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Review

The rise and fall of polyanionic inhibitors of the human immunodeficiency virus type 1

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ABSTRACT

Infection by the human immunodeficiency virus type 1 (HIV-1) is an ordered, multistep process involving binding and entry, reverse transcription, integration, viral gene transcription, translation, processing, and finally assembly. Numerous therapeutic and preventive compounds, which are currently available for clinical use or are under preclinical and clinical development, act on at least one of these steps. Polyanionic HIV-1 inhibitors comprise a family of compounds that are generally considered entry inhibitors. The main mechanism of anti-HIV-1 activity associated with these compounds involves electrostatic interactions with HIV-1 glycoprotein 120 that ultimately prevent binding of the virus to target cells. A number of these compounds have been considered for systemic use and for use as microbicides, which are products designed to prevent sexual HIV-1 transmission. These compounds have been studied extensively using in vitro assays of activity, cytotoxicity, and mechanism of action, ex vivo models of HIV-1 transmission, and animal models of in vivo efficacy and toxicity. Three of these polyanionic compounds – cellulose sulfate, carrageenan, and PRO 2000 – were advanced into clinical trials of microbicide safety and efficacy. Although phase I and phase II clinical trials showed these compounds to be safe and well tolerated, none of the phase III trials provided any evidence that these compounds were effective against heterosexual HIV-1 transmission. Furthermore, clinical and in vitro results suggest enhancement of HIV-1 infection in the presence of polyanionic compounds. We discuss the preclinical development of polyanionic HIV-1 inhibitors, the clinical trials of polyanionic compounds used systemically and as topical vaginal microbicides, and the prospects for the future development of these compounds as inhibitors of HIV-1 infection.

Contents

1. Introduction

The global impact of the acquired immune deficiency syndrome (AIDS) epidemic is clearly evident. Around the world, approximately 33.4 million people are now infected with the human immunodefi-

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ciency virus type 1 (HIV-1) (WHO/UNAIDS, 2009). In this population of HIV-1-infected individuals, approximately 2.7 million were newly infected in 2008 alone. Despite the continued spread of HIV-1 around the globe, however, the number of people living with HIV-1 has leveled over the last few years. This effect is due, in part, to advances in the development of an armamentarium of effective anti-HIV-1 drugs and, in particular, to the successful use of combination therapies, which are collectively referred to as highly active antiretroviral therapy (HAART). However, because of the emergence of drug-resistant HIV-1 strains and side effects associated with chronic antiretroviral drug use, a continued need remains for safe and effective systemic antiviral agents that can be used in effective therapeutic (or preventative) treatment strategies.

The slowed expansion of the AIDS epidemic, however, has not benefited all populations at risk for HIV-1 infection. Since the beginning of the epidemic, women have become increasingly involved in the spread of HIV-1. Globally, women represent about half of all HIV-1 infections. However, in sub-Saharan Africa, where women are at great risk for infection, approximately 60% of all adults living with HIV-1 in 2008 were women (Garcia-Calleja et al., 2006; WHO/UNAIDS, 2009). Efforts to control the transmission of HIV-1 have been thwarted by social stigmas and by beliefs and attitudes involving the status of women, particularly with regard to sex. The increased numbers of women infected with HIV-1, combined with societal limitations involving protective measures for women, have spurred the development of preventive products, called microbicides, that are capable of reducing or eliminating the risk of HIV-1 sexual transmission. Microbicides, which will provide an important, female-controlled preventive approach for combating the global spread of HIV-1, also rely on the availability of agents that serve as effective inhibitors of HIV-1 infection. The discovery and development of antiviral compounds for use in microbicides present particular challenges because these agents need to be nontoxic, safe for repeated use, and highly effective against HIV-1, if not against a wide variety of other sexually transmitted pathogens. In addition, they should be suitable for use within the cervicovaginal and rectal environments, because the risk of sexual HIV-1 transmission encompasses both vaginal- and anal-receptive intercourse.

Drug development efforts in both fields have followed similar paths. Following the identification of HIV-1 as the etiologic agent of AIDS, some of the early efforts to develop systemic anti-HIV-1 drugs explored the use of polyanionic compounds that inhibited HIV-1 infection. Recognizing the necessity of targeting specific steps within the HIV-1 replication cycle, investigators pursued the development of systemic drugs specifically targeted to the HIV-1-specific proteins reverse transcriptase, integrase, and protease. Later drug discovery efforts led to the exploitation of other targets in the replication cycle, including the process of binding and entry, which in turn led to the development and approval of the CCR5 inhibitor maraviroc (Selzentry) for systemic use (Meanwell and Kadow, 2007; Yost et al., 2009). Similarly, microbicide development efforts that followed failed trials of surfactant-based microbicides focused on the use of polyanionic compounds that blocked HIV-1 entry. However, the clinical failures of three products based on the polyanionic molecules cellulose sulfate, carrageenan, and naphthalene sulfonate have now placed greater focus on the use of antiretroviral drugs as microbicides. This approach was validated by the recent demonstration that a tenofovir-containing microbicide effectively reduced the risk of HIV-1 acquisition in uninfected women (Baleta, 2010).

In both fields, polyanionic compounds were considered for use as antiviral agents and then abandoned after clinical trials of these compounds failed to demonstrate efficacy against HIV-1 and, in some cases, revealed problems associated with their administration or application. This review provides an overview of the rise and fall of polyanionic molecules as inhibitors of HIV-1 infection.

2. The discovery and development of polyanionic HIV-1 inhibitors

Since the beginning of the HIV/AIDS epidemic, approximately 26 years of research have been focused on understanding the mechanisms of HIV-1 pathogenesis from the initial host–pathogen encounter to the onset of life-threatening disease. This single virus has arguably posed one of the biggest challenges to scientists in terms of developing effective treatments and preventive measures in the 20th and 21st centuries. Within the first few years of the eventual pandemic, several compounds with different mechanisms of action were discovered and utilized as anti-HIV-1 agents (Hirsch and Kaplan, 1985). One such compound was suramin (De Clercq, 1987) (Fig. 1). In the late 1970s, suramin was discovered to have activity against reverse transcriptase in animal retroviruses (De Clercq, 1979). The identification of HIV-1 as a retrovirus led scientists to test suramin as an anti-HIV-1 agent. Investigations into the activity of suramin demonstrated that it could block in vitro infection by cell-free HIV-1 (Mitsuya et al., 1984). Additionally, it was discovered that suramin could also block the cytopathic effects of cell-associated viral spread (Mitsuya et al., 1984). As such, suramin was the first compound to be recognized as an anti-HIV-1 agent (Mitsuya et al., 1984) and, additionally, was the first to be used clinically for the treatment of HIV-1 infection (Broder et al., 1985; Levine et al., 1986). When suramin was administered to patients infected by HIV-1, plasma drug concentrations were achieved that inhibited HIV-1 replication in vitro (Broder et al., 1985; Collins et al., 1986).

Although the mechanism of action was believed to be inhibition of reverse transcriptase, experiments indicated that its mechanism of action was nonspecific (De Clercq, 1987). Further research revealed that suramin, a polysulfonated compound, in fact acted to block viral adsorption (Schols et al., 1989). Its use as a treatment was complicated by the fact that suramin binds to plasma protein, such as albumin, thereby reducing the ability of suramin to inhibit HIV-1 binding and entry (Bos et al., 1990). Although the activity of this compound (and other polysulfonated compounds) is affected by the sequestration of the compound by plasma proteins (Yao et al., 1991), suramin was an effective inhibitor of Rauscher leukemia virus in mice, providing justification for the continued development of anionic compounds as HIV-1 inhibitors (De Clercq, 1987; Ruprecht et al., 1985).

Closely following studies of suramin, investigations of other polyanionic compounds as HIV-1 inhibitors were initiated. Previous studies had shown that polyanionic compounds exhibited activity against human herpes simplex virus (HSV) (De Somer et al., 1968; Nahmias and Kibrick, 1964). Studies of polyanionic compounds as agents active against HIV-1 increased following the discovery that dextran sulfate and heparin were potent inhibitors of HIV-1 (Ito et al., 1987) (Fig. 1). Dextran sulfate had a history of safe use as both an anticoagulant and an antilipemic agent (Mitsuya et al., 1988). Early studies of dextran sulfate demonstrated that the compound had a high level of efficacy against HIV-1 as well as a low level of toxicity (Ueno and Kuno, 1987a,b).

