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In vitro profiling of the vaginal permeation potential of anti-HIV microbicides and the influence of formulation excipients

Carolien Grammen, Patrick Augustijns, Joachim Brouwers *

Laboratory for Pharmacotechnology and Biopharmacy, KU Leuven, Belgium

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ABSTRACT

In the search for an effective anti-HIV microbicidal gel, limited drug penetration into the vaginal submucosa is a possible reason for failed protection against HIV transmission. To address this issue in early development, we here describe a simple in vitro strategy to predict the tissue permeation potential of vaginally applied drugs, based on solubility, permeability and flux assessment. We demonstrated this approach for four model microbicides (tenofovir, darunavir, saquinavir mesylate and dapivirine) and additionally examined the influence of formulation excipients on the permeation potential. When formulated in an aqueous-based HEC gel, high flux values across an HEC-1A cell layer were reached by tenofovir, as a result of its high aqueous solubility. In contrast, saquinavir and dapivirine fluxes remained low due to poor permeability and solubility, respectively. These low fluxes suggest limited in vivo tissue penetration, possibly leading to lack of efficacy. Dapivirine fluxes, however, could be enhanced up to 30-fold, by including formulation excipients such as polyethylene glycol 1000 (20%) or cyclodextrins (5%) in the HEC gels. Alternative formulations, i.e. emulsions or silicone elastomer gels, were less effective in flux enhancement compared to cyclodextrin-HEC gels. In conclusion, implementing the proposed solubility and permeability profiling in early microbicide development may contribute to the successful selection of promising microbicide candidates and appropriate formulations.

1. Introduction

With about 7000 new HIV infections each day, half of them occurring in women, there is an urgent need for a female controlled HIV prevention method such as anti-HIV microbicides (WHO, 2012). These are compounds that are applied vaginally or rectally in order to prevent the transmission of HIV. Since non-specific first generation microbicide candidates (nonoxynol-9, Savy®, Carraguard®, cellulose sulfate, PRO-2000, BufferGel®) all failed in phase 3 clinical trials, a shift has occurred towards more specific anti-retroviral drugs (ARV) such as (non-)nucleoside reverse transcriptase inhibitors ((N)NRTIs), protease inhibitors (PIs) and entry-inhibitors. The CAPRISA 004 trial, testing a gel of the NRTI tenofovir (1%) in African women, provided proof of concept that vaginally applied anti-HIV microbicides can reduce transmission of the virus (Abdool Karim et al., 2010). For small molecular ARV drugs, it is imperative that they are taken up into the tissue where HIV transmission takes place, i.e. the vaginal submucosa containing most HIV target cells (Adams and Kashuba, 2012; Hladik and Doncel, 2010). In addition to activity or toxicity issues, poor tissue uptake of microbicides can thus be a possible cause of failed protection against HIV transmission.

The importance of the pharmacokinetic disposition of microbicides upon their application in the vaginal or rectal lumen has long been underrated in microbicide research. Nowadays however, several in vitro and in vivo model systems are being used to evaluate key issues in microbicide disposition. Drug release properties of vaginal formulations are often studied in in vitro set-ups using large amounts of isopropanol/water mixtures. Obviously, these high volumes of non-aqueous liquid are not representative for the human vaginal fluid, limiting the in vivo relevance of these systems (Kiser et al., 2012; Woolfson et al., 2010). To study actual tissue uptake, several model systems are available such as the MatTec EpiVaginal™ tissue model, human ectocervical explants and animal models (primarily rabbit, sheep or macaque) (Ayehunie et al., 2006; Clark and Friend, 2012; Dobard et al., 2012; Kiser et al., 2012). While very useful in the final stages of preclinical microbicide development, these techniques are expensive, time consuming, not readily available (in case of ectocervical explants) and/or labor intensive. Consequently, neither of these approaches is suitable as a screening tool to evaluate the tissue permeation potential of numerous microbicide candidates in early development, or to select appropriate formulations.

* Corresponding author. Address: Laboratory for Pharmacotechnology and Biopharmacy, KU Leuven, Gasthuisberg O&N 2 – Herestraat 49, Box 921, 3000 Leuven, Belgium. Tel.: +32 16 330310; fax: +32 16 330305.
E-mail address: joachim.brouwers@pharm.kuleuven.be (J. Brouwers).

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To address these limitations, we propose a relatively simple in vitro strategy to assess the vaginal permeation potential of microbicides, based on fundamental principles of drug disposition across epithelial layers (van de Waterbeemd et al., 2003). The strategy mainly focuses on microbicides formulated in aqueous based vaginal gels. Since these gels are usually intended for immediate, coitus-dependent protection and since they only have limited retention time in the vaginal lumen, they should enable fast microbicide uptake into the submucosa. According to Fick's first law of diffusion, the permeation rate of a microbicide across the vaginal mucosa will depend on the microbicide concentration dissolved in the vaginal lumen and the permeability of the vaginal mucosa for the microbicide. Therefore, our in vitro approach is based on the determination of both microbicide solubility and permeability. Solubility is determined as the maximum microbicide concentration in solution when equilibrium has been reached between the solid and dissolved state. Permeability assessment is based on microbicide transport across an in vitro layer of the established HEC-1A cell line, originating from a human endometrial carcinoma. This dual chamber model has previously been used as a tool to assess possible epithelial toxicity caused by microbicides or formulations (Gali et al., 2010a,b; Rohan et al., 2010; Dezzutti et al., 2012).

