Reverse Transcriptase Inhibitors as Potential Colorectal Microbicides[†]

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We investigated whether reverse transcriptase (RT) inhibitors (RTI) can be combined to inhibit human immunodeficiency virus type 1 (HIV-1) infection of colorectal tissue ex vivo as part of a strategy to develop an effective rectal microbicide. The nucleotide RTI (NRTI) PMPA (tenofovir) and two nonnucleoside RTI (NNRTI), UC-781 and TMC120 (dapivirine), were evaluated. Each compound inhibited the replication of the HIV isolates tested in TZM-bl cells, peripheral blood mononuclear cells, and colorectal explants. Dual combinations of the three compounds, either NRTI-NNRTI or NNRTI-NNRTI combinations, were more active than any of the individual compounds in both cellular and tissue models. Combinations were key to inhibiting infection by NRTI- and NNRTI-resistant isolates in all models tested. Moreover, we found that the replication capacities of HIV-1 isolates in colorectal explants were affected by single point mutations in RT that confer resistance to RTI. These data demonstrate that colorectal explants can be used to screen compounds for potential efficacy as part of a combination microbicide and to determine the mucosal fitness of RTI-resistant isolates. These findings may have important implications for the rational design of effective rectal microbicides.

To date, the field of microbicides has focused its efforts largely on the development of products designed to prevent vaginal transmission of human immunodeficiency virus (HIV). While there is a clear and urgent need for vaginal microbicides (34, 73), they may provide little or no protection for men and women against rectal transmission of HIV. Furthermore, since receptive anal intercourse (RAI) between serodiscordant couples is associated with the highest probability of HIV transmission (40, 43, 66, 80), even a low frequency of RAI may be an important confounder in the interpretation of results from current vaginal microbicide trials. The relatively high vulnerability of the colorectal tract to HIV type 1 (HIV-1) transmission is likely due to histological and immunological differences between the intestinal and genital mucosae. Rectal mucosa has a single-cell columnar epithelium, in contrast to the pluristratified squamous epithelium of the lower female genital tract. Moreover, the intestinal lamina propria contains an abundance of highly activated target cells for HIV infection (3, 42, 69), is capable of transferring infectious virus to the underlying lymphoid tissue (2, 64), and is the major site of viral replication and CD4⁺ T-cell depletion during acute infection (9).

Although a number of candidate vaginal microbicides have progressed into phase III clinical trials, assessment of their safety and efficacy for rectal use has been significantly delayed, and currently the most advanced rectal microbicide products are only in phase I. Furthermore, differences in the dynamics of drug absorption, distribution, local retention, and clearance

between the two compartments make it highly unlikely that the same formulation will be safe and effective for both vaginal and rectal application. The anatomical and physiological characteristics of the colorectal tract, together with the fact that the gut is the main site for HIV replication, mean that an effective rectal microbicide will have to protect not only a larger area, but also a higher number of potential target cells, than a vaginal microbicide. Therefore, protection against HIV transmission via the colorectum may require the use of highly potent drugs, including reverse transcriptase inhibitors (RTIs) already used in effective highly active antiretroviral (ARV) therapy.

The assessment of potential RTI candidates for development as effective colorectal microbicides should include not only their inhibitory activities against wild-type HIV-1 isolates but also their activities against prevalent resistant strains. The ideal candidate(s) should have a high genetic barrier to resistance (requiring more than a single mutation) and ideally should not induce cross-class resistance to other therapeutic drugs. This would be particularly important for single-agent RTI microbicides, where escape mutants could emerge by the generation of natural genetic variation in the virus and/or by the selection of drug-resistant variants in infected individuals during therapy. Indeed, in the United States and Europe, it is estimated that 10% to 20% of new infections are caused by HIV-1 strains harboring resistance to at least one of the three main types of ARV drugs: entry/fusion inhibitors, RTIs, or protease inhibitors (6, 11, 12, 30, 46, 58, 70, 77). Although levels of RTI resistance in the developing world are not fully characterized (26, 48, 49, 63, 72), they are likely to increase with the scale-up of ARV therapy. The development of combination ARV rectal microbicides might provide an important defense against rectal transmission of resistant strains of HIV-1 in regions where treatment of chronic HIV infection has resulted in the emergence of ARV resistance.

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Therefore, we investigated the inhibitory activities of three RTIs as potential components of a rectal microbicide: the nucleotide RTI (NRTI) PMPA, also referred to as tenofovir (currently in phase II trials as a vaginal microbicide), and two nonnucleoside RTIs (NNRTIs), UC-781 and TMC120 (dapi-virine) (both currently in phase I trials). We assessed the activities of these RTI compounds individually and in dual combinations against wild-type HIV-1 and against RTI-resistant isolates. Their activities in cellular models and colorectal explants suggest that RTI combinations could be key to the rational design of effective colorectal microbicides.

MATERIALS AND METHODS

Reagents and plasmids. 9-[R-2-(Phosphonylmethoxy)propyl] adenine monohydrate (PMPA, or tenofovir) was donated by Gilead Sciences, Inc. (Foster City, CA); UC-781 was donated by Biosyn, Inc. (Huntington Valley, PA); and TMC120 (dapivirine) was donated by the International Partnership for Microbicides (Silver Spring, MD).

