Evaluation of PD 404,182 as an Anti-HIV and Anti-Herpes Simplex Virus Microbicide

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PD 404,182 (PD) is a synthetic compound that was found to compromise HIV integrity via interaction with a nonenvelope protein viral structural component (A. M. Chamoun et al., Antimicrob. Agents Chemother. 56:672–681, 2012). The present study evaluates the potential of PD as an anti-HIV microbicide and establishes PD's virucidal activity toward another pathogen, herpes simplex virus (HSV). We show that the anti-HIV-1 50% inhibitory concentration (IC_{50}) of PD, when diluted in seminal plasma, is ~1 µM, similar to the IC_{50} determined in cell culture growth medium, and that PD retains full anti-HIV-1 activity after incubation in cervical fluid at 37°C for at least 24 h. In addition, PD is nontoxic toward vaginal commensal *Lactobacillus* species (50% cytotoxic concentration [CC_{50}], >300 µM), freshly activated human peripheral blood mononuclear cells (CC_{50}, ~200 µM), and primary $CD4^{+}$ T cells, macrophages, and dendritic cells (CC_{50}, >300 µM). PD also exhibited high stability in pH-adjusted Dulbecco's phosphate-buffered saline with little to no activity loss after 8 weeks at pH 4 and 42°C, indicating suitability for formulation for transportation and storage in developing countries. Finally, for the first time, we show that PD inactivates herpes simplex virus 1 (HSV-1) and HSV-2 at submicromolar concentrations. Due to the prevalence of HSV infection, the ability of PD to inactivate HSV may provide an additional incentive for use as a microbicide. The ability of PD to inactivate both HIV-1 and HSV, combined with its low toxicity and high stability, warrants additional studies for the evaluation of PD's microbicidal candidacy in animals and humans.

Since its discovery in humans in 1981, human immunodeficiency virus (HIV), the causative agent of AIDS, has infected over 60 million people worldwide and caused more than 25 million deaths (1). Although highly active antiretroviral therapy (HAART) can significantly reduce the viral load and prolong patients' life expectancy, these therapies are not curative (2). Worldwide, nearly half of all individuals living with HIV are women, most of whom acquire the virus after sexual intercourse with HIV-positive men. As receptive partners, women are twice as likely as their male counterparts to acquire HIV during sex (3). Despite the knowledge of effective prevention strategies, such as the ABC approach (abstinence, be faithful, and use of condoms), the rate of HIV transmission remains high in developing countries (4). Moreover, many women cannot reliably negotiate safe sex practices, leaving them vulnerable to sexually transmitted infections. Thus, the development of a safe, effective, and acceptable topical microbicide capable of retarding or preventing the sexual transmission of HIV could empower women to take personal responsibility to prevent HIV acquisition from their infected partners (5).

Topical microbicides are agents able to inhibit the transmission of viral infections when applied to the vagina, penis, and/or lower gastrointestinal (GI) tract via the rectum. An ideal anti-HIV microbicide should fulfill most or all of the following criteria: (i) inhibit transmission of wild-type and drug-resistant virus (6); (ii) be stable and potent in seminal fluids and vaginal secretions; (iii) lack toxicity to the vaginal epithelium and commensal bacterial flora; (iv) be able to interfere with multiple transmission modes (e.g., as cell-free virus versus cell-associated virus), given the unknowns in the exact mode of HIV transmission *in vivo*; (v) possess a high genetic barrier to resistance development; (vi) preferably act through a mode of action distinct from the modes of action of existing therapeutics (7); and (vii) lack proinflammatory activity and immunotoxicity. The last consideration derives from the presence of rare preexisting drug-resistant viral variants, as well as drug-resistant HIV variants, from patients who underwent previous antiretroviral treatment that can bypass the microbicidal barrier and transmit to target cells. Most current anti-HIV microbicide candidates in clinical trials are formulated on the basis of existing antiretroviral drugs and target well-studied viral proteins such as HIV protease (PR), reverse transcriptase, and HIV envelope protein (Env) (6–10). In the CAPRISA 004 clinical trial involving 1% tenofovir gel, HIV type 1 (HIV-1) acquisition was reduced by ~38% in all woman and by 54% in woman who used the gel 80% or more of the time (11). Interestingly and unexpectedly, in the same trial, tenofovir gel was found to also inhibit HSV-2 acquisition by 51% (11). The recently FDA-approved anti-HIV prophylactic therapeutic Truvada comprises two nucleoside

analogs, tenofovir and emtricitabine (12). Truvada offered a 44% reduction in HIV transmission during initial clinical trials (12). However, since both tenofovir and emtricitabine are currently used in the clinic for HIV treatment as part of a HAART drug cocktail, concerns were raised about the potential for the spread of drug-resistant variants when the drug is used by individuals with unknown or positive HIV status. This issue becomes more significant when the drug is used on a large scale, generating an extra incentive to identify new and specific anti-HIV microbicidal compounds with unique modes of action. In addition, a recently completed comprehensive HIV prevention trial among African women known as VOICE (Vaginal and Oral Interventions to Control the Epidemic) involving tenofovir failed to provide protection against HIV, underscoring the need for additional HIV prevention options that incentivize patient usage and adherence (6).

Recently, our laboratory discovered a synthetic small molecule, PD 404,182 (PD), that possesses virucidal activity toward retroviruses, including HIV (13). PD is structurally and mechanistically distinct from existing HIV microbicides (6, 7) and inhibits a broad range of primary isolates of HIV and simian immunodeficiency virus (SIV) at submicromolar to micromolar concentrations with minimal cytotoxicity to human cells (50% cytotoxic concentration [CC_{50}]/50% inhibitory concentration [IC_{50}], >300). Previously, we found that PD (i) is effective against a broad range of primary HIV-1 isolates as well as HIV-2 (IC_{50}, ~1 μM) in TZM-bl cells; (ii) is fully active in cervical fluids; (iii) exhibits low toxicity in 7 different human cell lines, including cervical cancer cells (CC_{50}, >300 μM); (iv) is effective against both cell-free and cell-associated virus and inhibits the transmission of dendritic cell-associated HIV to T cells; (v) retains antiviral potency in cell culture for at least 8 h prior to the addition of HIV to the cells; and (vi) exhibits rapid antiviral action (HIV becomes >99% inactivated after a 5-min incubation with PD) (13).

