Virology

Development and in vitro evaluation of chloroquine gels as microbicides against HIV-1 infection

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ABSTRACT

The potential success of a microbicide candidate in resource-poor countries will depend to a large extent on its availability and cost. Chloroquine is an inexpensive antimalarial drug that also exerts anti-HIV activity. The purpose of this study was to develop and characterize a vaginal formulation for chloroquine with preservation of its anti-HIV-1 activity. Gels containing the nonionic polymer hydroxyethyl cellulose were loaded with concentrations of the diphosphate salt of chloroquine (0.3–30 mg/g), that were 10^2- to 10^4-fold higher than typical in vitro anti-HIV-1 IC_{50}-values of chloroquine (ca. 6 µg/ml). The gels were clear and homogeneous and displayed an osmolality of 300 mOsm/kg, a pH of 4.6 and a viscosity of 1.4 Pa s. Gel characteristics were preserved for at least 3 months at 40 °C and 75% relative humidity. Importantly, the chloroquine gels exerted a dose-dependent anti-HIV-1 activity in vitro (mean IC_{50} from 23 to 0.4 mg gel/ml) and the intrinsic activity of chloroquine was not affected by formulation factors. The in vitro efficacy of the chloroquine gel formulations warrants further testing of this drug as an anti-HIV-1 microbicide candidate.

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Introduction

The development of microbicides is currently one of the most emphasized prevention strategies in the effort to slow the spread of HIV (Balzarini and Van Damme, 2007; Klasse et al., 2008). Microbicides are antiretroviral agents that prevent HIV transmission when topically applied in the vagina or rectum. In contrast to for instance condom use, microbicides should empower women to protect themselves. Especially in sub-Saharan Africa, where three times more 15- to 24-year-old women are now HIV-1 infected than men of the same age, an effective microbicide will have an important impact on the HIV epidemic (Quinn and Overbaugh, 2005). In addition to its safety, acceptability and efficacy, the affordability of a microbicide will determine its success in resource-poor countries. For some state-of-the-art microbicide candidates performing very well in preclinical studies (e.g. protein- and peptide-based microbicide candidates), a high manufacturing cost may be the most important hurdle hindering their real-world success (Klasse et al., 2006). Therefore, available and inexpensive antiretroviral drugs, including the 4-aminoquinoline chloroquine, should not be ignored in the search for a useful microbicide.

Chloroquine has been used for decades as the primary and most successful drug against malaria. Concomitant with the emergence of chloroquine-resistant *Plasmodium* strains and a subsequent decrease in the use as antimalarial drug (Wellems and Plowe, 2001), other applications of chloroquine and its hydroxyl analogue hydroxychloroquine have been investigated. In vitro activity of (hydroxy)chloroquine against a wide variety of bacterial, fungal and viral infections has been shown (Rolain et al., 2007). In addition, chloroquine exerts immunomodulatory effects (Savarino et al., 2003), which may be beneficial to treat autoimmune diseases (e.g. rheumatoid arthritis) or to mediate inflammatory responses in microbial infections (e.g. malaria; Ramos-Avila et al., 2007).

Many studies have focused on the activity of (hydroxy)chloroquine against HIV (Savarino et al., 2001a). The presence of chloroquine at noncytotoxic concentrations, either prior or during incubation with HIV, inhibits in vitro HIV infection of different HIV permissive cells, including T cell lines and CD4$^+$ T lymphocytes or monocytes from peripheral blood (Boelaert et al., 1999; Pardridge et al., 1998; Sperber et al., 1993). (Savarino et al., 2001b) reported a broad spectrum anti-HIV activity of chloroquine, not only against laboratory strains of HIV-1 clade B but importantly also against primary isolates from HIV-1 clades A, B, C, D, E and against HIV-2; the antiviral activity of chloroquine does not depend on coreceptor (CXCR4/CCR5) usage (Savarino et al., 2001b).

