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Antiviral Research 55 (2002) 319–329

Antiviral Research

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Evidence of dual sites of action of dendrimers: SPL-2999 inhibits both virus entry and late stages of herpes simplex virus replication

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Received 29 November 2001; accepted 5 April 2002

Abstract

Dendrimers are macromolecules with broad-spectrum antiviral activity and minimal toxicity effective in animal models in preventing transmission of herpes simplex virus (HSV) infection. In order to further understand the mechanism of action, and toxicity profiles of the dendrimer SPL-2999 against HSV, we investigated in vitro activities as follows: modified plaque reduction assays for SPL-2999 showed that 50% effective concentrations (EC_{50}) determined by pre-treatment of cells with SPL-2999 were 0.5 μg/ml (30 nM) for HSV-2 and 1 μg/ml (60 nM) for HSV-1, respectively. SPL-2999 was not toxic to Vero cells at concentration up to the highest tested (CC_{50} greater than 1000 μg/ml). SPL-2999 appears to completely inhibit both viral adsorption and penetration to Vero cells at concentrations of higher than 3 μg/ml. Additionally, virus yield reduction assay showed that SPL-2999 was effective on cells already infected with HSV with EC_{90}s (effective concentration giving 90% virus yield reduction) approximately 29.2 μg/ml for HSV-1 and 6.7 μg/ml for HSV-2. When Vero cells were infected with HSV at moi (multiplicity of infection) of 0.01 pfu/cell, the infected cells could be completely protected from viral cytopathic effect (CPE) by SPL-2999 with EC_{90}s (effective concentration that protects 90% of cells from virus lysis) of 15 μg/ml for HSV-1 and 10 μg/ml for HSV-2. Results from Southern blot hybridization indicated that SPL-2999 inhibited DNA synthesis in HSV infected cells. We conclude that SPL-2999 inhibits both HSV entry into susceptible cells and late stages of HSV replication. Our data indicate that SPL-2999 is a potent inhibitor of both HSV-1 and -2 with the potential for further development as either a topical microbicide or a therapeutic agent.

Keywords: Dendrimer SPL-2999; Herpes simplex virus; Pre-clinical evaluation

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0166-3542/02/$ - see front matter
PII: S0166-3542(02)00054-2

1. Introduction

Herpes simplex virus (HSV) is a lipid-enveloped DNA virus whose replication cycle has been well characterized (for review see Roizman and Sears, 1996; Whitley, 1996; Boehmer and Lehman, 1997). The entry of HSV into cells is a complex process involving the interactions between HSV attachment proteins and cell surface heparan sulfate proteoglycan receptors, followed by fusion of the viral envelope with the host cell plasma membrane (Shieh et al., 1992). Several of the virus envelope glycoproteins (g) participate in the entry process. The initial attachment of virus to cell surface heparan sulfate is mediated by gB and gC (WuDunn and Spear, 1989; Herold et al., 1991, 1994; Immergluck et al., 1998; Laquerre et al., 1998). The fusion of viral envelope with cell membrane is mediated by gB, gD, and the gH/gL complex (Cai et al., 1988; Campadelli-Fiume et al., 1988; Herold and Spear, 1994; McLain and Fuller, 1994; Saharkhiz-Langroodi and Holland, 1997; Geraghty et al., 2000; Muggeridge, 2000). Several lines of evidence suggest that multiple receptors/co-receptors on the cell surface mediate HSV entry. These include a novel member of the TNF/NGF receptor family known as herpesvirus entry mediator (HveA), which is the principal receptor for infection of human lymphoid cells (Montgomery et al., 1996), and HveB, a member of poliovirus receptor-related protein (Geraghty et al., 1998; Warner et al., 1998).

After membrane fusion, the virus is uncoated, and viral nucleocapsids are transported to and dock at the nuclear pore, releasing viral DNA into the nucleus. Immediate-early genes are transcribed and the mRNAs are transported into the cytoplasm for translation (α-proteins). These proteins are then imported back to the nucleus, where they activate transcription of early genes. The early gene products (β-proteins) function primarily in DNA replication and production of substrates for DNA synthesis. DNA replication produces long concatemeric DNA molecules which are the templates for late gene expression. Late proteins (γ-proteins) are primarily virion structural proteins needed for virus assembly and particles egress. Assembled virions are transported from the nucleus for processing in the Golgi complex, and finally released from the cellular membrane surface (for review see Roizman and Sears, 1996; Boehmer and Lehman, 1997).

