STUDIA UBB CHEMIA, LVI, 3, 2011 (p. 221 – 230)

X-RAY CRYSTAL STRUCTURE AND DYNAMICS REVEAL HIV-1 PROTEASE DRUG INTERACTIONS

YONG WANG[a], TAMARIA G. DEWDNEY[a], ZHIGANG LIU[a], SAMUEL J. REITER[a], JOSEPH S. BRUNZELLE[b], IULIA A. KOVARI[a], LADISLAU C. KOVARI[a]

ABSTRACT. The dynamic movement of HIV-1 protease is an important feature for inhibitor design. The wide-open form of multi-drug resistant HIV-1 protease solved by our group exhibits an increase in flap distance. Stabilizing the protease flaps could be a strategy to overcome drug resistance. A peptidic inhibitor stabilizing the protease-inhibitor complex and a structural novel inhibitor targeting the wide-open form protease have been identified as a new scaffold for drug development.

Keywords: *multi-drug resistance, HIV-1 protease, drug design,*

INTRODUCTION

Drug resistance is a major obstacle in the long-term treatment of HIV/AIDS patients. The drug-resistance mutations accumulated on HIV-1 protease during antiretroviral therapy change the protease conformation and decrease the efficacy of protease inhibitors. While most HIV-1 protease inhibitors are designed to mimic the substrate cleavage intermediates, structurally diverse inhibitors are needed to maintain efficacy against the highly flexible protease [1]. The discovery of new inhibitor scaffolds is the first step to increase the diversity of HIV-1 protease inhibitors. The HIV-1 protease structures greatly facilitate the discovery of new inhibitors.

Few ligand-free 3-dimensional structures of HIV-1 protease have been deposited to the Protein Data Bank. We have previously reported one such structure of an uncomplexed multi-drug resistant (MDR) HIV-1 protease, MDR 769 [2]. As shown in Figure 1, the available ligand-free HIV-1 protease can be classified into two classes, wide-open form and curled

[a] *Department of Biochemistry and Molecular Biology, School of Medicine, Wayne State University, Detroit, MI, U.S.A., kovari@med.wayne.edu*

[b] *Department of Molecular Pharmacology and Biological Chemistry, Feinberg School of Medicine, Northwestern University, Evanston, IL, U.S.A.*

form [3]. Considering the flexibility and dynamic movement of the protease, the structure of unbound HIV-1 protease is as important as the ligand-bound structure in successful drug design.

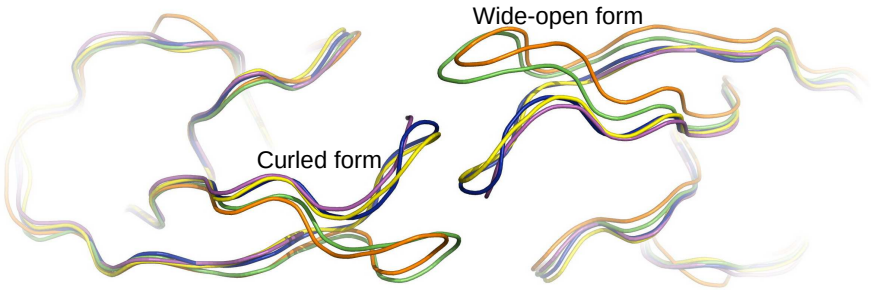

Figure 1. Apo HIV-1 protease structures. The structures include the wild-type (WT) HIV-1 protease NL4-3 (PDB ID: 2HB4, blue), the HIV-1 protease with mutations L24I, M46I, F53L, L63P, V77I, and V82A (PDB ID: 2HB2, magenta), the WT HIV-1 protease isolate BRU (PDB ID: 1HHP, yellow), another WT HIV-1 protease (PDB ID: 2PC0, green), and the HIV-1 protease MDR 769 with mutations L10I, M36V, M46L, I54V, I62V, L63P, A71V, V82A, I84V, and L90M (PDB ID: 1TW7, orange)

