Synthesis, characterization and preclinical formulation of a dual-action phenyl phosphate derivative of bromo-methoxy zidovudine (compound WHI-07) with potent anti-HIV and spermicidal activities*

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In a systematic effort to develop a microbicide contraceptive capable of preventing transmission of human immunodeficiency virus (HIV), as well as providing fertility control, we have previously identified novel phenyl phosphate derivatives of zidovudine (ZDV) with 5-halo 6-alkoxy substitutions in the thymine ring and halo substitution in the phenyl moiety respectively. Here, we describe the synthesis, characterization, and successful preclinical formulation of our lead compound, 5-bromo-6-methoxy-3\(^{-}\)azidothymidine-5\(^{-}\)-(p-bromophenyl)methoxyalaninyl phosphate (WHI-07), which exhibits potent anti-HIV and sperm immobilizing activities. The anti-HIV activity of WHI-07 was tested by measuring viral p24 antigen production and reverse transcriptase activity as markers of viral replication in HIV-1 infected human peripheral blood mononuclear cells (PBMC). WHI-07 inhibited replication of HIV in a concentration-dependent fashion with nanomolar IC\(_{50}\) values. The effects of WHI-07 on human sperm motion kinematics were analysed by computer-assisted sperm analysis (CASA), and its effects on sperm membrane integrity were examined by confocal laser scanning microscopy (CLSM), and high-resolution low-voltage scanning electron microscopy (HR-LVSEM). WHI-07 caused cessation of sperm motility in a concentration- and time-dependent fashion. The in-vitro cytotoxicities of WHI-07 and nonoxynol-9 (N-9) were compared using normal human ectocervical and endocervical epithelial cells by the MTT cell viability assay. Unlike N-9, WHI-07 had no effect upon sperm plasma and acrosomal membrane integrity. N-9 was cytotoxic to normal human ectocervical and endocervical cells at spermicidal doses, whereas WHI-07 was selectively spermicidal. The in-vivo vaginal absorption and vaginal toxicity of 2% gel-microemulsion of WHI-07 was studied in the rabbit model. The sperm immobilizing activity of WHI-07 was 18-fold more potent than that of N-9. Over a 10 day period, there was no irritation or local toxicity to the vaginal epithelia or systemic absorption of WHI-07. Therefore, as a potent anti-HIV agent with spermicidal activity, and lack of mucosal toxicity, WHI-07 may have the clinical potential to become the active ingredient of a vaginal contraceptive for women who are at high risk for acquiring HIV by heterosexual vaginal transmission.

Key words: contraceptives/HIV/microbicide/spermicide/ZDV

Introduction

Human immunodeficiency virus (HIV) is the aetiological agent of acquired immunodeficiency syndrome (AIDS) (Weiss, 1993). Sexually active women represent the fastest growing HIV/AIDS risk group (Osborn, 1991; Celum and Watts, 1996). Worldwide, heterosexual transmission accounts for >90% of all new HIV infections (Celum and Watts, 1996; Silver, 1997). AIDS now represents the third leading cause of death among all American women aged 25–44 years, and it is the leading cause of death among African–American women in this age group (Lindsay, 1996; Makulowich, 1997; Silver, 1997; Steinbrook, 1997). By the year 2000, a total of 13 million women of the 40 million HIV-infected individuals worldwide will be women (Mann and Tarantola, 1995). In the absence of an effective prophylactic HIV vaccine, there is a urgent need for female-controlled intravaginal microbicides for curbing the mucosal and perinatal HIV transmission (Potts, 1994; Ho, 1995).

The fact that sexual transmission is the dominant mode of the epidemic spread of HIV-1 implies that the virus must cross the protective epithelial barrier that lines the genital tract of both sexes. The ability of mononuclear cells isolated from semen of HIV-positive men to infect lymphocytes, as well as cervical epithelial cells \textit{in vitro}, supports the notion that trafficking HIV-infected mononuclear cells in semen contribute to the sexual transmission of HIV (Phillips, 1994; Phillips \textit{et al.}, 1994; Quayle \textit{et al.}, 1997). The continued use of detergent-based vaginal contraceptives could therefore enhance the risk of vaginal transmission of HIV by mucosal erosion and local inflammation.
At present, all commercially available spermicides have detergent ingredients that disrupt the cell membrane (Feldblum and Fortney, 1988; Feldblum et al., 1988). The widely used detergent microbiocide/spermicide, nonoxynol-9 (N-9) has been in use for >30 years in over-the-counter gels, foams, creams, sponges, and films (US Food and Drug Administration, 1980; Chantler, 1992). Because N-9 was shown to inactivate HIV in vitro (Hicks et al., 1985; Polsky et al., 1988), it is the only topical microbicide accessible to young women (Bird, 1991). However, recent in-vivo efficacy trials have shown that vaginal contraceptives containing N-9 had no protective effect against HIV/AIDS and other sexually transmitted diseases (Hira et al., 1997; Rowe, 1997; Roddy et al., 1998). Furthermore, there is a growing concern that chemical irritation that disrupts the vaginal mucosa might actually increase the risk of HIV transmission in sexually active women (Kreiss et al., 1992; Stafford et al., 1998). In addition to its membrane disruptive properties (Schill and Wolf, 1981; Wilborn et al., 1983), ability to damage the cervicovaginal epithelium (Niruthisard et al., 1991; Rekart, 1992; Roddy et al., 1993; Weir et al., 1995), and to cause an acute tissue inflammatory response (Tryphanos and Buttar, 1984), thereby enhancing the likelihood of HIV infection, N-9 also has a high contraceptive failure rate. Several large studies have demonstrated first-year pregnancy rates of 11–31%, making N-9 ~75% effective in preventing pregnancy (Trussell and Trost, 1987). Furthermore, its continued use can alter the vaginal flora permanently, and lead to an increased risk of developing urinary tract and gynaecological infections (Stafford et al., 1998). Such opportunistic infections are known to enhance the susceptibility of the ectocervical epithelium and the endocervical mucosa to HIV-1 infection (Augenbraun and McCormack, 1994). Therefore, new, effective, safe, and female-controlled topical dual-function microbicides lacking detergent-type membrane toxicity should have clinical advantage over the currently available vaginal contraceptives.