Unlike the small molecular structures of most antivirals, dendrimers are a novel class of polyanionic macromolecules (Holan et al., 2000). The dendrimers are synthesized from a core molecule by repetitive reactions using identical monomeric building blocks with multiple branches. The resulting precisely defined macromolecules possess an outer surface which carries a number of functional groups that are able to interact with biological surfaces or receptors. The active surface group for dendrimer SPL-2999 is naphthyl 3,6-disulfonic acid sodium salts. A large number of polyanionic dendrimers have been synthesized which exhibit broad-spectrum antiviral activities with high efficacy and minimal toxicity against HSV (Bourne et al., 2000), hepatitis B virus (Brent Korba, personal communication), human immunodeficiency viruses (Witvrouw et al., 2000), respiratory viruses (Barnard et al., 1997), and human papillomaviruses (Ian Frazer, personal communication). The mechanisms of action of the dendrimers have not been examined for all these activities. Thompson and Schengrund (1998) reported that the oligosaccharide-derivatized dendrimers inhibited the adherence of cholera toxin and the heat-labile enterotoxin of *Escherichia coli* to cell surface. Witvrouw et al. (2000) showed that dendrimers inhibit attachment or fusion of HIV to MT-4 cells and reverse transcriptase/integrase activities. Here we present in vitro evaluations of the efficacy, mechanism of action, and toxicity of SPL-2999 against HSV.

2. Materials and methods

2.1. Compounds

Dendrimer SPL-2999 was synthesized by Starpharma Limited, Melbourne, Australia. The chemical formula of SPL-2999 is BHAlys15lys16(NHCSNHNaphth-3,6-di$SO_3Na)_{32}$ (BHA: benzhydrylamine) with molecular weight of 16,615 Da. The structure of SPL-2999 is de-

scribed elsewhere (Bourne et al., 2000). Dextran sulfate (MW 500,000) was purchased from Sigma. The stock solutions of SPL-2999 and dextran sulfate were prepared in culture medium (Minimum Essential Medium GIBCO/BRL supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin).

2.2. Virus and cells

HSV reference strains F (HSV-1) and G (HSV-2) (ATCC: American Type Culture Collection) were used in the experiments. A HSV susceptible cell line, Vero cells (African green monkey kidney cells, ATCC), was used in the virus plaque reduction assays. Delta 333 is a laboratory HSV-2 TK^- (thymidine kinase) mutant strain with phenotypic resistance to both penciclovir and acyclovir (a kind gift from Dr Donald Coen, Harvard Medical School, Boston, MA).

2.3. Viral plaque reduction assay

Confluent cells in a 6-well plate were washed with PBS and subsequently infected with either HSV-1 or -2 (200 pfu/well) for 1 h at 37 °C. After viral inoculum was removed, the infected cells were washed with PBS and overlaid with 0.5% methylcellulose in culture medium containing no or increasing concentrations of SPL-2999. The cells were incubated at 37 °C until plaque sizes were adequate. The cells were then fixed with 10% formalin and stained with 0.5% crystal violet.

2.4. Virus yield reduction assay

Confluent cells were washed with PBS and infected with either HSV-1 or -2 at moi of 5 pfu/cell for 1 h at 37 °C. The infected cells were washed with PBS and covered with culture medium containing no or increasing concentrations of SPL-2999 for 2 days. The infected cells were then scraped into culture medium. The intracellular HSV was released through three cycles of freeze-thaw and centrifugation to remove cell debris. The total virus yield in each well was titrated by plaque assay.

2.5. Pre-treatment of cells with SPL-2999

Confluent Vero cells in 6-well plates were incubated with different concentrations of SPL-2999 at 37 °C for 1 h. Two hundred pfu virus was then added to the cells in the presence of test compound and the samples were incubated at 37 °C for 1 h. The infected cells were washed with PBS and overlaid with methylcellulose (no drug) for plaque assay.

2.6. Pre-mixing SPL-2999 with virus

0.5 ml of 200 pfu virus was mixed with 0.5 ml of various 2 × final concentrations of SPL-2999. The mixtures were incubated at 37 °C for 1 h, then used to inoculate confluent Vero cells in a 6-well plate. After incubating at 37 °C for 1 h, the infected cells were washed with PBS and overlaid with methylcellulose (no drug) for plaque assay.

2.7. Cytopathic effect inhibition assay

Confluent Vero cells in a 96-well plate were infected with HSV at moi of 0.01 pfu/cell at 37 °C for 1 h. Following removal of virus inoculum, the infected cells were washed with PBS and covered with medium containing either no or increasing concentrations of SPL-2999 for 3 days at 37 °C. The cells protected from cytopathic effect (CPE) was measured using neutral red uptake assay (Schmidt and Korba, 2000).

