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Recent Advances in Anogenital Antiretroviral Microbicides and Multimodal Delivery Systems

Osmond J. D'Cruz[1]* and Fatih M. Uckun[1,2]*

[1]Microbicide Development Laboratory, Developmental Therapeutics Program, Children's Center for Cancer and Blood Diseases, Children's Hospital Los Angeles & Department of Pediatrics, CA, USA
[2]University of Southern California Keck School of Medicine, Los Angeles, CA, USA

Abstract

The predominant route of human immunodeficiency virus type 1 (HIV-1) transmission is across the vaginal and rectal epithelia during sexual intercourse. Yet the development of safe and clinically effective vaginal and rectal antiretroviral microbicides remains an unmet challenge despite more than 25 years of accelerated product development. The clinical failure of seven microbicide candidates points to major unresolved challenges associated with target specificity, safety, potency as well as drug-delivery of validated candidates undergoing preclinical and clinical development as anogenital microbicides. Currently, additional antiretroviral agents are sought as anogenital microbicides to provide potential anti-HIV protection by directly inactivating HIV-1, preventing HIV-1 from attaching, entering or replicating in susceptible target cells, and/or by hindering the dissemination of HIV-1 to the host cells that line the vaginal/rectal walls along with targeting novel HIV-host dependency factors. Among the several types of anti-HIV microbicides currently in preclinical and clinical development, only one clinical trial of an HIV-1 reverse transcriptase inhibitor has shown clinical promise as a potential microbicide. This article reviews the preclinical/clinical efficacy and safety profiles of current antiretroviral microbicide candidates (Tenofovir, Stampidine, UC-781, Dapivirine/TMC120, MIV-150, HI-443, CCR5 antagonists, neutralizing antibodies, targeted RNA interference, RNA-based aptamers, Aptamer-siRNA-chimeric RNA), as well as advances in multimodal microbicide delivery systems (nanocarriers - liposomes, dendrimers, polymeric, solid lipid and metal nanoparticles, nanospheres, nanocapsules, intravaginal rings and recombinant lactobacilli delivery strategies). The clinical failure of first-generation antiretroviral gels is spearheading efforts to evaluate new mechanism-based antiretrovirals with a rational design and engineering of long-acting and novel delivery systems more appropriate to curb anogenital HIV transmission.

Keywords: AIDS; Human Immunodeficiency Virus-1; Microbicide; Vaginal; Rectal; Receptive anal intercourse; Reverse transcriptase

Introduction

Sexual transmission through vaginal and rectal mucosal surfaces has been the most common route of HIV-1 spread throughout the world [1]. The latest estimates by the Joint United Nations Program on HIV/AIDS (UNAIDS) indicate that more than 33.3 million people worldwide are living with HIV-1 infection or AIDS [1]. Worldwide, new HIV-1 infections (approximately 3 million per year) occur, mostly through heterosexual intercourse. Heterosexual transmission of HIV-1 now accounts for over 80% of adult infections worldwide and male-to-female transmission of HIV-1 is approximately eight times more frequent than female-to-male transmission [2]. Receptive anal sex is the predominant mode of HIV acquisition among men who have sex with men (MSM) [3,4] and a significant independent risk factor for HIV infection among women [5,6]. Unprotected receptive anal intercourse (RAI) has the highest per act risk of HIV acquisition with an unadjusted probability of 0.08 per contact for RAI [7] as compared to 0.001 per coital act for vaginal intercourse [8]. Furthermore, there is increasing epidemiological evidence that women as well as men in both the developed [9-11] and developing world [12-14] practice RAI. Clearly, both vaginal and rectal microbicides should be seen as an important HIV prevention technology for all individuals who practice RAI. However, the move for the development of a safe and effective anogenital microbicides remains an unmet challenge despite more than 25 years of accelerated product development. Early strategies to prevent the spread of sexual transmission of HIV-1 with first-generation vaginal antiviral agents led to the failure of 11 clinical trails with 6 microbicide candidates, nonoxynol-9, SAVVY®/C31G, cellulose sulfate, Carraguard®/PC-515, PRO 2000 and BufferGel® [15-19]. In addition, the recent failure of rectal microbicide candidates can be attributed to the use of vaginally formulated microbicide gels that failed in clinical vaginal efficacy and safety studies.

Multiple Mechanisms of Sexual Transmission of HIV

Sexual male to female transmission of HIV-1 can occur *via* multiple alternate pathways involving a variety of target cells in the host vaginal/rectal mucosa and cell surface receptors/co-receptors, and both cell-free and cell-associated virus [20-23]. HIV-1 entry into target cells (T cells, macrophages, dendritic cells, and mucosal Langerhans cells) involves a sequential, multi-step process that includes viral attachment to the host receptor, binding to host coreceptors, and fusion of the viral and host cell membranes [24]. HIV can enter the human cell in three important steps: (i) Attachment of the HIV surface envelope (ENV) glycoprotein (gp) 120 to the cellular CD4 receptor expressed by the monocyte derived macrophages and T-lymphocytes; (ii) Interaction of the gp120 protein and CD4 complex with either the CCR5 or CXCR4 coreceptor

***Corresponding authors:** Fatih M. Uckun, University of Southern California Keck School of Medicine, Los Angeles, CA, USA, E-mail: fmuckun@chla.usc.edu

Osmond J. D'Cruz, Microbicide Development Laboratory, Developmental Therapeutics Program, Children's Center for Cancer and Blood Diseases, Children's Hospital Los Angeles & Department of Pediatrics, CA, USA, E-mail: odcruz@chla.usc.edu

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(iii) Virus-cell membrane fusion mediated by conformational changes in the transmembrane gp41 protein [24-27]. Upon internalization, the viral enzymes, viz. reverse transcriptase (RT) and integrase are released into the cytosol of the host cell. The viral RNA is then transcribed into a double-stranded DNA with the help of RT, followed by integration of viral DNA into the host genome resulting in formation of a provirus [28,29]. Provirus formation is followed by a transcription step, wherein the unspliced viral RNA leaves the nucleus and, with the help of the host translation machinery, viral proteins are formed from unspliced transcript [30]. HIV-1 exploits several host proteins, also known as HIV-dependency factors (HDFs) during multiple steps of infection, including viral entry, viral integration, and viral transcription in cells expressing human CD4 and CXCR4 [31-34].

Microbicides Targeting Mucosal Infection and Virus Dissemination

HIV-1 appears to exploit multiple mechanisms to transit different epithelial barriers and gain access to susceptible target cells and the opportunity to establish a systemic infection. Due to different receptors and HIV-1 entry pathways, effective microbicides may need to target both localized mucosal infection and virus dissemination to draining lymph nodes [35]. HIV-1 remains localized in the genital mucosa for about a week defining the "eclipse phase" of the infection [36], which provides a window opportunity for intervening in order to prevent the establishment of the infection. Single-genome amplification and sequencing of the earliest detectable viruses showed that they are usually derived from a single transmitted virus which infects a small founder population of $CD4^+$ T cells [37,38]. The finding that R5, X4, and dual-tropic R5X4 HIV-1 isolates can all infect mitogen-activated cervical tissues [39], suggests that immune activation may increase susceptibility to additional HIV-1 phenotypes. Blockade of cell surface receptors (CD4, CCR5, and CXCR4) within the mucosa may be sufficient to prevent localized infection of T cells, macrophages, and dendritic cells (DCs). However, HIV-1 can attach to mucosal LCs and to DCs *via* the binding of HIV-1 gp120 to syndecan-3, a heparan sulfate moiety, as well as to DC-SIGN, a C-type lectin receptor, expressed on the surface of these cells, and may be more resistant to microbicides than the establishment of localized infection [40]. These cells can mediate the efficient transfer of HIV-1 to $CD4^+$ T cells across a gap termed the virological synapse [41]. Additionally, epithelial cells that line the female genital tract can endocytose HIV-1 and transfer virus to $CD4^+$ T cells and DCs in the underlying lamina propria in a process termed transcytosis [42]. Moreover, the virus could also remain unmodified within the cytoplasm of LCs for several days before transmission to T cells. Rectally administered microbicides have the potential to reach local nodes through lymphatic drainage [43]. The interior iliac lymph nodes are known to be a site of early virus replication and have common drainage of the female genital tract and rectum. Thus, effective microbicides should be able protect against vaginal as well as rectal HIV-1 infection and transmission. The best approach likely involves a combinatorial approach to targeting both cell-free and cell-associated virus, and also to protect the vaginal and rectal mucosa with compounds that impart resistance to infection.

Role of Mucosal Barriers and Inflammatory Mediators

The vaginal and rectal compartments are equipped with a variety of physical barriers, innate and adaptive immune responses important for preventing HIV-1 infection [44-48]. The multilayered squamous epithelium of cervico-vaginal mucosa is a significant anatomical barrier and challenge for the virus in the female genital tract to come in contact with susceptible $CD4^+$ target cells in the epithelium or superficial submucosae. The mucosal epithelium also contributes to innate defenses that have antiviral activity such as the production and secretion of microbicidal defensins, antimicrobial peptides, and secretory inhibitors deposited within the lumen of the genital tract [45,47-50]. A single cell layer of columner epithelium separates the rectal mucosa with abundant CD4-bearing target cells ($CD4^+$ T cells, DCs, and macrophages) in close proximity to the basolateral surface of the intestinal epithelium [43]. Therefore, it is critical that topical microbicides do not interfere with natural protective host immune mechanisms to sexually transmitted infections (STIs), but inhibit pathogen host cell interactions that facilitate spread. Exposure of the vaginal-ectocervical and rectal tissue to chemical insult can cause damage and/or inflammation at the site of application [51,52]. The public health risk caused by such reactions is significant due to increased rates for STIs such as HIV-1 [45]. The increased STI susceptibility is due to: (a) compromised tissue barrier which allows viral entry, (b) recruitment of susceptible target cells to the site of inflammation [44,45] and/or (c) induction of inflammatory cytokines that can activate HIV long terminal repeat (LTR) *via* the nuclear factor kappa B (NFκB) pathway [46,53]. Consequently, less intrusive microbicides, which could be used by women and male receptive partners, would be highly desirable [54]. Despite extensive preclinical research, five large-scale phase IIB/III clinical trials of candidate microbicides (i.e., Nonoxynol-9 [N-9], cellulose sulfate, Savvy [C31G], Carraguard, BufferGel and PRO-2000) showed no reduction or even an increased risk of acquiring HIV-1 [15-19]. These candidate microbicides were considered safe in preliminary short-term phase I safety trials and had shown activity against HIV-1 [55-58].

Developmental Requirements for Anogenital Microbicides

Microbicides are hoped to provide anti-HIV protection by directly inactivating HIV, preventing HIV from attaching, entering or replicating in susceptible target cells, and/or by hindering the dissemination of HIV to the host cells that line the vaginal wall. Unlike several first-generation microbicides that have failed in recent clinical trials [15-19], an ideal microbicide must be safe and effective following vaginal and rectal administration and it should cause minimal or no local inflammation following long-term repeated use. The desired criteria for an optimal microbicide include: (1) Rapid virucidal activity without requiring metabolic activation, (2) Ability to rapidly cross membrane barriers, (3) Prolonged or irreversible inhibition of HIV-1 enzyme activity, (4) Sustained antiviral activity under acidic and alkaline conditions, (5) Stability under various climatological temperatures, (6) Minimal binding to genital tract and rectal components, (7) Long-acting or sustained prophylactic activity, (8) Lack of systemic absorption that might contribute drug resistance, (9) Lack of pro-inflammatory effects, and (10) Lack of adverse effects on the healthy normal vaginal and rectal microbiomes.

