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Development and *in vitro* evaluation of a vaginal microbicide gel formulation for UAMC01398, a novel diaryltriazine NNRTI against HIV-1

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ABSTRACT

Diaryltriazines (DATAs) constitute a class of non-nucleoside reverse transcriptase inhibitors (NNRTIs) that are being investigated for use as anti-HIV microbicides. The aim of the present study was (1) to assess the biopharmaceutical properties of the DATA series, (2) to select the lead candidate as vaginal microbicide and (3) to develop and evaluate gel formulations of the lead candidate. First, the vaginal tissue permeation potential of the different DATAs was screened by performing permeability and solubility measurements. To obtain a suitable formulation of the lead microbicide candidate, several hydroxyethylcellulose-based gels were assessed for their cellular toxicity, stability and ability to enable UAMC01398 epithelial permeation. Also, attention was given to appropriate preservative selection. Because of its favourable *in vitro* activity, safety and biopharmaceutical profile, UAMC01398 was chosen as the lead microbicide candidate among the DATA series. Formulating UAMC01398 as a vaginal gel did not affect its anti-HIV activity. Safe and chemically stable gel formulations of UAMC01398 (0.02%) included a non-solubilizing gel and a gel containing sulfobutyl ether-β-cyclodextrin (SBE-βCD, 5%) as solubilizing excipient. Inclusion of SBE-βCD in the gel formulation resulted in enhanced microbicide flux across HEC-1A epithelial cell layers, to an extent that could not be achieved by simply increasing the dose of UAMC01398. The applied rational (pre)formulation approach resulted in the development of aqueous-based gel formulations that are appropriate for further *in vivo* investigation of the anti-HIV microbicide potential of the novel NNRTI UAMC01398.

1. Introduction

Because of the increased burden of the HIV epidemic for women in developing countries, a female-controlled preventive tool such as vaginal microbicides is urgently needed (WHO, 2013). The development of a successful microbicide requires both a potent anti-HIV agent and a formulation strategy that ensures disposition of the active agent at the site of action upon vaginal application. Of the many existing vaginal drug delivery strategies, including vaginal rings, films and tablets, gel formulations remain the most commonly used, since this dosage form is cheap, highly accepted and easy to manufacture (Gafos et al., 2010; Vallely et al., 2012).

Recently, improved analogues of previously described diaryltriazine (DATA) compounds, a class of non-nucleoside reverse transcriptase inhibitors (NNRTIs), have been synthesized to be investigated as potential anti-HIV microbicides (Ludovici et al., 2001). Several of these DATA analogues, referred to as UAMC (University of Antwerp Medicinal Chemistry) compounds, possess antiviral activities in the low nanomolar range against both subtype B and C HIV-1. They also show an excellent safety profile towards peripheral blood mononuclear cells, vaginal cell lines and the vaginal microflora (Ariën et al., 2013). To translate the promising *in vitro* activity of the DATAs into *in vivo* efficiency as vaginal HIV preventive, their disposition upon vaginal application is critical. HIV transmission initiates with a small fraction of HIV virions or even a single virion migrating into the vaginal submucosa and founding an initial viral infection of subepithelially located target cells. This viral founder population can only establish systemic infection after local viral expansion (Miller et al., 2005). To target this early stage of infection, microbicides

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must be able to migrate into the vaginal submucosa and exert their activity at the level of the primary HIV target cells (Pillay et al., 2012). To assist in selecting the most appropriate DATA for further development as a microbicide, we therefore performed an *in vitro* profiling of biopharmaceutical properties that are major determinants of a compound's vaginal tissue uptake. A previously demonstrated approach, consisting of solubility determination in relevant media and permeation assessment across a layer of epithelial HEC-1A cells, was applied to compare the tissue permeation potential among a subset of DATAs. Dapivirine (DPV), an NNRTI belonging to the related class of diarylpyrimidines and currently in phase 3 clinical trials as a microbicidal vaginal ring, was included as a reference.

In addition to previously reported *in vitro* activity, toxicity and resistance data (Ariën et al., 2013), the biopharmaceutical profiling was employed to select the most promising DATA. Subsequently, a vaginal gel formulation was developed and evaluated *in vitro*. Since we previously demonstrated that the formulation of hydrophobic compounds in vehicles such as an emulsion and a silicone elastomere gel led to poor drug release, we here focused on aqueous-based vaginal gels (Grammen et al., 2012). Due to their limited vaginal retention time, gels are mainly intended for instant, coitus-dependent drug delivery. Therefore, formulations were optimized to provide rapid tissue uptake of the microbicide by screening several formulation excipients for their potential to enhance DATA flux across HEC-1A cell layers. Additionally, gels were assessed for their *in vitro* antiviral activity, toxicity and chemical stability.

2. Materials and methods

2.1. Drugs and excipients

The DATAs were synthesized and provided by the Laboratory of Medicinal Chemistry of the University of Antwerp (Belgium) (Ariën et al., 2013; Ludovici et al., 2001). DPV was obtained from Axon Medchem (Groningen, The Netherlands). The following chemicals were used in the formulations: hydroxyethylcellulose (HEC; Natrosol 250 HHX Pharm, Hercules, Wilmington, DE, USA), sodium sulfobutyl ether-β-cyclodextrin (SBE-βCD; Captisol, CyDex Pharmaceuticals Inc., Lenexa, KS, USA), 2-hydroxypropyl-β-cyclodextrin (2-HP-βCD), 2-hydroxypropyl-γ-cyclodextrin (2-HP-γCD), glycerin, nonoxynol-9 (tergitol, N-9), polyethylene glycol 300 (PEG300) (Sigma–Aldrich, St. Louis, MO, USA), polyethylene glycol 1000 (PEG1000; Acros Organics, Geel, Belgium), Noveon AA-1 and carbopol 1382 (Lubrizol Advanced Materials Europe, Brussels, Belgium). The preservatives sorbic acid, methylparaben and propylparaben were purchased from Sigma–Aldrich and propionic acid was obtained from Janssen Chimica (Geel, Belgium). The following media for cell culture and permeation experiments were obtained from Lonza (Basel, Switzerland): Hanks' balanced salt solution (HBSS), McCoy's 5A medium, penicillin–streptomycine, fetal bovine serum (FBS), and 4-(2-hydroxyethyl)piperazin-1-ethanesulfonic acid (HEPES). D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS) was received from Eastman Chemical Company (Kingsport, TN, USA).

