Discovery of 2,5-dimethoxy-substituted 5-bromopyridyl thiourea (PHI-236) as a potent broad-spectrum anti-human immunodeficiency virus microbicide

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The increased risk of heterosexual transmission of human immunodeficiency virus-1 (HIV-1) has prompted the search for safe and effective female-controlled vaginal microbicides. Because endogenous reverse transcription is implicated in augmenting the sexual transmission of HIV-1, potential microbicides should have the inherent ability to optimally inhibit both wild-type and drug-resistant mutant strains of HIV-1. N-[2-(2,5-dimethoxyphenylethyl)-N'-[2-(5-bromopyridyl)]-thiourea (PHI-236) is a rationally designed non-nucleoside inhibitor of HIV-1 reverse transcriptase (NNRTI) that was deduced from changes in binding pocket size, shape and residue character that result from clinically observed NNRTI resistance mutations. PHI-236 displayed high-binding affinity (Ludi $K_i = 0.07 \mu M$) for HIV-1 RT and robust anti-HIV activity against the wild type ($IC_{50} = <0.001 \mu M$) as well as primary clinical isolates ($IC_{50} = 0.009–0.04 \mu M$) carrying multiple RT gene mutations associated with NRTI and NNRTI resistance. PHI-236 displayed high-selectivity index against human vaginal and cervical epithelial cells and did not affect human sperm functions. In the humanized severe combined immunodeficient mouse model for HIV/acquired immune deficiency syndrome (AIDS), pretreatment of HIV-1 (BaL)-infected human monocytes and semen with PHI-236 prevented the systemic infection via the vaginal route. PHI-236 has particular clinical utility as a non-steroidal microbicide as well as a prophylactic antiviral agent to inactivate cell-free and cell-associated HIV-1 in semen before assisted reproductive technology procedures.

Key words: AIDS or HIV/drug resistance/intravaginal/microbicide/non-nucleoside inhibitor/reverse transcriptase/sperm/thymidine analogue mutations

Introduction

Newly acquired human immunodeficiency virus-1 (HIV-1) infections are largely the result of heterosexual transmission (Mujeeb and Altaf, 2003; Centers for Disease Control, 2004a). Currently, about half of the 42 million people living with HIV/acquired immune deficiency syndrome (AIDS) are women (UNAIDS, 2003; National Institute of Allergy and Infectious Diseases, 2004b). In the United States, the proportion of women living with HIV/AIDS has increased from 8% of all cases during 1981–1987 to 17% during 1993–1995 and 28% in 2003 (Centers for Disease Control, 2004b). Worldwide, more than 90% of all adolescent and adult HIV infections have resulted from heterosexual intercourse (Newman, 2004). Women are particularly vulnerable to heterosexual transmission of HIV owing to substantial mucosal exposure to seminal fluids (European Study Group on Heterosexual Transmission of HIV, 1992). Also, male-to-female transmission of HIV is more efficient than female-to-male transmission (Nicolosi et al., 1994).

The emergence of HIV/AIDS as a disease spread through sexual intercourse has prompted the search for safe and effective female-controlled vaginal and rectal microbicides for curbing mucosal viral transmission via semen (D'Cruz and Uckun, 2002, 2004a,b). Microbicides can provide protection by inactivating viruses or preventing viruses from replicating either in semen or in the infected host cells that line the vagina/rectal wall. Microbicides that are currently being investigated are directed mainly at preventing pregnancy as well as protection against sexually transmitted infections (STIs; Uckun and D'Cruz, 1998; D'Cruz et al., 2000b, 2002, 2004b). However, the availability of a non-steroidal microbicide is equally important for (i) sexually active women to allow pregnancy while protecting both mother and her fetus or infant from HIV-1 and (ii) as a prophylactic antiviral agent to curb the transmission of HIV via semen during assisted reproductive procedures.

The success of non-nucleoside inhibitors (NNI) of HIV-1 reverse transcriptase (NNRTI) for the clinical treatment of AIDS has led to the computer-aided design and chemical synthesis of second-generation of potent NNRTIs (De Clercq, 1999, 2001; Pedersen and Pedersen, 1999; Campiani et al., 2002). NNRTIs bind to an allosteric site of HIV-1 RT, which is ~10 Å away from the polymerase active site (Schaefer et al., 1993; Smerdon et al., 1994; Ren et al., 1998). NNRTI binding induces rotamer conformation changes in amino acid residues (Y181 and Y188) and makes the ‘thumb’ region of the enzyme more rigid (Kohlstaedt et al., 1992; Ren et al., 1995). Both events alter the substrate-binding mode and/or affect the translocation of the double strand, which are critical for the polymerase function, thereby leading to a non-competitive inhibition of the enzyme (Ding et al., 1995; Esnouf et al., 1995). Most mutations conferring resistance to NNRTIs are directly in contact with the NNI molecule and thus are associated
with changes in the binding of NNI to HIV-1 RT (Larder, 1994). Dozens of mutant strains have been characterized as resistant to NNRTIs, including L100I, K103N, V106A, E138K, Y188I/C and Y188H (Artico, 1996; Schinazi et al., 1997). In particular, the K103N and Y181C mutants are the most difficult to treat, because they are resistant to most of the NNRTIs that have been examined (Casado et al., 2000; Clevenbergh et al., 2002).

