reviews

molecular pharmaceutics

Dendrimers as Drugs: Discovery and Preclinical and Clinical Development of Dendrimer-Based Microbicides for HIV and STI Prevention

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Received April 6, 2005

Abstract: Starpharma focuses on the use of dendrimers as drugs in their own right, in contrast to dendrimers as drug delivery vehicles or diagnostics. This contextual review describes how dendrimers offer a unique platform for exploring chemical diversity on the nanoscale and how the production of dendrimer libraries covering a diverse array of macromolecular structures can be used in drug discovery and development. Using Starpharma's work on the prevention of HIV and sexually transmitted infections (STIs) through the development of microbicide candidates as an example, the process from which SPL7013 emerged as a development candidate is described. Following a range of preclinical studies, Starpharma submitted an investigational new drug application (IND) for SPL7013 gel (VivaGel) to the United States Food and Drug Administration (FDA) in June 2003, the first such submission for a dendrimer-based drug. The first clinical trial under this IND was completed in 2004.

Keywords: Nanoscale; nanotechnology; chemical diversity; molecular diversity; dendrimer; SPL7013; VivaGel; antiviral; HIV; HSV-2; microbicide

Introduction

Modern drug discovery has been revolutionized by the advent of combinatorial chemistry and high-throughput screening such that modern pharmaceutical companies screen vast compound libraries in the search for biologically active molecules. Concepts such as chemical or molecular diversity have evolved, and although modern compound library design considers these principles, they are almost exclusively applied to small molecules. While there is no doubt that many future drugs will continue to be unearthed using this science, to date this approach has not yielded the quantum leap forward in newly approved medicines. Perhaps that is because the synthetic chemistry efforts of modern drug discovery have been focused on the drive to discover orally bioavailable small-molecule drugs[1] and have failed to fully appreciate that many of the biological targets are in fact macromolecules which rely heavily on polyvalent/multivalent interactions in their binding and signaling cascades.[2,3] In the language of nanotechnology, clearly most biological targets are in the nanoscale (1−100 nm) whereas even with all the chemical and molecular diversity of the huge number of small molecules screened to date, few of these encroach on the nanoscale. This mismatch in scale and absence of polyvalent or multivalent modes of binding within the libraries of molecules screened to date may be because prior to the advent of dendrimers there was a lack of a "nanoscale

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10.1021/mp050023q CCC: $30.25
Published on Web 06/22/2005

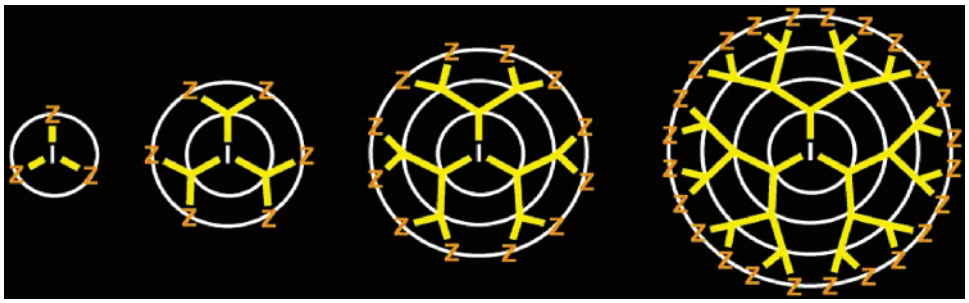

Figure 1. Dendrimer molecular diversity. Dendrimers are composed of molecules built up from a diverse array of initiators (I) and branching units (shown in yellow). As pharmaceuticals, dendrimers offer a unique single-molecule scaffold for the presentation of surface groups (Z) attached to the dendrimer scaffold through a variety of linkers (not shown).

synthetic chemistry tool box" which could be brought to bear in exploring the chemical and molecular space on a nanoscale. This review introduces how dendrimers can fill that nanoscale synthesis void and, with HIV antiviral properties as a context, provides some examples of how, with these molecules included in a compound library, new patterns of biological activity can be unearthed. Using the development of SPL7013 as an example, we describe the concept of "dendrimer developability", which is an important concept for the field to embrace in order for this class of macromolecules to make a significant contribution to improvements in human health.