As a part of our programme on structure-based design and synthesis of novel anti-HIV agents, we have synthesized a number of phenyl phosphate derivatives of zidovudine (ZDV) as dual-action agents for fertility control (D’Cruz et al., 1998b). Since physiological fertilization depends on the ability of the ejaculated spermatozoa to swim, bind the zona pellucida, and penetrate the egg, and is primarily dependent on sperm motility (Yanagimachi, 1988), adding spermicidal function to the currently used anti-HIV drug, ZDV, could be an effective way both to curb heterosexual vaginal transmission of HIV and prevent conception. The bioactivation (phosphorylation) of ZDV is dependent on cellular thymidine kinases (Mitsuya and Broder, 1987). Because the thymidine kinase activity of monocyte/macrophage cells is known to be low or lacking, ZDV by itself is ineffective in inhibiting HIV replication in these cells. Therefore, to bypass the dependence of kinase-mediated activation, new phenyl phosphate derivatives of ZDV have been synthesized (Kumar et al., 1994). They exhibit enhanced lipophilicity, superior pharmacokinetics, and retain full activity in thymidine kinase-deficient cells when compared with ZDV (McGuigan et al., 1993a; Wang et al., 1996; Vig et al., 1998ab). The antiviral action of phenyl phosphate derivatives of ZDV is particularly relevant to leukocytes and germ cells in semen containing associated virus because of their ability to bypass the dependency on thymidine kinase-mediated activation (McGuigan et al., 1993b). We have synthesized a novel phenyl methoxylanylinyl phosphate derivative of 5-bromo-6-methoxy-ZDV, compound WHI-07, that exhibits potent anti-HIV as well as spermicidal activities (D’Cruz et al., 1998b). Here, we describe the scaled-up synthesis, characterization, and successful preclinical formulation of this compound.

Materials and methods

Reagents and instrumentation

All the anhydrous solvents and chemical reagents, except ZDV, were purchased from Aldrich Chemical Co. Zidovudine was purchased from Toronto Research Chemicals Inc (Ontario, Canada). Proton nuclear magnetic resonance, $[^1]H$-nuclear magnetic resonance (NMR), and carbon nuclear magnetic resonance, $[^13]C$-NMR, spectra were recorded on a Varian Oxford 300 MHz spectrometer using CDCl$_3$ as solvent. Chemical shifts are reported as δ values in parts per million downfield from tetramethylsilane (δ = 0.0 ppm) as internal standard or from the residual chloroform signal (δ = 7.24 ppm for $[^1]H$-NMR or δ = 77.0 ppm for $[^13]C$-NMR). Splitting patterns are designated as follows: s = singlet; d = doublet; t = triplet; m = multiplet; br = broad peak. Fourier Transform (FT)-infrared spectra were obtained on a FT-Nicolet Model Protege 460 spectrometer (Nicolet Instrument Corporation, Madison, WI, USA) and the values are reported in per cm on KBr plates. Mass spectra analyses were performed using a Hewlett Packard Matrix Assisted Laser Desorption Time-of -Flight (MALDI-TOF) spectrometer (Model G 2025A) (Hewlett Packard Co, Wilmington, DE, USA) or on a FINNIGAN MAT 95 system (University of Minnesota Mass Spectroscopy Facility).

Chemical synthesis and characterization of compound WHI-07

Both the small scale and multigram synthesis of compound WHI-07, 5-bromo-6-methoxy-3′-azidothymidine 5′-(p-bromophenyl) methoxylaninyl phosphate, was accomplished in four steps (Figure 1). Compound 1 was prepared from the commercially available p-bromophenol in two steps (McGuigan et al., 1993b). Condensation of ZDV and compound 1 afforded compound 2 at a 95% yield. The bromo and methoxy groups were added to the C-5 and C-6 double bond in the thymidine moiety of compound 2 by treating it with methyl hypobromite solution to furnish WHI-07.

Preparation of WHI-07

For the small scale synthesis of WHI-07, liquid bromine (0.24 ml) was added to anhydrous methanol (10 ml) and the resulting orange-brown methyl hypobromite solution was stirred at room temperature in the dark for 10 min prior to use. Freshly prepared methyl hypobromite solution (7.4 ml) was added to a stirring solution of 3′-azidothymidine 5′-(p-bromophenyl methoxylaninyl phosphate) (0.654 g, 1.22 mM) (compound 2) in anhydrous methanol (13.0 ml). The reaction mixture was stirred at room temperature in the dark under N$_2$ atmosphere. Progress of the reaction was monitored by thin layer chromatography (TLC) (silica gel, 4% MeOH/CHCl$_3$). After 90 min of stirring, the pH of the reaction mixture was adjusted to 6.5 with saturated methanolic NaOH. The solvent was then removed by rotary evaporation to afford a dark brown mixture of viscous oil and solid as crude product. Purification of the crude product by
column chromatography containing silica (silica gel 230–400 mesh; eluent 100% CHCl₃ and 1.3% MeOH in CHCl₃) gave a nearly pure diastereomeric mixture of WHI-07.

For the scaled-up synthesis of WHI-07, a freshly prepared solution of 3'-azidothymidine-5'-[(p-bromophenyl) methoxyalaninyl phosphate (58.7 g, 0.1 M) in anhydrous methanol (1 l) with stirring until the light yellow colour of the reaction mixture persisted. The reaction was allowed to proceed for 1 h until the formation of product as monitored by TLC. To this reaction mixture, a solution of methanolic sodium hydroxide was added carefully to adjust the pH to 6.0 and the solvent was removed under vacuum. The resulting residue was redissolved in methanol (30–50 ml) and was passed through a silica gel column (20 g). The resulting solution was concentrated under vacuum and was chromatographed over a silica gel column by elution with chloroform-methanol (95:5, v/v) to obtain WHI-07 as a yellow viscous oil; the yield using this synthetic scheme was 73% with 98% purity.

Characterization of WHI-07
Infrared (undiluted)
3218, 3093, 2850, 1712, 1484, 1378, 1241, 1153, 1010, 877, 721, 673, 598, 579, 423 cm⁻¹.

