Development of Dual-Acting Pyrimidinediones as Novel and Highly Potent Topical Anti-HIV Microbicides

RUNNING TITLE: Development of Pyrimidinedione Topical Microbicides


ImQuest BioSciences Inc., 7340 Executive Way, Suite R, Frederick, Maryland 21704

*Corresponding author. Mailing Address: ImQuest BioSciences, Inc., 7340 Executive Way, Suite R, Frederick, MD 21704. Phone: (301)-696-0274. Fax: (301)-696-0381. E-mail: rbuckheit@imquestbio.com.
ABSTRACT

In the absence of an effective vaccine against the human immunodeficiency virus, topical microbicides to prevent the sexual transmission of HIV represent an important strategy to prevent the continued spread of infection. The recent trend in the development of new microbicide candidates includes the utilization of FDA approved therapeutic drugs that target the early stages of the HIV life cycle including entry inhibitors and reverse transcriptase inhibitors.

We have investigated twelve pyrimidinedione compounds with potent HIV activity and the ability to inhibit both virus entry and reverse transcription in an effort to determine a lead microbicide for product development. The candidate compounds were evaluated for efficacy against subtype B, C and E clinical virus strains in fresh human peripheral blood mononuclear cells and against CCR5-tropic virus strains in both monocytes-macrophages and dendritic cells.

Microbicide-specific biological assays and toxicity evaluations were also performed in a variety of established and fresh human cells as well as against Lactobacilli strains common to the vaginal environment. These evaluations resulted in the identification of congeners with cyclopropyl and cyclobutyl substituents at the N-1 of the pyrimidinedione as the most active molecules in the structure-activity relationship series. The pyrimidinediones represent excellent microbicide candidates in light of their significantly high efficacy against HIV-1 (sub-nanomolar concentration range), potency (TI > 1 million), solubility profiles, and dual mechanism of antiviral action which includes two early steps of virus replication prior to the integration of the virus considered most important for microbicidal activity.
INTRODUCTION

A recent report issued by UNAIDS on the global AIDS epidemic sighted significant gains in the prevention of new HIV infections in a number of countries that are most affected by the AIDS epidemic[34]. Many of these countries have seen changes in sexual behavior followed by a decline in the number of new HIV infections. These behavioral changes include increasing condom use among young people with multiple partners and encouraging signs that young people are waiting longer to have sexual intercourse in some of the most heavily affected countries[34]. However, the report also states that despite these declines in new HIV infections, the AIDS epidemic is far from over and that the rates of new HIV infections continue to rise in many other countries. HIV/AIDS continues to be the leading cause of death in Africa [34].

Despite intensive research, a vaccine to prevent HIV infection has not yet been successfully developed. In the absence of a prophylactic vaccine, antiretroviral therapy (ART) has been the primary mechanism utilized to prevent disease progression and prolong the survival of infected individuals. A wide variety of antiretroviral (ARV) agents are currently approved for use, targeting numerous steps in HIV replication such as attachment, fusion, reverse transcription, integration, and proteolytic processing. With the use of combination therapy strategies, resulting in the evolution of highly active antiretroviral therapies, or HAART, profound suppression of plasma viral load for prolonged periods of time have resulted in significant declines in AIDS-related morbidity and mortality in developed countries where the therapies can be afforded [23, 29]. Although HAART regimens have improved the prognosis for HIV-infected individuals, challenges to effective use of these therapeutic strategies still remain, including issues of adherence, side effects and toxicities, drug resistance and persistent viral replication in latent reservoirs. These challenges necessitate the development of alternative strategies to combat the
spread of HIV including prevention strategies in the form of vaginal and rectal topical microbicides.

With the incidence of HIV infections in women on the rise, especially in underdeveloped countries [34], the development and clinical evaluation of topical microbicides has achieved world-wide focus. It has been suggested that a microbicide with 60% efficacy introduced into 73 low-income countries, could prevent 2.5 million HIV infections over 3 years [36]. For these reasons, topical microbicides are an ideal HIV prevention strategy allowing a woman to take responsibility for her own protection from sexually transmitted disease [3, 33] especially in societies where women are unempowered. Minimally, a microbicide product should be safe, acceptable by the user, affordable, stable and effective without compromising existing physical barriers to STI transmission [33]. Microbicides are currently being developed in gel, cream, film, suppository, sponge and intravaginal ring formats, providing both a physical barrier which the virus needs to penetrate as well as specifically targeting critical steps of the virus replication cycle such as virus entry and reverse transcription [32]. Six microbicides have progressed to late stage clinical development, however, these products, including Nonoxynol-9, C31G (Savvy), Cellulose Sulphate (Ushercell), BufferGel, PRO2000, and Carageenan (CarraGuard), failed to demonstrate efficacy in clinical trials. However, most recently, a successful Phase 3 clinical trial of the anti-retroviral agent tenofovir formulated as a gel product resulted in protection of women and energized the microbicide field, suggesting that highly potent ARVs might result in highly successful microbicide products, especially when combined into co-formulated microbicide products[1]. Additionally, it has recently been reported that systemic prophylactic administration of the highly potent ARV Truvada (a combination product consisting of
tenofovir and emtricitabine) to men who have sex with men (MSM) provided protection from HIV transmission[19].

The recent failures of the initial microbicides focused critical attention on continued microbicide development and the types of products that must be developed. The recent trend in the development of new microbicide candidates includes the utilization of FDA approved drugs for HIV therapy that target the early stages of the HIV life cycle including entry inhibitors and reverse transcriptase inhibitors [15, 24]. As of October 2009 there were 38 ongoing and planned clinical trials for the development of microbicides and six (6) of these included ARV candidates [10]. ImQuest has licensed and has begun development of the pyrimidinedione (PYD) series of ARVs that have been shown to have potent activity against the HIV-1 reverse transcriptase and also possess a second mechanism of action targeting virus entry that extends their range of action to HIV-2 [5, 9]. The molecules have been evaluated in a wide variety of anti-HIV in vitro efficacy and toxicity assays and the results have defined twelve microbicide candidates [4, 5]. These 12 superior compounds have been further evaluated in more specific microbicide assays in an effort to rationally prioritize the various PYD molecules for advanced preclinical and clinical development. The data generated from these experiments and included in this manuscript indicate that the pyrimidinediones as a class have similar, if not superior, in vitro activity profiles to the ARV microbicide candidates currently being developed [17, 18, 26]. These results, combined with the dual mechanism of action which targets virus entry and reverse transcription, makes the PYD series of molecules highly attractive candidates as topical microbicides. Our results would suggest that PYD analogs with cyclopropyl and 3-cyclopenten-1-yl substitutions at the N1 of the pyrimidinediones represent highly attractive candidates for further development as topical anti-HIV microbicides.
MATERIALS AND METHODS

