Shen J, Chen K, Jiang H, et al: Determining PPAR-ligand binding affinity using fluorescent assay with cis-parinaric acid as a probe. *Science in China Ser B Chemistry* 2005;48:1-10.

Address reprint requests to:
Xu Shen
Drug Discovery and Design Center
State Key Laboratory of Drug Research
Shanghai Institute of Materia Medica,
Chinese Academy of Sciences
Shanghai 201203

E-mail: xshen@mail.shcnc.ac.cn or hljiang@mail.shcnc.ac.cn