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Susceptibility to PNU-140690 (Tipranavir) of Human Immunodeficiency Virus Type 1 Isolates Derived from Patients with Multidrug Resistance to Other Protease Inhibitors

STEFANO RUSCONI,* SIMONA LA SETA CATAMANCIO, PAOLA CITTERIO, SEMIR KURTAGIC, MICHELA VIOLIN, CLAUDIA BALOTTA, MAURO MORONI, MASSIMO GALLI, AND ANTONELLA D'ARMINIO-MONFORTE

Istituto di Malattie Infettive e Tropicali, Universitá di Milano, Ospedale Luigi Sacco, Milan, Italy

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In our study we examined the anti-human immunodeficiency virus type 1 (anti-HIV-1) activity of a novel HIV-1 protease inhibitor, PNU-140690 (tipranavir), against patient-derived isolates resistant to multiple other protease inhibitors (PIs). The aim of our experiments was to investigate the genotypes and the in vitro phenotypes of drug resistance of PNU-140690. We carried out drug susceptibility tests with peripheral blood mononuclear cells and a fixed amount of infectious virus (1,000 50% tissue culture infective doses) to determine the 50% inhibitory concentration (IC_{50}) and IC_{90}, PCR assays for the detection of drug resistance mutations in RNA in plasma, and direct sequencing of PCR products. Phenotypic resistance to PIs was invariably related to genotypic mutations. The substitutions among the amino acid residues of the protease included L10I, K20R, L24I, M36I, N37D, G48V, I54V, L63P, I64V, A71V, V77I, V82A, I84V, and L90M. Isolates from all of the patients had developed a maximal degree of resistance to indinavir, ritonavir, and nelfinavir (IC_{50}s, >0.1 μM). We also compared these mutations with the amino acid changes previously described in association with in vivo tipranavir administration. The mutations included the following: I15V, E35D, N37D, R41K, D60E, and A71T. Infections with IIIB, 14aPre, and N70 were inhibited by an average drug IC_{90} of 0.18 ± 0.02 μM in multiple experiments. The average mean ± standard error of mean IC_{90} for the entire group of multidrug-resistant isolates derived from the mean values for two culture wells with p24 antigen supernatant appeared to be 0.619 ± 0.055 μM (range, 0.31 to 0.86 μM). Tipranavir retained a sustained antiviral activity against PI-MDR clinical isolates and might be useful in combination regimens with other antiretroviral agents for patients who have already failed other PI-containing therapies.

Protease inhibitors (PIs) have significantly changed the prognosis of human immunodeficiency virus (HIV) type 1 (HIV-1) infection. Their use is, however, associated with the appearance of a pattern of many mutations in the protease gene which favor the development of resistance to multiple other compounds within the same class of drugs (for reviews, see references 1 and 6). Condra and colleagues (4) gave the first virological demonstration of cross-resistance to PIs among patients subjected to indinavir (IDV) monotherapy. Together with the progressive accumulation of mutations selected by IDV monotherapy, these patients harbored viruses that became resistant to other PIs, such as saquinavir (SQV) and amprenavir (VX-478 or 141W94) (4). The follow-up to that study evidenced that the extent of IDV resistance and the cross-resistance to other PIs depended upon the single amino acid substitutions, their numbers, and the combination in which they appeared (5). Ritonavir (RTV) had also been described as potentially inducing a large extent of unique resistance or cross-resistance within the PI class (17).

The complex picture of the emergence of cross-resistance to PIs was reported in several studies performed both in vitro (12, 20) and in vivo (13, 16; V. Miller, K. Hertogs, M.-P. de Bethune, R. Pauwels, T. Ivens, H. Azijn, C. Schlecht, B. Nolde, M. Sturmer, B. Morgenstern, M. Kortenbusch, and S. Staszewski, Abstr. Int. Workshop HIV Drug Resistance, Treatment Strategies Eradication, abstr. 82, p. 75–76, 1997). Additional data on PIs-related resistance following sequential treatment with several compounds within this class of drugs are becoming available. Winters and colleagues (21) have recently reported that an initial treatment with SQV may cause a full development of cross-resistance to IDV and nelfinavir (NFV) in patients who receive such regimens as part of an active anti-HIV-1 combination therapy. Duliousti and colleagues (9) observed that previous therapy with SQV followed by therapy with IDV allowed a viral evolution that maintained the initial selection of drug resistance and adapted the viral population to the IDV pressure. These results suggest that HIV-1 quasispecies would likely adapt to the presently available drugs which target the same enzyme.

