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Resistance and cross-resistance profile of the diaryltriazine NNRTI and candidate microbicide UAMC01398

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Objectives: The resistance development, cross-resistance to other NNRTIs and the impact of resistance on viral replicative fitness were studied for the new and potent NNRTI UAMC01398.

Methods: Resistance was selected by dose escalation and by single high-dose selection against a comprehensive panel of NNRTIs used as therapeutics and NNRTIs under investigation for pre-exposure prophylaxis of sexual HIV transmission. A panel of 27 site-directed mutants with single mutations or combinations of mutations involved in reverse transcriptase (RT) inhibitor-mediated resistance was developed and used to confirm resistance to UAMC01398. Cross-resistance to other NNRTIs was assessed, as well as susceptibility of UAMC01398-resistant HIV to diarylpyrimidine-resistant viruses. Finally, the impact of UAMC01398 resistance on HIV replicative fitness was studied.

Results: We showed that UAMC01398 has potent activity against dapivirine-resistant HIV, that at least four mutations in the RT are required in concert for resistance and that the resistance profile is similar to rilpivirine, both genotypically and phenotypically. Resistance development to UAMC01398 is associated with a severe fitness cost.

Conclusions: These data, together with the enhanced safety profile and good solubility in aqueous gels, make UAMC01398 an excellent candidate for HIV topical prevention.

Introduction

Antiretroviral-based microbicides may offer a means to reduce HIV sexual transmission in circumstances where systematic condom use is difficult and oral pre-exposure prophylaxis (PrEP) is not available. Whereas early-generation microbicides failed to demonstrate efficacy, the newer candidates are based on more potent and HIV-specific products.[1,2] The NNRTI dapivirine and the NRTI tenofovir are the most advanced in clinical development.[3] Other NNRTIs, such as UC781, MIV-150 and MC1220, are under preclinical evaluation.[4] We previously identified a new NNRTI, UAMC01398, that is currently being evaluated as topical prophylaxis in non-human primates.[5,6]

When antiretroviral-based microbicides are introduced, the potential impact on current and future therapeutic strategies needs to be considered. This is important because several drugs for topical (and oral) PrEP are already in use for first- and second-line treatment or are chemically related to products used in therapy. If a microbicide reduces treatment success because of resistance induction, the long-term implications could be dramatic. That this concern is not merely theoretical was shown with the use of nevirapine for the prevention of mother-to-child transmission.[7] Resistance can occur under two scenarios. The first is the transmission of resistant viruses in communities with widespread treatment. Topical application is generally done with doses many times higher than the required inhibitory concentration during oral delivery, but it remains unclear whether resistant viruses would survive these high concentrations. The second scenario that could lead to resistance is the continued use of a topical product by an HIV-infected individual. Although the potential for resistance upon topical use of a compound and absent or extremely low systemic levels remains controversial, resistance development is much more likely to occur when subinhibitory levels of the product penetrate systemically.

Here we report on the activity of UAMC01398 against dapivirine-resistant HIV, the resistance profile of UAMC01398, how this affects cross-resistance to other NNRTIs and the impact of resistance mutations on viral replicative capacity.

Materials and methods

Compounds

The NNRTIs dapivirine (TMC120), etravirine (TCM125) and rilpivirine (TMC278) were obtained from Janssen Infectious Diseases, Beerse, Belgium. The phenylethylthioureathiazole (PETT) MIV-170 was obtained from Medivir AB, Sweden. Efavirenz and nevirapine were obtained through the NIH AIDS Reagent Program. Lersivirine and UAMC01398 were synthesized by the Medicinal Chemistry Group of Antwerp University according to previously reported procedures.[8–10] All compounds were dissolved in DMSO as 10 mM stock solutions and stored at −20°C.

Viruses, cells and in vitro resistance selection

The prototypic subtype B CCR5-tropic virus Bal (NIH AIDS Reagent Program) and a subtype C CCR5-tropic primary isolate, VI829 (from our own collection),[11] were used to assess antiviral activity and for resistance induction. Resistance was selected in both HIV strains by dose escalation with UAMC01398, dapivirine, etravirine and rilpivirine. PBMCs were isolated from buffy coats from HIV-seronegative blood donors (provided by the Antwerp Blood Transfusion Centre) using density gradient centrifugation with Lymphoprep® (Axis-Shield, Oslo, Norway). PBMCs were subsequently stimulated for 48 h with 2 μg/mL phytohaemagglutinin (PHA) (Remel, Kent, UK) in RPMI 1640 medium containing 10% FBS, 50 μg/mL gentamicin and 2 μg/mL Polybrene (Sigma-Aldrich, Bornem, Belgium). Next, PBMCs were activated for 24 h with 1 ng/mL IL-2 (Gentaur, Brussels, Belgium) in RPMI 1640 medium supplemented with 10% FBS, 50 μg/mL gentamicin, 2 μg/mL Polybrene and 5 μg/mL hydrocortisone (Calbiochem, Leuven, Belgium).

One million PBMCs were infected with 1000 $TCID_{50}$ (10^{-3} moi) of Bal or VI829 in a 24-well cell-culture plate. Drug dosing started at the 50% effective concentration (EC_{50}) of each compound and was gradually increased (2–5-fold) until a 1000-fold change in EC_{50} was reached. Viral replication was monitored every 3–4 days with an in-house p24 antigen-capture ELISA. Drug concentrations were increased only when the p24 level in the resistant culture was >50% of the p24 level in the control cultures without compound.[11] When the predefined level of resistance was obtained, large stocks (30 mL) of cell-free virus were generated, aliquoted and stored at −80°C.

TZMbl cells were used for drug susceptibility assays, using a previously described set-up.[5] Briefly, 10^4 cells per well were incubated in 96-well plates and exposed to serial dilutions of compound for 30 min prior to infection with HIV. Subsequently, 200 $TCID_{50}$ of WT or resistant virus was added and cultures were incubated for 48 h at 37°C/7% CO_2 before luciferase activity was quantified. Each condition was tested in three to six replicate wells. Antiviral activity was expressed as the percentage of viral inhibition compared with that of the untreated controls and plotted against the compound concentration. Non-linear regression analysis was used to calculate the EC_{50}. Fold changes in EC_{50} were calculated relative to the EC_{50} value of the WT viruses.

