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Inhibition of Human Immunodeficiency Virus Type 1 Infection by the Candidate Microbicide Dapivirine, a Nonnucleoside Reverse Transcriptase Inhibitor▽

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Heterosexual transmission of human immunodeficiency virus (HIV) remains the major route of infection worldwide; thus, there is an urgent need for additional prevention strategies, particularly strategies that could be controlled by women, such as topical microbicides. Potential microbicide candidates must be both safe and effective. Using cellular and tissue explant models, we have evaluated the activity of the nonnucleoside reverse transcriptase inhibitor (NNRTI) dapivirine as a vaginal microbicide. In tissue compatibility studies, dapivirine was well tolerated by epithelial cells, T cells, macrophages, and cervical tissue explants. Dapivirine demonstrated potent dose-dependent inhibitory effects against a broad panel of HIV type 1 isolates from different clades. Furthermore, dapivirine demonstrated potent activity against a wide range of NNRTI-resistant isolates. In human cervical explant cultures, dapivirine was able not only to inhibit direct infection of mucosal tissue but also to prevent the dissemination of the virus by migratory cells. Activity was retained in the presence of semen or a cervical mucus simulant. Furthermore, dapivirine demonstrated prolonged inhibitory effects: it was able to prevent both localized and disseminated infection for as long as 6 days posttreatment. The prolonged protection observed following pretreatment of genital tissue and the lack of observable toxicity suggest that dapivirine has considerable promise as a potential microbicide candidate.

The human immunodeficiency virus (HIV) pandemic has entered its third decade, and almost 7,000 new infections are still reported to occur daily across the world (51). Globally, more than 33.2 million people are now infected with HIV type 1 (HIV-1) (51), and heterosexual intercourse is the major route of transmission. Furthermore, there is a growing discrepancy in infection rates between men and women, most notably in sub-Saharan Africa, where 61% of people living with HIV are women (51) and one in four women is infected by the age of 22 (52).

Current prevention methods include behavioral strategies, such as abstinence, monogamy, and reducing the number of sexual partners, and the use of barrier methods, such as the male and female condoms. There is also evidence to suggest that treatment of other sexually transmitted diseases (STDs), in particular ulcerative STDs, can help reduce HIV-1 transmission rates within "at-risk" populations (14). Condom use currently offers the best method for preventing HIV transmission through sexual intercourse, and consistent condom use is reported to be 80 to 87% efficacious (potential range, 60 to 96% for HIV-serodiscordant couples) (13). However, there is substantial evidence suggesting that consistent condom use is low in many settings, especially in primary partnerships (15). Furthermore, gender inequalities mean that abstinence is not a realistic choice for many women, and condom use requires partner consent (43). Monogamy offers no protection to women whose partners are unfaithful. In some cultures, there is strong social pressure for women to have children. Therefore, condom use is not a viable option for women where the imperative to have children outweighs concerns about HIV infection (43). It is possible that the gap between the requirement for protection and the inability to use condoms consistently could be filled by microbicides (13). Vaginal microbicides are topically applied formulations designed to prevent the transmission of HIV-1 and potentially other STDs. Such products could represent an important new prevention option for women (18).

Here we have tested dapivirine (also known as TMC120), a nonnucleoside reverse transcriptase inhibitor (NNRTI) made available to the microbicide field by a landmark agreement between Tibotec and the International Partnership for Microbicides (IPM) (International Partnership for Microbicides, IPM will take over the development of Tibotec's promising microbicide to help the prevention of the sexual transmission of HIV, press release, 2004 [http://www.ipm-microbicides.org/news_room/english/press_releases/2004/2004_0329_tibotec.htm]). Dapivirine, a substituted diarylpyrimidine analogue, is one of a new generation of NNRTIs that can accommodate some mutations within the NNRTI binding site without significant loss of activity (25–27). Thus, dapivirine displays improved activity against both wild-type strains of HIV-1 and strains harboring different mutations inducing resistance to other NNRTIs, and data show that dapivirine has a resistance profile superior to those of existing NNRTIs such as nevirapine

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(NVP) (31), delavirdine (DLV) (42), and efavirenz (EFV) (56). A short-term clinical trial evaluating dapivirine monotherapy demonstrated remarkable 1.44 and 1.51 log reductions in viral loads in treatment-naïve patients receiving 50 or 100 mg dapivirine, respectively (M.-P. de Bethune, K. Andries, D. Ludovici, P. Lewi, H. Azijn, M. de Jonge, J. Heeres, M. Kukla, P. Janssen, and R. Pauwels, presented at the 8th Conference on Retroviruses and Opportunistic Infection, Chicago, IL, 2 to 4 February, 2001, abstr. 304; B. Gruzdev, A. Horban, A. Boron-Kaczmarska, D. Gille, G. Van't Klooster, and R. Pauwels, presented at the 8th Conference on Retroviruses and Opportunistic Infections, Chicago, IL, 2001, abstr. 13). Extensive nonclinical and clinical testing has shown dapivirine to have a favorable safety profile when administered orally as a therapeutic drug for HIV/AIDS or intravaginally as a microbicide for the prevention of HIV infection.

To evaluate the activity of dapivirine, we have utilized a range of cellular models, as well as a nonpolarized cervical explant culture model (10, 17, 20), to mimic the ex vivo mucosal tissue initially exposed to the virus during heterosexual transmission in vivo. We have demonstrated previously that an intact stratified epithelium presents a barrier to infection (17), and there are clear physiological reasons for such barrier effects: the genital mucosa consists of a multilayered stratified squamous epithelium with an apical layer of keratinized cells (45); the epithelium has limited permeability to particles with diameters greater than 3 nm (44); and epithelial integrity is maintained through the presence of intercellular desmosomes and amorphous lipoidal material (23, 44). Furthermore, studies with the macaque challenge model suggest that transmission and primary infection are associated with epithelial microtrauma, which provides access to underlying susceptible cells in the submucosa (32); such microtrauma can be found in 61% of women following consensual sexual intercourse (37). Thus, we model a "worst-case scenario" where the virus has a maximal chance of establishing infection, allowing it to reach all potential susceptible cells within the epithelium and underlying submucosal tissue. We demonstrate not only that dapivirine is biocompatible with various cell types and tissue explants but also that dapivirine is active in T-cell cultures, prevents both localized and disseminated viral infection of cervical explants, and is active in the presence of semen and mucin. Furthermore, dapivirine demonstrates prolonged inhibitory activity in genital tissue.