The major mechanism for the anti-HIV effect of chloroquine seems to be the inhibition of glycosylation of the gp120 viral envelope protein, resulting in newly produced virions with severely reduced infectivity (Savarino et al., 2004, 2006). Presumably, the weak base chloroquine accumulates in acidic organelles, including endosomes

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and the trans-Golgi network, so that the activity of glycosylating enzymes may be impaired by the increased pH or by a specific interaction with chloroquine. The mechanism of action of chloroquine has some consequences. First, as glycosylation in host cells is crucial for the replication of many viruses, it is not surprising that chloroquine is not only active against various HIV strains, but also against other viruses (e.g. SARS coronavirus; Keyaerts et al., 2004) among which also other sexually transmitted infections (e.g. hepatitis B virus and herpes simplex viruses; Rolain et al., 2007). Secondly, the fact that chloroquine clearly affects host cellular enzymes (Naarding et al., 2007) may suggest that the likelihood of emergence of viral resistance against chloroquine is rather low. Finally, considering that the anti-HIV mechanism of chloroquine differs from mechanisms of commonly used antiretroviral drugs, combination can result in additive effects. This has been illustrated for the combination of chloroquine with hydroxyurea, the reverse transcriptase inhibitors zidovudine and didanosine and protease inhibitors (Boelaert et al., 2001; Savarino et al., 2004).

The use of (hydroxy)chloroquine in relatively inexpensive treatment regimens for HIV-1 infected patients in developing countries has been suggested; these regimens may delay the initiation of highly active antiretroviral therapy (HAART). Small-scale clinical trials have shown a reduction of the viral load in HIV-1 infected patients upon treatment with (hydroxy)chloroquine, either alone (Sperber et al., 1995, 1997) or in combination with a reverse transcriptase inhibitor (Joshi et al., 2004; Paton and Aboulhab, 2005).

The broad spectrum anti-HIV activity of chloroquine, its unique mechanism and its low cost warrant the investigation of chloroquine as affordable microbicide candidate. After characterization of the antiviral activity profile, an essential step in developing a microbicide involves evaluating the potential of the compound to be delivered in an active form at the vaginal or rectal cavity (Garg et al., 2003). In the present study, we developed and characterized a gel formulation containing chloroquine with preserved anti-HIV-1 activity, enabling further testing of chloroquine as an anti-HIV microbicide candidate.

Results and discussion

Development and characterization of a gel formulation for chloroquine

In order to explore the potential of chloroquine as an inexpensive microbicide, an appropriate dosage form is required to apply chloroquine to the cervicovaginal cavity. The most straightforward formulation strategy for microbicides are gels or creams, to be applied in the vagina before sexual intercourse (Garg et al., 2003). To formulate chloroquine, we prepared an aqueous gel composed of the gelling polymer hydroxyethyl cellulose (HEC), the humectant glycerol, the preservatives propyl- and methylparaben and lactic acid as acidifier. This placebo formulation was loaded with the diphosphate salt of chloroquine at different concentrations. HEC is a stable, nonionic linear polymer with a good safety profile, shear-thinning rheology and moderate mucoadhesive properties (Grabovac et al., 2005; Tien et al., 2005). Three independent batches of the gels were characterized with respect to fundamental properties, including appearance, pH, osmolality, viscosity and chloroquine loading.

Appearance

Both the placebo and chloroquine loaded formulations were clear, homogeneous, odorless and non-greasy gels. All these properties are in favor of the acceptability of the formulations, a critical issue for the real-world success of a microbicide.

pH

The pH of the gels amounted to 4.6±0.1, in the range of the normal, premenopausal vaginal pH (Owen and Katz, 1999). As the gels do not possess significant buffering capacity, alterations in vaginal pH, induced by e.g. the hormonal cycle or the presence of semen (pH 7–8; Owen and Katz, 2005), will not be buffered by the gels. However, due to the absence of ionizable groups in the gelling polymer HEC, important gel characteristics including rheology and mucoadhesion are independent of pH (Rowe et al., 2003).

Osmolality

The osmolality of the gels amounted to 300±3 mOsm/kg. Although many commercial vaginal products are hyperosmolar, it has been reported that hyperosmolar gels induce severe denudation of rectal (and possibly also cervicovaginal) epithelia, which may cause an increased transmission of HIV (Fuchs et al., 2007). Therefore, the osmolality of microbicide formulations preferably falls within the range typical of physiological fluids (including vaginal fluid; Owen and Katz, 1999).