Analytical HPLC retention times
Retention times for high-performance liquid chromatography (HPLC) were 39.1, 40.1, and 40.8 min.

In-vitro assays of anti-HIV activity
The HIV-1 strain, HTLV-IIIb, used in this study was obtained from Vanderbilt University (Nashville, TN, USA) and was propagated in CCRF-CEM cells. The virus stocks obtained from cell-free supernatants of infected cells were titrated using MT-2 cells. Cell-free supernatants of HTLV-IIIb-infected CCRF-CEM cells were harvested and frozen in 1 ml aliquots at −70°C. Periodic titration of stock virus was performed using MT-2 cells and the cytotoxic effect of the virus used in this study was typical of HIV-1 on MT-2 cells.

For in-vitro assays of the anti-HIV-1 activities of the test drugs, WHI-07, ZDV, and N-9, normal human peripheral blood mononuclear cells (PBMCs) from HIV-1 seronegative donors were cultured for 72 h in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% (v/v) heat-inactivated fetal calf serum, 3% interleukin-2, 2 mM -glutamine, 25 mM HEPES, 2 g/l NaHCO₃, 50 µg/ml gentamicin, and 4 µg/ml phytohaemagglutinin (PHA-P) prior to exposure to HIV-1 at a multiplicity of infection of 0.1 during a 1 h adsorption period at 37°C in a humidified 5% CO₂ atmosphere. Stock solutions (10 mM) of WHI-07 and ZDV were prepared in dimethylsulphoxide (DMSO). N-9 (IGEPAL CO-630; Rhone Poulenc, Cranbury, NJ, USA) was diluted in culture medium. Cells were cultured for 7 days in 96-well microtitre plates (100 µl/well; 2×10⁶ cells/ml, triplicate wells) in the presence and absence of various concentrations (0.001–100 µM) of the anti-HIV agents. Cells from non-infected controls were handled in the same way except the virus was omitted from the preparation. Aliquots of culture supernatants were removed from the wells on the 7th day after infection for p24 antigen and reverse transcriptase assays, as previously described (Zarling et al., 1990; Uckun et al., 1998a).

Immunoassay of the p24 antigen
An unmodified kinetic assay which was available commercially (Coulter Corporation/Immunotech Inc, Westbrook, ME, USA) was employed. A murine monoclonal antibody (mAb) to the HIV core protein was coated onto microwell strips to which the antigen present in the test culture supernatant sample was able to bind (Uckun et al., 1998a).
were obtained after informed consent and in compliance with the
Research Inc., Danvers, MA, USA) (D'Cruz
Optical System (IVOS), version 10 instrument (Hamilton Thorne
were scanned for analysis using a Hamilton Thorne Integrated Visual
m Microcell chamber set at 37°C. At least 5–8 fields per chamber
field was recorded for 30 s. The computer calibrations were set at 30
'swim-up' method (D'Cruz
(Conception Technologies, San Diego, CA, USA) centrifugation and
viability were examined by a microculture tetrazolium assay (MTA),
replication of HIV-1 in cell
culture, by 50%. In parallel, the effects of various treatments on cell
viability were examined by a microculture tetrazolium assay (MTA), using 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5[phenyl-amino]-carboxyl]-carbonyl]-2H-tetrazolium hydroxide (XTT).

Sperm immobilization assay (SIA)
To evaluate the spermicidal effects of WHI-07 and ZDV in comparison
with N-9, highly motile aliquots of pooled donor spermatozoa
(n = 4) were prepared by discontinuous (90–45%) Percoll gradient
(Conception Technologies, San Diego, CA, USA) centrifugation and
'swim-up' method (D'Cruz et al., 1996). All donor sperm specimens
were obtained after informed consent and in compliance with the
guidelines of the Hughes Institute Institutional Review Board. Motile
spermatozoa (≥10x10⁶/ml), were suspended in 1 ml of Biggers–
Whitten–Whittingham medium (BWW) containing 25 mM HEPES
(Irvine Scientific, Santa Ana, CA, USA), and 0.3% bovine serum albumin (BSA) in the presence and absence of serial two-fold dilutions
of test substance (300–2.3 µM) in 0.3% DMSO. The stock solutions
of WHI-07 and ZDV were prepared in DMSO (100 mM) and diluted
in DMSO to yield the desired concentrations. Corresponding volume
of DMSO (0.3%) was added to control tubes. N-9 was diluted in
BWW-0.3% BSA (pH 7.4) to yield the desired concentrations (2.3–
300 µM). After 3 h of incubation at 37°C, the percentage of motile
spermatozoa was evaluated by computer-assisted sperm analysis
(CASA). The percentage motilities were compared with sham-treated
control suspensions of motile spermatozoa. The spermicidal activity
of test compounds was expressed as the EC₅₀ (the final concentration
of the compound in medium that decreases the proportion of motile
spermatozoa by 50%).

To test the effect of duration of incubation on SIA in the presence
of WHI-07 or N-9, motile aliquots of spermatozoa (10⁷/ml) were
incubated at 37°C in 1 ml of BWW–0.3% BSA in the presence of
100 µM WHI-07, 100 µM N-9 or 0.1% DMSO alone. At timed
intervals, duplicate aliquots (4 µl) were transferred to a 20 µm
Microcell chamber (Conception Technologies) and sperm motility
was assessed by CASA. CASA was performed for a duration of
140 min.