Understanding the structural diversity of the target protease is essential for inhibitor development. The plasticity of the HIV-1 protease flap region is critical in binding substrates and inhibitors [3]. The increased flap flexibility may enhance drug cross-resistance by altering the binding pocket of protease inhibitors. Flap movement distorts the binding cavity and therefore reduces the binding affinity to inhibitors. Direct measurement using pulsed double electron-electron resonance (DEER) has confirmed a larger distance between the flaps of (MDR) HIV-1 protease as compared to the wild-type structure [4]. Therefore, the wide-open form of HIV-1 protease can serve as a nontraditional model to develop inhibitors targeting the open form protease. A crystal structure (PDB ID: 3BC4) has shown that the HIV-1 protease with open-flap conformation binds two symmetric pyrrolidine diester inhibitors [5].The range of opening and conformational flexibility of protease flaps demonstrate the importance of protein flexibility in structure-based drug design. Using ensembles of protein conformations as receptors for docking is a powerful method to increase accuracy [6,7]. Designing inhibitors based on both the open and closed form of HIV-1 MDR protease partially represents the HIV-1 protease dynamics and improves drugs' adaptation to the flexible movement of the protease.

RESULTS AND DISCUSSION

Four multi-drug resistant HIV-1 protease isolates are resistant to inhibitors at various levels

Table 1. Sequences of HIV-1 protease variants

HIV-1 protease	Sequences*
NL4-3	PQITLWKRPL VTIKIGGQLK EALLDTGADD TVLEEMNLPG RWKPKMIGGI
	GGFIKVRQYD QILIEICGHK AIGTVLVGPT PVNIIGRNLL TQIGCTLNF
769	PQITLWKRPI VTIKIGGQLK EALLDTGADD TVLEEVNLPG
	RWKPKLIGGI GGFVKVRQYD QVPIEICGHK VIGTVLVGPT
	PANVIGRNLM TQIGCTLNF
807	PQITLWKRPI VTIKIGGQLK EALLDTGADD TVLEEMNLPG
	KWKPKIIVGI
	GGFTKVRQYD NVQIEICGHK VIGAVLIGPT PANIIGRNLL TQLGCTLNF
1385	PQITLWKRPF VTIKIGGQLK EALLDTGADD TVLEEIDLPG
	RWKPKIIGGI
	GGFIKVKQYD QIPIEICGHK VIGTVLVGPT PTNIIGRNMM TQLGCTLNF
3761	PQITLWKRPI VAIKVGGQII EALLDTGADD TVLEEMNLPG
	RWKPKIIGGI
	GGFIKVRQYD QIPVEICGHK IITTVLVGST PVNVIGRNLM TQLGCTLNF

*The polymorphic changes are underlined. The drug-resistance mutations are highlighted in red.

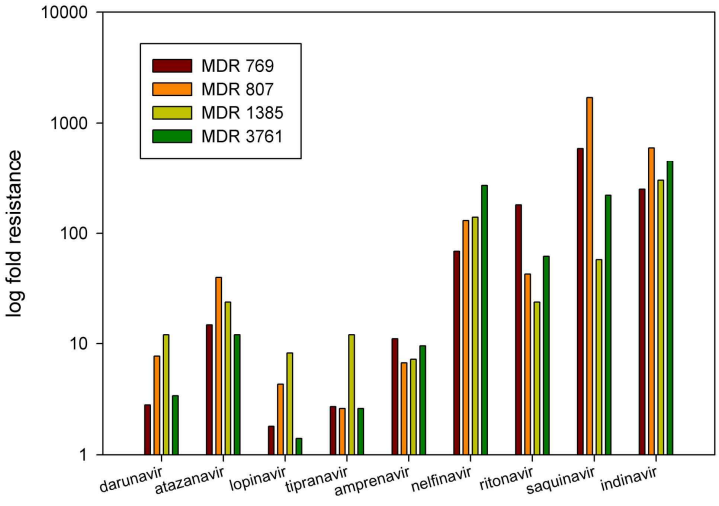

Figure 2. Fold resistance of four multi-drug resistant HIV-1 protease variants.

