In Vitro Microbicidal Activity of the Nonnucleoside Reverse Transcriptase Inhibitor (NNRTI) UC781 against NNRTI-Resistant Human Immunodeficiency Virus Type 1

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The nonnucleoside reverse transcriptase inhibitor (NNRTI) UC781 is under development as a microbicide to prevent sexual transmission of the human immunodeficiency virus type 1 (HIV-1). However, NNRTI-resistant HIV-1 is increasingly prevalent in the infected population, and one of the concerns for NNRTI-based microbicides is that they will be ineffective against drug-resistant virus and may in fact selectively transmit NNRTI-resistant virus. We evaluated the microbicidal activity of UC781 against UC781-resistant (UCR), efavirenz-resistant (EFVR), and nevirapine-resistant (NVPR) strains in a variety of microbicide-relevant tests, including inactivation of cell-free virus, inhibition of cell-to-cell HIV-1 transmission, and the ability of UC781 pretreatment to protect cells from subsequent infection in the absence of exogenous drug. UC781 was 10- to 100-fold less effective against NNRTI-resistant HIV-1 compared to wild-type (wt) virus in each of these tests, with UC781 microbicidal activity against the various virus strains being $wt \geq NVPR > UCR \geq EFVR$. Breakthrough experiments using UC781-pretreated cells and mixtures of wt and NNRTI-resistant HIV-1 showed that UC781-pretreatment selected for NNRTI-resistant HIV-1. However, the efficacy of UC781 was dose dependent, and 25 μ M UC781 provided essentially equivalent microbicidal activity against NNRTI-resistant and wt virus. The amount of UC781 in topical microbicide formulations under current development is approximately 100-fold greater than this concentration, so transmission of NNRTI-resistant virus may not be an issue at these microbicide formulation levels of UC781. Nonetheless, the reduced microbicidal activity of UC781 against NNRTI-resistant HIV-1 suggests that additional antiviral agents should be included in NNRTI-based microbicide formulations.

Sexual transmission of the human immunodeficiency virus type 1 (HIV-1) is the principal mode of spread of HIV throughout the world (18, 47, 51). Appropriate and effective strategies to prevent HIV-1 transmission are essential to minimize the continued spread of the virus. A prophylactic anti-HIV-1 vaccine would be ideal in this respect, but no such vaccine is available despite more than a decade of intensive development effort. Another potential mechanism to reduce the transmission of HIV-1 is the use of vaginal and/or rectal topical microbicides (20, 32, 35).

We suggest that the ideal microbicide should fulfill a number of criteria, including having high potency against HIV-1, having the ability to directly inactivate the virus, showing efficacy against a wide range of HIV strains, preventing cell-to-cell virus transmission of HIV, and providing a barrier to viral infection of uninfected cells. A variety of compounds have been proposed as potential topical anti-HIV microbicides (16, 21, 40, 41, 44, 54–57). One of these is the nonnucleoside reverse transcriptase inhibitor (NNRTI) UC781, which we and others have shown to possess excellent anti-HIV-1 microbicidal activity against wild-type (wt) HIV-1 in vitro (7, 10, 15, 22, 39, 42, 43). However, a significant concern with NNRTI-based microbicides is that these might be ineffective against NNRTI-resistant HIV-1 and thus might select for the transmission of

NNRTI-resistant virus strains. Such strains of HIV-1 are increasingly prevalent in infected individuals, not just in developed countries but also in developing nations, due to the use of single-dose nevirapine (NVP) to prevent maternal-fetal transmission of the virus (19, 23, 24, 31, 38). We therefore evaluated the efficacy of UC781 against several HIV variants with high-level resistance to NNRTI using a variety of microbicide-relevant tests, including inactivation of cell-free virus, inhibition of cell-to-cell HIV-1 transmission, and the ability of UC781 pretreatment to protect cells from subsequent infection in the absence of exogenous drug. Not surprisingly, UC781 was in most cases less effective against NNRTI-resistant HIV-1 compared to wt virus in each of these tests. However, UC781 was fully active against the NNRTI-resistant virus strains at concentrations of 25 μ M, a level well below the amount of UC781 in microbicide formulations under current clinical testing. Nonetheless, our results suggest that microbicides based on UC781 alone may not be ideal and that formulations comprising combinations of active agents are preferable.