We previously developed a novel model of the NNI-binding pocket of HIV-1 RT, which was constructed by superimposing nine individual NNI-RT crystal structures and generating a van der Waals surface that encompassed all the overlaid ligands (Vig et al., 1998; Mao et al., 1999a,b, 2000). This ‘composite-binding pocket’ surprisingly revealed a different and unexpectedly larger NNRTI-binding site than shown in or predictable from any of the individual structures and served as a probe to more accurately define the potentially usable space in the binding site. We used the composite NNRTI-binding pocket model to design potent NNRTIs against wild-type RT and drug-resistant RT mutants (Sudbeck et al., 1998, 1999; Mao et al., 1999a,b, 2000; Uckun et al., 1999; D’Cruz et al., 2004a). Molecular modelling and score functions were used to analyse how drug-resistant mutations would change the RT-binding pocket shape, volume and chemical make-up, and how these changes could affect NNI binding (Mao et al., 1999a,b, 2000; D’Cruz et al., 2004). The use of our composite-binding pocket led to the synthesis of a series of phenethylthiourea NNRTIs with potent anti-HIV activity (D’Cruz et al., 2000, 2004a). Molecular modelling studies revealed that the pyridyl group was an important determinant of the binding affinity for HIV-1 RT (Mao et al., 1999b).

Here, we show that PHI-236 exhibits robust anti-HIV activity against drug-resistant HIV-1 strains and genotypic and/or phenotypically nucleoside analogue RT and NNRTI-resistant primary clinical isolates. PHI-236 was remarkably more potent than the standard NNRTI drugs against the problematic multidrug-resistant HIV-1 strains with mutations involving RT residues M41L, L74V, K103N, V106A, Y181C or T215Y. PHI-236 prevented the vaginal transmission of a CCR5-dependent HIV-1 strain (Ba.L) in the humanized severe combined immunodeficient (Hu-SCID) mouse model for HIV/AIDS. PHI-236 was non-cytotoxic to normal human female genital tract epithelial cells and did not affect human sperm functions. PHI-236 has particular clinical utility as a non-spermicidal microbicide as well as a prophylactic antiviral agent to inactivate cell-free and cell-associated viruses in semen before assisted reproductive technology procedures.

Materials and methods

Synthesis and characterization of substituted phenethylthiourea compounds

The synthesis of 14 novel pyridyl thiourea compounds as inhibitors of HIV-1 RT was based on structural and computer modelling studies using the composite-binding pocket constructed from nine individual crystal structures of RT–NNI complexes (Mao et al., 1999a,b, 2000; D’Cruz et al., 2004a). Modelling studies for rational drug design included the analyses of surface complementarity between NNRTIs and HIV-1 RT, and application of inhibitory constants (Ludi $K_i$ values) combined with a docking procedure involving the novel thiourea compounds (Mao et al., 1999a,b, 2000; D’Cruz et al., 2004a). The calculated $K_i$ values of the positioned thiourea compounds were evaluated by using the Ludi score function (Bohm, 1994; Molecular Simulations Inc, 1996). The Insight II program (Accelrys, San Diego, CA, USA) used for docking and Ludi scoring employed a calibration procedure during its establishment, which involved calculation of the $K_i$ values of 45 protein–ligand complexes having known $K_i$ values and known crystal structures and comparing the calculated $K_i$ values to the experimentally determined $K_i$ values.

Our computational approach using phenethylthiazolylthiourea (PETT) NNRTIs (Bell et al., 1995; Cantrell et al., 1996) allowed the identification of several ligand derivatization sites for the generation of more potent thiourea compounds (Mao et al., 1999a,b, 2000; D’Cruz et al., 2004a). First, the pyridyl ring of trovirdine {N-[2-(pyridyl)]-N'-[2-(5-bromopyridyl)]-thiourea} was replaced with a substituted phenyl group that fits well with the Wing 2 region of the butterfly-shaped NNI-binding pocket of HIV-1 RT (Figure 1). Second, 14 phenyl substituted pyridyl thiourea compounds were synthesized according to the general scheme, as described previously (Mao et al., 1999a,b). The substitutions on the phenyl ring included bromo, chloro, fluoro, hydroxyl, methyl, methoxy or nitro groups at ortho (2,5), meta (3) or para (4) positions on the phenyl ring.

The pyridyl thioureas were synthesized by condensing 2-amino-5-bromopyridine with 1,1-thiocarbonyl diimidazole to furnish the precursor thiocarbonyl derivative. Further reaction of the product with substituted phenylethyl amine in dimethyl formamide yielded the target compound in good yields. Trovirdine was synthesized, according to literature procedure (Bell et al., 1995). The derivatives were purified by column chromatography. Purity was determined by proton ($^1$H), carbon ($^{13}$C) and fluorine ($^{19}$F) nuclear magnetic resonance spectroscopy (Varian Oxford 300 MHz spectrometer; Varian Associates, Palo Alto, CA, USA), Fourier transform infrared spectroscopy (FT-Nicolet Model Protege 460 instrument; Nicolet Instrument Corp., Madison, WI, USA), mass spectrometry (Hewlett Packard matrix-assisted laser desorption spectrometer.

![Figure 1](https://academic.oup.com/molehr/article-abstract/11/10/767/995059/86565307/13-December-2018)
Model G2025A, Wilmington, DE, USA) and ultraviolet spectrophotometry (Beckmann Model 3 DU 7400 UV-Visible spectrophotometer; Beckmann Instruments, Fullerton, CA, USA). Compounds with a purity of >99% were used for the preclinical studies.

**Anti-HIV assays**

Phenylthiourea NNRTIs were tested in enzyme assays and cell-based assays against wild-type Human T cell Lymphotropic Virus and a panel of NRTI- and NNRTI-resistant strains carrying clinically relevant mutations. Zidovudine (ZDV), trovirdine, nevirapine and delavirdine were used as reference compounds.

