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Evaluation of dendrimer SPL7013, a lead microbicide candidate against herpes simplex viruses

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This paper is dedicated to the memory of Professor Stephen L. Sacks, our cherished colleague, mentor and great man to be around.

Abstract

Dendrimers are a novel class of polyanionic macromolecules with broad-spectrum antiviral activities and minimal toxicities. A new generation of amide dendrimer, SPL7013, was evaluated as a lead microbicide candidate against herpes simplex viruses (HSV). The plaque reduction assays showed that the 50% effective concentrations (EC_{50}) determined by pre-treatment of cells were 2.0 μg/ml for HSV-1 and 0.5 μg/ml for HSV-2. Inhibitory effects were also observed on HSV-infected cells with EC_{50}s of 6.1 μg/ml for HSV-1 and 3.8 μg/ml for HSV-2. These are the mean values from the test results of six batches of SPL7013. SPL7013 was also shown to be equally potent against HSV drug-resistant strains. SPL7013 completely inhibited viral adsorption to Vero cells at concentrations of higher than 3 μg/ml. Analyzed by a LightCycler assay after treatment of HSV-infected cells for 17 h, SPL7013 showed strong inhibition of HSV DNA synthesis with EC_{50}s of approximately 6.2 and 2.0 μg/ml for HSV-1 and HSV-2, respectively. SPL7013 retained its anti-HSV activity even after treatment at acidic pHs 3.0 and 4.0 for 2 h. The presence of 10% human serum proteins did not affect the anti-HSV activity of SPL7013. SPL7013 was not toxic to Vero cells up to the highest concentration tested (10,000 μg/ml). Effects on cell proliferation were tested on two epithelial cell lines in both stationary and dividing phases. The 50% cytotoxic concentrations (CC_{50}) in all cases were greater than 10,000 μg/ml. Our data indicate that SPL7013 is a promising candidate for development as a vaginal microbicide and a therapeutic agent.

Keywords: Dendrimer SPL7013; Herpes simplex viruses; Antiviral activity; Microbicide

1. Introduction

Genital herpes infection is one of the world's most prevalent sexually transmitted diseases (STDs). It is typically the outcome of infection by HSV-2; although HSV-1 also causes genital infection, it is more commonly the cause of labialis or 'cold sores'. Both HSV-1 and HSV-2 infections are life-long infections with frequent recurrences (for reviews see Boehmer and Lehman, 1997; Roizman and Knipe, 2001; Whitley, 2001). It is estimated that several hundred million individuals worldwide are infected with STDs including HSV, human immunodeficiency virus (HIV) and human papillomaviruses (HPV). The development of safe topical microbicides is of great importance in the field of STD prevention (The International Working Group on Vaginal Microbicides, 1996; Potts, 2000). One approach to the control of transmission of STDs is the use of topically applied, female-controlled microbicides that: (i) inactivate the relevant pathogens by disrupting the organism cell membrane or by dissociating organism proteins (Krebs et al., 1999); (ii) block the receptor–ligand interactions essential for infectivity (Herold et al., 1997); or (iii) alter the vaginal environment and reduce susceptibility to infection (e.g., buffering gels). Spermicidal preparations of surfactants, such as nonoxynol-9 (N-9), that inactivate enveloped viruses like HSV, have been investigated as vaginal microbicides (Pope et al., 1998). Unfortunately N-9 was found to increase the rate of HIV transmission by 50%.

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This result can be rationalized in hindsight based on N-9's mode of action and is now not recommended as an effective means of HIV prevention (Mauck, 2001; Van Damme et al., 2002). Consequently, there is an urgent need to develop novel, non-toxic compounds that can efficiently reduce sexually transmitted infections.

Dendrimers are a new class of precisely constructed macromolecules that are characterized by multiple layers of branched subunits emanating from a central core and are constructed by the repeated stepwise addition of branched subunits to a reactive core (McCarthy et al., 2005; Bernstein et al., 2003; Bourne et al., 2000; Gong et al., 2002; Witvrouw et al., 2000). We have previously shown the dual sites of action of dendrimer SPL-2999, an inhibitor of HSV internalization and an effective agent for the treatment of HSV-infected cells (Gong et al., 2002). The new generation of lead dendrimer, SPL7013, has been proven to have better activities against HSV (Boune et al., 2001; Bernstein et al., 2003) and is active against HIV (McCarthy et al., 2005; Dezzutti et al., 2004), shows strong protection in a macaque SHIV vaginal challenge model (Jiang et al., 2005) and is easy to synthesize. It is therefore an ideal microbicide for the prevention and treatment of HSV infections.