MATERIALS AND METHODS

Reagents. UC781 was provided by Crompton Inc. (now Chemtura, Middlebury, CT). NVP and efavirenz (EFV) were obtained from the NIH AIDS Research and Reference Reagent Program. Stock solutions of NNRTI were prepared in dimethyl sulfoxide and stored at -20°C . Human lymphocytoid CEM, MT2, MT4, and H9 cell lines were obtained from the NIH AIDS Research and Reference Reagent Program and were cultured in RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) with 10% fetal bovine serum (Cellgro, Herndon, VA), 1% glutamine (Cellgro), and 1% penicillin and streptomycin (Gibco,

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TABLE 1. Antiviral activity of UC781 against wt and NNRTI-resistant HIV-1

HIV-1 strain	Genotype	Antiviral activity of UC781 (EC ₅₀ [nM]) ^a	Fold resistance
NL4-3	Wild type	7.0 ± 2.7	
UCR	V106A + I135R + Y181C	3,260 ± 440	466
EFVR	L100I + K103N	9,280 ± 670	1,325
NVPR	Y181C	52.0 ± 8.1	7.5

^a All values are the means ± standard deviation for three separate experiments each carried out in triplicate.

Grand Island, NY). HIV-1 p24 antigen assay kits were obtained from SAIC—Frederick (Frederick, MD).

In vitro generation of NNRTI-resistant HIV-1. HIV-1 resistant to UC781, EFV, or NVP was developed by serial passage of HIV-1 NL4-3 in the presence of increasing concentrations of the NNRTI. Briefly, 5-ml cultures of MT2 cells (4×10^5 cells/ml) were infected by HIV-1 (NL4-3) in the presence of $2 \times$ the 50% effective concentration of UC781, EFV, or NVP. The initial virus inoculum was $>1,000$ times the 50% tissue culture infective dose (TCID₅₀). The course of infection was monitored by microscopic evaluation of syncytium formation until the HIV-induced cytopathic effect (CPE) was 75% or greater. Virus-containing cell supernatants were obtained by centrifugation of the infected cultures and used to infect fresh MT2 cells. The drug concentration was increased twofold with each successive passage of virus to enhance the selective pressure on the virus. Virus able to replicate in the presence of a minimum of $200 \times$ the initial 50% inhibitory concentration was considered resistant. Mutations in the reverse transcriptase (RT) gene associated with resistance to the various NNRTI were determined by clonal sequencing of proviral DNA as previously described (8, 45).

H9 cells persistently infected with wt (NL4-3) or NNRTI-resistant (UCR, EFVR, and NVPR for UC781, EFV, and NVP resistant, respectively) HIV-1 were generated essentially as previously described (48). Virus stocks were prepared from cell supernatants from persistently infected H9 cells or CEM cells acutely infected with HIV-1NL4-3 and NNRTI-resistant HIV-1 strains. Cultures were cleared from cellular debris by low-speed centrifugation and filtered through 0.45- μ m membranes (Millipore, Billerica, MA), and the filtrate was concentrated by centrifugation at 3,500 rpm for 30 min using Amicon Ultra-15 centrifugal filter devices with a cutoff of 100 kDa (Millipore). Stock virus infectivity was determined by end-point dilution in MT2 cells. Virus-induced CPE was scored 7 days postinfection, and the TCID₅₀ was calculated as described previously (49).

