Pre-clinical safety evaluation of novel nucleoside analogue-based dual-function microbicides (WHI-05 and WHI-07)

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Compounds WHI-05 [5-bromo-6-methoxy-5,6-dihydro-3′-azidothymidine-5′-(p-methoxyphenyl)-methoxyalaninyl phosphate] and WHI-07 [5-bromo-6-methoxy-5,6-dihydro-3′-azidothymidine-5′-(p-bromophenyl)-methoxyalaninyl phosphate] are aryl phosphate derivatives of zidovudine (ZDV) with anti-HIV and contraceptive activity. WHI-05 and WHI-07 differ fundamentally from currently used surfactant microbicides that are cytotoxic to genital tract epithelial cells at spermicidal concentrations. These drugs were rationally designed to bypass the thymidine kinase dependency of ZDV activation in genital tract secretions, as well as to achieve spermicidal activity. WHI-05 and WHI-07 were formulated via a non-toxic gel-microemulsion for intravaginal use as potential anti-HIV spermicides. Pre-clinical safety studies of intravaginally administered WHI-05 and WHI-07 gel-microemulsions were performed in mice and rabbits to mimic closely the intravaginal application of a microbicidal preparation in women. In addition, systemic toxicity studies were performed in mice and non-human primates. The LD₁₀ doses for WHI-05 and WHI-07 when administered intravenously or intraperitoneally were >500 mg/kg for mice. Female cynomolgus monkeys treated with 20 mg/kg WHI-05 and WHI-07 intravenously developed no grade 2–4 systemic toxicities. Repetitive intravaginal administration of 2% WHI-05 and WHI-07 via a gel-microemulsion to achieve concentrations as high as 6.1 × 10⁴ and 5.7 × 10⁶ times their respective in vitro anti-HIV IC₅₀ values, and 1200 and 5700 times their spermicidal EC₅₀ values, for up to 13 weeks, was not associated with mucosal, systemic or reproductive toxicity. Furthermore, long-term (2 years) intravaginal administration of 2% WHI-07 gel-microemulsion was not associated with systemic toxicity or increased carcinogenicity in mice. The improved potency, as well as the lack of mucosal, systemic and reproductive toxicity of WHI-05 and WHI-07, means that these compounds have clinical potential as safe, prophylactic contraceptives in addition to their microbicidal activity to curb the sexual transmission of HIV.

Keywords: HIV/AIDS, microbicide, spermicide, antiviral drugs, nucleoside analogues

Introduction

Sexual transmission of human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, continues to be the predominant mode of the pandemic spread of HIV/AIDS.¹ Worldwide, >80% of all adult HIV infections have resulted from heterosexual intercourse.² Approximately 48%, or 17.6 million, of the 37.2 million adults living with HIV/AIDS worldwide are women.³,⁴ Women are 4–16 times more likely to contract HIV from infected males than vice versa, and young women are especially vulnerable.⁵ Increasingly, HIV-1 infection in the USA is being spread through heterosexual activity, rather than through same-sex contacts or intravenous drug use.⁶,⁷ The proportion of AIDS cases among US women more than tripled from 7% in 1985 to 25% in 2000.² HIV/AIDS is currently the leading cause of death for African-American women between the ages of 25 and 44, and the sixth leading cause of death for all American women in this age group.⁸ The emergence of AIDS as a disease spread through sexual intercourse has prompted the search for new, effective, safe and dual-function female-controlled vaginal microbicides for curbing mucosal viral transmission, as well as providing fertility control.⁹ Furthermore, prophylactic contraception is fundamentally important for HIV-infected women for the prevention...
of HIV transmission and pregnancy, especially since >80% of women with AIDS in the US are of childbearing age.4,5,10

Currently available spermicidal microbicides, such as nonoxynol-9 (N-9), octoxynol-9, sodium docusate, chlorhexidine, menfegol and benzalkonium chlorides, are cytotoxic to genital tract epithelial cells at spermicidal concentration.9 Frequent use of N-9, the most widely used vaginal contraceptive, has been associated with increased risks of vaginal irritation or ulceration.11–13 Clinical studies have confirmed that detergent-type spermicides alter normal vaginal bacteria or flora, and lead to increased risks of contracting sexually transmitted diseases.14–18 There is a growing concern that chemical irritation that disrupts the vaginal mucosa/flora might actually increase the risk of HIV transmission in sexually active women.19,20 N-9 also has a high contraceptive failure rate.21 Consequently, the development of new, safe, non-detergent-type, dual-function vaginal spermicidal microbicides has become the focal point in translational anti-HIV microbicide research.9