In the present study, we have evaluated the antiviral efficacy, mechanism of action, and toxicity of SPL7013, a lead microbicide against HSV infections. We also report the generation and phenotypic characterization of dendrimer-resistant HSV-1. The antiviral efficacies of SPL7013 in different pHs and in the presence of human serum proteins were also evaluated.

2. Materials and methods

2.1. Test compound

Dendrimer SPL7013 was synthesized by Starpharma Limited, Melbourne, Australia. The chemical formula of SPL7013 is BHA.lys15lys16(NHCOCH2O)1-(3,6-naphth(SO_3Na)$_{32}$ (BHA: benzhydrylamine) and it has a molecular weight of 16,581 Da. The structure of SPL7013 was described by McCarthy et al. (2005).

2.2. Virus and cells

HSV reference strains F (HSV-1) and G (HSV-2) were purchased from American Type Culture Collection, Manassas, VA (ATCC). Strain delta 333 is a laboratory HSV-2 TK$^-$ (thymidine kinase) mutant with phenotypic resistance to both penciclovir and acyclovir. Strain 615.8 is a HSV-1 DNA polymerase mutant with phenotypic resistance to foscarnet. Both were gifts from Dr Donald Coen, Harvard Medical School, MA. The Vero cells (African green monkey kidney cells) were purchased from ATCC and used in the virus plaque reduction assays. The culture medium for Vero cells was Minimum Essential Medium supplemented with 5% fetal bovine serum (MEM/5%FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Two epithelial cell lines, Hela-229 and Hep-2, were also purchased from ATCC. The culture media for these cell lines were as recommended by ATCC.

2.3. Virus infectivity assays

Viral plaque reduction assays were performed according to the guidelines of the NCCLS (National Committee for Clinical Laboratory Standards). The cytopathic effect (CPE) inhibition assay, virus adsorption assay, time-of-addition experiment, and cytotoxicity assays were performed as previously described (Gong et al., 2002).

2.4. Effect on HSV DNA synthesis

Confluent Vero cells were infected with either HSV-1 or HSV-2 at a multiplicity of infection (moi) of 0.01 at 37 °C for 1 h. Following removal of virus inoculum, infected cells were washed with PBS and overlaid with medium containing SPL7013 at concentrations of 0, 0.01, 0.1, 1, 10, and 100 μg/ml. At 17 h post-infection (p.i.), the medium was aspirated and the cells were washed with PBS. Four hundred microliters of lysis buffer (0.01 M Tris–HCl, pH 7.8, 0.001 M EDTA and 0.5% SDS) was added to each well. The lysates from triplicate wells were pooled. The cell lysates were then digested with 500 μg/ml of proteinase K at 37 °C for 3 h. The DNA was extracted by using a standard phenol/chloroform method (Sambrook et al., 1989).

A real-time PCR assay (Lightcycler) was used for quantitation of viral DNA. The reaction in a volume of 20 μl contained 2 mM $MgCl_2$, 0.5 μM each of the primers HSV 5911 (5′-CAG TAC GGC CCC GAG TTC GTG A-3′) and HSV 6367 (5′-GTA GAT GGT GCG GGT GAT GTT-3′), 0.2 μM of the fluorescein-labeled hybridization probe HSV 6045F (5′-CGT GTG GGA CAT AGG CCA GAG CCA CTT-3′), 0.4 μM of the LC-Red 640 labeled hybridization probe HSV 6073R (5′-CAG AAG CGC AGC AAG ATA AAG GTG AAC GG-3′), 1.0 U of uracil DNA glycosylase, and 2.0 μl of LC-FastSTART DNA Master Hybridization probe (Roche Biochemicals, Canada). A HSV plasmid DNA construct containing part of the HSV DNA polymerase gene was diluted serially and used as a standard for quantitative analysis. The cycling conditions were as follows: 1 cycle at 95 °C for 10 min, followed by 45 cycles at 95 °C for 5 s, 57 °C for 10 s, 60 °C for 15 s, 72 °C for 20 s.