Four MDR HIV-1 protease variants were isolated by Palmer *et al.* from patients failing protease inhibitor-containing antiretroviral regimens (Table 1) [8]. The drug-resistance profiles of four clinical MDR HIV-1 protease isolates were identified using enzyme inhibition assays. Four MDR protease variants were resistant to all FDA-approved HIV-1 protease inhibitors at various levels (Figure 2). The second generation of HIV-1 protease inhibitors (darunavir, atazanavir, lopinavir, and tipranavir) encountered relatively lower fold resistance. Among the four MDR protease variants tested here, MDR 807 and MDR 1385 were more resistant to the second generation HIV-1 protease inhibitors. According to the guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents, darunavir and atazanavir are preferred first-line antiretroviral drugs [9]. Our results showed that there was higher resistance to atazanavir comparing to darunavir. These results confirmed the *in vitro* resistance observed of the clinical isolates of HIV-1 protease.

Potent inhibitors induced flap closure of the multi-drug resistant HIV-1 protease

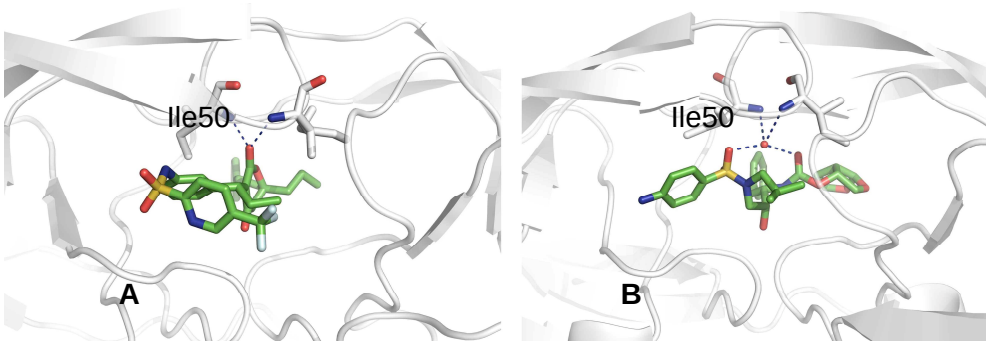

Figure 3. The hydrogen bonds between HIV-1 protease flaps and inhibitors. (A) tipranavir-MDR 769 complex structure. (B) darunavir-MDR 769 complex structure. The hydrogen bonds are represented as blue dash lines. The bridging water molecule is represented in a red dot.

Darunavir and tipranavir are among the protease inhibitors exhibiting the most potency and least fold resistance. The tipranavir-MDR 769 82T complex crystallized in the hexagonal space group $P6_1$ while the darunavir-MDR 769 82T complex crystallized in the orthorhombic space group $P2_12_12_1$. The crystal structure was determined at 1.24 Å resolution and 2.87 Å resolution, respectively. The other seven FDA-approved HIV-1 protease inhibitors crystallized with MDR 769 in an open form. The binding of tipranavir or darunavir effectively closes the open flaps of MDR 769. Tipranavir directly interacts with Ile50 while darunavir interacts with Ile50 through a bridging

water molecule (Figure 3). Therefore, incorporating a chemical group to contact the protease flaps is a useful strategy to increase inhibitor potency and decrease fold resistance. From the drug modification point of view, the tipranavir-like direct protease flap contact stabilizes protease-inhibitor complex, which is concluded based on a protease complex denaturation experiment [10].

A peptidic inhibitor reduced fold resistance by stabilizing the multi-drug resistant HIV-1 protease complexes

Table 2. The half maximal inhibitory concentration (IC_{50}) of mutated CA-p2 peptides

