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Early identification of availability issues for poorly water-soluble microbicide candidates in biorelevant media: A case study with saquinavir

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ABSTRACT

In the search for a successful HIV microbicide, many poorly water-soluble antiviral agents are currently being investigated. Unfortunately, solubility and precipitation issues may limit intravaginal concentrations and thus availability of these agents upon application of an aqueous gel formulation. In the present study, we evaluated the in vitro precipitation behavior of the HIV protease inhibitor saquinavir in vaginal and seminal fluid simulants (VFS and SFS). Despite its limited solubility, the mesylate salt of saquinavir enables formulation of sufficiently high concentrations (2.5 mM, i.e. ca. 10^5-fold in vitro IC_{50} values) in a standard aqueous vehicle. While saquinavir stays in solution upon dilution with VFS, SFS induces precipitation of saquinavir, resulting in a 5-fold reduced availability and antiviral potency. Inclusion of the solubilizing excipients polyethylene glycol 1000 (12%) and hydroxypropyl-β-cyclodextrin (2.5%) was required to avoid saquinavir precipitation in SFS and to restore the antiviral potency of the formulation. This study illustrates the importance of identifying solubility and precipitation issues of microbicide candidates in biorelevant media and provides a simple in vitro procedure to implement this evaluation in early microbicide development.

1. Introduction

After more than 15 years of research, the first clinical success in the quest for a microbicide that prevents vaginal transmission of HIV was presented in 2010. A gel formulation of the nucleotide reverse transcriptase inhibitor tenofovir succeeded in reducing the risk on HIV transmission in a large-scale clinical trial (CAPRISA 004 study, risk reduction by 39%) (Abdool Karim et al., 2010; Ariën and Vanham, 2010), whereas 6 other microbicide candidates (nonoxynol-9, Savvy®, Carraguard®, cellulose sulfate, PRO-2000, BufferGel®) previously failed. Despite this success, experts in the field have become aware of the fact that the continuation of the search for successful microbicides requires a more careful selection of promising microbicide candidates and their formulations in early development, guided by the results of predictive non-clinical assays (Doncel and Clark, 2010). Only in this way, the efficiency in microbicide development can be improved and costly failures in clinical trials can be minimized.

Early development procedures mainly consist of various in vitro assays (with variable biorelevance) that allow characterization of microbicide candidates with respect to their intrinsic antiviral activity and cervicovaginal toxicity (Lackman-Smith et al., 2008). In contrast, biopharmaceutical properties of microbicide candidates, including solubility and mucosal penetration, typically receive less appreciation in early development. Nevertheless, it is important to realize that the in vivo efficiency of a potential microbicide will not only depend on its intrinsic anti-HIV activity, but also on its biopharmaceutical availability, i.e. the ability to reach the primary target cells for HIV infection upon vaginal application of the microbicide (Garg et al., 2003a; Hladik and Doncel, 2010). Therefore, the implementation of in vitro availability assays in early microbicide development is gaining momentum.

One of the variables that affect microbicide availability upon application of a vaginal gel is its concentration in the vaginal lumen. Because of various reasons (limited cost, ease-of-manufacture, flexible composition, ease-of-application...), aqueous vaginal gels are still the most popular dosage form for the application of microbicides (Garg et al., 2003b). As microbicide gels are intended for immediate protection and have only limited retention in the vaginal tract (hours), they should ensure fast microbicide availability in the (sub-)epithelial tissue of the cervicovaginal tract. This requires a high intravaginal microbicide concentration, which is the driving force for penetration into underlying tissue (by virtue of Fick's first law of diffusion). Obviously, the amount of microbicide dissolved in the gel formulation will dictate initial intravaginal

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concentrations. The presence of vaginal and seminal fluid in the cervicovaginal tract, however, may cause the microbicide to dissolve (in the case of a suspension gel) or precipitate, thereby affecting the availability upon application. The importance of high luminal microbicide concentrations is obvious when considering the reported ratio between effective concentrations in vivo (i.e. applied in the vagina of macaques) versus in vitro (i.e. directly applied onto target cells) for some microbicide candidates under investigation: from 10^3 for certain virus-binding entry inhibitors (Klasse et al., 2006; Veazey et al., 2005), 1.7×10^4 for the NRTI tenofovir (Cranage et al., 2008), 2.5×10^5 for the NNRTI MIV-150 (Turville et al., 2008), up to 3×10^6 for the NNRTI MC-1220 (Stolte-Leeb et al., in press). In practice, this means that, even with in vitro activities in the nM range, luminal microbicide concentrations upon vaginal gel application should reach μM–mM range. Achieving this may be quite challenging considering the limited aqueous solubility of many microbicide candidates, especially small molecular and often rather lipophilic inhibitors of HIV reverse transcriptase, integrase or protease. Hence, it is of crucial importance that potential solubility issues of microbicide candidates are identified in early development to assist in candidate selection and guide formulation strategies.

