A Conserved Hydrogen-Bonding Network of P2 *bis*-Tetrahydrofuran-Containing HIV-1 Protease Inhibitors (PIs) with a Protease Active-Site Amino Acid Backbone Aids in Their Activity against PI-Resistant HIV

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In the present study, GRL008, a novel nonpeptidic human immunodeficiency virus type 1 (HIV-1) protease inhibitor (PI), and darunavir (DRV), both of which contain a P2-*bis*-tetrahydrofuranyl urethane (*bis*-THF) moiety, were found to exert potent antiviral activity (50% effective concentrations [EC₅₀s], 0.029 and 0.002 μ M, respectively) against a multidrug-resistant clinical isolate of HIV-1 (HIV_{A02}) compared to ritonavir (RTV; EC₅₀, >1.0 μ M) and tipranavir (TPV; EC₅₀, 0.364 μ M). Additionally, GRL008 showed potent antiviral activity against an HIV-1 variant selected in the presence of DRV over 20 passages (HIV_{DRV}^R_{P20}), with a 2.6-fold increase in its EC₅₀ (0.097 μ M) compared to its corresponding EC₅₀ (0.038 μ M) against wild-type HIV-1_{NL4-3} (HIV_{WT}). Based on X-ray crystallographic analysis, both GRL008 and DRV showed strong hydrogen bonds (H-bonds) with the backbone-amide nitrogen/carbonyl oxygen atoms of conserved active-site amino acids G27, D29, D30, and D30' of HIV_{A02} protease (PR_{A02}) and wild-type PR in their corresponding crystal structures, while TPV lacked H-bonds with G27 and D30' due to an absence of polar groups. The P2' thiazolyl moiety of RTV showed two conformations in the crystal structure of the PR_{A02}-RTV complex, one of which showed loss of contacts in the S2' binding pocket of PR_{A02}, supporting RTV's compromised antiviral activity (EC₅₀, >1 μ M). Thus, the conserved H-bonding network of P2-*bis*-THF-containing GRL008 with the backbone of G27, D29, D30, and D30' most likely contributes to its persistently greater antiviral activity against HIV_{WT}, HIV_{A02}, and HIV_{DRV}^R_{P20}.

Human immunodeficiency virus type 1 (HIV-1) protease (PR) is a critical viral component that is required for viral maturation and infectivity (1, 2). Due to rapid and error-prone viral replication, drug-resistant HIV-1 variants are inevitably selected during therapy with all currently available antiretroviral agents (3). Accumulation of mutations in the protease-encoding gene results in the emergence of multidrug-resistant (MDR) HIV-1 variants carrying a protease with an altered three-dimensional structure (4). Darunavir (DRV) (Fig. 1), the latest FDA-approved protease inhibitor (PI), which contains *bis*-tetrahydrofuranyl urethane (*bis*-THF) as the P2 moiety, has been shown to have a high genetic barrier (5, 6), a feature of a drug or regimen that delays or prevents the occurrence of genetic evolution of HIV-1 to acquire drug resistance-associated mutations, allowing the virus to overcome the antiretroviral activity of the very drug or regimen and to become capable of propagating despite treatment with the very drug or regimen. However, HIV-1 also ultimately develops high levels of resistance to DRV both *in vitro* and *in vivo* (7, 8). In order to suppress the propagation of such PI-resistant HIV-1 protease variants, the development of novel PIs with greater antiviral activities and higher genetic barriers is urgently needed.

Previously, two structurally related nonpeptidic PIs, GRL007 and GRL008, were designed based on the crystal structure of wild type HIV-1 protease (PR_{WT}) in complex with DRV (PDB ID 4HLA) to replace one of the crystallographic bridging water molecules seen between the P2' aniline moiety of DRV and G48' of

PR_{WT} (9). Both GRL007 and GRL008 contain *bis*-THF as the P2 moiety, while the former has benzene carboxylic acid and the latter has benzene carboxamide as the P2' moiety. Both compounds were evaluated against wild-type HIV-1 (HIV_{WT}) (9). While both GRL007 and GRL008 showed greater enzyme inhibitory activities than the parent compound (DRV), GRL007 failed to inhibit the replication of HIV_{WT} at up to 1 μ M due to its poor cell penetration capability. On the other hand, GRL008 achieved higher intracellular concentrations as DRV comparably did and showed favorable antiviral activity against HIV_{WT} (9).

In the present study, GRL008 (Fig. 1) was evaluated against an MDR clinical isolate of HIV-1 (HIV_{A02}) (10, 11) that contained eight amino acid substitutions, L10I, K45R, I54V, L63P, A71V, V82T, L90M, and I93L, in its protease (PR_{A02}). The antiviral activity of GRL008 was also evaluated against HIV-2_{ROD} and an

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FIG 1 Structures of protease inhibitors used in this study. GRL008 is a novel experimental HIV-1 PI, while DRV, tipranavir, lopinavir, saquinavir, indinavir, and ritonavir are FDA-approved PIs. Both GRL008 and DRV contain *bis*-THF as the P2 moiety. GRL008 and DRV contain benzene carboxamide and aniline, respectively, as the P2' moieties.

HIV-1 variant selected with DRV over 20 passages (HIV_{DRV P20}^R) (7). In addition, GRL008, DRV, ritonavir (RTV), and TPV were individually cocrystallized with PR_{A02}, and a structure-function evaluation of each agent was performed. Crystal structures of PR_{WT} in complex with GRL008, DRV, RTV, and TPV were published previously, with the following Protein Data Bank (PDB) identification codes: **4I8Z**, **4HLA**, **1HXW**, and **2O4P**, respectively.

MATERIALS AND METHODS

Protease inhibitors. Indinavir (IDV), lopinavir (LPV), RTV, saquinavir (SQV), and TPV were provided by the NIH AIDS Research and Reference Reagent Program. DRV was synthesized as described previously (12). GRL008 was synthesized by Arun K. Ghosh and coworkers (details of the synthetic procedures will be published separately).

