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A dual chamber model of female cervical mucosa for the study of HIV transmission and for the evaluation of candidate HIV microbicides

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Abstract

A dual chamber system was established to model heterosexual HIV transmission. Cell-associated, but not cell-free HIV, added to a confluent layer of cervical epithelial cells in the apical chamber, reproducibly infected monocyte-derived dendritic cells (MO-DC) and $CD4^+$ T cells in the basal compartment. Only minimal epithelial transmigration of HIV-infected mononuclear cells (HIV-PBMCs) was observed. Most evidence points to transepithelial migration of virus, released from HIV-PBMCs after their activation by epithelial cells.

We used this model for evaluation of the therapeutic index of various potentially preventive antiviral compounds, including non-nucleoside reverse transcriptase inhibitors (NNRTIs, including UC781 and various diaryltriazines and diarylpyrimidines), poly-anionic entry inhibitors (including PRO2000, cellulose sulphate, dextrane sulphate 5000 and polystyrene sulphonate) and the fusion inhibitor T-20. The epithelium was pre-treated with compound and incubated with HIV-PBMCs for 24 h. Afterwards the apical chamber was removed and MO-DC/$CD4^+$ T cell co-cultures were further cultured without compound. NNRTIs, including a TMC120 gel, blocked infection of the sub-epithelial targets at sub-micromolar concentrations. Polyanionic entry inhibitors (up to 100 μg/ml) and T-20 (up to 449 μg/ml) failed to inhibit transmission. Moreover, whereas the NNRTIs used interfered with epithelial integrity with cervical epithelium only at very high concentrations, the evaluated entry inhibitors showed toxicity at concentrations that did not prevent infection.

Keywords: Microbicides; Sexual HIV transmission; NNRTI; Entry inhibitors; Dendritic cells; In vitro model

1. Introduction

The HIV/AIDS pandemic continues its spread, with over 40 million people already infected and over 10,000 new infections per day according to UNAIDS estimates (UNAIDS Global Fact Sheet, 2005). More than 90% of new infections are acquired through sexual HIV transmission. Since prophylactic anti-HIV vaccination will not be available in the near future, there is an urgent need for effective HIV prevention strategies. Especially in developing countries, women and girls are more vulnerable to HIV infection for several reasons: (1) inadequate knowledge about HIV transmission and AIDS, (2) inherent higher biological susceptibility to transmission, (3) insufficient access to HIV prevention services, (4) inability to negotiate condom-use and safe sex and (5) lack of female-controlled HIV prevention methods (Blocker and Cohen, 2000; Elias and Coggins, 1996; Mayer and Anderson, 1995; Padian et al., 1997).

For all these reasons, the development of potent and safe intravaginal/intrarectal topical formulations of anti-HIV agents, referred to as HIV microbicides, which are able to block sexual HIV transmission has become a major priority in HIV research. In order to evaluate candidate microbicides in a pre-clinical stage, several in vitro and ex vivo models are currently being explored, including cell suspensions, tissue explant systems and

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a SCID mouse model (Collins et al., 2000; Di Fabio et al., 2003; Greenhead et al., 2000; Palacio et al., 1994; Shattock and Moore, 2003; Veazey et al., 2003).

In vivo, the lower female genital tract is comprised of different anatomical regions, including the vaginal mucosa, the ectocervix and the endocervix (Pudney et al., 2005). While the stratified squamous epithelium of the vagina and ectocervix constitutes a barrier to infection of subepithelial target cells, the single columnar epithelium of the endocervix is inherently more vulnerable (Greenhead et al., 2000). Especially, the squamocolumnar junction or cervical transformation zone, where the monolayered epithelium of the endocervix abruptly changes into the multilayered epithelium of the ectocervix, is believed to be most susceptible to HIV infection as it is also the main target of infection with the human papilloma virus and the major inductive and effector site for cell-mediated immunity in the lower female tract (Pudney et al., 2005). Moreover, this transformation zone harbours the highest concentration of lymphocytes and antigen-presenting cells of the genital tract, the latter including $CD1a^+$ dendritic cells (Pudney et al., 2005).

Combined evidence from in vitro and in vivo studies suggests that dendritic cells, either intra-epithelial Langerhans cells or sub-epithelial (interstitial) dendritic cells are very early targets for sexual HIV transmission (Hu et al., 2000; Kawamura et al., 2000; Sugaya et al., 2004). Interstitial dendritic cells and $CD4^+$ T lymphocytes are major targets for HIV infection, after the virus has passed the epithelium (Miller et al., 1992; Spira et al., 1996). Moreover, dendritic cells (DCs) have a key role in the induction of immune-responses (Banchereau and Steinman, 1998; Servet et al., 2002). Clearly, candidate microbicides should be able to block infection in DC/$CD4^+$ T cell co-cultures, but they should neither interfere with the integrity of the epithelial layer, nor with physiological DC-T cell interactions, responsible for induction of anti-HIV immune responses.

Several compounds, acting at different stages of the retroviral cycle, are being considered for development as microbicides. These include acid-buffering agents (e.g. Buffergel), polyanionic entry inhibitors (e.g. PRO2000, cellulose sulphate, etc.), surfactants (e.g. C32G) and reverse transcriptase inhibitors (e.g. PMPA, UC-781, TMC120) (D'Cruz and Uckun, 2004; McCormack et al., 2001; Turpin, 2002).

We developed a dual chamber in vitro model in which the apical chamber contains a confluent layer of ME-180 cervical epithelial cells and the basal chamber consists of co-cultures of monocyte-derived dendritic cells (MO-DC) and $CD4^+$ T cells. As such, we closely mimic the site where sexual HIV transmission is most likely to occur. The dual chamber in vitro model was used for the evaluation of a representative series of HIV entry inhibitors and non-nucleoside reverse transcriptase inhibitors (NNRTI), some of which are currently being evaluated in clinical trials. The evaluated NNRTIs included a series of highly potent compounds, belonging to the family of diaryltriazine (DATA) and diarylpyrimidine (DAPY) analogues (Andries et al., 2004; Janssen et al., 2005; Ludovici et al., 2001a,b). Additionally, a gel formulation with the DAPY NNRTI TMC120 (R147681) was evaluated.

2. Materials and methods

2.1. Epithelial and mononuclear cells

The human cervical epithelial cell line ME-180 was obtained from the American Type Culture Collection (ATCC-LGC Promochem, Teddington, UK) and cultured in RPMI-1640 culture medium (Bio-Whittaker, Verviers, Belgium), supplemented with 10% bovine fetal calf serum (FCS) (Biochrom, Berlin, Germany), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Roche Diagnostics, Mannheim, Germany), further referred to as complete medium.

Monocytes and lymphocytes were separated from donor buffy-coat peripheral blood mononuclear cells (PBMCs) by counter-flow elutriation. Monocyte-derived dendritic cells (MO-DC) were differentiated from the monocyte-containing fractions and $CD4^+$ T cells were purified from the lymphocyte fractions, according to previously described methods (Van Herrewege et al., 2002).

2.2. Evaluation of epithelial confluence by confocal microscopy and flow cytometry

The polycarbonate membrane of the apical chamber of a dual chamber Transwell system (pore size 3 μm and diameter 6.5 mm) (Corning Costar Corp., MA) was coated with 200 ng/ml of laminin (Sigma–Aldrich, St. Louis, Missouri) and air-dried. Five hundred thousand ME-180 cells in 100 μl were seeded in the apical chamber, whereas the basal chamber contained 900 μl of complete medium. The ME-180 cells were cultured in this system either for 1 day or for 3 days (to constitute, respectively, a non-confluent and a confluent layer). To evaluate epithelial confluence and the preservation of the tight intercellular contacts between the epithelial cells (further referred to as 'epithelial integrity'), the apical chamber was removed and the cells were fixed using 4% formaldehyde (VWR, Heverlee, Belgium)/250 mM Hepes (Cambrex, MD) for 10 min. Cells were rinsed twice in phosphate buffer saline (PBS, 20 mM, pH 7.4) and quenched in 50 mM NH_4Cl (UCB, Brussels, Belgium) in PBS for 10 min. Cells were subsequently rinsed twice in PBS and incubated with Alexa Fluor® 594 phalloidin (Molecular Probes, Leiden, The Netherlands), a red fluorescent dye for labelling F-actin, for 30 min at 37 °C (absorption and fluorescence emission maxima 590, respectively, 617 nm). The probe was diluted 1/5, in Tris–HCl (Dako, Glostrup, Denmark). As nuclear counterstain, DAPI (Molecular Probes, Leiden, The Netherlands) was used at a concentration of 1/5000 (absorption and fluorescence emission maxima 358, respectively, 461 nm). After 2 min, the membranes with the cells were washed, removed from the apical chamber and mounted on microscope slides with Vectashield (Vector laboratories, Orton Southgate, Peterborough, UK). Samples were analysed using a Bio-Rad Radiance 2100 Blue Diode CLSM system with a 60× plan apo oil immersion objective. Emission filters used were HQ442/45 for DAPI and E570LP for Alexa 594. Images were taken with Bio-Rad Lasersharp 2000 software (Bio-Rad, Hampstead, Hertfordshire, UK) using a sequential scanning method and processed with ImageJ (Ras-

band, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2006.).