Taking into account the rapid occurrence of PI cross-resistance, clinicians who are treating patients with HIV-1 infection will need new active PIs in the near future.

In the present study we examined the in vitro anti-HIV-1 activity of a novel HIV-1 PI, PNU-140690 (tipranavir) (15), which retained potent activity against RTV-resistant clinical isolates (2). We used isolates from patients with resistance to multiple other PIs: SQV and/or NFV, in addition to IDV and RTV. We have analyzed the genotypic pattern and the phenotypic drug resistance of the new compound in vitro.

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* Corresponding author. Mailing address: Istituto di Malattie Infettive e Tropicali, Universitá di Milano, Ospedale Luigi Sacco, Via GB Grassi 74, 20157 Milano, Italy. Phone: 39.02.35799676. Fax: 39.02.3560805. E-mail: rusconi@mailserver.unimi.it.

TABLE 1. Characteristics of patients in study cohort[a]

Clinical isolate	Date of isolation (day-mo-yr)	Ongoing treatment	No. of CD4 cells/μl	HIV-1 RNA load (no. of copies/ml)	Previous treatments	Time (mo) on PI therapy
001	11-11-1997	ZDV + ddI + IDV	562	10,240	ZDV, ddI	16
002	3-10-1997	d4T + ddI + IDV	508	11,620	ZDV, ddC, 3TC	11
003	5-12-1997	d4T + 3TC + RTV + SQV	28	36,170	ZDV, ddC, IDV	20
004	21-11-1997	d4T + 3TC + ddC + RTV + SQV	407	383,700	ZDV, ddC, RTV	29
005	25-07-1998	ddI + d4T + NVP + NFV	1,210	100,000	ZDV, ddI, 3TC, IDV, RTV	23
006	25-02-1997	d4T + ddI + RTV	20	77,500	ZDV, ddC	7
007	1-02-1997	ZDV + 3TC + IDV	175	490,000	ddI, DLV	0
008	1-01-1998	d4T + ddI + IDV	227	660	ZDV, 3TC, ddI, DLV	11
009	10-06-1998	d4T + SQV + NFV	410	140,000	ZDV, ddI, 3TC, IDV, RTV	25
010	17-10-1997	d4T + 3TC + IDV	224	184,800	ZDV, ddC, RTV, SQV	15

[a] Abbreviations: ZDV, zidovudine; ddI, didanosine; d4T, stavudine; 3TC, lamivudine; ddC, zalcitabine; NVP, nevirapine; DLV, delavirdine.

MATERIALS AND METHODS

Sampling and analytical protocol. We studied a number of PI-experienced subjects who had failed their current treatment (defined as a 1 $\log_{10}$ increase in viral load with or without clinical worsening or a decrease in the $CD4^+$ cell count of ≥20%) and for whom genotypic characterization of the HIV-1 protease indicated multiple amino acid substitutions. Then, we conducted the phenotypic test before the beginning of the PI treatment and at the time of the sequencing, and at this point susceptibility to PNU-140690 was determined.

Cells. Peripheral blood mononuclear cells (PBMCs) from HIV-1-seronegative donors were obtained by Ficoll-Hypaque density-gradient centrifugation of heparinized venous blood. The PBMCs were treated with phytohemagglutinin (PHA-P; 2 μg/ml; Difco Laboratories, Detroit, Mich.), propagated in R-20 medium (RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum [Sigma Chemical, St. Louis, Mo.], 50 U of penicillin per ml, 50 μg of streptomycin per ml, 2 mM L-glutamine, and 10 mM HEPES buffer) supplemented with 10% interleukin-2 (Human T-Stim; Collaborative Research Inc., Bedford, Mass.), and incubated at 37°C in 5% CO_2. MT-2 cells, a lymphoblastoid cell line, were provided by R. C. Gallo (Institute of Human Virology, Baltimore, Md.) and were maintained in R-10 medium (which is identical to R-20 medium except that it contains only 10% heat-inactivated fetal calf serum).

