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In Vitro Antiviral Activity and Cross-Resistance Profile of PL-100, a Novel Protease Inhibitor of Human Immunodeficiency Virus Type 1 ▽

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Despite the success of highly active antiretroviral therapy, the current emergence and spread of drug-resistant variants of human immunodeficiency virus (HIV) stress the need for new inhibitors with distinct properties. We designed, produced, and screened a library of compounds based on an original L-lysine scaffold for their potentials as HIV type 1 (HIV-1) protease inhibitors (PI). One candidate compound, PL-100, emerged as a specific and noncytotoxic PI that exhibited potent inhibition of HIV-1 protease and viral replication in vitro (K_i, ~36 pM, and 50% effective concentration [EC_{50}], ~16 nM, respectively). To confirm that PL-100 possessed a favorable resistance profile, we performed a cross-resistance study using a panel of 63 viral strains from PI-experienced patients selected for the presence of primary PI mutations known to confer resistance to multiple PIs now in clinical use. The results showed that PL-100 retained excellent antiviral activity against almost all of these PI-resistant viruses and that its performance in this regard was superior to those of atazanavir, amprenavir, indinavir, lopinavir, nelfinavir, and saquinavir. In almost every case, the increase in the EC_{50} for PL-100 observed with viruses containing multiple mutations in protease was far less than that obtained with the other drugs tested. These data underscore the potential for PL-100 to be used in the treatment of drug-resistant HIV disease and argue for its further development.

At the end of 2005, an estimated 38.6 million people worldwide were living with human immunodeficiency virus (HIV), with approximately 4.1 million cases of new infections and 2.8 million deaths due to AIDS (32). Highly active antiretroviral therapy (HAART) has resulted in durable virological suppression and a marked decrease in morbidity and mortality associated with HIV, bearing testimony to the success of HAART (17, 31, 33) in Western countries, in which access to therapeutic drugs is guaranteed. However, the development of viral resistance is a major cause of treatment failure (2, 12, 21, 31, 34). Mutated, drug-resistant HIV type 1 (HIV-1) strains emerge through the combined effects of the lack of proofreading activity of the viral reverse transcriptase (RT), recombination between coinfecting isolates (3, 27), and the high replication rate of HIV in vivo (9, 35). Drug-resistant HIV is a major clinical problem, not only for patients for whom therapy fails, but for drug-naïve patients, as well. In North America and Europe, it is estimated that approximately 10% of new HIV infections harbor drug-resistant mutations (15, 25, 37). Thus, novel therapeutic drugs with activity against resistant strains are needed. Other barriers to effective treatment are the toxicity of the drugs taken daily for the rest of a patient's life and the correlated lack of adherence to treatment. Therefore, new compounds should be highly specific, potent, and sufficiently bioavailable to limit the pill burden, in addition to being nontoxic.

HIV-1 protease (PR) has been recognized as a therapeutic target since the approval of the first PR inhibitor (PI) in 1995. Inhibition of this 99-amino-acid homodimeric enzyme prevents the proteolytic processing of the Gag and Gag-Pol viral polyproteins into the structural proteins (p17, p24, p2, p7, p1, and p6) and the viral enzymes (PR, RT, and integrase), thereby blocking viral infectivity (14). Hence, PIs have become cornerstones in the treatment of AIDS as components of HAART both for first-line medications in treatment-naïve patients and in patients with a long history of antiretroviral therapy. However, HIV can develop resistance to specific PIs through selection of amino acid substitutions in PR itself. Many mutated residues have been shown to decrease the enzyme's binding affinity for the inhibitors while the ability of PR to cleave its substrates is preserved. Distinct key or signature mutations have been associated with resistance to specific PIs (12, 18). In addition to these so-called primary mutations, other mutations, generally further away from the catalytic site, also play significant roles in resistance. However, the exact roles of these so-called compensatory or secondary mutations is not always clearly defined, although a role in enzymatic and viral fitness has been demonstrated for some of them.

Moreover, some mutations in PR confer cross-resistance among multiple PIs. Often, drug selective pressure may drive the accumulation of several primary mutations against a background of particular secondary mutations to favor the emergence of cross-resistance (12). This mainly involves amino acid substitutions in PR at positions 10, 32, 46, 54, 82, 84, and 90 (8, 12). Thus, a priority in antiretroviral-drug research is now the development of new HIV inhibitors that exhibit distinct resistance profiles to provide patients with alternatives in combi-

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nation therapy. To tackle this challenge, a drug discovery program was established that integrated viral resistance directly into the screening process (26, 28–30). We present the biochemical and virological characterization of a new PI, termed PL-100, that emerged from this program. PL-100 is a novel, specific, and noncytotoxic inhibitor of the HIV-1 PR that shows good antiviral activity against both wild-type laboratory strains and a wide spectrum of PI-resistant isolates.