In the present study, the profiling of solubility and permeability as biopharmaceutical properties critical for microbicide disposition was demonstrated for 4 model microbicides: the NRTI tenofovir (TFV), the NNRTI dapivirine (DPV), and the PIs darunavir (DRV) and saquinavir mesylate (SQV). In addition, the effect of formulation excipients on both solubility and permeability was investigated to illustrate how the gained knowledge may guide efficient formulation development.

2. Material and methods

2.1. Drugs and excipients

The model drugs TFV, SQV, DPV and DRV were obtained from Watson International (Kunshan City, China), Hetero Drugs Ltd (Hyderabad, India), Axon Medchem (Groningen, The Netherlands) and the NIH AIDS Research and Reference Reagent Program (Germantown, MD, USA), respectively. Powder particle sizes amounted to 1.1 ± 0.4 μm (TFV), 2.3 ± 0.8 μm (SQV), 96 ± 71 μm (DRV) and 84 ± 47 μm (DPV). Following chemicals were used in the formulations: hydroxyethylcellulose (HEC; Natrosol 250 HHX Pharm, Hercules, Wilmington, DE, USA), 2-hydroxypropyl-β-cyclodextrin (HPβCD), 2-hydroxypropyl-γ-cyclodextrin (HPγCD; Sigma–Aldrich, St. Louis, MO, USA), sodium sulfobuthyl ether-β-cyclodextrin (SBEβCD; Captisol, CyDex Pharmaceuticals Inc, Lenexa, KS, USA), polyethylene glycol 1000 (PEG1000; Acros Organics, Geel, Belgium), Cremophor EL (Sigma–Aldrich, St. Louis, MO, USA), cetostearyl alcohol (Alpha Pharma, Waregem, Belgium), Captex 300 EP (Abitec Corporation, Janesville, WI, USA). ST-Elastomer 10 and cyclomethicone were kindly provided by Dow Corning® (Midland, MI, USA).Following media were used for cell culture and permeation experiments: Hanks' balanced salt solution (HBSS), McCoy's 5A medium, penicillin–streptomycine (10.000 IU/ml), fetal bovine serum (FBS), 4-(2-hydroxyethyl)piperazin-1-ethanesulfonic acid (HEPES) (Lonza, Basel, Switzerland), D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS1000; Eastman Chemical Company, Kingsport, TN). Phophatase, alkaline type from bovine mucosa was obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium acetate and sodium chloride were obtained from VWR (Leuven, Belgium); calcium chloride, bovine serum albumin, glycerol and D-glucose from Sigma–Aldrich (St. Louis, MO, USA); tetrabutyl ammonium hydrogen sulfate, methanol, lactic acid and urea from Acros Organics (Geel, Belgium); magnesium sulfate and calcium hydroxide from UCB (RPL, Leuven, Belgium), and potassium hydroxide from Riedel-de-Haën (Seelze, Germany). Water was purified with a Maxima system (Elga Ltd., High Wycombe Bucks, UK).

2.2. Solubility assessment

The solubility of the 4 model microbicides in the different formulation media (acetate buffer 50 mM pH 4.2, in absence or presence of formulation excipients) was determined by adding an excess of drug powder (0.2–3 mg, depending on the compound) to the respective media (200 μl). Samples were incubated during 24 h to reach equilibrium (IKA KS 4000i control, 37 °C and 175 rpm). Afterwards, samples were centrifuged (21,000*g*, 37 °C, 15 min) to remove any undissolved drug powder and an appropriate dilution of the supernatant was injected into the HPLC system (see Section 2.6.). An identical procedure was followed for determining solubilities in presence of vaginal fluid simulant (VFS; 5/2 dilution, which corresponds to 2.5 mL of formulation diluted by 1 mL of VFS). VFS was prepared as described by Owen and Katz (1999).

2.3. Permeability assessment

Permeability experiments were performed in a dual chamber system, consisting of an apical and basal chamber separated by a layer of HEC-1A cells (Fig. 1). HEC-1A cells were kindly donated by prof. G. Vanham (Institute of Tropical Medicine, Antwerp, Belgium). Cells were cultured in McCoy's 5A medium, supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin, at 37 °C in presence of 5% CO_2 and at a relative humidity of 90%. Cells were passaged every 3–4 days (at 80–90% confluence). For permeation experiments, 100,000 cells were seeded in Costar® Transwell membrane inserts (12 well plate, 3 μm pore diameter, 12 mm diameter; Corning Inc., USA) and were used 9–10 days after seeding.