Replication-competent site-directed mutants (SDMs)

A panel of replication-competent molecular clones was made containing individual or combinations of NNRTI resistance-associated mutations (RAMs). Site-directed mutagenesis was performed on the WT pNL4.3-VifA[12] and using QuickChange II Site-Directed Mutagenesis technology (Agilent Technologies). All mutants were confirmed by nucleotide sequencing. Plasmid DNA (1 μg) was transfected in HEK293T producer cells in 6-well tissue culture plates (2×10^5 cells/well) using FuGENE®-6 (Promega). Cell-free supernatant containing the virus was collected 48 h post-transfection, aliquoted and frozen at −80°C. One vial was thawed to check viral infectivity and replication. SDMs were used in susceptibility assays with TZMbl cells, as described above.

Genotyping of NNRTI-resistant viruses and SDMs

Mutations in the reverse transcriptase (RT) coding region associated with resistance to NNRTIs were determined by population sequencing, as previously described.[11] Briefly, viral RNA (vRNA) was extracted from cell-free culture supernatant using the QIAamp Viral RNA kit (Qiagen) according to the manufacturer's instructions. Next, vRNA was reverse transcribed using Expand RT (Roche Applied Science) and primer SJH12a (5′-CCTAA TGCATACTGTGAGTCTG-3′). Amplification of the protease (PR) and complete RT/p51 coding regions was done by nested PCR using the Expand High Fidelity PCR system (Roche Applied Science). First-round PCR primers were SJH12a and SJH11A (5′-AAAAGGGCTGTTGGAAATGTGG-3′), resulting in a 2 kb Gag-Pol fragment (HxB2 nucleotide positions 2018–4060). Hemi-nested PCR was performed on 1 μL of the first-round product, using primers SJH13a (5′-GAGAGACAGGCTAATTTTTTAGGG-3′) and SJH12a. Amplicons were purified using Wizard SV Gel and PCR Clean-Up Start-Up (Promega) and sequenced at BaseClear, Leiden, The Netherlands, using primers SJH13a, 28Polup (5′-GAACCAGTACATGGAGTRTATTATGACCC-3′), H1P3339 (5′-TCCCATTCAGGAATCCAGGTGGC-3′) and H1P1697 (5′-AGAG CCAACAGCCCCACCAG-3′).

Dual infection/competition assay

Dual infection/competition assays were performed as previously described.[11,13,14] Briefly, pNL4.3 clones carrying UAMC01398 RAMs were competed against WT pNL4.3 in a 1:1 ratio, using equal moi (2×10^{-3} to 1×10^{-4}, depending on the virus) of each virus as input. PHA/IL-2-stimulated PBMCs (7.5×10^4) were seeded in each well of a 96-well plate and exposed to a 1:1 mixture of two viruses for 4 h. Next, virus was washed away and culture supernatant was collected at peak viraemia (day 8 post-infection). vRNA was extracted from cell-free culture supernatant and reverse transcribed in cDNA using primer H1Env6154 (5′-AGAGTGGGGTTAATTTTACACATGG-3′) and subsequently PCR amplified in combination with primer H1P4235 (5′-CCCTACAAT CCCCAAAGTCAAGG-3′). The PCR fragment was treated with ExoSAP-IT (Affimetrix) prior to nucleotide sequencing with primer H1P4327 (5′-TAAGACAGCAGTACAAATGGCAG-3′). Variant quantification was done according to Lanxon-Cookson *et al.*[15] using the web-based tool Chromat Quantitator (http://indra.mullins.microbiol.washington.edu/cgi-bin/chromatquant.cgi).

Results

Resistance induction

Two methods of selecting for UAMC01398-resistant HIV were followed: a gradual dose escalation and a single high-dose exposure. In the first method, PBMCs were infected with 200 $TCID_{50}$ of subtype B (Bal) and subtype C (VI829) HIV-1. These viruses were exposed to the EC_{50} of UAMC01398 and virus growth was followed over time with p24 antigen-detection ELISA. Compound concentration was doubled every time the virus clearly resumed replication. Resistance developed slowly, within 147 and 176 days for Bal and VI829, respectively (Figure 1). RAMs were mapped by nucleotide sequencing covering the entire PR (99 amino acids) and RT (amino acid 1–440) coding regions (GenBank KU351842–KU351845). The same viruses were passaged simultaneously in the absence of compound to control for spontaneous mutations *in vitro*. Four concurrent mutations were found in Bal (V90I, V106A, V179M and F227C) and five in VI829 (V75I, V90I, E138K, H221Y and M230I) (Figure 1). All of the mutations found have been reported previously in the context of NNRTI resistance.[16] The only exception is V75I, which is implicated in multi-NRTI resistance.[17,18]

