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TMC278, a Next-Generation Nonnucleoside Reverse Transcriptase Inhibitor (NNRTI), Active against Wild-Type and NNRTI-Resistant HIV-1▽†

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Nonnucleoside reverse transcriptase inhibitors (NNRTIs) have proven efficacy against human immunodeficiency virus type 1 (HIV-1). However, in the setting of incomplete viral suppression, efavirenz and nevirapine select for resistant viruses. The diarylpyrimidine etravirine has demonstrated durable efficacy for patients infected with NNRTI-resistant HIV-1. A screening strategy used to test NNRTI candidates from the same series as etravirine identified TMC278 (rilpivirine). TMC278 is an NNRTI showing subnanomolar 50% effective concentrations (EC_{50} values) against wild-type HIV-1 group M isolates (0.07 to 1.01 nM) and nanomolar EC_{50} values against group O isolates (2.88 to 8.45 nM). Sensitivity to TMC278 was not affected by the presence of most single NNRTI resistance-associated mutations (RAMs), including those at positions 100, 103, 106, 138, 179, 188, 190, 221, 230, and 236. The HIV-1 site-directed mutant with Y181C was sensitive to TMC278, whereas that with K101P or Y181I/V was resistant. *In vitro*, considerable cross-resistance between TMC278 and etravirine was observed. Sensitivity to TMC278 was observed for 62% of efavirenz- and/or nevirapine-resistant HIV-1 recombinant clinical isolates. TMC278 inhibited viral replication at concentrations at which first-generation NNRTIs could not suppress replication. The rates of selection of TMC278-resistant strains were comparable among HIV-1 group M subtypes. NNRTI RAMs emerging in HIV-1 under selective pressure from TMC278 included combinations of V90I, L100I, K101E, V106A/I, V108I, E138G/K/Q/R, V179F/I, Y181C/I, V189I, G190E, H221Y, F227C, and M230I/L. E138R was identified as a new NNRTI RAM. These *in vitro* analyses demonstrate that TMC278 is a potent next-generation NNRTI, with a high genetic barrier to resistance development.

The first-generation nonnucleoside reverse transcriptase inhibitors (NNRTIs), efavirenz (EFV) and nevirapine (NVP), are common components of first-line, highly active antiretroviral (ARV) therapy for patients infected with human immunodeficiency virus type 1 (HIV-1). Both have proven long-term efficacy, generally good tolerability, and a low pill burden (21, 52). EFV-based regimens have demonstrated viral suppression to <50 plasma HIV-1 RNA copies/ml after 96 weeks and for up to 7 years of treatment (32, 41). However, the clinical use of EFV and NVP can be limited due to their relatively low genetic barrier to resistance, the cross-resistance between them, and tolerability issues (2, 3, 18).

Various strategies have been developed to identify next-generation agents with activity against NNRTI-resistant viruses; one of these strategies successfully identified etravirine (ETR; TMC125) as a diarylpyrimidine NNRTI with activity against a broad spectrum of wild-type and first-generation NNRTI-resistant HIV-1 viruses (1). Briefly, lead compounds identified by structure-activity and structure-metabolism relationships were evaluated for antiviral activity against both wild-type HIV-1 strains and mutants harboring various combinations of NNRTI resistance-associated mutations (RAMs). In addition, functional HIV-1 reverse transcriptase (RT)-binding assays and tests for antiviral activity against a large panel of HIV-1 recombinant clinical isolates resistant to NNRTIs were performed. ETR has recently received approval for use in combination with other ARV agents for treatment-experienced patients (19, 29, 33, 47). The same strategy was used to characterize TMC278 (rilpivirine) as a second NNRTI of the diarylpyrimidine family (Fig. 1) (6, 17, 25), and its potential as a successful clinical candidate is under evaluation in treatment-naïve patients. The phase III TMC278-C209 (ECHO; NCT00540449) and TMC278-C215 (THRIVE; NCT00543725) trials are ongoing.

MATERIALS AND METHODS

Compounds. EFV, NVP, and the protease inhibitors (PIs), with the exception of atazanavir (ATV), tipranavir (TPV), and darunavir (DRV), were extracted from commercial formulations. ATV, TPV, DRV, emtricitabine (FTC), zidovudine (AZT), lamivudine (3TC), didanosine (ddI), enfuvirtide (ENF), plerixafor (AMD-3100), BMS-806, TMC278, and ETR were synthesized at Tibotec. Stavudine (d4T) and zalcitabine (ddC) were purchased from Sigma, St. Louis, MO. Abacavir (ABC) and tenofovir diphosphate (TDF) were provided by Virco, Mechelen, Belgium. Emivirine was obtained from Johnson & Johnson Pharmaceutical Research and Development, Beerse, Belgium, and Raritan, NJ. For the combination experiments, ETR, raltegravir (RAL), and maraviroc (MVC), were supplied by the Southern Research Institute (SRI) (Frederick, MD).

Cells and viruses. The MT4 human T-lymphoblastoid, CEM-SS, and MAGI-CCR5 cell lines were incubated in RPM 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 μg/ml gentamicin at 37°C under a humidified 5% CO_2 atmosphere.

Fresh human peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteer blood donors seronegative for HIV and hepatitis B virus. Cells

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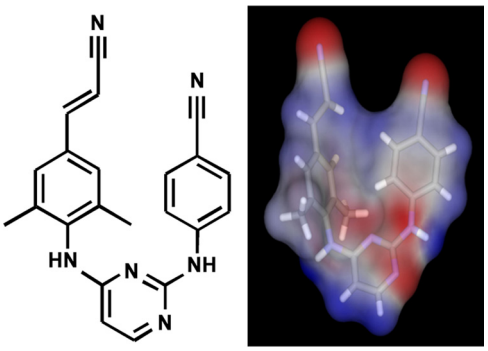

FIG. 1. Chemical structure and 3-dimensional model of TMC278. The chemical structure of TMC278, $C_{22}H_{18}N_6$, or 4-[[4-[[4-[(1*E*)-2-cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzonitrile, is shown. Red represents CN groups or the nitrogen of the pyrimidine and the nitrogen of the aniline (highest density of charge in the structure), and blue represents the aromatic group (no charge).

were pelleted/washed 2 to 3 times by low-speed centrifugation, resuspended in phosphate-buffered saline (PBS), layered over 14 ml of lymphocyte separation medium (Cellgro; Mediatech, Inc., Manassas, VA) in a 50-ml centrifuge tube, and centrifuged for 30 min at 600 × *g*. Banded PBMCs were gently aspirated, washed twice with PBS, and resuspended at 1×10^6/ml in RPMI 1640 supplemented with 15% FCS, 2 mM L-glutamine, and 4 μg/ml phytohemagglutinin (PHA) (Sigma). PBMCs were incubated for 48 to 72 h at 37°C under a humidified 5% CO_2 atmosphere, centrifuged, and resuspended in culture medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 20 U/ml recombinant human interleukin 2 (R&D Systems, Inc., MN). The PBMCs were maintained at a concentration of 1×10^6 to 2×10^6/ml and were kept in culture for a maximum of 2 weeks. HIV-1 and HIV type 2 (HIV-2) cell-free supernatants were stored at −80°C.

All HIV-1 primary isolates, except for the HIV-1 clinical isolate WEJO, were obtained from the NIAID AIDS Research and Reference Reagent Program, Rockville, MD. WEJO was obtained from a pediatric patient attending the AIDS Clinic at the University of Alabama at Birmingham (10). Low-passage-number stocks of the viruses were prepared using fresh human PBMCs, as described above. These stocks were titrated and stored.

HIV-1 site-directed mutants (SDMs) either were constructed in a pGEM vector encompassing the RT and protease (PR) coding sequences of HIV-1 HXB2 by using the QuikChange SDM kit (Stratagene, CA) or were obtained from a commercial supplier (Eurofins Medigenomix GmbH, Martinsried, Germany). Mutant viruses were generated by the recombination of mutant RT-PR sequences with an RT-PR-deleted HIV-1 HXB2 proviral clone, which was transfected into MT4 human T-lymphoblastoid cells (23). All SDM virus RT sequences were confirmed by DNA sequencing.

HIV-1 recombinant clinical isolates were generated by the recombination of HIV-1 RNA RT-PR sequences from clinical samples with an RT-PR-deleted HIV-1 HXB2 proviral clone (23), which was transfected into MT4 human T-lymphoblastoid cells (23).