Antiviral assays. Antiviral assays were performed as described previously (9). Briefly, human MT-4 cells were cultured in RPMI 1640 (supplemented with 10% fetal bovine serum) and were exposed to HIV_{WT} (HIV-1_{NL4-3}), HIV_{A02}, HIV-2_{ROD}, or HIV_{DRV P20}^R in the presence of var-

ious concentrations (1 μ M, 100 nM, 10 nM, and 1 nM) of the PIs. Fifty TCID₅₀ doses (the TCID₅₀ is the inoculum size of HIV-1 that causes a cytopathic effect in 50% of the target cells, i.e., the 50% tissue culture infective dose) were used to infect the MT-4 cells. Infected cells were cultured for 5 days in the presence of PIs. An MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide)-based colorimetric assay was performed to assess cell viability on day 5. Assays were conducted in triplicate. The antiviral data are summarized in [Table 1](#).

Expression and purification of PR_{A02}. Expression and purification of PR_{A02} was performed as described previously (9). Briefly, the inclusion bodies containing PR_{A02} were extracted with 3 M guanidine HCl (GnCl), the mixture was centrifuged, and the supernatant was loaded on a Sephadex 200 column that was preequilibrated with 4 M GnCl. Protease-containing fractions were pooled and further purified by using a reverse-phase column. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the purity of PR_{A02} was determined to be >95%.

Protease refolding and cocrystallization with PIs. Protease refolding was performed as described previously (9). Briefly, lyophilized PR_{A02} was

TABLE 1 Anti-HIV^a activity of GRL008 in comparison with activities of FDA-approved protease inhibitors

PI	Mean EC ₅₀ (μM) ± SD (fold change) ^b			
	HIV-1 _{NL4-3} ^c	HIV-2 _{ROD}	HIV-1 _{A02}	HIV-1 _{DRV} ^R _{P20}
GRL008	0.038 ± 0.01	0.03 ± 0.006	0.029 ± 0.001 (0.8)	0.097 ± 0.028 (2.6)
DRV	0.001 ± 0.001	0.0057 ± 0.0028	0.002 ± 0.001 (2.0)	0.05 ± 0.004 (50)
TPV	0.128 ± 0.09	1.8 ± 0.3	0.364 ± 0.14 (2.8)	0.34 ± 0.03 (2.7)
LPV	0.029 ± 0.01	0.014 ± 0.002	0.474 ± 0.22 (16.3)	>1 (>34.0)
IDV	0.039 ± 0.02	ND	0.526 ± 0.15 (13.5)	ND
RTV	0.034 ± 0.02	0.26 ± 0.03	>1 (>29.0)	>1 (>29.0)
SQV	0.017 ± 0.01	ND	0.107 ± 0.08 (6.3)	ND

^a The amino acid substitutions identified in the proteases of HIV-1_{A02} and HIV-1_{DRV}^R_{P20} compared to the wild-type HIV-1_{NL4-3} were as follows: L101/K45R/I54V/L63P/A71V/V82T/L90 M/I93L and L101/I15V/K20R/L24I/V32I/M36I/M46L/L63P/A71T/V82A/L89M, respectively.

^b The fold change is the ratio of the EC₅₀ of the inhibitor against HIV-1_{A02} or HIV-1_{DRV}^R_{P20} and the corresponding EC₅₀ against HIV-1_{NL4-3}. ND, not determined.

^c The EC₅₀s of GRL008, TPV, and SQV against wild-type HIV-1 (HIV-1_{NL4-3}) were obtained from data previously published (9).

dissolved in 1 ml of a 50% acetic acid solution and then added dropwise to 29 ml of refolding buffer (50 mM sodium acetate [pH 5.2], 5% ethylene glycol, 10% glycerol, 5 mM dithiothreitol, and a 10- to 20-fold molar excess of PI) while stirring on ice. Refolding was continued at 4°C with constant stirring overnight. The refolded protease-drug complex was concentrated using Amicon filters (3-kDa molecular mass cutoff) by centrifugation at 4,000 × *g*. The final protease concentration was determined to be 2 mg/ml. The hanging drop vapor diffusion method was used for co-crystallization of PR_{A02}-drug complexes. Two microliters of the PR_{A02}-drug complex was mixed with 2 μl of well solution per drop. Ammonium sulfate and sodium chloride grid screens (Hampton Research, CA) were used to obtain preliminary crystallization hits. Crystals were usually obtained in 1 to 2 days at room temperature (298 K). Cocrystals of PR_{A02} in complex with GRL008, DRV, TPV, or RTV were obtained using 1.6 M ammonium sulfate (in 0.1 M citric acid buffer at pH 5.0), 1 M sodium chloride (in 0.1 M citric acid buffer at pH 5.0), 2.4 M ammonium sulfate (in 0.1 M HEPES buffer at pH 7.0), and 3 M sodium chloride (in 0.1 M HEPES buffer at pH 7.0), respectively. Cocrystals were obtained as clusters of plates that were carefully dissociated using microtools, and individual crystals were picked up into nylon loops. Glucose (30%) was used as cryoprotectant for all the cocrystals. Cryo-coated cocrystals were instantaneously frozen in liquid nitrogen.