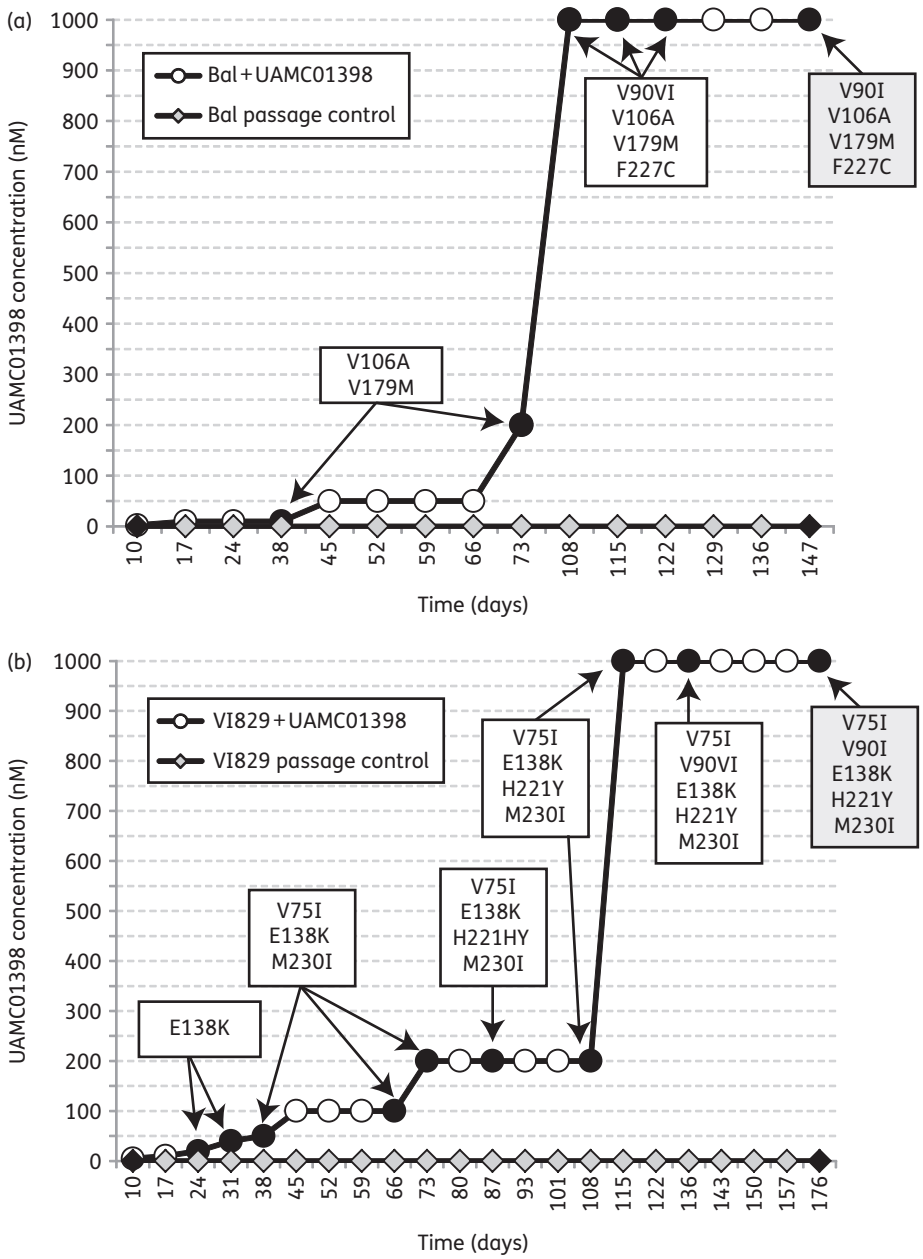

Figure 1. *In vitro* selection of UAMC01398-resistant viruses. Subtype (a) B (Bal) and (b) C (VI829) viruses were grown in the presence of increasing amounts of the diaryltriazine UAMC01398 in activated PBMC cultures. Resistance induction was started at the EC_{50} (1 nM) and gradually increased to 1 μM. Black circles indicate timepoints used for nucleotide sequencing. Mutations are indicated in boxes. Control cultures in the absence of compound were done in parallel (indicated by diamonds) and the nucleotide sequence was verified at the start and on days 147 and 176 for Bal and VI829, respectively.

The second approach involved a single high-dose exposure of Bal and VI829 to UAMC01398 and resembled as closely as possible the real-life circumstances of a microbicide gel application. Similarly to the previous approach, PBMCs were first infected with 200 $TCID_{50}$ of either virus and subsequently exposed to 10×, 25× and 50×EC_{50} of UAMC01398. Control experiments involved exposure at similar concentrations of nevirapine, efavirenz, dapivirine, etravirine, rilpivirine, lersivirine and MIV-170. Virus growth was monitored weekly using p24 antigen-detection ELISA and cultures were maintained for 65 days. The only condition where breakthrough virus replication was found was in the culture with nevirapine at the lowest concentration tested (i.e. 1 μM or only 1.4×EC_{50} of nevirapine) (Figure S1, available as Supplementary data at *JAC* Online). None of the cultures with the other NNRTIs showed HIV replication. These results show that a single exposure as low as 10×EC_{50} of nevirapine, efavirenz, dapivirine, etravirine, rilpivirine, lersivirine, MIV-170 and UAMC01398 abrogates viral infection and does not result in resistance selection.

Cross-resistance to other NNRTIs

Next, the UAMC01398-resistant viruses were tested for cross-resistance to a series of NNRTIs used for the treatment of HIV infection (i.e. efavirenz, nevirapine, etravirine and rilpivirine), as well as NNRTIs in (pre)clinical development for topical prevention (i.e. dapivirine, lersivirine and MIV-170). Dapivirine-, etravirine- and rilpivirine-resistant viruses, obtained during previous resistance inductions,[11] were also included in the cross-resistance analysis (Figure 2).