UC781 inactivation of cell-free virus. HIV-1 virions (300 ng p24) were incubated with various concentrations of UC781 in 0.5 ml RPMI 1640 containing 10% fetal bovine serum (FBS) for 1 h at 37°C. The virus samples were then diluted with drug-free medium to a volume of 12 ml and concentrated by ultrafiltration as previously described (10, 33, 39). The dilution and concentration steps were repeated a total of three times. Analysis of antiviral activity of the final virus-free wash medium confirmed that the washing procedure resulted in complete removal of the exogenous UC781 from the treated HIV-1 virions, in confirmation of our previous results (10, 39). The final exogenous drug-free virus was suspended in RPMI 1640 containing 10% FBS and stored at -80°C until use.

Effect of UC781 pretreatment of persistently infected H9 cells on subsequent infectivity of cell-free, cell-associated, and nascent HIV-1 produced by these cells. Cells persistently infected with wt or NNRTI-resistant HIV-1 were incubated with 25 μ M UC781 for 18 h at 37°C. Cells and virus-containing supernatants were separated by low-speed centrifugation, and the cells were washed several times with medium to remove residual drug. The virus in the cell-free supernatant was concentrated by ultrafiltration, and extracellular drug was removed by dilution and concentration as previously described (10, 39).

The washed persistently infected cells were suspended in drug-free medium (at 2×10^5 cells/ml) and cultured at 37°C. After every 24-h culture period, cell supernatants were obtained by low-speed centrifugation. The cells were again suspended in drug-free medium (at 2×10^5 cells/ml) and cultured at 37°C. This process was repeated for three sequential 24-h incubations.

The effect of UC781 treatment on subsequent cell-to-cell virus transmission was assessed by incubating the UC781-treated persistently infected H9 cells with uninfected MT4 cells in a ratio of 1:200. Virus replication was assessed by measurement of HIV-1 p24 antigen levels 7 days after initiation of coculture. To assess the infectivity of virions produced by the persistently infected H9 cells following removal of UC781, the virus in the cell culture supernatants obtained

after each 24-h culture period was isolated and washed free of any exogenous UC781 by ultracentrifugation through a 20% sucrose cushion. Aliquots of isolated HIV-1 virions corresponding to 250 pg p24 were used to infect 5×10^4 MT4 cells. Infectivity was assessed by measurement of HIV-1 p24 antigen levels 7 days postinfection.

Determination of the genotype of breakthrough variants of HIV-1 from UC781-pretreated cells exposed to mixtures of wild-type and UC781-resistant HIV-1. Uninfected CEM T4 cells were incubated with 25 μ M UC781 for 18 h at 37°C. Extracellular drug was removed by multiple washings of the cells with drug-free RPMI 1640 containing 10% FBS. Treated cells (2×10^5 cells) were then infected with various ratios of wt to NNRTI-resistant HIV-1 ranging from 90:10 to 10:90 based on equivalent TCID₅₀ values. Infectivity was monitored by daily microscopic examination of CPE. Cells were passaged every 2 days, and supernatants were collected for subsequent analysis of HIV-1 24 content. Positive virus breakthrough was considered when CPE was 30% or greater. When CPE was greater than 80%, cell culture supernatants were isolated and stored at -80°C . Virions in the cell culture supernatants were pelleted by ultracentrifugation, and viral RNA was prepared by the guanidinium isothiocyanate method (17). A 1,775-bp fragment containing the entire HIV-1 RT gene was amplified by from isolated viral RNA using the Superscript First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA). The PCR product was resolved by electrophoresis on 1% agarose and purified by Genelute Minus ethidium bromide spin columns (Sigma, Saint Louis, MO). The purified PCR product was cloned into the pSTBlue-1 Acceptor vector (Novagen, Madison, WI). Positive colonies were selected by blue/white screening of recombinants. Plasmid DNA was isolated from single colonies using QIAprep Spin Miniprep kits (QIAGEN Inc., Valencia, CA). The presence of NNRTI resistance mutations in the RT was verified by sequencing.

