Spermicides, Microbicides and Antiviral Agents: Recent Advances in the Development of Novel Multi-Functional Compounds

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Abstract: Non-ionic surfactants have been proposed as dual action anti-viral and spermicidal agents to tackle viral infections, namely HIV. Given very promising *in vitro* results, nonoxynol-9 has been widely used. However, toxic effects were reported, paradoxically increasing the incidence of transmission of HIV/Sexually Transmitted Diseases *in vivo*. Thus, there has been a growing interest in identifying and evaluating a new generation of accessible and easy-to-use molecules with simultaneous spermicidal and microbicide action. Different biochemical compounds and mechanisms of action are currently being studied. This article reviews the diverse strategies and mechanisms of action of these novel compounds, as well the necessary systematic studies needed to evaluate their possible toxicity.

Keywords: HIV infection, sperm, contraception, spermicides, microbicides, antiretroviral agents, female reproductive tract.

INTRODUCTION

The continuous increase of infection rates by the human immunodeficiency virus (HIV) is an important worldwide health issue [1-5]. It is estimated that more than 40 million people live with acquired immunodeficiency syndrome (AIDS), and approximately 5 million are newly infected [6-7]. It is also known that the main mechanism of HIV transmission is heterosexual intercourse, and nearly half of all individuals infected are now women [3, 8].

At the same time the exponential increase of the global population growth rate is an additional cause for concern, especially in less developed regions [9-10]. Around 6.4 billion people inhabit the planet and statistical studies reveal that the total world population will reach 9 billion by 2050 [10]. Moreover, inflated population growth in developing countries is often accompanied by poverty, hunger and the spread of infectious diseases, such as sexually transmitted diseases (STDs) and AIDS [11]. This suggests that both problems, increasing rates of HIV infection and exponential world population growth, cannot be addressed separately.

If one also factors in that women are the most affected gender, it is clearly urgent to develop and implement new female-controlled methods, such as topical multi-function spermicides/microbicides, that can be widely available, and are both effective and safe [12]. This review provides a summary of the most recent compounds developed for these purposes currently involved in clinical trials and preclinical investigations, discussing the molecular mechanisms and possible targets involved.

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HIV INFECTION – AN OVERVIEW

There are numerous alerts in terms of an HIV/AIDS pandemic [13]. Furthermore, the virus has shifted to a predominantly heterosexual transmission for the majority of new infections [14]. The causal agent of AIDS has been thoroughly studied and it is well known that this retrovirus uses the host cell membrane to form a lipid bilayer envelope [15] that contains both cell-derived proteins (histocompatibility antigens- CD59; intracellular adhesion molecules- ICAM; actin and ubiquitin) and virus-encoded proteins (external glycoprotein - gp120; transmembrane glycoprotein - gp41). All the above proteins have, in one way or another, important roles for the identification, binding and fusion of the virion with target cells [16].

The exact mechanisms underlying HIV infection are still not fully understood. However, and focusing on the molecular mechanism of heterosexual HIV transmission, once the virion is in the genital tract, it can penetrate cells thanks to gp120, which binds to its target cell receptor and co-receptors, namely CD4 (primary receptor; a molecule found on the surface of CD_4^+ cells) and CCR5 and CXCR4 (chemokines). The ligand-receptor binding triggers a conformational change in gp41, allowing the insertion of its hydrophobic N-terminus into the target cell membrane, causing bilayer disruption and promoting membrane fusion between the viral envelope and the cell plasma membrane [17].

HIV can infect reproductive system tissues as both free virus or cell-associated virus. In cell-associated infection HIV binds to its receptor and co-receptor present in mucosal Langerhans cells and seminal leukocytes. The infected cells, localized in the external part of mucosa, lead to the infection of subepithelial target cells by epithelial transmigration. In the free virion infection HIV accesses the subepithelial space through epithelial breaks caused by ulcerations or abrasions. Once in the internal part of the mucosa, it attaches preferentially to immunologically-relevant dendritic cells, CD_4^+ lym-

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phocytes and macrophages [18] (Fig. **1**). Infection thus directly results in immunologic deficiency, and consequent incapability to fight opportunistic diseases [6].