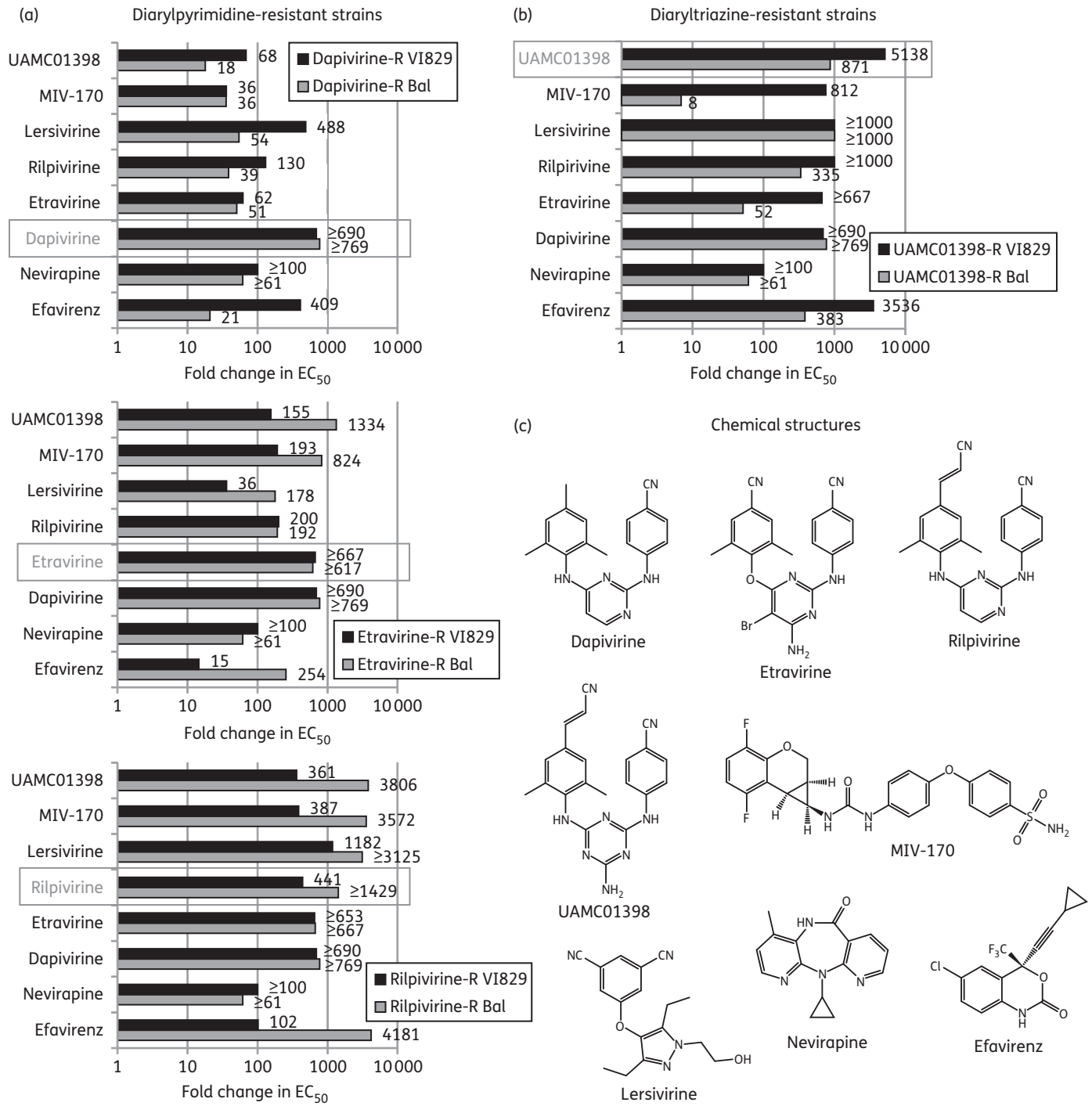

Figure 2. Drug activity evaluation with HIV strains resistant to (a) dapivirine (Bal: K101E, K103K/N, V108V/I, E138Q, V179M, Y181C; VI829: L100I, E138K, T369I), etravirine (Bal: V179M, Y181C; VI829: E138A, Y181C, M230L), rilpivirine (Bal: K101E, Y181C, M230L; VI829: E138K, Y181C, Y188H, M230L) and (b) UAMC01398 (Bal: V90I, V106A, V179M, F227C; VI829: V75I, V90I, E138K, H221Y, M230I). Values shown are fold changes in EC_{50}. Exact values are given alongside each bar; '≥' indicates that EC_{50} fold changes are equal to or larger than could be measured in the range of drug concentrations tested. (c) Chemical structures of the compounds used. R, resistant.

As expected, dapivirine-, etravirine-, rilpivirine- and UAMC01398-resistant viruses showed high-level resistance (at least 400-fold change in EC_{50}) to dapivirine, etravirine, rilpivirine and UAMC01398, respectively. Interestingly, dapivirine-resistant Bal was still moderately susceptible (<100-fold change) to inhibition by efavirenz, etravirine, rilpivirine, lersivirine, MIV-170 and UAMC01398. Dapivirine-resistant VI829 showed more pronounced cross-resistance (≥100-fold change) to efavirenz, nevirapine, rilpivirine and lersivirine, but was still moderately susceptible to etravirine, MIV-170 and UAMC01398. Etravirine-resistant Bal showed high-level cross-resistance to all other NNRTIs tested, whereas the etravirine-resistant VI829 was still moderately susceptible (<100-fold change) to efavirenz and lersivirine, but showed higher cross-resistance to all other NNRTIs tested. Rilpivirine-resistant viruses, both Bal and VI829, showed high-level cross-resistance to all of the tested NNRTIs. The UAMC01398-resistant Bal virus showed low-level resistance (<10-fold change) to MIV-170, intermediate-level resistance to nevirapine and etravirine and high-level resistance to all others, whereas UAMC01398-resistant VI829 showed high-level cross-resistance to all tested NNRTIs (Figure 2).

Site-directed mutagenesis

A panel of 27 replication-competent viruses was made, carrying single or combinations of two to five NNRTI RAMs. These viruses also had a signature sequence in Vif allowing easy variant quantification in dual infection/competition fitness assays[12] (Figure 3). This panel of RT mutants was used to confirm the impact of the mutations found during the resistance induction experiments, as well as to assess cross-resistance to dapivirine, etravirine, rilpivirine, lersivirine and MIV-170.