2.8. Effect on early stages of virus replication

To investigate if SPL-2999 inhibits the early stages of virus replication, confluent Vero cells were pre-treated with different concentrations of SPL-2999 at 4 °C for 1 h. One hundred pfu virus was subsequently added to the medium, on ice, and the plates were incubated at 4 °C for 2 h to allow virus binding to the cell surface. Unbound virions were removed by washing three times with PBS. The cells were subsequently upshifted to 37 °C for 1 h to allow virus uptake. The uninternalized virions were stripped from cell surface and inactivated by addition of 1 ml acidic glycine solution (0.1 M glycine in saline, adjust pH to 2.2

with HCl) for 1 min. Following two washes with PBS and MEM (no serum), the cells were overlaid with methylcellulose for plaque assay.

2.9. Virus adsorption assay

To examine if SPL-2999 inhibits the adsorption of HSV to cells, confluent cells were cooled to 4 °C. Different concentrations of cold SPL-2999 were added to the cells and incubated at 4 °C for 1 h. Four hundred pfu virus was then added (time 0) to the cells which were placed on ice. At various time points (at 0, 15, 30, 45, 60, 90, and 120 min), the cells were washed twice with PBS, and once with MEM (no serum) to remove unadsorbed virions. Adsorbed virions were allowed to internalize into the cells by addition of warm medium and incubation at 37 °C for 90 min. The cells were then covered with methylcellulose (no drug) for plaque assay.

2.10. Virus penetration assay

The rate of penetration of HSV-2 was measured in the presence of SPL-2999 as described (Dargan, 1998). Briefly, confluent cells in 6-well plates were cooled to 4 °C. After washing with cold PBS, 1 ml of cold virus dilution containing 500 pfu of HSV-2 was added to each well. The cells were incubated at 4 °C for 2 h to allow maximum adsorption of virus. Unbound virus was removed by washing with cold PBS. Different concentrations of SPL-2999 in warm medium were then added to the cells (time 0) and incubated at 37 °C to allow virus penetration. At various times (0, 15, 30, 45, 60, 90, 120, and 150 min), the cells were washed three times with PBS, acidic glycine (pH 2.2) for 1 min, and MEM (no serum), respectively. The cells were then overlaid with methylcellulose to allow virus plaques to develop.

2.11. Time-of-addition experiments

To analyze the stage of replication cycle at which the dendrimer acts, SPL-2999 was added to confluent Vero cells in 6-well plates both before and after virus infection. For the time point before infection, the cells were pre-treated with various concentrations of SPL-2999 for 2 h as described in Section 2.5. For other time points, confluent cells in 6-well plates were infected with 100 pfu HSV-2 per well at 37 °C for 1 h. After inoculum was removed by washing with PBS, the cells were covered with culture medium. At different time points: 0 (virus and SPL-2999 were added at same time), 2, 4, 6, and 20 h postinfection (p.i.), the medium was replaced with methylcellulose containing different doses of SPL-2999 for plaque assay.

2.12. Southern blot hybridization

Confluent Vero cells in 6-well plates were infected with HSV-2 at moi of 0.1 pfu/cell at 37 °C for 1 h. Following removal of virus inoculum, the infected cells were washed with PBS and overlaid with medium containing varying doses of SPL-2999. At 18 h p.i., the infected cells were washed with PBS and the DNA was extracted with DNAzol (GIBCO/BRL) as described by manufacturer.

The extracted DNA was digested with Hind III, separated by using a 0.7% agarose gel, and transferred onto a nylon membrane. Southern blot hybridization was then performed essentially as described by Sambrook et al. (1989). The ^{32}P-labeled probe was prepared from PCR products that contained part of HSV DNA polymerase gene and that were cleaned up by QIAquick PCR purification column. After hybridization, the blot was washed with 2 × SSC/0.1%SDS three times at room temperature, followed by two stringent washes, 20 min each, in 0.1 × SSC/0.1%SDS at 65 °C. The blot was then exposed to a X-ray film. The regions containing hybridized signal were excised from the membrane and radioactivity was measured by a liquid scintillation counter.

2.13. Cytotoxicity assay

The cytotoxicity of SPL-2999 was examined using Vero cells. Confluent cells in a 96-well plate were washed with PBS. One hundred microliter of culture medium containing SPL-2999 at concentrations of 0, 250, 500, and 1000 μg/ml was added to each well. The cells were incubated at 37 °C for 2 days. The cytotoxicity of SPL-2999 was then

examined using neutral red uptake assay (Schmidt and Korba, 2000).