It is significant that the efficiency of HIV transmission may vary depending on the morphology of the epithelium targeted. In the vagina a stratified squamous epithelium is found, lacking HIV surface receptors (only located in a subepitheliar region), rendering it somewhat more protected from possible infection. However, when we reach the endocervical junction there is a shift to single-layer columnar epithelium, a much more vulnerable region for HIV infection, not only because of reduced thickness, but also because it has primary receptors for HIV on the cell surface [19].

It is also imperative to highlight that the incidence of HIV transmission has gender-based differences; in fact, male-to-female sexual transmission is more efficient than the reverse, contributing to a vulnerability of the female sex [8]. Additionally, there is a positive correlation between the presence of other STDs and facilitated HIV dissemination [20], which leads, again, to an increase in the susceptibility of women, since STDs are more difficult to diagnose, recognize and treat in this gender [21]. If this is combined with the fact that women in developing regions often do not have the social and economic power to negotiate sexual choices, the main reasons for heightened vulnerability of women to HIV infection become clearer [22].

To address these issues a variety of topical microbicides have been developed. These molecules may act at the vaginal/cervical/rectal mucosa (as a physical barrier or enhancing protective vaginal flora, for example), at the cell or virus surface (HIV cell receptor "blockers", for example) and inclusively inside the cell, interfering with the viral replication cycle [23] (Fig. **2**). However, and usually due to non-scientific reasons related to lack of financial funds or difficult usage of the product, the rapid development of an ideal and effective agent seems always to be delayed [24].

CONTRACEPTION – AN OVERVIEW

Recent data suggest an alarming world population growth rate [10]. At the same time, even though there is an increment in the use of contraceptives worldwide, a high percentage of couples still have limited access to this technology, contributing to an increase in unwanted pregnancies [10]. More than 400.000 maternal deaths related with unwanted pregnancies were directly linked to risky abortions, especially in developing countries between 1995 and 2000 [25]. Thus, it seems necessary to develop new, cheap but efficient, contraceptive formulations that can be easily distributed [9]. As observed previously, reductions in maternal death rates (related to unsafe abortions), child mortality rates, and demographic growth rates are positively correlated with the use of contraception [26]. These results are very appealing, mainly if we consider the population growth problem in poorer regions.

Currently the available contraceptive methods can be divided into definitive (tubectomy and vasectomy), natural (safe periods, coitus interruptus), barriers (condoms, spermicides, intra-uterine devices), reproductive-endocrine interventions (steroidal contraceptives) and post-coital methods. The implementation of efficient contraception methods, such as the condom (the only method offering a dual anti-microbial and anti-viral protection), has made spermicides seem less attractive. However, their potential action against both HIV and other STD pathogens, and, especially, the possibility that they can be female-controlled, brought these compounds again to the forefront of the debate [27]. Yet, it must be noted that all spermicidal compounds must possess specific requirements to be considered effective and safe. Namely, they need to rapidly kill or immobilize sperm on