2.5. Generation of dendrimer-resistant HSV

The SPL7013-resistant viruses were obtained by serial passage of reference strains in the presence of increasing concentrations of SPL7013 (Andrei et al., 2000). Confluent Vero cells in a T75 flask were infected with HSV-1 or HSV-2 at a moi of 0.002 at 37 °C for 2 h. The infected cells were washed with PBS, and then covered with culture medium containing SPL7013 at the concentration of the EC_{50}s. With every passage of the virus, the dendrimer concentration was increased twofold. The cultures were terminated at generation 21. The resistant viruses were then plaque-purified, and the antiviral susceptibilities were tested by a plaque reduction assay.

2.6. Evaluation of antiviral efficacy of SPL7013 in different pH

Phosphate buffers with pHs ranging from 3 to 9 were prepared by mixing different ratios of 1 M Na_2HPO_4 and 1 M NaH_2PO_4. A stock solution of dendrimer (40× final concentrations) was prepared in sterile water and 200 μl was added to 1800 μl of each pH buffer. The mixture was incubated at 37 °C for 2 h and 1 ml of PBS was then added to buffer unexpected pH changes which otherwise might affect the cell growth. Two milliliter of 2× MEM was used to dilute the drug and make final concentrations of 0.3, 1, 3, 10, and 30 μg/ml, respectively. Mock treatment (without drug) was performed in parallel and received PBS. The pH-treated dendrimer (1 ml) was then incubated with confluent Vero cells at 37 °C for 1 h. HSV-2 or HSV-1 (200 pfu/well) was subsequently added to the cells and incubated at 37 °C for 1 h. After the inoculum was removed, the cells were overlaid with methylcellulose (no drug) for a plaque assay.

2.7. Activity of SPL7013 in the presence of human serum proteins

Plaque reduction assays were used to test the efficacy of SPL7013 against HSV in the presence of human serum proteins. The dendrimer SPL7013 was prepared in medium supplemented with either 10% human serum (obtained from HSV negative donors) or 10 mg/ml human albumin (Sigma). Additional experiments were performed by incubating varying doses of SPL7013 in human serum at 37 °C overnight, which was then used to pre-treat the cells and treat infected cells as described.

2.8. Effect of SPL7013 on cell proliferation measured by uptake of radiolabeled thymidine

The effect of SPL7013 on cell proliferation was measured by uptake of radiolabeled ^{3}H-thymidine as described (Cheetham et al., 1998). Two epithelial cell lines, Hela-229 (cervical origin) and Hep-2, were seeded in 24-well plates at 50 and 100% confluence. The cells were washed with PBS and 200 μl of culture medium containing SPL7013 at concentrations of 0, 250, 500, 1000, and 10,000 μg/ml was added to each well. The cells were incubated at 37 °C for 24 h, and then 2.0 μCi of ^{3}H-thymidine (ICN) was added to each well and incubated for a further 24 h. After removal of medium, the wells were washed twice with PBS, 200 μl of 7.5% cold TCA (trichloroacetic acid) was added to the test wells and the plate was placed at 4 °C for 1 h to ensure precipitation. The supernatant was removed and the wells were washed with 200 μl fresh, cold TCA. Then 200 μl of 4% NaOH was added and the plate was left at room temperature for 2 h. The NaOH digests were transferred to scintillation vials. The wells were washed with 200 μl of 4% HCl then pooled with respective scintillation vials. The radioactivity was counted by a scintillation counter.

2.9. Data analysis

The effects of the dendrimer at various concentrations were expressed as percent of control (the mean plaque counts in drug-treated wells/the mean plaque counts in control wells without drug). EC_{50}s were calculated using computer program Statview™ using a linear regression.

3. Results

3.1. The antiviral activity of SPL7013 against HSV

The ability of SPL7013 to block the HSV entry process was examined in susceptible cells that were incubated with various concentrations of SPL7013 for 1 h prior to virus infection. Viral infection was performed in the presence of SPL7013. After incubating at 37 °C for 1 h, viral inoculum and SPL7013 were removed by washing with PBS. Antiviral activity was then determined by plaque reduction assay and the EC_{50}s are summarized in Table 1. The results (Fig. 1A) showed that the dendrimer SPL7013 was highly active at preventing HSV infection. The EC_{50}s determined by pre-treatment of cells were 2.0 μg/ml (standard deviation 0.48) for HSV-1 and 0.5 μg/ml (standard deviation 0.08) for HSV-2. These data were obtained from the test results of six batches of SPL7013. The antiviral activities were slightly better against HSV-2 than HSV-1.

