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Optimizing siRNA Delivery to the Genital Mucosa

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Abstract

RNA interference (RNAi) describes a highly conserved pathway, present in eukaryotic cells, for regulating gene expression. Small stretches of double-stranded RNA, termed small interfering RNAs (siRNAs), utilize this pathway to bind homologous mRNA, resulting in site-specific mRNA cleavage and subsequent protein degradation. The ubiquitous presence of the RNAi machinery, combined with its specificity and efficacy, makes it an attractive mechanism for reducing aberrant gene expression in therapeutic settings. However, a major obstacle to utilizing RNAi in the clinic is siRNA delivery. Administered siRNAs must make contact with the appropriate cell types and, following internalization, gain access to the cytosol where the RNAi machinery resides. This must be achieved so that silencing is maximized, whilst minimizing any undesirable off-target effects. Recently, the utility of siRNAs as a microbicide, usually applied to the genital mucosa for preventing transmission of sexually transmitted diseases including HIV-1 and HSV-2, has been investigated. In this review we will describe these studies and discuss potential strategies for improving gene silencing.

Introduction

Sexually transmitted diseases of viral etiology, in particular HIV-1 and HSV-2, affect millions of people worldwide. A recent UNAIDS report lists the total number of people living with HIV-1 at over 33 million (UNAIDS, 2009). Sub-Saharan Africa accounts for over 60% of infections and women are particularly susceptible as they are often unable to negotiate safe sex (Garcia-Calleja *et al.*, 2006; Pilcher, 2004). HSV-2 is a major co-factor for HIV-1 (Freeman *et al.*, 2006; Wald and Link, 2002). In the U.S. more than 20% of the population is infected with HSV-2, and the infection rate in some areas of sub-Saharan Africa has been recorded to be as high as 90% (Corey *et al.*, 2004). HSV-2 infection is the leading cause of genital ulcers. The ulcers, even when healed, are a major source of inflammatory cells, and it is thought that this creates a permissive environment for transmission of HIV-1 (Celum *et al.*, 2004). There is no cure for HIV-1 or HSV-2, therefore a method to prevent transmission would make a significant impact on slowing the spread of these diseases. Furthermore, a successful strategy that prevents HSV-2 infection could result in a concomitant decrease in HIV-1 transmission. A vaccine that confers long-lived protection would be an ideal strategy. There are several vaccine candidates for HIV-1 and HSV-2. However, these have shown no protection, limited protection, and in some cases resulted in increased infection (Dudek and Knipe, 2006; McElrath *et al.*, 2008; Rerks-Ngarm *et al.*, 2009).

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In the absence of an effective vaccine, microbicides are being developed to prevent HIV-1 and HSV-2 transmission. Microbicides are compounds that, when applied to the genital mucosa, decrease viral transmission by inhibiting viral uptake or replication. There are more than 50 microbicides under development for HIV-1 (complete listing on www.microbicide.org). Disappointingly, most of the trials that have proceeded through phase III testing have not shown any significant decrease in the rate of HIV-1 transmission. However, one recent study reported decreased HIV-1 transmission rates of almost 40% when an HIV-1 inhibitor was vaginally applied in a gel formulation (Abdool Karim *et al.*, 2010). This result is encouraging and demonstrates that an effective microbicide is an achievable goal, not merely an unproven concept.

We initiated studies to determine whether RNA interference (RNAi) could be utilized to prevent HSV-2 transmission. RNAi is a pathway, found in many species, that regulates gene expression. Originally described in plants and *Caenorhabditis elegans*, RNAi is also functional in mammalian cells (Elbashir *et al.*, 2001; Fire *et al.*, 1998). The RNAi pathway is mediated through small non-coding double stranded RNA species, termed small interfering RNAs (siRNAs). The siRNA binds to homologous target mRNA, resulting in mRNA cleavage and subsequent protein degradation. Due to the specificity of siRNA-mediated mRNA cleavage, gene silencing via the RNAi pathway has become an attractive method to target diseases, including those of viral etiology. We have used siRNAs that target either HSV-2-specific viral genes or host-encoded viral entry receptors to prevent transmission in a mouse model of vaginal HSV-2 infection (Palliser *et al.*, 2006; Wu *et al.*, 2009). We will review the studies that demonstrate the feasibility of using siRNAs in a topical microbicide. We will also discuss how a detailed knowledge of the mechanisms involved in delivery and uptake of a vaginal microbicide will likely be required to allow judicious optimization of siRNAs formulated for vaginal delivery.