2.2. Permeability assessment

To estimate the potential of DPV and the DATAs to permeate vaginal tissue, an *in vitro* dual chamber set-up containing a HEC-1A cell layer, was used. HEC-1A cells were obtained from the American Type Culture Collection (ATCC-LGC Promochem, Teddington, UK). Cell culture conditions and experimental procedures for permeability assessment were applied as previously described (Grammen et al., 2012). Briefly, 100,000 cells were seeded in Costar Transwell membrane inserts (12 well plate, 3 mm pore diameter, 12 mm diameter; Corning Inc., USA) and used for permeability assessment 9–10 days after seeding. Solutions of the DATAs (4 μM) in acidified HBSS (25 mM glucose, 50 mM acetate buffer, pH 4.2) were prepared from a DMSO stock solution, after which the samples were diluted with vaginal fluid simulant (VFS, dilution factor 5:2). VFS was prepared as described by Owen and Katz (1999). Following an equilibration step with acidified HBSS, full-grown HEC-1A cell layers were incubated with the DATA solutions (0.5 mL) at their apical side and with 1.5 mL neutral HBSS (25 mM glucose, 10 mM HEPES, pH 7.4), supplemented with TPGS (0.2%) to enhance solubility and prevent back flux, as the basolateral buffer. After 1 h, samples were taken from the apical and basal chamber. To measure intracellular compound accumulation, HEC-1A cells were rinsed and microbicides were extracted using 0.5 mL methanol–water (70:30%), while sonicating. Apical, basal and intracellular compound concentrations were determined using HPLC-UV/fluorescence (Section 2.9). Results were expressed as the compound fraction (in %) that was present apically, basolaterally and intracellularly in the dual chamber system after 1 h. To verify the integrity of the HEC-1A cell layer, transepithelial electric resistance values (TEER) were measured with a Millicell-ERS Volt-Ohm meter (Millipore, Bedford, MA, USA) before and after each experiment.

2.3. Solubility assessment

The solubilizing capacity of different formulation media (acetate buffer 50 mM pH 4.2, in the absence or presence of solubilizing excipients) for the DATAs and DPV was determined by adding an excess of compound to 200 μL of the respective media. Samples were shaken during 24 h (IKA KS 4000i control, 37 °C and 175 rpm). Subsequently, samples were centrifuged (21,000*g*, 37 °C, 12 min) to remove undissolved compound and an appropriate dilution of the supernatant was injected into the HPLC system (see Section 2.9).

2.4. Gel preparation of UAMC01398

Aqueous gels of UAMC01398 were prepared in an acetate buffer (50 mM, pH 4.2), containing a selected preservative (sorbic acid 0.2%, propionic acid 0.2% or the combination methylparaben 0.2%–propylparaben 0.05%), and sodium chloride for isotonicity. Cyclodextrins (CDs, 5% w/w) or PEG1000 (10% w/w) were added as solubilizing excipients if applicable. UAMC01398 was included at doses of 0.02%, 0.1% and 1% (corresponding to a theoretical concentration of 0.5, 2.6 and 26 mM, respectively). The pH was adjusted to 4.25 ± 0.05. After sonicating, media were incubated and shaken during 24 h (IKA KS 4000i control, 37 °C and 175 rpm) to reach equilibrium solubilities. Finally, HEC (1.8% w/w) was added to allow gel formation. Osmolality was measured using an Advanced Osmometer 3250 (Advanced Instruments, Norwood, MS). The composition and osmolality of the evaluated UAMC01398 gel vehicles is summarized in Table 2. Rheological properties of the gels at room temperature were assessed with the Rheomat 115 rotational viscometer using the DIN-114 measuring system. For comparison purposes, the commercial gels Replens® (Wolfs, Belgium) and Mithra Intim® (Mithra Pharmaceuticals, Belgium) were included. For the rheology and toxicity assays, a number of reference gels were prepared. The universal placebo (UP) gel consisted of sorbic acid (0.1%), sodium chloride (0.85%) and HEC (1.5%) (Tien et al., 2005). The DPV vehicle gel contained PEG300 (35%), HEC (1.5%), Noveon AA-1 (0.65%), carbopol 1382 (0.6%), methylparaben (0.2%) and propylparaben (0.05%) (composition as reported by Nel et al. (2010)). For the tenofovir (TFV) vehicle gel, only glycerin (20% and 5%), HEC (1.5%) and the conserving agent (methylparaben

0.18%–propylparaben 0.02%) were included, since the detailed composition is not reported in literature (Dezzutti et al., 2012). For both gels, the neutralized vehicles, i.e., the formulations without active compound, were used.

2.5. Flux assessment

The UAMC01398 flux from the different gel formulations across HEC-1A cell layers was assessed in the same *in vitro* set-up as described for the permeability experiments (Section 2.2). Prior to flux assessment, $CaCl_2$ (186 mg/L) and $MgSO_4$ (200 mg/L) were included in the formulations to maintain tight junctions between HEC-1A cells. Subsequently, gels were diluted with VFS (5:2) at the apical side of HEC-1A cell layers, while the basal chamber contained HBSS with TPGS (0.2%). After 1 h, samples were taken and analyzed to calculate fluxes, i.e., the amount of compound migrating across the HEC-1A layer per unit of time and surface area.