Table 1 shows the antiviral activity of SPL7013 in HSV-infected cells. Vero cells were infected with either HSV-1 or HSV-2 and then treated with increasing doses of SPL7013. Antiviral activity was determined by plaque reduction assay. The results (Fig. 1B) showed that SPL7013 had a strong inhibitory effect on viral replication in cells that were already infected with either HSV-1 or HSV-2. The EC_{50}s in this treatment were 6.1 μg/ml (standard deviation 1.49) for HSV-1 and 3.8 μg/ml (standard deviation 1.15) for HSV-2. To confirm these findings, a CPE inhibition assay was performed. The cells were infected with HSV and then covered with medium containing no or increasing concentrations of SPL7013 and cultured until the CPE was ∼100% in control wells (no drug). The viability of protected cells was measured using a neutral red dye uptake assay. The results showed a concentration-dependent protection of cells from viral lysis (data not shown). The EC_{50}s (effective concentration that protects 50% of cells from virus lysis) were 7.5 μg/ml for HSV-1 and 4.3 μg/ml for HSV-2. The positive con-

Table 1
The EC_{50}s (μg/ml) of six batches of SPL7013 against HSV measured by plaque reduction assay using Vero cells

Batch number	HSV-1		HSV-2	
	PT	INF	PT	INF
PK813-013	1.5	6.5	0.5	3.3
PK1-36	1.6	7.6	0.5	4.6
IDT104626	1.7	7.8	0.5	4.8
IDT104119	2.2	4.7	0.5	4.6
IDT103794	2.8	5.6	0.5	3.6
IDT104359	1.9	4.2	0.7	1.8
Mean EC_{50}	2.0	6.1	0.5	3.8
Standard deviation	0.48	1.49	0.08	1.15

PT: pre-treatment of cells with SPL7013; INF: treatment of infected cells.

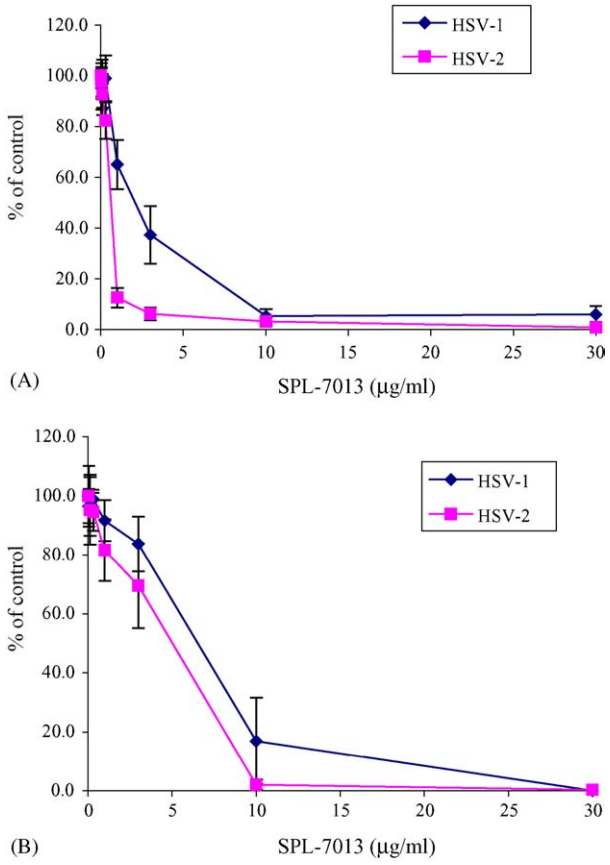

Fig. 1. The antiviral effect of SPL7013 on HSV determined by pre-treatment of cells with SPL7013 (A) and treatment of HSV-infected cells (B).

trol, acyclovir at concentrations of 10 and 50 μM, completely protected cells from CPE.

3.2. Effect of SPL7013 on virus adsorption

To examine whether SPL7013 inhibits HSV adsorption to its cellular receptors, the adsorption curve of HSV-2 was measured in the presence of various concentrations of SPL7013. The results (Fig. 2) indicated that the attachment of HSV-2 to Vero cells was completely inhibited by SPL7013 at concentrations of 3 and 10 μg/ml.