2.14. Data analysis

All data were generated from duplicate or triplicate wells in at least one repeating experiment. The effect of SPL-2999 at varying concentrations was expressed as % of control (the mean plaque counts in drug-treated wells/the mean plaque counts in control wells). The EC_{50} was calculated by linear regression using a computer program Statview™ (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Antiviral activity of SPL-2999

The antiviral activity of the dendrimer SPL-2999 on HSV was initially examined by modified plaque reduction assays. These assays included pre-incubation of the compound with susceptible cells and subsequent challenged with virus; mixing the compound at various concentrations with virus prior to infection of susceptible cells; and incubation of HSV infected cells with SPL-2999. Dextran sulfate, a polysulfated carbohydrate known to inhibit HSV entry into cells (Herold et al., 1997), was used as a positive control.

The results showed that the dendrimer SPL-2999 was effective against both HSV-1 (data not shown) and -2 (Fig. 1A, 'PT') infections examined by pre-treatment of the cells with various concentrations of SPL-2999 prior to virus infection. The EC_{50}s in this treatment were approximately 0.5 μg/ml (30 nM) for HSV-2 and 1 μg/ml (60 nM) for HSV-1. A similar inhibitory effect was obtained by pre-mixing HSV-1 (data not shown) or -2 (Fig. 1A, 'PM') with SPL-2999 at 37 °C for 1 h. However, SPL-2999 at a concentration of 3 μg/ml (sixfold higher than its EC_{50}) had no inhibitory effect on cells that were already infected with HSV-2 (Fig. 1A, 'INF'; data not shown for HSV-1). A similar inhibitory pattern to SPL-2999 was observed by plaque reduction assays for the positive control, dextran sulfate, in pre-treatment of cells, pre-mixing with HSV-2, and treatment of HSV-2 infected cells (Fig. 1B, PT, PM, and INF, respectively; data not shown for HSV-1). Dextran sulfate, determined by pre-incubation of the cells, was also active against HSV with EC_{50}s of 0.2 μg/ml (0.3 nM) for HSV-2 and 0.7 μg/ml (1.4 nM) for HSV-1. These results suggest that SPL-2999 may inhibit the early stages of HSV replication, possibly in the virus adsorption or penetration stage. Further experiments by plaque reduction assay demonstrated that at higher concentrations the dendrimer SPL-2999 was also effective on HSV infected cells (Fig. 1C) with EC_{90}s approximately 30 μg/ml (1.8 μM) for HSV-2 and 60 μg/ml (3.6 μM) for HSV-1, suggesting that the dendrimer SPL-2999 may also act on late stages of virus replication. The antiviral effect of SPL-2999 on HSV infected cells was confirmed by a virus yield reduction assay and a CPE inhibition test. The EC_{90}s measured by virus yield reduction assay were approximately 29.2 μg/ml for HSV-1 and 6.7 μg/ml for HSV-2. The EC_{90}s (effective concentrations that protect 90% of cells from virus lysis) tested by CPE inhibition assay were approximately 15 μg/ml for HSV-1 and 10 μg/ml for HSV-2.

3.2. Effect on early stages of virus replication

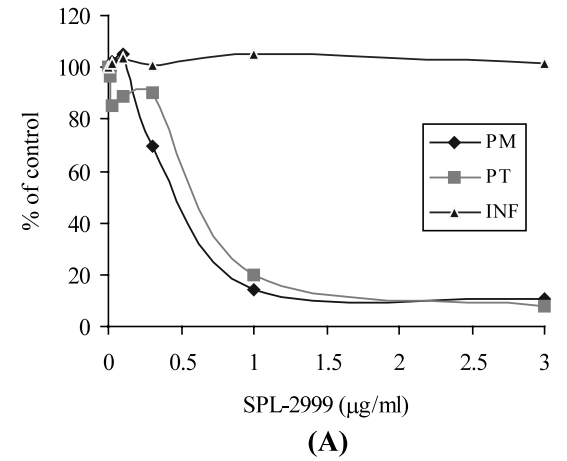
Initial experiments suggest that SPL-2999 may act on the early stages of virus replication, i.e. attachment and penetration. To examine this possibility, the viral attachment was performed at 4 °C. At this temperature, virions can bind to cellular receptors, but cannot penetrate the plasma membrane. If the dendrimer acts on these stages, attachment and penetration would be blocked. Unbound virions blocked by the dendrimer were removed by washes. Bound virions were taken up by the cells when the temperature was raised to 37 °C, and uninternalized virions were stripped by acidic pH. The results showed that SPL-2999 at concentration of 3 μg/ml could completely block virus entry (Fig. 2). This indicates that SPL-2999 has inhibitory effect at the early stages (virus adsorption or fusion stage) of HSV replication.

