TMC310911, a Novel Human Immunodeficiency Virus Type 1 Protease Inhibitor, Shows *In Vitro* an Improved Resistance Profile and Higher Genetic Barrier to Resistance Compared with Current Protease Inhibitors[▽][†]

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TMC310911 is a novel human immunodeficiency virus type 1 (HIV-1) protease inhibitor (PI) structurally closely related to darunavir (DRV) but with improved virological characteristics. TMC310911 has potent activity against wild-type (WT) HIV-1 (median 50% effective concentration [EC_{50}], 14 nM) and a wide spectrum of recombinant HIV-1 clinical isolates, including multiple-PI-resistant strains with decreased susceptibility to currently approved PIs (fold change [FC] in EC_{50}, >10). For a panel of 2,011 recombinant clinical isolates with decreased susceptibility to at least one of the currently approved PIs, the FC in TMC310911 EC_{50} was ≤4 for 82% of isolates and ≤10 for 96% of isolates. The FC in TMC310911 EC_{50} was ≤4 and ≤10 for 72% and 94% of isolates with decreased susceptibility to DRV, respectively. *In vitro* resistance selection (IVRS) experiments with WT virus and TMC310911 selected for mutations R41G or R41E, but selection of resistant virus required a longer time than IVRS performed with WT virus and DRV. IVRS performed with r13025, a multiple-PI-resistant recombinant clinical isolate, and TMC310911 selected for mutations L10F, I47V, and L90M (FC in TMC310911 EC_{50} = 16). IVRS performed with r13025 in the presence of DRV required less time and resulted in more PI resistance-associated mutations (V32I, I50V, G73S, L76V, and V82I; FC in DRV EC_{50} = 258). The activity against a comprehensive panel of PI-resistant mutants and the limited *in vitro* selection of resistant viruses under drug pressure suggest that TMC310911 represents a potential drug candidate for the management of HIV-1 infection for a broad range of patients, including those with multiple PI resistance.

The introduction of more efficacious, convenient, safer, and better-tolerated antiretroviral (ARV) agents for the treatment of human immunodeficiency virus type-1 (HIV-1) infection over the past several years has significantly improved treatment outcomes, especially for highly treatment-experienced patients (30). Despite this remarkable success, emergence of resistance to these new ARV agents remains a critical factor in highly active antiretroviral therapy (HAART) failure. Hence, there remains a need for new, safer, more convenient ARV agents without significant drug-drug interactions and with high genetic barriers to resistance to further improve long-term treatment efficacy for patients with multidrug-resistant HIV-1 infection (36).

Darunavir (DRV) is used extensively as a first-line HIV-1 protease inhibitor (PI) for the treatment of drug-naive HIV-1-infected patients (22) and treatment-experienced patients (1, 21), including those who are resistant to multiple PIs. DRV has been reported to possess a high genetic barrier to the development of resistance (6) and to exhibit activity against HIV-1

isolates with a high number of PI resistance-associated mutations (RAMs). Analyses of the influence of baseline mutations on virological response and on *in vitro* susceptibility to DRV, and of mutations in patients experiencing virologic failure in the POWER 1, 2, and 3 and the DUET 1 and 2 trials, identified a set of 11 DRV RAMs (V11I, V32I, L33F, I47V, I50V, I54L and I54M, T74P, L76V, I84V, and L89V) that may reduce the susceptibility to DRV when present in combinations of 3 or more (7, 8).

Although the population with high treatment experience is on the decline due to the increased effectiveness of treatment using recently approved ARVs, highly treatment-experienced patients with multidrug-resistant virus who fail multiple PI regimens have limited therapeutic options. Therefore, an internal HIV-1 protease research program was initiated to discover novel PIs with improved resistance profiles and limited selection of resistant virus under drug pressure. A series of fused heteroaromatic sulfonamides that showed extension into the P2′ pocket of the HIV-1 protease and exhibited exceptional activity against a panel of highly PI-cross-resistant mutants was discovered (32). The present report gives a virological characterization of TMC310911 (Fig. 1), a 2-(substituted-amino) benzothiazole sulfonamide, including details of antiviral activity, resistance profile for PI-resistant recombinant clinical isolates, and *in vitro* selection of resistant virus under drug pressure.

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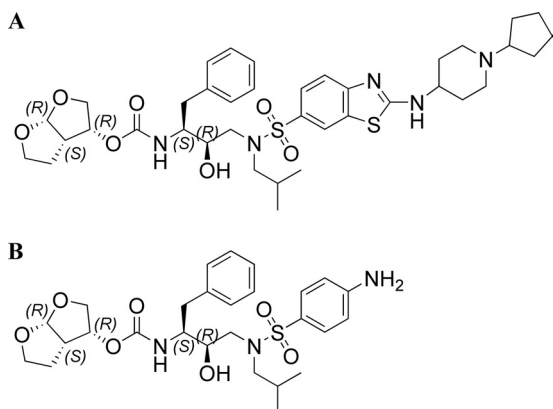▽ Published ahead of print on 6 September 2011.

A

B

FIG. 1. Structures of (A) TMC310911 and (B) darunavir.

MATERIALS AND METHODS

Compounds. TMC310911 was prepared as reported previously for related structures (32). DRV was prepared as reported previously (33). Atazanavir (ATV) and tipranavir (TPV) were synthesized in-house. Amprenavir (APV), indinavir (IDV), lopinavir (LPV), and saquinavir (SQV) were purified from commercially available formulations.

Cells and viruses. MT4 cells are human lymphoblastoic T cells that are permissive for HIV-1 infection and show a rapid and strong cytopathic effect (CPE). MT4-LTR-EGFP cells were generated by transfecting MT4 cells with a selectable construct encompassing the coding sequences for the HIV long terminal repeat (LTR) as a promoter for expression of enhanced green fluorescent protein (EGFP). Through subsequent selection, a stably transfected cell line was obtained. MT4-CMV-EGFP cells constitutively expressing the EGFP reporter under the control of a CMV promoter (the cytomegalovirus immediate-early [IE] promoter) were obtained by selection for permanently transformed MT4 cells with a CMV-EGFP reporter gene.