Sperm kinematic parameters
For CASA, 4 µl of each sperm suspension were loaded into a 20-
µm Microcell chamber set at 37°C. At least 5–8 fields per chamber
were scanned for analysis using a Hamilton Thorne Integrated Visual
Optical System (IVOS), version 10 instrument (Hamilton Thorne
Research Inc., Danvers, MA, USA) (D'Cruz et al., 1998a,b). Each
field was recorded for 30 s. The computer calibrations were set at 30
frames at a frame rate of 30/s. Other settings were as follows:
minimum contrast 8; minimum size 6; low-size gate, 1.0; high-size
gate, 2.9; low-intensity gate, 0.6; high-intensity gate, 1.4; phase-
contrast illumination; low path velocity at 10 µm/s, and threshold
straightness at 80%; magnification factor, 1.95.
The sperm kinematics evaluated included numbers of motile (MOT)
and progressively (PRG) motile spermatozoa; curvilinear velocity
(VCL; a measure of the total distance travelled by a given spermato-
zoan during the acquisition divided by the time elapsed); average
path velocity (VAP; the spatially averaged path that eliminates the
wobble of the sperm head), straight line velocity (VSL; the straight-
line distance from beginning to end of track divided by time taken),
beat cross frequency (BCF; frequency of sperm head crossing sperm
average path), the amplitude of lateral head displacement (ALH; the
mean width of sperm head oscillation) and the derivatives, straightness
(STR = VSL/VAP×100); linearity (LIN = VSL/VCL×100; departure
of sperm track from a straight line). Data from each individual cell
track were recorded and analysed. At least 200 motile spermatozoa
were analysed for each aliquot sampled.

Confocal laser scanning microscopy (CLSM)
The percentages of spermatozoa with an intact acrosome following a
3 h treatment with and without WHI-07 were evaluated by CLSM.
In positive control spermatozoa, the acrosomal loss was induced by
incubating the sperm suspension with either 10 µM calcium ionophore
(Cal) A23187 (Sigma) or 100 µM N-9. Ethanol-permeabilized and
air-dried sperm smears were stained sequentially with the three
fluorescent markers, fluorescent isothiocyanate (FITC)–Pisum sativum
lectin, TOTO-3 iodide, and Nile Red (Molecular Probes, Eugene,
OR, USA) because their targets are different (acrosome, nucleus, and
plasma membrane respectively). Samples were examined under a
BioRad MRC-1024 Laser Scanning Confocal Microscope (BioRad
Laboratories, Hercules, CA, USA) equipped with a krypton/argon
mixed gas laser (excitation lines 488, 568, and 647 nm) and mounted
on a Nikon Eclipse E800 series upright microscope. The fluorescence
emission of fluorescein, TOTO-3 iodide, and Nile Red from the
acrosomal region, nucleus, and the plasma membrane of spermatozoa
after ethanol permeabilization were simultaneously detected using the
598/40 nm, 522 DF32, and 680 DF32 emission/filter respectively.
Confocal images were obtained using a Nikon ×100 (NA 1.35)
objective lens and Kalman collection filter. Digitized data was
processed using Lasersharp (Bio-Rad), saved on a Jaz disk (Imagina
Corporation, Roy, UT, USA) and further processed with the Adobe
Photoshop softwares (Adobe Systems, Mountain View, CA, USA).
Final images were printed using a Fuji Pictography 3000 (Fuji Photo
Film Co, Tokyo, Japan) colour printer.

High-resolution low-voltage scanning electron microscopy
(HR-LVSEM)
HR-LVSEM was used for topographical imaging of different mem-
brane domains over the sperm head (Earlsden et al., 1989; D'Cruz
and Uckun, 1998). Aliquots (20x10⁶) of motile spermatozoa were
incubated with DMSO alone (0.1%), or 100 µM of WHI-07 or 10
µM Cal in 0.1% DMSO for 3 h at 37°C. Spermatozoa were fixed in
1% paraformaldehyde and 1% glutaraldehyde in 0.14 M sodium
cacodylate buffer for 3 h and post-fixed in 1% osmium tetroxide
(OsO₄) containing 0.1% ruthenium red in 0.14 M cacodylate buffer
for 1 h at 4°C. Samples were dehydrated through an ascending
euthanol series, critical point-dried, and coated with ~2 nm of
platinum using ion beam sputtering with argon (4 mA at 10 kV;
Ion Tech Ltd, Middlesex, England) and examined using a Hitachi
S-900 SEM at an accelerating voltage of 2 keV. Spermatozoa were
observed under low magnification (×2000–5000) and representative
spermatozoa were photographed under intermediate magnification ($\times18,000$–$25,000$). In each specimen evaluated, at least 200 spermatozoa were scanned for the intactness of the acrosomal region.

**Cytotoxicity assay**

The cytotoxicity of WHI-07 in comparison to N-9 against normal human ectocervical and endocervical epithelial cells (Clonetics Corporation, San Diego, CA, USA), was analysed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Boehringer Mannheim Corporation, Indianapolis, IN, USA) (Uckun et al., 1998b). Briefly, exponentially growing ectocervical and endocervical epithelial cells were seeded onto 96-well plates at a density of $2\times10^3$ cells/well and incubated for 24 h at $37^\circ$C prior to drug exposure. On the day of treatment, culture medium was carefully aspirated from the wells and replaced with fresh medium containing the WHI-07 or N-9 at concentrations ranging from 3.9 to 1000 $\mu$M. Triplicate wells were used for each treatment. The cells were incubated with the various compounds for 24 h at $37^\circ$C in a humidified 5% CO$_2$ atmosphere. To each well, 10 $\mu$l of MTT (0.5 mg/ml final concentration) was added and the plates were incubated at $37^\circ$C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized overnight at $37^\circ$C in a solution containing 10% sodium dodecyl sulphate in 0.1 M HCl. The absorbance of each well was measured in a microtitre reader at 540 nm and a reference wavelength of 690 nm. To translate the OD$_{540}$ values into the number of live cells in each well, the OD$_{540}$ values were compared with those of standard OD$_{540}$ versus cell number curves generated for each cell line. The percentage survival was calculated using the formula:

$$\% \text{ survival} = \frac{\text{live cell number (test)}}{\text{live cell number (control)}} \times 100.$$  

The IC$_{50}$ values were calculated by non-linear regression analysis.