In the present study, we present a simple, straightforward in vitro assay to identify solubility and precipitation issues in biorelevant media (vaginal and seminal fluid simulants) during early microbicide development. The value of the assay is illustrated in (pre)formulation of the HIV protease inhibitor saquinavir as a microbicide candidate. Saquinavir is a first-generation HIV protease inhibitor that has been used as a component of oral highly active antiretroviral therapy since 1995 (Bragman, 1996; la Porte, 2009). Notwithstanding the fact that protease inhibitors act on the post-integration stage of HIV's lifecycle and are therefore unable to prevent initial infection, their potential as microbicide is currently being investigated (Evans, 2010; Stefanidou, 2010). When used in combination, protease inhibitors may enhance the effect of sub-optimal infection prevention by reverse transcriptase or integrase inhibitors by reducing amplification of infection in primary HIV target cells.

2. Materials and methods

2.1. Drug and excipients

The mesylate salt of saquinavir (MW 766.96 g/mol) was kindly provided by Roche (Hertfordshire, United Kingdom). The following chemicals were used in the formulation vehicles: sorbic acid and glycerol (Sigma–Aldrich, St. Louis, MO), NaCl and sodium acetate trihydrate (VWR, Leuven, Belgium), NaOH (Merck, Darmstadt, Germany), polyethylene glycol 1000 (Acros Organics, Geel, Belgium), hydroxypropyl-β-cyclodextrin (Fluka, Buchs, Switzerland) and hydroxyethylcellulose (Natrosol 250 HHX Pharm, Hercules, Wilmington, DE, USA).

2.2. Viruses and cell cultures

The HIV-1 T-tropic (X4) molecular clone NL4.3 (HIV-$1_{NL4.3}$) was obtained from the National Institute of Allergy and Infectious Disease AIDS Reagent Program (Bethesda, MD). Its viral stocks were collected from the culture supernatant of HIV-infected MT-4 cells. The $CD4^+$ T-cell line MT-4 was obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker Europe, Verviers, Belgium) and 2 mM L-glutamine (Gibco BRL). Cell cultures were maintained at 37 °C in a humidified, CO_2-controlled atmosphere, and subcultivations were done every 2–3 days.

Table 1
Composition and characteristics of the vehicles used for microbicide formulation of saquinavir.

	Universal placebo	Standard	Buffering	Solubilizing
Sorbic acid (%)	0.1	0.1	0.1	0.1
NaCl (%)	0.85	–	–	–
Glycerol (%)	–	2.5	1.5	–
Sodium acetate (%)	–	–	0.82	–
PEG 1000 (%)	–	–	–	12
HPβCD (%)	–	–	–	2.5
pH (adjusted with NaOH)	4.6	4.6	4.6	4.6
Osmolality (mOsm/kg)	289	294	324	319
Ionic strength (mM)	309	4.5	74	4.5
Solubility of saquinavir mesylate (mM)	0.037 ± 0.001	2.9 ± 0.3	2.5 ± 0.1	6.0 ± 0.1

2.3. Formulation vehicles and biorelevant media

The composition and main characteristics of the vehicles used to formulate saquinavir are given in Table 1. The osmolality was determined using an Advanced Osmometer 3250 (Advanced Instruments, Norwood, MS). Upon addition of the gelling agent hydroxyethylcellulose (HEC, 1.6%) and mixing for 2 h (37 °C), homogeneous and clear gels could be formed. As biorelevant media, vaginal and seminal fluid simulants (VFS and SFS) were prepared according to Owen and Katz (1999, 2005). Their composition and major characteristics are summarized in Table 2.

2.4. Solubility assessment

The solubility of saquinavir mesylate in the formulation vehicles (in absence of HEC) was determined by mixing an excess of saquinavir mesylate powder (2–4 mg) in the respective media (0.25 ml) on a 3D Rocking Platform STR9 (Stuart Scientific, Staffordshire, UK) at 37 °C. After 24 h, samples were centrifuged (21,000*g*, 37 °C, 10 min). An appropriate dilution of the supernatant was injected into the HPLC system (see below).

