Preclinical studies on thiocarboxanilide UC-781 as a virucidal agent

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Background: Thiocarboxanilide UC-781 is a highly potent and selective non-nucleoside reverse transcriptase inhibitor (NNRTI) of HIV-1, which also has virucidal properties. Recent studies have shown that UC-781 would seem an ideal candidate for application as a vaginal virucide.

Objective: To investigate the antiviral potency and stability of UC-781 in a lipophilic gel formulation.

Methods: UC-781 was formulated in replens gel at different concentrations and administered intravaginally to rabbits at 5% in replens gel for 10 days. UC-781 was also exposed to temperatures of 4, 37 and 50°C, and to low pH (6.0, 4.3, 2.0 and 1.2). A number of microorganisms were exposed in culture to serial dilutions of UC-781.

Results: The drug was stable under low pH conditions and did not lose its antiviral potency upon 4 h exposure to pH 3.5 (the estimated vaginal pH). UC-781 can be easily formulated into a lipophilic gel (replens; up to 5%) and proved fully stable at 50°C for 30 days. There was no effect on the growth of microorganisms (i.e., *Candida* and *Lactobacillus* strains) that are present in the vaginal flora. Neither systemic side-effects, nor local inflammation or damage of the vaginal mucosa or epithelium were observed in rabbits to which 5% UC-781 in replens gel had been administered. UC-781, formulated as 0.5, 0.2 and 0.05% replens gel, and UC-38, α-APA and zidovudine, formulated as 0.5 or 0.2% replens gel, were effective in protecting CEM cells in the very beginning against productive HIV-1 replication. This points to an efficient diffusion of the drugs from the lipophilic gel to the hydrophilic culture medium. However, subsequent subcultivations at a dilution rate of 1 : 10 every 3–4 days resulted in a rapid breakthrough of virus with all drugs except UC-781 in its 0.5 and 0.2% gel formulation. These cultures were fully protected against HIV-1 and remained completely cleared from virus for at least 10 subcultivations.

Conclusions: The virus that emerged under 0.05% UC-781 remained highly sensitive to the NNRTI, including UC-781, in cell culture, suggesting a lack of resistance development under our experimental conditions.

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Introduction

In an attempt to control AIDS with chemotherapy, the majority of research efforts have focused on the development of antiviral drugs that are administered, preferentially via the oral route, to HIV-infected individuals. The major targets of the anti-AIDS drugs that are currently used in the clinic are HIV-encoded reverse transcriptase (RT) and protease [1–3]. Although a variety of other compounds have been found to act at other targets such as HIV adsorption/fusion, integration and transactivation (*tat*), none of these compounds have yet been approved for clinical use. Because of the increasing number of infected people and the daily incidence of new infections, additional effective strategies need to be developed to prevent the further spread of the AIDS epidemic.

Female-controlled methods (i.e., vaginal virucide) to prevent or reduce the risk of HIV infection by sexual intercourse is one of the strategies that should be considered with high priority. Thus, the development of safe and effective vaginal microbicides is a high ranking priority for HIV and sexually transmitted disease prevention [4–6]. An ideal vaginal virucide should be safe and effective, non-toxic, stable in harsh climates, and easily affordable. It should inhibit a broad range of HIV strains and, preferably, also cell-associated HIV transmission. Moreover, the agent should not be inhibitory to the normal vaginal flora, it should not be irritating to the vaginal epithelium and mucosa, and it should be active in vaginal fluids or in physiologically relevant conditions [7,8]. Most of the currently available microbicides contain nonoxynol-9, a non-ionic surfactant. Despite its *in vitro* HIV activity [9,10], there are no conclusive data regarding its effectiveness *in vivo* [11–13]. A randomized controlled trial with vaginal contraceptive film containing 70 mg nonoxynol-9 recently finished in Cameroon; however, there was no protection observed against HIV, gonorrhoea or chlamydia [14]. This might be due to a lack of solubility of the film in the vagina, so that the total amount of nonoxynol-9 was not released. The incidence of genital lesions was higher in the active group than the placebo group, although the difference was not significant. It could be that the benefit of the anti-HIV activity was counteracted by the occurrence of lesions that may enhance HIV transmission. Although one more nonoxynol-9 effectiveness trial is ongoing and more are planned, its dose-dependent toxicity may always be a limiting factor. For this reason the development of potent new vaginal microbicides is clearly needed.

Recently, it has been reported that thiocarboxanilide UC-781 may have considerable promise as a virucidal agent in preventing the spread of HIV from infected to non-infected individuals [15–17]. UC-781 belongs to the class of (thio)carboxanilide derivatives, the prototype of which (UC-84 or Uniroyal Senior) was originally reported by Bader *et al.* [18] to inhibit HIV-1 replication in cell culture. The thiocarboxanilide UC-781 is a non-nucleoside RT inhibitor (NNRTI), and is extremely potent as an inhibitor of HIV-1 in cell culture [50% effective concentration (EC_{50}), approximately 3 ng/ml]. It is targeted at HIV-1 RT and effective against a variety of HIV-1 strains that contain NNRTI-characteristic mutations in their RT genome [19–23]. UC-781 proved to be a tight-binding inhibitor of HIV-1 RT with median inhibitory concentration (IC_{50}) values in the lower nanomolar range [17,22]. In addition, UC-781 was found to markedly attenuate the infectivity of nascent virus produced by UC-781-treated HIV-1-infected cells after removal of exogenous drug, and to abolish the infectivity of virus produced by drug-treated peripheral blood lymphocytes [15]. In addition, pretreatment of uninfected cells with UC-781 rendered these cells refractory to subsequent HIV infection in the absence of extracellular drug for several days following the removal of the drug [15]. We have now investigated a number of characteristics that are of crucial importance for the further development of UC-781 as a virucidal agent.