An Unmet Need

Although there are many microbicides in clinical trials, only one has demonstrated efficacy in reducing HIV-1 transmission (summarized in Hladik and Doncel, 2010). Microbicides can be categorized by their mode of action. Non-specific compounds have the potential to inhibit multiple types of virus. However, to date, these microbicides have not proven to be effective. Nonoxynol-9 (N9), a detergent that disrupts the viral envelope, was found to effectively inhibit HIV-1 replication *in vitro*. However, in clinical trials, vaginal application of N9 resulted in increased HIV-1 transmission. This enhanced susceptibility was attributed to N9 causing disruption of the vaginal epithelium, resulting in inflammation (Van Damme *et al.*, 2002). Recently, glycerol monolaurate (GML; also a detergent) was reported to protect macaques from vaginal SIV infection (Li *et al.*, 2009). GML was evaluated in a macaque model for safety (Schlievert *et al.*, 2008). Multiple criteria were reported, including normal mucosal integrity and no induction of inflammation. Although these results are encouraging, a study using a murine HSV-2 susceptibility model reported that vaginal application of GML resulted in increased levels of HSV-2 virus in the vaginal cavity (Moench *et al.*, 2010). Which safety models are most predictive of an outcome in humans is not known and will need to be determined.

Specific microbicides are designed to inhibit a distinct viral interaction, *e.g.*, CCR5 antagonists that prevent HIV-1 binding its co-receptor. Tenofovir is an adenosine nucleotide analogue that is used for treating HIV-1 infection. A clinical trial tested the efficacy of a 1% concentration of tenofovir in a gel formulation as a vaginal microbicide and found that HIV transmission was reduced by 39% (Abdool Karim *et al.*, 2010). This figure rose to 54% in women who better adhered to the gel application regimen. A 51% reduction in HSV-2 infection was also reported. However, HIV-1 acquisition did not correlate with HSV-2

infection — the efficacy of tenofovir gel was similar, irrespective of HSV-2 status. This study suggests that a topically applied gel can effectively reduce transmission of HIV-1 and HSV-2. These results are encouraging; however, issues regarding dosing, long-term tolerability, and cost need to be addressed. It may be that other compounds, or combinations of compounds, may yield better results and should be evaluated.

siRNAs as a Vaginal Microbicide

siRNAs are considered attractive therapeutic agents because of their ability to potentially target any disease for which the genetic basis is known. Clinical trials (see http://clinicaltrials.gov/) targeting respiratory syncytial virus (DeVincenzo *et al.*, 2008; DeVincenzo *et al.*, 2010) (Alnylam), liver cancers (Alnylam, 2011) (Alnylam), melanoma (Davis *et al.*, 2010) (Calando), and hypercholesterolemia (Zimmermann *et al.*, 2006) (Tekmira) highlight some of the diverse diseases that are being targeted using RNAi. HIV-1 was one of the first viruses found to be amenable to siRNA-mediated cleavage in mammalian cells (Capodici *et al.*, 2002; Coburn and Cullen, 2002; Jacque *et al.*, 2002; Lee *et al.*, 2002; Novina *et al.*, 2002; Surabhi and Gaynor, 2002). Subsequent studies showed that the RNAi pathway could be used to target viruses including polio, influenza, and HSV (Bhuyan *et al.*, 2004; Ge *et al.*, 2004; Gitlin *et al.*, 2002). Therefore, siRNAs can effectively inhibit viral replication *in vitro*, following transfection of either chemically synthesized siRNAs or vector-encoded short hairpin RNA (shRNA). However, to be effective as a topical microbicide, siRNAs must be formulated so that they remain available for cellular uptake. They must be designed to resist degradation by nucleases, inactivation due to the low pH vaginal environment, or entrapment in the mucosal layer. siRNAs must gain access to the appropriate cells targeted by the virus, *e.g.*, CD4+ T cells, macrophages, and DCs for HIV-1 and epithelial cells and neurons for HSV-2. The mechanism of cellular uptake must result in delivery of siRNAs into the cytoplasm, where the RNA induced silencing complex (RISC) resides. As an siRNA must circumvent such a large and diverse set of obstacles following vaginal application, siRNA formulation is critical in determining how effectively viral transmission is inhibited (summarized in Figure 1; for a general review discussing siRNA delivery, see Manjunath and Dykxhoorn, 2010).