2.5. Precipitation assessment in biorelevant media

Fig. 1 schematically depicts the procedure to assess precipitation of microbicide candidates in biorelevant fluids. In the present study, saquinavir mesylate was dissolved at a low (0.15 mM) or high (2.5 mM) concentration in (1) standard, (2) buffering and (3) solubilizing vehicles (in absence of HEC, Table 1). These saquinavir formulations were divided into aliquots of 50 μl in a 96-well plate, which was incubated at 37 °C in an orbital shaker (600 rpm) (Thermostar, BMG Labtech, Offenburg, Germany). VFS (20 μl) and SFS (70 μl) were added after 10 and 130 min, respectively. The dilution factor (5:2:7) corresponds to 2.5 g of gel applied into the vagina, diluted with 1 ml of vaginal fluid and, upon coitus, 3.5 ml of semen; these volumes represent mean values reported in the literature (Owen and Katz, 1999, 2005). Samples (50 μl) were taken after 10 (prior to dilution with VFS), 20, 40, 70, 100, 130 (prior to dilution with SFS), 140, 160, 190 and 250 min (one time point/well). All samples were immediately centrifuged (21,000*g*, 37 °C, 10 min) upon which 20 μl supernatant was diluted in 0.5 ml of a mixture of acetate buffer (37 mM, pH 4.0) and methanol (25:75 v/v) to precipitate any proteins in the sample. After centrifugation (5 min at 21,000*g*), an appropriate dilution of the supernatant was injected into the HPLC system (see below) to determine saquinavir concentrations.

Using a modified, semi-quantitative version of this procedure, solubilizing excipients were screened for their capacity to limit

Table 2
Composition and characteristics of vaginal and seminal fluid simulant.

	Vaginal fluid simulant (VFS)	Seminal fluid simulant (SFS)
Composition (g/l)	NaCl (3.5), KOH (1.4), $Ca(OH)_2$ (0.22), BSA[a] (0.018), lactic acid (2.0), acetic acid (1.0), glycerol (0.16), urea (0.4), glucose (5.0)	$NaH_2PO_4.H_2O$ (0.049), Na_2HPO_4 (0.43), sodium citrate (8.1), KCl (0.91), KOH (0.88), fructose (2.72), glucose (1.02), lactic acid (0.62), urea (0.45), BSA (50.4), $CaCl_2.(H_2O)_2$ (1.01), $MgCl_2.(H_2O)_8$ (0.92), $ZnCl_2$ (0.34)
pH	4.2	7.7
Osmolality (mOsm/kg)	232	330
Ionic strength (mM)	88	238

[a] BSA, bovine serum albumin

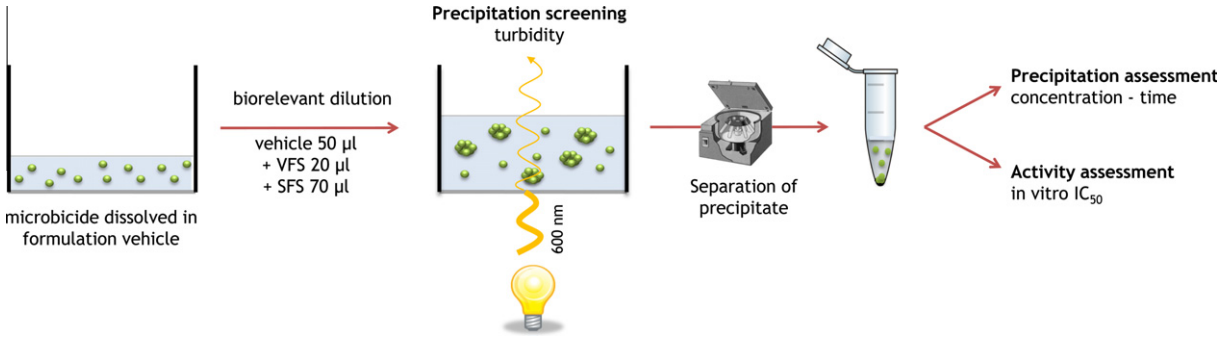

Fig. 1. Protocol to identify availability issues of microbicide candidates in biorelevant media. Microbicide candidates are dissolved in an appropriate formulation vehicle. In a 96-well plate, 50 µl of the microbicide vehicle is diluted with either VFS (20 µl) or VFS and SFS (70 µl). For semi-quantitative screening of precipitation, the turbidity of the diluted vehicle can be monitored by measuring its optical density at 600 nm. For a quantitative analysis of precipitation, the precipitate is separated by centrifugation, followed by concentration assessment as a function of time in the supernatant. Finally, the impact of precipitation on the in vitro antiviral activity of the vehicle can be assessed.

