

Diaryltriazine non-nucleoside reverse transcriptase inhibitors are potent candidates for pre-exposure prophylaxis in the prevention of sexual HIV transmission

Kevin K. Ariën^{1*}, Muthusamy Venkatraj², Johan Michiels¹, Jurgen Joossens², Katleen Vereecken¹, Pieter Van der Veken², Saïd Abdellati³, Vicky Cuylaerts³, Tania Crucitti³, Leo Heyndrickx¹, Jan Heeres², Koen Augustyns², Paul J. Lewi^{2†} and Guido Vanham^{1,4}

¹Department of Biomedical Sciences, Institute of Tropical Medicine, B-2000 Antwerp, Belgium; ²Laboratory of Medicinal Chemistry, University of Antwerp, B-2000 Antwerp, Belgium; ³Department of Clinical Sciences, Institute of Tropical Medicine, B-2000 Antwerp, Belgium; ⁴Department of Biomedical Sciences, University of Antwerp, B-2000 Antwerp, Belgium

*Corresponding author. Tel: +32-3-2476631; Fax: +32-3-2476333; E-mail: karien@itg.be

†This article is dedicated to the memory of Dr Paul J. Lewi (1938–2012), former vice-president of the Janssen Center for Molecular Design and Guest Professor at the KU Leuven, the Vrije Universiteit Brussel (VUB) and the Universiteit Antwerpen (UA). Dr Lewi's enthusiasm for science remains a source of inspiration for all of us.

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Objectives: Pre-exposure prophylaxis and topical microbicides are important strategies in the prevention of sexual HIV transmission, especially since partial protection has been shown in proof-of-concept studies. In search of new candidate drugs with an improved toxicity profile and with activity against common non-nucleoside reverse transcriptase inhibitor (NNRTI)-resistant HIV, we have synthesized and investigated a library of 60 new diaryltriazine analogues.

Methods: From this library, 15 compounds were evaluated in depth using a broad armamentarium of *in vitro* assays that are part of a preclinical testing algorithm for microbicide development. Antiviral activity was assessed in a cell line, and in primary human cells, against both subtype B and subtype C HIV-1 and against viruses resistant to therapeutic NNRTIs and the candidate NNRTI microbicide dapivirine. Toxicity towards primary blood-derived cells, cell lines originating from the female reproductive tract and female genital microflora was also studied.

Results and conclusions: We identified several compounds with highly potent antiviral activity and toxicity profiles that are superior to that of dapivirine. In particular, compound UAMC01398 is an interesting new candidate that warrants further investigation because of its superior toxicity profile and potent activity against dapivirine-resistant viruses.

Keywords: triazines, NNRTIs, microbicides, cytotoxicity, lactobacilli

Introduction

In the absence of a preventive vaccine and in circumstances where systematic and correct condom use is difficult, an effective microbicide and oral pre-exposure prophylaxis (PrEP) may be the best way to offer a discreet method for protection against HIV infection.^{1–3}

The nucleotide reverse transcriptase inhibitor tenofovir was the first antiretroviral shown to prevent HIV infection in women, although the effect was only partial, when applied as a 1% gel intravaginally and using a specific, coitus-dependent dosing scheme.⁴ Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are widely used to treat HIV infection and some have also undergone

preclinical evaluation as topical (dapivirine, UC781, MIV-150 and MC1220)^{5–9} or intramuscular PrEP (rilpivirine, TMC278).¹⁰ Dapivirine (TMC120), a diarylpyrimidine (DAPY), is the most advanced in terms of (pre)clinical development.¹¹ The structurally different DAPYs etravirine (TMC125) and rilpivirine are used for HIV therapy. NNRTIs currently in development as microbicides are all extremely active against wild-type viruses and against some strains resistant to first-generation NNRTIs. By contrast with their favourable *in vitro* activity and toxicity profile, efficacy studies with UC781, MIV-150 and MC1220 in non-human primates have shown only partial protection upon vaginal and/or rectal challenge.^{7,8,12–15} No data from non-human primate efficacy studies with dapivirine have to date been reported, but a Phase 3 clinical

trial with a dapivirine ring started in early 2012 (<http://www.mtnstopshiv.org/node/4546>).

Occasional and incorrect usage of topically applied products might result in cross-resistance towards drugs used for HIV treatment.^{16,17} Therefore, NNRTIs dedicated exclusively to topical use are to be preferred. For this purpose we have investigated a series of novel diaryltriazine (DATA) analogues as potential intravaginal microbicides. DATAs are structurally different from the DAPYs dapivirine, etravirine and rilpivirine.

Here, we report on the anti-HIV activity and cellular toxicity profile of novel DATA analogues. We identified several highly potent compounds with a toxicity profile superior to that of the most advanced NNRTI-based microbicide candidate, dapivirine.

Materials and methods

Compounds

The NNRTIs dapivirine, rilpivirine and etravirine were obtained from Janssen Infectious Diseases, Beerse, Belgium. The phenylethylthioureathiazole (PETT) MIV170 was obtained from Medivir AB, Sweden. Lersivirine and the library of 60 triazine analogues were synthesized by the Medicinal Chemistry Group of Antwerp University, as per previously reported procedures.^{18–20} All compounds were dissolved in DMSO as 10 mM stock solutions and stored at -20°C .

Viruses

The prototypic subtype BCCR5-tropic (R5) virus Bal was used throughout the entire screening of the NNRTI library. A selection of compounds was further tested against an R5 subtype C primary isolate (VI829) and against NNRTI-resistant viruses (i.e. Bal-V106A, VI829-Y181C, Bal-L100I+K103N, Bal-K101E+K103KN+V108VI+V179M+Y181C+E138Q, VI829-L100I+E138K+T369I; as described previously by Selhorst *et al.*¹⁷). A replication-competent site-directed mutant (SDM) virus carrying the prototypic dapivirine resistance-associated mutation E138K was made, starting from wild-type pNL4.3-VifA²¹ and using QuickChange II Site-Directed Mutagenesis technology (Agilent Technologies).