3.3. The stage of HSV replication cycle blocked by SPL7013

A time-of-addition experiment was performed to examine the stage(s) at which SPL7013 acts. The cells were treated with varying doses of SPL7013 at 1 h prior to virus infection (−1 h), and at 0, 2, 4, 8, 16, and 20 h p.i. Strong inhibition of viral replication (Fig. 3) was observed when SPL7013 was added at either −1 h (pre-treatment of cells) or 0 h (virus and dendrimer added at the same time). When SPL7013 was added to cells at 2 to 16 h p.i., strong inhibition of virus replication was also detected, but the concentrations required to achieve the EC_{50}s increased with the time of addition. When HSV-infected cells were treated at 20 h p.i., a slight inhibitory effect was still seen, but a concentration of 100 μg/ml was able to only achieve approximately 50% inhibition. This experiment confirmed that SPL7013 inhibited virus entry and stages of HSV replication after virus internalization.

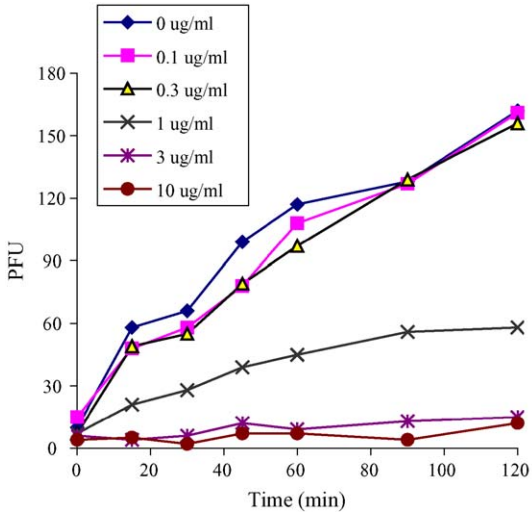

Fig. 2. The adsorption rate of HSV-2 in the presence of SPL7013. PFU: plaque forming units.

3.4. Effect of SPL7013 on HSV DNA synthesis

The effect of SPL7013 on HSV DNA synthesis was analyzed quantitatively using a real-time PCR assay (the Roche LightCycler™ technology). The infected cells were treated with SPL7013 for 17 h. Total DNA was extracted from the cells and quantitated using the LightCycler assay. A significant reduction in HSV DNA synthesis was detected in SPL7013-treated HSV-infected cells (Fig. 4). The EC_{50}s (effective concentration giving 50% reduction in DNA copy numbers) were approximately 6.2 and 2.0 μg/ml for HSV-1 and HSV-2, respectively.

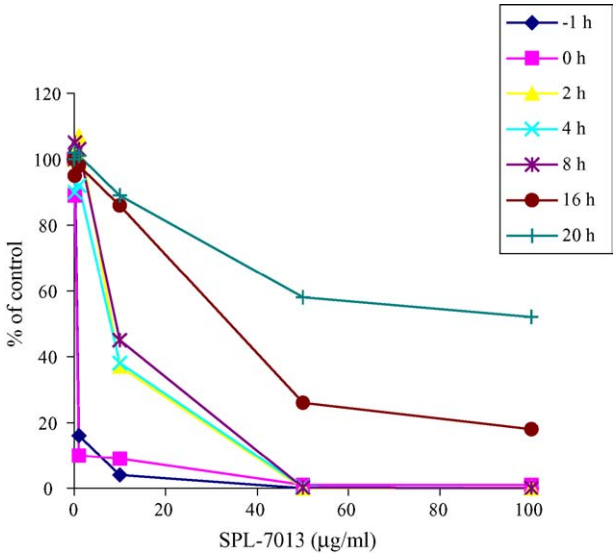

Fig. 3. Time-of-addition experiment. Varying doses of SPL7013 were added to the culture medium and/or the methylcellulose overlay for plaque assays, 1 h before infection (−1 h), and 0 (dendrimer and virus added at same time), 2, 4, 8, 16, and 20 h post-infection.

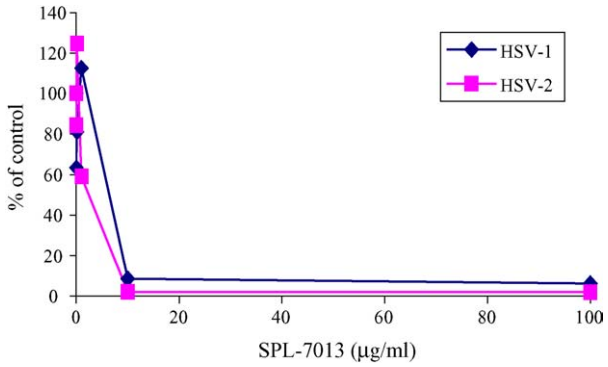

Fig. 4. The inhibition of HSV DNA synthesis by SPL7013 quantitated using a real-time PCR assay (LightCycler).