X-ray diffraction data collection and processing details. X-ray diffraction data were collected at the Advanced Photon Source (APS), Argonne National Laboratory IL. Diffraction data for PR_{A02}-GRL008 and PR_{A02}-RTV were collected at the SER-CAT (Southeast Regional Collaborative Access Team) facility, beam line 22-ID (insertion device; wavelength, 1.0 Å) equipped with a Mar300 charge-coupled-device (CCD) detector. Diffraction data for PR_{A02}-TPV were collected at the LS-CAT (Life Sciences Collaborative Access Team) facility, beam line 21-ID (wavelength, 0.98 Å) equipped with an MX225 CCD detector, while the diffraction data for PR_{A02}-DRV were collected using a Rigaku 007 HF rotating anode X-ray generator (Cu K_α wavelength, 1.54 Å) equipped with multilayer focusing mirrors and a Saturn A200 CCD detector. Sample temperature, crystal to detector distance, and frame width were 100 K, 200 mm, and 1°, respectively, at the synchrotron, while they were 95 K, 100 mm, and 0.5°, respectively, on the Rigaku generator. Diffraction data for PR_{A02}-GRL008 and PR_{A02}-RTV were processed and scaled using HKL2000 (13); data for PR_{A02}-TPV and PR_{A02}-DRV were processed using iMOSFLM (14) and scaled using SCALA (15) through the CCP4 interface (16, 17). Details of diffraction data processing results are given in Table 2.

Structure solutions and refinement. Structure solutions were obtained by the molecular replacement (MR) method. Initial MR was performed using MOLREP (18) through the CCP4 interface for PR_{A02} with the protease taken from PDB ID 4HLA as a search model. Amino acid substitutions were modeled into 4HLA by using Maestro (version 9.3; Schrödinger, LLC, New York, NY). Final MR solution of PR_{A02} was obtained using BALBES (19), an automated molecular replacement pipeline

available from the York Structural Biology Laboratory server (<http://www.york.ac.uk/chemistry/research/ysbl>). Structure solutions were directly refined using REFMAC5 (20) through the CCP4 interface. Initial coordinates for GRL008, DRV, RTV, and TPV were taken from crystal structures (PDB IDs 4I8Z, 4HLA, 1HXW, and 2O4P, respectively). The PIs were fit into the electron density by using ARP/wARP ligands (21, 22) through the CCP4 interface. Refinement libraries for GRL008 were prepared using the PRODRG server (23) as described previously (9). Solvent molecules were built using the ARP/wARP solvent-building module through the CCP4 interface. After building water molecules, the final models were refined using the simulated annealing method from phenix.refine (Phenix, version 1.8.2-1309) (24) on the NIH Biowulf Linux cluster. Details of the refinement statistics are given in Table 2. The final refined structures were used for structural analysis. Hydrogen bonds (H-bonds) were calculated by using cutoff values for distance (maximum distance between the donor and acceptor heavy atoms was 3.0 Å) and angles (minimum donor, 90°, and minimum acceptor, 60°). H-bonds with a distance of >3.0 Å were considered as weak interactions. Hydrophobic contacts were calculated between two carbon atoms (one from PI and one from PR_{WT} or PR_{A02}) with a 4-Å distance cutoff.

Protein structure accession numbers. The final refined coordinates for the crystal structures of PR_{A02}-GRL008, PR_{A02}-DRV, PR_{A02}-TPV, and PR_{A02}-RTV were deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) under accession IDs 4NJS, 4NJT, 4NJU, and 4NVJ, respectively.

RESULTS

GRL008 is highly active against HIV_{A02}. Cell-based antiviral assays using human MT-4 cells exposed to HIV_{A02} revealed that GRL008 is highly active against HIV_{A02}, with an EC₅₀ of 0.029 μM. GRL008 and DRV, both of which contain a P2 *bis*-THF moiety, had <1- and 2-fold changes in their EC₅₀s against HIV_{A02} compared to their corresponding EC₅₀s against HIV_{WT} (Table 1). In contrast, as shown in Table 1, the EC₅₀s of RTV against HIV_{WT} and HIV_{A02} were 0.034 μM and >1 μM, respectively, resulting in a >29-fold increase in the EC₅₀ against HIV_{A02}. Similarly, LPV and IDV showed >16- and >13-fold increases in their EC₅₀s, respectively, against HIV_{A02} in comparison to their corresponding EC₅₀s against HIV_{WT}. Although lesser changes in the EC₅₀s were seen for IDV than for LPV, the EC₅₀ of IDV (0.526 μM) was still greater than that of LPV (0.474 μM) against HIV_{A02}. TPV and SQV showed 2.8- and >6-fold changes, respectively, in their EC₅₀s against HIV_{A02} compared to their corresponding EC₅₀s against HIV_{WT}. However, SQV had greater antiviral potency against HIV_{A02} (EC₅₀, 0.107 μM) than TPV (EC₅₀, 0.364 μM). Among the seven PIs tested, based on their antiviral activities,

TABLE 2 X-ray diffraction data and structure refinement details for PR_{A02} in complex with GRL008, DRV, TPV, or RTV

Parameter	PR _{A02} -GRL008	PR _{A02} -DRV	PR _{A02} -TPV	PR _{A02} -RTV
PDB entry	4NJS	4NJT	4NJU	4NJV
Diffraction data				
Resolution range (Å)	35.37–1.80	28.83–1.95	34.62–1.80	34.81–1.80
Unit cell				
a (Å)	44.632	44.95	45.35	45.952
b (Å)	57.995	57.60	57.54	58.219
c (Å)	88.111	86.48	86.70	86.866
α (°)	90.000	90.000	90.000	90.000
β (°)	90.030	90.020	90.020	90.020
γ (°)	90.000	90.000	90.000	90.000
Space group	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁
Solvent content (%)	53.34	52.27	52.96	54.02
No. of unique reflections	41,155 (1,909) ^a	31,996 (4,519)	41,189 (5,915)	40,844 (1,408)
Mean [I/σ(I)]	23.1 (1.9)	7.6 (2.1)	10.1 (2.9)	18.71 (1.8)
<i>R</i> _{merge} ^b	0.090 (0.478)	0.097 (0.472)	0.089 (0.456)	0.089 (0.398)
Data redundancy	3.8 (2.2)	3.2 (2.8)	4.2 (4.1)	3.4 (2.1)
Completeness (%)	98.9 (92.3)	98.5 (95.7)	99.1 (98.6)	96.6 (67.0)
Structure refinement data				
Resolution range (Å)	35.37–1.80	22.47–1.95	34.62–1.80	34.81–1.80
No. of reflections used	40,957	31,875	41,166	40,761
<i>R</i> _{cryst} ^c	0.1823	0.1996	0.1840	0.1868
<i>R</i> _{free} ^d	0.2161	0.2425	0.2211	0.2160
No. of PR _{A02} dimers/AU	2	2	2	2
No. of protein atoms/AU	3,044	3,064	3,044	3,064
No. of ligand molecules/AU	2	2	2	4
No. of ligand atoms/AU	80	76	84	200
No. of water molecules	378	420	375	359
Mean temp factors				
Protein (Å ²)	22.538	18.339	20.142	21.705
Main chains (Å ²)	20.390	16.818	17.930	19.213
Side chains (Å ²)	24.870	19.966	22.542	24.373
Ligand (Å ²)	17.672	15.725	16.28	22.574
Water molecules (Å ²)	32.114	26.305	30.263	31.957
RMSD bond length (Å)	0.007	0.009	0.007	0.009
RMSD bond angle (Å)	1.160	1.261	1.185	1.279
Ramachandran plot				
Most favored (%)	97.8	96.5	98.1	97.8
Additional allowed (%)	1.9	3.5	1.9	2.2
Generously allowed (%)	0.3	0.0	0.0	0.0
Disallowed (%)	0.0	0.0	0.0	0.0

