Inhibition of vaginal transmission of HIV-1 in hu-SCID mice by the non-nucleoside reverse transcriptase inhibitor TMC120 in a gel formulation

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Objective: The development of drugs that can be used as topical microbicides is currently recognized as a priority area of research.

Design: A preclinical evaluation of the potential effectiveness of TMC120, a non-nucleoside reverse transcriptase inhibitor (NNRTI), as a topical microbicide to prevent vaginal HIV-1 transmission in a humanized severe combined immunodeficient (hu-SCID) mouse model.

Methods: Reconstituted mice received an intravaginal application of a TMC120-containing gel 20 min prior to a non-invasive vaginal challenge with cell-associated HIV. The possible cytotoxic effect of TMC120-containing-gel on lymphocytes was assessed and their *in vivo* migration was followed using fluorescently labelled human lymphocytes. Systemic infection was monitored by p24 antigen detection in culture supernatant from cocultured intraperitoneal cells using antigen capture enzyme-linked immunosorbent assay test and by the presence of integrated proviral HIV-1 DNA in DNA extracted from spleen cells. *In vivo* migration of labelled lymphocytes was examined by analysis of cells isolated from regional lymph nodes.

Results: In this model, systemic infection was successfully inhibited by the presence of TMC120-containing gel at vaginal level. The *in vivo* migration of human lymphocytes from the vagina to regional lymph nodes, following the deposition of TMC120-containing gel, excluded the possibility that inhibition of systemic infection was a result of NNRTI toxicity.

Conclusions: Vaginal transmission of HIV was successfully prevented by the application of a gel formulation containing TMC120. This is the first evidence of the *in vivo* effectiveness of a microbicide preparation containing an NNRTI against cell-associated HIV.
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Introduction

The identification of an anti-HIV compound to prevent virus transmission between sexual partners is a priority for controlling the spread of AIDS in both developed and developing countries. Women are at particular risk of HIV transmission since male-to-female transmission is more efficient than female to male [1,2]. Although condoms can provide good protection against a broad range of sexually transmitted diseases and HIV if used correctly and consistently [3], a number of factors limit their practical usefulness and efficacy.

One approach to the development of a preventive method against HIV and other sexually transmitted diseases is based on intravaginal administration of a microbicide [4,5]. Vaginal microbicides are anti-infective agents for topical administration, under the control of woman, to protect against the transmission of HIV. Additionally, microbicides may have the potential to reduce the transmission rate of HIV and other sexually transmitted infections for both homosexual and heterosexual contacts. Modelling studies have demonstrated the strong impact that even a microbicide with moderate efficacy could have on reducing the numbers of new infections. It has been estimated that a 60% effective product, used in countries with high prevalence of HIV at 20% coverage, could prevent 2.5 million new HIV infections over 3 years, with strong impact on development and poverty [6].

A microbicide needs to be active against both free and cell-associated virus, mainly against R5 viruses [7–9]. So far, several experiments based on *in vitro* cell lines or explants have yielded a large number of candidate microbicides, most of which are active on cell-free virus [10,11]. However, no preclinical studies *in vivo* have been done to evaluate the efficacy and safety of antiretroviral agents, such as tight-binding non-nucleoside reverse transcriptase inhibitor (NNRTI), that are capable of blocking cell-associated HIV infection. This should be part of the selection process before going into clinical trials [5,12,13].

TMC120 is a potent inhibitor of HIV-1 reverse transcriptase in cell culture assays, with an EC_{50} and EC_{90} (concentrations reducing viral replication by 50% or 90%, respectively, as measured in a cell-based assay) of 0.9 and 2.6 nmol/l, respectively, for the wild-type (LAI) strain. The CC_{50} (50% inhibitory concentration in cell culture) is 2.15 μmol/l, resulting in a selectivity index (SI) of 2400. Additionally, TMC120 has a good anti-HIV activity on NNRTI-resistant recombinant clinical isolates: 80% of strains with typical mutations for NNRTI resistance have an EC_{50} below 0.1 μmol/l, with 50% of these strains showing less than 10-fold increase in EC_{50} compared with wild type [14]. Moreover, the emergence of HIV-1 resistance to TMC120 is delayed compared with that seen with first-generation NNRTI drugs and usually requires the presence of two mutations. TMC120 has been evaluated in antiretroviral-naive HIV-infected patients, a mean fall in viral load of 1.5 $\log_{10}$ copies/ml was observed upon oral administration (50 or 100 mg twice daily) of the inhibitor as monotherapy for 7 days [15].

