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RESEARCH ARTICLE

Broad-Spectrum Anti-HIV Microbicide Activity of the Non-Nucleoside Reverse Transcriptase Inhibitor UC781

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Abstract

Background: The tight-binding nonnucleoside reverse transcriptase inhibitor (NNRTI) UC781is under development as a topical microbicide to prevent sexual transmission of the human immunodeficiency virus type 1 (HIV-1). However, there are concerns whether UC781 will be broadly effective against different HIV subtypes as well as against the increasingly prevalent NNRTI-resistant HIV strains.

Methods: A variety of *in vitro* tests were used to evaluate the microbicidal efficiency of UC781 against 25 different HIV-1 clades and subtypes, and drug-resistant HIV-1 variant. Anti-HIV microbicide activity of UC781 was assessed both in single cycle infectivity assays as well as in virus spreading assays using activated human peripheral blood mononuclear cells (PBMCs), CD4+ T cells and monocyte-derived macrophages (MDM).

Results: For 20 of the 25 HIV-1 strains assessed, including several multi-drug-resistant HIV-1 strains, the mean EC_{50} for UC781 was 0.008 μM (range, 0.003 to 0.026 μM) in single cycle infectivity assays. UC781 was 60-fold less effective against one of the nine HIV-1 subtypes tested, subtype O (strain MVP5180). Not unexpectedly, UC781 was 465-fold, 1325-fold and 7-fold less effective against NNRTI-resistant HIV: UC781-resistant (UCR), efavirenz-resistant (EFVR), and nevirapine-resistant (NVPR), respectively. Nonetheless, pretreatment of PBMC, CD4+ T cells and MDM (induced by both M-CSF and GM-CSF) with UC781 concentrations of 25 μM or greater completely prevented subsequent infection of these cells by any of the virus strains in the absence of exogenous drug.

Conclusion: Reduced potency of UC781 against NNRTI-resistant virus variants is well below those in current microbicide formulations due to the "memory effect" exerted by pretreatment. Thus, UC781 demonstrated potent and genetic-subtype independent anti-HIV activity swhich suggests that UC781-based microbicides will likely be efficacious in different geographical regions.

Keywords

AIDS/HIV, UC781, Microbicide, NNRTI-resistant, HIV-1 subtypes, Multi-drug resistant, NRTI-resistant

Glossary

AIDS: Acquired Immunodeficiency Syndrome; HIV-1: Human Immunodeficiency Virus type 1; NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitor; PBMC: Peripheral Blood Mononuclear Cell; MDM: Monocyte Derived Macrophages; MDR: Multi-Drug Resistant; UCR: UC781-Resistant; EFVR: Efavirenz-Resistant; NVPR: Nevirapine-Resistant; NRTI: Nucleoside-Reverse Transcriptase Inhibitor; M-CSF: Macrophage Colony Stimulating Factor; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; PHA: Phytohemagglutinin

Introduction

Despite the availability of antiviral drugs that can reduce the viral transmission and slow the progression of diseases, human immunodeficiency virus type-1 (HIV-1) is still a leading cause of death, and it remains a major global health issue. Currently, about 39 million people globally are living with HIV-1 and 46% of all new HIV infections were among women and girls [1]. There are 1.3 million people became newly infected with HIV in 2022 and majority of the infection through heterosexual transmission [2,3]. Sexual transmission of the HIV-1 is the predominant mode of spread of HIV throughout the world [4,5]. Transmitted HIV-1 is more virulent in heterosexual individuals than men-who-have sex with men [6]. A subtype-B variant of HIV-1 has been circulating within the Netherlands during the past

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two decades with exceptionally high virulence reported by Wymant, et al. [7]. In a recent report, the HIV/AIDS pandemic have not been ended in low- and middle-income countries [8].

To halt the spread of the virus, an appropriate and effective strategy is essential to prevent HIV-1 transmission. A prophylactic anti-HIV-1 vaccine would be a preventative method to control the spread of the virus [9,10]. Due to lack of efficacy of HIV vaccine the key trials have been stopped [11,12]. However, there is hope that COVID-19 vaccine strategy can be applied to HIV vaccine development. What concentration of neutralizing antibodies are needed for lifelong protection [13], and now, several mRNA-based HIV vaccines are undergoing clinical trials, but their safety and efficacy is still unclear [14]. In a recent report, by combining vaccination with a topical microbicide that can potentiate vaccine-induced immunity [15].

Vaginal and/or rectal topical microbicides may provide an alternative potential mechanism to reduce the transmission of HIV-1 [16-18] and can be used clinically in HIV infected patient suffering from HIV/AIDS [19]. Topical anti-HIV microbicides are products designed to block or prevent the transmission of HIV when applied in the vagina/rectum or week mucosal surfaces sensitive to HIV infection [20-22]. An ideal vaginal microbicide should fulfill a variety of criteria including high potency against HIV-1, efficacy against a wide range of HIV strains, the ability to directly inactivate the virus, prevention of cell-to-cell transmission of HIV, provide a barrier to viral infection of uninfected cells [23]. A variety of compounds have been proposed as potential topical anti-HIV microbicides [24-27] and some are under clinical trial [28,29]. Nonnucleoside reverse transcriptase inhibitor (NNRTI) UC781 is a tight-binding inhibitor with high antiviral potency against wild-type HIV-1 [30-33] and some NNRTI-resistant HIV-1 *in vitro* [23,33]. Moreover, the expansion of distinct viral subtypes (clades A, B, C, D, E, F, G, K, and O) in different geographical regions is a problem of drug resistance [34]. However, HIV drug-resistance is a limiting factor in the development of a NNRTI and NRTI-based anti-HIV vaginal microbicides [35]. Therefore, evaluation of anti-HIV microbicide potency against subtypes, clades, and drug-resistant viruses are urgent.

In previous studies, chemical barriers to HIV-1 and retroviricidal activity of UC781 were tested [30,36,37] and it was proposed that UC781 could be an excellent candidate for anti-HIV-1 topical microbicides. Recently, a variety of microbicide-relevant tests were performed including testing antiviral activity of UC781 in pretreated cells to protect cells from subsequent infection in the absence of exogenous drug, inactivation of cell-free and cell-associated virus, and inhibition of cell-to-cell HIV-1 transmission [33]. However, there are concerns whether UC781 will be broadly effective against different HIV subtypes as well as against the increasingly prevalent NNRTI-resistant HIV strains. We therefore evaluated UC781 microbicidal activity against a wide variety of HIV-1 strains in both single cycle infectivity assays as well as in virus spreading assays using activated human peripheral blood mononuclear cells (PBMCs), CD4+T cells and monocyte-derived macrophages (MDM).