RESULTS

Inhibitory activity of UC781 against wt and NNRTI-resistant HIV-1. The antiviral activity of UC781 against wt and NNRTI-resistant HIV-1 strains is shown in Table 1. UC781 is a very potent inhibitor of wt HIV-1, with half-maximal inhibitory concentrations of less than 10 nM (Table 1), as has been previously shown (10, 39). The NNRTI-resistant HIV-1 strains

FIG. 1. Inactivation of cell-free HIV-1 particles following exposure to different concentrations of UC781. NNRTI-resistant HIV (UCR, EFVR, and NVPR) and wt NL4-3 were incubated with the indicated concentrations of UC781 for 1 h at 37°C. Excess drug was then removed by repeated dilution and concentration by ultrafiltration as described in Materials and Methods. Infectivity was then assessed by addition of equivalent aliquots of the different virus strains (normalized for p24 content) to CEM cells. HIV-1 p24 antigen content of cell culture supernatants was measured 5 days postinfection. ∇ , wt HIV-1 NL4-3; $\bullet$, NVPR HIV-1; $\circ$, UCR HIV-1; $\blacktriangle$, EFVR HIV-1. $\square$, antiviral activity of the final virus wash medium. Data shown are the means ± standard deviation of triplicate determinations.

FIG. 2. Effect of UC781 treatment on transmission of cell-associated HIV-1. H9 cells persistently infected with wt or UCR HIV-1 were incubated with 25 μ M UC781 for 18 h at 37°C. Exogenous UC781 was removed from the treated cells and from the virions produced by the cells during the 18-h incubation. (A) Infectivity of the virions produced by persistently infected H9 cells incubated for 18 h in the absence (gray bars) or the presence (black bars) of 25 μ M UC781. Data are the means $\pm$ standard deviation of at least three individual determinations. (B) Persistently infected H9 cells were incubated in the absence or presence of 25 μ M UC781 for 18 h, and the cells were then washed several times to remove exogenous drug. The cells were then cultured in drug-free medium, and the virus produced during each sequential 24-h period was isolated and evaluated for the ability to infect CEM cells. ∇ , wt HIV-1, cells not exposed to drug; $\circ$, UCR HIV-1, cells not exposed to drug; $\blacktriangledown$, wt HIV-1, cells pretreated with 25 μ M UC781; $\bullet$, UCR HIV-1, cells pretreated with 25 μ M UC781. Data are the means $\pm$ standard deviation of at least three individual determinations. (C) Persistently infected H9 cells were incubated in the absence or the presence of 25 μ M UC781 for 18 h, and the cells were then washed

selected under dose-escalating drug pressure showed a variety of mutations. NVPR HIV-1 possessed the Y181C mutation in RT. UC781 showed only a 5- to 10-fold reduction in potency against the NVPR virus. UCR HIV-1 possessed three mutations, V106A, I135R, and Y181C. This combination of mutations provided nearly 500-fold resistance to the drug. UC781 was significantly less active against EFVR HIV-1. This virus possessed the L100I and K103N mutations in RT, which provided over 1,300-fold resistance to UC781.

UC781 inactivation of cell-free HIV-1. Cell-free wt and NNRTI-resistant virions were exposed to various concentrations (0.0025 to 25.0 μ M) of UC781 for 1 h, and the virions were then separated from exogenous drug by repeated dilution and concentration by ultrafiltration. Dose-dependent inactivation of wt HIV-1 NL4-3 was observed (Fig. 1), and the virus was completely inactivated by incubation with 250 nM UC781, in confirmation of our previous data with HIV-1_{IIB} (10). Significantly higher concentrations of UC781 were needed to inactivate NNRTI-resistant HIV-1, especially UCR and EFVR virus. However, NNRTI-resistant HIV-1 strains were completely inactivated when exposed to 25 μ M UC781 (Fig. 1).