The high affinity and tight-binding characteristics of NNRTI [16–18], together with the high potency of TMC120, stimulated the choice of this molecule for *in vivo* testing as a potential microbicide.

The present study describes the potential effectiveness of TMC120-containing-gel in the prevention of sexual transmission of cell-associated HIV-1 in an *in vivo* humanized severe combined immunodeficient (hu-SCID) mouse model [19]. This hu-SCID mouse model was chosen because its ability to reproduce HIV vaginal infection using monotropic R5 or dualtropic laboratory-derived R5X4 HIV strains, which are the phenotypes most commonly transmitted sexually [20,21].

Methods

Animals

CB.17 SCID/SCID female mice (Charles River, Milan, Italy) of reproductive age (4–5 weeks old) were used and kept under controlled pathogen-free conditions. SCID mice were housed in microisolator cages. Food, water and bedding were autoclaved prior to use. The animal studies were performed in biosafety level 3 facility.

Reconstitution of mice with human peripheral blood lymphocytes

Human peripheral blood lymphocytes (hu-PBL) were obtained from the peripheral blood of healthy donors. All donors were screened for HIV-1 and hepatitis prior to donation. The hu-PBL were obtained by Ficoll–Hypaque density gradient centrifugation (Nycomed Pharma AS, Oslo, Norway) and resuspended in RPMI 1640 medium (EuroClone, Wetherby, UK), 40×10^6 cells in 0.3 ml injected intraperitoneally.

Inoculation of mice with cell-associated HIV-1

Hu-PBL were stimulated with phytohaemagglutinin A (2 μg/ml; Sigma-Aldrich, Milan, Italy) and after 12 h were infected *in vitro* with HIV-1_{SF162} or with HIV-$1_{1/BX08}$ (0.01 multiplicity of infection). After 3 days of culture, cells were washed twice and resuspended in fresh phosphate-buffered saline (PBS) (EuroClone). The fraction of cells infected by both viruses was previously standardized in a set of experiments assessed by fluorescent activated cell sorting (FACS) analysis using anti-p24, and ranged between 15 and 30% (data

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not shown). Fifteen minutes after the treatment with gel, a sterile plastic pipette was used to deliver 25 μl of cell suspension (2×10^6 cells infected with HIV-1_{SF162} or with HIV-$1_{1/BX08}$) into the vagina of each animal.

Composition of microbicides used

Two water-soluble polymers, carbopol 940 and hydroxyethylcellulose (HEC), were used to prepare the formulations containing TMC120 at concentrations ranging from 0.0225 mmol/l to 1000 mmol/l and a placebo gel, which did not contain TMC120. Gels containing the hydrophilic polymer were prepared at rate of viscosity of 1 : 1 and 2 : 3. The concentration of the hydrophilic polymer in a 2 : 3 gel is 67% of that of a 1 : 1 gel. All ingredients have a generally recognized as safe (GRAS) status.

Pretreatment with gel containing TMC120 and vaginal infection

All the animals used in this study were treated subcutaneously with progesterone (5 mg) (Prontogest; AMSA Rome, Italy) and reconstituted intraperitoneal with hu-PBL. Four days following reconstitution, during which hu-PBL colonize the lymphoid organs [22], mice were divided into three groups according to different dosing schedules. The negative controls in each group were reconstituted not infected and the positive controls were reconstituted and infected with intraperitoneally with HIV-1; placebo gel was the same gel composition and viscosity but no TMC120.

Set 1 contained a total of 43 female mice divided into five groups (A–E) and received a single vaginal deposition of 25 μl TMC120-containing gel with carbopol at a rate of viscosity (1 : 1) or the placebo gel:

A, 14 mice, TMC120 0.225 mmol/l
B, 14 mice, TMC120 0.0225 mmol/l
C, 6 mice, placebo
D, negative control
E, positive control.

Set 2 contained a total of 34 female mice divided into seven groups (A–G) and received 25 μl TMC120-containing gel with carbopol or HEC at low viscosity (2 : 3) or the placebo gel:

A, 6 mice, TMC120 0.0225 mmol/l
B, 4 mice, placebo
C, 6 mice, TMC120 0.0225 mmol/l
D, 6 mice, TMC120 0.00225 mmol/l
E, 4 mice, placebo.
F, negative control
G, positive control.