UC781 shows potent antiviral activity against virtually all the HIV-1 as well as against a variety of drug-resistant virus variants. Although UC781 potency is substantially reduced against NNRTI-resistant virus variants, the “memory effect” exerted by pretreatment of cells with concentrations of UC781 well below those in current microbicide formulations of UC781 can completely abrogate subsequent infection by all HIV-1 strains tested, including NNRTI-resistant virus and all subtypes of HIV-1. UC781-based microbicides will likely be efficacious in different geographical regions.

Materials and Methods

Reagents

The thiocarboxanilides, UC781 was provided by Crompton Inc., (now Chemtura, Middlebury, CT) and stock solutions of UC781 were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. PBMCs were isolated from buffy coats obtained from blood center, University of Pittsburgh, Pittsburgh, Pennsylvania. PBMCs were isolated from healthy blood donors by Ficol-Hypaque (Histopaque, Sigma-Aldrich, St. Louis, Missouri, USA) gradient centrifugation. HIV-1 p24 antigen assay kits were obtained from SAIC-Frederick (Frederick, Maryland, USA).

Virus stocks

Twenty-five HIV-1 strains were used in the study (Table 1) [38-47]. Nine primary isolates, $HIV_{92RW009}$, $HIV_{92BR014}$, $HIV_{98IN017}$, $HIV_{92UG001}$, $HIV_{93TH051}$, $HIV_{93BR020}$, HIV_{RU570}, HIV_{YBF30}, $HIV_{MVP5180}$ were provided by AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Maryland, USA, and grown in Phytohemagglutinin (PHA)-stimulated PBMCs. Two laboratory adapted wild-type HIV-1 strains: LAI, NL4-3 was grown in the CEM cell line. Five macrophage-tropic HIV-1 strain: Ada-M, BaL, JR-CSF, JR-FL, SF-162 were produced by infecting monocyte-derived macrophages (MDMs). UC781-resistant (UCR; V106A+Y181C), Efavirenz-resistant (EFVR; L100I + K103N) and Nevirapine-resistant (NVPR; Y181C) were generated by serial passage of HIV-1 infected cells in the presence of increasing concentration of UC781 [23,33]. Nucleoside reverse transcriptase inhibitor (NRTI)-resistant HIV-1: AZT-3 (M41L, L210W and T215Y), AZT-4 (D67N, K70R, T215F and K219Q) and AZT-9 (M41L, D67N, K70R, L210W, T215Y and K219Q) were generated by site-directed mutagenesis. Multi-

Table 1: Inhibition of different HIV-1 strains by UC781 in P4/R5 cells and determination of IC_{50}.

HIV-1 Strains	Genetic Subtype	Origin	Co-receptor Usage	Biological Phenotype	IC_{50s} of UC781 (μM)	Authors
92RW009	A	Rwanda	R5/X4	SI	0.017 ± 0.0015	[38]
92BR014	B	Brazil	R5/X4	SI	0.008 ± 0.0028	[38]
98IN017	C	India	X4	SI	0.004 ± 0.0017	[38]
92UG001	D	Uganda	R5/X4	SI	0.026 ± 0.0085	[38]
93TH051	E	Thailand	R5/X4	SI	0.015 ± 0.0045	[38]
93BR020	F	Brazil	R5/X4	SI	0.01 ± 0.0075	[38]
RU570	G	Russia	R5	NSI	0.004 ± 0.0015	[38]
YBF30	N	Cameroon	R5	NSI	0.015 ± 0.0084	[38]
MVP5180	O	Cameroon	R5/X4	SI	0.316 ± 0.018	[38]
Ada-M	B	USA	R5	NSI	0.007 ± 0.0043	[39]
Bal	B	USA	R5	NSI	0.004 ± 0.001	[39]
JR-CSF	B	USA	R5	NSI	0.003 ± 0.0002	[39]
JR-FL	B	USA	R5	NSI	0.005 ± 0.0047	[39]
SF-162	B	USA	R5	NSI	0.007 ± 0.003	[39]
89.6	B	USA	R5/X4	SI	0.007 ± 0.003	[40]
LAI	B	USA	X4	SI	0.006 ± 0.0014	[41]
NL4-3	B	USA	X4	SI	0.007 ± 0.002	[42]
AZT^R(3)	B	USA	X4	SI	0.006 ± 0.0027	[43]
AZT^R(4)	B	USA	X4	SI	0.007 ± 0.002	[44]
AZT^R(9)	B	USA	X4	SI	0.008 ± 0.0029	[45]
MDR-1	B	USA	X4	SI	0.006 ± 0.0076	[46]
MDR-5	B	USA	X4	SI	0.004 ± 0.0037	[47]
EFV^R	B	USA	X4	SI	9.28 ± 0.675	[33]
NVP^R	B	USA	X4	SI	0.052 ± 0.008	[33]
UC^R	B	USA	X4	SI	3.26 ± 0.443	[33]

drug resistant (MDR-1: K70G, M184V, T69K, V75I, F77L, F116Y and Q151M) and (MDR-5: M41L, D67N, L210W, T215Y, M184V, T69D, E44D, V118I) were obtained from the NIH AIDS Research and Reference Reagent Program. Virus-induced cytopathic effect (CPE) or p24 antigen was monitored 7 days post-infection and the $TCID_{50}$ was calculated with the Reed and Muench equation [48]. The characteristics of the various viral strains are shown in Table 1.