Prevalence of Non-B Subtypes and Recombinant Forms

HIV-1 entry and fitness may also play a role in HIV-1 transmission, spread in the human population, and global evolution. The current HIV-1 epidemic is a mixture of old and contemporary lineages. High mutation frequencies coupled with plasticity of functional *env* glycoproteins have now resulted in extreme *env* diversity observed among different subtypes (>15% predicted amino acid diversity) and between isolates of the same subtype (10 to 15%). HIV-1 subtype B is the prevalent variant in North America and West Europe. However, non-subtype B and recombinants (non-B variants) are responsible for

90% of the 33 million infections worldwide [59]. These variants have an increasing prevalence and heterogeneity in developed countries [60]. Regardless of the human ethnicity of the host cell, subtype C HIV-1 isolates are significantly less fit in terms of relative replication efficiency than any other group M isolates (e.g., subtypes A, B, D, and E) [61], and yet subtype C now dominates the worldwide epidemic.

The coexistence of multiple HIV-1 variants in the same region favors the recombination between them after coinfection and/or superinfection events. At least 20% of HIV-1 isolates sequenced worldwide are inter-subtype recombinants that can be divided into two categories: CRFs (circulating recombinant forms) and URFs (unique recombinant forms) [62]. Currently, more than 40 CRFs and 100 URFs have been identified worldwide http://www.hiv.lanl.gov. Recombinant CRF02_AG, a CRF derived from subtype A and G, is the most prevalent strain in West and West Central Africa. Also, the prevalence of newly diagnosed patients infected with non-B subtypes and CRFs is very high in some European countries (43.9% in UK) and is traditionally associated with immigration [63]. Genotypic diversity among HIV-1 subtypes and CRFs may lead to distinct pathways to drug resistance. Non-B variants present clade-specific substitutions in positions related to drug-resistance [64] that could accelerate the emergency of drug-resistant viruses, change or induce alternative pathways of resistance, affect the drug-binding affinity or accelerate disease progression. Notably, HIV-1 subtype C viruses rapidly develop K65R resistance to tenofovir in cell culture [65]. Thus, the continuous spread of HIV-1 recombinants may have serious implications in the effort to control the AIDS pandemic with future microbicide trials, and could potentially represent one of the highest barriers to HIV-1 eradication [66].

Antiretroviral Microbicides in Development

Currently there are six classes of drugs act¬ing at various stages of the viral life cycle, nucleos(t)ide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), CCR5 coreceptor antagonists, fusion inhibi¬tors and integrase strand transfer inhibitors. Protection against HIV transmission *via* the colorectum requires the use of highly potent antiretroviral (ARV) agents with high solubility. Nucleoside/tide analog and non-nucleoside reverse-transcriptase inhibitors (NRTIs and NNRTIs) are of greatest interest because of their preintegration activity, long half-lives, safety profiles, and success in preventing infection in animal models [67-75]. HIV-1 can only evolve in those host cells that provide the essential microenvironment required for its life cycle. A recent genome-wide siRNA-mediated single gene knockdown study provided elegant evidence that HIV-1 exploits several host proteins, also known as HIV-dependency factors during multiple steps of infection, including viral entry, viral integration, and viral transcription in HeLa cells expressing human CD4 and CXCR4 [31-34]. Accordingly, identifying new agents capable of both preventing and treating HIV-1 infection by leveraging the dependency of HIV on host factors as well as the viral RT enzyme for infecting and replicating in human cells will be superior approach for developing effective antiretroviral microbicides.

Virus targeting entry inhibitors

HIV-1 entry into target cells involves a sequential, multi-step process that includes viral attachment to the host receptor, binding to host coreceptors and fusion of the viral and host cell membranes [25]. Since viral infection is mediated by a single type of protein cluster on the virus surface, inhibition of the initial entry of HIV-1 into host cells has been a compelling means to prevent infection and spread of the virus [26]. Each virus Env spike consists of a trimer of two non-covalently associated glycoproteins, an inner gp41 transmembrane protein and a gp120 exterior protein. Viral entry is dependent on the ability of the virus envelope protein spike of Env to interact with specific cell receptors (CD4 as well as a coreceptor CXCR4 or CCR5) in a multistage process that triggers conformational rearragements in Env and consequent fusion of virus and cell membrane to deliver virus contents to the host [76-80]. Agents that could either block virus Env-host cell receptor interactions or inactivate the Env spike before cell encounter would provide virus-targeted molecular weapons for prevention of HIV-1 transmission [81].

C-C chemokine receptor 5 (CCR5) antagonists: CCR5 is the coreceptor almost exclusively used by HIV-1 isolates involved in the initial viral transmission [82]. Nearly all newly infected individuals have primarily CCR5-tropic viruses in the blood; however, CXCR4-tropic viruses can emerge as HIV disease progresses [37,80,81]. CCR5 is expressed on a large number of $CD4^+$ T lymphocytes, usually activated, present in the vaginal, rectal, and foreskin epithelia. Humans bearing homozygous *CCR5* mutations that abrogate CCR5 function are resistant to HIV infection and do not lead to any significant immune dysfunction [83,84]. RANTES is a natural chemokine that binds to CCR5; this binding subsequently leads to the internalization of the receptor, and as a result, prevents HIV binding and infection [85]. These findings imply that blocking HIV-1 binding to CCR5 is a viable strategy to prevent HIV-1 transmission.

CCR5 antagonists have already proven useful at preventing HIV transmission in nonhuman primates [86-88]. PSC-RANTES, a chemically modified version of RANTES with anti-HIV-1 blocking and CCR5 agonist properties, has been shown to prevent vaginal SHIV-162P infection of rhesus macaques [86]. In spite of the potential of gp120 antagonists for HIV-1 prevention, progress has been limited due to such factors as low potency of CCR5 inhibitors, the high cost and potential toxicity of protein inhibitors and the potential risk of infection enhancement with CD4-mimicking ligands. Human vaginal *Lactobacillus jensenii* are being engineered to secrete wild-type (wt) RANTES as well as its CCR5 antagonist analogue, C1C5 RANTES [89,90]. Both proteins exert strong anti-HIV-1 activity in $CD4^+$ T cells and macrophages, the two major target cells for HIV-1. Viral resistance against CCR5 inhibitors, primarily through isotype conversion to CXCR4 for entry is also a concern. CCR5 antagonism is crucial to prevent mucosal inflammation. Therefore, while blocking HIV-1 entry, RANTES derivatives (e.g. PSC-RANTES, 5P12 RANTES) should not activate CCR5 that can trigger proinflammatory activity and mucosal inflammation that could enhance HIV-1 transmission. CCR5 activation together with persistent elimination of CCR5 from the cell surface can perturb the function of CCR5 in host physiology. Also, internalization of CCR5 following RANTES exposure is short-lived thereby leading to renewed CCR5 surface expression. Although CCR5 antagonists effectively block localized infection unlike RT inhibitors they are unable to inhibit dissemination by migratory cells [91]. Since HIV-1 transmission can occur days after the initial exposure to HIV-1 in seminal fluid [42], underscoring the importance of advancing microbicides that can prevent the binding and internalization of HIV-1.

The activity of CCR5 antagonists is limited to patients with virus that uses only CCR5 for entry (R5 virus). Viruses that use both CCR5 and CXC chemokine receptor 4 (CXCR4; termed dual/mixed [D/M]) or only CXCR4 (X4 virus) do not respond to treatment with CCR5 antagonists [92]. Virologic failure of these drugs frequently is associated with outgrowth of D/M or X4 virus from a preexisting minority population present at levels below the limit of assay detection [92-95].

The main determinants of HIV-1 coreceptor usage are located in the V3-loop of gp120, although mutations in V2 and gp41 are also known. Mutations in HIV-1 gp120 that allow the virus to bind to the drug-bound form of CCR5 have been described in viruses from some patients whose virus remained R5 after virologic failure of a CCR5 antagonist [95-97]. Most of these mutations are found in the V3 loop, the major determinant of viral tropism. Some CCR5 antagonist-resistant viruses selected *in vitro* have shown mutations in gp41 without mutations in V3 [97-99]. The sensitivity to R5 entry inhibitors is closely related to HIV-1 fitness, entry efficiency, and more specifically, to CCR5 binding [100-102]. Diversity in the viral envelope gene likely results in variable sensitivity to entry inhibition [100,102,103]. In the African countries the predominant HIV subtypes are A, C, and D. In the US, subtype B predominates. A relevant biological difference is the binding avidity of HIV subtypes for CCR5 receptors, which are important mechanisms for entry into LCs, and are the predominant HIV-1 co-receptor in foreskin immune cells [104]. Subtype B has a greater binding avidity for CCR5 receptors than subtype C [105], which could represent decreased sensitivity to R5 entry inhibitors (RANTES derivatives).

Neutralizing antibodies: Enhancing anti-HIV-1 humoral immunity at the mucosal cell surface by local expression of anti-HIV-1 broadly neutralizing antibodies (BnAbs) that block HIV-1 entry would provide an important new intervention that could slow the spread of HIV. The major targets for HIV-1 neutralizing antibodies are the viral envelope glycoprotein trimers on the surface of the virus that mediate receptor binding and entry [106,107]. HIV-1 has evolved many mechanisms on the surface of envelope glycoproteins, gp120 and gp41 to evade antibody-mediated neutralization, including the masking of conserved regions by glycan, quaternary protein interactions and the presence of immunodominant variable elements. Human BnAbs against highly variable viral pathogens are much sought after to treat or protect against global circulating viruses. A growing number of human BnAbs including, b12, 2G12, 2F5, 4E10, Z13e1, VRC01, HJ16, PG9 and PG16 are capable of potently neutralizing a broad range of primary HIV-1 isolates [108-111]. The monoclonal antibodies 2G12, PG9 and PG16, which neutralize HIV-1 from multiple clades, bind to glycosyl moieties or V2 and V3 of gp120 [106-111]. The use of antibody fragments, such as FabV and scFv molecules, preserves the high degree of specificity and the orientation of the binding region, while immune reactions are reduced since Fc receptor-mediated phagocytosis by cells of the mononuclear phagocyte system is avoided. On the other hand, the binding avidity might be lost or decreased, although, coupling the fragments onto liposomes would lead to multivalent binding and can restore avidity [112]. Nonetheless, the sequence variability, glycosylation and mobility make the envelope a moving target, which complicates the search for molecules that bind with high specificity.

