Antiviral Activity of Retrocyclin RC-101, a Candidate Microbicide Against Cell-Associated HIV-1

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Abstract

Microbicides have been evaluated mostly against cell-free HIV-1. Because semen contains both cell-free and cell-associated HIV-1, HIV-1 transmission could occur via either or both sources. Therefore, it is important to examine the antiviral activity of microbicides against cell-associated HIV-1. The cyclic antimicrobial peptide retrocyclin RC-101 has been shown previously to have antiviral activity against cell-free HIV-1, with no associated cellular toxicity. In this article we have examined the antiviral activity of RC-101 against cell-associated HIV-1. The results demonstrate potent antiviral activity of RC-101 against cell–cell HIV-1 transmission in both CD4-dependent and CD4-independent assays against CCR5- and CXCR4-tropic HIV-1, with no cellular toxicity. Furthermore, this antiviral activity was retained in the presence of human seminal plasma. The potent antiviral activity of RC-101 against cell-associated HIV-1 reported here, and the previously reported antiviral activity in cervical tissues, suggest that RC-101 is an excellent and promising microbicide candidate against HIV-1.

Introduction

HETEROSEXUAL TRANSMISSION ACCOUNTS for more than 80% of the worldwide spread of HIV-1. Unprotected vaginal intercourse is the most common route through which women are infected with HIV-1. Consistent condom use, one cornerstone of primary HIV-1 prevention, is not always feasible for many receptive partners^{1–3} for various reasons, including religious and cultural taboos that are present in many areas of the world. Consequently, women urgently need access to preventive measures that are within their complete personal control. Thus, in the absence of an effective anti-HIV-1 vaccine, it is now recognized that an effective vaginal microbicide that can provide such protection against HIV-1 infection is of critical importance.

Heterosexual transmission is initiated by exposure to HIV-1 in semen. Because semen contains both cell-free and cell-associated HIV-1,^{4–7} HIV-1 transmission could occur via either or both cell-free and cell-associated HIV-1. Using a cervical tissue-derived organ culture model, we have demonstrated significant levels of transmission from both cell-free and cell-associated macrophage-tropic R5 and T cell-tropic X4

HIV-1 across the mucosa of cervical tissue, although transmission efficiency was highest with cell-free macrophage-tropic virus.⁸ Therefore, microbicides must be active against both cell-free and cell-associated HIV-1 of R5 and X4 tropisms.

A number of compounds have been evaluated both *in vitro* and *in vivo* as candidates for microbicides. Several reverse transcriptase (RT)-inhibiting microbicides, including both the nucleotide analog 9-[2-(phosphonomethoxy)propyl]adenine (MPA; tenofovir) and nonnucleoside analogs UC781 and TMC120, are currently in clinical trials.^{9–12} Although in one study vaginal application of 1% tenofovir gel was found to provide partial protection against HIV-1 infection,¹³ a later study found no efficacy for tenofovir gel (Microbicide Trials Network, September 2011 bulletin; www.mtnstopshiv.org/node/3909). The mode of action of other microbicide candidates, alone or in combination, occurs via their ability to block the initial viral attachment to CD4 and/or coreceptors (CCR5 and CXCR4), or by blocking HIV-1 gp41-mediated fusion. Cellular coreceptor antagonists, such as CMPD167 and aminooxypentane (AOP)-RANTES (CCR5 inhibitors), and AMD3465 (X4 inhibitor), are now being evaluated in humans.^{14,15} Although microbicides have been evaluated

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against cell-free HIV-1, only a few of them have been evaluated against cell-associated HIV-1.¹⁶

The cyclic antimicrobial peptide retrocyclin RC-101, which interacts with gp41 and prevents viral fusion, has been shown to exert antiviral activity against cell-free HIV-1 with no toxicity in cell lines and tissues.^{17–19} RC-101, applied cervicovaginally, was safe and nontoxic to pigtail macaques and retained *ex vivo* anti-HIV-1 activity even several days post-application.²⁰ RC-101 induces little resistance in HIV-1, which could be overcome with only a 5- to 10-fold increase in drug concentration.²¹ In this article we evaluated the antiviral activity of RC-101 against cell-associated HIV-1 in the absence and presence of semen. These data demonstrate that RC-101 is active against cell-associated R5 and X4 HIV-1 with no cellular toxicity and remains active in the presence of semen.