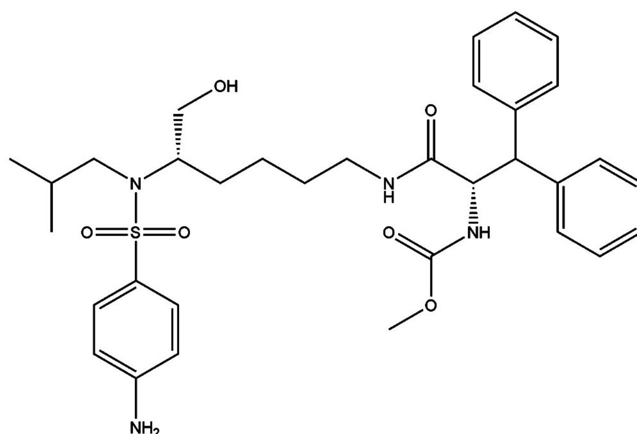

FIG. 1. Chemical structure of PL-100.

MATERIALS AND METHODS

Cells and viral strains. MT-4 and H9/IIIb cells (22–24), as well as HIV-1 NL-4.3 and HIV-1_{4596} proviral clones (1, 4) and HIV-1_{SaqR} (10), were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μg penicillin/ml, and 50 μg streptomycin/ml. HIV-1 NL-4.3 was generated by transfecting the proviral DNA into MT-4 cells by the DEAE-dextran method (6). All HIV stocks were propagated in MT-4 cells and titrated in the same cell line by the endpoint titration method (5, 11).

Materials. Saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), lopinavir (LPV), and atazanavir (ATV) were purified by preparative high-performance liquid chromatography from the marketed drug products purchased in Canada. Synthesis of PL-100 has been described elsewhere (28). Compounds were prepared as 20 mM solutions in dimethyl sulfoxide and stored at −20°C. Stock solutions were thawed and diluted in cultured medium on the day of assay.

Determination of the K_i of PL-100 on purified HIV-1 PR. The inhibitor constant (K_i) of each PI was determined by incubating bacterially expressed, purified HIV-1 PR (Bachem) at 31°C in reaction buffer (1 M NaCl, 100 mM Na acetate, 1 mM EDTA, 1 mM dithiothreitol, and 1 mg/ml bovine serum albumin, pH 5.2) containing increasing concentrations of test inhibitor. After a 5-min preincubation, the reaction was initiated by the addition of the fluorogenic substrate Arg-Glu (EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys (DABCYL)-Arg (Molecular Probes) at a final concentration of 10 μM. Cleavage of the substrate was monitored by measuring the increase in the fluorescence intensity at 490 nm after excitation at 340 nm (16) using an FL600 fluorometer (BioTek). K_i values were obtained by fitting the initial velocity data to the tightly binding inhibition equation as described by Williams and Morrison (36). Alternatively, 50% inhibitory concentrations (IC_{50}s) were determined from a dose-response curve and converted to K_is using the equation $IC_{50} = K_i(1 + [S]/K_m)$, where $[S]$ is the substrate concentration and K_m, the Michaelis-Menten constant.

Determination of the K_i of PL-100 on purified cathepsin D and pepsin. The inhibition of human cathepsin D (Calbiochem) and porcine pepsin (Roche) proteolytic activities by PL-100 was determined using a fluorescent substrate (Bachem). Briefly (7), cathepsin D (5 nM) was preincubated at 37°C in the presence or absence of the inhibitor in the assay buffer (50 mM glycine-HCl, 0.001% Triton X-100, pH 3.5). The reaction was initiated by the addition of the fluorogenic substrate at a final concentration of 5 μM. The enzyme activity was evaluated by measuring the fluorescence at 485 nm after excitation at 340 nm using an FL600 fluorometer (BioTek). The IC_{50} was determined from a dose-response curve using the initial velocity against the inhibitor concentration. The same procedure was used to evaluate the inhibition of pepsin. In the presence of the same fluorescent substrate (Bachem no. M-2295), pepsin was used at a final concentration of 1 nM in an assay solution consisting of 10 mM HCl, pH 2.0 (13).

Viral Gag precursor protein processing. H9/IIIb cells and H9 cells (for a negative control) were cultured for 5 days in the presence of various concentrations of PL-100 or 0.1 μM of SQV. On day 5, each culture supernatant was spun at 47,000 rpm for 2 h in an SW 50.1 rotor, and the pellet was resuspended in lysis buffer and then stored at −70°C. Fifty micrograms of each protein extract was loaded on a 3 to 12.5% polyacrylamide gel. After electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transfer to a blotting membrane (Hybond ECL; Amersham), p24 viral protein was detected using an anti-HIV-1 p24 monoclonal antibody (Perkin-Elmer) as the primary antibody and anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Amersham) as the secondary antibody. After the membrane was washed, horseradish peroxidase activity was detected using the ECL Western blotting analysis system (Amersham) and subjected to autoradiography.

Drug susceptibility and cytotoxicity assays. The antiviral activities of the compounds were determined by a cytoprotection assay measuring the inhibition of the cytopathic effects induced by HIV-1 in MT-4 cells. Briefly, 1×10^4 MT-4 cells per well were incubated in 96-well culture plates for 2 hours at 37°C in 5% CO_2 in the presence of various concentrations of inhibitors. The cells were then infected with HIV-1 at a multiplicity of infection sufficient to induce complete cell killing at 6 days postinfection. Typically, the multiplicity of infection was less than 0.06 50% tissue culture infective dose per cell. Six days postinfection, the inhibition of viral replication was measured by MTT colorimetric assay (19). The cytotoxicities of the compounds were tested in parallel using the same assay but without adding virus to the MT-4 cells. The results of at least three experiments with different viral stocks were used to determine the 50% effective concentration (EC_{50}) and the 50% cytotoxic concentration (CC_{50}).