Dendrimer-Based Drug Discovery: Dendrimer Diversity

So how do dendrimers fit into the drug discovery landscape,[4,5] whose language is dominated by concepts of chemical and molecular diversity? To start to explain the role of dendrimers in this process it is useful to go back to the definition of what dendrimers are: macromolecules constructed through the sequential addition of branching units radiating out from an initiating point.[6] While dendrimers have applicability across a range of industries, as pharmaceuticals, dendrimers of a variety of sizes and shapes offer a unique platform or scaffold for the presentation of surface groups in a polyvalent or multivalent array. The principle of polyvalency or multivalency is now well recognized as a tool to both uncover and optimize new biological activities.[3,4] In addition, dendrimers can be tailored to improve a therapy's pharmacokinetic profile and introduce either active or passive targeting components and both boron and gadolinium dendrimers applied to neutron capture therapy against cancer,[7,8] all within a *single molecular species*. It is this single molecule character along with their desirable cost of manufacture, toxicology profile, and biocompatibility which differentiates dendrimers from many of the other nanotechnology species which might be used for polyvalent or multivalent drug discovery. These other species include traditional or hyperbranched polymers whose very method of synthesis cannot help but give a mixture of molecules.

Perhaps it is not well understood that dendrimers provide a chemically diverse array of compounds, since the literature is dominated by the application of a limited family of commercially available dendrimers such as PAMAM and Astromol (also called PPI or DAB-Am). To illustrate the molecular diversity of dendrimer-based pharmaceuticals it is worthwhile to explore the four components of which they are composed (Figure 1).

Initiators. Dendrimers can be grown from initiators of a variety of valencies. In addition, other functionalities can be incorporated for the construction of multifunctional dendrimers. For example, the initiator can include a site for antibody conjugation.[9] The valency of the core determines how rapidly a polyvalent or multivalent platform is prepared and contributes significantly to the functional group density of the final dendrimer construct.

Branching Units. Similarly, dendrimers can be prepared with a range of internal chemistries by the use of a diverse array of branching units. Three well-known examples give dendrimers with an all-amine interior (DAB-Am = PPI = Astromol)[6], a mixed amine/amide interior (PAMAM),[6] or an all-amide interior (L-lysine dendrimers).[6] But there are many more types of branching units, like gallate or resorcinolate examples,[10] Fréchet's branching unit to prepare ester-

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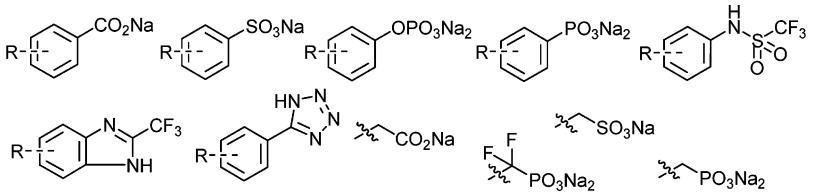

Figure 2. A subset of chemically diverse anionic dendrimer surface groups.

based dendrimers,[11] and those based on TRIS.[12] If you are developing dendrimers as drugs, the important issue is finding those dendrimer branching units which are suitable for pharmaceutical applications from a chemistry manufacturing and control (CMC), pharmacokinetic, toxicity, and efficacy point of view.

Linkers and Dendrimer Surface. By far the greatest diversity in dendrimer structures is built into the choice of surface group that is presented on a dendrimer scaffold and the type of linker that is used. Perhaps the easiest way to think about this is to imagine the dendrimers as scaffolds for the generation of macromolecular combinatorial libraries.

Through the interplay of these parameters, dendrimers can be prepared which represent a diverse array of sizes, shapes, internal chemistry, and surface presentation for use in a dendrimer drug discovery program. This is best illustrated by some simple mathematics. With only a relatively narrow selection of five initiators, three types of branching units assembled to the level of generations 3−5, with no mixing of the type of branching units within any given dendrimer, 10 choices of surface groups attached by two types of linkers, then $5 \times 3 \times 3 \times 10 \times 2 = 900$ structurally unique dendrimers can be prepared.