Materials and Methods

Cells and viruses

GHOST-X4/R5 cells; HIV-1IIIB (X4) and HIV-1Ba-L (R5); HIV-1 international strains UG/92/037 (Clade A, X4), RW/92/008 (Clade A, R5), IN/93/999 (Clade C, R5), and TZ/98/013 (Clade C, R5); and infectious molecular clone JRCSF were obtained from the National Institutes of Health (NIH, Bethesda, MD) AIDS Research and Reference Reagent Program. Primary isolates 33015 (Clade B, R5) and 30562 (Clade B, X4) were isolated from a symptomatic HIV-1-infected subject and a patient with AIDS from the Pitt Men's Study of the Multicenter AIDS Cohort Study (Pittsburgh, PA). JRCSF virus was isolated by transfection of JRCSF cloned DNA into 293 T cells. All cell-free viruses were grown in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) or CD8-depleted PBMCs from seronegative persons and titered in the same cells. HIV-1 SK1 was obtained as previously described.^{22,23} ME-180 cells were obtained from the American Type Culture Collection (Manassas, VA). HeLa-R5-16 cells were a gift from Roche (Palo Alto, CA). All cell lines were maintained in the recommended culture media with or without selection antibiotics. Seminal fluids were obtained by centrifuging pooled whole semen from uninfected subjects at 1200 × g for 10 min at 4°C.

RC-101 formulation as intravaginal films

The 18-amino acid RC-101 peptide was prepared on a 0.25-mmol scale with an ABI 431A peptide synthesizer (Applied Biosystems, Foster City, CA), using FastMoc chemistry. The purified RC-101 was subsequently oxidized and cyclized as described elsewhere.¹⁷ The peptides were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry to confirm homogeneity and that the measured mass agrees well with its expected mass. RC-101 was formulated by a solvent-casting technique as a quick-dissolving polymeric 27.5 mm × 33.5 mm vaginal film, composed of 2.0 mg of RC-101, 6% polyvinyl alcohol (Kuraray America, New York, NY), 0.12% (hydroxypropyl)methyl cellulose (HPMC, 6 cP; Sigma-Aldrich, St. Louis, MO), and 3% glycerin (Dow Chemical, Midland, MI) as described by Sassi and colleagues.²⁴ All films were stored in polyethylene terephthalate (PET)/aluminum foil pouches (Amcor Flexibles Healthcare, Mundelein, IL) until use.

Testing antiviral activity against cell-free HIV-1 in PBMCs

Phytohemagglutinin (PHA)-stimulated PBMCs were plated at 2×10^5 per well in a 96-well plate and centrifuged at $1000 \times g$ for 5 min to pellet the cells. Cell pellets were incubated with various concentrations of RC-101 for 2 h. Without removing the drug, 100 μ l of HIV-1 (50,000 pg/ml p24 equivalent) was then added to the cells and RC-101 mixture. As a control, HIV-1 was added to control cells incubated for the same period of time with medium. After 5 days of incubation at 37°C, production of HIV-1 was monitored by measuring HIV-1 p24 in the culture supernatant, using an antigen capture assay (Dupont/Perkin-Elmer, Boston, MA). At the end of the incubation, cell viability was also measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Studies were conducted in triplicate on at least two occasions.

CCR5- and CXCR4-tropic CD4-dependent cell-associated HIV-1 transmission

Inhibition assays. CD4-dependent cell-associated HIV-1 transmission inhibition assays using the CD4-positive GHOST (3) X4/R5 cell line were performed as described previously.¹⁶ Briefly, H9 cells chronically infected with the SK1 isolate of HIV-1 (CXCR4-tropic assay), or MOLT-4/CCR5 cells chronically infected with the HIV-1_{JR-CSF} molecular clone (CCR5-tropic assay), were used as the transmitting cells. The transmitting cells were treated with mitomycin C (200 μ g/ml; Sigma-Aldrich) for 60 min at 37°C, washed once, and incubated with target cells and drug for 4 h. After incubation medium was removed by washing three times, and incubation was continued for an additional 16 h. At the endpoint, the assay medium was removed, cell lysates were prepared with 0.5% Triton X-100, and intracellular p24 content was determined by enzyme-linked immunosorbent assay (ELISA). The control compounds TAK-779 and AMD3100 were used to confirm CCR5 or CXCR4 coreceptor specificity.