Genotyping and subtype determination. Genotypic analyses were performed according to the vircoTYPE (51) methodology based on automated population-based DNA sequence analysis (ABI Prism BigDye Terminator cycle sequencing). Sequencing results were reported as amino acid changes compared with the wild-type reference sequence of HIV-1 $HXB2_{CG}$ (27). HIV-1 subtypes of the recombinant clinical isolates were determined on the basis of the PR and RT region, which is particularly relevant for this study, by performing local sequence alignments using the Smith-Waterman algorithm. This algorithm identifies similarities between the HIV-1 recombinant clinical isolate and the HIV-1 $HXB2_{CG}$ nucleotide sequences (44).

Mechanism-of-action studies. A time-of-addition assay was conducted in order to evaluate the stage at which TMC278 acts in the HIV-1 replication cycle. ENF (a fusion inhibitor), AMD-3100 (a CXCR4 entry inhibitor) (43), BMS-806 (a CD4/gp120 attachment inhibitor) (31), and EFV (an NNRTI) were used as references. MT4-long terminal repeat (LTR)-luciferase cells at a concentration of 3.5×10^5/ml were infected with HIV-1 IIIB at a multiplicity of infection (MOI) of 0.4. Compounds were added to each culture after 30 min or every 60 to 90 min for the next 11 h postinfection. The final concentration in the culture was 50 nM TMC278, 1 μM ENF, 1 μM AMD-3100, 10 μM BMS-806, or 1 μM EFV. At 24 h postinfection, the antiviral activity of each compound was calculated by determination of the induction of luminescence in the cell cultures (Luclite; Perkin-Elmer, CT).

The inhibitory effect of TMC278 on HIV-1 RT activity was determined by an enzymatic assay (Quan-T-RT; Amersham) described previously (14). Briefly, the inhibition of incorporation of [^{3}H]TTP and TTP by reverse transcription in a p(rA)p(dT)primer/template was measured via biotin/streptavidin linkage.

Drug sensitivity assays. The antiviral activity of each compound against laboratory-adapted HIV strains, HIV-1 SDMs, and HIV-1 recombinant clinical isolates was measured in MT4 cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and green fluorescent protein (GFP) assays. The MTT assay was performed as previously described (23). Briefly, using a calibrated automated pipetting station, the test compound was serially diluted in 96-well plates prefilled with cell culture medium. MT4 cells (1.5×10^5/ml) and HIV (MOI, 0.01) were added to a final volume of 200 μl and were incubated for 5 days at 37°C under a 5% CO_2 atmosphere. Cell viability was determined by the tetrazolium colorimetric MTT methodology. The GFP assay exploits the inducibility of the HIV-1 LTR by the HIV-1-encoded Tat gene product. The ongoing replication of HIV-1 in MT4 LTR-enhanced GFP cells was measured using the specific interaction of the HIV-1 Tat protein, coupled to the GFP reporter gene, with the HIV-1 LTR. The test compound was serially diluted, and MT4 LTR-enhanced GFP cells were infected with HIV (MOI, 0.01) and added to the 96-well plates as described above. The cell cultures were then divided into 4 wells of a 384-well plate and were incubated at 37°C under a 5% CO_2 atmosphere for 3 days. GFP production was measured by fluorescence at 488 nm (35).

The antiviral activity of each compound against HIV primary isolates from groups M and O was measured in human PBMCs by the SRI (University of Alabama). Briefly, pooled PHA-stimulated cells were diluted in fresh medium to a final concentration of 1×10^6/ml and were plated in a 96-well microplate at 50 μl/well (5×10^4 cells/well). Assessment of cytotoxicity in the presence and absence of the compound tested showed that PBMC viability remained high throughout the incubation period. TMC278, EFV, and ETR were evaluated at concentrations ranging from 0.05 to 500 nM. The virus stock, at a final MOI of 0.1, was added to each test well. The PBMC cultures were maintained at 37°C under 5% CO_2 for 7 days following infection. After this period, cell-free supernatant samples were collected for analysis of HIV-1 RT activity (11).

Determination of resistance. Resistance to a given drug was based on current biological cutoffs (BCO), which determine the cutoff fold change (FC) value for the sensitivity (28) of NNRTIs reported on the Antivirogram (version 2.5.00) (50). These values are 3.3 for EFV, 3.2 for ETR, and 6.0 for NVP (17). A BCO of 3.7 was calculated and used to determine resistance for TMC278. Of note, the BCO value for TMC278 is preliminary and may be subject to change over time. This preliminary BCO for TMC278 was determined as the 97.5 percentile of FC measurements, after outlier removal, from 2,796 wild-type HIV-1 recombinant clinical isolates from a 3-year period (February 2004 to February 2007). Outliers are the values outside the interval comprising the mean FC plus 3 standard deviations in the log domain. Wild-type isolates are defined as recombinant clinical isolates containing none of the mutations that are both present in the public domain and identified by the Virco linear modeling algorithm (update from November 2007) (48).

HIV-1 *in vitro* antiviral activity in the presence of human serum proteins. The GFP assay was performed to determine the effects of human serum proteins on the activities of TMC278, EFV, ETR, and NVP against HIV-1 IIIB in MT4 cells. HIV-1 *in vitro* antiviral activity assays were performed in standard media containing 10% FCS, to which 50% human serum, 45 mg/ml human serum albumin (HSA), or 1 mg/ml alpha-1-acid glycoprotein (AAG) was added. The ratio of the 50% effective concentration (EC_{50}) in the presence of serum protein to that in the absence of serum protein was calculated.

Selection of drug-resistant viruses. For high MOIs and fixed drug concentrations, MT4 cells were infected with wild-type HIV-1 or with HIV-1 strains harboring mutations associated with resistance to RT inhibitors (RT RAMs) at an MOI of 0.1 to 1.0 in the absence (control) or presence of different concentrations of the inhibitor. Cell cultures were maintained by repeat passages up to a maximum of 32 days in the presence of the initial concentration of each inhibitor and were examined for signs of viral replication. Assessment of cytopathic effect was used to determine viral replication.

For low MOIs and escalating drug concentrations, MT4 cells were infected at an MOI of 0.001 to 0.01 with wild-type HIV-1 (HIV-1 IIIB), NNRTI-resistant recombinant clinical HIV-1 isolates of various subtypes, or various NNRTI-resistant HIV-1 HXB2 SDMs in the presence of TMC278 at initial concentrations ranging from 1 to 10 nM. The cultures were maintained by repeat cell passages, and cells were examined for signs of viral replication. At 100% cyto-

TABLE 1. *In vitro* antiviral activities of TMC278, EFV, ETR, and NVP against HIV-1 IIIB, HIV-2, and a range of HIV-1 group M recombinant clinical isolates

Viral type (subtype)	Median EC_{50} (Q1–Q3)[a]			
	TMC278	EFV	ETR	NVP
HIV-1 (IIIB)	0.73 (0.39–0.98) ($n = 403$)	1.73 (1.14–2.42) ($n = 889$)	2.73 (2.06–3.49) ($n = 927$)	34.09 (26.23–44.90) ($n = 512$)
HIV-1 (A1)	0.44 ($n = 1$)	0.72 (0.60–0.87) ($n = 2$)	1.39 ($n = 1$)	9.32 ($n = 1$)
HIV-1 (AE)	0.21 (0.20–0.21) ($n = 2$)	0.53 (0.43–0.65) ($n = 2$)	0.65 (0.63–0.66) ($n = 2$)	8.94 ($n = 1$)
HIV-1 (AG)	0.13 (0.10–0.17) ($n = 2$)	0.53 (0.34–0.81) ($n = 2$)	0.46 (0.32–0.66) ($n = 2$)	14.40 ($n = 1$)
HIV-1 (BG)	0.22 (0.20–0.24) ($n = 2$)	0.70 (0.46–1.06) ($n = 2$)	0.67 (0.60–0.75) ($n = 2$)	25.99 ($n = 1$)
HIV-1 (C)	0.30 (0.18–0.52) ($n = 2$)	0.31 (0.31–0.31) ($n = 2$)	0.52 (0.28–0.97) ($n = 2$)	<4.88 ($n = 1$)
HIV-1 (D)	0.30 (0.27–0.33) ($n = 2$)	1.19 (0.96–1.47) ($n = 2$)	1.13 (1.01–1.26) ($n = 2$)	24.17 ($n = 1$)
HIV-1 (F1)	0.21 (0.18–0.26) ($n = 2$)	0.66 (0.58–0.76) ($n = 2$)	0.35 (0.13–0.99) ($n = 2$)	14.88 ($n = 1$)
HIV-1 (G)	0.14 (0.06–0.34) ($n = 2$)	0.31 (0.23–0.42) ($n = 2$)	0.24 (0.21–0.26) ($n = 2$)	24.63 ($n = 1$)
HIV-1 (H)	0.24 (0.22–0.25) ($n = 2$)	0.49 (0.31–0.77) ($n = 2$)	0.51 (0.26–0.98) ($n = 2$)	34.14 ($n = 1$)
HIV-2 (ROD)	5.22 (2.51–10.83) ($n = 2$)	24.83 (14.49–32.00) ($n = 28$)	5.67 (3.10–7.34) ($n = 30$)	>31.25 ($n = 9$)