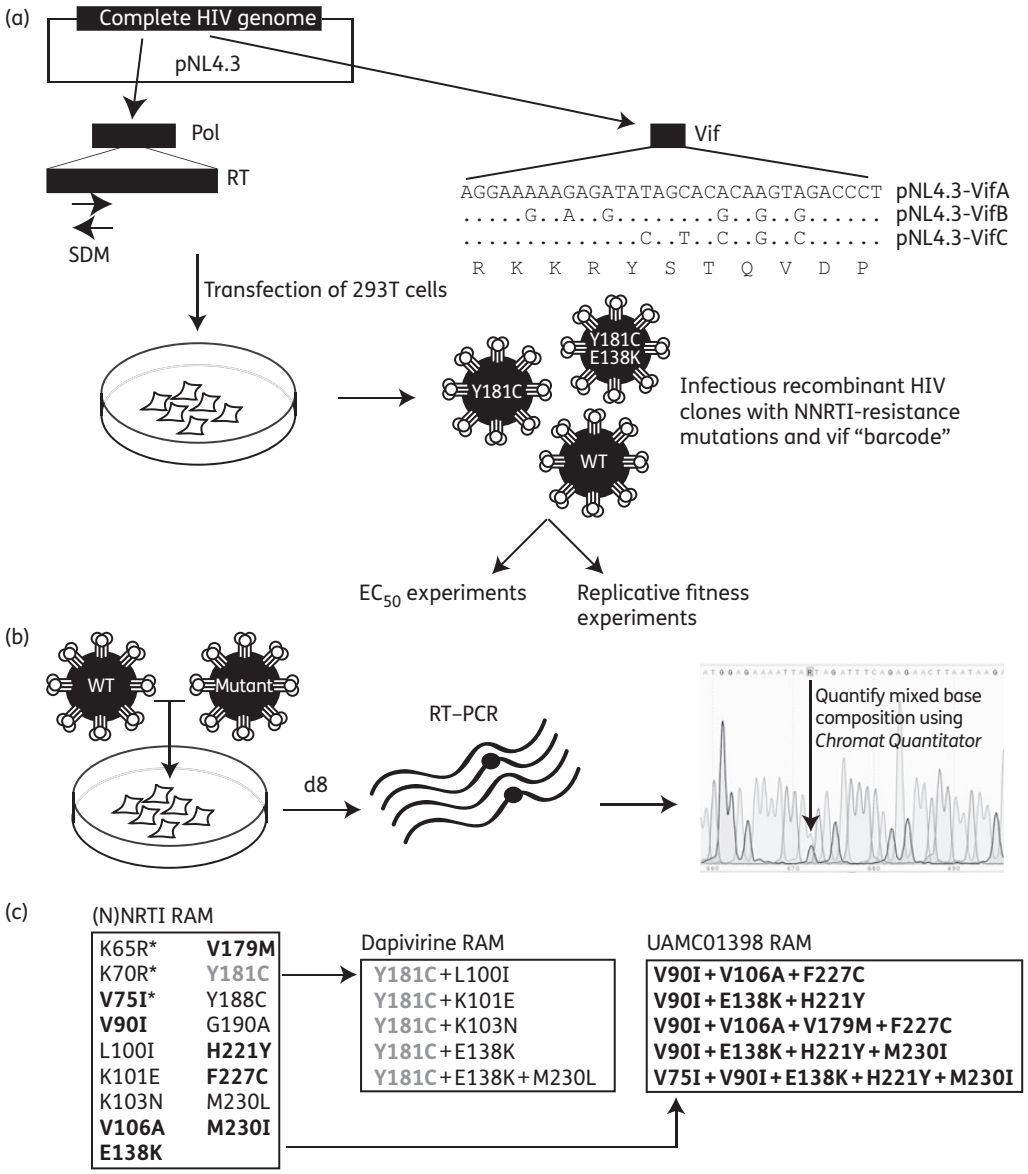

Figure 3. (a) Cloning strategy for the panel of replication-competent SDMs. (b) Competition fitness assay with SDMs. (c) List of (N)NRTI RAMs available as replication-competent virus in the pNL4.3 backbone. Asterisk, NRTI RAMs; bold, UAMC01398 RAMs; grey, key NNRTI RAM and combinations of this RAM with other NRTI RAMs.

Figure 4 compares the antiviral activity of UAMC01398 with that of dapivirine, the reference anti-HIV product currently furthest advanced in clinical development for PrEP. UAMC01398 and dapivirine retained very good activity against all of the SDMs with single mutations (maximum 10-fold change in EC_{50}). UAMC01398 performed markedly better than dapivirine against the L100I, K103N, Y181C and Y188C mutations. Interestingly, UAMC01398 retained good activity against dapivirine RAMs Y181C–L100I, Y181C–K101E, Y181C–K103N, Y181C–E138K and Y181C–E138K–M230L. Both UAMC01398 and dapivirine lost activity against the UAMC01398 RAMs V90I–V106A–F227C, V90I–E138K–H221Y, V90I–V106A–V179M–F227C, V90I–E138K–H221Y–230I and V75I–V90I–E138K–H221Y–M230I.

Figure 5 compares the antiviral activity of UAMC01398 with the chemically related therapeutics etravirine and rilpivirine. Whereas

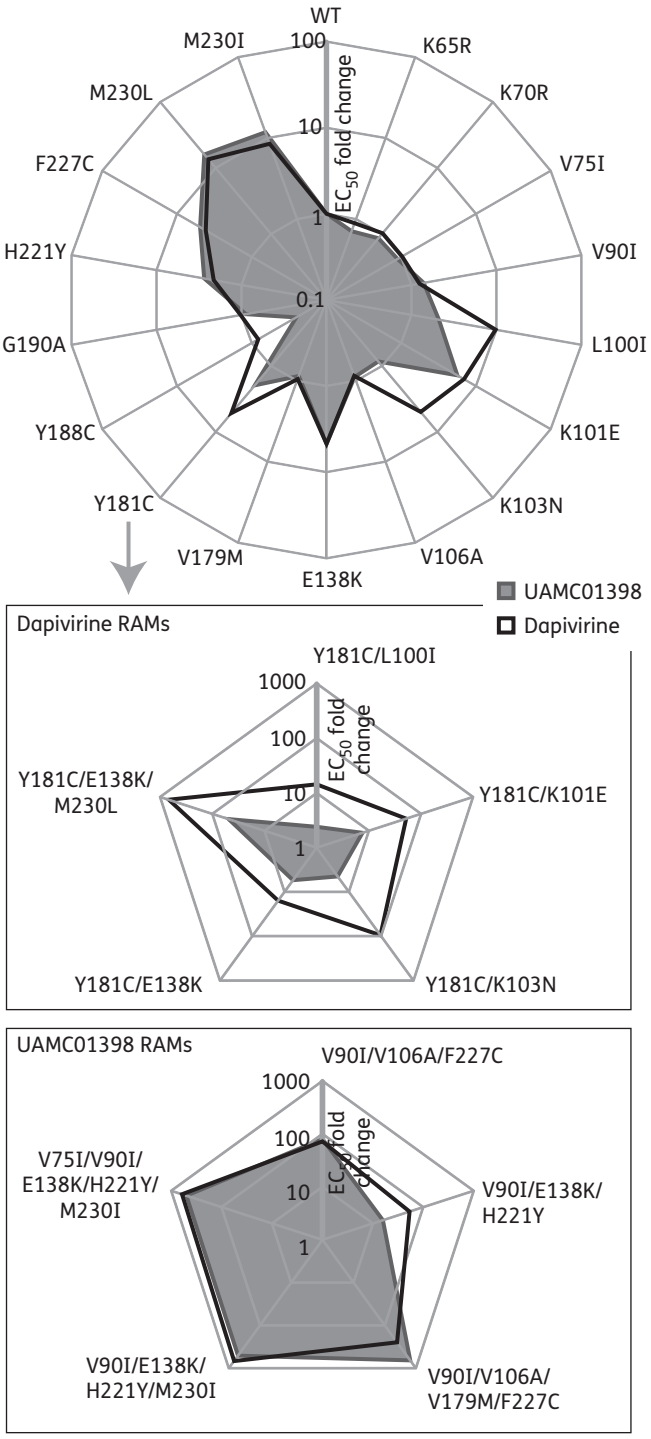

Figure 4. Fold change in antiviral activity of dapivirine and UAMC01398 against a series of single RT SDMs, dapivirine RAMs and UAMC01398 RAMs.