2.6. Toxicity evaluation

For toxicity assessment, 25,000 HEC-1A cells were seeded in 96-well plates (BD Falcon™, USA) and cultured for 2 days in McCoy's medium. Two colorimetric assays were used for the evaluation of the different formulations: the water-soluble tetrazolium-1 (WST-1) test and the lactate dehydrogenase (LDH) test. For the WST-1 test, gels were diluted (1:2) with McCoy's medium (supplemented with HEPES) and the pH was neutralized. N-9 (0.1%) in McCoy's medium and the universal placebo gel (UP, 1:2 dilution with McCoy's medium) were used as toxic and non-toxic controls, respectively. The neutralized gel vehicles of DPV and TFV were included as references. Cells were incubated during 24 h after which the WST-1 test was performed according to the manufacturer's instructions (Roche, Belgium). Briefly, cells were washed twice with neutral HBSS followed by a 3 h incubation period with the WST-1 reagent. Absorbance at 450 nm was measured using a microplate reader (Tecan Infinite M200, Tecan, Belgium). For the LDH assay, formulations were evaluated in absence of the gelling agent since supernatant needs to be collected after incubation. Formulations were diluted (1:2) with HEPES–buffered HBSS (pH 7.4) and were neutralized. Identical control and reference formulations as used for the WST-1 test were included. After a 24 h incubation period, the LDH assay was performed according to a previously described protocol (Ops Diagnostics, 2013). In brief, 50 μL of cell supernatans was collected and added to 150 μL of the LDH reagents mixture. After 5 min, absorbance was measured at 490 nm.

2.7. Antiviral activity assay

The antiviral activity of UAMC01398 gel formulations was tested in comparison with free UAMC01398 in solution and the gels without compound (blank gels). Serial dilutions of UAMC01398 gels, compound in solution and blank gels were incubated with 10^4 TZM-bl cells in a 96-well plate for 30 min at 37 °C and 7% CO_2. Next, 200 $TCID_{50}$ of HIV-1 Bal was added to each well and cultures were incubated for 48 h before luciferase activity was quantified. Antiviral activity was expressed as the percentage of viral inhibition compared to the untreated control and plotted against the compound concentration. Next, non-linear regression analysis was used to calculate the EC_{50}.

2.8. Stability of UAMC01398

For chemical stability assessment of UAMC01398 (0.02% dose) in the gel formulations, closed tubes were stored at 40 °C and samples were collected at 0–3 months. UAMC01398 was extracted from the gels as follows: the internal standard DPV was added to the gels from a DMSO stock solution, followed by extraction using methanol–water (50:50), centrifugation (21,000*g*, 37 °C, 5 min) and quantification using LC/UV (Section 2.9). The extraction procedure was validated at 200 μM and 700 μM, with accuracy and repeatability errors below 7% and 3%, respectively.

2.9. Analytical methods

Quantitative analysis of DPV and DATAs was performed by reversed phase HPLC using a Hitachi LaChrom Elite system with a Waters Nova-Pak® RP-18 column (100 × 8 mm, 4 μm) under radial compression (Waters, Milford, MA, USA) and UV/fluorescence detection. An injection volume of 50 μl and flow rate of 1 ml/min was used. The mobile phases contained methanol and acetate buffer (37 mM, pH 4.0) in a ratio of 89:11 for UAMC00889, 73:27 for UAMC01030 and UAMC01398, 83:17 for UAMC00847, UAMC00898, UAMC01009, UAMC01019, UAMC01020, UAMC 01047 and DPV, and 78:22 for all other DATAs. Detection occurred by UV (289 nm) absorbance measurement or, in case of DATA permeability assessment, by fluorescence measurement (excita-

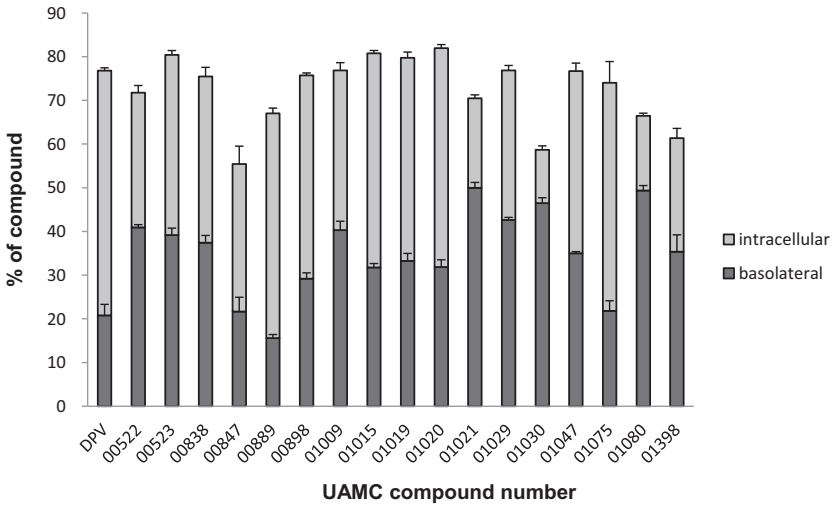
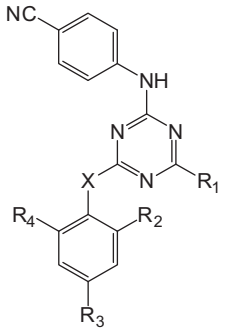


Fig. 1. Fraction of the DATAs present in the basal compartment and inside the HEC-1A cells of the dual chamber system after a 1 h incubation period with DATA solutions (4 μM). Data represent mean ± SD (n = 3).

Table 1
Structural characteristics and log*D* values of DATAs.