Fresh human peripheral blood mononuclear cells (PBMCs) were isolated from donors seronegative for HIV and hepatitis B virus (HBV) (Biological Specialty Corporation, Colmar, PA). Informed consent was not required (donors were compensated and identities were blinded). For use in drug susceptibility assays, PBMCs were purified and stimulated with phytohemagglutinin (4 μg/ml) for 48 to 72 h and further cultured in the presence of human interleukin 2 (20 U/ml) as described previously (9). Mature monocytes and macrophages (M/Ms) were isolated from PBMCs by adhesion as described previously (29). All cells were cultured in RPMI 1640 medium supplemented with fetal bovine serum combined with penicillin and streptomycin in a humidified incubator with a 5% CO_2 atmosphere at 37°C. Virus stocks of HIV-1 and HIV-2 strains were harvested from MT4 cells, except for HIV-1 BaL, which was grown in M/Ms.

Recombinant clinical isolates. Recombinant viruses, derived from clinical samples, were constructed by cotransfection of MT4 cells with patient-derived viral protease (PR) and reverse transcriptase (RT) coding sequences and an HIV-1 HXB2-derived proviral clone with deletions in the same regions as previously described (11).

Antiviral assays. The activity of compounds against laboratory-adapted HIV strains and patient-derived recombinant viruses was evaluated using MT4-LTR-EGFP cells and a reporter gene assay (RGA). In short, various concentrations of test compounds were added to wells of a flat-bottom microtiter plate. Subsequently, virus and MT4 cells were added for final concentrations of 200 to 250 50% cell culture infectious doses ($CCID_{50}$)/well and 30,000 cells/well, respectively. After 3 days of incubation (37°C and 5% CO_2), the relative fluorescence of treated cultures was measured and compared with the relative fluorescence of untreated cultures. The results of antiviral assays were expressed as EC_{50} values, representing the concentration of a compound achieving 50% inhibition of infection compared to the drug-free control results, or as EC_{90} values, representing the dose achieving 90% inhibition of infection. In some cases, a fold change (FC) in susceptibility was calculated by dividing the EC_{50} for the tested virus by the EC_{50} for the wild-type (WT) virus (HIV-1/LAI) tested in parallel. The cytotoxicity of the test compound was determined in parallel with the antiviral activity. In short, compounds were added to MT4-CMV-EGFP cells and fluorescence was measured after 3 days of incubation (37°C and 5% CO_2). Toxicity results were expressed as CC_{50} values representing the concentration of

a compound that resulted in a 50% reduction in cell viability compared to the drug-free control results. The selectivity index (SI) was calculated as CC_{50}/EC_{50}.

The antiviral assays with PBMCs and M/Ms were carried out as previously described (9, 29). PBMCs were infected with HIV-1/LAI and HIV-1/SF2. Primary M/Ms were acutely infected with HIV-1/BaL at a multiplicity of infection (MOI) of ± 0.01. The viral replication was measured by a p24 enzyme-linked immunosorbent assay (ELISA).

Genotyping. Genotypic analysis was performed by automated population-based sequencing. Sequence data were aligned to the WT HIV-1/HXB2CG reference (available in the GenBank database at http://www.ncbi.nlm.nih.gov/nuccore/K03455) and are reported as amino acid changes along the PR (11, 19).

In vitro resistance selection (IVRS). HIV-1 was subjected to serial passages in MT4-LTR-EGFP cells in the presence of increasing concentrations of HIV-1 PIs. In short, cells were acutely infected with HIV-1 at an MOI of 0.001 to 0.01. Cells were resuspended in 10 ml of complete medium at a density of 1.5 × 10^5/ml. The concentrations of the compounds at passage 1 were 1 to 5 times the respective EC_{50} values. The cultures were subcultured and scored microscopically with respect to virus-induced fluorescence and cytopathicity every 3 to 4 days. Cultures were maintained in the presence of the same concentration of compound until the full virus CPE was observed and subsequently at a higher compound concentration to select for variants able to grow in the presence of the highest possible PI concentration.

Protein crystallography. The WT HIV-1 protease used for crystallization was expressed, isolated, and purified from inclusion bodies as previously described (14). WT HIV-1 protease was further purified using a Pharmacia Superdex 75 fast-performance liquid chromatography column equilibrated with 0.05 M sodium acetate buffer at pH 5.5, containing 10% glycerol, 5% ethylene glycol, and 5 mM dithiothreitol, just before crystal trays were set. Cocrystals of the inhibitor with the WT protease were grown at room temperature by the hanging-drop vapor diffusion method. A protease concentration of 1.6 mg/ml with a 3-fold molar excess of inhibitors was used to set the crystallization drops, with the reservoir solution consisting of 126 mM phosphate buffer at pH 6.2, 63 mM sodium citrate, and 24 to 29% ammonium sulfate.

Intensity data on the protease crystals were collected at −80°C on an in-house Rigaku X-ray generator equipped with an R-axis IV image plate system. A total of 180 frames with an angular separation of 1° and without overlap between frames were collected. The data processing of the frames was carried out using the programs DENZO (23) and ScalePack (28). The crystal structure was solved and refined with the programs within the CCP4 interface (4). A structure solution for the WT protease-TMC310911 complex was obtained using the molecular replacement package AMoRe (27) and 1F7A as the starting model. After the solution was obtained, the molecular replacement phases were further improved using ARP/wARP (25) to build solvent molecules into the unaccounted regions of electron density. Model building was performed using the interactive graphics programs O (13) and Coot (10). Conjugate gradient refinement was performed using Refmac5 (26) by incorporating the Schomaker and Trueblood tensor formulation of TLS (translation, libration, screw-rotation) parameters (18). The working R (Rfactor) and its cross-validation (Rfree) were monitored throughout the refinement. The data collection and refinement statistics can be found in Table S1 in the supplemental material. The hydrogen bonding figure was made using PyMOL software (The PyMOL Molecular Graphics System, version 1.2r3pre; Schrödinger, LLC.).

Protein structure accession number. The final coordinates and structure factors for TMC310911 in complex with WT HIV-1 protease have been deposited with the Protein Data Bank (accession number 3R4B).