Materials and methods

Test compounds

The thiocarboxanilides UC-781 and UC-38 were supplied by Uniroyal Chemical Ltd (Guelph, Ontario, Canada) and their synthesis will be published elsewhere. The structural formula of UC-781 is shown in Fig. 1. Nevirapine was from P. Ganong (Boehringer, Ingelheim, Germany). Delavirdine was synthesized by Dr R. Kirsch (Hoechst AG, Frankfurt, Germany) and kindly provided by Dr J-P. Kleim (Hoechst AG). α-APA (anilino-acetamide; Loviride) was provided by Dr K. Andries (Janssen Foundation, Beerse, Belgium). Zidovudine (ZDV) was obtained from Sigma (St Louis, Missouri, USA). Lamivudine was provided by Dr J. Cameron (Glaxo Wellcome, Stevenage, Hertfordshire, UK).

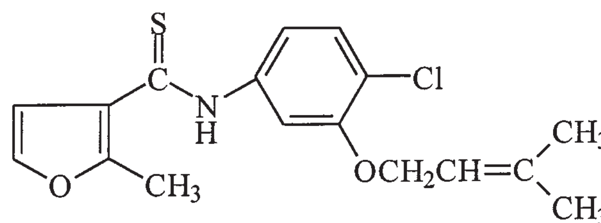

Fig. 1. Structural formula of UC-781.

Gel formulation of UC-781 and other test compounds

UC-781, UC-38, α-APA and ZDV were formulated in replens gel containing glycerine (3%), sorbic acid (0.08%), carbopol 940 (1%), liquid paraffin (4%), NaOH 1 N (16%) and H_2O. The pH of the replens gel was approximately 10. Addition of the drugs to the gel preparation did not markedly alter the pH (a trend to a lower pH value of 9.15–9.65 was noticed). Replens gel was chosen because it is currently being used to evaluate the virucidal properties of nonoxynol-9 in humans [24] upon intravaginal administration. The following concentrations were prepared: UC-781 at 10, 5, 0.5, 0.2 and 0.05%; UC-38 at 0.2%; α-APA at 0.2%; and ZDV at 0.5 and 0.2%. Control gel contained the same ingredients without test compound.

UC-781 was also prepared at 5 and 2% in orabase, containing gelatin (16.7%), pectin (16.7%), sodium carboxymethethylcellulose (16.7%), polythene (mean molecular weight, 32 000; 2.5%) and liquid paraffin (47.4%). Orabase was chosen because this type of formulation is commonly used to locally apply drugs on the epithelium of the mouth because of its favourable adhering properties to the mucus layer.

Cells

Human lymphocyte CEM cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA), and grown in RPMI-1640 medium supplemented with 10% (vol/vol) inactivated fetal calf serum (Gibco, Grand Island, New York, USA), 2 mmol/l L-glutamine (Flow Laboratory, Irving, Scotland, UK), and 0.075% (vol/vol) $NaHCO_3$ (Flow Laboratories). Cells were subcultured every 3–4 days. Molt4/clone 8 cells were a kind gift of Dr N. Yamamoto (Tokyo Medical and Dental University, Tokyo, Japan). C8166 cells were obtained from Dr P. La Colla (Università degli Studi di Cagliari, Cagliari, Italy).

Viruses

HIV-1_{IIIB} was kindly provided by Dr R.C. Gallo (National Cancer Institute, Bethesda, Maryland, USA) [25].

Antiviral activity of the test compounds

CEM cells were suspended at 250 000 cells/ml culture medium and infected with 100 median cell culture infective doses ($CCID_{50}$) of HIV-1_{IIIB} or HIV-1 strains that had emerged in the presence of UC-781. Then, 100 µl of the infected cell suspension was added to 200 µl microtitre plate wells containing 100 µl of an appropriate dilution of the test compounds (i.e., 100, 20, 4, 0.8, 0.16, 0.032, and 0.006 µg/ml). The inhibitory effect of the test compounds on HIV-1-induced syncytium formation in CEM cells was examined on day 4 after infection as described previously [19,20]. The EC_{50} was determined as the compound concentration required to inhibit giant cell (syncytium) formation by 50%.

Exposure of drug-containing replens gel preparations to HIV-1-infected CEM cell cultures

Two-milligram gel ointments containing several concentrations of UC-781 (0.5, 0.2 and 0.05%), UC-38 (0.2%), α-APA (0.2%) and ZDV (0.5, 0.2%) were added to 5 ml HIV-1-infected CEM cell cultures. Twice weekly these cell cultures were subcultured at a dilution of 1 : 10 without administration of additional drug-containing gel. Early signs of virus breakthrough, microscopically visualized by giant cell formation in the cell cultures, was recorded at the time of subcultivation. The time of virus breakthrough (at 50% cytopathicity) was defined as the number of days post-initiation of the experiment, that allowed the virus to afford 50% cytopathicity (i.e., approximately 100 giant cells per microscopic field) in the cell cultures.