In this study, we further evaluated the potential of PD as an anti-HIV microbicide and show that PD (vii) is stable and effective at both acidic and neutral pH for at least 48 h, (viii) remains fully active in the presence of seminal plasma and after incubation in cervical fluids for at least 24 h, (ix) retains full potency when stored in Dulbecco's phosphate-buffered saline (DPBS) under acidic pH at 42°C for at least 8 weeks, (x) can be formulated in hydroxyethyl cellulose (HEC) gel under acidic conditions, (xi) is nontoxic to the vaginal commensal bacterium *Lactobacillus* (CC_{50}, >300 μM) and freshly activated peripheral blood mononuclear cells (PBMCs; CC_{50}, >200 μM), (xii) is active in PBMCs against HIV-1 clinical isolates representing different viral subtypes and tropisms (average IC_{50} = 0.55 μM), and (xiii) does not foster the emergence of resistant variants when HIV-1-positive TZM-bl cells are passaged at subinhibitory concentrations of PD in cell culture for 60 days. Finally, we show that PD effectively inactivates human herpes simplex virus 1 (HSV-1) and HSV-2 at submicromolar concentrations (200 nM). In the United States alone, 16.2% of the population is estimated to be infected with HSV-2 (15). Infection with HSV-1 or -2 is an important risk factor for susceptibility to HIV-1 transmission *in vitro* (16). These new findings further underscore PD as a promising new HIV microbicide. In addition, PD is a small molecule that can be easily synthesized (17) and thus can potentially be manufactured at low cost on a large scale for use in developing countries.

MATERIALS AND METHODS

Cells, media, and reagents. PD 404,182 was purchased from Sigma-Aldrich (St. Louis, MO). PD was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 30 to 40 mM, aliquoted, and stored at −20°C. DPBS and penicillin-streptomycin (pen-strep) were purchased from Thermo Scientific HyClone (Logan, UT) and Lonza (Walkersville, MD), respectively. Unless otherwise stated, the complete growth medium for all cell culture work was Dulbecco modified Eagle medium (DMEM) containing 4,500 mg/liter glucose, 4.0 mM L-glutamine, and 110 mg/liter sodium pyruvate (Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1× nonessential amino acids (Thermo Scientific HyClone, Logan, UT). 293T cells were from Life Technologies (Grand Island, NY). Vero cells were obtained from ATCC (Manassas, VA). The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from John C. Kappes, Xiaoyun Wu, and Tranzyme Inc.; HIV-1 isolates 92RW016, 92RW021, 92TH006, 92TH026, 93TH053, 93BR020, 93BR021, 93BR029, 98IN017, 98IN022, 92UG001, 92UG005, 92UG024, and 92UG029 from The UNAIDS Network for HIV Isolation and Characterization (18); HIV-1 92HT599 from Neal Halsey; HIV-1 96USNG31 from D. Ellenberger, P. Sullivan, and R.B. Lal (19); HIV-1 RU132 from A. Bobkov and Jonathon Weber; HIV-1 93IN101 from Robert Bollinger; and HIV-1 Jv1083 from Alash'le Abimiku (20). All primary HIV isolates were amplified in activated human PBMCs. HIV NL4-3 was obtained from the NIH AIDS Research and Reference Program. HSV-1 (strain Syn 17) and HSV-2 (strain 333) were obtained from Theo Geijtenbeek (21) and amplified, and titers were determined in Vero cells.

Lentiviral pseudoparticle production. Pseudotyped lentiviruses were produced by cotransfecting 293T cells with plasmids carrying HIV *gag-pol* (22), a provirus (pV1-B1 [13], pTRIP-Gluc [23], or NL4-3.Luc [NIH AIDS Reagent Program]), and vesicular stomatitis virus glycoprotein (VSV-G) (22). TransIT reagent (Mirus, Madison, WI) was used to perform the transfection following the manufacturer's protocol. The supernatants containing the pseudoparticles were collected at 48 h posttransfection, filtered (0.45-μm pore size), and stored at −80°C until use.

PD stability. As a gauge of compound stability, we determined the antiviral activity of PD after storage under different conditions for different periods of time. PD was diluted in buffered DPBS (pH 4, 6, 8, 10) or cervical fluids (a pool of fluids from 3 donors 5-fold diluted in DPBS; Lee Biosolutions, St. Louis, MO) to achieve a final concentration of 30 μM. DPBS was buffered to the desired pH using hydrochloric acid or sodium hydroxide. Diluted drug was incubated at the desired temperature for 0, 8, 24, or 48 h. After the temperature incubation, the drug mixture was further diluted to 1, 0.1, and 0.05 μM in complete growth medium and used for incubation with VSV-G lentiviral pseudoparticles (VSV-Gpp; harboring either pTRIP-Gluc or NL4-3.Luc viral supernatant diluted 500-fold in complete growth medium) at 37°C for 30 min. Huh-7.5 cells ($2 × 10^4$ cells/well) or 293T cells that had been seeded 24 h earlier were inoculated with the PD-treated virus at 4°C for 2 h, thoroughly washed to remove unbound viruses and drug, replenished with complete growth medium containing 1× pen-strep, and returned to 37°C in 5% CO_2. Viral infectivity was quantified 48 h later by measuring supernatant *Gaussia* luciferase (Gluc) levels using a BioLux *Gaussia* luciferase assay kit (New England BioLabs, Ipswich, MA) or a firefly luciferase assay kit.

To study the long-term stability of PD, the compound was diluted to 5 μM in DPBS buffered at pH 4 and 7 using acetic acid (0.1%) and HEPES (2.5 mM), respectively, aliquoted, and incubated at 4°C, room temperature (RT), or 42°C. Each week an aliquot was removed and tested for antiviral activity as previously described above. Similar experiments were conducted with PD (5 μM) or vehicle control (0.02% DMSO) diluted in DPBS (adjusted to pH 4) containing 1.5% HEC.

PD stability in seminal plasma. TZM-bl cells (10^5 cells/well, 50 μl) were seeded in a flat-bottom 96-well plate. On the next day, PD dilutions were prepared at a 2× concentration in seminal plasma (pooled from 10 HIV-seronegative donors [Lee Biosolutions] 2-fold diluted in DMEM),

and 100 µl of the 2×-concentrated mixtures was added to the wells. Fifty microliters of a predetermined dilution of HIV stock (X4 NL4-3, 1 ng of p24, also in 50% seminal plasma) was placed in each test well. The cultures were incubated at 37°C in 5% CO_2 for 4 h and washed with complete growth medium to remove unbound viruses and compound, fresh growth medium was replaced, and the cultures were returned to the incubator. Infection was scored 48 h later by β-galactosidase activity.