Viscosity

The viscosity of microbicide gels will affect both their distribution and retention in the vaginal cavity (Kieweg and Katz, 2007; Owen et al., 2000). In the present study, we used a vibrational viscometer to measure the viscosity of both the chloroquine gels and six commercially available vaginal gels or creams. As reported in Table 1, the viscosity of the chloroquine gels (1.4±0.2 Pa s) fell within the lower viscosity range of the commercial products. Obviously, the viscosity can easily be raised by increasing the concentration of the gelling polymer HEC; however, it should be noted that too viscous gels are more difficult to apply and may hinder proper distribution in the vaginal cavity, which is crucial for microbicide effectiveness (Owen et al., 2007).

Chloroquine loading

The rather moderate antiretroviral activity of chloroquine (typical in vitro IC_{50}-values in the order of μg/ml) and the observation that effective microbicide concentrations in vivo are typically 10^2- to 10^6-fold higher than in vitro (Klasse et al., 2006) necessitate the application of high doses of chloroquine in the vaginal cavity. We loaded the gels with concentrations of chloroquine diphosphate ranging from 0.3 to 30 mg/g. As chloroquine diphosphate is freely soluble in aqueous media (solubility ≥100 mg/ml; Verbeeck et al., 2005), these concentrations could easily be dissolved in the gels. Moreover, no precipitation was observed upon increasing the pH to 7.5 (data not shown). For quality control purposes, we developed an analytical methodology to assess the chloroquine concentration after preparation of the gel. At all loadings, the maximum deviation between the measured and theoretical concentrations was below 4%, not exceeding the analytical error (see Materials and methods).

Stability of the gel formulation

The ability to store a microbicide without loss of characteristics in the often tropical climate of certain target regions, will be crucial for its success. Therefore, samples of the placebo gel and the gel loaded with chloroquine diphosphate 3 mg/g were examined after storage for 3 months at 40 °C and 75% relative humidity; the maximum relative changes in mass, pH, osmolality, viscosity and chloroquine concentration are reported in Table 2. The results clearly show the stability of the active

Table 1
Viscosity of chloroquine gels and commercially available vaginal gels/creams

Vaginal gel/cream	Use	Viscosity (Pa s)[a]
Chloroquine gels	Microbicide	1.4±0.2[b]
Canasten®	Antimycotic	>5
Gyno-Daktarin®	Antimycotic	1.8
KY Jelly®	Lubricant	2.6
Lacta-Gynecogel®	Acidifying gel	>5
Phyto-Soja®	Moisturizer	0.1
Replens®	Moisturizer	2.8

[a] The viscosity was measured at 37 °C with a vibrational viscometer.
[b] Mean±sd of 3 independent batches.

Table 2
Stability of chloroquine gel (3 mg/g) after 3 months at 40 °C and 75% relative humidity

Variable	Maximum relative change
Appearance	No visual change
Mass	−2%
pH	−7%
Osmolality	+1%
Viscosity	−14%
Chloroquine concentration	±3%

ingredient chloroquine in the formulation. In addition, the maximum relative changes in mass, pH and osmolality fell within the range of experimental error, while the decrease in viscosity (14%) was similar to previously reported observations for HEC-based gels (Tien et al., 2005).

In vitro activity of the gel formulations against HIV-1

As described in the introduction, the anti-HIV activity of chloroquine has been extensively studied. Obviously, this activity should be preserved in the gel formulations of chloroquine to warrant further investigation of these gels as microbicide formulations. Therefore, the activity of the gels against infection of MT-4 cells by HIV-1$_{NL4.3}$ was investigated. In Fig. 1, the average gel concentration required for 50% reduction in HIV replication (IC$_{50}$) is reported in function of the chloroquine loading. Table 3 lists detailed results for one representative batch of gels.

All chloroquine containing gels were able to inhibit HIV-1 infection of MT-4 cells. Fig. 1 clearly shows an increase in activity of the gels with increasing chloroquine loading. The IC$_{50}$-values of formulated chloroquine (calculated based on the IC$_{50}$-values of the gels and the corresponding chloroquine loading, Table 3), were comparable to the IC$_{50}$ of native chloroquine (6.3 µg/ml). In addition, no significant anti-HIV-1 activity was observed upon incubation with the placebo gel (Table 3). These observations demonstrate that chloroquine can be formulated in the gels with preservation of its anti-HIV-1 activity.