The full-length, replication- and infection-competent proviral HIV-1 clones pYU2 (44, 45) and pNL4-3 (1) and the infectious RTI-resistant clones HIV-1_{IIIB} A17 (with the K103N and Y181C mutations in the RT domain) (65), HIV-1 71361-1 (K65R), and HIV-1 8415-2 (K65R and M184V) (29) were provided by the NIH AIDS Research and Reference Reagent Program (<http://www.aidsreagent.org/>). R8-BaL was kindly provided by T. Hope at Northwestern University (Chicago, IL).

Cell and virus culture conditions. All cell cultures were maintained at 37°C under an atmosphere containing 5% CO₂. TZM-bl cells (25, 68, 83) were grown in Dulbecco's minimal essential medium (Sigma-Aldrich, Inc., St. Louis, MO) containing 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (100 U of penicillin/ml and 100 µg of streptomycin/ml). Peripheral blood mononuclear cells (PBMCs) were isolated from multidonor buffy coats from healthy HIV-seronegative donors by centrifugation onto Ficoll-Hypaque, mitogen stimulated as previously described (37), and maintained in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, antibiotics (100 U of penicillin/ml and 100 µg of streptomycin/ml), and 100 U of interleukin-2/ml.

The molecular clones and the laboratory-adapted HIV-1_{RF}, HIV-1_{IIIB}, and HIV-1_{BaL} isolates were passaged through activated PBMCs for 11 days.

Patients and tissue explants. Surgically resected specimens of intestinal tissue were collected at St George's Hospital, London, United Kingdom, after receipt of signed informed consent. All patients were HIV negative. All tissues were collected under protocols approved by the Local Research Ethics Committee. Colorectal tissue was obtained from patients undergoing rectocele repair and colectomy for colorectal cancer. Only healthy tissue obtained 10 to 15 cm away from any tumor was employed. Following transport to the laboratory, muscle was stripped from the resected tissue, which was then cut into 2- to 3-mm³ explants comprising both epithelial and muscularis mucosae as described previously (33). Colorectal explants were maintained with Dulbecco's minimal essential medium containing 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (100 U of penicillin/ml, 100 µg of streptomycin/ml, and 80 µg of gentamicin/ml) at 37°C under an atmosphere containing 5% CO₂.

Infectivity and inhibition assays. The infectivities of virus preparations were estimated in TZM-bl cells (by luciferase quantitation of cell lysates [Promega, Madison, WI]) and PBMCs (by measurement of the p24 antigen content in cell culture supernatants). Briefly, TZM-bl cells were seeded at 3×10^5 /well 24 h prior to infection with HIV isolates. After incubation for 2 days, the cells were washed with phosphate-buffered saline (PBS) and lysed with 100 µl of luciferase cell culture lysis reagent (39). Fifty microliters was transferred to a white, opaque assay plate for luciferase quantification in a Synergy HT Multi-Detection microplate reader (BioTek Instruments, Inc., Burlington, VT) using 50 µl of luciferase assay reagent. The extent of luciferase expression was recorded in relative light units. The p24 antigen content in culture supernatants was measured using an in-house enzyme-linked immunosorbent assay (ELISA) that includes an affinity-purified sheep polyclonal antibody (D7320; Aalto BioReagents, Dublin, Eire) as a capture antibody and has a sensitivity limit of 3 to 10 pg of recombinant p24 per 100 µl, as previously described (57, 62).

Inhibition assays were performed using a standardized viral inoculum. Cells or tissue explants were incubated with serial dilutions of RTIs for 1 h at 37°C. For cellular infection, virus was then added to cells and left in culture for the duration of the experiment. For tissue explant studies, explants were incubated with virus for 2 h and then washed four times with PBS to remove unbound drug and virus

TABLE 1. Sensitivities of test isolates to RTIs in TZM-bl cells

Isolate	Coreceptor usage	IC ₅₀ (nM) ^a of:		
		PMPA	UC-781	TMC120
RF	X4	4,027.42 ± 886.29	6.01 ± 0.63	0.84 ± 0.12
IIIB	X4	2,296.40 ± 364.67	7.99 ± 0.41	0.53 ± 0.09
BaL	R5	2,518.67 ± 389.67	9.34 ± 2.86	0.80 ± 0.06
YU.2	R5	3,338.58 ± 1,075.76	5.87 ± 2.49	0.15 ± 0.07
NL4.3	X4	6,689.74 ± 2,791.86	10.21 ± 9.80	0.37 ± 0.30
R8.BaL	R5	4,591.30 ± 1,982.56	5.86 ± 2.44	0.19 ± 0.11

^a Data are means ± standard deviations derived from three independent experiments performed in triplicate.

before further culture of the explants on gel foam rafts (Welbeck Pharmaceuticals, United Kingdom) as previously described (33). Negative controls (without virus or drug) were included to confirm the HIV-negative status of the donors. For explant studies, approximately two-thirds of the culture supernatant was harvested on days 3, 7, 11, and 15, and the cultures were refed with fresh medium. Levels of p24 in tissue explant supernatants were quantified with the Innostest HIV antigen kit (Innogenetics, Belgium) according to the manufacturer's instructions. Each experiment was performed in triplicate, using tissues from different donors.