**Purified RT assays for anti-HIV activity**

Fourteen thiourea and reference compounds were tested for RT inhibitory activity [IC_{50 RT}] against purified HIV-1 recombinant RT (rRT) by using the cell-free QuickRT-RT system (Amersham, Arlington Heights, IL, USA), which utilizes the scintillation proximity assay (SPA) principle (Bosworth and Towers, 1989). In the assay, a DNA–RNA template is bound to SPA beads via a biotin–streptavidin linkage. The primer DNA is a 16-mer oligo(DT), which is annealed to a poly(A) template. The primer template is bound to a streptavidin-coated SPA bead. [3H]TTP is incorporated into the primer by reverse transcription. In brief, [3H]Thymidine Triphosphate (TTP), at a final concentration of 0.5 μCi/sample, was diluted in RT assay buffer [49.5 mM Tris–Cl (pH 8.0), 80 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, 2.5 mM EGTA, 0.05% Nonidet P-40] and added to annealed DNA–RNA bound to SPA beads. The compound being tested was added to the reaction mixture at 0.001–100 μM concentrations. Addition of 10 μl of HIV rRT and incubation at 37°C for 1 h resulted in extension of the primer by incorporation of [3H]TTP. The reaction was stopped by adding 0.2 ml of 120 mM EDTA. The samples were counted in an open window by using a Beckman LS 7600 instrument (Beckman Instruments), and IC_{50 RT} was calculated by comparing the measurements to untreated samples.

**p24 assays for anti-HIV activity**

Normal human peripheral blood mononuclear cells (PBMCs) from HIV-negative donors were cultured for 72 h in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, 3% interleukin-2 (IL-2), 2 mM l-glutamine, 25 mM HEPES, 2 g/l NaHCO₃, 50 μg/ml gentamicin and 4 μg/ml phytohaemagglutinin (PHA) before the exposure to HIV-1 at a multiplicity of infection (MOI) of 0.1 during a 1 h adsorption period. Subsequently, cells were cultured in 96-well plates (100 μl/well, triplicate wells) as described above, addition of 10 μl of HIV rRT and incubation at 37°C for 1 h resulted in the p24 antigen release. The anti-HIV activity was calculated by comparing the p24 values from the test substance-treated infected cells with p24 values from untreated infected cells (i.e. virus controls).

In parallel, the effects of various treatments on cell viability were also examined, as previously described (Zarling et al., 1990; Erice et al., 1993). In brief, non-infected PBMCs were treated with compound for 7 days under identical experimental conditions. A microculture tetrazolium assay (MTA), using 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide, was performed to quantitate cellular proliferation.

**Sperm function assays**

**Sperm motility using computer-assisted sperm analysis**

The effect of PHI-236 on human sperm motility was tested using semen and swim up fractions. Donor semen specimens were obtained after informed consent and in compliance with the guidelines approved by the Parker Hughes Cancer Center’s Institutional Review Board. For computer-assisted sperm analysis (CASA), highly motile fraction of sperm was prepared from normospermic semen (n = 8) by discontinuous (45–90%) gradient centrifugation followed by a ‘swim up’ of the washed and pelleted fraction in Biggers–Whitten–Whittingham medium (BW; Irvine Scientific, Santa Ana, CA, USA) containing 3% bovine serum albumin (BSA) (D’Cruz et al., 1998, 1999, 2000a). The supernatant containing highly motile fraction of sperm was recovered in BW medium with 0.3% BSA and resuspended in the same medium. Sperm aliquots (>10 × 10⁶/ml) were exposed to serial two-fold dilutions of PHI-236 (312.5–1000 μM) in BW medium. The stock solutions of PHI-236 were prepared in dimethyl sulphoxide (DMSO) and diluted in medium to obtain the desired concentrations. After 3 h incubation with PHI-236 at 37°C, the motilities of sperm were compared with those of vehicle-treated (0.5% DMSO) control suspensions of motile sperm.
Sperm kinetic parameters

Sperm motion parameters were evaluated using a Hamilton Thorne Integrated Visual Optical System (IVOS), version 10.9i instrument (Hamilton Thorne Research, Danvers, MA, USA), as described previously (D’Cruz et al., 1998, 1999, 2000a). The parameters that were determined included numbers of progressively motile (MOT) sperm, curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), beat-cross frequency (BCF), the amplitude of lateral head displacement (ALH), and the derivatives, straightness (STR = VSL/VAP × 100) and linearity (LIN = VSL/VCL × 100). Data from each individual cell track were recorded and analysed. At least 200 motile sperm were analysed for each aliquot sampled.

Sperm motility using bovine cervical mucus penetration assay

The bovine cervical mucus penetration test was performed using the Penetrak kit (Biochem Immunosystems, Allentown, PA, USA) essentially, according to the manufacturer’s instructions. Briefly, cut end of two capillary tubes filled with estrus cervical mucus were placed vertically in a vial containing 0.2 ml aliquots of liquefied normospermic donor semen (n = 4) treated with and without 250, 500 and 1000 μM of PHI-236 and incubated at room temperature for 2 h. The capillary tubes were then placed on a calibrated microscope slide, and the distance travelled by the vanguard sperm was recorded to the nearest millimetre.

Human-peripheral blood leukocyte-SCID mouse model

The in vivo microbiode efficacy of PHI-236 was evaluated in the human-peripheral blood leukocyte-SCID (Hu-PBL-SCID) mouse model for human AIDS (Di Fabio et al., 2001). CB.17 SCID mice (6–8 weeks of age) were obtained from Taconic Laboratories (Germantown, NY, USA) and maintained in our BL-3 containment facility under specific pathogen-free conditions. Mice were housed in microisolator cages (Allentown Caging Equipment, Allentown, NJ, USA, or Lab Products, Maywood, NY, USA) containing autoclaved food, water and bedding. Trimethoprim–sulfamethoxazole (Bactrim) was added to the drinking water once a week. Microbicide efficacy studies in SCID mice were approved by the Parker Hughes Institute Animal Care and Use Committee and all animal care procedures conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington DC, 1996).