Cell Lines, Virus and Bacteria

The CEM-SS [28], HeLa-CD4-LTR-β-Galactosidase [21], GHOST X4/R5 [25] and H9 [31] cell lines, as well as the HIV-1mb (CXCR-4-tropic)[31] and CCR5-tropic clinical virus isolates (Clade B: US/92/727 and HIV-1Bal; Clade C: ZA/97/003, 93/MW/959, 93/IN/101; Clade E: — CMU06, 92/TH/020, 93/TH/07) were obtained from the NIAID AIDS Research and Reference Reagent Program (Rockville, MD) to include The ME180 [20, 30] cells and Lactobacillus strains (L. crispatus ATCC # 33820, L. jensenii ATCC #25258 and L. acidophilus ATCC #11975) were purchased from the American Type Culture Collection (Manassas, VA). The HIV-1SK-1[8, 13, 20, 30] used for developing chronically infected H9 cells (H9/SK-1) was gifted to ImQuest BioSciences from Duke University (Durham, NC). Human peripheral mononuclear blood cells (PBMCs), monocytes-macrophages and dendritic cells were derived from human blood which was purchased from Biological Specialty Corporation (Colmar, PA). The cell lines were propagated as recommended and stored in liquid nitrogen; titered stocks of HIV-1mb and clinical virus strains were stored at -80°C prior to being used in the antiviral assays.

Materials

Twelve of 68 active pyrimidinedione compounds [5] were used in these evaluations (Figure 1). These compounds were defined as the most promising potential microbicides based on *in vitro* evaluations. Each compound was obtained from Samjin Pharmaceutical Company, LTD as dry white powders. The compounds were solubilized at 1 or 40 mM in 100% DMSO and stored at 4°C. AZT [27], Dextran Sulfate (500,000 MW) [2] and Chicago Sky Blue [12] were used as experimental control compounds and were obtained from Sigma-Aldrich Corporation (St. Louis).
Cytopathic Effects (CPE) Inhibition Assay

The CPE assay was performed as previously described [6]. Briefly, serially diluted compound was added to a 96-well round bottom microtiter plate in triplicate. CEM-SS cells at a concentration of $2.5 \times 10^3$ cells per well and HIV-1\textsubscript{HIV} at the appropriate pre-determined titer were sequentially added to the microtiter plate. The cultures were incubated at 5% CO\textsubscript{2}/37°C for six days. Following the incubation, the microtiter plates were stained with XTT tetrazolium dye (Sigma, St. Louis, MO) to evaluate the efficacy and toxicity of the test compound(s). AZT was evaluated in parallel as a positive control for the assay.

Virus Entry Inhibition Assay

The assay was performed as previously described [35]. Twenty-four hours prior to compound and virus addition, HeLa-CD4-LTR-β-Galactosidase (MAGI) cells diluted in DMEM medium supplemented with 10% FBS and antibiotics were plated in 96-well flat bottomed microtiter plates at $1 \times 10^4$ cells per well in a volume of 100 μL and incubated overnight at 37°C/5% CO\textsubscript{2}. Following the overnight incubation, test compound was serially diluted and added to the cells in triplicate in a volume of 50 μL per well. HIV-1\textsubscript{HIV} was diluted to a pre-determined titer in assay medium and added to the microtiter plate. The cultures were incubated for 2 hours at 37°C/5% CO\textsubscript{2}. Following the incubation, the monolayers were washed three times with RPMI1640 (without additives) to remove residual extracellular compound and unbound virus, and incubated at 37°C/5% CO\textsubscript{2} for an additional 48 hours. Following the incubation, toxicity plates were evaluated by XTT staining, as previously described [6] and efficacy plates were evaluated by chemiluminescence detection using Gal-Screen (Applied Biosystems) according to the manufacturer’s instructions. Chicago Sky Blue was evaluated in parallel as a positive control for the assay.
RT Inhibition Assay

The assay was performed as previously described [35]. A 2X buffer solution was prepared that contained 2M Tris pH 8.0, 3M KCl, 1 M MgCl2, 2 M DTT, 25 U/mL rCdG, 1 mM dGTP and 800 Ci/mM alpha p32:dGTP. The compounds were diluted serially 10-fold in water and 30 µL was added in triplicate to a 96-well round bottom tissue culture plate. Fifty microliters (50 µL) of the 2X buffer was added to each sample including the positive and negative controls. Twenty microliters (20 µL) of HIV-1 wild type RT enzyme (CHIMERx) diluted in water containing BSA (1:100) and Triton-X (1:1000) was added to all wells except the negative control. The plate was incubated for 50 minutes at 37°C. Following the incubation 10 µL of 10 mg/mL DNA (MB-grade fish sperm) was added to each well, followed by the addition of 150 µL of cold 10% TCA. The plate was incubated at room temperature for approximately 15 minutes in order to allow for precipitation of the samples. Following the incubation the contents were transferred to filter plates containing DEAE filter paper and contents were filtered using a vacuum manifold. The plate was rinsed two additional times with 200 µL of 10% TCA as above. The plate was transferred to a reading cassette and 20 µL of Wallac Supermix Scintillant was added to each well. The plate was covered with a plate sealer and read on a Wallac MicroBeta Scintillation counter. The NNRTI UC-38 was evaluated in parallel as a positive control for the assay.

Anti-HIV Assay in Fresh Human Peripheral Blood Mononuclear Cells

PBMC based anti-HIV assays were performed as previously described [35]. Briefly, PHA-stimulated PBMCs cultured in the presence of IL-2 were suspended at 1 x 10^6 cells/mL and were added to a 96-well round-bottom plate. Serially diluted test materials were added to the plate in triplicate followed by the appropriate pre-titered strain of HIV. The culture was incubated for 7 days at 37°C/5% CO₂. Following the incubation, supernatants were collected for analysis of virus replication by supernatant RT
activity and cells analyzed for viability by XTT dye reduction. AZT was used as an internal assay standard. Clinical subtypes of virus that represent HIV strains found in geographic locales where microbicide products are most likely to be clinically evaluated (clades B, C and E) were utilized.