Statistical and mathematical analyses. Fifty percent inhibitory concentrations (IC₅₀s) were calculated from sigmoid curve fits (GraphPad Prism). For all IC₅₀ data presented, R² is >0.7.

RESULTS

Inhibitory activities of PMPA, UC-781, and TMC120 in TZM-bl cells. The inhibitory activities of nonformulated PMPA, UC-781, and TMC120 against a panel of HIV-1 clade B isolates were assessed using TZM-bl indicator cells. Both NNRTIs, UC-781 and TMC120, showed high inhibitory activities against X4 (HIV-1 RF, HIV-1 IIIB, HIV-1 NL4.3) and R5 (HIV-1 BaL, HIV-1 YU.2, HIV-1 R8-BaL) isolates, with IC₅₀s ranging from 10.21 to 5.86 nM for UC-781 and from 0.84 to 0.15 nM for TMC120, as shown in Table 1 (see also Fig. S1 in the supplemental material). PMPA inhibited the six isolates with IC₅₀s in the micromolar range (2.3 to 6.7 µM) (Table 1) (see also Fig. S1 in the supplemental material). None of the compounds demonstrated significant differences in IC₅₀s against laboratory-adapted isolates and molecular clones. No cytotoxicity was observed by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assay at the concentrations tested (data not shown).

NRTI-NNRTI or NNRTI-NNRTI combinations are more active than individual drugs in TZM-bl cells. To explore the possibility that PMPA, UC-781, and TMC120 could be used in combination as part of an effective microbicide, we compared the inhibitory activities of NRTI-NNRTI (PMPA plus UC-781 or PMPA plus TMC120) or NNRTI-NNRTI (UC-781 plus TMC120) combinations with the activity of each single compound. The molecular weights of the three compounds are similar (287.22 for PMPA, 335.85 for UC-781, and 329.41 for TMC120), so drug concentrations for dual-combination studies were based on the 1:1 ratio of inhibitory potency (IC₅₀) for each individual drug (Table 1) in TZM-bl cells; for example, the average IC₅₀ of PMPA is 347 times that of UC-781, so the fixed PMPA/UC-781 IC₅₀ ratio is 347:1. The IC₅₀ ratios employed to inhibit the panel of clade B isolates were 347:1 for PMPA-UC-781, 4,167:1 for PMPA-TMC120, and 12:1 for UC-781-TMC120 combinations. When PMPA was used in

TABLE 2. Reductions in the IC₅₀s of drugs used in combination in TZM-bl cells

Drug B and isolate	% Reduction in the IC ₅₀ of the following drug (drug A) in combination with drug B ^a :		
	PMPA	UC-781	TMC120
PMPA			
RF		50.94	72.57
IIIB		51.19	67.17
BaL		56.47	71.74
YU.2		47.85	34.15
NL4.3		51.29	33.37
R8.BaL		23.78	32.21
Mean ± SD		46.92 ± 11.67	51.87 ± 20.50
UC-781			
RF	74.59		82.10
IIIB	41.06		63.65
BaL	43.95		60.41
YU.2	68.14		15.81
NL4.3	74.18		54.78
R8.BaL	66.22		24.92
Mean ± SD	61.36 ± 14.99		50.28 ± 25.08
TMC120			
RF	76.20	70.01	
IIIB	68.18	70.82	
BaL	62.78	59.49	
YU.2	80.64	61.86	
NL4.3	80.68	80.04	
R8.BaL	83.24	70.15	
Mean ± SD	75.29 ± 8.11	68.73 ± 7.33	

^a The percentage of reduction in the IC₅₀ of each compound when used in combination was calculated as follows: $\{1 - [\text{IC}_{50}(\text{drug A} + \text{drug B})/\text{IC}_{50}(\text{drug A})]\} \times 100$. The data are means derived from three independent experiments performed in triplicate.

combination with either of the two NNRTIs, the IC₅₀ of each of the compounds in the combination was lower than the IC₅₀ of that compound used alone. The inhibitory activity of PMPA was augmented when it was used with UC-781 or TMC120, with an average reduction in the IC₅₀s against all isolates of 61.36% (±14.99%) or 75.29% (±8.11%), respectively (Table 2). The IC₅₀ of each NNRTI used in combination with PMPA was reduced by approximately 50% in comparison to the activity of the drug alone (46.92% for UC-781 and 51.87% for TMC120). The combination of UC-781 with TMC120 was also positive, with a decrease of 68.73% in the IC₅₀ of UC-781 and 50.28% in that of TMC120 (Table 2). Thus, any combination between two of the three compounds studied was more active than any of the individual drugs alone when evaluated in TZM-bl cells.

PMPA, UC-781, and TMC120 inhibit HIV-1 clade B infection in PBMCs. The three compounds were next tested against several HIV-1 isolates in activated PBMCs obtained from multiple healthy donors. The inhibitory activities of all three compounds demonstrated a dose response for all viruses evaluated in PBMC cultures (see Fig. S2 and Table S1 in the supplemental material). The IC₅₀s measured in activated PBMCs were similar to those obtained in TZM-bl (see Table S1 in the

supplemental material). The order of potency for the three compounds (highest for TMC120, intermediate for UC-781, and lowest for PMPA) was maintained, with IC₅₀s in the nanomolar range for TMC120 and UC-781 and in the micromolar range for PMPA. Despite a slight decrease in IC₅₀s for PMPA and an increase in IC₅₀s for TMC120, these values were within the same range for each compound. As with TZM-bl cells, no cytotoxicity in PBMCs was detected by an MTT assay for any of the compounds in their nonformulated versions (data not shown).