Preparation of PBMCs and monocytes

PBMCs were isolated from healthy blood donors by Ficoll-Hypaque (Histopaque, Sigma-Aldrich, St. Louis, Missouri, USA) gradient centrifugation as previously described [49]. Monocytes were isolated from freshly separated PBMCs, using positive selection with anti-CD^{14}-coated magnetic beads (Dynal) according to the manufacturer instructions. Alternatively, to isolate $CD14^{pos}$ monocytes, the Miltenyi anti-CD14 microbeads and mini MACS system were used according to the manufacturer's protocol (Miltenyi Biotec, Auburn, California, USA). Purity of isolated monocytes was evaluated by the methods as described previously [50]. To detect surface expression of CD14 in human monocytes, 1×10^6 cells were washed once with FACS buffer (1% BSA and 0.02% NaN_3 in PBS). Cells were then incubated with PE-conjugated anti-human CD14 (BD Biosciences, San Jose, California, USA) or FITC-conjugated anti-human CD14 (BD Biosciences) antibodies on ice for 45 minutes. After the incubation, cells were washed twice with FACS buffer and resuspended in 0.5 ml FACS buffer with 1% paraformaldehyde. Cytofluorography was performed on a FAC Scan (BD Biosciences) as described previously by Marimuthu, et al. [51]. FACS analysis of the cells showed that more than 94% of these cells were CD14+ monocytes.

Single-cycle HIV infectivity assays

The P4/R5 is a HeLa cell line carrying both CXCR4 and CCR5 as co receptors stably transfected with a Tat-activated β-galactosidase gene under the control of an HIV-long terminal repeat promoter [52]. The P4/R5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Cellgro, Herndon, Virginia, USA), 100 U/ml penicillin (Invitrogen),100 μg/ml streptomycin (Invitrogen), and 0.5 μg/ml puromycin (Clontech, Palo Alto, CA). Single-cycle infectivity assay was performed

using P4/R5 cells. Briefly, cells were seeded in 96-well flat-bottom white tissue culture treated plates (Corning, Corning, NY) at 3×10^3 cell/well in Dulbecco's modified Eagle's medium (DMEM) complete medium containing 10% fetal bovine serum, 100 U/ml penicillin (Invitrogen),100 µg/ml streptomycin (Invitrogen), 0.5 µg/ml puromycin and incubated overnight at 37°C in a 5% CO_2 incubator. Cells were infected by replacing the fresh DMEM complete medium containing HIV-1 (0.01 MOI) and serial dilutions of UC781 for IC_{50} determination. After incubating an additional 48 h, cells were lysed in lysis buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgSO_4$, 2% Triton × 100), and β-galactosidase was detected using 4-Methylumbelliferyl β-$_D$-galactoside (Sigma, St. Louis, MO) fluorescence substrate. Data was obtained using Spectra Max Gemini XS luminometer, (Molecular Devices Corporation, Sunnyvale California USA).

Inhibition of HIV-1 replication in PBMC culture

PBMCs were stimulated with 5 µg/ml PHA (Sigma) for 48 hours before exposure to the drug. Cells (2×10^6) were exposed to 0-25 µM UC781 in a total volume of 1 ml of RPMI -1640 medium (BioWhittaker, Walkersville, Maryland, USA) with 10% fetal bovine serum (Cellgro), 1% glutamine (Cellgro), and 1% penicillin and streptomycin (Gibco, Grand Island, New York, USA) and incubated at 37°C in a CO_2 incubator for 18 hrs. Excess extracellular drug was then removed by pelleting the cells by centrifugation at 300 × g for 10 min. and removal of the medium. The cell pellet was washed by suspension in 10 ml of medium followed by centrifugation. This washing step was repeated additional four times. At the final wash, cells were counted by trypan blue exclusion method and resuspended in growth medium containing RPMI-1640 (BioWhittaker) medium supplemented with 10% FBS (Cellgro) and 10 U/ml interleukin-2 (Roche, Indianapolis, USA). Cells were seeded in 96 well flat-bottom tissue culture plates (Millipore, Bedford, Massachusetts, USA) at a density of 2×10^5 PBMCs/200 µl medium. Cells were infected with t-tropic, m-tropic and dual-tropic HIV-1 strains at a multiplicity of infection 0.01. Media was replaced at 3 days post-infection with fresh media containing IL-2. On day 7, cell-free supernatants were harvested and HIV-1 p24 antigen was measured using an HIV-1 p24 antigen capture assay kit (SAIC-Frederick).

Inhibition of HIV-1 replication in UC781 pretreated CD4$^+$ T cells

CD4+ T cells were purified by Dynabeads as previously described [49]. Briefly, after depletion of monocytes from whole PBMCs, cells were mixed with magnetic beads coated with anti-CD4 monoclonal antibodies (Dynal, New York, USA) and incubated for 20 min. at 4°C on a shaker incubator. Magnetic separation of CD4$^+$ T cells was performed using a magnetic particle concentrator (Dynal) and washed twice as recommended by the manufacturer. Purified CD4$^+$ T cells were mixed with detaches a bead for 45 min. at room temperature and magnetic beads were depleted from the cell surface. CD4$^+$ T cells were pre-activated for 3 days with 5 µg/ml PHA (Sigma) and 10U/ml IL-2 (Roche). These cells are referred to as PHA/IL-2-activated CD4$^+$ T cells. Cells were exposed to varying concentration of UC781 (0-25 µM) for 18 hr at 37°C in a CO_2 incubator. Extra-cellular drug was removed by several washes in RPMI medium. Cells were seeded in a 96 well flat-bottom plate at a density of 2×10^5/well in 200 µl RPMI 1640 (BioWhittaker) medium containing 10% FBS (Cellgro) and 10 U/ml IL-2 (Roche). Cells were infected with wild-type NL4-3 and UCR at MOI of 0.01. Culture media were replaced after 3-4 days of incubation with fresh medium containing IL-2 (Roche). HIV-1 replication was measured as p24 antigen in the culture supernatants on day 7 after infection, the time point at which peak of virus production was observed.