RESULTS

Activity against WT HIV. The *in vitro* activity of TMC310911 against laboratory HIV strains was evaluated using acutely infected MT4 cells, PBMCs, and M/M cells. Results are presented in Table 1. The TMC310911 EC_{50} values against WT HIV-1 ranged from 2.2 to 14 nM, and the corresponding EC_{90} values ranged from 5.0 to 94.7 nM. The TMC310911 CC_{50} for MT4 cells was 9.9 μM (interquartile ranges [IQR] = 8.57 to 12.19), with a resulting SI of 692. The TMC310911 EC_{50} values increased by a median factor of 7 in the presence of 50% human serum (I. Dierynck; data not shown).

Activity against a panel of PI-resistant recombinant clinical isolates. To assess the activity of TMC310911 against PI-resis-

TABLE 1. Activity of TMC310911 against WT HIV[a]

Virus/strain	Cell	Assay	EC$_{50}$ (nM)			EC$_{90}$ (nM)		
			n	Median	IQR	n	Median	IQR
HIV-1/LAI	MT4	RGA[b]	60	14.2	7.9–19.0	57	94.7	27.5–311.1
HIV-1/LAI	PBMC	p24[c]	1	2.2	NA	1	7.0	NA
HIV-1/SF2	PBMC	p24	1	2.3	NA	1	5.0	NA
HIV-1/BaL	M/M	p24	1	13.9	NA	1	72.7	NA
HIV-2/ROD	MT4	RGA	2	2.7	2.3–2.9	1	6.4	NA

[a] n, number of determinations; IQR, interquartile range; NA, not applicable.
[b] RGA, determination of inhibition of HIV LTR-driven reporter gene expression as measured in a reporter gene assay.
[c] p24, determination of inhibition of p24 antigen accumulation.

tant HIV-1, TMC310911 was used to challenge 3,444 recombinant clinical isolates of HIV-1 submitted for routine clinical testing with various degrees of genotypic diversity and phenotypic resistance as determined by the VircoType HIV-1 and Antivirogram methods, respectively. Since DRV was extensively evaluated in highly pretreated patients with PI mutant viruses, the composition of this panel of recombinant clinical isolates was biased toward viruses with genotypic and/or phenotypic determinants of resistance to DRV. The median number (range) of major PI RAMs (12) in the panel of clinical isolates was 4 (0 to 9), with 0 major PI RAMs in 13% of isolates, 1 to 2 major PI RAMs in 14% of isolates, 3 to 6 major PI RAMs in 66% of isolates, 7 major PI RAMs in 6% of isolates, and 8 to 9 major PI RAMs in 1% of isolates. Individual major PI RAMs were present in 3% (D30N), 33% (V32I), 66% (M46I/L), 22% (I47A/V), 5.8% (G48V), 8.7% (I50L/V), 31% (I54L/M), 21% (Q58E), 11% (T74P), 6.5% (L76V), 47% (V82A/F/S/L/T), 46% (I84V), 0.8% (N88S), and 62% (L90M) of the clinical isolates.

Among the analyzed samples with an Antivirogram phenotype available for all currently approved PIs (APV, ATV, DRV, IDV, LPV, SQV, and TPV; $n = 2,402$), 2,011 (84%) showed decreased susceptibility to at least one of these PIs, with an EC$_{50}$ FC of >10. Only 3.8% (76/2,011) of these samples showed an EC$_{50}$ FC of >10 for TMC310911, in comparison to 81%, 90%, 59%, 86%, 94%, 72%, and 21% for APV, ATV, DRV, IDV, LPV, SQV, and TPV, respectively (Fig. 2). The multiple-PI-resistant viruses with a TMC310911 EC$_{50}$ FC > 10 contained ≥11 PI RAMs.

In further analyses of the antiviral activity of TMC310911, an EC$_{50}$ FC > 4 and an EC$_{50}$ FC > 10 were used as criteria to describe potential resistance to TMC310911. The TMC310911 EC$_{50}$ FC was ≤4 for most (82%; $n = 1,658$) of the 2,011 isolates with decreased susceptibility (FC > 10) to at least one of the current PIs and was between 4 and 10 for 14% ($n = 277$) of isolates (Fig. 2). For isolates with decreased susceptibility to the most-prescribed PIs, namely, DRV (EC$_{50}$ FC > 10, representing the clinical cutoff [CCO]), LPV (EC$_{50}$ FC > 10; CCO), and ATV (EC$_{50}$ FC > 2.2, representing the biological cutoff [BCO]), 71% (879/1,233) and 94% (1,155/1,233) had TMC310911 EC$_{50}$ FC values of ≤4 and ≤10, respectively.

TMC310911 showed a more comprehensive antiviral inhibition of the diverse set of clinical isolates than the currently prescribed PIs, including DRV. As shown in Fig. 3A (data shown represent 2,781 samples with a DRV and TMC310911 phenotype available), TMC310911 exhibited EC$_{50}$ FC values of ≤4 and ≤10 for 72% (882/1,229) and 94% (1,157/1,229),

respectively, of samples with decreased susceptibility to DRV (EC$_{50}$ FC > 10; CCO).

The antiviral activity of TMC310911 was affected to a small extent by the presence of an increasing number of major PI RAMs or DRV RAMs (12) compared with other PIs (Fig. 4). While the median FC value for DRV exceeded 10 (i.e., the DRV CCO) in the presence of 3 or more DRV RAMs, the median FC value for TMC310911 remained below 4 in the presence of 6 DRV RAMs and below 10 in the presence of 8 DRV RAMs (Fig. 4A). The median FC values for TMC310911 were 2.3 and 4.8 in the presence of 8 major PI RAMs and DRV RAMs, respectively. The increase in the median in the TMC310911 EC$_{50}$ FC with increasing numbers of major PI RAMs was also lower than that observed for TPV (Fig. 4B), a PI that also has shown activity against many HIV-1 strains with multiple PI RAMs (20). TMC310911 had EC$_{50}$ FC values ≤ 10 and ≤ 4 for 95% (901/948) and 79% (750/948), respectively, of clinical isolates with an EC$_{50}$ FC value > 3 (i.e., the TPV CCO) for TPV (Fig. 3B; data represent 2,427 samples with TPV and TMC310911 phenotype available).