Cross-resistance analysis. The in vitro susceptibilities of HIV-1 strains to PL-100 were assessed using a replication-defective reporter gene-based phenotypic assay (PhenoSense HIV assay; Monogram Biosciences, South San Francisco, CA) (20). In brief, 63 constructs containing patient-derived PR and RT sequences from PI-experienced patients were tested against PL-100, ATV, APV, IDV, LPV, NFV, and SQV. RTV was not tested because it is not used clinically except as a pharmacological boosting agent. Tipranavir (TPV) (Aptivus) and darunavir (DRV) (TMC-114; Prezista) were not available at the time the phenotypic testing was performed. Treatment histories of the patients from whom the PR and RT sequences were derived are not available. The genotypes of the selected strains encompass a wide variety of mutational patterns showing high-level resistance to the approved PIs, as well as mutations known to confer resistance to other second-generation PIs (TPV and DRV) that are currently approved for use in salvage therapy. In the present study, the primary PI mutations were D30N, V32I, L33F, M46I/L, I47A/V, G48V, I50L/V, V82A/F/L/S/T, I84V, N88D/S, and L90M (12). Mixed genotypes in the samples were not considered. Susceptibility data are expressed as the change (n-fold) in the EC_{50} of the patient isolate compared to that of the reference strain, NL-4.3.

Comparison of the cross-resistance patterns between PL-100 and the approved PIs was performed using linear regression of log-transformed change values. Statview version 5 (SAS Institute, Cary, NC) was used to generate the scatter plots and to calculate the R^2 values.

RESULTS

The present study describes the biological activity of PL-100 (Fig. 1), a lysine sulfonamide peptidomimetic drug resulting from a discovery effort focusing on the drug resistance of the HIV PR.

Activities of PL-100 against HIV-1 PR and other aspartyl PRs. PL-100 is a novel L-lysine derivative HIV PI that inhibited purified HIV-1 PR with a mean K_i of 36 pM, as determined by a tightly binding inhibitor plot (Table 1) (36). With the exception of IDV (K_i, ~2 nM), all of the PIs tested demonstrated K_i values in the same picomolar range (14 to 306 pM).

The selectivity of PL-100 was evaluated by testing its ability to inhibit the activities of other aspartyl PRs, namely, pepsin

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TABLE 1. Comparison of PL-100 activity against HIV-1 protease and wild-type virus and cytotoxicity with those of commercial PIs

Inhibitor	K_i ± SD (nM)[a,b]	EC_{50} ± SD (μM)[a,c]	CC_{50} ± SD (μM)[a,d]	SI[e]
PL-100	0.036 ± 0.024	0.016 ± 0.003	37 ± 16	2,313
APV	0.117 ± 0.043	0.051 ± 0.014	>100	>1,961
LPV	0.014 ± 0.010	0.019 ± 0.001	28 ± 8	1,474
ATV	0.039 ± 0.011	0.004 ± 0.001	71 ± 19	17,750
IDV	1.945 ± 0.358	0.067 ± 0.010	>100	>1,493
NFV	0.306 ± 0.081	0.029 ± 0.004	8 ± 2	276
RTV	0.034 ± 0.019	0.061 ± 0.003	26 ± 6	426
SQV	0.080 ± 0.022	0.012 ± 0.002	19 ± 7	1,629

[a] Mean values of at least three experiments. Each determination was done in triplicate.
[b] K_i s were determined by Morrison's tightly binding plot.
[c] EC_{50}s were obtained by MTT assay 6 days after infection of MT-4 cells with the wild-type NL-4.3 strain.
[d] The CC_{50} was obtained by MTT assay of MT-4 cells after 6 days.
[e] SI, selectivity index (SI = C_{50}/EC_{50}).

and cathepsin D. In these assays, PL-100 showed no inhibitory activity (its IC_{50}s were >50 μM for both cathepsin D and pepsin, whereas APV's IC_{50}s were 15.2 μM and 0.28 μM, respectively.

These results demonstrate that PL-100 is a potent and selective competitive HIV-1 PI.

Inhibition of Gag polyprotein processing by PL-100. HIV PR is an essential enzyme for the processing of Gag and Gag-Pol polyproteins into the mature structural proteins and replication enzymes required for viral replication. To determine whether PL-100 effectively blocks the proteolytic processing of HIV-1 p55 polyprotein, H9 cells chronically infected with HIV-1 IIIB were incubated in the presence of various concentrations of PL-100, and the p55 cleavage product p24 was detected by Western blotting. Figure 2 shows that PL-100 effectively prevented the processing of the p55 precursor protein, as demonstrated by a dose-dependent decrease in the amount of p24 product.