RT assay

The reaction mixture (50 µl) contained 50 mmol/l Tris–HCl (pH 7.8), 5 mmol/l dithiothreitol, 300 mmol/l glutathione, 500 µmol/l EDTA, 150 mmol/l KCl, 5 mmol/l $MgCl_2$, 1.25 µg bovine serum albumin, 2.5 µmol/l [8-^{3}H]dGTP (specific radioactivity, 15.6 Ci/mmol; 2 µCi per assay), a fixed concentration (0.1 mmol/l) of the template/primer poly(C)–oligo(dG), 0.06% Triton X-100, 10 µl inhibitor solution (UC-781, UC-38, nevirapine, delavirdine and α-APA, containing various concentrations of the compounds), and 1 µl of the RT preparation [26]. The reaction mixtures were incubated at 37°C for 15 min, at which time 100 µl calf thymus DNA (150 µg/ml), 2 ml $Na_2P_2O_7$ (0.1 mol/l in 1 mol/l HCl), and 2 ml trichloroacetic acid (10% vol/vol) were added. The solutions were kept on ice for 30 min, after which the acid-insoluble material was washed and analysed for radioactivity. For the experiments in which the inhibitory effect of UC-781 on RT was evaluated under different pH conditions, Tris–HCl buffer was adjusted to pH values of 8.0, 7.2, 6.6, 6.0 and 5.4.

pH stability of UC-781

Solutions of 100 µg/ml UC-781 in 20% dimethyl sulphoxide (DMSO) and 80% sodium phosphate buffer 10 mmol/l pH 6.0 or 4.3, and solutions of 100 µg/ml UC-781 in 20% DMSO and 0.01 N HCl (pH 2.0) or 0.1 N HCl (pH 1.2) were incubated for 1 or 4 h at 37°C. Then the drug solutions at pH 6.0 and 4.3 were neutralized by adding an equal volume of 100 mmol/l sodium phosphate buffer pH 7.5, and the drug solutions at pH 2.0 and 1.2 were neutralized by adding an equal volume of NaOH at 0.015 and 0.15 N, respectively. The neutralized solutions were then analysed on a LiChroCard reverse-phase RP-8 column

(Merck, Darmstadt, Germany) by high performance liquid chromatography (HPLC), using the following gradient: 0–2 min, 5% acetonitrile in H_2O; 2–22 min, linear gradient to 90% acetonitrile in H_2O; 22–27 min, 90% acetonitrile in H_2O; 27–35 min, linear gradient to 5% acetonitrile in H_2O; 35–40 min, equilibration at 5% acetonitrile in H_2O. Under these experimental conditions, the retention time of UC-781 was 21.8 min. The wavelength used for detection of the compound was 290 nm.

Temperature stability of UC-781

UC-781 at 5 or 2% in replens gel or orabase was incubated at 4°C, 37°C and 50°C for 30 days. After the indicated incubation period, the test compound (and potential conversion products) were extracted from the replens gel or orabase formulations by chloroform ($CHCl_3$) or dichloromethane (CH_2Cl_2), and the extract was analysed on an RP-8 column by HPLC, using the acetonitrile/H_2O gradient described above.

Intravaginal treatment of rabbits with 5% UC-781 replens gel

Four female rabbits (weighing approximately 1200 g; age, 10 weeks) were daily treated intravaginally with 100 μl control replens gel (one rabbit) or 100 μl 5% UC-781 replens gel (three rabbits) for 10 consecutive days. The gel preparation was brought into the rabbit vagina at 1 cm depth with 1 ml syringes. After a 10-day treatment period, the rabbits were killed by CO_2 inhalation, and the vaginas were prepared for macroscopic and microscopic inspection. The internal and external genitalia including ovaries, tubae, uterus, vagina and vulva were removed in one procedure together with the rectosigmoid and perineum. The anterior side of the vagina was longitudinally opened and photographs were taken. For histology, transversal tissue blocks including the total vagina perimeter were taken at three equidistant levels: level A, 1 cm proximal to the vaginal opening; level B, middle; and level C, just underneath the cervix. Four-micron sections were prepared in a standard way, stained with haematoxylin–eosin and examined by light microscopy. From the vaginal epithelium the number of mitoses per perimeter was counted as an index of reaction to injury.

Exposure of HIV-1-infected CEM cell cultures to replens gel containing HIV inhibitors

Two milligrams of replens gel containing 0.5, 0.2 or 0.05% UC-781, 0.2% UC-38, 0.2% α-APA, 0.5% ZDV or 0.2% ZDV, or control gel (without drug) were brought into 25 cm^2 culture bottle flasks. Then, 5 ml culture medium containing CEM cells (approximately 300 000 cells/ml) freshly infected with 200 $CCID_{50}$ HIV-1 were added. The HIV-1-infected cell cultures exposed to the gel formulations were incubated at 37°C for 4 days, and subcultured every third or fourth day. Subcultivation was performed at 1 : 10 ratios by adding 0.5 ml of the HIV-1-infected gel-exposed cell culture to 4.5 ml fresh culture medium. The appearance of HIV-1-induced giant cell formation was followed daily, and monitored microscopically. As soon as virus that broke through in the gel-exposed HIV-1-infected cell culture caused 100% cytopathicity (approximately 200 giant cells per microscopic view), the supernatant was frozen in aliquots until the virus was further investigated on its drug sensitivity and potential appearance of drug-related mutations in its RT gene.

