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Alginate microbead-encapsulated silver complexes for selective delivery of broad-spectrum silver-based microbicides

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ABSTRACT

In sub-Saharan Africa, human immunodeficiency virus (HIV) infections are predominantly acquired via heterosexual contact, and women are at greatest risk of being infected. This region also has the highest rates of sexually transmitted infections (STIs) per capita worldwide; STIs are strongly associated with increased HIV transmission. Therefore, there is an urgent requirement for microbicides that are active against HIV and STIs. Silver compounds exhibit broad antimicrobial activity, making them potentially ideal broad-spectrum microbicides. However, for silver compounds to be effective microbicides, they must be active within seminal fluid and the delivery vehicle used must protect the silver microbicide from vaginal fluid components but selectively release it during intercourse and/or following ejaculation. In this study, silver complexes were synthesised from the ligands saccharin, benzimidazole and 8-hydroxyquinoline and their microbicidal activity was assessed. We show that a silver saccharinate–benzimidazole complex (AgSB) exhibited activity against HIV-1, herpes simplex virus type 2 (HSV-2) and *Neisseria gonorrhoeae* at concentrations significantly below LD_{50} levels for the vaginal mucosal cell line SiHa. Furthermore, we show that alginate microbeads are stable in vaginal fluid simulant but rapidly dissolve in seminal fluid simulant. Finally, we have established that microbead-encapsulated AgSB, dissolved in seminal fluid simulant, is active against the above pathogens, albeit at higher concentrations for HIV-1. This research therefore highlights, for the first time, the potential use of silver complexes encapsulated in alginate microbeads as a novel system for the delivery and selective release of broad-spectrum silver-based microbicides within the vaginal milieu during sexual intercourse/after ejaculation.

1. Introduction

In sub-Saharan Africa, human immunodeficiency virus (HIV) infections are predominantly acquired via heterosexual contact, and women are at greatest risk of being infected, accounting for 60% of HIV infections [1]. Currently there is no effective vaccine against HIV and therefore the use of topical microbicides for the prevention of viral entry at the cervico-vaginal surface is an important alternative means of preventing HIV infection via vaginal intercourse [2]. Sub-Saharan Africa also has the highest rates of sexually transmitted infections (STIs) per capita worldwide and the contribution of STIs to HIV transmission remains high in this region, with 50% or more of HIV transmission attributed to STIs [3,4]. STIs are understood to increase susceptibility to HIV infection by: (i) the recruitment (and activation) of HIV-susceptible inflammatory cells to the site of infection and (ii) weakening of the genital mucosal barrier, particularly by genital ulcer diseases such as herpes simplex virus type 2 (HSV-2) [5]. There is therefore a requirement for broad-spectrum microbicides that are active both against HIV and STIs, particularly HSV-2, which strongly contributes to HIV spread in countries with mature HIV epidemics [5].

To address this requirement, we have investigated the efficacy of silver-based broad-spectrum microbicides for the rapid inactivation of HIV-1 as well as viral and bacterial sexually transmitted infective agents. The use of silver nanoparticle preparations as microbicides has been previously investigated [6], but because of concerns with the use of silver nanoparticles in therapeutics,

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specifically their ability to be easily endocytosed and rapidly oxidised intracellularly, resulting in the uncontrolled delivery and accumulation of toxic doses of silver within subcellular compartments [7], we therefore restricted our initial research to the use of silver(I) complexes.

Topical intravaginal application of silver compounds prior to sexual intercourse offers the following advantages: (i) silver compounds exhibit broad antimicrobial activity, making them ideal broad-spectrum microbicides as opposed to molecules that target only HIV [8]; (ii) silver has an excellent safety track record; only high oral intake has been associated with toxicity (renal, hepatic) and argyria [8]; (iii) silver compounds have an excellent therapeutic track record having been extensively used as topical anti-infectives [8]; and (iv) the silver ion binds to multiple groups on biological molecules (carbonyl, phosphate, imidazole, amino and thiol); pathogen binding at multiple sites results in no to low resistance for silver and its compounds. Indeed, recent studies indicate that silver co-treatment restores resistant pathogen susceptibility to antibiotics [9]. However, for silver-based microbicides to be successfully utilised, the following challenges need to be overcome. (A) Stability: silver compounds are often light-sensitive and easily reduced to metallic silver; formulations must therefore be stable or readily stabilised. (B) Cytotoxicity: silver compounds can be cytotoxic; silver-based microbicide candidates must exhibit antimicrobial activity at doses significantly below those that exhibit mucosal cell cytotoxicity. (C) Sequestration/selective delivery: the ability of silver ions to bind to biological molecules also results in rapid sequestration by proteins etc. in biological fluids, effectively reducing available silver for pathogen inactivation. Therefore, the delivery vehicle needs to stably 'insulate' the silver-based microbicide to prevent its sequestration by vaginal fluid components prior to intercourse but selectively release the microbicide during intercourse and/or following ejaculation. (D) Maintenance of activity in seminal fluid: seminal fluid is a complex multicomponent medium that contains high concentrations of citrate and polyamines which exhibit both a high buffering capacity (effectively neutralising the low pH of vaginal fluid) and high metal chelating capacity. Seminal fluid also contains reducing agents, which promote an antioxidant environment. Microbicides utilised must therefore be designed to be active within this protective environment.