For permeability experiments, following media were prepared. Acidified HBSS (25 mM glucose, 50 mM acetate buffer, pH 4.2) was used as the apical buffer and neutral HBSS (25 mM glucose, 10 mM HEPES, pH 7.4), containing 0.2% TPGS to enhance microbicide solubility and prevent backflux, was used as the basolateral buffer. Formulation excipients (HPβCD (5% w/w); HPγCD (5% w/w); SBEβCD (5% w/w) and PEG1000 (20% w/w)) were dissolved in the apical medium to study their impact on permeation. Microbicides were added from a DMSO stock solution (final DMSO concentration <1%) to obtain initial apical concentrations below the maximal solubility: 1.0 mM for TFV, 15 μM for SQV, 100 μM for DRV and 4 μM for DPV. Prior to the actual experiment, cells were equilibrated with the media containing no active compound for 5 min. Apical microbicide solutions were diluted with VFS (dilution factor 5:2) before initiating the experiment. Plates were incubated during 1 h and shaken at 300 rpm, 37 °C (Thermostar, BMG Labtech, Ortenberg, Germany) after which samples were taken from

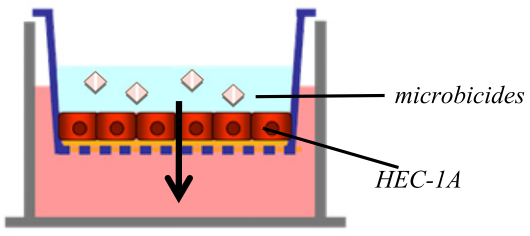

Fig. 1. Transwell dual chamber model. (Adapted from Gali et al., 2010a).

the apical and basal chamber. To assess intracellular drug accumulation, cells were rinsed 2 times with acidified HBSS and microbicides were extracted using methanol–water (70:30%, except for TFV: 30:70%) while sonicating. In case of TFV, cells were first incubated during 30 min with a phosphatase solution (25 units/mL, in HBSS, pH 9.8) at 37 °C to dephosphorylate any possibly present TFV-diphosphate. It should be noted that the technique to determine intracellular drug accumulation does not necessarily allow complete separation of strongly adsorbed extracellular drug molecules. Apical, basal and intracellular drug concentrations were determined using HPLC-UV or -MS/MS (described in Section 2.6.). Results were expressed as the permeated fraction, i.e. the percentage of compound (relative to the applied dose) that reached the basal chamber after 1 h, and as the intracellular fraction, i.e. the percentage of compound (relative to the applied dose) present in the HEC-1A cells after 1 h.

For DPV, the release of the intracellular fraction, present after the 1 h incubation period, towards the basal chamber was additionally assessed. After the incubation period with DPV, cells were rinsed and incubated apically with acidified HBSS and basolaterally with neutral HBSS containing TPGS. After 1 h, the apical, intracellular and basolateral concentrations of DPV were determined.

Transepithelial electric resistance values (TEER) were measured with a Millicell-ERS Volt-Ohm meter (Millipore, Bedford, MA, USA) before and after each experiment to verify the epithelial integrity.

2.4. Preparation of gels

Aqueous HEC gels of the model microbicides were prepared by adding the microbicide compound to an acetate buffer (50 mM, pH 4.2) containing $CaCl_2$ (186 mg/L) and $MgSO_4$ (200 mg/L), both to maintain tight junctions between epithelial cells, and glucose (40 g/L) to ensure isotonicity and as nutrition for the cells. CDs (5% w/w) or PEG1000 (20% w/w) were present if applicable. After mixing, media were incubated and shaken during 24 h (IKA KS 4000i control, 37 °C and 175 rpm) to reach equilibrium concentrations. HEC (1.5% w/w) was added followed by a 3 h incubation period to allow gel formation. All water-based gels were isotonic except for the gel containing 20% of PEG1000 (720 mOsm/kg).

Emulsions consisted of cremophor EL (2.2% w/w), cetostearylalcohol (7.3% w/w), captex 300 (34% w/w) and isotonic acetate buffer (56.5% w/w). DRV and DPV were dissolved in the oil phase, while TFV and SQV were added to the water phase. After heating, the water phase was slowly added.

Silicone elastomer gels were prepared by mixing ST-Elastomer 10 and cyclomethicone (60/40% w/w) after which the compound was added. After mixing, gels were incubated during 24 h. Microbicide concentrations in all gel formulations amounted to 35 mM for TFV and 2 mM for SQV, DRV and DPV.

2.5. Flux assessment

For the gel formulations, flux experiments were performed using the same in vitro set-up as described for the permeability experiments (Section 2.4.). VFS and gel (2/5 dilution) were applied in the apical chamber, while the basal chamber contained HBSS with TPGS. After 1 h, samples were taken and analyzed to calculate fluxes, i.e. the amount of compound migrating per unit of time and surface area. TEER values were measured before and after each experiment.

2.6. Analytical methods

Microbicide concentrations were determined by reversed phase HPLC with UV detection. An Hitachi LaChrom Elite HPLC system was used consisting of an L-2130 pump, an L-2200 autosampler, an L-2400 UV detector and EZChrom Elite software (VWR, Leuven, Belgium). The used column was a Waters Nova-Pak® RP-18 (100 × 8 mm, 4 μm) inserted into a radial compression module (Waters, Milford, MA, USA). For all samples, an injection volume of 50 μl was used. The flow rate was set at 1 ml/min, except for DRV for which a flow rate of 1.3 ml/min was used. The mobile phases for the different microbicide compounds were composed as follows: phosphate buffer (10 mM KH_2PO_4, 2 mM tetrabutylammoniumchloride, pH 6.0) and methanol (26:73, v/v) for TFV; acetate buffer (25 mM, pH 5.5) and methanol (68:31, v/v) for DRV; acetate buffer (37 mM, pH 4.0) and methanol (84:16, v/v) for SQV; acetate buffer (37 mM, pH 4.0) and methanol (83:17, v/v) for DPV. Microbicides were detected by UV absorbance measurement at following wavelengths: 259 nm for TFV, 268 nm for DRV, 240 nm for SQV and 289 nm for DPV. Calibration curves were linear over the following concentration ranges: from 250 μM to 250 nM for TVF and DRV, from 130 μM to 130 nM for SQV and from 250 μM to 16 nM for DPV. The presence of formulation excipients or VFS did not interfere with microbicide detection. Precision and accuracy were assessed by analyzing standard samples ($n = 5$). Intraday repeatability and mean bias were below 6% at all concentrations.