**Gel-microemulsion formulation of WHI-07**

Due to the lipophilic nature of WHI-07, a microemulsion-based formulation strategy was developed. Initially, several microemulsion compositions were screened for their drug solubility, particle size, stability, and responses to in-vivo and in-vitro biological models. The ingredients tested were: medium chain triglycerides, purified soya phospholipid, Pluronic F-68, ethoxylated castor oil, propylene glycol, polyethylene glycol, and water. A microemulsion-based system with high solubilizing capacity for WHI-07 was identified through systematic mapping of ternary phase diagrams, and drug solubilization study (Eccleston, 1992; Ritschel, 1993). Various polymeric gels were screened to produce a gel with desirable viscosity. Polymer suspensions of xanthan gum or carrageenan were selected as additives to the microemulsion-based system to obtain a gel with desirable viscosity containing 1–2% WHI-07 with high thickening capability and compatibility with microemulsions. These polymers did not cause drug precipitation or alter the microemulsion particle size. The gel-microemulsion was found to be very stable at ambient temperature. Particle size determination was made using Nicomp Model 380 dynamic light scattering particle sizer equipped with a 15 mW power laser diode source (Particle Sizing Systems, Santa Barbara, CA, USA). Measurements of drug concentrations were carried out by HP 1100 series HPLC and Beckman DU7500 UV-visible spectrophotometer. Viscosity measurements were made using the Brookfield Viscometer (Brookfield Engineering Laboratories, Spoughton, MA, USA).

**In-vitro spermicidal activity of WHI-07 cream formulation**

The spermicidal activity of 2% cream formulation of WHI-07 was tested using the modified Sander–Cramer test (Sander and Cramer, 1941). Aliquots (0.1 ml) of freshly liquefied semen were rapidly mixed with an equal volume of freshly prepared 2% gel-microemulsion of WHI-07. A 4 $\mu$l sample was transferred to a 20 $\mu$m Microcell chamber and examined immediately under a phase contrast microscope (Olympus BX-20; Olympus Corporation, Lake Success, NY, USA) attached to a CCD camera (Hitachi Denashi Ltd, Tokyo, Japan) and a videomonitor. The time required for sperm immobilization was recorded in seconds. This test was performed in six separate trials, with semen obtained from three different donors.

**Vaginal irritation model**

Specific-pathogen-free adult New Zealand White female rabbits (4–5 kg) were purchased from Charles River Laboratories (Wilmington, MA, USA). They were maintained in an animal facility approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and caged as required by the current US Department of Agriculture guidelines. Five does were administered intravaginally
Figure 3. Spermicidal activity of 5-bromo-6-methoxy-3'-azidothymidine-5'-((p-bromophenyl) methoxyalaninyl phosphate (WHI-07). (A) Comparison of concentration-dependent inhibition of sperm motility by WHI-07 and nonoxynol-9 (N-9). Highly motile aliquots of spermatozoa were incubated for 3 h with increasing concentrations (2.3–300 µM) of WHI-07, N-9 or 0.3% dimethyl sulphoxide (DMSO) in the assay medium, and the percentage of motile spermatozoa were evaluated by computer-assisted semen analysis (CASA). Each data point represents the mean from three or more independent experiments. (B) Time-dependent sperm immobilization in the presence of WHI-07 and N-9. Motile spermatozoa were incubated at 37°C in assay medium in the presence of 100 µM of WHI-07, 100 µM N-9 or 0.1% DMSO alone. At timed intervals sperm motility was assessed by CASA. Each data point represents the mean ± SD from three independent experiments.

Figure 4. Effect of 5-bromo-6-methoxy-3'-azidothymidine-5'-((p-bromophenyl) methoxyalaninyl phosphate (WHI-07) on sperm motion parameters. (A) Concentration-dependent inhibition of sperm motility parameters. Highly motile fraction of spermatozoa were incubated for 3 h with increasing concentrations (2.3–37.4 µM) of WHI-07 or 0.3% dimethyl sulphoxide (DMSO) in the assay medium, and their effects on progressive motility (PRG MOT), curvilinear velocity (VCL), average path velocity (VAP), and straight line velocity (VSL) were evaluated by CASA. Each data point represents the mean from three independent experiments. (B) Time-dependent effect of WHI-07 on sperm kinematics. Motile fractions of spermatozoa were incubated in assay medium in the presence of 100 µM WHI-07 and the motility characteristics were determined as described in the text. Values: percentage for progressive motility; µm/s for VCL, VAP, VSL.

with 1 ml of a 2% WHI-07 in microemulsion daily for a 10 day period. Three does treated with control gel-microemulsion served as control group. Animals were killed on day 11 and the reproductive tract was examined grossly and microscopically. The ovaries, uteri, and vaginal tissues were rapidly removed and weighed. Parts of the caudal, mid-, and distal regions of the vagina were fixed in buffered formalin for histochemistry and pathological examination, the remainder was frozen in dry ice for solvent extraction and analytical HPLC.
Spermicidal zidovudine derivative

Figure 5. (A, B, C) Laser scanning confocal fluorescence images of spermatozoa. Triple labelling of spermatozoa with fluorescein isothiocyanate (FITC)–*Pisum sativum* lectin for acrosome (green), TOTO-3 iodide for DNA (blue), and Nile Red for membrane lipid (red). In acrosome-intact spermatozoa, the acrosomal region of the sperm heads exhibited a uniform, bright green fluorescence. In acrosome-reacted spermatozoa, green fluorescence was either absent or restricted to the equatorial segment of the sperm heads. Spermatozoa exposed to (A) 0.1% dimethylsulphoxide (DMSO) alone, and (B) 5-bromo-6-methoxy-3′-azidothymidine-5′- (p-bromophenyl) methoxyalaninyl phosphate (WHI-07) did not show an increased acrosome reaction at 3 h of incubation. (C) Spermatozoa exposed to 100 µM of N-9 revealed only acrosome-reacted spermatozoa. (D, E, F) HR-LVSEM of spermatozoa. HR-LVSEM of spermatozoa incubated in the (D) absence and (E) presence of 100 µM WHI-07 and (F) 10 µM CaI for 3 h (original magnification ×18 000). The acrosomal surface is delineated from the post-acrosomal region by a equatorial band (D). Spermatozoa exposed to WHI-07 (E), revealed intact acrosomes and plasma membranes, whereas CaI-treated spermatozoa reveal blebbing, or vesiculation, fenestration, and loss of plasma and acrosomal membranes (F).