Evaluation of RC-101 in seminal plasma or pH transition. The cell-associated and cell-free CD4-dependent HIV-1 transmission inhibition assays were modified as described earlier¹⁶ to include human seminal plasma or a pH transition from 4.0 to 7.0. Cell-free virus or HIV-1-infected transmitting cells were mixed with seminal plasma and added to target cells, which were preincubated for 15 min with RC-101, yielding a final concentration of 12.5 or 25% seminal plasma, respectively, for the cell-free or cell-associated assays. The compound quinobene²⁵ served as an additional assay performance control because it is consistently inactivated at a concentration of 100 μ g/ml in the presence of seminal plasma. For pH transition assays, compounds prepared in assay medium at pH 4 were added to target cells followed by addition of virus (cell-free assay) or effector cells (cell-associated assays) in medium that buffers the assay medium to neutral pH.

CD4-independent cell-associated HIV-1 transmission inhibition assay

The assay was performed similarly to the CD4-dependent assay described previously, except that ME-180, a CD4-negative cervical cell line that contains human papillomavirus DNA, was used as the target cell.^{26,27} ME-180 cells were plated at a density of 5×10^3 cells per well 48 h before the assay. On

day 2, serially diluted compound and 5×10^4 mitomycin C-treated H9/HIV-1SK1 cells were added to each well. After a 4-h incubation, compound and effector cells were removed by washing the plate three times with phosphate-buffered saline (PBS). Additional washing steps were performed at 24 and 48 h postinfection. On day 6, supernatants were collected and evaluated for cell-free HIV-1 Gag p24 antigen expression by ELISA. Dextran sulfate and dextran were used as the positive and negative control compounds, respectively.

CCR5- and CXCR4-tropic fusion assays. The fusion assays were performed as described earlier.¹⁶ The assay is sensitive to inhibitors of gp120 to CD4/CCR5/CXCR4 binding and inhibitors of gp41-mediated fusion. In this assay HeLa R5-16 cells (expressing HIV-1 Tat and CCR5-tropic Env from HIV-1 92US715) were incubated with target cells MAGI-CCR5 (expressing CD4 and CCR5) for 40 to 48 h, after which fusion was monitored by the measurement of β -galactosidase (β -Gal) enzyme expression, detectable by chemiluminescence (Tropix Gal-Screen β ; Applied Biosystems). TAK-779 and AMD3100 were used as control compounds to demonstrate specificity for the CCR5 or CXCR4 coreceptor, and T20 served as a fusion inhibitor control.

Data analysis

Data were analyzed as described previously.¹⁶

Results

Evaluation of antiviral activity of RC-101 against cell-free HIV-1 of various clades in PBMCs

RC-101 has been found to have potent antiviral activity against a variety of R5- and X4-tropic HIV-1 in various transformed cell lines. In this study we have extended the previous work by evaluating RC-101 against a number of HIV-1 isolates of various clades and of various tropisms in PBMCs, which are more relevant cells than transformed cell lines. Because a number of studies have reported that microbicides may show lower antiviral activity against clinical isolates rather than laboratory-adapted isolates, we included two Clade B clinical isolates: 33015 (R5) and 30562 (X4). We also included two well-characterized laboratory-adapted isolates, HIV-1 Ba-L (R5) and IIIB (X4), as controls. In addition, we used international isolates from four countries with various clades and tropism. They were as follows: Clade A X4 virus from Uganda (UG/92/037), Clade A R5 virus from Rwanda (RW/92/008), a Clade C R5 virus from Tanzania (TZ/98/013), and a Clade C R5 virus from India (IN/93/999). Results presented in Fig. 1 indicate that RC-101 showed potent antiviral activity against Clade B isolates regardless of whether they are R5 or X4 and clinical or laboratory-adapted isolates (Fig. 1A). RC-101 demonstrated antiviral activity against all international isolates, although somewhat lower antiviral (60–75%) activity against isolates from Rwanda, Tanzania, and India. RC-101 exhibited potent antiviral activity (more than 80%) against the Ugandan HIV-1 isolate (Fig. 1B).

Antiviral activity of film-formulated RC-101 against cell-free HIV-1

Because microbicides will be applied in the vagina/cervix as a formulated product, we examined the antiviral activity of

FIG. 1. Antiviral activity of RC-101 against cell-free HIV-1 of various clades in peripheral blood mononuclear cells (PBMCs). **(A)** Activity against laboratory (IIIB and Ba-L) and clinical isolates (33015, Clade B, R5; and 30562, Clade B, X4). **(B)** Activity against various clades: UG/92/037 (Clade A, X4), RW/92/008 (Clade A, R5), IN/93/999 (Clade C, R5), and TZ/98/013 (Clade C, R5).

a self-dissolving film-formulated RC-101 in PBMCs. This film, containing 2000 μ g of RC-101, is dissolved in less than 2 h as determined in *in vitro*²⁴ and *in vivo* studies in monkeys.¹⁷ For this purpose film containing 2000 μ g of RC-101 was dissolved in 1 ml of medium and evaluated for its antiviral activity in PBMCs at various dilutions against HIV-1 Ba-L. We used HIV-1 Ba-L, an R5-tropic HIV-1, because most of the transmitted circulating viruses are R5 tropic. As shown in Fig. 2, film-formulated RC-101 exhibited potent antiviral activity in PBMCs compared with placebo-formulated film.

