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RESEARCH ARTICLE

(4-Benzyloxy-Phenylmethylene)-Bis-Diisopentanoyl Phloroglucinol: Evaluation of Anti-HIV-1 Properties and Use as HIV-1 Microbicide Candidate

Sudeep Sabde [a*], Sanjay Toshniwal [b]

[a]Shri Jagdishprasad Jhabarmal Tibrewala University, Vidyanagari, Jhunjhun-Churu Road, Jhunjhunu, Rajasthan, India 333001

[b]Vidarbha Institute of Pharmacy, Washim, Maharashtra, India 444505

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ABSTRACT

HIV-1 transmission occurs mostly through sexual contact with infected individual. HIV crosses vaginal mucosa and HIV-1 can infect dendritic cells, macrophages and T lymphocytes in the submucosa and then target circulating CD4+ T cells. In the developing world, women have limited freedom to choose sexual situations or to insist on condom use. In such scenario, microbicide would prove an effective prevention tool. Therefore, the development of an anti-HIV microbicide is extremely important. 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 (RT) inhibitors. We have evaluated 4-BPMDP, a potent reverse transcriptase inhibitor, for its spectrum of anti-HIV-1 activity in hPBMCs. 4-BPMDP significantly inhibited several strains of HIV-1 including X4, R5 and X4R5 dual tropic viruses. It also inhibited replication of subtype C R5 tropic clinical isolates at low micromolar concentrations. Further we investigated 4-BPMDP as microbicide candidate. 4-BPMDP is nontoxic to vaginal epithelial cells as evident from cytokine analysis. In dual chamber model system of cervical epithelial cells, 4-BPMDP moderately inhibited transmission of HIV-1_{NL4-3}.

Our data indicate that 4-BPMDP displays potent anti-HIV-1 activity and could serve as tools for the identification of novel anti-HIV targets. However further chemical modification would be needed to enhance its HIV-1 microbicidal properties.

Keywords: HIV-1; 4-BPMDP, Tenofovir; Azidothymidine; Microbicide, cytokines.

1. INTRODUCTION

Transmission of HIV-1 occurs primarily through sexual contact in which mucosal surfaces of the genital are exposed to HIV and/or HIV-infected cells. Epithelial cells in genital and gastrointestinal tracts do not express CD4, but HIV-1 can bind to the cell membrane using galactosyl-ceramide [1]. After crossing the epithelial barrier, HIV-1 can infect dendritic cells, macrophages and T lymphocytes in the submucosa and then target circulating CD4+ T cells. HIV then disseminates, initially to the lymph node, and subsequently to secondary lymphoid organs, to generate a systemic infection. Once in the systemic circulation, virus targets cells of the immune system and induce cell death leading to AIDS[2].

The introduction of highly active antiretroviral therapy (HAART) has significantly decreased morbidity and mortality among patients infected with human immunodeficiency virus type 1 (HIV-1). Although HAART regimens have improved the prognosis for HIV-infected individuals, challenges to effective use of these therapeutic strategies remain, including issues of adherence, side effects and toxicities, drug resistance, and persistent viral replication in latent reservoirs [3;4]. In the developing world, effective prevention strategies are lacking, often because women have limited freedom to choose sexual situations or to insist on condom use. Therefore, the development of an anti-HIV microbicide is extremely important[5].

*Corresponding author: Sudeep Sabde | Shri Jagdishprasad Jhabarmal Tibrewala University, Vidyanagari, Jhunjhun-Churu Road, Jhunjhunu, Rajasthan, India 333001| Email: sudeepsabde@gmail.com

In past few years several molecules have been evaluated as HIV-1 microbicide candidate. The disappointing results of several phase III microbicide effectiveness trials evaluating the spermicide nonoxynol-9, followed by SAVVY (C31G), cellulose sulfate, Carraguard, BufferGel, and finally PRO 2000, were a major setback for development of HIv-1 microbicide [6;7].

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 (RT) inhibitors[8].

In 2010, CAPRISA trial for the first time showed that use of tenofovir, a reverse transciptase inhibitor as 1% microbicide gel can prevent sexual transmission of HIV.