Viruses. We evaluated several drug-resistant isolates derived from patients who were undergoing anti-HIV-1 combination therapy. Different clinical isolates and laboratory-adapted strains of HIV-1 were used as controls. 14aPre was derived from an HIV-1-seropositive individual before any therapy (Massachusetts General Hospital, Boston, Mass.); N70 was an isolate derived from a clinically asymptomatic patient and was a gift from D. D. Ho (Aaron Diamond AIDS Research Center, New York, N.Y.). The IIIB prototype strain of lymphotropic HIV-1 was obtained from R. C. Gallo (Institute of Human Virology). Viral titers ranged from 2×10^4 to 4.5×10^4 50% tissue culture infective doses ($TCID_{50}$s)/10^6 PBMCs for the new clinical isolates and from 1.5×10^5 to 3×10^5 $TCID_{50}$s/10^6 million PBMCs for the control isolates.

Compounds. The sulfonamide-containing nonpeptidic PI PNU-140690 was provided by Pharmacia-Upjohn (Kalamazoo, Mich.). IDV was obtained from Merck Research Laboratories (West Point, Pa.). RTV was obtained from Abbott Laboratories (Abbott Park, Ill.). NFV was obtained from Agouron Pharmaceuticals, Inc. (La Jolla, Calif.).

Drug susceptibility test. The concentrations of PNU-140690 that inhibited the viruses were evaluated in PBMCs. In all the experiments, uninfected cell controls were maintained for determination of drug toxicity. Cell proliferation and viability were assessed by the trypan blue dye exclusion method. Virus replication were measured in cell-free culture supernatants by an HIV-1 p24 antigen enzyme-linked immunosorbent assay (DuPont-NEN Research Products, Boston, Mass.). We maintained virus without cells or drug for the entire duration of the experiments in order to take into account the viral carryover.

To determine virus-inhibitory concentrations in PBMCs, uninfected 3-day PHA-P-stimulated PBMCs (1.5×10^6 cells) were resuspended in 1.5 ml (final volume) of R-20–interleukin-2 medium in 24-well plates (Costar, Cambridge, Mass.). Drug susceptibilities were determined with a fixed amount of infectious virus (1,000 $TCID_{50}$s). An equal viral input, as calculated by the method of Reed and Muench (8), was used to infect 10^6 PHA-P-stimulated PBMCs that were drug-free (control wells) or that had been pretreated with five drug concentrations in duplicate wells. PNU-140690 concentrations ranging from 0.05 to 0.8 μM were derived starting from a working solution of 10 mM in dimethyl sulfoxide. Drugs and viruses were simultaneously added to the culture. On day 4, the cells were resuspended and passaged at a 1:3 ratio in fresh R-20–interleukin-2 medium containing drugs at their original concentrations. On days 4 and 7, cell-free supernatants were harvested for HIV-1 p24 antigen production, and cell counts were determined. The MT-2 test for determination of the capacity for syncytium formation was performed according to the recommendations of the AIDS Clinical Trials Group (11).

The 50% inhibitory concentrations (IC_{50}s) and the subsequent IC_{75}s, IC_{90}s, IC_{95}s, and IC_{99}s were determined by the dose-effect analysis of Chou and Talalay (3) with Systat computer software program for Macintosh, version 5.1 (Evanston, Ill.).