UC781 inactivation of cell-associated HIV-1 and inhibition of cell-to-cell virus transmission. HIV-infected patient seminal and vaginal fluids contain both free virus particles and virus-infected cells (36), and cell-associated virus has been proposed to be a more significant source for HIV transmission than cell-free virus (4, 28, 29). We compared the ability of UC781 treatment of H9 cells persistently infected with wt or UCR HIV-1 on the subsequent transmission of infection in the absence of exogenous drug. Persistently infected H9 cells were incubated with 25 μ M UC781 for 18 h. The cells and the virus produced by the cells during the 18-h incubation were separated by low-speed centrifugation, and the virions were then washed free of exogenous UC781 by repeated dilution and concentration by ultrafiltration. The recovered virus was then tested for infectivity. As can be seen in Fig. 2A, both wt and UCR HIV-1 produced in the presence of UC781 were completely attenuated in infectivity.

The UC781-treated persistently infected H9 cells were washed extensively to remove exogenous drug, and the cells (2×10^5 cells/ml) were then cultured in drug-free medium. Every 24 h, the cell supernatants were collected and the cells (adjusted to 2×10^5 cells/ml) were again cultured in drug-free medium. The cell supernatant samples (normalized for p24 content) were used to infect MT4 cells in the absence of UC781. As shown in Fig. 2B, virus produced by UC781-treated H9 cells

several times to remove exogenous drug. The cells were then cultured in drug-free medium. At 24-h intervals, cells were isolated and then cocultured with uninfected MT4 cells at a ratio of 10^3 persistently infected H9 cells to 2×10^5 uninfected MT4 cells. The extent of cell-to-cell transmission of virus was evaluated by measuring HIV-1 p24 antigen in cell supernatants obtained 4 days postinfection. Gray bars, infectivity of persistently infected H9 cells after 24 h of culture in drug-free medium; white bars, infectivity of persistently infected H9 cells after 48 h of culture in drug-free medium; black bars, infectivity of persistently infected H9 cells following 72 h of culture in drug-free medium. Data are the means $\pm$ standard deviation of at least three individual determinations.

FIG. 3. Effect of UC781 pretreatment of cells on subsequent HIV-1 infection in the absence of exogenous drug. CEM cells were incubated with the indicated concentrations of UC781 for 18 h at 37°C, and then exogenous drug was removed by repeated washing with RPMI 1640 medium containing 10% FBS. The pretreated cells were then exposed to equivalent inocula of wt or NNRTI-resistant HIV-1 (normalized for p24). HIV-1 replication was evaluated by measuring p24 antigen in the culture supernatants 7 days postinfection. ∇ , wt HIV-1 NL4-3; $\bullet$, NVPR HIV-1; $\circ$, UCR HIV-1; $\blacktriangle$, EFVR HIV-1. Data are the means $\pm$ standard deviation of triplicate determinations.

persistently infected with wt HIV-1 remained attenuated in infectivity over the time period of the experiment. Even virus produced by these cells 3 days following removal of UC781 was markedly reduced in infectivity. In contrast, although virus produced by UC781-treated H9 cells persistently infected with UCR HIV-1 during the first 24-h period after removal of the drug showed substantially reduced infectivity relative to the control, virus produced in subsequent 24-h periods showed increasing infectivity such that full infectious potential was noted 72 h following removal of the drug.

The UC781-treated H9 cells persistently infected with wt NL4-3 or UCR HIV-1 isolated at each 24-h interval of culture in drug-free medium were cocultured with uninfected MT4 cells at a ratio of 1:200 persistently infected H9 cells to uninfected MT4 cells. Infection of the MT4 cells was evaluated by microscopic assessment of HIV-1-induced CPE and measurement of HIV-1 p24 levels in cell-free culture supernatants 4 days following the initiation of the coculture. As shown in Fig. 2C, UC781 pretreatment completely prevented cell-to-cell transmission of wt HIV-1 for up to 72 h following removal of UC781. In contrast, UC781 pretreatment was ineffective at preventing cell-to-cell transmission of UCR HIV-1.