Hu-PBL-SCID mice were generated by reconstituting CB.17 SCID mice with an i.p. inoculum of 5 × 10^7 Hu-PBLs obtained from an HIV-negative blood donor (Uckun et al., 2002b). Mice were treated s.c. with 2.5 mg progesterone (Depo-Provera; Upjohn, Kalamazoo, MI, USA) on the same day to synchronize the estrus cycle and to facilitate viral transmission by the vaginal route. Ten days before HIV infection, PBLs from a different HIV-negative blood donor were stimulated with 2 μg/ml PHA and after 12 h were infected (0.1 MOI) with the monotypic HIV-1 strain BaL. Seven days after reconstitution, progesterin-treated Hu-PBL-SCID mice were anaesthetized with isoflurane and challenged with an intravaginal inoculum of 10^6 HIV-infected PBLs resuspended in tissue culture medium and mixed with human semen and pretreated for 1 h with vehicle or 1 and 2 μM PHI-236. The total volume of the inoculum was 100 μl of which 20 μl was semen. The inoculum was introduced into the vaginal vault in 25 μl aliquots and held in place for 5 min. After vaginal inoculation with HIV-infected PBLs, mice were monitored for 2 weeks for overall health and survival and then selectively killed to determine the plasma viral RNA load by the Nuclisens™ HIV-1 QT assay (Organon Teknika, Boxtel, The Netherlands), as described previously (Uckun et al., 2002b).

Statistical analysis

Results are presented as the mean or mean ± SD values from independent measurements. Non-linear regression analysis was used to find IC_{50}, EC_{50} and CC_{50} values from concentration-effect curves using the GraphPad PRISM version 4.0a software (San Diego, CA, USA). Correlations between two variables were examined using Pearson’s correlation coefficient and linear regression. Paired t-tests were performed to test for differences between mean IC_{50} values for PHI-236 and ZDV for the primary clinical isolates. The statistical significance of the treated group mean with that of control group with respect to sperm function parameters was analysed by a one-way analysis of variance, followed by Dunnett’s multiple comparison test. Fisher’s exact probability test was used to compare the number of mice infected between test and control groups. P values below 0.05 were deemed significant.

Results

Identification of PHI-236 as a tight-binding inhibitor of HIV-RT

Molecular modelling studies and computational approach allowed the identification of several ligand derivatization sites for the generation of more potent PETT-derived NNRTIs. Docking studies with the PETT derivative, trovirdine revealed multiple sites, which could be used for the incorporation of larger functional groups. The docked trovirdine molecule showed abundant sterically allowed usable space surrounding the pyridyl ring, the ethyl linker, and near the 5'-bromo position (Figure 1). Based on the hypothesis that addition of strategically designed functional groups should yield more potent anti-HIV agents, a series of substituted phenyl thiourea compounds where synthesized in which one of the nitrogen atoms of the thiourea was attached to a phenyl moiety through an ethyl bridge, and the other nitrogen atom was attached to a 5'-bromo-substituted pyridyl ring.

The energetically favoured docked position of the thioureas in the NNI-binding pocket was determined, and a Ludi score was assigned, and an estimation of the inhibition constant (K_i value) was determined (Bohm, 1994; Mao et al., 1999a,b). The accuracy of the predictions of the modelling studies was evaluated in anti-HIV assays. Fourteen rationally designed thioureas were synthesized and assayed for their RT inhibitory activity in cell-free assays using purified recombinant HIV-1 RT [reported as IC_{50(RT)}] as well as for their ability to inhibit HIV-1 replication in normal human PBMCs infected with the HIV-1 strain HTLV[1] [reported as IC_{50(p24)}]. Based on modelling studies, the trend of calculated Ludi K_i values for compounds 1–14 (0.07 to >100 μM) were predictive of the general trend of their measured IC_{50} values for rRT (0.1 to >100 μM) and p24 (<0.001 to >100 μM) inhibition (Table I). The trend of the calculated Ludi K_i values based on the modelling studies predicted the trend of the experimentally determined IC_{50} values with surprising accuracy. Compounds that better fit the composite-binding pocket when compared with trovirdine had lower calculated Ludi K_i values and rRT IC_{50} as well as p24 IC_{50} values (Table I).

Among the 14 phenyl ring-substituted pyridyl thiourea compounds, functionalization of the phenyl ring with 2.5-dimethoxy, 2-fluoro, 2-fluoro and 2-chloro was associated with enhanced anti-HIV activity [IC_{50(RT)} = 0.1–0.7 μM; IC_{50(p24)} = <0.001 μM; Table I]. In addition, substitutions with 3-methoxy, 4-methyl and 4-chloro functional groups also resulted in potent inhibitors of HIV-1 [IC_{50(RT)} = 0.1–2.5 μM; IC_{50(p24)} = 0.001–0.007 μM]. By comparison, the 4-hydroxyl- and 2-nitro-substituted phenyl thioureas were inactive with IC_{50(RT)} values of >80 μM and IC_{50(p24)} values of >100 μM, respectively. Thus, the substitution of the phenyl ring with various functional groups had a major impact on the anti-HIV activity of pyridyl thioureas. The lead compound, PHI-236, abrogated HIV replication at nanomolar concentrations [IC_{50(p24)} = <0.001 μM] without evidence of cytotoxicity and with an unprecedented selectivity index of >100 000. These results confirmed the hypothesis that the binding interactions predicted based on our modelling studies largely account for the superior anti-HIV activity of our lead compound, PHI-236.