**Anti-HIV Assay in Fresh Human Monocytes-Macrophages**

Monocyte-macrophage based anti-HIV assays were performed as previously described [35]. Briefly, monocyte-macrophage derived from human PBMCs (4x10^6 cells/well) were plated for 5 to 7 days at 37°C/5% CO2. On the day of assay, the cell monolayer was gently washed with HBSS or DPBS several times to eliminate residual PBMCs. After the final wash, 50 mL of supplemented RPMI 1640 was placed in each well and 100 µL of compound prepared at 2 times the high test concentration was transferred to the flat-bottom 96-well plate containing the cells. A pre-determined titer of HIV-1BaL was added to the appropriate wells in a 50 µL volume. Following the 24 hour incubation, virus was removed by washing with HBSS or DPBS. After 7 days in culture, HIV-1 replication was quantified by the measurement of HIV-1 p24 using a commercial antigen capture ELISA for the HIV p24 antigen (Perkin Elmer). Compound cytotoxicity was evaluated using the tetrazolium dye XTT. AZT was evaluated in parallel as a control for the assay.

**Anti-HIV Assay in Monocyte-Derived Dendritic Cells (MO-DCs)**

Dendritic cell-based anti-HIV assays were performed as previously described [35]. MO-DCs derived from fresh human PBMCs were suspended at 1x10^7 cells/mL in RPMI 1640 with 10% FBS, 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin and incubated for 2 hours at 37°C, 5% CO2 with HIV-1BaL at a multiplicity of infection (MOI) of 50-150 TCID50. Following the incubation, cells were washed six times and resuspended at 1x10^6 cells/mL in complete medium. One hundred microliters (100 µL) of MO-DCs were dispensed in
a 96-well round bottom plate. Compounds to be evaluated (100 μL) prepared in a half-log
dilution series in complete medium, were added immediately after infection. Following 7 days
in culture at 37°/5% CO₂, HIV-1 replication was quantified by the measurement of HIV-1 RT
activity. Compound cytotoxicity was determined using the tetrazolium dye XTT. AZT was
evaluated in parallel as a positive control for the assay.

Microbicide Transmission Sterilization Assay (MTSA)
The MTSA was performed as previously described [35]. Briefly, CEM-SS cells at a
concentration of 5 x 10⁵ cells per well and the appropriate pre-determined titer of HIV-1IIIb were
cocultured for 1 hour at 37°C in a 96 well round bottom microtiter plate. Following the initial
infection, the cells and virus suspension were transferred to a T25 tissue culture flask containing
5 mL of tissue culture medium. Five or six concentrations of test or control compound were
added to appropriate individual flasks. Three days following the initial infection, cell-free
supernatant samples collected from each flask were evaluated for virus content by the RT assay
[6] and the cells were subcultured by transferring 20% of the existing culture (1 mL of
resuspended cells and supernatant) to 4 mL of fresh cells (at a cell density of 1 x 10⁵ cells/mL) in
fresh tissue culture medium containing test compound at the identical fixed compound
concentration. Each sub-culturing performed at three day intervals was considered a new
passage. Sub-culturing of the infected cultures was performed every third day for a total of 15
passages. Cells in passages 11 through 15 were cultured in the absence of test compound to
confirm complete virus sterilization of the culture. The results of each assay are reported as the
number of passages determined to be positive for virus replication in the flask (i.e., a 0 value
indicates complete virus sterilization, whereas a value of 15 indicates no inhibition of virus
replication).
CD4-Independent Virus Transmission Inhibition Assays

The assay was performed as previously described [35]. ME180 cells resuspended in RPMI1640 medium with 10% FBS and antibiotics were plated at a density of \( 5 \times 10^3 \) cells per well in a 96-well flat-bottomed microtiter plate and incubated overnight at 37°C/5%CO₂. Following the incubation, test compound was serially diluted in assay medium and added in triplicate to the cells. Thoroughly washed, mitomycin C-treated chronically HIV-infected H9 cells (H9/SK-1) cells were added at \( 2 \times 10^4 \) cells/well and the plates were incubated at 37°C/5% CO₂ for 4 hours. The mitomycin C concentration employed was that which resulted in complete killing of the chronically infected cells within 24 hours to prevent virus production from the H9/SK-1 inoculum from contributing to the endpoint of the assay. Immediately following the infection period, and again at 24 and 48 hours post-infection, the cell monolayers were washed with RPMI1640 (without additives) to remove residual compound and virus-infected cells. Following a 6-day incubation at 37°C/5% CO₂, cell free supernatant samples from the cell cultures were evaluated for virus content using the HIV-1 p24 Antigen ELISA Kit (Beckman Coulter) according to the manufacturer’s instructions. Duplicate assay plates were evaluated for cellular toxicity by XTT staining. Dextran Sulfate was evaluated in parallel as a positive control for the assay.

Cell-Free Virus and CD4 Dependent Transmission Inhibition Assay

The assay was performed as previously described [35]. GHOST X4/R5 cells diluted in DMEM medium supplemented with 10% FBS and antibiotics were incubated in a 96-well flat-bottom microtiter plate (\( 5 \times 10^3 \) cells per well) overnight prior to assay initiation. Following the incubation the cultures were washed to remove non-adherent cells. Serially diluted test compound was added in triplicate wells, and the cells were infected with a pre-determined titer
of HIV-1IIIB for 4 hours at 37°C. Following the incubation, residual virus and test material was removed by washing with RPMI1640 (without additives). The cultures were incubated for 6 days at which time antiviral activity was assessed by evaluating cell-free supernatants for virus content by reverse transcriptase (RT) assay as described previously [6]. Cytotoxicity was evaluated in parallel using XTT dye reduction. Dextran Sulfate was evaluated in parallel as a positive control for the assay.