Dual-RTI combination studies in PBMCs. NRTI-NNRTI and NNRTI-NNRTI combination ratios were calculated using the IC₅₀ obtained for each individual compound against HIV infection of PBMCs. The proportions of compounds were 88:1 for PMPA-UC-781, 735:1 for PMPA-TMC120, and 8:1 for UC-781-TMC120 combinations. The three combinations tested showed a decrease in the IC₅₀ for each compound present in the combination compared with that for the compound alone. The increase in the inhibitory activity of each compound in combination was remarkably similar to that seen in TZM-bl cells, with average reductions in the IC₅₀s between 60.60% and 74.01% (see Table S2 in the supplemental material). The decrease in the IC₅₀ for each compound was unaffected by the coreceptor phenotype (X4 or R5). The similarity of the results obtained with PBMCs and TZM-bl cells validated the use of the reporter cell line for screening potential RTI microbicides and combinations.

Replication capacities of R5 and X4 HIV-1 isolates in colorectal explants. Before testing of the activities of the three RTIs against HIV infection of colorectal tissue, the replication kinetics of several HIV-1 isolates in colorectal explants were determined. R5 isolates (BaL and YU.2) replicated efficiently in colorectal explant cultures, as indicated by increasing supernatant p24 concentrations. The same trend was observed for the R5-tropic molecular clone R8.BaL, a chimera of an R5 (BaL) Env with an X4 (NL4.3) backbone. In contrast, the X4 virus NL4.3 did not replicate in colorectal tissue (Fig. 1).

Activities of NRTI-NNRTI and NNRTI-NNRTI combinations in colorectal tissue explants. The results obtained with TZM-bl cells and PBMCs allowed us to design the experimental conditions to test the inhibitory activities of nonformulated

FIG. 1. Kinetics of BaL, YU.2, NL4.3, and R8-BaL replication in human colorectal explants. Colorectal explants were incubated with BaL (△), YU.2 (◆), NL4.3 (■), or R8.BaL (○) for 2 h, washed four times in PBS, and cultured on gel foam for 15 days. Supernatants were harvested at different time points, and p24 concentrations were measured by ELISA. Data are means (± standard deviations) of at least three independent experiments performed in triplicate.

FIG. 2. Activities of dual combinations of PMPA, UC-781, and/or TMC120 against YU.2 and BaL in colorectal explants. Colorectal explants were treated for 1 h in the presence or absence of PMPA and/or UC-781 (a and d), PMPA and/or TMC120 (b and e), or UC-781 and/or TMC120 (c and f). YU.2 (a, b, and c) or BaL (d, e, and f) was added for 2 h before four washes with PBS. Explants were then transferred to gel foam rafts and cultured for 15 days. The concentrations of p24 in the harvested supernatants were quantified by ELISA, and the extent of inhibition by each compound or combination of compounds was calculated. The percentage of inhibition was normalized relative to the p24 values obtained for explants not exposed to virus (0% infectivity) and for explants infected with virus in the absence of compound (100% infectivity). Data are means ($\pm$ standard deviations) from three independent experiments performed in triplicate.

PMPA, UC-781, and TMC120 individually and in combination in the colorectal tissue explant model. Based on the higher level of replication for R5 isolates (Fig. 1) and the prevalence of R5 viruses following transmission by RAI, we used the molecular clone YU.2 and the laboratory-adapted isolate BaL to perform combinatorial studies in the colorectal explant model. The activities of compounds alone and in double combinations were compared by plotting the dose-response curve for each of the two drugs alone and the dose-response curve for each of the two drugs in the double combination. All combinations tested produced, for at least one of the compounds, a positive change in the dose-response curve, as shown in Fig. 2 and Table 3. However, this finding does not represent an actual shift in potency but merely reflects the fact that the presence of the more active drug in the combination reduced the amount of the less active drug required to produce the inhibitory effect. For example, the amount of PMPA required to reach 50% inhibition when used alone was reduced by 82.86% when PMPA was used in combination with UC-781 and by 93.03% when it was used with TMC120. Therefore, the

dose-response curve for PMPA when used in combination with an NNRTI was improved due to the high inhibitory activity of UC-781 or TMC120. When the two NNRTIs were used in combination, the shift in inhibitory activity was slightly higher for UC-781 than for TMC120 (as shown in Table 3), reflecting the relative potencies of the individual drugs. None of the compounds affected colorectal tissue viability when used alone (as previously reported by Fletcher et al. [33]) or in combinations at the highest dose tested, as assessed by an MTT assay (data not shown).