^a Values in parentheses are for the highest-resolution shell.^b $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$.^c $R_{\text{cryst}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$.^d A test set of 5% of the reflections was used for the *R*_{free} analysis.

RTV, LPV, IDV, and TPV were the least effective against HIV_{A02}, all with EC₅₀s in the high nanomolar concentration range (364 nM to >1,000 nM). The substantial changes in the EC₅₀s for these drugs demonstrate the multidrug resistance profile of HIV_{A02}. Comparative analysis of the EC₅₀s against HIV_{A02} given in Table 1 shows that the antiviral potency of GRL008 is >34-fold higher than RTV, >16-fold higher than LPV, >18-fold higher than IDV, 3.7-fold higher than SQV, and >12-fold higher than TPV. These results suggest that GRL008 has a desirable genetic barrier similar to that of DRV and can effectively inhibit multi-PI-resistant strains of HIV-1, such as HIV_{A02}.

GRL008 shows favorable antiviral activities against HIV-2_{ROD} and HIV_{DRV}^R_{P20}. The favorable antiviral activity of GRL008 against HIV_{WT} and HIV_{A02} prompted us to further evaluate it

against HIV-2_{ROD} and HIV_{DRV}^R_{P20}. Antiviral assays using human MT-4 cells exposed to either HIV-2_{ROD} or HIV_{DRV}^R_{P20} showed that GRL008 was equipotent against HIV_{WT} (EC₅₀, 0.038 μM) and HIV-2_{ROD} (EC₅₀, 0.03 μM). LPV showed slightly better antiviral activity (EC₅₀, 0.014 μM) than GRL008 against HIV-2_{ROD}. Both TPV and RTV showed lesser antiviral activities against HIV-2_{ROD}, with EC₅₀s of 1.8 μM and 0.26 μM, respectively. While DRV showed a 50-fold increase in its EC₅₀ against HIV_{DRV}^R_{P20} (0.05 μM) compared to its EC₅₀ against HIV_{WT} (0.001 μM), GRL008 showed only a 2.6-fold increase in its EC₅₀ against HIV_{DRV}^R_{P20} (0.097 μM) compared to its EC₅₀ against HIV_{WT} (0.038 μM). TPV showed a 2.7-fold increase in its EC₅₀ against HIV_{DRV}^R_{P20} (0.34 μM) compared to its EC₅₀ against HIV_{WT} (0.128 μM). However, GRL008 showed a 3.5-fold-greater antiviral activity

FIG 2 Hydrogen bonding profiles of GRL008, DRV, and TPV. H-bonds formed by GRL008, DRV, and TPV with PR_{WT} (a, b, and c, respectively) and with PR_{A02} (d, e, and f, respectively) in their corresponding crystal structures are shown as yellow dashed lines. In all panels, the carbon atoms of the inhibitors are shown as white thick sticks, while the corresponding carbon atoms of PR_{WT} and PR_{A02} residues are shown as green and cyan thin sticks, respectively. Nitrogen, oxygen, sulfur, and fluorine atoms are shown as blue, red, yellow, and light cyan sticks, respectively. Crystallographic water molecules are shown as red spheres. All H-bonds were calculated as the distances between two heavy atoms, with a maximum distance cutoff of 3.0 Å and minimum angle cutoff for donor of 90° and for acceptor of 60°. The P2 *bis*-THF moiety of GRL008 and DRV shows three conserved H-bonds with the backbone amide nitrogen atoms of D29 and D30 in both PR_{WT} (a and b) and PR_{A02} (d and e). While TPV shows similar three H-bonds with the backbone amide nitrogen atoms of D29 and D30 in PR_{WT} (c), the sulfonyl oxygen of TPV shows two H-bonds, one each with the backbone amide nitrogen atoms of D29 and D30 and one H-bond with the side chain δ-oxygen atom of D30 from PR_{A02} (f). Additionally, the P2' moieties of GRL008 and DRV show one conserved H-bond, each with the backbone of D30' (a, b, d, and e). Both GRL008 and DRV have a direct H-bond with the backbone carbonyl oxygen atom of G27 (a, b, d, and e). No H-bonds with D30' or G27 were seen with TPV; instead, three direct H-bonds, one each with the backbone carbonyl oxygen atom of G48 and the backbone amide nitrogen atoms of I50 and I50', were seen with TPV (c and f). In the cases of GRL008 and DRV, bridging H-bonds were seen with the backbone amide nitrogen atoms of I50 and I50' via a conserved water molecule (a, b, d, and e). While the P2' benzene carboxamide moiety of GRL008 showed a conserved bridging H-bond with G48' from both PR_{WT} and PR_{A02} via a water molecule (a and d, respectively), such conserved bridging H-bonds were not seen for the P2' aniline moiety of DRV with G48' of PR_{A02} (e). Overall, no significant changes in the H-bonding profiles were seen for GRL008, DRV, and TPV against PR_{A02} compared to their profiles against PR_{WT} in their respective crystal structures.

against HIV_{DRV}^R_{P20} than TPV. RTV failed to inhibit HIV_{DRV}^R_{P20} even at a 1.0 μM concentration. These results suggest that GRL008 has a greater genetic barrier than most of the PIs evaluated in this study.