CA-p2 mutagenesis	Sequences	IC_{50} (nM)		Fold resistance
		Wild-type	MDR 769	
P1'F	Arg-Val-Leu-Phe-Glu-Ala-Met	2.6	4.4	1.7
P1'F, P1F	Arg-Val-Phe-Phe-Glu-Ala-Met	30.7	142	4.6
P1'F, P1A	Arg-Val-Ala-Phe-Glu-Ala-Met	4873	5020	1.0
P1'F, P2F	Arg-Phe-Leu-Phe-Glu-Ala-Met	1143	770	-
P1'F, P2A	Arg-Ala-Leu-Phe-Glu-Ala-Met	78.2	74.1	-
P1'F, P2'F	Arg-Val-Leu-Phe-Glu-Phe-Met	1010	1263	1.3
P1'F, P2'A	Arg-Val-Leu-Phe-Ala-Ala-Met	232.3	233.7	1

The cleavage of the CA-p2 site is a rate-limiting step of gag polyprotein maturation. Mutations were introduced to the CA-p2 heptapeptide with a reduced scissile peptide bond to test the inhibitory efficacy. One of the peptidic inhibitors, CA-p2 P1'F, displayed high inhibitory efficacy and low resistance. CA-p2 P1'F inhibited both wild-type (WT) HIV-1 protease and MDR 769 at low nanomolar concentration (Table 2). The combination of phenylalanine and leucine analogues at the P1 and P1' site could be employed to the peptidomimetic inhibitors.

Based on the crystal structure of MDR 769 in complex with CA-p2 P1'F, the homologous complex models were built for MDR 807, MDR 1385, MDR 3761, and NL4-3. After 10 ns molecular dynamics simulation, the movement of CA-p2 P1'F was analyzed for the last 40 ps of simulation. The root-mean-square deviation (RMSD) of each residue of CA-p2 P1'F was plotted in Figure 4. The RMSD values of CA-p2 binding to MDR proteases were significantly lower compared to that of CA-p2 binding to the WT HIV-1 protease. The lower RMSD value suggests a tighter substrate-protease complex. This result indicates that CA-p2 P1'F stabilizes MDR protease complexes and could serve as a template to be further developed as a resistance overcoming peptidomimetic inhibitor.

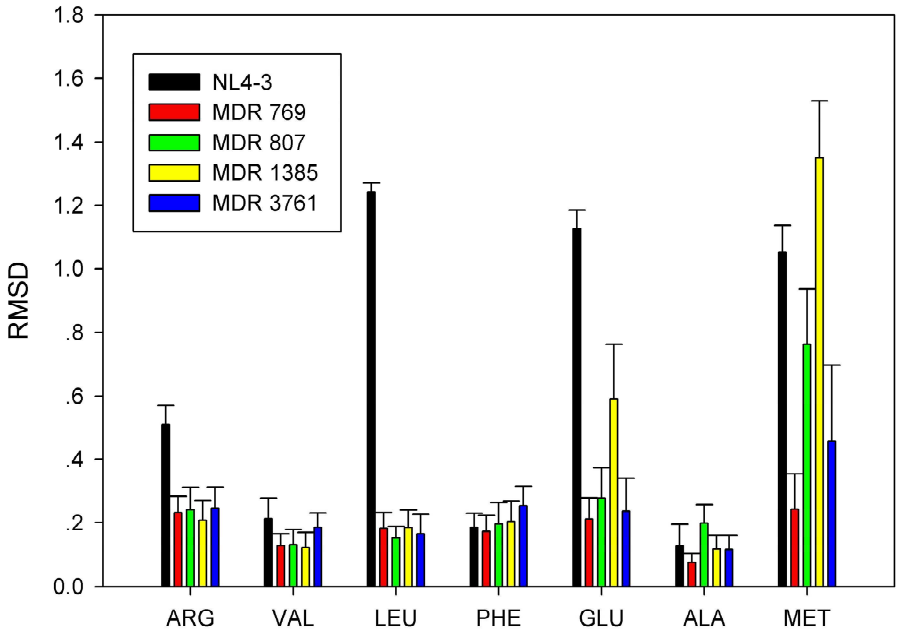

Figure 4. The root-mean-square deviation (RMSD) of CA-p2 P1'F binding to HIV-1 protease variants.

Based on the open and closed form HIV-1 protease structures, a resistance overcoming inhibitor candidate was identified using virtual screening.