For a microbicide to be an effective anti-HIV agent in genital tract secretions, it is essential that the drug is able to inactivate HIV replication in lymphocytes, epithelial cells and sperm, irrespective of the metabolic state of the cell. Inasmuch as trafficking HIV-infected mononuclear cells in semen contribute to the sexual transmission of HIV via cell-to-cell transmission of HIV,22,23 the anti-HIV microbicide should be metabolized with equal efficiency by seminal cells as well as genital tract epithelial cells of the cervico-vaginal region. Because of the dependence of nucleoside analogues such as zidovudine (ZDV) on intracellular thymidine kinase (TK) activation,24,25 we synthesized novel aryl methoxy alaninyl phosphate derivatives of 3′-azido-3′-deoxythymidine (ZDV, AZT), which undergo intracellular hydrolysis to yield monophosphate derivatives that are further phosphorylated by thymidylate kinase to give the bioactive triphosphate derivatives in a TK-independent fashion.26–30 Aryl phosphate derivatives of 5-bromo-6-methoxy-zidovudine with an alanine methyl ester side chain and a methoxy substitution [compound WHI-05; 5-bromo-6-methoxy-5,6-dihydro-3′-azidothymidine-5′-(p-methoxyphenyl)-methoxyalaninyl phosphate] or a bromo substitution in the phenyl ring [compound WHI-07; 5-bromo-6-methoxy-5,6-dihydro-3′-azidothymidine-5′-(p-bromophenyl)-methoxyalaninyl phosphate] were identified as the lead dual-function agents with potent anti-HIV and spermicidal activities (Figure 1). WHI-05 and WHI-07 displayed potent anti-HIV activity (IC50[RT] = 0.04 and 0.009 µM and IC50[p24] = 0.02 and 0.005 µM, respectively) when compared with N-9 (IC50[p24] = 2.2 µM). WHI-05 was active against both ZDV-sensitive and -resistant HIV-1 strains.28 The spermicidal EC50 values were 24 and 5 µM for WHI-05 and WHI-07, respectively, when compared with that of N-9 (EC50 = 81 µM).

Structure–activity function studies of these novel ZDV derivatives established that the C-5 bromo and C-6 methoxy functionalization on the thymine ring, an alanine side chain and a bromo group on the C-5 position of the phenyl ring are essential for the potent anti-HIV as well as spermicidal activities of ZDV derivatives.26,31,32 Topological imaging and ultrastructural studies of WHI-05- and WHI-07-treated sperm by high-resolution, low-voltage scanning, as well as transmission electron microscopy, demonstrated that the spermicidal property of WHI-05 and WHI-07, unlike N-9, was not associated with membrane disruption.26,29

Safety studies in cell systems

Cytotoxicity studies

The MTT cell proliferation and viability assay was used to test the potential cytotoxicity of WHI-05 and WHI-07 in comparison with N-9 against normal human female ectocervical and endocervical epithelial cells.28,29,33 N-9 exhibited significant cytotoxicity to these cells at spermicidal concentration.
Pre-clinical safety evaluation of WHI-05 and WHI-07

(mean IC\textsubscript{50} values of 19 and 11 \(\mu\)M, respectively). By comparison, the mean IC\textsubscript{50} values of the WHI-05 and WHI-07 dose survival curves were >1000 \(\mu\)M for ectocervical cells and >300 \(\mu\)M for endocervical cells. Unlike N-9, which was spermicidal only at cytotoxic concentrations [EC\textsubscript{50} value: 81 \(\mu\)M; selectivity indices (SI): 0.23 and 0.13 for ectocervical and endocervical cells, respectively], WHI-05 and WHI-07 showed high SI against these cells (EC\textsubscript{50} value: 24 \(\mu\)M for WHI-05; SI: >41 for ectocervical cells and >12 for endocervical cells; EC\textsubscript{50} value: 5 \(\mu\)M for WHI-07; SI: >200 for ectocervical cells and >60 for endocervical cells). Thus, WHI-05 and WHI-07 were significantly less active against genital tract epithelial cells.