Inhibition of HIV-1 replication in UC781 pretreated macrophages cultures

Monocytes were seeded in 96 well flat-bottom tissue culture plates at a density of 1×10^5/100 µl RPMI 1640 medium (BioWhittaker) containing 10% FBS (Cellgro), 1% glutamine (Cellgro), 1% penicillin and streptomycin (Gibco), 100U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage colony stimulating factor (M-CSF) (R&D Systems, Minneapolis, Minnesota, USA) and cells were allowed to differentiate for 7 days and differentiated human monocyte-derived macrophages as described previously [39]. Culture media were replaced after 3-4 days of incubation with fresh medium containing GM-CSF or M-CSF (R&D Systems). On day 7, Macrophage function was assessed by the ability of cells to phagocytose 0.8 microns latex beads (Sigma). Latex beads were added to cells in culture medium at a ratio of 1000 beads per cell and agitated at 37°C for 30 min. Phagocytosed macrophages were enumerated with light microscopy. Culture medium was removed from the well and cells were washed once in phosphate-buffered saline (PBS, Gibco) at room temperature to remove non-adherent cells. Macrophages were exposed to different concentration of UC781 for 18 hrs in a 37°C humidified CO_2 incubator. Cells were washed several times with fresh medium (200 µl/well) with a multi-channel pipette to remove extra-cellular drug. Adherent cells were cultured in 200 µl macrophage culture medium and m-tropic viruses were added in each well at a MOI of 0.01. Media was replaced at 3 days post-infection with fresh media containing GM-CSF or M-CSF. After infection culture supernatants were collected on day 7 and HIV-1 p24 antigen production in culture supernatant was quantified using a p24 antigen ELISA (SAIC-Frederick).

Statistical analysis

IC50 of UC781 against 25 virus strains was calculated

using sigma plot software program. Mean and standard deviation were computed using Microsoft Excel 2016 after collection of data from each experiment.

Results

Inhibitory activity of UC781 against various HIV-1 strains in a single cycle infection assay

The anti-HIV activity profile of UC781 summarized in Table 1. UC781 effectively inhibited single-round infection of various HIV-1 strains in P4/R5 cells. Of the 25 HIV-1 isolates used in this investigation, seventeen (68%) were subtype B. Antiviral activity of UC781 evaluated against 9 different HIV-1 clinical isolates: 7 from group M (Major), O (Outlier), and N (non-M, non-O) [38]. Six out of 8 group M and O subtype utilized both CCR5 and CXCR4 co-receptors for viral entry into the cells. All nine subtypes tested in fluorescence-based P4/R5 MAGI assay were susceptible to UC781 and IC_{50} ranged from 0.004 - 0.026 µM expect subtype O which showed 60-fold less effective than others (IC_{50}, 0.316 µM). Five macrophage-tropic (m-tropic) HIV-1 isolates: HIV-1_{ADA}, HIV-1_{BAL}, HIV-1_{JR-CSF}, HIV-1_{JR-FL}, HIV-1_{SF162} utilized CCR5 as entry co-receptors [39], and the mean IC50 of UC781 against them was 0.005 µM (range, 0.003-0.007 µM). Dual-tropic 89.6 [40] showed similar IC50 (~ 0.007) as of each of the five m-tropic HIV. Two laboratory-adapted wild-type HIV_{NL4-3} [42] and HIV_{LAI} [41]; 3 nucleoside-reverse transcriptase inhibitor (NRTI)-resistant strains: zidovudine (AZT)-resistant, AZT-3 [43], AZT-4 [44] and AZT-9 [45]; 2 multi-drug resistant HIV: MDR-1 [46] and MDR-5 [47], all of them were originated in U.S. and utilized CXCR4 as co-receptor showed anti-HIV activity with a median IC_{50} of 0.006 µM (range from 0.004 - 0.008 µM). Antiviral activity of UC781 against NNRTI-resistant HIV-1 strains UCR, EFVR, and NVPR showed 465, 1325 and 7-fold less effective respectively, compared to that of HIV_{NL4-3} [33].

Inhibition of HIV-1 subtypes by UC781 pretreated human PBMCs

The ability of UC781 pretreated cells to protect from subsequent infection in the absence of exogenous drugs has been tested in previous studies [30,33,37]. To test the effect of UC781 pretreated cells against HIV-1 clinical isolates, PBMCs were exposed to varying concentration of UC781 (0 - 25 µM) and exogenous drug was removed by several wash in PBS. Cells were infected with HIV-1 subtypes A, B, C, D, E, F, G, N and O (Table 1) and cell-free supernatants were collected on day 7. HIV-1 infectivity in the culture supernatants were measured using HIV-1 p24 antigen assay kit (SAIC-Frederick). Interestingly, all nine HIV-1 clinical isolates were potently inhibited by UC781 pretreated PBMCs (Figure 1). Viruses were completely inhibited by 25 µM concentration of UC781 and inhibition was reduced at higher to lower concentration of drug in a dose dependent manner. Although, the greatest differences in viral sensitivities were observed for HIV-1MVP5180, which was 60-fold less sensitive (IC_{50}, 0.316)