UAMC	X	R_1	R_2	R_3	R_4	log*D*[a]
00522	NH	NH_2	Me	Me	Me	3.71
00523	NH	NH_2	Br	Me	Br	4.26
00838	NH	NH_2	Br	Me	Me	3.99
00847	NH	NH_2	Br	Me	H	3.47
00889	NH		Br	Me	Br	3.18
00898	NH	$NH(CH_2)_3OMe$	Br	Me	Br	4.69
01009	O	NH_2	Me	Me	Me	5.35
01015	O	NH_2	Br	Me	Br	5.87
01019	O	NH_2	Me	Cl	Me	5.44
01020	O	NH_2	Me	Br	Me	5.60
01021	O	NH_2	Me	H	Me	4.84
01029	O	NH_2	Cl	Me	H	4.93
01030	O	NH_2	OMe	Me	OMe	4.02
01047	NMe	NH_2	Me	Me	Me	4.43
01075	NH	NH_2	Me	Br	Me	3.97
01080	NH	NH_2	Me	H	Me	3.20
01398	NH	NH2	Me	CH=CHCN (E)	Me	3.59
DPV						4.71

[a] Log*D* values were computationally calculated at pH 4.2 using MarvinSketch (version 5.9.3, ChemAxon, www.chemaxon.com).

tion 289 nm/emission 324 nm, except for UAMC01398: excitation 289 nm/emission 400 nm). For each compound, the analytical method was validated for the used concentration range. The presence of formulation excipients or VFS did not interfere with compound detection. Intraday repeatability and accuracy were assessed by analyzing standard samples ($n = 5$) and were below 5% at all concentrations.

2.10. Data analysis

Unless otherwise specified, data are presented as the mean ± standard deviation (SD) of three replicates. To compare multiple conditions, a one way Anova test followed by Dunnett's post test was performed. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Biopharmaceutical profiling of DATA compounds

3.1.1. Permeability assessment

In order to reach the primary HIV target cells, among which CD4+ T cells that are mainly located in the vaginal submucosa, NNRTI microbicides have to permeate across the multilayered vaginal epithelial tissue (Adams and Kashuba, 2012). As a biopharmaceutical selection criterion, we therefore assessed the permeability of a HEC-1A cell layer for the different DATAs, using DPV as the reference compound. Although the HEC-1A cell line originates from an endometrium carcinoma, it shows a limited multilayered structure and forms tight junctions between the cells, which is comparable to the vaginal epithelium (Gali et al., 2010a). Fig. 1 depicts the fractions of compound that permeated into the HEC-1A cells and across the epithelial layer towards the basal compartment during a 1 h apical incubation with DATA solutions at pH 4.2, i.e., the pH of the vaginal lumen. No large differences in the total permeated fraction (i.e., intracellular + basal fraction) could be observed among the different DATAs and DPV. Importantly, all DATAs and DPV exhibit excellent permeability (permeated fraction between 50% and 80%) when compared to some previously assessed microbicide candidates, including darunavir (basal fraction of 10%, intracellular fraction of 2%) and tenofovir (basal fraction of 2%) (Grammen et al., 2012). The relatively high permeability of the DATAs can be explained by their hydrophobicity ($\log D_{pH\ 4.2} > 3$, Table 1).

3.1.2. Solubility assessment – selection of UAMC01398 as lead compound

Since Fig. 1 indicates fast permeation of dissolved DATAs into and across epithelial cell layers, tissue uptake will mainly be determined by the DATA concentrations in solution that can be achieved in the vaginal lumen. Consequently, the ideal DATA microbicide candidate should possess a sufficiently high solubility in the vaginal gel that is used for application. Fig. 2 indicates that 4 DATAs, UAMC00522, UAMC01075, UAMC01080 and UAMC01398 were significantly better soluble than the reference compound DPV. However, in comparison with the solubility of, for instance, TFV (40 mM) (Grammen et al., 2012), DATA solubilities are low (1–100 μM range). Therefore, we investigated the capacity of formulation excipients to improve the solubility of these hydrophobic compounds. Addition of the cyclodextrins 2-HP-βCD, 2-HP-γCD and SBE-βCD (10%) or the cosolvent PEG1000 (20%) all significantly increased the solubility of the DATAs by, on average, 33-, 15-, 34- and 19-fold, respectively. It should be noted that the best soluble DATAs in the simple buffer solution, also reached the highest solubilities in presence of the excipients. Because of its favourable activity, toxicity and biopharmaceutical profile and, in particular, its superior activity against NNRTI-resistant viruses (Ariën et al., 2013), UAMC01398 was selected as the lead compound for further development of a microbicide gel. The increase in solubility of UAMC01398 in presence of formulation excipients is shown in

Table 2
Composition and characteristics of the evaluated gel vehicles for UAMC01398. UAMC01398 was included at doses of 0.02%, 0.1% or 1%.

	Non-solubilizing gel	2-HP-bCD gel	SBE-bCD gel	PEG1000 gel
HEC (%)	1.8	1.8	1.8	1.8
Solubilizing excipients (%)	–	2-HP-βCD (5%)	SBE-βCD (5%)	PEG1000 (10%)
Preservative (%)	All gels: propionic acid (0.2%) [also sorbic acid (0.2%) and methylparaben/propylparaben (0.2%/0.05%) were evaluated]			
NaCl (%)	0.6	0.5	0.2	–
Sodium acetate trihydrate (%)	0.68	0.68	0.68	0.68
pH	4.25	4.25	4.25	4.25
Osmolality (mOsm/kg)	338	356	350	377