MATERIALS AND METHODS

Cell and virus culture. All "complete" media were supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) unless otherwise stated. PM-1 cells (AIDS Reagent Project, NIBSC, United Kingdom) and MT-4 cells were grown in continual culture in complete RPMI medium. TZM-bl cells (NIH AIDS Research and Reference Reagent Program) were grown in continual culture in complete Dulbecco's modified Eagle medium and were treated with 1× trypsin-EDTA for cell passage. Primary human macrophages were prepared and purified from peripheral blood mononuclear cells (3) and were cultured in complete RPMI medium containing 20% fetal calf serum.

Wild-type strains of HIV-1, both CCR5 (R5) utilizing (HIV-1_{BaL}) and CXCR4 (X4) utilizing (HIV-1_{RF} and HIV-1_{IIIB}), were grown either in phytohemagglutinin-stimulated peripheral blood mononuclear cells or in PM-1 cells. A panel of site-directed mutants and recombinant clinical isolates, containing strains with single and double mutations associated with resistance to NNRTIs, was prepared as previously described (19). Viral phenotyping was performed by the cell-based antivirogram assay method, using viability measurements from a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (46).

Drug substances. Dapivirine was obtained from Tibotec Pharmaceuticals Ltd. (Mechelen, Belgium). Marketed anti-HIV compounds (EFV, NVP, and DLV) were extracted and purified by high-pressure liquid chromatography from commercial formulations (1). Stock solutions of all drugs were made in dimethyl sulfoxide and stored at −20°C. All compound stocks were diluted in complete medium immediately prior to use.

Antiviral activity of dapivirine. The activities of dapivirine (and those of other NNRTIs) against a broad panel of HIV-1 isolates of different origins were determined using several cellular assays. In all cases, inhibition of viral growth was determined by comparing drug-treated wells with untreated control wells.

(i) TZM-bl luciferase reporter assay. TZM-bl cells (5×10^4/well) cultured overnight were treated with dapivirine for 1 h prior to exposure to HIV-1_{BaL} or HIV-1_{IIIB} (200 50% tissue culture infective doses [$TCID_{50}$]/ml). After 24 h, cells were washed and lysed, and luciferase units were determined using the luciferase assay kit (Stratagene, United Kingdom). The potential effects of 12.5% (final concentration) whole semen (WS; obtained with written consent according to the local Research Ethics Committee) or cervical mucus (CM) simulant (5) on dapivirine activity were also evaluated. For WS, virus was pretreated with WS prior to application to dapivirine-pretreated cells. For CM, cells were pretreated first with CM and then with dapivirine prior to exposure to virus.

(ii) T-cell line assay. The activity of dapivirine was evaluated against both HIV-1_{BaL} and HIV-1_{RF} (200 $TCID_{50}$/ml) using PM-1 cells (4×10^4/well). Following 7 days in culture in the presence of dapivirine, viral replication was assessed by measurement of viral reverse transcriptase (RT) in culture supernatants (40).

(iii) Acutely infected macrophage assay. Following pretreatment with dapivirine for 1 h, primary macrophages were exposed to HIV-1_{BaL} (300 $TCID_{50}$/ml) for 2 h. Excess virus was removed, and cells were cultured in the presence of dapivirine for 14 days, with medium/dapivirine replacement every 2 days. Viral replication was assessed by measurement of p24 in culture supernatants (Abbott Laboratories, Pomezia, Italy).

(iv) Profiling against primary and resistant HIV isolates. The activities of compounds (dapivirine, EFV, NVP, DLV) against laboratory-adapted strains (HIV-1_{IIIB}), site-directed mutants, and clinically derived recombinant viruses (200 $TCID_{50}$/well) were tested in MT-4 cells (3×10^4/well) cultured for 3 to 5 days by using an MTT colorimetric assay (1, 19, 39).

Supply and culture of human genital tract tissue explants. Cervical tissue was obtained from women undergoing planned therapeutic hysterectomies at St George's, St Helier's, and Kingston Hospitals (London, United Kingdom). (Written consent was obtained according to the local Research Ethics Committee.) Cervical tissue was cut into 2-to-3-mm^3 explants as previously described (17, 20). Explants were treated with dapivirine for 1 h prior to exposure to HIV-1_{BaL} (two 50% tissue infectious doses, equivalent to 10^4 $TCID_{50}$ as determined on PM-1 cells) for 2 h at 37°C in the presence of the compound. Explants were washed four times with phosphate-buffered saline (PBS) and transferred to fresh culture plates. Following overnight culture, explants were transferred to fresh culture plates and cultured for 12 to 14 days, with 50% medium feeds every 2 to 3 days. Cells that had spontaneously migrated out of the tissue explants (20) during overnight culture were washed (twice with PBS), transferred to fresh plates, and cocultured with 4×10^4 PM-1 cells/well to assess the blockade of virus transfer by migrating cells. At the end of the assay, HIV-1 infection was determined by the measurement of p24 in culture supernatants (explant supernatants [lower detection limit, 15 pg/ml; Beckman Coulter] and migratory coculture supernatants [lower detection limit, 300 pg/ml; AIDS Vaccine Program, National Cancer Institute, Frederick, MD]) by an enzyme-linked immunosorbent assay. Tissue was assessed for the presence of proviral HIV-1 DNA (long terminal repeat) and β-actin by multiplex quantitative real-time PCR, with a sensitivity of 10 copies/10^5 cells, as previously described (17). To determine the duration of protection by dapivirine in the absence of the compound, cervical explants were pretreated with dapivirine for 2 or 24 h. The compound was then removed by washing (4 volumes of PBS), and tissue was transferred to fresh culture plates and exposed to virus (as previously described) on day 0, 2, 4, or 6 posttreatment. Virus was removed, and explants were cultured as described above. There was no detectable drug toxicity for any of the concentrations tested under any of the experimental conditions throughout the culture period (data not shown).