Cell lines and primary cells

TZMbl cells (obtained through the NIH AIDS Research and Reference Reagent Program, Germantown, MD, USA) were used for the evaluation of the anti-HIV activity of a series of triazines. TZMbl cells were cultured in Dulbecco's minimum essential medium (DMEM) (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 $\mu\text{g}/\text{mL}$ of gentamicin, and incubated at 37°C in a humidified 7% CO_2 environment. Cells were split twice a week and plated at 10^6 cells in tissue culture flasks and at 10^4 cells in 96-well plates.

The human endometrial adenocarcinoma cell line HEC-1A (obtained through the ATCC, Teddington, UK) was maintained in McCoy's 5A modified medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% FBS, 100 U/mL of penicillin and 100 $\mu\text{g}/\text{mL}$ of streptomycin.

The human vaginal epithelial cell line VK2/E6E7 (provided by Dr R. Fichorova, Harvard Medical School, USA) was maintained in keratinocyte serum-free medium (Invitrogen) supplemented with 100 U/mL of penicillin, 100 $\mu\text{g}/\text{mL}$ of streptomycin, 0.05 mg/mL of bovine pituitary extract (BPE) (Invitrogen) and 0.1 ng/mL of epidermal growth factor (EGF) (Invitrogen), as described previously.²²

Human peripheral blood mononuclear cells (PBMCs) were used to propagate HIV stocks and to determine the antiviral activity of a selection of compounds. PBMCs were isolated from buffy coats from HIV-seronegative blood

donors (provided by the Antwerp Blood Transfusion Centre) using Ficoll density gradient centrifugation. PBMCs were stimulated for 48 h with 2 $\mu\text{g}/\text{mL}$ of phytohaemagglutinin (PHA) (Remel, Kent, UK) in RPMI 1640 medium containing 10% FBS, 50 $\mu\text{g}/\text{mL}$ of gentamicin and 2 $\mu\text{g}/\text{mL}$ of polybrene (Sigma-Aldrich, Bornem, Belgium). Subsequently, PBMCs were activated for 24 h with 1 ng/mL interleukin 2 (IL-2) (Gentaur, Brussels, Belgium) in RPMI 1640 medium supplemented with 10% FBS, 50 $\mu\text{g}/\text{mL}$ of gentamicin, 2 $\mu\text{g}/\text{mL}$ of polybrene and 5 $\mu\text{g}/\text{mL}$ of hydrocortisone (Calbiochem, Leuven, Belgium). PHA/IL-2-activated PBMCs were plated at 10^5 cells per well in 96-well plates for the antiviral activity assays.

For co-cultures of dendritic cells (DCs) and autologous CD4+ T lymphocytes, PBMCs were isolated as described above. Next, monocytes were separated from these PBMCs by magnetic bead isolation using CD14 MicroBeads (Miltenyi Biotec, Leiden, the Netherlands), according to the manufacturer's instructions. Part of the PBMCs was frozen as a source for autologous CD4+ T lymphocytes. Monocytes were differentiated in DCs by treatment with 20 ng/mL each of granulocyte-macrophage colony stimulating factor (GM-CSF) (Leucomax, Novartis, Belgium) and interleukin 4 (IL-4) (Immunosource, Zoersel, Belgium) for 7 days. Autologous CD4+ T cells were isolated from the frozen PBMC fraction using CD4 Dynabeads (Invitrogen) following the manufacturer's instructions. DCs and CD4+ T cells were co-cultured at a 1:3 ratio.

293T cells were cultured in DMEM (Sigma-Aldrich) containing 1% L-glutamine, 10% heat-inactivated FBS and 50 $\mu\text{g}/\text{mL}$ of gentamicin, and were incubated at 37°C in 5% CO_2 . In 6-well plates, 2×10^5 293T cells were transfected with 1 μg of pNL4.3-VifA-E138K DNA using FuGENE[®]-6 (Promega) 24 h after plating the cells. Supernatant was harvested 48 h later and passed over a 0.45 μm filter.

Antiviral activity assay

The antiviral activity of a series of 60 triazine analogues and the reference NNRTIs dapivirine, rilpivirine, etravirine, lersivirine and MIV170 was determined by pre-incubating 10^4 TZMbl cells per well in a 96-well plate for 30 min at 37°C in 7% CO_2 with or without a serial dilution of compound. Next, 200 TCID₅₀ (50% tissue culture infective dose) of virus (wild-type, resistant or SDM) was added to each well and cultures were incubated for 48 h before luciferase activity was quantified. Each compound was tested in triplicate and each experiment was repeated in at least three independent runs. Antiviral activity was expressed as the percentage of viral inhibition compared with the untreated control and plotted against the compound concentration. Next, non-linear regression analysis was used to calculate the 50% effective concentration (EC₅₀).

A selection of 15 triazines and the 5 reference compounds were assayed for antiviral activity in PBMCs and co-cultures of DCs and CD4+ T lymphocytes.

Antiviral activity in cervico-vaginal lavage (CVL) fluid

The antiviral activities of dapivirine, etravirine, rilpivirine, lersivirine, MIV170 and UAMC01184 were also evaluated in the presence of CVL fluid. CVL fluid was obtained from healthy HIV-negative Caucasian women, and was pooled and stored at -80°C .²³ Compound dilutions were incubated in pure CVL fluid for 2 h or 24 h at 37°C in 7% CO_2 . Subsequently, compound/CVL fluid mixtures were added to TZMbl cells with a final concentration of CVL fluid of 10%. Next, 200 TCID₅₀ of virus was added to each well and cultures were incubated for 48 h before luciferase activity was quantified. Each compound was tested in triplicate. Antiviral activity was expressed as the percentage of viral inhibition compared with the untreated control and plotted against the compound concentration. Next, non-linear regression analysis was used to calculate the EC₅₀.