Antimicrobial activity of UC-781

A variety of Gram-positive and Gram-negative bacteria and two yeast species (Table 1) were grown in 2 ml liquid medium (Bacto-peptone 10 g/l, beef extract 3 g/l, NaCl 5 g/l; pH 6.5) in 6 ml tubes and containing serial dilutions of UC-781 (30, 15, 7.5, 3.75, 1.87, 0.93 and 0.46 μmol/l). Therefore, these tubes were inoculated with 20 μl of a microbial suspension (10^7 colony-forming units/ml) of the microorganisms indicated. All cultures contained 2% DMSO during growth. Since the *Lactobacillus* strains (Table 1) did not grow well in BD broth, they were grown in thioglycolate medium (Difco, Detroit, Michigan, USA) containing 0.5% agar

Table 1. Antimicrobial activity of UC-781.

	MIC_{50}* (μmol/l)
Yeasts	
Candida albicans	> 30
Candida tropicalis MUCL 28.180	> 30
Gram-positive bacteria	
Bacillus cereus NCTC 8035	3.75–30†
Bacillus subtilis ATCC 6633	> 30
Micrococcus flavus ATCC 10240	> 30
Micrococcus lysodeicticus NCTC 2665	> 30
Staphylococcus aureus	> 30
Sarcina lutea ATCC 9241	> 30
Lactobacillus casei ATCC 7469‡	> 25
Lactobacillus sp. from cervix 37 isolate‡	> 25
Lactobacillus sp. from vagina 14 isolate‡	> 25
Lactobacillus sp. from vagina 53 isolate‡§	≥ 25
Lactobacillus sp. from vagina 121 isolate‡¶	> 25
Gram-negative bacteria	
Escherichia coli NCIB 8743	> 30
Pseudomonas aeruginosa	> 30
Serratia marcescens	> 30

*Determined by measurement of the absorbance of the cultures at 540 nm on a Multiscan MCC/340P version 2.33. †At concentrations between 3.75 and 30 μmol/l, 50% inhibition of bacterial growth was observed; at 1.875 μmol/l, no growth inhibition occurred. ‡The bacteria were diagnosed as *Lactobacillus* sp. based on their genital origin, α-haemolysis, Gram-positive character, bacillus-shape, non-motile, catalase-negative, and vancomycin resistance (i.e., cervix 37 and vagina 121 isolates); growth inhibition was determined in thioglycolate medium; the cultures containing the highest drug concentration contained 3% dimethyl sulphoxide (DMSO) during growth; in the next drug concentrations, DMSO was serially diluted out (duplo dilutions); cultures were exposed to the drug for 24 h in 6 ml tubes containing 3 ml thioglycolate culture medium at 37°C. §Bacterial growth was markedly inhibited by DMSO at 3 and 1.5%, but not at 0.75% in both the aerobic and anaerobic areas of the cultures. ¶Bacterial growth was markedly inhibited by DMSO at 3 and 1.5% but not at 0.75% in the anaerobic area of the cultures only.

and resazurine as an O_2 indicator. These strains were inoculated in fresh 3 ml thioglycolate medium-containing 6 ml tubes, and a variety (25, 12.5, 6.25, 3.12, 1.56 and 0.78 μmol/l) of UC-781 concentrations. Incubation was performed at 37°C in a CO_2-controlled incubator. The tubes containing the highest drug concentration (25 μmol/l) also contained 3% DMSO. Control cultures were performed without drug but in the presence of 3, 1.5 or 0.75% DMSO to examine the potential inhibitory effect of DMSO on bacterial growth in the thioglycolate medium. At 24 h post-inoculation, bacterial growth in the presence of different concentrations of UC-781 was recorded by visual inspection and compared with untreated control. In addition, a 200 μl suspension of each culture tube was brought into the wells of a 96-well microtitre plate and the bacterial density was measured in a Multiscan MCC/340P version 2.33 (Lab Systems, Life Sciences International, Belgium) by absorbance at 540 nm. The microbial density of the original inoculum that was kept at 4°C during the 24 h incubation period of the drug-treated cultures, was also measured and subtracted from the absorbance values found for the drug-treated cultures. The median minimal inhibitory concentration was defined as the drug concentration that reduced the absorbance value (540 nm) of the control cultures by 50%.

Results

Physical properties of UC-781

Both temperature and pH stability were examined for UC-781. The drug, formulated as 10% in replens gel or in orabase, was exposed to 4°C, 37°C and 50°C for up to 30 days. After the incubation period, the compound was extracted from the gel with $CHCl_3$ or CH_2Cl_2 and subjected to HPLC analysis on an RP-8 column. In no case was there any significant breakdown of UC-781, pointing to the high chemical stability of the drug at a variety of temperatures.

The drug was also exposed to varying pH conditions for 1 or 4 h. At pH 6.0, 4.3 or 2.0, no breakdown of UC-781 was found, irrespective of the incubation time. Only at pH 1.2, UC-781 was converted to another derivative with more polar properties (retention time, 11.1 min on an HPLC reverse-phase RP-8 column compared with 21.8 min for the parent UC-781 compound), the conversion being approximately 7% after 1 h and approximately 16% after 4 h of incubation. We have not yet been able to identify this breakdown product. However, neutralization of all the samples to pH 7.0 and subsequent evaluation of their antiviral potency against HIV-1 in CEM cell cultures revealed no significant loss of the inhibitory effect of the samples against HIV-1 (EC_{50}, 0.001–0.004 μg/ml; data not shown).