To quantify SQV in samples obtained in permeability experiments, an HPLC-MS/MS method was used. The MS/MS detector was a TSQ Quantum with Xcalibur as the software program (Thermo-Electron, San Jose, CA, USA). ESI (electron spray ionization) was applied in the positive polarity mode, the collision energy was 31 V and the detected ion of SQV had an m/z of 570.21. The column, a Kinetex C18 (50 × 3 mm, 1.7 μm; Phenomenex, Utrecht, The Netherlands), was maintained at a temperature of 30 °C. An injection volume of 10 μL was used and the flow was set at 400 μL/min. A gradient elution was applied using methanol/buffer (ammonium acetate 5 mM, pH 3.5) mixtures: 5/95% (0–1 min); 92/8% (1–3.5 min) and 5/95% (3.4–4.5 min). The calibration curve was linear from 1.3 μM to 2.5 nM. Precision and accuracy were below 8%.

2.7. Data presentation and statistics

Unless otherwise specified, data are presented as the mean ± standard deviation (SD) of 3 replicates. To compare multiple conditions, a one way Anova test followed by Dunnett's post

Table 1
Physicochemical properties and typical in vitro anti-HIV EC50 values of the model microbicides used in this study.

Drug	Molecular weight	pKa[a]	logP[b]	logD at pH 4.2[c]	EC50
Tenofovir	287.2	1.3*; 7.9*; 3.0; 5.3	−1.1	−3.6	0.04–8.5 μM
Saquinavir mesylate	766.9	0.5 and 8.5	3.2	−0.3	1–10 nM
Darunavir ethanolate	593.7	2.4	2.8	2.8	1.2–8.5 nM
Dapivirine	329.4	5.6	5.2	4.7	1–5 nM

logP, logD and pKa values were computationally determined using MarvinSketch (version 5.9.3, ChemAxon, www.chemaxon.com). EC50 values were obtained from EPAR reports, except for dapivirine (Fletcher et al., 2009).

[a] pKa values for basic groups, except when indicated (* = acidic group).

[b] Partition coefficient: concentration ratio of unionized compound between octanol and water at equilibrium.

[c] Distribution coefficient: concentration ratio of ionized and unionized compound between octanol and water (pH 4.2) at equilibrium.

test was performed. Differences were considered statistically significant at $p < 0.01$.

3. Results and discussion

In the present study, solubility and permeability were evaluated for 4 model microbicides as critical factors for the compounds' potential to reach the HIV target cells in vaginal tissue. These model compounds are listed in Table 1, along with some key physicochemical properties that may affect this potential, i.e. molecular weight, pKa values determining the molecular charge at pH 4.2, and the octanol–water partition coefficient logP and distribution coefficient logD (at pH 4.2) as measures of hydrophobicity.

3.1. Solubility assessment

Solubility assessment of a microbicide candidate is essential given that a drug must be dissolved to permeate cellular layers and reach the HIV target cells. In case of coitus dependent vaginal gels, this uptake should occur fast, requiring relatively high concentrations in the gels. For this reason, we determined aqueous solubilities of 4 model microbicide candidates at pH 4.2, resembling the normal vaginal pH and corresponding to the pH of most vaginal gel formulations. Fig. 2 shows a large range in solubility among the different compounds: TFV (39.1 mM) > SQV (3.1 mM) > DRV (0.51 mM) > DPV (4 μM). It can be noticed that the compounds' solubility decreases with increasing hydrophobicity (logD) at pH 4.2 (Table 1). The high solubility of TFV enables the vaginal application of a high TFV concentration using a simple aqueous based gel. In contrast, the 10,000 times lower solubility of DPV may limit its driving force for permeation.

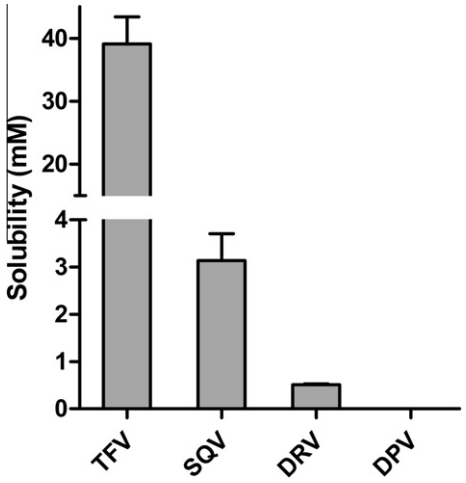

Fig. 2. Solubility of model microbicides in acetate buffer pH 4.2. Data represent mean ± SD ($n = 3$, SQV: $n = 2$).