Genotoxicity/mutagenicity studies in cell systems

The effect of WHI-07 on genomic stability was tested in the yeast DEL recombination assay.\textsuperscript{34,35} This assay measures the frequency of intrachromosomal recombination between two partially deleted his\textit{3} alleles on chromosome XV. The his\textit{3} delta alleles share \(~400\) bp of overlapping homology, and are separated by an intervening \textit{LEU2} sequence. Homologous recombination between the his\textit{3} delta alleles results in the deletion of the intervening \textit{LEU2} sequence (DEL) and the reversion to histidine prototrophy. Exposure of exponentially growing yeast cells to increasing concentrations of WHI-07 (0.01–100 \(\mu\)M) was not found to increase the DEL recombination frequency of yeast cells.

Since the genotoxic stress response of human cells can be detected at the transcriptional level, we measured the activation of eight stress response specific promoters or regulatory elements (p53RE, GADD 45, FOS, HSP70, HMT\textit{IIa}, NFBRE, GST\textit{Y} and GRP\textit{78}) in HepG2 cell lines using the CAT-Tox(L) assay.\textsuperscript{36,37} When eight separate cell lines carrying the promoter–reporter constructs and control (HepG2) cells were simultaneously treated with increasing concentrations of WHI-07 (0.01–100 \(\mu\)M) for 48 h, no significant activation of these reporter genes was evident as measured by ELISA. These studies in cell systems clearly established that WHI-07 does not induce primary DNA damage in human cells.

Safety of systemic exposure in mice and non-human primates

The toxicological potential of WHI-05 and WHI-07 has been evaluated in two species, non-human primates and rodents. In subacute intraperitoneal toxicity studies performed in mice, no significant toxicological alterations were seen. The LD\textsubscript{10} of WHI-05 and WHI-07 when administered intravenously or intraperitoneally was >500 mg/kg for mice.

WHI-05 and WHI-07 were administered to female cynomolgus monkeys either as a single intravenous (iv) injection or multiple (five) iv injections for 2 days. At doses of 20 mg/kg, WHI-05 and WHI-07 were non-toxic to cynomolgus monkeys (Tables 1 and 2). Blood for haematological (red blood cells, haemoglobin, white blood cells, packed cell volume (PCV), mean corpuscular volume, mean cell haemoglobin, mean cell haemoglobin concentration, red cell distribution width, mean platelet volume, Diff., platelets) and clinical chemistry [Na, K, Ca, Cl, HCO\textsubscript{3}, anion gap, glucose (GLU), blood urea nitrogen (BUN), creatinine (CRE), total bilirubin (TBIL), alanine aminotransferase (ALT), amylase (AMY), creatinine kinase (CK) and albumin (ALB)] determinations collected from cynomolgus monkeys before (day 1) and after (on days 2, 8, 13, 26 and 33 for WHI-05 and on days 2, 3, 5, 9, 17 and 24 for WHI-07) drug treatment did not reveal

Table 1. Summary of WHI-05 toxicity grading in cynomolgus monkeys\textsuperscript{a}

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pre-treatment day 1</th>
<th>WHI-05 post-treatment</th>
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<tbody>
<tr>
<td></td>
<td>day 2</td>
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<tr>
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</tr>
<tr>
<td>Hb</td>
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</tr>
<tr>
<td>Platelets</td>
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<td>0</td>
</tr>
<tr>
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<td>2</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Urea nitrogen</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Creatinine</td>
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<td>0</td>
</tr>
<tr>
<td>Total bilirubin</td>
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</tr>
<tr>
<td>Alanine aminotransferase</td>
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<td>1</td>
</tr>
<tr>
<td>Albumin</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Cynomolgus monkey was treated with 20 mg/kg of WHI-05 at day 1 with iv bolus injection. The toxicity grading system was adapted from the Children’s Cancer Group toxicity criteria for testing new agents in patients.
significant differences. Furthermore, toxicological grading performed post-treatment of monkeys showed no clinical evidence of significant toxicity (toxicity grades 0 to <2) (Tables 1 and 2).

### Mucosal safety studies in mice and rabbits

The local tissue alterations and inflammatory response to repetitive intravaginal application of WHI-05 and WHI-07 gel versus N-9 gel were evaluated in mice and rabbits (Table 3). Subgroups of mice were treated for 5 or 20 consecutive days with 5% WHI-07 gel, 5% N-9 gel or gel control, and the cervicovaginal tissue sections were examined for histopathological changes and influx of inflammatory cells by immunofluorescence assay with anti-neutrophil antibodies. Similar to control mice, none of the 10 mice treated with WHI-07 for 5 days or 20 days revealed any inflammatory response or membrane disruption of the squamous epithelia (Table 3). By contrast, disruption of the epithelial lining, and an inflammatory response with influx of neutrophils in the squamous epithelia of cervicovaginal crypts, were evident in 90% of mice given N-9 intravaginally.