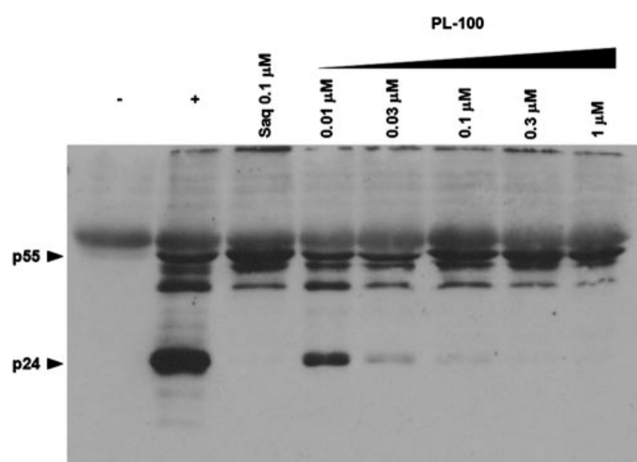

FIG. 2. PL-100 inhibition of the processing of the Gag precursor protein by HIV-1 PR. Chronically infected H9/IIIb cells were cultured for 5 days in the absence (Ctrl+) or presence of various concentrations of PL-100 or 0.1 μM of SQV. On day 5, the pelleted culture supernatants were resuspended in lysis buffer, and equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using an anti-HIV-1 p24 monoclonal antibody.

These results confirm that PL-100 is an effective inhibitor of the HIV-1 PR in infected cells.

In vitro antiviral activity and cytotoxicty of PL-100. The in vitro antiviral activity of PL-100 was tested by a standard MTT cytoprotection assay. PL-100 potently suppressed viral replication of the laboratory-adapted strain NL-4.3 in MT-4 cells with an average EC_{50} of 0.016 ± 0.003 μM and an EC_{90} of 0.023 ± 0.005 μM, while the FDA-approved PIs gave EC_{50}s that ranged between 0.004 and 0.067 μM (Table 1).

Furthermore, the cytotoxicity of PL-100, with a CC_{50} of 37 ± 16 μM and a selectivity index of 2,313, was similar to those of commercially available PIs (Table 1).

In vitro susceptibilities of HIV-1-resistant strains and cross-resistance profile. (i) Phenotypic analysis. Our initial screening strategy involved the evaluation of the antiviral activities of the test compounds against two laboratory-adapted HIV strains containing mutations in the PR gene. Both the SQV-resistant strain, HIV-1_{SaqR} (10), carrying the primary mutations required to confer resistance to SQV (G48V and L90M), and a virus produced from the molecular clone HIV-1_{4596} (4), carrying five mutations (L10R, M46I, L63P, V82T, and I84V) known to confer cross-resistance to several PIs, remained sensitive to PL-100 in the MTT assay, with changes of 2.4- and 1.2-fold, respectively.

The antiviral activity of PL-100 was further evaluated in parallel with a series of commercially available PIs, using a panel of 63 constructs containing PR and RT gene sequences from PI-experienced patients in a replication-defective reporter-gene based phenotypic assay (PhenoSense HIV assay; Monogram Biosciences). The selection criteria for the 63 NL-4.3-based constructs included high-level loss of susceptibility to specific PIs and high-level loss of susceptibility to multiple PIs. The following primary PI mutations were considered in the study: D30N, V32I, L33F, M46I/L, I47A/V, G48V, I50L/V, V82A/F/L/S/T, I84V, N88D/S, and L90M, as defined by the International AIDS Society—USA (12). The numbers of constructs in the panel harboring zero, one, two, three, four, five, or six of these primary PI mutations were 2, 4, 7, 30, 15, 4, and 1, respectively. Hence, the majority of the constructs in this panel harbored three or four primary PI mutations; this emphasizes the strength of the panel. The representation of each primary PI mutation in the panel of 63 constructs tested in the cross-resistance profiling of PL-100 was as follows: 2 constructs

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TABLE 2. Cross-resistance profile of PL-100[a]