Taking into account both in vitro anti-HIV-1 activity and cytotoxicity towards MT-4 cells, a loading of 3 mg chloroquine diphosphate/g gel performed best in the current study. Applied at the highest concentrations (≥20 mg gel/ml), the 3 mg/g gel completely blocked HIV-1 infection in our model system (100% inhibition), without being cytotoxic for MT-4 cells. Although the gels with higher chloroquine loading (10
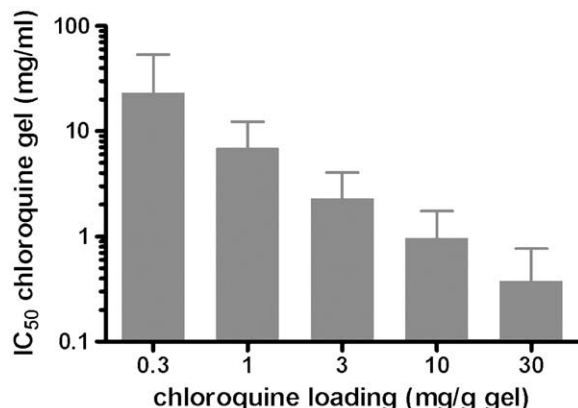

Fig. 1. Anti-HIV-1 activity of chloroquine gels in function of chloroquine loading. HEC gels were loaded with different concentrations of the diphosphate salt of chloroquine (i.e., 0.3, 1, 3, 10 and 30 mg/g) and evaluated for their antiviral potency. MT-4 cells were infected with HIV-1$_{NL4.3}$ in the presence of a serial dilution of the gels. The IC$_{50}$-values are the gel concentrations (mg gel/ml cell culture medium) required to reduce viral HIV-1 replication by 50% as calculated from the p24 content in the supernatant of infected cells at the end of the experiment (day 5). The placebo gel (no chloroquine) did not reduce HIV-1 replication at the concentrations tested (up to 100 mg gel/ml). Data represent mean ± sd of 3 independent experiments.

Table 3
Anti-HIV-1 activity (IC$_{50}$) and cytotoxicity (CC$_{50}$) of chloroquine gels in MT-4 cells[a]

Chloroquine loading (mg/g)	CC$_{50}$	IC$_{50}$	
	Gel (mg/ml)	Gel (mg/ml)	Chloroquine (µg/ml)[b]
Placebo	>100	>100	–
0.3	>100	44.7	13.4
1	>100	8.3	8.3
3	>100	1.9	5.6
10	44.7	0.8	7.9
30	9.6	0.3	8.1

[a] Values represent one representative batch of gels.
[b] The IC$_{50}$-values for chloroquine were calculated based on the IC$_{50}$-values for the gels and the chloroquine loading.

and 30 mg/g) were still significantly selective in their antiviral effect (based on Table 3, the selectivity indices (CC$_{50}$/IC$_{50}$) amounted to 56 and 32, respectively), increased cytotoxicity was observed (reduced CC$_{50}$).

Although cytotoxicity towards MT-4 cells is not representative for toxicity towards the cervicovaginal epithelium, potential epithelial damage should be considered by evaluating the safety profile of high doses of chloroquine in appropriate model systems of the lower genital tract. In addition, uptake of chloroquine from vaginal gels into the systemic circulation should be assessed. Although plasma concentrations will presumably not exceed the non-toxic levels obtained after oral dosing (Rolain et al., 2007), the long-term presence of low concentrations of chloroquine may increase the risk on resistance induction of *Plasmodium*. Interference with the antimalarial use of chloroquine should be avoided as much as possible.

Conclusion

The broad spectrum anti-HIV activity of chloroquine, its unique mechanism of action and its low cost warrant the investigation of chloroquine as affordable microbicide candidate. In the present study we investigated the formulate-ability of chloroquine as an anti-HIV-1 microbicide. We successfully developed a stable gel formulation for application of chloroquine in the cervicovaginal tract. Gels can be loaded with chloroquine in concentrations that are 10^2- to 10^4-fold higher than the in vitro anti-HIV-1 IC$_{50}$. We showed that the chloroquine gel formulations exerted a clear, dose-dependent anti-HIV-1 activity in vitro. The efficiency to prevent HIV-1 transmission and the safety of the chloroquine microbicidal gels can now be investigated in appropriate model systems, including animal models.