Dendrimer-Based Antivirals

With the ability to prepare a diverse array of dendrimers, patterns of biological activity can be investigated where subtle changes in the dendrimer design parameters—type of initiator, branching unit type, dendrimer generation, linker, and surface—may have a bearing on the biological properties of the individual dendrimers. To illustrate this idea, the topic of this contextual review is dendrimers drawn from our dendrimer-based-antiviral program. One subset of this program investigated the antiviral properties of dendrimer-based polyanions.[13] Those close to the evolution of polyanions as inhibitors of HIV and other enveloped viruses will know that sulfated carbohydrates and traditional polymer-based polyanions (e.g., polystyrene sulfonate) have shown biological activity against these viruses.[14] However, the molecular diversity of naturally occurring sulfated carbohydrates is

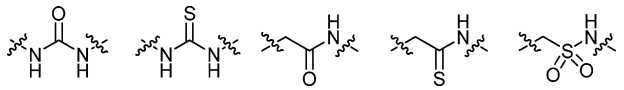

Figure 3. A subset of chemically diverse dendrimer linker groups.

limited and the synthesis of these types of species is very complex. Polymer-based polyanions are also complex mixtures of materials due to the difficulty of obtaining discrete molecular species using this synthetic approach, and as a result it is difficult to align the observed biological activity with the precise structural components of the mixture. We felt that, with the ability to prepare dendrimer-based polyanions as *single* molecular species, structure−activity relationships would emerge from which specific dendrimers could be taken forward into development based on the unique biological properties of well-defined molecular entities. Figures 2 and 3 illustrate how through the interplay of two dendrimer-design parameters a diverse selection of dendrimer-based polyanions can be prepared and assayed for their biological activity. By this means, this class of dendrimers offers the opportunity to tap into a wider diversity of polyanion structures, unearth unusual biological activities, and study cellular uptake mechanisms in much the same way as traditional small-molecule medicinal chemists do in their drug discovery programs.

To illustrate this point, the contrasting biological activity of two dendrimers, SPL2923 and SPL6195, is presented.[15] Both are based on the PAMAM branching unit of the same generation but either built up from an ammonia (SPL2923)

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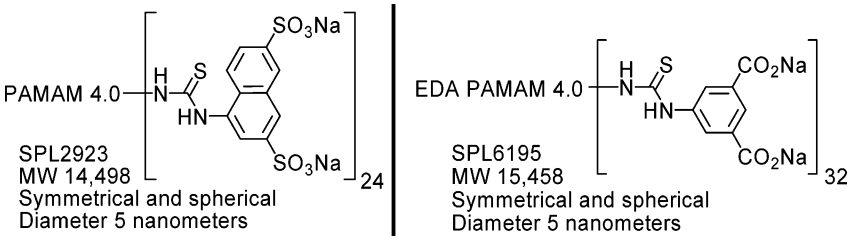

Figure 4. Chemical structure and properties of SPL2923 and SPL6195.

Table 1. In Vitro Antiviral Activity of SPL2923 and SPL6195 against a Range of HIV-1 Strains in a Variety of Cell Types, Relative to Dextran Sulfate (DS) Control

		EC_{50} (μg/mL)[a]		
strain	cells	SPL2923	SPL6195	DS
IIIB	MT-4	0.3 ± 0.2	0.1 ± 0.06	0.4 ± 0.3
IIIB	PBMC	2.2 ± 1.5	9.1 ± 4.2	0.4 ± 0.2
NL4.3	MT-4	0.4 ± 0.3	0.4 ± 0.2	0.4 ± 0.4
RF	MT-4	0.1 ± 0.002	0.1 ± 0.02	0.8 ± 0.3
ROD	MT-4	0.7 ± 0.5	1.7 ± 0.6	0.04 ± 0.03
EHO	MT-4	0.01 ± 0.0008	2.1 ± 0.7	10.6 ± 4.8
MAC251	MT-4	0.08 ± 0.2	1.2 ± 0.6	3.5 ± 1.9

[a] Concentrations of each compound required to inhibit the cytopathic effect (CPE) of HIV-1 in cell culture by 50%.

or ethylenediamine (EDA) core (SPL6195). For SPL2923, naphthalenedisulfonic acid groups are attached to the dendrimer surface by a thiourea bond, and for SPL6195, benzenedicarboxylic acid groups are attached, again using the same type of thiourea linker (Figure 4).