PCR assay for drug resistance mutations in RNA. Plasma RNA was extracted with a nucleic acid extraction kit (QUIAamp Viral RNA kit; Quiagen, Inc., Chatsworth, Calif.) according to the manufacturer's recommendations. The *pol* gene was reverse transcribed with the antisense primer P8 (7). RNA was denatured together with the P8 antisense primer at 70°C for 5 min. The reaction mixture (30 μl) contained 1× reaction buffer, 0.5 mM each deoxynucleoside triphosphate, (dNTP), 1.6 μM P8 primer, 30 U of RNAsin, and Moloney murine leukemia virus reverse transcriptase (200 U). The reverse transcription reaction started with 42°C for 60 min, followed by 95°C for 5 min. The HIV-1 protease-coding sequences were amplified by a PCR assay. The reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 8.3), 25 mM KCl, 1 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.), and 25 pmol of primers P8 and P7 (7). The reaction was initiated with 1 cycle at 95°C for 9 min and then with 35 cycles at 94°C for 30 s, 68°C for 1 min, and 72°C for 2 min, followed by incubation at 72°C for 6 min. The second amplification was performed with 10 μl of the first amplification product. The reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 8.3), 25 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer), and 25 pmol of primers Pro-F (5′-TGTAAAACGACGGCCAGTACAACAACTCCCTCTCA-3′) and Pro-R (5′-CAGGAAACAGCTATGACCAATGGCCATTGTTTAAC-3′) (the M13 sequence is underlined). The conditions that we used were 95°C for 9 min for 1 cycle, then 94°C for 50 s, 45°C for 50 s, and 72°C for 2 min repeated for 2 cycles, and then 35 cycles at 94°C for 30 s, 55°C for 50 s, and 72°C for 2 min, followed by incubation at 72°C for 10 min. The final PCR products were analyzed on a 2% agarose gel.

Sequencing procedures. Unincorporated primers and nucleotides were removed with the QUIAQUICK Gel Extraction Kit (Quiagen, Inc.), and the PCR products were sequenced directly by using dye-labeled M13 primers and ABI sequencing kit reagents (Applied Biosystems, Inc., Foster City, Calif.) in the presence of 10% glycerol. An automatic DNA sequencer (373A; Applied Biosystems, Inc.) was used. The sequence pattern was confirmed by sequencing both the positive and negative strands and was derived from two independent sequencing reactions. They were aligned with the HIV-1 IIIB consensus sequence and were analyzed by using the Sequence Navigator software program, version 1.0.1 (Applied Biosystems, Inc.).

Nucleotide sequence accession numbers. All sequences have been submitted to GenBank (Bethesda, Md.) and have been given accession nos. AF154954 to AF154963.

RESULTS

Baseline characteristics. Eight isolates were obtained from patients who received IDV, RTV, or SQV (mean ± standard error of the mean [SEM] treatment duration, 18.25 ± 2.61 months) and were demonstrated to be both IDV and RTV resistant. These subjects had failed antiviral treatment, defined as a ≥1 $\log_{10}$ increase in viral load with or without clinical worsening or a decreasing $CD4^+$ cell count of ≥20%. Two isolates were obtained from a single patient with a previous exposure to NRTIs and NNRTIs at the beginning and the end of PI treatment (11 months of therapy). A summary of the patients' features, including the CD4 counts (range, 28 to 1,210/μl) and HIV-1 loads (range, 660 to 490,000 copies/ml), is shown in Table 1.

Genotypic and phenotypic profiles related to previous PI treatment. A total of 10 primary HIV-1 isolates obtained from

TABLE 2. Genotypic and phenotypic characteristics of clinical isolates resistant to IDV and NFV[a]

Clinical isolate	IDV and RTV IC_{50} (μM) at baseline (before PI treatment)	IDV, RTV, and NFV IC_{50} (μM) after PI treatment	Protease genotype after PI treatment
002	0.001 ± 0.0002 (0.001 ± 0.0003)[b]	>0.1	K20R, L24L/I, M36I, M46M/L, L63P, V82A
003	0.01 ± 0.0005 (0.001 ± 0.0001)	>0.1	L10L/I, K20R, M36I, I54V, L63P, A71I/V, V82A, I84I/V, L90M
006	0.001 ± 0.0001 (0.001 ± 0.0004)	>0.1	Fragment not amplified
001	0.001 ± 0.0004 (0.001 ± 0.0006)	>0.1	L10I, L63S, V77I
010	0.001 ± 0.0002 (0.001 ± 0.0005)	>0.1	L10I, G48V, I54V, L63P/S, V77I, V82A, L90M
004	0.001 ± 0.0004 (0.001 ± 0.0006)	>0.1	L10I, L24I, M36I, M46I, I54V, L63C, A71V/A, V82A, I84V
007	>0.1 (0.001 ± 0.0002)	Not applicable	L10L/I, N37D, L63P
008	Not applicable	>0.1	L10I, M36I, N37D, G48V, I54V, L63P, A71V, V82A, L90M
005	0.01 ± 0.0009 (0.001 ± 0.0002)	>0.1	L10I, K20R, M36I/M, I54V, L63Q, I64I/V, A71V, V82A, L90M
009	0.01 ± 0.0007 (0.001 ± 0.0005)	>0.1	L10I, K20R, M36I, F53F/L, I54V, L63L/P, I64V, V82A