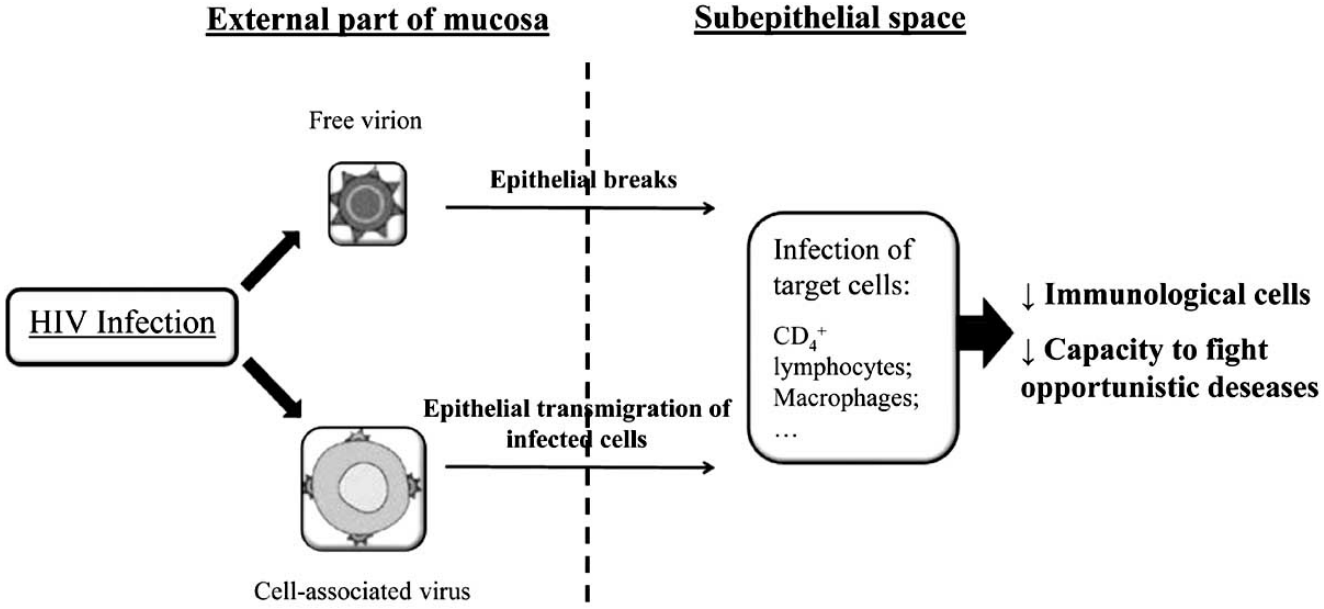

Fig. (1). Potential pathways of HIV mucosal epithelium infection. HIV can infect mucosal tissues by free virions or as cell-associated virus. The virus enters in the subepithelial region by epithelial breaks or transmigration, respectively. In the subepithelial space HIV specific target cells can be found, such as CD_4^+ lymphocytes, among others, leading to a decrease in immunological defenses.