To determine the degree of inflammation and membrane integrity of squamous epithelia, conventional paraffin-embedded sections (6 µm) were prepared and stained with haematoxylin and eosin and observed under ×300 magnification with a Leica (Milton Keynes, UK) microscope interfaced with an image analysis system. The images were captured using the ImagePro Plus program (Media Cybernetics, Silver Spring, MD, USA) in conjunction with a 3CCD camera (DAGE-MTI Inc, Michigan City, KS, USA), and images were transferred to Adobe Photoshop software for observation and analysis.

**HPLC determination of WHI-07 and its metabolites**

For tissue retention studies, the thawed tissue was rinsed in PBS, blotted, weighed, and homogenized using a Polytron (PT-MR2000) homogenizer (Kinematical AG Littau, Switzerland). WHI-07 and its metabolite(s) were extracted from tissue homogenate with methanol. After centrifugation, 50 µl of the supernatant was injected into a Hewlett Packard series 1100 HPLC equipped with an automated electronic degasser, a quaternary pump, an autosampler, an automatic thermostatic column compartment, diode array detector, and a computer with a Chemstation software program for data analysis. A 250×4 mm Lichrospher 100, RP-18 (5 µm) analytical column and a 4×4 mm Lichrospher 100, RP-18 (5 µm) guard column were used. For the analysis of WHI-07, the linear gradient mobile phase was 100% A/0% B/0% C at 0 min, 80% A/20% B/0% C at 20 min, 0% A/50% B/50% C at 30–50 min, in which A is 10 mM ammonium phosphate buffer (pH 3.7); B is acetonitrile; and C is water containing 0.1% acetic acid. The mobile phase was degassed automatically by the electronic degasser system. The column was eluted under gradient conditions using a flow rate of 1.0 ml/min at ambient temperature. The wavelength of detection was set at 270 nm. Peakwidth, response time, and slit were set at >0.03 min, 0.5 s and 8 nm respectively. The retention times of WHI-07 and its known metabolites (ZDV, alaninyl-ZDV-monophosphate, and ZDV monophosphate) were determined with a sensitivity of 25 picomoles.

The systemic absorption of intravaginally applied 2% WHI-07 gel-microemulsion was monitored by analytical HPLC of blood plasma extracts. Blood (~0.5 ml) was collected from ear venipuncture prior to and at 5, 10, 15, 30, 45 min and 1, 1.5, 2, 3, and 4 h after intravaginal administration of WHI-07 (8.5 mg/kg). All blood samples were heparinized and centrifuged at 7000 g in a microcentrifuge for 10 min to obtain plasma. Following precipitation of plasma proteins by methanol (1:3), the samples were extracted twice with ethyl acetate.
HPLC chromatograms were compared with control plasma extracts validated WHI-07 pharmacokinetics with mouse plasma samples. The subjected to HPLC analysis as described above using previously determined by the MTT assay. The data points represent the mean mM for 24 h in 96-well plates and the percentage cell viability was resuspended in methanol, clarified by centrifugation and 50 µl and the aqueous layer was isolated and lyophilized. The residue was assited sperm analysis (CASA).

Effect of WHI-07 on sperm activity

The spermicidal effects of WHI-07 were compared with ZDV and N-9 using CASA. Exposure of the highly motile fraction of human spermatozoa to ZDV at concentrations as high as 300 µM did not alter the sperm motion parameters (data not shown). Compound WHI-07 exhibited concentration-dependent spermicidal activity with a median inhibitory concentration (EC50) value of 4.82 µM and IC50 (reverse transcriptase) values for WHI-07 for HIV-1 replication were well below 0.01 µM [IC50 (p24) = 0.005 µM and IC50 (reverse transcriptase) = 0.009 µM respectively] (Figure 2C). In comparison, the IC50 reverse transcriptase) values for ZDV were 0.02 µM against HTLV-III (not shown), whereas the IC50 value for N-9 was 2.195 µM in p24 antigen production assay. Thus, WHI-07 was 4-fold more potent than ZDV and 439-fold more potent than N-9 as an inhibitor of HIV-1 replication. The observed reduction in p24 production and reverse transcriptase activity by WHI-07 were not due to decreased cell numbers or cytotoxic effects of the new agent since cell viability was >70% at 10 µM (IC50 (MTA) = 27.8 µM) after 7 days of culture.

Statistical analysis

Values are presented as the mean ± SD values from independent measurements. A two-tailed Student’s t-test was used to analyse the differences between test groups; P < 0.05 was considered to be significant. Non-linear regression analyses were used to find IC50 and EC50 values from the concentration effect curves using GraphPad Software (San Diego, CA, USA).

Results

Effect of WHI-07 on viral replication

The anti-HIV-1 activity of WHI-07 was evaluated by measuring the viral p24 antigen production and RT activity as markers of viral replication in PHA-stimulated acutely infected PBMCs. As shown in Figure 2, WHI-07 inhibited both the viral p24 antigen production (Figure 2A) and reverse transcriptase activity (Figure 2B) of HTLV-III in PBMC in a concentration-dependent fashion. The median inhibitory concentration (IC50) values of WHI-07 for HIV-1 replication were well below 0.01 µM [IC50 (p24) = 0.005 µM and IC50 (reverse transcriptase) = 0.009 µM respectively] (Figure 2C). In comparison, the IC50 reverse transcriptase) values for ZDV were 0.02 µM against HTLV-III (not shown), whereas the IC50 value for N-9 was 2.195 µM in p24 antigen production assay. Thus, WHI-07 was 4-fold more potent than ZDV and 439-fold more potent than N-9 as an inhibitor of HIV-1 replication. The observed reduction in p24 production and reverse transcriptase activity by WHI-07 were not due to decreased cell numbers or cytotoxic effects of the new agent since cell viability was >70% at 10 µM (IC50 (MTA) = 27.8 µM) after 7 days of culture.

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<table>
<thead>
<tr>
<th>Region</th>
<th>Control placebo (n = 3)</th>
<th>WHI-07 group (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region 1b</td>
<td>Region 2</td>
<td>Region 3</td>
</tr>
<tr>
<td>Epithelial ulceration</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lamina propria thickness</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Leukocyte infiltration</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Vascular congestion</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total score</td>
<td>2 ± 0</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

*Each of five rabbits were administered intravaginally with 1 ml of gel microemulsion containing 2% WHI-07.