[a] Results are expressed as means ± SEMs; three independent experiments were performed to derive the mean.
[b] Values in parentheses are IC_{50}s of NFV (in micromolar).

nine patients treated with SQV, RTV, or IDV were genotypically sequenced and evaluated for their sensitivities to IDV, RTV, and NFV (Table 2). The results showed that all the isolates had a reduced sensitivity with a maximal degree of resistance (IC_{50}, >0.1 μM) to IDV, RTV, and NFV when they were tested. Four isolates come from patients who had received only IDV, two were from patients who had received combination therapy with SQV and RTV, one was from a patient who had received combination therapy with SQV and NFV, one was from a patient who had received NFV alone, and one was from a patient who had received RTV alone. Patients 003, 005, 009, and 010 received sequential therapy with different PIs, without achieving control of their viremia or the maintainance of a stable $CD4^+$ cell count. All the resistant strains had at least three mutations, which were localized either at the active site of the enzyme or outside the known binding cleft.

Phenotypic resistance to PIs was invariably related to genotypic mutations, as previously pointed out by Condra (6). The substitutions among the amino acid residues of the protease gene included L10I, K20R, L24I, M36I, N37D, G48V, I54V, L63P, I64V, A71V, V77I, V82A, I84V, and L90M. Among the viral isolates described in this study, four expressed nine mutations, one expressed eight mutations, one expressed seven mutations, one expressed six mutations, and two expressed three mutations (one protease fragment was not amplified).

Prevalence of mutations described in association with PNU-140690 treatment. Following the recent report of genotypic resistance to PNU-140690 in a phase II clinical trial by Wang and colleagues (Y. Wang, W. W. Freimuth, C. L. Daenzer, M. T. Borin, C. M. Tutton, A. A. Piergies, R. M. Wurtz, H. I. Li, J. W. Davis, D. J. Crampton, and the PNU-140690 Team, Abstr. 2nd Int. Workshop on HIV Drug Resistance and Treatment Strategies, abstr. 5, p. 5, 1998), we analyzed in our clinical isolates the prevalence of the amino acid changes whose occurrence was previously described during in vivo PNU-140690 administration. The mutations included the following: I15V, E35D, N37D, R41K, D60E, and A71T. Virus from one single patient showed multiple substitutions at positions E35, N37, and D60 on both occasions (pre- and posttherapy [007 and 008, respectively]), and viruses from the other subjects exhibited variable substitution patterns which included various mixtures of mutations at selected codons. Viruses from two patients (patients EB and SU) did not show any PNU-140690-related change. The highest prevalence was represented by the E35D amino acid substitution (60%), followed by the D60E and N37D (30%) and the R41K (20%) changes.

PNU-140690 phenotypic susceptibility. PNU-140690 suppressed the replication of either laboratory and clinical viral strains. Infections with laboratory-adapted viral strains such as IIIB, 14aPre, and N70 were inhibited by the drug, with an average IC_{90} of 0.18 ± 0.02 μM in repeat experiments. The IC_{50}s and the IC_{90}s for the multidrug-resistant clinical isolates are shown in Table 3. The PNU-140690 IC_{50}s ranged from 0.046 to 0.383 μM, and a dose-response to the drug was shown for all the clinical isolates. The average mean ± SEM IC_{90} for the entire cohort of multidrug-resistant isolates from the patients was derived from the mean values for two culture wells with p24 antigen supernatant and appeared to be 0.619 ± 0.055 μM (range, 0.31 to 0.86 μM). We did not observe any characteristic pattern in the phenotypic susceptibilities compared to the genotypic resistance profile.