Activity profiles of PMPA, UC-781, and TMC120 against RTI-resistant isolates in TZM-bl cells. The studies described above were all performed with NRTI- and NNRTI-sensitive X4 and R5 isolates. The potential success of any colorectal microbicide may depend not only on its activity against wild-type isolates but also on its activity against possible RTI-resistant isolates. A wide range of mutations can emerge in RT, conferring resistance to NRTIs and/or NNRTIs, and many may be prevalent in populations with high levels of treatment. We studied two representative NRTI- or NNRTI-resistant isolates

TABLE 3. Reductions in the IC₅₀s and IC₉₀s of drugs used in combination in colorectal explants

Drug B and isolate	% Reduction in the IC ₅₀ (IC ₉₀) of the following drug (drug A) in combination with drug B ^a :		
	PMPA	UC-781	TMC120
PMPA			
BaL		61.04 (64.96)	32.97 (0.11)
YU.2		12.72 (14.15)	18.38 (15.87)
Mean ± SD		36.88 ± 34.17 (39.56 ± 35.93)	25.68 ± 10.31 (7.99 ± 11.14)
UC-781			
BaL	72.58 (67.74)		75.06 (55.53)
YU.2	93.15 (75.55)		38.95 (33.81)
Mean ± SD	82.86 ± 14.54 (71.64 ± 5.52)		57.01 ± 25.53 (44.67 ± 15.36)
TMC120			
BaL	91.31 (74.92)	82.67 (71.38)	
YU.2	94.75 (73.73)	49.99 (27.46)	
Mean ± SD	93.03 ± 2.43 (74.33 ± 0.84)	66.33 ± 23.11 (49.42 ± 31.05)	

^a The percentage of reduction in the IC₅₀ or IC₉₀ of each compound when used in combination was calculated as follows: $\{1 - [\text{IC}_{50/90}(\text{drug A} + \text{drug B})/\text{IC}_{50/90}(\text{drug A})]\} \times 100$, where IC_{50/90} stands for IC₅₀ or IC₉₀. The values shown are means derived from three independent experiments performed in triplicate.

and a double NRTI escape mutant. HIV-1 A17 is highly resistant to inhibition by NNRTIs, including UC-781 (47). The resistance of A17 arises from two point mutations, K103N and Y181C, in RT. K103N is the mutation that emerges predominantly in patients receiving NNRTIs (4, 16, 22, 24, 25, 38, 76), and Y181C is often accompanied by other mutations, such as K103N. The NRTI-resistant HIV-1 isolate 71361-1 contains a K65R mutation that can be selected in vitro by PMPA (81) and during monotherapy intensification with PMPA (61). K65R is generally detected in association with other mutations, such as M184V (36), which by itself emerges rapidly during 3TC (lamivudine) monotherapy (7, 41, 71, 75, 82). The HIV-1 isolate 8415-2 contains both K65R and M184V mutations.

The individual activities of the three compounds against the resistant viruses were evaluated in TZM-bl cells. The NNRTI escape mutant, isolate A17, was fully resistant to UC-781 (as shown in Fig. S3a in the supplemental material) and partially resistant to TMC120, with an IC₅₀ of 8.34 ± 0.56 nM, representing an average 17-fold increase in the IC₅₀. The dose-response curve of PMPA was not affected by the two mutations in the RT of A17. As expected, the activity of PMPA against the NRTI-resistant isolate 71361-1 (K65R) was significantly impaired (see Fig. S3b in the supplemental material), with an increase in the IC₅₀ of at least 1 log unit (IC₅₀, >30,000) relative to that for wild-type isolates (see Table S1 in the supplemental material). This isolate was fully resistant to the NNRTI UC-781 and partially resistant to TMC120, with a ≥ 1 -log-unit increase in the IC₅₀. When the M184V mutation was coexpressed with the K65R mutation in the 8415-2 isolate, the activity of PMPA was partially recovered (see Fig. S3c in the supplemental material), with an IC₅₀ of approximately 8,200 nM. This increase in susceptibility to PMPA, measured in TZM-bl cells, for the double mutant (with the K65R and M184V mutations) has been reported previously for patients receiving tenofovir and lamivudine therapy (67, 84). This iso-

late was sensitive to both NNRTIs tested (see Fig. S3c in the supplemental material).

Activities of NRTI-NNRTI and NNRTI-NNRTI combinations against RTI-resistant isolates in TZM-bl cells. The three compounds were then tested for their inhibitory activities in dual combinations against the escape mutants in TZM-bl cells. The NNRTI-resistant isolate A17 was fully sensitive to PMPA; therefore, combinations of PMPA with either UC-781 or TMC120 were able to inhibit infection in TZM-bl cells (Fig. 3a and b). A17 was only partially resistant to TMC120, and the mixture of the two NNRTIs, UC-781 and TMC120, did not reach higher levels of inhibition than TMC120 alone and did not reduce the IC₅₀ of TMC120 (Fig. 3c and Table 4). When the three dual combinations (NRTI-NNRTI and NNRTI-NNRTI combinations) were tested against the NRTI escape mutant 71361-1, containing the K65R mutation in RT, the levels of inhibition (up to 83% inhibition) reached with the mixtures of two RTIs were higher than the levels reached individually by any of the compounds included in the mixture (57% for PMPA, 7% for UC-781, and 61% for TMC120) (Table 4; see also Fig. S4a, b, and c in the supplemental material). The 8415-2 isolate, containing the K65R and M184V mutations, was only partially resistant to PMPA, and all combinations tested were more active than PMPA, UC-781, or TMC120 alone (Table 4; see also Fig. S4d, e, and f in the supplemental material). These results with resistant isolates in TZM-bl cells demonstrate the importance of considering combinations of compounds with different inhibitory mechanisms in the design of microbicides, even if they target the same step of the viral replication cycle.