From a cursory glance, the two dendrimers appear to be very similar with respect to structure and polarity. Indeed, during the initial in vitro HIV screen they each appeared to have essentially the same level of antiviral activity using a range of HIV-1 strains and cell types (Table 1).[15]

It was only when our collaborators at the REGA Institute conducted time of addition studies that subtle differences in the mode of action of these two dendrimers emerged.[15] These types of studies are used to probe a compound's antiviral mode of action. For HIV, the life cycle includes the following processes: virus attachment to and fusion with the host, formation of a DNA copy of the viral RNA using the viral enzyme reverse transcriptase, integration of this DNA copy into the host's DNA, mRNA formation and protein expression, processing of viral proteins, formation of new virus particles, and then their escape from the host cell. This whole process takes time, and so in an appropriately established time of addition experiment, adding the antiviral compound at certain times before and after the virus comes into contact with the target cell helps determine at what stage of the virus life cycle the compound acts. The validity of the experiment is demonstrated by the inclusion of certain control compounds. For example the sulfated carbohydrate dextran sulfate (DS) exhibits an antiviral effect only for a window of time close to when the virus and cells are combined, as the mode of action of DS is inhibition of virus attachment. The drug AZT, which inhibits the viral enzyme reverse transcriptase, acts for a time only until DNA copies have been made of the viral RNA. If it were added after this time, "the horse has bolted", and the DNA copies of the viral RNA have been prepared and no amount of AZT is going to be effective. Later in time, a range of other events such as DNA integration, mRNA formation, and protein expression are taking place and drugs such as the protease inhibitor Ritonavir have a window in which to act. In such a time of addition experiment, SPL6195 at 20 μg/mL was shown to act on virus attachment and fusion in much the same way as the control compound dextran sulfate, itself a macromolecular polyanion. Even if the concentration of SPL6195 were increased 5-fold, this situation did not change and the only mode of action that was evident was inhibition of virus attachment and fusion. This result was not too surprising because the early stages of HIV infection, namely, virus attachment and fusion, are taking place on the outer surface of the host cell, and a compound like SPL6195 would have a chance to act on the later mechanisms only if it were to have made its way into the cell. At first glance this would seem unlikely for a compound of this size and overall charge. For SPL2923, again at 20 μg/mL, the picture appeared the same as for SPL6195: inhibition of virus attachment and fusion, but no effect on intracellular events. What was surprising was that, when the concentration was increased to 100 μg/mL, the compound seemed to also have an effect on incorporation of the viral RNA into the host's DNA. SPL2923 must be getting into the cell, and further studies indicated that the predominant intracellular mode of action was in fact inhibition of reverse transcriptase and viral integrase, the enzymes responsible for the formation of DNA copies of the viral RNA and integration of these viral DNA copies into the host's DNA.[15] The cell penetration of SPL2923 when compared to SPL6195 was shown in a variety of confocal microscope studies using fluorescein-labeled materials and also in cell incubation/lysis studies. This work showed that there seemed to be differing cell penetration and resulting modes of action for dendrimers that on first glance appear very similar, but which differ in the initiator and type of anionic surface. These contrasting results for the two dendrimers are summarized in Table 2.

Dendrimer-Based HIV Antivirals: Treatment or Prevention? The Need for a Microbicide

With a library of polyanion-coated dendrimers, some good biological activity and an idea of mode of action attention turned to developing a commercial product based on this technology. One of the first decisions to make was whether

Table 2. Different Mode of Action for SPL2923 at 100 μg/mL When Compared to SPL2923 at 20 μg/mL or SPL6195 at Either Concentration Relative to a Dextran Sulfate (DS) Control

compound	concentration	surface group	attachment/ fusion	reverse transcriptase	integrase	protease
DS	n/a	sulfated hydroxyl	yes	no	no	no
SPL6195	20 μg/mL	benzene dicarboxylate	yes	no	no	no
SPL6195	100 μg/mL	benzene dicarboxylate	yes	no	no	no
SPL2923	20 μg/mL	naphthalene disulfonate	yes	no	no	no
SPL2923	100 μg/mL	naphthalene disulfonate	yes	***yes***	***yes***	no