Tenofovir now represents the first vaginal microbicide proven to be safe and efficacious in the primary prevention of HIV in women. This proof-of-concept has invigorated the field with renewed optimism[9].

Several studies are underway investigating nonnucleoside reverse transcriptase inhibitors (NNRTI) anti-HIV microbicides such as dapivirine and UC781. UC-781 is a thiocarboxanilide nonnucleoside reverse transcriptase (RT) inhibitor (NNRTI). It binds to RT with very high affinity. The DABO compounds, UC-781, MIV-150 readily cross membrane barriers and irreversibly inactivate RT [9;10]. Development of effective microbicide will likely require a combination of drugs that target different steps in the HIV life cycle.

In our continuous efforts to find out new molecules to fight against AIDS, we have recently reported anti-HIV-1 activity of several dimeric phloroglucinols. Seven dimeric phloroglucinols have shown potent activity against HIV-1NL4-3 in $CD4^+$ CEM-GFP T cells.

1-[3-Isopentanoyl-5-[(3,5-diisopentanoyl-2,4,6-trihydroxyphenyl)-4-benzyloxyphenyl-methyl]-2,4,6-trihydroxyphenyl]-3-methylbutan-1-one (compound 24, hereafter referred as (4-benzyloxy-Phenylmethylene)-bis-diisopentanoyl phloroglucinol or 4-BPMDP) has shown potent HIV-1 RT inhibition with IC_{50} 1.03µM[11].

We were interested in the study of the anti-HIV-1 activity of (4-benzyloxy-Phenylmethylene)-bis-diisopentanoyl phloroglucinol or 4-BPMDP and its evaluation as microbicide candidate.

In this report we have investigated spectrum of anti-HIV-1 activity of 4-BPMDP in primary culture of human peripheral blood mononuclear cells against X4 and R5 tropic viruses and subtype C clinical isolates of HIV-1. Further we demonstrated the effect of 4-BPMDP on secretion of proinflammatory cytokines in human cervical epithelial cells. The ability of 4-BPMDP to reduce transmission of HIV-1 across epithelial barrier was assessed.

2. METHODS:

Compounds: 1-[3-Isopentanoyl-5-[(3,5-diisopentanoyl-2,4,6-trihydroxyphenyl)-4-benzyl oxyphenyl-methyl]-2,4,6-trihydroxyphenyl]-3-methylbutan-1-one (compound 24 in earlier report, hereafter referred as (4-benzyloxy-Phenylmethylene)-bis-diisopentanoyl phloroglucinol or 4-BPMDP) was synthesized as reported earlier [11]. Zidovudine (AZT) was obtained from Sigma Aldrich (USA). Tenofovir was obtained from National Institute of Health AIDS Research and Reference Reagent Program, USA.

Cells and viruses:

ME180 cells[12] were obtained from the cell repository of National Centre for Cell Science, India. ME180 cells were propagated in McKoy's 5a medium containing 10% FBS and 0.1% penicillin and streptomycin. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors and purified using the Histopaque (Sigma, USA) density gradient centrifugation. The cells were then stimulated with 2µg/ml PHA for further 36h.

Viruses: HIV-1 CXCR4 tropic molecular clone $NL_{4.3}$ and IIIB, R5 tropic strains HIV-1 ADA, HIV-1 and JRCSF, X4-R5 dual tropic strains 89.6 and Yu-2 were obtained from NIH AIDS reagent program, USA. HIV-1 Indie C1, a full length molecular clone of HIV-1 subtype C Indian isolate was a kind gift of Dr M Tatsumi, Japan[13]. Primary subtype C isolates VB51 and VB52 were obtained from National AIDS Research Institute, India.

Cell Viability assay: To assess toxicity of compounds on hPBMCs, 10^4 cells were added to each well of 96 well plate just before addition of compounds. For assessment of toxic effects of compound on vaginal epithelial cells, 10^4 Me180 cells/well were seeded in 96 well plate and incubated overnight at 37^0C CO_2 incubator. Cells were then treated with compounds. Viability of PBMCs and Me180 cells was assayed using MTT Kit (Roche) according to the manufacturer's protocol as described earlier [14;15].