Exposure of several gel formulations of test compounds to HIV-1-infected CEM cell cultures

It is important to reveal whether a drug that is appropriately formulated in the gel can also easily be released from the gel preparation in order to be taken up by the target (virus-infected and uninfected) cells. Therefore, the following experiment was performed. The thiocarboxanilides UC-781 and UC-38, and α-APA and ZDV were formulated in replens gel at different concentrations (0.5, 0.2, 0.05%; Table 2). A 2 mg gel ointment was exposed to 5 ml of an HIV-1-infected CEM cell culture. Then the cell cultures were subcultured every 3–4 days at a dilution of 1 : 10. This means that the initial total drug concentrations were 2, 0.8 and 0.2 μg/ml in the cell culture medium, for the cell cultures exposed to 0.5, 0.2 and 0.05% gel, respectively, assuming that the drugs were completely released from the gels. With each cell culture passage, the drug concentrations decreased 10-fold. Therefore, at a 100% release of the drugs from the gel into the cell culture medium, a maximum of 0.2, 0.08 and 0.02 μg/ml of drug may be present after 4 days; 0.02, 0.008 and 0.002 μg/ml of drug after 7 days; and 0.002, 0.0008 and 0.0002 μg/ml of drug after 10 days in the cell cultures. Thus, after 10 days all drug concentrations were reduced to levels that were equal (for UC-781 at 0.5% in replens gel) or well below (for UC-781 at 0.2 and 0.05% in replens gel) their antiviral EC_{50} values.

ZDV, α-APA and UC-38 failed to markedly delay virus breakthrough under our experimental conditions. In contrast, UC-781 completely cleared the infected cell cultures from virus if applied at 0.5 and 0.2% in replens gel. Even the lowest UC-781 gel concentration

Table 2. Virus breakthrough in HIV-1-infected CEM cell cultures upon exposure of replens gel-containing test compounds.

Compound	Compound concentration in replens gel (%)	Time (days) of virus breakthrough* (50% cytopathicity)
Experiment 1		
UC-781	0.5	> 60†
	0.2	> 60†
	0.05	30
None‡	0	4
Control§	–	3
Experiment 2		
UC-781	0.5	> 50†
	0.2	> 50†
	0.05	16
UC-38	0.2	11
α-APA	0.2	11
Zidovudine	0.5	11
	0.2	11
None‡	0	7

*Virus breakthrough was inspected microscopically at each day of the subcultivation as giant cell (syncytium) formation; full cytopathicity was obtained when approximately 200 giant cells were visible in one microscopic field. †Cell cultures were cleared from virus. ‡HIV-1-infected cell cultures in the presence of replens gel without drug. §HIV-1-infected cell cultures in the absence of replens gel.

(0.05%; initial maximum concentration in the cell culture medium, 0.2 μg/ml) was able to significantly delay virus breakthrough (Table 2). It was ascertained that the prevention of virus breakthrough was not due to a toxic activity of UC-781 in the CEM cell cultures. Indeed, no morphological changes to the UC-781 gel-exposed cells were observed, and the growth rate of the cells during the several subcultivations proceeded at a speed that was comparable to the growth rate of mock-infected cell cultures. In a second and independent set of experiments, the 0.5 and 0.2% UC-781 gel formulations again afforded full protection against the virus, and the cultures were again, as observed in the first experiment, cleared from virus. Treatment of HIV-1-infected cell cultures with the lowest UC-781 concentration (0.05%) in the replens gel resulted in a marked delay of virus breakthrough compared with the control (Table 2).

Drug-sensitivity of the HIV-1 strains that emerged under 0.05% UC-781-containing replens gel

The virus strain that emerged in the HIV-1-infected CEM cell culture with UC-781 at 0.05% in replens gel was evaluated for its sensitivity towards a variety of NNRTI and nucleoside RT inhibitors. Compared with the original HIV-1 strain that was used to infect CEM cell cultures, UC-781-containing replens gel-treated virus showed identical sensitivity to the variety of test compounds (Table 3). In particular, UC-781 showed an EC_{50} as low as 0.002 μg/ml against both virus strains. Sequencing of the RT gene of the drug-treated virus strain revealed no mutations within the 50–270 amino-acid region of RT, an observation that is in agreement with the drug sensitivity data.

Table 3. Sensitivity of wild-type and UC-781-containing replens gel-exposed HIV-1 strains to non-nucleoside and nucleoside reverse transcriptase inhibitors.

	EC_{50}* (μmol/l)		
		UC-781 replens gel-exposed virus	
Compound	Wild-type	Experiment 1[†]	Experiment 2[‡]
UC-781	0.005	0.013	0.005
α-APA	0.01	0.020	0.02
Nevirapine	0.03	0.11	0.05
Delavirdine	0.009	0.03	0.03
MKC-442	0.01	0.02	0.01
TSAO-m^3T	0.07	0.08	0.08
Quinoxaline (HBY 097)	0.001	0.005	0.002
8-Chloro-TIBO	0.006	0.02	0.01
Zidovudine	0.002	0.018	0.007
Didanosine	5	20	10
Lamivudine	0.05	0.20	0.13

*Median (50%) effective concentration, or compound concentration required to inhibit HIV-1-induced syncytium formation in CEM cell cultures by 50%. [†]Virus that emerged from HIV-1-infected CEM cell cultures exposed to 0.05% UC-781-containing replens gel from experiment 1 (Table 2). [‡]Virus that emerged from HIV-1-infected CEM cell cultures exposed to 0.05% UC-781-containing replens gel from experiment 2 (Table 2).