Because lower Ludi K_i values of thiourea NNRTIs were invariably associated with improved anti-HIV activity, a possible correlation was sought between the calculated Ludi K_i values and the experimental IC_{50} values obtained with enzyme-based and cell-based anti-HIV assays. Indeed, a comparison of 14 pyridyl thiourea NNRTIs revealed that the observed rRT IC_{50} values significantly correlated with Ludi K_i values (r^2 = 0.942; P = <0.0001; Figure 2A). Similarly, the p24 antigen values correlated significantly with Ludi K_i values (r^2 = 0.981; P = <0.0001; Figure 2B).
Table 1. Calculated Ludi $K_i$ values and experimental human immunodeficiency virus-1 (HIV-1) recombinant reverse transcriptase (rRT) and p24 antigen inhibitory values for 14 substituted phenyl thiourea compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>$K_i$ (μM)*</th>
<th>$IC_{50RT}$ (μM)†</th>
<th>$IC_{50p24}$ (μM)‡</th>
<th>SI§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trovirdine</td>
<td>NA</td>
<td>0.7</td>
<td>0.8</td>
<td>0.007</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>1</td>
<td>4-OH</td>
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<td>88</td>
<td>&gt;100</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>2</td>
<td>2-NO$_2$</td>
<td>&gt;84</td>
<td>&gt;100</td>
<td>&gt;100</td>
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</tr>
<tr>
<td>3</td>
<td>2-F</td>
<td>0.8</td>
<td>0.6</td>
<td>&lt;0.001</td>
<td>&gt;100 000</td>
</tr>
<tr>
<td>4</td>
<td>2-Cl</td>
<td>0.5</td>
<td>0.7</td>
<td>&lt;0.001</td>
<td>&gt;100 000</td>
</tr>
<tr>
<td>5</td>
<td>4-F</td>
<td>7.8</td>
<td>6.4</td>
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</tr>
<tr>
<td>6</td>
<td>4-Br</td>
<td>6.3</td>
<td>0.9</td>
<td>0.07</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>7</td>
<td>4-Cl</td>
<td>4.7</td>
<td>2.5</td>
<td>0.001</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>2-OMe</td>
<td>1.2</td>
<td>1.0</td>
<td>0.01</td>
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</tr>
<tr>
<td>9</td>
<td>3-Cl</td>
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<td>3.1</td>
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</tr>
<tr>
<td>10</td>
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<tr>
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<tr>
<td>12</td>
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<td>0.4</td>
<td>0.003</td>
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</tr>
<tr>
<td>13</td>
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<td>0.1</td>
<td>0.007</td>
<td>ND</td>
</tr>
<tr>
<td>PHI-236</td>
<td>2,5-(OMe)$_2$</td>
<td>0.07</td>
<td>0.1</td>
<td>&lt;0.001</td>
<td>&gt;100 000</td>
</tr>
</tbody>
</table>

NA, not applicable; ND, not determined; PHI-236, N-[2-(2,5-dimethoxyphenylethyl)]-N’-[2-(5-bromopyridyl)]-thiourea.

* Ludi $K_i$ values were calculated based on the empirical score function in the Ludi program (Bohm, 1994).
† $IC_{50RT}$ values are drug concentration inhibiting recombinant HIV-1 RT activity by 50%.
‡ $IC_{50p24}$ values are drug concentration inhibiting HIV-p24 antigen production by 50%.
§ The selectivity index (SI) is equal to the ratio of $CC_{50MTA}$ to $IC_{50p24}$. Because the $CC_{50}$ values for the inhibitors tested were >100 μM, only the minimum SI estimates could be determined for these non-cytotoxic agents.

Figure 2. Correlation between lower Ludi $K_i$ values and human immunodeficiency virus-1 (HIV-1) reverse transcriptase (RT) $IC_{50}$ or p24 $IC_{50}$ values. The data point represent the calculated Ludi $K_i$ values and experimental recombinant RT (rRT) (A) and p24 antigen (B) inhibitory values for 14 rationally designed phenyl ring-substituted pyridyl thiourea compounds. Ludi $K_i$ values were calculated based on the empirical score function in the Ludi program. Ideal H-bond distances and angles between compounds and proteins are assumed in all cases for Ludi $K_i$ calculations.
**Activity of PHI-236 against drug-resistant HIV-1 mutants**

Table II summarizes the anti-HIV activity profile of PHI-236 against laboratory and primary clinical isolates with variable resistance to thymidine analogues (ZDV/STV) currently used for the treatment of HIV-1-infected patients. The activity of PHI-236 against genotypic NNRTI-resistant HIV-1 strains (A17, A17 variant) carrying clinically relevant mutations (Y181C and K103N + Y181C, respectively) was superior when compared with the activity of the three currently marketed NNRTIs (nevirapine, delavirdine or efavirenz) (Table II). p24 antigen assays revealed that PHI-236 was not only more potent than the above NNRTIs (nevirapine, delavirdine or efavirenz) (Table II). p24 superior when compared with the activity of the three currently marketed NNRTIs (nevirapine, delavirdine or efavirenz) (Table II).