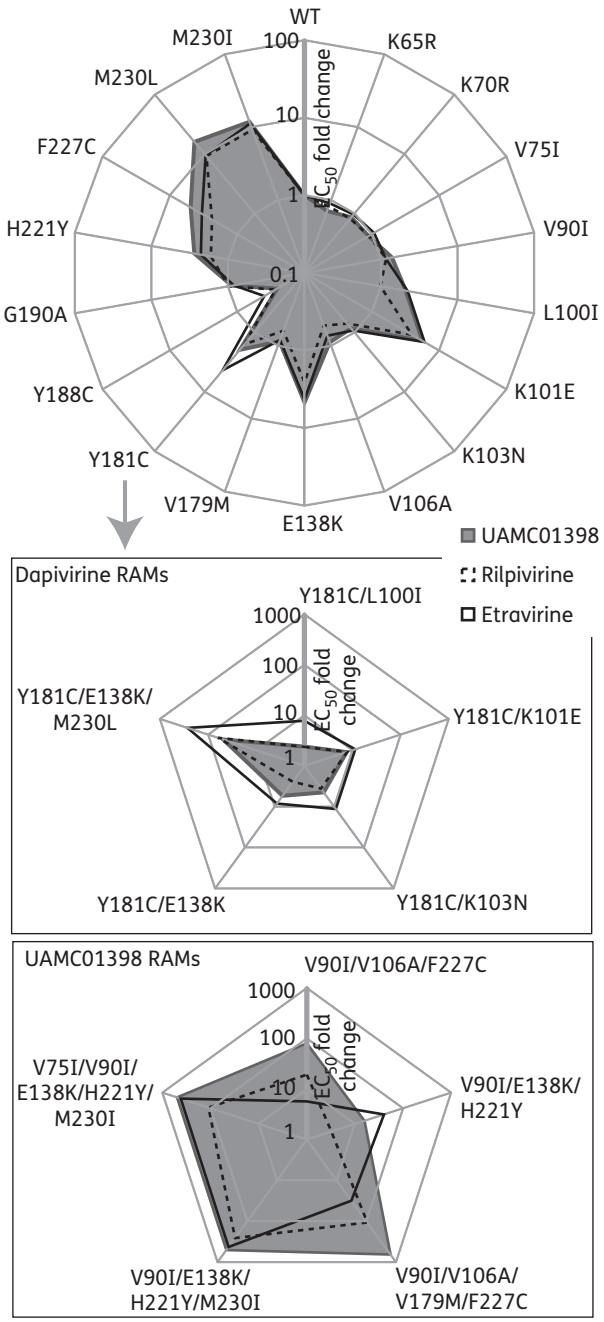

Figure 5. Fold change in antiviral activity of etravirine and rilpivirine in comparison with UAMC01398 against a series of single RT SDMs, dapivirine RAMs and UAMC01398 RAMs.

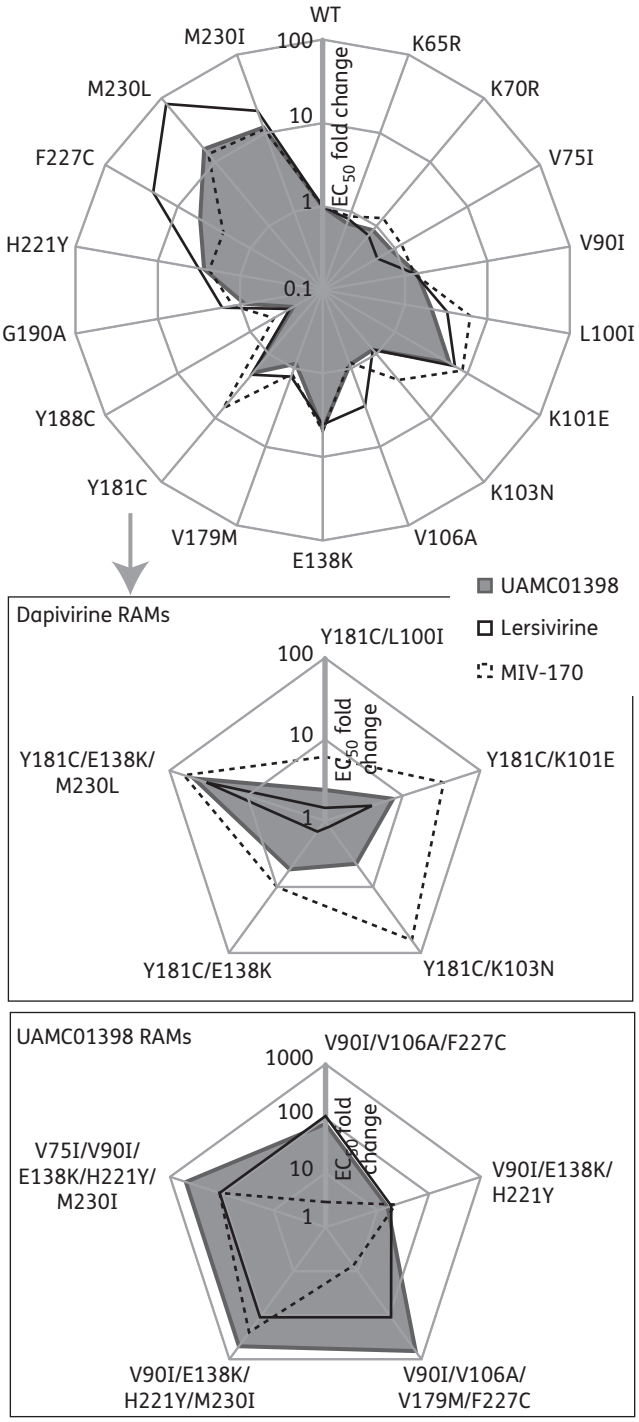

Figure 6. Fold change in antiviral activity of lersivirine and MIV-170 in comparison with UAMC01398 against a series of single RT SDMs, dapivirine RAMs and UAMC01398 RAMs.

the three compounds showed similar good activity against the SDMs with a single RAM, UAMC01398 and rilpivirine performed equally well and slightly better than etravirine against the SDMs with dapivirine RAMs. Both etravirine and rilpivirine lost activity against the SDMs carrying UAMC01398 RAMs.