Key mutations[b]	Change (n-fold) in EC_{50} vs reference						
	ATV	APV	IDV	LPV	NFV	SQV	PL-100
24I/33F/54V/82A/84V	ND[d]	13.2	10.4	43.4	31.4	85.4	4.2
24I/33F/46I/54L/82A	ND	7.7	3.3	16.9	8.1	1.5	6.4
54V/82A/84V/90M	ND	9.7	29.3	46.5	33.1	125.3	7.0
33F/54V/73S/82A/84V/90M	ND	68.8	32.8	130.1	172.1	400[e]	37.2
33F/46L/54V/82A/84V/90M	ND	20.9	11.4	59.7	31.4	61.4	18.4
46I/82T/84V	ND	2.8	7.2	8.1	1.6	1.0	1.2
46I/82T/84V	ND	5.9	12.9	20.1	5.2	3.2	2.5
46I/82T/84V/90M	ND	4.5	31.4	17.2	39.9	105.7	4.8
33I/46I/84V/88D/90M	ND	8.7	38.5	28.3	71.7	192.8	6.3
84V/90M	ND	9.1	8.1	8.5	12.1	27.0	2.3
33F/50V	ND	36.8	1.3	12.8	3.6	1.7	2.7
48V/54V/82A/90M	ND	3.9	53.4	70.4	68.2	400[e]	1.9
30N/88D/90M	ND	1.8	2.3	1.2	42.1	6	1.4
32I/47A[c]	1.0	19.7	3.8	65.1	3.1	0.2	1.4
46I/47V/84V[c]	7.1	20.1	3.6	19.5	5.1	11.0	4.3
46I/47A/84V	9.5	47.2	7.9	151.8	10.4	1.1	14.8
46I/88S	13.5	0.3	10.3	1.7	28.9	1.9	0.8
46I/88S	3.1	0.2	2.0	0.7	4.6	0.6	0.4
46I/88S	9.9	0.9	11.3	2.6	25.2	2.0	1.1
33F/54L/88S/90M	65.0	5.9	8.0	6.0	41.4	31.7	3.3
33F/54L/88S/90M	18.9	3.3	3.1	3.0	14.5	18.5	1.7
33I/46I/84V/88D/90M	16.4	5.7	26.2	9.5	39.6	133.9	5.6
33F/46I/84V/88D/90M	38.0	21.0	6.7	42.5	27.9	400[e]	8.8
30N/33F/46L/54L/84V/88D	66.1	106.3	9.3	37.9	400[e]	237.3	23.2
32I/46I/47V/50L	81.2	7.9	2.1	2.9	3.5	0.5	1.1
33F/54L/82A/90M	10.7	20.9	6.4	8.9	13.6	4.0	51.5
33F/54L/82A/90M	7.5	13.6	7.5	13.1	10.4	3.2	30.3
33F/54V/73S/82A/90M	11.9	6.4	18.5	52.9	20.0	16.7	5.3
32I/46L/47V/84V	25.0	89.4	21.7	133.9	7.8	0.8	0.6
33F/54L/82A/84V	44.5	54.5	6.2	44.5	17.2	37.6	31.2
33F/54V/82A/84V	5.5	37.9	18.7	119.6	5.8	7.3	3.4
33F/46I/53L/82A/84V	18.3	20.8	6.0	21.1	11.1	23.2	17.8
33F/54V/82T/84V/90M	14.1	5.0	26.8	19.2	14.9	51.0	3.4
33F/46L/53L/54V/82A	22.7	15.3	16.8	65.9	28.3	14.0	5.5
46L/54M/82L/84V/90M	52.3	116.8	42.5	73.7	43.8	50.3	84.0
46L/54V/82A/90M	11.8	9.2	5.9	17.2	14.1	41.5	3.2
46I/54V/82A/84V	5.8	9.9	8.2	45.1	8.8	6.7	3.7
46L/54V/73C/84V/90M	55.2	26.6	93.2	36.9	82.6	400[e]	10.8
46I/82T/84V/90M	32.0	7.6	30.2	15.2	27.1	79.3	10.3
46I/82A/84V/90M	3.8	7.3	7.7	5.7	11.2	9.9	10.1
54V/82A/84V/90M	50.5	38.5	37.4	103.3	36.1	133.9	28.3
33F/54V/82A/84V/90M	24.7	18.6	8.1	33.6	21.1	85.5	9.6
48V/54V/82A/90M	28.0	1.1	23.1	24.4	25.4	400[e]	1.1
46L/48V/82A/84V/90M	91.6	35.6	36.5	44.7	43.6	400[e]	13.4
48V/82A/84V/90M	57.7	24.0	34.0	18.7	27.3	400[e]	13.5
46L/48V/82A/84V	21.5	8.6	7.4	5.4	5.9	36.0	1.2
46L/48V/82A/90M	35.4	8.9	36.1	10.0	30.8	320.7	7.5
46L/50L/54V/82A	68.9	8.3	4.1	10.6	11.8	2.8	3.2
46I/50L/54V/82A	67.5	3.2	7.2	6.8	14.0	2.2	4.0
33I/46I/84V/90M	78.2	10.8	73.7	18.1	165.1	270.8	3.4
33I/46I/84V/90M	7.5	10.8	30.3	5.4	28.5	44.4	3.6
37S/41K/70E	1.4	0.7	1.2	1.0	1.6	1.2	0.9
37T/41K/70E	1.5	1.9	1.4	1.3	1.9	1.2	1.9
46I/53L/82T/90M	33.7	7.3	33.5	17.9	32.9	64.1	8.3
54V/84V	8.3	4.3	5.2	5.4	15.0	12.2	0.8
54V/84V	33.2	14.9	19.5	28.5	32.9	102.1	1.2
54V/82A	3.9	1.4	4.8	10.1	12.1	2.1	1.8
46L	0.9	1.2	0.8	0.8	1.7	0.9	1.1
46I	0.7	10.4	4.6	8.7	1.6	0.8	1.5
46I/50V/54V	2.4	23.5	2.8	41.0	12.7	32.2	0.5
46I/50V/90M	1.2	19.4	4.7	9.8	19.4	14.0	1.4
46I/50V	0.4	12.4	0.7	3.8	1.3	0.4	0.8
33F/46I/84V/88D/90M	14.7	26.3	11.3	81.0	10.0	29.7	5.6

[a] The in vitro susceptibilities of HIV-1 strains to the tested PIs were determined using the PhenoSense HIV assay (Monogram Biosciences). The PIs were ATV, APV, IDV, LPV, NFV, and SQV, in addition to PL-100. Susceptibility data are expressed as the change in the EC_{50} of the patient isolate compared to the reference strain, NL-4.3. The mean EC_{50}s for the tested PIs against the wild-type NL-4.3 reference construct were 5.3 ± 1.8 nM for PL-100, 10.7 ± 2.5 nM for APV, 1.5 ± 0.2 nM for ATV, 7.8 ± 1.1 nM for IDV, 2.8 ± 0.4 nM for LPV, 5.8 ± 0.9 nM for NFV, and 1.8 ± 0.2 nM for SQV.