saquinavir precipitation upon dilution with biorelevant fluids. Instead of concentration assessment, the turbidity of the diluted saquinavir formulations (see above) was determined in absence or presence of excipients, by measuring the optical density of the mixtures at 600 nm (Tecan Infinite M200 microplate reader, Tecan Benelux, Mechelen, Belgium). The following excipients were screened: polyethylene glycol (PEG) 400, 1000 and 3350, hydroxypropyl methylcellulose (HPMC), Chremophor EL®, D-α-tocopheryl polyethyleneglycol 1000 succinate (TPGS), polysorbate 80, hydroxypropyl-β-cyclodextrin (HPβCD), and sulfobutylether-β-cyclodextrin (SBEβCD).

Finally, the turbidity measurement was also applied to assess precipitation in saquinavir gels (i.e. standard and solubilizing vehicles with HEC as gelling polymer) at 10, 30 and 60 min after dilution of 50 mg gel with 20 µl VFS and 70 µl SFS.

2.6. Analytical assay for saquinavir

Saquinavir concentrations were determined by reversed phase HPLC and UV detection. A volume of 50 µl was injected into a Hitachi LaChrom Elite HPLC system consisting of an L-2130 pump, an L-2200 autosampler, an L-2400 UV detector and EZChrom Elite software (VWR, Leuven, Belgium). The column used was a Purospher® Star RP-18 (150 × 4.6 mm, 5 µm) and the mobile phase consisted of acetate buffer (37 mM, pH 4.0) and methanol (25:75, v/v). The flow was maintained at 0.9 ml/min. Saquinavir was detected by absorbance monitoring at 240 nm; its retention time amounted to 5.7 min. Calibration curves of saquinavir in mobile phase were linear over the concentration range from 0.1 to 100 µM. All samples were diluted to fit in this range. At the appropriate dilutions, the presence of excipients, vaginal or seminal fluid simulant did not interfere with saquinavir detection. Precision and accuracy were assessed by analyzing standard samples ($n = 5$) of 0.1, 3 and 100 µM. Intraday repeatability and mean bias were below 6% at all concentrations.

2.7. In vitro activity of saquinavir formulations against HIV-1

The impact of VFS and SFS on the in vitro activity of saquinavir formulations against the HIV-1 strain $X4_{NL4.3}$ was evaluated in MT-4 cells. Saquinavir was dissolved in standard and solubilizing vehicle at low (0.15 mM) and high (2.5 mM) concentrations. Vehicles were diluted with VFS (5:2) or with both VFS and SFS (5:2:7). Upon incubation (1 h, 37 °C) and centrifugation, samples were frozen (−20 °C) until activity assessment. Placebo samples were prepared in the same way, but did not contain any saquinavir. Subsequently, the anti-HIV-1 activity of both native saquinavir (solution in DMSO) and the saquinavir formulations (diluted with VFS and/or SFS) was evaluated using a 5-fold dilution series of the samples with cell culture medium (100 µl) in 96-well flat-bottomed plates (International Medical, Brussels, Belgium). To each well, 7.5×10^4 MT-4 cells were added in 50 µl of medium, followed by 50 µl (500 pg/ml p24 Ag) of diluted HIV-1 stock. Cytopathic effect (CPE) induced by the virus was checked microscopically at regular times. When strong CPE was observed (mostly after 4 or 5 days of incubation) in untreated HIV-infected cells, the supernatant of all samples was collected simultaneously and stored at −20 °C. We assessed productive HIV-1 infection by measuring p24 Ag concentration in culture medium using a p24 Ag ELISA commercial kit (Perkin Elmer, Boston, MA). Finally, the IC_{50} value of the samples was determined as the vehicle concentration required for 50% reduction in HIV replication as measured by the p24 antigen production.