Set 3 contained 46 female mice divided into eight groups (A–H) and received 25 μl TMC120-containing-gel with carbopol or HEC at low viscosity (2 : 3) or the placebo gel:

A, 8 mice, TMC120 0.0225 mmol/l
B, 8 mice, TMC120 0.00225 mmol/l
C, 3 mice, placebo
D, 8 mice, TMC120 0.0225 mmol/l
E, 8 mice, TMC120 0.0225 mmol/l
F, 4 mice, placebo
G, negative control
H, positive control.

Sample collection

SCID mice were sacrificed 14 days after vaginal inoculation and cells were collected from peritoneal cavity following peritoneal lavage with 3 ml PBS, and from spleen. Spleens were disrupted with the blunt end of a 5 ml syringe plunger. After lysing erythrocytes by osmotic shock, the cells were resuspended in complete RPMI 1640 medium containing 10% inactivated fetal calf serum (FCS) (EuroClone), 20 U/ml interleukin-2 (Becton Dickinson Labware, Two Oak Park Bedford, Massachusetts, USA), 250 U/l penicillin, 2 mmol/l L-glutamine and 250 μg/ml streptomycin (Sigma-Aldrich, Milan, Italy), at a final concentration of 2×10^6 cells/ml. The cell suspensions were washed extensively in RPMI 1640, analysed by FACS (data not showed) and counted at 1×10^6 (dry pellet).

Determination of p24

Peritoneal lavage was used to collect cells from the peritoneal cavity and 1×10^5 cells were cocultured with 1×10^5 huPBL at final volume of 200 μl, in complete medium (RPMI 1640) in 96-well tissue culture plates. The cocultures were incubated for 14 days at 37°C, under 5% CO_2, being fed with fresh culture medium and feeder on day 7. Measurement of HIV-1 p24 Gag protein was performed on culture supernatant using antigen capture enzyme-linked immunosorbent assay test (ELISA) (NEN[th] Life Science Products HIV-1-p24, Boston, Massachusetts, USA) with limits of detection of 12 pg/ml. Samples that showed an absorbance of > 0.200 were scored as positive.

Quantification of provirus HIV-1 DNA

A dry pellet of 1×10^6 human lymphocytes recovered from spleen were lysed with 250 μl lysing solution in the presence of proteinase K. The DNA obtained was prepared using the QIAamp DNA tissue mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations for tissue. Briefly, the DNA of lysed cells was absorbed onto a silica matrix, washed and eluted with 100 μl elution buffer by centrifugation. Polymerase chain reaction (PCR) was performed on total DNA using primers specific for the *gag* region of HIV-1 and from reference samples containing 5, 50, 500 and 5000 copies of HIV DNA

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using the Quanti-Kin detection system kit (Symbiosis, Asti, Mi, Italy) according to the manufacturer's recommendations. Briefly, 25 μl of template DNA was added to the reaction mixture; PCR was performed in a final volume of 75 μl in a Perkin Elmer 5600 thermal cycler (Crawley, UK). The amplified DNA, labelled with biotin at the 5′ end, was hybridized by adding 1 μl specific probe carrying a molecule of fluorescein. The hybrids were immobilized in 96-well microtitre plates in a final volume of 100 μl and detected with an antibody antifluorescein conjugated with horse radish peroxidase and a substrate. After 30 min, the reaction was stopped by adding 100 μl of stop solution. The cut-off value for the negative samples showed an absorbance value < 0.250. Reference controls (50, 500 and 5000 DNA copies/10^6 cells) were used to calculate the DNA copy number present in the experimental sample.

Labelling of lymphocytes

Freshly isolated hu-PBL were labeled with 5-carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probe, Eugene, Oregon, USA) as described elsewhere [19]. Fluorescence was verified by fluorescent microscopy analysis (Leitz, Diaplan) and by FACSCalibur (Becton Dickinson Immunocytometry System, San Jose, California, USA).