Water-soluble tetrazolium-1 (WST-1) proliferation assay

The WST-1 cell proliferation assay is based on the cleavage of the tetrazolium salt WST-1 to a formazan dye by a complex cellular mechanism. Because this bioreduction is dependent on the glycolytic production of NAD(P)H in viable cells, the amount of formazan dye formed correlates directly with the number of viable cells in a culture. Quantification is achieved by measuring absorbance at 450 nm in a multi-well plate reader. TZMbl cells (10^4 cells/well), VK2 cells (10^4 cells/well), HEC-1A cells (7×10^3 cells/well) or PBMCs (7.5×10^4 cells/well) were plated in a 96-well plate and a serial dilution of compound was added. Cell proliferation reagent was added 24 h (VK2 and HEC1A), 48 h (TZMbl) or 1 week (PBMCs) later, and cell viability was measured compared with untreated control cultures. Cell viability was plotted against the compound concentration and non-linear regression analysis was performed to calculate the 50% cytotoxic concentration (CC_{50}).

Bacterial growth inhibition assay

Bacterial strains were obtained from the Laboratorium voor Microbiologie, Universiteit Gent, Belgium, the University of Göteborg, Sweden and the laboratory collection (Table S1, available as Supplementary data at JAC Online). The strains were grown on Columbia agar (Oxoid, Erembodegem, Belgium) supplemented with 5% horse blood in an anaerobic atmosphere using the Anaerocult A (VWR International, Leuven, Belgium) at $35 \pm 2^\circ\text{C}$. Tenfold serial dilutions were tested of the reference NNRTIs dapivirine, lersivirine and MIV170 and the triazines UAMC00838, UAMC01009 and UAMC01184 in trypticase soy agar (TSA) (Becton Dickinson, Erembodegem, Belgium) supplemented with 5% horse blood. The strains suspended in Mueller–Hinton broth (Becton Dickinson) were inoculated (10^4 cfu) using a multipoint inoculator (Elscolab, Kruibeke, Belgium) on the TSA blood agar. The plates were incubated at $35 \pm 2^\circ\text{C}$ in an anaerobic atmosphere for 48 h. In addition, TSA blood plates without compounds were inoculated and incubated under the same conditions to control for growth of the strains. The MIC was determined as the lowest concentration of compound at which growth of the microorganism was inhibited.

Results

Antiviral activity

Screening a library of DATA NNRTIs

A series of 60 DATA analogues was synthesized and investigated for anti-HIV activity in a primary screening assay using TZMbl reporter cells. A rapid but basic toxicity evaluation was done on all active compounds using a cell proliferation-based assay (WST-1 assay) and selectivity indices (SIs) were calculated.

The structurally related DAPYs etravirine, rilpivirine and dapivirine, the structurally different PETT MIV170, and the pyrazole lersivirine were used as comparators (Figure S1, available as Supplementary data at JAC Online). It was investigated whether the replacement of the *p*-methyl group in the prototypic DATA UAMC00529 (Figure S2, available as Supplementary data at JAC Online) by a cyanovinyl group could increase the antiviral activity, analogous to the cyanovinyl substitution in the DAPY rilpivirine, and whether the addition of an amino group in the 6-position of the central triazine ring could decrease cellular toxicity.

The structure, EC_{50} , CC_{50} and SI of the five reference compounds are shown in Figure S1 (available as Supplementary data at JAC Online). As expected from previous studies, the three DAPYs, MIV170 and lersivirine proved to be highly active *in vitro*, with

EC_{50} values below 10 nM. The highest SIs were found for MIV170 (SI=100000) and lersivirine (SI=31250). Of note, the SI of the microbicide candidate dapivirine (SI=1438) was substantially lower than that of the other two DAPYs used for HIV therapy, etravirine (SI=3177) and rilpivirine (SI=10747).

All of the 60 experimental triazines showed anti-HIV activity (with $EC_{50} < 10 \mu\text{M}$), of which 31 had very potent activity ($EC_{50} < 10 \text{ nM}$) comparable to the anti-HIV activity of dapivirine ($EC_{50} = 2.0 \text{ nM}$). The library was divided into nine subgroups (A–I), based on the substitution pattern (Figure S2A–I, available as Supplementary data at JAC Online).

All but two of the group A compounds had potent antiviral activity ($EC_{50} < 10 \text{ nM}$) comparable to the reference compounds dapivirine, etravirine, rilpivirine, MIV170 and lersivirine. Compound UAMC01008 showed anti-HIV activity with an EC_{50} of $\sim 1 \mu\text{M}$, suggesting that a chloro group in the X position combined with 'O' in the Y position significantly decreases the activity. Comparison of the cytotoxicity (CC_{50}) values of UAMC00529 (a triazine scaffold) and dapivirine (a pyrimidine scaffold) showed that UAMC00529 is ~ 10 -fold less toxic than dapivirine. Interestingly, replacing the 'H' by 'NH₂' in the X position and maintaining 'NH' in the Y position further decreased the CC_{50} value (UAMC00522; $CC_{50} = 4.4 \times 10^4 \text{ nM}$), suggesting that these substitutions are beneficial with regard to cytotoxicity.

Compounds from group B had a more diverse spectrum of antiviral activity and cytotoxicity. Of note, the introduction of bromo substituents at positions 2 and 6 of the left aromatic ring only minimally affected antiviral activity but did cause an increase in cytotoxicity and hence lowered the SI of the otherwise highly potent compounds UAMC00545, UAMC00523 and UAMC01015.

In group C, the influence of the cyano group on antiviral activity was studied. Removal of the cyano group from the *para* position ($R_3 = \text{H}$) and moving it to the *meta* position ($R_4 = \text{CN}$) decreased the antiviral activity. For example, compounds UAMC01096 and UAMC01099 were 60-fold to 100-fold less active than the group A compound UAMC01009.

Group D contained four analogues with a bromo group in position 2 of the left aromatic ring, all having low nanomolar activity but unfortunately also considerable cytotoxicity.

Group E contained six analogues, of which UAMC00847, UAMC01024 and UAMC01029 were highly potent (1.5–3.5 nM EC_{50}) and had an excellent cytotoxicity profile ($CC_{50} > 100 \mu\text{M}$). Interestingly, all three compounds bear an 'NH₂' group in the X position.

