Pre-incubation of cell-free HIV-1 group M isolates with non-nucleoside reverse transcriptase inhibitors blocks subsequent viral replication in co-cultures of dendritic cells and T cells

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This work is dedicated to the memory of Paul AJ Janssen, founder of Janssen Pharmaceutica and the Centre for Molecular Design

In order to study the inhibitory effect of various reverse transcriptase inhibitors (RTIs) on cell-free HIV, we adapted a recently described *in vitro* system, based on co-cultures of dendritic cells and resting CD4 T cells, modelling early target cells during sexual transmission. The compounds tested included the second-generation non-nucleoside RTI (NNRTI) TMC-120 (R147681, dapivirine) and TMC-125 (R165335, travertine), as well as the reference nucleoside RTI AZT (zidovudine), the nucleotide RTI PMPA (tenofovir) and the NNRTI UC-781. The virus strains included the reference strain HIV-1_{Ba-L} and six primary isolates, representative of the HIV-1 group M pandemic. They all display the non-syncytium-inducing and CCR5 receptor-using (NSI/R5) phenotype, important in transmission. Cell-free virus was immobilized on a poly-L-lysine (PLL)-treated microwell plate and incubated with compound for 1 h. Afterwards, the compound was thoroughly washed away; target cells were added and cultured for 2 weeks, followed by an extended culture with highly susceptible mitogen-activated T cells. Viral production in the cultures was measured on supernatant with HIV antigen ELISA. Negative results were confirmed by showing absence of proviral DNA in the cells.

TMC-120 and TMC-125 inhibited replication of HIV-1_{Ba-L} with average EC_{50} values of 38 nM and 117 nM, respectively, whereas the EC_{50} of UC-781 was 517 nM. Complete suppression of virus and provirus was observed at compound concentrations of 100, 300 and 1000 nM, respectively. Inhibition of all primary isolates followed the same pattern as HIV-1_{Ba-L}. In contrast, pre-treating the virus with the nucleotide RTI PMPA and AZT failed to inhibit infection even at a concentration of 100 000 nM. These data clearly suggest that NNRTIs inactivate RT enzymatic activity of different viral clades (predominant in the epidemic) and might be proposed for further testing as a sterilizing microbicide worldwide.

Introduction

Women are at a greater risk of acquiring HIV than men [1] and they account for nearly 50% of all people living with HIV worldwide, according to the 2004 UNAIDS report [2]. In some parts of Africa, the incidence of HIV is alarmingly high in younger women (15–25 years old). Although effective preventive measures (condoms) exist, they are often not a feasible option for women, due to the need for consent from male partners. In view of this situation, a new focus in prevention is on the development of microbicides that, when applied topically, should substantially reduce transmission of HIV. An ideal microbicide should be able to block infection in an early stage, at least before integration of the virus in the host cell DNA.

Nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), respectively, act as competitive and non-competitive inhibitors of reverse transcriptase (RT) [3], but the latter have an advantage in microbicide development since NNRTIs do not need cellular activation to be effective against HIV. Hence, these compounds can act directly on the RT molecules of cell-free virus and have an equal activity in proliferating or resting cells. UC-781, an NNRTI of the carboxyaniline class,

was the first NNRTI considered as a topical microbicide following the demonstration of its virucidal effects [4,5]; it is presently in a clinical trial for development into a microbicide [6].

TMC-120 and TMC-125 are potent new NNRTIs that belong to the diarylpyrimidine (DAPY) analogues. Importantly, they are active against HIV strains resistant to older NNRTIs: it was shown that 80% of the strains with typical mutations for NNRTI resistance remain highly sensitive to the DAPY compounds (EC_{50} below 0.1 μmol/l) [7]. DAPY compounds can adopt multiple stable modes of binding to wild-type and mutant HIV-1 RT [8,9]; this property could delay emergence of resistance to DAPY compounds as compared with the first generation of NNRTI compounds.