The open form MDR 769 structure was used as a receptor to identify potential inhibitor scaffolds from commercially available compounds through virtual screening. One compound, GR346 (Figure 4), showed the best docking scores against the open form MDR 769. The binding of GR346 was then confirmed by docking it to the closed formed MDR 769. The docking conformation of GR346 is shown in Figure 5. GR346 was originally synthesized as a SIRT2 inhibitor (IC_{50}=56.7 μM) [11]. The HIV-1 protease inhibitory efficacy was tested using *in vitro* enzyme assays (Figure 6). GR346 inhibited a larger percentage of MDR 769 enzyme activity at various concentrations comparing to its inhibition against the WT HIV-1 protease. At the lower compound concentration (6.25 μM), GR346 did not inhibit the WT HIV-1 protease but inhibited MDR 769 activity by 10%. The wide-open conformation of MDR 769 flaps allowed the bulky compound, GR346, to easily enter into the protease active site. This result suggested a possibility of designing large flexible inhibitors could fit better to the expanded active site of MDR HIV-1 protease.

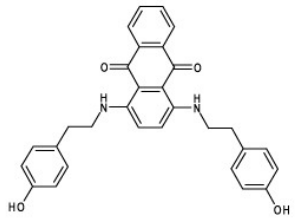

Figure 4. The structure of compound GR346

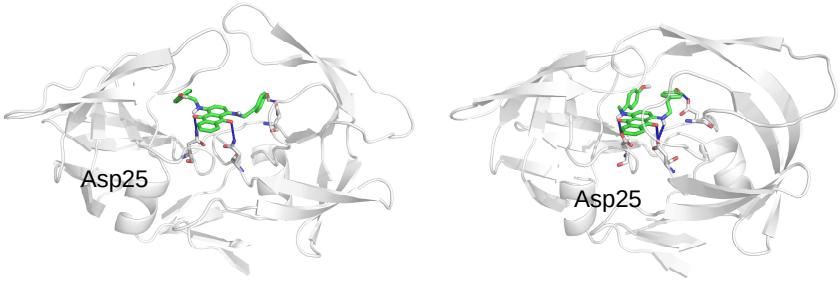

Figure 5. The docking conformation of GR346 in the active site of MDR 769. (A) GR346 was docked to the open form MDR 769. (B) GR346 was docked to the closed form MDR 769. The blue line represents possible hydrogen bonds.

CONCLUSIONS

Protein flexibility is an important feature to be considered in drug design. The movement of HIV-1 protease flaps may interrupt drug binding and cause resistance. Designing drugs that make contacts with the protease flaps to reduce protease flexibility, and targeting the open form protease could be options to overcome drug resistance caused by protease flap conformation and flexibility.

EXPERIMENTAL SECTION

Protein expression and purification

Table 1 lists the protein sequences of MDR proteases. Active and inactive MDR protease genes were codon optimized for *E. coli* expression, synthesized by GENEART, Inc. (Regensburg, Germany), and inserted into the pET21b plasmid. To prevent auto-proteolyses, Q7K mutation was introduced into the active MDR genes. The A82T mutation was introduced to facilitate crystallization. The protein expression, purification, and refolding procedures were described previously [12]. The final protein buffer was 20 mM sodium acetate, pH 5.0, 1 mM dithiothreitol (DTT), and 10% (v/v) glycerol. The proteases prepared for crystallization were concentrated to 1.5 mg/ml using Amicon concentrators with 5 kDa molecular mass cut-off (Millipore Corporate, Billerica, MA, USA).