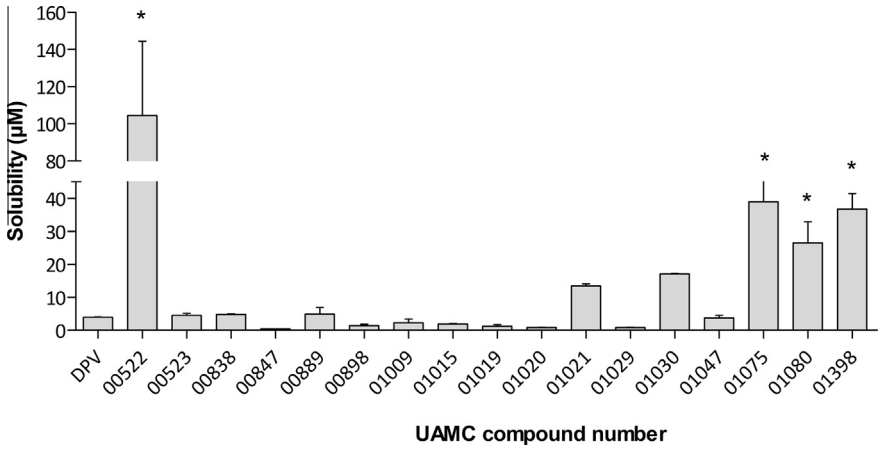


Fig. 2. Solubility of the DATAs in acetate buffer at pH 4.2. Data represent mean ± SD ($n = 3$). Statistical significant differences with the solubility of the reference compound DPV were evaluated using a one way Anova test followed by Dunnett's post test ($^*p < 0.05$).

Fig. 3. These UAMC01398 solubilities in the different formulations were found to be stable for at least 3 months. The most efficient solubilizing excipients, 2-HP-βCD, SBE-βCD and PEG1000 were selected for further evaluation.

3.2. In vitro evaluation of UAMC01398 gel formulations

As depicted in Table 2, UAMC01398 was formulated at different doses (0.02%, 0.1%, and 1%) in four different gel vehicles at pH 4.2: a non-solubilizing vehicle and three solubilizing vehicles, comprising 2-HP-βCD, SBE-βCD or the cosolvent PEG1000. Three candidate preservatives were assessed, of which propionic acid was finally selected for reasons that will be discussed in Section 3.2.6. In different *in vitro* set-ups, these UAMC01398 gel formulations were characterized with respect to rheology, microbicide disposition, antiviral activity, toxicity and stability.

3.2.1. Rheology

Fig. 4 depicts the shear stress and viscosity in function of shear rate of the four gel vehicles, compared to two commercial gels, Replens® and Mithra Intim®. The rheological assessment was also performed for the universal placebo gel (UP) and reference vehicles for DPV and TFV (5% or 20% glycerine) that have been used in clinical assessment (Nel et al., 2010; Abdool Karim et al., 2010). All gels displayed non-Newtonian, pseudoplastic behaviour. The viscosity of the four evaluated gels was comparable to that of Mithra Intim®, while the UP and the TFV vehicles showed lower viscosity values similar to Replens®. In contrast, the DPV vehicle was more viscous at low shear rates.

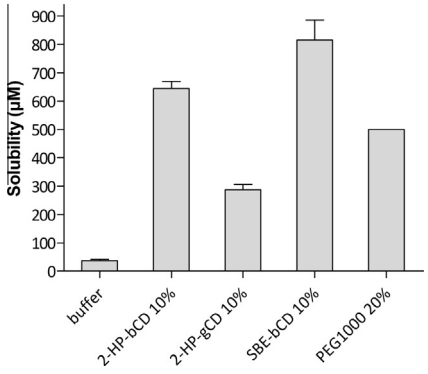


Fig. 3. Solubility of UAMC01398 at pH 4.2 in absence and presence of the formulation excipients 2-HP-bCD (10%), 2-HP-gCD (10%), SBE-bCD (10%), and PEG1000 (20%). Data represent mean ± SD ($n = 3$).

3.2.2. Flux assessment

Since the relatively limited retention time of coitus-dependent vaginal gels necessitates sufficiently fast tissue drug uptake, the main evaluation criterion comprised the UAMC01398 flux from the gel formulation across HEC-1A epithelial cell layers (i.e., the amount of UAMC01398 migrating per unit of time and surface area).

Firstly, the effect of different UAMC01398 doses (0.02%, 0.1% and 1%) suspended in the non-solubilizing HEC gel, was evaluated. As reported in Fig. 5, increasing compound loading enhanced the UAMC01398 flux. This finding is in agreement with the previously reported observation that higher drug doses of maraviroc in aqueous suspension gels generated increased plasma concentrations in macaques (Malcolm et al., 2013). For UAMC01398, however, solubilizing the microbicide by inclusion of CDs (5%) or PEG1000 (10%) in the low-dose gel (0.02% ≈ 500 µM) was a much more efficient approach to enhance compound flux than increasing the dose by 50-fold (Fig. 5; $p < 0.05$). This effect can be explained by the fact that only compound in solution can permeate into and across epithelial cells; simply enhancing the dose in suspension without any solubilizing strategy still requires the microbicide to dissolve prior to permeation. Consequently, inclusion of solubilizing excipients could allow a drastic reduction of drug loading in aqueous vaginal gels, while maintaining/enhancing drug uptake.

3.2.3. Toxicity evaluation

Ever since the failure of the N-9 gel in phase 3 clinical trials, due to increased risk on HIV transmission, safety towards the vaginal epithelial mucosa has become a central issue in the microbicide field (Morris and Lacey, 2010). To identify possible toxicity risks of the different proposed formulations of UAMC01398, we used two types of toxicity assays on the HEC-1A epithelial cell model. Firstly, the WST-1 test indicates toxic effects on mitochondrial metabolism since it is based on the cleavage of the tetrazolium salt WST-1 to a formazan dye by mitochondrial dehydrogenases in viable cells. In contrast, the LDH assay enables identification of the disruption of cell membranes since it measures the activity of LDH enzyme that has been released in the culture supernatant. Figs. 6 and 7 depict the viability/toxicity results of HEC-1A cells after a 24 h incubation with the evaluated formulations as determined by the WST-1 and LDH assay, respectively. In both tests, the addition of N-9 (0.1%) as toxic control resulted in a clear