Cell-Associated Virus Transmission Inhibition Assay

The assay was performed as previously described [35]. GHOST X4/R5 cells diluted in DMEM medium supplemented with 10% FBS and antibiotics were incubated in a 96-well flat-bottom microtiter plate (5 x 10^3 cells per well) overnight prior to assay initiation. Following the incubation the cultures were washed to remove non-adherent cells. Serially diluted test compound was added in triplicate wells. Thoroughly washed, mitomycin C-treated chronically HIV-infected H9 cells (H9/SK-1) cells were added at 2x10^4 cells/well and the plates were incubated at 37°C/5% CO₂ for 4 hours. The mitomycin C concentration employed was that which resulted in complete killing of the chronically infected cells within 24 hours to prevent virus production from the H9/SK-1 inoculum from contributing to the endpoint of the assay. Immediately following the infection period, and at 24 and 48 hours post-infection, the cell monolayers were washed with RPMI1640 (without additives) to remove residual compound and virus-infected cells. The cultures were incubated for 6 days at which time antiviral activity was assessed by evaluating cell-free supernatants for virus content by reverse transcriptase (RT) assay as described previously [6]. Cytotoxicity was evaluated in parallel using XTT dye reduction. Penicillin/Streptomycin was evaluated in parallel as a positive control for the assay.

Lactobacillus Toxicity Assay
The assay was performed as previously described [22]. Briefly, in a 15 mL conical tube 10 mL of MRS media was inoculated with a stab from a frozen glycerol stock of *Lactobacillus crispatus*, *jenseni*, or *acidophilus* and was incubated for 24 hours at 37°C in an anaerobic chamber. The overnight culture was diluted in MRS media until an absorbance of 0.06 at 670 nm was obtained. Six serial ½ log dilutions of the compound were performed and were added in a volume of 100 µL to the plate followed by the addition of 100 µL of the diluted bacteria. The plates were placed in an anaerobic chamber and were incubated at 37°C for 24 hours. Following the incubation the plates were read spectrophotometrically at 490 nm on a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Statistical Software Used to Generate EC$_{50}$ and TC$_{50}$ concentrations.

For each assay, raw data was collected by the Softmax Pro 4.6 (or higher) software or generated from the Microbeta Trilux software and was imported into a Microsoft Excel spreadsheet for analysis by 4-parameter or linear curve fit calculations. Both antiviral efficacy and cellular toxicity with a graphical representation of the data were provided in a Plate Analysis Report (PAR) summarizing the activity of the experimental and control compounds and are expressed as an EC$_{50}$ or TC$_{50}$. 

RESULTS

Efficacy of Lead Candidate Pyrimidinediones (PYDs)

Twelve (12) PYD congeners (Figure 1) from a structure activity relationship series which included 68 PYD molecules were evaluated in relevant primary topical microbicide assays to assess their potential as prevention agents. The twelve molecules in the series were chosen based on their relative antiviral activity against HIV-1 and HIV-2, as well as their relative ability to inhibit virus entry and reverse transcription using wild-type infectious virus or enzyme, respectively. The most active compounds in CEM-SS cells against HIV-1IIIB included compounds IQP-0406, IQP-0407, IQP-0528, IQP-0558, IQP-0410 and IQP-1187, which exhibited sub-nanomolar protection against HIV replication with 50% efficacy concentrations (EC\textsubscript{50}’s) that ranged from 0.2 to 0.5 nM. The most active compounds against HIV-2ROD were compounds IQP-0406, IQP-0528, IQP-0549 and IQP-0558 which exhibited EC\textsubscript{50}’s ranging from 100 to 500 nM. When evaluated against wild-type HIV-1 RT in a biochemical RT inhibition assay, compounds IQP-0405, IQP-0406, IQP-0529 and IQP-0558 were found to be the most active agents with EC\textsubscript{50}’s ranging from 12 to 20 nM. As expected based on mechanistic studies performed with the pyrimidinediones (unpublished data) none of the compounds were active in enzymatic assays against purified HIV-2 RT. Compounds IQP-0405, IQP-0407, IQP-0528, IQP-0554 and IQP-0558 were found to be the most active inhibitors against HIV-1IIIB entry with EC\textsubscript{50}’s ranging from 6 to 12 nM. In addition to evaluating the compounds based on concentrations which inhibit HIV replication, we also ranked the most potent compounds based on their observed \textit{in vitro} therapeutic index (TI) which takes into account both efficacy against virus replication and toxicity to target cells evaluated in parallel with the same cells over the same period of time. Using TI values to compare the PYDs, the most active HIV-1 inhibitors in
CEM-SS cells were IQP-0405, IQP-0407, IQP-0528, IQP-0565, IQP-0410 and IQP-1187. The compounds with greatest TI values in the entry inhibition assay were the same except that IQP-0558 outperformed IQP-1187. Against HIV-2 in CEM-SS cells, IQP-0405, IQP-0407 and IQP-0528 were still superior with the greatest TI values but IQP-0531, IQP-0549 and IQP-0558 also outperformed the other PYDs. These comparative data are presented in Table 1 and provide comprehensive efficacy data to allow the prioritization of the active pyrimidinedione microbicide candidates.

Efficacy of Pyrimidinediones Against Subtype Viruses Relevant to Microbicide Development

In standardized fresh human PBMC assays the 12 PYDs were evaluated against HIV-1 subtypes A through G and O and were found to be highly efficacious against viruses from each subtype, with EC$_{50}$’s in the low to sub-nanomolar concentration range for each of the most active compounds (data not shown). A more extensive evaluation was performed with the twelve PYDs against low passage, clinical HIV-1 subtypes C and E since these are the predominant strains found in regions of the world where microbicides will most likely be primarily used. These antiviral data are presented in Figure 2. The activity of the 12 molecules against clinical subtype B virus (strain US/92/727) ranged from 0.13 to >10 nM with the most active compounds being IQP-0405, IQP-0406, IQP-0407, IQP-0528, IQP-0410 and IQP-1187 (Figure 2A). The compounds were also evaluated against three strains of clinical subtype C viruses and exhibited activity ranging from 0.09 nM to 8.9 nM, with compounds IQP-0406, IQP-0407, IQP-0528, IQP-0558, IQP-0410 and IQP-1187 being the most active (Figure 2B). When tested against three clinical subtype E subtype viruses the most active compounds were IQP-0405, IQP-0406, IQP-0407, IQP-0528, IQP-0558, IQP-0410, and IQP-1187. EC$_{50}$’s for all twelve PYDs ranged from 0.07 nM to 31.4 nM against clinical subtype E viruses (Figure 2C).
Efficacy of Pyrimidinediones in Monocytes-Macrophages and Monocyte-Derived Dendritic Cells

In addition to evaluation of test compounds in fresh human PBMCs, it is also important that the activity of potential microbicides be evaluated for activity in other relevant target cell types found within the vaginal mucosa, including monocytes-macrophages and dendritic cells. Thus, we evaluated the activity of the PYDs in these two cell types infected with HIV-1BaL. The activity of the PYDs in monocyte-macrophages ranged from 0.20 nM to >10 nM with the most active compounds being IQP-0528, IQP-0558, IQP-0410, and IQP-1187 (Figure 3A). The antiviral activity of the test molecules extended into the sub-picomolar range in dendritic cells with EC_{50}'s ranging from 0.0003 nM to 0.72 nM. The most active molecules in the series were IQP-0405, IQP-0406, IQP-0528, IQP-0565, IQP-0410, and IQP-1187 (Figure 3B).