To increase the driving force for permeation, one may enhance the solubility of microbicides by including solubilizing formulation excipients. The selection of excipients for the present study took into account both their solubilizing properties, as well as potential safety issues, recently identified by Gali et al. (2010b). PEG1000 can enhance the solubility of poorly water soluble drugs by cosolvency while the selected cyclodextrins (CDs) HPβCD, HPγCD and SBEβCD, are cyclic oligosaccharides that can solubilize lipophilic drugs by complexation in their hydrophobic cavity. In spite of their good solubilizing capacity, surfactants were not used in this study because of well-known safety issues (cell membrane disruption) (Hillier et al., 2005). From Fig. 3, which depicts the solubility increase of the model microbicides in presence of PEG1000 and CDs, it is clear that the compounds with the lowest aqueous solubility experienced the strongest impact of adding solubilizing formulation excipients. In case of TFV, the presence of CDs had no effect on the solubility, while PEG1000 even caused a significant decrease. In contrast, the solubility of DPV could be enhanced up to 55-fold by the addition of SBEβCD (5%). This indicates that even hydrophobic drugs can be applied at high concentrations in aqueous based gels upon inclusion of solubilizing excipients.

3.2. Solubility in presence of VFS

In vivo, the applied gel will be diluted by vaginal fluid, which may cause precipitation of microbicides dissolved in the gel, thus reducing the driving force for permeation into the submucosa. We therefore examined the solubilities of the 4 compounds (with or without solubilizing excipients) in presence of VFS (5/2 dilution; data not shown). TFV solubilities were not affected by the presence of VFS. In absence of formulation excipients, thermodynamic solubilities of DRV and SQV were reduced by 24% and 87% respectively, while no change was found for DPV. The strong decrease in case of

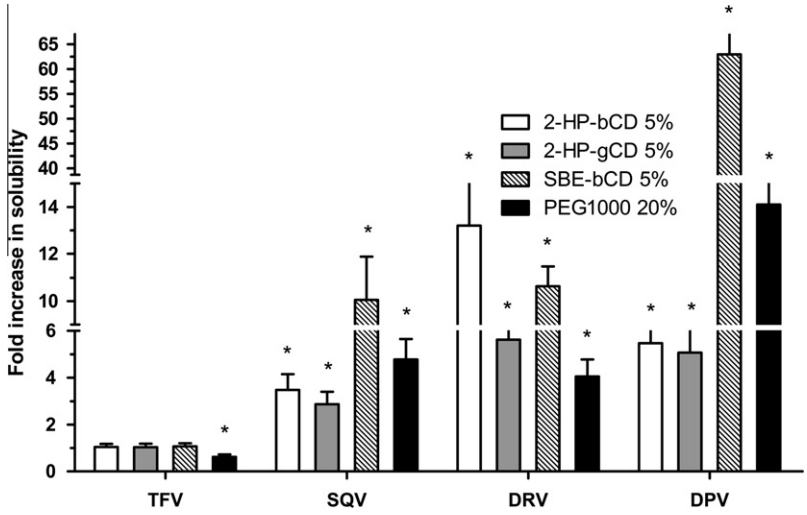

Fig. 3. Excipient induced increase in solubility of model microbicides compared to solubilities in acetate buffer at pH 4.2 (Fig. 2). Data represent mean ± SD ($n = 3$, SQV: $n = 2$). Statistical significant difference with the control condition was evaluated using a one way Anova test followed by Dunnett's post test (*$p < 0.01$).

SQV is probably due to the increased ionic strength in presence of VFS which is detrimental for the solubility of the mesylate salt. However, it has previously been shown that, if a (gel) solution of SQV is diluted with VFS, SQV stays in solution at supersaturated but metastable concentrations (Brouwers et al., 2011). In presence of formulation excipients, VFS induced a decrease in solubility (50–85% for SQV; 30–40% for DRV and DPV), which can be ascribed to the dilution of the solubilizing excipients. Nevertheless, for SQV and DRV in presence of formulation excipients, solubilities remained higher than 2 mM, i.e. the concentrations applied in flux experiments described in Section 3.4. Consequently, no precipitation of SQV or DRV would occur during flux experiments.

3.3. Permeability assessment

The amount of microbicide that migrates into the submucosa will not only depend on its concentration in the vaginal lumen but also on the permeability of the cervicovaginal epithelial layer for the microbicide. To assess permeability, an in vitro dual chamber model was used in which an apical and basolateral chamber are separated by a layer of HEC-1A cells (Fig. 1) (Gali et al., 2010a). We first determined the permeated and intracellular fractions for the 4 model microbicides after a 1 h incubation period in absence of any formulation excipient. To avoid confounding solubility effects, microbicides were applied at concentrations below their solubility in acidified HBSS diluted with VFS as the apical solvent. As reported in Fig. 4A, following permeated fractions were found: 20.8% (DPV) > 10.1% (DRV) > 1.85% (TFV) > 0.26% (SQV). For TFV, the observed permeated fraction corresponds to an apparent permeability coefficient ($P_{app,}$ i.e. the amount of drug migrating per unit of time, surface area and donor concentration) of 1.9×10^{-6} cm/s, which is comparable to the permeability through ectocervical explant tissue reported by Dezzutti et al. (2012). The poor permeability of TFV and SQV is probably due to their low logD values and, in particular, the fact that they are fully charged at pH 4.2 (Table 1), restricting the ability to permeate across the phospholipid bilayer of cell membranes. DPV and DRV show higher permeated fractions since both are only partially loaded at vaginal pH and possess higher logD values (increased hydrophobicity).