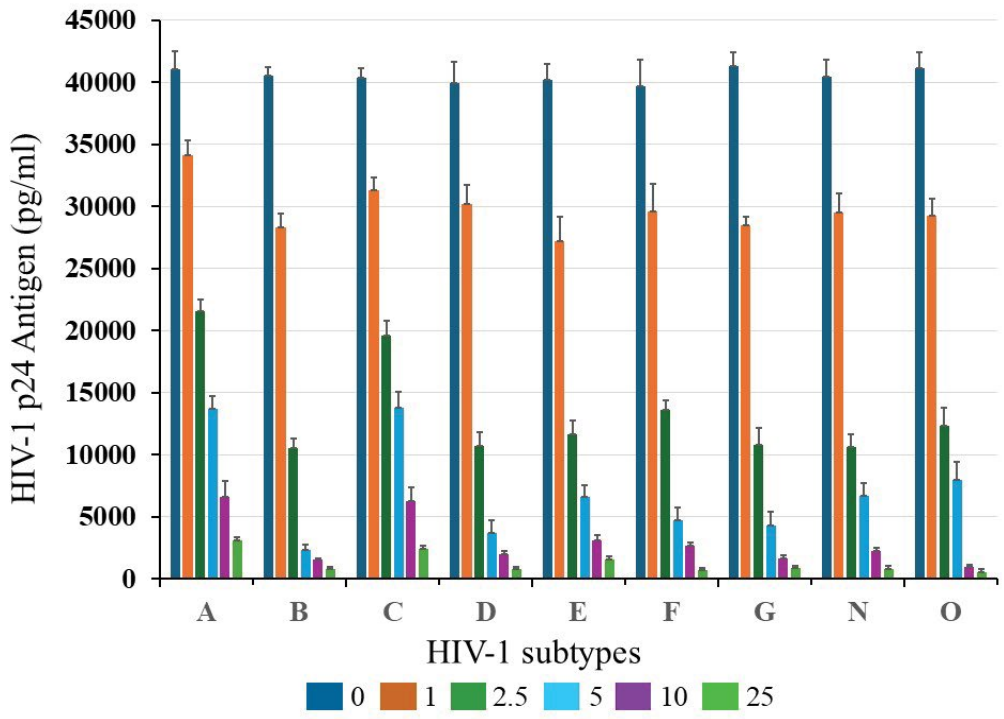

Figure 1: Inhibition of different HIV-1 subtypes by UC781 pretreated PBMCs: PHA-stimulated PBMCs were exposed to different concentrations of UC781 for 18 hr at 37°C, extra-cellular drugs were removed by several wash in RPMI medium. Cells were exposed to 0.01 MOI of HIV-1 subtypes A-G, N and O. HIV-1 replication was measured as p24 antigen in the culture supernatants on day 7 after infection. Results represent mean p24 antigen (± standard deviation) of an experiment that is representative of three independent experiments.

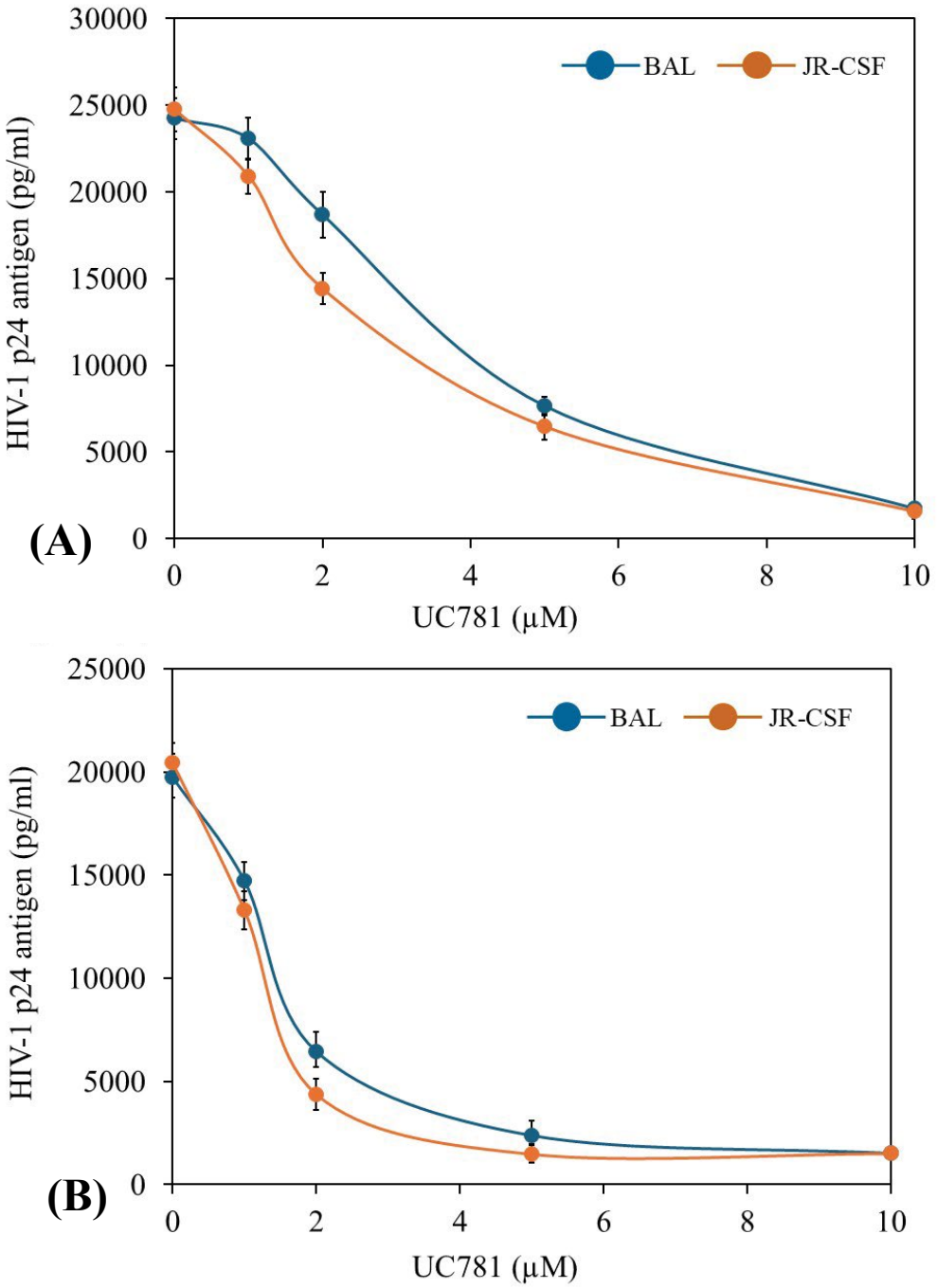

Figure 2: Inhibition of m-tropic HIV-1 infection in UC781 pretreated M-CSF induced MDMGM-CSF induced MDM: HIV-1 replication was monitored in UC781 pretreated: A) M-CSF induced MDM; and B) GM-CSF induced MDM. Monocytes were cultured in the presence of M-CSF or GM-CSF for 7 days before infection. Cells were exposed to varying concentration of UC781 for 18 hrs at 37°C, extra-cellular drugs were removed by several wash in RPMI medium and infected with 0.01 MOI of BaL, JR-CSF. After 7 days, HIV-1 p24 antigen production in culture supernatants was quantified using a p24 antigen ELISA. Data represent mean ± standard deviation of triplicate determinants in each experiment.

to UC781 compared to other subtypes in P4/R5 cells (Table 1). However, all HIV subtypes were completely inhibited due to pretreatment of cells with UC781 at 25 µM concentration. There were no genetic-subtype-dependent differences in inhibition of HIV-1 infection in UC781 pretreated human PBMC.