Inoculation of mice, cell recovery and analysis

One untreated and not reconstituted mouse was used as negative control. Three groups of two SCID female mice each, were pretreated with 25 μl of HEC-based formulations at 0.225 mmol/l, 0.0225 mmol/l and 0.00225 mmol/l TMC120, respectively. After 15 min, labeled lymphocytes suspension (2×10^6 cells/25 μl) were delivered into the vagina of each animal. One mouse received the labelled cells only and was used as positive control. After 48 h, mice were killed, and lymph nodes were extracted separately Single-cell suspensions were prepared for cell-surface staining and FACS analysis. CFSE-labelled hu-PBL, recovered from regional lymph nodes, were washed twice, counted and resuspended at 0.5×10^6 cells/100 μl in PBS containing 2% FCS. Cells were incubated for 30 min at 4°C in the presence of anti-CD3–fluoroscein isothiocyanate (FITC) monoclonal antibody (Becton Dickinson). The cells were washed twice with PBS prior to FACS analysis and were analysed for the presence of CD3 cells within the CFSE-positive population. Cells incubated with FITC–antimouse immunoglobulin and phycoerythrin–antimouse immunoglobulin were always used as controls for all analyses. Stained samples were analysed using a FACScalibur flow cytometer (Becton Dickinson) with CELLQuest software (Becton Dickinson). At least 1×10^5 events were collected for each sample.

Results

The composition of the gels used is shown in Table 1.

The effect of pretreatment with TMC120 gel with carbopol (1 : 1) on vaginal infection by cell-associated HIV-1$_{SF162}$

The most likely candidates for inhibition of cell-associated HIV, gels made from two different hydrophilic polymers (carbopol or HEC), were examined at vaginal level. Systemic infection was investigated 2 weeks after vaginal infection by collecting peritoneal and spleen cells.

HIV replication was monitored by p24 antigen detection on culture supernatant by ELISA using samples from the mice in set 1. Overall, p24 antigen was detected in culture supernatants of cell coculture assays from 2 of 14 mice in group A (0.225 mmol/l), 3 of 14 in group B (0.0225 mmol/l), 6 of 6 receiving placebo, and 5 of 5 positive controls (Table 2).

The animals showing markers of HIV infection were also positive when the presence of proviral DNA was investigated. The number of HIV proviral copies present in the experimental samples among the HIV-infected mice varied from a minimum of 5700 DNA copies/10^6 peripheral blood mononuclear cells (PBMC) to a maximum of 45 700 DNA copies/10^6 PBMC ($\pm$ 22 720) (groups A and B) and was consistently $>$ 50 000 DNA copies/10^6 PBMC in the placebo group. The number of HIV proviral copies present in the intraperitoneally infected (positive control) samples ranged between 278 000 and 928 000 DNA copies/10^6 PBMC ($\pm$ 565 500). Table 2 shows the results presented as a global analysis of treated mice instead of presenting an analysis of individual animals.

Table 1. Composition of the intravaginal gel formulations.

Components of gels	Viscosity (1 : 1)	Viscosity (2 : 3)
Carbopol based TMC120		
	Desired concentration[a]	Desired concentration[a]
Ethanol (ml)	2	2
Carbopol 940 (g)	0.25	0.17
1,2-Propanediol (g)	7.5	7.5
Na_2-EDTA (g)	0.025	0.025
Triethanolamine (g)	0.625	0.42
Water (g)	Ad 50.0	Ad 50.0
Hydroxyethylcellulose based TMC120		
	Desired concentration[a]	Desired concentration[a]
Hydroxyethylcellulose (g)	1.0	0.67
Ethanol (ml)	2	2
Glycerin (g)	5.0	5.0
Water (g)	Ad 50.0	Ad 50.0

EDTA, ethylenediaminetetraacetic acid.
[a]Placebo is no added TMC120.

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Table 2. Ability of TMC120 to reduce vaginal transmission of cell-associated R5 (SF162) HIV-1 in hu-PBL–SCID mice.

Formulation in carbopol (1 : 1) TMC120 (mmol/l)[a]	No. mice positive for HIV p24[b]/total No. mice pretreated and exposed to HIV	No. mice with detectable *gag* DNA[c]/ total No. mice pretreated and exposed to HIV
A, 0.225	2/14	2/14
B, 0.0225	3/14	3/14
C, placebo	6/6	6/6
D, Positive controls	5/5	5/5

hu-PBL, human peripheral blood lymphocytes; SCID, severe combined immunodeficient.
[a]Mice from set 1.
[b]The cocultures were monitored for the presence of HIV $p24^{gag}$ antigen in the supernatants for 14 days.
[c]Expression of HIV *gag* in cells isolated from spleen. The level of proviral DNA was analysed by semiquantitative polymerase chain reaction using specific primers amplifying the *gag* region of HIV.