Several cell surface receptors and molecules can facilitate HIV-1 entry into epithelial cells allowing passage through the mucosal barrier. Syndecans are found to be exploited by HIV-1 to cross the mucosal epithelium by transcytosis [113-115]. It has been reported that the Arg298 in gp120 mediates HIV-1 binding to syndecans, and the human b12 anti-HIV gp120 neutralizing antibodies can block this interaction [116-118]. Macaques treated with b12 IgG1 by intravaginal application were shown to be protected against SHIV infection by the vaginal route [119]. Also, mAb 2G12, a potent neutralizing anti-HIV-1 IgG, which binds to a constellation of high mannose-type carbohydrates on gp120, has been shown to protect macaques against vaginal transmission upon chimeric simian HIV challenge [116,119,120]. Another novel microbicide strategy to protect against sexual transmission of HIV-1 is by adeno-associated virus (AAV) transfer of broadly neutralizing antibody genes to cervico-vaginal epithelial stem cells that could replenish human b12 anti-HIV gp120 BnAb secreting cells through multiple menstrual cycles [121]. However, most humans-unlike macaques-possess CD8 T-cell responses specific for the AAV capsid due to prior exposure; these responses may clear the vector too rapidly for it to be effective.

Cyanovirin-N: High-mannose N-linked glycans recognized by carbohydrate-binding agents are potential targets for topical microbicides. HIV-1 Env gp120 is a highly glycosylated protein, with approximately 24 N-linked carbohydrates accounting for as much as 50% of its mass. Cyanovirin-N (CV-N) is an 11-kDa cyanobacterial lectin that prevents virus-to-cell fusion by blocking gp120 interaction with CD41 and cell-associated CCR5 coreceptor [122,123]. This antiviral activity is attributed to two homologous carbohydrate binding sites that specifically bind high mannose glycosylation present on envelope glycoproteins such as HIV-1 gp120 [124]. CV-N is currently being investigated for, including gels, suppositories, and *in vivo Lactobacillus* delivery [125-127]. The efficacy of either 1% or 2% recombinant CV-N formulated into a carboxyethylcellulose gel matrix as a topical microbicide has been tested in male macaques that were rectally challenged with a chimeric SIV/HIV-1 virus (SHIV89.6P) [125]. In this study, all of the untreated macaques were infected and experienced high viremia and CD4+ T cell depletion while none of the macaques that received either 1% or 2% CV-N gel showed evidence of SHIV89.6P infection. In the vaginal challenge model, 0.5, 1, and 2% CV-N gels were effective in blocking vaginal transmission of cell-free SHIV89.6P in macaques [126]. All of the placebo-treated and untreated control macaques became infected while 83% of CV-N treated macaques remained uninfected.

Among the anti-HIV carbohydrate-binding agents investigated, lectins with higher mannose binding sites are more effective inhibitors of HIV-1 than CV-N which has only four binding sites [124,128]. Also, CV-N has been shown to enhance viral replication levels at suboptimal concentrations with pronounced mitogenic/stimulatory effects on human peripheral blood mononuclear cells (PBMCs) [129-131]. CV-N has the capacity to promote secretion of pro-inflammatory cytokines and chemokines from human PBMCs, activate quiescent $CD4^+$ T-cells, and promote T-cell proliferation. CV-N affects the cell morphology of PBMCs and enhances the expression of the cellular activation markers CD25, CD69 and HLA-DR. Consequently, the use of CV-N may be accompanied by various stimulatory effects that may compromise its application for microbicidal use. In addition, HIV-1 resistance to CV-N, by deletion of multiple high-mannose *N*-linked glycosylation sites, has been well described [129,132]. Although there are three glycan clusters on gp120, a single deglycosylation on the glycan trimer regardless of subtype and tropism can limit the anti-HIV activity of these carbohydrate binding lectins [128,132,133].

Peptides: Retrocyclins are circular 18-residue, tetracyclic peptides with three cysteine disulfide bonds [134]. RC-101 (GICRCICGKGICRCICGR), a cationic retrocyclin exhibits activity against X4 and R5 strains of HIV-1 *in vitro* [135]. RC-101 prevents viral entry by blocking 6 helix bundle formation and binds to gp41 with high affinity. Mutations in gp41 have a greater effect on retrocyclin's anti-HIV-1 binding activity than gp120 mutations. RC-101 has a low therapeutic index with potential for hemolytic activity or cytolytic activity at 100-fold above its antiviral activity. Retrocyclins were shown to protect primary T cells from X4 and R5 strains of HIV-1 *in vitro*; protect primary $CD4^+$ cells against infection by clinical HIV-1 isolates from multiple clades. RC101 lacks inflammation potential and retains

anti-HIV activity in the presence of vaginal fluids. RC-101 formulated as a quick-dissolve film was found to be safe and retained antivirally property following repeated topical vaginal application in pigtailed macaques [136]. However, RC-101 is far less potent than NNRTIs requiring micromolar concentrations for preventing HIV-1 infection *in vitro*.

RTI-based microbicides

Reverse transcriptase (RT) inhibitors (RTIs) are the most advanced compounds used as potential components of vaginal and rectal microbicides because they are very specific and potent and potentially have a long-term inhibitory effect [137]. Candidate microbicides from all three major categories of RT inhibitors are being explored: (i) 2',3'-dideoxynucleoside analogs designated nucleoside RT inhibitors (NRTIs); (ii) acyclic nucleoside phosphonate analogs designated nucleotide RT inhibitors (NtRTIs), and (iii) non-nucleoside RT inhibitors (NNRTIs).

NtRTI-based microbicide: (Tenofovir/PMPA, [9-(R)-{2-(phosphonomethoxy)propyl}adenine] or PMPA, is a nucleotide analog of deoxyadenosine monophosphate that inhibits HIV-1 RT [138]. Tenofovir (TFV), a widely prescribed ARV drug in combination with other ARV agents for the management of HIV-1 infection [139]. The oral lipophilic prodrug, tenofovir disoproxil fumarate (TDF) is hydrolyzed to TFV intracellularly and phosphorylated to the active metabolite, tenofovir diphosphate (TDP). Multiple nonclinical studies have demonstrated the *in vitro* and *in vivo* efficacy of TFV for preventing HIV transmission [140-144]. TFV gel has also been proven effective in preventing vaginal SHIV transmission in macaques [72]. TFV 1% gel has been found to be well tolerated in women and men [144-146]. Some systemic absorption of TFV was reported following 14-day administration of 1.0% vaginal microbicide gel [144,147]. Tenofovir was detected in the sera of 56% of tested women.

Tenofovir is the first vaginal microbicide shown in a clinical trial to possibly provide a safe and effective way to prevent sexual transmission of HIV. In a double-blind, randomized, placebo controlled clinical trial in women (CAPRISA 004), vaginal application of 1% TFV gel was shown to reduce HIV acquisition by an estimated 39% (P = 0.017) overall, and by 54% (P = 0.025) in women with high gel adherence (>80%) [148]. Several other safety and effectiveness studies of 1% TFV gel as an HIV prevention strategy are ongoing [http://www.avac.org/ht/a/GetDocumentAction/i/3109]. Following the CAPRISA 004 study which demonstrated that Tenofovir gel used before and after sex reduced HIV infection by 39%, there was high hope that the VOICE (Vaginal and Oral Interventions to Control the Epidemic) study of daily Tenofovir gel would show similar promising results. The VOICE study is designed to test whether antiretrovirals, either as tablets or as gels, are safe and effective in preventing sexual transmission of HIV involving 5029 women from South Africa, Zimbabwe and Uganda. The Tenofovir tablet component of the VOICE study was discontinued after interim results showed that it was no better than placebo in preventing HIV in the study women [149]. Furthermore, VOICE study data revealed that the incidence rate of HIV infection in the women assigned to daily Tenofovir gel was 6.0% compared to 6.1% in women assigned to placebo gel [150]. Based on the unfavorable outcome, the Tenofovir gel component of the VOICE study was also discontinued while the Tenofovir/Emtricitabine (Truvada) tablet component is continuing to study completion [150]. Vaginal acquisition of HIV may require a stronger barrier to infection than that provided by oral dosing with a Tenofovir/Emtricitabine combination [151].

The major risks of daily TFV/TDF use include: (i) mitochondrial toxicity [152,153], (ii) loss of bone mineral density due to reduced phosphate absorption [154-156], (iii) renal injury due to tubular dysfunction [157,158], and (iv) development of secondary ARV resistance in treated persons [159-165]. Resistance mutation (K65R) selected by TDF confers a reduced susceptibility to TDF [160]. K65R is frequently associated with M184V mutation. Tenofovir (TDF) resistance occurs in the presence of K65R, the 69 insertion complex, or at least three thymidine analog mutations (TAMs) [159-165].

NRTIs-based microbicides: The nucleoside analogues bind to the active site of the RT enzyme and can be incorporated into the growing DNA chain. However, further elongation is not possible, as they lack the 3'-OH group normally present in the substrate. This causes premature termination of the growing viral DNA strand.

Stampidine (5'-[4-bromophenyl methoxylaninylphosphate]-2',3'-didehydro-3'-deoxythymidine) is a novel aryl phosphate derivative of stavudine with a unique mechanism of action as an epigenetic modulator of HIV infection-associated gene expression [166,167]. Stampidine was rationally designed novel prodrug of stavudine (STV)/d4T that is being developed as a promising new microbicide candidate against ARV-resistant HIV [75]. NRTI form the backbone of contemporary combination ARV therapy regimens. The 5'-triphosphates of the NRTI family, which are generated intracellularly by the action of nucleoside and nucleotide kinases, are capable of competing with the natural deoxynucleoside triphosphates for binding to the RT primer:template complex and represent the biologically active form of NRTI responsible for their anti-HIV activity [168]. The rate-limiting step for the generation of the bioactive NRTI triphosphates is the conversion of the NRTI to their monophosphate derivatives. Stampidine was developed in an attempt to overcome the dependence of the NRTI stavudine on intracellular nucleoside kinase activation [169]. Stampidine is a much more potent anti-HIV agent than STV and was active against phenotypically and/or genotypically NRTI-resistant HIV with low nanomolar to subnanomolar IC_{50} values [170-174]. The superior anti-HIV-1 activity of Stampidine was attributed to the rapid formation of its active metabolite Ala-STV-MP [169,175,176]. Cellular metabolic studies revealed that the *p*-Br group in the phenyl moiety of Stampidine contributes to its ability to undergo rapid hydrolysis yielding the key metabolite Ala-STV-MP in a thymidine kinase (TK)-independent fashion [169]. The potency of Stampidine against genotypically and phenotypically NRTI HIV-1 resistant isolates is attributed to its rapid kinetics of the generation of its active triphosphate metabolite yielding much higher inhibitor concentrations at the catalytic site sufficient to overcome the binding restrictions imposed by the NRTI resistance-associated RT mutations. As a lipophilic aryl phosphate derivative of STV, Stampidine can enter target cells easier than STV, which could also contribute to higher inhibitor concentrations at the catalytic site of HIV RT. In addition, the presence of Ala side chain may promote the binding and/or incorporation of the triphosphate metabolite of these prodrugs. Drug metabolism studies conducted in multiple animal species have provided experimental evidence that Stampidine is rapidly biotransformed to two active metabolites, Ala-STV-MP and STV with favorable pharmacokinetics [175-178].