Determination of compound toxicity. The viability of cells (TZM-bl and PM-1 T cells) and cervicovaginal tissue (in the absence of viral infection) following treatment with the compound was determined by the principle of MTT dye reduction (11, 12). The viability of macrophages following 14 days of exposure to dapivirine was determined using trypan blue exclusion. All data are expressed as the percentage of viability for compound-treated wells compared to untreated

TABLE 1. Activities of dapivirine in cell-based cultures[a]

Assay system	CC_{50} (nM)[a]	IC_{50} (nM)[a]		Selectivity index	
		R5-HIV-1	X4-HIV-1	R5-HIV-1	X4-HIV-1
TZM-bl cells	20,400 ± 1,200	2.2 ± 0.3	1.0 ± 0.5	9,273	20,400
T cells					
MT-4	2,150	ND	0.9		2,389
PM-1	11,000 ± 1,400	6.0 ± 1.4	2.4 ± 1.6	1,833	4,583
Monocyte-derived macrophages	>20,000	2.4	ND	>8,333	

[a] CC_{50} and IC_{50} were determined in the continued presence of dapivirine for equivalent time periods. Viral replication was assessed by luciferase activity (for TZM-bl cells), an MTT colorimetric assay (for MT-4 cells), RT activity (for PM-1 cells), or p24 in culture supernatants (for monocyte-derived macrophages). Data are means or means ± SD for ≥2 independent experiments where each condition was tested in triplicate or more. ND, not determined. R5-HIV-1, HIV-1_{BaL}; X4-HIV-1, HIV-1_{IIIB} (in TZM-bl or MT-4 cells) or HIV-1_{RF} (in T cells).

cell control wells, and the 50% cytotoxic concentration (CC_{50}) is defined as the concentration of the drug at which the cell/tissue viability was reduced to 50% of the drug-free control value.

Data manipulations and statistical analyses. Unless otherwise stated, data were analyzed to produce either medians, means, or means with standard deviations (SD) (for variation within an individual experiment) or standard errors of the means (SEM) (used for the means of ≥3 individual donors/experiment) (35) using Microsoft Excel or GraphPad Prism (GraphPad Software, Inc.). The results of drug susceptibility assays were expressed as the 50% inhibitory concentration (IC_{50}), defined as the concentration of the drug at which the level of infection was 50% of that with a drug-free control. IC_{50} and CC_{50} were calculated by nonlinear regression analysis using GraphPad Prism. In some cases, the change in susceptibility was calculated by dividing the (mean or median) IC_{50} for the test isolate by the (mean or median) IC_{50} for the wild-type isolate (HIV-1_{IIIB}), tested in parallel. Data were analyzed by analysis of variance (with Bonferroni posttests) and Student *t* tests to determine the effect of the presence of WS or CM on the activity of dapivirine. The selectivity index (calculated as the CC_{50}/IC_{50} ratio) was determined as an indicator of the specificity of the antiviral effect and was calculated using IC_{50} and CC_{50} values determined in the same time period.

RESULTS

In vitro toxicity of dapivirine. The in vitro toxicity of dapivirine was evaluated using cervical TZM-bl cells, a T-cell line (PM-1), and macrophages. In all cases, even following 14 days of exposure to the compound, the CC_{50} was in the range of 10 to 20 μM (Table 1). Additional studies with ex vivo human cervical tissue explants demonstrated no significant toxicity at the maximum concentration tested (1 mM) over a 24-h period (Fig. 1). This experiment was designed to mimic once-daily dosing with a formulated product where a bolus of drug would be applied in a gel formulation and the drug concentration would decline over the following 24-h period. As such, these data suggest that daily dosing with 1 mM dapivirine is likely to show good tissue biocompatibility. This was in contrast to the significant toxicity observed when tissue was treated with nonoxynol-9 for only 2 h (CC_{50}, 0.07% after 2 h of exposure and 0.003% after 24 h of exposure).

Antiviral activity of dapivirine in T-cell cultures. Dapivirine potently inhibited infection by both X4- and R5-utilizing HIV-1 strains in cell-based assays. There was no significant difference in the activity of dapivirine against R5- versus X4-utilizing viruses: similar results were obtained in the TZM-bl reporter single-round infection assay and the more traditional T-cell infection assay using the PM-1 T-cell line (Table 1). Equivalent activity was also observed against acute infection of primary monocyte-derived macrophages. These results are consistent with previous reports of dapivirine activity in cell-based assays (48, 53). Further experiments were completed to ensure that dapivirine retained its anti-HIV activity in the presence of biologically relevant fluids such as WS or CM. TZM-bl cells were exposed to HIV-1_{BaL} and dapivirine in the presence of 12.5% WS or CM. Importantly, statistical analysis (analysis of variance with Bonferroni posttests) determined that the activity of dapivirine was not significantly affected by the presence of WS or CM simulant in cultures (Table 2). Higher concentrations of WS or CM could not be evaluated due to their toxicity in these assays.