In addition, a flow cytometric evaluation was done. In this experiment, 100 μl of a 1/100 dilution of yellow-green fluorescent sulphate microspheres (fluospheres, diameter 0.02 μm, excitation/emission at, respectively, 505 and 515 nm) (Molecular Probes, Leiden, The Netherlands) was added to the apical chamber. Cells (with fluospheres) were incubated for 2 h (37 °C, 5% CO_2) in the apical chamber. Afterwards, 20 μl of medium was harvested from the apical chamber and diluted 1/10 with complete medium. Also, 200 μl of culture medium was harvested from the basal chamber. All samples were analysed by FACScan (BD Immunocytometry Systems, San Jose, CA).

2.3. Preparation of cell-free and cell-associated HIV

We used the non-syncytium inducing, CCR-5 using (NSI/R5) HIV-1 strain Ba-L. Activated donor PBMCs were used to prepare a stock of cell-free virus and to determine its infectious titer. PBMCs were activated with PHA (0.5 μg/ml; Innogenetics, Ghent, Belgium) and IL-2 (5 ng/ml; Immunosource, Belgium) in complete medium for 3 days. Cells were seeded in 96-well plates (7.5×10^4 cells/well) and infected with a serial one-third dilution of viral stock in six-fold. Infectious titers were determined after 7 days of culture and expressed as CCID50 (50% cell culture infectious dose), according to the method of Reed and Muench (1938) as modified by Peden and Martin (1995).

The Ba-L strain was also used to prepare stocks of cell-associated virus. To this end, freshly isolated, non-stimulated PBMCs were infected with HIV-1 Ba-L at a multiplicity of infection (MOI) of 10^{-2}, calculated based on the $CCID_{50}$, as described in the previous paragraph. After co-incubation of HIV and resting PBMCs during 24 h at 37 °C, remaining free virus was washed and the cell-associated virus or "HIV-PBMCs" was aliquotted and stored in liquid nitrogen until use.

2.4. Passage of cell-free versus cell-associated HIV Ba-L in the dual chamber model

One hundred thousand HIV-PBMCs or cell-free HIV Ba-L (at 10^{-2} or 10^{-3} MOI) were added in different laminin-coated (200 ng/ml) apical chambers of a dual chamber system, which contained confluent or non-confluent layers of ME-180 epithelial cells. Subsequently, the apical chamber was inserted in a 24-well cup (basal chamber) with co-cultures of 2×10^5 MO-DC and 1×10^6 $CD4^+$ T cells. After 24 h, the apical chamber was removed from the dual chamber system and the cells in the basal chamber were further cultured for 14 days in complete medium without added cytokines. Twice weekly, 0.5 ml of the culture medium was harvested for analysis in HIV p24 Ag ELISA and replaced by 0.5 ml of complete medium. At day 14, supernatants of primary cultures were harvested, while cells were used for secondary cultures in order to monitor viral rescue. Secondary cultures were set-up by adding 0.5×10^6 uninfected, PHA/IL-2-stimulated blasts to the primary cultures. Supernatants of these secondary cultures were harvested 1 week later. The system is schematically represented in Fig. 1.

2.5. HIV p24 antigen ELISA

HIV p24 antigen was detected using a modified in-house monoclonal p24 (HuMab-HIVp24) (Biomaric NV, Ghent, Belgium) enzyme-linked immunosorbant assay (ELISA) (Beirnaert et al., 1998). The 100% inhibitory concentration of compound was defined as the concentration of compound that inhibited viral transmission and replication below the detection limit of the ELISA (20 pg HIV p24 Ag/ml), as measured in the primary culture supernatants and confirmed by the secondary cultures supernatants. The upper detection limit of the ELISA is 25,000 pg/ml. In previous experiments, we showed that the ELISA result of the secondary cultures was fully predictive of the presence or absence of proviral DNA (Van Herrewege et al., 2002, 2004a).

2.6. Evaluation of transmigration of fluorescent beads or HIV-PBMCs by confocal microscopy

A confluent layer of ME-180 cells, present in the laminin-coated (200 ng/ml) apical chamber of the dual chamber system, was incubated with 100 μl of a 1/100 dilution of yellow-green fluorescent sulphate microspheres with a size of 100 nm (Molecular Probes, Leiden, The Netherlands) or with 100 μl of HIV-PBMCs, which were previously labelled with 5 μl of the green fluorescent cell tracer DiO during 5 min (Vybrant™ DiO cell labelling solution; Molecular Probes, Leiden, The Netherlands) (absorption and fluorescence emission maxima 484, respectively, 501 nm). The basal chamber contained MO-DC/$CD4^+$ T cell co-cultures. After 24 h, the apical chambers were removed and the cells were prepared for confocal microscopy analysis, using the same labelling protocol as for the evaluation of the epithelial integrity.

2.7. Cytospin analysis

After a similar experiment as described in the previous paragraph, the content of the basal chamber, containing MO-DC/$CD4^+$ T cell co-cultures and possibly fluorescent beads or DiO-labelled HIV-PBMCs (if they had migrated through the epithelial layer of the apical chamber), was harvested and centrifuged (5 s, 1000 rpm) using a Shandon Cytospin Centrifuge. Resulting cytospins were collected on Superfrost PLUS microscopic slides (Menzel Glaser, Braunschweig, Germany) and subsequently labelled with Alexa Fluor® 594 and DAPI, using the same protocol as for the preparation of the samples for confocal microscopy.

2.8. Evaluation of transmigration of HIV-PBMCs by FACS

To investigate the potential transmigration of cell-associated virus through an epithelial layer, we used 1×10^6 HIV-PBMCs/ml, which were incubated for 5 min with 2.5 μl of the red fluorescent cell tracer DiI (Vybrant™ DiI cell-labelling solution; Molecular Probes, Leiden, The Netherlands) (absorption and fluorescence emission maxima 549, respectively, 565 nm). DiI-labelled HIV-PBMCs (DiI-HIV-PBMCs) were washed two times and a 100 μl suspension of 1×10^6 cells/ml was added to the ME-180 epithelial layer in laminin-coated