Owing to the lipophilic nature of WHI-05 and WHI-07, we developed a microemulsion-based formulation to achieve as much as 2% of the drug in submicron (30–80 nm) particle size for intravaginal use as potential anti-HIV spermicides. Microemulsions appear to have the ability to deliver larger amounts of topicaly applied agents into the mucosa than traditional vehicles because they provide a better reservoir for a poorly soluble drug through their capacity for enhanced solubilization. In the Sander Cramer test, sperm motility in the control gel-microemulsion base was unaffected even after 15 min, whereas gel-microemulsion containing 2% WHI-05 or WHI-07 immobilized all sperm in human semen in <3 min. The spermicidal activity was stable in the gel-microemulsion formulation. The decrease from rapidly progressive motility to slow, non-progressive motility occurred in <60 s.

The rabbit vaginal irritation studies were performed by daily intravaginal administration of 1 mL of gel-microemulsion with and without 2% WHI-05 or WHI-07 compared with the standard 4% N-9 gel for 10 consecutive days. Histological evaluation of three distinct areas of the cervico-vaginal region showed no vaginal irritation or inflammation. None of the rabbits that received WHI-05 or WHI-07 gel intravaginally had vaginal erythema, oedema, exudate, leucocyte influx or epithelial disruption characteristic of inflammation (individual score 0–2 and total score <4; acceptable range) when
compared with N-9 gel (individual scores 1–3; total score 9; marginal irritation). Thus, evidence obtained by histopathological and immunofluorescence studies indicated that unlike N-9 treatment, intravaginal application of WHI-05 or WHI-07 via a gel-microemulsion at a dose 1200 and 5700 times higher than their respective in vitro spermicidal EC₅₀ values, and 6.1 × 10⁴ and 5.7 × 10⁹ times higher than their anti-HIV IC₅₀ values, did not cause any membrane disruption or acute inflammatory response in the cervico-vaginal epithelial crypts.²⁸,²⁹

The systemic absorption of 2% WHI-05 and WHI-07 gel-microemulsion applied intravaginally was also investigated in NZW rabbits. Following intravaginal application of 2% WHI-05 and WHI-07, blood was collected at timed intervals for up to 4 h. Plasma WHI-05/07 and their major metabolites (ZDV, alaninyl-ZDV-monophosphate and ZDV-monophosphate) were analysed by a validated HPLC procedure with a detection limit of 25 pmol in plasma samples. Following intravaginal application of a 2% WHI-05 and WHI-07 gel-microemulsion, WHI-05/07 and their metabolites were undetectable (<25 pmol) in all blood samples throughout the 4 h sampling period.²⁹ The concentration of WHI-05 and WHI-07 and their metabolites in the vaginal tissue 24 h after 10 days of daily intravaginal application of 2% WHI-05 or WHI-07 in a gel-microemulsion base was assayed after tissue homogenization, solvent extraction and analytical HPLC. In all rabbit vaginal tissues analysed (cervico-vagina, mid-vagina and uro-vagina), WHI-05/07 and their known metabolites were undetectable.²⁸,²⁹ These results indicated that WHI-05 and WHI-07 have very low capacity to be absorbed through the vaginal epithelium after repetitive intravaginal administration.

Subchronic (13 weeks) toxicity studies in mice

Because vaginal microbicides would probably be used repeatedly over decades, an ideal microbicide should have an established safety record and lack local and systemic toxicity and carcinogenic potential. It was anticipated that under the conditions of their intended use as an intravaginal/rectal microbicide, individuals would be exposed to WHI-05 or WHI-07 on short-term or long-term repeated use. Because WHI-05 and WHI-07 are non-cytotoxic and are not absorbed systemically when given intravaginally,²⁸,²⁹ they are unlikely to have adverse effects on the general well-being of women. However, because oral administration of the parent compound, ZDV, has been shown to result in reversible haematological toxicity in man and experimental animals,⁴⁰–⁴³ it was prudent to test the potential local and systemic side effects of these dual-function ZDV derivatives. Therefore, we determined the effects of repeated intravaginal exposure to WHI-05 and WHI-07.