*Region 1 = caudal; Region 2 = middle; Region 3 = distal.

Semiaquantitative scoring based on (Eckstein et al., 1969). Individual score: 0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = intense.

Total score: <8 = acceptable, 9–10 = marginal, and >11 = unacceptable.
remained stable (97 ± 2% compared to baseline) throughout the 140 min monitoring period.

The concentration- (Figure 4A) and time-dependent (Figure 4B) sperm motility loss induced by WHI-07 was associated with significant changes (P < 0.001) in the movement characteristics of the surviving spermatozoa particularly with respect to the track speed (VCL), path velocity (VAP), and straight line velocity (VSL). The sperm motion parameters of control spermatozoa showed no significant changes during the 140 min incubation period.

**Effect of WHI-07 on sperm membrane activity**

We examined the effects of WHI-07 on sperm head acrosomal membrane integrity by triple staining (FITC–*Pisum sativum* lectin for acrosome, TOTO-3 iodide for nuclear DNA, and Nile red for membrane lipids) of spermatozoa using CLSM. There was an intense acrosomal staining with FITC-lectin (green), nuclear staining with TOTO-3 (blue), and membrane staining (red) with Nile Red, respectively (Figure 5, upper panels). In acrosome-intact spermatozoa, more than half of the sperm head (the acrosomal region) exhibited a uniform, bright green fluorescence in spermatozoa exposed to vehicle (i.e. 0.1% DMSO) alone (Figure 5A) or 100 µM WHI-07 (Figure 5B) for 3 h. By comparison, spermatozoa exposed to 100 µM of N-9 as a positive control under identical conditions showed no green fluorescence due to disruption of membrane integrity and acrosomal loss (Figure 5C). Despite complete immobilization of spermatozoa in the presence of WHI-07, the vast majority (96 ± 3%) remained acrosome-intact after 3 h of incubation.

These findings were confirmed by topographical imaging of WHI-07-treated sperm head by HR-LVSEM (Figure 5, lower panels) which revealed intact acrosomes in spermatozoa exposed to vehicle (Figure 5D) and WHI-07 (Figure 5E), whereas spermatozoa treated with 10 µM Cal as a positive control revealed blebbing, or vesiculation, fenestration, as well as loss of plasma and outer acrosomal membranes (Figure 5F).

**Cytotoxicity of WHI-07**

The MTT assay measuring cell proliferation and viability was used to assess the in-vitro cytotoxicity of WHI-07 in comparison to N-9 against confluent monolayers of normal human ectocervical and endocervical epithelial cells. Cells were exposed to these compounds at doses ranging from 3.9 µM to 1 mM for 3 or 24 h. The concentration–response cell survival curves for WHI-07 and N-9 for these cells measured by the MTT assay were compared with spermicidal activity measured by CASA (Figure 6). In MTT assays, N-9 exhibited significant cytotoxicity to ectocervical epithelial and endocervical epithelial cells with mean IC$_{50}$ values of 19 and 11 µM respectively. By comparison, the IC$_{50}$ values of the WHI-07 dose survival curves were >1 mM for ectocervical epithelial cells and 312 µM for endocervical epithelial cells (Figure 6).

Thus, N-9 was spermicidal only at cytotoxic concentrations (EC$_{50}$ value: 86 µM; selectivity indices: 0.22 and 0.12 for ectocervical and endocervical cells respectively), whereas, WHI-07 showed high selectivity indices against these cells (SI > 200 for ectocervical cells and 64 for endocervical cells). Thus, WHI-07 was significantly less active against these reproductive tract cells.

**Gel-microemulsion formulation of WHI-07**

Since WHI-07 is lipophilic, we developed a microemulsion-based formulation to achieve as much as 2% of the drug in
submicron (30–80 nm) particle size. In the Sander–Cramer test, sperm motility in the control gel microemulsion base was unaffected even after 15 min, whereas the gel-microemulsion containing 2% WHI-07 immobilized all spermatozoa in semen in <2 min (mean 1.2 ± 0.1 min; n = 6; P < 0.05). The spermicidal activity was stable in the gel microemulsion formulation. The decrease from rapidly progressive motility (3+) to slow, non-progressive motility occurred in <60 s. The spermotostatic effects of WHI-07-gel was unaffected following the addition of glucose to the incubation mixture.

**Effect of WHI-07 on rabbit vaginal tissue histology**

Histological evaluation of vaginal tissue obtained after repetitive intravaginal application of 2% WHI-07 in gel-microemulsion (at doses 500–1000 times higher than its spermicidal potency) for a duration of 10 days showed no vaginal irritation when compared with control rabbits. None of the five rabbits which received WHI-07 intravaginally had vaginal erythema, oedema, exudate, leukocyte influx, or epithelial disruption (acceptable ranges <4; total scores <8) as assessed by macroscopic and histological evaluations (Table I).

**Systemic absorption and tissue retention of vaginal WHI-07 in the rabbit**

The systemic absorption of 2% WHI-07 in gel-microemulsion applied intravaginally was studied in vivo in New Zealand White rabbits. Following intravaginal application of 2% WHI-07 (8.5 mg/kg), blood was collected at timed intervals for up to 4 h. Plasma WHI-07 and its major metabolites (alaninyl ZDV monophosphate and ZDV) were analysed by a validated HPLC procedure with a detection limit of 25 picomoles using mouse plasma samples. Figure 7 shows the representative HPLC chromatograms of WHI-07 (Figure A) and its in-vivo metabolites, alaninyl ZDV monophosphate and ZDV following i.v. injection of WHI-07 (Figure B). Following intravaginal application of 8.5 mg/kg of WHI-07 gel microemulsion, WHI-07 and its metabolites were undetectable (<25 pmol) in all blood samples throughout the 4 h sampling period (Figure C). The concentration of WHI-07 and its metabolites in the rabbit vaginal tissue 24 h after 10 days of repetitive intravaginal administration of 2% WHI-07 in a gel-microemulsion base was assayed after tissue homogenization, solvent extraction, and analytical HPLC. In all five rabbit vaginal tissues (caudal, middle, and distal regions) analysed, WHI-07 and its known metabolites were undetectable (Figure 7D).