FIG. 2. Antiviral activity of film-formulated RC-101 against HIV-1.

Antiviral activity of RC-101 against CD4-dependent cell-cell transmission of HIV-1

Because HIV-1 transmission could occur via cell-free or cell-associated HIV-1 present in semen, we evaluated RC-101 in both cell-free and cell-associated HIV-1 transmission assays. As shown in Fig. 3A, RC-101 exhibited potent antiviral activity against CCR5-tropic cell-cell transmission with a median inhibitory concentration (IC_{50}) of $0.19 \mu\text{g/ml}$. Figure 3B shows that although there was a reduction in activity, RC-101 remained active against cell-associated R5-tropic HIV-1 in the presence of 25% seminal plasma, with an IC_{50} of $2.4 \mu\text{g/ml}$. This concentration of seminal plasma was selected on the basis of previous reports that levels higher than 25% exert cytotoxic effects in cell culture.²⁸ No significant cell cytotoxicity of RC-101 was observed in the absence and presence of seminal plasma. Similar results were obtained using cell-free virus in the absence and presence of seminal plasma (IC_{50} , 0.64 and $3.73 \mu\text{g/ml}$, respectively; data not shown). Antiviral activity was also noted with CXCR4-tropic cell-cell transmission, with an IC_{50} of $2.1 \mu\text{g/ml}$ (Fig. 4), which was higher than the inhibitory concentration observed against CCR5-tropic HIV-1 (Fig. 3). Taken together, the activity of RC-101 against cell-cell transmitted virus in the nanomolar range and the broad-spectrum activity of RC-101 against multiple clinical isolates of HIV-1 from diverse clades strongly suggests further development of RC-101 as a topical microbicide.

FIG. 3. Antiviral activity of RC-101 in a CD4-dependent, CCR5-tropic, cell-cell transmission assay alone (A) and in 25% seminal plasma (B). The mean and percent standard deviations of the triplicate data were calculated and are displayed graphically. Cytotoxicity was evaluated in parallel along with the efficacy. The IC_{50} was calculated by linear regression analysis. The percentage of virus control (%VC) and percentage of cell control (%CC) are used to show antiviral activity and cellular cytotoxicity, respectively.

FIG. 4. Antiviral activity of RC-101 in a CD4-dependent, CXCR4-tropic, cell-cell transmission assay with mean and percent standard deviations of the triplicate data displayed graphically. The IC_{50} was calculated as described in the legend to Fig. 3. The percentage of virus control (%VC) and percentage of cell control (%CC) are used to show antiviral activity and cellular cytotoxicity, respectively.

Evaluation of antiviral activity of RC-101 in a cell-cell fusion assay

HIV-1 gp120-mediated cell fusion between infected and uninfected cells has been reported to be a mechanism for cell-cell transmission. Therefore, we examined the antiviral property of RC-101 for cell-cell viral transmission in a cell fusion assay. As shown in Fig. 5 RC-101 exhibited potent antiviral activity against CCR5-tropic HIV-1-mediated cell fusion with an IC_{50} of $0.33 \mu\text{g/ml}$, which was similar to those observed in a CD4-dependent cell-cell transmission assay. No toxicity was observed at the highest concentration of the drug tested in this assay ($10 \mu\text{g/ml}$).

Evaluation of RC-101 in a CD4-independent cell-cell transmission assay

Epithelial cells in the vagina and cervix do not express HIV-1 receptor CD4 and coreceptors CCR5/CXCR4. The mechanism by which HIV-1 crosses the epithelium to set up the infection is currently unknown. From *in vitro* experiments using transformed epithelial cells, investigators speculated that CD4-independent cell-cell transmission may be responsible for viral transmission across the cervical mucosa.

FIG. 5. Antiviral activity of RC-101 in a CCR5-tropic cell fusion assay with mean and percent standard deviations of the triplicate data displayed graphically. The IC_{50} was calculated as described in the legend to Fig. 3. The percentage of virus control (%VC) and percentage of cell control (%CC) are used to show antiviral activity and cellular cytotoxicity, respectively.