Stampidine is a promising microbicide candidate because of it exhibits (a) remarkable subnanomolar to low nanomolar *in vitro* ARV potency against genotypically and phenotypically NRTI-resistant primary clinical HIV isolates, NNRTI-resistant HIV-1 isolates, clinical non-B subtype HIV-1 isolates (subtypes A, C, F, and G) originating from South America, Asia, and sub-Saharan Africa with resistance to

stavudine, adefovir and tenofovir, as well as recombinant HIV clones containing common patterns of RT mutations responsible for NRTI resistance such as multiple TAMs plus M184V, multiple TAMs plus T69 insertion, and Q151 complex [170-174] (b) favorable pharmacokinetics profile in mice, rats, dogs, and cats with 25 mg/kg or 50 mg/kg tolerable dose levels yielding micromolar plasma concentrations of Stampidine in mice, cats, and dogs, which are 1,000-fold higher than its *in vitro* IC_{50} value against HIV [175-180], (c) favorable, safety profile in mice, rats, dogs, and cats [177-181], (d) *in vivo* anti-retroviral activity in Hu-PBL-SCID mice as well as FIV-infected domestic cats [179,180], and (e) lacks adverse effects on human sperm functions and vaginal mucosa following prolonged exposure [182-184]. In a placebo-controlled Phase I study involving 30 therapy-naïve adult HIV-infected patients, formulated GMP-grade oral Stampidine capsules did not cause dose-limiting toxicity at single dose levels ranging from 5 to 25 mg/kg [185].

Additionally, Stampidine epigenetically modulates the host transcriptome in a unique manner which prevents HIV infection from distorting and disrupting key cellular transcriptional networks. As a new dual-function agent, Stampidine has the potential for preventing and treating HIV infection by leveraging the dependency of HIV on host HIV-dependency factors as well as the viral RT enzyme for infecting and replicating in human cells. Unlike available treatments for HIV that attempt to disrupt a specific step in the life-cycle of HIV, as a microbicide, Stampidine has the potential to completely abrogate all steps in the life cycle of HIV.

NNRTI-based microbicides: Non-nucleoside inhibitors of HIV-1 reverse transcriptase (NNRTIs) are allosteric inhibitors that indirectly interfere with the catalytic mechanism of the enzyme. NNRTIs blocks reverse transcription in cells which virus has entered but not yet established productive infection. Some NNRTIs also possess virucidal properties *in vitro*. Rationally prepared formulations of "mechanism-based" broad-spectrum anti-HIV compounds, especially the membrane permeable, "tight-binding" NNRTIs have emerged as promising anti-HIV-1 microbicide candidates due to their documented ability (unlike NRTIs) to block mucosal HIV-1 infection without a need for further metabolic activation [67,70,71,75,186-194]. The rationale for the development of "tight-binding NNRTI class of compounds as microbicides is the fact that unlike nucleoside analog RT inhibitors (NRTIs) they do not require metabolic activation to elicit antiviral activity. Unlike NRTIs, NNRTIs can directly exert their antiviral action against cell-free and cell-associated HIV-1 within the vaginal cavity [188-191]. The key criteria for an NNRTI to be an optimal microbicide include: (1) Ability to rapidly cross membrane barriers, (2) Prolonged or irreversible inhibition of HIV-1 RT activity, (3) Rapid virucidal activity without metabolic activation, (4) High genetic barrier to resistance, (5) Potent activity against drug-resistant strains, (6) Sustained antiviral activity under acidic and alkaline conditions, (7) Long-acting antiviral activity following drug removal, (8) Lack of systemic absorption that might contribute drug resistance, (9) Lack of pro-inflammatory effects, and (10) Lack of adverse effects on normal vaginal microbiome. Four NNRTIs (i.e., UC-781, TMC120, MIV-150, HI-443) are currently being developed as candidate microbicides.

UC-781, (N-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furancarbo-thioamide), is a tight binding thiocarboxanilide, originally developed as a crop protection agent. UC-781 has been shown to protect pretreated cells from subsequent infection with HIV-1 in the absence of drug. In addition, UC-781 treatment of infected cells results in release of attenuated virus [190]. The compound may have direct virucidal activity [195]. Other groups have also failed to demonstrate direct virucidal activity for UC-781 [196]. UC-781 vaginal gel potently inhibits viral replication in indicator T cells when present during viral exposure [91]. UC-781 does not prevent binding of virus to DC-SIGN-positive cells (mannose binding C-type lectin receptors) and unlikely to prevent HIV-1 capture and endosomal internalization in vivo. UC781 has a low genetic barrier to resistance with rapid emergence of one (Y181C) or two (V108I/K103T) most prevalent mutations *in vivo* [197]. This would suggest the failure of UC781 to protect against the tested NNRTI-resistant viruses. The instability of UC-781 in aqueous conditions as well as difficulties encountered combining UC-781 with Tenofovir and in alternative formulations has led to discontinuation of UC-781 development in favor of pursuing other microbicide candidates.

Dapivirine/TMC120 [4-[[4-[2,4,6-trimethylphenyl)amino]-2-pyrimidinyl]-amino]-benzonitrile)], is a substituted diarylpyrimidine derivative currently in clinical development in multiple vaginal dosage forms including gels and rings for the prevention of HIV transmission to women [197-202]. Dapivirine is a potent inhibitor of HIV-1 replication *in vitro* and *in vivo* and exhibits potent antiviral activity against multiple clades of HIV as well as both wild-type virus and strains harboring various resistance-inducing mutations [203,204]. Dapivirine is a tightly binding lipophilic NNRTI that is active against cell-free and cell-associated HIV-1 [205]. Dapivirine vaginal gels and rings have been tested in phase 1 and phase 1/2 clinical safety trials [198-202]. Phase 1 and 2 studies indicate that twice daily administration of the gel for 42 days was safe and well-tolerated. A series of mutations has been observed among Dapivirine-resistant viruses (L100I, K101E, K103N, V108I, E138K/Q, V179M/E, Y181C, and/or F227Y) [197]. An amino acid change at position 138 is indicative of the development of cross-resistance between Dapivirine and both first and second generation inhibitors of the NNRTI family of drugs [197]. E138K confers resistance to Etravirine and the most recently approved NNRTI, Rilpivirine since Etravirine and Rilpivirine due to their structural and functional relationship to Dapivirine [206-209]. E138K was the most common mutation with Dapivirine alone and with Dapivirine plus Tenofovir [210]. Thus, suboptimal concentrations of Dapivirine and Dapivirine plus Tenofovir permit the emergence of more RT mutations. The development of Y181C under sub-optimal use of a Dapivirine-containing microbicide is also a concern.

MIV-150 [(1S;2S)-N-(cis-6-fluoro-2-hydroxy-3-propionyl-phenyl) cyclopropyl]-N'-(5-cyanopyrid-2-yl)urea], a phenylethylthiazolylurea compound has been shown to inactivate free virus *in vitro* [196, 197]. MIV-150 has a higher genetic barrier than UC781 requiring a combination of at least two or three RAMs (L100I, K103N, Y181C, and/or M230L) [2]. MIV-150 showed potent activity against SHIV in monkeys and prevented infection when dosed after SHIV inoculation and showed a good profile in pre-clinical safety and toxicology [71]. However, loss of antiretroviral activity of MIV-150 was apparent when higher viral challenge dose was used for macaque mucosal efficacy studies [71]. MIV-150 lacked identifiable toxicity in mice, rats, dogs, and monkeys in the dose range studied. Notably, viral isolates from subtypes B, C, and CRF02_AG are resistant to three NNRTIs currently under development as potential microbicides. Furthermore, low-level systemic absorption observed for the three most advanced RTI-based microbicide candidates (UC781, Dapivirine, and Tenofovir) could possibly result in the development of RTI-resistant genital reservoirs. Significant systemic absorption of RTI-based microbicides could lead to suboptimal drug pressure and could potentially promote the selective transmission of RTI-resistant viruses, contributing to an already increasing public health problem in developing countries.

These findings demonstrate the importance of pursuing alternative NNRTI compounds with superior activity against both NNRTI- and NRTI-resistant isolates. Therefore, novel bifunctional inhibitors combining the functionalities of a chain-terminating NRTI and a tight binding NNRTI could bind very tightly and specifically to RT and could be effective in preventing HIV transmission.. Success and overall protection against HIV infection and/or spread may best be achieved through the combined effects of bifunctional inhibitors that exhibit different mechanisms of action.

HI-443 (*N'-[2-(2-thiophene)ethyl]-N'-[2-(5-bromopyr idyl)] thiourea*, is a rationally designed thiophene thiourea NNRTI using a computer model of the NNRTI binding pocket of RT and high resolution crystal structure information from 9 individual RT-NNRTI complexes [211-213]. HI-443 was identified through an integrated multidisciplinary effort involving structure-based drug design, molecular docking studies of HIV-1–NNRTI complexes in the NNRTI binding pocket of RT, chemical synthesis, and extensive biological evaluation [70,187,214-216]. HI-443 was designed based on changes in NNRTI binding pocket size, shape, and amino acid residues that result from clinically-observed NNRTI resistance mutations [70,187,213-215]. HI-443 is a first-in-class thiophene thiourea compound that tightly fits the NNI binding pocket of HIV RT and exhibits subnanomolar to low nanomolar activity against primary HIV isolates with multidrug resistance. HI-443 was active against clinical isolates (subtypes A, B, F, G) from diverse geographic areas at nanomolar to sub-nanomolar IC_{50} values [68,75,216,217]. HI-443 exhibited nanomolar to low micromolar IC_{50} values against genotypically and/or phenotypically NRTI/NNRTI-resistant primary HIV-1 isolates with 2–7 TAMs [68,75,217,218]. Hi-443 has been formulated in a self-emulsifying nonspermicidal gel formulation which offers rapid dispersion and enhanced solubilization of the active drug substance, lack of proinflammatory effects, and lack of systemic absorption [219]. HI-443 is capable of preventing vaginal transmission of a drug-resistant clinical HIV-1 isolate in the Hu-PBL-SCID mouse model [220]. HI-443 lacked toxicity following repeated oral, intraperitoneal, intravenous, and intravaginal administration at doses in excess of those predicted to be clinically effective – the microbicide gel formulation causes no vaginal inflammation in rabbits or pigs [184,221].

As a nonspermicidal microbicide, HI-443 exhibits (i) Potent and broad-spectrum activity against multidrug-resistant clinical non-B subtype HIV-1 isolates, (ii) Ability to prevent vaginal transmission of clinical HIV-1 isolate in the Hu-PBL-SCID mouse model, (iii) Lack of adverse effects on fertility outcome following semen pretreatment and artificial insemination, (iv) Favorable toxicity profile after repeated oral, intraperitoneal or intravenous administration in mice and rats, (v) Favorable pharmacokinetics following oral, intraperitoneal, and intravenous dosing in mice, and (vi) Favorable safety profile after repeated intravaginal dosing *via* a gel formulation in rabbits and pigs. Finding a vaginal gel that protects women against HIV but still allows them to get pregnant has long been sought by AIDS researchers, because it can be used covertly by women without having to negotiate with their partners. The discovery of HI-443 as a non-spermicidal broad-spectrum anti-HIV agent represents a significant step forward in the development of a microbicide for curbing heterosexual HIV transmission. Based on extensive preclinical data and unique mode of action, HI-443 is a superior NNRTI microbicide candidate to prevent vaginal transmission of HIV-1 and post-exposure development of systemic HIV-1 infection.