In initial studies we used a mouse model of HSV-2 transmission as a proof-of-concept to determine whether siRNAs could inhibit viral infection across the genital mucosa (Palliser *et al.*, 2006). siRNAs targeting viral genes, essential for viral viability, were complexed with a cationic lipid and applied to the vaginal mucosa both prior to and following challenge with HSV-2 virus. Mice were protected from HSV-2 transmission. However, protection was transient; siRNAs had to be administered within a few hours of viral challenge for mice to be protected. siRNAs were observed in the vaginal epithelial cells and were detectable deep into the lamina propria. Furthermore, targeting an endogenous gene resulted in more durable silencing, with reduced expression of mRNA and protein observed for at least 7 days (Palliser *et al.*, 2006; Zhang *et al.*, 2006). A similar observation had been reported for siRNA-mediated inhibition of HIV-1 replication in macrophages *in vitro*. Knockdown of the HIV co-receptor, CCR5, was sustained for several days and coincided with decreased levels of HIV replication, whereas silencing HIV viral genes inhibited viral replication only if administered within hours of viral challenge (Song *et al.*, 2003).

Although the preliminary results are encouraging, the transient nature of the protection observed is not optimal for a microbicide. The CAPRISA 004 trial reported low adherence rates for tenofovir gel, with compliance possibly decreasing further over time, concomitant with a reduction in gel efficacy (Abdool Karim *et al.*, 2010). A microbicide that confers more durable protection could circumvent this problem.

Obstacles Along the Way

Histological analysis of vaginal tissue isolated from mice treated with lipid-complexed siRNAs showed no evidence of inflammatory infiltrates and induction of interferon-related genes was not detected (Palliser *et al.*, 2006). However, in follow-up studies using additional criteria, lipid-related toxicity was observed (Woodrow *et al.*, 2009; Wu *et al.*, 2009). These results emphasize the need to use assays that accurately determine whether a microbicide, both the active component and the carrier, can induce undesirable responses. As discussed above, this is not a trivial undertaking. When microbicides such as N9, originally reported to be safe, were evaluated in a mouse model, disruption of the vaginal epithelium, significant inflammatory responses, and increased susceptibility to HSV-2 were reported (Mesquita *et al.*, 2009; Moench *et al.*, 2010).

Due to their short length, siRNAs were originally believed not to induce interferon-related responses. However, activation of immune responses via toll-like receptors (TLR) and retinoic acid inducible gene-I (RIG-I) by siRNAs has been reported (Robbins *et al.*, 2009). Some of these responses are sequence or structure dependent and can therefore be avoided when designing siRNAs. Chemical modification of siRNAs is routinely used to prevent induction of immune responses. 2′-O-methyl and 2′-fluoro substitutions effectively negate immune stimulation. Not all ribonucleotides need to be replaced with 2′-O-methyl or 2′-fluoro. Two substitutions in the siRNA sense strand can be sufficient to ameliorate immunostimulatory activity. Minimal alterations to the siRNA backbone are preferable to maintain silencing efficiency of the siRNA (Robbins *et al.*, 2009).

In vivo, degradation of siRNAs by nucleases shortens the half-life of unmodified siRNAs to minutes. Unmodified siRNAs can be protected from nucleases, for example, by complexation in a lipid or nanoparticle (Wu *et al.*, 2009). However, as these carriers can induce toxic effects, it may be preferable to use siRNAs that are nuclease-resistant. In addition to decreasing immune stimulation, siRNAs are stabilized by modification with 2′-O-methyl or 2′-fluoro (Robbins *et al.*, 2009). Incorporation of phosphorothioate (PS) groups also extends siRNA half-life (Soutschek *et al.*, 2004; Vaishnaw *et al.*, 2010).

miRNA-like off-target effects have been reported following siRNA treatment (Birmingham *et al.*, 2006; Jackson *et al.*, 2003). This occurs if the siRNA contains sufficient homology to non-targeted mRNA in the open reading frame or the 32 untranslated region. Algorithms used to identify active sequences usually exclude siRNA that share a degree of homology with non-targeted mRNAs. If homologous sequences need to be chosen, the sense and/or antisense strands can be chemically modified to minimize off-target silencing (Jackson *et al.*, 2006). *In vitro* microarray data has shown down-regulation of a large number of transcripts following siRNA transfection (Jackson and Linsley, 2010). Whether the decrease in mRNA reported is sufficient to significantly modulate protein expression is not clear. Furthermore, miRNA-like off-target effects are often minimal at low concentrations of siRNA (picomolar to low nanomolar range), the dose at which potent siRNAs are effective (Vaishnaw *et al.*, 2010).