Toxicity of Pyrimidinediones to the Normal Vaginal Flora Lactobacillus

The presence of the normal hydrogen peroxide producing Lactobacillus flora of the vagina is critical to a healthy vaginal environment. Each PYD was evaluated for toxicity to three strains of Lactobacilli normally found in the vagina, including Lactobacillus acidophilus, Lactobacillus jensenii and Lactobacillus crispatus. The PYDs were found to be nontoxic to these normal vaginal microorganisms at concentrations 10^4 to 10^6 times higher than those concentrations required for antiviral activity. Concentrations which inhibited the growth of the Lactobacilli ranged from 40 μM to >500 μM. IQP-0405 and IQP-0558 were the least toxic compounds with 50% toxicity concentrations (TC_{50}'s) of >500 μM against all strains of Lactobacillus evaluated. IQP-0407 and IQP-1187 were found to possess the greatest toxicity, however, their defined average TC_{50}'s were 66.1 μM and 48.3 μM, respectively, well above the antiviral activity of the molecules. The remaining compounds had only marginal toxicity with TC_{50}'s ranging from
101.8 μM to >500 μM. Thus, the PYDs are considered to have little to no toxicity to the normal vaginal flora. These toxicity data are presented in Table 2.

Toxicity of Pyrimidinediones to Fresh and Established Human Cells

In order to further assess the relative toxicity of the PYDs, each compound was exposed to fresh and established human cells for 24 hours and/or 6 days (Figure 4). Cell growth inhibitory concentrations ranged from 23.9 μM to >500 μM. Compounds IQP-0406, IQP-0549, IQP-0554 and IQP-0558 appeared to have the greatest impact on cellular replication with most TC\textsubscript{50}’s ranging from 23.9 to 114.7 μM. Compounds IQP-0405, IQP-0407, and IQP-0410 were the least toxic with all TC\textsubscript{50}’s ranging from 269.5 to >500 μM. The results from the 24 hour studies are more likely to mimic typical microbicide use (short term exposure). The 6 day results are presented to highlight potential effects of chronic microbicide use. Again, it should be noted that the concentrations impacting cell growth were 10^4 to 10^6 higher than those which inhibit virus replication, consistent with the high therapeutic index of the PYDs. Also, in nearly all cases the toxicity of the compounds has been found to be related to their overall solubility in aqueous medium as opposed to direct toxic effects on the target cells.

Efficacy in the Microbicide Transmission and Sterilization Assay

The twelve PYDs were evaluated in parallel for their potential to completely sterilize a virus infected culture. This assay quantifies the concentration of the microbicide necessary to completely suppress virus transmission and yielding sterilization of the cell culture. For each test compound a dose-response range of five to six concentrations spanning the TI window of each compound (selected based on the defined EC\textsubscript{50} and TC\textsubscript{50} of the compound in the CPE inhibition assay) were evaluated. The results are presented as the number of passages which were positive for virus replication at each compound concentration with a 0 representing...
complete sterilization of the culture and a 15 representing no effect on virus replication [35]. The concentrations chosen to be evaluated for each compound included 10, 50, 250, 1250, 6250 and 31,250 times the EC50 that was defined in the CPE assay. Compounds IQP-1187, IQP-0405 and IQP-0528 performed the best with complete sterilization at 50, 250 and 250 times the EC50, respectively. IQP-0406 and IQP-0531 were slightly less effective with complete sterilization at concentrations of 1250 and 6250 times the EC50. IQP-0407, IQP-0529, IQP-0549, IQP-0554, IQP-0558, IQP-0565 and IQP-0410 were the least effective with complete sterilization achieved at 31,250 or greater than 31,250 times the EC50 in the CPE assay. These data are graphically presented in Figure 5.

Cell-Free and Cell-Associated Virus Transmission

Based on the performance in the efficacy and toxicity assays, seven PYDs were chosen for further evaluation as microbicide candidates. The lead PYDs were evaluated in parallel with a standard control compound (dextran sulfate) in assays measuring the inhibition of cell-free and cell-associated virus transmission. The activity against the cell-free virus transmission of HIV-1 strains in GHOST X4/R5 cells ranged from 0.17 to 0.38 μM for all of the tested PYDs. The activity of dextran sulfate was 0.31 μg/mL. The PYDs also effectively inhibited virus transmission from chronically-infected H9 cells (HIV-1SK-1 virus) to GHOST X4/R5 cells at concentrations ranging from 0.016 to 0.21 μM, with IQP-0406 having the greatest activity at an EC50 concentration of 0.016 μM. Dextran sulfate was active at 0.78 μg/mL. These data are presented in Table 3.

The seven PYDs were also evaluated in assays evaluating CD4-independent and CD4-dependent virus transmission. Consistent with mechanistic data indicating that CD4 was necessary for the activity of the PYDs, all of the compounds tested were unable to inhibit the CD4-independent transmission of virus from chronically infected cells to the CD4- ME180
cervical cell line. The control compound dextran sulfate was active in the CD4-independent transmission inhibition assay at a concentration of 0.63 μg/mL. The PYDs were able to inhibit the transmission of virus from chronically infected cells to CD4+ GHOST X4/R5 cells at concentrations ranging from 0.016 to 0.21 μM. IQP-0406, IQP-0407, IQP-0528 and IQP-1187 were the most active with EC₅₀’s ranging from 0.016 μM to 0.04 μM. IQP-0405 and IQP-0410 were 2 to 5-fold less active with EC₅₀’s of 0.072 μM and 0.08 μM, respectively. IQP-0558 was the least active with an EC₅₀ of 0.21 μM. Dextran sulfate was active at 0.78 μg/mL. The data are presented in Table 4.