The combination of different ARV compounds in one microbicide could not only diminish the possibility of drug resistance selection but also increase the residual activity of these microbicides against preexisting drug-resistant HIV (either NRTI or NNRTI resistant) from an infected partner. A bifunctional inhibitor combining the functionalities of a chain-terminating NRTI and a tight binding NNRTI could bind very tightly and specifically to RT and could be effective in the treatment of AIDS. Success and overall protection against HIV infection and/or spread may best be achieved through the combined effects of bifunctional inhibitors that exhibit different mechanisms of action.

RNA interference (RNAi)-based microbicides

RNA interference (RNAi) is a highly conserved gene silencing mechanism that uses small noncoding RNAs (typically 21-23-nucleotides) to guide the sequence-specific inhibition of gene expression [222-224]. By mimicking endogenous small regulatory RNAs, small interfering RNAs (siRNAs) can harness the cellular RNAi machinery for the targeted silencing of gene expression. Experimental introduction of siRNAs can harness the RNAi pathway to guide the sequence specific cleavage of target mRNA. siRNAs target HIV genes, the host receptors (CD4, CXCR4, CCR5), as well as host dependency factors (HDFs) required for HIV replication in cells [225-228]. Suppression of HIV infection via RNAi-mediated silencing has been tested in tissue culture models, primary $CD4^+$ T cells and monocyte-derived macrophages, vaginal explants and humanized mouse models [229-236]. siRNA treatment silenced gene expression up to 7 days in $CD4^+$ T cells and over three weeks in terminally differentiated monocyte-derived macrophages (MDMs). The most widely used systems for delivering siRNA are liposomal nanoparticle-based delivery systems.

The incorporation of siRNAs into a microbicide is mainly focused on the CCR5 co-receptor which is essential for HIV-1 infection through all routes of transmission. CCR5 co-receptor can be successfully targeted by RNAi [231,233,235]. The use of CCR5 siRNAs alone would not be an adequate strategy for effective HIV gene therapy as they will not protect against X4 or dual tropic strains of HIV-1. Additionally, the development of safe, easy to administer, and efficient delivery systems that achieve sustained target gene silencing is of substantial clinical importance. The failure of harnessing the RNAi technology can be attributed to several limitations: (i) development of therapeutically relevant delivery of siRNAs to the appropriate target cells in sufficient quantities to efficiently silence target gene expression, (ii) inability to deliver siRNAs to the cytoplasm of target cell types important in viral pathogenesis, (iii) reducing off-target and other undesired systemic effects, (iv) identification of potent and broad spectrum siRNAs that can target diverse viral strains, (v) and lack of *in vivo* characterization of the efficacy and safety of the siRNA mediated silencing technologies [237,238]. The clinical application of RNAi has been hindered by several challenges, particularly the potential for viral escape [239,240]. For improved long-range efficacy, an ideal combinatorial vector for HIV gene therapy should incorporate anti-HIV genes targeted to both viral and cellular targets to minimize the development of escape mutants. Such strategies are currently under development.

RNA-based aptamer microbicides

Aptamers are single-stranded synthetic oligonucleotides that are selected from random sequences and then expose them to the target bits of protein to identify the tight binding RNA sequences [241,242]. Repeated rounds of the process - known as *in vitro* selection or systematic evolution of ligands by exponential enrichment (SELEX) - can yield aptamers with improved affinities for their targets [243-245]. The ability of aptomers to fold into a variety of complex, sequence-

specific tertiary conformations means enables them to bind a wide range of targets and rival antibodies in their potential diversity. A neutralizing aptamer against the HIV-1 Env gp120 [246], is currently being developed for use as a potential microbicide [247]. While RNA-based aptamers are capable of neutralizing a broad spectrum of clinical HIV-1 isolates in cell culture, they are highly susceptible to different nucleases in the vaginal/cervical or rectal milieus that are able to rapidly degrade 2'-F-modified RNA. Presence of Zn (2+) cations has been shown to have some protective activity from nucleases [247]. The use of aptameric modulators in cell culture appears straightforward, however, their *in vivo* applicability is currently limited by their instability, bioavailability, and transmembrane delivery, at least when targeting intracellular proteins.

Aptamer-siRNA-based chimeric microbicides

Although siRNAs hold promise as a new weapon for blocking mucosal HIV transmission, efficient, targeted, systemic or mucosal delivery of siRNAs *in vivo* remains a major challenge for clinical translation. Consequently, the ability of aptamers to target specific cell surface proteins are being used to deliver siRNAs to target a distinct cell type, to minimize off-target effects and unwanted side effects. Cell type-specific aptamers are being combined with siRNAs to achieve cell-specific delivery of the siRNAs for selective target mRNA knockdown [248]. Such chimeric RNAs is an alternative for *in vivo* gene knockdown [249]. Aptamer-siRNA chimeras efficiently transfect and knock down gene expression in cells bearing the surface receptor recognized by the aptamer. Fusion of an anti-HIV *tat/rev* siRNA to an aptamer directed to the surface gp120 protein on HIV-infected cells has led to cell type-specific delivery of the siRNA [250]. The antiviral activity of aptamer was enhanced by aptamer-mediated delivery of an anti–HIV-1–delivered siRNA. Chimeric-siRNAs containing an aptamer that recognizes HIV-gp120 inhibits HIV replication in already infected cells *in vitro* and *in vivo* [249-253]. Human CD4 specific chimeric siRNAs are being engineered to prevent HIV transmission [251-253]. CD4-siRNA chimeras are thought to inhibit HIV infection in 2 ways: by blocking viral entry via binding to CD4 and by RNAi knockdown of viral genes (gag and vif), host receptors (CCR5), or other host genes required for viral replication. Such polyfunctional molecules inhibit HIV infection in primary $CD4^+$ T cells and macrophages, vaginal explants and humanized mice. siRNAs targeting HIV could be delivered specifically to HIV infected cells in culture and in humanized mice using an aptamer-specific for the HIV-1 Env gp160. However, since each aptamer can only deliver a single siRNA molecule, a major limiting step for this technology is the accumulation of sufficient siRNAs in the cytoplasm of the target cells to effectively inhibit gene expression. To overcome this obstacle, lipid nanoparticles (LNPs) encapsulating ample (~4,000) siRNA molecules/particle conjugated to CD4 aptamer are being explored [254].

Thus far, most of the anti-HIV gene therapy strategies revolve around targeting the viral genome with a focus on inhibiting HIV replication. Since HIV is continuously evolving, even targeting multiple viral regions cannot safe-guard against escape mutants. Despite substantial progress, no aptamer-based siRNA delivery approach has moved to the clinic. Until now, RNA-based microbicide combinatorial approaches are being tested only in humanized mouse models that support HIV-1 replication. However, before aptamer and aptamer-siRNA chimeras can be a practical way to prevent HIV-1 transmission, extensive tests including biodistribution, pharmacokinetics, dose-response, effects on drug-resistant and latent viral infections and potential toxicity due to off-target sites is a prerequisite. Receptor (viz, CCR5) expression can be down regulated by specific siRNA treatment or the gene can be disrupted by nucleases targeting the receptor gene. However, these strategies will not protect against CXCR4 tropic HIV infection and will not be a successful treatment strategy in individuals with high viral loads of CXCR4 tropic or dual tropic viruses. These finding encourage the development of a "library" of targets and drugs that can be further tailored toward specific steps in the life cycle of HIV-1.

Coitally-dependent Delivery Systems

Gel-based anogenital microbicides

A major goal in HIV prevention strategies is to simultaneously and independently target HIV-1 virions and HIV-1-infected cells but protect uninfected target cells in the mucosal tissues of the anogenital tract. Yet, the development of a safe and effective anogenital microbicide is still in its early stages. A major concern for topical delivery is the retention time of the formulation. The effectiveness of a microbicide is dependent on the bioadhesion of the formulation and the bioavailability of the drug. Clinical trials of vaginal microbicides have generally used 5 mL of gel or less, which is considered adequate to provide vaginal protection, the rectum requires at least 3-fold greater volume to achieve the same degree of coverage as in the vagina [255,256]. A clinical trial suggested that up to 35 mL of a gel applied intrarectally before RAI would be acceptable to the majority of men [257]. Suppositories are an alternate mode of delivery of a microbicidal agent intrarectally [258,259]. Rectally administered microbicides have the potential to reach local nodes through lymphatic drainage [260]. The interior iliac lymph nodes are known to be a site of early virus replication and have common drainage of the female genital tract and rectum [142,261]. In order to achieve this, sufficient levels of the drug must remain at the target mucosal sites and draining lymph nodes to block HIV-1 infection and viral dissemination by migratory cells [262]. Therefore, prior to performing microbicide efficacy studies in humans, it is critical to determine whether levels of microbicide that can be recovered after vaginal or rectal dosing are substantially in excess of the concentrations needed to block viral replication in the absence and presence of semen. In addition, elucidating antiretroviral levels that can be recovered as a function of time post application is equally important for predicting the timing of pre-and post-coital dosing schedules.

Vaginal specific microbicides: Several dosage forms have been developed as vaginal delivery systems, such as gels, creams, films, foams, suspensions, suppositories, and tablets and all have short residence time [263]. Bioadhesive polymers such as polycarbophil, sodium carboxymethyl cellulose, polyacrylic acid polymer Carbopol® 974P are incorporated to control the rate of drug release from, and extend the residence time of vaginal formulations [264]. A significant decrease in drug release can be expected from gel formulations as the polymer concentration is increased. In addition, effective vaginal microbicide drug delivery can be limited due to the low pH and presence of proteolytic enzymes in the female genital tract [146,265]. Further, the active and inactive ingredients in microbicidal formulations should not irritate or disrupt the mucosal epithelium as evidenced by the early clinical trials of N-9, Carraguard, Savvy and cellulose sulfate [266,267]. In the past 15 years, 11 clinical trials with six candidate microbicides has led to negative or inconclusive findings despite the fact that their development path followed the guidelines and recommendations proposed for the nonclinical development of microbicide candidates [268]. The six candidate microbicides tested previously include N-9, SAVVY® (C31G), cellulose sulfate (CS), Carraguard® (PC-515), PRO 2000, and BufferGel® [15-19]. None of these proven antiretroviral

products had a protective effect against HIV in a clinical setting, with two (N-9 and CS) paradoxically showing a trend towards increased risk of HIV infection. These clinical trials revealed three essential components of a desired microbicide: undesirable local effects on epithelial integrity, inflammatory response and immune functions [269-271].

More recently, following the CAPRISA 004 study which demonstrated that pre-and post-coital use of 1% Tenofovir loaded poly(acrylic acid) (Carbopol®) and HEC polymer-based gel reduced HIV infection by 39% [148], there was high hope that the confirmatory VOICE (Vaginal and Oral Interventions to Control the Epidemic) study of daily Tenofovir gel would show similar promising results. The VOICE study was designed to test whether antiretrovirals, either as tablets or as gels, would be safe and effective in preventing sexual transmission of HIV involving 5029 women from South Africa, Zimbabwe and Uganda [149]. The VOICE study data revealed that the incidence of HIV infection in women assigned to daily Tenofovir gel was 6.0%, virtually identical to a rate of 6.1% in women assigned to a placebo gel [150]. Based on this disappointing outcome, the Tenofovir gel component of the VOICE study has been discontinued [150].