In Fig. 4B, the percentages of compound present inside the HEC-1A cells after the 1 h incubation period are depicted. Mainly the high intracellular fraction of the hydrophobic DPV (56%) can be observed. An additional release experiment demonstrated that 54% of the intracellular DPV was released to the basal chamber during the next hour. This good permeability for DPV presumably plays a key role in the considerable vaginal drug uptake as observed by Nel et al., 2009a,b, despite DPV's limited solubility.

Regarding the choice of HEC-1A cells to study vaginal drug permeation, it should be noted that, although this cell line originates from an endometrium carcinoma, its epithelial layer shows a multilayered structure with tight junctions between the cells, a cellular organization similar to the vaginal epithelium (Gali et al., 2010a). Furthermore, the use of an established in vitro cell line is relatively cheap and very practical compared to other techniques such as ectocervical explants, the MatTec EpiVaginal™ tissue model or animal models. Though, it should be noted that the latter model systems are structurally more representative of the in vivo situation.

As demonstrated above, formulation excipients may be included to increase the solubility of microbicides, leading to an enhanced driving force for permeation. To investigate whether these excipients may also affect the permeability, we assessed their influence in the dual chamber model. Again, microbicides were applied at concentrations below their solubility, to exclude any impact of the solubilizing effect of the excipients. In Fig. 5, the permeated fractions of the model microbicides in presence of the solubilizing excipients are expressed relative to the permeated fraction in absence of excipients. For TFV, inclusion of PEG1000 but not CDs, decreased its permeation. The permeated fractions of SQV, DRV and DPV were all decreased by adding the solubilizing excipients. In case of CDs, only free drug molecules are able to permeate cellular layers; the fraction of the dissolved drug that is included in the hydrophobic CD cavity may not be readily available for permeation (Loftsson and Brewster, 2011). In presence of PEG1000, the higher affinity of the hydrophobic microbicides for the apical medium presumably results in a reduced permeation towards the basolateral compartment. Hence, while enhancing the solubility of hydrophobic compounds, the presence of excipients may decrease the permeability. Consequently, finding a balance between these two opposite effects will be critical to obtain an optimal microbicide flux into the submucosa.

3.4. Flux assessment

In vivo, microbicide permeation into the vaginal submucosa will be the combined result of both solubility and permeability. Therefore, microbicide fluxes from HEC-based gel formulations

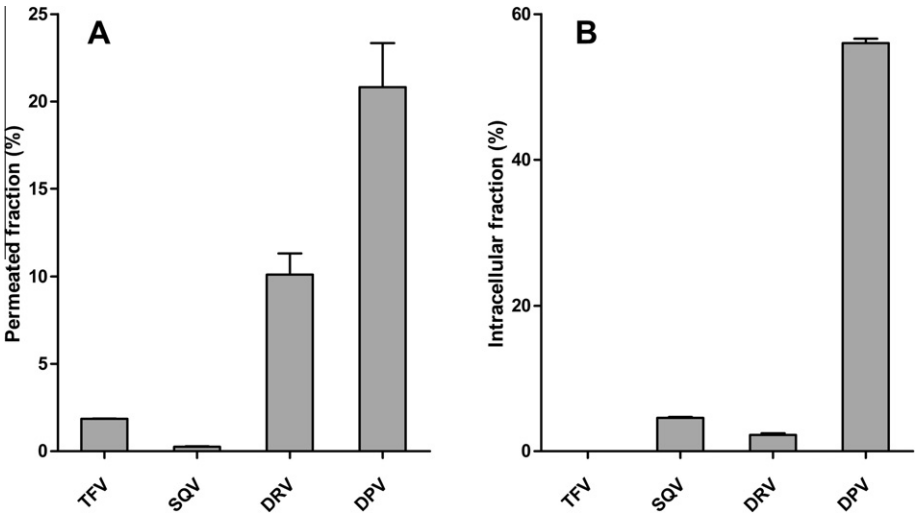

Fig. 4. (A) Permeated fraction, i.e. the percentage of compound that reached the basal chamber after 1 h incubation of the different microbicides, at pH 4.2 and in absence of any formulation excipient. (B) Intracellular fraction, i.e. the percentage of compound present in the HEC-1A cells after 1 h, at pH 4.2 and in absence of any formulation excipient. Data represent mean ± SD ($n = 3$).

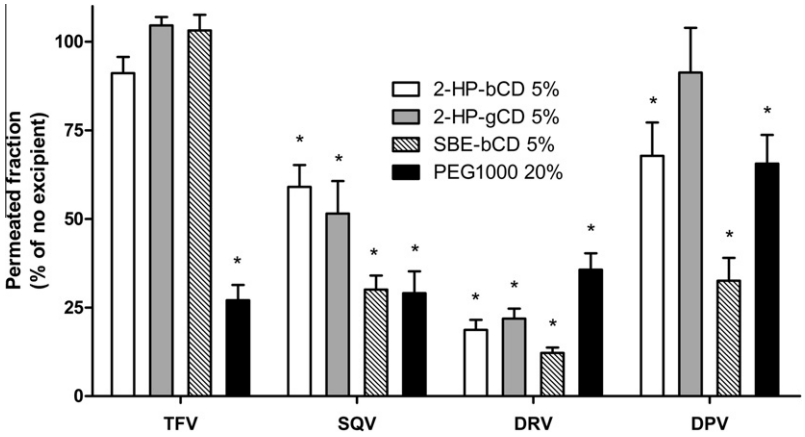

Fig. 5. Excipient induced decrease in permeated fraction of model microbicides compared to the permeated fraction without excipients at pH 4.2 (Fig. 4). Data represent mean ± SD ($n = 3$). Statistical significant difference with the control condition was evaluated using a one way Anova test followed by Dunnett's post test ($^*p < 0.01$).