Although most authors agree that mucosal dendritic cells (DCs) have an important role in the initial stage of heterosexual transmission, there is disagreement as to the type of DC involved. Intra-epithelial Langerhans cells seem ideally positioned to capture HIV. Nevertheless, elegant animal studies point to interstitial DCs as more important targets. This might be related to the high expression of DC-SIGN, a molecule that binds gp120 and protects HIV from degradation [10–13]. For this conceptual reason, as well as for practical ones, we decided to build our *in vitro* model on monocyte-derived DCs (MO-DCs), which more closely resemble interstitial DCs, rather than Langerhans cells. In previous papers, we and others showed that incubation of MO-DCs with cell-free (or cell-associated) NSI/R5 HIV and co-culture with resting autologous CD4 T cells results in a productive infection, without the need for added cytokines. Moreover, we extensively demonstrated that various NRTIs and NNRTIs, added to the cells before, during and/or after infection, could block HIV production [14–16].

In order to evaluate if certain compounds are able to inactivate the cell-free virus before it infects the target cells, we pre-coated plates with positively charged poly-L-lysine (PLL) to enhance binding of the negatively charged virus envelope to the surface of the plastic plate. We then explored the ability of the immobile virus to still infect target cells and documented the antiviral effects of the DAPY analogues, in comparison with the reference compounds UC-781, zidovudine (AZT) and tenofovir [9-(*R*)-2-(phosphonomethoxypropyl)adenine] (PMPA).

Materials and methods

Generation of monocyte-derived cells and CD4+ T cells

Monocytes and lymphocytes were purified from buffy coats. The former were cultured with granulocyte–monocyte colony stimulating factor (GM-CSF) and interleukin (IL)-4 to induce DC differentiation. CD4 T cells were purified from the cryopreserved lymphocytes. The protocol was based on Sallusto *et al.* [17,18,20], with modifications previously described [15].

HIV strains, virus titration

A first series of experiments was carried out with the NSI/R5, monotropic strain HIV-$1_{Ba\text{-}L}$, kindly provided by the National Institute of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD, USA). Six NSI/R5 primary isolates obtained from seropositive African patients consulting at the Institute of Tropical Medicine during the period 1989–1993 were used in this study (Table 1). Subtype information was obtained by *gag*/*env* sequencing and included HIV-1 group M subtypes A, A/G, B, C and CRF02_AG, which represent the relevant circulating subtypes of the HIV pandemic. The 50% tissue culture infectious dose ($TCID_{50}$) was determined by titrating virus stock in MO-DC and CD4+ T cell co-cultures [21,22].

Treatment and replication inhibition (primary culture)

Compound stocks were prepared by dissolution in dimethyl sulphoxide (DMSO) at 10 or 100 mM. Poly-L-lysine (100 μg/ml) (Sigma-Aldrich, Bornem, Belgium) in phosphate buffered saline (PBS) (BioWhittaker, Verviers, Belgium) was coated onto 96-well flat-bottomed polystyrene plates (Microtest^{TM}96, Becton Dickinson France SA, Meylan Cedex, France) at room temperature. After 1 h, plates were washed three times with PBS. To these plates, we added 100 μl of virus at a multiplicity of infection (MOI) of 10^{-4} and 100 μl of compound. The latter was prepared in a sevenfold dilution (final concentration of 100 000 nM–0.1 nM) in complete medium, for example, RPMI 1640 (BioWhittaker) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS) (Biochrom KG, Berlin, Germany). After an incubation period of 1 h at room temperature, plates were washed thoroughly (6×) with PBS to remove excess compound and unbound virus. The plate-bound and

Table 1. Panel of six primary isolates, origin, subtype and patient information

Virus code	*gag*/*env**	Origin†
VI 820	A/A	Democratic Republic of Congo
VI 191	G/A	Belgium
CI 22	B/B	Côte d'Ivoire
VI 882	C/C	Belgium
CI 20	CRFO2	Côte d'Ivoire
CA 18	CRFO2	Cameroon

*Subtype of virus was determined by sequencing the *gag* and *env* genes.
†Patient's country of origin.

compound-pretreated virus was then seeded with 100 µl MO-DC (4×10^5 cells/ml) and 100 µl autologous resting CD4+ T cells (2×10^6 cells/ml) in complete medium. Each compound concentration was tested sixfold. Half of the culture medium (without cytokines and compound) was replaced twice every week during the primary culture phase of 14 days (Figure 1).