IVRS performed with WT HIV-1/LAI. In order to study the development of resistance in the presence of TMC310911, IVRS experiments with WT HIV-1/LAI under pressure from
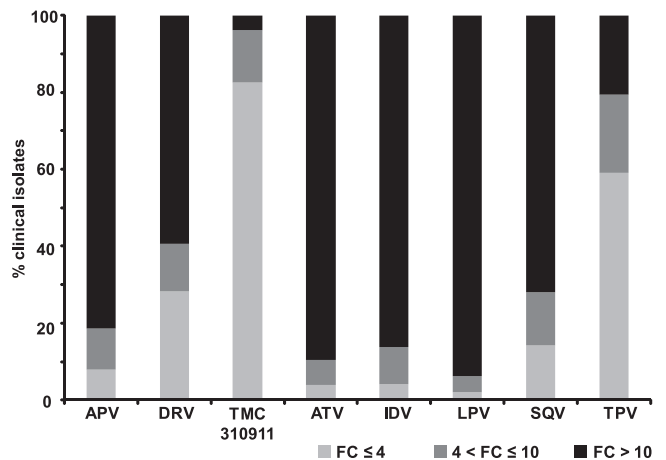

FIG. 2. Activity of different PIs against a series ($n = 2,011$) of recombinant clinical isolates with various levels of genotypic diversity and phenotypic susceptibility and an EC$_{50}$ FC > 10 for at least 1 of the currently approved PIs (APV, ATV, DRV, IDV, LPV, SQV and TPV). Antivirogram clinical cutoff (CCO): DRV FC = 10, LPV FC = 10, TPV FC = 3. Antivirogram biological cutoff (BCO): APV FC = 2.2, ATV FC = 2.1, IDV FC = 2.3, SQV FC = 1.8.
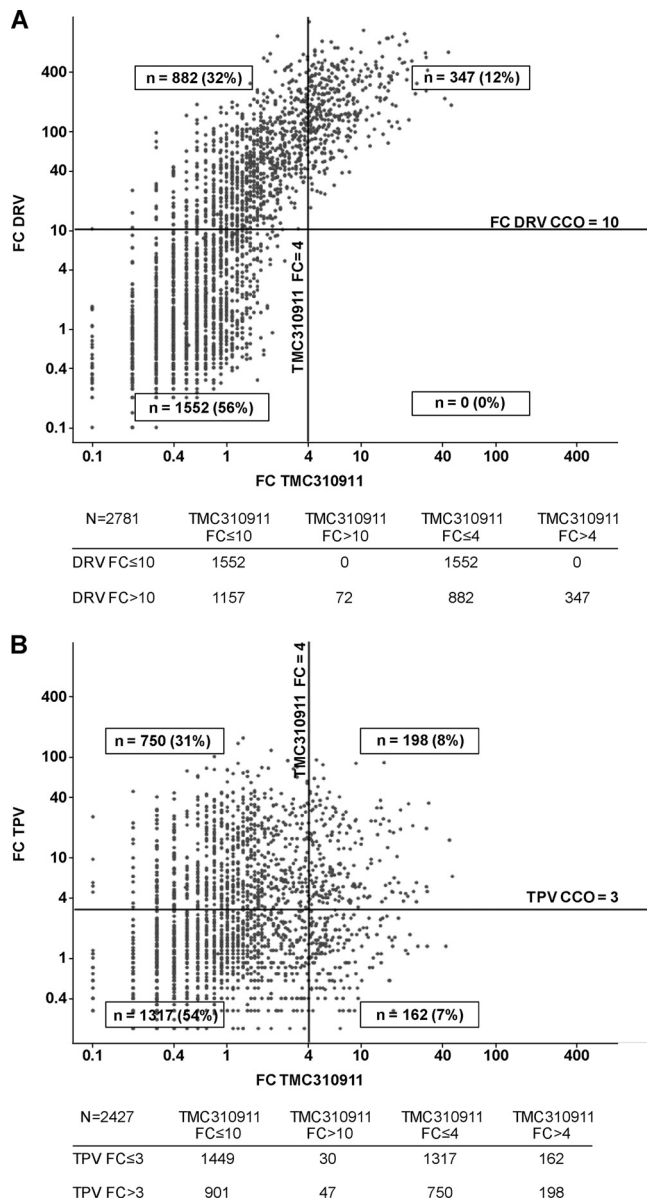
N=2781	TMC310911 FC≤10	TMC310911 FC>10	TMC310911 FC≤4	TMC310911 FC>4
DRV FC≤10	1552	0	1552	0
DRV FC>10	1157	72	882	347

N=2427	TMC310911 FC≤10	TMC310911 FC>10	TMC310911 FC≤4	TMC310911 FC>4
TPV FC≤3	1449	30	1317	162
TPV FC>3	901	47	750	198

FIG. 3. Comparison of the activity of TMC310911 against DRV (A) and TPV (B) determined with a series of recombinant clinical isolates ($n = 2,781$ [A] and 2,427 [B]) with various levels of genotypic diversity and phenotypic susceptibility (censored values are not shown). The Antivirogram clinical cutoff (CCO) for DRV (FC = 10) and TPV (FC = 3) are represented by horizontal lines; the TMC310911 FC value of 4 is represented by vertical lines.

FIG. 4. Activity (FC in EC_{50}) against a series of recombinant clinical isolates with various levels of genotypic diversity and phenotypic susceptibility (censored values not shown) according to the number of (A) major PI RAMs for TMC310911, DRV, LPV and TPV ($n = 1,388$) and (B) DRV RAMs for TMC310911 and DRV ($n = 2,781$). Each box plot depicts the median value (horizontal lines), lower (Q1) and upper (Q3) quartile (squared bar), upper (largest value ≤ Q3 + 1.5 IQR) and lower (lowest value ≥ Q1 − 1.5 IQR) adjacent values (vertical lines), and outlier values (dots). The Antivirogram clinical cutoff (CCO) values for DRV and LPV (FC = 10) and TPV (FC = 3) and the FC value of 4 for TMC310911 are represented by horizontal lines. DRV RAMs: V11I, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V, and L89V (12). Major PI RAMs: D30N, V32I, M46I/L, I47A/V, G48V, I50L/V, I54L/M, Q58E, T74P, L76V, V82A/F/L/S/T, I84V, N88S, and L90M (12).