Ability of UC781 pretreatment of uninfected cells to protect against subsequent HIV-1 infection in the absence of exogenous drug. CEM cells were incubated with various concentrations of UC781 for 18 h, and then the residual exogenous drug was removed and cells were infected with wt or NNRTI-resistant HIV-1. As shown in Fig. 3, UC781 pretreatment was very effective at preventing subsequent infection by wt HIV-1, with virtually complete protection noted in cells pretreated with 1 μ M UC781. UC781 pretreatment was less effective at preventing subsequent infection by NNRTI-resistant HIV-1, with protection efficacy being NVPR > UCR $\geq$ EFVR. However, pretreatment of cells with 25 μ M UC781 was able to afford

protection against subsequent infection by all NNRTI-resistant HIV-1 strains in the absence of exogenous drug.

Effect of UC781 pretreatment of uninfected cells on subsequent infection by mixtures of wt and NNRTI-resistant HIV-1. CEM cells were treated with 25 μ M UC781 for 18 h, and residual exogenous drug was then removed by extensive washing. Cells were then inoculated with different ratios of wt to NNRTI-resistant HIV-1. All virus inocula were normalized for p24 content. As shown in Fig. 4A to C, UC781 pretreatment effectively blocked breakthrough of wt HIV-1. As the ratio of NNRTI-resistant virus in the inoculum was increased, the time to breakthrough became progressively shorter.

Does UC781 pretreatment of uninfected cells select for breakthrough of NNRTI-resistant HIV-1? The genotype of the breakthrough virus populations obtained 14 days postinfection in the experiment shown in Fig. 4 was determined by sequencing analysis of the viral RNA, as described in Materials and Methods. The ratio of wt to NNRTI-resistant HIV-1 in the breakthrough virus population obtained from untreated cells was virtually identical to that in the virus inoculum used to infect these cells (Table 2). In contrast, the breakthrough virus population obtained from UC781-pretreated cells was highly enriched for the NNRTI-resistant virus (UCR or EFVR). These data suggest that UC781 pretreatment of cells does indeed select for infection by NNRTI-resistant HIV-1.

DISCUSSION

In our opinion, an ideal microbicide to prevent HIV-1 transmission should possess high potency against HIV-1, have the ability to directly inactivate the virus without the need for metabolic activation, have efficacy against a wide range of HIV strains, prevent cell-to-cell virus transmission of HIV, and provide a barrier to viral infection of uninfected cells. We, and others, have shown that the NNRTI UC781 fulfills all of these criteria against wild-type HIV-1 (1, 6, 10, 12, 39, 50, 56) and have therefore proposed that UC781 is a logical candidate for development as a microbicide to prevent sexual transmission of HIV-1. There is concern, however, that NNRTI-based microbicides might be ineffective against NNRTI-resistant HIV-1 and thus might select for the transmission of NNRTI-resistant virus strains. Such strains of HIV-1 are increasingly prevalent in infected individuals, not just in developed countries with access to highly active antiretroviral therapy (HAART) but also in developing nations, due to the use of single-dose nevirapine to prevent maternal-fetal transmission of the virus. The latter protocol has unfortunately resulted in the rather significant appearance of NNRTI-resistant HIV-1 strains both in the treated mothers and in the offspring (23, 27).

Resistance to UC781 correlates with mutations in the NNRTI binding pocket of HIV-1 RT (11, 53). However, our data show that UC781 maintains potent antiviral and microbicide inhibitory activity against HIV strains and RT with single mutations in the NNRTI binding pocket, such as Y181C. NNRTI-resistant virus strains appearing in individuals in response to single-dose nevirapine exposure generally possess single NNRTI resistance mutations. We therefore suggest that microbicide formulations containing UC781 as the active agent may still be very useful in such infected patient populations.