These results demonstrate that, in our *in vivo* model, TMC120 was able to reduce the rate of vaginal transmission of cell-associated HIV by 70–80%. All mice became infected when treated with placebo, suggesting that the gel with carbopol per se had no anti-HIV activity.

The effect of pretreatment with TMC120 gel with carbopol or hydroxyethylcellulose (2 : 3) on vaginal infection by cell-associated HIV-1_{SF162}

It is essential that there is a uniform distribution of topical microbicides into the irregularities of the vaginal mucosa in order to form an effective barrier against infectious agents. However, in the first set of experiments, the high viscosity of the gel made it difficult to apply in the vagina of the mice. Two different polymers (carbopol and HEC) at a lower viscosity (2 : 3) were examined using mice in set 2 to see if this could achieve a uniform distribution over the entire vagina/cervix.

The carbopol-based formulation containing 0.0225 mmol/l TMC120 protected 100% of the animals from HIV infection. The same was true for the HEC-based formulation containing an even lower TMC120 concentration (0.00225 mmol/l). However, the more concentrated HEC-based formulation (0.0225 mmol/l TMC120) protected 70% of the animals from HIV infection. This may be related to a defect of retention in the vagina of the gel formulation, which can occasionally happen, rather than a lack of inhibitory properties of TMC120. The placebo gels did not confer any protection, again demonstrating that the observed positive effects are from the TMC120. The results of this study are summarized in Table 3.

The effect of pretreatment with TMC120 gel with carbopol or hydroxyethylcellulose (2 : 3) on vaginal infection by cell-associated HIV-$1_{1/BX08}$

The same experimental setting was used with set 3 mice to monitor the efficiency of TMC120-containing gel formulations in preventing vaginal infection by cell-associated HIV-$1_{1BX/08}$, a dual-tropic R5X4 strain. The results are summarized in Table 4. Vaginal transmission of HIV-1 was blocked in 100% of animals through a single treatment with all tested TMC120-containing formulations.

In vivo lymphocytes migration

To exclude the possibility that the inhibition observed with TMC120-containing-gels on the vaginal transmission of HIV could have resulted from the toxicity of the inhibitor against hu-PBL, the migration of fluorescently labelled human lymphocytes from the vagina to regional lymph nodes (external iliac) after application of TMC120- containing gels was examined *in vivo*. The concentration of TMC120 in the formulations used in these studies was approximately 0.00225 mmol/l, or exceeding the CC_{50} of the compound observed in *in vitro* cell cultures (2.15 μmol/l).

Table 3. Ability of TMC120 to reduce vaginal transmission of cell-associated R5 (SF162) HIV-1 in hu-PBL–SCID mice.

Formulation[a]	No. mice positive for HIV p24[b]/No. mice pretreated and exposed to HIV	No. mice with detectable *gag* DNA[c]/No. pretreated and exposed to HIV
In carbopol (2 : 3)		
A, TMC120 0.0225 mmol/l	0/6	0/6
B, Placebo	4/4	4/4
In hydroxyethylcellulose (2 : 3)		
C, TMC120 0.0225 mmol/l	2/6	2/6
D, TMC120 0.00225 mmol/l	0/6	0/6
E, Placebo	4/4	4/4
F, Positive controls	5/5	5/5

hu-PBL, human peripheral blood lymphocytes; SCID, severe combined immunodeficient.
[a]Mice from set 2.
[b]The cocultures were monitored for the presence of HIV $p24^{gag}$ antigen in the supernatants for 14 days.
[c]Expression of HIV *gag* in cells isolated from spleen. The level of proviral DNA was analysed by semiquantitative polymerase chain reaction using specific primers amplifying the *gag* region of HIV.

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Table 4. Ability of TMC120 to inhibit vaginal transmission of cell-associated R5X4 (1BX/08) HIV-1 in hu-PBL–SCID mice.