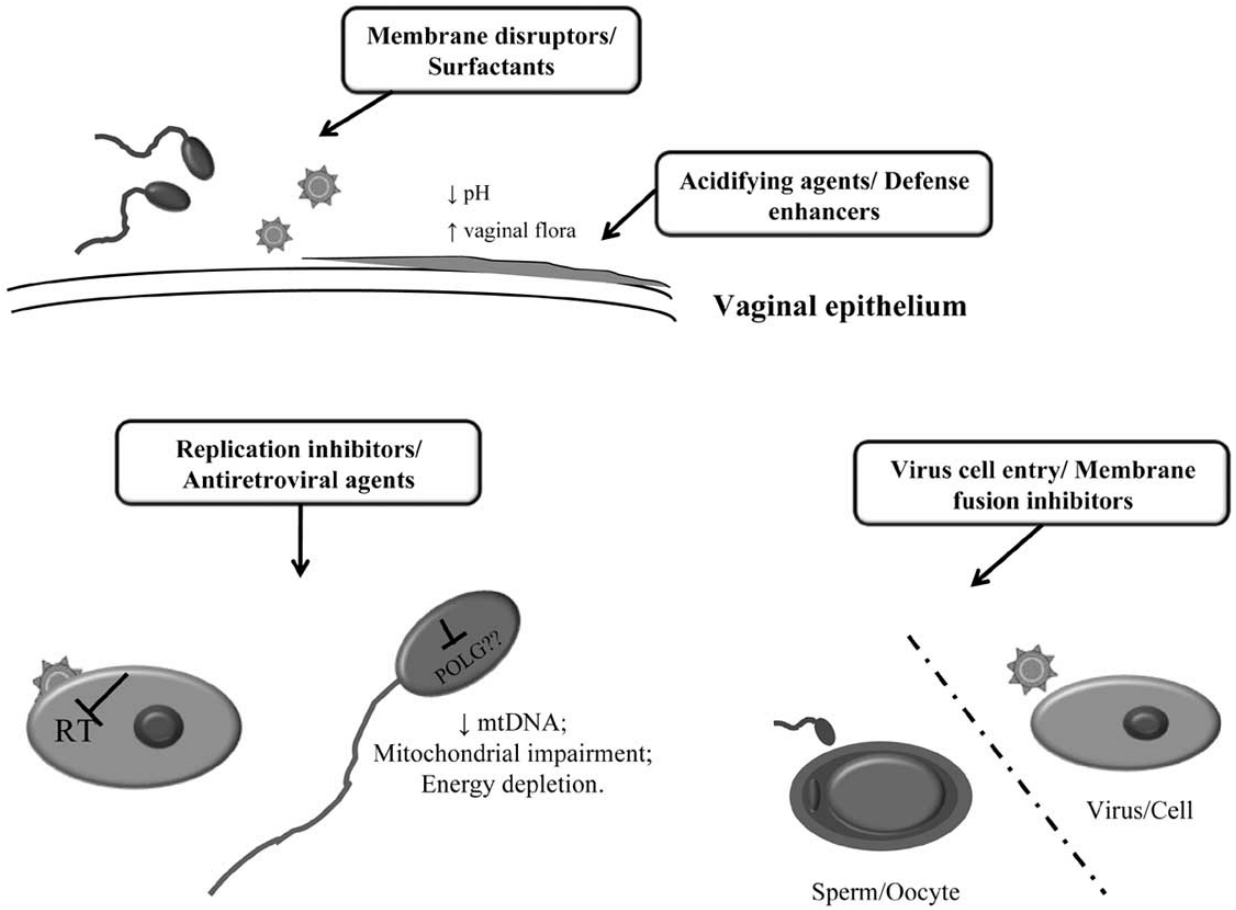

Fig. (2). Several mechanisms through which multi-function compounds may act. Surfactants disrupts the sperm and pathogen biomembranes, causing damage and inactivation. Acidifying agents enhance vaginal defenses, through the maintenance of a protective mucosal pH. Viral cell entry/Membrane fusion inhibitors block attachment and fusion of pathogens/sperm into target cells, through the interaction with important entry/fusion receptors and co-receptors. Antiretroviral agents inhibit viral DNA replication, leading to the inactivation of virus. Although it has been proven that replication inhibitors block viral RT, they can also inhibit cellular DNA polymerases, particularly POLG (DNA polymerase Gamma), which can potentially lead, in sperm, to the depletion of mtDNA, with spermicidal effects such as loss of motility (probably due to energy depletion).

contact, or otherwise render them incapable of fertilizing an oocyte. They should also have no adverse effects on a putative embryo/fetus. Finally, non-irritating properties against vaginal and penile mucosa and the long-term absence of toxicity are other important requirements [28].

Spermicidal agents have to undergo a screening process involving several types of tests. Among others one should consider the Sander-Cramer test (to examine effects on sperm motility), the hypoosmotic swelling test – HOST (to determine changes in sperm membrane integrity), the cervical mucus penetration test (to evaluate sperm penetration and survival in cervical mucus) and sperm viability tests [29-31]. Viability can be assessed essentially using two techniques: the optical microscopy-based eosin-nigrosin assay and the fluorescence-based live-dead assay using the dyes propidium iodide and SYBR-14 [32-33]. Both methods correlate very well [34-35]. It should also be noted that some compounds can affect several aspects of sperm activity and act as *de facto* spermicides, even though sperm may retain some functional properties (e.g. motility).

COMMON CELLULAR MECHANISMS AS IDEAL TARGETS FOR THE MULTI-FUNCTION COMPOUNDS

To develop compounds that would both inactivate HIV or other STD agents and, at the same time, disable sperm, there are two logical strategies: either to design safe and effective spermicides that will also interfere with pathogenic microorganisms [36]; or use well-known anti-HIV/STD drugs and test their effects on sperm function [37-38].