Since subtype B is not the most common subtype in African and Asian countries, a panel of clinically derived recombinant viruses from different geographical regions was established (7). The panel represents strains from HIV-1 group M subtypes A, B, C, D, F, and H, as well as circulating recombinant forms (CRF) CRF01_AE, CRF02_AG, and CRF05_DF and HIV-1 group O. The activity of dapivirine was determined in parallel with those of NVP, DLV, and EFV. All group M viruses were sensitive to dapivirine, with IC_{50}s below 3 nM (the IC_{50} for the wild-type was 0.9 nM), and the changes in susceptibility (defined as the IC_{50} for the test virus divided by the IC_{50} for wild-type HIV-1_{IIIB}, tested in parallel) were below fourfold

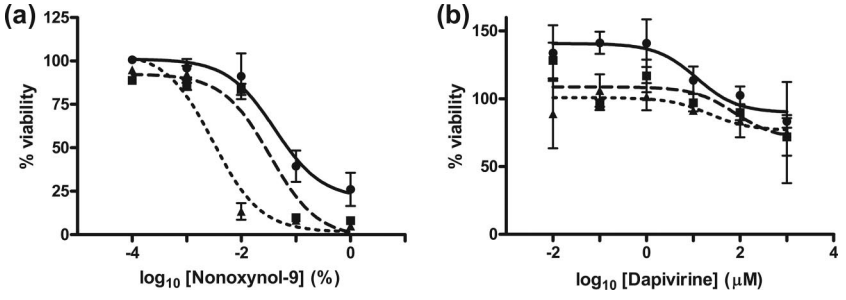

FIG. 1. Toxicity of dapivirine in cervical tissue. Ectocervical explants were exposed to nonoxynol-9 (a) or dapivirine (b) for 2 or 24 h. Explant viability was then determined using the principle of MTT dye reduction as described in Materials and Methods. Compound-treated explants were compared to unexposed controls, and the percentage of viable tissue was calculated per milligram. Data are means ± SEM for four (dapivirine) or three (nonoxynol-9) independent donors where each condition was tested in triplicate. Viability was determined either immediately following a 2-h exposure (●), after overnight culture following a 2-h exposure to the compound (■), or immediately following exposure to the compound for 24 h (▲).

TABLE 2. Activities of dapivirine in the presence of biological fluids[a]

Condition	IC_{50} (nM)	IC_{90} (nM)
Dapivirine alone	1.46 ± 0.76	10.23 ± 4.25
Dapivirine + WS[b]	1.75 ± 0.08	16.7 ± 4.34
Dapivirine + CM[c]	8.32 ± 2.08	41.6 ± 27.6

[a] TZM-bl cells were exposed to HIV-1_{BaL} in the presence of dapivirine, and viral replication was assessed by measurement of luciferase activity following 24 h. Data are means ± SD for three independent experiments, where each condition was tested in triplicate.

[b] HIV-1 was preincubated with 25% WS prior to infection of TZM-bl cells in the presence of dapivirine (final concentration, 12.5% WS).

[c] TZM-bl cells were preexposed to 25% CM simulant immediately prior to exposure to dapivirine for 1 h. Exposure to HIV-1_{BaL} occurred in the presence of both a CM simulant and dapivirine (final concentration of CM, 12.5%).

(Table 3). Eight of the group M viruses carried mutations in the RT coding region at positions associated with NNRTI resistance (positions 98, 101, 106, and 179). The one group O virus tested (V029524) naturally harbored amino acids associated with NNRTI resistance in HIV-1 strains from group M (positions 98 [G], 179 [E], and 181 [C]). This virus displayed significantly reduced sensitivity to NVP (89-fold reduced), DLV (140-fold), EFV (42-fold), and dapivirine (150-fold) (Table 3).

The antiviral activities of dapivirine, NVP, DLV, and EFV were also evaluated against a panel of NNRTI-resistant strains obtained by introducing well-defined resistance mutations into a wild-type strain using site-directed mutagenesis. In comparison with currently approved drugs (NVP, DLV, EFV), dapivirine demonstrated higher potency in vitro against most of the strains tested. The change in the IC_{50} of dapivirine was below 10-fold for 5/10 strains evaluated, compared to just 2/10 for NVP and EFV and 0/10 for DLV (Table 4). Furthermore, more than 400 ($n = 421$) clinically derived, NNRTI-resistant, recombinant HIV-1 strains were tested for their susceptibilities to dapivirine, NVP, DLV, and EFV. NNRTI resistance was defined as resistance to at least one NNRTI. Of these isolates, 2, 10, and 11% had <4-fold changes in susceptibilities to NVP, DLV, and EFV, respectively, compared with 31% for dapivirine. Similarly, 96, 85, and 82% of the isolates had >10-fold changes in their susceptibilities to NVP, DLV, and EFV, compared with 54% for dapivirine (data not shown).

Inhibition of infection of human cervical tissue and dissemination of virus by migratory cells. The potential of dapivirine to inhibit infection of the female genital mucosa was investigated ex vivo using mucosal tissue explants obtained from seronegative women undergoing planned therapeutic hysterectomies (10, 17, 20). Ectocervical explants, cultured in a non-

TABLE 3. Activities of dapivirine compared to those of other NNRTIs against a range of HIV-1 subtypes

Virus ID	Pol subtype	NNRTI mutations[b]	Fold change in IC_{50}[a]			
			Dapivirine	NVP	DLV	EFV
V022808	CRF02_AG	None	1	0.8	0.6	0.9
V022825	CRF02_AG	None	0.8	0.7	1.9	0.8
V022826	CRF02_AG	None	1	0.4	1.3	0.4
V022830	CRF02_AG	None	1.6	1.3	0.9	1.2
V022820	CRF01_AE	None	0.9	1.3	3.3	0.8
V022821	CRF01_AE	None	1.7	1.2	2.3	1.3
V022822	CRF01_AE	None	0.9	0.8	2.7	0.7
V029521	CRF01_AE	None	3.5	1.4	4.4	1.6
V029525	CRF01_AE	None	2.6	7	20	2.1
V022807	B	None	1.1	3.4	3.6	1.4
V022809	B	None	1.5	5	2.6	2.8
V022810	B	A98(S/A), K101R	1.6	1.7	2.7	0.8
V022811	B	None	3.1	3.4	3.7	1.5
V022812	B	V179I	2	0.8	6	0.3
V022813	B	None	0.7	1	0.7	0.6
V022814	B	None	0.8	2.4	1.9	0.7
V022815	B	None	2.2	1	4.9	2.9
V022816	C	None	1.8	3.8	5.3	0.6
V022817	C	None	0.7	ND	5.1	1.5
V022829	C	None	1	0.6	2.7	0.4
V022831	C	None	0.8	0.9	2.6	0.6
V022832	D	None	0.7	0.9	1.1	0.3
V022818	D	None	1.4	0.5	0.9	0.4
V022819	D	None	1.5	1	2.3	0.5
V022823	CRF05_DF	None	0.7	0.9	0.6	0.8
V022824	CRF05_DF	V106I	0.7	3.1	1.3	0.3
V022833	CRF05_DF	V179I	1	1.7	0.6	0.9
V029522	F	None	1.5	2.1	3.1	1
V022827	H	V179I	0.9	0.4	0.3	0.3
V022828	H	K101Q, V179I	1.5	1.1	2	0.5
V029523	H	K101Q, V179I	1.4	1.4	2.6	0.8
V029524	O	A98G, V179E, Y181C	150	89	140	42