[a] EC_{50}, 50% effective concentration in a cell-based assay; Q1–Q3, first- to third-quartile values. Values are given in nanomolar concentrations for HIV-1 and in micromolar concentrations for HIV-2. *n*, number of experiments. For MT4 cells, the 50% cytotoxic concentrations in cell-based assays (with first- to third-quartile values in parentheses) were 5.91 μM (5.02 to 8.31 μM) for TMC278 in 381 experiments, 40.02 μM (36.46 to 43.06 μM) for EFV in 805 experiments, >64.00 μM for ETR in 820 experiments, and >32.00 μM for NVP in 457 experiments.

pathic effect, virus supernatants were collected and used to infect fresh cells at 0- to 5-fold-incremented concentrations of TMC278.

In vitro susceptibility testing was performed according to Antivirogram Phenotype methodology (50), using either the MTT or the GFP assay. Briefly, from the selected viruses, a virus stock was grown in the absence of the inhibitor; titration was performed; and the newly obtained virus stock was used for susceptibility testing. Genotypic analyses were performed as described in the paragraph on genotyping and subtype determination.

Drug combination assays. Combinations of TMC278 with the nucleoside reverse transcriptase inhibitors (NRTIs) 3TC, ABC, AZT, d4T, FTC, and ddI; the nucleotide reverse transcriptase inhibitor (NtRTI) TDF; the PIs amprenavir (APV), ATV, DRV, indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and TPV; the NNRTIs EFV, ETR, and NVP; and the fusion inhibitor ENF were tested against HIV-1 IIIB in MT4 cells, as described above, at three different molar ratios of their estimated respective EC_{50} values: 3/1, 1/1, and 1/3. Each combination was tested in triplicate in three independent experiments. A combination index (CI) at 50% protection was calculated as $(D_A/d_A) + (D_B/d_B)$, where D_A and D_B are the EC_{50} values of the compounds when used alone and d_A and d_B are the EC_{50} values of the compounds when used in the drug combination (7, 13). A median CI of <0.8, ≥1.2, or ≥0.8 and <1.2 denotes, respectively, synergy, antagonism, or additivity between drugs. The statistical significance of the results was established when at least two of the three experiments gave the same score. Control combinations were AZT-AZT and the combination of AZT and emivirine, which has previously been reported to be synergistic (9).

Combinations of TMC278 with MVC, ETR, and RAL were tested in a checkerboard plate format using five concentrations of MVC, ETR, or RAL in all possible combinations with eight concentrations of TMC278. Combination antiviral activity was evaluated in triplicate, and control wells included cells and virus only. The antiviral activity of TMC278 with MVC was measured as the inhibition of β-galactosidase expression, and the antiviral activity of TMC278 with ETR and RAL was measured with the anti-HIV-1 cytoprotection assay and the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay (26). Combinations of TMC278 with MVC were tested in MAGI-CCR5 cells infected with the CCR5-tropic virus strain HIV-1 Ba-L (NIH AIDS Research and Reference Reagent Program). MAGI-CCR5 cells were plated at 1.0×10^4 per well and were incubated at 37°C overnight prior to infection with HIV-1 Ba-L at an MOI of 0.001. At 48 h postinfection, supernatants were aspirated and washed. Gal-screen reagent (Tropix, Bedford, MA) for chemiluminescent detection of β-galactosidase activity was added per the manufacturer's instructions and was incubated at room temperature for 90 min. Chemiluminescence was read spectrophotometrically at 593 nm. Combinations of TMC278 with ETR and RAL were tested in CEM-SS cells infected with HIV-1 IIIB, resuspended, and diluted into tissue culture medium to obtain 85 to 95% cell death at 6 days postinfection (MOI, 0.01) as assessed using the MTS assay. Assay plates were stained with 25 μl MTS per well (CellTiter reagent; Promega, WI), incubated for 4 to 6 h at 37°C, and read spectrophotometrically at 490/650 nm. The drug combination assay data were then analyzed according to the method of Prichard and Shipman (40) using the MacSynergy II program for data analysis and statistical evaluation. Statistical significance was established when the 3-dimensional profile gave a uniform score over the concentration range analyzed.

Summary of the data. Data are presented as medians and interquartile ranges (Q1 to Q3) due to the nonparametric (non-Gaussian) distribution of the data set used.

RESULTS

***In vitro* antiviral activities of TMC278 against HIV-1 IIIB, a range of HIV-1 group M subtype recombinant clinical isolates, and HIV-2.** The antiviral activities of TMC278 against HIV-1 IIIB and HIV-2 ROD were compared with those of other NNRTIs, EFV, ETR, and NVP (Table 1). The median EC_{50} for TMC278 against HIV-1 IIIB observed in the MT4 T-cell line was in the subnanomolar range (0.73 nM). This median EC_{50} was lower than those obtained for EFV, ETR, and NVP by factors of 2.4, 3.7, and 46.7, respectively. The antiviral activity of TMC278 was tested in parallel with those of EFV, ETR, and NVP against a panel of nine group M recombinant HIV-1 clinical isolates (16) of subtypes A1, AE, AG, BG, C, D, F1, G, and H (Table 1). All the HIV-1 subtypes were sensitive to TMC278, EFV, ETR, and NVP. Like ETR, TMC278 showed higher antiviral activity against HIV-2 than EFV and NVP (Table 1). A selectivity index (ratio of the 50% *in vitro* cytotoxic concentration [CC_{50}] to the EC_{50}) greater than 8,000 was calculated for MT4 cells, indicating that TMC278 is a potent and specific inhibitor of HIV-1 RT.

***In vitro* antiviral activity of TMC278 against primary HIV-1 isolates.** A summary of the antiviral activities of TMC278 against HIV-1 primary isolates of group M subtypes A, B, C, D, E, F, and G and group O is provided in Table 2. There was no apparent difference in the antiviral activity of TMC278 based on virus tropism. Similarly, except for HIV-1 group O, there was no apparent difference in the antiviral activity of the compound based on HIV-1 subtype. TMC278 was on average approximately 10- to 15-fold less active against the HIV-1 group O isolates than against the HIV-1 group M isolates. The median EC_{50} of TMC278 against all the HIV-1 primary clinical isolates tested in PBMC cultures was 0.26 nM (Q1 to Q3, 0.15 to 0.52 nM).

TABLE 2. *In vitro* anti-HIV-1 activities of TMC278, EFV, and ETR against primary HIV-1 isolates

HIV-1 strain	Subtype	Tropism[a]	EC_{50} (nM)[b]		
			TMC278	EFV	ETR
92UG029	A	CXCR4	0.44	0.62	1.45
92UG037	A	CCR5	0.24	1.11	1.42
92RW020	A	CCR5	0.07	0.30	0.48
JR-CSF	B	CCR5	0.51	0.76	1.02
93BR021	B	CCR5	0.23	1.17	1.35
WEJO	B	CXCR4	0.08	0.16	0.43
92BR025	C	CCR5	0.33	2.08	1.70
93IN101	C	CCR5	0.53	1.47	2.72
93MW959	C	CCR5	0.11	0.19	1.01
92UG001	D	DUAL	0.26	0.53	0.90
92UG024	D	CXCR4	0.38	0.72	3.18
92UG035	D	CCR5	0.07	0.34	0.44
92TH006	E	CCR5	0.08	0.48	0.50
93TH073	E	CCR5	0.07	0.39	0.21
CMU08	E	CXCR4	1.01	0.78	3.47
93BR019	F	CXCR4	0.95	0.15	0.46
93BR020	F	DUAL	0.16	0.74	1.08
93BR029	F	CCR5	0.19	0.42	1.06
G3	G	CCR5	0.25	0.12	0.66
JV1083	G	CCR5	0.26	0.70	1.45
RU132	G	CCR5	0.51	0.52	1.70
BCF01	O	CCR5	3.13	72.8	16.4
BCF02	O	CCR5	2.88	57.2	52.8
BCF03	O	CCR5	8.45	194	149

[a] CCR5, chemokine receptor 5; CXCR4, CXC chemokine receptor 4.
[b] EC_{50}, 50% effective concentration in a cell-based assay. The median EC_{50} values for TMC278, EFV, and ETR were 0.26, 0.66, and 1.22 nM, respectively, and the corresponding first- to third-quartile values were 0.15 to 0.52, 0.38 to 1.13, and 0.62 to 1.96 nM, respectively.