Anti-HIV efficacy evaluation in fresh human PBMCs. Testing of the efficacy of PD against HIV-1 in PBMCs (Biological Specialty Corporation, Colmar, PA) was performed at the Southern Research Institute as described previously (24, 25). Briefly, phytohemagglutinin-stimulated cells from at least two healthy donors were mixed together, diluted in fresh medium to a final concentration of 1×10^6 cells/ml, and plated in a 96-well round-bottom microplate at 50 µl/well (5×10^4 cells/well). Test drug dilutions were prepared at a 2× concentration in microtiter tubes, and 50 µl of the 2×-concentrated mixtures was added to the wells. Fifty microliters of a predetermined dilution of virus stock was placed in each test well (final multiplicity of infection [MOI], ~0.1). Separate plates were prepared identically without virus for drug cytotoxicity studies. The PBMC cultures were maintained for 7 days following infection at 37°C in 5% CO_2. After this period, cell-free supernatant samples were collected for analysis of reverse transcriptase activity (26), and compound cytotoxicity was measured by addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; CellTiter 96 reagent; Promega) to the separate cytotoxicity plates for determination of cell viability. Wells were also examined microscopically, and any abnormalities were noted.

***Lactobacillus* toxicity testing.** Reference strains of *Lactobacillus crispatus* and *L. jensenii* were obtained from ATCC (Manassas, VA) and cultured on Columbia blood agar plates at 35°C in air enriched with 6% CO_2. Bacterial suspensions were prepared in saline or ACES buffer (27) to a density of 2 McFarland units (~2×10^8 bacteria/ml) and exposed to PD (300 µM) or DMSO (10%) for 30 min at 37°C. After incubation, the cells were serially diluted in ACES buffer, pH 7.0 (Sigma-Aldrich, St. Louis, MO), and plated on blood agar plates to quantify the number of CFU per ml (27).

Primary cell toxicity testing. Primary cells were seeded at 6×10^3 cells/well in flat-bottom 96-well plates in triplicate in the presence of increasing concentrations of PD. We used the permeabilization agent saponin (0.1%) as a positive control. After 0, 7, and 14 days, the amounts of lactate dehydrogenase (LDH) in the cell culture medium were quantified using an LDH cytotoxicity assay kit (Cayman Chemical, Ann Arbor, MI).

Cell death can occur either by apoptosis or by necrosis. Necrosis is accompanied by mitochondrial swelling and increased plasma membrane permeability, whereas apoptosis involves an articulated breakdown of the cell into membrane-bound apoptotic bodies. LDH is a soluble cytosolic enzyme that is released into the culture medium following the loss of membrane integrity resulting from either apoptosis or necrosis. LDH activity, therefore, can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from chemical compounds or environmental toxic factors. Cayman's LDH cytotoxicity assay kit measures the LDH activity present in the culture medium using a coupled two-step reaction. In the first step, LDH catalyzes the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate. In the second step of the reaction, diaphorase uses the newly formed NADH and H^+ to catalyze the reduction of a tetrazolium salt [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride] to highly colored formazan, which absorbs strongly at 490 to 520 nm.

HSV infection assays. Vero cells (2×10^5 cells/well) were seeded in a 24-well plate. On the next day, these cells were infected with increasing titers (MOI range, 0.0001 to 1) of HSV-1 (strain Syn 17) or HSV-2 (strain 333) in the presence of PD (2 µM and 200 nM) or DMSO (0.01%) control prepared in DMEM in the absence of serum. At 2 days postinoculation, cells were harvested, fixed with 5% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), stained with antibodies against HSV glyco-

TABLE 1 PD's anti-HIV efficacy in seminal plasma[a]

HIV isolate	Clade	Coreceptor usage	IC$_{50}$ (µM)	IC$_{90}$ (µM)
92RW021	A	R5	0.58 ± 0.04	4.62 ± 0.32
92UG029	A	X4	1.33 ± 0.02	4.71 ± 0.26
92TH026	B	R5	0.43 ± 0.02	2.95 ± 0.18
92HT599	B	X4	1.93 ± 0.03	6.31 ± 0.52
93IN101	C	R5	1.76 ± 0.02	5.36 ± 0.37
98IN017	C	X4	0.45 ± 0.05	2.09 ± 0.19
92UG005	D	R5	1.32 ± 0.02	5.57 ± 0.44
92UG024	D	X4	0.42 ± 0.03	1.58 ± 0.20
92TH006	E	R5	1.96 ± 0.01	6.73 ± 0.51
93TH053	E	X4	1.67 ± 0.02	5.56 ± 0.48
93BR029	F	R5	0.85 ± 0.01	3.72 ± 0.26
93BR020	F	X4	1.61 ± 0.02	7.19 ± 0.62
RU132	G	R5	0.74 ± 0.01	3.28 ± 0.22
Jv1083	G	R5	1.27 ± 0.01	4.22 ± 0.39

[a] TZM-bl cells (1×10^5 cells/ml) were exposed to the indicated HIV isolates (1 ng of p24) in the presence of PD or DMSO diluted in 50% seminal plasma. Cells were washed at 4 h postinoculation, and fresh growth medium was added. Infection was scored 48 h later by β-galactosidase activity. Values and errors represent the means and standard deviations, respectively, of 2 independent experiments carried out in duplicate.

protein gD (Novus Biological, Littleton, CO), and analyzed by flow cytometry (21).

HSV sedimentation assay. HSV was concentrated by loading 30 ml HSV-1-infected Vero cell supernatant on a 20% sucrose cushion and centrifuging in an SW 28 rotor at 20,000 rpm for 1 h at 4°C. Pelleted viruses (~20 µg/ml) were resuspended in 1 ml PBS, exposed to PD (200 nM) or DMSO (0.01%) for 30 min at 37°C, and immediately loaded over a 20 to 70% sucrose density gradient (11 ml). After ultracentrifugation at 30,000 rpm for 24 h in an SW 41 T rotor at 4°C, fractions of 1 ml were collected and analyzed for HSV gB content by enzyme-linked immunosorbent assay (ELISA) using homemade rabbit polyclonal antibody. The density of each fraction from the sucrose gradient was determined by measuring the refractive index.

Statistical analysis. Statistical significance between different samples was evaluated using a two-tailed Student's *t* test in the Microsoft Excel program. A *P* value of 0.05 was considered statistically significant.