Crystal structures of PR_{A02} in complex with PIs. Crystal structures of PR_{A02} in complex with GRL008, RTV, or TPV were solved to a resolution of 1.8 Å, while the structure of PR_{A02} in complex with DRV was solved to a resolution of 1.95 Å (Table 2). All structures were solved in the space group *P*₂₁, with two dimers of PR_{A02} per asymmetric unit (AU), in agreement with the predicted number of PR_{A02} dimers per Matthew's coefficient values (25), which were precalculated before molecular replacement. As shown in Fig. S1a, in the supplemental material, the overall *R* factors (*wR*_{fac}), as calculated using MOLREP, were high, with low scores when one PR_{A02} dimer per AU was obtained as a solution. When two dimers of PR_{A02} per AU were obtained as a solution, the *wR*_{fac} values decreased, with a relatively proportional increase in the corresponding scores (see Fig. S1a) for each of the structure solutions. No significant root mean square deviation (RMSD) in the C_α atoms of the two PR_{A02} dimers within the AU was observed for any of the four structure solutions (see Fig. S1b). RMSD values of <1.0 Å were considered biologically insignificant. Continuous

difference electron density was observed for GRL008, DRV, RTV, and TPV, with map correlation coefficient values of 0.94, 0.89, 0.78, and 0.88, respectively, in their corresponding maps. Based on the electron density maps, one molecule of GRL008, DRV, or TPV was fit into the active site of each PR_{A02} dimer. On the other hand, two molecules of RTV were fit into the active site of each PR_{A02} dimer. Thus, two molecules of GRL008, DRV, or TPV were fit per AU, while four molecules of RTV were fit per AU. The *R*_{cryst} and *R*_{free} values improved significantly (see Fig. S1c and d, respectively) with the two-step refinement of structure solutions containing two PR_{A02} dimers per AU. The RMSDs in bond lengths and bond angles significantly improved (Table 2) by using the libraries for ligands that were geometry optimized through the semiempirical quantum mechanical method of refinement, eLBOW-AM1 (26) during refinement conducted using phenix.refine. Ramachandran plots showed no significant deviations for the protein, suggesting an overall good quality of stereochemistry.

GRL008, DRV, and TPV showed minor changes in their binding profiles in the active site of PR_{A02} compared to their interactions with PR_{WT}. Crystal structures of PR_{A02} in complex with GRL008, DRV, or TPV showed two PR_{A02} dimers per AU, with each PR_{A02} dimer bound to one molecule of the PI. No major

FIG 3 Hydrophobic interactions of GRL008 and DRV. (a and b) Profiles of hydrophobic interactions for GRL008 (a) and DRV (b) in the active site of PR_{A02} and PR_{WT}. The protease binding pockets (S2, S1, S1', and S2') are shown as arcs and are labeled. The corresponding protease amino acids that form the hydrophobic contacts with either GRL008 or DRV are listed for each binding pocket accordingly. Residues shown in green are from PR_{WT} structures (PDB IDs 4I8Z and 4HLA for GRL008 and DRV, respectively), while the residues in red are from PR_{A02}. In both panels, the amino acid residues are labeled 1 to 99 for monomer 1 and 1' to 99' for monomer 2. Both GRL008 and DRV showed slightly altered but persistent contacts in all the binding pockets that were comparable to their corresponding wild-type profiles, thus supporting their higher antiviral activities.

differences in the binding profiles of three PIs were seen between the two PR_{A02} dimers (within the AU) in their corresponding structures. As shown in Fig. 2, the P2 *bis*-THF moiety of GRL008 (panel d) and DRV (panel e) showed three strong H-bonds with the backbone amide nitrogen atoms of D29 (two contacts) and D30 (one contact). In the case of TPV, two strong H-bonds, one each with the backbone amide nitrogen atoms of D29 and D30, were seen (Fig. 2f). TPV showed two additional H-bonds in the S2 binding pocket of PR_{A02}, one with the backbone carbonyl oxygen atom of G48 and one with the side chain δ -oxygen atom of D30 (Fig. 2f). One strong H-bond with G27 was seen for both GRL008 and DRV but not for TPV. The P2' moieties, benzene carboxamide (GRL008) and aniline (DRV), showed one strong H-bond each with the backbone amide nitrogen and the backbone carbonyl oxygen atoms of D30', respectively. TPV, due to lack of P2' polar atoms, did not show any H-bonds in the S2' binding pocket of PR_{A02}. The transition-state mimic hydroxyl group of all three PIs showed at least one H-bond each with the δ -oxygen atoms from the side chains of both D25 and D25' (Fig. 2d, e, and f). Both GRL008 and DRV showed two H-bonds each, with a conserved bridging water molecule, which in turn had H-bonds with the backbone amide nitrogen atoms of the PR_{A02} flap residues, I50 and I50' (Fig. 2d and e). TPV showed direct H-bonds with I50 and I50' (Fig. 2f) without a bridging water molecule, as seen in its corresponding wild-type protease structure (PDB ID 2O4P) (Fig. 2c). In addition, the P2' moieties of GRL008 and DRV showed H-bonds with two and one water molecules, respectively. In the case of GRL008, one of the water molecules, interacting with its P2' moiety, showed a bridging H-bond with the backbone amide nitrogen atom of G48' (Fig. 2d). As shown in Fig. 2a, a similar profile was seen in the crystal structure of PR_{WT} in complex with GRL008 (PDB ID 4I8Z). As shown in Fig. 2b, DRV showed two water molecules and an H-bonding network between the P2' aniline moiety and G48' of PR_{WT} (PDB ID 4HLA). However, as shown in Fig. 2e, such an H-bonding network was not seen in the crystal structure of PR_{A02} in complex with DRV (PDB ID 4NJT). No major changes in the binding orientation were seen for any of the three PIs compared to their corresponding binding profiles against PR_{WT}.