TMC310911 were performed. As with IVRS performed with WT HIV-1 and DRV (6), selection of resistant viruses in the presence of TMC310911 was very difficult, as viruses were frequently found unable to replicate, resulting in the failure of the resistant mutant to propagate. A possible explanation might be the low replication capacity of the selected viruses. Although initial attempts to select for TMC310911-resistant virus were unsuccessful, IVRS performed with WT HIV-1/LAI subsequently resulted in selection of TMC310911-resistant virus. The results of a comparison of the data from this experiment to IVRS data from a DRV experiment are presented in
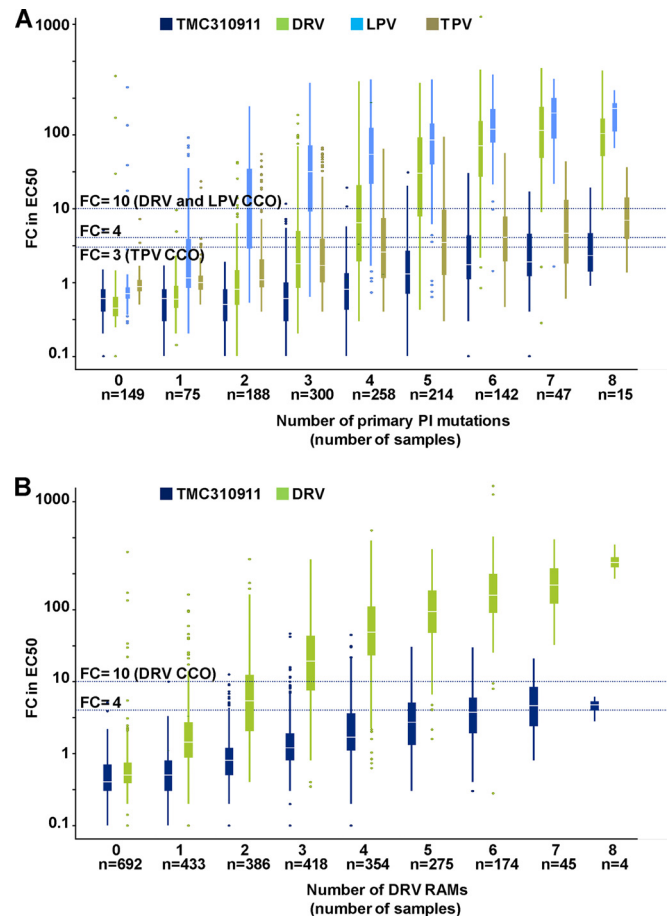
Fig. 5 and Table 2. Under selective pressure from TMC310911, viruses harboring the R41G mutation and those harboring the R41E mutation were isolated at 60 and 160 nM TMC310911, respectively. The concentration of TMC310911 could not be increased above 160 nM even after prolonged culture at this concentration due to the inability of the resistant virus to propagate at higher concentrations of drug, resulting in an EC_{50} FC increase of 12.

IVRS performed with r13025, a PI-resistant recombinant clinical isolate. In order to determine whether strains with decreased sensitivity to TMC310911 could be generated from viruses harboring mutations conferring resistance to currently approved PIs, IVRS was performed with r13025, a multiple-PI-resistant clinical isolate (Fig. 6). The r13025 virus contained
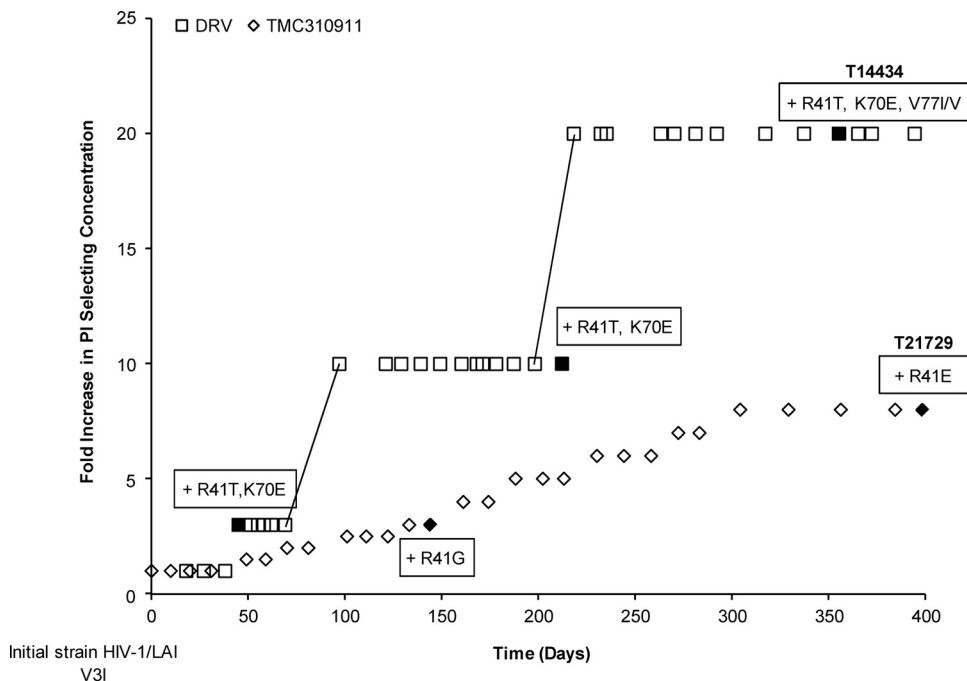
FIG. 5. *In vitro* selection of resistant HIV-1 starting from WT HIV-1/LAI in the presence of TMC310911 or DRV. Selection curves have been normalized, and starting selection concentrations were 20 and 10 nM for TMC310911 and DRV, respectively. Genotypes of virus strains selected at defined time points (indicated by filled symbols) list all changes from the starting strain HIV-1/LAI.