Mechanism of action. The antiviral activities of TMC278, ENF (a fusion inhibitor), AMD-3100 (a CXCR4 entry inhibitor) (43), BMS-806 (a CD4/HIV-1 gp120 attachment inhibitor) (31), and EFV (an NNRTI) were studied at different times of addition of the drugs to the infected HIV-1 IIIB cell culture. The data presented in Fig. 2 show that the activity profile of TMC278 was similar to that of the NNRTI EFV. A median

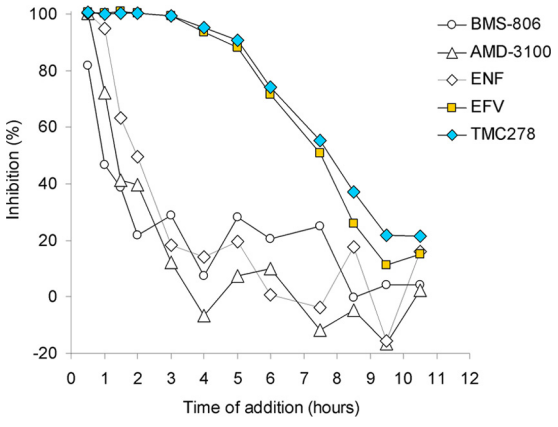

FIG. 2. Effect of the time of addition of TMC278 on its anti-HIV-1 activity compared with that of other ARVs. The antiviral activity of TMC278 was determined at various times of addition of the compound in order to estimate the viral replication stage at which TMC278 inhibits the HIV-1 replication cycle by using the known ARV inhibitors BMS-806, AMD-3100, ENF, and EFV as references.

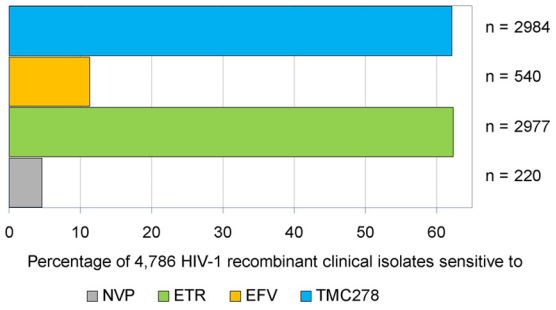

FIG. 3. Analysis of the prevalence of HIV-1 recombinant clinical isolates sensitive to TMC278, ETR, EFV, or NVP among those resistant to at least one first-generation NNRTI (i.e., EFV and/or NVP) (n = 4,786).

inhibitory activity of HIV-1 RT by TMC278 (50% inhibitory concentration [IC_{50}]) of 42 nM was determined using a primer extension-based scintillation proximity assay (14).

Antiviral activity of TMC278 against HIV-1 NNRTI-resistant recombinant clinical isolates. A large-scale evaluation of the antiviral activity of TMC278 was done on a panel of 4,786 HIV-1 recombinant clinical isolates chosen from a library of 10,990 on the basis of resistance to a first-generation NNRTI (i.e., FC greater than the BCO for either EFV or NVP or both). The percentages of HIV-1 recombinant clinical isolates with sensitivity (i.e., FC less than or equal to the BCO) to TMC278, EFV, ETR, and NVP are presented in Fig. 3. The data show that, *in vitro*, 62% of these HIV-1 recombinant clinical isolates, resistant to at least one first-generation NNRTI, retained sensitivity to TMC278 or ETR, compared with 11% with sensitivity to EFV and 5% with sensitivity to NVP.

Antiviral activities of TMC278 against HIV-1 HXB2 SDMs containing NNRTI RAMs. A series of HIV-1 SDMs were constructed in an HIV-1 HXB2 molecular clone backbone in order to study the *in vitro* resistance profile of TMC278. EFV, ETR, and NVP were also included in this analysis for purposes of comparison. The data presented in Table 3 show the levels of resistance of 55 HIV-1 HXB2 SDMs to NNRTIs. HIV-1 SDMs with two and three NNRTI RAMs were selected based on the current knowledge of the ETR resistance profile. Data are displayed for each isolate as median FC (Q1 to Q3 values). Of the 31 HIV-1 SDMs with a single NNRTI RAM, that with K101P was cross-resistant to all NNRTIs tested; that with Y181I or Y181V was cross-resistant to TMC278, ETR, and NVP; and that with Y181C was sensitive to TMC278 (FC [Q1 to Q3], 2.7 [1.9 to 3.6]) and EFV but resistant to NVP and ETR (a low level of resistance; FC [Q1 to Q3], 4.0 [3.2 to 5.4]). The HIV-1 SDM that had only the highly prevalent NNRTI RAM K103N was sensitive to TMC278. Overall, 3/31, 12/31, 7/31, and 14/30 of the HIV-1 HXB2 SDMs with a single NNRTI RAM were resistant to TMC278, EFV, ETR, and NVP, respectively. Of the 16 HIV-1 HXB2 SDMs with two NNRTI RAMS that were investigated, 7 retained sensitivity to TMC278; 8 were cross-resistant to TMC278, EFV, ETR, and NVP; and 1 was resistant to TMC278 but retained sensitivity to EFV (V179F, Y181I). Cross-resistance between TMC278, EFV, and NVP was observed for the ETR-resistant mutants with three NNRTI RAMs. Overall, among the 55 SDMs, a

TABLE 3. *In vitro* antiviral activities of TMC278, EFV, ETR, and NVP against 55 SDMs of HIV-1/HXB2 harboring one, two, or three NNRTI RAMs