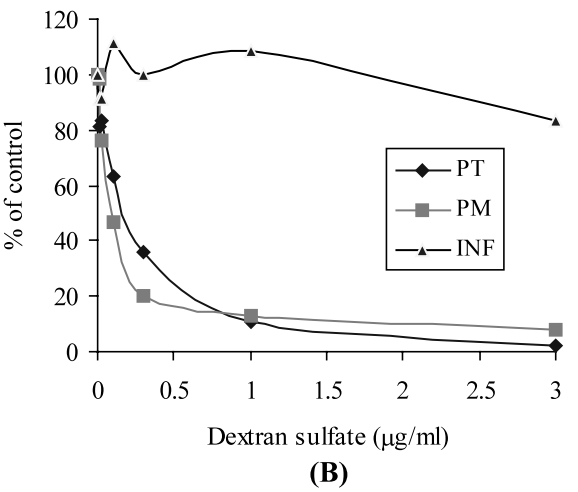
3.3. Effect on virus adsorption

Virus adsorption curves measure the amount of adsorbed virus to the cell surface with time. To further examine the inhibitory effect of SPL-2999 on HSV attachment to its cellular receptors, the adsorption rate of HSV-2 was measured in the presence of various concentrations of SPL-2999. The results (Fig. 3) indicated that the adsorption of HSV-2 to Vero cells was essentially abolished by SPL-2999 at concentrations of 3 and 10 μg/ml. It was estimated by pre-treatment of cells that 50 μg/ml of SPL-2999 could block approximately 2×10^4 pfu of HSV-2 attaching to cells (data not shown).

3.4. Effect on virus penetration

To examine the effect of SPL-2999 on HSV penetration of cells, the penetration rate of HSV-2 was measured in the presence of SPL-2999 (Dargan, 1998). Virus binding to Vero cells was performed at 4 °C for 2 h to allow maximum adsorption of virus. Unbound virions were re-





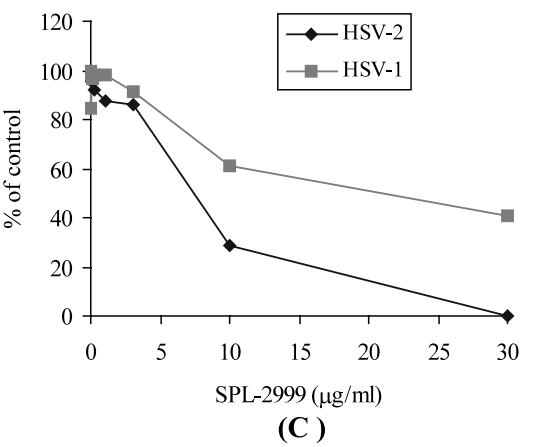


Fig. 1. The antiviral activities of SPL-2999 (A) and dextran sulfate (B), a positive control, on HSV-2 determined by plaque reduction assays. PT, pre-treatment of cells with test compound; PM, pre-mixing virus with test compound; INF, treatment of virus infected cells with test compound. (C) Comparison of the antiviral effect of SPL-2999 on HSV-1 and -2 infected cells.

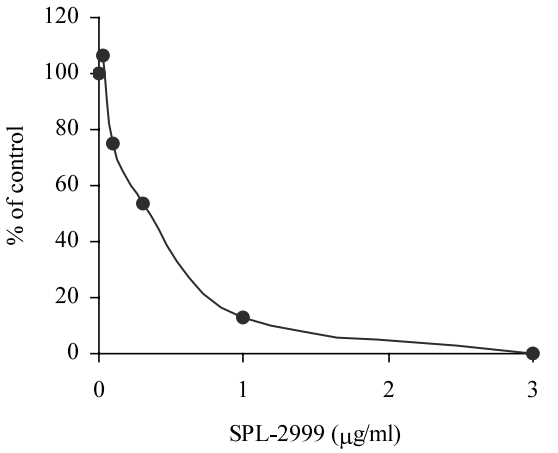


Fig. 2. The effect of SPL-2999 on the early stages of HSV-2 replication determined by plaque reduction assay.

moved by washing with cold PBS. Penetration of virus occurred after addition of warm medium containing increasing concentrations of SPL-2999 and temperature upshift to 37 °C. The results (Fig. 4) demonstrated that SPL-2999 could inhibit HSV-2 penetration at concentrations of higher than 3 μg/ml. By combining the data of absorption and penetration, it can be concluded that the