As shown in Fig. 3, both GRL008 and DRV showed conserved hydrophobic interactions with residues L23, V32, G49, T82, L23', A28', V32', T82', and I84' in the corresponding S1, S2, S1', and S2' binding pockets of PR_{A02}. GRL008 showed additional hydrophobic interactions with residues A28 and I50, while DRV showed additional hydrophobic interactions with residues D29 and P81'. Overall, the profiles of hydrophobic contacts for GRL008 and DRV were similar to their corresponding profiles against PR_{WT} structures, PDB IDs 4I8Z and 4HLA, respectively. TPV is involved in multiple hydrophobic interactions with residues R8, L23, A28, D29, V32, G49, I50, P81, T82, R8', G27', A28', V32', G49', I50', and I84' in the corresponding S1, S2, S1', and S2' binding pockets of PR_{A02}. No significant deviation was seen in the profile of hydrophobic contacts for TPV against PR_{A02} compared to its corresponding profile against PR_{WT} structure (PDB ID 2O4P). Thus, the binding profiles of GRL008, DRV, and TPV in the active site of PR_{A02} were comparable to their corresponding profiles in the active site of PR_{WT}.

RTV shows an altered binding orientation in the active site of PR_{A02}. The crystal structure of PR_{A02} in complex with RTV showed two PR_{A02} dimers per AU. As shown in Fig. 4a and b, the P2' thiazolyl moiety of RTV showed two alternate binding orientations, RTV-1 and RTV-2, in the active site of PR_{A02}. Superposition of RTV-1 onto RTV-2 is shown in Fig. S2 in the supplemental material for clarity. RTV-1 (Fig. 4a) showed a previously reported binding orientation seen in the active site of PR_{WT} (PDB ID 1HXW). RTV-2 (Fig. 4b) shows the altered binding of the P2' thiazolyl moiety, which contributes to a significant loss of contacts in the S2 and S2' binding pockets of PR_{A02}. In order to verify the alternate conformations of the P2' thiazolyl group of RTV, the average individual *B* factors of all five atoms (C1, C2, S3, C4, and N5) from the P2' thiazolyl group were analyzed (see Fig. S3 in the supplemental material) and it was found that the individual *B* factors support the two conformations.

Two conserved H-bonds, one with the backbone amide nitrogen atom of D29 and one with the backbone carbonyl oxygen atom of G48, were seen for both RTV-1 and RTV-2 in the S2 binding pocket of PR_{A02}. Two and one H-bonds were seen with the backbone carbonyl oxygen atoms of G27 and G27', respectively, for both RTV-1 and RTV-2. The

FIG 4 Hydrogen bonds and hydrophobic contacts for RTV in the active site of PR_{A02}. (a and b) H-bonds made by RTV-1 (a) and RTV-2 (b) (alternate conformations of the P2' thiazolyl moiety of RTV, highlighted by red circles) in the active site of PR_{A02}. In both panels, the carbon atoms of RTV are shown as white thick sticks, while the carbon atoms of corresponding amino acid residues from PR_{A02} are shown as green thin sticks. Nitrogen, oxygen, and sulfur atoms are shown as blue, red, and yellow sticks, respectively. Crystallographic water molecules are shown as red spheres, and the H-bonds are shown as yellow dashed lines. While RTV-1 (a) showed a profile comparable to its corresponding profile with PR_{WT} (PDB ID 1HXW), RTV-2 (b) showed an altered binding orientation for the P2' thiazolyl group, with an average root mean square deviation of 3 Å, resulting in loss of a critical H-bond with the backbone carbonyl oxygen atom of D30'. The P3 isopropyl group also showed a slightly altered binding orientation but was not biologically significant. (c) Profile of hydrophobic contacts made by RTV in the active sites of PR_{WT} and PR_{A02}. The binding pockets are represented as arcs and are labeled accordingly. The corresponding amino acids from PR_{WT} and PR_{A02} that are involved in hydrophobic interactions with RTV are shown in green and red, respectively. For PR_{A02}, only RTV-2 is shown here because the binding profile of RTV-1 in the active site of PR_{A02} is similar to that of its corresponding profile in the active site of PR_{WT}. RTV-2 showed significant loss of contacts in the S2 and S2' binding pockets of PR_{A02}, thus supporting its weaker antiviral activity. Superposition of RTV-1 and RTV-2 is shown in Fig. S2 of the supplemental material.

transition-state mimic hydroxyl group of both RTV-1 and RTV-2 showed one H-bond each with the side-chain δ -oxygen atoms of both D25 and D25'. The P2' thiazolyl group of RTV-2 showed a significant change in the binding orientation, with an average RMSD of 3 Å compared to that of RTV-1, resulting in loss of a critical H-bond with the backbone carbonyl oxygen atom of D30'. Both RTV-1 and RTV-2 showed a conserved water molecule that bridges H-bonds between RTV

and the backbone amide nitrogen atoms of I50 and I50'. Two additional water molecules were seen for both RTV-1 and RTV-2 that are involved in H-bonding with the P3 and P2 moieties of RTV. Overall, the profiles of H-bonds for both RTV-1 and RTV-2 were similar to that of the wild-type structure (PDB ID 1HXW). However, the P2' moiety of RTV-2 showed major conformational changes that resulted in the loss of a critical H-bond with D30'.