Anti-HIV activity assay in human PBMCs. Human PBMCs were used for anti-HIV activity assay as described previously[16]. Cells were infected with 0.05MOI of HIV-1_{NL4-3}. Cells were stimulated by the addition of 20U/ml recombinant human interleukin-2 (Roche, Germany) into the culture medium. Cells were added to 24 well plates and compounds were added at different concentrations. AZT was used as a positive control. Culture was continued for 7 days. To determine the level of virus inhibition, cell-free supernatant samples were collected for analysis of p24 protein by using HIV-1 p24 antigen core ELISA (Perkin Elmer Life Science, USA). For anti-HIV-1 activity against other X4, R5 and dual tropic viruses same procedure was followed.

For infection of PBMCs with primary isolates 5 ng of virus were used to infect 5 X 10^6 cells.

Evaluation of compounds as potential HIV-1 microbicde

Measurement of transepithelial resistance:

5 X 10^5 ME180 cells were added to laminin coated transwell inserts of plate (0.4µm polycarbonate, 6.5mm inserts, Costar). Transepithelial electrical resistance was measured on day 9 after addition of fresh medium, in presence or absence of compounds, using Millicel ERS potentiometer (Millipore, USA) and fluorescent beads were used to check confluence of epithelial layer of ME180 cells as described earlier[17].

Effect of compounds on secretion of proinflammatory cytokines

2×10^5 ME180 cells were cultured overnight at 37^0C in CO2 incubator. Medium was replaced and cells were treated with nontoxic concentrations of compounds for 24h. Culture supernatants were collected and then assayed for presence of proinflammatory cytokines like IL1b, IL6, IL8, IL10, RANTES and TNFα by using multiplex bead based array in Bioplex 200 system (BioRad, USA) according to manufacturer's protocol.

Transepithelial migration of HIV-1 NL4.3 virus in the dual chamber model system

Once ME180 cells reached confluence, HIV-1 NL4.3 virus (0.05MOI) and the compound mixture was added in different laminin-coated (200 ng/ml) apical chambers of a dual chamber system. Subsequently, the apical chamber was inserted in a 24-well cup (basal chamber) with co-cultures of 2×10^5 PBMCs per well. After 24 h, the apical chamber was removed from the dual chamber system and the cells in the basal chamber were further cultured for 10 days in complete medium. On day 5, 0.5 ml of the culture medium was harvested and replaced by 0.5 ml of complete medium. At day 10, supernatants of primary cultures were harvested, to assess p24 gag protein levels produced in control and treated wells. Tenofovir was used as a positive control in this experimental system.

3. RESULTS

4-BPMDP inhibits infection of hPBMCs

hPBMCs comprises monocytes, lymphocytes, macrophages, etc. hPBMCs are a critical component in the immune system to fight infection. HIV-1 after sexual transmission spread to systemic circulation and infect hPBMCs[18;19]. We first evaluated the cytotoxic effects of 4-BPMDP on hPBMCS. We treated hPBMCs with different concentrations of compound. 5.7µM was found to be nontoxic concentration. We have initially assessed the ability of 4-BPMDP to inhibit infection of hPBMCs by HIV-1_{NL4-3}. For this study, hPBMCs were infected with HIV-1_{NL4-3} virus and then treated with compound. 7 days post infection, cell free culture supernatants were assessed for production of HIV-1 virus particles by using HIV-1 p24 antigen ELISA. In earlier report, 4-BPMDP exhibited 70% inhibition of CEM-GFP T cells by HIV-1_{NL4-3}. We observed that compound exhibited better activity in hPBMCs culture than in CEM-GFP T cells. As shown in Table1, 4-BPMDP showed potent inhibition (80%) of infection at 5.7µM, AZT at 5 µM exhibited 96% inhibition of infection of hPBMCs by HIV-1_{NL4-3}. CC_{50} and IC_{50} concentrations of 4-BPMDP were found to be 21.65 µM and 2.5 µM respectively (Table1).