7 PI RAMs (L10I, M46I, Q58E, L63P, A71V, I84V, and L89V) and showed decreased susceptibility to APV (FC = 20), ATV (FC = 238), IDV (FC = 98), SQV (FC = 28), and TPV (FC = 15) but not to TMC310911 (FC = 1.4), DRV (FC = 1.9) or LPV (FC = 1.4) (Table 3). The r13025 virus was passaged in increasing concentrations of TMC310911 (starting from 20 nM); after 66 days, a 2-fold increase in drug concentration was found to be associated with the emergence of mutations L10F and I47I/V (TMC310911 EC$_{50}$ FC = 3.4). At the conclusion of IVRS (>200 days), the drug concentration was increased 20-fold and mutations L10F, I47V, and L90M appeared (strain T21217 in Fig. 6) and were found to be associated with a 12-fold increase in the TMC310911 EC$_{50}$ (EC$_{50}$ = 312 nM; FC = 16) in comparison with the value determined with the initial virus (Table 3). Strain T21217 showed a 4-fold or 7-fold decrease in susceptibility to DRV (FC = 7.4) or LPV (FC = 10), respectively, compared to the initial virus. IVRS performed with r13025 in the presence of TMC310911 was much slower than IVRS performed with r13025 and DRV (Fig. 6) or LPV (I. Dierynck; data not shown). Within a similar time frame of IVRS, the DRV concentration was increased >400-

fold, resulting in the development of mutations V32I, I50V, G73S, L76V, and V82I and a 30-fold increase in the DRV EC$_{50}$ (EC$_{50}$ = 670 nM; FC = 258) compared with the DRV EC$_{50}$ seen with the initial virus (Table 3). IVRS performed with r13025 and LPV resulted after 244 days in the selection of a virus containing the additional mutations L10F, L24I, V32I, I54V, G73S, and V82A (LPV FC = 324) and a 45-fold increase in drug concentration (I. Dierynck; data not shown).

Structural basis of TMC310911 and WT HIV-1 protease interaction. To enhance our understanding of the resistance profile of TMC310911 compared with DRV, we determined the crystal structure of TMC310911 in complex with the HIV-1 WT protease to 1.9Å. The TMC310911 complex crystallized in a $P2_12_12_1$ space group with one protease dimer in the asymmetric unit and with a final R_{factor} of 17.9 (R_{free}, 22.7) (see Table S1 in supplemental material). The inhibitor was modeled in two alternate conformations in the active site, with 50% occupancy for each conformation. Each of the alternative conformations had continuous electron density for the inhibitor, except for partial density of the piperidine ring and no density of the cyclopentyl ring that was exposed to the solvent. Because

TABLE 2. Phenotypic data showing PI susceptibility of the final selected virus strains starting from WT HIV-1/LAI as determined in IVRS experiments performed with TMC310911 and DRV

Strain (compound)	Emerging protease mutation(s)	EC$_{50}$ in nM (FC)[a]							
		TMC310911	DRV	IDV	SQV	APV	ATV	LPV	TPV
T21729 (TMC310911)	R41E	110 (12)	99 (13)	408 (8.0)	313 (8.0)	188 (3.8)	85 (8.0)	99 (10)	2,587 (6.4)
T14434 (DRV)	R41T,K70E,V77I/V	85 (22)	62 (23)	43 (3.2)	24 (4.3)	47 (3.2)	46 (14)	25 (6.4)	175 (4.0)

[a] Median values of at least three EC$_{50}$ determinations are presented. Values in parentheses represent FC in drug EC$_{50}$ relative to the EC$_{50}$ for HIV-1/LAI measured within the same antiviral experiment.
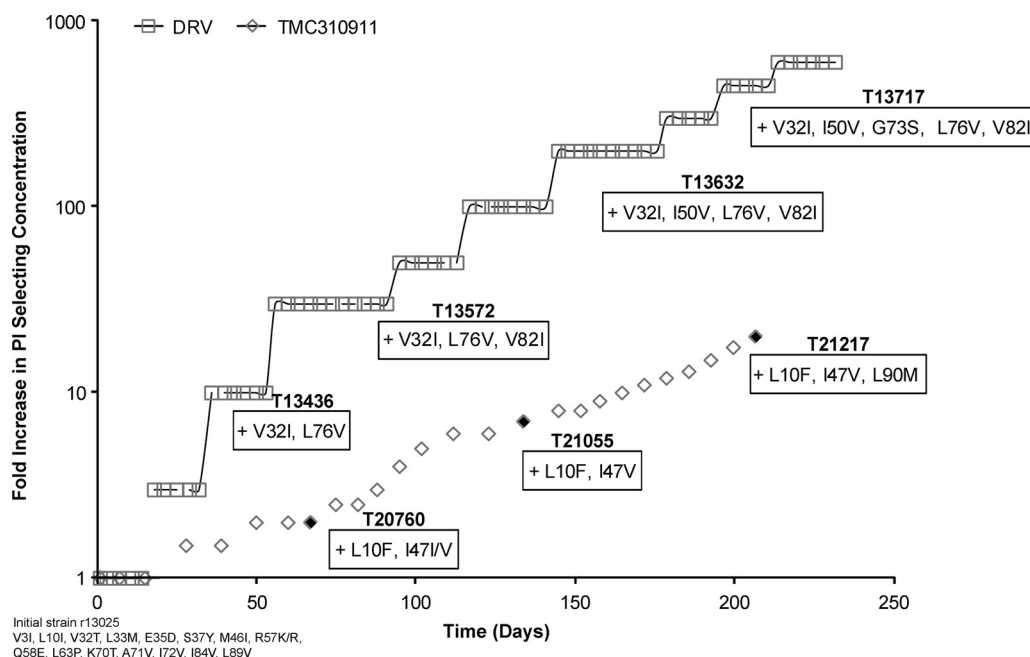
FIG. 6. *In vitro* selection of resistant HIV-1 starting from r13025, a PI-resistant recombinant clinical isolate, in the presence of TMC310911 or DRV. Selection curves have been normalized, and the starting selection concentration was 20 nM for TMC310911 and DRV. Genotypes of virus strains selected at defined time points (indicated by filled symbols) list the mutations that developed in the protease compared to the starting r13025 strain, which already contained 7 PI RAMs.

of the disorder of these two rings in the crystal structure, the interactions shown by the piperidine ring and the cyclopentyl ring were not considered in analyzing the interactions of TMC310911 with the protease.