The assay conditions consisted of gel-microemulsion with and without three increasing doses of WHI-05 and WHI-07. Female B₆C₃F₁ mice in subgroups of 10 and 20 were treated with intravaginal applications of 0, 0.5, 1 or 2% WHI-05 or WHI-07 gel-microemulsion, 5 days per week, for 13 consecutive weeks.⁴⁴ On a molar basis, these concentrations of WHI-05 and WHI-07 dose are 1200 and 5700 times higher than their respective in vitro spermicidal EC₅₀, and 6.1 × 10⁴ and 5.7 × 10⁹ times higher than their in vitro anti-HIV activity IC₅₀. Mortality did not occur in any mouse group and all animals were clinically healthy at the end of the study. Mean body weight gain and final mean body weight of the mouse group exposed to increasing doses of WHI-05 or WHI-07 were similar to those of the gel-microemulsion controls. Table 4 summarizes the toxicity parameters for mice given increasing concentrations of intravaginal WHI-07 for 13 weeks.

Complete blood cell counts in the mice revealed no biologically significant differences between WHI-05- or WHI-07-dosed and vehicle control mice.⁴⁴–⁴⁶ The values of

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<tr>
<th>Parameters</th>
<th>WHI-07 concentration (%)</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Number examined</td>
<td>30</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>100</td>
</tr>
<tr>
<td>Final body weight (% of concurrent control)</td>
<td>100</td>
</tr>
<tr>
<td>Individual differences in haemogram profile versus control (n)</td>
<td>–</td>
</tr>
<tr>
<td>Individual differences in plasma chemistry versus control (n)⁴</td>
<td>–</td>
</tr>
<tr>
<td>Individual differences in absolute organ weight versus control (n)⁴</td>
<td>–</td>
</tr>
<tr>
<td>Individual differences in relative organ weight versus control (n)</td>
<td>–</td>
</tr>
<tr>
<td>Incidental lesions (n)</td>
<td>4/25</td>
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<td>Test article-related lesion (n)</td>
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⁴No single mouse had multiple variables in plasma chemistry or organ weights.
Reproductive performance after intravaginal exposure in mice

The potential for reproductive toxicity of WHI-05 and WHI-07 was assessed in a series of experiments using CD-1 mice under the conditions of their intended use as intravaginal microbicides. The effects of intravaginally administered WHI-05 and/or WHI-07 gel on: (i) ovulation efficiency; (ii) in vivo fertilization and early embryonic fetal development; and (iii) reproductive outcome, including neonatal survival and pup development, were examined.44,45,47 WHI-05 and/or WHI-07 were administered intravaginally during superovulation, organogenesis and prior to mating for 5 and 10 consecutive days, and for 13 weeks, respectively. After 14 and 40 h post-hCG injection, mice were evaluated for ovulation efficiency and fertilization rate and cleavage, respectively. Pregnant mice were administered 2% WHI-07 intravaginally during gestation days (GD) 6–15, and measures of teratogenicity were evaluated on GD 17. In addition, CD-1 mice were given intravaginal treatment of gel-microemulsion containing 0%, 0.5%, 1.0% and 2.0% WHI-05 and WHI-07 for 13 weeks and then mated with untreated males to evaluate potential reproductive and developmental effects.

Table 5 summarizes the ovulation rates of placebo control versus 2% WHI-05- and WHI-07-treated CD-1 mice. In five independent experiments, the ovulation response was similar when PMSG/hCG-primed mice were given intravaginal gel-microemulsion with and without 2% WHI-05 or WHI-07 before and during superovulation for 5 consecutive days. All mice from the control and the WHI-07-treated groups ovulated. Also, the differences in the mean number of eggs recovered from the ampullae of their oviducts were not statistically significant (33.5 versus 35.4 and 29.8 for WHI-05 and WHI-07, respectively).

Table 6 summarizes the effects of repeated intravaginal application of 2% WHI-07 on in vivo fertilization and the recovery of total embryos and further development of the two-cell embryos to four cells or greater stage embryos. In three independent experiments, fertilization of eggs was similar when PMSG/hCG-primed mice were mated after daily exposure to 2.0% WHI-07 before and during superovulation for five consecutive days. There was no significant difference in the total number of eggs recovered and the proportion of eggs fertilized or cleaved between untreated control or placebo control and 2.0% WHI-07-treated test groups. The average of 61% (333/542) fertilized eggs obtained with mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Results</th>
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<tbody>
<tr>
<td>Placebo gel</td>
<td>100% ovulation response (1139 eggs; n = 34)</td>
</tr>
<tr>
<td>2% WHI-05 gel</td>
<td>94% ovulation response (1132 eggs; n = 34)</td>
</tr>
<tr>
<td>2% WHI-07 gel</td>
<td>100% ovulation response (1045 eggs; n = 35)</td>
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</table>

*Mice with eggs in the ampullae 14 h post-hCG injection.*

The potential for reproductive toxicity of WHI-05 and WHI-07 was assessed in a series of experiments using CD-1 mice.
with intravaginal treatment of gel-microemulsion was similar to the 53% (375/710) average obtained with mice treated with 2% WHI-07 gel-microemulsion intravaginally.