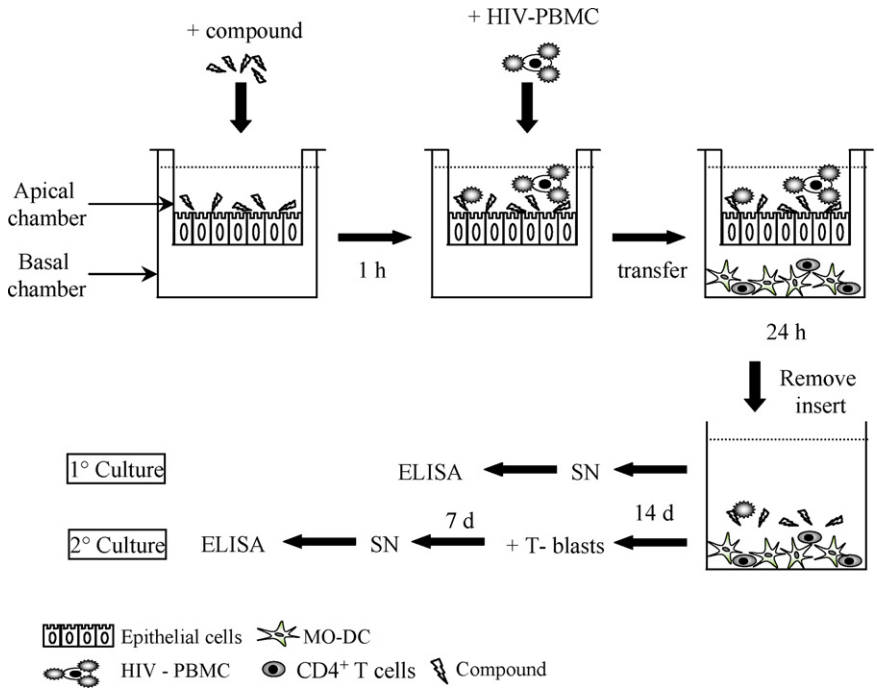

Fig. 1. In vitro dual chamber model of sexual HIV transmission for the evaluation of candidate microbicides. An apical chamber, containing a confluent (or non-confluent) layer of ME-180 cervical cells, is inserted into a 24-well cup (basal chamber). In drug-inhibition experiments, the ME-180 cells are pretreated for 1 h with compound. Next, cell-associated HIV-1 Ba-L (HIV - PBMC) is added to the apical chamber. This insert is immediately transferred into a new cup, containing co-cultures of monocyte-derived dendritic cells (MO-DC) and autologous $CD4^+$ T cells. After 24 h, the apical chamber is removed and the MO-DC/$CD4^+$ T cell co-cultures are further cultured for 14 days, without compound added (primary culture). After primary culture, supernatants (SN) are harvested, followed by addition of PHA/IL-2 stimulated blasts (T-blasts) for a 7-day secondary culture. Supernatants of primary and secondary culture are analysed by the HIV p24 antigen ELISA.

(200 ng/ml) apical chambers. The basal chamber contained co-cultures of MO-DC/$CD4^+$ T cells. After 24 h, supernatants of apical and basal chambers were harvested separately and centrifuged (8 s, 500 × *g*). Cell pellets were resuspended in 50 μl of 1 × PBS and incubated for 10 s with 5 μl of fluorescein-isothiocynate (FITC)-labelled anti-human CD3 monoclonal antibody (Becton Dickinson, Erembodegem, Belgium). Afterwards, cells were washed, fixed with 1% paraformaldehyde and analysed on a FACScan (BD Immunocytometry Systems, San Jose, CA).

2.9. Test compounds

Several entry-inhibitors were evaluated: PRO2000, kindly provided by Interneuron Pharmaceuticals Inc. (Lexington, MA); cellulose sulphate (CS) and polystyrene sulphonate (PSS), kindly provided by the TOPCAD program (Rush University, Chicago, IL) and high-molecular-weight dextran sulphate (DS-5000), kindly provided by Prof. J. Balzarini (Rega Institute for Medical Research, Leuven, Belgium).

A series of NNRTIs consisting of diaryltriazine (DATA) and diarylpyrimidine (DAPY) compounds were designed at Janssen Pharmaceutica NV (Spring House, NJ; Beerse and Vosselaar, Belgium) and were shown to be very active against HIV-1 group M, including common NNRTI-resistant mutants, at Tibotec (Mechelen, Belgium) (data not shown). We evaluated the DATA compound R153430 and the DAPY compounds R147681 (TMC120, dapivirine), R165335 (TMC125, etravirine), R278474 (TMC278, rilpivirine), R152929 and R151694, of which the structures were previously published (Van Herrewege et al., 2004b). The NNRTI UC-781 was kindly provided by Crompton Corp. (Uniroyal Chemical) (Middleburg, CT).

2.10. Formulation of placebo and TMC120 gels

Gels made of hydroxyethylcellulose (HEC) (Dow Chemical, MI), hydroxypropylcellulose (HPC) (Hercules Ltd., Lancashire, UK) and xanthan gum (CP Kelco, Beringen, Belgium) were prepared by dispersing the correct amount of polymer in a mixture of glycerol (10 g) (Febelco, Mechelen, Belgium), lactic acid (100 mg) (Febelco, Mechelen, Belgium) and 60 g of demineralised water. Gels made of hydroxypropyl methylcellulose (HPMC) (Dow Chemical, MI) were prepared by dispersing the correct amount of polymer in a mixture of glycerol (15 g) (Febelco, Mechelen, Belgium), lactic acid (100 mg) (Febelco, Mechelen, Belgium) and 60 g of demineralised water. Gels made of carbopol 934 (BF Goodrich, OH, USA) were prepared by dispersing the correct amount of polymer in a mixture of propylene glycol (20 g) (Febelco, Mechelen, Belgium), NaEDTA (100 mg) (Febelco, Mechelen, Belgium), lactic acid (100 mg) (Febelco, Mechelen, Belgium) and 50 g of demineralised water. For all gels, NaOH (1 M) (Febelco, Mechelen, Belgium) was added dropwise while stirring until pH 4.5. Finally, water was added until 100 g. The gels were sterilized by autoclaving.

A 0.25% HEC gel was used to prepare the formulations containing TMC120 at concentrations ranging from 0 to 10,000 nM.

2.11. Dual chamber infection inhibition experiments

Fifty microliter of a 10-fold dilution range of either colchicine (Sigma–Aldrich, St. Louis, MO), compound or TMC120 gel was added to a confluent ME-180 layer present in the laminin-coated (200 ng/ml) apical chamber of the dual chamber system. After 1 h, also 1×10^5 Ba-L infected, non-stimulated PBMCs were added to the apical chamber. Subsequently, the apical chamber was transferred to a cup of a 24-well plate (basal chamber) containing MO-DC/CD4$^+$ T cell co-cultures (ratio 1/5). Twenty-four hours later, the apical chamber was removed from the dual chamber system and the cells from the basal chamber were cultured for 2 weeks, with half of the culture medium (without colchicine, compound or TMC120 gel) being refreshed twice weekly (primary culture). At day 14, supernatants of primary cultures were harvested, while cells were used for secondary cultures in order to monitor viral rescue, as described in Section 2.4.

2.12. Evaluation of diffusion of TMC120 from the apical to the basal chamber

To evaluate the diffusion of compound from the apical to the basal chamber, a dilution series of the DAPY NNRTI compound R147681 (TMC120, dapivirine) was added to a confluent or non-confluent ME-180 epithelial layer in the laminin-coated (200 ng/ml) apical chamber. The basal chamber contained 1 ml of complete medium. After 24 h, supernatants of the apical and basal chamber were harvested, stored at −20 °C and analysed by HPLC in order to determine the residual concentration of TMC120. A dilution series of compound was directly analysed in HPLC and included as internal controls.

2.13. Evaluation of compound toxicity towards the epithelial layer

A dilution range of compound or TMC120 gel was added to a 24-well cup with a confluent layer of ME-180 cells. Cultures were examined microscopically 24 h later. Subsequently, culture plates were centrifuged and cell pellets were incubated with trypsin-EDTA (Bio-Whittaker, Verviers, Belgium). Detached cells were harvested and used for analysis in flow cytometry. To this end, 0.1×10^6 cells in 50 μl of complete medium were incubated 15 min at 4 °C with 20 μl of the nucleic acid dye 7-AAD (Via-Probe, Becton Dickinson, Erembodegem, Belgium), used for the exclusion of non-viable cells in flow cytometric assays. Cells were washed, fixed with 1% paraformaldehyde and analysed on a FACScan (BD Immunocytometry Systems, San Jose, CA).

3. Results

3.1. The in vitro dual chamber system as a model for sexual HIV transmission

The general set-up of the dual chamber model of sexual HIV transmission and for the evaluation of candidate microbicide compounds is shown in Fig. 1. Confocal microscopy analysis of the ME-180 layer showed that these cells predominantly grow as a tight confluent monolayer after 3 days (Fig. 2), while sometimes also the tendency to form a multilayered epithelium was found (Fig. 3). The confluence of the epithelial cell layer was also examined by fluospheres of 20 nm, added to the apical chamber. Flow cytometric analysis of the samples from the basal chamber after 2 h showed that fluospheres passed a non-confluent layer (grown for 1 day only), but not a confluent layer (data not shown).