Rectal specific microbicides: Unprotected RAI has the highest per act risk of HIV acquisition with an unadjusted probability of 0.08 per contact for RAI [7] as compared to 0.001 per coital act for vaginal intercourse [8]. Furthermore, there is increasing epidemiological evidence that women as well as men in both the developed [9-14] and developing world [12-14] practice RAI. Clearly, rectal microbicides should be seen as an important HIV prevention technology for all individuals who practice RAI. The differences between the microenvironments of the rectal and vaginal mucosal tissue require that different formulations be used for the two routes [272]. The earlier failure of rectal microbicide candidates can be attributed to the use of vaginally formulated microbicide gels that failed in clinical vaginal efficacy and safety studies [271-274]. Water-based gel formulations of UC781 (0.1% and 1.0%) have been assessed for pharmacokinetic and preclinical safety screening after repeated vaginal and rectal applications in the pig-tailed macaque models [274]. A reduced safety profile for the 1.0% UC781 gel was evident when applied rectally suggesting the differential sensitivities of the vagina and rectum to topical microbicides.

Unlike the cervicovaginal tract which is composed of a pluristratified squamous epithelium, the rectal mucosa has a single-cell columnar epithelium which is extremely receptive to injury and highly vulnerable to HIV-1 infection [275,276]. It is densely populated with activated memory T-cells expressing both CD4 and co-receptors CCR5 and CXCR4, DCs and macrophages capable of transferring infectious virus to the underlying lymphoid tissue, the major site of viral replication and $CD4^+$ T-cell depletion during acute infection [277-279]. Consequently, rectal transmission of HIV-1 is thought to be up to 200-times more likely per sexual act than vaginal transmission [280]. These differences may also increase rectal compared to vaginal susceptibility to microbicide-induced toxicity, potentially favoring HIV infection as seen with other STIs [281,282]. Therefore, knowledge of HIV coreceptor tropism at cervicovaginal and rectal sites is essential to better understand the molecular biology of HIV transmission from vaginal/rectal secretions and for developing effective anogenital microbicides. Coreceptor tropism in ectocervical tissues, rectal secretions, rectal biopsies and feces is being investigated to examine differences in HIV envelope gene (env), HIV receptors/coreceptors and drug-resistance profiles between plasma, vaginal and rectal secretions.

Microbicidal agents incorporated into gels and suppositories that could be applied to the rectal mucosa before intercourse have been proposed as a prevention tool [259,283]. The rectum is 10 cm in length and has surface area 300 cm2. Surface area without villi gives it a relatively small surface area for drug absorption [284]. Use of rectal-specific microbicides would require at least 3-fold greater volume to achieve the same degree of coverage as in the vagina [285,286]. Exogenously dosed autologous lymphocytes and HIV-sized particles have been found to migrate to similar locations and associate with the colonic tissue and within the rectosigmoid colon for 24 hrs [287]. Most rectal absorption of drugs is achieved by a simple diffusion process through the lipid membrane. Hydroxypropyl-beta-cyclodextrin is one of the preferred solubility enhancer for the development of liquid suppositories for poorly water-soluble drugs [288]. More recently, there have been attempts to develop microbicides whose properties are better suited for use in the rectal compartment (i.e., iso-osmolar, self-emulsifying systems). However, the overall effectiveness of a rectal microbicide will depend on efficacy, consistency of use and acceptability [289].

Intrarectal SHIV challenge of macaques pretreated with rectal microbicide gels is used a model to study their possible effects for preventing HIV transmission by anal intercourse. Since CCR5-using viruses are frequently associated with sexual transmission of HIV in humans [290,291], a pathogenic CCR5-specific chimeric envelope SHIV, is more appropriate for testing anogenital transmission in the macaque model [292,293]. In one study, a differential effectiveness of MIV-150-carrageenan gel was observed when tested both vaginally and rectally for protection from either vaginal or rectal challenge with RT-SHIV (SIVmac239) transmission in macaques [71,294]. MIV-150 gel provided either partial or complete protection against vaginal or rectal challenge, respectively, with RT-SHIV when applied at 30 min or 4 h. However, loss of antiretroviral activity of MIV-150 gel was apparent when higher viral challenge dose was used for macaque rectal efficacy studies [294]. Moreover, a single dose of either 1% or 2% recombinant CV-N gel has been shown to protect male macaques that were rectally challenged with a chimeric SIV/HIV-1 virus (SHIV89.6P) [125]. Rectal application of Tenofovir prior to virus exposure was shown to efficiently protect against subsequent intrarectal challenge with SIVmac251/32H, a virus that result in high cell-associated and plasma viral RNA loads shortly after a single application to naïve macaques [142]. The concentration of Tenofovir detectable in the plasma 15 min after rectal application was positively associated with protection. However, animals that received Tenofovir gel 2 h after virus exposure showed partial protection. One of the rate limiting steps to 100% protective efficacy can be the local uptake of Tenofovir [295].

Vaginal gel vs. oral tablet interventions: Pre-exposure prophylaxis (PrEP) is an evolving new approach to prevention of sexually transmitted AIDS that employs ARV agents prior to potential HIV-1 exposure in an attempt to reduce the likelihood of HIV infection post exposure [296]. Current PrEP strategies in clinical development rely on two clinically approved nucleotide (NtRTI)/nucleoside (NRTI) RT inhibitors TDF and Truvada (tenofovir plus emtricitabine, TDF/FTC) [297,298]. While promising clinical results were recently reported regarding the effectiveness of PrEP as an HIV-1 prevention strategy for MSM by the iPrEx Study Team [299], effective PrEP for women remains an unmet challenge, as emphasized by recent clinical failures of TFV (VOICE) and Truvada (FEM-PrEP) based PrEP strategies to reduce HIV infections in women [300-302].

In the iPrEX (Preexposure prophylaxis initiative) trial, a daily dosage of two ARV drugs given to HIV-seronegative MSM was shown

to reduce the HIV incidence by 44% compared to the placebo-treated control group [299]. Conversely, the FEM-PrEP, a large PrEP Phase III clinical trial of TDF/FTC (Truvada) using the same once daily drug regime failed to show any protection from HIV transmission in at-risk HIV-negative women resulting in the closure of the study [300-302]. However, preliminary data from two recent trials, the Center for Disease Control (CDC) TDF2 study (daily oral TDF/FTC) and the University of Washington Partners PrEP study (daily oral TDF or TDF/FTC), showed reduced risk for HIV infection among heterosexuals on PrEP [303,304]. Notably, TFV, one of the PrEP arms of VOICE study, an NIH funded HIV prevention trial of the Microbicide Trials Network involving more than 5,000 women in Africa and evaluating oral PrEP agents TDF and Truvada (TDF/FTC), a vaginal microbicide gel formulation of TFV, and combinations thereof, has been discontinued based on the interim review of the data by the NIAID Prevention Trials DSMB demonstrating that oral TDF did not reduce HIV infection in participants receiving it [300,301]

Antiretroviral therapies are known to have significant side effects, including mitochondrial cytotoxicity, loss of bone density, lipodystrophies, aggravation of renal impairments and increased risk for liver disease and diabetes, many of which increase with the length of time spent on ARVs [154,155,158,162,305,306]. Since it is unclear how long individuals need to stay on PrEP, these side effects could reduce the high levels of compliance necessary to maintain the efficacy of these approaches [154,155,158,162]. Incomplete adherence to these drugs could promote increased viral evolution and the selection of drug resistant viral strains [160-162]. Furthermore, long-term use is limited by emergence of resistant HIV strains while on therapy as well as the alarmingly increasing frequency of *de novo* resistant HIV strains in therapy-naïve heterosexual persons [161,162]. In particular, emergence of resistance (K65R mutation) diminishes TDF binding and incorporation into viral DNA, causing significant drug resistance [161,164,165]. Tenofovir resistance occurs in the presence of K65R, the 69 insertion complex, or at least three TAMs. These findings demonstrate the urgent need for developing innovative and effective antiviral agents that have minimal side effects and provide durable protection against drug-resistant HIV transmission. The identification of new ARV agents with potent activity against multi-drug resistant HIV remains an unmet and urgent challenge in the field of PrEP.

The differential clinical effectiveness of gel-based microbicide clinical trials implies that both coitally dependent and coitally independent strategies are required to increase user acceptability and clinical effectiveness of microbicides.

Antiretroviral nano-microbicides

An alternative approach for creating multivalency is to use a scaffold, such as polymers, lipids or nanomaterials, on which multiple copies of a ligand can be presented, thereby generating a multivalent ligand [307]. Nanocarriers include liposomes, dendrimers, polymeric nanoparticles (NPs), solid lipid NPs, and metal NPs as well as nanospheres, nanocapsules, or NPs based upon the dispersion of drug within the nanocarrier. Nanocarriers especially, NPs (solid colloidal particles of approximately 10-1000 nm) are being evaluated for mucosal delivery of ARV agents to prevent HIV transmission [308-313]. The critical characteristics of a NP related to its function include size, surface charge, encapsulation efficiency, release properties, and clearance. Two types of NP-based formulations are being explored: those where the therapeutic molecules are the NPs and those with the therapeutic molecules are directly coupled (functionalized, entrapped or coated to a carrier). Nanoparticles can be superior microbicide delivery vehicles due to their ability to encapsulate and release active therapeutic compounds in the vaginal or rectal tract. NPs offer more stability to the encapsulated drug in biological fluids and against enzymatic metabolism as compared to other colloidal systems, such as liposomes or micelles Encapsulation of anti-HIV agents in NPs results in higher concentration of the drug in the cells. Development of NPs formulated from polylactide homopolymers (PLA) and poly (lactide-*co*-glycolide) (PLGA) offers an advantage for the delivery of both hydrophilic and hydrophobic drugs in a sustained manner. PLGA and PLA are the FDA approved polymers for human use. Additionally, NPs can be engineered with surface antibodies, aptamers or siRNAs to develop combination microbicides targeting both cell-free virus as well as specific mucosal cell types to prevent cell-associated HIV-1 transmission [309,314].

Surface functionalization of nanocarrier with polyethylene glycol (PEG) is used to avoid reticuloendothelial system uptake [315,316]. Although PEG modification substantially increases the diffusion rates of otherwise nearly immobile NPs in cervicovaginal mucus, it can reduce NP uptake by mucosal cells. Mucoadhesive NPs can reduce the clearance of ARV agents and ensure their prolonged retention, resulting in improved absorption of poorly absorbable drugs. Polymers such as poloxamers, pectins, chitosans, polyacrylates, and their derivatives, are being used to impart vaginal or rectal mucoadhesive properties to the NPs by surface coating [317-319]. However, the residence time of mucoadhesive NPs in the cervicovagina and rectum can be affected by mucus turnover rate, mucosal site, physiologic conditions, and the presence of irritants. The NPs with neutral surfaces are transported faster through human mucus compared with unmodified NPs. PLGA nanoparticles encapsulated with PSC-RANTES revealed a four-fold greater uptake in the vitro human ectocervical tissue [320]. However, as a potential microbcide delivery system, antiretroviral NPs must be nontoxic, nonimmunogenic, display favorable pharmacokinetics and selective in drug targeting to specific tissue sites and while not activating the complement cascade.