The strategy focused on the development of dual active compounds has in its favor the fact that, although different receptors are involved, oocyte fertilization and genital infection by STDs share the same functional and anatomical context; namely the same route of transmission and anatomical environment in terms of membrane fusion/cell penetration, thus offering the possibility of simultaneous inhibitory action [11]. Furthermore, possible common targets have been actively researched. Among them, the structure and function of both sperm and HIV membranes are extensively mentioned, since both have lipid bilayers that can be physically dis-

rupted, for example by surfactant agents through formation of mixed micelles [39-40] (Fig. **3A**). Additionally, localized membrane changes, such as those achieved by the disruption of lipid rafts, can also lead to remarkable modifications in sperm or pathogen function [41-42]. Lipid rafts are microdomains in biological membrane, enriched in cholesterol, sphingolipids and glycosylphosphatidylinositol-anchored proteins [43], and are present in both sperm and HIV. In sperm, they seem to have a role in capacitation, the acrosome reaction and sperm motility [44], and the disruption of these domains (for example, by cholesterol depletion) can lead to dramatic changes in sperm function [45]. These domains are also very important for HIV infection and replication, since virions leave the host cells through lipid rafts, and their disruption causes a drop in viral infectivity [46-47].

Membrane oxidative damage may also play a role in this process. Even though low and controlled concentrations of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-), are crucial for proper sperm function, high amounts of ROS can cause sperm pathologies, including loss of motility and viability [48]. ROS have similar results on HIV, acting as potential anti-viral agents [49].

Besides membranes, other strategic events should be considered. One of them involves molecules that contribute for cell interaction and fusion, as both sperm and STD pathogens need to recognize and fuse with a target cell to achieve their goal (fertilize/infect) [50-51]. Thus, compounds that would interact with binding receptors, such as gp120/41 and CD_4 (in HIV infection), or zona pellucida or CD_9 receptors (in fertilization), may inhibit attachment [52-53]. Although the molecular mechanisms involved in virus-cell and cell-cell fusion are still not yet well elucidated, there have been some agents developed for this purpose, such as anionic polymers [54], and some were even included in clinical trials (see **Viral cell entry/membrane fusion inhibitors**).

CURRENT CLINICAL AND PRECLINICAL COMPOUNDS UNDER EVALUATION

Over 50 agents are being evaluated for the potential development of safer topical spermicides and/or microbicides, in a variety of trials [14, 36]. All of them are included in one of the compound classes described below. Preclinical and clinical trials are absolutely essential given that it is the only way to assess compound safety and effectiveness, as well as its level of acceptance. Before the product is widely available it should pass several steps/trials.

Briefly, studies can be divided into preclinical and clinical trials, the latter including Phase I, II and III evaluations, all defined and approved (in the USA) by the Food and Drug Administration (FDA) [55]. Preclinical trials provide the basis for clinical trial testing, which takes place in humans. Phase I clinical trials test a new drug using a small group of people (20-80) to evaluate its safety, determine a safe dosage range, and identify possible side effects. In Phase II clinical trials, the effectiveness and safety of the compound is assessed in a larger group of people (100-300). In Phase III clinical trials, the drug is given to even larger groups of people (1000-3000) in order to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the product to be used safely [56]. Obviously, each of these phases is tremendously expensive, with costs increasing in value with progressing stages. It is also relevant to note that some research groups are now trying to define improved methods to determine potential injury in these trials, given past examples of inadequate safety evaluation (namely the N-9 case).

In this section, we will focus on clinical trials still in evaluation. There are nine candidates microbicides in clinical trials; however, the number of ongoing and planned/funded clinical trials is, respectively, 15 and 16 [56]. The compounds involved in ongoing clinical trials are listed in Table **1**.