Figure 6 compares the antiviral activity of UAMC01398 with the chemically distinct lersivirine and MIV-170. Compared with UAMC01398, MIV-170 was slightly less active against the L100I, K103N and Y181C SDMs, but more active against F227C SDMs. Lersivirine showed considerably lower activity against the F227C and M230L SDMs compared with MIV-170 and UAMC01398. Of note, lersivirine had better activity against viruses with dapivirine-associated RAMs compared with MIV-170 and was also slightly more active than UAMC01398, except for the triple mutant E138K–Y181C–M230L. In contrast, MIV-170 retained better activity against two of the five viruses with UAMC01398 RAMs, compared with lersivirine, i.e. those with V90I–V106A–F227C. EC_{50} values and fold changes are presented in Table S1.

Replicative fitness of UAMC01398-resistant HIV

Next, we analysed the impact of UAMC01398 RAMs on viral replicative fitness, using a panel of pNL4.3 molecular clones carrying the UAMC01398 RAMs together with signature sequences in Vif to allow easy variant quantification in dual infection/competition assays (Figure 3). The viruses with UAMC01398-resistant genotypes, i.e. V90I–V106A–V179M–F227C, V90I–E138K–H221Y–M230I and V75I–V90I–E138K–H221Y–M230I, were competed against WT pNL4.3 and against each other. WT pNL4.3 completely outcompeted each of the UAMC01398-resistant pNL4.3 clones. The virus carrying V90I–V106A–V179M–F227C outcompeted the two other UAMC01398-resistant molecular clones. Interestingly, the pNL4.3 clone carrying V75I in addition to V90I–E138K–H221Y–M230I appeared slightly more fit than the variant lacking V75I. Although V75I was selected as part of the Q151M multi-NRTI resistance complex and has been described as emerging under selective pressure of the guanosine analogue aciclovir,[17,18] it has not been described in association with NNRTI-resistance mutations. V75I appeared to increase viral replication capacity in the context of V90I–E138K–H221Y–M230I mutations (Figure S2). Growth kinetic analysis of the UAMC01398-resistant Bal and VI829 viruses obtained on days 147 and 176 in the dose-escalation studies showed that the viruses replicated to high titre in the presence of drug, but were severely crippled in the absence of UAMC01398. Interestingly, UAMC01398-resistant virus replicated better in gradually increasing concentrations of compound (10-fold increases between 0.01 nM and 1 μM; data not shown).

Discussion

Because of the increased burden of the HIV epidemic for women in developing countries, a female-controlled biomedical prevention tool, such as a vaginal microbicide, is urgently needed. The development of a successful microbicide requires a potent antiretroviral agent and a formulation that ensures disposition of the active product at the site of viral transmission upon vaginal application. Many vaginal delivery strategies exist, such as rings, films, tablets and gels. General desirable characteristics of a microbicide further include good safety and tolerability, sufficiently high mucosal tissue penetration but low systemic penetration, activity against drug-resistant HIV variants, a high barrier to resistance selection and preferentially a resistance profile that is different from that of products used for the treatment of HIV infection.[1–4]

The CAPRISA004 trial with tenofovir gel presented the first, although modest, proof of principle for the NRTI-based topical

prevention of HIV transmission. RT inhibitors are conceptually interesting for use in (topical) prevention because they interfere with the pre-integration process of reverse transcription and can thus prevent HIV from establishing irreversible infection. In addition, several studies have shown proof of concept for NNRTIs in preventing SHIV infection by mucosal challenge in non-human primate models.[19–23] Several highly potent NNRTIs have been evaluated preclinically over the past decade, but few have progressed to *in vivo* studies in non-human primates and ultimately to clinical trials in humans.[1] Dapivirine, a diarylpyrimidine NNRTI donated to the International Partnership on Microbicides (IPM) by Tibotec BVBA, is currently the most advanced in clinical development, with an ongoing Phase 3 double-blind, randomized, placebo-controlled trial called ASPIRE that aims to evaluate a dapivirine ring in five African countries. Results on efficacy are expected in early 2016.

We previously identified the highly potent cyanovinyldiaryltriazine UAMC01398, a molecule with enhanced activity, safety and biopharmaceutical profile compared with dapivirine.[5,6,24] The current study reports on the activity of UAMC01398 against NNRTI-resistant viruses, including dapivirine-resistant strains, as well as on the resistance profile of UAMC01398, its cross-resistance profile and the effect of UAMC01398 RAMs on viral replicative fitness. A first and particularly interesting preliminary observation was that UAMC01398 retains low nanomolar antiviral activity against dapivirine-resistant HIV.[5] In the present study we selected resistance to UAMC01398 in prototypic subtype B and C strains by dose escalation. It took 147–176 days (21–25 weeks) to select UAMC01398-resistant viruses with a 1000-fold change in EC_{50} versus WT HIV. Previous dose-escalation studies with dapivirine in an identical experimental set-up resulted in dapivirine-resistant viruses after 12 weeks.[11] Schader *et al.*[25] also studied the *in vitro* resistance profile of dapivirine and continued drug pressure for 25 weeks, after which the RT genotype was determined. Unfortunately, this paper does not present genotypic and phenotypic data on timepoints prior to week 25. Our own experience with *in vitro* resistance selection against dapivirine, etravirine, rilpivirine and UAMC01398 in PBMCs suggests that resistance to these agents develops slowly and at similar rates. In terms of cross-resistance, we found that dapivirine-resistant viruses were still susceptible to inhibition by the structurally related diarylpyrimidines etravirine and rilpivirine, the diaryltriazine UAMC01398 and the chemically distinct MIV-170. There was clear cross-resistance between etravirine- and rilpivirine-resistant viruses, confirming earlier reports.[16,26,27]