The cytotoxicity of the samples was determined in MT-4 cells using a tetrazolium-based colorimetric assay. Briefly, after 5 days of incubation of the cells with the samples, cell viability was assessed spectrophotometrically via the *in situ* reduction of the tetrazolium compound MTS, using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI). The absorbance was then recorded at 490 nm with a 96-well plate reader (Molecular Devices, Sunnyvale, CA). Finally, the CC_{50} value

of the samples was determined as the formulation concentration required for 50% reduction in cell viability.

3. Results and discussion

Saquinavir's IC_{50}-value for the in vitro inhibition of infection of MT-4 cells by HIV-$1_{NL4.3}$ amounted to 16 ± 4 nM, within the range of previously reported anti-HIV-1 IC_{50}-values (1–30 nM) (Roberts et al., 1990; Bragman, 1996; Invirase EPAR, 2011). Assuming that effective concentrations in vivo need to be 10^4- to 10^5-fold higher than in vitro, intravaginal saquinavir concentrations should reach about 0.2–2 mM upon application of a gel formulation. From a physicochemical point of view, saquinavir is a lipophilic (XLog*P* = 4.2 (Cheng et al., 2007)), weakly basic (pK_a's ≅ 2 and 7) compound with a limited aqueous solubility (0.3 mM) (Buchanan et al., 2008). The mesylate salt of saquinavir, however, has an improved solubility in water (2.7 ± 0.2 mM). In the present study, we evaluated the feasibility to reach saquinavir concentrations within the target range of 0.2–2 mM, taking into account the effects of both formulation and biorelevant fluids on saquinavir availability.

3.1. Solubility assessment: selecting a starting vehicle for formulation

As initial intravaginal concentrations are dictated by the amount of saquinavir dissolved in the formulation, we determined the solubility of saquinavir mesylate in potential vehicles, depicted as universal placebo and standard vehicle in Table 1. As the separation of dissolved from non-dissolved drug is required for accurate solubility determination but not readily feasible in highly viscous gels, these vehicles did not contain a gelling agent. Evaluation of saquinavir availability in presence of the gelling agent HEC, using a semi-quantitative tool (turbidity measurement), will be discussed at the end of Section 3.2. A gel based on the universal placebo vehicle has previously been recognized as non-effective against HIV transmission and safe for vaginal use due to its compatibility with the normal vaginal environment (e.g. pH 4–5, physiological osmolality) and absence of any harmful agents (Tien et al., 2005). Therefore, it is an appropriate starting vehicle for microbicide formulation. However, the solubilizing capacity of the universal placebo for saquinavir mesylate was very limited (0.037 ± 0.001 mM), presumably due to the relatively high ionic strength. To reduce the ionic strength, NaCl was omitted from the vehicle; the use of a limited amount (2.5%) of the cosolvent glycerol preserved the physiological osmolality. In this standard vehicle, the solubility of saquinavir mesylate increased up to 2.9 ± 0.3 mM, enabling the vaginal application of saquinavir at concentrations that are about 10^5-fold higher than its in vitro anti-HIV activity. It should be noted that the use of glycerol may affect certain properties of vaginal gels, including rheology and solubilizing capacity (Rowe et al., 2009). However, in the present study, effects will be minimal due to the limited amount of glycerol used. In addition, recently reported safety issues and unacceptable side effects related to the use of glycerol in vaginal gels (Lacey et al., 2010) are only relevant in case of high concentrations causing hyperosmolality.

3.2. Precipitation assay: evaluating the impact of biorelevant fluids on saquinavir availability

Upon vaginal application, an aqueous microbicide gel will be diluted with vaginal fluid and, upon coitus, with semen. The shift in physicochemical conditions (e.g. pH, ionic strength…) may affect the solubility of poorly soluble microbicide candidates, including saquinavir. As schematically depicted in Fig. 1, we evaluated the in vitro behavior of formulated saquinavir upon dilution with vaginal fluid simulant (VFS) (Owen and Katz, 1999) and seminal fluid simulant (SFS) (Owen and Katz, 2005). These simulants, designed to reflect the composition of vaginal and seminal fluid, respectively, are relatively easy to prepare and therefore appropriate as biorelevant medium in early microbicide development. While they have been used to predict the impact of physiological fluids on gel rheology (Owen et al., 2003, 2007; Gupta et al., 2007; Lai et al., 2008; Mahalingam et al., 2010), microbicide release (Andrews et al., 2009; Gupta et al., 2007) and microbicide activity (Sassi et al., 2008), no reports have described their use in solubility and/or precipitation studies.