Table II

<table>
<thead>
<tr>
<th>HIV-1 strain or isolate</th>
<th>Anti-HIV activity IC₅₀ (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHI-236</td>
</tr>
<tr>
<td></td>
<td>Zidovudine</td>
</tr>
<tr>
<td></td>
<td>Trovirdine</td>
</tr>
<tr>
<td></td>
<td>Nevirapine</td>
</tr>
<tr>
<td></td>
<td>Delavirdine</td>
</tr>
<tr>
<td>HTLV V₅µ</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NNRTI-resistant isolates</td>
<td></td>
</tr>
<tr>
<td>A17 (Y181C)</td>
<td>0.1</td>
</tr>
<tr>
<td>A17 variant (K103N, Y181C)</td>
<td>8</td>
</tr>
<tr>
<td>RT–MDR (L74V, M41L, V106A, T215Y)</td>
<td>0.005</td>
</tr>
<tr>
<td>NNRTI/NNRTI-resistant clinical isolates</td>
<td></td>
</tr>
<tr>
<td>Primary isolates (n = 17)†</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>NRTI-resistant clinical isolates (n = 16)‡</td>
<td>0.009 ± 0.001</td>
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</table>

**PHI-236 is neither cytotoxic nor cytostatic**

In the mitochondrial-based MTT conversion assays, PHI-236 displayed high-selective index (SI) against normal human vaginal, ectocervical and endocervical epithelial cells (SI = >100 000; Figure 3A). In the BrDU cell proliferation assay, PHI-236-treated human monocytes yielded dose-response curves with IC₅₀ of >100 µM for 24, 48 and 72 h exposure points (SI = 10 000; Figure 3B).

**PHI-236 does not affect sperm motility and kinematics**

Exposure of a highly motile fraction of sperm to PHI-236, which inhibited HIV-1 replication in human PBMCs with an IC₅₀ value of <0.001 µM for p24 viral antigen production and 0.1 µM for RT activity, did not affect sperm motility even at concentrations as high as 1000 µM. Further, sperm motion kinematics using CASA confirmed that PHI-236 treatment (3 h) did not significantly alter the sperm motion parameters, such as the mean MOT, VCL, VAP, VSL, STR, LIN, BCF and ALH (Figure 4).

**PHI-236 does not impede functional motility in cervical mucus**

The bovine cervical mucus penetration test has been used to evaluate the in vitro fertilizing capacity of human sperm (De Geyter et al.,...
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Exposure of human semen to increasing concentrations of PHI-236 did not adversely affect the vanguard sperm penetration distance (Figure 5). The mean penetration distance of the vanguard sperm from fresh vehicle-treated specimens in estrus bovine cervical mucus was $34.2 \pm 5.9$ mm after $2$ h of migration, whereas the corresponding distances for sperm treated with $250, 500$ and $1000 \mu M$ PHI-236 were $35.4 \pm 7.8, 31.2 \pm 6.2$ and $32.2 \pm 7.2$ mm, respectively.

**PHI-236 prevents vaginal transmission of HIV-1**

Owing to its low $L_{D_{50}}$ value, PHI-236 is a tight-binding inhibitor of HIV-1 RT. Tight-binding NNRTIs have the unique property as molecular virucides owing to their ability to inactivate cell-free as well as cell-associated HIV-1 without metabolic activation (D’Cruz and Uckun, 1999; Pani et al., 2001; Zussman et al., 2003; Di Fabio et al., 2003; Van Herrewege et al., 2004; Njai et al., 2005). PHI-236 is lipophilic and, therefore, can readily enter membrane milieu such as the plasma membrane or the membrane envelope surrounding the HIV core. These attributes of PHI-236 are particularly attractive as an anti-HIV microbicide. Therefore, the virucidal efficacy of formulated PHI-236 was evaluated in the Hu-PBL-SCID mouse model of vaginally transmitted HIV-1. PHI-236 exhibited remarkable microbicidal activity against the HIV-1 monotropic R5 strain BaL. Pretreatment with vehicle-treated HIV-infected PBLs suspended in $20\%$ human semen led to systemic infection via the vaginal route. Plasma from $9$ of $10$ ($90\%$) vehicle-only treated SCID mice became HIV–PCR positive $2$ weeks following intravaginal inoculation with HIV-1-infected PBLs resulting in a mean viral load of $14\,755$ HIV-1 RNA copies/ml (range $3300–66\,000$ RNA copies/ml) (Table III). Pretreatment ($1\ h$) of HIV-1-infected PBLs and semen with PHI-236

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**Figure 3.** 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and 5-bromide-2′-deoxyuridine (BrdU) uptake dose-response curves for N-[2-(2,5-dimethoxyphenylethyl)]-N′-[2-(5-bromopyridyl)]-thiourea (PHI-236) against human female genital epithelial cells and monocytes. The viability of normal human vaginal, ectocervical and endocervical epithelial cells exposed to increasing concentrations (7.8–$1000 \mu M$) of PHI-236 for $24$ h was quantitated by the mitochondrial-based MTT assay (A). BrdU cell proliferation was assayed using cultured monocytes exposed to increasing concentrations (7.8–$250 \mu M$) of PHI-236 for $24, 48$ and $72$ h (B). Inhibition of cell growth is expressed relative to vehicle dimethyl sulphoxide (DMSO) controls. Each data point represent the mean of three separate experiments.

**Figure 4.** Sperm motion parameters are unaffected when human sperm are treated with N-[2-(2,5-dimethoxyphenylethyl)]-N′-[2-(5-bromopyridyl)]-thiourea (PHI-236). Motile fraction of sperm prepared from pooled semen specimens ($n = 8$) was incubated in Biggers–Whitten–Whittingham medium (BWW) medium with $0.3\%$ bovine serum albumin (BSA) in the presence of increasing concentrations of PHI-236 ($250–1000 \mu M$) or vehicle ($0.5\%$ dimethyl sulphoxide (DMSO)) for $3$ h at $37^\circ C$, and the motility characteristics were determined using the Hamilton Thorne Integrated Visual Optical System (IVOS) version 10.9i computer-assisted sperm analysis (CASA). Values are the mean ± SD from three to five independent experiments. ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; LIN, linearity; PRG, progressive motility; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.
Microbicide efficacy of N-[2-(2,5-dimethoxyphenylethyl)]-N'[2-(5-bromopyridyl)]-thiourea (PHI-236) with HIV-1-infected PBLs survived the 2-week post-infection period. Low viral load (3100 RNA copies/ml). All mice vaginally inoculated from only 1 of 20 (5%) PHI-236-treated mice inoculated with BaL-strain from systemic infection via the vaginal route (Table III). Plasma multiplicity of infection (MOI) with the HIV-1 monotropic R5 strain BaL. Seven days after reconstitution, progestin-treated Hu-PBL-SCID mice were vaginally challenged with 10^6 HIV-infected PBLs in medium mixed with human semen (4:1) and pretreated for 1 h with vehicle or PHI-236. Two weeks after vaginal inoculation, plasma viral RNA load was determined by the Nuclisens™ HIV-1 QT assay.