Formulation[a]	No. mice positive for HIV p24[b]/No. mice pretreated and exposed to HIV	No. mice with detectable *gag* DNA[c]/No. mice pretreated and exposed to HIV
In carbopol (2 : 3)		
A, TMC120 0.0225 mmol/l	0/8	0/8
B, TMC120 0.00225 mmol/l	0/8	0/8
C, Placebo	3/3	3/3
In hydroxyethylcellulose (2 : 3)		
D, TMC120 0.0225 mmol/l	0/8	0/8
E, TMC120 0.00225 mmol/l	0/8	0/8
F, Placebo	3/3	3/3

hu-PBL, human peripheral blood lymphocytes; SCID, severe combined immunodeficient.
[a]Mice from set 3.
[b]The cocultures were monitored for the presence of HIV $p24^{gag}$ antigen in the supernatants for 14 days.
[c]Expression of HIV *gag* in cells isolated from spleen. The level of proviral DNA was analysed by semiquantitative polymerase chain reaction using specific primers amplifying the *gag* region of HIV.

Figure 1 shows the results of a representative experiment with data from a negative control mouse, a mouse pretreated with the highest concentration of HEC-based formulation (0.225 mmol/l) and a positive control mouse. The results demonstrated that the labelled huPBL did migrate from the vagina to the regional lymph nodes and, therefore, were not affected by the presence of the TMC120-containing-gel, suggesting that there was no toxicity against human cells. No differences were observed between treated animals and the untreated control mouse.

Discussion

In this study an *in vivo* hu-SCID mice model of vaginal HIV-1 transmission was used to define the potential efficacy of a vaginal gel formulation containing the NNRTI TMC120 in preventing sexual transmission of cell-associated HIV-1.

Before proceeding to efficacy studies, two important parameters that could have limited the usefulness of TMC120 were excluded based on prior rabbit experiments. These were systemic absorption, which eventually could lead to the selection of resistant mutants or result in side effects and vaginal irritation, which would actually facilitate the risk of HIV infection.

No TMC120 could be detected in the plasma of rabbits, even by high pressure liquid chromatography, after a single intravaginal application (1 ml) of gel at the concentrations of TMC120 used for efficacy studies. However, very low systemic levels were observed after topical vaginal applications of very high concentrations of TMC120 in the formulation (data not shown).

The potential for vaginal irritation was also evaluated at drug concentrations of 100, 0.9, 0.45 and 0.225 mmol/l with both gel formulations. After 24 hours of exposure, rabbits were sacrificed and vaginas dissected. Neither vaginal trauma nor inflammation was observed when tissue embedded in paraffin, sectioned, and stained with haematoxylin and eosin was examined under the microscope (data not shown).

The first set of results obtained indicated that the microbicide TMC120 could represent a preventive measure to reduce the rate of sexual transmission of cell-associated HIV-1 by 70–80%. Indeed, according to the literature, a microbicide capable of preventing 70% of HIV transmission will have a significant impact on the course of the epidemic especially in developing countries [4].

Microbicides will also protect HIV-infected people against other sexually transmitted infections, and from the possibility of becoming infected with a more virulent or drug-resistant HIV strain; they could also contribute, indirectly, to a reduction in mother-to-child transmission of HIV [23,24].

When the gel formulation was given to the mice at lower viscosity (2 : 3), a maximum efficacy of 100% was obtained. This may suggest that lowering the viscosity could alter spreading of the gel over the vaginal surface, and that a more uniform distribution is an important factor in preventing cell-associated HIV-1 infection in our model.

The efficacy studies have addressed three parameters: the viscosity of the gel formulation, the concentration of the antiviral compound and the phenotype of infecting HIV. Our results demonstrated that lower viscosity gels played an important role in protection (100% in the majority of the cases), pointing to the importance of a good retention of the gel formulation