Materials and methods

Chemicals

The diphosphate salt of chloroquine (M.W. 515.8 g/mol, weak base pK$_a$ 8.1 and 10.4), glycerol, lactic acid, methyl- and propylparaben were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hercules (Wilmington, DE, USA) supplied Natrosol 250 HHX Pharm (hydroxyethyl cellulose). Acetonitrile and methanol were obtained from Fisher Scientific (Leicestershire, UK). BDH Laboratory Supplies (Poole, UK) provided KH$_2$PO$_4$ and NaOH. Water was purified with a Maxima system (Elga Ltd., High Wycombe Bucks, UK).

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, USA) 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 to 3 days.

Gel formulation of chloroquine

A placebo gel formulation was developed by adding a mixture of hydroxyethyl cellulose (HEC, final concentration 1.6%, w/w) and glycerol (2.5%) to a solution of methyl- (0.18%) and propylparaben (0.02%) in water. Upon mixing, a clear and homogeneous gel was formed. The pH was decreased by adding lactic acid (0.05%) and adjusted to 4.5 by addition of NaOH 1 M. To load the gels with chloroquine, an appropriate amount of the diphosphate salt of chloroquine (0.3, 1, 3, 10 or 30 mg/g gel) was added as powder to the placebo gel and mixed to ensure homogeneity and complete dissolution of chloroquine. After removing entrapped air under reduced pressure, gels were stored at 4 °C unless otherwise specified. Three batches were prepared independently allowing the estimation of batch-to-batch variability.

Characterization of the gel formulation

Upon preparation of the gels, the following variables were assessed to characterize the formulations: pH (combined pH electrode pHC3359-8, Radiometer Analytical, Villeurbanne Cedex, France), osmolality (upon 5-fold dilution of the gels in water; The Advanced Osmometer 3250, Advanced Instruments, Norwood, MS), viscosity (37 °C; SV-10 vibrational viscometer, A&D Company, Tokyo, Japan) and chloroquine loading.

To determine chloroquine loading, a sample of the gel was diluted 1/100 in phosphate buffer (KH_2PO_4 50 mM, pH 3.5) and the concentration of chloroquine diphosphate in this solution was 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 (150x4.6 mm, 5 µm) and the mobile phase consisted of KH_2PO_4-buffer (50 mM, pH 3.5), methanol and acetonitrile (81:9:10, v/v). The flow was maintained at 1 ml/min. As indicated by absorbance monitoring at 341 nm, the retention time of chloroquine amounted to 5.6 min. Calibration curves of chloroquine diphosphate in a mixture of phosphate buffer and 1% placebo gel were linear over the concentration range from 0.05 to 100 µg/ml. Precision and accuracy were assessed by analyzing standard samples ($n=5$) of 0.05, 1 and 100 µg/ml. Assessment of intraday repeatability resulted in relative standard deviations of 4.1, 0.2 and 0.1%, respectively. The mean relative error amounted to −5.9, −0.8 and +2.7%, respectively.

Stability of the gel formulation

To assess the stability of the gel formulations, samples of the placebo gel and gel containing chloroquine diphosphate 3 mg/g were stored in a capped syringe at 40 °C and 75% relative humidity. After 3 months, the gel samples were examined for any change in visual appearance, mass, pH, osmolality, viscosity and chloroquine loading (as described above).

In vitro activity of the gel formulations against HIV-1

The in vitro activity of the gel formulations against the HIV-1 strain $X4_{NL4.3}$ was evaluated in MT-4 cells. Briefly, 5-fold dilutions of the gels (starting at 100 mg gel/ml final concentration) or native chloroquine diphosphate powder in cell culture medium (100 µl) were added to 96-well flat-bottomed plates (International Medical, Brussels, Belgium). Then, to each well, $7.5 \cdot 10^4$ MT-4 cells were added in 50 µl of medium, followed by 50 µl (500 pg/ml p24 Ag, corresponding to twofold the 50% tissue culture infective dose TCID50) 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 compounds (i.e., the concentration of the compound required for 50% reduction in HIV replication as measured by the p24 antigen production) was calculated.

The cytotoxicity of the gels was determined in MT-4 cells using a tetrazolium-based colorimetric assay (Pauwels et al., 1988). Briefly, after 5 days of incubation of the cells with the gels, 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, Sunny Vale, CA). Finally, the CC_{50} value of the gels (i.e., the concentration of the gels required for 50% reduction in cell viability) was calculated.

Data presentation

Unless otherwise specified, data are presented as mean±sd for 3 independent batches of chloroquine gels.

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