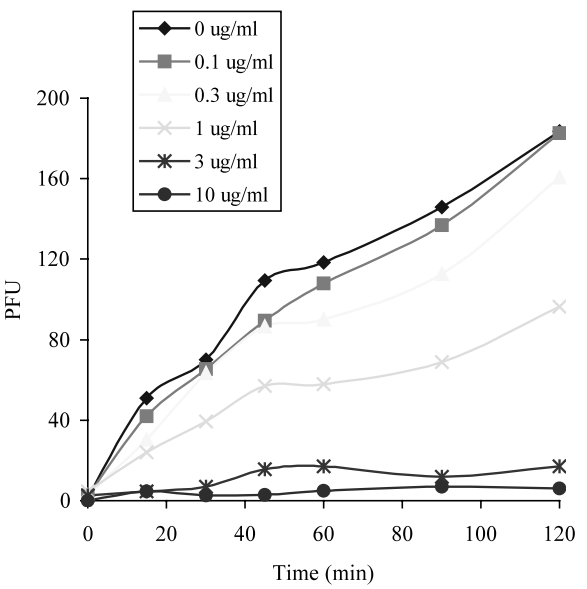


Fig. 3. The adsorption rate of HSV-2 in the presence of SPL-2999. PFU, plaque forming unit.

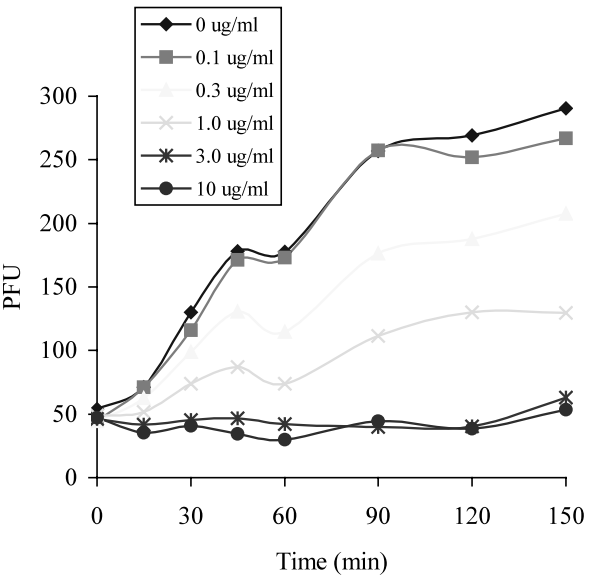


Fig. 4. The penetration rate of HSV-2 in the presence of SPL-2999. PFU, plaque forming unit.

dendrimer SPL-2999 inhibits HSV entry into susceptible cells.

3.5. The stage of HSV replication cycle blocked by SPL-2999 (time-of-addition experiment)

To examine the stage(s) that SPL-2999 may act on, a time-of-addition experiment was performed. The cells were treated with varying doses of SPL-2999 at 2 h before infection (pre-treatment of the cells), and at 0, 2, 4, 6, 20 h p.i. The results (Fig. 5) showed that SPL-2999 added at 2 h before infection dramatically inhibited HSV-2 virus plaque formation due to the block of virus entry. A similar result to the pre-treatment of cells was obtained when SPL-2999 was added at 0 h (virus and dendrimer added at same time). This is probably due to the immediate blockage of virus entry by SPL-2999. When SPL-2999 was added to cells at 2–6 h p.i., a stage at which virus was still in the early phase of replication cycle (α,β-protein expression, no virus assembly) (Boehmer and Lehman, 1997), it could inhibit the virus replication, but required approximately 10 times of the concentration to reach the same inhibitory effect. When HSV infected cells were treated at 20 h p.i.,

a stage at which virions were about to release (the eclipse phase for HSV is ~18–20 h) (Roizman and Sears, 1996), inhibitory effect was observed at higher concentrations (e.g. 10 and 30 μg/ml). This experiment confirms that SPL-2999 can inhibit not only virus entry, but also late stages of replication.

3.6. Effect on HSV DNA synthesis

To examine the effect of SPL-2999 on HSV DNA synthesis, cells were infected with HSV-2 and then treated with various concentrations of SPL-2999. The DNA was extracted from infected cells at 18 h p.i. Due to the large size of HSV genomic DNA (~150 kb), it is impossible to resolve in a regular agarose gel. The DNA was therefore digested with Hind III and separated on a 0.7% agarose gel. The DNA was then transferred onto a nylon membrane and detected by Southern blot analysis. The probe was a ^{32}P-labeled DNA fragment of 476 bp containing part of the DNA polymerase gene. After autoradiography, the regions containing hybridized probes were cut from the membrane and the radioactivity