- **Number of people living with HIV/AIDS (Total 42 million)**
  - Adults 38.6 million
  - ***Women 19.2 million***
  - Children under 15 years 3.2 million
- **People newly infected with HIV in 2002 (Total 5 million)**
  - Adults 4.2 million
  - ***Women 2 million***
  - Children under 15 years 800 000
- **AIDS deaths in 2002 (Total 3.1 million)**
  - Adults 2.5 million
  - ***Women 1.2 million***
  - Children under 15 years 610 000

Figure 5. Statistical breakdown of the state of the HIV/AIDS epidemic in 2002 around the time Starpharma decided to focus on the development of dendrimer-based HIV prevention (microbicide) strategies.

to focus on HIV prevention or treatment. The decision to concentrate on prevention strategies came when the reasons behind the startling 2002 HIV epidemic statistics were examined.[16] For an epidemic that in the initial stages was perceived in Western countries as being a disease of men who have sex with men or intravenous drug users, it was clear in 2002 that the vast majority of global HIV transmission occurred during heterosexual sex and that women were increasingly bearing the burden, which resulted in the disturbing associated transmission of HIV to the newborn (Figure 5). As a direct result of these alarming statistics, Starpharma is now part of an international effort surrounding the discovery and development of intravaginal products that women could use prior to sexual intercourse to protect themselves from infection by HIV and sexually transmitted infections (STIs). Such products are now termed microbicides.[17]

Dendrimer-Based Microbicides: Lead Optimization and Identification of a Clinical Candidate

In order to develop a dendrimer-based microbicide, our dendrimer medicinal chemistry efforts entered into a lead optimization phase, considering much the same parameters that you would if you were developing a small-molecule drug. These included expanding the structure−activity relationship, assessing in vivo activity, conducting formulation development and stability studies, developing and validating analytical and bioanalytical methods, carrying out a scale-up chemistry program, and conducting cost of manufacture analyses. The activities led us to the following conclusions:

(1) With the available dendrimers at that time, there was an initial increase in HIV inhibitory activity with dendrimer size, but a threshold seemed to be reached above which there was no apparent gain in antiviral activity.

(2) Certain regioisomers of naphthalenedisulfonic acid surface groups seemed to give the greatest biological activity.

(3) Utilizing an amide linker achieved the optimum stability profile.

With these parameters established, we concentrated on the optimum dendrimer branching unit for the presentation of the sodium 1-(carboxymethoxy)naphthalene-3,6-disulfonate surface group.[18] We compared the HIV antiviral activity of 32 of these surface groups attached through an amide bond to either a Generation 3 L-lysine, PAMAM, or PPI dendrimer, and as shown in Table 3, the biological activity was essentially the same within the error of the experiment.

However, as with the development of any drug, biological activity is not the sole determinant of which compound to take forward into formal preclinical development. When we concentrated solely on scale-up manufacture of the dendrimer-based drug substance (i.e., unformulated material) and drug product (dendrimer in the formulation to be administered in the clinic), distinct preferences emerged. We were concerned about the level of residual cobalt in the PPI-based dendrimer SPL7320 (from residual catalyst used to reduce the nitrile group during PPI dendrimer synthesis), and while cobalt-free material was able to be prepared, we thought there was a significant risk that this would be a difficult to control variable on large-scale manufacture. In microbicide development the dendrimer would be formulated into a pH 4 aqueous gel, and the target shelf life for such a product is years even when stored at elevated climatic temperatures. As a result we felt that the PAMAM dendrimer SPL7304, due to the risk of reverse Michael addition chemistry particularly in this acidic formulation, was not the optimum branching unit for this application. The L-lysine-based dendrimer SPL7013

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(17) (a) Turpin, J. A. Considerations and development of topical microbicides to inhibit the sexual transmission of HIV. *Expert Opin. Invest. Drugs* **2002**, *11*, 1077−1097. (b) D'Cruz, O. J.; Uckun, F. M. Clinical development of microbicides for the prevention of HIV infection. *Curr. Pharm. Des.* **2004**, *10*, 315−336. (c) www.microbicide.org.