RESULTS

Efficacy of PD in seminal plasma. Previously, we showed that PD effectively inhibits several isolates of HIV-1 and SIV in TZM-bl cells at submicromolar to low micromolar concentrations (IC$_{50}$, ~1 µM) when diluted in DMEM or cervical fluid (13). It has been shown that seminal plasma can enhance HIV infectivity (28, 29) and protect HIV against the action of microbicides (30–32). We therefore sought to test the antiviral activity of PD in seminal fluids. Briefly, CD4$^+$ CCR5$^+$ HeLa cells (TZM-bl cells [33–37]) that produce β-galactosidase in response to HIV infection were exposed to 14 different clinical and laboratory isolates of HIV-1, representing various subtypes that use either coreceptor CCR5 (R5 viruses) or coreceptor CXCR4 (X4 viruses), in the presence of PD or DMSO prepared in 50% seminal plasma. After 4 h of incubation of the virus and compound with the cells, cells were washed and the infection was scored 48 h later by β-galactosidase activity. The IC$_{50}$ and IC$_{90}$ of PD against the tested subtypes of HIV-1 ranged from 0.42 to 1.96 µM and 1.58 to 7.19 µM, respectively (Table 1). These values are consistent with those of PD's anti-HIV activity determined in DMEM (0.33 to 1.80 µM for IC$_{50}$ and 1.4 to 6.6 µM for IC$_{90}$) and in cervical fluid (0.61 to 2.30 µM for IC$_{50}$ and 1.80 to 7.50 µM for IC$_{90}$) (13), indicating that seminal plasma does not negatively impact PD's anti-HIV activity.

TABLE 2 Toxicity and efficacy of PD in PBMCs[a]

HIV isolate	Clade	Coreceptor usage	IC$_{50}$ (μM)	IC$_{90}$ (μM)	Therapeutic index
92RW016	A	R5	0.22	0.54	916
92UG029	A	X4	1.18	4.67	170
92HT599	B	X4	0.55	1.92	364
93BR021	B	R5	0.6	4.26	334
96USNG31	C	X4/R5	0.14	1.62	1,425
98IN022	C	R5	0.4	1.48	506
92UG001	D	X4/R5	1.11	1.89	181
RU132	G	R5	0.2	0.53	1,015

[a] The CC$_{50}$ was ~200 μM for all isolates. Freshly activated PBMCs (5×10^4 cells/well) were infected with HIV isolates (MOI = 0.1) in the presence of different concentrations of PD. At 7 days after infection, supernatants were collected and analyzed for reverse transcriptase activity. Compound toxicity was determined using an MTS assay in the parallel uninfected plates. Values represent the means of triplicate samples from one experiment using zidovudine as the positive control (data not shown).

TABLE 3 Toxicity of PD toward commensal *Lactobacillus* bacteria[a]

Bacterial strain	No. of CFU (10^5)/ml		Log(control)− log(PD)
	Control	PD	
Lactobacillus crispatus ATCC 33197	75.8 ± 0.19	72.6 ± 0.55	0.019
Lactobacillus jensenii ATCC 25258	99.8 ± 5.72	75.3 ± 16.8	0.122
Lactobacillus jensenii LBP 28Ab	105 ± 7.2	100 ± 0.124	0.021

[a] Triplicate bacterial suspensions (~2×10^8 bacteria/ml) were separately exposed to PD (300 μM) or 10% DMSO for 30 min at 37°C. After incubation, each suspension was serially diluted and plated on blood agar plates to quantify the number of CFU per ml. Values and errors represent the means and standard deviations, respectively, of triplicate samples.

Efficacy and toxicity of PD evaluated using primary cells. We previously evaluated the cytotoxicity of PD on 7 different human cell lines, including TZM-bl (HeLa) human cervical cells (13). In all cases, PD showed minimal cytotoxicity (CC$_{50}$, >300 μM), giving a therapeutic index (CC$_{50}$/IC$_{50}$) of >300 for HIV-1. In the current study, freshly activated human PBMCs pooled from multiple donors were infected with 8 HIV-1 clinical isolates representing different viral subtypes and tropisms in the presence of different concentrations of PD. The supernatant reverse transcriptase activity was determined 7 days later and used as an indication of HIV infection. The toxicity of PD was determined under identical conditions in the absence of HIV infection and replication. As shown in Table 2, PD exhibited antiviral activity toward all the viral isolates tested, with an average IC$_{50}$ of 0.55 μM (IC$_{50}$ range, 0.14 μM with HIV-1 96USNG31 to 1.18 μM with HIV-1 92UG029). A 48% reduction in cell viability was observed at the highest tested PD concentration (200 μM), resulting in a CC$_{50}$ of ~200 μM, indicating that PD is relatively nontoxic to freshly activated human PBMCs. The therapeutic index of PD ranged from 170 (for HIV-1 92USNG31) to 1,425 (for HIV-1 96USNG31).

To evaluate the toxicity of PD against other primary cells, increasing concentrations of PD were incubated with CD4$^+$ T lymphocytes, macrophages, and dendritic cells for up to 14 days. As

shown in Fig. 1, minimum toxicity was observed in all three types of primary cells (CC$_{50}$s, >300 μM), further pointing to the extremely low cytotoxicity of PD. We also conducted an 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay on these primary cells 7 days after PD exposure (200 μM) and did not observe any significant cytotoxic effect (data not shown).

Toxicity of PD to *Lactobacillus* normal vaginal flora. The vaginal microflora is a key component of the innate immune environment and plays an important role in reducing the risk of HIV infection (38–41). The dominant bacterial species in healthy woman is *Lactobacillus*, which produces lactic acid, hydrogen peroxide, bacteriocins, and other antimicrobial substances that inhibit the growth of pathogenic organisms in the vagina (38–42). PD was evaluated for toxicity toward three strains of *Lactobacillus* normally found in the vagina. These strains were incubated with 300 μM PD or the solvent DMSO (10%) at 37°C for 30 min and plated on blood agar plates to quantify the number of CFU per ml. A less than 1-log-unit difference between the control and test numbers of CFU is considered nontoxic (27). As shown in Table 3, no growth inhibition of any of the strains of bacteria was observed after incubation with PD at a concentration of 300 μM, indicating that PD is nontoxic to commensal *Lactobacillus* species.