Table 7 summarizes the reproductive parameters of pregnant mice on GD 17 following intravaginal administration of gel-microemulsion with and without 2% WHI-07 during GD 6–15. In three independent experiments, there were no significant group differences in the mean litter size (11 ± 9 versus 10.3 ± 6.8), mean fetal weight (0.58 ± 0.19 versus 0.55 ± 0.17) and percentage of live fetuses (93% versus 94%) in mice-administered gel-microemulsion alone or gel-microemulsion containing 2% WHI-07. Intravaginal administration of 2% WHI-07 during the period of major organogenesis did not result in a significant increase in external malformations in offspring when compared with the placebo control group. A low incidence of external anomalies (short limbs) and fetal skeletal malformations (absence of ribs and vertebral column) was found in both the placebo control and 2% WHI-07 treated mice.

Table 8 summarizes the fertility parameters for CD-1 mice given, intravaginally, increasing concentrations of WHI-07 for 13 weeks prior to mating. When gel-microemulsion control and WHI-05- or WHI-07-treated female CD-1 mice were evaluated for their fertility immediately after cessation of the 13 week intravaginal administration, neither concentration of WHI-05 or WHI-07 had a statistically significant effect on fertility parameters. Thirteen week treatment with WHI-05 and WHI-07 at concentrations as high as 1200 and 5700 times higher than their respective in vitro spermicidal EC₅₀ and 6.1 × 10⁴ and 5.7 × 10⁶ times higher than their in vitro anti-HIV activity IC₅₀ had no statistically significant effect on subsequent fertility, median litter size, neonatal survival, pup morphology or development.⁴⁴,⁴⁵,⁴⁷ The mean neonatal and pup weights from mice exposed to 0.5%, 1.0% and 2.0% WHI-05 or WHI-07 were not lower than that of the placebo control group at birth and on lactation day 5.

These studies demonstrated that repeated short-term intravaginal exposure of mice to 2% WHI-07 had no adverse effects on ovulation response, mean number of eggs recovered or the percentage of eggs fertilized or cleaved.⁴⁷ No evidence of reproductive toxicity, fetal toxicity or teratogenicity was found following repetitive intravaginal application of 2% WHI-07 during the period of organogenesis.⁴⁷ Furthermore, repeated intravaginal exposure of mice to 0.5–2.0% WHI-05 and WHI-07 for 13 weeks had no adverse effect on the subsequent reproductive capability, perinatal outcome, growth and development of the offspring.⁴⁴,⁴⁵,⁴⁷
Two year toxicity studies in mice

To evaluate the toxicity and carcinogenic potential of long-term exposure to WHI-07, groups of 50 female \( B_6C_{3F1} \) mice were given no treatment or exposed intravaginally to a gel-microemulsion formulation with and without 2.0% WHI-07, 5 days per week for up to 2 years. The endpoints that were evaluated included survival, body weight, haematological and clinical chemistry profiles, absolute and relative organ weights, and histopathology.

No significant difference in survival was observed among the groups of untreated control, placebo control and 2% WHI-07-treated mice at the end of the 2 year study (Table 9). At the completion of the 104 week study, the survival figures for the untreated control group, vehicle-only control group and 2% WHI-07-treated group females were 32/50 (64%), 40/50 (80%) and 31/50 (62%), respectively. There was no treatment-related effect on mortality rate. All animals survived beyond the first year treatment period (59 weeks) and >90% of mice from all three groups survived up to 85 weeks of the 2 year study. The spontaneous death rate in all groups was relatively low until week 90 of the study, when it rose in the untreated control and 2% WHI-07-treated groups in a similar manner. There were no statistically significant body weight differences among the groups of intravaginally exposed and untreated control mice at the completion of the 2 year intravaginal study. No compound-related clinical signs were observed.