Effects of mutations in RT on replication capacity in colorectal explants. The replication capacities of the three resistant isolates were tested in the colorectal explant model. The NNRTI escape mutant A17 was able to produce a sustained infection for 15 days in culture (Fig. 4). However, the two

FIG. 3. Activities of dual combinations of PMPA, UC-781, and/or TMC120 against the NNRTI-resistant isolate A17 in TZM-bl cells. TZM-bl cells were treated for 1 h in the presence or absence of PMPA and/or UC-781 (a), PMPA and/or TMC120 (b), or UC-781 and/or TMC120 (c). The cells were then exposed to virus. Luciferase expression (measured in relative light units) was determined after 48 h, and the extent of inhibition by each drug was calculated. The percentage of inhibition was normalized to the relative light units obtained for cells grown in the absence of virus (0% infectivity) and for cells infected with virus in the absence of drug (100% infectivity). Data are means ($\pm$ standard deviations) for three independent assays performed in triplicate.

NNRTI-resistant isolates containing the K65R mutation, 71361-1 and 8415-2, had impaired replication capacities, with average p24 concentrations of only 500 pg/ml in the culture supernatant at day 15. This decrease in replication capacity associated with the K65R mutation in colorectal tissue correlates with the loss in infectivity of emerging resistant isolates containing the same mutation detected in vitro and in plasma

from patients receiving tenofovir therapy (53, 55, 59, 85). This finding further supports the validity of the colorectal explant model for determining isolate infectivity and replication ability.

Combinations of PMPA, UC-781, and/or TMC120 against the NNRTI-resistant isolate A17 are active in colorectal tissue. Next, we tested the NNRTI-NNRTI and NNRTI-NNRTI combinations against the A17 isolate in colorectal tissue. The other two resistant strains were not evaluated, because they failed to replicate efficiently in colorectal tissue. Dose-response curves corresponding to the three dual combinations tested showed

TABLE 4. Reductions in the IC₅₀s and IC₇₀s of drugs used in combination in TZM-bl cells

Drug B and isolate	% Reduction in the IC ₅₀ (IC ₇₀) of the following drug (drug A) in combination with drug B ^a :		
	PMPA	UC-781	TMC120
PMPA			
A17		100 (100)	96.47 (100)
71361-1		100 (100)	53.77 (29.27)
8415-2		24.39 (20.44)	43.06 (76.55)
UC-781			
A17	17.13 (26.91)		32.69 (NA)
71361-1	62.13 (100)		20.19 (19.54)
8415-2	81.07 (82.51)		48.24 (80.45)
TMC120			
A17	40.59 (47.95)	100 (NA)	
71361-1	50.29 (100)	100 (100)	
8415-2	81.48 (82.31)	32.73 (32.93)	

^a The percentage of reduction in the IC₅₀ or IC₇₀ of each compound when used in combination was calculated as follows: $\{1 - [\text{IC}_{50/70}(\text{drug A} + \text{drug B}) / \text{IC}_{50/70}(\text{drug A})]\} \times 100$. The values shown are means derived from three independent experiments performed in triplicate. NA, not applicable (70% inhibition was not reached with the concentrations used).

FIG. 4. Kinetics of A17, 71361-1, and 8415-2 replication in human colorectal explants. Colorectal explants were incubated with A17 ($\diamond$), 71361-1 ($\blacksquare$), or 8415-2 ($\blacktriangle$) for 2 h, washed four times in PBS, and cultivated on gel foam for 15 days. Supernatants were harvested at different time points, and p24 concentrations were measured by ELISA. Data are means ($\pm$ standard deviations) from at least three independent experiments performed in triplicate.

FIG. 5. Combinations of PMPA, UC-781, and/or TMC120 in colorectal explants are more active against A17 than the individual compounds. Colorectal explants were treated for 1 h in the presence or absence of PMPA and/or UC-781 (a), PMPA and/or TMC120 (b), or UC-781 and/or TMC120 (c). A17 was added for 2 h, after which explants were washed four times with PBS. Explants were then transferred to gel foam rafts and cultured for 15 days. The concentrations of p24 in the harvested supernatants were quantified by ELISA, and the extent of inhibition by each compound per se or in combination was calculated. The percentage of inhibition was normalized relative to the p24 values obtained for explants not exposed to virus (0% infectivity) and for explants infected with virus in the absence of compound (100% infectivity). Data are means ($\pm$ standard deviations) for three independent experiments performed in triplicate.

increased activity for all of the compounds when used in combination (Fig. 5). Here, not only did the IC_{50} of each individual compound decrease, but the combination demonstrated higher levels of inhibition overall. Furthermore, while the NRTI-NNRTI combination was able to completely inhibit the NNRTI-resistant isolate (Fig. 5a and b), the NNRTI-NNRTI combination was also able to achieve up to 80% inhibition with the concentrations tested (Fig. 5c).