PD short-term stability. We sought to determine the short-term stability of PD under conditions that the compound is likely to encounter if used as a microbicide by measuring its antiviral activity at different times. The environment of the vagina is highly
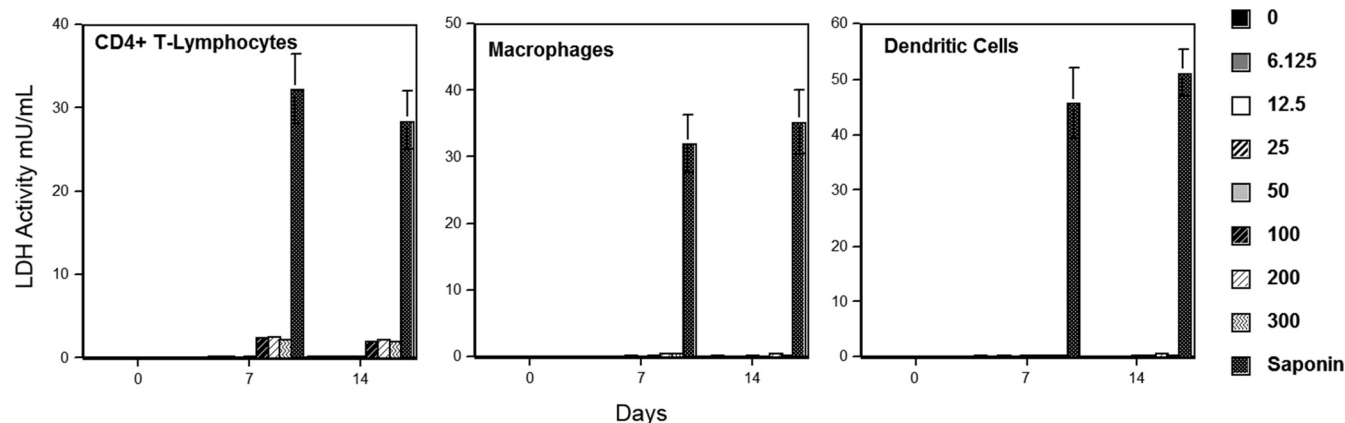

FIG 1 PD exhibits minimum toxicity against primary human cells. Increasing concentrations were incubated with primary CD4$^+$ T lymphocytes, macrophages, and dendritic cells at 37°C, and the amounts of lactate dehydrogenase (LDH) present in the culture medium were quantified after 0, 7, and 14 days. Values and error bars represent the mean and standard deviation, respectively, from two independent experiments.
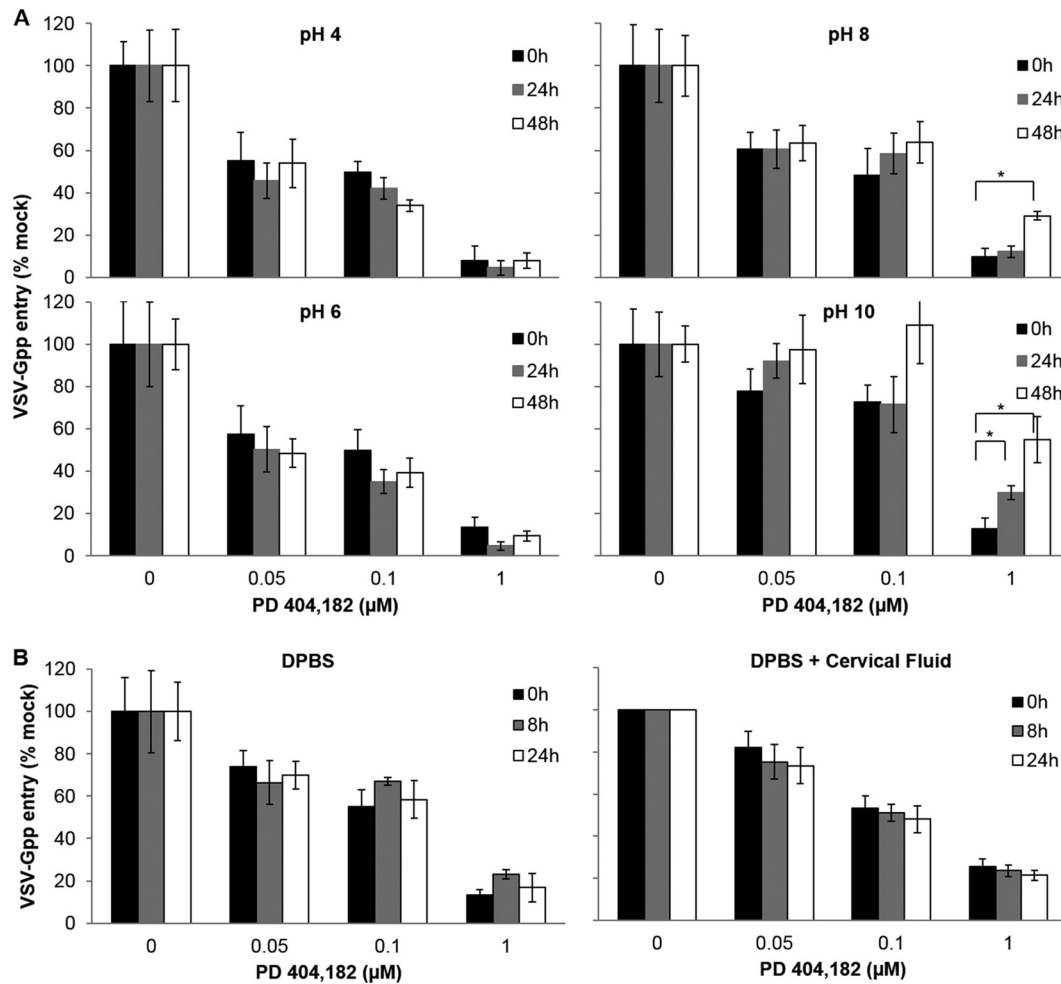
FIG 2 PD is stable and fully active at acidic pH and in cervical fluid. PD (30 µM) or DMSO (10%) was incubated at 37°C for 0, 8, 24, or 48 h in DPBS adjusted to pH 4, 6, 8, or 10 (A) or 20% cervical fluid (the diluent was DPBS) (B). These PD samples were then diluted to the desired concentration in complete growth medium containing VSV-Gpp (viral supernatant diluted 500-fold), and the PD-virus mixtures were incubated at 37°C for 30 min and used to inoculate naive Huh-7.5 or 293T cells at 4°C for 2 h prior to incubation at 37°C in 5% CO_2. The infectivity (virus entry into cells) was quantified by measuring the supernatant luciferase reporter activity at 48 h postinfection. Values and error bars represent the means and standard deviations, respectively, of at least two independent experiments carried out in duplicate. Statistical significance was determined by Student's t test (*, $P < 0.01$).

acidic (pH 3.5 to 4.9) due to the lactic acid produced by the commensal *Lactobacillus* bacteria (43). Exposure to seminal fluid (pH 7.2 to 8) (44) can raise the vaginal pH to 5.8 to 7.2 for several hours (45). Thus, we determined the stability of PD under different pHs at 37°C (Fig. 2A). To ensure that the loss of antiviral activity is not masked by an excess of compound, three different concentrations of PD were used, including two in the vicinity of the IC_{50}. PD retained full activity in acidic buffer at pH 4 and 6 at 37°C. Since PD targets a nonenvelope protein HIV-1 structural component (13), we used HIV-1 pseudotyped with VSV-G (VSV-Gpp) for these studies because this virus is easy to generate to high titers and can be handled in a biosafety level 2 environment. A basic pH of 8 or 10 was observed to compromise PD's activity, but only after extended exposure. For example, PD lost ~50% antiviral activity after incubation in pH 10 buffer for 48 h and lost ~20% activity when exposed to pH 8 for 48 h at 37°C. In contrast, no activity loss was observed for PD after 24 h of exposure to pH 8 buffer, and minimal (~20%) activity loss was seen after 24 h of exposure to pH 10. Taken together, these results indicate that PD will likely be

stable in highly acidic cervical fluid and should remain active for at least several hours upon contact with seminal fluid.