Saturation of the RNAi machinery can also mediate unwanted effects. Overexpression of shRNA in mouse hepatocytes following intravenous infusion resulted in liver injury and, in some cases, death. Morbidity correlated with decreased levels of liver-associated miRNAs, and saturation of exportin-5 was observed (Grimm *et al.*, 2006). Administration of siRNAs *in vivo* has been reported not to interfere with the miRNA pathway, even when multiple siRNAs were injected (John *et al.*, 2007). However, some studies report that, just as observed for shRNAs, high concentrations of siRNAs can perturb miRNA function (Khan *et al.*, 2009). Furthermore, injection of multiple siRNAs can lead to competition for RISC

resulting in less efficient silencing of one of the targeted genes (Bitko *et al.*, 2005; Castanotto *et al.*, 2007).

Improving siRNA Delivery

As the lipid reagent used in our initial studies displayed some toxicity, an alternative method to deliver the siRNAs was needed. Studies looking at siRNA delivery at another mucosal surface, the lung, reported siRNA uptake and gene-specific silencing using unmodified siRNAs formulated in saline, delivered intranasally (Alvarez *et al.*, 2009; Bitko *et al.*, 2005). However, we found that unmodified siRNAs were degraded within minutes following incubation with vaginal washes (Wu *et al.*, 2009). Substitution of one PS group on the sense and antisense strand of the siRNA was sufficient for protection from nucleases. However, the PS-siRNAs were not effective for gene silencing following vaginal delivery. Further modification of the siRNA sense strand by adding a cholesterol moiety resulted in siRNA uptake and silencing of the targeted gene (Soutschek *et al.*, 2004; Wu *et al.*, 2009).

Previous studies, including our own, suggested that targeting endogenous genes results in long-lived gene silencing (Palliser *et al.*, 2006; Song *et al.*, 2003; Wu *et al.*, 2009). Therefore, to increase durability of protection in the HSV-2 infection model, we targeted HSV-2 viral entry receptor genes. We used siRNAs modified for stability (PS-siRNA) that had a cholesterol group attached to the sense strand (C-siRNA) to circumvent a requirement for complexing the siRNA with a lipid transfection reagent (Soutschek *et al.*, 2004). When C-siRNA specific for a putative HSV-2 entry receptor, nectin-1, was vaginally administered, mice were protected from viral transmission for at least 7 days following C-siRNA application. Protection correlated with receptor knockdown, with no protection observed when C-siRNAs were given within hours of viral challenge. As observed in our previous study, application of C-siRNA specific for viral genes protected mice only when administered close to the time of viral challenge. When C-siRNAs specific for both viral genes and nectin-1 were combined, mice were protected from viral infection for 7 days, irrespective of time of challenge (Wu *et al.*, 2009).

From the studies outlined, targeting a combination of genes may confer better protection from viral transmission than knocking down one gene with a single siRNA. Using multiple siRNAs should result in fewer off-target effects and for an error-prone virus, such as HIV-1, targeting multiple genes should prevent emergence of RNAi-resistant variants that harbor mutations in the target sequence. Targeting both viral and endogenous genes involved in viral replication may be a good strategy for achieving durable protection that is effective within hours of application as well as several days later. However, targeting endogenous genes may not be feasible if knockdown results in altered cell function. As siRNAs targeting viral genes may be short-lived once inside the cell, particles designed for sustained release of siRNAs could be useful for providing sustained protection from viral transmission. In a recent study, nanoparticles were synthesized to release siRNAs over several weeks. Vaginally applied nanoparticles were observed in the epithelium and lamina propria, and the cervix. Endogenous genes could be silenced for 14 days, in the absence of any overt cellular infiltration or epithelial disruption (Woodrow *et al.*, 2009).

What Next?