NNRTI RAM(s)	Median FC (Q1–Q3)[a] in the antiviral activity of:			
	TMC278	EFV	ETR	NVP
V090I	1.7 (1.4–1.9) (n = 4)	1.6 (1.4–1.8) (n = 4)	1.5 (1.4–1.6) (n = 4)	4.4 (3.4–5.1) (n = 4)
L100I	0.9 (0.5–1.1) (n = 73)	20.3 (15.5–28.9) (n = 106)	1.3 (1.0–1.8) (n = 107)	7.3 (4.7–10.1) (n = 43)
K101E	2.4 (2.0–4.7) (n = 9)	3.8 (2.7–4.2) (n = 10)	2.7 (1.5–4.2) (n = 10)	ND
K101P	51.7 (34.7–64.1) (n = 48)	72.3 (39.0–96.3) (n = 45)	5.3 (4.6–6.4) (n = 48)	>166.1 (108.9–244.5) (n = 3)
K101Q	1.6 (0.9–2.2) (n = 8)	2.4 (1.7–3.5) (n = 8)	1.4 (1.1–2.5) (n = 8)	2.5 (1.9–3.6) (n = 5)
K103N	0.9 (0.6–1.2) (n = 72)	32.5 (23.9–43.0) (n = 113)	0.9 (0.7–1.1) (n = 110)	>42.1 (33.5–54) (n = 51)
K103S	1.6 (1–2) (n = 4)	4.8 (3.8–6.7) (n = 3)	0.8 (0.8–1.1) (n = 4)	59.4 (35.9–83.0) (n = 2)
V106A	0.6 (0.5–0.9) (n = 6)	2.0 (1.5–2.8) (n = 4)	0.5 (0.5–0.6) (n = 6)	49.0 (45.3–81.7) (n = 3)
V106M	0.9 (0.7–1.5) (n = 7)	2.6 (1.3–3.7) (n = 8)	0.9 (0.6–1.0) (n = 6)	6.3 (6.2–10.4) (n = 5)
V108I	0.7 (0.6–1.0) (n = 4)	1.5 (1.2–1.9) (n = 5)	0.7 (0.7–0.9) (n = 5)	3.1 (2.5–3.9) (n = 4)
E138A	2.5 (2.3–2.6) (n = 4)	1.6 (1.4–2.2) (n = 4)	2.9 (2.6–3.1) (n = 4)	2.3 (2.1–3.0) (n = 4)
E138G	1.6 (1.4–2.2) (n = 4)	0.9 (0.8–1.3) (n = 4)	2.4 (1.6–3.3) (n = 4)	2.8 (2.5–2.9) (n = 3)
E138K	2.8 (1.7–3.9) (n = 10)	2.0 (1.4–2.3) (n = 10)	2.6 (2.0–4.1) (n = 12)	1.1 (1.1–1.3) (n = 5)
E138Q	2.7 (2.1–3.9) (n = 7)	3.4 (2.8–5.6) (n = 8)	3.0 (2.5–3.7) (n = 7)	5.1 (3.9–6) (n = 6)
E138R	3.3 (2.9–3.6) (n = 4)	2.2 (2.0–2.5) (n = 4)	3.6 (3.2–4.2) (n = 4)	4.4 (4.1–4.7) (n = 4)
E138S	2.7 (2.5–2.8) (n = 4)	1.4 (1.1–1.7) (n = 4)	2.8 (2.1–3.5) (n = 4)	3.8 (2.0–5.9) (n = 4)
V179D	1.7 (1.1–2.4) (n = 6)	2.7 (2.5–2.9) (n = 6)	1.9 (1.4–2.5) (n = 6)	4.6 (3.7–4.9) (n = 3)
V179E	1.2 (0.7–1.9) (n = 7)	5.7 (4.0–7.6) (n = 9)	1.3 (1.2–3.1) (n = 9)	2.1 (1.3–3.2) (n = 4)
V179F	<0.1 (0.1–0.1) (n = 5)	<0.4 (0.2–0.6) (n = 6)	<0.2 (0.1–0.2) (n = 5)	0.6 (0.5–1.1) (n = 3)
V179T	1.5 (1.5–1.7) (n = 3)	1.0 (0.9–1.0) (n = 3)	0.7 (0.7–0.8) (n = 3)	0.8 (n = 1)
Y181C	2.7 (1.9–3.6) (n = 151)	2.1 (1.3–3.0) (n = 197)	4.0 (3.2–5.4) (n = 191)	>43.0 (34.7–76.7) (n = 60)
Y181I	15.3 (12.9–16.8) (n = 3)	1.6 (1.3–1.9) (n = 4)	12.5 (10.7–13.2) (n = 3)	>65.7 (60.6–71.5) (n = 3)
Y181V	12.2 (10.8–15.2) (n = 4)	3.0 (2.3–3.7) (n = 4)	15.1 (14.2–20.7) (n = 3)	2,155.9 (n = 1)
Y188L	2.8 (2.1–4.1) (n = 71)	42.6 (31.3–56.5) (n = 106)	1.1 (0.9–1.4) (n = 110)	>42.1 (33.1–65.4) (n = 45)
G190A	1.1 (0.8–1.2) (n = 12)	8.1 (6.3–10.1) (n = 17)	1.1 (0.8–1.4) (n = 14)	>86.6 (49.6–92) (n = 11)
G190S	0.2 (0.2–0.4) (n = 7)	94.8 (73.4–131) (n = 8)	0.2 (0.2–0.3) (n = 8)	96.9 (54.2–149.1) (n = 4)
H221Y	1.8 (1.3–2.2) (n = 4)	1.6 (1.3–1.9) (n = 4)	1.6 (1.1–2.2) (n = 4)	4.3 (3.7–4.6) (n = 4)
M230I	2.7 (2.5–3.7) (n = 3)	5.0 (4.6–5.9) (n = 10)	3.5 (3–4.1) (n = 9)	13.6 (10.8–18.6) (n = 8)
M230L	3.4 (2.6–4) (n = 4)	5.9 (3.9–11.1) (n = 9)	3.6 (2.6–5) (n = 8)	20.4 (16.8–21.3) (n = 7)
M230V	2.1 (1.5–3.5) (n = 4)	1.1 (1–1.3) (n = 11)	0.8 (0.8–1) (n = 8)	1.4 (1.2–1.7) (n = 8)
M236L	1.1 (1–1.3) (n = 4)	1.0 (0.6–1.7) (n = 4)	1.2 (1.0–1.4) (n = 4)	3.4 (n = 1)
L100I, K101E	2.1 (1.4–2.7) (n = 4)	49.1 (41.3–50.4) (n = 3)	0.9 (0.9–1.0) (n = 4)	18.1 (12.9–24.5) (n = 4)
L100I, K103N	7.0 (4.7–9.7) (n = 138)	>575.8 (56–1,172.2) (n = 149)	4.0 (2.9–5.3) (n = 178)	>42.5 (33.9–66.8) (n = 48)
K101E, K103N	2.0 (1.2–3.2) (n = 71)	56.4 (39–86.3) (n = 149)	1.8 (1.2–2.7) (n = 152)	>41.6 (29.9–59.3) (n = 85)
K103N, F227L	0.5 (0.4–0.6) (n = 3)	8.9 (7.1–16.2) (n = 3)	0.5 (0.4–0.6) (n = 3)	141.1 (109.0–173.1) (n = 2)
K103N, V108I	0.5 (0.5–0.8) (n = 3)	96.0 (71.1–107.8) (n = 4)	0.7 (0.6–0.7) (n = 4)	>205.2 (136.7–237.8) (n = 3)
K103N, Y181C	3.5 (2.1–4.8) (n = 246)	36.5 (25.2–50.7) (n = 420)	4.1 (2.8–5.5) (n = 426)	>41.8 (29.6–60.6) (n = 128)
K103N, Y181I	94.9 (80.0–117.5) (n = 3)	6.4 (5.5–7.5) (n = 3)	16.1 (13.5–21.8) (n = 3)	>71.5 (68.6–74.4) (n = 2)
E138K, M230L	22.6 (20.8–24.0) (n = 3)	18.7 (17.9–22.4) (n = 3)	19.3 (17.5–26.3) (n = 3)	>63.7 (61.9–70.5) (n = 6)
V179D, Y181C	6.0 (3.9–6.1) (n = 3)	9.3 (9.2–9.7) (n = 3)	10.8 (8.6–11.6) (n = 3)	>71.5 (68.6–74.4) (n = 2)
V179E, Y181C	6.8 (6.0–7.9) (n = 3)	13.1 (10.9–14.6) (n = 3)	30.6 (24.3–36.9) (n = 3)	>71.5 (68.6–74.4) (n = 2)
V179F, Y181C	8.7 (6.0–12.2) (n = 79)	4.6 (3.6–7.5) (n = 76)	158.9 (113.6–211.6) (n = 79)	>358.3 (267.4–459.3) (n = 7)
V179F, Y181I	11.4 (10.3–27.4) (n = 3)	1.0 (1.0–1.1) (n = 3)	122.8 (101.4–146.5) (n = 3)	>71.5 (68.6–74.4) (n = 2)
V179I, Y181C	3.7 (3.2–4.0) (n = 3)	1.3 (1.3–2) (n = 3)	4.6 (4.3–12.8) (n = 3)	139.9 (107.2–172.5) (n = 2)
Y181C, F227C	23.6 (17.3–24.7) (n = 3)	12.1 (11.5–13.5) (n = 4)	25.3 (18.6–31.5) (n = 3)	>77.3 (71.5–77.4) (n = 3)
Y181C, G190S	3.4 (3.3–4.3) (n = 3)	486.0 (363.8–604.7) (n = 3)	20.4 (17.0–26.8) (n = 3)	>71.5 (68.6–74.4) (n = 2)
Y181C, Y188L	30.2 (25.9–37.9) (n = 3)	237.5 (178.8–280.2) (n = 4)	6.0 (3.7–10.3) (n = 4)	>157.7 (134.0–181.5) (n = 2)
L100I, K103N, E138G	33.2 (23.6–62.7) (n = 6)	1,695.3 (1,453.9–1,796.3) (n = 6)	20.8 (10.9–24.3) (n = 5)	>58.1 (50.4–65.7) (n = 7)
L100I, K103N, T386A	22.8 (16.5–27.5) (n = 3)	10,866.5 (8,349.2–12,035.2) (n = 3)	12.2 (10.2–15.7) (n = 3)	>51.6 (n = 1)
L100I, K103N, V179L	46.1 (42.1–63.1) (n = 3)	5,660.6 (5,405.2–6,002.4) (n = 3)	13.4 (13.1–15.9) (n = 3)	>71.5 (68.6–74.4) (n = 2)
L100I, K103N, Y181C	80.8 (66.0–109.4) (n = 7)	1,812.0 (1,496.9–2,110.8) (n = 5)	58.1 (34.8–73.2) (n = 7)	468.1 (395.5–540.8) (n = 2)
L100I, V179I, Y181C	15.2 (9.1–22.1) (n = 4)	16.9 (14.6–17.2) (n = 4)	34.1 (27.0–42.6) (n = 4)	>205.2 (128.4–267.8) (n = 3)
K101P, K103N, V108I	>162.1 (115.8–314.9) (n = 3)	12,931.1 (11,343.6–13,462.3) (n = 3)	18.4 (17.3–19.7) (n = 3)	>51.6 (n = 1)
K103N, V179I, Y181C	10.5 (8.2–14.1) (n = 9)	16.7 (12.6–27.3) (n = 8)	14.5 (10.4–17.2) (n = 8)	>429.9 (252.5–445.1) (n = 7)
V179F, Y181C, F227C	553.8 (386.7–578.5) (n = 3)	25.7 (22.5–57.7) (n = 3)	638.6 (559.5–763.1) (n = 3)	>71.5 (68.6–74.4) (n = 2)

[a] Fold changes (FCs) were calculated as the ratio between the 50% effective concentration of the compound for the HIV-1 SDM and that obtained in the same experiment with HIV-1/IIIB. Q1–Q3, first- to third-quartile values; n, number of experiments; ND, not determined.

smaller proportion were resistant to TMC278 (36%) and ETR (49%) than to EFV (62%) or NVP (70%).