As shown in Fig. 4c, RTV-1 showed a profile of hydrophobic contacts similar to that of RTV, based on the PR_{WT} crystal structure (PDB ID 1HXW), while RTV-2 showed loss of hydrophobic contacts in the S1, S2, and S2' binding pockets of PR_{A02}. The isopropyl group from the P3 isopropylthiazolyl moiety showed minor changes in binding orientation, with an average RMSD of 1 Å, but still maintained hydrophobic contacts with Pro81. Nine amino acids (D29, G49, T82, I84, R8', G49' I50', P81', and T82') from the S3, S1, and S1' binding pockets of PR_{A02} showed conserved hydrophobic interactions with both RTV-1 and RTV-2. RTV-2 showed complete loss of hydrophobic contacts in the S2 and S2' binding pockets of PR_{A02}. Thus, the loss of contacts in the S1, S2, and S2' binding pockets of PR_{A02} should well explain the loss of antiviral activity of RTV against HIV_{A02}.

DISCUSSION

The present structure-function data provide insights into the structural basis for the greater antiviral activity of a P2 *bis*-THF-containing novel PI, GRL008, and should help us understand the drug resistance mechanism of the clinical isolate HIV_{A02} toward RTV and other PIs. HIV_{A02} was initially isolated at the National Institutes of Health Clinical Center from a treatment-experienced male patient (age 44 years) who had been heavily treated with the PIs IDV, RTV, and SQV, along with reverse transcriptase (RT) inhibitors abacavir (ABC), 3'-azido-2'-dideoxythymidine (AZT), 2',3'-dideoxyinosine (ddI), 3'-thiacytidine (3TC), 2',3'-dideoxycytidine (ddC), and 2',3'-didehydro-2',3'-dideoxythymidine (d4T), as a part of 39-month antiviral therapy (10). Antiviral assays with viral isolates obtained by culturing the peripheral blood mononuclear cells (PBMCs) from the patient's blood sample showed 4- to >5,000-fold-increased resistance against IDV, RTV, SQV, and the RT inhibitors mentioned above (10). In the present study, the antiviral assays showed that both GRL008 and DRV, which contain the *bis*-THF group as the P2 moiety, were highly active with a desirable genetic barrier against HIV_{A02}, with no significant change in their EC₅₀s against HIV_{A02} compared to those against HIV_{WT}. LPV was much less effective in inhibiting the replication of HIV_{A02} than inhibiting HIV_{WT} replication in the cell-based antiviral assays. The lower antiviral activity of LPV against HIV_{A02} could be partially attributed to its structural resemblance to RTV, which was used in the treatment regimen of the patient described above. Although TPV was able to inhibit the replication of HIV_{A02}, GRL008 was found to be >12-fold more potent than TPV in this cell-based antiviral assay. As predicted, HIV_{A02} was resistant to IDV, RTV, and SQV, in view of the fact that the patient failed the treatment with these three PIs. Achievement of higher anti-HIV-1 activity and a greater genetic barrier in combination with lower cytotoxicity and high oral bioavailability represent major critical goals in the design of novel PIs. For example, TMC-126, an analog of DRV that contains P2 *bis*-THF and P2' methoxy benzene groups, was previously shown to have a greater antiviral potency than DRV, with a desirable genetic barrier (27), but could not be used further due to its poor oral bioavailability. Similarly, GRL007 (an analog of GRL008) showed poor cell penetration properties in spite of its picomolar enzyme inhibitory activity (9). It is noteworthy that GRL008 has a desirable therapeutic window due to its greater antiviral activity, lower cytotoxicity (50% cytotoxic concentration, >100 μM), and greater cell penetration capability, similar to that of DRV (9), thus making

it a desirable compound to be further evaluated against multi-PI-resistant strains of HIV-1, including DRV-resistant strains.

It was previously noted that DRV resistance-associated amino acid substitutions are not clinically seen and the correlation for the occurrence of such DRV resistance-associated amino acid substitutions in conjunction with other PI resistance amino acid substitutions (such as L10I, I54V, V82T, and L90M, which are seen in PR_{A02}) is very low (8). However, we recently selected a highly DRV-resistant strain of HIV-1 *in vitro* by using a mixture of multi-PI-resistant (but DRV-sensitive) clinical HIV-1 strains as a starting viral population (7). In an attempt to examine the genetic barrier of GRL008, it was further evaluated against one of the DRV-resistant HIV-1 variants, HIV_{DRV^R P20} (7) and was found to be potent, with only a 2.6-fold increase in its EC₅₀ above that for HIV_{WT}. Although TPV showed a similar 2.7-fold increase in its EC₅₀ against HIV_{DRV^R P20}, the absolute EC₅₀ of GRL008 was 3.5-fold lower than that of TPV, suggesting that GRL008 is overall a better PI than TPV against HIV_{DRV^R P20}.

The P2 *bis*-THF moiety of both GRL008 and DRV showed strong H-bonds with the backbone of conserved active-site amino acids D29 and D30 of PR_{A02}. The P2' benzene carboxamide moiety of GRL008 was designed to replace one of the bridging water molecules seen with DRV (Fig. 2a and b) in the S2' pocket (9). In the present study, the crystal structure of PR_{A02} in complex with GRL008 showed a conserved profile of a bridging H-bond with G48' via a water molecule (Fig. 2d), as was seen in its corresponding interactions with PR_{WT} (PDB ID 4I8Z) (Fig. 2a). This suggests that the amino acid substitutions in PR_{A02} do not affect the binding profile of GRL008 in either the S2 or S2' binding pockets of PR_{A02}. On the other hand, of the two bridging water molecules in the S2' pocket of PR_{WT} in complex with DRV (PDB ID 4HLA) (Fig. 2b), only one was seen in the structure of PR_{A02} in complex with DRV (Fig. 2e). Taken together, the crystal structures confirmed that both GRL008 and DRV, bound in the active site of PR_{A02}, do not have notable changes in their binding orientations; this evidence thus supports their greater conserved antiviral activities. These structural analyses may further explain in part the conserved and greater antiviral activity of GRL008 against HIV_{DRV^R P20}.