Group F included 14 analogues, 10 of which had low nanomolar anti-HIV activity, with EC_{50} values ranging between 1.3 and 11.7 nM. Four analogues, having a chlorine and oxygen atom in positions X and Y, respectively, were clearly less potent. The cytotoxicity was fairly good, with CC_{50} values between 4.4 and 26.7 μM . Of note, compound UAMC01184 is of particular interest with an SI of 26655.

Groups G and H both consist of two analogues each. Only one compound (UAMC01030, group G) had low nanomolar activity and good cellular toxicity. Compounds UAMC01043 and UAMC01044 (Group H) were less active (μM EC_{50}) but also had a low cytotoxicity ($CC_{50} > 100 \mu\text{M}$).

Group I contained three compounds: UAMC00893 with low micromolar activity, and UAMC00894 and UAMC00951 with potent nanomolar antiviral activity, all associated with a good cytotoxicity profile.

In summary, <15 of 60 compounds from the library were found to have an SI superior to that of the microbicide candidate dapivirine and at least seven compounds had an SI >5000 (see Figure S2, available as Supplementary data at JAC Online). The compounds with an SI superior to that of the reference NNRTI dapivirine (SI = 1438) were selected for further analysis.

DATA NNRTIs are active against a range of HIV subtypes and in primary cells

The 15 selected compounds were further investigated for antiviral activity against a strain of HIV-1 subtype B and subtype C in TZMbl indicator cells. The EC₅₀ values of the five reference compounds and those of the 15 experimental compounds did not differ significantly for subtype B or C HIV-1, and all remained below 10 nM (Figure 1a).

Antiviral activity in PBMCs and in co-cultures of DCs and CD4+ T lymphocytes overall confirms the potent antiviral activity of the reference and experimental compounds. Of note, the EC₅₀ of lersivirine increased 7.6-fold to 24.4 nM in PBMCs and almost 4-fold to 12.0 nM in DC/lymphocyte co-cultures, compared with TZMbl cells. From the triazines, 11 compounds had decreased antiviral activity in PBMCs. The increase in EC₅₀ values in PBMCs versus TZMbl cells averaged around 16-fold (range: 8.3-fold to 29-fold), but still resulted in highly potent viral inhibition in the low nanomolar range. Interestingly, only 5 of the 11 compounds with increased EC₅₀ values in PBMCs also had higher EC₅₀ values in DC/lymphocyte co-cultures, although much more modestly (range: 2.8-fold to 5.2-fold). The remaining six compounds had EC₅₀ values comparable to the antiviral activities found in TZMbl cells (Figure 1b). UAMC00522, UAMC00523 and UAMC01184 were the most active under these different conditions.

Antiviral activity in CVL fluid

The effect of the cervico-vaginal environment on the anti-HIV activity of the reference compounds dapivirine, etravirine, rilpivirine, lersivirine and MIV170, and the DATA UAMC01184, was also evaluated using an adapted antiviral assay. Compounds were first incubated in CVL fluid for 2 h or 24 h at 37°C and residual antiviral activity was subsequently measured in the TZMbl assay, as described elsewhere in this article. Exposure of the microbicide candidate dapivirine to CVL fluid for 2 h or 24 h did not affect the anti-HIV activity (Figure 1c). Similarly, the activity of our triazine UAMC01184 did not change after CVL fluid exposure. Of note, small fold changes (FCs) in EC₅₀ were observed for etravirine (1.8-fold to 5.9-fold) and lersivirine (2.9-fold to 3.1-fold) upon incubation with CVL fluid, whereas rilpivirine and MIV170 remained equally active.

DATA analogues are highly active against nevirapine- and efavirenz-resistant HIV

With NNRTI being a current backbone of highly active antiretroviral therapy (HAART), new-generation NNRTIs should be evaluated for their activity against resistant viruses, regardless of whether they are developed for oral or topical use. In this respect, we tested our 15 triazines against a series of NNRTI-resistant viruses that we generated in an earlier study.¹⁷ Resistance was induced *in vitro* against the NNRTIs most commonly used for the treatment

of HIV infection, i.e. nevirapine and efavirenz. We performed EC₅₀ assays using two resistant strains, one with the V106A mutation and the other with the Y181C mutation. Whereas no changes in antiviral activity were seen for the reference compounds dapivirine, etravirine, rilpivirine and MIV170 against the V106A mutant strain, an EC₅₀ FC of 11 was observed for lersivirine. Very minor FCs (ranging between 3 and 8) were found for all the reference compounds against the Y181C mutant virus (Figure 1d).

All experimental compounds remained highly active against the V106A and Y181C mutant viruses, with nM and sub- μ M activities. For the V106A mutant, EC₅₀ FCs were <10, except for compound UAMC01030 (FC=31), but the antiviral activity remained <100 nM for all compounds. The Y181C mutant virus was also inhibited with EC₅₀ values <0.5 μ M by all triazine analogues. Interestingly, compound UAMC01184 remained single-digit nM active against both V106A (EC₅₀ = 1 nM; FC = 1) and Y181C (EC₅₀ = 4 nM; FC = 3) mutant viruses (Figure 1d).

Next, the inhibitory activity of all reference compounds and the 15 selected triazine analogues was evaluated against an HIV strain carrying the prototypic efavirenz-associated resistance mutations L100I and K103N. Significant FCs in activity were observed for dapivirine (FC=337) and MIV170 (FC=121), whereas etravirine (FC=9), rilpivirine (FC=4) and lersivirine (FC=28) lost less of their antiviral activity (Figure 1e). All but one of the triazines (UAMC01184) showed significant decreases in inhibitory activity against this L100I/K103N mutant virus. Compounds UAMC00847, UAMC01021, UAMC01024, UAMC01029, UAMC01030 and UAMC01080 lost all potency (EC₅₀ > 10 μ M), whereas a few compounds such as UAMC00838 (EC₅₀ = 290.3 nM), UAMC00523 (EC₅₀ = 601.2 nM) and UAMC01184 (EC₅₀ = 15 nM) were still able to inhibit the L100I/K103N mutant at sub-micromolar EC₅₀ values (Figure 1e).