In Fig. 2, the saquinavir concentration is shown as a function of time upon dilution of formulated saquinavir (in absence of the gelling agent HEC) in VFS and SFS. When dissolved at a high concentration in the standard vehicle (2.5 mM, i.e. below the equilibrium solubility), saquinavir stayed in solution upon dilution with VFS (dilution ratio 5:2): the observed saquinavir concentrations were as expected based on the initial concentration and dilution factor. Remarkably, the saquinavir concentrations upon dilution with VFS (about 1.9 mM) were 6-fold higher than the thermodynamic solubility of saquinavir mesylate in a mixture (5:2) of the standard vehicle and VFS (0.29 ± 0.02 mM). This illustrates the importance of a time-dependent precipitation assay rather than a simple solubility measurement: microbicide concentrations may exceed its thermodynamic solubility, thereby creating a metastable, supersaturated solution that improves microbicide availability for penetration into the (sub)epithelium. The importance of supersaturation as a driving force for permeation of poorly soluble drugs has been recognized in the fields of topical (Pellett et al., 1997) and, more recently, oral drug delivery (Brouwers et al., 2009).

While saquinavir remained available in VFS, further dilution of the standard vehicle with SFS (vehicle:VFS:SFS 5:2:7) caused saquinavir to precipitate: concentrations dropped more than 5-fold below the expected concentration. This suggests that, in vivo, coitus may cause saquinavir formulated in a standard aqueous vehicle, to precipitate, thereby reducing its availability as microbicide. The observed precipitation in SFS is presumably due to a combination of increased ionic strength (detrimental for the solubility of the mesylate salt) and increased pH (Table 2). While the weak base saquinavir (p*K*a 7) is completely ionized in the vehicle (pH

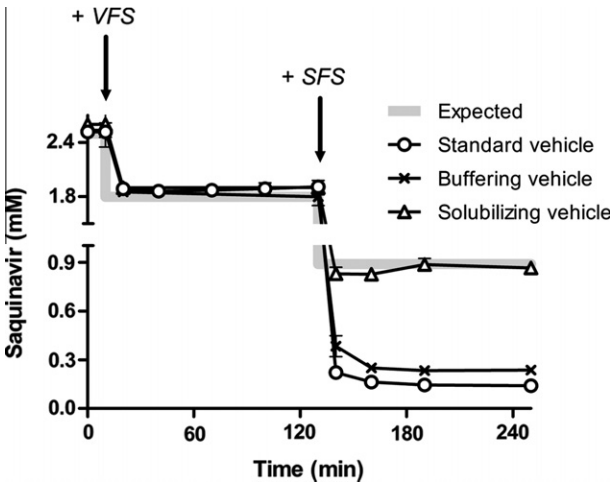

Fig. 2. Saquinavir concentration as a function of time upon dilution of saquinavir formulations with VFS and SFS. Saquinavir was dissolved at 2.5 mM in standard (○), buffering (×) and solubilizing (Δ) vehicles (composition: see Table 1). VFS (20 μl) and SFS (70 μl) were added to 50 μl of vehicle after 10 and 130 min, respectively. The grey line represents the expected concentration, based on the dose and dilution factors. An observed concentration below the expected concentration indicates saquinavir precipitation. Data represent mean ± SD (n = 3).

4.6) and upon dilution with VFS (pH of dilution = 4.5), a significant fraction is unionized and less soluble upon adding SFS (pH of dilution = 7.5). To limit the pH increase upon dilution with SFS, we dissolved saquinavir mesylate (2.5 mM) in an alternative, buffering vehicle (Table 1) and evaluated its precipitation behavior. However, despite a reduced pH increase (up to 5.6), saquinavir still precipitated upon dilution with SFS (Fig. 2), suggesting a major role for the increased ionic strength of SFS in precipitation induction.

To avoid SFS-induced saquinavir precipitation, we screened various solubilizing excipients for their potential to maintain saquinavir in solution, including cosolvents, surfactants and cyclodextrins. Based on high-throughput turbidity measurements (Fig. 1), the combination of the cosolvent polyethylene glycol 1000 (PEG-1000, 12%) with hydroxypropyl-β-cyclodextrin (HPβCD, 2.5%) was selected to be further investigated as solubilizing vehicle for saquinavir (Table 1). As depicted in Fig. 2, saquinavir, dissolved in this solubilizing vehicle (2.5 mM), remained in solution, even upon dilution with SFS; this ensures that saquinavir remains available for penetration into the (sub)epithelium. The use of HPβCD as an excipient in dosage forms for intravenous, oral and topical (including vaginal) application has previously been described (Cal and Centkowska, 2008; Davis and Brewster, 2004; Francois et al., 2003); its solubilizing properties are attributed to the formation of dynamic, non-covalent and water-soluble inclusion complexes with poorly water-soluble drugs.