Effect of UC-781 and other NNRTI on HIV-1 RT activity

Thiocarboxanilide UC-781, nevirapine, delavirdine and α-APA were evaluated for their inhibitory effect against recombinant HIV-1 RT using poly rC–dG as the template primer and [^{3}H]dGTP as the radiolabelled substrate. The IC_{50} values of UC-781, nevirapine, delavirdine and α-APA were 0.005, 1.26, 0.32 and 0.35 μg/ml, respectively. Inhibition of UC-781 was non-competitive against HIV-1 RT with respect to dGTP (data not shown). When evaluated at a variety of pH values (pH 8.0, 7.8, 7.18, 6.62 and 6.0), UC-781 inhibited HIV-1 RT at 0.005, 0.008, 0.008, 0.009 and 0.07 μg/ml, respectively. At pH 6.0, it should be mentioned that the activity of the enzyme was markedly decreased (n10% of control) compared with the higher pH values.

Inhibitory activity of UC-781 against yeasts, bacteria and viruses other than HIV

UC-781 was evaluated for its inhibitory effect against a variety of yeast (*Candida*) and bacteria (12 Gram-positive and three Gram-negative) strains (Table 1). In no case did UC-781 display a marked inhibitory effect against microbial growth at 25–30 μM, except for *Bacillus cereus* growth, which was inhibited at approximately 50% at UC-781 concentrations ranging from 3.75 to 30 μM. Of particular interest was the clear lack of inhibitory effect of UC-781 at 30 μM on a variety of clinical *Lactobacillus* sp. isolates. The latter bacteria are part of the vaginal microbial flora. The thiocarboxanilide UC-781 was also evaluated against a variety of virus strains including the DNA viruses herpes simplex virus type 1 (strain KOS) and type 2 (strain G), and vaccinia virus in human embryonic lung fibroblast E_6SM cell cultures and the RNA viruses vesicular stomatitis virus in E_6SM cell cultures and sindbis virus, coxsackie virus B4, reovirus-1, Punta Toro and parainfluenza type 3 virus in monkey kidney Vero cell cultures. UC-781 had no inhibitory effect on the replication of these viruses at 100 μM, the highest concentration tested (data not shown).

Intravaginal exposure of 5% UC-781 in replens gel to female rabbits

Four female rabbits were given a daily intravaginal inoculation of 100 μl replens gel (one control rabbit) or replens gel containing 5% UC-781 (three rabbits). After

Table 4. Number of epithelial mitoses per transverse section of the vagina.

Level	Control	Animal 1	Animal 2	Animal 3
A	2	9	0	2
B	1	1	2	1
C	7	9	1	0

10 days, the rabbits were killed and dissected, and the vaginas prepared for macroscopic and microscopic examination, as described in Materials and methods. Simple visual inspection revealed no differences between the vaginal mucosa of control (Fig. 2a) and treated (Fig. 2b) animals. Light microscopic examination revealed a normal non-keratinized squamous epithelium in the control animals. At levels A and B, mitoses were sparse and exclusively located in the basal layer. Few plasma cells and an occasional lymphocyte was seen (Fig. 3a, level A). At level C, a normal transition to endocervical mucosa was seen (data not shown). Histology was normal in control as well as UC-781-treated animals. There was no systematic increase of the number of inflammatory cells. The number of epithelial mitoses remained constant, namely one or two per transverse section, except in one treated animal (animal 1) in which up to nine mitoses were counted. This was not associated with inflammation or cellular atypia (Fig. 3b, level A).

One hour after the last application of UC-781, a blood sample of each rabbit (one control and three drug-treated animals) was taken, and the serum split into two parts. The first part was subjected to HPLC analysis to determine UC-781 levels in the plasma. The other part of the serum was used for a bioassay, in which the serum was serially diluted and added to HIV-1-infected CEM cell cultures to detect any antiviral effect of the test compound (if present) in the serum. HPLC analysis on 200 μl samples did not enable us to detect any trace of UC-781 in the serum. Since the detection limit of UC-781 was approximately 2 nmol (600 ng), lack of detection of UC-781 by HPLC means that the plasma contained UC-781 levels lower than 10 nmol/ml (3 μg/ml). Likewise, the bioassay did not reveal any HIV-1-inhibitory activity in a serum sample diluted 1 : 5. Since a UC-781 concentration as low as 3 ng/ml (i.e., EC_{50} of UC-781 against HIV-1 in CEM cell cultures) could be detected in our bioassay, the plasma (serum) levels of UC-781 must have been lower than 15 ng/ml (data not shown).