Taken together, these data demonstrate the potential of dual- and single-modality RTI combinations in the formulation of rectal microbicides, providing activity against multiple strains of HIV-1 and against RT-resistant HIV-1 variants that may arise in HIV-infected partners of uninfected individuals. Such combinations may play a key role in the design of an effective rectal microbicide.

DISCUSSION

Efforts to develop effective rectal microbicides have been considerably delayed relative to those for vaginal microbicides, where several compounds are or will be tested in phase III clinical trials (<http://www.microbicide.org>). Furthermore, of the compounds evaluated here, PMPA is already in phase II/IIB and UC-781 and TMC120 in phase I clinical trials as formulated vaginal microbicides. While much of the technology developed for vaginal microbicides may be important for the development of effective rectal microbicides, it is not clear whether the same formulations will provide protection against both routes of HIV transmission. Indeed, anatomical differ-

ences, including the number and accessibility of target cells, the dynamics of drug absorption, and the surface area, make it highly likely that effective prevention of HIV transmission via RAI (56) will require the design of rectum-specific formulations.

Previous studies have demonstrated that the individual drugs evaluated here (the NRTI PMPA and the two NNRTIs UC-781 and TMC120) exhibit dose-dependent activity against R5 and X4 isolates (19, 20, 31, 32, 78) in cellular and cervical explant models. In this study, we have evaluated, side by side, their activities alone and in dual combinations in two cellular models and in colorectal explants. The order of potency and the range of IC_{50} s for all three compounds were the same in all models tested (TZM-bl cells, PBMCs, and colorectal tissue) against a panel of HIV-1 clade B isolates. TMC120 was more active than UC-781, with IC_{50} s in the nanomolar order, and PMPA was the least potent, with IC_{50} s in the μ M range. To assess the combinatorial activity (synergy/additivity/antagonism) of drugs, it was not possible to use the Chou-Talalay equation (15) included in the Calcsyn analysis software. In order for this equation to be applied correctly, the slopes of all the titration curves compared must be parallel. This was not possible to achieve, due to donor-to-donor variation in the explant model and the use of RTI-resistant isolates. Hence, to provide a quantitative indication of the potential increase or decrease in activity, we chose a concept similar to "dose reduction" (14) and calculated the reductions in the IC_{50} , IC_{70} , and/or IC_{90} of one drug when it was used in combination with

another drug. While this method does not provide a numerical indication (combination index) of the combinatorial effects, it does allow the classification of combinations as "positive" or "negative." Double combinations of the three compounds in NRTI-NNRTI or NNRTI-NNRTI mixtures were more potent than the individual compounds titrated alone in both cellular and tissue models. Importantly, none of the compounds lost activity when titrated in a combination. Moreover, the IC_{50} of each compound was reduced when it was included in any of the dual combinations tested (Tables 2 and 3; see also Table S2 in the supplemental material). These results are particularly encouraging, considering that the range of concentrations used in the *in vitro* models are significantly lower than those already included in vaginal gels tested in clinical trials.

The remarkably similar results obtained with TZM-bl cells, activated PBMCs, and colorectal tissue prove the value of the reporter cell line in the screening of candidate microbicides, providing a sensitive and cost-effective tool for quickly assessing drug activity alone and in combination. Recently, TZM-bl cells have been shown to be contaminated with replication-competent gamma-retroviruses, most likely an ecotropic murine leukemia virus (MLV) (74). The presence of contaminating ecotropic MLV would not substantially affect our results or conclusions. MLV does not encode a viral transactivator required for reporter readout and hence TZM-bl cells demonstrate very low background reporter expression. Furthermore, MLV does not interfere with HIV entry and/or transactivation as evidenced by the high reporter expression following HIV infection. Moreover, endogenous MLV reverse transcriptase activity in TZM-bl cells is highly unlikely to have influenced the activity of RT inhibitors against HIV-1, as reflected by similar activity of the evaluated drugs in TZM-bl cells, PBMCs, and explant cultures.

In addition to determining the efficacies of individual drugs and combinations against wild-type virus, assessment of activity against resistant strains is of critical importance. NNRTI resistance is increasingly prevalent in HIV-infected populations in the developed and developing worlds. Furthermore, different clades may develop resistance more or less rapidly against NRTIs (10) and NNRTIs. Hence, combinations of compounds that inhibit the virus by different mechanisms may provide more-effective coverage against rectal transmission where resistant isolates are already prevalent.

To this end, dual combinations of PMPA, UC-781, and TMC120 were tested against NNRTI- and NRTI-resistant isolates. In the TZM-bl assay, the NNRTI-resistant isolate A17 was fully resistant to UC-781 and partially resistant to TMC120 (Fig. 3). Not surprisingly, the combination of UC-781 with TMC120 was no better than TMC120 alone. A combination of PMPA with either UC-781 or TMC120 restored activity against the NNRTI escape mutant A17. However, neither combination was more active than PMPA alone against this virus. While this would be expected for UC-781, it is interesting that the low activity of TMC120 against this virus did not enhance the activity of PMPA when these drugs were used in combination. We cannot exclude the possibility that, in formulations where the dose of TMC120 could be increased, the titration curve of TMC120 might reach higher levels of inhibition against A17 alone or in combination with PMPA.

The K65R mutation is associated with NRTI resistance.