Since cervical fluid is a complex mixture, we next determined the stability of PD in cervical fluid. As shown in Fig. 2B, no activity loss was observed after PD was incubated in 20% cervical fluid for 24 h at 37°C. These results indicate that once-a-day application of PD should be adequate to provide protection against HIV infection. The ability of PD to retain its antiviral potency at a nearly neutral pH suggests that PD may also be formulated as a rectal gel.

PD long-term stability. To evaluate long-term stability, PD was diluted in DPBS buffered at pH 4 or 7 and incubated at 4°C, room temperature, or 42°C. An aliquot was taken every week for determination of antiviral activity. As shown in Fig. 3A, PD was extremely stable when stored in pH 4 buffer and retained full antiviral potency even after 8 weeks at 42°C. At pH 7, PD was stable only at room temperature and 4°C. Storage at 42°C and pH 7 significantly compromised PD activity after 2 weeks.

We also determined the stability of PD formulated in HEC gel at pH 4, as PD is not stable in the presence of HEC gel at pH 7 (data
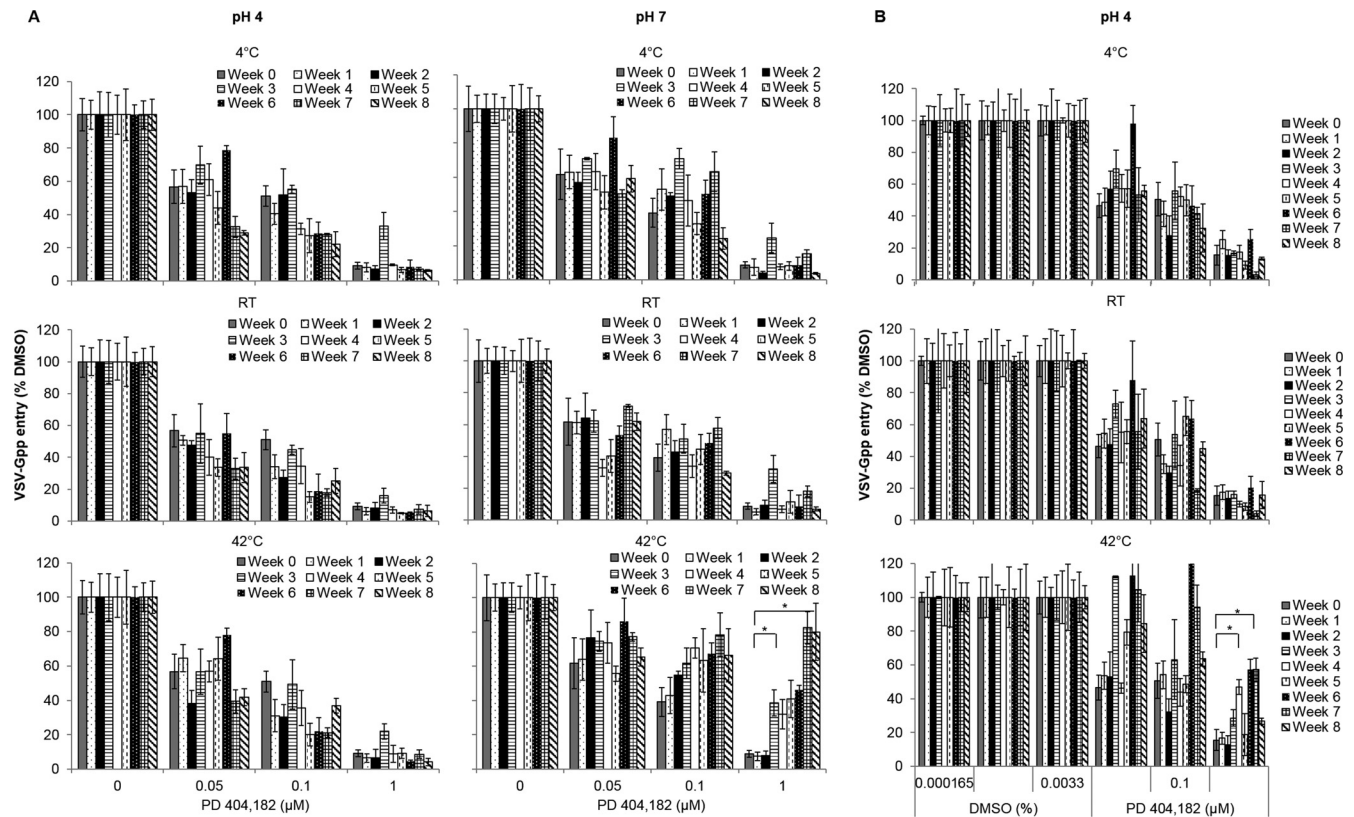
FIG 3 Long-term stability of PD. PD (5 μM) or DMSO (1.67%) was diluted in pH-adjusted DPBS (pH 4 or 7) (A) or 1.5% HEC in DPBS (pH 4) (B) and stored at the indicated temperature for 8 weeks. An aliquot was removed each week, diluted to the desired concentration in complete growth medium containing VSV-Gpp (500-fold diluted), incubated at 37°C for 30 min, and used to infect naive Huh-7.5 cells at 4°C for 2 h prior to incubation at 37°C in 5% CO_2. The viral infectivity was quantified by measuring the supernatant Gluc reporter activity at 48 h postinfection. Values and error bars represent the means and standard deviations, respectively, of duplicate samples. Statistical significance was determined by Student's t test (*, $P < 0.05$).

not shown). PD retained full potency at pH 4 in 1.5% HEC gel at 4°C and RT for at least 4 weeks. However, PD was not stable under the same buffer conditions if stored at 42°C (Fig. 3B) for more than 2 weeks, despite its stability in pH 4 DPBS buffer. Work is under way to determine other suitable formulation conditions for PD storage at high temperatures.