3.5. Antiviral effects of SPL7013 on drug-resistant HSV

The antiviral effects of SPL7013 on HSV drug-resistant strains were evaluated. The cells were incubated with SPL7013 at 37 °C for 1 h prior to infection with drug-resistant viruses, Delta 333 and strain 615.8. The antiviral effects were subsequently determined by plaque reduction assay. The results (Table 2, PT) showed that SPL7013 was highly active against delta 333 and 615.8, by blocking virus entry into cells, with EC_{50}s of approximately 0.3 and 1.7 μg/ml, respectively. Thus, SPL7013 was equally potent against HSV reference and drug-resistant strains.

To test the antiviral activity of SPL7013 on drug-resistant HSV-infected cells, a plaque reduction assay was performed. The results (Table 2, INF) showed that SPL7013 could completely inhibit Delta333 and 615.8 virus replication with EC_{50}s of approximately 2.3 and 5.1 μg/ml, respectively. This activity is similar to that observed with HSV reference strains and suggests that SPL7013 can potentially be used as an alternative for patients who are clinically resistant to acyclovir and penciclovir therapies. Acyclovir and foscarnet were used in parallel as controls and results are shown in Table 2.

3.6. Generation of dendrimer-resistant viruses

To generate SPL7013-resistant HSV, reference strains F and G were serially passaged in the presence of increasing concentrations of SPL7013 supplemented in culture medium. The HSV-2 resistant mutants to SPL7013 were unable to raise because SPL7013 at concentrations higher than that used in generation 7 inhibited virus replication completely. SPL7013-resistant HSV-1 (generation 21) was plaque-purified and the virus susceptibility profiles were analyzed by plaque reduction assays. The EC_{50}s of the resistant viruses are listed in Table 3, along with those of their parental strains for comparison. In the pre-treatment of cells, SPL7013 was equally active against these dendrimer-resistant strains and their parental strains. Generation 21 HSV-1 mutants resisted post-infection treatment with SPL7013 with EC_{50}s ranging from 138 to 324 μg/ml. Phenotypically, these SPL7013-resistant strains produced larger plaques than the wild-type F strain.

Table 3
The EC_{50}s of dendrimer-resistant HSV strains and their parental strains

Treatments	HSV strains	EC_{50} (μg/ml)
PT	HSV-1/wildtype	1.1
	HSV-1/Resistant generation 21	0.9
INF	HSV-1/wildtype	4.0
	HSV-1/Resistant generation 21	138–324[a]

PT: pre-treatment of cells; INF: on infected cells.
[a] A range of 24 drug-resistant mutants.

3.7. Antiviral activity of SPL7013 at different pHs

The antiviral activity of a vaginal microbicide can be influenced by the special physiological properties of the vagina, including the pH, protein composition, osmolarity, and the presence of cervical mucous. In evaluating potential vaginal microbicides, these parameters must be considered as they may influence drug efficacy. To examine the activity of SPL7013 against HSV at different pHs, various concentrations of SPL7013 were treated with buffers of differing pH for 2 h. The mixtures were then used to pre-incubate cells, followed by infection with either HSV-1 or HSV-2. Antiviral activities were measured using plaque reduction assays.

The results, summarized in Table 4, demonstrated that SPL7013 retained its activity against HSV after incubation at pHs 3.0 and 4.0 for 2 h, but was less efficacious at acidic pH than at neutral pH.

3.8. Evaluation of the antiviral efficacy of SPL7013 in the presence of human serum proteins

Three sets of experiments were performed to test the antiviral efficacy of SPL7013 against HSV in the presence of human

Table 2
Comparison of activities of SPL7013, acyclovir, and foscarnet against HSV drug-resistant strains

Test compounds	Drug resistant HSV strains	Treatments	EC_{50} (μg/ml)
SPL7013	615.8-Pol mutant	PT	1.7
		INF	5.1
	Delta333-TK negative mutant	PT	0.3
		INF	2.3
Acyclovir	Delta333-TK negative mutant	INF	19.4
Foscarnet	615.8-Pol mutant	INF	148.1

PT: pre-treatment of cells; INF: on infected cells.