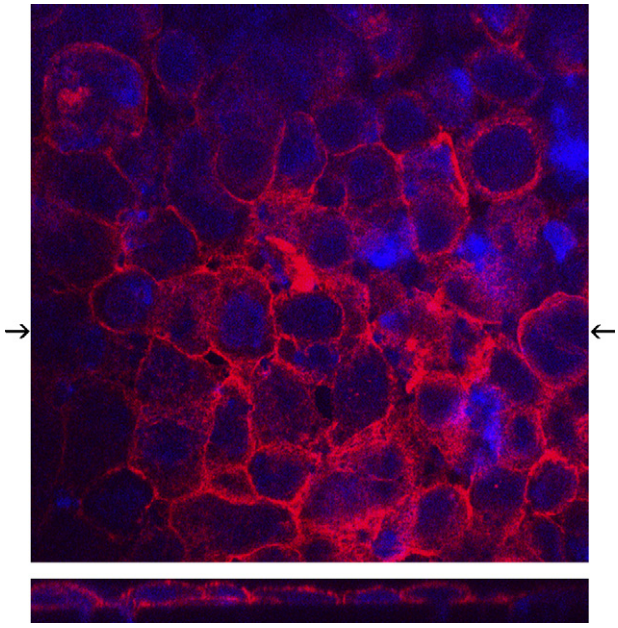

Fig. 2. Microscopic evaluation of the confluence of the epithelial layer. ME-180 epithelial cells were cultured for 3 days, labelled with the red F-actin marker Alexa Fluor 594 and the blue nuclear marker DAPI and subsequently analysed by confocal microscopy. A top view of the epithelial layer is shown, together with a transverse section of which the position is indicated on the top view by the arrows. Box size = 119.7 μm.

In this model, we first compared cell-associated and cell-free HIV-1 Ba-L, added to the apical chamber, for their respective efficiency to infect the MO-DC/CD4$^+$ T cell co-cultures in the basal chamber over a confluent ME-180 layer. Addition of 10^5 non-stimulated, infected PBMCs (HIV-PBMCs) resulted in a reproductive infection of MO-DC/CD4$^+$ T cell co-cultures in the basal compartment. In contrast, no infection was measurable if cell-free HIV-1 Ba-L was directly applied to the epithelial layer at a multiplicity of infection (MOI) of 10^{-3}. With a higher concentration (10^{-2} MOI) of cell-free virus, infection was observed in two out of four experiments (data not shown). Titration of both the HIV-PBMCs and cell-free virus in PHA-activated PBMCs, showed that the infectious titer of 10^5 HIV-PBMCs corresponded to 40 CCID50 doses, whereas 10^{-2} MOI cell-free virus corresponded to 1000 CCID50 doses (data not shown). Therefore, since infection with cell-associated virus was more efficient, we decided to concentrate on experiments with HIV-PBMCs.

3.2. HIV-infected PBMCs are in close contact with epithelial cells and can migrate to a limited extent over a confluent epithelial layer

Fluorescently labelled HIV-PBMCs were added to a confluent layer of ME-180 in the apical chamber and studied by confocal microscopy after 24 h. At multiple sites, very close contact between the PBMCs and ME180 was observed, but there was no image suggesting actual passage of PBMCs through the epithelial layer (Fig. 3). In addition, cytospins of the cells in the basal chamber were prepared. Only the MO-DC and T lymphocytes were detected but not so far the labelled HIV-PBMCs (data not shown).

As a more quantitative approach, also flow cytometric analysis was used. In these experiments, DiI labelled HIV-PBMCs were added to either a non-confluent or a confluent ME180 layer in the apical chamber. After 24 h, cells from both apical and basal chambers were additionally labelled with anti-CD3. The results indicated that passage of DiI labelled HIV-PBMCs through a confluent epithelial layer was very limited (<0.5%, corresponding to <500 HIV-PBMCs) (Fig. 4). Moreover, when HIV-PBMCs were directly added to the MO-DC/CD4$^+$ T cell co-cultures in the basal chamber, at least 2500 HIV-PBMCs were required for a consistent infection of the MO-DC/CD4$^+$ T cell co-cultures (data not shown).

In view of these results, it seems unlikely that passage of HIV-infected PBMCs over a confluent epithelial layer is exclusively responsible for the reproducible HIV infection of the MO-DC/CD4$^+$ T cell co-cultures in the basal chamber.

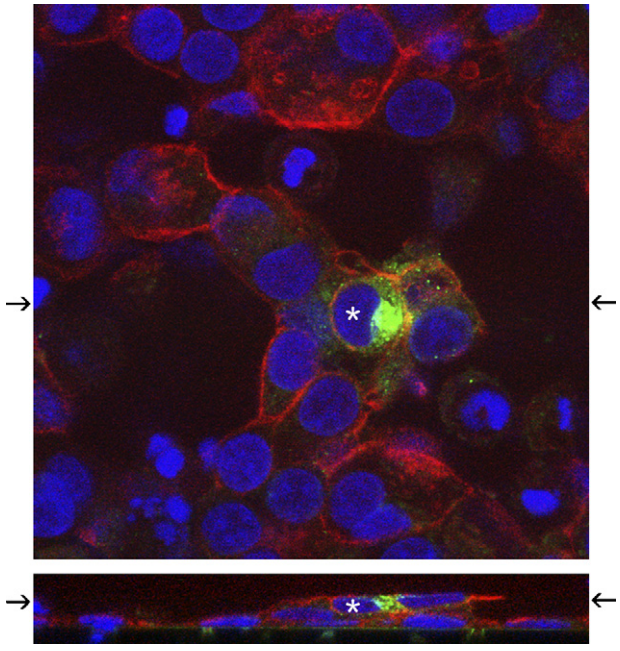

Fig. 3. HIV-PBMC are in close contact with ME-180 epithelial cells. HIV-PBMC, labelled with the green cell tracer DiO, were added to a confluent layer of ME-180 epithelial cells, present in the apical chamber of the dual chamber model. After 24 h, all cells were labelled with the red F-actin marker Alexa Fluor 594 and the blue nuclear marker DAPI and subsequently analysed by confocal microscopy. A top view of the epithelial layer is shown, together with a transverse section of which the position is indicated on the top view by the arrows. The presence of HIV-PBMC, in close contact with the epithelial layer, is indicated by an asterisk. Box size = 100 μm.

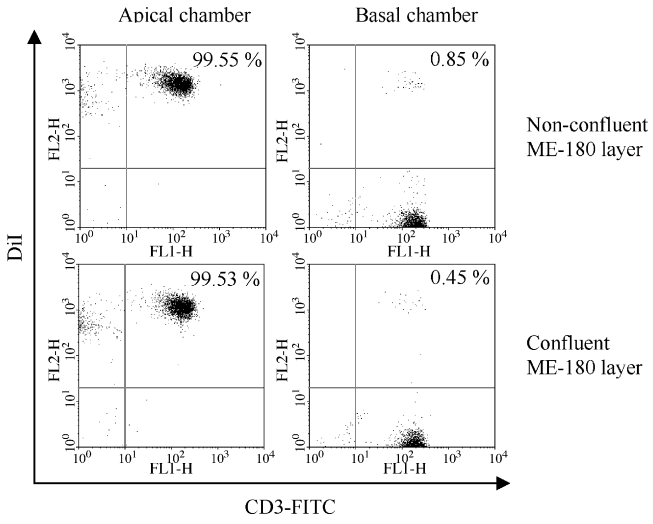

Fig. 4. Passage of DiI labelled, HIV-infected PBMC over the cervical layer. One hundred thousand DiI labelled, HIV-infected PBMC were added to the apical chamber, containing a non-confluent (upper panels) or a confluent layer (lower panels) of ME-180 epithelial cells. The basal chamber contained MO-DC/CD4$^+$ T cell co-cultures. After 24 h, the content of apical and basal chamber was harvested, cells were incubated with CD3-FITC antibodies and analysed by flow cytometry (left panels: apical and right panels: basal chamber). CD3$^+$DiI$^-$ cells in the basal chamber derive from the MO-DC/CD4$^+$ T cell co-cultures, whereas CD3$^+$DiI$^+$ cells derive from the labelled PBMC added to the apical chamber. The percentage of DiI$^+$ cells is indicated in each graph.