[a] NNRTI activity was evaluated using MT-4 cells. Data are mean changes (n-fold) in the IC_{50} (medians for ≥3 experiments), calculated as the IC_{50} for the tested virus divided by the IC_{50} for the wild-type virus (HIV-1_{IIIB}), tested in parallel. The IC_{50}s for the wild-type virus were 0.9 nM (dapivirine), 77.1 nM (NVP), 16.1 nM (DLV), and 1.0 nM (EFV). ND, not determined.

[b] Mutations at amino acid positions associated with NNRTI resistance (positions 98, 100, 101, 103, 106, 108, 179, 181, 188, 190, 234, 225, 227, and 236) are listed.

TABLE 4. Activities of dapivirine compared to those of other NNRTIs against infection by resistant laboratory and recombinant HIV-1 strains

Strain	Fold change in IC_{50}[a]			
	Dapivirine	NVP	DLV	EFV
SM002-181C	8	222	83	2
SM007-103N	5	22	105	39
SM012-108I	1	1	2	1
SM024-190A	1	96	2	11
SM026-103 + 181	63	222	621	43
SM030-100I	18	5	215	38
SM034-188L	47	222	12	138
SM051-100I + 103N	2,389	22	621	10,000
SM052-101E + 103N	17	22	90	183
SM058-227L + 106A	8	22	10	39

[a] NNRTI activity was evaluated using MT-4 cells. Data are changes (*n*-fold) in the IC_{50} (medians for ≥3 experiments), calculated as the IC_{50} for the tested virus divided by the IC_{50} for the wild-type virus (HIV-1_{IIIB}), tested in parallel. The IC_{50}s for the wild-type virus were 0.9 nM (dapivirine), 77.1 nM (NVP), 16.1 nM (DLV), and 1.0 nM (EFV). A total of 10 strains for each compound were tested for changes in resistance. The number of strains with <10-fold changes in resistance was five for dapivirine, two for NVP, none for DLV, and two for EFV.

polarized manner, were pretreated with dapivirine for 1 h prior to exposure to HIV-1_{BaL} in the presence of the compound. Explants were then cultured in the absence of dapivirine. Viral infection was evaluated by the release of p24 into culture supernatants and by quantitative real-time PCR for proviral DNA (long terminal repeat) within the tissue. No drug toxicity was detected at any of the concentrations tested throughout the culture period (data not shown). Dapivirine potently inhibited HIV-1_{BaL} infection of human ectocervical explant tissue in a dose-dependent manner, as evaluated by the reduction in both p24 release and provirus content in cultured explants (Fig. 2a and b). Supernatant p24 levels produced by tissue explants suggested that dapivirine inhibited productive infection at concentrations as low as 0.1 nM (Fig. 2a). Background levels represent residual p24 input (<200 pg/ml) resulting from nonspecific adsorption to tissue. This is verified by the observed >99% inhibition of provirus formation at concentrations down to 1 nM and the fact that the calculated IC_{50} was 0.2 nM (Fig. 2b), a concentration less than that observed for T-cell cultures.

Dapivirine was also evaluated for its ability to prevent the dissemination of HIV-1 by $CD4^+$ dendritic cells (DCs) that spontaneously migrate out of cervical explants following viral exposure (20). These cells were harvested from the same cultures exposed to HIV-1 and dapivirine and were cultured overnight in the absence of drug. Cells migrating out of the tissue following overnight culture were first washed to remove any unbound virus and then cultured with permissive T cells (the PM-1 T-cell line). Dapivirine inhibited the transmission of virus to permissive T cells in a dose-dependent manner (Fig. 2c), with an IC_{50} of 0.1 nM. Complete inhibition of infection was observed at 100 nM dapivirine, and >90% inhibition was still observed with 10 nM dapivirine.

Further experiments were completed to ensure that dapivirine retained its anti-HIV activity in the presence of the biologically relevant fluids WS and CM. Cervical explants were exposed to HIV-1_{BaL} and dapivirine in the presence of 25% WS or CM for 2 h. After viral exposure, excess virus, dapivirine, and the WS or CM were removed by washing, and explants were cultured as previously described. The activity of dapivirine against HIV-1_{BaL} infection of cervical tissue and dissemination of virus via migratory cells in the presence of WS or CM (final concentration, 12.5%) was not significantly different from that of the compound tested alone (data not shown).

Prolonged inhibitory effect of dapivirine following pretreatment of cervical tissue. To determine the duration of protection afforded by pretreatment of tissue with dapivirine, cervical explants were preexposed to dapivirine for 2 or 24 h. Following compound removal, explants were either exposed immediately to HIV-1_{BaL} in the absence of compound or cultured for 2, 4, or 6 days in the absence of compound prior to exposure to the virus in the absence of dapivirine. A 2-h pretreatment of explant tissue with dapivirine resulted in significant inhibition of HIV infection when explants were challenged with virus immediately (90% inhibition at 100 nM), and inhibition was still apparent when virus challenge was delayed by 2 (90% inhibi-