Table 4
The antiviral activity of SPL7013 after pH treatment

pH treatment	EC_{50} (μg/ml)
HSV-1	
pH 3.0	2.6
pH 4.0	2.5
pH 5.0	2.5
pH 6.0	2.4
pH 7.0	1.6
pH 8.0	0.9
pH 9.0	0.8
HSV-2	
pH 3.0	1.2
pH 4.0	1.2
pH 5.0	0.7
pH 6.0	0.6
pH 7.0	0.5
pH 8.0	0.7
pH 9.0	0.5

serum proteins (Table 5). In the first set of experiments, the 5% fetal bovine serum (FBS) in regular Vero cell culture medium was replaced with 10% human serum (obtained from HSV negative donors) in the plaque reduction assay. The results showed that the presence of 10% human serum did not significantly affect the anti-HSV activity of SPL7013.

The second set of experiments was performed by incubating varying doses of SPL7013 in human serum obtained from HSV negative donors at 37 °C overnight, prior to using it to pretreat cells and treat infected cells in the plaque reduction assay (Table 5). The results demonstrated that the anti-HSV activity of SPL7013 was still seen after incubation in human serum at 37 °C overnight, but was lower than in MEM/5%FBS.

In the third set of experiments, the plaque reduction assay was performed in medium supplemented with 2% FBS and 10 mg/ml human albumin. The results (Table 5) indicated that the presence of 10 mg/ml of human albumin did not affect the anti-HSV activity of SPL7013.

Table 5
The effect of 10% human serum and albumin on SPL7013 antiviral activity

Treatments	EC_{50} (μg/ml)
10% Human serum	
HSV-1/PT	5.6
HSV-1/INF	13.1
HSV-2/PT	2.2
HSV-2/INF	8.7
10% Human serum (overnight incubation)	
HSV-1/PT	5.5
HSV-1/INF	10.6
HSV-2/PT	1.9
HSV-2/INF	9.3
10% Human albumin	
HSV-1/PT	1.0
HSV-1/INF	5.5
HSV-2/PT	0.3
HSV-2/INF	1.8

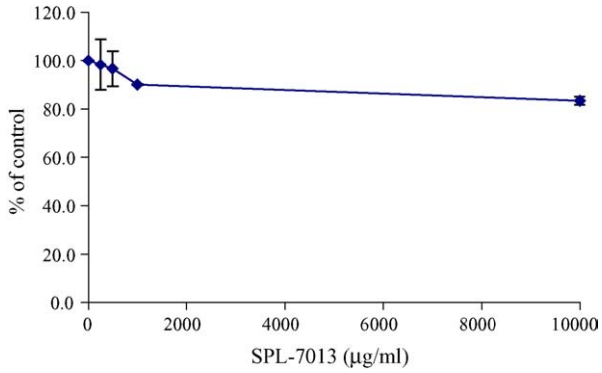

Fig. 5. The cytotoxicity profiles of three batches of SPL7013 (PK813-013, IDT104119 and IDT103794) measured by neutral red dye uptake assay.

3.9. The cytotoxicity of SPL7013 for Vero cells

The cytotoxicity of SPL7013 was initially examined on Vero cells using a neutral red dye uptake assay (Schmidt and Korba, 2000). Dendrimer SPL7013 at concentrations of 0, 250, 500, 1000, and 10,000 μg/ml was incubated with cells for 2 days. The results (Fig. 5) illustrate the cytotoxicity profiles of three batches of SPL7013 (PK813-013, IDT104119 and IDT103794) and show that the compound was not toxic to Vero cells at the highest concentration tested (10,000 μg/ml). The CC_{50}s (cytotoxic concentration giving 50% cell death) of all the batches tested were greater than 10,000 μg/ml.

3.10. Effect of SPL7013 on cell proliferation

To examine the cytotoxicity profiles of SPL7013 on multiple epithelial cell lines in both stationary and dividing phases, two epithelial cell lines, Hela-229 and Hep-2, were seeded to 50% and 100% confluency. The effect of SPL7013 on cell proliferation was determined by measuring the uptake of radiolabeled ^{3}H-thymidine (Cheetham et al., 1998). The results are shown in Fig. 6. No significant inhibition of ^{3}H-thymidine uptake by SPL7013 was observed in either stationary or dividing phase

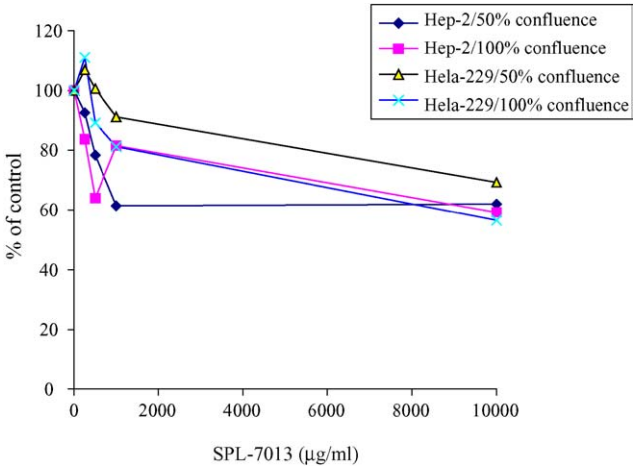

Fig. 6. The effect of SPL7013 on cell proliferation measured by incorporation of ^{3}H-thymidine (percent of control).