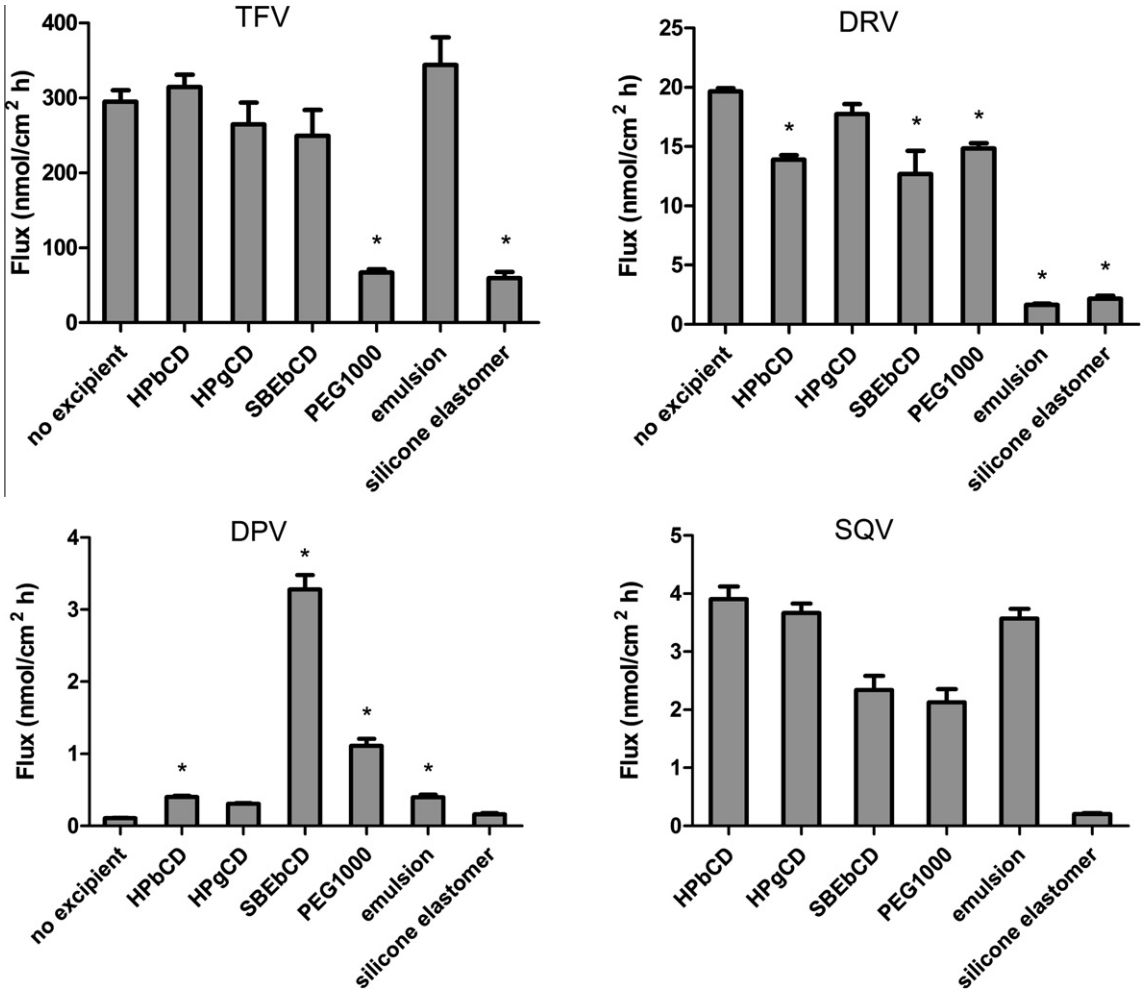

Fig. 6. Flux values of TFV (35 mM), DRV (2 mM), DPV (2 mM) and SQV (2 mM) from different formulations: buffered HEC gel (pH 4.2) in absence of excipients (control), buffered HEC gel in presence of cyclodextrins (5%) or PEG1000 (20%), an emulsion and a silicone elastomer gel. For SQV, the control condition could not be measured due to loss of epithelial integrity. Data represent mean ± SD ($n = 3$). Statistical significant difference with the control condition was evaluated using a one way Anova test followed by Dunnett's post test ($^*p < 0.01$).

(in absence or in presence of CDs or PEG1000) containing clinically relevant microbicide concentrations were evaluated in the dual chamber model (Fig. 6). For TFV, a 35 mM concentration was used corresponding to the 1% gel being tested in clinical trials (Abdool Karim et al., 2010). For DPV, DRV and SQV, a 2 mM concentration was used, corresponding to the 0.05% gel of DPV currently being evaluated in clinical trials (Clinicaltrials.gov, 2012), and taking into account the similar anti-HIV EC50 values of these three compounds (Table 1). At these concentrations, SQV and TFV were fully dissolved in all formulations except for the TFV-PEG1000

combination (suspension). DRV could only be fully dissolved in presence of CDs, while all DPV formulations were suspensions.