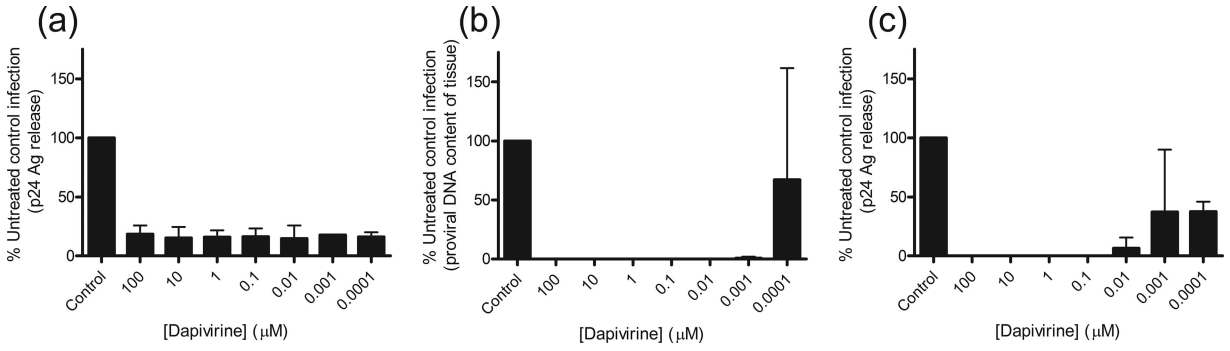

FIG. 2. Inhibition of infection of human cervical tissue explants and dissemination of virus by migratory cells. Ectocervical tissue explants were exposed to dapivirine for 1 h prior to exposure to HIV-1_{BaL} in the presence of the compound for 2 h. The virus and compound were removed, and the explants were cultured overnight. The explants were then separated from any cells that had migrated from the tissue and were cultured separately for 10 days. (a and b) Viral replication was determined by measurement of p24 culture release into the culture supernatant (a) and the relative copy number of HIV-1 proviral DNA in proteinase K-digested tissue (b). (c) Viral dissemination by migratory cells was determined by p24 release following coculture with indicator T cells. Data are means ± SD for two individual donors where each condition was tested in triplicate. Ag, antigen.

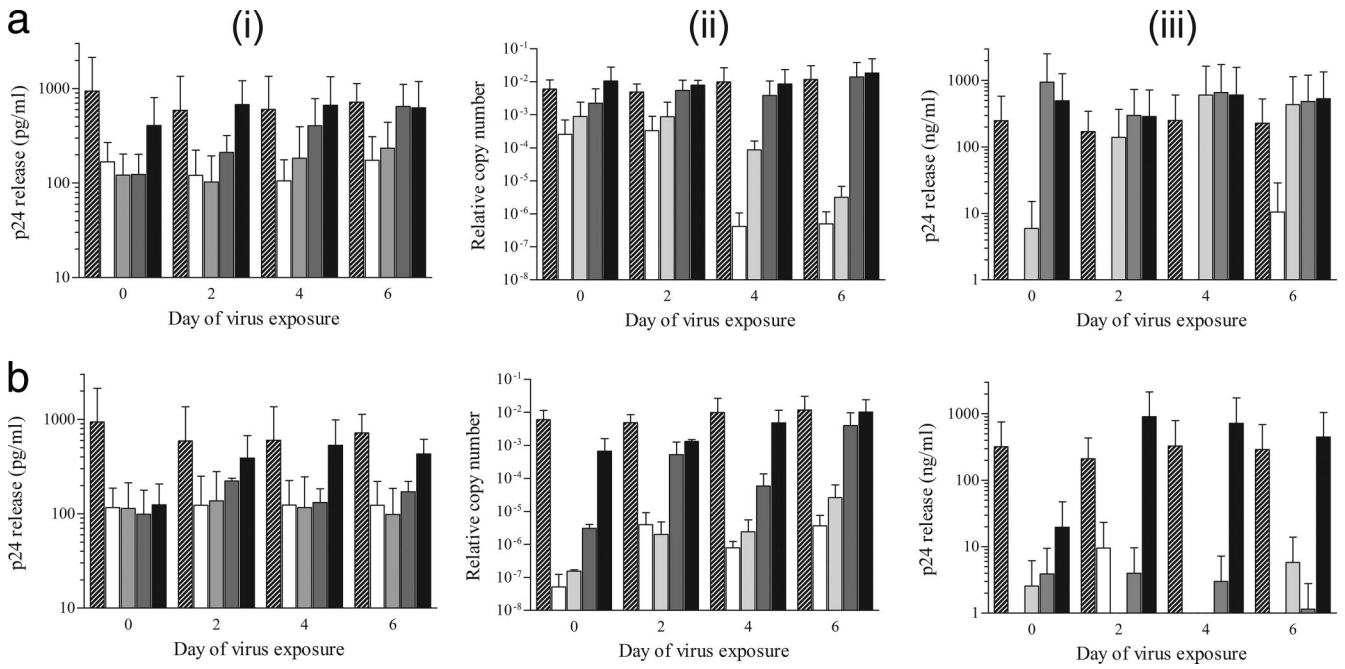

FIG. 3. Prolonged inhibitory effect of dapivirine following pretreatment of cervical tissue. Ectocervical explants were exposed to dapivirine for 2 h (a) or 24 h (b) at 37°C. Following compound removal, explants were exposed to HIV-1_{BaL} (2 h) either immediately (day 0) or following 2, 4, or 6 days in culture in the absence of compound. Virus was removed by washing and explants cultured as described in Materials and Methods. Ten days following virus exposure, infection was determined by the p24 content of explant culture supernatants (i), the HIV-1 proviral DNA content of proteinase K-digested explants (ii), and the p24 content of migratory-cell–T-cell coculture supernatants (iii). Data are means ± SEM for three individual donors (2-h exposure only) or means ± SD for two individual donors (24-h exposure only) where each condition was tested in triplicate. Bars represent dapivirine concentrations as follows: hatched bars, no-compound control; open bars, 10 μM; light shaded bars, 1 μM; dark shaded bars, 0.1 μM; filled bars, 0.01 μM.

tion at 1,000 nM), 4 (90% inhibition at 10,000 nM), and 6 days (80% inhibition at 10,000 nM) post-drug removal (Fig. 3ai [note log scale; background levels of residual p24 input were <200 pg/ml]). A similar trend was seen with 24-h dapivirine pretreatment of tissue (Fig.3bi), but dapivirine was 10-fold more active (90% inhibition at 10 nM [day 0] or 100 to 1,000 nM [day 2, 4, or 6]). A similar but more obvious dose response was seen by PCR (Fig.3ii). Furthermore, in addition to suppressing localized infection of mucosal tissue, dapivirine was also able to inhibit viral dissemination by migratory cells (Fig.3iii). This was apparent following both 2-h (90% inhibition on day 0 at 1,000 nM and on days 2, 4, and 6 at 10,000 nM) and 24-h (90% inhibition on day 0 at 10 nM and on days 2, 4, and 6 at 100 nM) pretreatment of tissue with the compound.