COMPOUND CLASSES AND CELLULAR TARGETS OF EXISTING MULTI-FUNCTION MICROBICIDES

Even though HIV infection and sperm-oocyte fusion are still not totally understood at the molecular level, there are several compounds being studied in preclinical investigations and clinical trials [36]. Those compounds imply diverse inhibitory strategies. Topically applied microbicides, an ideal approach for the development of female-controlled devices, can be subdivided into four main groups. Each will be described in detail below, including typical compounds and putative modes of action (Fig. **3B**).

SURFACTANTS

Surface active agents (i.e. detergents) were among the first compounds to be tested as microbicides. Their mechanism of action is believed to involve membrane disruption, causing damage in the phospholipid bilayer of target cells [57-58]. This disruption probably implies changes in the electrostatic potential at the membrane surface [59].

Another a possible mechanism of action more recently proposed for this class of compounds includes a possible toxic effect in the cell cytoplasm [60]. Surfactants insert their hydrophobic regions in the lipid bilayer and may then penetrate into the cell, quickly reaching equilibrium between the inner and extracellular space [61]. Once the detergent gets into the cell it may interact with cytoplasmic components and change their activity, for example causing changes in protein synthesis or inducing cellular death through apoptotic mechanisms by activating caspases [62]. Possible targets for surfactants inside the cell include nucleic acids (evidently targeted by cationic detergents, as DNA and RNA are negatively charged polymers) [63]. Indeed, a few studies have noted surfactant-induced DNA fragmentation [64], while others have attempted to discriminate genotoxic actions (direct action on the DNA) from the extragenomic cytotoxic action (indirect effect, also eventually leading to DNA fragmentation and subsequent cell death) [65].

Despite the putative mechanism of action, this class of compounds always acts non-specifically, as surfactants can kill not only sperm and STD pathogens but also vaginal and penile epithelium cells [66]. This feature can, unfortunately, lead to significant toxicity issues, which were observed in one of the most broadly used agent of this class: Nonoxynol-9 (N-9). N-9 was the first very promising microbicide com-