The initial studies outlined demonstrate that use of siRNAs as part of a microbicide is feasible. To achieve effective and durable protection from viral transmission, siRNA delivery must be optimized. As discussed above, off-target effects can be overcome using potent siRNAs that are chemically modified. Another potential source of toxicity is uptake of siRNAs by bystander cells. This problem could be alleviated by limiting siRNA delivery to cells targeted by virus. For example, the primary cells infected by HIV are macrophages,

dendritic cells, and CD4+ T cells. siRNAs have been delivered to these cells using antibody- and aptamer-based modalities. When attached to an antibody, protamine, a positively charged protein that binds nucleic acids, can be used to specifically deliver siRNAs to a receptor-positive cell. An HIV-specific gp120 antibody, fused to protamine, was used to deliver siRNAs to HIV-infected cells (*i.e.*, expressing HIV-gp120) (Song *et al.*, 2005). Growth of a melanoma, stably expressing gp120, was delayed when gp120 antibody-protamine bound to oncogene-specific siRNAs were injected intravenously into a mouse model. Histological analysis of tumors from mice injected with fluorescently-labeled siRNAs bound to gp120-antibody-protamine showed siRNA uptake only in gp120-positive tumors. This method has been used to deliver siRNAs to CD7+ and LFA-1+ cells using CD7− and LFA-1-specific antibodies, respectively (Kumar *et al.*, 2008; Peer *et al.*, 2007). CD7 antibody, fused to nine arginines, was used to deliver siRNAs specific for HIV-1 viral genes and the CCR5 coreceptor to T cells in a humanized mouse model. Intravenous injection of the antibody-bound siRNAs protected mice from HIV-1 challenge. This regimen was also effective for controlling HIV-1 infection and maintaining CD4+ T cells (Kumar *et al.*, 2008).

Similar to antibodies, aptamers bind specific ligands, and have been used to deliver siRNAs to receptor-positive cells. Aptamers are structured nucleic acids and ligand-specific aptamers can be selected following several round of selection, using a protein or cell type of interest (Yan and Levy, 2009). Aptamers have been selected with higher binding affinities, compared with antibodies (picomolar range). In addition, aptamers are easily synthesized and are amenable to chemical modification thereby protecting the nucleic acid from nucleases and limiting off-target responses. An anti-PSMA aptamer (prostate-specific membrane antigen) has been reported to deliver siRNAs to prostate specific membrane antigen (PSMA)-positive cells *in vitro* and *in vivo* (Chu *et al.*, 2006; Dassie *et al.*, 2009; McNamara *et al.*, 2006). A gp120 aptamer neutralizes HIV-1. Fusion of this aptamer to anti-*tat/rev* siRNA resulted in uptake of this construct only by HIV-1 infected cells, decreased expression of *tat/rev* transcripts, and better suppression of HIV-1, when compared to using gp120 aptamer alone (Zhou *et al.*, 2008). Injection of this construct into an HIV-1 infected humanized mouse model is reported to inhibit HIV-1 replication by several logs and prevent CD4+ T cell depletion normally associated with HIV-1 infection (Neff *et al.*, 2011). Modification of the gp120 aptamer to bind multiple siRNAs (using a GC-rich “sticky bridge”) has been used to deliver siRNAs targeting HIV-1 *tat/rev*, the HIV-1 receptor CD4, and HIV-1-dependency factor TNPO3 to HIV-1 infected cells *in vivo*. Competition for RISC entry by the siRNAs was observed. However, silencing was sufficient to suppress HIV-1 viral loads and stabilize CD4+ T cell numbers. As previously stated, for an error-prone virus such as HIV-1, targeting multiple genes should minimize the emergence of viral escape mutants (Zhou and Rossi, 2010; Zhou *et al.*, 2009).

Although delivery of siRNAs via specific receptors represents a method that should lower effective dose and limit toxicity, siRNA uptake does not necessarily equate with effective silencing. Following internalization, siRNAs must gain access to the cytosol where RISC resides (Zeng and Cullen, 2002). Receptor-mediated endocytosis would generally require siRNAs to cross the endosomal membrane to reach the cytosol. Studies have shown that this process can be very inefficient. However, strategies such as the inclusion of an endosomal escape peptide in an siRNA/ligand complex can facilitate siRNA entry into the cytosol and increase efficiency of silencing (Aouadi *et al.*, 2009; Oliveira *et al.*, 2007).