DISCUSSION

A successful microbicide should be capable of inhibiting the different infection pathways of HIV across the genital mucosa without inducing local or systemic toxicity or inflammation. While we have previously demonstrated inhibition of localized infection of mucosal T cells and macrophages through the blockade of cell surface receptors (CD4, CCR5, and CXCR4) (20), viral dissemination by migratory DCs, whether through "*cis*" or "*trans*" infection (22, 49), will also need to be prevented. Thus, an effective microbicide product will need to inhibit both localized mucosal infection and virus dissemination to draining lymph nodes (45).

Here we have shown that in the absence of cytotoxicity, dapivirine potently inhibits wild-type viral replication in various cell types (TZM-bl cells, T cells, and primary macrophages) when present during viral exposure (Table 1), in agreement with previous reports (48, 53). Furthermore, dapivirine demonstrates at least 10-fold greater activity than the structurally different NNRTI UC781 (10, 48, 54). Importantly, dapivirine activity was retained in the presence of biological fluids (CM and WS) (Table 2).

A particular concern in considering the use of compounds such as dapivirine as microbicides is their potential to act against viruses containing mutations associated with resistance to the family of NNRTI drugs. However, dapivirine retains its activity against a range of isolates with known resistance to other NNRTIs (Table 4) and was found to be more potent against this panel of resistant strains of HIV than the currently available NNRTIs EFV, NVP, and DLV. Furthermore, dapivirine demonstrates potent inhibitory activities against a broad panel of clinically derived isolates from a range of clades, including B, C, D, F, and H, and circulating recombinant forms AG, AC, and DF, and it demonstrated significantly reduced activity only against one clade O isolate, which harbored amino acids associated with NNRTI resistance in group M strains. Finally, dapivirine showed substantially better activity than NVP, DLV, and EFV against a random panel of clinically derived isolates from patients with NNRTI resistance. It is recognized that the therapeutic field continues to drive the production of newer NNRTIs with even better profiles than

dapivirine against resistant strains (6). There is much debate as to whether these newer drugs should be made available as potential microbicide candidates or reserved for salvage treatment for infected individuals harboring multiple-drug-resistant mutant viruses (16).

Dapivirine inhibited infection of cervical explant tissue when the tissue was exposed to the compound prior to and during viral exposure; 10 nM was sufficient to completely inhibit infection as determined by the presence of proviral DNA. Interestingly, while for many compounds the concentrations required to inhibit HIV infection of cervical tissue explants are greater than those required in cell-based assays (10, 12), this appears not to be the case with dapivirine, which demonstrated better activity in tissue experiments (IC_{50}, 0.2 nM [Fig. 2b]) than in T cells (IC_{50}, 6 nM [Table 1]). Investigation into the ability of dapivirine to inhibit viral dissemination by DCs that migrate out of tissue explants following viral exposure also demonstrated that a 100 nM concentration was sufficient to completely inhibit this pathway. Furthermore, the reduced dose of 10 nM dapivirine was still able to prevent the dissemination of about 90% of the virus. Interestingly, other groups have evaluated the ability of dapivirine to inhibit HIV infection in monocyte-derived DC and peripheral blood mononuclear cell cocultures and have determined that 100 nM was sufficient to completely inhibit infection by both cell-free and cell-associated viruses (48, 53).

The mechanism by which dapivirine inhibits the dissemination of virus by DCs is not clear. The role of DCs in mucosal HIV infection and dissemination is proposed as a two-phase transfer model: (i) DC-endosomal transfer, a rapid process (occurring within hours) where HIV is captured and internalized in endosomes, and then localized T cells are *trans*-infected, and (ii) DC replication-dependent transfer, where the transfer of virus to T cells is reliant on primary infection of the DCs, a process that reportedly takes 24 to 48 h (50). The hydrophobic nature of dapivirine means that it was not possible to reliably remove drug associated with the viral envelope; therefore, we have been unable to demonstrate definitively that dapivirine is directly able to inactivate free virions through interaction with intravirion RT prior to fusion and uncoating (34) (data not shown). Nevertheless, it is likely that sufficient drug may be associated with the virus to prevent infection. In contrast, it is unlikely that dapivirine prevents either the capture of virus, by DC-specific intercellular adhesion molecule 3-grabbing nonintegrin or other mannose C-type lectin receptors, or the endosomal internalization of virus. However, since dapivirine is able to prevent HIV infection of cervical tissue, even when the compound has been washed away, it is probable that the hydrophobic nature of dapivirine allows sufficient drug to remain associated with tissue and cells to prevent both localized DC endosomal and DC replication-dependent *trans*-infection of localized T cells. This is supported by other studies using in vitro-derived DCs (53). Interestingly, dapivirine, along with EFV and TMC125, is also reported to inhibit late stages of HIV-1 replication through enhanced processing of Gag and Gag-Pol polyproteins, leading to an associated decrease in viral particle production (9). Although not directly addressed here, this would be expected to have an additional impact on replication-dependent DC transfer to T cells.

Dapivirine demonstrated a potent, prolonged inhibitory effect in genital tissue exposed to the compound for either 2 or 24 h. A relatively short pretreatment of tissue explants with dapivirine reduced (90%) both localized and disseminated viral infection, even when viral exposure occurred as long as 6 days following compound treatment. It was noted that a 24-h pretreatment of tissue gave better protection against infection than a 2-h pretreatment; lower concentrations of dapivirine had a more obvious effect following the longer pretreatment. This suggests that the increased exposure time allows better distribution of dapivirine in tissues.