Dextran is a high molecular weight polysaccharide produced from sucrose by several bacteria (Witvrouw and De Clercq, 1997). Dextran is then sulfated to produce dextran sulfate, which typically contains 2–3 sulfates per glucose group. The varying molecular weights of dextran sulfate stem from the differences in the chain length and branching of dextran (Witvrouw and De Clercq, 1997). Early structure–activity relationship investigations identified a correlation between anti-HIV-1 activity and the num-

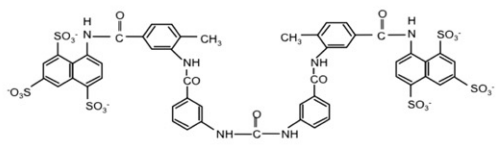
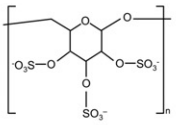
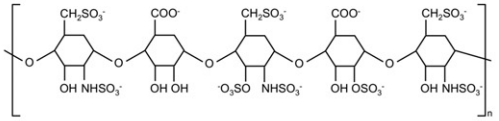
Compound	Structure	Comments	References
suramin		Although originally proposed to be a RT inhibitor, further studies revealed it to be able to block viral adsorption. Viral activity reduced because suramin can also bind to and be sequestered by plasma proteins.	Bos et al., 1990; De Clercq, 1987; Schols et al., 1989; Yao et al., 1991
dextran sulfate		Originally proposed to be an RT inhibitor, subsequently found to block syncytia formation with activity at the level of viral binding. However, usefulness diminished because of interference with blood coagulation and difficulty standardizing the compound.	Astrup and Galsmar, 1944; Busso and Resnick, 1990; De Clercq, 1995; Mitsuya et al., 1988
heparin		Compound shown to inhibit HIV-1 infection at the level of viral binding at nontoxic concentrations. However, usefulness diminished because of interference with blood coagulation and difficulty standardizing the compound.	Astrup and Galsmar, 1944; Busso and Resnick, 1990; De Clercq, 1995; Ito et al., 1988

Fig. 1. Polyanionic compounds assessed for anti-HIV-1 activity as systemic agents. Three polyanionic compounds were extensively studied for use as systemic therapeutics in HIV-1-infected individuals. These compounds include suramin, dextran sulfate, and heparin.

ber of sulfate groups per monosaccharide unit. Studies with non-sulfated dextran demonstrated no activity against HIV-1 (Mitsuya et al., 1988). As was the case with suramin, mechanism studies originally attributed the activity of dextran sulfate to its ability to inhibit reverse transcriptase (Ueno and Kuno, 1987a,b). Additional investigations, however, revealed that the formation of syncytia was completely blocked by dextran sulfate, thus suggesting that the activity of dextran sulfate appeared to be at the level of viral binding (Mitsuya et al., 1988). In contrast, azidothymidine (AZT), which is a specific inhibitor of reverse transcriptase and does not inhibit viral binding, had no effect on formation of syncytia (Mitsuya et al., 1988). In a similar manner, heparin (Fig. 1) was also shown to inhibit HIV-1 infection at the level of viral binding at nontoxic concentrations (Ito et al., 1987). The usefulness of these compounds, however, was diminished by the fact that these compounds also interfered with blood coagulation (Astrup and Galsmar, 1944). In addition, variations in the range of molecular weights and varying degrees of sulfation made standardization of these compounds difficult. The large degree of variability within the V3 loop of gp120 also made the activity of these compounds variable against varied strains of HIV-1 (Busso and Resnick, 1990; De Clercq, 1995).

The antiviral activity of polyanionic polysaccharides derived from marine algae has also been well characterized and has continued to be studied for the past five decades (Gerber et al., 1958). Initially, one such polysaccharide, carrageenan, was reported to be active against mumps virus and influenza B virus (Gerber et al., 1958). Further studies described various carrageenans as inhibitors of herpes simplex virus (Ehresmann et al., 1977). Later studies further characterized a compound in the λ-carrageenan family as an inhibitor of the retrovirus avian myeloblastosis virus, while having almost no cytotoxicity (Nakashima et al., 1987a). Following the classification of HIV-1 as a retrovirus, multiple compounds in the carrageenan family, including λ-, κ-, and ι-carrageenan, were examined for efficacy and subsequently demonstrated to be active against HIV-1 (Baba et al., 1988; Nakashima et al., 1987b) (Fig. 2). The κ- and λ-carrageenan families are typically identified by the position of the sulfate and by the absence or presence of anhydrogalactose (Witvrouw and De Clercq, 1997). Additionally, the levels of sulfation also differ, with the degree of sulfation for κ- and λ-carrageenan determined to be 25% and 35%, respectively (Morris et al., 1977). The degree of sulfation, which refers to the number of sulfate groups per monosaccharide residue, plays a major role in the level of antiviral activity for a given sulfated polysaccharide (Ghosh et al., 2009; Witvrouw and De Clercq, 1997). Previous studies have shown a direct correlation between the degree of sulfation and the efficacy of the compound against HIV-1 (Jurkiewicz et al., 1989; Witvrouw and De Clercq, 1997). Therefore, the differences in the anti-HIV-1 activity between κ- and λ-carrageenan can be explained by their differences in sulfation, with the highly sulfated λ-carrageenan shown to have greater activity relative to κ-carrageenan, which has a lower level of sulfation (Witvrouw and De Clercq, 1997). Because in vitro assays that compared the anticoagulant and antiviral properties of λ-carrageenan demonstrated that its EC_{50} value was more than 100-fold lower than the anticoagulant threshold, λ-carrageenan was thought to be especially promising as an antiviral agent (Witvrouw and De Clercq, 1997).

Studies involving sulfated and sulfonated polysaccharides were followed with the discovery of many other polyanionic compounds that demonstrated varying levels of activity as well as toxicity. These compounds included cellulose sulfate, poly(styrene) sulfonate, and naphthalene sulfonate (Fig. 2). Studies involving cellulose sulfate have been ongoing for more than 50 years (Doring, 1954; Hildebrandt, 1956; Morrow et al., 1952). Cellulose sulfate was originally suggested for use in food, drugs, and cosmetics (Morrow et al., 1952). Early studies demonstrated that cellulose sulfate was not absorbed from the gastrointestinal tract and was characterized by low toxicity, indicating that cellulose sulfate was safe for consumption (Morrow et al., 1952). The activity of cellulose sulfate against HIV-1 was determined while studying the effect of sulfation on the level of activity. Cellulose sulfate can vary in molecular weight depending on the length of the backbone and degree of sulfation. However, the most active version of cellulose sulfate has a molecular weight of at least 500,000 daltons and is

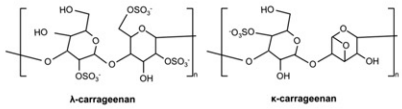
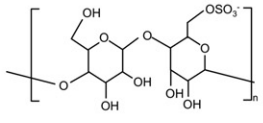
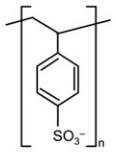
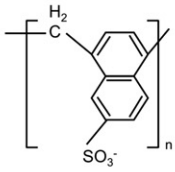
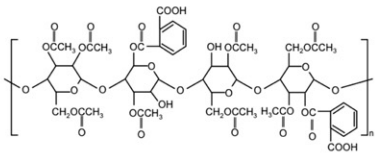
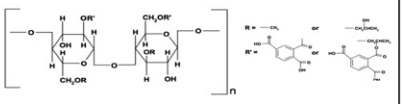
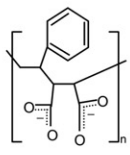
Compound	Structure	Comments	References
carrageenan		Found to be active against mumps virus, influenza B, herpes simplex virus, AMV, and HIV-1. Direct correlation found between levels of sulfation and anti-HIV activity. Carraguard (1:1 κ- to λ-carrageenan) made it through, but failed, Phase III clinical trials.	Gerber et al., 1958; Ehresmann et al., 1977; Nakashima et al., 1987a b; Baba et al., 1988; Ghosh et al., 2009; Witvrouw and De Clercq, 1997; Jurkiewicz et al., 1989; Skoler-Karpoff et al., 2008;
cellulose sulfate		Found to be active against HSV-1, HSV-2, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, HPV, and HIV-1. Also demonstrates contraceptive properties. Most active version is maximally sulfated with 17% sulfation by weight. Ushercell (cellulose sulfate) did not complete Phase III clinical trials because of lack of efficacy and concerns over enhanced transmission.	Anderson, 2000; Christensen et al., 2001; Astrup and Alkjaersig, 1950; Halpern et al., 2008; Van Damme et al., 2008
polystyrene sulfonate		Demonstrated activity against HIV-1 by a mechanism involving the viral glycoproteins gp120 and gp41. Found to be active against against HSV-1, HSV-2, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, influenza A, and HIV-1. Also demonstrates contraceptive properties. However, large amounts of compound are required to inhibit HIV-1 infection, which increases the likelihood of non-specific binding and reduced compound effectiveness	Anderson, 2000; Neurath et al., 2002b; Herold et al., 2000; Zaneveld et al., 2002; Mauck et al., 2004
naphthalene sulfonate		Mechanism determined to involve binding to gp120 and blocking binding to CD4. Shown to inhibit a large range of HIV-1 isolates in a variety of cell types. Its activity in vivo may be hampered by the binding of the compound to serum components, the inability to reach sites of viral replication, and toxicity associated with therapeutic concentrations after prolonged exposure. PRO 2000 (naphthalene sulfonate), failed Phase III clinical trials when tested at two different concentrations (0.5% and 2%).	Mohan et al., 1991; Rusconi et al., 1996; Meylan et al., 1994; Alliance for Microbicide Development, 2008; Abdool Karim et al., February 9, 2009; Microbicide Trials Network, February 9, 2009 a, b; Cohen et al., December 19, 2009; Warren and Marshall, December 19, 2009
cellulose acetate phthalate		A pharmaceutical excipient with a long history of human consumption. Found to inhibit HSV-1, HSV-2, bacterial vaginosis, and HIV-1. Its mechanism of action is suggested to involve either gp120 or gp41. However, it is insoluble at the low pH of the vaginal tract and as such is formulated as a micronized compound.	Lee, 1994; Gyotoku et al., 1999; Neurath et al., 2000; Neurath et al., 2001; Neurath et al., 2002a, b
hydroxypropyl methylcellulose trimellitate		A pharmaceutical excipient that is structurally similar to cellulose acetate phthalate, except it is able to remain soluble and molecularly dispersed at much lower pH values. Has demonstrated activity against HIV-1.	Rando et al., 2006
polystyrene maleic acid		Has a structure similar to PSS, which also includes a styrene-based backbone, however derives its negative charge from maleic acid rather than sulfation or sulfonation. Demonstrates equal efficacy against X4 and R5 HIV-1 viruses, and efficacy against several different HIV-1 subtypes. Also, does not demonstrate viral enhancement of infection following compound removal.	Pirrone et al., 2010