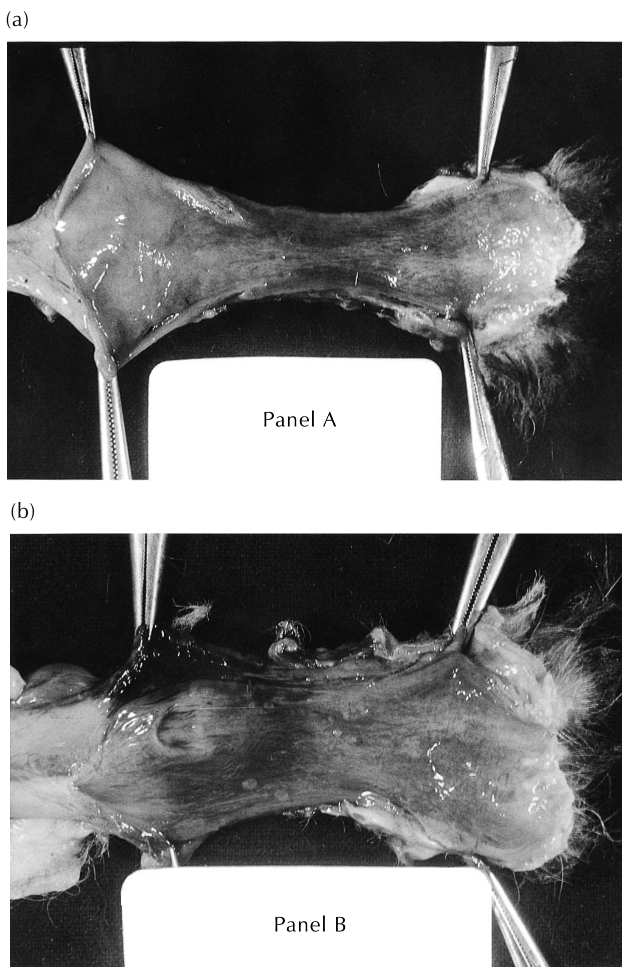

Fig. 2. Visual inspection of the vaginal mucosa from (a) a control (replens gel-treated) rabbit and (b) a UC-781 (5%)-containing replens gel-treated rabbit.

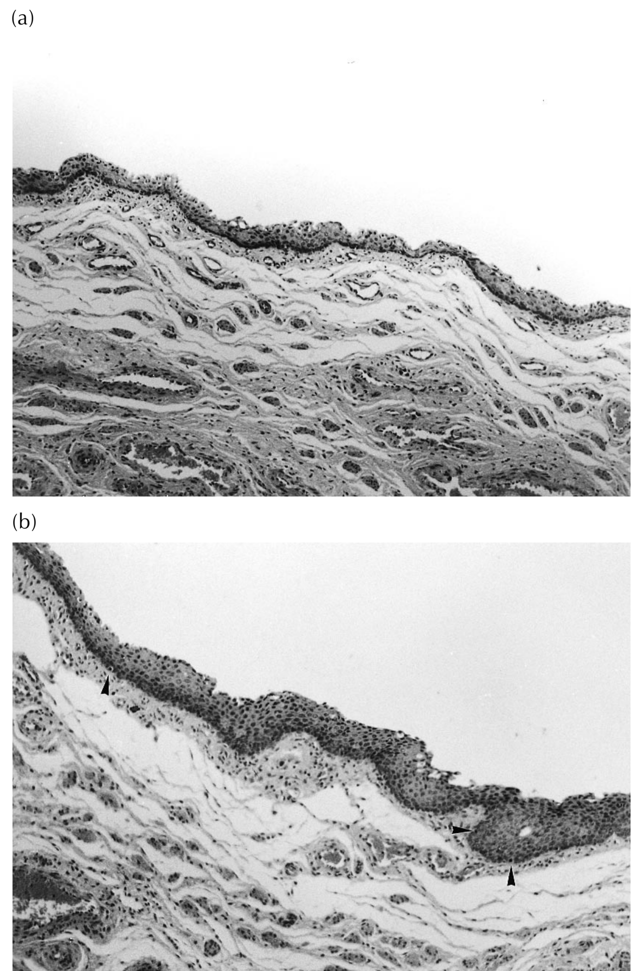

Fig. 3. Light-microscopic examination of the vagina epithelium from (a) a control (replens gel)-treated rabbit and (b) a UC-781 (5%)-containing replens gel-treated rabbit.

Discussion

Vaginal microbicides should be clinically evaluated for both safety and efficacy before being administered to individuals at risk for HIV infection. Safety studies are necessary because irritation of vaginal and cervical mucosa, inflammation and ulcerations might increase HIV transmission upon sexual intercourse. Thus, any candidate microbicides for vaginal application should not be irritating for the vaginal mucosa. Efficacy studies are obviously needed to assess prevention of HIV infection, and such trials can only be conducted with products that have been evaluated for safety and appear to be non-toxic. It is generally recommended that both the active agent and the clinical formulation of the product be tested in a rabbit vaginal irritation model (at a standard 10-day application) early in the development process. The safety study with 5% UC-781 replens gel in rabbits indicated the absence of any signs of irritation or toxicity. It should be emphasized that the drug-containing replens gel was applied at the vaginal mucosa, but not at the cervical mucosa. Therefore, the drug administration closely mimics the application of a microbicidal preparation in humans. However, if significant diffusion of the drug from the replens gel to the cervical mucosa had occurred (a situation that is presently unknown), it did not appear to have affected the integrity of the cervical mucosa.

Replens gel has been used already as a vehicle for 4% nonoxynol-9 in Phase I/II clinical trials to evaluate the virucidal properties of this compound [24], and is considered to be safe and without side-effects on the vaginal or cervical mucosa. UC-781 was also found to lack any significant inhibitory activity against the growth of a variety of yeasts and bacteria, including *Lactobacillus casei*, which is considered a surrogate for the normal vaginal flora as well as several clinical *Lactobacillus* isolates from patients. The drug concentrations that were used for antimicrobial testing did not exceed 25–30 μM due to insolubility of UC-781 at higher concentrations. Therefore, although these concentrations are much lower than those used in the replens gel formulation, they are relevant since the drug will never exceed these concentrations in soluble form in the vaginal environment. The lack of antimicrobial and antifungal activity is not surprising in view of the highly specific nature of inhibition and mechanism of action of UC-781 against HIV-1. UC-781 also lacks inhibitory activity against closely related HIV-2 or simian immunodeficiency virus strains and any other DNA or RNA viruses. Thus, the high specificity and selectivity of UC-781 (as an NNRTI) by itself guarantees a lack of significant biological activity against other microorganisms. In the rabbits, UC-781-containing replens gel did not cause any toxic side-effects. Nor had UC-781 any influence on vaginal commensals such as *Candida* spp. and *Lactobacillus* spp. This justifies further investigations on the potential of UC-781 as a vaginal virucide. Unfortunately, due to the high specific nature of UC-781, no reliable intravaginal infection animal model is currently available to investigate the antiviral efficacy of the drug in the *in vivo* setting.