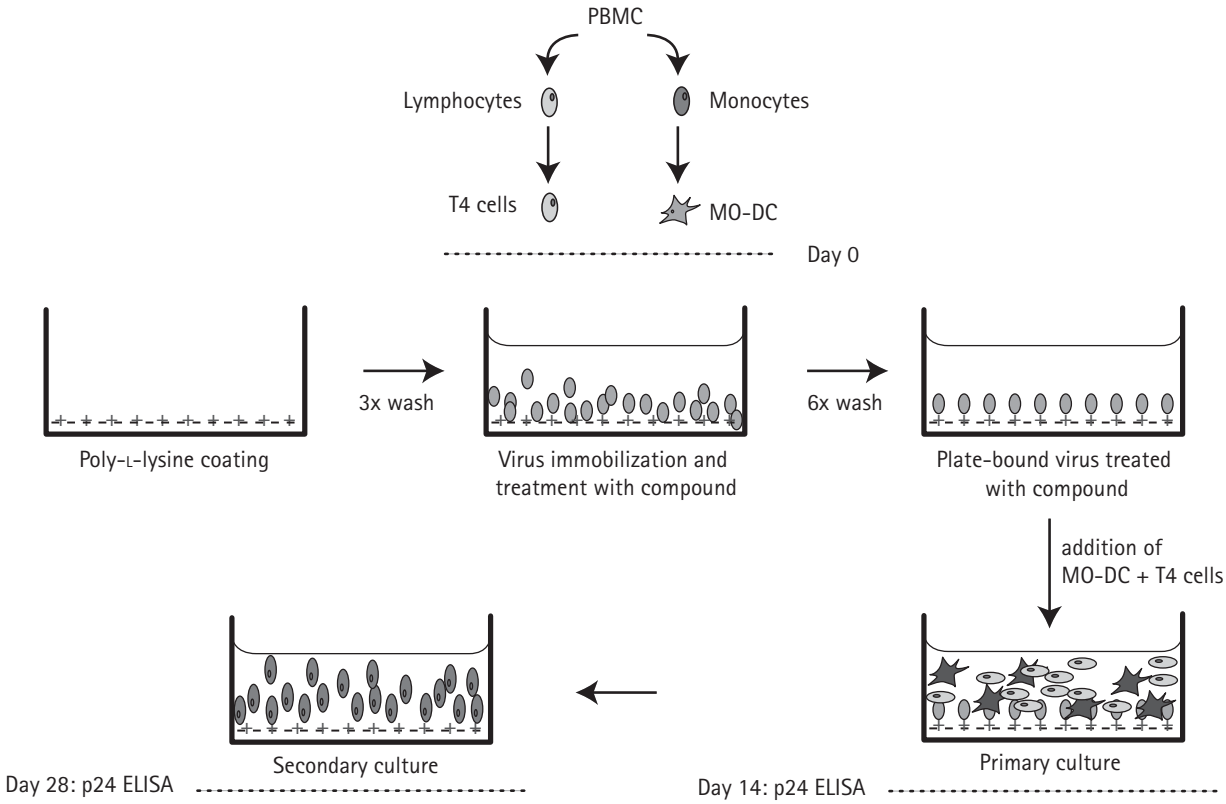
Rescue of latent virus phytohaemagglutinin/interleukin-2-stimulated PBMCs (secondary culture)

Three days before the end of the primary culture, PBMCs from a different donor were stimulated with phytohaemagglutinin (PHA) at 0.5 µg/ml and interleukin (IL)-2 at 10 U/ml. At day 14 of the primary culture, cells were washed 3× (by centrifugation of the plates at 400 ×*g* for 10 mins) to remove cell-free virus in the supernatant. The PHA/IL-2-stimulated PBMCs (hereafter referred to as PHA T BLAST) were washed and resuspended at 0.5×10^6 cells/ml in complete medium with IL-2 at 10 U/ml. 200 µl of this suspension was added to all the wells of the MO-DC/CD4+ T cells (DC/T4) cultures for a secondary culture of another 14 days. Half of the culture medium (including IL-2, but without compound) was replaced twice every week.