Complete blood cell counts in the \( B_6C_{3F1} \) mice revealed no biologically significant differences between the groups of untreated control, placebo control and 2% WHI-07-treated mice at the end of the 2 year study. The mean values of haematological parameters for red cells (total count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean cell haemoglobin, mean cell haemoglobin concentration and red cell distribution width) were essentially identical. The mean neutrophil count was highly variable in the 2% WHI-07 group due to their marked increase in individual mice (4 of 28 (14.2%)). However, other differentials (lymphocytes, monocytes, eosinophils and basophils) were similar. A slight increase in mean platelet count was observed in the placebo control and 2% WHI-07-treated groups. However, the mean platelet volume in all groups was within normal limits.

Analysis of 19 blood chemistry parameters for female \( B_6C_{3F1} \) mice given no treatment or exposed intravaginally to a gel-microemulsion with and without 2.0% WHI-07 for 2 years revealed no significant treatment-related differences among the three groups, except for ALP and TBIL levels, which were slightly lower in the placebo control and 2% WHI-07-treated groups than in the untreated control group. These minor differences were not considered clinically significant, nor were they related to treatment with WHI-07. In general, the indicators tested for kidney function (BUN and CRE), liver function (TBIL, AST, ALT, ALP, CHO and TG), pancreas function (AMY, GLU), immunological function (G), nutritional status (TP), calcium (Ca), phosphorous (P) and plasma electrolytes (Na, K and Cl) were not affected adversely by repeated intravaginal exposure of gel-microemulsion with or without 2% WHI-07.

There were no statistically significant treatment-related differences in absolute and relative organ weights of brain, thymus, heart, lung, intestine, liver, pancreas, spleen, kidney, genital tract or urinary bladder among the three groups, or between vehicle-only control and 2% WHI-07 treatment groups that were considered to be related to WHI-07 exposure. A variety of non-neoplastic lesions were observed in both untreated and intravaginally treated groups. These lesions were considered to be incidental, related to ageing or procedural related to the intravaginal route of administration.

Light microscopic examination of brain, heart, intestine, lung, lymph node, pancreas, skeletal muscle, skin, spinal cord, stomach, thymus, cervico-vagina and urinary bladder specimens taken from the untreated control, vehicle-only control and 2% WHI-07-treated \( B_6C_{3F1} \) mice demonstrated no significant increases in the severity index above that seen for the untreated group. The most frequent non-neoplastic lesions observed in all three groups included: fibro-osseous process in the bone, nephritis in the kidneys, hepatitis, lym-
phadenitis, ovarian cyst, ovarian inflammation, pancreatitis, cystitis, endometrial hyperplasia and metritis. A higher incidence of metritis of the uterus and inflammation of the ovaries was noted in vehicle-only control (28.8–32.5%) and 2% WHI-07-treated (18.6–37.5%) mice compared with untreated controls (7.1–10.8%). No significant differences in the increased total incidences of non-neoplastic lesions of the kidney (26.6% versus 25.5%), liver (35.5% versus 32.5%) and uterine specimens (64.4% versus 51.1%) were observed between the vehicle-only control and the 2% WHI-07-treated mice. There was a slight numerical increase in the incidence of moderate medullary hypoplasia (5/20) and moderate Type A (4/20) and Type B (3/20) subcapsular hyperplasia in the limited adrenal gland tissues obtained from the 2% WHI-07 treatment group versus the vehicle control group (2/16 and 1/45).

Two year carcinogenicity studies in mice

A variety of neoplastic lesions were observed in both untreated and intravaginally treated groups. These lesions were considered incidental or related to ageing. Microscopic evaluation of the various organs from controls and the WHI-07-treated group did not show any treatment-related increase in neoplasms. Tumours were found in the adrenal, bone marrow, intestine, kidney, liver, lung, lymph node, ovaries, pancreas, skeletal muscle, skin, spinal cord, spleen, stomach, thymus, uterus, cervico-vagina and urinary bladder of both untreated and intravaginally treated mice. No evidence of neoplastic lesions was observed in the brain and heart of untreated and treated groups. There were no statistically significant differences between the untreated control and treatment groups in overall tumour incidence.