Besides being evaluated against HIV-1_{IIIB}, UC-781 has also been evaluated for its antiviral activity against the clinical HIV-1 isolate (HE) in CEM cells, against HIV-1_{IIIB} in freshly isolated peripheral blood lymphocytes from healthy donors and against HIV-$1_{Ba\text{-}L}$ in monocyte/macrophages. In all cases, UC-781 had an antiviral activity ranging between 0.001 and 0.08 μg/ml [13]. It would also be useful to evaluate UC-781 on its inhibitory properties of HIV-1 isolates that belong to virus clades other than type B before it is administered to HIV-1-infected patients.

The marked temperature and pH stability of UC-781 is another highly desirable property of UC-781. The temperature stability of UC-781 in replens gel for at least 1 month at 4°C, 37°C, and particularly at 50°C is important since the drug-containing gel formulations may be exposed to unusually high temperatures in warmer climatological areas of the developing countries. In addition, UC-781 has proven to be stable at pH 2.3 for at least 4 h, a pH value that is well below the mean pH of the human vagina (pH 3.5). Even if UC-781 was exposed to pH 1.2 for a limited time period, it did not show significant loss of antiviral activity.

It should also be mentioned that UC-781 virtually kept its full inhibitory potential against recombinant HIV-1 RT when the assays were performed at lower pH values such as pH 7.18, 6.62 and 6.0 (IC_{50}, 0.002–0.004 μg/ml; data not shown). At pH 5.4, the enzyme reaction proceeded at a velocity that was less than 5% of control (pH 7.8), and therefore, the IC_{50} value of UC-781 under these experimental conditions could not be accurately measured. Thus, UC-781 retained its biological activities at high temperatures and low pH values.

Our data on HIV-1-infected CEM cell cultures exposed to UC-781-containing replens gel are of particular interest. The remarkable protection demonstrated with the 0.5 and 0.2% UC-781 gel formulations are indicative for a rapid release of active UC-781 from the replens gel vehicle into the hydrophilic cell culture medium. The fact that the 0.5 and 0.2% UC-781-containing replens gels cleared HIV-1-infected cell cultures from the virus under the experimental conditions where α-APA, UC-38 and ZDV failed to do so, could also be seen as the consequence of the virucidal properties of UC-781 reported by Parniak and collaborators [15,16]. Under our experimental conditions, UC-781, α-APA, UC-38 and ZDV concentrations reached lower levels in the HIV-1-infected

cell cultures than their EC_{50} values for HIV-1 at day 10 of subcultivation. Remarkably, UC-781, but not α-APA, UC-38 or ZDV, protected CEM cell cultures for 10 subcultivations against the cytopathic effect of HIV-1, which is again in agreement with the virucidal properties of UC-781.

Since not much more than 1 ml gel per intravaginal administration is recommended in humans, 50 mg UC-781 present in 1 ml of a 5% UC-781-containing gel can readily be exposed to the vaginal/cervical environment (this drug concentration is at least seven orders of magnitude higher than the EC_{50} of UC-781 in cell culture).

Because we were unable to detect any drug in plasma following intravaginal application of UC-781 in rabbits, one may presume that virus drug resistance would not readily develop. In addition, our *in vitro* studies with UC-781-containing replens gel demonstrated that the drug did not induce the selection of NNRTI-specific resistant virus strains at a concentration that was only 2.5-fold below the drug concentration that cleared the virus from the HIV-1-infected cell cultures. The virus that emerged in the presence of 0.05% UC-781-containing replens gel at the start of the experiment remained highly sensitive to UC-781 and did not contain any of the known NNRTI-specific mutations in its RT gene. These findings are important in view of the fear that application of an NNRTI as a vaginal virucide may select for NNRTI-characteristic resistance in the drug-treated individuals. In fact, since neither our HPLC analyses nor bioassays revealed significant UC-781 levels in plasma from rabbits that were treated intravaginally with 5% UC-781-containing replens gel argues against potential selection of UC-781-resistant virus strains in plasma.

Moreover, it should be kept in mind that if NNRTI-resistance should show up in UC-781 gel-treated patients, such mutant virus strains retain, as a rule, marked sensitivity to nucleoside RT inhibitors such as ZDV and lamivudine, as well as protease inhibitors. Since triple-drug combination therapy with ZDV, lamivudine and a protease inhibitor has recently proven to be highly effective in suppressing HIV replication over an extended time period, NNRTI-resistant virus strains, if emerging, would still be fully susceptible to these triple-drug combination therapies. In addition, it has been previously shown that UC-781, in cell culture, selects for drug-resistant virus strains that invariably show low-level resistance to UC-781, and thus retain sufficient susceptibility to UC-781 and several other NNRTI [22].

In conclusion, our physicochemical examinations, preliminary toxicity studies and antiviral findings with UC-781-containing replens gel formulations have revealed that UC-781 has highly desirable properties as a virucidal agent, thus warranting the further exploration of UC-781 as a vaginal virucide in the clinical setting.

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