HIV-1 does not acquire resistance to PD after 60 days. With the high rate of HIV mutation, an ideal microbicide should have a high threshold for viral resistance development. To gauge the ability of HIV-1 to acquire resistance to PD, TZM-bl cells were infected with HIV-1 at an estimated MOI of 0.001 to 0.005 and passaged in the presence of 1, 5, and 10 μM PD for 60 days. A similar method was previously employed to successfully evolve HIV-1 resistance against multiple HIV protease/reverse transcriptase inhibitors (46–48) and in resistance evolution of other viruses (49–53). For a classical HIV-1 protease and reverse transcriptase inhibitor with a known mechanism, the supernatant capsid p24 level is usually undetectable for the first 1 to 2 weeks but increases to 1 to 50 ng/ml after 4 weeks. No detectable p24 was measured in the supernatant even after 60 days (see Fig. S1 in the supplemental material), suggesting that no PD-resistant variants evolved during this time frame and also suggesting that PD has a high resistance barrier. The inability of HIV-1 to escape PD inactivation further underscores the potential of PD as an HIV-1 microbicide. We chose TZM-bl cells for the resistance study because these cells can

be passaged for an extended period and remain viable for months even in the presence of viral replication. A similar experiment was performed using freshly activated human PBMCs (from two donors), and no emergence of viral resistance was observed (data not shown). However, HIV-1-infected PBMCs were cultured for only 12 days because significant cell death was observed after this period. We also cultured HSV-1-infected Vero cells in the presence of subinhibitory concentrations of PD for 2 weeks and were not able to detect any viral capsid by ELISA in the supernatant (data not shown).

PD inactivates HSV-1 and HSV-2. We investigated the ability of PD to inactivate HSV-1 and HSV-2. Vero cells were infected with HSV-1 and HSV-2 in the presence of PD (2 μM and 0.2 μM) or DMSO (0.01%). These concentrations were selected on the basis of their closeness to the in vitro IC_{50} of PD against HIV-1. Infection was quantified by the cell surface expression of HSV gD. PD was found to inhibit both HSV-1 and HSV-2 infection at low to intermediate MOIs (MOI range, 0.0001 to 0.1) and exhibited partial protection at an MOI of 1 (Fig. 4A). The data from the lowest concentration are presented. At 2 μM, similar results were obtained, except that the protection curve shifted to the right, leading to full protection at an MOI of 1 and partial protection at an MOI of 10.

Previously, we showed that PD compromises the integrity of retroviruses (13). We thus asked whether PD can disrupt the
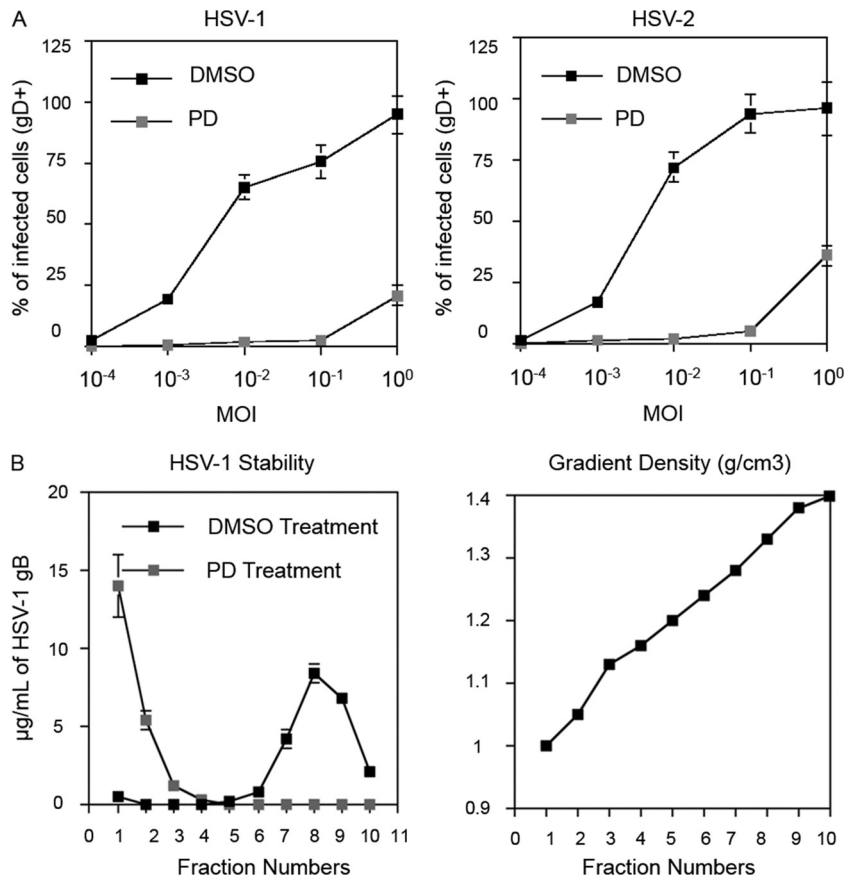
FIG 4 PD is effective against HSV. (A) PD blocks HSV infection. Vero cells were seeded in a 24-well plate and infected with HSV-1 (strain Syn 17) or HSV-2 (strain 333). PD (200 nM) or DMSO (0.01%) control was added to the target cells immediately after viral inoculation. Two days later, the cells were harvested, stained with antibody against HSV glycoprotein gD, and analyzed by flow cytometry. Error bars represent standard deviations of triplicate samples. Results are representative of two independent experiments. (B) PD destabilizes HSV particles. Concentrated HSV ($\sim$20 μg/ml) was incubated with DMSO (0.01%) or PD (200 nM) for 30 min at 37°C, and the mixture was immediately loaded over a 20 to 70% sucrose density gradient. The amount of HSV glycoprotein gB in each fraction was analyzed by ELISA. The sucrose gradient density of each fraction was determined by measuring the refractive index. Results are representative of two independent experiments. Values and error bars represent the means and standard deviations, respectively, of triplicate samples.

structure of HSV virions. Purified HSV-1 virions were resuspended in PBS and incubated with PD (0.2 μM) or DMSO (0.01%) for 30 min at 37°C. After the incubation, the mixture was immediately loaded over a 20 to 70% sucrose density gradient and centrifuged at 30,000 rpm in an SW 41 T rotor for 24 h. Each gradient fraction was analyzed for HSV glycoprotein gB by ELISA. DMSO-treated HSV-1 sediments at a density of 1.24 g/cm³. However, with PD-exposed HSV, all gB distributed to the top of the gradient (Fig. 4B). This result indicates that, like with HIV-1, PD inactivates HSV by compromising virion structural integrity.