3.3. Colchicine blocks HIV transmission over the epithelial layer

Next, we investigated if viral transmission over the epithelial layer and subsequent infection of the MO-DC/CD4$^+$ T cell co-cultures could be blocked by the presence of the microtubules depolymerising compound colchicine in the apical chamber. As is evident from Fig. 5, colchine inhibited infection of the target cells in the lower chamber in a dose dependent manner. In separate experiments (not shown), we demonstrated that up to 50 μM of colchicine was not toxic to the epithelial cells. This result is similar to previous findings which suggested that virus, originating from HIV-infected PBMCs, was predominantly transmitted over an intact epithelial layer through an intracellular mechanism involving microtubule-mediated transcytosis (Meng et al., 2002; Bomsel, 1997), whereby it was shown that colchicine had no effect on virus production by the infected cells (Pearce-Pratt et al., 1994).

In parallel, we also investigated if fluorescently labelled beads with a size of 100 nm, as a model of cell-free virus, could pass the confluent epithelial layer of the dual chamber system in a time period of 24 h and in this way reach the MO-DC/CD4$^+$ T cell co-cultures present in the basal chamber. (Clearly, these fluorescent beads are similar to those used for confirming confluence, see Section 2.2, but in those experiments much smaller beads were used and their passage was already evaluated after 2 h). In the present experiment, confocal microscopy analysis of the

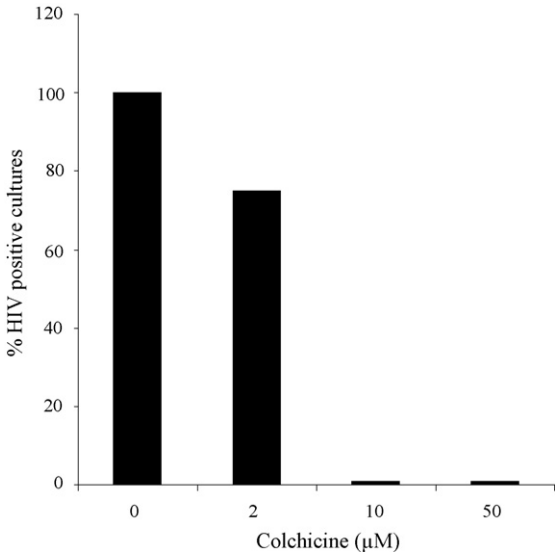

Fig. 5. Colchicine blocks viral transmission in the dual chamber model. A serial dilution of colchicine was added to the apical chamber of a dual chamber system, prior to infection with HIV-PBMC. Supernatants of the MO-DC/CD4$^+$ T cell co-cultures in the basal chamber were analysed in HIV p24 antigen ELISA after secondary culture. The total percentage of HIV-positive secondary cultures in two independent experiments (with each condition tested in duplicate) is shown.

apical chamber after 24 h revealed the presence of beads inside the epithelial cells (Fig. 6A). Additionally, cytospin analysis of the cells from the basal chamber clearly indicated the intracytoplasmatic accumulation of beads in the MO-DC, which made contact with surrounding CD4$^+$ T cells (Fig. 6B).

3.4. Antiretroviral compounds block productive infection of sub-epithelial target cells

Several classes of compounds were added to the apical chamber and evaluated for their capacity to block reproductive infection of the sub-epithelial MO-DC/CD4$^+$ T cell co-cultures. A first group of compounds consisted of non-nucleoside reverse transcriptase inhibitors (NNRTIs) and included the reference thiocarboxanilide derivative UC-781 and a series of diaryltriazine (DATA) and diarylpyrimidine (DAPY) compounds. The DATA compound R153430 and the DAPY compounds R147681 (TMC120), R165335 (TMC125), R152929, R278474 (TMC278) and R151694 have already proved to be highly efficient inhibitors of HIV replication in vitro (Van Herrewege et al., 2004b). A second group of compounds consisted of the polyanionic entry inhibitors PRO2000, cellulose sulphate (CS), high-molecular-weight dextran sulphate (DS-5000), polystyrene sulphonate (PSS) and the fusion inhibitor T-20.

All NNRTIs efficiently blocked HIV infection of the sub-epithelial target cells. The most potent compound, R278474 (TMC278), completely blocked HIV infection after addition of 50 μl of a 100 nanomolar (nM) compound solution to the apical chamber. All other NNRTIs blocked HIV at a 10-fold higher concentration of 1000 nM (Fig. 7). Based on the percentage of HIV positive cultures after addition of 100 nM compound, R278474 (TMC278) was clearly most potent, followed

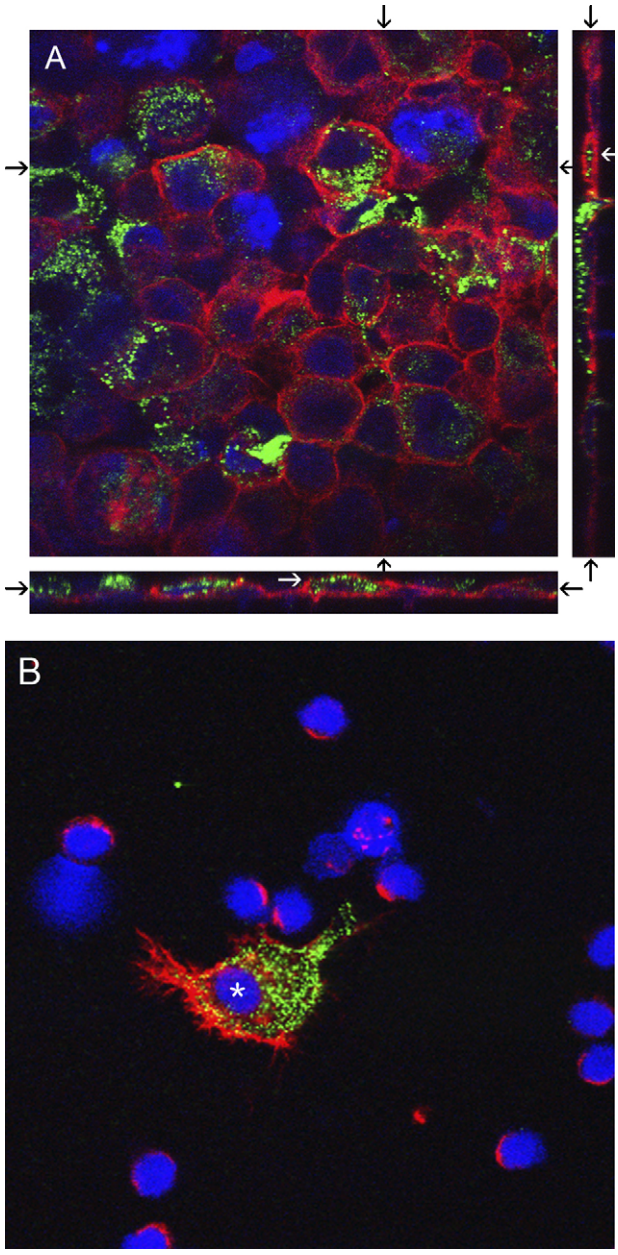

Fig. 6. Transmigration of fluorescent beads over the ME-180 epithelial layer. Green fluorescent beads were added to a confluent layer of ME-180 epithelial cells, present in the apical chamber. The basal chamber contained MO-DC/CD4$^+$ T cell co-cultures. After 24 h, the cells from the apical chamber were labelled with the red F-actin marker Alexa Fluor 594 and the blue nuclear marker DAPI and subsequently analysed by confocal microscopy. (A) A top view of the epithelial layer is shown, together with a horizontal and vertical transverse section of which the position is indicated on the top view by the black arrows. The presence of the beads, inside the epithelial cells, is indicated by a white arrow. Box size = 119.7 μm. (B) MO-DC/CD4$^+$ T cell co-cultures, present in the basal chamber, were harvested after 24 h of incubation with the apical chamber. Cytospins were made and cells were labelled with the red F-actin marker Alexa Fluor 594 and the blue nuclear marker DAPI before analysis by confocal microscopy. Beads are accumulated by MO-DC (indicated by the asterisk), which are surrounded by CD4$^+$ T lymphocytes. Box size = 77 μm.