Discussion
The rationally designed tight-binding thiourea NNRTI, PHI-236, was found to be more potent against HIV-1 than the three classes of NNRTIs currently in clinical use to treat HIV-1 infections. PHI-236 displayed unprecedented potency against NNRTI-resistant HIV-1 strains harbouring K103N, V106A or Y181C mutations. Furthermore, exposure of human sperm to PHI-236 at doses 10^6 times higher than those that yield effective concentrations against the AIDS virus had no adverse effect on sperm motility, kinematics, cervical mucus penetrability or the viability of genital epithelial cells. In preliminary studies, in the presence of genital fluids, PHI-236 prevented the vaginal transmission of a CCR5-dependent HIV-1 strain in the humanized SCID mouse model of vaginal transmission of HIV/AIDS. These findings warrant further development of PHI-236 as a potential non-spermicidal intravaginal microbicide as well as a prophylactic antiviral agent to inactivate cell-free and cell-associated viruses in semen before assisted reproductive technology procedures.

X-Ray crystal structures of HIV-1 RT/NNI complexes have shown a common configuration resembling a butterfly-like shape where the wings are usually occupied by π-electron systems that can interact with aromatic amino acid residues within the NNI-binding site. The Wing 2 region of the NNRTI-binding pocket contains multiple aromatic residues (Das et al., 1996; Ren et al., 2001). Y181C, Y188C and Y188H mutations in drug-resistant HIV-1 strains result in larger unoccupied volume in the binding pocket and a different interaction environment (Debyser et al., 1993; Deval et al., 2004). Preferred NNRTIs should maximize the occupancy in the Wing 2 region of the binding site of RT. Most mutations conferring resistance to NNRTIs are associated with changes in the binding of NNRTIs to HIV-1 RT (Tantillo et al., 1994; Larder et al., 1999). Dozens of mutant strains have been characterized as resistant to NNRTIs, including L100I, K103N, V106A, E138K, Y188C and Y188H (Larder 1999; Larder et al., 1999; Joly et al., 2004). In particular, the K103N and Y181C mutants are the most difficult to treat, because they are resistant to most of the NNRTIs that have been examined (Marcelin et al., 2004). For example, primary mutations associated with resistance to nevirapine involve residues K103N, V106A, V108I, Y181C, Y188C, G190A and K238S, which have van der Waals contact with the NNRTI (Abrahao-Junior et al., 2001; Hachiya et al., 2004). The mutations of these residues lead to the weakening of the NNRTI binding to RT. Delavirdine and nevirapine are less active against RT with primary mutations K103N or Y181C (Hsiou et al., 1998; Albrecht et al., 2001; Parietti et al., 2004). Efavirenz is less active against the primary mutation K103N (Ding et al., 1995).

In the NNRTI-resistant A17 strain (Y181C mutation), the Wing 2 region of the mutant becomes larger when Y181 is mutated to a cysteine residue, and the impact of NNRTI inhibiting the RT region of the mutant becomes larger when Y181 is mutated to a cysteine residue.

Table III. Microbicide efficacy of N-[2-(2,5-dimethoxyphenylethyl)]-N'[2-(5-bromopyridyl)]-thiourea (PHI-236) in the human-peripheral blood leukocyte-severe combined immunodeficient (Hu-PBL-SCID) mouse model of vaginal human immunodeficiency virus-1 (HIV-1) infection

<table>
<thead>
<tr>
<th>Pretreatment*</th>
<th>Number of infected/total challenged (%)</th>
<th>Survival on day 14 (%)</th>
<th>Plasma HIV-1 RNA (copies/ml)</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (+ semen)</td>
<td>9/10 (90)</td>
<td>100</td>
<td>14 755 ± 1 387‡</td>
<td>0.0011</td>
</tr>
<tr>
<td>PHI-236 (+ semen)</td>
<td>1/10 (10)</td>
<td>100</td>
<td>3100</td>
<td>Not applicable</td>
</tr>
<tr>
<td>1000 nM</td>
<td>0/10 (0)</td>
<td>100</td>
<td>Not applicable</td>
<td></td>
</tr>
</tbody>
</table>

*Hu-PBL-SCID mice were generated by reconstituting CB.17 SCID mice with an i.p. inoculum of 5 × 10^7 human PBLs and treated s.c. with 2.5 mg Depo-Provera. Ten days before the HIV infection, PBLs from a different HIV-negative donor were stimulated with 2 μg/ml phytohaemagglutinin (PHA) and infected [0.1 multiplicity of infection (MOI)] with the HIV-1 monocistronic R5 strain BaL. Seven days after reconstitution, progestin-treated Hu-PBL-SCID mice were vaginally challenged with 10^6 HIV-infected PBLs in medium mixed with human semen (4:1) and pretreated for 1 h with vehicle or PHI-236. Two weeks after vaginal inoculation, plasma viral RNA load was determined by the Nuclisens™ HIV-1 QT assay.
†Fisher’s exact probability test.
‡Mean ± SD (range = 3300–66 000 RNA copies/ml)
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by directly inactivating HIV-1 RT thereby preventing HIV-1 from replicating either in semen or in the infected host cells that line the vaginal wall. Using a monocyte/macrophage tropic laboratory strain HIV-1 BaL and Hu-PBL-SCID mouse model for AIDS, we generated preliminary evidence that PHI-236 can significantly prevent vaginal transmission of R5 strain in the presence of human semen.