In all three groups, tumours of the liver (15.5–20.9%), lung (11.6–20.9%), lymph node (26–62.5%), ovaries (8.1–20%), spleen (4.6–28.8%), thymus (11.4–15.7%) and uterus (6.6–11.6%) were the most frequent, followed by urinary bladder (2.7–10.8%) and cervico-vagina (5.2%). The overall incidence of ovarian neoplastic lesions for untreated and vehicle control and 2% WHI-07-treated mice were 8.1%, 20% and 20%, respectively. Some of the more common findings included: lymphoma of the liver, lung, lymph node, ovary, pancreas, thymus, uterus, cervico-vagina and urinary bladder. Other spontaneous neoplastic lesions typical for the age and strain of the mouse were observed occasionally. All of the neoplastic lesions observed in the liver, lung, lymph node, ovaries, spleen and uterus were considered spontaneous for female B6C3F1 mice and did not exceed the incidences observed for the control group when compared with a historical database. Very low incidence of neoplastic lesions occurring sporadically in the various groups was also detected in adrenal, bone marrow, large and small intestine, kidney, pancreas, skeletal muscle, skin, spinal cord and stomach (2.2–7.1%).

Microscopic evaluations of mice that died during the second year indicated that the predominant cause of death in all groups was malignant lymphoma. At the end of the 2 year study, lymphocytic lymphoma accounted for 88.2%, 62.0% and 88.2% of tumour-bearing mice from untreated control, vehicle-only control and the 2% WHI-07 treatment group, respectively. Generalized lymphoma was frequently encountered in the liver, lung, lymph nodes, ovaries, thymus and uterus, and less frequently in other organs. The cumulative incidence of microscopic lesions in various organs showed that malignant lymphoma was the major cause of death in ageing female B6C3F1 mice, the incidence of which was unaffected by intravaginal treatment. Table 10 summarizes the total numbers of malignant tumours in female B6C3F1 mice killed or dying during the duration of the 2 year study. The incidental neoplastic lesions occurred with similar frequency in the untreated and 2% WHI-07-treated group and were slightly more pronounced in the vehicle-only group, although the differences were not statistically significant. The total number of malignant tumours as well as the type of malignant tumours in all three groups was comparable. Thus, long-term intravaginal administration of WHI-07 is not associated with systemic toxicity or increased carcinogenicity in mice.

Summary

WHI-05 and WHI-07, aryl phosphate derivatives of 5-bromo-6-methoxy-zidovudine, are dual-function agents with potent anti-HIV and contraceptive activity. WHI-05 and WHI-07 differ fundamentally from currently used membrane-active surfactant spermicides that are cytotoxic to genital tract epithelial cells at spermicide concentration. These drugs

Table 10. Summary of 2 year intravaginal carcinogenicity data in B6C3F1 mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Untreated control</th>
<th>Placebo gel</th>
<th>2% WHI-07 gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective animals</td>
<td>43</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>Animals with malignant tumours</td>
<td>17 (39.5%)</td>
<td>21 (46.6%)</td>
<td>17 (39.5%)</td>
</tr>
<tr>
<td>Total no. of malignant tumours</td>
<td>69</td>
<td>51</td>
<td>79</td>
</tr>
<tr>
<td>Types of malignant tumour</td>
<td>5</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>
were rationally designed to bypass the TK dependency of ZDV activation in genital tract secretions as well as to achieve spermicidal activity. WHI-05 and WHI-07 were formulated via a non-toxic gel-microemulsion for intravaginal use as potential anti-HIV spermicides. Pre-clinical safety studies of intravaginally administered WHI-05 and WHI-07 gel-microemulsions were performed in mice and rabbits to mimic closely the intravaginal application of a microbicidal preparation in women. In addition, systemic toxicity studies were performed in mice and non-human primates. The LD_{10} doses for WHI-05 and WHI-07 when administered intravenously or intra-peritoneally were >500 mg/kg for mice. Female cynomolgus monkeys treated with 20 mg/kg of either WHI-05 or WHI-07 intravenously developed no grade 2–4 systemic toxicities. Repetitive intravaginal administration of 2% WHI-05 or 2% WHI-07 via a gel-microemulsion to achieve toxicities. Repetitive intravaginal administration of 2% WHI-05 or 2% WHI-07 via a gel-microemulsion to achieve concentrations as high as 6.1 × 10^{4} and 5.7 × 10^{6} times their respective in vitro anti-HIV IC_{50} values, and 1200 and 5700 times their spermicidal EC_{50} values, for up to 13 weeks, was not associated with mucosal, systemic, or reproductive toxicity. Furthermore, long-term (2 years) intravaginal administration of 2% WHI-07 gel-microemulsion was not associated with systemic toxicity or increased carcinogenicity in mice. The improved potency, as well as the lack of mucosal, systemic and reproductive toxicity of WHI-05 and WHI-07, means that these compounds have clinical potential as safe, prophylactic contraceptives in addition to their microbicidal activity to curb the sexual transmission of HIV.

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References