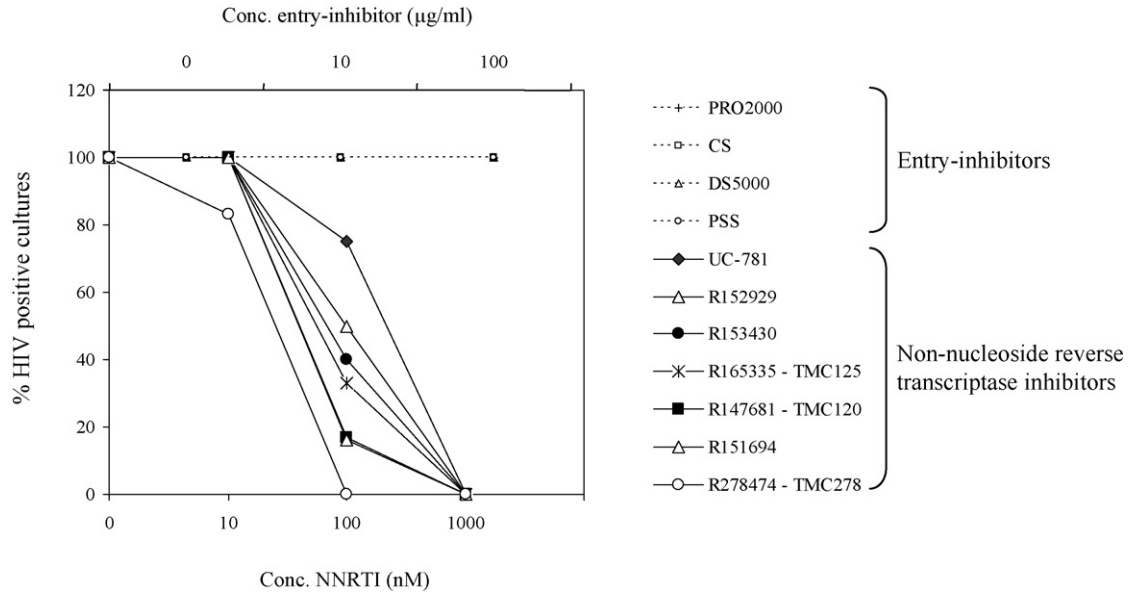

Fig. 7. HIV blocking capacity of entry inhibitors and NNRTIs in a dual chamber model. A serial dilution of compound was added to the apical chamber of a dual chamber system prior to infection with HIV-PBMC. Supernatants of the basal chamber were analysed in HIV p24 antigen ELISA after secondary culture. The total percentage of HIV positive secondary cultures in at least two independent experiments is shown for each concentration of compound. For the NNRTIs, the number of HIV-infected cultures at a concentration of 100 nM was: UC-781 (6/8), R152929 (3/6), R153430 (2/5), R165335-TMC125 (2/6), R147681-TMC120 (3/18), R151694 (1/6) and R278474-TMC278 (0/6). For the entry inhibitors, the number of HIV infected cultures at the highest concentration (100 μg/ml) was: PRO2000 (9/9), CS (4/4), DS5000 (4/4) and PSS (2/2).

by R147681 (TMC120) and R151694 which both blocked 84% of the HIV infections, whereas UC-781 was the weakest, blocking only 25% of the cultures (Fig. 7). Statistical analysis using a two-sided Fisher's Exact Test indicated that the antiviral potency of R278474 (TMC278) and R147681 (TMC120) was significantly higher compared to UC-781, with a *p*-value of 0.008 and 0.010, respectively. No significant differences were found between the different DATA and DAPY compounds (*p*-value: 0.315).

Surprisingly, none of the evaluated polyanionic entry-inhibitors were able to block viral transmission and replication, at concentrations up to 100 μg/ml (Fig. 7). Similarly, no blocking activity was found, up to a concentration of 100 000 nM of the fusion inhibitor T-20 (three independent experiments, data not shown).

3.5. TMC120-gel is effective as an HIV microbicide

To mimic the in vivo situation, we also used gel formulations in our model. First, several placebo gels of different viscosity levels were evaluated for their capacity to block transepithelial viral migration. A 0.25% hydroxyethyl cellulose (HEC) gel, a 0.5% hydroxypropyl cellulose (HPC) gel, a 2.5% hydroxypropylmethyl cellulose (HPMC) gel and a 0.5% Xanthan gum gel allowed productive HIV-l infection of the MO-DC/CD4$^+$ T cell co-cultures. In contrast, a 0.5% HEC gel and a 1% HPC gel blocked viral transmission in one out of two cultures, whereas a 0.25% and a 0.5% Carbopol gel as well as a 1% Xanthan gum gel blocked viral transmission in both duplicates (data not shown).

We therefore decided to use a 0.25% HEC gel for the formulation of different concentrations of the NNRTI TMC120. In these experiments 100 nM of TMC120, either in solution or in a gel, completely blocked transmission whereas 10 nM TMC120 blocked transmission in <40% of the cultures (Fig. 8).

3.6. The NNRTI TMC120 (R147681) diffuses through a ME-180 epithelial layer

When added to a non-confluent epithelial layer, all concentrations of TMC120 completely equilibrated between the apical and basal chamber after a 24 h incubation period. In contrast, if a confluent epithelial layer was used, compound concentration in the basal chamber was approximately 60% of that in the apical chamber, indicating that a confluent epithelial layer delays, but does not block, passage of the highly lipophilic TMC120 (Table 1).

3.7. Polyanionic entry inhibitors interfere with epithelial integrity at non-cytotoxic concentrations

ViaProbe labelling of ME-180 cervical cells, treated for 24 h with the DAPY NNRTI TMC120, showed cellular toxicity, occurring at concentrations of 10,000 nM or higher, being at least 100 times higher than the concentration that completely blocked infection. This finding was confirmed by microscopical evaluation of the TMC120-treated epithelium, of which a representative example is given (Fig. 9).

ME-180 cervical cells treated with a placebo HEC gel showed a minor increase in the percentage of cell death compared to untreated cells. HEC gel formulations containing a dilution series of TMC120 showed no higher toxicity compared to the

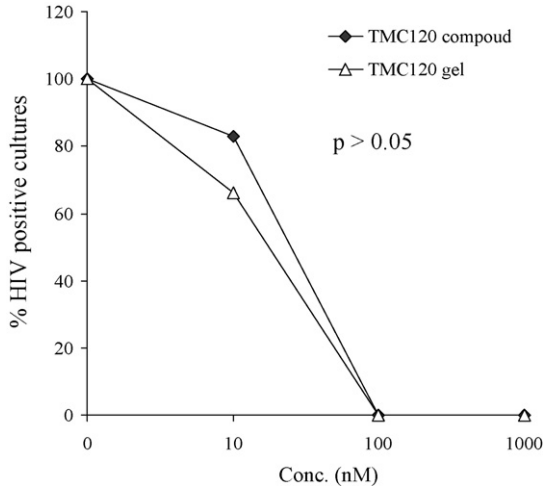

Fig. 8. HIV-blocking capacity of a gel formulation of TMC120 (R147681). Either a 10-fold serial dilution of TMC120 or HEC gel formulations containing different concentrations of TMC120 were added to the apical chamber of the dual chamber system prior to infection with HIV-PBMC. In the case when pure TMC120 compound was used, concentration '0' represents medium without compound, in the case when TMC120 gel was used, concentration '0' represents 0.25% HEC placebo gel. Supernatants of the basal chamber were analysed in HIV p24 antigen ELISA after secondary culture. The total percentage of HIV positive secondary cultures in three independent experiments (with each condition tested in duplicate in each experiment) is shown for each concentration of compound. The number of HIV-infected cultures at a compound concentration of 10 nM was: TMC120 compound (five out of six), TMC120 gel (four out of six). Statistical analysis was done using a two-sided Fisher's Exact Test, of which the *p*-value is indicated.

Table 1
Diffusion of TMC120 from the apical to the basal chamber

Amount added (ng)[a]	Chamber	Concentration detected (ng/ml)[b]
Non-confluent layer		
24.7	Apical	13.0–13.2
/	Basal	11.9–13.0
8.2	Apical	4.0–5.1
/	Basal	3.9–4.6
2.7	Apical	1.3–1.5
/	Basal	1.1–1.4
0	Apical	0–0
/	Basal	0–0
Confluent layer		
24.7	Apical	17.3–17.8
/	Basal	9.0–9.5
8.2	Apical	4.5–4.9
/	Basal	2.7–2.9
2.7	Apical	1.4–1.8
/	Basal	1.0–1.0
0	Apical	0–0
/	Basal	0–0

[a] A dilution range of TMC120 was added to the apical chamber, containing a confluent or non-confluent layer of ME-180 cervical cells. Twenty-four hours later, supernatants of apical and basal chamber were harvested and analysed in HPLC for the presence of compound.