HIV antigen and EC_{50} of antiretroviral compounds determination

100 µl of day 7, day 14 (primary culture), day 21 and day 28 (secondary culture) supernatants were collected and inactivated with an equal volume of Nonidet P-40 (NP40) (Calbiochem, EMD Biosciences, Inc., Darmstadt, Germany). HIV antigen in the supernatants was measured using a modified in-house monoclonal p24 (HuMab-HIVp24; Biomaric NV, Ghent, Belgium), enzyme-linked immunosorbent assay (ELISA) [23]. Optical density (OD) readings at 450 nm were transformed into HIV antigen concentrations, using a standard curve of HIV-$1_{Ba\text{-}L}$ stock dilutions, the p24 content of which was previously determined with a commercial kit (Innogenetics, Ghent, Belgium). HIV antigen concentration was plotted against compound concentration (Microplate Manager PC program; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and regression analysis was performed on the linear part of

Figure 1. Poly-L-lysine based *in vitro* model to study inhibitory capacity of NNRTIs



T4, CD4+ T cells; MO-DC, monocyte-derived dendritic cells; NNRTI, non-nucleoside reverse transcriptase inhibitor.

the curve to calculate the EC_{50} value (equivalent to the compound concentration required to reduce HIV antigen production by 50%).

Quantification of provirus HIV-1 DNA

Provirus DNA was quantified with a commercial kit according to the manufacturer's instructions (Amplicor HIV-1 amplification kit; Roche Molecular Systems, Branchburg, NJ, USA) [24]. The cut-off value for a negative sample was 10 DNA copies/1×10^6 cells.

Residual compound quantification in supernatant after washing

Several serial dilutions of compound were added to PLL-coated plates and left to incubate at room temperature for 1h. Plates were then subjected to a series of six washing steps, and supernatants of the 3rd and 6th wash were collected for analysis. High performance liquid chromatography (HPLC) (performed at Janssen Pharmaceutica) was used to quantify the residual compound concentration in the supernatant collected. A series of known concentrations of compounds was injected onto the HPLC for detection and their chromatograph gave a series of peaks that correlated with the concentration of the compound injected. A linear calibration curve was generated using Excel (Microsoft Corp, Redmond, WA, USA). From this curve, unknown concentrations injected in the HPLC were calculated. Supernatant before washing was used as positive control and negative control included complete culture medium.

Residual compound quantification in PLL-coated plates

Radiolabelled ^{14}C-TMC-120 and ^{14}C-AZT were used to evaluate whether compound had bound to PLL-coated plates and/or associated with cell-free, immobilized virus. Plates were coated with 100 µg/ml PLL for 1 h and different concentrations of ^{14}C-labelled compounds were added in the presence or absence of cell-free HIV virus. Radioactivity was measured before washing, after 3× washing and after 6× washing, using a scintillation counter (Top count; Canberra-Packard, Zellik, Belgium) and residual amount of compound on plate was expressed as counts per minute (cpm).

Results

Treatment and replication inhibition of HIV-$1_{Ba\text{-}L}$ virus

In a series of preliminary experiments, we used various infective doses of the viruses (10^{-3}, 10^{-4} and 10^{-5} MOI) to coat the PLL plates. After washing away the unbound virus and addition of MO-DC and CD4 T cells, it was evident that coating with 10^{-3} and 10^{-4} MOI always resulted in high-level productive infection after 2 weeks, whereas the infection was less consistent at lower concentrations (data not shown). Therefore, we decided to choose 10^{-4} MOI for all further experiments.

Inhibitory capacities of compounds on cell-free HIV-$1_{Ba\text{-}L}$ were studied using a wide concentration range (100 000 nM–0.1 nM) of TMC-120, TMC-125, UC-781, PMPA and AZT. Each compound concentration was duplicated six times, incubated with the cell-free virus for 1 h and thoroughly washed away before addition of DC and CD4 T cells. In the primary DC/T4 culture, pretreatment with TMC-120 and TMC-125 more potently inhibited virus replication than UC-781 by factors of 11 and 4, respectively (see EC_{50} values in Table 2). The minimal concentration to inhibit HIV-$1_{Ba\text{-}L}$ virus production below the detection limit of the ELISA was 100 nM for TMC-120, 300 nM for TMC-125 and 1000 nM for UC-781. Remarkably, pre-treatment of the virus with the NRTI AZT and the nucleotide RTI (NtRTI) PMPA, failed to inhibit infection at the highest concentrations used (100 000 nM).

To investigate if any subliminal or latent infection was present in the ELISA-negative primary cultures, a secondary culture was initiated by adding PHA T BLAST, known to be the most sensitive target cells for HIV. From cultures, of which the supernatant remained ELISA-negative after the secondary culture, cells were additionally checked for the presence of proviral DNA. In fact, if HIV-$1_{Ba\text{-}L}$ virus had originally been pretreated with NNRTIs, very few negative primary cultures turned positive after the secondary cultures.