Description of HIV-1 mutant strains selected in the presence of TMC278. After 32 days in culture, no viral replication was detected in cells infected with HIV-1 IIIB at a high MOI at any concentration of TMC278 greater than or equal to 40 nM (Fig. 4) (17). The same results were also observed for recombinant clinical HIV-1 isolates of group M subtypes A1, AE, AG, BG, C, D, F1, G, and H (Table 4) and for two HIV-1 HXB2 SDMs containing either the NNRTI RAM K103N (Fig. 5A) or Y181C (Fig. 5B). Together these data showed that a 40 nM concentration of TMC278 was sufficient to completely prevent the replication of these HIV-1 strains.

The results from these high-MOI selection experiments also showed that 10 nM TMC278 was a suboptimal permissive concentration at which new HIV-1 strains emerged from subtypes A1, B, BG, and D and from the two HIV-1 SDMs resistant to TMC278. These emerging HIV-1 strains were sequenced and found to harbor combinations of the NNRTI RAMs L100I, K101E, V106I, Y181C, Y181I, and/or M230I.

For the low-MOI and escalating drug concentration experiments, mutant strains resistant to TMC278 were isolated from cell cultures containing initial TMC278 concentrations ranging from 5 nM to 16 μM starting from wild-type and NNRTI-resistant strains from various group M subtypes. The resistance profiles of these 12 HIV-1 strains are presented in Table 5. The

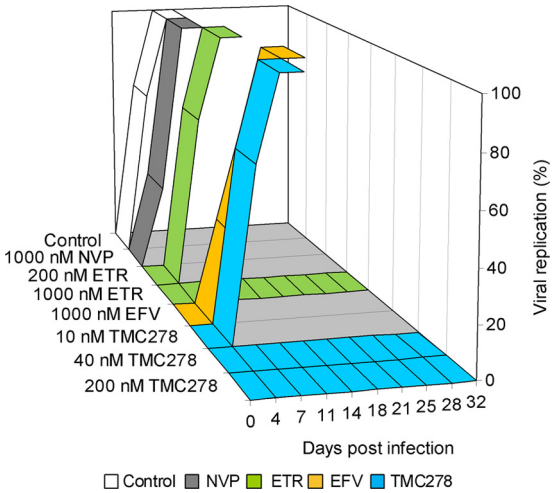

FIG. 4. Selection of viruses resistant to TMC278, ETR, EFV, or NVP starting from wild-type HIV-1 IIIB. The time to breakthrough of resistant viruses was determined in cell culture, over a 32-day period, under selective pressure from various concentrations of TMC278, ETR, EFV, and NVP, or in the absence of NNRTIs (control).

data presented are limited to the emerging strains that were resistant to TMC278 (TMC278 FC, 3.8 to 13,920.7). These isolates exhibited as many as five NNRTI RAMs. A greater number of emerging RAMs was associated with a higher TMC278 FC. The HIV-1 strain emerging from r13817 (subtype AE) harbored E138G in combination with M230I and H221Y. In this experiment, E138G evolved under further selective pressure to E138R; this final alteration from glycine to arginine resulted in significant increases in the FC for TMC278 (from 66.8 to >1,932.0), EFV (from 268.3 to 4,849.9), and ETR (from 180.2 to >3,787.9). The increase in FC for TMC278, EFV, and ETR in these isolates cannot be attributed solely to the presence of E138R, because the HIV-1 SDMs containing E138R had FC values of 3.2, 2.4, and 3.7 for TMC278, EFV, and ETR, respectively. The E138R mutation was also observed to emerge in combination with H221Y from the mutant strain SM041, which contained E138K at day zero.

Genotypic analyses of the viral RT of the strains emerging under selective pressure from TMC278, which were performed with a range of viruses of different origins and genotypic profiles, suggest that the *in vitro* resistance profile of TMC278 may include the mutations V90I, L100I, K101E, V106A/I, V108I, E138G/K/Q/R, V179F/I, Y181C/I, V189I, G190E, H221Y, F227C, and M230I/L.

TABLE 4. Time to viral replication of HIV 1 group M subtypes in the presence of three doses of TMC278

HIV-1 subtype	Days to viral replication breakthrough with the following dose of TMC278:		
	10 nM	40 nM	200 nM
A1	10	>31	>31
AE	>31	>31	>31
AG	>31	>31	>31
B	7	>31	>31
BG	10	13	>31
C	>31	>31	>31
D	20	>31	>31
F1	>31	>31	>31
G	>31	>31	>31
H	>31	>31	>31

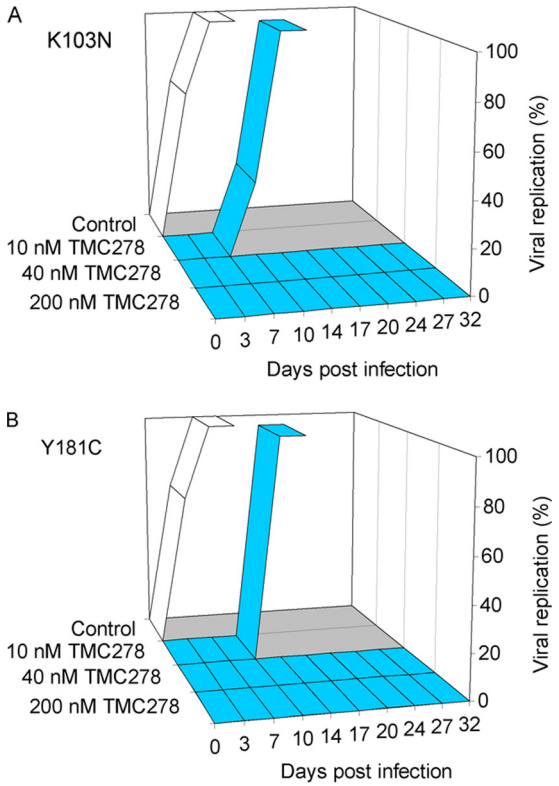

FIG. 5. Selection of viruses resistant to TMC278, EFV, ETR, or NVP, starting from recombinant viral strains containing the NNRTI RAM K103N (A) or Y181C (B). The time to breakthrough of resistant viruses was determined in cell culture, over a 32-day period, under selective pressure from various TMC278 concentrations.

Antiviral activity of TMC278 in the presence of human serum proteins. The antiviral activity of TMC278 was compared to those of other NNRTIs in the presence or absence of human serum proteins by the GFP assay. The data presented in Table 6 show a reduction in the antiviral activity of TMC278 in the presence of 50% human serum and 45 mg/ml HSA, as demonstrated by median EC_{50} ratios of 18.5 and 39.2, respectively. Comparable results were obtained for EFV.

Antiviral activity of TMC278 in combination with other ARVs. Combinations of TMC278 with the NRTIs 3TC, ABC, AZT, d4T, FTC, and ddI; the NtRTI TDF; the PIs APV, ATV, DRV, IDV, LPV, NFV, RTV, SQV, and TPV; the NNRTIs EFV, ETR, and NVP; the integrase inhibitor RAL; the fusion inhibitor ENF; and the binding inhibitor MVC were studied. On the basis of the methodologies used, all combinations were scored as additive, except for the combinations with 3TC, AZT, and RAL, which showed low levels of synergy (see the supplemental material).