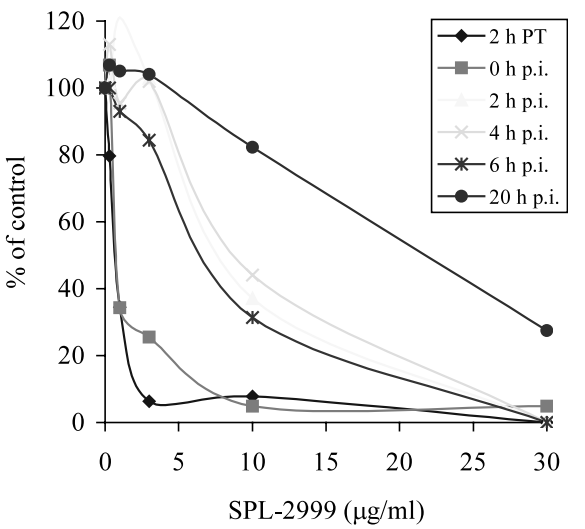


Fig. 5. Time-of-addition experiment of SPL-2999. At 2 h before infection (PT, pre-treatment of cells), 0 (dendrimer and virus added at same time), 2, 4, 6, and 20 h p.i., the medium was replaced with methylcellulose containing varying doses of SPL-2999 for plaque assay.

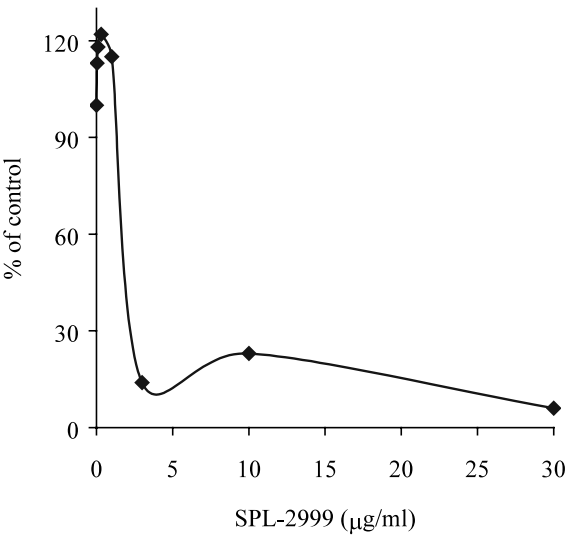


Fig. 6. Qualification of HSV-2 DNA synthesis by Southern blot hybridization after treatment of SPL-2999. Vero cells were infected with HSV-2 at moi of 0.1 pfu/cell, then covered with medium containing different doses of SPL-2999. The cells were harvested at 18 h p.i. The DNA was extracted and cut with Hind III. The blot was hybridized with ^{32}P-labeled DNA probe from the viral polymerase gene. The regions containing hybridized bands were excised and the radioactivity was counted by a liquid scintillation counter. % of control: the mean cpm (counts per minute) in drug treated wells/the mean cpm in control wells).

was counted with a liquid scintillation counter. The results showed that SPL-2999 at 3 μg/ml could significantly reduce HSV-2 DNA synthesis in dendrimer-treated HSV-2 infected cells (Fig. 6).

3.7. The cytotoxicity of SPL-2999

The cytotoxicity of SPL-2999 was examined on Vero cells using neutral red dye uptake assay (Schmidt and Korba, 2000). Different concentrations of dendrimer SPL-2999 were incubated with cells for 2 days. The maximal concentration tested was 1000 μg/ml. No significant cell death was observed before neutral red staining. The results of neutral red uptake (Fig. 7) showed that dendrimer SPL-2999 was not toxic to cells at highest concentration tested. The CC_{50} (cytotoxic concentration giving 50% of cell death) was therefore greater than 1000 μg/ml. Higher concentrations were not tested.

4. Discussion

We have shown that the polyanionic dendrimer SPL-2999 is highly effective against both HSV-1 and -2 with EC_{50}s approximately 1 and 0.5 μg/ml, respectively, examined by pre-incubation of the cells with the compound. It is also effective on HSV infected cells with EC_{90}s of 29.2 μg/ml for HSV-1 and 6.7 μg/ml for HSV-2, determined by virus yield reduction assay. Additionally, SPL-2999 is active on drug-resistant strain delta 333 virus infected cells with a similar EC_{90} to wildtype G strain measured by plaque reduction assay (data not shown). It appears that SPL-2999 is slightly more potent against HSV-2 than -1. The cytotoxicity profile measured with a neutral red dye uptake assay showed that dendrimer was not toxic to Vero cells up to the highest concentration tested (1000 μg/ml). Thus, the selectivity index (SI) of SPL-2999 for HSV-2 is greater than 2000, while for HSV-1 is greater than 1000.