DATA UAMC01184 retains potent activity against dapivirine-resistant virus

To further map the antiviral activity of our experimental compounds we evaluated the potency of a selection of compounds against an E138K SDM and against dapivirine-resistant strains. Virus was grown in the presence of escalating doses of dapivirine, and two resistant viruses were generated – both carrying a prototypic dapivirine resistance-associated mutation at position E138; Bal (K101E, K103KN, V108VI, E138Q, V179M, Y181C) and VI829 (L100I, E138K, T369I).¹⁷ The compounds that inhibited nevirapine- and efavirenz-resistant viruses with EC₅₀ values \leq 1 μ M were further tested for inhibition of E138K SDM virus and dapivirine-resistant strains. For all compounds tested against the E138K SDM, FCs in EC₅₀ were typically small (ranging between 2.5 and 7; Figure 1f). Whereas the EC₅₀ of dapivirine increased at least 500-fold to >1 μ M, etravirine (EC₅₀ = 76 nM and 93 nM), rilpivirine (EC₅₀ = 27 nM and 130 nM), lersivirine (EC₅₀ = 174.3 nM and 2.35 μ M) and MIV170 (EC₅₀ = 31.9 nM and 35.5 nM) could still potentially inhibit replication of the dapivirine-resistant viruses (Figure 1g).

Compounds UAMC00523, UAMC00838 and UAMC01184 retained sub-micromolar activity, whereas compound UAMC01009 had difficulty inhibiting the L100I/E138K/T369I mutant virus. Notably, compound UAMC01184 potently inhibited both dapivirine-resistant viruses with EC₅₀ values of 22.8 nM and

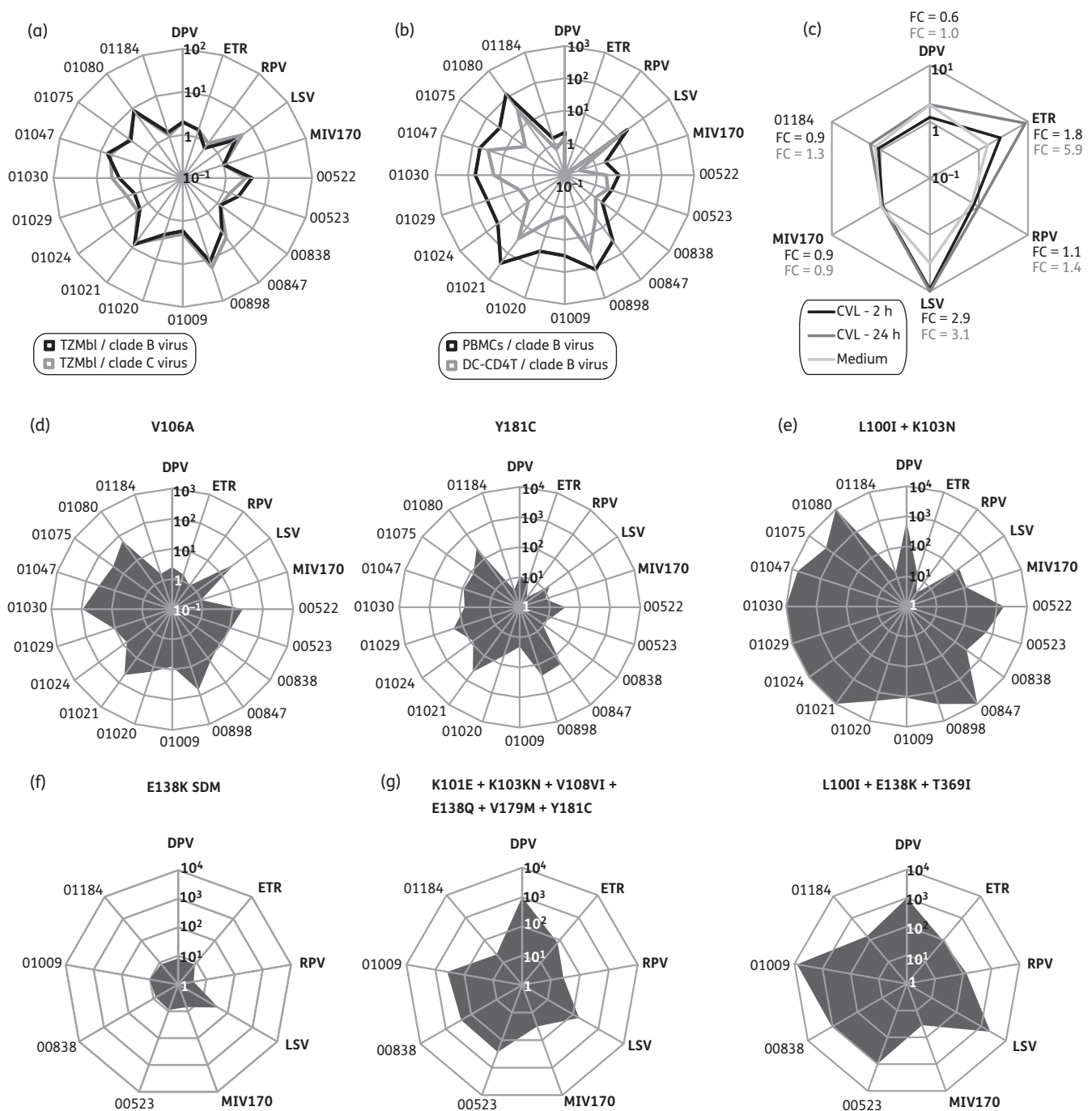


Figure 1. Antiviral activity (EC_{50}) of the reference compounds and the 15 most active triazine analogues against subtype B and subtype C HIV-1 in (a) TZMbl cells and (b) human PBMCs and co-cultures of DCs and autologous CD4+ T lymphocytes. (c) Anti-HIV activity in the presence of CVL fluid. Antiviral activity was determined in a TZMbl assay, after a 2 h or 24 h exposure of compound to CVL fluid, compared with cell culture medium alone. Corresponding EC_{50} (nM) values and FCs relative to the medium control are plotted. Antiviral activity (EC_{50}) of the reference compounds and 15 triazines against mutant HIV-1 carrying one or more mutations in the reverse transcriptase gene: V106A (d), Y181C (d) and L100I+K103N (e). The reference compounds and the four triazine analogues with sub-micromolar activity against the L100I+K103N mutant virus were also tested against an E138K SDM (f) and two dapivirine-resistant viruses (g). The axis shows drug concentrations in nM. Reference compounds are in bold. DPV, dapivirine; ETR, etravirine; RPV, rilpivirine; LSV, lesivirine.