Gold and silver nanoparticles

Both gold (Au) and silver (Ag) NPs have received considerable attention as potential microbicides due to their activity against a wide range of HIV-1 strains *in vitro*, including laboratory strains, clinical isolates, M and T tropic strains, and resistant strains [321-323]. Au NPs serve as an efficient multivalent scaffold that significantly enhances the apparent affinity of ligands [324]. Small molecule-coated Au NPs are effective inhibitors for HIV fusion [322,325]. Multivalent Env-targeting gold (Au)-based NPs (AuNPs) exhibit direct virucidal activity specific for HIV-1 [325]. Peptide triazole AuNPs (dual receptor site gp120 antagonists) display *in vitro* antiviral activity against a broad range of HIV-1 subtypes [325]. TAK-778, a CCR5 inhibitor-derived SDC-1721 gold NPs effectively inhibited HIV-1 fusion to human T lymphocytes, while free SDC-1721 had no inhibitory activity [321]. Galactosyland glucosyl-functionalized Au NPs exhibit 300 times better binding to gp120 [322]. Carbohydrate ligands conjugated to Au NPs exhibit affinities up to five orders of magnitude higher than those of the corresponding monomeric ligands with lectins [324].

Silver NPs (AgNPs) act as viral entry inhibitors by binding to gp120 and thus preventing CD4-mediated viral membrane fusion to host cells and subsequent infectivity [326,327]. They are also found to inhibit post-entry stages of HIV-1, indicating that AgNPs act at multiple stages of the HIV life cycle. Polyvinylpyrrolidone (PVP) AgNPs mixed in a topical gel rapidly inhibit the transmission of infection when applied to the human cervical tissue in a model for explants, at a non-toxic range [323]. Consequently, combination of AgNPs with neutralizing

antibodies to viral envelope glycoprotein trimers exhibited an additive effect, increasing the inhibitory effect of AgNPs and neutralizing antibodies against cell-associated HIV-1 transmission/infection [328].

Although AgNPs and AuNPs are colloidally stable, their stability which is highly dependent on parameters such as concentration of the colloid, pH, total ionic strength or susceptibility to proteases in the genital tract need to be optimized for their usefulness at sites of microbicidal intervention. The *in vivo* safety and efficacy of gold and silver NPs remains to be demonstrated.

Polyanionic dendrimer

Dendrimers are "nanoscale" macromolecules characterized by highly-branched, well-defined, three-dimensional structures (5-20 nm) that provide a high degree of surface functionality and versatility is the active ingredient of a topical microbicide [329]. Dendrimers cannot only act as carriers of ARV agents, but can also themselves act as ARVs. Dendrimers with inherent ARV activity can be synthesized by incorporating certain functional groups on their surface that can interfere with the binding of the virus to the cell. SPL7013, the active ingredient in VivaGel (3% w/w SPL7013 in Carbopol®-based aqueous gel) is a fourth-generation polylysine dendrimer and contains a specifically designed polyanionic surface. SPL7013 is comprised of a divalent benzylhydrylamine core, four generations of L-lysine branches radiating from the core, with the outermost branches capped with 32 naphthalene disulfonic acid (DNAA) surface groups which impart hydrophobicity and a high anionic charge to the dendrimer surface [330]. Productive HIV entry is dependent on gp120 binding to CD4 and chemokine receptors. This highly charged polyanionic structure allows SPL7013 to attach to targets on viruses, blocking viral attachment and/or adsorption to cells thereby preventing infection. SPL7013 is thought to bind gp120 proteins on the surface of the virus, through which the virus normally attaches to CD4 receptors on human cells.

SPL7013 [3% (w/w)] has been formulated in a mucoadhesive Carbopol®-based aqueous gel (VivaGel®) for use as a topical vaginal microbicide [329-331]. In a macaque model, SPL7013 Gel was protective against vaginal challenge with a CXCR4 using SHIV (SHIV89.6P) in a dose-related manner and inhibited replication of the CCR5 using SHIV162P3 strain in macaque and human PBMCs [332,333]. Phase I safety studies of SPL7013 gel as a candidate vaginal microbicide has been evaluated in populations with different characteristics [334-337]. The HIV-1 inhibitory levels of SPL7013 gel in the female genital tract were retained over a 24 h period. An expanded Phase I study assessed the safety and tolerability of VivaGel® versus placebo gel in healthy women. Twice daily application for 14 days revealed genitourinary adverse events and colposcopic findings consistent with mild epithelial irritation and inflammation among women in the VivaGel arm [336]. A second Phase I study assessed the safety, adherence, acceptability, and effect on vaginal microflora of 3% SPL7013 Gel (VivaGel) and two placebo gels applied twice daily for 14 consecutive days [337]. Although, VivaGel was generally well tolerated, Exposure to VivaGel and VivaGel placebo resulted in minor shifts in the vaginal microflora with a higher incidence of low-grade related genital adverse events compared to the HEC placebo gel.

The ARV activity of sulfated oligosaccharides is very low [338]. However, sulfated oligosaccharides when attached to a dendrimer show high ARV activity due to cluster effects [339]. Anionic polymers and dendrimers through ionic interactions with the V3 loop of gp120 interfere with viral-host cell interactions [331,339,340]. SPL7013 is believed to prevent the attachment of HIV to human T-cells by binding gp-120. SPL7013 exhibits virucidal against HIV-1 strains that utilize the CXCR4 coreceptor but not viruses that solely use CCR5. SPL7013 has HIV-1 virucidal activity against X4 and R5X4 but not R5 HIV-1 strains. Also, the mode of action against X4 and R5X4 strains appears to differ from R5 strains of HIV-1 [341]. Nevertheless, in the absence of potent R5 virucidal activity, the ability of a microbicide to colocalize with HIV-1 at target cells in the lower epithelial layers and submucosa becomes more critical in the context of preventing the sexual transmission of HIV-1.

Liposomes

Liposomes consist of amphiphilic lipid molecules that self-assemble to form vesicles, encapsulating a nanoscale aqueous payload within a lipid bilayer. Liposomes can range from approximately 50 nm to μm in diameter, although diameters 100-200 nm are often desirable for drug delivery applications [342,343]. Conventional, passive or active targeted liposomes have been used to enhance the half-life and solubility of drugs and to decrease their toxicity. Liposomes can bind to the HIV-1 virus [344] and modulate HIV infectivity [345]. Liposomes have the ability to deliver drugs into cells or inside individual cellular compartments. The lipid composition of liposomal membranes can affect the rate and extent of HIV-1 fusion [346], and the infectivity of HIV-1 in cell culture [347]. Conventional or unmodified liposomes composed of phospholipids undergo rapid disintegration and only a fraction of the original formulation reaches the genital tissue and allows for intracellular uptake of the drug by direct binding to cell surface proteins. The physicochemical properties of liposomes, such as net surface charge, hydrophobicity, size, fluidity, and packing of the lipid bilayers, influence their stability and the type of proteins that bind to them [348,349]. Surface modifications either with glycolipids or hydrophilic polymers, such as polyethylene glycol (PEG) substantially prolong their half-life *in vivo* [350-352]. Effective liposomal formulations could be introduced intravaginally/intrarectally prior to coitus. A nonphospholipid liposome carrier (Novasomes 7474) provided a non-specific but robust protection against vaginal challenge in macaques with a CCR5-tropic SHIV (162P3) when compared with a synthetic chemokine (-2 RANTES), formulated Novasomes 7474 [353]. In addition, a liposomal gel loaded with NNRTI MC1220 was shown to provide partial protection against vaginal challenge of macaques with RT-SHIV [354].

Polymeric micelles composed of block copolymers have been utilized for improving aqueous solubility, membrane permeability, and site-specific delivery of several drug moieties. However, current limitations for mucosal delivery incude: (i) physico-chemical and biological stability, (ii) limited hydrophilic drug-loading capacity due to the small volume of the core (approximately 15 μL), rapid clearance rate, and (iii) slow cell penetration precludes their use for sustained drug delivery applications [355]. In addition, the complement cascade can be activated by both negatively charged and positively charged liposomes in man: negatively charged liposomes activate the complement system via the classical pathway, while positively charged liposomes activate it via the alternative pathway [356-359].

Nano (micro)-emulsions

Lipid-based systems are a promising choice for the delivery of hydrophobic molecules. These systems could be lipid solution, emulsions, microemulsions, self-emulsifying drug delivery systems (SEDDS), self-microemulsifying drug delivery systems (SMEDDS), or micellar systems. They help improve the bioavailability of hydrophobic drugs through several mechanisms, e.g., facilitation of *in vivo* dispersion

through the added surfactant, lipolysis of constituent lipids, increased lymphatic transport, etc. Micellar and microemulsion systems, being the most dispersed of all, appear the most promising.

Microemulsions are thermodynamically stable, isotropically clear dispersions of two immiscible liquids, such as oil and water, stabilized by an interfacial film of surfactant molecules [360]. The surfactant may be pure, a mixture, or combined with other additives. The role of surfactant is stabilization of the microemulsion, for instance, by decreasing the interfacial tension. The microemulsion has oil-in-water (o/w), water-in-oil (w/o), or a bicontinuous structure. Water-in-oil (W/O) microemulsions have been widely utilized for the solubilization and increased bioavailability of bioactive molecules. Polymer-based gelmicroemulsions are suitable as carriers for both water-soluble and lipo-soluble drugs [361-366]. Oil-soluble drugs can be formulated in o/w microemulsions, whereas, water-soluble drugs are better suited for w/o systems. If the microemulsion has a bicontinuous structure, the composition is suitable as carrier for both water-soluble and oil-soluble drugs. The droplet size is typically in the range of 1–100 nm. Microemulsions are superior to simple micellar solutions in terms of solubilization potential, and their thermodynamic stability offers advantages over unstable dispersions, such as emulsions and suspensions, and has a long shelf-life [367]. Drug delivery advantages offered by microemulsions include improved drug solubilization and protection against enzymatic hydrolysis, as well as the potential for enhanced absorption afforded by surfactant-induced membrane fluidity and thus permeability changes.

Microemulsions have great potential as intravaginal/rectal drug delivery vehicles for lipophilic microbicides because of their high drug solubilization capacity, increased absorption, and improved clinical potency [368]. A novel, lipophilic, submicron (30-80 nm)-particle size gel-microemulsion, GM-144, prepared from pharmaceutical excipients commonly used in topical, oral, and injectable medications, was found to exhibit potent spermicidal activity, although these excipients by themselves exhibit little or no spermicidal activity in human semen [369].

The use of microemulsions for intravaginal or intrarectal administration imposes rigorous demands on the nontoxicity of the formulation and its bioavailability. Two antiretroviral spermicides, WHI-05 [5-bromo-6-methoxy-5,6-dihydro-3'-azidothymidine-5'-(p-methoxyphenyl)-methoxyalaninyl phosphate] and WHI-07 [5-bromo-6-methoxy-5,6-dihydro-3'-azidothymidine-5'-(p-bromophenyl)-methoxyalaninyl phosphate] formulated via gel microemulsion have undergone extensive preclinical testing for intravaginal delivery as dual-function microbicides [370-376].