**Discussion**

In this study, we report the synthesis, characterization and gel formulation of WHI-07, a novel phenyl phosphate derivative of bromo-methoxy-ZDV, which was identified as a potent inhibitor of HIV-1 reverse transcriptase, with spermicidal properties. In antiviral assays, WHI-07 had an IC₅₀ value that was virtually identical to that of ZDV and was 439-fold lower than that of the detergent spermicide N-9. The spermicidal activity of WHI-07 was 18-fold more potent than that of N-9, suggesting it is a virucidal spermicide with potent anti-HIV activity. Furthermore, the spermicidal activity of WHI-07 was not caused or accompanied by membrane disruption, since despite the cessation of sperm motility, the acrosomal structures remained intact. Compared with N-9, WHI-07 was not cytotoxic to normal human ectocervical and endocervical epithelial cells. When applied topically as a 2% gel-microemulsion base, WHI-07 did not induce vaginal irritation in the rabbit model. Unlike the detergent-type contraceptives, that are cytotoxic at spermicidal doses due to their ability to disrupt membranes, inhibitors of HIV replication such as nucleosides or non-nucleoside analogues and protease inhibitors work by inhibiting viral replication in mucosal cells. These molecules have been proposed by the World Health Organization as potential candidates for intravaginal microbicides (Cookson, 1993). However, preclinical studies of their in-vivo efficacies are not yet available. Polyanionic compounds, e.g. dextran sulphate and other compounds such as N-dodecanol (a 22-carbon straight-chain alcohol), were shown to either inhibit the adhesion of virus infected cells to cervical epithelial cells *in vitro*, or to prevent vaginal transmission of SIV in the SIV/macaque model (Miller et al., 1995). Currently, however, only sulphated polysaccharides such as dextrin sulphate and anionic surfactant (sodium dioctyl sulphosuccinate) that inhibit lymphocyte-to-epithelial transmission of HIV *in vitro* (McClure et al., 1991; Phillips and Pratt-Pearce, 1996) are being evaluated as vaginal applications in humans (Elias and Coggins, 1996; Martindale, 1996; Stafford et al., 1997). However, in-vitro studies suggest that these non-specific products appear to interfere only generally with viral adhesion to cells. Also, preliminary clinical studies evidence of inflammation and abrasion attributable to product use was apparent (Elias et al., 1997; Rosenstein et al., 1998).

We have previously shown that WHI-07 has in-vivo spermicidal activity. Preliminary results demonstrated that intravaginal application of 2% WHI-07 via a cream base prior to artificial insemination with epididymal spermatozoa led to drastic reduction (by 81%) in in-vivo fertilization rates in hormonally primed CD-1 mice (D’Cruz et al., 1998b). Furthermore, repetitive intravaginal applications of WHI-07 versus N-9 administered intravaginally for 5 and 20 consecutive days with 5% WHI-07 or 5% N-9 via a cream base in the mouse model, had no adverse effects on the cervicovaginal histology tissue when compared with N-9-treated sections which revealed disruption of the squamous epithelia and influx of neutrophils (D’Cruz et al., 1998b). The present study confirmed the lack of mucosal toxicity of WHI-07, using the highly sensitive rabbit epithelium in the in-vivo vaginal irritation model. Despite daily application of 2% WHI-07 in gel-microemulsion for 10 days, the vaginal epithelium remained intact with no evidence of tissue oedema, leukocyte influx, or vascular congestion. In addition, tissue absorption and retention studies, clearly demonstrated that WHI-07 has very low capacity to be absorbed through the vaginal epithelium, low potential to produce systemic toxicity following intravaginal applications, and therefore, does not appear to be toxic to the female reproductive tract and the developing conceptus. We postulate that, because of its potent anti-HIV activity, spermicidal efficacy, and lack of mucosal toxicity, WHI-07 may be useful...
as a dual-action vaginal contraceptive for women who are at high risk of acquiring HIV/AIDS by heterosexual transmission.

Although WHI-07 is highly active and promising ZDV derivative with potent spermicidal activity, its further development as a dual-function contraceptive or an intravaginal/rectal microbicide with anti-HIV activity will depend on detailed preclinical evaluations of its safety and activity in relevant animal models. We have scaled up the production of WHI-07 in multigram amounts for preclinical evaluation. Based on our preliminary preclinical studies, it is highly unlikely that WHI-07 when administered intravaginally, will have adverse systemic side effects. Notably, the LD\(_{50}\) dose for WHI-07 when administered i.v. or i.p. was >500 mg/kg for mice. In addition, cynomolgus monkeys treated with 20 mg/kg of WHI-07 i.v. displayed potent anti-HIV activity in serum detectable even after 4 h, and no grade 2–4 systemic toxicities were evident when monitored for up to 24 days (F.M. Uckun et al., unpublished data). Experiments to formally test the safety of the intravaginal formulations of WHI-07 with special emphasis on the long term reproductive health of test animal species are currently in progress.

The identification of compound WHI-07 as a dual function anti-HIV agent with potent spermicidal activity represents a significant step forward in the development of a new vaginal microbicide for curbing heterosexual HIV transmission. Reasonably high levels (2%) of WHI-07 have been successfully formulated in gel-microemulsion base using components suitable for topical application. The promising results reported herein illustrate that WHI-07 shows unique clinical potential to become the active ingredient of a new female-controlled topical virucidal vaginal contraceptive for women who are at high risk for acquiring HIV by heterosexual vaginal transmission. At nanomolar ranges, WHI-07 may also reduce the risk of HIV transmission during conception by assisted reproductive technologies. The further development of this dual-action ZDV derivative as a novel vaginal microbicide may provide the basis for a new strategy aimed at preventing the sexual transmission of HIV while providing fertility control for women.

Acknowledgements

We thank Barbara Waurzniak DVM (Department of Experimental Pathology, Hughes Institute) for the histological grading of rabbit cervicovaginal tissues.

References


Spermicidal zidovudine derivative


Received on June 2, 1998; accepted on January 25, 1999.