149 nM (Figure 1g). These activities are similar to the activities found for the reference compounds etravirine, rilpivirine and MIV170.

Cellular toxicity

In addition to cytotoxicity measurements in TZMbl cells (see SI in Figure S2, available as Supplementary data at JAC Online), the

reference NNRTIs and the selection of 15 highly potent triazine analogues were further evaluated in different cytotoxicity assays. Given the past failure of several microbicide candidates in clinical trials, owing to unforeseen mucosal toxicity that occasionally increased susceptibility for HIV acquisition, a thorough toxicity evaluation is now mandatory. Here, we have investigated cytotoxic effects on PBMCs and on epithelial cells originating from the female genital tract, as well as the capacity to interfere with the growth kinetics of the most important microbial species colonizing the female genital tract.

Cytotoxicity towards primary blood cells and female genital tract epithelial cells

Serial dilutions of each compound were incubated with PHA/IL-2-activated PBMCs from the same blood donor, with VK2 cells (of vaginal origin) and with HEC-1A cells (of uterine origin). Cells were exposed to compound continuously for 7 days, followed by microscopic examination and a WST-1 cellular proliferation assay on day 7 post-exposure. In general, the reference NNRTIs lersivirine and MIV170 had a better cytotoxicity profile than the three DAPY NNRTIs (Figure 2a). Whereas in our assays there was apparently no cytotoxicity associated with lersivirine, the CC_{50} values of the PETT compound MIV170 were at least an order of magnitude lower. Interestingly, dapivirine appears to be less toxic than etravirine and rilpivirine on cells derived from the female genital tract.

From the DATA analogues, compounds UAMC00522, UAMC00847, UAMC00898, UAMC01024, UAMC01029, UAMC01030, UAMC01080 and UAMC01184 all have an excellent cytotoxicity profile. Of note, for compound UAMC00847 we could not demonstrate cytotoxicity in any of our assays. The SIs of UAMC00847, UAMC01024, UAMC01029 and UAMC01184 are at least 10-fold higher than that of dapivirine (Figure 2a). In conclusion, introduction of an 'NH₂' group to the triazine structure decreased cellular toxicity.

Bacterial growth inhibition

Growth inhibition of the vaginal microflora was not observed for the three reference NNRTIs, or for the DATA UAMC01184 (Figure 2b). Of note, due to limited solubility and solvent toxicity, the maximum dapivirine concentration that could be assessed was 20 μ M, whereas the other compounds could be tested at 100 μ M. The growth of *Lactobacillus iners* was somewhat inhibited by UAMC00838, UAMC01009 and UAMC01184, and the reference compound MIV170 tested at the highest concentration. UAMC00838 and UAMC01009 also interfered with the growth of *Lactobacillus vaginalis*, and the non-lactobacillus strains *Gardnerella vaginalis* and *Atopobium vaginae*. Furthermore, UAMC01009 affected the growth of the *Lactobacillus jensenii* clinical isolate only at the highest concentration tested, and not the reference *L. jensenii* strain (Figure 2b). Etravirine and rilpivirine were not tested because they are used exclusively for therapy and are not under development for intravaginal application to prevent transmission.

UAMC01184 geometric isomer separation

The most active triazine from our screen, UAMC01184, consisted of a mixture of both Z- and E-isomeric forms in an approximate 20/80

ratio. Z- and E-isomers were separated (UAMC01411 and UAMC01398, respectively) and both isomers were assayed for antiviral activity against wild-type HIV-1 (clade B and C) and against the panel of drug-resistant viruses described earlier (Figure 3). Interestingly, compared with the isomer mixture (UAMC01184), the E-isomer was equally active against wild-type HIV-1 and slightly more active against NNRTI-resistant viruses. Whereas the Z-isomer was equally active against wild-type HIV-1, its activity against resistant viruses was substantially decreased compared with the E-isomer and the isomer mixture. The E-isomer antiviral activity against the NNRTI-resistant strains was 4.6-fold to 18.1-fold better than that of the Z-isomer. Anti-HIV activity of the E-isomer in PBMCs and DC/CD4 T lymphocyte co-cultures was comparable to that of the isomer mix UAMC01184 (data not shown).

Cytotoxicity in TZMbl cells was also determined for both isomers, but no significant differences in CC_{50} values were observed (Figure 3).

Altogether, we present solid data showing that DATA analogues are potentially excellent microbicide candidates that may retain potent activity against dapivirine-resistant HIV, with a large SI and no significant bacterial toxicity, with good chemical stability and anti-HIV activity in conditions specific to the female genital tract.

Discussion

Since the success of the CAPRISA 004 trial with tenofovir gel, and of several oral PrEP trials with Truvada[®], reverse transcriptase inhibitor (RTI)-based regimens are regarded as a potentially promising biomedical tool in the prevention of HIV infection.⁴

Many extremely potent NNRTIs have been identified over the years and several of these have been tested in animal models for prevention of the sexual transmission of simian-human immunodeficiency virus (SHIV).^{5-9,12-15,24-26} Dapivirine, a DAPY compound previously owned by Tibotec BVBA (now Janssen Infectious Diseases) and donated to the International Partnership for Microbicides (IPM), is currently the most advanced NNRTI in development as a microbicide.²⁷ This development is supported by a substantial body of knowledge from cell cultures,²⁸⁻³¹ tissue explant cultures,^{6,32} small animal studies^{33,34} and Phase 1/2 clinical trials in humans.³⁵⁻³⁸ Although there are no publications on dapivirine use in non-human primates, IPM started with a Phase 3 clinical trial called ASPIRE, which will enrol ~3500 women at 17 sites in 5 African countries (<http://www.mtnstopshiv.org/studies/3614>).