DISCUSSION

Four major types of vaginal HIV microbicides have been developed with various degrees of clinical success: surfactants, entry inhibitors, vaginal milieu protectors, and reverse transcriptase inhibitors (6). Surfactants nonspecifically disrupt membranes and were the first molecules to enter clinical trials as candidate HIV microbicides. However, these surfactants were found to be toxic to the cervicovaginal mucosa and resulted in an increased rate of HIV infection in phase III clinical trials (54, 55). Entry inhibitors prevent HIV from binding to or entering cells and encompass a wide range of molecules, including CCR5 inhibitors (56–58) and

fusion inhibitors (59, 60). Many polyanions have also been developed to inhibit HIV entry, and some have been extensively tested in phase III trials, including PRO 2000 (61), cellulose sulfate (62), and Carraguard (63). However, most of these candidates have failed to show *in vivo* efficacy in preventing HIV transmission, partly due to the complexity of the mucosal environment as well as the interference of semen (32, 64). Vaginal milieu protectors are designed to maintain or enhance the protective acidic pH of the vaginal environment through the use of strong buffering agents, such as Carbopol 974 (65), or genetically engineered lactobacilli (66). An agent that is being considered for HIV microbicidal applications in clinical trials, tenofovir, is a nucleotide analogue that inhibits the reverse transcriptase of HIV (12). A 1% tenofovir gel applied before and after sexual intercourse was 39% effective overall in preventing HIV infection in women and 54% effective among highly adherent users of the gel (11). These data are encouraging but nevertheless show that the efficacy of tenofovir microbicidal therapy alone is limited.

In contrast to existing anti-HIV microbicidal candidates, PD inactivates HIV via a novel mechanism. PD is the only nonsurfactant small molecule reported to physically compromise the integrity of HIV, thus rendering the extracellular virus noninfectious

(Table 1 and 2). In addition, PD exhibits low toxicity toward several human cell lines (13), freshly activated PBMCs (Table 2), primary CD4^{+} T lymphocytes, macrophages, and dendritic cells (Fig. 1), and lactobacilli found in the normal vaginal flora (Table 3). It should be noted, nevertheless, that we have yet to test the cytotoxicity of PD under conditions that mimic the high level of basal inflammation typically seen in developing countries. For example, it has been reported that increased levels of immune activation were observed in the genital tract of healthy young women from sub-Saharan Africa (67).

The antiviral potency of PD is not affected by the presence of seminal plasma (Table 1) or exposure to cervical fluid at 37°C for 24 h (Fig. 2B), indicating the potential for a once-a-day application of PD for HIV prophylaxis. The very high stability of PD in acidic pH at both room temperature and 42°C and in neutral pH at room temperature (Fig. 3A) indicates that PD can be easily formulated for convenient transportation and storage in developing countries lacking refrigeration facilities. In the current study, we evaluated the stability of PD when formulated in 1.5% HEC gel. Surprisingly, PD was not stable when formulated in HEC gel at pH 7 (data not shown), although PD formulated in HEC gel at pH 4 retained full potency after 4 weeks at ambient temperature (Fig. 3B). Studies are under way to investigate the reason for PD's instability in HEC gel at neutral pH. One hypothesis that we are exploring is that PD reacts with the deprotonated hydroxyl group in HEC.

Genital herpes has been found to increase the vulnerability to HIV-1 infection by compromising the integrity of the mucosal barrier (68–70). Most genital herpes is caused by HSV-2 infection, although in some cases it can also be caused by HSV-1 (71–73). In one study, 50 to 90% of HIV-1-infected patients tested seropositive for HSV-2 (74), and HSV-2 infection was found to increase the rate of HIV-1 acquisition by 3-fold (72). Due to the synergy between HIV and HSV, a topical microbicide with dual action against both pathogens may more effectively reduce HIV transmission.

In addition to being an HIV microbicide, PD could also inactivate HSV at nanomolar concentrations. Genital herpes is one of the most prevalent sexually transmitted diseases worldwide and is the common cause of genital ulcers (75). Ulcerations can disrupt the mucosal barrier and abrogate the protective barrier function of the epithelium, allowing HIV-1 to reach the subepithelial dendritic cells susceptible to HIV-1 infection (16) and increasing the risk of HIV acquisition (76). Currently, there is no approved vaccine for HSV, and therapeutic treatment for genital herpes involves repeated dosing of antiviral drugs. Development of topical microbicides that are effective against both HIV and HSV may provide a more effective strategy to prevent HIV-1 infection/transmission. We showed that 0.2 μM PD physically compromises the integrity of extracellular HSV and effectively blocks the infection of both HSV-1 and 2 in vitro, in a manner similar to PD's action on HIV-1 (Fig. 4).

In the past 5 years, several broad-spectrum antiviral compounds have been discovered. For example, the rigid amphipathic fusion inhibitors (RAFIs) (77, 78) and lysophosphatidylcholine (79) inhibit virus-host fusion by inducing positive membrane curvature, while LJ001 compromises virion integrity by intercalating into the viral lipid membrane (80). There are others, like the C5A amphipathic peptide derived from HCV NS5A protein (81, 82) and alkylated porphyrins (83), whose mechanisms of virucidal action remain somewhat mysterious.

Finally, it is worth noting that a number of factors tend to raise

the vaginal pH. For example, the seminal plasma can raise the vaginal pH to 7.2 to 8 after intercourse (44). Women diagnosed with *Trichomonas vaginalis* infection or bacterial vaginosis (BV) have vaginal pHs that range from 4 to 7 (84, 85). However, as shown in Fig. 2, PD retains most of its potency at pH 8 for over 24 h at 37°C. Thus, PD should remain effective in preventing HIV/HSV infection even in these individuals.

In summary, we demonstrated that the virucidal small molecule PD possesses several attributes that lend support to its use as a microbicide for combating HIV spread. These attributes include full activity and high stability in the fluids and pHs encountered physiologically, a lack of toxicity to freshly activated human PBMCs and vaginal commensal bacteria, and activity toward another virus—HSV—that exacerbates the pathogenicity of HIV. Future studies will focus on (i) formulation of PD into a topical form that promotes high PD activity and stability and (ii) evaluation of the toxicity and efficacy of PD in animals and humans.

ACKNOWLEDGMENTS

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