TFV clearly showed the highest flux of all compounds (about 300 nmol/cm^2 h), as a result of its very high aqueous solubility. In view of TFV's relatively high EC50 value (Table 1), this elevated flux value might be necessary in order to be active against HIV transmission. In accordance with the observed solubility and permeability data (Figs. 3 and 5), CDs did not alter the TFV flux. The presence of PEG1000, however, significantly reduced the flux. For DRV, the highest flux was obtained in absence of any solubilizing excipient (20 nmol/cm^2 h), indicating that the gain in solubility upon inclusion of CDs or PEG1000 could not compensate for the loss in permeability. In contrast, the DPV flux, which was extremely low (0.1 nmol/cm^2 h) due to DPV's poor water solubility, was significantly enhanced by including CDs and PEG1000; the best result was obtained by including SBEβCD (30-fold increase). SQV fluxes in absence of formulation excipients could not be determined since this condition disrupted the epithelial cell layer integrity, as indicated by a drop in TEER of 80%. In presence of formulation excipients, however, the epithelial cell layer remained intact, illustrating that CDs can act in a protective manner (Cal and Centkowska, 2008). As a result of its poor permeability, fluxes of SQV were relatively low (up to 4 nmol/cm^2 h).

To improve the formulation of hydrophobic microbicides, emulsions and silicone elastomer gels have been proposed as alternatives for aqueous HEC gels (D'Cruz and Uckun, 2007; Forbes et al., 2011; Stolte-Leeb et al., 2011). Hydrophobic compounds such as DRV and DPV are expected to have higher solubilities in these non-aqueous formulations, possibly resulting in an elevated flux. Therefore, we compared the observed microbicide fluxes from HEC gels with fluxes from an emulsion and a silicone elastomer gel (Fig. 6). Although both DRV and DPV were fully dissolved in the emulsion formulation, only a small increase in flux was observed for DPV and even a clear decrease in flux for DRV. This clearly indicates that, despite the higher solubility of hydrophobic compounds in emulsions, their release from the oil phase into the vaginal mucosa can be problematic. For the more water soluble compounds TFV and SQV, formulation in an emulsion did not alter the flux since these drugs are presumably mainly present in the outer water phase of the oil-in-water emulsion. Fluxes from the silicone elastomer gel were comparable (DPV) or even substantially lower (TFV, DRV and SQV) to the HEC gel formulation without any solubilizing excipient. It should be noted that silicone elastomer gels may have some advantages over HEC gels such as their longer intravaginal retention time or the fact that they do not need to include any preservative (Forbes et al., 2011). However, they may preclude significant permeation of hydrophobic compounds, a disadvantage which has not been observed to the same extent by using CDs.

During flux experiments, TEER values were measured to verify epithelial integrity. In absence of solubilizing excipients, TEER recoveries varied from 76% to 88%, except for SQV (20%). Comparable results were found for the emulsion and silicone formulation. Formulations containing HPβCD (5% w/w) or PEG1000 (20% w/w) were less compatible with the HEC-1A layer (TEER recoveries ranging from 35% to 80%). The CDs SBEβCD and HPγCD (5% w/w) in contrast, appeared to have a protective effect on epithelial layer integrity; TEER recoveries were 103% ± 20.0 and 97% ± 1.3 respectively. Gali et al. (2010b) also reported the highest CC50 values for these 2 types of CDs, compared to other formulation excipients.

In contrast to most pharmacokinetic studies with microbicides, we opted to determine microbicide fluxes instead of intracellular cell/tissue concentrations. First, it appeared impossible to fully remove gel and undissolved microbicide particles from the cells, preventing the reliable determination of compound taken up intracellularly versus adhered compound. As a side note, this issue may be underestimated when attempting the assessment of tissue concentrations during microbicide PK studies. Secondly, intracellularly acting microbicides should be taken up inside the primary HIV target cells, mainly CD4+ T cells in the deepest layers of the multilayered mucosa or in the submucosa (Miller et al., 2005). To reach these cells, permeation across epithelial cell layers is necessary. Therefore, a limited microbicide flux across HEC-1A cell layers strongly suggests pharmacokinetic issues in vivo.

4. Conclusion

This study describes a simple in vitro strategy, consisting of solubility and permeability assessment, to appraise the potential of microbicide candidates to reach the cervicovaginal submucosa. Our results indicate a large variation in in vitro permeation potential among different microbicide compounds, presumably leading to differences in in vivo tissue penetration and, consequently, anti-HIV efficacy. Since high vaginal concentrations enable fast tissue permeation, hydrophilic microbicides with sufficient aqueous solubility (e.g. TFV) are ideally suited for vaginal application using aqueous-based gels, such as commonly used HEC gels. By including solubilizing formulation excipients (e.g. cyclodextrins), even hydrophobic microbicides (e.g. DPV) can be applied by means of aqueous-based gels. When using solubilizing excipients, care should be taken to balance their effects on solubility (typically increase) and permeability (typically decrease) in order to obtain the optimal flux. In terms of enabling permeation of hydrophobic microbicides, cyclodextrin-HEC gels outperform alternative formulations, including emulsions and silicone elastomers. In conclusion, the proposed biopharmaceutical profiling allows including permeation potential as a criterion in the early selection of promising microbicide candidates and appropriate formulations. This approach enables the early identification of possible pharmacokinetic issues and may, as such, reduce failure of intrinsically potent antiviral compounds in more complex ex vivo or in vivo models due to limited tissue permeation.

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