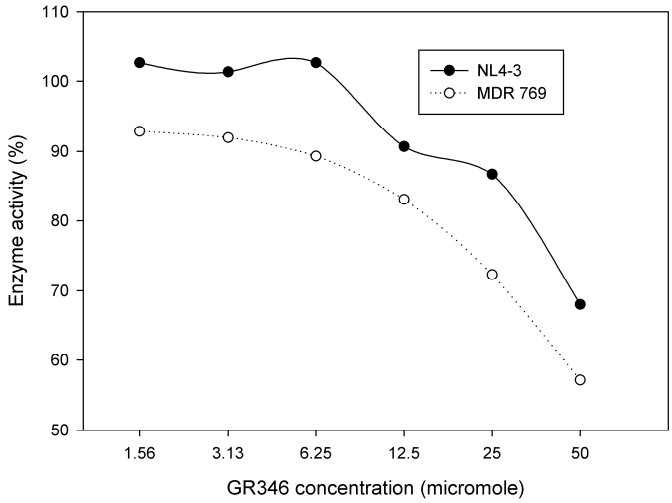

Figure 6. The enzyme inhibition of GR346

Protease inhibition assays

HIV protease Forster Resonance Energy Transfer (FRET) substrate I, purchased from AnaSpec, Inc. (Fremont, CA, USA), was used in the IC_{50} determination experiments. The fluorescence emitted by substrate cleavages was monitored by a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA) at a 340 nm excitation wavelength with an emission wavelength of 490 nm. The HIV-1 protease reaction buffer was adjusted to pH 4.7 [0.1 M sodium acetate, 1.0 M sodium chloride, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mM DTT, 10% dimethylsulfoxide (DMSO), and 1mg/ml bovine serum albumin (BSA)]. In the reaction buffer containing 5 μM FRET substrates, the protease concentration was adjusted to a substrate cleavage velocity of 5 Relative Fluorescence Units (RFU)/min. The active proteases and inhibitors were pre-incubated at 37℃ for 20 min prior to signal monitoring. An inhibitor-free control was used to determine the background fluorescent signal. The progress of the reaction was monitored over 20 min sampling at 1 min intervals. The FRET data were plotted with the software SoftMax Pro V5.2 (Molecular Devices, Sunnyvale, CA, USA) to determine the IC_{50} values.

Crystallization and data collection

Inhibitors (tipranavir and darunavir) or substrates (CA-p2) were co-crystallized with the MDR769 inactive protease by the hanging drop vapor diffusion method. The protease and ligand were pre-mixed at a molar ratio of 1:20 before crystallization set-up. The protease-ligand mixture was then mixed at 2:1 v/v ratio with the precipitant solution. The optimal crystallization was screened from a set of precipitant solution (0.1 M citric acid/MES/HEPES

and 0.8~2.8 M ammonium sulfate at pH 4.0~6.0). The reservoir volume was 750 µl. The co-crystal grew within a week to a suitable size for diffraction. The crystals were placed in a 30% w/v glucose cryoprotectant before being frozen in liquid nitrogen. Diffraction data were collected at the Life Sciences Collaborative Access Team (LS-CAT) at the Advanced Photon Source (APS) Sector 21, Argonne National Laboratory (Argonne, IL, USA) and processed with HKL2000 program suite [13].

Virtual screening and protein-ligand docking

The large scale screening was conducted using a fast pre-screening tool, LASSO (Ligand Activity in Surface Similarity Order) 2009 [14]. The smaller library with thousands of compound was further validated using eHiTS (Electronic High Throughput Screening), a fast and automated docking program [15]. Compound libraries were downloaded from the ZINC database of commercially-available compounds (zinc.docking.org). To confirm the lead compound from virtual screening, the compound with the best eHiTS score was docked into the binding pocket of HIV-1 protease using AutoDock4 [16].

Molecular dynamics simulation

The MD simulation was performed using Scaling NAno Molecular Dynamics (NAMD) V 2.7b [17]. The protease complex model was solvated in an orthogonal TIP3P water box. The cut-off of non-bonded interactions was 10 Å. The systems were minimized and gradually heated from 70 K to 310 K in 200 ps. Simulations were conducted at 310K and 1.0 atm for 10 ns using the CHARMM force field23 and a timestep of 2 fs.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (R21AI65294). Use of the Advanced Photon Source was supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor for the support of this research program (Grant 085P1000817).

REFERENCES

1. N. Ozer, C. A. Schiffer, T. Haliloglu, Rationale for more diverse inhibitors in competition with substrates in HIV-1 protease, *Biophysical journal*, **2010**, *99*, 1650.
2. P. Martin, J. F. Vickrey, G. Proteasa, Y. L. Jimenez, Z. Wawrzak, M. A. Winters, T. C. Merigan, L. C. Kovari, "Wide-open" 1.3 A structure of a multidrug-resistant HIV-1 protease as a drug target, *Structure*, **2005**, *13*, 1887.