Fig. 2. Polyanionic compounds assessed for use in anti-HIV-1 microbicides. Seven polyanionic compounds were assessed for efficacy and safety as topically applied microbicides. These compounds included carrageenan, cellulose sulfate, polystyrene sulfonate, naphthalene sulfonate, cellulose acetate phthalate, hydroxypropyl methylcellulose trimellitate, and polystyrene maleic acid.

maximally sulfated with approximately 17% sulfation by weight (Anderson et al., 2000). Additional in vitro studies demonstrated that cellulose sulfate also has activity against HSV type 1 (HSV-1) and HSV type 2 (HSV-2) (Anderson et al., 2000), *Neisseria gonorrhoeae* (Anderson et al., 2000), *Chlamydia trachomatis* (Anderson et al., 2000), and human papillomavirus (Christensen et al., 2001). In addition to its antiviral and antimicrobial activity, cellulose sulfate also has contraceptive activity, with the ability to hinder sperm penetration through cervical mucus (Anderson et al., 2000), the ability to stimulate acrosomal loss (Anderson et al.,

2000), and the ability to inhibit hyaluronidase activity (Astrup and Alkjaersig, 1950).

Like cellulose sulfate, polystyrene sulfonate (PSS) (Fig. 2) combines antiviral and contraceptive activity (Anderson et al., 2000). PSS also has demonstrated activity against HIV-1 and is suggested to act by a mechanism that involves interactions with the viral glycoproteins gp120 and gp41 that inhibit viral fusion with the cell membrane (Neurath et al., 2002b). The activity of PSS is broad: It inhibits *C. trachomatis*, *N. gonorrhea*, influenza A virus, and HSV-1 and -2 (Herold et al., 2000). The spermicidal activity is attributed to its ability to inhibit hyaluronidase and acrosin and its capacity to prevent fertilization by affecting penetration of mucus (Zaneveld et al., 2002). PSS seemed promising for use as a microbicide because it was shown to target multiple sexually transmitted infections without affecting the resident *Lactobacillus* species (Zaneveld et al., 2002). There also appeared to be no in vitro or in vivo toxicity associated with compound application. Studies have generally focused on the use of this compound as an active agent in a microbicide as opposed to a systemic agent. One drawback of PSS is that large amounts of compound are required to inhibit HIV-1 infection, which consequently increases the likelihood of nonspecific binding and reduced compound effectiveness (Mauck et al., 2004; Zaneveld et al., 2002).

Naphthalene sulfonate (Fig. 2) is representative of a polyanionic family of compounds that binds to gp120 and blocks binding to CD4 in an extremely efficacious manner, thereby demonstrating, in vitro, the ability to block HIV-1 infection (Mohan et al., 1991). The compound, studied primarily for its activity against HIV-1, is composed of a synthetic, long-chain polymer with alternating 2-naphthalene sulfonic acid sodium salt and methylene units (Abdool Karim and Baxter, 2009). The compound inhibits infection of a large range of HIV-1 isolates in a wide variety of cell types, including $CD4^+$ T-cell lines, human peripheral blood mononuclear cells, and cells of the monocyte–macrophage lineage (Rusconi et al., 1996). The activity of this compound appears to differ significantly from that of dextran sulfate, which has been shown to be inactive against several R5 (CCR5-using) HIV-1 strains (Meylan et al., 1994). At non-cytotoxic concentrations, naphthalene sulfate completely inhibited infection with no viral breakthrough after 28 days in culture (Rusconi et al., 1996). In addition, when combined with zidovudine, antiviral synergy was observed (Rusconi et al., 1996). However, the compound only transiently suppressed HIV-1 replication in cells that were infected prior to compound application (Rusconi et al., 1996), suggesting that the compound is best suited as a potential microbicide rather than as a therapeutic agent. Additionally, the activity of naphthalene sulfonate in vivo may be hampered by the binding of the compound to serum components, the inability to reach sites of viral replication, and toxicity associated with therapeutic concentrations after prolonged exposure (Rusconi et al., 1996).

In general, the early polyanionic compounds evaluated for antiviral activity had their charges associated with sulfation or sulfonation. In parallel with these preclinical efforts, studies were also performed to evaluate the activities of polyanionic compounds with charges derived from nonsulfate groups. One such compound was cellulose acetate phthalate (CAP) (Fig. 2). CAP is a pharmaceutical excipient that has been used for over four decades as an enteric coating on tablets and capsules (Lee, 1994). CAP is generally regarded as nontoxic because of a long history of human consumption in commonly used capsules and tablets. CAP inhibits infection not only by HIV-1 but also by HSV-1 and -2, as well as organisms associated with bacterial vaginosis (Gyotoku et al., 1999; Neurath et al., 2000). The higher molecular weight of this compound suggests that its mechanism of action is confined to the viral surface, involving interactions with either gp120 or gp41 (Neurath et al., 2001). Studies of micronized CAP, which derives its anionic charges from carboxylic acid groups instead of sulfate or sulfonate residues, also demonstrated the ability to remove from physiological fluids infectious virus by adsorption onto the micronized particles, an effect not seen with other polyanionic compounds tested (Neurath et al., 2002b).

Over the last decade, this compound has also been evaluated for use as a microbicide. However, the chemical nature of the compound, with a pK_a of 5.3, is such that CAP is insoluble at the low pH typically encountered in the vaginal environment. To circumvent this obstacle, CAP was formulated as a micronized compound (Neurath et al., 2001). Micronized CAP is able to rapidly bind both R5 and X4 HIV-1 in physiological fluids by adsorption, as demonstrated in ELISA assays showing binding of R5 and X4 HIV-1 to micronized CAP. The interaction between micronized CAP and cell-free virions effectively inhibits HIV-1 infection by preventing productive contacts with HIV-1-susceptible cells (Neurath et al., 2002a,b).

The insolubility of CAP at low pH prompted the study of other pharmaceutical excipients with similar antiviral activities combined with the ability to remain soluble at low pH. One such compound was hydroxypropyl methylcellulose trimellitate (HPMCT) (Rando et al., 2006) (Fig. 2). Although this compound is structurally similar to cellulose acetate phthalate, it remains soluble and molecularly dispersed at much lower pH values due to the replacement of the phthalate group with a trimellityl moiety (Rando et al., 2006). The degree of trimellitate substitution plays an important role, because molecules with varied trimellitate content differed with respect to their antiviral activities. In addition, the retention of antiviral activity at pH values as low as 4.0 suggests the importance of the ability of HPMCT to remain molecularly dispersed at low pH values (Rando et al., 2006).

Poly(styrene-*alt*-maleic acid) (*alt*-PSMA) is another polyanionic molecule that does not derive its negative charges from sulfation or sulfonation (Fig. 2). *alt*-PSMA, which is an alternating copolymer with a 1:1 ratio of styrene and maleic acid, has a structure similar to that of PSS, which also includes a styrene-based backbone. However, the anionic charge of *alt*-PSMA is derived from maleic acid groups that are directly attached to the backbone. In contrast, PSS derives its negative charges from sulfonic acid groups attached to the aromatic rings of the styrene backbone. In contrast to other polyanionic compounds, *alt*-PSMA inhibits infection by both R5- and X4 strains of HIV-1 with equal effectiveness (Pirrone et al., 2010). In addition, *alt*-PSMA lacks a potentially detrimental characteristic possessed by other polyanionic compounds. In assays designed to assess persistent or "memory" antiviral activity (Thakkar et al., 2010), application and removal of λ-carrageenan, PSS, or cellulose sulfate prior to infection with cell-free HIV-1 resulted in increased levels of infection relative to cells infected in the absence of polyanionic compound exposure (Pirrone and Krebs, submitted for publication). This effect was observed up to 7 h after compound exposure and removal. In contrast, *alt*-PSMA application and removal had no effect on HIV-1 infection (no enhancement or persistent inhibition). These results may be relevant to the clinical failures of microbicides containing λ-carrageenan and cellulose sulfate, suggesting that antiviral activity in the presence of these compounds may be offset by enhanced susceptibility to HIV-1 infection once in vivo concentrations of the products have been diminished by either dilution or product loss from the cervicovaginal environment.