Treatment and replication inhibition of prevalent HIV-1 group M primary subtypes

Several experiments were performed on the HIV-$1_{Ba\text{-}L}$ virus first and then later extended to primary isolates. The inhibitory capacities of the DAPY compounds

Table 2. Treatment of immobilized cell-free HIV-$1_{Ba\text{-}L}$ virus with compounds: replication inhibition in primary and secondary cultures

Compound*	Class	1^o culture EC_{50}, nM	2^o culture EC_{50}, nM
TMC-120	NNRTI	37 (±26)†	38 (±26)
TMC-125	NNRTI	103 (±86)	117 (±131)
UC-781	NNRTI	442 (±182)	517 (±193)
PMPA	NtRTI	>100 000	>100 000
AZT	NRTI	>100 000	>100 000

*Cell-fee HIV-$1_{Ba\text{-}L}$ (10^{-4} MOI) was immobilized and pre-incubated with compound for 1 h, washed, seeded with MO-DC and autologous T4 cell and cultured for 2 weeks, without adding compound (primary culture). Day 14 cells were washed (3×) and fresh PHA T BLAST added and cultured for another 14 days (secondary culture). †Mean ±SD (eight experiments for TMC-120, six experiments for UC-781, four experiments for TMC-125 and two experiments for PMPA and AZT). EC_{50}, 50% effective concentration, compound concentration required to inhibit HIV-1 replication in 1^o (primary) and 2^o (secondary) cultures; NNRTI, non-nucleoside reverse transcriptase inhibitor; NtRTI, nucleotide reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor.

were similar for the primary isolate panel (see Figure 2). Replication inhibition by the different compounds showed no clear-cut subtype specificity, CI22 (subtype B) was the most susceptible virus to TMC-120, VI882 (subtype C) was the most susceptible to TMC-125 and CI20 (subtype CRF02) was the most susceptible to UC-781. In the primary DC/T4 culture, both TMC-120 and TMC-125 were more potent than UC-781 by a factor of 5 (see Table 3). The minimal concentration to inhibit all virus clades below the detection limit of the ELISA was between 30–100 nM for TMC-120, 10–300 nM for TMC-125 and 1000–3000 nM for UC-781.