The two conformations of TMC310911 form similar hydrogen bond interactions with the protease, as shown in Fig. 7. The TMC310911-WT HIV-1 protease interactions were compared with those of DRV and WT HIV-1 protease as reported previously by us (15). Table 4 lists all the hydrogen bond interactions observed for DRV and the two conformations of TMC310911. Given the structural resemblance between DRV

and TMC310911, the enzyme-inhibitor interactions were comparable. The major difference between the interactions of the WT protease with TMC310911 or DRV is that residue Asp30 supports two hydrogen bonds with the amine group of DRV whereas it forms one hydrogen bond with the nitrogen atom of benzothiazole in TMC310911 and another with the nitrogen atom of the amino group attached to the benzothiazole fragment. Although the interactions of TMC310911 are comparable to those of DRV, the additional interactions that can be formed by the piperidine ring may account for the improved resistance profile or limited selection of TMC310911-resistant virus over time.

TABLE 3. Phenotypic data showing PI susceptibility of the selected virus strains starting from r13205, a PI-resistant recombinant clinical isolate, in IVRS experiments performed with TMC310911 or DRV

Strain	Emerging PR mutations[b]	EC_{50} in nM (FC)[a]							
		TMC310911	DRV	IDV	SQV	APV	ATV	LPV	TPV
r13205	NA	26 (1.4)	22 (1.9)	6,274 (98)	818 (28)	1,601 (20)	4,000 (238)	28 (1.4)	729 (15)
TMC310911 expt									
T20760	L10F, I47I/V	65 (3.4)	39 (3.4)	3,530 (55)	653 (23)	1,496 (18)	8,325 (496)	112 (5.8)	2,087 (4.4)
T21055	L10F, I47V	308 (16)	122 (11)	5,969 (94)	1,915 (67)	6,372 (78)	9,601 (572)	426 (22)	13,903 (29)
T21217	L10F, I47V, L90M	312 (16)	84 (7.4)	5,406 (85)	5,408 (188)	3,650 (44)	6,884 (410)	198 (10)	11,800 (25)
DRV expt									
T13436	V32I, L76V	5.1 (1.3)	49 (21)	28 (1.9)	2.8 (0.5)	2,037 (71)	110 (33)	263 (42)	180 (2.9)
T13572	V32I, L76V, V82I	1.5 (3.7)	234 (99)	61 (4.1)	10 (1.8)	4,816 (169)	317 (99)	511 (81)	240 (3.7)
T13632	V32I, I50V, L76V, V82I	42 (10)	624 (265)	107 (7.1)	1.08 (0.2)	12,111 (425)	75 (20)	1,597 (254)	92 (1.4)
T13717	V32I, I50V, G73S, L76V, V82I	68 (17)	670 (258)	320 (27)	4.2 (0.8)	30,000 (1500)	89 (34)	1,900 (413)	86 (1.3)

[a] Median values of at least three EC_{50} determinations are presented. Values in parentheses represent FC in drug EC_{50} relative to the EC_{50} for HIV-1/LAI measured within the same antiviral experiment.
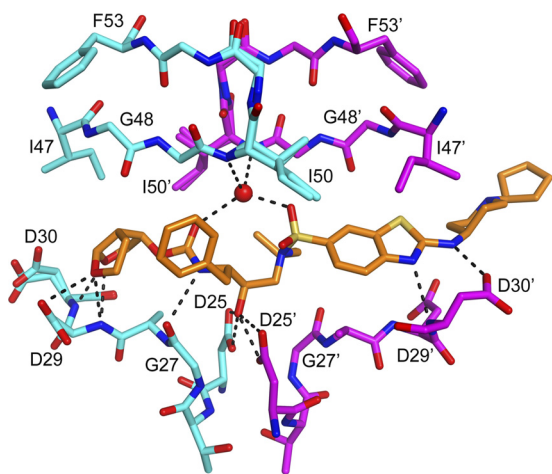[b] Mutations that developed under selective drug pressure.

FIG. 7. The hydrogen bond network formed between TMC310911 and the WT HIV-1 protease. The hydrogen bonds formed by one conformation of TMC310911 are shown here. A second conformation resulted in a similar hydrogen-bonding network. The piperidine and cyclopentyl rings are not well ordered in either conformation.

DISCUSSION

The high degree of cross-resistance between most HIV-1 PIs often limits their sequential use in patients on PI-containing regimens who show virological failure. The introduction of DRV and TPV, two HIV-1 PIs with a high genetic barrier to development of resistance and activity against a wide range of PI-resistant HIV-1 strains, has significantly improved response rates in patients with a history of multiple PI failure. However, highly treatment-experienced patients failing a DRV-containing regimen have limited PI treatment options. TPV often exhibits activity against DRV-resistant isolates, and DRV often exhibits activity against TPV-resistant isolates, but in contrast to DRV, the poor tolerability of TPV has limited its use in treatment regimens (2). Hence, there is a need for well-tolerated PIs with activity against DRV- and TPV-resistant isolates that may prolong the use of this class of ARV agents.

In this study, we characterized the antiviral activity, resistance profile, and development of resistance to TMC310911, a novel PI belonging to the same chemical class as DRV. TMC310911 is a potent PI, with activity against WT HIV-1 at concentrations ranging from 2.2 to 14 nM. With few exceptions, this compound retained potent activity against a comprehensive panel of recombinant clinical isolates with decreased susceptibility to currently approved PIs, including DRV and TPV. The TMC310911 EC_{50} FC was >10 for only 3.8% of the multiple-PI-resistant viruses that contained ≥11 PI RAMs. The reduction in susceptibility corresponding to increasing numbers of major PI RAMs was limited and lower in magnitude for TMC310911 than for other PIs such as DRV and TPV with broad activity against multiple-PI-resistant strains. The median TMC310911 EC_{50} FC values were 2.3 and 4.8 in the presence of 8 major PI RAMs and DRV RAMs, respectively.