In this proof-of-concept study, silver complexes were synthesised from the ligands saccharin, benzimidazole and 8-hydroxyquinoline and their microbicidal activity was assessed against HIV-1, HSV-2 and *Neisseria gonorrhoeae*. The use of alginate microbead encapsulation of silver complexes was also assessed as a novel system for the delivery and selective release of silver microbicides within the vaginal milieu during sexual intercourse/after ejaculation for the inactivation both of HIV and sexually transmitted infective agents.

2. Materials and methods

2.1. Reagents

All reagents were from Sigma-Aldrich (St Louis. MO), all culture reagents were from Gibco (Life Technologies, Carlsbad, CA) and plasticware was from Corning (Corning, NY).

2.2. Bacterial and viral strains and cell lines

The HIV-1 strain Ba-L was obtained through the NIH AIDS Reagent Program [Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH)] from Dr Suzanne Gartner, Dr Mikulas Popovic and Dr Robert Gallo [10]. The HSV-2 strain MS and the *N. gonorrhoeae* strain ATCC 49226 were provided by Prof. David Lewis [Sexually Transmitted Infections Reference Centre (STIRC), National Institute for Communicable Diseases (NICD), Johannesburg, South Africa]. The vaginal mucosal cell line SiHa (ATCC cat # HTB-35) was purchased from the American Type Culture Collection (Manassas, VA). Vero cells were provided by the Viral Diagnostic Unit (NICD). MOLT 4/CCR5 cells were obtained through the NIH AIDS Reagent Program (NIAID, NIH) from Dr Masanori Baba, Dr Hiroshi Miyake and Dr Yuji Iizawa [11].

2.3. Synthesis of silver complexes

Silver saccharinate (AgS), synthesised as previously described [12], was combined with equimolar quantities of benzimidazole or 8-hydroxyquinoline in dimethyl sulfoxide (DMSO) and was stirred for 15 min, after which the reaction mixture was precipitated with water to yield a white, finely crystalline, silver saccharinate–benzimidazole complex (AgSB) or off-white/tan amorphous silver saccharinate–8-hydroxyquinoline complex (AgSHQ), which were extensively washed with water and then ethanol to remove excess ligand. Crystals of the above, suitable for analysis by single-crystal X-ray diffraction, were obtained by water vapour diffusion of DMSO solutions over a period of ca. 3 weeks. Diffraction intensity data for both samples were collected at $-100 \pm 2\,^{\circ}C$ on a Bruker D8 Venture PHOTON 100 CMOS area detector diffractometer (Bruker, Billerica, MA) with curved crystal monochromated Mo Kα radiation (50 kV, 30 mA). Data reduction was carried out using the program *SAINT+* and face indexed absorption corrections were made using the program *XPREP*. Both crystal structures were solved by direct methods using SHELXS-97 and were refined using SHELXL-97. Non-hydrogen atoms were first refined isotropically followed by anisotropic refinement (when possible) by full matrix least-squares calculations based on F^2. The CIF files for these structures have been deposited at the Cambridge Crystallographic Data Centre (CCDC) (http://www.ccdc.cam.ac.uk) and can be obtained using the link: http://www.ccdc.cam.ac.uk/services/structures?access=referee&searchdepnums=1006595,1006596&searchauthor=Fernandes. Silver–dibenzimidazole nitrate (AgDB) was synthesised as previously described [13]. Silver–8-hydroxyquinoline was prepared from equimolar quantities of silver nitrate and 8-hydroxyquinoline in DMSO, precipitation with water and extensive washing with water and then ethanol.

2.4. MTT assays

MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assays for silver complexes dissolved in DMSO or alginate-encapsulated were performed using the vaginal epithelial cell line SiHa as previously described [14], and LD_{50} values (50% lethal dose) were obtained from four parameter dose–response curves using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA). Briefly, cells in 100 μL of DMEM/F12 medium were seeded (8000/well) into round-bottom 96-well plates and were then exposed to dilutions of DMSO-solubilised complexes (in 100 μL of medium) for 18 h, after which 10 μL of MTT solution (5 mg/mL) was added to each well. After 3 h the medium was removed and MTT formazan was solubilised by the addition of 100 μL of DMSO to each well. Wells were then read on a multiwell spectrophotometer (VersaMax™ Microplate Reader; Molecular Devices, Sunnyvale, CA) at 562 nm. MTT assays were also carried out for alginate-encapsulated AgSB. AgSB-encapsulated beads containing 0.24 mg AgSB/mL of wet beads (see Section 2.9) were dissolved in an equal volume of seminal fluid simulant, which was then further diluted with DMEM/F12 medium and used as above.