Therefore, we examined the antiviral activity of RC-101 in a cell–cell transmission assay involving epithelial cells (ME-180) and HIV-1-infected H9 cells. As shown in Fig. 6, RC-101 exhibited potent antiviral activity against CXCR4-tropic HIV-1 SK1 in this assay, with an IC_{50} of 2.6 μ g/ml. Furthermore, in a separate experiment, pretreatment of cells for 4 h, followed by washout, resulted in no change in IC_{50} between the standard assay (5.53 μ g/ml) and the washout assay (5.48 μ g/ml; data not shown). Little cytotoxicity was observed at the highest concentration of RC-101 tested in this assay (10 μ g/ml), where cell viability was $\geq 90\%$.

Discussion

A limited number of anti-HIV-1 microbicides that can be applied topically in the vagina have been developed. Although these compounds inactivate HIV-1 on contact, several have been found to be either quite toxic in genital tissue or ineffective in blocking HIV-1 transmission. In contrast to other microbicide development platforms, the specific molecular targeting of HIV-1 attachment, fusion, and reverse transcription in the context of sexual transmission represents a rational, attractive, and timely approach to microbicide development. Blockage of initial attachment to CD4, of coreceptor (CCR5 and CXCR4) interactions, or of gp41-mediated fusion by compounds alone or in combination, may provide a highly effective strategy for preventing localized mucosal infection. Unlike surfactant-based microbicides, such an approach is highly unlikely to perturb the protective effects of resident microflora, or have contraceptive potential. The antimicrobial peptide retrocyclin RC-101, which interacts with gp41 and prevents fusion, is an attractive candidate microbicide because of its anti-HIV-1 activity against a wide variety of HIV-1 of various clades and tropisms, and because it has no toxicity in cell culture.^{17–19}

However, the antiviral activity of RC-101 against diverse U.S. isolates has been evaluated mostly against cell-free virus in transformed T cell lines. There are concerns that these transformed cell-based assays may have less relevance to *in vivo* transmission. Therefore, in this article we have examined the antiviral activity of RC-101 in a PBMC-based assay

against a wide array of global isolates. We showed that RC-101 had potent antiviral activity against clinical as well as international isolates of HIV-1 in PBMCs. Furthermore, a film-formulated RC-101 suitable for vaginal application retained potent antiviral activity against HIV-1 in PBMCs, indicating the usefulness of formulated RC-101 for development as a potential microbicide. The fact that transmission of HIV-1 could occur via cell-free and cell-associated HIV-1 present in semen raises the importance of examining microbicides against cell-associated HIV-1. In this article we examined and demonstrated the antiviral activity of RC-101 in various cell–cell transmission assays, including CD4-dependent and CD4-independent assays. The latter assay has important implications when one considers that HIV-1 transmission across the cervical/vaginal epithelium occurs via an CD4-independent pathway.²⁹ The antiviral activity of RC-101 in the cell–cell fusion assay further confirms the antiviral activity of RC-101 against cell-associated HIV-1.

Seminal fluid has substantially higher osmolality than blood plasma, slightly alkaline pH (7.8), and the presence of other binding proteins with the potential to inactivate a peptide-based microbicide, such as RC-101.³⁰ RC-101 did retain activity in the presence of seminal plasma, although its potency was reduced by 6- to 13-fold against CCR5-tropic cell-free and cell-associated virus, respectively. The reason for the reduced antiviral activity of RC-101 in seminal plasma is unknown. It is possible that peptide RC-101 binds to some component of seminal plasma and thereby its effective concentration becomes reduced. We have not observed an enhancing effect of seminal plasma on HIV infection as described by Munch and colleagues.³¹ One reason for the lack of enhancement might be due to the high multiplicity of infection and relatively low level (25%) of seminal plasma used in the assay.

In summary, RC-101 has been shown to have strong antiviral activity and low cytotoxicity in cell culture, tissues, and recently in nonhuman primate models. The present study demonstrates its potent antiviral activity against cell–cell transmission, making RC-101 an excellent candidate microbicide for clinical trials. However, the clinical relevance of the antiviral activity of RC-101 against cell-associated HIV-1 must be further evaluated *in vivo* in an appropriate nonhuman primate model.

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Author Disclosure Statement

No competing financial interests exist.

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FIG. 6. Antiviral activity of RC-101 in a CD4-independent cell–cell transmission assay with mean and percent standard deviations of the triplicate data displayed graphically. The IC_{50} was calculated as described in the legend to Fig. 3. The percentage of virus control (%VC) and percentage of cell control (%CC) are used to show antiviral activity and cellular cytotoxicity, respectively.

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