Given that dapivirine is a tight-binding inhibitor of HIV RT and is particularly hydrophobic, it is likely that this prolonged inhibitory effect is due to the association of sufficient drug with the tissue following compound removal. This attribute of the product may make it more forgiving than products that are exclusively coitally dependent, providing a potential advantage over current polyanion-based microbicide candidates evaluated in completed or ongoing phase IIb/III clinical trials for efficacy (http://clinicaltrials.gov/ct2/show/record/NCT002621066; http://clinicaltrials.gov/ct/show/NCT00213083; http://www.mtnstopshiv.org/node/352). Assuming that the in vitro efficacy of dapivirine can be translated to the in vivo situation, it is possible that tight-binding NNRTIs could be formulated for once-daily use and be applied independently of coitus. Microbicidal gel formulations of dapivirine are currently being evaluated in phase I clinical trials (21; http://www.ipm-microbicides.org/clinical_activities/english/trials.htm), and early studies suggest that a dapivirine gel (0.001, 0.002, or 0.005%), applied vaginally twice daily over 7 to 42 days, is generally well tolerated (21, 36; A. Nel, P. Coplan, S. Smythe, K. Douville, J. Romano, and M. Mitchnick, presented at Microbicides 2008, New Delhi, India, 2008, abstr. 563; S. Smythe, M. Ferreira, S. Kapiga, G. Masenga, J. Moyes, H. Rees, J. van de Wijgert, C. Von Mollendorf, J. Vyankandondera, and A. Nel, presented at Microbicides 2008, New Delhi, India, 2008, abstr. 546).

As a low-molecular-weight, hydrophobic molecule, dapivirine is an appropriate candidate for formulation into silicone elastomer intravaginal rings (IVRs) (4, 29, 30, 55). In fact, IVRs containing dapivirine have already entered early phase I clinical trials for safety, pharmacokinetics, and acceptability (http://www.ipm-microbicides.org/clinical_activities/english/trials.htm). Once-daily gels and IVRs, both used independently of coitus, could provide significant advantages for user compliance. Furthermore, the availability of a microbicide product in different dosage forms would provide users some choice as to which type of product they use, which may accommodate regional variations in cultural preferences and sexual practices.

Perhaps one of the biggest questions in microbicide research is whether in vitro efficacy data can be translated to the in vivo situation. Interestingly, dapivirine (225 and 22.5 μM) demonstrated protection (85.7 and 78.6%, respectively) against a cell-associated HIV_{SF162} challenge 20 min following vaginal application in the hu-SCID mouse model (8), suggesting that dapivirine has the potential to block infection following topical application. While it is not yet known how much vaginally delivered drug would be absorbed across the mucosa to reach target cells, it has been shown in vitro that significant amounts of dapivirine are able to diffuse through a confluent epithelial layer (54). Clinical trials are currently evaluating the safety of a 0.05% gel, equivalent to 1.5 mM dapivirine. This delivers

>100,000 times more dapivirine than is required to inhibit in vitro infection of genital tissue in the presence of the compound (Fig. 2). A recent study, evaluating the tissue dapivirine levels following vaginal administration to macaques (38), found that dapivirine remained associated with the vagina and cervix in female macaques for at least 48 h. The drug was found on the epithelial surface or within the keratinized layer of the epithelium, extending into the superficial cellular layers. Furthermore, the drug concentration associated with these tissues was reported to be higher than that required for 99% inhibition of infection in human genital tissue cultured ex vivo (38).

Systemic absorption of dapivirine following vaginal application is low, whether dosing occurs with a vaginal gel (0.16 ng/ml detected in 75% of women by day 7) (21) or an IVR (<0.2 ng/ml detected in all subjects within 4 h of insertion) (A. Nel, A., S. Smythe, K. Young, K. Malcolm, Z. Rosenburg, and J. Romano, presented at the Conference on Retroviruses and Opportunistic Infections, Boston, MA, 2008). Interestingly, the plasma dapivirine levels observed following vaginal dapivirine application are still 1,000 times lower than the lowest trough plasma level following 7 days of monotherapy with oral dapivirine (G. Van't Klooster, B. Gruzdev, A. Horban, et al., presented at the 14th International Conference on Antiviral Research, Seattle, WA, 2001). The low systemic absorption of topically applied dapivirine may minimize both the potential for systemic toxicity and the potential for the development of drug resistance if the product is used by someone unaware of her HIV status.

While dapivirine demonstrates activity against a range of isolates with known resistance to other NNRTIs, Tibotec has recently developed newer NNRTIs with even greater activity against NNRTI-resistant HIV-1. Etravine (TMC125) has an in vitro resistance profile superior to that of dapivirine and has demonstrated efficacy for NNRTI-resistant patients (24, 28). Rilpivirine (TMC278) also has a better in vitro resistance profile than dapivirine and has recently entered phase III clinical development with antiretroviral-therapy-naïve patients (33, 41). While these newer drugs have promising activities against NNRTI-resistant HIV-1, their activities against wild-type HIV-1 are similar to that of dapivirine (1, 2, 25, 33). Their development as microbicides in preference to dapivirine would require significant preclinical (formulation, toxicity, pharmacology) and early clinical safety studies before they could be evaluated in efficacy trials, with no guarantee that they might not fail at any stage in the development process. Furthermore, their superior resistance profiles might provide a strong argument for restricting their use to second-line antiretroviral therapy after failure of initial NNRTI-based regimens in a resource-poor setting (16, 47). Electing to wait for the development of one of these newer compounds as a potential microbicide would result in an unacceptable delay (several years) given the urgent need for an effective microbicide. As is common in all drug development, improvements will always follow, but currently, dapivirine represents the most advanced microbicide candidate in the NNRTI class. The data presented in this study favorably support the continued development of dapivirine as a topical vaginal microbicide against HIV-1 transmission.

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