DISCUSSION

Twelve PYD molecules were identified from a SAR series as having highly desirable properties as potential vaginal topical microbicide candidates. These properties included high potency, activity against both HIV-1 and HIV-2, and inhibitory activity against two steps of HIV infection and replication that would be beneficial for a microbicide product (inhibition of virus entry and RT). In an effort to define and prioritize a lead microbicide candidate for development, the PYDs were further evaluated using a series of in vitro anti-viral and toxicity evaluations relevant to the development of microbicides. Based on all the results obtained herein, we can summarize that the PYDs have significant efficacy against laboratory and clinical virus strains (Clade B, C and E) when evaluated in CEM-SS cells, PBMCs, monocytes-macrophage and dendritic cells at concentrations ranging from 0.1 to 10 nM with therapeutic indices approaching and exceeding one million. In more relevant topical microbicide assays that evaluate cell-free and cell-associated virus transmission the compounds were active in the sub-micromolar concentration range. The molecules were found to be inactive against HIV transmission to cell lines devoid of CD4, consistent with their proposed entry inhibitory mechanism which
demonstrated a requirement for CD4 for the compounds to exert entry inhibition (unpublished data). In target cells that express cell surface CD4, antiviral activity was observed in the high nanomolar concentration range. In the MTSA, a transmission inhibition assay which quantifies the ability of microbicide candidates to completely suppress virus transmission (cell-to-cell and cell-free transmission events), the PYDs had varying levels of activity with the best compound requiring only 50-fold more compound than the experimentally determined EC$_{50}$ to totally suppress virus infection, while the worst performing PYD required >35,000-fold more compound to completely “sterilize” the culture. We believe the MTSA may reflect the relative ability of a microbicide candidate to prevent virus transmission events in a microbicide environment and thus may be an important screening tool to prioritize candidate compounds.

Thus, our data would suggest that compounds IQP-1187, IQP-0405 and IQP-0528 would likely represent the best pyrimidinediones for continued development and these pyrimidinediones were found to be more active than PRO2000, Tenofovir and SPL7013 (observed results). The toxicity of the molecules to established and fresh human cells and to the natural normal flora \textit{Lactobacillus} was significantly higher (one thousand to one million-fold greater concentrations) than the defined efficacy concentrations. The experimentally determined toxicity in most cases was attributable to precipitation of the compound, as opposed to overt toxicity to the cells or bacteria.

Without an approved and/or highly successful microbicide product, there currently does not exist a “gold-standard” for use as a model for the development of a superior microbicide. In choosing inhibitors to prioritize for continued development, algorithms exist which assist with the definition of the microbicidal properties of the compounds in comparison with others that have progressed to human trials or are currently in development. PRO2000 has been in
development since the mid-1990s and until recently was the most advanced compound in human clinical trials. PRO2000 is a HIV inhibitor that targets entry, specifically the CD4/gp120 interaction, and possesses sub-micromolar antiviral activity and was robust and potent enough in the in vitro assay systems used to advance compounds in order to move forward to human clinical trial. Unfortunately, results from a Phase III clinical trial data indicated that PRO2000 was not protective against the vaginal transmission of HIV[14]. This was an unexpected result and focused attention on what properties a successful microbicide candidate will need to possess for success in the clinic. The antiretroviral agent tenofovir, a nucleotide analog which inhibits viral reverse transcriptase, recently completed a Phase III human clinical trial (CAPRISA 004) with marginal levels of protection. In this trial, tenofovir vaginal gel had moderate protection when administered before and after sexual intercourse, exhibiting a 39% lower risk of HIV acquisition overall and a 54% reduction among women with the highest level of adherence to the trial protocol [37]. The results from our evaluations would suggest that the most potent PYDs are significantly more active inhibitors of HIV than tenofovir and that the PYDs would likely work potently in combination with tenofovir since they exert antiviral activity by complementary and non-overlapping mechanisms of action. The NNRTI dapivirine is also approved for human therapeutic use and is in development for use as a microbicide[18]. The PYDs have similar potency and antiviral characteristics as dapivirine but may have the advantage of being less toxic and more soluble in formulated form. Additionally, the experimental NNRTI UC781 (thiocarboxanilide) has progressed to advanced stages of microbicide development. UC781 possesses nanomolar concentration activity and a TI of approximately 62,000[7] but has a yellow color and sulfur odor, which are not ideal characteristics for a microbicide. Additionally, unlike the lead pyrimidinediones, UC781 appears to be more unstable in aqueous solutions[16].
NNRTI-resistant virus strains which preexist in the population and the relative ease of selection of highly NNRTI-resistant viruses to the compound render UC781 a problematic microbicide for use unless it was a component of a microbicide combination product. Thus, the PYDs represent a class of compounds that meet all of the advantageous *in vitro* characteristics of a microbicide and might be superior to each of these two NNRTIs. The PYDs are colorless molecules that have nanomolar to sub-nanomolar activity and little to no cellular toxicity, yielding TIs>100,000 and in some cases >1,000,000. The FDA approved CCR5-antagonist Maraviroc (Selzentry), exhibits activity against CCR5-tropic strains of virus in the low nanomolar concentration range. Although possessing similar *in vitro* activity to the PYDs, maraviroc is only active against CCR5-tropic strains of virus. Although it is known that a large percentage of the sexually transmitted viruses have CCR5 specificity it has not been conclusively shown that CXCR4-tropic viruses are not sexually transmitted. The PYDs are highly active against CXCR4-, CCR5- and dual-tropic (CXCR4/CCR5) viruses and also possess an entry inhibitory mechanism, yielding antiviral properties that might make them equivalent or superior to maraviroc. As noted above, the combination of maraviroc and a PYD would represent an effective combination product with complementary and non-overlapping antiviral mechanisms of action.