Indeed, a low rate of K65R mutant emergence has been observed in clade B-infected subjects during tenofovir-based therapy (5, 8, 13, 35, 50–52, 55, 79). Clinical trial data for the emergence of K65R mutants in subjects infected with other clades are less clear and require further study (18, 28, 54, 60). However, *in vitro* studies suggest that resistance might occur more quickly with clade C viral isolates (10). When the NRTI-resistant isolate 71361-1, which carries the K65R point mutation in RT, was tested, the activities of all three combinations were better than that of each drug alone, the most potent combination being PMPA plus TMC120. Interestingly, the presence of an additional mutation in RT, M184V, partially restored sensitivity to PMPA. This resensitization has also been reported during therapy (67, 84), suggesting that the emergence of certain mutations in an infected partner might be an unplanned benefit for the development of microbicides for the prevention of rectal transmission. Again, all combinations were more active against the 71361-1 isolate than any single drug used alone.

Interestingly, neither of the two NRTI-resistant isolates containing the K65R mutation could establish productive infection in colorectal explants (Fig. 4). Therefore, this resistance mutation appears, at least *in vitro*, to come with a fitness cost for the virus. It is currently unclear whether isolates containing the K65R mutation have a lower frequency of transmission via the rectal route than wild-type virus *in vivo*. However, were this to be the case, it might in itself reduce the transmission of resistant strains in populations where the use of RTI-based microbicides may become common. In contrast, while the NNRTI-resistant isolate A17 was able to replicate effectively in colorectal tissue, all combinations, both NRTI-NNRTI and NNRTI-NNRTI, were active against this virus. In summary, the combination of an NRTI with an NNRTI that provided the widest coverage against potential resistant isolates was PMPA plus TMC120, which demonstrated more-potent activity than PMPA plus UC-781 or UC-781 plus TMC120. Studies in progress will determine whether triple combinations of these drugs further improve activity.

These data suggest that dual NRTI-NNRTI combinations are likely to reduce the possibility that exposure to preexisting drug-resistant HIV (either NRTI or NNRTI resistant) from an infected partner might overcome a microbicide, even if only one of the two component drugs were active against the resistant isolate. Therefore, should a dual-RTI-based microbicide prevent infection with a resistant HIV isolate, there would be no further potential for the development of resistance in the exposed subject. The likely requirements that individuals be prescreened for HIV-1 infection prior to having access to an ARV rectal microbicide and that they undergo regular follow up testing should reduce the likelihood of the use of such products by seropositive individuals unaware of their status, thereby reducing the possibility of selecting for resistance in an infected individual. However, were an individual to become infected due to a lack of product efficacy or due to inconsistent use, the risk for the development of drug resistance is currently unknown. This is particularly hard to calculate given the likely rapid reemergence of the wild-type virus upon cessation of product use (23), the time between infection and the initiation of therapy (often a period of years), the development of second-line therapy active against common ARV escape muta-

tions, and the observation that ARV therapy still provides immunological and virological benefits by suppressing nonresistant virus (27). Ultimately, the possible selection for drug resistance by the use of ARV-containing microbicides, and the clinical as well as epidemiological consequences, can be addressed only in clinical trials.

The inhibitory activities of PMPA, UC-781, and TMC120, tested alone and in combination, in the colorectal explant model provide key data on the potential tissue drug concentrations required to prevent rectal transmission of HIV. It will be important to determine whether such tissue concentrations equate to protection in vivo (79). Key to this will be the development of combination formulations specifically targeted for rectal administration. As a first step toward this goal, we have recently demonstrated, in a simian immunodeficiency virus rectal challenge model, that protection in vivo was associated with the drug concentration in vivo and with ex vivo protection of colorectal explants (17). Further work is needed to determine whether the drug concentrations shown to inhibit HIV replication in colorectal explants can provide a surrogate biomarker of in vivo efficacy. To this end, ex vivo challenge of rectal biopsy specimens is currently under evaluation in an ongoing phase I rectal microbicide trial of formulated UC-781 used at two different doses (0.1 and 0.25%) (J. Elliott, I. McGowan, A. Adler, E. J. Johnson, K. Tanner, D. Cho, T. Saunders, E. Khanukhova, C. Mauck, and P. Anton, presented at Microbicides 2008, New Delhi, India). Should this trial distinguish between the placebo formulation and the two different doses of drug, this may provide the first indication that such a strategy may have utility in wider microbicide trials.

In conclusion, this study supports the rationale for the further development of RTI combinations and the investigation of mixtures with other types of anti-HIV compounds, including protease inhibitors and entry/fusion inhibitors, as part of a comprehensive strategy to develop effective colorectal microbicides. Furthermore, our data suggest that some RTI escape mutations may come with a fitness cost for rectal mucosal transmission, an important issue that requires further investigation. This study further validates the use of the recently developed colorectal explant system as a tool for the preclinical evaluation of potential microbicides and as a model with which to assess the infectivities and replication capacities of wild-type and ARV-resistant viral isolates. Although these studies demonstrate in vitro that RTI combinations used prior to exposure to virus provide coverage against HIV-1 isolates containing RT escape mutants, the predictive value of inhibitory tissue concentrations in vitro can be validated only by detailed tissue pharmacokinetics, ex vivo challenge studies in nonhuman primate models, and human clinical trials.

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