Aguilar et al. (1999) reported that the polysulfonic compound suramin could block adsorption of HSV-1 to cell membranes and lateral diffusion in Vero cells. The mechanism was prevention of binding of viral glycoproteins to heparan sulfate proteoglycans. Langeland et al. (1988) showed that polylysine and polyarginine block the binding of HSV-1 to receptors by interfering with the cellular receptor function. As predicted from the polyanionic structure, SPL-2999 may block binding of HSV to its cellular receptors. We therefore examined the ability of SPL-2999 to inhibit the early stages of HSV replication, i.e. adsorption and penetration. Based on the results from: (i) pre-treatment of cells with dendrimer; (ii) binding of virus to the cells at 4 °C with uptake at 37 °C, followed by stripping of uninternalized virus at low pH; (iii) adsorption curves; (iv) penetration curves; and (v) a time-of-addition experiment, we conclude that the dendrimer SPL-2999 inhibits the early stage of HSV replication by blocking virus entry into cells.

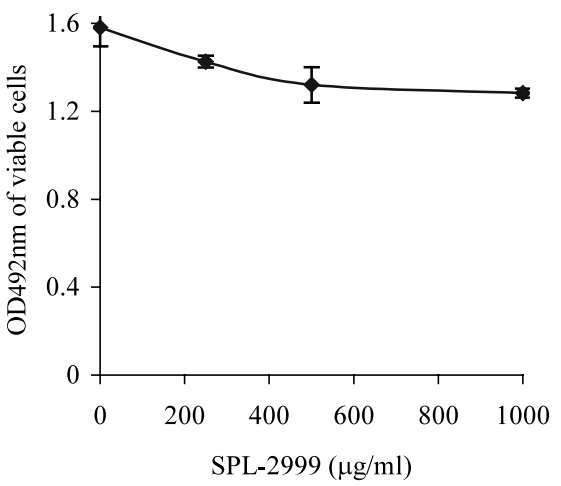


Fig. 7. Cytotoxicity of SPL-2999 on Vero cells determined by neutral red uptake assay.

Both plaque reduction and virus yield reduction assays indicate that SPL-2999 was effective on HSV infected cells. The CPE inhibition test showed that 90–100% of HSV-infected cells could be protected from virus CPE. The time-of-addition experiment confirmed that SPL-2999 inhibited HSV virus plaque formation if added 2–20 h p.i. Taking these data together with the inhibitory effect on HSV DNA synthesis, we conclude that SPL-2999 can also inhibit the late stages of HSV replication. The mechanism of this inhibition remains to be investigated. Dendrimers can function as an effective delivery system for DNA transfection (Bielinska et al., 1996; Kukowska-Latallo et al., 1996), indicating that they can enter cells. Shen et al. (1985) showed that polylysine could be endocytosed into cells by a non-receptor-mediated pathway. Witvrouw et al. (2000) showed that another polyanionic dendrimer (SPL-2923) could be internalized into cells using fluorescein labeled dendrimer and confocal microscopy. SPL-2923 could inhibit HIV reverse transcriptase and integrase activities in vitro. Based upon these observations, we speculate that SPL-2999 can be internalized into cells and inhibit HSV replication. The ability of dendrimer to inhibit a penciclovir- and acyclovir-resistant HSV mutant strain (thymidine kinase negative) indicates that thymidine kinase is not the primary target for SPL-2999's anti-HSV activity.

The global incidence, morbidity, and mortality of sexually transmitted diseases (STDs) caused by HIV, HSV, and other pathogens are very significant, estimated at several hundred millions indi-

viduals worldwide. One approach to control of transmission of STDs is the use of topically applied, female-controlled microbicides that inactivate the relevant pathogens. Consequently, the development of new, safe, topical micobicides for the prevention of STDs is a high priority. One class of such microbicides is virus entry inhibitors, i.e. sulfated carbohydrate (Herold et al., 1997) and *n*-docosanol (Pope et al., 1998). SPL-2999 is not only an HSV entry inhibitor, but also an effective agent for the treatment of HSV infected cells, and has been successfully employed in mice as a preventive agent for intravaginal HSV infection (Bourne et al., 2000). It is therefore a promising candidate for the development of a topical microbicide for the prevention and treatment of HSV infections.

Acknowledgements

This project was supported by Starpharma Limited, 343 Royal Parade, Parkville, Victoria 3052, Australia. The authors would like to thank Dr John Tavis at St. Louis University School of Medicine for critical reading of the manuscript.

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