Inhibition of m-tropic HIV-1 replication in UC781 pretreated macrophages

To test the ability of UC781 to inhibit the replication of m-tropic HIV in monocyte-derived macrophages (MDM), 1×10^4 monocytes were seeded in a flat bottom 96 well plate and the cells allowed to differentiate for 6 days in the presence of M-CSF or GM-CSF separately. MDM was exposed to varying concentration of UC781 (0-25 µM) at 37 °C for 18 hours and extra-cellular drugs were removed by washing in PBS several times. Cells were infected with m-tropic HIV-1 strains: HIV-1_{BAL}, HIV-1_{JR-CSF} and supernatants were collected on day 7. HIV-1 p24 antigen was assayed at 7 days post-infection. Pretreatment of MDM with UC781 concentrations of 25 µM or greater completely prevented subsequent

infection of these cells by m-tropic virus strains in the absence of exogenous drug (Figure 2). Interestingly, we found that the antiviral activity of UC781 pretreatment is dramatically reduced in macrophages stimulated with M-CSF, but not in those stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF). The minimal concentration of UC781 to inhibit m-tropic viruses was 5.0 µM in GM-CSF treated macrophages whereas 2-fold higher concentration is required to inhibit the same viruses in macrophages stimulated with M-CSF (Figure 2A and Figure 2B).

Effect of UC781 pretreated PBMCs and autologous CD4⁺ T cells on NNRTI-UCR

The antiviral activity of UC781 against cell-free and cell-associated wild-type (wt) and NNRTI-resistant UCR strains have been evaluated in the previous studies [33]. To study the inhibitory effect of UC781 pretreated PBMCs and autologous CD4+ T cells in HIV infection, activated cells were exposed to 0-25 µM concentration of UC781. Extra-cellular drugs were removed by extensive washing in PBS, cells were infected with wild-type NL4-3 and UC781-resistant HIV (UC^R), supernatants were collected on day 7 and viral infection was measured by HIV-1 p24 antigen assay in culture supernatants. At 25 µM concentration, NL4-3 and UCR were almost completely inhibited by UC781 pretreated PBMC and CD4+ T cells compared to the no drug control (Figure 3). On the other hand, no inhibition of UCR variant was found at decreasing concentration of UC781 compared to wild-type NL4-3 in both PBMC and CD4+ T cells. However, UC781 pretreated PBMC and CD4 T cells was significantly less effective in inhibition of UCR virus infection which possessed three mutations, V106A, I135R and Y181C. The concentration of three mutation showed 466-fold resistant to UC781 [33].

Inhibition of multi-drug-resistant HIV by UC781 pretreated PBMCs

Multi-drug resistant HIV (MDR-1) carrying K70G, M184V, T69K, V75I, F77L, F116Y and Q151M mutations showed reduction in susceptibility 61 fold to ZDV, 3.9 fold to d4T, 2.3 fold to TDF, 7.7 fold to ABC and 2.4 fold to ddI; Similarly, MDR-5 carrying M41L, D67N, L210W, T215Y, M184V, T69D, E44D, V118I mutations displayed more than 261 fold to ZDV, 11 fold to d4T, 2.4 fold to TDF, and 23 fold to ddI. The IC50 of UC781 against MDR-1 and MDR-5 were 0.006 and 0.004 µM in HIV-1 single cycle replication experiment which is similar to the wild-type virus. Thus, the ability of UC781 to inhibit the replication of MDR-1 and MDR-5 (Johnston et al., 2005) in human PBMC exposed to 10 µM UC781 was tested. Pre-activated PBMCs were exposed to 0-10 µM concentration of UC781 and extra-cellular drugs were removed by extensive washing in PBS, cells were infected with wild-type NL4-3, MDR-1 and MDR-5, supernatants were collected on day 7, and viral infection was measured by p24 antigen assay in culture supernatants.

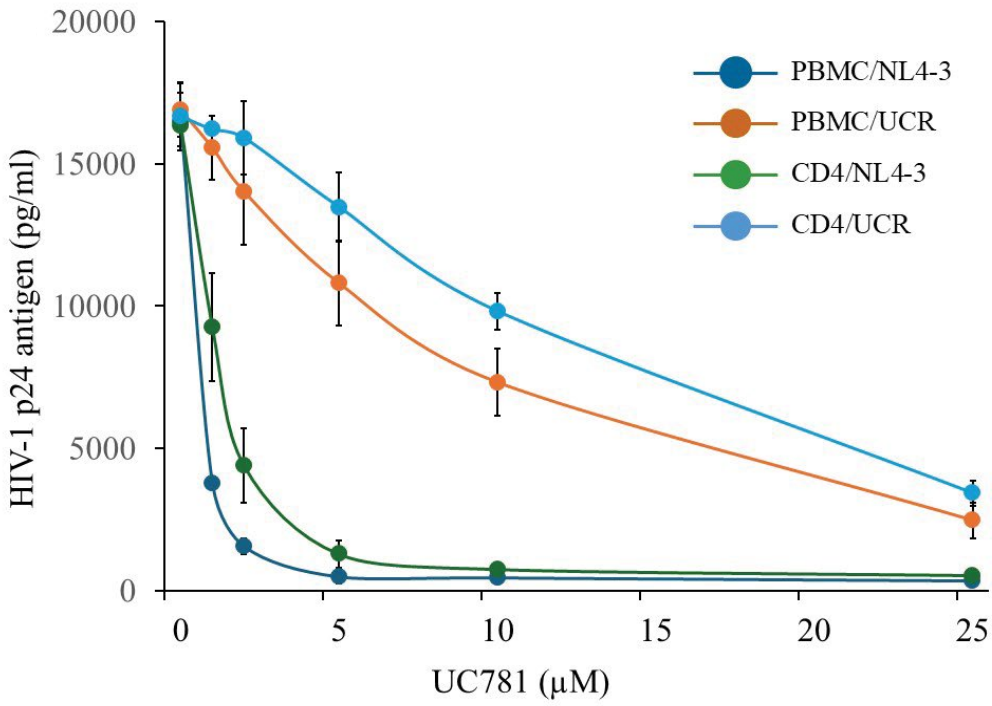

Figure 3: Comparison of HIV-1 infection by NL4-3 and UCR in UC781 pretreated PBMCs and CD4+ T cells: A serial dilution of UC781 (0-25 µM) was added to PHA/IL-2-activated PBMCs and CD4+ T cells and incubated for 18 hrs at 37°C, excess drug was washed away by diluting in RPMI medium several times. PBMCs were exposed to NL4-3 and UCR; autologous CD4+ T cells were infected with wild-type NL4-3 and UCR. HIV-1 replication was measured as p24 antigen production in cell-free culture supernatants on day 7 after infection. Data represent mean ± standard deviation of triplicate determinations in each experiment.