[b] Key mutations (primary and other important mutations) that were present, unmixed, in each sample are listed, other mutations can be present in the genotype but are not indicated.

[c] Non-B subtype.

[d] ND, not determined.

[e] Fifty percent inhibition was not reached at the highest drug concentration tested; an arbitrary change value (400-fold) was assigned for statistical calculation purposes.

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TABLE 3. Summary of phenotypic susceptibility results[a]

PI	Change (n-fold) in EC_{50} vs reference				% with change of:			Total no. of isolates	% of isolates with lowest change[b]
	Median	Mean	Minimum	Maximum	>2.5-fold	>10-fold	>50-fold		
ATV	15.6	25.7	0.4	91.6	84	62	22	50	6
APV	9.7	18.5	0.2	116.8	85.7	47.6	7.9	63	13
IDV	8.1	16.5	0.7	93.2	87.3	46	4.8	63	19
LPV	17.9	31.3	0.7	151.8	90.5	63.5	19	63	3
NFV	15	31.7	1.3	400[c]	90.5	73	9.5	63	0
SQV	23.2	85	0.2	400[c]	73	60.3	36.5	63	17
PL-100	3.6	8.7	0.4	84	63.5	23.8	3.2	63	46

[a] The in vitro susceptibilities of HIV-1 strains to the tested PIs were determined using the PhenoSense HIV assay (Monogram Biosciences). The PIs were ATV, APV, IDV, LPV, NFV, and SQV, in addition to PL-100. All PIs were tested against 63 isolates, except ATV, which was tested against 50.
[b] The percentage of the total number of isolates that gave the lowest change value for each PI tested.
[c] See Table 2 footnote *e*.

with D30N, 3 with V32I, 21 with L33F, 38 with M46I/L, 5 with I47A/V, 6 with G48V, 7 with I50L/V, 33 with V82A/F/L/S/T, 35 with I84V, 11 with N88D/S, and 31 with L90M. Mixed genotypes in the samples were not considered. The mean EC_{50} of PL-100 in this assay was 5.3 ± 1.8 nM against the wild-type NL-4.3 reference construct compared to 10.7 ± 2.5, 1.5 ± 0.2, 7.8 ± 1.1, 2.8 ± 0.4, 5.8 ± 0.9, and 1.8 ± 0.2 nM for APV, ATV, IDV, LPV, NFV, and SQV, respectively. The PI susceptibility of each patient-derived resistance test vector was compared to that of the wild-type NL-4.3 reference construct; the results were expressed as the change in EC_{50} (n-fold) in relation to the reference (Tables 2 and 3). For this analysis, samples were considered to be susceptible to an inhibitor when the change in the EC_{50} was below 2.5-fold, arbitrarily chosen as a threshold for susceptibility pending clinical analysis.

As expected from the genotypes in the panel, there was broad cross-resistance among the approved PIs tested (Table 3), with a range in the median change of 8.1- to 23.2-fold. Between 73% and 91% of the viruses had >2.5-fold-reduced susceptibility to the approved PIs, and 46% to 73% had changes of >10-fold. Overall (Table 3), PL-100 showed the lowest median change (3.6- versus 8.1- to 23.2-fold for the other PIs tested) and the lowest mean change (8.7- versus 16.-5 to 85-fold for the other PIs tested).

The number of constructs with changes in EC_{50} to PL-100 of <2.5-fold was also the highest (36% with changes of <2.5-fold versus 9% to 27% for the other PIs). We also observed that only 3% of the tested strains showed a change above 50-fold for PL-100 compared to 5% to 37% for the other PIs. Notably, PL-100 was the PI with the lowest change in EC_{50} in 46% of the samples; IDV was the PI with the next-lowest change in 19% of the samples (Table 3).

The 63 constructs from the panel were clustered into six groups on the basis of the number of primary PI mutations in their PR sequences, and the median changes for each PI were compared (Table 4). As expected, the level of PI resistance generally increased as the number of primary PI mutations accumulated in the constructs. Strains with zero or one mutation generally gave lower changes, but four of the tested PIs still gave a change above 2.5-fold (Table 4) against constructs with only one PI mutation; the changes for PL-100 and SQV for all constructs with zero to two PI mutations were below 2.5-fold. Furthermore, the median change for PL-100 was the lowest in all the groups of samples bearing one to four primary PI mutations.

The 63 constructs of the panel were also clustered into seven groups on the basis of the number of tested PIs with changes of >2.5-fold (Table 5). Thus, looking at the increase in the cross-resistances of the constructs, PL-100 showed a median change below 2.5-fold in groups in which zero to five of the other six PIs were above that threshold. Even among samples with changes of >2.5-fold for six or seven PIs tested, the median PL-100 change remained below those of other drugs. These data clearly indicate that PL-100 has a favorable cross-resistance profile.