3. H. Heaslet, R. Rosenfeld, M. Giffin, Y. C. Lin, K. Tam, B. E. Torbett, J. H. Elder, D. E. McRee, C. D. Stout, Conformational flexibility in the flap domains of ligand-free HIV protease, *Acta crystallographica. Section D, Biological crystallography*, **2007**, *63*, 866.
4. L. Galiano, F. Ding, A. M. Veloro, M. E. Blackburn, C. Simmerling, G. E. Fanucci, Drug pressure selected mutations in HIV-1 protease alter flap conformations, *Journal of the American Chemical Society*, **2009**, *131*, 430.
5. J. Bottcher, A. Blum, S. Dorr, A. Heine, W. E. Diederich, G. Klebe, Targeting the open-flap conformation of HIV-1 protease with pyrrolidine-based inhibitors, *ChemMedChem*, **2008**, *3*, 1337.
6. E. S. Bolstad, A. C. Anderson, In pursuit of virtual lead optimization: pruning ensembles of receptor structures for increased efficiency and accuracy during docking, *Proteins*, **2009**, *75*, 62.
7. S. Y. Huang, X. Zou, Ensemble docking of multiple protein structures: considering protein structural variations in molecular docking, *Proteins*, **2007**, *66*, 399.
8. S. Palmer, R. W. Shafer, T. C. Merigan, Highly drug-resistant HIV-1 clinical isolates are cross-resistant to many antiretroviral compounds in current clinical development, *AIDS*, **1999**, *13*, 661.
9. Panel on Antiretroviral Guidelines for Adults and Adolescents, *Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents*, **2011**, 41.
10. Y. Wang, Z. Liu, J. S. Brunzelle, I. A. Kovari, T. G. Dewdney, S. J. Reiter, L. C. Kovari. The higher barrier of darunavir and tipranavir resistance for HIV-1 protease, *Biochemical and Biophysical Research Communications*, **2011**, *412*, 737
11. P. H. Kiviranta, J. Leppanen, S. Kyrylenko, H. S. Salo, M. Lahtela-Kakkonen, A. J. Tervo, C. Wittekindt, T. Suuronen, E. Kuusisto, T. Jarvinen, A. Salminen, A. Poso, E. A. Wallen, N,N'-Bisbenzylidenebenzene-1,4-diamines and N,N'-Bisbenzylidenenaphthalene-1,4- diamines as Sirtuin Type 2 (SIRT2) Inhibitors, *Journal of Medicinal Chemistry*, **2006**, *49*, 7907.
12. J. F. Vickrey, B. C. Logsdon, G. Proteasa, S. Palmer, M. A. Winters, T. C. Merigan, L. C. Kovari, HIV-1 protease variants from 100-fold drug resistant clinical isolates: expression, purification, and crystallization, *Protein Expression and Purification*, **2003**, *28*, 165.
13. C. W. Carter, R. M. Sweet, "Methods in Enzymology", Academic Press, Inc., New York, **1997**, chaper 20.
14. A. Simon, D. Reid, B. S. Sadjad, Z. Zsoldos, LASSO-ligand activity by surface similarity order: a new tool for ligand based virtual screening, *Journal of Computer-Aided Molecular Design*, **2008**, *22*, 479.
15. Z. Zsoldos, D. Reid, A. Simon, S. B. Sadjad, A. P. Johnson, eHiTS: a new fast, exhaustive flexible ligand docking system, *Journal of Molecular Graphics and Modelling*, **2007**, *26*, 198.
16. G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson, AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility, *Journal of computational chemistry*, **2009**, *30*, 2785.
17. J. C. Phillips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R. D. Skeel, L. Kale, K. Schulten. Scalable molecular dynamics with NAMD. *Journal of Computational Chemistry*, **2005**, *26*, 1781.