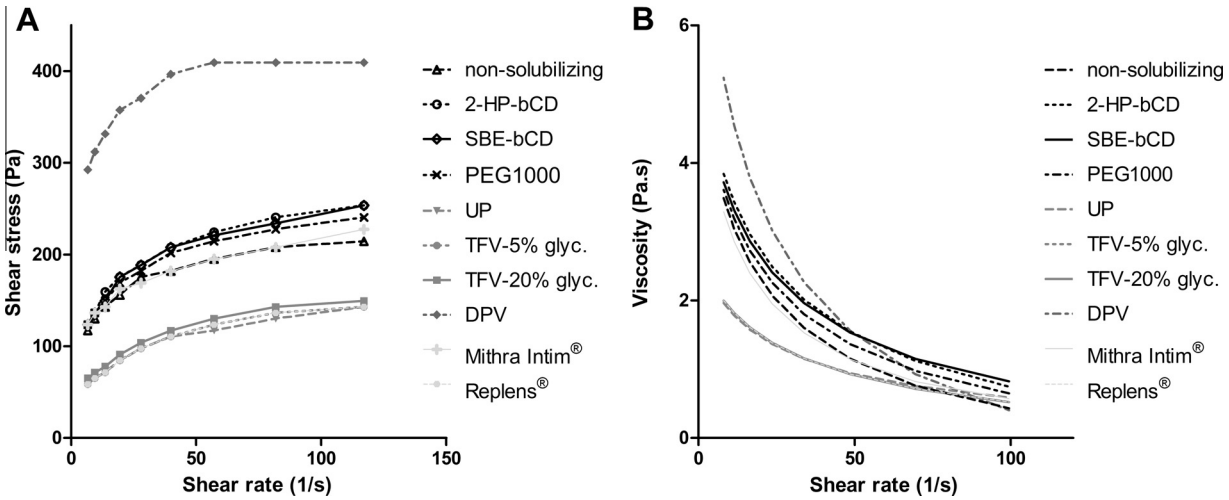


Fig. 4. Shear stress (A) and viscosity (B) in function of shear rate for the four evaluated gel vehicles of UAMC01398. Additional gels were included for comparison purposes: the commercial gels Replens® and Mithra Intim®, the universal placebo gel (UP) and the reference vehicles of TFV (two different concentrations of glycerin) and DPV that have been tested in clinical trials.

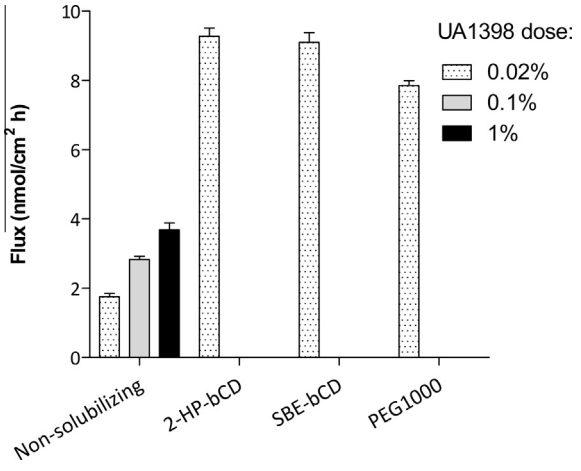


Fig. 5. UAMC01398 flux values from different formulations: non-solubilizing HEC gel with increasing UAMC01398 dose (0.02%, 0.1% and 1%) and HEC gels in presence of cyclodextrins (5%) or PEG1000 (10%) containing 0.02% UAMC01398 dose. The preservative used was propionic acid (0.2%). Data represent mean ± SD ($n = 3$).

reduction of viability, while the universal placebo as non-toxic control was found to be safe.

For formulating UAMC01398, the non-solubilizing vehicle and the solubilizing vehicles with SBE-βCD (5%) or PEG1000 (10%) were found to be safe *in vitro*. The vehicle containing 2-HP-βCD (5%) exerted toxic effects in both tests and was no longer considered appropriate. This observation is in accordance with the relatively low CC_{50} values of 2-HP-βCD that have been reported by Gali et al. (2010b). Including UAMC01398 at doses up to 1% did not reduce cell viability in the WST-1 assay. In contrast, the LDH assay did indicate an enhanced toxic effect for the 1% dose, presumably caused by physical rupture of the cell membranes due to the presence of a large amount of non-dissolved particles.

For comparison purposes, formulation vehicles that have been used for clinical evaluation of DPV and TFV, were included in the toxicity studies (Nel et al., 2010; Abdool Karim et al., 2010). Both DPV and TFV gel vehicles strongly decreased cell viability as measured by the WST-1 assay. This effect was observed to a lesser extent in the LDH assay. The observed toxicity may result from the use of parabens for preservation or from the hyperosmotic nature of the gels (1986 mOsm/kg as measured for the DPV vehicle; 836 mOsm/kg (5% glycerine) and 3111 mOsm/kg (20% glycerine) in case of the TFV gels, as reported by Dezzutti et al. (2012)). These *in vitro* observations suggest a smaller risk on *in vivo* toxicity for the proposed isotonic UAMC01398 formulations as compared to the DPV and TFV vehicles.

The DPV gel used in this study was evaluated in a phase 1 clinical trial and was considered safe and well tolerated, but no details about possible vaginal adverse events were mentioned (Nel et al., 2010). It is possible that the higher viscosity of this vehicle has limited the oxygen and nutritional uptake in the performed WST-1 assay. Despite the use of the TFV vehicles in clinical trials, the observed *in vitro* toxicity in the present study is not entirely surprising. The original TFV gel containing 20% of glycerin was considered safe based on *in vitro* toxicity tests even though viability assessment of cell lines (HEC-1A and Caco-2) and ectocervical or colorectal explants clearly demonstrated toxic effects even for a 1:5 dilution of the gel (Rohan et al., 2010). In a phase 1 clinical trial, a 2-week course of the TFV gel (20% glycerin) applied twice daily was considered well tolerated, although 92% of the participating women reported at least one adverse event (including vaginal discharge, pruritus or a burning sensation) (Mayer et al., 2006). To reduce the hyperosmotic nature of the original TFV gel, a 5% glycerin gel was proposed. Although strong dilutions (1:5–1:10) of this reformulated TFV gel did not cause toxicity towards cell line monolayers or colorectal and ectocervical tissues (Dezzutti et al., 2012), an *in vivo* evaluation in rabbits demonstrated vaginal mucosal damage for both TFV formulations (Clark and Friend, 2012). These findings question the relevance of using strongly diluted gels for *in vitro* safety evaluation; in the current study, gels were therefore only diluted 2-fold with culture medium.