DISCUSSION

The current *in vitro* data demonstrate that TMC278, an inhibitor of HIV RT, is highly potent against wild-type HIV

TABLE 5. Evolution of viral variants selected with TMC278, starting from wild-type or mutant HIV-1 strains, from subtypes AE, AG, B, C, and D[a]

Subtype	Strain	Expt	Day[b]	Concn (nM)[c]	FC[d]			Mutation(s) in HIV-1 reverse transcriptase[e]
					TMC278	EFV	ETR	
Wild types								
AE	r13816[f]		0	0	0.4	1.5	0.9	
		1	69	10	3.8	7.4	5.0	**E138K**
		2	109	40	5.9	13.1	24.3	**E138K**, R358G
	r13817[f]		0	0	0.7	1.6	0.7	
			107	40	66.8	268.3	180.2	**E138G**, **V189I**, **H221Y**, **M230I**
			293	5,000	>1,932.0	4,849.9	>3,787.9	I050T, **E138R**, **V189I**, K219E, **H221Y**, **M230I**, E297K
AG	r13813[f]		0	0	0.7	0.8	0.9	
			108	40	5.4	13.4	26.2	E006A, **E138Q**, I178M, T200V, V245E
B	IIIB[g]		0	0	1.0	1.1	0.9	
		1	69	80	12.0	NA	17.9	**E138K**, **G190E**
			90	600	114.1	>12,345.8	787.4	E006K, **E138K**, **G190E**
			121	5,000	>1,830.2	>15,923.6	>3,906.3	E006K, **L100I**, **E138K**, **G190E**, K219N
		2	66	62	34.6	19.7	45.3	**K101E**, **Y181C**, T386A
			108	4,000	5,490.6	3,895.1	NA	**L100I**, **K101E**, **Y181C**, **F227C**, T386A
			122	16,000	13,920.7	>21,240.8	4,878.0	**L100I**, **K101E**, A158T, **V179F**, **Y181C**, **F227C**, T386A
		3	34	62	100.1	173.5	286.0	**V179F**, **Y181C**, **F227C**, **M230I**
			59	4,000	7,716.3	546.1	7,381.9	**V179F**, **Y181C**, **F227C**, **M230I**
		4	34	5	5.0	5.4	5.7	E040K, **Y181C**
			62	80	315.4	427.8	1,722.2	E040K, **V179F**, **Y181C**, **F227C**, **M230I**
			90	1,500	626.9	783.6	244.8	E040K , V060A, **V090I**, **Y181C**, **F227C**, **M230I**
		5	32	40	23.7	30.9	27.1	E040K, **K101E**, **V108I**, **Y181C**, **H221Y**
			52	200	144.4	402.8	117.1	E040K, **K101E**, **V108I**, **Y181C**, K219E, **H221Y**, R358K
			70	5,000	2,154.6	1,688.0	2,566.5	E040K, **K101E**, **V108I**, **Y181C**, **F227C**, **M230I**
		6	116	2,000	2,022.6	1,025.2	112.0	**L100I**, **K101E**, **Y181C**, P225L, **F227C**
C	v071130[f]		0	0	1.9	0.9	1.6	
			128	80	15.9	174.8	19.9	**E138K**, V241M, E399G
			276	5,000	>1,467.1	6,191.1	>759.9	**V108I**, **E138K**, **M230L**, Y232F, V241 M, G335Y, E399G
D	v071038[f]		0	0	0.4	0.3	0.3	
			93	80	26.0	287.8	224.0	L074V, **L100I**, T139K, **Y181C**, T240I, R284K
			139	3,000	978.9	2,455.1	2,796.7	R072K, L074V, **L100I**, T139K, **Y181C**, T240I, E396K
			163	5,000	>1,984.1	15,027.6	>2,883.5	L074V, **L100I**, **V106A**, **V108I**, T139K, **Y181C**, T240I, V241M
Mutants								
B	SM041[h]		0	0	3.1	1.5	2.0	**E138K**
			90	40	13.2	6.4	3.1	**E138R**, K173Q, **H221Y**, G273E
			160	150	185.6	27.5	60.7	**L100I**, **E138R**, K173Q, **V179I**, **H221Y**, V241I, G273E
			212	5,000	>3,004.8	159.9	>41.3	**L100I**, **V108I**, I135T, **E138R**, K173Q, **V179I**, L214F, **H221Y**, V241I, G273E
	SM051[h]		0	0	0.7	17,097.0	6.1	**L100I**, **K103N**
			93	5,000	>1,830.2	>15,923	333.1	**L100I**, **K103N**, **E138G**, **V179I**, G196R, P225Y
	SM052[h]		0	0	3.2	348	1.7	**K101E**, **K103N**
			118	5,000	>1,947.0	4,238.0	269.4	E028K, **K101E**, **K103N**, **V108I**, **E138G**, **V179I**, **Y181C**, K219R, L228R, T376S
	SDM020001[h]		0	0	3.1	1.8	9.1	**V179I**, **Y181C**
			161	5,000	970.2	130.6	1674.2	K030I, **V106I**, **V108I**, **E138K**, T165A, **V179I**, **Y181C**, **V189I**, L214F, **H221Y**, N348I, E370K
	SDM020059[h]		0	0	8.0	4.5	184.0	**V179F**, **Y181C**
			171	5,000	>2,784.0	>24,630.3	>3,041.4	**L100I**, **V108I**, **V179F**, **Y181C**, L214F, N348I, E370G, V381I
	r9602[f]		0	0	3.2	ND	3.2	K102Q, **K103N**, E122K, **Y181C**, T200I, L214F, S322T, D324E, K366R, T400A
		1	28	40	7.4	102.2	ND	T069I, K102Q, **K103N**, E122K, **E138G**, S163N, **Y181C**, **V189I**, T200I, L214F, S322T, D324E, K366R, T386A, T400A
			52	200	237.7	91.7	409.4	K102Q, **K103N**, E122K, **E138G**, **V179I**, **Y181C**, T200I, L214F, S322T, D324E, N348I, V365I, K366R, T386A, T400A
			63	1,000	156.9	26.1	159.6	K102Q, **K103N**, E122K, **E138G**, **V179I**, **Y181C**, T200I, L214F, S322T, D324E, N348I, K366R, T386A, T400A
		2	74	1,000	142.4	312.8	485.1	L074I, K102Q, **K103N**, E122K, **E138G**, **V179I**, **Y181C**, T200I, L214F, S322T, D324E, N348I, K366R, T386A, T400A
			109	10,000	460.3	332.8	635.2	M041I, L074I, K102Q, **K103N**, **V108I**, E122K, **E138G**, **V179I**, **Y181C**, T200I, E203K, L214F, S322T, D324E, N348I, K366R, T386A, T400A

[a] The experiments for which results are presented are those in which viruses resistant to TMC278 (FC, >3.7) were obtained.
[b] Number of days in cell culture.
[c] TMC278 concentration at which the resistant isolate was selected.
[d] Calculated as the ratio between the 50% effective concentration of the compound for the emerging HIV-1 strain and that obtained in the same experiment with HIV-1/IIIB. Each result is the mean for a single experiment run in duplicate. NA, not applicable; ND, not done.
[e] The first 400 amino acids of the reverse transcriptase were sequenced. Emerging mutations are underscored, and NNRTI resistance-associated mutations are in boldface.
[f] Recombinant HIV-1 clinical isolate.
[g] Laboratory derived HIV-1 strain.
[h] Site-directed mutant derived from HIV-1 HXB2.

TABLE 6. Influence of human serum proteins on *in vitro* anti-HIV activities of TMC278, EFV, ETR, and NVP

NNRTI	Median EC_{50} ratio[a] (Q1–Q3)		
	1 mg/ml AAG	45 mg/ml HSA	50% human serum
TMC278	1.8 (1.5–2.1) (n = 13)	39.2 (25.7–54.1) (n = 12)	18.5 (11.0–40.4) (n = 13)
EFV	3.8 (3.2–4.1) (n = 5)	17.3 (13.2–19.4) (n = 5)	12.3 (10.2–16.2) (n = 5)
ETR	3.4 (2.0–4.4) (n = 17)	4.9 (4.0–5.9.) (n = 17)	5.7 (4.9–12.1) (n = 16)
NVP	2.0 (1.5–2.9) (n = 7)	2.9 (1.5–3.3) (n = 7)	3.8 (1.8–5.1) (n = 7)

[a] Ratio of the 50% effective concentration (EC_{50}) in the presence of serum proteins to that in the absence of serum proteins. Q1–Q3, first- to third-quartile values; n, number of experiments.

and NNRTI-resistant mutants that emerge after treatment with EFV or NVP. The inhibitory activity of TMC278 on the HIV-1 RT was comparable to that observed for ETR but higher than that of EFV or NVP (5). The *in vitro* antiviral activity of TMC278 against HIV-1 IIIB was in the subnanomolar concentration range and was overall greater than that of the other NNRTIs investigated. A selectivity index of >8,000 *in vitro* indicated that TMC278 is a potent and specific inhibitor of HIV-1. Crystal structures of the binding site between TMC278 and the HIV-1 RT complex, as well as freedom-of-motion analyses, revealed that TMC278 binds to the HIV-1 RT and adapts to changes in the NNRTI-binding pocket. TMC278 thereby compensates for the presence of drug resistance mutations, which further explains the mechanism of the increased genetic barrier to resistance to this compound (15, 20).