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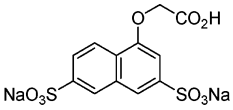
Table 3. HIV Antiviral Activity of Three Different Dendrimers, Each Presenting 32 Sodium 1-(Carboxymethoxy)naphthalene-3,6-disulfonate Surface Groups Each Linked by an Amide Bond

Linker = amide, surface =  **, n=32**

	SPL7013 (μg/mL)		SPL7304 (μg/mL)		SPL7320 (μg/mL)	
branching unit:	L-lysine		PAMAM		PPI	
virus isolate	IC_{50}[a]	TI[b]	IC_{50}	TI	IC_{50}	TI
HIV-1 clade A (RW/92/016)	1.68[c]	>60	0.40	>250	1.02	>98
HIV-1 clade B (302056)	0.73	>137	0.72	>139	1.17	>85
HIV-1 clade C (BR/92/025)	1.73	>58	0.81	>123	1.40	>71
HIV-1 clade D (UG/92/046)	2.35	>43	0.82	>122	0.72	>139
HIV-1 clade E (CMU02)	1.51	>66	1.71	>58	1.60	>63

[a] Concentration required to reduced the degree of HIV infection by 50% in peripheral blood mononuclear cells (i.e., primary cells). [b] Concentration required to reduced cell viability by 50%/concentration required to reduced the degree of HIV infection by 50%. [c] 1 μg/mL = 61 nM.

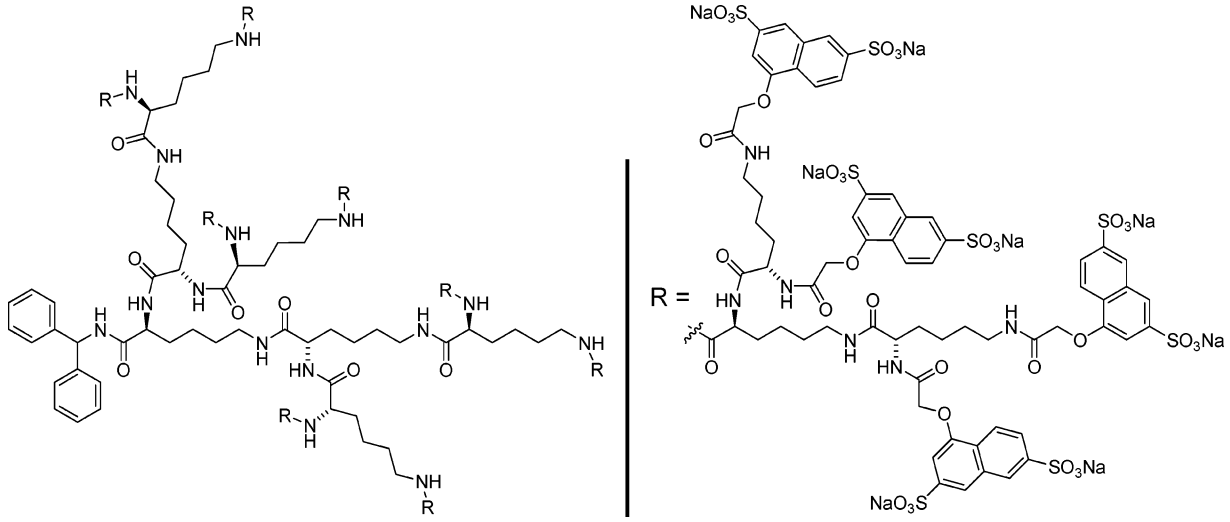


Figure 6. Chemical structure of SPL7013, the dendrimer antiviral in VivaGel.

on the other hand had the optimum formulation compatibility and both drug substance and drug product stability profiles. As with any drug development, these studies were supported by the appropriate stability-indicating analytical methods which have been validated under GLP. In addition, after intensive investigations SPL7013 emerged as by far the easiest of three dendrimers to prepare on large scale as a single molecular species. Finally, again after an intensive method development phase, bioanalytical methods for SPL7013 were validated under GLP for the detection of SPL7013 in human and a variety of animal plasmas.