It should be noted that, due to the limited multilayered structure of the HEC-1A epithelium, this cell line can be considered as a sensitive tool to detect possible toxicity issues. In comparison to the HEC-1A cell model used in the present study, more structurally similar *in vitro* models of the vaginal mucosa such as ectocervical explants or the MatTek EpiVaginal™ tissue model as well as *in vivo* animal models such as rabbit and macaque are available for microbicide toxicity and pharmacokinetic assessment (Ayehunie et al., 2006; Dezzutti et al., 2012; Kiser et al., 2012). However, those systems are difficult to obtain and/or expensive which makes them less appropriate to compare multiple formulations in early microbicide development. As a next step, our *in vitro* observations are currently being evaluated *in vivo* in a macaque PK study.

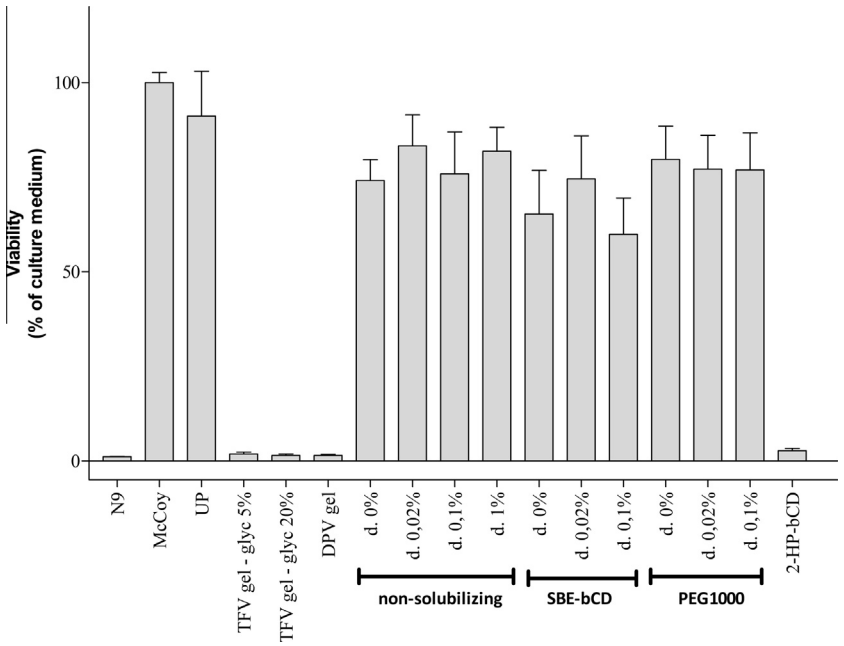


Fig. 6. Viability of HEC-1A cells after a 24 h incubation period with different aqueous gels (1:2 dilution with McCoy's culture medium), measured by the WST-1 assay. N-9 (0.1%) and UP were included as toxic and non-toxic controls, respectively. The reference vehicles of TFV (two different concentrations of glycerin) and DPV represent gels that have been tested in clinical trials, but do not include an active drug. The investigated UAMC01398 formulations (all HEC gels) varied in composition (no solubilizing excipients versus cyclodextrins (5%) or PEG1000 (10%)) and UAMC01398 dose (0%, 0.02%, 0.1% and 1%). The preservative used was propionic acid (0.2%). Data represent mean ± SD ($n = 4$).