[b] The concentration detected in the apical and basal chamber is shown (duplicate measurements).

Table 2
Compound toxicity towards ME-180 cells

Compound	Concentration	% Cell death
TMC120	1000 nM	0
	10,000	15
	100,000	57
	Placebo	6
	0.5 nM	6
TMC120	5	6
HEC gel	50	4
	500	6
	5000	12
UC-781	2000 nM	0
	20,000	4
	200,000	6
PRO2000	2.5 μg/ml	0
	25	0
	250	0
	2500	8
PSS	2.5 μg/ml	0
	25	9
	250	10
	2500	25
DS5000	2 μg/ml	0
	20	0
	200	0
	2000	0

A dilution range of either compound or TMC120 formulated in a HEC gel was added to an ME-180 cervical cell layer. Twenty-four hours later, cells were harvested, labelled with 7-AAD (ViaProbe) and analysed in FACS for the percentage of cell death compared to untreated control cells. Values are given as the geometric mean of at least two experiments.

placebo HEC gel (Table 2). The NNRTI UC-781 showed no toxicity at the evaluated concentrations.

Evaluating polyanionic inhibitors with ViaProbe revealed very limited direct cytotoxicity: only PSS at the highest concentration used (2.5 mg/ml) induced some cell death (Table 2). However, microscopic examination clearly indicated that polyanionic entry inhibitors interfered with epithelial layer integrity at non-cytotoxic concentrations. Epithelial layers showed decreased intercellular contacts after a 24 h treatment with 250 μg/ml of PRO2000 and even completely detached from the culture plate after treatment with higher concentrations (Fig. 9).

4. Discussion

The development of a safe and effective HIV microbicide is a major aim in current anti-AIDS research. Since in vitro evaluation of new compounds is necessary before formulations can be used in vivo, we propose a dual chamber system of ME-180 cervical epithelial cells, present in the apical chamber, and MO-DC/CD4$^+$ T co-cultures, present in the basal chamber, as a relevant model that closely mimics the cervix, a susceptible site for heterosexual transmission (Clemetson et al., 1993).

ME-180 cells originate from a cervical epidermoid carcinoma and are frequently used as a model of cervical epithelial cells (Dezzutti et al., 2001; El Messaoudi et al., 1999; Pearce-Pratt

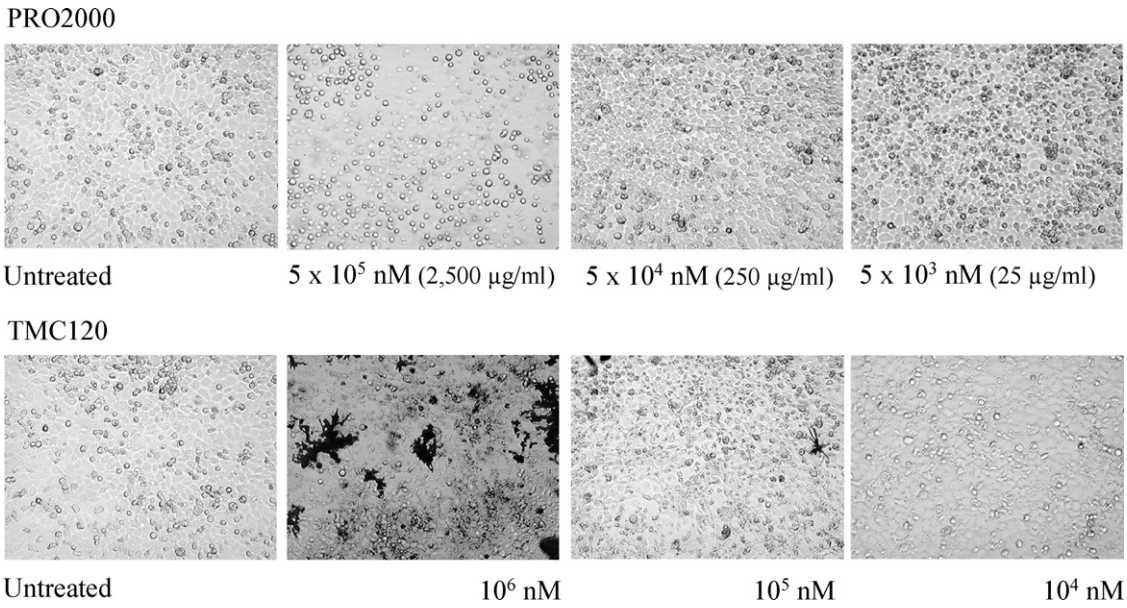

Fig. 9. Effect of the NNRTI TMC120 and the polyanionic entry inhibitor PRO2000 towards ME-180 epithelial cells. ME-180 cells were incubated with a dilution range of compound. Twenty-four hours later, epithelial integrity of the cells was examined microscopically.

et al., 1994; Tan et al., 1993). Our microscopic results show that these cells predominantly grow as a tight monolayer, while sometimes also the tendency to form a dual layer can be detected. Moreover, earlier studies reported the presence of numerous desmosomes and epidermal filaments, which indicates that these cells display the typical ultrastructural characteristics of the cervical epithelial cells from which they were derived (Tan et al., 1993).

Semen and cervicovaginal secretions from HIV-infected subjects contain cell-free virions as well as HIV-infected lymphocytes (Hart et al., 1999; Pilcher et al., 2004; Quayle et al., 1997; Vernazza et al., 1997). It remains unclear whether cell-free virus, HIV-infected cells or both are important for sexual transmission (Chan, 2005). In our dual chamber model, adding HIV-infected resting PBMCs (HIV-PBMCs), but not cell-free virus, to the apical part of the epithelial layer resulted in reproducible infection of the sub-epithelial MO-DC/CD4$^+$ T cell co-cultures.

Confocal microscopy showed that HIV-PBMCs make close contact with the confluent epithelial layer, which is similar to the interactions of uninfected cells with an epithelial layer (Pearce-Pratt and Phillips, 1993). Despite the adherence of HIV-PBMCs to the ME-180 epithelial layer, no actual migration of HIV-PBMCs over the epithelial layer could be observed and no infected cells were found in cytospin preparations from the basal chamber. Since we used in these experiments the non-syncytium-inducing HIV-1 strain Ba-L, fusion of the HIV-PBMCs with the epithelial cells is unlike but cannot be completely ruled out. More sensitive and quantitative flow cytometric studies with fluorescently labelled HIV-PBMCs, on the other hand, indicated that passage of these cells over the epithelial layer did occur, but it was probably too limited to explain the consistent infection of MO-DC/CD4$^+$ T cell co-cultures, as at least a five times higher amount of HIV-PBMCs was needed to directly infect the MO-DC/CD4$^+$ T cell co-cultures in titration experiments. Therefore, we hypothesize that T cells, containing HIV, are activated by the close contact with the allogenic cervical layer. As a result, new virions bud from the infected cell, are taken up by the epithelial cells and subsequently migrate from the apical to the basal pole of the cervical epithelium, followed by infection of the underlying target mononuclear cells. This hypothesis is supported by two additional experiments. Twenty-four hours after addition of fluorescent microbeads with the size of a virus, to the ME-180, confocal microscopy revealed the presence of microbead clusters inside both the ME-180 epithelial cells, present in the apical chamber, and the MO-DC, present in the basal chamber, suggesting an endocytic uptake and transcytosis of HIV over the epithelial layer. In infection experiments with cell-associated virus, we showed that 10 μM of colchicine blocked viral transmission to the target cells in the basal chamber (without an antiretroviral compound being added to the basal chamber), a feature also reported by others and consistent with the characteristics of microtubule-dependent transcytosis (Bomsel et al., 1989; Bomsel and Mostov, 1991; Bomsel, 1997). Moreover, it was shown before that colchicine had no effect on HIV production by the infected cells (Pearce-Pratt et al., 1994). Additionally, incubation of the ME-180 cells with the fusion-inhibitor T-20 could not prevent transfer of the virus over the epithelial cells to the MO-DC/CD4$^+$ T cell co-cultures present in the basal chamber, indicating that the uptake and transfer of virus did not involve classical gp41-mediated fusion between the virus and the epithelial cell. This result is consistent with earlier studies, in which transfer of HIV over intestinal epithelial cells to CD4- and coreceptor expressing Hela cells was not inhibited by incubation of the epithelial cells with T-20 (Meng et al., 2002).