3. Systemic use of polyanionic compounds

Polyanionic compounds were advanced into clinical trials because of their potent in vitro activities against HIV-1. The first clinical study of a polyanionic compound effective against HIV-1

involved treating HIV-1-infected patients with orally administered dextran sulfate (Abrams et al., 1989). These trials were quickly terminated when it became apparent that the drug was poorly absorbed (Lorentsen et al., 1989), with dextran sulfate levels in the plasma shown to be undetectable. This result contrasted with the intravenous administration of dextran sulfate, which resulted in detection of the compound in both the plasma and urine (Lorentsen et al., 1989). More recent studies with dextran sulfate, however, determined that orally administered dextran sulfate can, in fact, be absorbed in HIV-1-infected individuals, with dextran sulfate being detected in plasma as well as in peripheral blood lymphocytes (Hiebert et al., 1999). Unfortunately, these trials did not examine the association between compound administration and the course or degree of HIV-1 infection. In this regard, additional studies were performed to address the postadministration pharmacokinetics, toxicity, and activity of dextran sulfate. In one study, dextran sulfate was administered by continuous intravenous infusion adjusted to maximize drug concentration and duration of treatment while maintaining a tolerable level of thrombostasis. As a result, dextran sulfate plasma concentrations achieved following administration were up to 200-fold higher than the concentrations needed in vitro to inhibit HIV-1 infection by 50% (Flexner et al., 1991). Although significant levels of dextran sulfate were present in the plasma, the systemic concentration of HIV-1 p24 antigen increased in all patients who received the drug for more than 3 days. This increase was highly significant when compared to untreated historical control patients. In addition to the increase in HIV-1 p24 antigen, dextran sulfate administration also appeared to cause toxic side effects, including reversible thrombocytopenia and reversible alopecia (Flexner et al., 1991). Furthermore, dextran sulfate and similar compounds have the additional disadvantage of poor central nervous system penetration and a short systemic half-life (Lorentsen et al., 1989). These cumulative results indicated that dextran sulfate was not suitable for further clinical development. Additional caution was advised with regard to the further development of all polysulfated polyanionic compounds for systemic administration (Flexner et al., 1991).

4. Polyanionic compounds as microbicides

All preventive or therapeutic agents intended for use in humans must undergo a series of rigorous clinical trials before they can be approved for widespread distribution and use. Traditionally, there are three levels of clinical testing: phase I, phase II, and phase III. Phase I trials are used to demonstrate agent safety in humans. Low-risk volunteers are observed over a course of weeks to months to determine adverse affects associated with compound use. This phase is completed for drugs designed for systemic use as well as microbicide products developed for topical application. Phase II trials are expanded safety trials. Phase II trials involve a larger number of volunteers followed over a longer period of time. In a microbicide phase II trial, recruitment efforts can involve volunteers whose risk of infection more closely resembles those who will be enrolled in subsequent efficacy trials. Phase IIb trials, which are considered "proof of concept" microbicide clinical trials (Global Campaign for Microbicides), are often larger than a normal phase II trial but smaller than a full efficacy trial. This trial is completed to provide initial indications of product efficacy and justification for a larger efficacy trial. A phase III microbicide clinical trial is a full efficacy trial with thousands of enrolled volunteers at multiple trial sites. The trial offers the final determination as to whether a compound demonstrates efficacy in preventing HIV-1 transmission (Global Campaign for Microbicides). Because of the time involved in planning a study, performing the trial, and analyzing the results, each trial can span multiple years. The clinical trials of three microbicides containing polyanionic compounds were conducted over a period of approximately 15 years (Fig. 3).

4.1. Carrageenan (Carraguard)

Although polyanionic compounds were deemed unsuitable for systemic use, their effectiveness as HIV-1 entry inhibitors suggested a role for these compounds as active ingredients in topical microbicides. One of the first polyanionic compounds to enter clinical trials as a microbicide was carrageenan. In 1995, ι-carrageenan, referred to as PC213, entered into clinical trials with 25 women from 5 different sites participating in a phase I trial (Fig. 3). The women applied 5 mL of a 2% formulated gel once daily for 7 consecutive days. During this time interval, the women were instructed to abstain from sexual intercourse. The results of this trial indicated that ι-carrageenan was not associated with significant irritation (Elias et al., 1997). However, given the small size of the trial, it was concluded that careful assessments of compound safety needed to be included in larger studies of this formulation.

Around the same time period, λ-carrageenan was studied in phase I clinical trials as PC503, with 35 women from 5 participating sites (Fig. 3). Similar to ι-carrageenan, this product was also administered as 5 mL of a 2% gel to be used once daily for 7 consecutive days. The women included in this study were characterized as healthy, HIV-1-negative, low-risk, and sexually abstinent. The results of this trial indicated that λ-carrageenan application did not cause significant adverse effects (Coggins et al., 2000). However, this trial was also limited by a small sample size.

Various iterations of carrageenan were subsequently developed and tested. In 1996, phase I clinical trials of another carrageenan-based product began (Fig. 3). This product, Carraguard, consisted of a combination of κ- and λ-carrageenan formulated at a 3% total concentration. This trial included healthy, HIV-1-negative women from six countries. Women participating in this trial found the product to be easy to use as well as acceptable, and none experienced significant irritation or changes in vaginal flora resulting from product use (Coetzee et al., May 12–15, 2002; Ramjee et al., 2007). Trials were also conducted among 55 low-risk, sexually active Thai couples. Among these couples, adherence was high, with 80% of couples using the product in over 95% of all vaginal sex acts. This study showed that this product was safe and acceptable to men as well as to women (Kilmarx et al., 2008; Whitehead et al., 2006).

To demonstrate the safe use of Carraguard in HIV-1-infected individuals, a study was initiated in South Africa, enrolling HIV-1 positive women and men. Although a product such as Carraguard would not be marketed for use in HIV-1-infected individuals, safety in this population is important because of the potential for use by those who are unaware of their HIV-1 status or those who use the product despite their infection. In this trial, the women were instructed to apply Carraguard (or placebo) once daily for 14 consecutive days. Sexually abstinent and sexually active women were included in the study. The men in this study, however, were sexually abstinent and were instructed to apply the product to the penis once daily for 7 consecutive days. Participants were assessed for visual changes to the vaginal, cervical, and penile epithelia, the effects of product application on vaginal HIV-1 shedding, changes in genital inflammation markers, epithelial sloughing, and the presence of microhemorrhages. The results derived from these trials suggested that Carraguard was not associated with abnormal genital symptoms, adverse changes in genital inflammation markers, epithelial sloughing, or the occurrence of microhemorrhaging. In addition, self-reported symptoms in both men and women, as well as changes in vaginal flora and genital shedding of HIV-1 RNA in women, remained unremarkable (van de Wijgert et al., 2007).

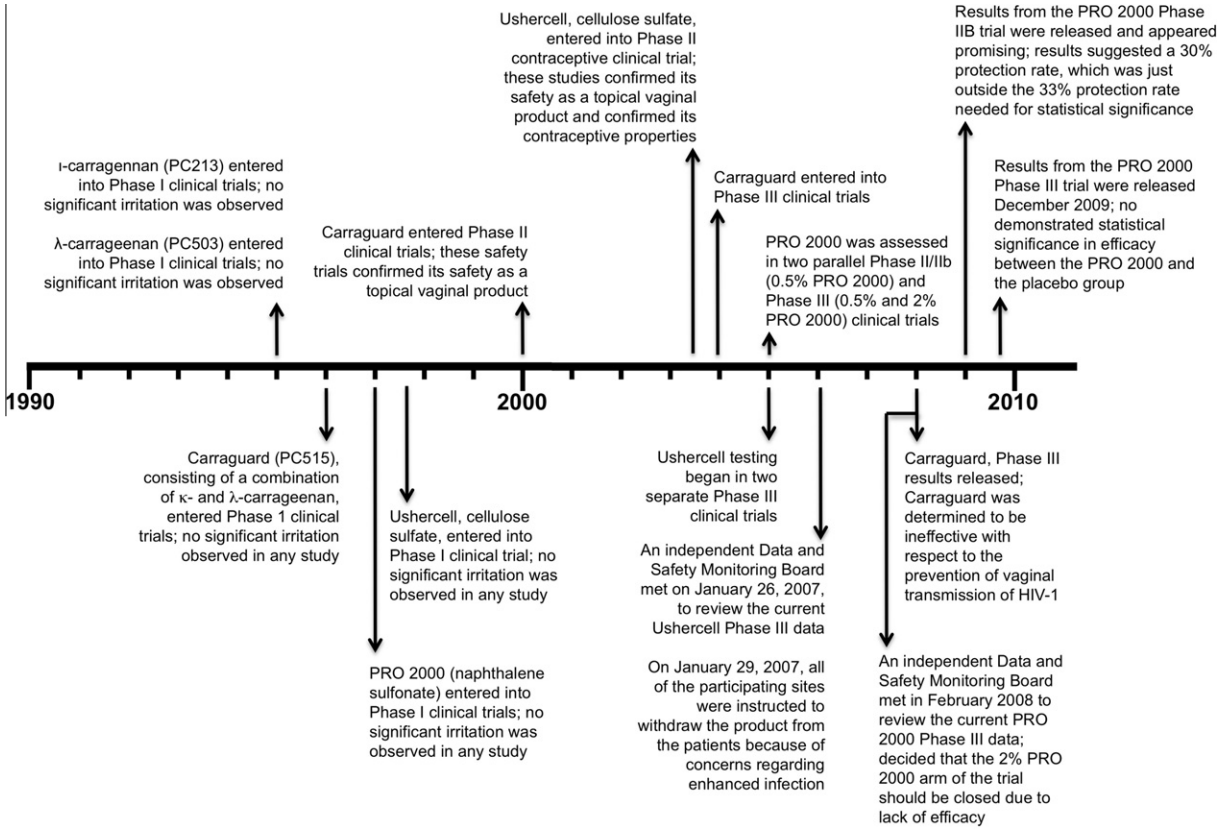


Fig. 3. Timeline of clinical trials involving microbicides containing polyanionic compounds. Topically applied microbicides were assessed for safety in phase I and phase II clinical trials. More extensive safety tests as well as initial efficacy tests were completed in phase II/IIb clinical trials. Compounds with demonstrated safety were advanced into phase III efficacy trials. To date, three microbicides containing polyanionic compounds have entered into and progressed through phase III clinical trials: carrageenan (Carraguard), cellulose sulfate (Ushercell), and naphthalene sulfate (PRO 2000). None of these microbicides were found to be effective against HIV-1 transmission.