Hep-2 and Hela-229 cells. The CC_{50}s in all cases were greater than 10,000 μg/ml.

4. Discussion

We have screened dozens of dendrimer compounds in order to select a lead compound for the development of a topical microbicide against HSV, HIV, and other pathogens. The dendrimer SPL7013 showed potent activity against both HSV-1 and HSV-2 in vitro and in guinea pig and mouse models of HSV infection (Bernstein et al., 2003). It was also active against HIV (McCarthy et al., 2005; Dezzutti et al., 2004) and shows strong protection in a macaque SHIV vaginal challenge model (Jiang et al., 2005). Accordingly, it was selected as a lead microbicide for further development.

SPL7013 showed strong anti-HSV activity by inhibiting virus internalization of both HSV-1 and HSV-2. This was demonstrated by incubation of cells with SPL7013 prior to the addition of the virus inoculum. The experiment simulates the topical application of drug into the female genital tract. The observation was confirmed by measuring the absorption rate of virus in the presence of dendrimer compound, which showed that SPL7013 at concentrations of greater than 3 μg/ml abolished virus absorption to Vero cells. Time-of-addition experiments showed that SPL7013 added 1 h prior to infection dramatically inhibited HSV-2 virus plaque formation. This effect was also seen when dendrimer and virus were added to cells at the same time. Because the CC_{50} of SPL7013 is greater than 10,000 μg/ml, the therapeutic indices (the ratio of the median toxic dose to the median effective dose) in this pre-infection treatment are greater than 5000 and 20,000 for HSV-1 and HSV-2, respectively.

SPL7013 also showed strong post-exposure activity on HSV-infected cells. The EC_{50}s obtained for this post-infection treatment were higher than those for pre-treatment of cells. This activity was initially tested by plaque reduction assays, but confirmed by CPE inhibition assay, time-of-addition experiments, and inhibition of DNA synthesis. This provides strong evidence that SPL7013 can also be used as a therapeutic agent. The therapeutic indices for post-infection treatment were greater than 1639 and 2632 for HSV-1 and HSV-2, respectively.

We have successfully generated SPL7013-resistant HSV-1, but were unable to generate drug-resistant HSV-2. The reason for this is unknown. The resistant viruses were plaque-purified and phenotypically characterized by plaque reduction assays. Pre-treatment of cells showed SPL7013 to be equally active against dendrimer-resistant strains and their parental strains. When cells were treated post-infection, one clone of HSV-1 resisted SPL7013 up to an EC_{50} of 324 μg/ml. The observation that the drug-resistant viruses are susceptible to pre-treament, but not post-infection treatment suggests a change in DNA replication. The genotypic characterization of these drug-resistant viruses remains to be investigated, but should provide valuable information on the mechanism of action of dendrimers.

Topical microbicides are applied directly to the genital tract or rectum prior to intercourse to protect against the acquisition of sexually transmitted infections. The activity of vaginal microbicides may be affected by the unique physiological properties of the vagina. We therefore tested the anti-HSV activity of SPL7013 at various pHs and in the presence of proteins. The results showed that these treatments did not significantly reduce the activity of SPL7013 against HSV-1 and HSV-2. SPL7013 did not show any significant toxicity for either the stationary or dividing phases of multiple epithelial cell lines.

In summary, SPL7013 is highly active against both HSV-1 and HSV-2 in both the prevention and treatment of HSV infection. It is also equally active against HSV reference and drug-resistant strains. Anti-HSV activity of SPL7013 was not significantly affected by acidic pH or the presence of human serum proteins and human albumin. Dual sites of action were observed: inhibition of virus attachment and entry and inhibition of later stages of HSV replication. The advantages of SPL7013 over traditional entry inhibitors are that it is nontoxic, has a broad spectrum of antiviral activities and, most importantly, is not only an entry inhibitor, but also a therapeutic agent.

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