FIG. 4. Effect of UC781 pretreatment of cells on subsequent infection by mixtures of wt and NNRTI-resistant HIV-1 in the absence of exogenous drug. CEM cells were incubated with 25 μ M UC781 for 18 h at 37°C and then washed free of residual exogenous drug. Cells were infected with different ratios of wt NL4-3 and (A) UCR HIV-1, (B) EFVR HIV-1, or (C) NVPR HIV-1. Aliquots of cell culture supernatants were assessed for HIV-1 p24 antigen levels every 2 days postinfection. The ratios of wt to NNRTI-resistant HIV-1 in the inocula were as follows: ●, wt only; ▽, 10% NNRTI resistant; ■, 25% NNRTI resistant; ◇, 50% NNRTI resistant; ▲, 75% NNRTI resistant; ○, 90% NNRTI resistant; ●, 100% NNRTI-resistant. ▽, untreated cells infected with 100% wt or 100% NNRTI-resistant HIV-1. Data are the means $\pm$ standard deviation of triplicate determinations.

High-level HIV-1 resistance to UC781 requires multiple mutations in the RT NNRTI binding pocket (11, 58). While such virus strains are increasingly common in developed countries due to the widespread use of NNRTI in HAART, HIV-1

possessing multiple NNRTI mutations is less predominant in non-HAART populations as found in developing countries.

HIV-1 strains developed under increasing UC781 drug pressure in vitro show a variety of different mutations in the RT NNRTI binding pocket. Balzarini et al. (8) observed that UC781-resistant HIV-1 possessed V106A plus F227L plus L100I in RT, whereas Buckheit et al. (15) noted Y181C plus V108I plus K101E. In the present study, we identified a different mutation set, V106A plus I135R plus Y181C, providing 500-fold resistance to UC781 (Table 1). The variety of different mutations identified in resistance to UC781 may be related to the intrinsic flexibility of the molecule. UC781 is a significantly more flexible molecule than either EFV or NVP and thus may be capable of binding to the NNRTI binding pocket in multiple conformations. The conformational flexibility of the UC series of NNRTI may be necessary to provide potent inhibition of HIV-1 RT. We have previously shown that the conformationally different UC NNRTI UC84 and UC38 interact with different mechanistic forms of HIV-1 RT (25). Virus resistant to these two NNRTI possess different mutations in the NNRTI binding pocket (G. Borkow and M. A. Parniak, unpublished data). Unlike UC38 and UC84, UC781 inhibits all mechanistic forms of HIV-1 RT (26). This characteristic may be important for the increased potency of UC781 compared to the other UC NNRTI. However, the flexibility of the molecule may also be detrimental in that mutations arising anywhere in the NNRTI binding pocket of RT may provide some degree of resistance to the drug.

Of considerable concern for the use of UC781-based microbicides in populations with access to HAART are mutations associated with resistance to efavirenz, since this NNRTI is widely used in NNRTI-containing therapeutic regimens. We identified the mutations L100I plus K103N in RT of HIV-1 strains developed under EFV drug pressure in vitro (Table 1). These mutations are identical to those found by others (5), as might be expected given the relative inflexibility of EFV compared to UC781. The L100I plus K103N mutations led to nearly 1,500-fold resistance to UC781 (Table 1), substantially greater than the resistance provided by mutations arising directly from resistance to UC781. Nonetheless, our data show that UC781 retains microbicidal activity against HIV-1 strains with either the mutation in RT V106A plus I135R plus Y181C or L100I plus K103N (Fig. 1 and 3).

TABLE 2. Breakthrough of NNRTI-resistant HIV-1 in UC781-pretreated cells subsequently exposed to HIV-1 in the absence of exogenous drug

Virus mixture (ratio)	No. of clones analyzed	No. (%) of NNRTI-resistant HIV-1 strains/no. tested in breakthrough population
No UC781 pretreatment		
wt/UCR (50:50)	12	6/12 (50)
wt/UCR (90:10)	30	2/30 (7)
wt/EFVR (50:50)	30	15/30 (50)
Cells pretreated with 25 μ M UC781		
wt/UCR (50:50)	13	12/13 (92)
wt/UCR (90:10)	31	22/31 (71)
wt/EFVR (50:50)	30	26/30 (87)