As a consequence of positive phase I clinical trial results, Carraguard was advanced into a phase II trial (Fig. 3). Again, this trial took place in Thailand. In this trial, 165 women were recruited into the trial and received either Carraguard or the methylcellulose placebo gel. The women were instructed to apply the formulated compound a minimum of 3 times per week independent of sexual intercourse and also to use the gel with condoms during every sexual encounter. Safety of the compound was assessed by visual inspection of the vagina and cervix, changes in normal vaginal flora, and self-reported symptoms at 2 weeks, 1 month, and thereafter monthly up to 1 year. Results from this trial indicated that Carraguard use was not associated with the appearance of abnormal vaginal flora, clinical symptoms/findings, or Papanicolau test results. Those adverse symptoms that were reported were generally mild and were not attributed to compound use. The compound was found to be highly acceptable among participants (Kilmarx et al., 2006). Collectively, the results of all Carraguard safety trials confirmed its safety as a topical vaginal product.

The demonstrated safety of Carraguard provided justification for advancing this product into phase III efficacy trials. In 2004, Carraguard was assessed for efficacy against HIV-1 transmission and infection (Fig. 3). Trials began in South Africa and were conducted through the collaboration of three institutions: the Medical Research Council of South Africa, the University of Limpopo–Medunsa campus, and the University of Cape Town. The 6202 volunteers recruited into the study were sexually active HIV-1-negative women, aged 16 years and older. Women were randomly assigned to one of two groups: Carraguard and condom or placebo and condom. The participants were instructed to insert the gel up to 1 h prior to vaginal intercourse and were also instructed to use condoms. The participants were followed for 2 years; the outcome was time to seroconversion. The results concerning HIV-1 incidence in this trial were 3.3 per 100 woman-years in the Carraguard group (equaling 134 events) and 3.8 per 100 woman-years in the placebo group (151 events). Although these results were suggestive of efficacy, statistical analyses revealed no significant difference between the two groups with respect to time to conversion. Therefore, Carraguard was determined to be ineffective with respect to the prevention of vaginal transmission of HIV-1 (Skoler-Karpoff et al., 2008). However, the results of this trial may have been compromised by a number of factors, including infections due to anal intercourse, the use of methylcellulose as a placebo that may provide a barrier to HIV-1 infection, and the incidence of use (Coetzee et al., 2001; Kilmarx and Paxton, 2003). In this trial, the applicators were examined for the presence of vaginal proteins to determine if the applicators were actually inserted into the vagina (Wallace et al., 2004, 2007). A large discrepancy existed between the number of self-reported uses of the product and the number of applicators that tested positive for insertion: 95–96% of women self-reported gel use while applicator testing estimated gel usage at 41–43% (Skoler-Karpoff et al., 2008). Although Carraguard is apparently not an effective microbicide, the gelling properties of carrageenan in combination with its in vitro activities against HIV-1 and other pathogens provide support for its continued use in microbicide formulations. Preclinical studies have continued to evaluate Carraguard as a partner in a number of combination microbicides (Fernandez-Romero et al., 2007).

4.2. Cellulose sulfate (Ushercell)

The second polyanionic compound to enter into clinical trials was cellulose sulfate (Ushercell). Cellulose sulfate underwent

extensive safety and acceptability trials (Fig. 3). In an initial trial, a total of 48 healthy women between the ages of 18 and 50 were enrolled. In this trial, 6% cellulose sulfate gel was examined at two different application volumes (2.5 and 5.0 mL) (Mauck et al., 2001b). In addition to cellulose sulfate, 2.5 mL of Conceptrol (a nonoxynol-9 containing product) and 2.5 mL of K–Y Jelly were also assessed. The women were instructed to insert the product before bedtime, to remain recumbent for at least 15 min, and to refrain from voiding the bladder or bowels for at least 1 h after application. The women used the product for 6 consecutive nights. Results from this trial indicated that women experienced less irritation after application of either volume of cellulose sulfate when compared to either Conceptrol or K–Y Jelly. However, application of cellulose sulfate was also associated with increased leakage of the product. Although product leakage could have resulted in a decrease in irritation, this hypothesis could not be tested because of the limited number of women enrolled in the trial. Among women who used cellulose sulfate, no colposcopic findings were observed that involved disruption of the epithelium or epithelial blood vessels (Mauck et al., 2001b). In this study, however, acceptability appeared to be low but was consistent across all of the test groups, with only half of the women stating that they would purchase such a product. A large number of those women stated that they would be unwilling to purchase it because they would have no need for such a product. The acceptability profile might be different in women who need such a product for contraceptive or antimicrobial purposes (Mauck et al., 2001b). However, the results of the safety trial of cellulose sulfate indicated that it was safe and that further development was warranted.

In addition to examining women as the target population for the product, investigators also tested Ushercell in men to assess the amount of penile irritation associated with 7 consecutive days of application (Mauck et al., 2001a). Aside from being an important indicator of product toxicity, penile irritation might also signal the presence of a microbicide and prevent the use of the product without the knowledge of the male partner. In this trial, 36 healthy HIV-1-negative circumcised and uncircumcised men were enrolled. These men abstained from both masturbation and sexual intercourse for the duration of the study. Men received either cellulose sulfate or Conceptrol to apply directly to the penis (Mauck et al., 2001a). Little evidence of irritation was evident following application of either cellulose sulfate or Conceptrol. Additionally, one out of 24 men in the cellulose sulfate group experienced tingling or stinging shortly after application, whereas 3 out of 12 men in the Conceptrol group experienced similar symptoms (Mauck et al., 2001a). Differences were observed between circumcised and uncircumcised men, with uncircumcised men generally experiencing irritation. Although men generally found both products acceptable, cellulose sulfate was found to be less messy and faster drying. This study showed that cellulose sulfate was not more irritating to the male genitalia, and men generally found the product to be acceptable (Mauck et al., 2001a).

Expanded phase I safety studies were performed to assess the safety and acceptability of the compound in more relevant geographic locations (Fig. 3). In this study, 180 healthy women at low risk for HIV-1, both sexually abstinent and sexually active, were enrolled in India, Nigeria, and Uganda, and received either 3.5 mL of 6% cellulose sulfate gel or K–Y Jelly twice daily for 7 consecutive days (Malonza et al., 2005). The women in the sexually active groups were instructed to insert the product 5–15 min before vaginal intercourse to simulate the intended use of the product. Safety assessments were expanded to include signs and symptoms of irritation, adverse effects, and changes in vaginal health. The women from the sexually abstinent groups were enrolled and examined first, and their results were evaluated before the women from the sexually active groups were enrolled. Results suggested that application of 6% cellulose sulfate twice daily for 7 consecutive days was safe and potentially less irritating than a similar regimen of K–Y Jelly (Malonza et al., 2005). These results mimicked those of the first trial, which took place in the United States. Although product retention was not directly assessed in this trial, a small number of participants reported leakage. Lastly, approximately 80% of women in both groups stated that they would purchase the product if it were available (Malonza et al., 2005).

An additional study examined the safety and acceptability of cellulose sulfate in two groups of HIV-1-infected women: those who were sexually abstinent and those who were sexually active (El-Sadr et al., 2006). In this study, the women in the sexually abstinent group applied the product either once or twice daily and those in the sexually active group applied it once daily. A total of 59 women were enrolled and were instructed to use the product for 7 consecutive days. Adverse effects were observed less often in women who used cellulose sulfate than in those who used the placebo. There was also no increase in adverse effects following sexual intercourse. In this trial, cellulose sulfate caused minimal irritation and was acceptable to the female participants and their male partners (El-Sadr et al., 2006).