For all these reasons, SPL7013 (Figure 6) was selected to enter formal preclinical development.[19,20] This particular dendrimer is built up from a divalent core, the benzhydrylamine amide of L-lysine. Successive additions of four L-lysine layers leads to a dendrimer with 32 amine groups on the surface; 16 α-amines and 16 ϵ-amines from the outer L-lysine layer. The final step in the synthesis involves the last amide-bond-forming reaction to attach 32 sodium 1-(carboxymethoxy)naphthalene-3,6-disulfonate groups to the surface via amide linkers. This manufacturing process is controlled by HPLC and LC/MS in-process controls and delivers SPL7013 as a *single molecular entity* as determined by HPLC, capillary electrophoresis (CE), and electrospray mass spectral analysis. We have scaled key SPL7013 intermediates to 5−100 kg quantities, and our projected cost of commercial scale manufacture is in line with our business model for the

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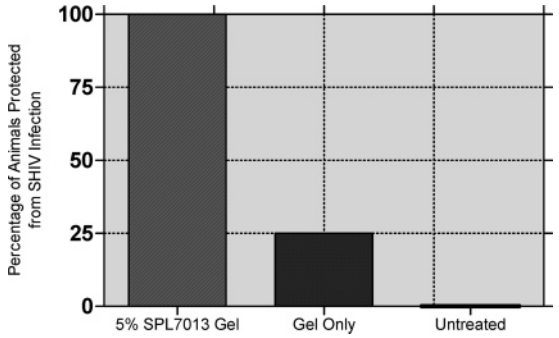

Figure 7. Protection from intravaginal SHIV infection in macaques by a single intravaginal dose of 5% w/w SPL7013 gel ($n = 6$) relative to gel only ($n = 4$) and untreated control groups ($n = 4$).

microbicide opportunity. The clinical drug product of SPL7013 is now called VivaGel and is a relatively simple water-based Carbopol gel buffered to a physiologically compatible pH. VivaGel can be packed either into a single-use applicator or in large tubes with a reusable applicator.

In pivotal nonhuman primate efficacy studies (Figure 7) conducted in collaboration with the United States National Institute of Allergy and Infectious Diseases (NIAID) and investigators at the University of Washington at Seattle, a single intravaginal dose of the clinical formulation containing 5% w/w SPL7013 protected all pig-tailed macaques (i.e., monkeys) from a single intravaginal infection by a strain of simian-human immunodeficiency virus (SHIV).[21]

An investigational new drug application (IND) was submitted to the United States Food and Drug Administration (FDA) in June 2003 and to our knowledge represented the first time a dendrimer-based drug has been submitted to the FDA. We feel that this is a milestone in the development of nanotechnology-based solutions to human health conditions and the development of dendrimer-based drugs. An initial phase I clinical trial involving 36 healthy women was recently completed in 2004 and showed that the safety profile of VivaGel containing 0.5−3.0% w/w SPL7013 was comparable with that of the placebo gel following once daily intravaginal dosing for seven consecutive days.[22] Significantly, SPL7013 was not absorbed into the systemic circulation following intravaginal dosing. This is an important characteristic as it is essential that SPL7013 is retained in the vaginal lumen following dosing in order to inhibit HIV infection immediately. The lack of systemic exposure of SPL7013 also significantly simplifies the toxicology aspects for this drug. The clinical program for VivaGel will expand in 2005 and if successful would represent a significant demonstration of the power of nanotechnology and dendrimers in particular to yield important public health benefits to society.

Acknowledgment. We thank Professor Erik De Clercq and colleagues (REGA Institute, Leuven, Belgium), Professors Larry Stanberry and Nigel Bourne (UTMB, Galveston, TX), Professor David Bernstein (Cincinnati Children's Hospital, Cincinnati, OH), Professor Earl Kern (University of Alabama at Birmingham, Birmingham, AL), and the National Institute of Allergy and Infectious Diseases (National Institutes of Health, Bethesda, MD) for assistance with our in vitro and in vivo antiviral studies and our development of microbicides.

MP050023Q

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