IVRS performed with WT HIV-1/LAI and the multiple-PI-resistant r13025 clinical isolate confirmed the limited emergence of resistant virus under pressure from TMC310911 over time. Starting with WT HIV-1, selection of resistant HIV

strains by the use of APV, ATV, LPV, NFV, SQV, or TPV is usually easy to achieve, resulting in the emergence of strains carrying PI RAMs (6, 24), including mutations observed in the clinic with patients failing PI-containing treatment regimens. In contrast, drug concentrations could not be increased rapidly in IVRS performed with WT HIV-1 and TMC310911 or DRV, and it was not possible to observe virus replication at concentrations higher than 160 nM and 200 nM TMC310911 and DRV, respectively. Viruses containing a mutation at position 41 were selected for using both TMC310911 (R41E or R41G) and DRV (R41T) but have not been found to be associated with decreased susceptibility to PIs in clinical studies (12) and are present in only <0.05% of samples submitted for routine clinical testing (Virco BVBA; personal communication). The viruses selected under drug pressure from TMC310911 or DRV exhibited reduced susceptibility to both TMC310911 and DRV (FC > 10). It was shown using site-directed mutagenesis that the individual PR mutations selected during IVRS had no effect on susceptibility to DRV. However, several mutations accumulated in the GAG gene of the selected viruses and were found to be associated with decreased susceptibility to DRV in the corresponding GAG-PR recombinant viruses (5). These findings suggest that mutations in the GAG gene could be an implicating factor in the cross-resistance observed between DRV and TMC310911-selected viruses.

Starting from r13025, a multiple-PI-resistant clinical isolate, the development of resistant viruses in the presence of TMC310911 was reduced compared with DRV or LPV: the increase in the drug selection concentration was slower and fewer mutations appeared in the presence of TMC310911 compared with DRV or LPV. The mutations L10F, I47V, and L90M are known PI RAMs, and the resulting virus showed decreased susceptibility to all investigated PIs. Interestingly, strain T13717, selected under pressure from DRV, did not acquire resistance to IDV, ATV, SQV, or TPV but was more susceptible to those PIs than the initial r13025 strain. This could be explained by the selection of L76V, which can cause resensitization to ATV, SQV, and TPV and also to IDV, depending on the genetic mutation profile of the virus (3, 34, 35).

TABLE 4. Overview of the hydrogen bond interactions observed between the HIV-1 WT protease and DRV or two alternative TMC310911 conformers

Protease atom or molecule	Inhibitor atom	Distance (Å) for:		
		DRV	TMC310911-1	TMC310911-2
Asp 29 N	O26	3.1	3.3	3.4
Asp 30 N	O26	3.1	3.2	3.4
Asp 29 N	O28	2.9	3.0	2.9
Asp 29 OD2	O28	3.3	3.2	3.5
Water	O22	2.8	2.9	3.0
Gly 27 O	N20	3.1	3.3	3.4
Asp 25 OD1	O18	2.6	2.6	3.0
Asp 25 OD2	O18	3.0	2.6	2.8
Asp 25′ OD1	O18	3.0	2.7	2.7
Asp 25′ OD2	O18	2.5	3.2	2.9
Water	O9	2.9	2.4	2.5
Water	N1	3.0		
Asp 30′ N	N1	3.4	3.2	3.0
Asp 30′ O	N1	3.1		
Asp 30′ OD2	N24		2.5	3.0

TPV exhibits a resistance profile distinct from that of other currently available PIs (20); therefore, it was important to determine whether TMC310911 retained activity against TPV-resistant isolates. Our results showed that TMC310911 was active (FC ≤ 4) against the majority (79%) of clinical isolates with decreased susceptibility to TPV, highlighting TMC310911 as a potential drug candidate for treatment-experienced patients with resistance to TPV.

The genotypic and phenotypic determinants of DRV resistance that have been previously reported were confirmed in our experiments: phenotypic susceptibility to DRV decreased (FC > 10) in the presence of 3 or more DRV RAMs in a broad panel of recombinant clinical isolates, while most of the mutations (V32I, I50V, G73S, and L76V) that were selected when a multiple-PI-resistant clinical isolate was grown under pressure from DRV had previously been shown to be associated with reduced susceptibility to DRV (7, 8, 12). Mutation V82I does not affect susceptibility to DRV, but other mutations at this position (V82F and V82L) have been reported in a commercial database to confer resistance to DRV (31). Recently, selection of DRV-resistant variants by propagation of a mixture of HIV-1 variants isolated from patients failing a DRV-containing regimen was reported (16). Here, we showed that DRV-resistant virus could be generated using a single HIV-1 clinical isolate. The improved resistance profile or even reduced *in vitro* selection of viruses resistant to TMC310911 compared to DRV might be explained by an additional backbone interaction with Asp30 compared to DRV as observed in the crystal structure of the TMC310911–WT HIV-1 protease complex. Koh et al. have reported that, in addition to inhibiting the proteolytic activity of mature HIV-1 protease, DRV also inhibits the HIV-1 protease dimerization *in vitro* (17). This dual anti-HIV-1 function of DRV might play a role in its potent activity against multiple-PI-resistant isolates. Interactions of DRV involving Asp29, and not Asp30, were suggested to be linked with the observed dimerization inhibition. The effect of TMC310911 on HIV-1 protease dimerization has not been studied.

In addition to improved activity against multiple resistant viruses and a higher genetic barrier to the development of resistance, new HIV-1 PIs should also have an acceptable oral pharmacokinetic profile in order to lower pill burden, thereby improving convenience and adherence, as is often the case for recently approved ARVs. A good safety and tolerability profile is also desired, but this study did not address the safety and tolerability of TMC310911. Data from a proof-of-concept trial indicate the potential for once-daily dosing with TMC310911, and clinical studies evaluating this are ongoing (R. Verloes, unpublished data).

In conclusion, our findings suggest that TMC310911 exhibits broader *in vitro* activity against a diverse set of recombinant clinical isolates, including multiple-PI-resistant samples, and has a higher genetic barrier to the development of resistant virus *in vitro* than any of the currently approved PIs, including DRV. These properties support the evaluation of TMC310911 as a candidate drug suitable initially for highly treatment-experienced patients and subsequently for first-line treatment.

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