The dogma surrounding potential microbicide candidates has previously involved the compound exhibiting anti-HIV activity at sub-micromolar concentrations and having a mechanism of action that targeted the early pre-integration steps in virus replication including attachment, entry and reverse transcription [33]. That viewpoint and focus however is changing with the introduction of integrase and protease inhibitors into the microbicide pipeline [11]. Not only do many of the compounds in development specifically target virus replication as traditional anti-retroviral agents (ARVs) but some of the compounds now in development are already...
approved for use as therapeutic agents [10, 15, 24]. The data presented herein summarizes the initial in vitro efficacy and toxicity evaluations needed to define and prioritize the PYD congeners for continued microbicide development. Although the activity of the compounds is relatively similar, there are seven compounds from this subset of highly active pyrimidinedione analogs that we believe have properties that provide a rationale for the performance of additional evaluations as a means to define the optimal lead candidate. These highly active and potent microbicide candidates include IQP-0406, IQP-0407, IQP-0528, IQP-0558, IQP-0410 and IQP-1187. Of these seven molecules, the first four are structurally related (see Figure 1) with a cyclopropyl substituent at the N-1 of the pyrimidinedione. In preliminary experiments, these molecules have been determined to have much higher chemical and metabolic stability than the molecules with the 3-cyclopenten-1-yl substitution (IQP-0410 and IQP-1187), although they have similar efficacy against HIV. IQP-0410 has progressed to an IND submission as a potential therapeutic agent for the treatment of HIV infection. Additional studies that are more relevant to the vaginal microenvironment during virus transmission are in progress with these seven molecules, including the performance of antiviral evaluations in the presence of additives such as vaginal fluids, seminal plasma, and mucin, as well as a pH transition assay. Additionally, the lead PYD compounds are being evaluated in combination with other potential microbicide products such as tenofovir and are being comparatively evaluated in human cervical explant efficacy and toxicity assays. Stability and pre-formulation of the molecules is also being performed. From these additional data we will define a lead PYD candidate microbicide for late stage development.

ACKNOWLEDGMENTS
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REFERENCES

14. CONRAD. CONRAD Statement on MDP 301 Results. 2009.


Figure Legends

Figure 1: Structure of candidate pyrimidinedione microbicides

aThe twelve representative pyrimidinediones with the chemical substituents noted at each side chain position (denoted R, R1, R2, R3 and X).

bAbbreviations: Et = Ethyl; Me = Methyl; iPr = Isopropyl.

Figure 2: Efficacy of Pyrimidinediones Against HIV-1 Clade Viruses in PBMCs. The results presented were obtained from representative antiviral assays with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC₅₀. In each individual assay, mean efficacy values are derived from a minimum of three replicate wells. One Clade B strain and three Clade C and Clade E strains were evaluated.

Figure 3: Efficacy of Pyrimidinediones Against HIV-1Bal in Monocyte Macrophages(A) and Dendritic Cells (B). The results presented were obtained from representative antiviral assays with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC₅₀. In each individual assay, mean efficacy values are derived from a minimum of three replicate wells.

Figure 4: Toxicity of Pyrimidinediones to Fresh and Established Human Cells. The results presented were obtained from representative antiviral assays with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple toxicity assays averaged less than 10% of the respective mean TC₅₀. In each individual assay, mean toxicity values are derived from a minimum of three replicate wells.
Figure 5: Sterilization Concentration Determination of Pyrimidinediones in a Microbicide Transmission and Sterilization Assay. The pyrimidinediones were evaluated in the MTSA and the results are presented as the number of passages which were positive for virus replication at each compound concentration. The results of one representative replicate assay are presented. Each compound was evaluated at concentrations that were 10, 50, 250, 1250, 6250 and 31250 times the EC₅₀ that was defined in the CPE inhibition assay. All of the concentrations tested were significantly below the defined TC₅₀ for CEM-SS cells. Passages which were positive for virus production were defined by detection of virus in the cell-free supernatant by RT assay. Cells were passaged for 10 passages and in the absence of the compound for an additional 5 passages. Passages with 0 values represent those that were completely sterilized; passages with values of 15 represent concentrations of the test compounds which had no impact on virus replication in the cultures.
Figure 1: Structure of candidate pyrimidinedione microbicides

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>X</th>
<th>R</th>
</tr>
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<tr>
<td>IQP-0405</td>
<td>Et</td>
<td>Me</td>
<td>Me</td>
<td>O</td>
<td>Cyclopropyl</td>
</tr>
<tr>
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<td>iPr</td>
<td>Me</td>
<td>Me</td>
<td>O</td>
<td>Cyclopropyl</td>
</tr>
<tr>
<td>IQP-0407</td>
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<td>Me</td>
<td>Me</td>
<td>C=O</td>
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<td>Me</td>
<td>C=O</td>
<td>Cyclopropyl</td>
</tr>
<tr>
<td>IQP-0529</td>
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<td>Me</td>
<td>Me</td>
<td>S</td>
<td>Cyclobutyl</td>
</tr>
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<td>Me</td>
<td>Me</td>
<td>O</td>
<td>Cyclobutyl</td>
</tr>
<tr>
<td>IQP-0549</td>
<td>Et</td>
<td>Me</td>
<td>Me</td>
<td>O</td>
<td>Phenyl</td>
</tr>
<tr>
<td>IQP-0554</td>
<td>iPr</td>
<td>Me</td>
<td>Me</td>
<td>S</td>
<td>1-Cyclopenten-1-yl</td>
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<td>IQP-0558</td>
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<td>C=O</td>
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<td>IQP-0565</td>
<td>Et</td>
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<td>Me</td>
<td>O</td>
<td>3-Cyclopenten-1-yl</td>
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<tr>
<td>IQP-1187</td>
<td>iPr</td>
<td>Me</td>
<td>Me</td>
<td>C=O</td>
<td>3-Cyclopenten-1-yl</td>
</tr>
</tbody>
</table>
Figure 2: Efficacy of Pyrimidinediones Against HIV-1 Clade Viruses in Fresh Human Peripheral Blood Mononuclear Cells

A. Clade B

B. Clade C
C. Clade E

![EC50 Concentration (nM) Diagram]

- CMU06
- 92/TH/020
- 93/TH/072

Compounds: (List of compound names not visible in the image)
Figure 3: Efficacy of Pyrimidinediones in Monocytes and Monocyte Derived Dendritic Cells

A. Monocytes Macrophage

B. Monocyte-Derived Dendritic Cells
Figure 4: Toxicity of Pyrimidinediones to Fresh and Established Human Cells
Figure 5: Sterilization Concentration Determination of Pyrimidinediones in a Microbicide Transmission and Sterilization Assay
Table 1: Efficacy of Candidate Microbicides Against HIV-1 and HIV-2