We used dapivirine as the primary benchmark in our study, along with the related DAPYs etravirine and rilpivirine and the experimental NNRTIs lersivirine and MIV170. Several investigators have developed test algorithms, which include all of the tests described in this manuscript, to prioritize inhibitors for continued development as topical products for HIV prevention.³⁹⁻⁴²

We have investigated a series of novel triazines as potential intravaginal microbicides. The rationale for doing this was 3-fold. First, NNRTIs are conceptually interesting for use in topical prevention because they interfere with the pre-integration process of reverse transcription and can thus prevent infection from establishing irreversibly. Second, the partial successes

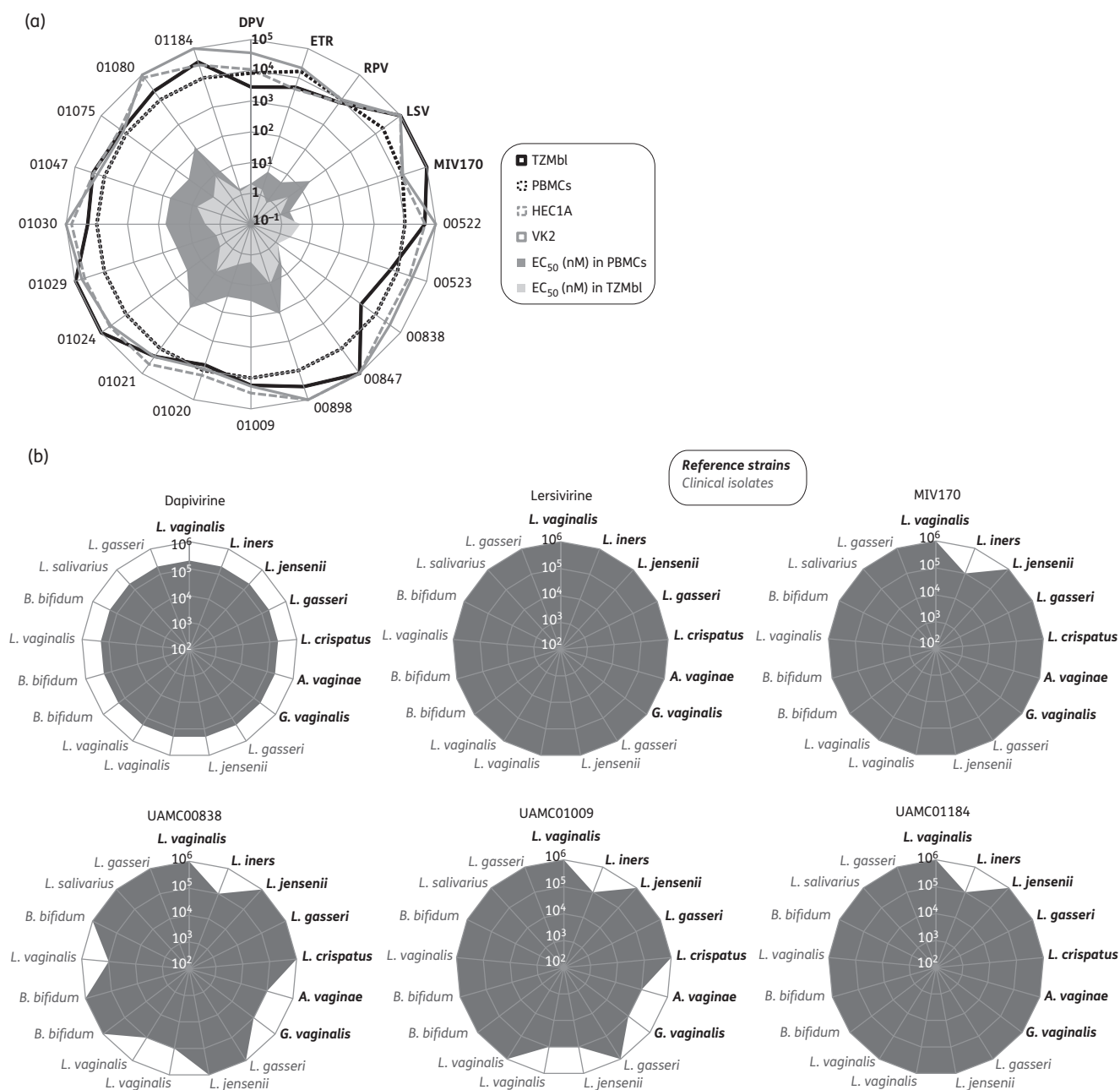


Figure 2. (a) Cytotoxicity (CC_{50}) results of the reference compounds and 15 triazines in TZMbl cells, PBMCs and female genital tract-derived cells (VK2 and HEC-1A). Corresponding EC_{50} values in TZMbl cells and PBMCs are also shown for convenience. The axis shows drug concentrations in nM. DPV, dapivirine; ETR, etravirine; RPV, rilpivirine; LSV, lersivirine. (b) Interference of the candidate microbicides dapivirine, lersivirine and MIV170 and triazine analogues UAMC00838, UAMC01009 and UAMC01184 with the growth of normal microflora from the female genital tract. The MIC was determined for each of the compounds. Drug concentrations are depicted in nM. Compounds were tested against seven reference strains of different *Lactobacillus*, *Atopobium* and *Gardnerella* (in black) and against 10 isolates of mainly *Lactobacillus* and *Bifidobacterium* (in grey). Strains are listed in Table S1, available as Supplementary data at JAC Online.

with NNRTI in preventing SHIV infection by mucosal challenge in non-human primates justifies further research to comprehend the reasons underlying incomplete protection. Investigating new molecules with a superior selective index and enhanced physico-chemical and formulation properties can hence give rise to improved bioavailability and higher tissue concentrations.

Third, with NNRTIs being a backbone for therapy in HIV clinical management and the relative ease with which HIV develops resistance, compounds with non-overlapping resistance profiles that are exclusively dedicated to either therapeutic or preventive usage should be developed.

