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 can 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.html]). 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.
(NVP) (31), delavirdine (DLV) (42), and efavirin (EFV) (56). A short-term clinical trial evaluating dapivirine monotherapy demonstrated remarkable 1.44 and 1.51 log reductions in viral loads in treatment-naive patients receiving 50 or 100 mg dapivirine, respectively (M.-P. de Bethune, K. Andries, D. Lu-dovici, 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. Gruzed, A. Horban, A. Boron-Kaczmarska, D. Gille, G. Van’t Klooster, and R. Pauwels, presented at the 8th Conference on Retroviruses and Opportu-nistic Infections, Chicago, IL, 2001, abstr. 13). Extensive nonclini-cal 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 under-lying 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 1-glutamin (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 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-1BaL) and CXCR4 (X4) utilizing (HIV-1adap and HIV-1inmac) 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-dimeth-ylthiazol-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-1BaL or HIV-1inmac (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) simultaneously (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-1BaL and HIV-1inmac (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 superna-tant (RT-PCR).

(iii) Acutely infected macrophage assay. Following pretreatment with dapivirine for 1 h, primary macrophages were exposed to HIV-1inmac (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-1inmac), site-directed mutants, and clinically derived recombinant viruses (200 TCID_{50}/well) were tested in MT-4 cells (3×10^5/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 Commit-tee.) 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-1inmac (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 deter-mined 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 assayed 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 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 macropuffs 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 controls.
TABLE 1. Activities of dapivirine in cell-based cultures
c

<table>
<thead>
<tr>
<th>Assay system</th>
<th>CC_{50} (nM)</th>
<th>IC_{50} (nM)</th>
<th>Selectivity index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R5- HIV-1</td>
<td>X4- HIV-1</td>
<td>R5- HIV-1</td>
</tr>
<tr>
<td>TZM-bl cells</td>
<td>20,400 ± 1,200</td>
<td>2.2 ± 0.3</td>
<td>1.0 ± 0.5</td>
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<tr>
<td>T cells</td>
<td>2.150</td>
<td>ND</td>
<td>0.9</td>
</tr>
<tr>
<td>MT-4</td>
<td>11,000 ± 1,400</td>
<td>6.0 ± 1.4</td>
<td>2.4 ± 1.6</td>
</tr>
<tr>
<td>PM-1</td>
<td>&gt;20,000</td>
<td>2.4</td>
<td>ND</td>
</tr>
<tr>
<td>Monocyte-derived macrophages</td>
<td>&gt;20,000</td>
<td>2.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

*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_{R5}; X4-HIV-1, HIV-1_{X4}; (in TZM-bl or MT-4 cells) or HIV-1_{RF} (in T cells).*
(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).

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**TABLE 2. Activities of dapivirine in the presence of biological fluids**

<table>
<thead>
<tr>
<th>Condition</th>
<th>IC_{50} (nM)</th>
<th>IC_{90} (nM)</th>
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<tbody>
<tr>
<td>Dapivirine alone</td>
<td>1.46 ± 0.76</td>
<td>10.23 ± 4.25</td>
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<tr>
<td>Dapivirine + WS</td>
<td>1.75 ± 0.08</td>
<td>16.7 ± 4.34</td>
</tr>
<tr>
<td>Dapivirine + CM</td>
<td>8.32 ± 2.08</td>
<td>41.6 ± 27.6</td>
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</table>

a TZM-bl cells were exposed to HIV-1_{HIV}, 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_{HIV}, occurred in the presence of both a CM simulant and dapivirine (final concentration of CM, 12.5%).

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

<table>
<thead>
<tr>
<th>Virus ID</th>
<th>Pol subtype</th>
<th>NNRTI mutations</th>
<th>Fold change in IC_{50}</th>
<th>Dapivirine</th>
<th>NVP</th>
<th>DLV</th>
<th>EFV</th>
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<tr>
<td>V022808</td>
<td>CRF02_AG</td>
<td>None</td>
<td>1</td>
<td>0.8</td>
<td>0.6</td>
<td>0.9</td>
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</tr>
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<tr>
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<td>1.6</td>
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<td>1.7</td>
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<td>2.3</td>
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<td>B</td>
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<td>A98(S/A), K101R</td>
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<td>V179I</td>
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<tr>
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<td>K101Q, V179I</td>
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<tr>
<td>V029523</td>
<td>H</td>
<td>K101Q, V179I</td>
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<td>A98G, V179E, Y181C</td>
<td>150</td>
<td>89</td>
<td>140</td>
<td>42</td>
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</table>

| 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_{HIV}), tested in parallel. The IC_{50} 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, 101, 103, 106, 108, 179, 181, 188, 190, 234, 225, 227, and 236) are listed.
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. 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.

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<sub>BaL</sub> 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<sub>BaL</sub> 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<sub>BaL</sub> 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-
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 sup-
pressing 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% inhibi-
tion 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.

Here we have shown that in the absence of cytotoxicity,
dapivirine potently inhibits wild-type viral replication in vari-
ous cell types (TZM-bl cells, T cells, and primary macro-
phages) when present during viral exposure (Table 1), in
agreement with previous reports (48, 53). Furthermore, dapi-
virine 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, dapi-
virine 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

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-1std. (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.

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 pre-
vented. Thus, an effective microbicide product will need to
inhibit both localized mucosal infection and virus dissemina-
tion to draining lymph nodes (45).
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 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 (IC₅₀, 0.2 nM [Fig. 2b]) than in T cells (IC₅₀, 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 inactive 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 nonavon-based microbicide candidates evaluated in completed or ongoing phase Ib/III clinical trials for efficacy (http://clinicaltrials.gov/ct2/show/record/NCT002621066; http://clinicaltrials.gov/ct2/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. Vykandondera, 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 HIV₁₁₁₆₂ 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
detected in 75% of women by day 7) (21) or an IVR (tion is low, whether dosing occurs with a vaginal gel (0.16 ng/ml was reported to be higher than that required for 99% inhibi-
levels following vaginal administration to macaques (38),
vitro infection of genital tissue in the presence of the com-
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ng/ml detected in all subjects withi n4ho f insertion) (A. Nel,

microbicide candidate in the NNRTI class. The data presented in

is common in all drug development, improvements will always

potential microbicide would result in an unacceptable delay (sev-
ond-line antiretroviral therapy after failure of initial NNRTI-
dominant regimens in a resource-poor setting (16, 47). Electing to

While dapivirine demonstrates activity against a range of iso-
lates with known resistance to other NNRTIs, Tibotec has re-
dlately altered vaginal application are still 1,000 times lower than the lowest trough plasma level following 7 days of monotherapy with oral dapi-

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REFERENCES

1. Andries, K., H. Aziin, T. Thielemans, D. Ludovici, M. Kukla, J. Jeeres, P. Janssen, B. De Corte, J. Vingerhoets, R. Pauwels, and M. P. de Bethune. 2004. TMC125, a novel next-generation nonnucleoside reverse transcriptase inhibitor active against nonnucleoside reverse transcriptase inhibitor-resis-


roviral activity of different drugs on macrophages. Methods Mol. Biol. 304:


19. Hertogs, K., M. P. de Bethune, V. Miller, T. Ivens, P. Schel, A. Van Cau-