<table>
<thead>
<tr>
<th>Compound</th>
<th>CEM-SS/HIV-1 \text{III}</th>
<th>CEM-SS/HIV-2 \text{ROD}</th>
<th>Wild-Type RT Inhibition</th>
<th>HIV-1 \text{III} Entry Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*EC_{50} (nM)</td>
<td>TI</td>
<td>*EC_{50} (nM)</td>
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</tr>
<tr>
<td>AZT</td>
<td>0.007</td>
<td>&gt;71.4</td>
<td>0.005</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Chicago Sky Blue (μg/mL)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>UC38</td>
<td>---</td>
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</tr>
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<td>IQP-0405</td>
<td>1</td>
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<td>600</td>
<td>&gt;2,099</td>
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<tr>
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<td>40</td>
</tr>
<tr>
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<td>100</td>
<td>&gt;13,030</td>
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<td>&gt;250</td>
<td>1700</td>
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<tr>
<td>IQP-0531</td>
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<td>&gt;50</td>
<td>7200</td>
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<td>4</td>
<td>&gt;125</td>
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<td>500</td>
<td>57.4</td>
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<td>IQP-0558</td>
<td>0.5</td>
<td>&gt;1000</td>
<td>100</td>
<td>122</td>
</tr>
<tr>
<td>IQP-0565</td>
<td>2</td>
<td>&gt;250</td>
<td>19,000</td>
<td>&gt;3.2</td>
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<tr>
<td>IQP-0410</td>
<td>0.2</td>
<td>&gt;2500</td>
<td>16,000</td>
<td>&gt;71</td>
</tr>
<tr>
<td>IQP-1187</td>
<td>0.4</td>
<td>&gt;1250</td>
<td>15,500</td>
<td>&gt;75</td>
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*The results presented were obtained from a single representative antiviral assay with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC_{50} and TC_{50} (data not shown). In each individual assay, mean efficacy and toxicity values are derived from a minimum of three replicate wells. The TI represents the ration of the EC_{50}/TC_{50}.
Table 2: Toxicity to the Normal Vaginal Flora *Lactobacillus*

<table>
<thead>
<tr>
<th>Compound</th>
<th>L. acidophilus TC50 (µM)</th>
<th>L. jensenii TC50 (µM)</th>
<th>L. crispatus TC50 (µM)</th>
</tr>
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<tr>
<td>IQP-0405</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
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<tr>
<td>IQP-0406</td>
<td>222.8</td>
<td>295.7</td>
<td>329.3</td>
</tr>
<tr>
<td>IQP-0407</td>
<td>94.2</td>
<td>58.0</td>
<td>46.2</td>
</tr>
<tr>
<td>IQP-0528</td>
<td>190.7</td>
<td>108.4</td>
<td>101.8</td>
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<tr>
<td>IQP-0529</td>
<td>135.3</td>
<td>187.0</td>
<td>146.6</td>
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<tr>
<td>IQP-0531</td>
<td>401.4</td>
<td>192.7</td>
<td>120.5</td>
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<tr>
<td>IQP-0549</td>
<td>&gt;500</td>
<td>329.7</td>
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<td>IQP-0554</td>
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<td>&gt;500</td>
<td>&gt;500</td>
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<tr>
<td>IQP-0565</td>
<td>138.3</td>
<td>83.7</td>
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<td>IQP-0410</td>
<td>198.4</td>
<td>235.6</td>
<td>157.0</td>
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<tr>
<td>IQP-1187</td>
<td>58.8</td>
<td>40.0</td>
<td>46.0</td>
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* Toxicity of Pyrimidinediones to the normal vaginal flora *Lactobacillus*. The results presented were obtained from representative toxicity assays with appropriate control compounds evaluated in parallel selected from a minimum of three toxicity assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean TC50. In each individual assay, mean toxicity values are derived from a minimum of three replicate wells.
## Table 3: Evaluation of Cell Free and Cell Associated Virus Transmission

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell-Free Virus Transmission $^a$EC$_{50}$ (µM)</th>
<th>Cell-Associated Virus Transmission $^a$EC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran Sulfate (µg/mL)</td>
<td>0.31</td>
<td>0.78</td>
</tr>
<tr>
<td>IQP-0405</td>
<td>0.34</td>
<td>0.072</td>
</tr>
<tr>
<td>IQP-0406</td>
<td>0.17</td>
<td>0.016</td>
</tr>
<tr>
<td>IQP-0407</td>
<td>0.17</td>
<td>0.024</td>
</tr>
<tr>
<td>IQP-0528</td>
<td>0.21</td>
<td>0.034</td>
</tr>
<tr>
<td>IQP-0558</td>
<td>0.38</td>
<td>0.21</td>
</tr>
<tr>
<td>IQP-0410</td>
<td>0.21</td>
<td>0.08</td>
</tr>
<tr>
<td>IQP-1187</td>
<td>0.31</td>
<td>0.04</td>
</tr>
</tbody>
</table>

$^a$The results presented were obtained from a single representative antiviral assay with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC$_{50}$. In each individual assay, mean efficacy values are derived from a minimum of three replicate wells. Toxic concentrations were greater than the highest concentration evaluated in each assay (data not shown).
Table 4: Evaluation of CD4 Independent and CD4 Dependent Virus Transmission

<table>
<thead>
<tr>
<th>Compound</th>
<th>CD4 Independent Virus Transmission EC_{50} (µM)</th>
<th>CD4 Dependent Virus Transmission EC_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran Sulfate (µg/mL)</td>
<td>0.63</td>
<td>0.78</td>
</tr>
<tr>
<td>IQP-0405</td>
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<td>&gt;100</td>
<td>0.024</td>
</tr>
<tr>
<td>IQP-0528</td>
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<td>IQP-0558</td>
<td>&gt;100</td>
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<tr>
<td>IQP-0410</td>
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</tr>
<tr>
<td>IQP-1187</td>
<td>&gt;100</td>
<td>0.04</td>
</tr>
</tbody>
</table>

a The results presented were obtained from a single representative antiviral assay with appropriate control compounds evaluated in parallel selected from a minimum of three antiviral assays. We have demonstrated that the standard error among multiple antiviral assays averaged less than 10% of the respective mean EC_{50}. In each individual assay, mean efficacy values are derived from a minimum of three replicate wells. Toxic concentrations were greater than the highest concentration evaluated in each assay (data not shown).