To mimic the conditions under topical prophylaxis, a single high dose (10×, 25× or 50×EC_{50}) of each compound was added to HIV-infected PBMCs and virus growth was measured weekly for 65 days (9 weeks). Even with the lowest dose of 10×EC_{50}, breakthrough infection did not occur for nevirapine, efavirenz, dapivirine, etravirine, rilpivirine, lersivirine, MIV-170 and UAMC01398. These observations are in contrast to an earlier report, where viral breakthrough was observed with relatively low doses of nevirapine (~30×EC_{50} WT), efavirenz (~500×EC_{50} WT), etravirine (~65×EC_{50} WT) and rilpivirine (~10×EC_{50} WT) over a 32 day period.[25] No viral replication was observed at higher doses of etravirine (>300×EC_{50} WT) and rilpivirine (>30×EC_{50} WT). Differences in the experimental set-up, i.e. choice of susceptible cell (MT4 cells instead of PBMCs) and assessment of cytopathic effect instead of p24 ELISA quantification likely explain the different results. Our assay with primary activated PBMCs most likely resembles the *in vivo* situation more closely.

Given that multiple mutations are required in concert for resistance to UAMC01398 and the low frequency of most of these mutations in WT HIV (Stanford University HIV Drug Resistance Database), it is unlikely that resistance selection will occur upon exposure to a single high dose of compound in the context of a topical application. In practice, the concentration of an antiviral in a microbicide will be at least 10^3–10^5-fold higher than the EC_{50} of the product. However, more lengthy exposure to subinhibitory concentrations may select for resistance over time, especially when pre-existing NNRTI-resistance mutations are present, e.g. in an individual on an NNRTI-containing regimen. Resistance mutations may persist for a variable time following transmission, but almost all come with a fitness cost.[28,29] Several large studies investigating transmitted drug resistance in Europe have found prevalence rates of around 10%, including NRTI, NNRTI and PI resistance.[30–33] A recent study in Barcelona showed low frequencies of the NNRTI RAMs K101E (0.5%), K103N (1.6%), E138A (1.6%–5.4%, depending on testing period), Y181C (1.6%) and G190A (0.5%) in acute and recently infected individuals.[33] In any case, we show here that UAMC01398 retains potent antiviral activity and is superior to dapivirine against viruses (and viral clones) that carry individual NNRTI RAMs and combinations thereof (Figure 4).

All of the mutations found during the dose-escalation studies with UAMC01398 have been reported before in the context of other NNRTIs (Stanford University HIV Drug Resistance Database).[16] M230I is an extremely rare mutation selected *in vitro* by rilpivirine,[26] and also by the structurally related diaryltriazine UAMC01398. Its effects on NNRTI susceptibility have not been well studied. Here, we show that the M230I mutation induces a ≥10-fold change for dapivirine, etravirine, rilpivirine, UAMC01398, lersivirine and MIV-170. The effect observed is similar to the fold change found with M230L. A recent study with doravirine and MK-1439, another new NNRTI that is currently in Phase 2b clinical trials, also reported the selection of M230I upon *in vitro* resistance induction with rilpivirine.[34] Doravirine resistance is mediated by V106A and either F227I or L234I. The study showed that doravirine-resistant viruses were susceptible to rilpivirine and efavirenz and mutants selected by rilpivirine and efavirenz were still susceptible to doravirine. Of note, two mutations in RT were sufficient for high-level resistance to doravirine, whereas at least four mutations were required for complete resistance to UAMC01398.

Acquiring resistance to UAMC01398 came with a severe fitness cost to the virus. When resistant virus was propagated to obtain larger volumes for subsequent antiviral experiments, it was noticed that the viruses only grew to very low titres in the absence of compound. This phenomenon was more pronounced for VI829, the virus carrying five mutations, than for Bal, with four mutations. Growth experiments in the presence of serial concentration dilution of UAMC01398 revealed that the resistant viruses replicated better in the presence of compound. Very poor replication and resulting low titres were observed between 0.01 and 100 nM of UAMC01398, whereas high titres were obtained when >100–1000 nM of UAMC01398 was added. Using SDMs combining the UAMC01398 RAMs, we could clearly show the replication disadvantage of resistant virus over WT HIV. Similar observations have been made with other antiretrovirals.[35,36] Of note, the clone

with the V75I mutation, a mutation not typically found with NNRTI resistance, was slightly more fit than the mutant clone lacking this mutation, suggesting a compensatory role. V75I was noticed only in the subtype C virus (VI829) and appeared early in resistance development (first detected on day 38). Although V75I is selected as part of the Q151M multi-NRTI resistance complex and has been described to emerge under selective pressure of the guanosine analogue aciclovir,[17,18] it has not been described in association with NNRTI-resistance mutations. Our experiments with a V75I SDM showed that this mutation by itself does not impact the antiviral activity of dapivirine, etravirine, rilpivirine, UAMC01398, lersivirine and MIV-170.

In summary, the data presented here show that UAMC01398 has potent activity against dapivirine-resistant HIV, that four or five mutations must be selected in concert for resistance to occur and that the resistance profile is similar to that of rilpivirine, both genotypically and phenotypically. Resistance development against UAMC01398 is associated with a severe fitness cost. This, together with its enhanced safety profile and good solubility in aqueous gels,[5,6,24] leads us to believe that UAMC01398 is an excellent candidate for HIV topical prevention.

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Transparency declarations

J. H. is a former employee of the Janssen Center for Molecular Design, a Johnson & Johnson company, and is currently a Johnson & Johnson shareholder. J. H. is co-inventor of dapivirine, etravirine and rilpivirine. K. K. A., M. V., J. J., P. V. d. V., K. A. and G. V. are co-inventors of UAMC01398. All other authors: none to declare.

Supplementary data

Table S1 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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