In yet another study of Ushercell, sexually abstinent and sexually active women applied either 6% cellulose sulfate or K–Y Jelly twice daily for 14 consecutive days. In this study, slightly more women in the Ushercell group had at least one sign of genital irritation seen by colposcopic examination. However, this finding was deemed insignificant because previous work had indicated that gross epithelial breaches were the only cervicovaginal finding associated with HIV-1 transmission (Schwartz et al., 2006; Van Damme et al., 2002). A separate study examined the use of Ushercell in conjunction with the diaphragm. Women enrolled in this study received either a diaphragm with 6% Ushercell, a diaphragm with K–Y Jelly, or 6% Ushercell gel alone. Within the context of this study, 80% of the women indicated genital irritation associated with product usage. However, because this study lacked a K–Y Jelly-only arm, the contribution of the gel and diaphragm alone could not be assessed (van der Straten et al., 2007). Additionally, the incidence of deep epithelial disruption among the diaphragm users was a cause for concern (Van Damme et al., 2002; van der Straten et al., 2007). Because the effects of Ushercell gel were deemed to be not statistically different from the effects observed in the K–Y jelly arm, Ushercell was concluded to be safe for use alone or in conjunction with a diaphragm (van der Straten et al., 2007).

As a consequence of demonstrated safety in the phase I and II trials, two separate phase III trials were begun in 2005 to assess the efficacy of Ushercell against the transmission of HIV-1 (Fig. 3). One trial took place at 5 different locations, including 3 sites in Africa (Benin, Uganda, and South Africa) and 2 sites in India (Chennai and Bangalore). A total of 1398 women were enrolled in the study and randomized into two groups: one that received 6% Ushercell gel and one that received a placebo (Van Damme et al., 2008). The placebo used in this trial (and in many clinical trials of gel-formulated microbicides) was the hydroxyethylcellulose (HEC)-based universal placebo (Tien et al., 2005). Concurrently, a second large phase III trial was also conducted in Nigeria, where 1644 women were enrolled and randomized into a 6% Ushercell group or a placebo group (Halpern et al., 2008). As a result of lessons learned during efficacy trials involving N-9, an independent data monitoring committee was assigned responsibility for tracking the progress of the trial until its completion. Such a committee is charged with the task of stopping a trial in the event of futility or harm as a consequence of the trial (i.e., an increase in HIV-1 transmission in the product arm). When the committee convened on January 26, 2007 to review the data through December 18, 2006, they found approximately 35 new HIV-1 infections, with 24 occurring in the Ushercell group and 11 in the placebo group, giving a

hazard ratio of 2.23 ($P = 0.02$) (Van Damme et al., 2008). The committee recommended that the trial be stopped; on January 29, 2007, the individual sites were instructed to withdraw the product from participants. Once all of the data were analyzed, the researchers noted a total of 41 new infections, with 25 in the Ushercell groups and 16 in the placebo group. The new results indicated no significant difference between the Ushercell and placebo arms, with a hazard ratio of 1.61 ($P = 0.13$) (Van Damme et al., 2008). However, the trend suggested a greater risk for infection associated with the use of Ushercell. A concurrent study in Nigeria was also terminated early on the basis of these results. However, the partial results of the Nigeria trial did not show a similar trend toward increased risk associated with product use. In Nigeria, fewer infections in the Ushercell arm were noted (10 new infections as compared to 13 new infections in the placebo arm) (Halpern et al., 2008). Again, these differences were not significant, indicating a lack of effect.

At present, no scientific explanation exists for the differences between the 5-site trial and the Nigeria trial or for the trend toward increased risk for infection associated with Ushercell use. It has been suggested, however, that inflammatory reactions, immune dysfunction, or disruption of the vaginal flora may have played roles in the differences between the trials and the apparent adverse effect of Ushercell (Aidsmap.com, 2007; Van Damme, 2007). These issues continue to be investigated.

4.3. Naphthalene sulfonate (PRO 2000)

Clinical trials of a third polyanionic compound were also conducted. In 1997, naphthalene sulfonate, known clinically as PRO 2000, entered into phase I clinical trials at two different concentrations (0.5% and 4.0%) (Fig. 3). The initial studies were conducted in London, UK, and Antwerp, Belgium. In these trials, 73 healthy, sexually abstinent women were enrolled, randomized, and provided with either PRO 2000 at 0.5%, PRO 2000 at 4%, or the placebo (Van Damme et al., 2000). Women were instructed to insert the product (2 mL) once daily for 14 consecutive days. To assess the safety of the product, changes in the genital health were assessed by colposcopy; microscopic inflammation was assessed by high vaginal biopsy; blood levels of PRO 2000 were measured, laboratory safety tests were performed; and vaginal microflora were assessed (Van Damme et al., 2000). Results indicated that repeated vaginal application of PRO 2000 up to 4% was safe for vaginal application, both locally (as assessed by colposcopy) and systemically (as assessed by blood tests). There was also no impact on vaginal microflora following compound application. Although the sample number in this trial was small, the minor indications of toxicity associated with application of 4% PRO 2000 suggested that this concentration was approaching the upper limit for tolerance in the cervicovaginal environment (Van Damme et al., 2000).

Because of indications of toxicity associated with 4% PRO 2000, trials were then completed to determine the highest tolerated concentration of PRO 2000. This study was completed in sexually active HIV-1-negative women as well as in sexually abstinent HIV-1-positive women. HIV-1-negative women were given either 2% or 4% PRO 2000 and instructed to apply the compound either once or twice daily for 14 consecutive days depending on grouping (Mayer et al., 2003). This trial was open-label and escalated with regard to concentration and dosing, with elevation to the next level only once the lower level was established as safe. The women in these groups engaged in sexual intercourse at least twice per week. HIV-1-infected abstinent women were also tested with 4% PRO 2000 applied twice daily for 14 consecutive days. The end point of these studies focused on toxicity as determined by measuring microscopic indications of irritation, ulceration, abrasion, erythema, or edema (Mayer et al., 2003). In general, PRO 2000 at both concentrations and dosing regimens was well tolerated and safe in HIV-1-negative women, and the 4% twice daily PRO 2000 was deemed safe and tolerated in HIV-1-positive women. Although adverse effects were reported by 73% of women, these effects were considered to be mild, including some discomfort, bleeding, or increased vaginal discharge, usually associated with product leakage. Although both 2% and 4% PRO 2000 were deemed safe, the trend toward an increase in adverse effects suggested that future studies should be limited to the lower 2% concentration (Mayer et al., 2003). The acceptability of PRO 2000 was also assessed in this trial, with considerations of the compound's impact on sex and ease of use. Women from both sites stated that they found the product acceptable.

Additional studies were conducted to directly measure the concentration of PRO 2000 in cervicovaginal lavages and in plasma as an indication of bioavailability as well as systemic exposure linked to possible toxicity. Women received either 0.5% or 4% PRO 2000 twice on the first day of the study and then once for the next 12 consecutive days. Following application, PRO 2000 did not appear to be absorbed systemically, whereas the cervicovaginal lavage concentrations exceeded 25 μg/mL after the first dose (Lacey et al., 2006). However, levels in excess of the target concentration were present 12 h after repeated dosing in more women who applied the 4% PRO 2000 than in those who applied the 0.5% PRO 2000 (Lacey et al., 2006). This result suggested that a greater window for efficacy exists with application of a product containing a higher concentration of active ingredient. Continued safety trials were conducted using 0.5% PRO 2000. Sexually active, HIV-1-negative women from Pune, India were categorized as either high risk or low risk for infection and were given 0.5% PRO 2000 to be applied twice per day for 14 consecutive days. Following application, no adverse effects due to product application were noted (Smita et al., 2006). Additionally, no PRO 2000 appeared in the blood of the women following 14 days of application. These results, added to the previous findings of safety, indicated that 0.5% PRO 2000 was safe for vaginal application (Smita et al., 2006).

The previous failure of nonoxynol-9 (N-9) as a microbicide suggested that one method for determining microbicide safety should involve assessment of post-application cytokine levels (Doncel et al., 2004; Fichorova, 2004; Fichorova et al., 2001, 2004). In these studies, N-9 application was associated with large increases in proinflammatory cytokines following application (Doncel et al., 2004; Fichorova, 2004; Fichorova et al., 2001, 2004). As a result, trials were designed to examine cytokine release in the vaginal environment observed after PRO 2000 application. In these studies, healthy, sexually abstinent women applied 0.5% PRO 2000 or a placebo once daily for 14 consecutive days (Keller et al., 2007). Cervicovaginal lavage samples were collected on days 0, 7, 14, and 21. In these studies, no increases in proinflammatory cytokine release were observed, and protective factors, including interleukin-1 receptor antagonist, were slightly decreased. All levels returned to normal by day 21 (7 days after the last application) and did not appear to be related to any reduction in intrinsic antimicrobial activity (Keller et al., 2007).

A novel trial was conducted to examine the ability of 0.5% PRO 2000 to inhibit HIV-1 or HSV-2 infection following vaginal application. In these studies, cervicovaginal lavages were collected from female participants 1 h after product application. The ability of the post-application lavage to inhibit HIV-1 and HSV-2 infection was compared to a cervicovaginal lavage taken 48 h prior to compound application (Keller et al., 2006). In these trials, the post-application lavage inhibited infection by both viruses with potency at least 1000-fold greater than the pre-application lavage. Additionally, no difference was noted in the levels of cytokines released between the pre- and post-application lavage samples. These results suggested that PRO 2000 remains bioavailable, retains its

antiviral activity post-application, and does not cause a significant increase in inflammation above the levels of the placebo itself (Keller et al., 2006).