Conclusion

Initial studies support the feasibility of using siRNAs as part of a microbicide to prevent sexually transmitted diseases, such as HIV-1 and HSV-2. Vaginally applied siRNAs were

observed in epithelial cells and deep in the lamina propria. This level of siRNA uptake was sufficient to protect mice from a lethal HSV-2 infection (Palliser *et al.*, 2006; Wu *et al.*, 2009). Lipid-based reagents confer some toxicity, and it is unclear whether lipid-formulated siRNAs are taken up effectively by the initial cells infected by HIV-1. Therefore, receptor-mediated siRNA delivery may represent a feasible strategy for microbicide development. The expense associated with antibody production may limit their utility as part of a microbicide, with aptamer-mediated delivery potentially being more cost-effective. Increased binding affinity, ease of manufacture, ability to modify nucleic acids, and possible lack of immune-stimulating responses are additional attractive features associated with aptamer technology (Yan and Levy, 2009).

In addition to ensuring delivery of siRNAs to appropriate cells, durability of protection is an important factor to consider in terms of overall cost of each application and, as has been reported in microbicide trials, compliance (Abdool Karim *et al.*, 2010). Silencing endogenous genes may result in long-term (days) inhibition of protein expression (Palliser *et al.*, 2006; Song *et al.*, 2003; Wu *et al.*, 2009). If knockdown of host genes is not feasible, reagents such as nanoparticles could be used to release siRNAs over a period of days to weeks (Woodrow *et al.*, 2009). To ensure uptake of nanoparticles by appropriate cells, the particles could be modified to bind antibodies or aptamers (Peer *et al.*, 2008). Such modification should also serve to reduce the effective siRNA dose and limit toxicity.

As has been emphasized throughout this review, determining any toxic effects associated with an siRNA or its carrier is critical for synthesizing a microbicide that does not fail in initial safety trials, due to undesirable side effects. A major problem in evaluating potential microbicides is a lack of models to predict safety. Recent work has identified new biomarkers that are consistent with safety outcomes seen in clinical trials (Mesquita *et al.*, 2009; Moench *et al.*, 2010; Wilson *et al.*, 2009).

Moving forward, increased efficacy and safety of topically applied siRNAs will likely require receptor-mediated delivery and possibly sustained siRNA release. These technologies are available and with the rapid pace with which siRNA-mediated clinical trials are proceeding, an siRNA-based microbicide should be a viable option.

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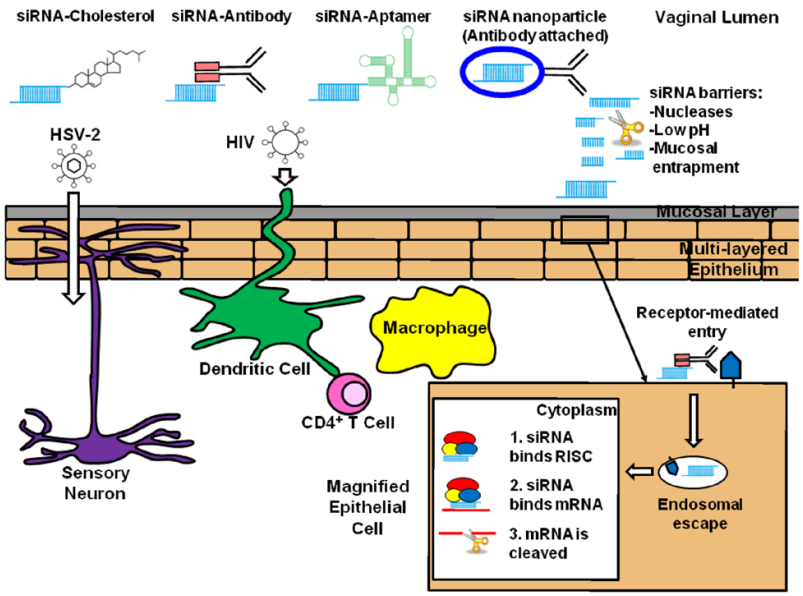

Figure 1.

Initial cellular targets for viral entry of HSV-2 and HIV-1 and methods of delivering siRNAs that could be used to prevent transmission of these viruses. HSV-2 infects epithelial cells and neurons. HIV-1 infects CD4+ T cells, macrophages, and dendritic cells. siRNAs delivered to the vaginal lumen must be protected from nucleases, low pH environment, and avoid mucosal entrapment. Upon uptake by a cell, such as an epithelial cell, siRNAs must escape the endosome and gain entry to the cytoplasm. Once inside the cytoplasm, siRNA is incorporated into the RNA-induced silencing complex (RISC). The siRNA then binds, via sequence homology, to its target mRNA, resulting in site-specific cleavage of the mRNA by the RNase III enzyme argonaute 2.