When selecting solubilizing excipients to be included in microbicide formulations, their solubilizing capacity should not be the only criterion. As recently demonstrated by Gali et al. (2010), many of these excipients may exert detrimental effects on the integrity and viability of cervicovaginal epithelial layers, potentially causing increased HIV transmission. For this reason, we selected HPβCD over the surfactants Cremophor EL and TPGS that performed equally well in solubilizing saquinavir, but showed greater toxicity (Gali et al., 2010). As hyperosmolar vaginal gels may cause unacceptable side effects and may harm the cervicovaginal epithelium (Fuchs et al., 2007; Lacey et al., 2010), it is also important to note that none of the proposed vehicles for saquinavir are significantly hyperosmolar (Table 1).

In the above mentioned experiments, saquinavir was dissolved in liquid vehicles instead of semi-solid gels that will be applied in vivo. Precipitation kinetics may be affected by saquinavir's diffusion coefficient in the vehicles, which is obviously lower in gels. Therefore, we also assessed precipitation upon dilution of saquinavir gels (i.e. the standard and solubilizing vehicles gelled with hydroxyethylcellulose 1.6%) with VFS and SFS. Due to difficulties in separating dissolved and precipitated saquinavir in highly viscous gels, concentration monitoring was not feasible. However, as reported in Fig. 3, turbidity measurements clearly indicated that, within 10 min after dilution with VFS/SFS, saquinavir precipitated from the standard gel (increased turbidity), but not from the solubilizing gel (remained clear). These results confirm the precipitation behavior of saquinavir.

3.3. In vitro anti-HIV activity of formulated saquinavir upon dilution with biorelevant fluids

In addition to saquinavir concentration monitoring, we assessed the impact of biorelevant fluids on the in vitro anti-HIV-1 activity of the saquinavir formulations (Fig. 1). In Fig. 4, the vehicle concentration required for 50% reduction in HIV replication (IC_{50}), is reported for undiluted and diluted saquinavir formulations (corrected for the dilution factor). The precipitation, observed when diluting high-dose saquinavir (2.5 mM) in the standard vehicle with VFS/SFS (Fig. 2), resulted in a 4-fold decrease in antiviral potency as compared to the undiluted vehicle. It has previously been reported that seminal fluid may directly affect the antiviral activity of microbicide candidates (in particular polyanionic compounds) (Neurath et al., 2006; Patel et al., 2007; Lackman-Smith et al., 2008). In the present study, however, SFS did not interfere with the intrinsic antiviral activity of saquinavir but reduced the available saquinavir concentration. Indeed, inclusion of the solubilizing excipients PEG-1000 and HPβCD restored the antiviral potency, in agreement with the lack of precipitation (Fig. 2). Note that a placebo vehicle, containing PEG-1000 and HPβCD but no saquinavir, did not reduce the anti-HIV-1 activity at the concentrations tested (data not shown). Finally, neither precipitation (data not shown) nor a reduction in antiviral activity was observed when diluting low-dose saquinavir (0.15 mM in standard vehicle) with VFS/SFS.

Based on the activity of the formulations and their initial saquinavir concentration, the intrinsic anti-HIV-1 activity of formulated saquinavir was calculated. In samples without

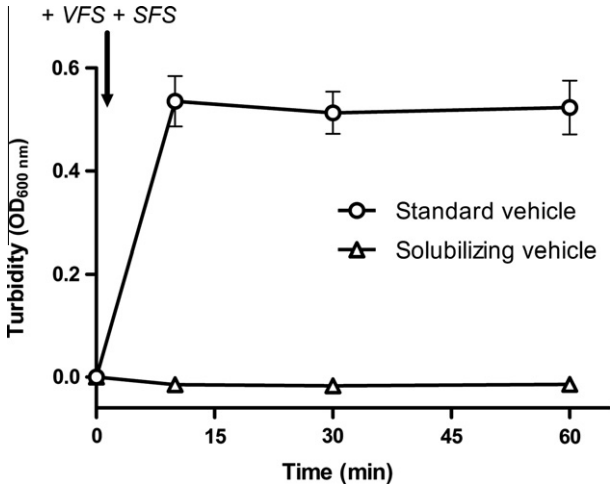

Fig. 3. Turbidity of saquinavir gels as a function of time upon dilution with VFS and SFS. Saquinavir was dissolved at 2.5 mM in standard (○) and solubilizing (Δ) gel (i.e. vehicle (Table 1) + HEC 1.6%). VFS (20 μl) and SFS (70 μl) were added to 50 mg of gel. Turbidity was assessed as the optical density at 600 nm. Data represent mean ± SD (n = 3).