With clinical trial safety results suggesting that PRO 2000 at concentrations up to 4% was safe for application, PRO 2000 advanced to clinical trials of efficacy to determine the ability of PRO 2000 to inhibit HIV-1 transmission and infection. PRO 2000 was initially assessed in parallel phase II/IIb and phase III clinical trials (Fig. 3). One of the trials was referred to as the HPTN 035 study, which was initially sponsored and conducted by the HIV Prevention Trials Network (HPTN). During the clinical trial, a collaboration was developed between HPTN and Microbicides Trials Network (http://www.mtnstopshiv.org) that continued through the duration of the trial. This study was conducted at multiple sites in Africa and at one site in the United States. The trial was designed to examine 0.5% PRO 2000 and BufferGel against a placebo and also against a no-gel arm (Abdool Karim et al., February 9, 2009; Microbicide Trials Network, February 9, 2009a,b). This trial was the first to include a no-gel arm. Although it was suggested that women would not remain in the trial if they were given a condom without a gel product, retention in the no-gel arm was similar to that in the other arms of the trial. Researchers thought this arm was important because they believed that a gel alone could supply some level of protection through a barrier effect created by the viscosity of the gel. They speculated that the true activity of the compound might be lost in comparison to a placebo gel. Additionally, results could be confounded by any changes in the environment caused by the placebo. Results from this trial demonstrated no apparent difference in the number of HIV-1 infections in the no-gel and the placebo groups, suggesting that the placebo itself had no effect on HIV-1 transmission (Abdool Karim et al., February 9, 2009; Microbicide Trials Network, February 9, 2009a,b). The purpose of the trial was to compare PRO 2000 (and BufferGel) to the placebo and the no-gel arms. In this study, PRO 2000 was found to be safe for use in women and 30% effective in preventing HIV-1 transmission (Abdool Karim et al., February 9, 2009; Microbicide Trials Network, February 9, 2009a,b). A total of 3099 individuals took part in this trial. A total of 194 HIV-1 seroconversions occurred: 36 seroconversions in the 0.5% PRO 2000 arm, 54 in the BufferGel arm, 51 in the placebo arm, and 53 in the no-gel arm. Although the difference between the PRO 2000 and the control groups did not quite reach statistical significance, the results were nevertheless encouraging and provided renewed hope that an effective microbicide would be developed (Abdool Karim et al., February 9, 2009; Microbicide Trials Network, February 9, 2009a,b).

The parallel phase III trial of PRO 2000 was referred to as the MDP 301 study (Fig. 3). Supported by the Microbicides Development Programme (http://www.mdp.mrc.ac.uk/), the trial was designed to compare 0.5% PRO 2000, 2% PRO 2000, and a placebo (Microbicides Development Programme, February 14, 2008). This trial was the largest vaginal microbicide trial ever undertaken. This trial, which was conducted between September 2005 and September 2009, enrolled 9385 women at 6 research centers in South Africa, Tanzania, Uganda, and Zambia. Like the Ushercell trials, a Data Safety Monitoring Board was assigned to monitor the trial. Following a data review in February 2008, the Data Safety Monitoring Board for the MDP 301 study decided that the 2% PRO 2000 arm of the trial should be closed because there appeared to be no possibility of the 2% arm demonstrating an effect compared to the placebo arm (Aidsmap.com, February 18, 2008). It was postulated that the 2% arm did not show any effect because the protective effect of the compound might have been masked by localized irritation caused by the compound (Microbicides Development Programme, February 14, 2008). During the trial, researchers noted 86 new HIV-1 infections in the 2% PRO 2000 arm, 70 new infections in the 0.5% PRO 2000 arm, and 77 new infections in the placebo arm. The possibility remained, however, that the 0.5% arm of the trial would show efficacy. The results garnered from the HPTN 035 trial established excitement for the completion and release of the MDP 301 results utilizing 0.5% PRO 2000. Because the MDP 301 trial contained many more participants, any effect would likely be demonstrated with statistical significance. Results of the trial were released on December 14, 2009. Although the HPTN 035 trial gave hope that PRO 2000 would demonstrate some level of activity against HIV-1 infection, this sadly was not the case (Cohen, December 14, 2009; Warren and Marshall, December 14, 2009). During this trial, 145 new HIV-1 infections occurred in individuals in the 0.5% PRO 2000 arm and 143 new HIV-1 infections in those in the placebo arm, demonstrating no statistical significant difference in efficacy between PRO 2000 and the placebo. In addition, there were no differences in primary safety end points. Many investigators now believe that the results of this trial signaled the end of polyanion-based microbicides used to prevent HIV-1 transmission.

5. After the clinical trials

Even a cursory review of the microbicide development field reveals a significant gap between preclinical compound development and clinical trial results. All of the polyanion-based microbicides examined to date in clinical trials were shown to have high levels of efficacy in preclinical evaluations and apparent safety in both preclinical and early clinical testing. Despite these results, however, only one compound was shown to have even a suggestion of efficacy in humans. It has become apparent that there is still much to learn with respect to preclinical assays that are more predictive of clinical outcomes and factors that may affect the in vivo safety and efficacy of microbicide products. Factors relating to both safety and efficacy, including activity in the presence of seminal plasma, compound concentrations, and compound retention, are the focus of current investigations.

One area that has received considerable attention with regard to blocking HIV-1 transmission is the pH of the vaginal environment as well as that of the compound. The normal pH of the vaginal environment is between 4.0 and 4.5, a level of acidity that has been shown to inactivate HIV-1 (Kempf et al., 1991). Studies have also shown that, in addition to inactivating cell-free HIV-1, low pH conditions prevent transmission of cell-associated HIV-1 (Olmsted et al., 2005). With a pH value between 4.0 and 4.5, the vaginal environment appears to be an antiviral environment, and the risk of HIV-1 infection should be innately minimized by the protective effect of low pH. However, the introduction of semen during sexual intercourse dramatically increases the local pH. Following intercourse, the pH rises to between 6.5 and 7.5 (Levin, 1998), creating a more hospitable environment for HIV-1 transmission. Several areas of research have focused on creating a compound that would maintain the acidity of the vaginal environment during sexual intercourse. It has even been suggested that the failures of compounds in clinical trials may have been directly correlated with the higher pH associated with the compounds (Neurath, 2008). However, recent clinical trials suggest that pH alone may not be an effective deterrent to HIV-1 transmission. The recent HPTN 035 trials included BufferGel, which was intended to boost the natural acidity of the vagina in the presence of seminal plasma (Olmsted et al., 2005). In this trial, BufferGel did not appear to block transmission of HIV-1. Fifty-four seroconversions occurred in the women who were randomized to the BufferGel arm of the trial, while in the control arms there were 51 in the placebo group and 53 in the no-gel group (Microbicide Trials Network, February 9, 2009a,b). Although pH may still be shown to play a role in transmission, the maintenance of low pH alone does not appear to

decrease the incidence of infection. Additional investigations are required before the role of pH in HIV-1 transmission is fully defined.

As indicated by studies of CAP (Neurath et al., 2001, 2002a,b), pH can also dictate the choice of compounds to be used in the cervicovaginal environment. With respect to polyanionic compounds, the effect of pH can be twofold. First, compounds need to be soluble and bioavailable over a wide range of pH values. Second, given the relationship between molecular charge and pH, molecules that rely on charge for antiviral activity need to remain charged throughout the variations in pH that take place during vaginal-receptive intercourse.

Investigators are also considering the role of seminal plasma in HIV-1 transmission. Studies have focused on the ability of components of seminal plasma to inactivate polyanionic compounds in vitro. The inhibitory activities of a number of polyanionic compounds, including several examined in clinical trials, were greatly diminished in the presence of seminal plasma (Neurath et al., 2006). The compounds examined included CAP (solubilized and micronized), carrageenan (κ and λ combined at a 1:1 ratio), cellulose sulfate, naphthalene sulfonate, and PSS. The inhibitory activity of each of these compounds, with the exception of micronized CAP, was significantly diminished in the presence of seminal plasma. Micronized CAP retained its activity in the presence of seminal plasma. The significance of these results is not clear in light of the concentrations of agents used in clinical trials compared with the concentrations examined in vitro. Typically, the concentrations of the compounds in clinical trials are many orders of magnitude higher than those assessed using in vitro assays (Neurath, 2008). However, recent clinical-based studies appear to corroborate the in vitro findings. In studies in which PRO 2000 was applied by women in the absence or before sex, the act of coitus had two effects: reductions in PRO 2000 recovered in cervicovaginal fluid samples and significant decreases in the antiviral activity of PRO 2000 (Keller et al., 2010). These studies clearly indicate the need for more investigations focused on the effects of seminal plasma (and cervicoaginal secretions) on microbicide effectiveness. To address these effects, future investigations involving mouse and macaque models of HIV-1 infection may be augmented by the introduction of semen or seminal plasma along with infectious virus and formulated microbicide products.