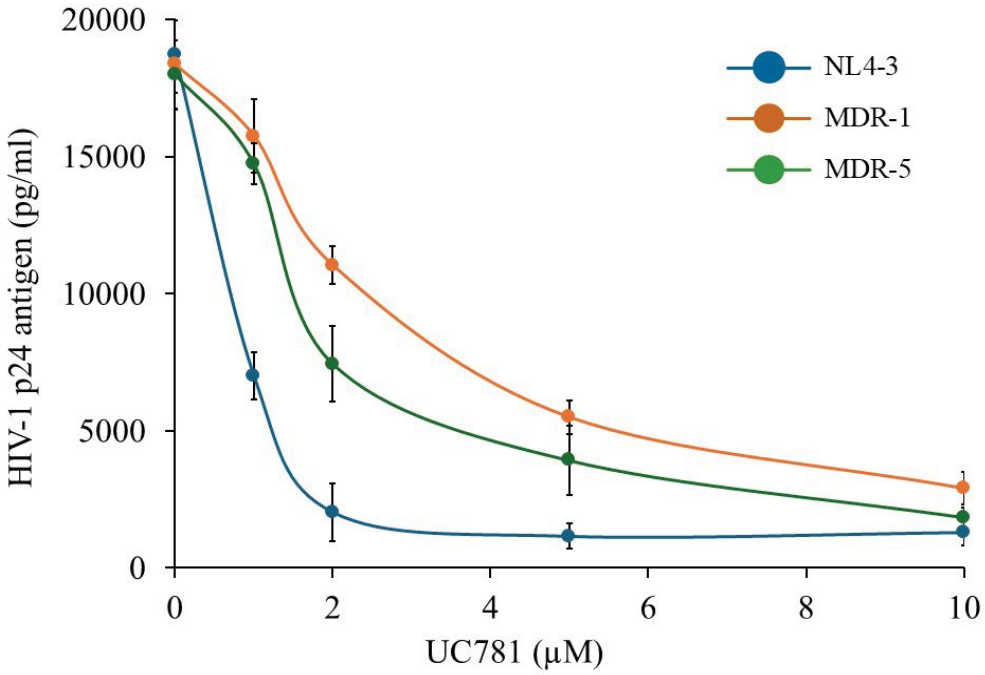

Figure 4: Inhibition of multi-drug-resistant HIV in UC781 pretreated PBMCs: Multi-drug-resistant HIV inhibitory effect of UC781 pretreated PBMCs was evaluated by exposing a serial dilution of UC781 (0-10µM) to activate PBMCs. Extra-cellular drug was removed by repeated washing in RPMI medium. Extra-cellular drug free 2 × 10^5 PBMCs were seeded in a 96 well plate and infected with 0.01 MOI of wildtype NL4-3, MDR-1 and MDR-5. HIV-1 p24 antigen was assessed at 7 days post-infection. Values represent the mean ± standard deviation for triplicate determination.

We observed complete inhibition of NL4-3, MDR-1 and MDR-5 by UC781 pretreatment at concentration of 10 µM. We found minimal decrease in inhibition of MDR-1 and MDR-5 at lower concentration of UC781. Therefore, our data suggested that exposure of primary cells to UC781 results in blocking of subsequent HIV-1 infection of multidrug resistant virus. However, multidrug resistant HIV-1strains were completely inhibited when primary cells exposed to 10 µM UC781 (Figure 4).

Discussion

The main goal of this study was to evaluate NNRTI UC781 as a potential candidate for anti-HIV microbicides. In our hypothesis, to prevent HIV-1 transmission, an ideal microbicide should possess high potency against HIV-1, should have efficacy against a wide range of HIV-1 strains, have the ability to directly inactivate the virus without the need for metabolic activation, be able to make a barrier to viral infection of uninfected cells, and prevent cell-to-cell transmission of HIV. While it has been proposed that UC781 is a potential candidate for development as a microbicide to prevent sexual transmission of HIV-1, it has also been shown that UC781 fulfills all the criteria for anti-HIV microbicide against wild-type HIV-1 [21,23]. However, there are concerns whether UC781 will be broadly effective against different HIV subtypes as well as against the increasingly prevalent drug-resistant HIV-1 strain. The viruses are classifiable into distinct subtypes or clades and new HIV-1 strains are constantly emerging through dynamic genetic evolutionary processes. The viral diversity in different geographical region has implications for differential rates of disease progression, responses to antiretroviral therapy, anti-HIV microbicide, and vaccine development [34,38,53].

UC781 is a tight binding non-nucleoside reverse transcriptase inhibitor (NNRTI) with broad spectrum anti-HIV activity *in vitro*. This study investigated the potential utility of UC781 as anti-HIV microbicides. Twenty-five different HIV-1 strains were tested to evaluate anti-HIV microbicide activity of UC781 by single cycle HIV-1 infection assay in P4/R5 cells, activated human peripheral blood mononuclear cells (PBMCs), and monocyte-derived macrophages (MDMs). The ability of UC781 to protect cells from HIV-1 infection was tested by pretreatment of target cells with different concentration of UC781 and cells were challenged with various HIV-1 strains usage with different co-receptors CXCR4, CCR5 or both in the absence of exogenous drugs. The findings of these studies have practical implications for designing and developing anti-HIV microbicides.

Increasing diversity of HIV-1 subtypes is a major problem for current HIV prevention strategies [54]. An ideal microbicide should be effective against a broad spectrum of HIV-1 subtypes; in particular, those that are found in different geographical regions. In a fluorescence-based P4/R5 MAGI assay, nine HIV-1 clinical isolates A, B, C, D, E, F, G (group M) and N (group N) were susceptible to UC781 and IC50 ranged (0.004 - 0.026 µM) except the isolate O (group O) which was 60-fold less sensitive than others (IC_{50},0.316 µM) (Table 1).