CODE	Highest noncytotoxic Concentration	% Inhibition	IC $_{50}$ µM	CC $_{50}$ µM
4-BPMDP	5.7	80.66 ± 1.7	2.5 ± 0.3	21.65 + 1.7
AZT	5	96.28 ± 1.5	1.17 ± 0.07	44.06 + 0.57

Table 1: Cytotoxicity and anti-HIV-1 activity of 4-BPMDP in hPBMCs infected with HIV-1_{NL4-3}

HIV-1 inhibition activity of 4-BPMDP against X4, R5 and X4R5 dual tropic viruses

HIV-1_{NL4-3} is a X4 tropic HIV-1 virus. To further evaluate the anti-HIV-1 activity of 4-BPMDP, we used X4 tropic, R5 tropic and dual X4R5 tropic virus strains. The compound exhibited broad spectrum of anti-HIV-1 activity against all virus. 4-BPMDP inhibited replication of all viruses by 70-80% in hPBMCs with highest activity 84.65 observed against subtype C R5 tropic viruses JRCSF (Table 2).

4-BPMDP exhibits potent activity against clinical isolates

To be an effective anti-HIV drug, the compound should show inhibition of infection by clinical viral isolates. In order to see effect of 4-BPMDP, hPBMCs were infected with subtype C VB51 and VB52 viral isolates. 4- BPMDP showed potent activity against these isolates. It inhibited infection of PBMCs by VB51 and VB52 virus isolates upto 88.63 and 92.63% respectively. AZT at 5 µM was also found to inhibit VB51 and VB52 infection upto 95% (table 2).

Our data suggest that the 4-BPMDP possess potent anti-HIV activity and is effective against various strains of HIV-1 and clinical isolates as well.

		X4 tropic	R5 tropic subtype C			X4 and R5 dual tropic viruses		Clinical Isolates	
Code	Highest concentration (µM)	IIIB	ADA	JRCSF	Indie C	89.6	YU2	VB51	VB52
4-BPMDP	5.7	72.6 ± 3.4	74.8 + 1.7	84.6 + 1.2	81.3 ± 1.5	71.93 ± 2.1	78.59 + 0.9	88.63 ± 3.2	92.63 ± 2.7
AZT	5	92.93 + 5.1	92.93 + 2.8	92.95 + 1.9	87.1 + 4.4	92.54 + 0.7	82.89+ 1.7	95.64 + 1.2	95.93 + 0.1

Table 2: 4-BPMDP inhibits infection of hPBMCs by X4, R5 and X4-R5 dual tropic strains and subtype C isolates of HIV-1

Evaluation of 4-BPMDP as microbicide candidate

Effect of 4-BPMDP on vaginal epithelial cells

Several candidate microbicides have shown cytotoxicity to epithelial layer resulting in increased rate of transmission of HIV[20;21]. In order to see the cytotoxicity of the compound on cervical epithelial cell line, ME180, we first analyzed the CC50 of the compound using MTT assay. ME180 cells were treated with 4-BPMDP and tenofovir at different concentrations. The Cytotoxicity data indicated that CC_{50} of 4-BPMDP and tenofovir were 27µM and 272 µM respectively (figure 2a).

We then evaluated the effect of these compounds on secretion of pro-inflammatory cytokines by ME180 cells. ME 180 cells were treated with 5.7 µM of 4-BPMDP and 8 µM of Tenofovir. Triton X-100 was used as cytotoxic control. As shown in figure 1, addition of 4-BPMDP compounds did not exert significant effect on secretion of inflammtory cytokines like IL1b, IL6, IL8, IL10, RANTES and TNFα after 24h. The positive control Triton X-100 showed a significant induction of cytokines compared to the basal levels. Overall the data indicates that 4-BPMDP is nontoxic and safe for evaluation as potential microbicide candidate.