Although the HIV-1 group M subtype B continues to predominate in Europe and the United States, the prevalence of non-B subtypes is increasing over time in these areas and in other regions of the world (4, 30, 37; HIV-1 Sequence Database, 2008 [http://www.hiv.lanl.gov/components/sequence/HIV/new_geography/geography.comp?region=world&form=all]). We demonstrated comparable antiviral activities against wild-type HIV-1 recombinant and primary clinical isolates from group M and O subtypes, with median EC_{50} values ranging from 0.07 to 1.01 nM and 2.88 to 8.45 nM, respectively. TMC278 was on average approximately 10- to 15-fold less active against the HIV-1 group O isolates than against the HIV-1 group M primary isolates. However, TMC278 retained greater activity against the group O isolates than did EFV and ETR (which were, on average, approximately 90-fold and 40-fold less active, respectively, against the HIV-1 group O isolates). *In vitro* antiviral activity of TMC278 in the micromolar range was also detected against HIV-2, although the clinical significance of this activity is unknown. These observations indicate that a broad spectrum of HIV strains are sensitive to TMC278, a member of the next-generation NNRTIs, among which ETR is so far the only one approved for the treatment of NNRTI-experienced patients.

Analysis of sensitivity to TMC278 in a panel of 4,786 HIV-1 recombinant clinical isolates resistant to at least one of the first-generation NNRTIs (EFV or NVP) showed that the cross-resistance that exists between EFV and NVP is not shared to the same extent by TMC278 and ETR, with the majority of the isolates (62%) retaining sensitivity to these novel NNRTIs.

More detailed information on the resistance profile of TMC278 was obtained from *in vitro* experiments using a series of HIV-1 HXB2 SDMs. We observed that TMC278 retained antiviral activity against a number of HIV-1 strains containing NNRTI RAMs. Analysis of the SDMs containing a single NNRTI RAM showed that the presence of K101P, Y181I, and Y181V resulted in phenotypic resistance to TMC278, while the other 28 SDMs exhibited FC values below the current BCO of 3.7 for TMC278. The HIV-1 SDMs with the most common NNRTI RAMs, K103N and Y181C, were sensitive to TMC278. The sensitivity to TMC278 of an HIV-1 SDM containing Y181C has been described recently (24, 45). Of note, however, the variant carrying Y181C alone that emerged in one of the passage experiments with HIV-1 IIIB had a TMC278 FC of 5.0. This apparent discordance between the SDM and emerging variant results on the impact of Y181C could be explained by the fact that, in a selection experiment setting, other variants present at frequencies below the sensitivity of population sequencing techniques could have contributed to the phenotypic TMC278 FC observed. In this particular case, three additional NNRTI mutations, V179F, F227C, and M230I, that were detected at subsequent time points could have been present when Y181C was the only detectable mutation. However, it remains possible that the Y181C mutation plays a more significant role in TMC278 susceptibility than that observed in our SDM experiments. Ongoing phase III clinical studies will help to answer this question. Cross-resistance between TMC278, EFV, and NVP was observed with most mutants carrying two NNRTI RAMs and all mutants carrying three NNRTI RAMs, at least for those presented in Table 3, which were generated on the basis of prior knowledge about ETR resistance. SDMs containing the V179F RAM in combination with other mutations showed lower levels of resistance to TMC278 than to ETR, while those containing K101P showed a higher level of resistance to TMC278 than to ETR. This observation points out potential differences in the resistance profiles of TMC278 and ETR. While high levels of resistance to EFV and NVP often occurred with strains containing one NNRTI RAM, most of these strains retained sensitivity to both TMC278 and ETR. Of interest, some of the NNRTI RAMs present in the SDMs that showed resistance to TMC278 are uncommon in NNRTI treatment-experienced patients, as previously reported in an analysis of >100,000 HIV-1 recombinant clinical isolates received for routine clinical testing (42, 46). In these analyses, prevalences of 2.04%, 1.05%, and 0.63% were found for K101P, Y181I, and Y181V, respectively.

Comparative *in vitro* viral replication of wild-type and mutant HIV-1, in the presence of various concentrations of TMC278 and other NNRTIs, provided insight into the potential *in vitro* pathways of resistance of HIV-1 to TMC278. A TMC278 concentration of 40 nM, lower than that needed for any of the other NNRTIs studied, was sufficient to prevent the

replication of various wild-type HIV-1 group M subtypes inoculated at high MOIs and cultured for 32 days in the presence of a fixed concentration of TMC278. A preliminary *in vitro* resistance profile was determined for TMC278 from selection over extended periods of resistant strains from various viral isolates inoculated at low MOIs and cultured in the presence of increasing TMC278 concentrations. The data showed that in the RTs of variant strains, emerging NNRTI RAMs were detected in various combinations of one to five. The FC values for TMC278 increased with the number of NNRTI RAMs, and the NNRTI RAMs emerging under selective pressure from TMC278 included V90I, L100I, K101E, V106A/I, V108I, E138G/K/Q/R, V179F/I, Y181C/I, V189I, G190E, H221Y, F227C, and M230I/L. E138R was identified as a novel NNRTI RAM. Based on our data obtained with SDMs, none of the E138 alleles, on their own, caused resistance to TMC278, EFV, ETR, or NVP. However, it has been reported that E138K can cause resistance to some experimental HIV-1 RT inhibitors (36). In our experiments, we observed the emergence of mutations at position E138 in the isolates resistant to TMC278, obtained under TMC278 selective pressure. We noted that E138G/R mutations were always present in combination with other NNRTI RAMs known to induce a high level of resistance to NNRTIs, such as L100I and M230I, either at the time of testing or at the next time point analyzed. Furthermore, an interaction between E138 and TMC278 has been reported at the molecular level (15), but no explanation was provided as to why mutations at position E138 do not confer resistance on their own. Future work with TMC278 will help elucidate the role of mutations at position E138 in NNRTI resistance.

Comparison of the TMC278 and ETR FC values, as well as the genetic profiles of strains emerging under TMC278 pressure (49) and results obtained with SDMs, suggests that considerable cross-resistance exists between these two compounds. Studies are in progress to further characterize the impact of specific combinations of NNRTI RAMs, which are found in viruses emerging from selection with TMC278, on the antiviral activity of ETR.

We investigated the antiviral activity of TMC278 in the presence of human serum proteins. Drugs, such as NNRTIs, can bind to plasma proteins, which may restrict their entry into cells and reduce their activity (8). The concentrations of the human serum proteins HSA and AAG added to the cell cultures were selected based on their respective physiological levels in healthy and HIV-1-infected individuals (12, 34). The antiviral activity of TMC278 in the presence of human serum proteins was comparable to that of EFV, which has demonstrated clinical efficacy. The latter finding suggests that the level of impact of human serum proteins on TMC278 activity is fully compatible with the observed clinical efficacy (22). Combination experiments showed that, *in vitro*, the antiviral activity of TMC278 was not altered by the presence of other ARVs investigated, except for low-level synergy with 3TC, AZT, and RAL.

Clinical trials of TMC278 have been initiated with treatment-naïve HIV-1-infected patients, with one phase IIB dose-finding study (TMC278-C204) and two phase III efficacy studies (TMC278-C209 [ECHO] and TMC278-C215 [THRIVE]). In the TMC278-C204 study (38, 39), 76% of patients receiving the 25-mg dose of TMC278, which is being studied in the phase III trials, achieved a viral load of <50 copies/ml at week 96 (TLOVR analysis) compared with 71% of patients receiving EFV. The proportions of patients receiving the 75-mg and 150-mg doses of TMC278 who achieved viral loads of <50 copies/ml at week 96 were 72% and 71%, respectively (38). TMC278 demonstrated generally better tolerability than EFV for as long as 96 weeks (38).

The *in vitro* results presented here suggest that TMC278 is a potent next-generation NNRTI, with a higher genetic barrier and a more robust resistance profile than EFV and NVP. As such, TMC278 has the potential to become a valuable drug in the treatment of HIV-1-infected patients.

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