In animal toxicity studies performed in mice, rabbits, cats, and/or pigs, intravaginal administration of gel microemulsion with or without 2.0% WHI-05 or WHI-07 was not associated with any mucosal, systemic, developmental, and/or reproductive toxicity [74, 221,377-383]. Topical application of WHI-07 as a single agent and in combination via a nontoxic gel microemulsion was shown to block vaginal as well as rectal transmission of feline AIDS (FAIDS) by chronically FIV-infected feline T cells in the natural host model [375]. Polymer-based antiretroviral WHI-05 and WHI-07 gel-microemulsions offers several benefits for vaginal delivery, including increased solubility, protection from enzymatic hydrolysis, increased bioavailability for prolonged contraceptive and antiretroviral effects, as well as decreased toxicity.

SMEDDS (self-microemulsifying drug delivery systems) are isotropic mixtures of oil, surfactant, cosurfactant (or solubilizer), and drug. The basic principle of this system is its ability to form fine oil-in-water (O/W) microemulsions. A novel polymeric nonspermicidal SMEDDS (viz Conceival) has been developed for formulating lipophilic ARV agents for mucosal delivery [219]. Conceival greatly enhanced the solubility of poorly water-soluble anti-HIV microbicide candidates. In the rabbit model, Conceival lacked mucosal toxicity following repeated intravaginal application and did not affect *in vivo* fertility and birth outcome when administered at the time of artificial insemination. Conceival was found to be a clinically useful, safe noncontraceptive vaginal vehicle for formulating lipophilic drugs as prophylactic microbicides [184].

Coitally-Independent Delivery Systems

Intravaginal rings (IVRs)

Long-acting ARV releasing intravaginal rings (IVRs) are being developed as coitally independent strategies to improve the user compliance and acceptability as microbicides. The flexible, torus-shaped, elastomeric IVR drug delivery devices originally developed for contraceptive and hormone delivery can provide long-term, either sustained or controlled release of compounds to the vagina for either local or systemic effect [384,385]. They are designed to be self-inserted and removed, and are positioned in the upper third of the vagina, generally adjacent to the cervix [386]. IVRs are generally only suitable for microbicides with very specific physicochemical characteristics [387], such as the hydrophobic, small molecule, NRTI/NNRTIs [202,388-391]. Currently, controlled release systems for vaginal administration are mainly used for contraceptive delivery ranging from 3 weeks to 3 months [391]. IVRs made from polymers (e.g., silicone, poly (ethylene-co-vinyl acetate) (PEVA)) have good track records for hormonal contraceptives. The delivery rate from current contraceptive ring is ~120 μg/day.

Vaginal rings for prevention of HIV transmission have focused primarily on delivery of small molecule ARV compounds, whose favorable physicochemical properties such as diffusion and solubility are conducive to potentially effective release rates [387,388]. Novel vaginal ring types are in development to overcome obstacles associated with more conventional designs and construction materials, particularly the limits placed on the permeation of ARV compounds candidates through conventional vaginal rings constructed from hydrophobic silicone and PEVA. 'Sandwich' and 'core' IVRs have been developed to provide constant daily release rates, resulting in linear cumulative release versus time profiles, and conforming to zero order release kinetics [389]. The release rates can be modified by changing the thickness of the rate-controlling membrane. Recent clinical studies have reported the high user acceptability of IVRs [202,391-393].

Dapivirine (TMC120) has been extensively tested in silicone elastomer vaginal rings [202,388-390,392]. Earlier clinical studies of reservoir-type ring with different dapivirine loadings (200 mg or 25 mg) within the ring cores after 7 days revealed plasma and cervicovaginal fluid levels levels were <50 pg/mL and >1,000 fold, respectively, above the EC_{50} of the drug for both ring types [200,202]. Levels of dapivirine in tissue biopsies were similar for each ring (>1000× EC_{50}). Additionally, reservoir-type IVR containing 400 mg dapivirine was shown to provide continuous and controlled *in vitro* release over the 71-day study period with an observed daily release rate of 140μg/day. Thus, similar levels and distribution of dapivirine were obtained with the reservoir rings, independent of the drug load. In contrast, the rank order of UC781 release composed of silicone < polyurethanes < ethylene vinyl acetate copolymer IVRs [393].

The major challenges facing microbicide-releasing IVRs include drug stability, the physical stability of the drug, mechanical properties of these devices [394] and potential local toxicity to mucosal tissues. Another important consideration is the amorphous to crystalline transformation of the drug substance which can occur at high drug loading (>10 wt %). Typical processing temperatures for creating polymeric elastomeric devices range from 130 to 190°C [391,393]. Therefore, ARVs incorporated within the device need to be stable under these conditions, at least for several minutes while the drug is being compounded into the polymer melt and then processed to form the final device. The high solubility of hydrophobic drug substances in the polymer at the high melt extrusion temperature creates an amorphous dosage form that is potentially thermodynamically unstable and may undergo an amorphous to crystalline transformation. IVRs should deliver ample amounts to ARVs locally to the cervicovaginal fluid/tissues to prevent mucosal HIV infection without altering the gene expression profiles of mucosal cells or systemic absorption to avoid the development of a resistant strain [387,389]. Although dapivirine silicone IVRs were generally safe and well tolerated, it revealed high systemic drug concentrations [200,202]. Most of the toxicity associated with Tenofovir is linked to delayed mitochondrial destruction. To date, only very limited mucosal toxicity data has been generated for drug-releasing IVRs. In particular, microbicide-releasing IVRs need to be tested for safety in long-term, repeated vaginal exposure models before they progress to the clinic. Current IVRs do not meet the delivery rate requirement for less potent ARVs (e.g., RANTES analogs, monoclonal antibodies) for HIV prevention.

Lactobacilli expressing antiviral biologics

The healthy human vagina is dominated by a variety of *Lactobacillus* species which play an essential role in protecting women from genital infection [395]. Lactobacilli colonize and persist on the mucosal surfaces where HIV-1 is transmitted [396]. *L. crispatus*, together with *L. jensenii*, are the most common species in vagina and in rectum [397,398]. The gut may function as a reservoir for vaginal colonization by Lactobacilli for the maintenance of a normal vaginal microflora [398,399]. Through the production of lactic and acetic acids, hydrogen peroxide, antimicrobial substances, and other prebiotic effects, these bacteria possibly contribute to the maintenance of colonization resistance [400-403]. Imbalance in the intestinal microflora favors the suppression of lactobacilli, which in turn leads to overgrowth of anaerobes in the vagina [404]. Rectal and vaginal co-colonization with hydrogen peroxide-producing lactobacilli is associated with the lowest prevalence of bacterial vaginosis [395,404]. A loss of lactobacilli frequently leads to bacterial vaginosis or recurrent genitourinary infection [396, 401,402]. *L. crispatus* is more prevalent in the vaginal flora of fertile women [398]. In fertile women, the vaginal lactobacilli can account for up to 10^7-10^9 colony-forming units per gram of vaginal fluid [398]. The identification of vaginal lactobacilli using phenotypic methods (sugar fermentation patterns and other biochemical tests) in microbicide safety studies has limited accuracy and reliability [405]. As a result, such tests may be unable to differentiate between closely related species. The highly sensitive culture-independent genomic methods of identification of lactobacilli using multiplex polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE) and DNA sequencing as well as pyrosequencing of tagged 16S rRNA gene amplicons has allowed the accurate identification of species-specific variations contributing to vaginal and rectal microbiomes [405-408].

Recombinant lactobacilli are being tested to deliver effective levels of antiviral proteins to mucosal surfaces as an alternative to topical applications. These antiviral proteins include: (i) the first two domains of human CD4 both as a secretory protein and as a *lactobacillus*-anchored moiety to block or capture the virus, respectively [403,409], (ii) fusion inhibitory peptides derived from the gp41 transmembrane Env glycoprotein, which exhibit virus-blocking properties [410-412], (iii) MIP-1β, another CCR5-ligand chemokine [99], (iv) a single-chain variable fragment (scFv) derived from an anti-intercellular adhesion molecule 1 (ICAM-1) monoclonal antibody (MAb) to block cell-associated HIV-1 transmission [109,413], and (v) cyanovirin-N lectin displaying anti-HIV-1 activity owing to its high-affinity recognition of gp120 carbohydrate moieties [127,414].

Nanobodies - single-domain antigen-binding fragments derived from Camelid heavy chain-only antibodies have been expressed constitutively in lactobacilli [109]. The domain antibody m36 binds to a highly conserved CD4-induced (CD4i) epitope on HIV-1 gp120 and exhibits broad neutralizing activity against a number of diverse primary HIV-1 isolates that is superior to the scFv antibody m9 [109]. Preclinical studies of MucoCept, an engineered recombinant *L. jensenii* producing cyanovirin-N showed 63% efficacy ($p < 0.004$) in a non-human primate model and a reduction in viral load ($p = 0.014$) [415]. Current efforts are directed at expressing potent and broadly neutralizing single domain antibodies/nanobodies (~11-15 kDa) directed against HIV. As commensal colonizers, lactobacilli can provide prolonged delivery of the antiviral biologic they are engineered to express and secrete, thereby reducing the frequency and burden of application, hopefully increasing compliance. Engineered lactobacilli have been shown to produce sufficient levels of active antiviral biologics to potentially achieve efficacy.

The use of bacteria for drug delivery is currently in clinical trials, and thus far been shown to be safe. However, despite their reported safety, the risk of immunogenicity by transformed lactobacilli and alternations of commensal bacteria in the vagina remains a concern in the long run. Since lactobacilli can efficiently present an antigen to the immune system, mucosal administration of these genetically engineered lactobacilli has the potential to elicit both systemic and mucosal immunity. The highest immune response is usually obtained with cell-wall anchored antigens exposed to the surface of lactobacilli. Consumer acceptance of genetically engineered lactobacilli microbicide remains a very significant hurdle. The future of recombinant lactobacilli delivery strategy requires clear demonstration of the efficacy and safety in human clinical trials.

Conclusion

Sexual transmission through vaginal and rectal mucosal surfaces has been the most common route of HIV-1 spread throughout the world Microbicides are being developed as a first line of defense to block the transmission of HIV-1 via the female and male genital tracts and rectum. The challenge exists to develop packaging systems that deliver optimal concentrations of microbicides to the mucosal tissues and also allow the penetration of active compounds through the epithelial lining to reach and protect susceptible target cells. Receptive anal sex is the predominant mode of HIV acquisition among MSM, and a significant independent risk factor for HIV infection among women. The differential sensitivities of the human vagina and rectum present a significant challenge for the design of microbicides. The clinical failure of first-generation microbicide candidates has propelled the field to mechanism-based candidates that act more specifically on viral receptors, viral enzymes, and host proteins. More than 40 compounds are being tested as topical vaginal microbicides, including 12 products currently in clinical trials. CAPRISA 004 clinical trial has given proof-

of-concept that a topical microbicide applied vaginally can decrease the chances of HIV transmission. Stopping sexual HIV-1 transmission will require a broad toolkit of products that address individual needs and preferences, including long-acting microbicides and multimodal delivery systems that could improve consistent use and adherence, and ultimately enhance effectiveness, while reducing the possibility of resistance. The two most advanced microbicide dose forms are gels and rings. The clinical failure of first-generation microbicides attests to the need for new guidelines and recommendations for the development of safe and efficacious anogenital microbicides. The desired safety profile should include lack of specific target organ/systemic toxicity, epithelial integrity, inflammatory response/immune functions, greater adherence and acceptability as a result of its overall safety, which is expected to be superior to the non-specific first generation microbicides.

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