Previous work also showed that the contact between chronically infected cells and intestinal, endometrial or cervical

derived epithelial cell lines induced massive budding of HIV virions at the contact site, followed by internalisation of the virions into epithelial endosome-like structures (Bomsel, 1997; Hocini and Bomsel, 1999; Phillips, 1994; Tan et al., 1993). Of note, it has also been demonstrated that transcytosis of HIV over an epithelial layer is not restricted to cell lines chronically infected by laboratory isolates but can also occur with PBMCs from HIV-1 infected patients (Bomsel, 1997). Similar to our present results, also in these studies, infection and subsequent transcytosis of cell-free HIV was not achieved (Bomsel, 1997; Phillips, 1994) or required inocula 100–1000-fold higher in HIV-1 p24 units compared to the inocula required for infection using cell-associated HIV-1, placed in direct contact with the epithelial cells (Hocini et al., 2001).

The membrane composition of infected cells can be modulated by contact of the infected cell and the uninfected target cell, which may explain the different efficiency of endo-trancytosis of cell-free versus cell-associated HIV (Fais et al., 1995). Moreover, Alfsen et al. (2005) reported that transcytosis of infectious virus across epithelial cells relies upon contact between HIV-infected PBMCs and the mucosal pole of endometrial and intestinal epithelial cells. These results suggest that the contact between HIV-1 infected PBMCs and uninfected epithelial cells leads to the formation of a mucosal epithelial synapse, which induces modifications in the cell membranes of both cell types permitting signal transduction from the infected cell to the epithelial cell and resulting in efficient HIV-1 endocytosis and transcytosis.

The antiretroviral potency of several compounds of different drug classes was evaluated in our dual chamber model. Non-nucleoside reverse transcriptase inhibitors, including UC-781 and several diaryltriazine (DATA) and diarylpyrimidine (DAPY) compounds, were shown to be highly active: complete blocking of HIV transmission and infection of the MO-DC/CD4$^+$ T co-cultures was possible if 100–1000 nM of compound was added to the apical chamber. In contrast, the polyanionic entry inhibitors PRO2000, cellulose sulphate (CS), dextran sulphate (DS-5000) and polystyrene sulphonate (PSS) could not efficiently block viral transmission at the concentrations evaluated (up to 100 μg/ml). One clear-cut difference between polyanionic entry inhibitors and NNRTIs is the hydrophilic nature of the former and the lipophilic nature of the latter which allows the NNRTIs to more easily penetrate through the cell membranes of either HIV-infected PBMCs, epithelial cells and/or MO-DC/CD4$^+$ T cell clusters. Moreover, it is known that polyanionic entry inhibitors are predominantly active against cell-free virus and particularly against CXCR4 co-receptor using HIV strains. They are less active against CCR5 co-receptor using HIV strains, of which the V3 loop of the envelope protein gp120 is in general less positively charged (Moulard et al., 2000). As a result, polyanionic entry inhibitors become only potent inhibitors of CCR5 co-receptor-using HIV strains after interaction of the viral gp120 with the cellular CD4 molecule. This interaction induces a conformational change of the gp120 molecule with consequently increased exposure of more polarized surfaces of gp120, which then become available for binding with the poly-anion.

From this viewpoint, the lack of potent activity of polyanionic entry inhibitors in our in vitro model, using cell-associated HIV-1 Ba-L, is less surprising: interaction of gp120 with CD4 molecules can only occur at the level of the basal chamber, containing the MO-DC/CD4$^+$ T cell co-cultures. Most probably polyanionic entry inhibitors are unable to penetrate the epithelial layer because of their size and hydrophilic nature, in contrast to small, low molecular weight lipophilic NNRTIs, which were shown to effectively cross the intact epithelial layer. This is in line with a previously reported phase I study with the polyanionic compound PRO2000, for which no evidence of systemic absorption was found after application of a PRO2000 gel formulation (Van Damme et al., 2000).

Recently, a PRO2000 gel showed antiviral activity in cervicovaginal lavage (CVL) samples (Keller et al., 2006). However, these findings were based on the antiviral activity of the compound against infection of Hela cells and human macrophages with cell-free, replication-defective HIV-1. This set-up is clearly different from our method which is based on the infection of physiologically relevant MO-DC/CD4$^+$ T cell co-cultures by replication-competent HIV-1 Ba-L, released by HIV-infected PBMCs. Additionally, the concentration of PRO2000 in the CVL samples varied from 115 to 342 μg/ml, while in our study we could only evaluate the antiviral activity of the compound up to a concentration of 100 μg/ml. Moreover, despite the higher concentration of PRO2000 and the use of single-cycle HIV-1, also in the former study infection was not consistently blocked with all CVL samples. While this is similar to the results we obtained, it also points out the importance of defining blocking concentrations of candidate microbicides rather than EC_{50} values.

The promising results we obtained with the TMC120 gel are similar to earlier observations made in a human SCID mice model, where intravaginal infection with cell-associated HIV-1 was prevented by a gel containing 2.25 μM of TMC120 (Di Fabio et al., 2003). A similar set-up was used to evaluate the potency of a 0.5% and 4% PRO2000 gel as a microbicide against rectal HIV transmission. Although the latter animal model is clearly different from the intravaginal model and from our in vitro system, it showed that even at concentrations of PRO2000 which are 50–400 times higher compared to the concentrations we use, complete prevention of cell-free or cell-associated HIV infection was not possible (S. Di Fabio, personal communication).

The NNRTI UC-781 and the NNRTI DAPY compound TMC120 showed favourable therapeutic indices, with some evidence of toxicity only occurring at concentrations well above their active concentration. Also HEC gel formulations containing TMC120 were found to be non-toxic at active concentrations of TMC120. This result confirms earlier studies in human SCID-mice, rabbits and slugs, where no toxicity or mucosal irritation by HEC was found (Dhondt et al., 2004; Di Fabio et al., 2003; Tien et al., 2005). Additionally, the HEC placebo gel formulation has recently been reported as an isotonic, non-ionic, non-virucidal and non-inflammatory formulation with minimal cytocidal activity (Moench et al., 2004; Tien et al., 2005). This reflects the “generally recognized as safe” (GRAS) status of

HEC and confirms its usefulness as a gel formulation for microbicide safety and efficacy trials in humans.

Polyanionic entry inhibitors predominantly interfered with epithelial integrity instead of influencing cellular viability. Disrupting the epithelial barrier might result in increased viral access and subsequent infection of submucosal target cells, as was found earlier with nonoxynol-9 (Phillips et al., 2000; Stafford et al., 1998; Van Damme et al., 2002). Therefore, since the polyanionic compounds showed no potent inhibitory activity in our in vitro dual chamber model and since the tested concentrations of compound in this model are close to their epithelium-disrupting concentrations, in vivo safety might be a problem for the polyanionic substances.

In conclusion, we developed an in vitro dual chamber model using representative target cells of sexual HIV transmission. Whereas in vivo, also intra-epithelial Langerhans cells are believed to play an important role in sexual HIV transmission, we focussed in our in vitro model on the infection of sub-epithelial DC and T cells by cell-associated HIV. The implementation of intra-epithelial Langerhans cells into the epithelial layer present in the apical chamber is currently being evaluated and will be addressed in ongoing studies. In the present paper, we provide evidence that the dominant mechanism of transmission in this model is the release of HIV from the infected PBMCs, transcytosis through the epithelial layer and subsequent infection to subepithelial MO-DC/CD4$^+$ T cell co-cultures. Several compounds were evaluated for use as microbicides. NNRTIs completely blocked HIV infection of the sub-epithelial MO-DC/CD4$^+$ T cell co-cultures at non-toxic concentration. Additionally, a TMC120 gel completely prevented viral transmission in this model at submicromolar concentrations. In contrast, polyanionic entry inhibitors failed to show potent antiviral activity and interfered with epithelial cell integrity. Therefore, it would be worthwhile to further investigate especially the use of NNRTIs for development as potent microbicides while the development of polyanionic entry inhibitors should be pursued with great prudence and vigilance.

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