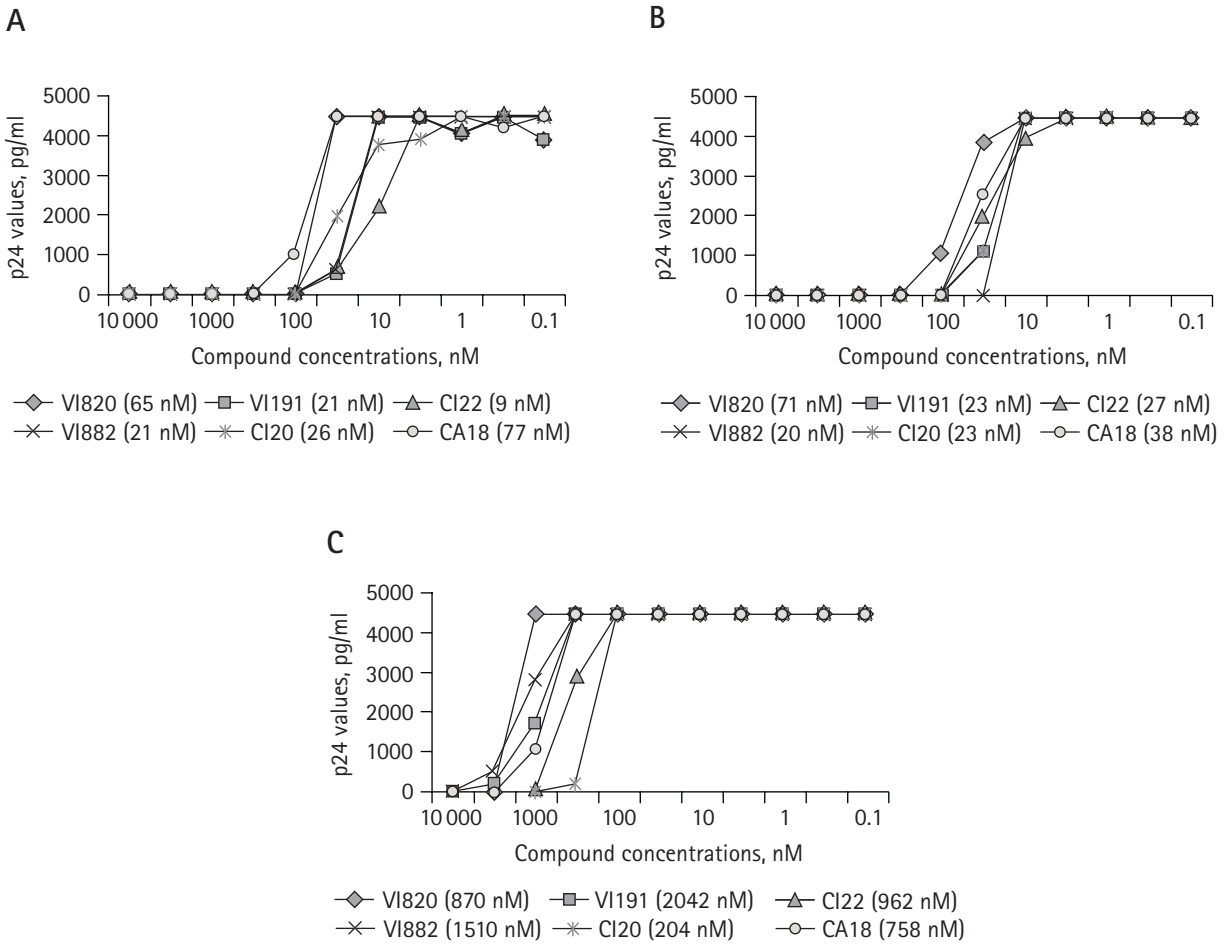
Primary isolates pre-treated with either TMC-120 or TMC-125 showed limited virus rescue in the secondary culture; however, sizeable numbers of primary cultures from isolates pretreated with UC-781 turned positive after the secondary cultures. This is reflected in the overall minimal rise of EC_{50} for TMC-120, a slightly higher rise for TMC-125 and a more pronounced fivefold increase for UC-781 (Table 3).

An overall comparison of EC_{50} values at the end of the secondary cultures, infected with HIV-1_{Ba-L}, indicates that TMC-120 is three times more potent than TMC-125 and 14 times more potent than UC-781. In contrast, on average the six primary isolates were equally sensitive to inhibition by TMC-120 and TMC-125, whereas UC-781 was 30 times less potent.

Residual compound in supernatant, bound on plates or virus

In order to know if any compound (TMC-120 and TMC-125) could remain present after pretreatment of

Figure 2. Treatment of immobilized, cell-free HIV-1 group M primary isolate viruses, replication Inhibition in secondary cultures



Antiviral activity of **(A)** TMC-120, **(B)** TMC-125 and **(C)** UC-781 on six primary isolates in secondary co-cultures. A serial dilution of compound was added to immobilized virus (10^{-4} MOI), excess compound was washed away and target cells added. Culture was kept for 14 days, a secondary culture was initiated for a further 14 days. HIV antigen (p24) was measured in the supernatants by ELISA and is represented in pg/ml. 50% effective concentration was (EC_{50}) calculated in nanomoles (nM) by linear regression analysis. Subtype information on the viruses used are as follows: VI820 (subtype A), VI191 (subtype A/G), CI22 (subtype B), VI882 (subtype C), CI20 (subtype CRF02_AG), and CA18 (subtype CRF02_AG).

Table 3. Treatment of immobilized, cell-free HIV-1 group M primary isolates virus with compounds: replication inhibition in primary and secondary cultures

	TMC-120*		TMC-125		UC-781	
	1° culture, nM	2° culture, nM	1° culture, nM	2° culture, nM	1° culture, nM	2° culture, nM
VI820	63.5	65.0	13.4	71.1	261.4	870.9
VI191	20.9	21.2	<10.0	23.5	588.3	2042.0
CI22	7.1	9.8	18.9	27.3	192.2	962.1
VI882	19.0	21.6	11.8	20.0	67.5	1510.9
CI20	20.7	26.9	18.9	23.2	34.4	204.3
CA18	74.4	77.2	<10.0	38.1	84.0	758.2
EC_{50} ±SD	34 (±25)	36 (±24)	14 (±4)	33 (±17)	204 (±188)	1058 (±582)