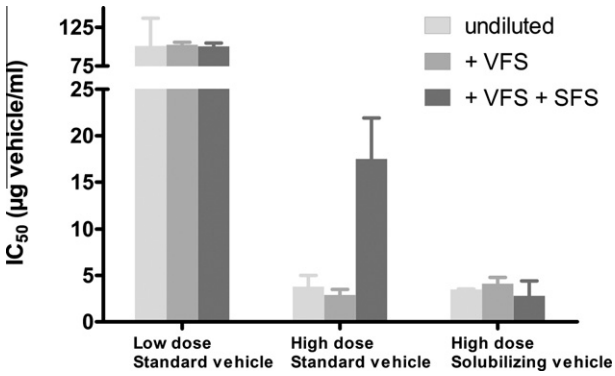

Fig. 4. Anti-HIV-1 activity of saquinavir formulations before and after dilution with VFS and SFS. Saquinavir was dissolved at low (0.15 mM) or high (2.5 mM) concentration in standard and solubilizing vehicles (composition: see Table 1). Vehicles (50 μl) were tested undiluted, diluted with VFS (20 μl) or diluted with VFS and SFS (70 μl). The anti-HIV-1 activity is reported as IC_{50}-values of the formulations (corrected for dilution) to inhibit infection of MT-4 cells by HIV-$1_{NL4.3}$. Placebo vehicles (no saquinavir) did not reduce the anti-HIV-1 activity at the concentrations tested. The cytotoxicity of the solubilizing vehicles was 5-fold higher compared to the standard vehicles. However, in all cases, cytotoxicity was only observed at concentrations at least 1.6×10^3-fold higher than corresponding IC_{50}-values. Data represent mean ± SD (n = 2–5).

precipitation (all except SFS-diluted, high dose saquinavir in standard vehicle), the calculated saquinavir IC_{50} varied between 7 and 15 nM, comparable to the activity of native saquinavir (16 ± 4 nM). This indicates that formulating saquinavir in either the standard or solubilizing vehicle does not affect its intrinsic activity. In contrast, the calculated IC_{50} of saquinavir formulated at high concentration in standard vehicle and diluted with VFS and SFS, was 3-fold higher (44 ± 10 nM) than the IC_{50} of native saquinavir. This decreased activity reflects the reduced availability of saquinavir in standard vehicle upon dilution with SFS.

4. Concluding remarks

In the present in vitro study, we evaluated solubility and precipitation issues of the microbicide candidate saquinavir in biorelevant media. Despite the limited solubility of saquinavir, its mesylate salt enables formulation at sufficiently high concentrations (2.5 mM, i.e. $>10^5$-fold the in vitro anti-HIV-1 activity) in a standard aqueous vehicle for vaginal application. However, our results suggest that saquinavir may precipitate in the vaginal environment, in particular in presence of semen. Solubilizing excipients are required to avoid precipitation and preserve the antiviral potency of the saquinavir formulation.

Considering the lipophilic nature of many microbicide candidates (most protease, reverse transcriptase and integrase inhibitors), this study illustrates the importance of investigating potential solubility/precipitation issues in biorelevant fluids during development of aqueous vaginal gels. The straightforward evaluation procedure presented here (Fig. 1) employs common laboratory tools and relatively simple but biorelevant media (VFS/SFS), and enables high (turbidity screening) to medium throughput (concentration and activity assessment). In addition, only a limited amount of active ingredient is required. Consequently, the procedure is appropriate for implementation in early microbicide development where it may contribute to the biopharmaceutical profiling of microbicide candidates. As microbicide availability is crucial to translate intrinsic antiviral activity to in vivo efficacy, a biopharmaceutical profile is a valuable instrument to guide formulation strategies and to assist in early selection of microbicide candidates. While the procedure presented here focuses on intravaginal availability, approaches to implement tissue penetration in the early biopharmaceutical profiling of microbicide candidates are currently being investigated.

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