Following the removal of cellulose sulfate from clinical trials, investigations were also directed toward explaining the failure of this compound to affect HIV-1 transmission. Recent results have suggested that cellulose sulfate has biphasic antiviral activity in vitro; at high concentrations, it effectively inhibits HIV-1 infection, whereas at low concentrations it actually enhances infection (Tao et al., 2008). These observations suggest that in vivo product dilution or loss may decrease the concentration of the compound to a level that may cause enhancement of infection instead of inhibition. This enhancement was observed in the absence of cellular activation, apoptosis, or decreases in target cell viability. The relevance of these results, however, remains in question because the concentrations of cellulose sulfate assessed in clinical trials were far in excess of the concentrations shown to cause in vitro enhancement of infection (Neurath, 2008). Furthermore, it was stated that similar results were observed with PRO 2000 (Tao et al., 2008). However, with the low-dose arm of the PRO 2000 trial showing a trend toward protection from infection, more appears to be involved in the failure of compounds than just enhancement of HIV-1 infection at low concentrations.

An additional area of research involves compound retention. In trials involving cellulose sulfate, compound retention appeared to be a problem. In these trials, product leakage was reported, even in trials in which product leakage was not being directly assessed (Malonza et al., 2005). To address the effect of compound loss (or dilution), we have used an experimental design that would normally be considered for demonstrating persistent or "memory" antiviral activity after compound removal (Thakkar et al., 2010). In this assay, a compound is applied for a set period of time and then removed from the target cells by extensive washing. Cells are then infected with HIV-1 at various time points following compound washout. In experiments involving four polyanionic compounds, application of cellulose sulfate, carrageenan, and PSS resulted in enhancement of HIV-1 infection (Pirrone and Krebs, submitted for publication). This effect, demonstrated in a continuous cell line and primary human peripheral immune cells, appeared to be concentration-dependent, with the higher concentrations resulting in greater enhancement of infection. The limitation of this assay design is that compound concentrations in vivo generally decrease gradually over time instead of dropping precipitously after removal by washing. However, local concentrations of active compound may drop faster due to dilution by semen, uneven product distribution and redistribution during intercourse, and product loss during the act of intercourse. This paradigm for product loss (or redistribution) and the method of assessment should continue to be investigated as a possible mechanism for product failure, not only for polyanionic compounds but also for any compounds considered for use as microbicides. In light of the continued interest in carrageenan as a microbicide formulation excipient and a microbicide effective against other sexually transmitted pathogens (Anonymous, 2010; Buck et al., 2006; Marais et al., 2010; Roberts et al., 2007), investigations into the mechanisms that underlie the clinical failure of Carraguard should receive particular emphasis.

It is also possible that mechanisms not obviously connected with microbicide safety or efficacy may have played roles in the failures of polyanion-based microbicides. One such mechanism was highlighted in studies of the effects of microbicide agents on epithelial barrier integrity (Mesquita et al., 2009). A series of in vitro experiments demonstrated reductions in epithelial integrity (as measured by trans-epithelial resistance) and concomitant increases in permeability to infectious HIV-1 as a consequence of epithelial cell monolayer exposure to cellulose sulfate or N-9. In addition, cellulose sulfate exposure triggered increased rates of HIV-1 replication, presumably through activation of the nuclear transcription factor NF-κB. These effects were agent-specific, however, as exposure to tenofovir or PRO 2000 did not alter epithelial resistance or virus permeability. These results provide yet another factor that may have contributed to the clinical failure of Ushercell.

While all of these leads are suggestive, definitive explanations for the failures of the polyanion-based microbicides have remained elusive. Investigations directed toward polyanion-based microbicide development have provided a considerable volume of pre-clinical data from in vitro and in vivo studies, and an equally large body of clinical trial results. However, there exists a direct conflict between the pre-clinical and early clinical results, which indicated both safety and efficacy, and the late clinical trials, which failed to demonstrate effectiveness. This fact strongly suggests that the strategies used to advance polyanionic compounds toward human trials were not adequate to predict their inabilities to inhibit HIV-1 transmission. Indeed, only in post-trial investigations, such as those described above, have experimental findings provided evidence that polyanionic molecules may not have been suitable for use in anti-HIV-1 microbicides. These latter investigations clearly indicate that microbicide development strategies need to evolve to incorporate new experimental approaches that ask questions that were not previously addressed.

Retrospective analyses of studies focused on polyanionic compounds and microbicides illustrate a critical gap in the field of microbicide development: the need for common threads between pre-clinical studies and clinical trials that facilitate comparisons

and correlations between the two sets of results. For example, pre-clinical studies of microbicide agent efficacy provide detailed assessments of agent activity against defined viral strains and subtypes over a range of low and high concentrations, while clinical studies are generally performed using a relatively high concentration of the antiviral agent in populations that are at risk for infection by viral strains that are genotypically and phenotypically heterogeneous (but likely the same subtype) and generally not sampled in conjunction with the clinical trial. Similarly, in vitro evaluations of compound safety rely on quantitative assays of cell cytotoxicity and tissue toxicity, whereas safety assessments associated with clinical trials typically involve physical examinations that only document gross changes in cervicovaginal health and patient reports of adverse events associated with microbicide use. While each pre-clinical and clinical approach provides a measure of microbicide efficacy or safety, meaningful comparisons are difficult to make because of the considerable dichotomy in variables and measured endpoints.

Steps to close this gap, which involve bringing pre-clinical studies and clinical trials in closer alignment, are already being taken. For example, amidst growing evidence that significant cervicovaginal toxicity was associated with the use of N-9-based microbicides (Hillier et al., 2005), in vitro investigations indicated that patterns of cytokine release accompanied the cytotoxicity associated with N-9 exposure (Fichorova et al., 2001, 2004). This work was the basis for extensive efforts focused on validating the use of biomarkers, such as cytokines, as important predictors of safety during the pre-clinical development of candidate microbicide agents and as potential indicators of product safety during clinical trials (Doncel et al., 2004; Doncel and Clark, 2010; Trifonova et al., 2007). Similarly, recognizing the need for pre-clinical approaches that better predict microbicide efficacy in late clinical trials, investigators are now incorporating clinical samples, such as semen and cervicovaginal fluids collected before and after the topical application of candidate microbicides, into in vitro experiments designed to better emulate conditions under which microbicides can be expected to function in humans (Herold et al., 2011; Keller et al., 2011; Keller et al., 2010). Implementation of this latter type of approach was in direct response to lessons learned regarding the impact of semen on the antiviral activities of polyanionic compounds. These and other advances are providing much needed connectivity between pre-clinical studies and clinical trials, and the means to identify antiviral agents that will likely succeed as microbicides in late stage clinical trials.

By far, the most significant advance toward greater connectivity between pre-clinical microbicide development and clinical trials has been the demonstrated effectiveness of tenofovir as a topical microbicide (Abdool Karim et al., 2010). Prior to the successful outcome of the CAPRISA 004 trial (see below), all microbicides advanced into clinical trials had failed to provide protection against HIV-1 infection despite numerous indications to the contrary in pre-clinical experiments. In effect, the microbicide development field lacked a positive control compound. Following the successful clinical trial of a tenofovir-based microbicide, investigators are now incorporating tenofovir as a "gold standard" microbicide (Keller et al., 2011), against which other candidate antiviral agents can be assessed. As more pre-clinical and clinical data concerning tenofovir as a microbicide are collected, the value of this compound as a positive control in pre-clinical microbicide development efforts will increase.

6. Prospects for the future

With the prominent clinical failures of the polyanion-based products, the microbicide field has turned its attention to other classes of compounds, including compounds known to inhibit the activity of HIV-1 reverse transcriptase. Recent clinical trial results indicate the potential promise of reverse transcriptase inhibitors as microbicides. The CAPRISA 004 study, which was a Phase IIb clinical trial of a 1% gel formulation of tenofovir, demonstrated a 39% reduction in the overall incidence of HIV-1 infection among women who used the gel, and a 54% reduction in incidence among women who used the gel with a high level of adherence to the application regimen (Abdool Karim et al., 2010). Although additional studies are needed to corroborate the findings of this trial, the early conclusion from this trial is that greater levels of specificity and potency, such as those provided by reverse transcriptase inhibitors, are required of compounds to be used as microbicides. Microbicides formulated using polyanionic molecules may have failed to achieve significant levels of efficacy because polyanionic molecules are generally characterized by low specificity and efficacy relative to compounds like tenofovir.

Given the varied results involving polyanionic compounds as inhibitors of HIV-1 infection, any future consideration of polyanionic compounds must progress with due diligence. The use of polyanionic compounds as systemic agents to combat HIV-1 infection is apparently no longer a viable option. The lack of efficacy of these compounds and, in some instances, increases in systemic viral load have contraindicated their systemic use and cleared the way for more specific inhibitors of HIV-1 infection to be developed and used clinically. Some of these compounds, however, are still being considered for topical uses. For example, microbicides containing carrageenan will be studied in upcoming clinical trials as inhibitors of human papillomavirus (HPV) infection (2010), because carrageenan has been shown in vitro and in vivo to be an effective HPV inhibitor (Buck et al., 2006; Roberts et al., 2007). The clinical failures of microbicides containing polyanionic compounds and indications of potential problems associated with their use as inhibitors of HIV-1 transmission suggest that continued efforts to develop new compounds of this type should proceed cautiously using the lessons learned during the rise and fall of their predecessors.

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