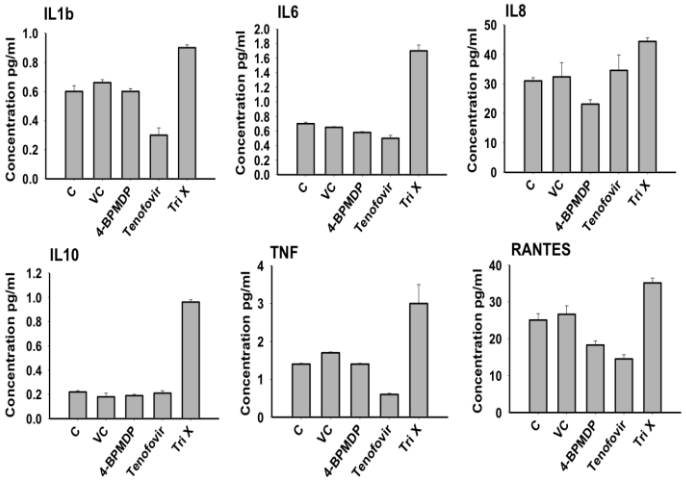

Figure 1: Effect of 4-BPMDP on cytokine secretion by ME180 cells.

ME180 cells were treated with 5µM of 4-BPMDP and 8 µM of tenofovir and Triton X-100 for 24 h. Culture supernatants were collected and assessed for cytokine levels using bead based multiplex assay.

Prevention of transmission of HIV-1 across epithelial barrier by 4-BPMDP

HIV-1 is thought to be sexually transmitted by genital epithelium transmigration followed by dendritic cell and/or langerhans cell mediated capture and transmission to $CD4^+$ T cells [22;23]. In order to see effect of 4-BPMDP on transepithelial cell migration and infection of monocytes and T cells, we used dual chamber model comprising an apical chamber having ME180 epithelial cell layer and hPBMCs in basal chamber. ME180 cells form confluent monolayers when grown on insert chambers. They are characterized by numerous desmosomes connecting adjacent cells, and prominent epidermal filaments. These morphological characteristics are typical for stratified squamous epithelia such as those which line the cervix, and urethra.

Completely confluent ME180 cells in apical chamber were incubated with activated human PBMCs in basal chamber. HIV-1 NL4-3 virus (0.1MOI) was added to apical chamber in presence or absence of compounds. After 6 h of infection, apical chambers were removed and culture supernatants collected on day 5 from hPBMCs were assayed for presence of p24 antigen. We found that 4-BPMDP showed 30% inhibition of infection of hPBMCs while tenofovir inhibited infection upto 86.49% (figure 2b).

As evident from our results, 4-BPMDP moderately inhibited HIV-1 transmission across epithelial cells.

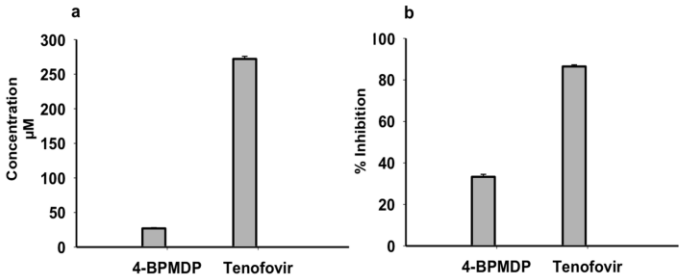

Figure 2: Cytotoxicity and Microbicidal activity of 4-BPMDP.

a, ME180 cells were exposed to different concentration of 4-BPMDP and tenofovir to assess Cytotoxicity. CC50 concentration for both compounds was calculated. 4-BPMDP showed CC_{50} 27 µM and tenofovir displayed CC_{50} 272 µM. **b**, In dual chamber model system, ME180 cells in apical chamber were grown to confluence and then treated with compounds 4-BPMDP (5µM) and Tenofovir 8 µM and HIV-1_{NL4-3}. Infection of hPBMCs by transmitted HIV-$1_{NL4-3,}$ in basal chamber was assessed using p24 antigen ELISA according to manufacturer's protocol. % inhibition was calculated by comparing p24 values of compound treated cells to untreated control cells.