Due to high genetic variation, group O isolate showed some phenotypic differences compare to subtypes of group M. High frequency of group O in the HIV-1 infected populations in Cameroon and Gabon are a leading cause of challenges for HIV-1 treatment strategies. It has been reported that more than 60% of population infected with group O HIV-1 are naturally resistant to NNRTI, such as EFV, NVP [55]. Group O clinical isolates having a cysteine at position 181 in the NNRTI binding pocket of reverse transcriptase (RT) are intrinsically resistant to NNRTI inhibitors. However, decreased sensitivity of group O isolates to UC781 correlates with mutations in the NNRTI binding of HIV-1 RT in group O HIV-1 [56].

We examined susceptibility of nine clinical isolates in virus spread assay using PHA stimulated human PBMCs. While UC781 was 60-fold less effective against subtype O (strain MVP180), infectivity was inhibited by UC781 pretreatment of PBMC (Figure 1). The results are consistent with previous findings that Nevirapine resistant HIV-1 possessed Y181C mutation in the NNRTI binding pocket completely inhibited by UC781 pretreated cells [33]. Moreover, UC781 diminished virus replication in X4-tropic or X4/R5-tropic HIV-1 clinical isolates infected PBMC cultures. We therefore suggest that microbicide formulations containing UC781 as an inhibitory agent may still be very useful in such infected patient population bearing group O HIV-1.

Macrophages are known as a primary target for HIV-1 infection in the body. The ability of HIV-1 entry into the cells and their replication depends on cytokine mediated cell differentiation. To evaluate anti-HIV microbicide activity of UC781, M-CSF or GM-CSF induced human MDMs were exposed to various concentration of UC781 and thoroughly washed away extracellular drug then infected with macrophage-tropic HIV-1_{BAL} and HIV-1_{JRCSF}. Important findings of these studies are that UC781 inhibits the HIV-1 replication potentially in GM-CSF compared to M-CSF (Figure 2A and Figure 2B). The data of our current studies are consistent with reduction of antiviral activity in M-CSF stimulated macrophages compared to GM-CSF induced macrophages [57]. M-CSF induced macrophages increase the CD4 receptor expression on the cell surface resulted in the enhancement of HIV-1 infection [57] and the suppression of HIV-1 replication in macrophages induced by GM-CSF reported by others [58]. It has been reported that UC781 inhibit HIV-1 BAL replication completely at concentrations 50 and 100 µM in the explant model [59]. Nevertheless, the reduced microbicidal activity of UC781 in M-CSF induced macrophage culture may need additional antiviral agents to suppress HIV-1 replication in full.

Since semen or vaginal and cervical secretions containing infected lymphocytes that spread HIV-infection through heterosexual transmission [60,61]. As a results, the principal mode of HIV infection is heterosexual transmission [4,62]. An ideal microbicide should be effective in the mucosal environment to prevent cell-to-cell HIV transmission. We have assessed the anti-HIV microbicide activity of UC781 against UC781-resistant HIV in PHA-stimulated CD4 T cells and PBMCs. Pre incubation of uninfected cells in the presence of 25 µM concentration of UC781 are not enough to complete inhibition of UC781-resitant HIV-1 in the absence of exogenous drug (Figure 3). However, CEM cells were protected against infection in the absence of exogenous drugs when exposed to 50 µM concentration of UC781 [33]. UC781 in formulation under preclinical trial as an anti-HIV microbicide is 60-fold greater than the concentration of UC781 required for fully active microbicide activity against highly NNRTI-resistant HIV-1. Therefore, reduced potency of UC781 against NNRTI-resistant virus variants is well below those in current microbicide formulations due to the "memory effect" exerted by pretreatment.

UC781, a tight-binding NNRTI, have potential utility of anti-HIV microbicidal applications. UC781 binds to HIV-1 RT firmly than any other inhibitors and acts as a tight-binding inhibitor. Therefore, when UC781 penetrates the HIV-1 envelope membrane and capsid cores then binds to RT, its process of dissociation from the RT is very slow and is therefore trapped within the virion. Thus, RT remains inhibited for prolonged period of time after binding; this property imparts to UC781 the potential as a topical anti-HIV microbicide. Brief exposure of cell-free virions to UC781 inactivated HIV-1, chronically infected with HIV-1 incubated in the presence of UC781 led to the subsequent production of nascent virus with attenuated infectivity and prevented cell-to-cell transmission of wild-type HIV-1 [30,33]. Additionally, exposure of uninfected human lymphoblastoid cell lines to UC781 prevented subsequent virus infection by "chemical barrier system" in the absence of extra-cellular drug [30,33,37]. UC781 shows potent antiviral activity against virtually all of HIV-1 as well as against a variety of drug-resistant virus variants. Although UC781 potency is substantially reduced against NNRTI-resistant virus variants, the "memory effect" exerted by pretreatment of cells with concentrations of UC781 well below those in current microbicide formulations of UC781 can completely abrogate subsequent infection by all HIV-1 strains tested, including NNRTI-resistant virus and all subtypes of HIV-1. UC781-based microbicides will likely be efficacious in different geographical regions.

However, anti-HIV microbicidal activity of UC781 is not HIV-1 strains specific. At 25 µM or greater, UC781 inhibited NNRTI-resistant virus and all subtypes. Macrophage-tropic: HIV-1_{BAL} and HIV-1_{JR-CSF}, and dual-tropic 89.6 were inhibited by UC781 in MDM induced by both M-CSF and GM-CSF. Inhibition of NRTI-resistant HIV and multi-drug resistant HIV, suggesting that UC781 is an excellent microbicide candidate.

Conclusion

The ability of UC781 to inhibit primary isolates of HIV in PBMCs is of paramount importance in the development of anti-HIV microbicides.UC781 shows potent antiviral activity against all subtypes of HIV-1. While UC781 was 60-fold less effective against subtype O (strain MVP180), infectivity was inhibited by UC781 pretreatment of PBMC. UC781 at 10 µM completely inhibited Bal and JR-CSF in MDM induced by both M-CSF and GM-CSF. Reduced potency of UC781 against NNRTI-resistant virus variants is well below those in current microbicide formulations due to the "memory effect" exerted by pretreatment. UC781 at 25 µM or greater inhibited NNRTI-resistant virus and all subtypes of HIV-1. UC781 shows potent antiviral activity against all subtypes of HIV-1 as well as against a variety of drug-resistant virus variants. UC781-based microbicides will likely be efficacious in different geographical regions.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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