*Cell-free primary isolates (10^{-4} MOI) were immobilized and pre-incubated with compound for 1 h, washed, seeded with MO-DC and autologous T4 cells and cultured for 2 weeks, without adding compound (1° culture). Day 14 cells were washed (3×) and fresh PHA T BLAST added and cultured for another 14 days (2° culture). EC_{50}, 50% effective concentration, compound concentration required to inhibit HIV-1 replication in primary and secondary cultures.

the cell-free virus and extensive washes, the supernatants after the sixth wash were subjected to high-pressure liquid chromatography (lower detection limit of 8 nM). No traces were detectable even if the highest concentration 10 000 nM was used (data not shown).

In addition, we evaluated residual plate-bound TMC-120 and AZT, using ^{14}C-labelled compounds. At inhibitory concentrations of TMC-120 (10–30 nM), less than 0.22% of ^{14}C-TMC-120 is bound to the plate (in the absence of virus). At concentrations well above the inhibitory capacity of the TMC-120 (100 nM–10 000 nM), an average of 2.7% of ^{14}C-TMC-120 is bound to the plate (data not shown). There was no difference in residual ^{14}C-TMC-120 in the presence or absence of virus. In contrast, ^{14}C-AZT could be washed away completely from the PLL-coated plate with or without virus.

Discussion

We evaluated the direct effect of various RTIs on immobilized, cell-free virus and clearly show that all NNRTIs have the capacity to completely prevent subsequent cellular infection (at nanomolar and micromolar concentrations).

This pretreatment model mimics the scenario where a drug acts on the virus before it can enter the epithelial layer and reach the subepithelial target cells (represented in our model by MO-DC and CD4 T cells). The six primary isolates (HIV-1 group M subtypes A, A/G, B, C and CRF02_AG) used in this study were mostly from seropositive African individuals and represent the relevant circulating subtypes. Primary HIV-1 group M isolates were similarly as sensitive as HIV-$1_{Ba\text{-}L}$ to inhibition by NNRTI. The fact that sterilizing capacities of the NNRTI tested showed no subtype specificity strongly suggests, but obviously does not prove, that they could be used as a sterilizing microbicide.

The diarylpyrimidine compounds TMC-120 and TMC-125 were consistently more potent than the structurally unrelated carboxyaniline UC-781. In addition, viral rescue was infrequently observed when primary isolates were pretreated with the DAPY compounds, but occurred more after UC-781 pretreatment. Collectively, these data indicate that DAPY compounds inactivate RT enzymatic activity in a more efficient manner than UC-781, hence preventing the virus from being integrated in the host cell genome.

A remarkable observation was that PMPA (a classic NtRTI) and AZT (an NRTI) failed to inhibit virus. In previous studies, PMPA and AZT have been shown to efficiently abort HIV infection when they remain present during the first 24 h of cultures of MO-DC and CD4 T cells, with EC_{50} values of 440 nM and 25 nM, respectively [16]. This apparent discrepancy can be explained by the fact that NtRTIs and NRTIs need cellular activation to display RTI activity whereas the DAPY compounds (NNRTIs) are directly inhibitory on viral RT [3].

We observed that a small percentage of the highly hydrophobic DAPY compounds was sticking to the wells, despite extensive washing. Therefore, it is not excluded that the residual compound, bound to the plate prolongs the pre-treatment effect. This 'stickiness' could also be an advantage *in vivo*, as demonstrated by Shattock's experiments [25]. When a cervical explant tissue was treated with TMC-120 or UC-781 for 2 h, HIV infection was blocked up to 6 days after exposure to compound, creating a significant anti-HIV 'memory effects' [25].

In conclusion, our *in vitro* model allows rapid screening of direct antiviral effects on cell-free HIV in a system using primary HIV-1 isolates and primary

MO-DC/T4 cells closely mimicking targets involved in HIV transmission. The new DAPY compounds, TMC-120 and TMC-125, show high antiviral activity against different viral clades (predominant in the epidemic), suggesting that both compounds could be good candidate microbicides. However, in the absence of any microbicide of proven potency in women, no direct conclusion can be drawn from any *in vitro* system and careful preclinical testing in animal models of candidate microbicides remains mandatory.

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