DISCUSSION

The consecutive use of different PIs, which can also be due to the different times of approval of PIs for clinical use, can be the most frequent reason for the development of resistance and the consequent therapeutic failure and has frequently occurred in HIV-1-infected patients (10, 18, 19, 21). The in vivo pharmacological pressure of a PI-containing regimen may theoretically be able to influence the in vitro susceptibility to other PIs; in fact our multidrug-resistant isolates presented with a common background of marked resistance to PIs. This phenomenon was seen in our isolates, in which a cross-resistant genotypic pattern of drug cross-resistance was present at the

TABLE 3. Phenotypic susceptibilities to PNU-140690 of clinical isolates

Clinical isolate	IC_{50} (μM)	IC_{90} (μM)
006	0.191 ± 0.01	0.860 ± 0.04
003	0.280 ± 0.02	0.560 ± 0.05
007	0.046 ± 0.01	0.310 ± 0.02
008	0.097 ± 0.04	0.650 ± 0.01
004	0.315 ± 0.01	0.740 ± 0.07
010	0.103 ± 0.07	0.340 ± 0.02
001	0.355 ± 0.05	0.670 ± 0.01
002	0.363 ± 0.03	0.660 ± 0.08
009	0.301 ± 0.09	0.668 ± 0.04
005	0.383 ± 0.05	0.730 ± 0.07

[a] Results are expressed as means ± SEMs; three independent experiments were performed to derive the mean.

baseline for IDV, RTV, and NFV but not for PNU-140690. Of note, it has been reported that treatment with a second PI selected for the emergence of HIV-1 strains with reduced sensitivity to more than one PI (9). The results indicated that initial therapy with one PI might induce genotypic pressure, with the emergence of further mutations in the protease-coding region following the exposure to other compounds within the same class.

The reports by Winters et al. (21) and Dulioust et al. (9) underlined the fact that the emergence of cross-resistance to PIs in vivo is a dynamic process that cannot be adequately predicted by standard phenotypic and genotypic assays (J. H. Condra, D. J. Holder, D. J. Graham, M. Shivaprakash, D. T. Laird, W. A. Schleif, J. A. Chodakewitz, and E. A. Emini, Abstr. Int. Workshop HIV Drug Resistance, Treatment Strategies and Eradication, abstr. 47, p. 48–49, 1997). Nevertheless, it is widely recommended that the antivirologic efficacy of a combination regimen be tested before its eventual replacement and, more appropriately, prior to the introduction of a new therapeutic agent, e.g., PNU-140690.

We performed sequence analysis of the multidrug-resistant isolates recovered from plasma samples during PI therapy and before the in vitro PNU-140690 susceptibility tests and found various patterns of resistance. These amino acid substitutions may be selected by PNU-140690 administration (week 12 data from a phase II trial; Y. Wang, personal communication). The substitutions and their prevalences evidenced in our samples (I15V, 1 of 10 isolates; E35D, 6 of 10 isolates; N37D, 3 of 10 isolates; R41K, 2 of 10 isolates; D60E, 3 of 10 isolates; and A71T, 1 of 10 isolates) did not correlate with the phenotypic susceptibility to PNU-140690. Among the patients who participated in the phase II protocol mentioned above, only D60E A71T, and V77G were not seen at the baseline but were seen at week 12. The mutations reported by Wang and us have not been studied by in situ mutagenesis, and their role in determining PNU-140690 resistance is not yet clear. PNU-140690 retained activity against our clinical isolates that harbored those mutations. As for other PIs (4, 12, 14), resistance to PNU-140690 might require multiple amino acid subsitutions and specific codon changes that could reduce its proteolytic activity. These genotypic characteristics have not yet emerged from the currently available in vivo resistance data.

Despite the variety of PNU-140690 IC_{90}s, PNU-140690 concentrations achieved a constant reduction of viral replication under the culture condition used for the PI-MDR isolates examined in this study. In contrast, the antiviral effects of IDV, RTV, and NFV, when tested, had been suppressed, allowing viral replication, despite the presence of high drug concentrations capable of limiting infection with PI-sensitive isolates and reference HIV-1 strains.

In conclusion, from the results of our study, the possibility has emerged that PI-MDR isolates can be treated with PNU-140690, most likely in combination with other RT inhibitors and possibly with other PIs, especially after taking into consideration the therapeutic approach of combining PNU-140690 with RTV as treatment against RTV-resistant isolates (2), as recently reported. The efficacy of a potent PI like PNU-140690 as part of combination regimens against HIV-1 warrants further in vitro studies and in vivo confirmation in clinical trials.

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