The DATA analogues described in this work are novel structures that are chemically different from dapivirine. Whereas the potent

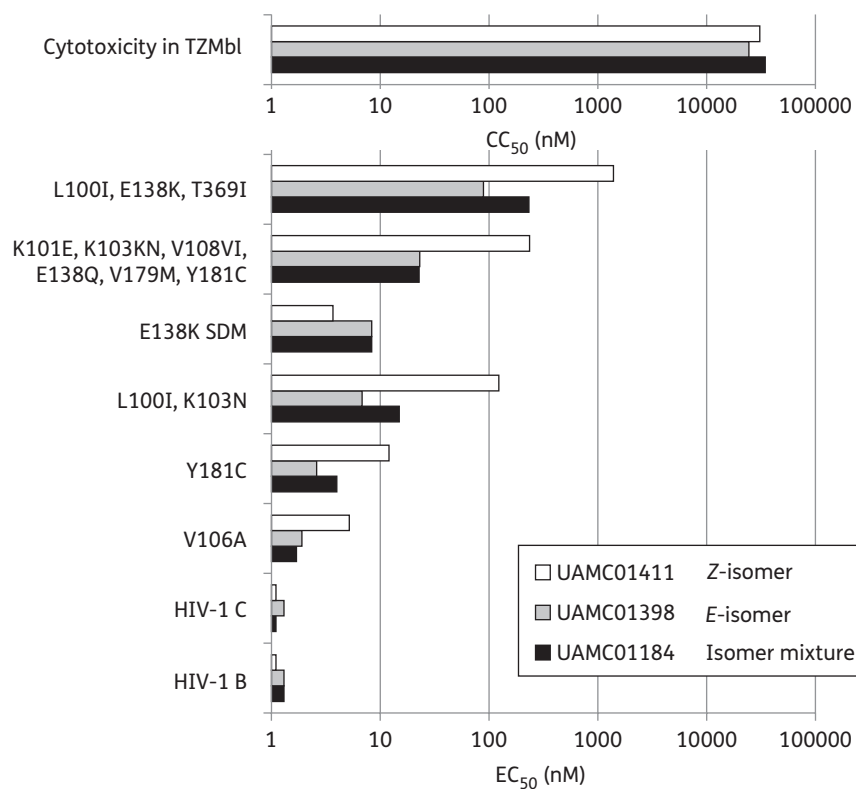


Figure 3. Antiviral activity and cellular toxicity of UAMC01398 (*E*-isomer) and UAMC01411 (*Z*-isomer) in comparison with UAMC01184 (isomer mixture). Antiviral activity (EC₅₀) against wild-type and drug-resistant viruses is plotted, as well as cellular toxicity (CC₅₀) in TZMbl cells.

anti-HIV activity is retained, many of the DATA analogues have an improved cellular toxicity profile, including in cells derived from the female genital tract, compared with the microbicide candidate dapivirine. A bacterial growth inhibition assay revealed that compound UAMC01184 does not interfere with the metabolism of the normal female genital tract microbiome, a pre-requisite for advancing a molecule in development for intravaginal use.

A particularly interesting observation concerns the activity of the DATA analogues UAMC00523, UAMC00838, UAMC01009 and UAMC01184 against two dapivirine-resistant viruses. Only compound UAMC01184 retained low nanomolar activity against these dapivirine-resistant viruses, with comparable or slightly better EC₅₀ values than etravirine and rilpivirine. Further purification of the *E*-isomer (UAMC01398) from the mixture revealed that the activity against NNRTI-resistant viruses was even slightly better compared with the isomer mixture. The chemical synthesis procedure has further been optimized to produce pure *E*-isomer (99% pure by liquid chromatography–mass spectrometry analysis).

An interesting series of pyrimidinediones (PYDs) have shown potent activity against the HIV-1 reverse transcriptase of different viral subtypes, and they also possess a second mechanism of action that targets viral entry.^{43,44} Johnson *et al.*⁴⁵ recently formulated IQP-0529 (PYD1) and IQP-0532 (PYD2) at different loading concentrations in a polyurethane intravaginal ring to determine PYD vaginal biodistribution in pigtail macaques. Kinetic analysis of vaginal proinflammatory cytokines, native microflora and drug levels suggested that all formulations were safe, but only the high-

loaded PYD2 (14wt%) intravaginal ring demonstrated consistently high PYD concentrations in vaginal fluid and tissues over the 28 day study. Unfortunately, no activity data on NNRTI-resistant viruses have been published. For the further development of this class of molecules as topical treatments to prevent HIV transmission, PYD activity against dapivirine-resistant HIV is required, given the lead in development of dapivirine as a microbicide over any other NNRTI.

We have reported previously on the cross-resistance of NNRTIs used in therapy and those developed for topical prevention.¹⁷ We showed that UC781 and MIV160, both structurally different from the DAPY scaffold, had difficulty inhibiting dapivirine-resistant virus, whereas MIV170 and the NRTI tenofovir were still active.

Although the results from the Phase 3 clinical trial with a dapivirine ring are not expected until late 2014 or early 2015, expectations for success are high and it is anticipated that dapivirine will raise the standards for microbicide development. In this respect, UAMC01398 warrants further investigation because of its superior toxicity profile, excellent chemical stability and activity in CVL fluid, and because it is active against dapivirine-resistant viruses, which might start circulating once dapivirine rings are used widely among high-risk populations. Together, our current data suggest that UAMC01398 is potentially an excellent successor to dapivirine. Ongoing research on UAMC01398 includes solubility determination, gel and ring formulation, up-scaling production, and pharmacokinetic/pharmacodynamic and vaginal challenge studies in macaques.

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Transparency declarations

J. H. and P. J. L. are former employees of the Janssen Center for Molecular Design, a Johnson & Johnson company; J. H. is currently a shareholder of Johnson & Johnson and P. J. L. was a shareholder of Johnson & Johnson. J. H. and P. J. L. are co-inventors of the DAPYs dapivirine, etravirine and rilpivirine. All other authors: none to declare.

Supplementary data

Table S1, Figure S1 and Figure S2 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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