4. DISCUSSION

HIV-1 RT is one of the major targets of the currently available anti-retroviral drug therapies for the treatment of HIV / AIDS. Non-nucleoside reverse transcriptase inhibitors (NNRTI's) are proving to be an important component of highly active anti-retroviral therapy (HAART). In particular, the use of NNRTIs in combination with nucleosides is an excellent first line therapy for HIV / AIDS patients and it has been used extensively in recent years. However HAART is often associated with drug resistance and hepatic and renal toxicity[24;25].

Therefore, it is imperative to look for the new scaffold having broad spectrum activity against a variety of X4, R5 tropic isolates of HIV.

To begin with, we first explored anti-HIV-1 properties of (4-benzyloxy-Phenylmethylene)-bis-diisopentanoyl phloroglucinol in primary culture of hPBMCs. 4-BPMDP

showed potent activity against HIV-1_{NL4-3} in hPBMCs with IC_{50} 2.5µM. The data indicated that anti-HIV-1 activity of 4-BPMDP is independent of cell type.

In order to further explore spectrum of activity of 4-BPMDP, we used X4 tropic, R5 tropic and X4R5 dual tropic viruses. 4-BPMDP exhibited inhibition of replication of all these viruses in hPBMCs. 4-BPMDP showed more or less similar anti-HIV activity against all the virus strains used independent of their co receptor use. The activity of 4-BPMDP was similar to that of AZT.

In order to see the effects of 4-BPMDP on replication of clinical isolates, we used subtype C clinical isolates VB51 and VB52. Subtype C HIV isolates are more prevalent in Asia, Africa [26;27]. 4-BPMDP exhibited significant inhibition of replication of these isolates in hPBMCs culture.

Our data suggest that 4-BPMDP; a dimeric phloroglucinol possesses potent anti-HIV-1 activity against several strains of HIV-1 isolates.

The anti-HIV microbicides currently undergoing clinical evaluation are non-specific surfactants or non-specific anionic polymers that variably interfere with virus binding/fusion/entry [28;29]. Insights into sexual transmission of HIV and its spread to systemic infection has helped the development of compounds that target specific viral–host cell interactions and has allowed for a more tailored approach to microbicide development. With the success of antiretroviral therapy in the treatment of HIV disease, interest has grown in using these more targeted drugs for prevention of the sexual transmission of HIV. A major challenge has been to design mechanism-based microbicides that are highly effective against HIV-1 and maintain intact vaginal mucosa. This is especially important because currently available over-the-counter detergent-based spermicidal microbicides have been shown to damage the cervicovaginal epithelium, cause an acute inflammatory tissue response, and enhance the risk of promoting opportunistic infections in the genito-urinary tract [30;31].

Preclinical safety testing includes the assessment of toxicity in cell based assays e.g. epithelial cell lines, peripheral blood mononuclear cells[32].

In order to investigate 4-BPMDP as microbicide candidate, we first assessed the Cytotoxicity of compound using vaginal epithelial cells ME180. 4-BPMDP was found to be less toxic to ME180 cells than hPBMCs.

Thorough investigation of the toxicity and irritation potential of microbicide formulations is critical, since in the past some microbicides that have caused irritation to genital tissue also led to higher infection rates in women compared to placebo[32;33]. Hence we assessed effect on secretion of cytokines by ME180 cells upon treatment with 4-BPMDP.

Neither 4-BPMDP nor tenofovir were found to activate cytokine secretion by ME180 cells, however triton X-100 induced marked secretion of cytokines.

In an *In vitro* dual chamber model for evaluating microbial activity, 4-BPMDP exhibited moderate microbicidal activity. From our data, it is clear that 4-BPMDP moderately affected transmission of HIV-1 and infection of hPBMCs.

In conclusion, we demonstrated that 4-BPMDP is effective against infection by a broad spectrum of HIV-1 strains, including both Indian clinical isolates and laboratory-adapted HIV-1 strains with IC50 at low µM levels. 4-BPMDP has no effect on cytokine secretion by epithelial cells however at non cytotoxic concentration it is able to reduce transmission of HIV-1 moderately. 4-BPMDP scaffold can be considered for future development of new molecules with high potency and efficacy to treat HIV-infection.

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