As an example, exposure to 500 nM UC781 completely inactivates wild-type HIV-1 virions, whereas 50-fold-higher concentrations are needed to inactivate EFV- or UC781-resistant HIV-1 (Fig. 1). Similarly, while preincubation of uninfected cells with 1 μ M UC781 rendered these cells refractory to subsequent infection in the absence of exogenous drug, this concentration provided little protection against infection by UC781-resistant HIV-1. However, cells were protected against infection by pretreatment with 50 μ M UC781 (Fig. 3). The lowest level of UC781 in formulations under current preclinical development is 0.1%, which corresponds to a potential concentration of 3,000 μ M, 60-fold greater than the concentration of UC781 needed for microbicidal activity against highly NNRTI-resistant HIV-1. We therefore propose that concerns about the transmission of NNRTI-resistant HIV-1 due to use of UC781-based microbicides may be unfounded.

UC781 has the interesting property of being a "tight-binding" inhibitor of HIV-1 RT (9, 12, 13, 39). In other words, UC781 binds rapidly to RT, but once bound, dissociates very slowly, even in the absence of exogenous unbound compound. This contrasts with nevirapine, a rapid equilibrium inhibitor, which can inhibit RT DNA synthesis only when there is a sufficient exogenous concentration of the drug to ensure continued occupancy of the NNRTI binding pocket on RT. Nevirapine is ineffective in any of the in vitro tests used to evaluate microbicide candidates (10, 30, 39; this paper). Because of this, we previously proposed that the tight-binding characteristic is an essential parameter for microbicidal activity of NNRTI (10, 39). Our present data suggest that this is not necessarily the case. It therefore seems that properties other than (or perhaps in addition to) tight binding must contribute to the microbicidal activity of UC781.

We have suggested that UC781 may accumulate in some cellular compartment that enables subsequent access to infecting HIV-1 (3, 39). However, the chemical properties of UC781 are similar to those of EFV and NVP, all of which have similar hydrophobicity indices (logP), ionization, etc. While EFV has pronounced serum protein binding (2, 34, 37, 52), which could impact microbicidal activity, NVP and UC781 do not (14, 46). Furthermore, NVP has no microbicidal activity, whereas both EFV and UC781 do. Thus, the properties of UC781 that provide its exceptional utility in microbicide applications remain unclear.

While UC781 is less effective against NNRTI-resistant HIV-1 than against wt virus, the potential utility of UC781 as an anti-HIV microbicide is strengthened by our findings that formulation-attainable concentrations of the compound retain microbicidal properties against NNRTI-resistant HIV-1. This is perhaps a surprising finding, but one that further underscores the exceptional promise of this drug for use as a microbicide to prevent HIV-1 transmission. Nevertheless, the reduced microbicidal activity of UC781 against NNRTI-resistant HIV-1 suggests that it would be expedient to include additional antiviral agents in NNRTI-based microbicide formulations. Ideally, these antiviral agents should be directed against a different HIV target from RT. These additional agents need not be additional expensive antiretroviral drugs, as this would substantially increase the cost per dose of the microbicide formulation and negatively impact their use in developing countries. Instead, readily available and inexpensive agents such as carrageenans or other anionic polysaccharides that can block HIV attachment may be considered. Such excipient

agents would be equally effective against wt and NNRTI-resistant strains of HIV-1 and could reduce the effective infective dose of virus to which cells are exposed. This would potentiate the antiviral activity of UC781 and possibly provide even greater protection against transmission of NNRTI-resistant virus.

Lessons learned from systemic treatment of HIV-1-infected patients show that drug combinations provide the most effective therapeutic approach. While UC781 alone may be effective as a microbicide against transmission of NNRTI-resistant HIV-1, we emphasize that only those microbicide formulations comprising combinations of active anti-HIV agents should be seriously considered for development in order to minimize the selective transmission of drug-resistant variants of HIV-1.

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