The crystal structure of PR_{A02} in complex with TPV did not show major changes in the binding orientation of TPV (Fig. 2f) compared to that with PR_{WT} (PDB ID 2O4P) (Fig. 2c). However, the EC₅₀ of TPV against HIV_{A02} was >12-fold greater than that of GRL008. The P2' region of TPV lacks polar groups, and hence no H-bonds were seen in the S2' binding pocket of either PR_{A02} or PR_{WT}, while both GRL008 and DRV showed persistent, strong H-bonds with the backbone of D30' in the S2' binding pocket of PR_{A02}. TPV has been shown to have a greater genetic barrier against multi-PI-resistant HIV-1 variants due to its unique mechanism of minor loss of binding affinity through entropy/enthalpy compensation against amino acid substitutions in the protease (28). In the present study, TPV showed a 2.8-fold change in its EC₅₀ against HIV_{A02} versus HIV_{WT}, while GRL008 and DRV showed <1- and 2-fold changes, respectively, in their EC₅₀s against HIV_{A02}. Furthermore, based on Lipinski's rule of five (29) and the logP values of TPV (7.0) and DRV (2.9) obtained from the PubChem database (<http://pubchem.ncbi.nlm.nih.gov/>), it is apparent that DRV and analogs such as GRL008 have favorable oral bioavailabilities in addition to greater antiviral potencies than TPV against HIV_{A02}.

In this study, IDV, RTV, and SQV showed much lower antiviral activities against HIV_{A02} than against HIV_{WT} (Table 1). In particular, RTV showed a >29-fold increase in its EC₅₀ against HIV_{A02} compared to its EC₅₀ against HIV_{WT}. In order to understand the RTV resistance mechanism of HIV_{A02}, the crystal structure of PR_{A02} in complex with RTV was solved and analyzed. Morissette et al. previously showed that RTV undergoes a conformational polymorphism (30) that lowers its solubility and compromises its anti-HIV-1 activity. In the present study, the EC₅₀ of RTV against HIV_{WT} (Table 1) was within 2-fold of the EC₅₀s of LPV, IDV, and SQV against HIV_{WT}, suggesting that the lowered solubility of RTV may not play a critical role in the loss of its activity against HIV_{A02}. The crystal structure of PR_{A02} in complex with RTV showed an altered binding orientation of the P2' thiazolyl moiety of RTV in the S2' binding pocket of PR_{A02} (Fig. 4a and b), with an RMSD of 3 Å. In order to understand the binding profiles of RTV, the crystal structure of PR_{A02} in complex with RTV was compared in detail to its corresponding wild-type structure (PDB ID 1HXW). In the case of the PR_{A02} structure, the H-bond between the P2' thiazolyl group of RTV and D30' was seen for RTV-1 (Fig. 4a), which closely resembled its corresponding binding orientation in the active site of PR_{WT} (PDB ID 1HXW). On the other hand, the H-bond between the P2' thiazolyl moiety of RTV-2 and D30' of PR_{A02} was lost (Fig. 4b). This loss of contact was not seen in the case of RTV interactions with PR_{WT} (PDB ID 1HXW), suggesting that the conformational polymorphism of RTV may not be the reason for loss of its antiviral activity against HIV_{A02}.

Based on the binding profile of RTV-1, one could hypothesize that RTV-1 (Fig. 4a) inhibits the replication of HIV_{A02} at least by 50% due to its close resemblance to its corresponding wild-type structure (PDB ID 1HXW). However, it has been shown previously that the presence of less than 25% of active viral protease can support viral propagation, due to the formation of mature and infectious viral particles (31). Additionally, mutations at codons 10, 54, 63, 71, 82, and 84 have been known to cause HIV-1 resistance against RTV (32), and PR_{A02} consists of amino acid substitutions L10I, I54V, L63P, A71V, and V82T. The combination of active-site and non-active-site amino acid substitutions has been shown to cause a significant loss of binding affinity to RTV (42- to 1,330-fold increase in the K_i values compared to that for PR_{WT}) due to loss of direct contacts as well as altered protease-flap dynamics (33, 34). As shown in Fig. 4b, RTV-2 has a complete loss of direct contacts in the S2 and S2' binding pockets of PR_{A02}. Furthermore, the substitution L63P has been previously shown to enhance viral fitness in combination with L90M (35). PR_{A02} contains both amino acid substitutions L63P and L90M. Thus, the enhanced replication fitness of HIV_{A02} in combination with loss of contacts for RTV in the active site of PR_{A02} may well explain the compromised activity of RTV (EC₅₀ >1 μM) against HIV_{A02} in the antiviral assays. In the case of patients, a study based on the Abbott M97-720 trial showed that low-level viremia could persist at least for 7 years in patients on an LPV/RTV regimen, partially due to latent viral reservoirs (36). Based on our structural analysis, amino acid substitutions (both active site and non-active site) in PR_{A02} caused an altered binding orientation of RTV in the active site, resulting in a significant loss of contacts in the S2 and S2' binding pockets of PR_{A02} and, consequently, causing HIV-1 resistance to RTV. Thus, our present data not only explain the mechanism for RTV resistance but also help explain the retained anti-

viral potency of a novel P2 *bis*-THF-containing PI (GRL008) against HIV_{A02}.

In conclusion, the conserved H-bonding network of P2 *bis*-THF-containing PIs, such as GRL008 and DRV, with the backbone of conserved active-site amino acids G27, D29, D30, and D30' of PR_{A02} and PR_{WT} most likely contributes to their antiviral activities against HIV_{A02} and HIV_{WT}, with minimal changes in the EC₅₀s. In particular, GRL008 proved to have a favorable antiviral activity against HIV_{DRV^R P20}, a finding that warrants further investigation.

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