It is often informative to compare patterns of cross-resistance between a novel inhibitor, such as PL-100, and approved PIs to determine whether novel inhibitors truly have distinct profiles. This was performed using linear regression of log-transformed change values, as shown in Fig. 3. The best cor-

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TABLE 4. Antiviral activities of PL-100 against isolates with increasing numbers of primary PI mutations[a]

No. of primary PI mutations (no. of strains)	Median change (n-fold) in EC_{50}						
	ATV	APV	IDV	LPV	NFV	SQV	PL-100
0 (2)	1.5	1.3	1.3	1.2	1.8	1.2	1.4
1 (4)	2.4	**5.9**	**4.7**	**9.4**	**6.9**	1.5	1.4
2 (7)	**3.1**	**9.1**	**3.8**	**8.5**	**12.1**	1.9	1.1
3 (30)	**11.9**	**9.8**	**8.0**	**18.0**	**16.1**	**14.0**	**3.7**
4 (15)	**24.9**	**8.7**	**26.8**	**18.7**	**27.1**	**79.3**	**7.5**
5–6 (5)	**52.1**	**26.3**	**11.3**	**44.7**	**31.4**	**237.3**	**13.4**

[a] The 63 isolates forming the panel were clustered into six groups on the basis of the numbers of primary PI mutations in their protease sequences, and the median change to each PI within each group was calculated. Median change values over 2.5-fold are in boldface.

TABLE 5. Antiviral activities of PL-100 against multi-PI-resistant isolates[a]

No. of PIs to which a construct showed resistance[b]	Median change (n-fold) in EC_{50}						
	ATV	APV	IDV	NFV	SQV	LPV	PL-100
0	1.4	1.2	1.2	1.7	1.2	1.0	1.1
2	1.8	1.8	2.0	**4.6**	0.6	1.2	0.8
3	**7.1**	**2.8**	**7.2**	1.6	1.0	**8.1**	1.2
4	**6.9**	**7.9**	**3.8**	**3.6**	1.7	**10.1**	1.4
5	**13.7**	**8.4**	**10.5**	**12.4**	**20.5**	**22.3**	1.7
6	**20.2**	**9.2**	**10.9**	**31.4**	**73.4**	**28.4**	**4.5**
7	**24.7**	**15.3**	**16.8**	**27.1**	**44.4**	**19.5**	**8.3**

[a] The sixty-three isolates forming the panel were clustered into seven groups on the basis of their cross-resistances, i.e., the numbers of FDA-approved PIs with changes of >2.5-fold. Median change values over 2.5-fold are in boldface.
[b] With a change in the EC_{50} of >2.5-fold. Increased cross-resistance is shown by increasing numbers from top to bottom.

relations between PL-100 and an approved PI were those with APV ($R^2 = 0.37$) and LPV ($R^2 = 0.27$). Weaker but significant correlations were also observed with ATV, NFV, IDV, and SQV (26, 28). Thus, PL-100's cross-resistance profile is unique, and this is probably attributable to its distinct chemical structure.

(ii) Genotypic analysis. An additional analysis focused on key mutations for approved PIs. We selected the samples from

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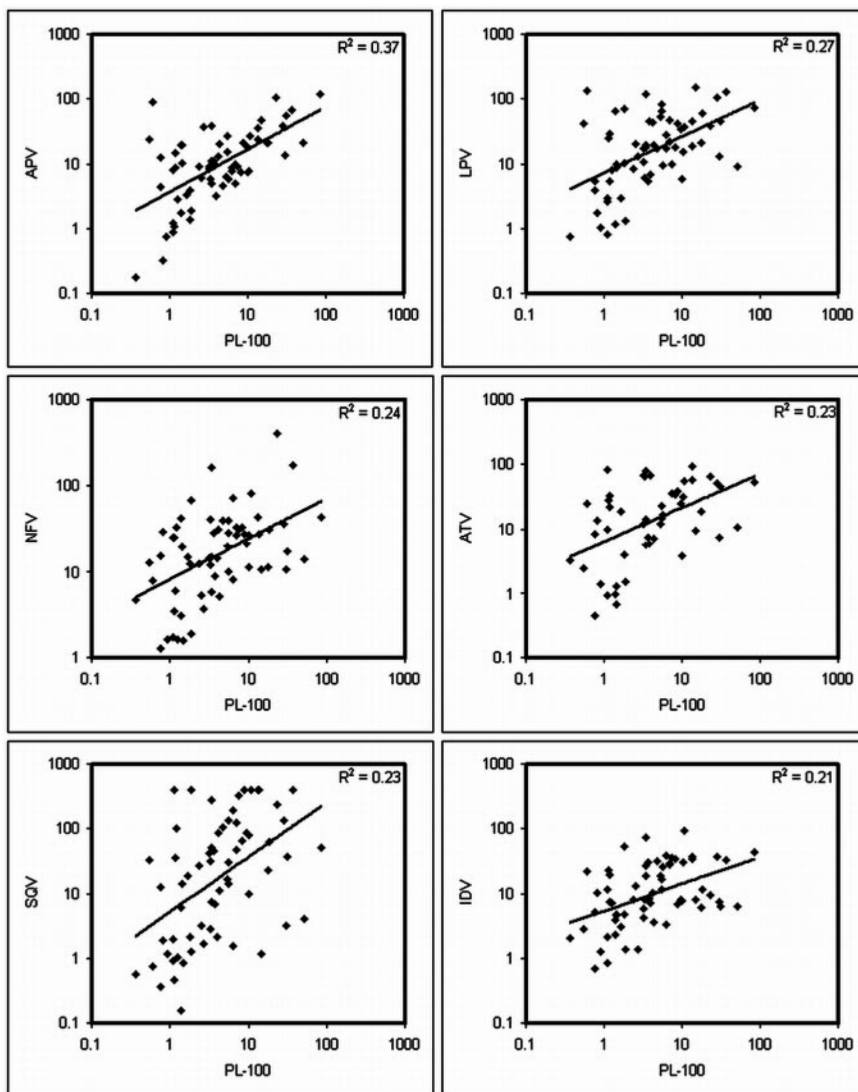