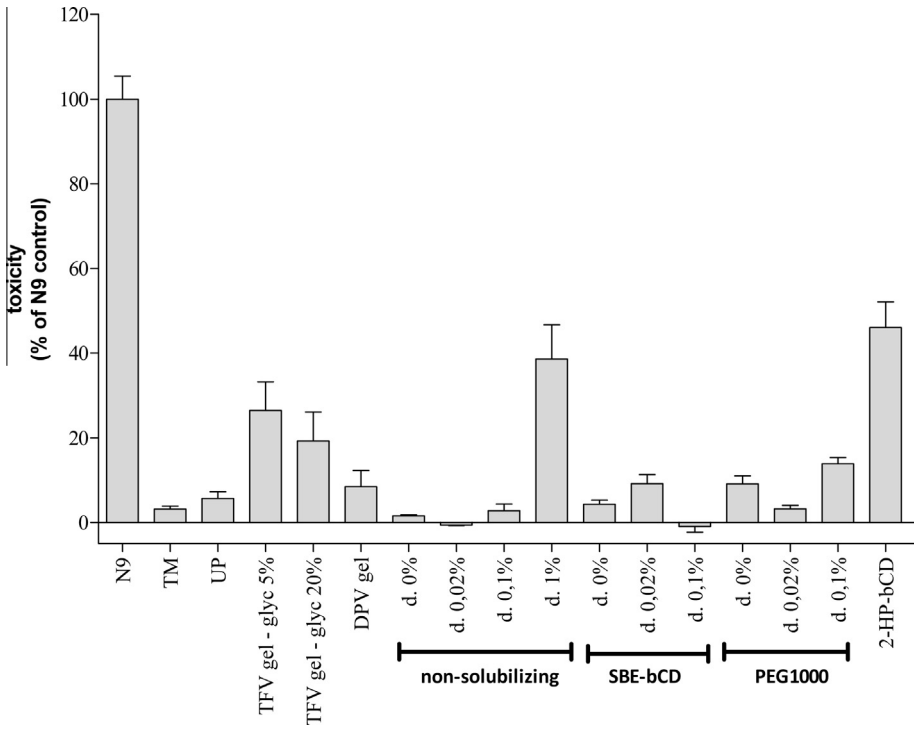


Fig. 7. Toxic effects of various formulations (at 1:2 dilution in HBSS) towards HEC-1A cells after a 24 h incubation period as measured by the LDH assay. N-9 (0.1%) and UP were included as toxic and non-toxic controls, respectively. The reference vehicles of TFV (two different concentrations of glycerin) and DPV represent gels that have been tested in clinical trials, but do not include an active drug. The investigated UAMC01398 formulations varied in composition (no solubilizing excipients versus cyclodextrins (5%) or PEG1000 (10%)) and UAMC01398 dose (0%, 0.02%, 0.1% and 1%). The preservative used was propionic acid (0.2%). Data represent mean ± SD ($n = 4$).

3.2.4. Stability of UAMC01398

UAMC01398 (0.02%) was found to be stable at 40 °C when formulated in both the non-solubilizing gel and the solubilizing gel containing SBE-βCD: the UAMC01398 recovery after 3 months of storage amounted to 101.8 ± 5.8% and 105.5 ± 3.9%, respectively. In adition, the viscosity and pH of these gels remained stable during storage. Also in the PEG1000 containing gel, no degradation of UAMC01398 was observed (109.1 ± 12%); however, this formulation was considered unstable because of a clear loss of viscosity (data not shown) and a decrease in pH to 3.3. It has previously been

Table 3
Antiviral activity measurement of pure UAMC01398 in solution and in gel formulations using the TZM-bl assay. The preservative used was propionic acid (0.2%). Data represent mean ± SD of three independent experiments.

Formulation	EC_{50} (nM)
Pure UAMC01398	1.5 ± 0.2
UAMC01398 in non-solubilizing gel	2.9 ± 0.1
UAMC01398 in SBE-bCD gel	0.9 ± 0.4

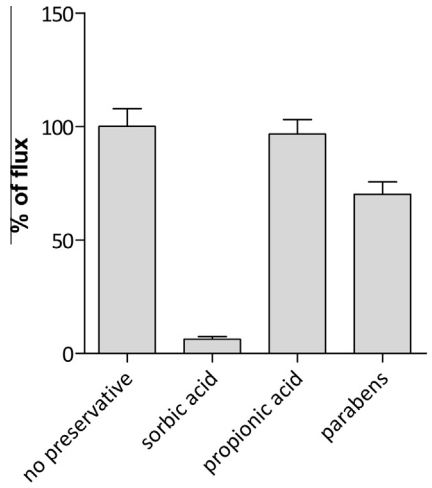


Fig. 8. The effect of preservatives (sorbic acid (0.2%), propionic acid (0.2%), parabens (methylparaben (0.20%)–propylparaben (0.05%)) on the UAMC01398 flux from the SBE-βCD (5%) containing gel. Data represent mean ± SD (n = 3).

demonstrated that trace levels of hydrogen peroxide present in PEGs may cause rapid degradation of the HEC-polymer (Dahl et al., 1998).

3.2.5. Antiviral activity assay

An antiviral activity test was performed for the safe and stable UAMC01398 gels, i.e., the non-solubilizing gel and the SBE-βCD (5%)-containing gel. As measured in TZM-bl cells, the EC_{50} values of formulated and native UAMC01398 are similar, which indicates that formulating UAMC01398 does not alter the *in vitro* potency of the compound (Table 3). In addition, no loss in antiviral activity (EC_{50} = 0.77–1.00 nM) was observed for these UAMC01398 gels after storage for 3 months.

3.2.6. Preservative selection

Preservatives may possibly impact the characteristics of a formulation. Therefore, the effect of three candidate preservatives (sorbic acid–propionic acid–paraben combination) on solubility, flux, toxicity and stability was evaluated. The commonly used preservatives sorbic acid and parabens, but not propionic acid, reduced the UAMC01398 flux across HEC-1A monolayers in presence of CDs, as illustrated in Fig. 8. A possible explanation is the competition between these preservatives and UAMC01398 for the formation of inclusion complexes with CDs (Zhang et al., 2010). This is confirmed by the impaired CD-based solubilization of UAMC01398 that was observed in presence of certain preservatives. Sorbic acid (0.2%) and the combination of methylparaben (0.2%) with propylparaben (0.05%) reduced the UAMC01398 solubility in presence of SBE-βCD by 90% and 41%, respectively. In contrast, propionic acid (0.2%) did not affect UAMC01398 solubility and was thus selected for the preservation of CD-containing gels.

In case of the non-solubilizing gel and the PEG1000 containing gel, sorbic acid promoted the degradation of UAMC01398, as revealed by stability studies, while the inclusion of the parabens in these gel formulations resulted in toxic effects as measured in *in vitro* viability tests (data not shown). As a result, also for these gels, propionic acid was selected as the preserving agent. The UAMC01398 fluxes and toxicity data reported so far (Figs. 5–7) are therefore shown for gels containing propionic acid (0.2%) as preserving agent. This study demonstrates the importance of careful preservative selection for microbicidal gels.

4. Conclusion

UAMC01398, which previously appeared to be very potent against HIV-1 with an excellent cellular safety profile and activity against NNRTI-resistant viruses, also proved to be one of the best soluble DATA compounds and was thus formulated into several aqueous based HEC gels. The non-solubilizing and the SBE-βCD (5%)-containing gel formulations of UAMC01398 with propionic acid as preservative were found to be stable up to 3 months as well as safe in *in vitro* toxicity assays. The inclusion of the solubilizing excipient SBE-βCD in the formulation significantly increased the UAMC01398 compound flux across HEC-1A cell layers suggesting enhanced tissue penetration without increasing compound loading. These *in vitro* findings are currently being evaluated in an *in vivo* macaque PK/PD study.

Declarations

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