Intrauterine insemination procedures performed among HIV-1 serodiscordant couples in Europe have clearly shown that HIV-1-seropositive men can have their own children without infecting their female partners or fetus (Semprini et al., 1992; Marina et al., 1998). Effective antiretroviral therapy before assisted reproductive technology procedures can reduce the viral burden in both blood and genital secretions thereby reducing the likelihood of sexual transmission of HIV-1 (Quinn et al., 2000). Inasmuch as HIV-1 can still be cultured from the genital secretions of some patients who are receiving antiretroviral therapy and who have undetectable levels of HIV-1 RNA in blood, pretreatment of semen with antiviral agents such as PHI-236 before assisted reproductive technology procedures is likely to further reduce the risk of sexual transmission of HIV-1 without impairing fertility.

In conclusion, therationally designed non-steroidal phenylthiourea NNRTI PHI-236, is a potent inhibitor of NRTI-resistant, NNRTI-resistant and multidrug-resistant HIV-1. PHI-236 will be a tremendous advantage for the development of a non-contraceptive broad-spectrum anti-HIV microbicide because drug-resistant HIV is common in newly infected patients. PHI-236 because of its broad-spectrum anti-HIV activity, non-interference with sperm function, favourable pharmacokinetics and lack of toxicity may be useful as a non-contraceptive microbicide for (i) sexually active women to allow pregnancy while protecting both mother and her fetus or infant from HIV-1 and (ii) as a prophylactic antiviral agent, especially for HIV-1 serodiscordant couples, to curb the transmission of HIV via semen.

Acknowledgements
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References

mutants would therefore be attenuated. Molecular modelling studies revealed that the addition of 2,5-dimethoxy groups in PHI-236 increases the molecular volume in the Wing 2 region of the binding site by 18 Å (Mao et al., 1999a,b). PHI-236, which has a maximum occupancy at the Wing 2 region and more closely in contact with residues L100 and L234, was predicted to have an advantage against Wing 2 mutants, such as the Y181C and Y188C mutants. An energy-minimized model of PHI-236 in the RT-binding site revealed the largest molecular surface area in contact with the protein and thus achieved the highest lipophilicity score (Sudbeck et al., 1999). Consequently, PHI-236 was substantially more potent than the standard NNRTI drugs against the NNRTI-resistant HIV-1 strains. PHI-236 was 270-fold more potent than ZDV against genotypically and phenotypically NNRTI-resistant non-subtype B HIV-1 isolates originating from South America, Asia and sub-Saharan Africa carrying two to seven TAMs associated with NRTI resistance. PHI-236 was 80 times more potent than delavirdine, and 1000 times more potent than nevirapine against the multidrug-resistant HIV-1 strain RT–MDR with mutations involving RT residues V106A, L74V, M41L and T215Y. PHI-236 was 500–1000 times more effective than delavirdine and nevirapine against the problematic NNRTI-resistant HIV-1 strain A17-mutant with an Y181C mutation. The anti-HIV activity of PHI-236 against A17 variant with K103N and Y181C mutations was also superior.

The higher potency of PHI-236 relative to standard NNRTIs is consistent with our previously reported hypothesis that NNRTIs containing larger and compatible functional groups at the Wing 2 region of the binding site can provide better inhibitor activity against these HIV-1 RT mutants (Vig et al., 1998; Mao et al., 1999a,b). This is particularly relevant because a high percentage of newly infected individuals harbour NRTI/NNRTI-resistant mutants with increased incidence of HIV subtypes (Huang et al., 1998; Kantor and Katzenstein, 2004). However, HIV-1 subtype B currently accounts for only 12% of the estimated 42 million HIV-infected individuals worldwide, and the vast majority of new infections are caused by non-subtype B HIV-1 strains (Gao et al., 1998; Kantor and Katzenstein, 2004).

The rationale for NNRTIs as potential anti-HIV microbicides include (i) ability to rapidly cross membrane barriers, (ii) prolonged or irreversible inhibition of wild-type and mutant HIV-1 RT activity without metabolic activation, (iii) retain antiviral potency in the presence of genital fluids, (iv) retain virucidal activity following drug removal, (v) lack systemic absorption to prevent drug resistance and (vi) does not perturb the vaginal mucosa and normal vaginal flora following repeated use. PHI-236 because of its high affinity for HIV-1 RT, broad-spectrum anti-HIV activity, favourable pharmacokinetics, and lack of toxicity as well as lack of systemic absorption, has clinical utility for the prevention and treatment of HIV-1 infection (Chen et al., 2001).

Semen is an important vehicle for sexual transmission of HIV-1 (Kalichman et al., 2001). The infectiousness of semen varies as the infection progresses. Symptomatic stage individuals are approximately 12 times more infectious than primary stage men and approximately 150 times more infectious than asymptomatic stage men (Rapatski et al., 2005). Although antiretroviral drugs can greatly reduce the HIV viral load in semen, they are not foolproof in preventing HIV infection via sexual contact (Quinn et al., 2000; Barroso et al., 2003). A substantial percentage of HIV-positive men have active, potentially infectious viruses in their semen, even after 6 months of therapy. Hence, significant proportions of men who undergo therapy and subsequently feel well remain potentially infectious and therefore continue to pose a public health risk. PHI-236 because of its lipophilic as well as tight-binding attributes could provide protection...


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