FIG. 3. Comparison of the cross-resistance profile of PL-100 with those of other PIs. The pattern of resistance of each PI tested was compared to that of PL-100 by linear regression analysis of log-transformed change values. The correlation factor R^2 is indicated for each pair.

the panel of 63 depending on the presence of only primary/signature mutations for each PI tested, as defined by the International AIDS Society—USA (12).

Thirty-four strains harbored the I50L, I84V, and/or N88S signature mutation associated with resistance to ATV (12). The median changes were 23.1-fold (range, 3.1- to 81.2-fold) and 3.9-fold (range, 0.4- to 84.0-fold) for ATV and PL-100, respectively (Table 4).

Thirty-nine isolates bore the APV resistance-conferring primary mutations I50V and/or I84V. The median changes were 18.6-fold (range, 2.8- to 16.8-fold) and 4.8-fold (range, 0.5- to 37.2-fold) for APV and PL-100, respectively.

The IDV primary PI mutations M46I/L, V82A/F/T, and/or I84V were present in 56 of the 63 isolates. The median changes were 10.4-fold (range, 0.7- to 93.2-fold) and 4.3-fold (range, 0.4- to 51.5-fold) for IDV and PL-100, respectively.

The LPV primary mutations V32I, I47A/V, and/or V82A/F/S/T were present in 37 of the isolates. The median changes were 20.1-fold (range, 2.9- to 151.8-fold) and 5.3-fold (range, 0.6- to 51.5-fold) for LPV and PL-100, respectively.

D30N and L90M are the signature mutations for resistance to NFV. Thirty-two strains harboring at least one of these two mutations were analyzed. The median changes were 31.1-fold (range, 10.0- to 400.0-fold) and 7.3-fold (range, 1.1- to 84.0-fold) for NFV and PL-100, respectively.

The last head-to-head comparison was between SQV and PL-100. A total of 32 isolates harbored at least one of the two SQV signature mutations, G48V and L90M. The median changes were 71.7-fold (range, 4.0- to 400.0-fold) and 6.7-fold (range, 1.1- to 84.0-fold) for SQV and PL-100, respectively.

In all cases, both the median change and the average change (data not shown) observed with PL-100 were the lowest of all PIs in these genotype-based comparisons.

At the time that this cross-resistance study was performed, the recently approved PIs TPV and DRV were not available for direct comparisons with PL-100.

DISCUSSION

This study describes the antiviral activity of a novel potent, noncytoxic, and selective HIV-1 PI, PL-100. The results from cross-resistance experiments highlight the excellent in vitro antiviral activity of PL-100 against viral isolates resistant to numerous other PIs. The complexity of the genotypes included in the panel, despite the relatively small number of isolates tested, indicates that PL-100 has superlative potential for activity against existing PI-resistant viruses commonly found in treatment-experienced patients.

Recently, lower and upper clinical cutoffs (shown in parentheses) have been defined for the following ritonavir-boosted (/r) PIs: APV/r (4 and 11.5), ATV/r (5.2 and 20), LPV/r (9 and 55), SQV/r (2.3 and 12), and IDV/r (10 and 50) (Monogram Biosciences). No clinical cutoff was defined for NFV/r, since it is not currently used in salvage therapy. The observed median changes for the commercial PIs in this cross-resistance study (Table 5) show that the panel used is clinically relevant, since the values obtained are above the lower clinical cutoffs of most boosted PIs and are above the upper clinical cutoffs of some of them. The only exception was IDV, for which the observed median change was slightly below the lower clinical cutoff of IDV/r (Table 5). Despite the impossibility of determining a clinical cutoff for PL-100, at this time the phenotypic susceptibilities to PL-100 of resistant viral strains in the panel are testimony to the potential clinical utility of PL-100.

Ultimately, the determination of a clinical cutoff for PL-100 will allow a better correlation between these in vitro susceptibility data and virological responses in patients. We are currently attempting to generate tissue culture-selected variants of HIV-1 that are resistant to PL-100 in order to shed light on mutational pathways that may be identified in the clinic.

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