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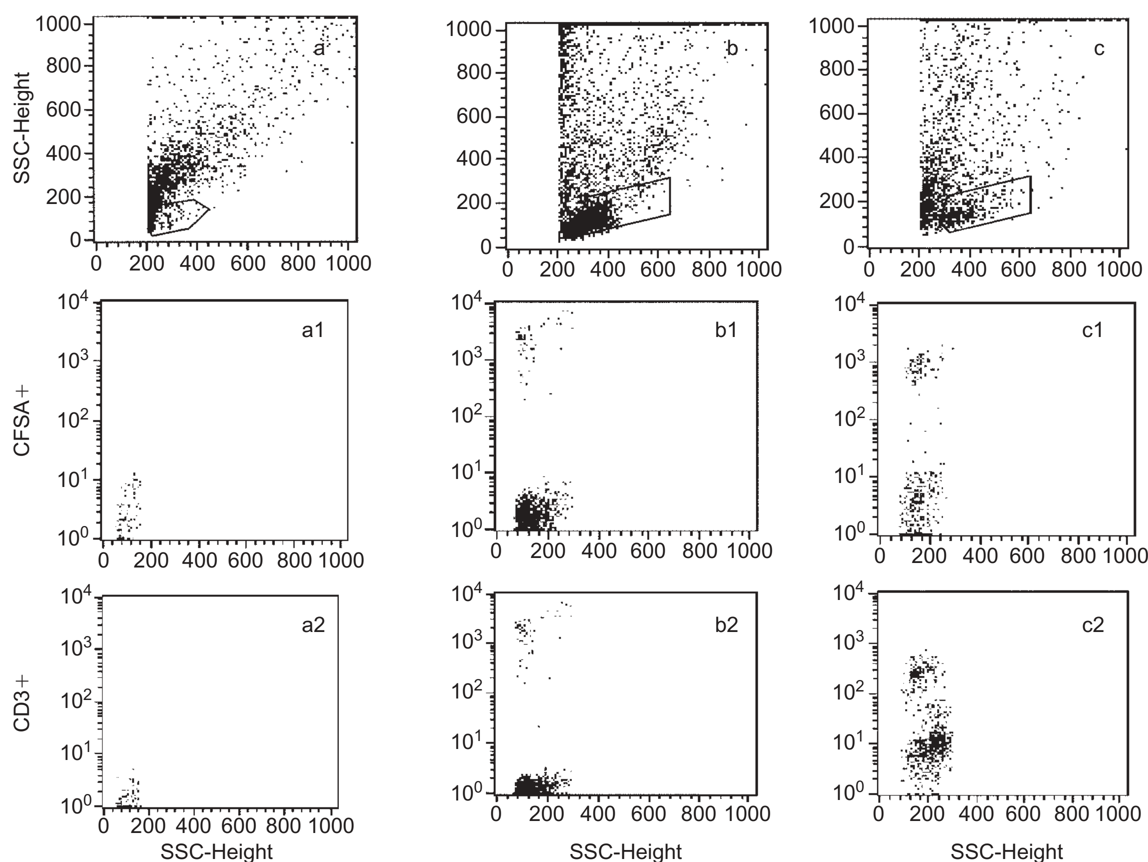

Fig. 1. Fluorescence profile measured by flow cytometry for *in vivo* migration of 5- (and 6-) carboxyfluorescein diacetate (CFSE)-stained human peripheral blood lymphocytes (hu-PBL). The hu-PBL were isolated from draining regional lymph nodes of SCID (humanized severe combined immunodeficient) mice and gated by scatter (a–c) and phenotypic (a1–c1, CFSA huPBL; a2–c2, CD3 huPBL) characteristics. Two-colour immunofluorescence was used for the identification of CFSE huPBL and CD3 huPBL. (a) Negative control: profile of cells recovered from draining lymph nodes of a not reconstituted, not treated SCID mouse. (b) Recovered cells at 48 h following vaginal application of a HEC-based formulation of TMC120 at 0.225 mmol/l and vaginal inoculation with CFSE-labelled hu-PBL. (c) Recovered cells at 48 h after vaginal inoculation with CFSE-labelled hu-PBL of untreated mice.

and uniform distribution into the irregularities of the vagina. At all TMC120 concentrations tested (0.225, 0.0225 and 0.00225 mmol/l), protection rates between 70 and 100% were observed. This protection resulted from TMC120, since placebo formulations containing no TMC120 failed to protect the animals from infection. Moreover, the effect observed results from the anti-HIV activity of TMC120, as the compound showed no toxicity for human cells in the same experimental setting using uninfected hu-PBL.

An ideal microbicide should be able to act against a variety of strains: wild type, drug resistant, laboratory derived and clinical, including (according to *in vitro* studies on the mucosal translocation of HIV-1) both R5 (CCR5-tropic) and R4 (CXCR4-tropic) viruses [25,26]. In our study, the efficacy of TMC120 was similar against both the R5 and the R5X4 laboratory-adapted strains used in our experiments.

This study provides the first *in vivo* data showing the potential of a microbicide gel incorporating an NNRTI in blocking cell-associated HIV-1 transmission at the vagina level. These results are relevant for the development of microbicides against HIV-1 since one of the key goals is to block the infection at the site of entry of the pathogen. Our strategy based on vaginal delivery of an NNRTI (TMC120) is potentially useful in humans and merits further evaluation.

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