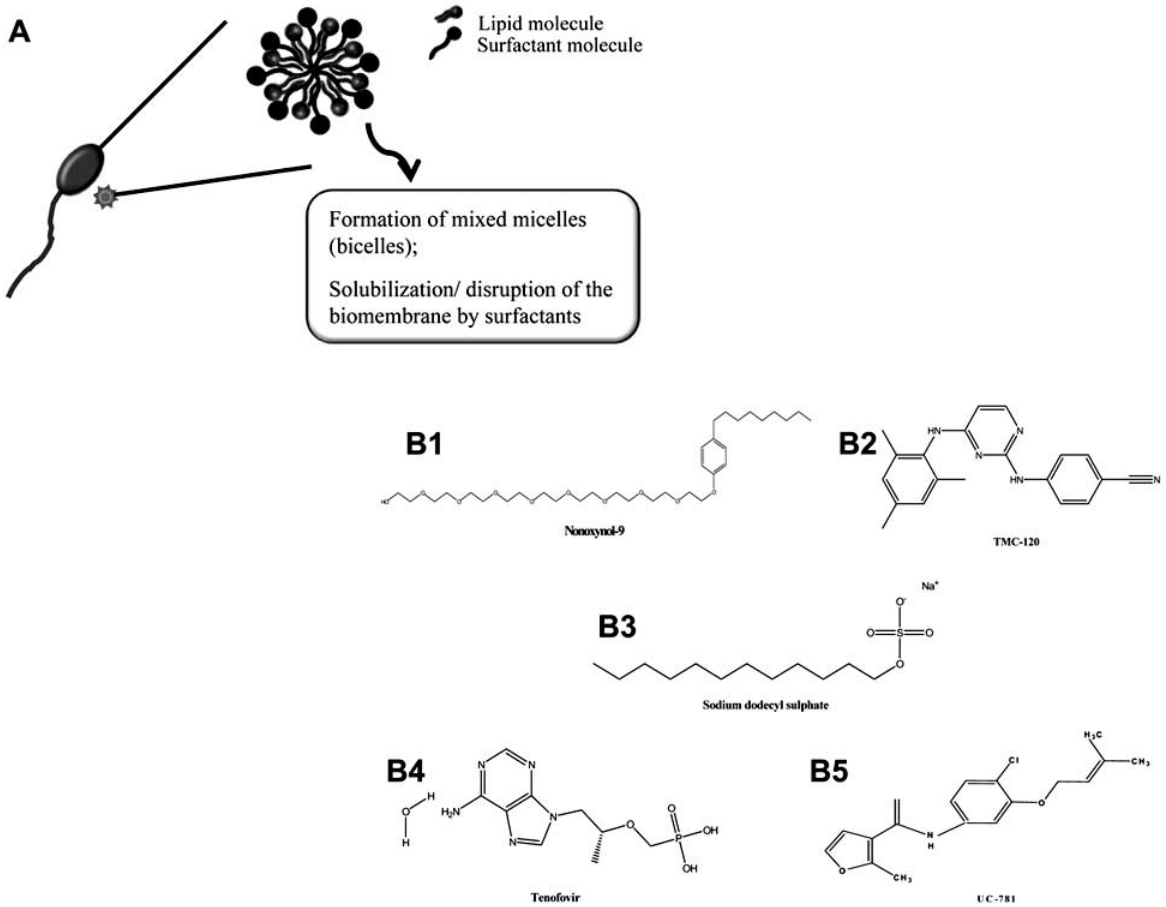

Fig. (3). A) **HIV and sperm biomembranes.** Both sperm and HIV have lipid molecules in their membranes or envelopes, which are a target for surfactants. These compunds disrupt membranes via the formation of mixed micelles, containing both surfactant and lipidic molecules. **B) Chemical structures representing some multi-function compounds.** Nonoxynol-9 (N-9) (**B1**); TMC-120 (**B2**); Sodium Dodecyl Sulphate (SDS) (**B3**); Tenofovir (**B4**); UC-781 (**B5**). See text for discussion.

pound designed to exert a "dual-action", since it has disruption properties at both sperm and HIV levels [40]. This non-ionic surfactant (an amphiphilic molecule with no charged groups in its polar head) has been used since the 1950's as a cheap, effective and widespread spermicide. N-9 has the typical mode of action of all surfactants, resulting in plasma membrane disruption [40, 57]. It also has a known broad-spectrum activity, acting through the formation of mixed micelles, causing sperm immobilization and death [67] and having similar effects on a wide variety of STD pathogens, such as HIV, *Herpes simplex*, *Neisseria gonorrhoeae* and *Treponema pallidum* [40].

Nevertheless, despite an *in vitro* reduction of HIV infectivity, this result was contradicted by *in vivo* tests. Over time N-9-induced toxic effects on the vaginal mucosa were detected with frequent use, paradoxically increasing the incidence of HIV [68]. Therefore, dose and frequency-dependent toxicity resulted in acute cervico-vaginal inflammatory responses (irritations and ulcerations), consequently increasing the risk of transmission of opportunistic diseases, due to a constant mucosal inflammation [5], leading to the recruitment of CD_4^+ and other preferred HIV target cells [5]. Taking these outcomes into account, the World Health Organization (WHO) ruled that the use of this substance by people with high risk of HIV infection should be discouraged [69].

Although N-9 was the most touted microbicide surfactant, there are some other compounds in this class that deserve attention. Molecules such as benzalkonium chloride [70], sodium lauryl sulfate [71-72], C31G [72], acylcarnitine analogues [73] and saponins [74] also showed efficacy in terms of promoting sperm death and inactivation of HIV and STD pathogens. Benzalkonium chloride, a known spermicide, is efficient against HIV and other STD infections, as was revealed by both *in vitro* and *in vivo* studies using animal models [75-76]. Sodium lauryl sulfate, also called sodium dodecyl sulphate (SDS) or *Invisible Condom* (Université Laval, Quebec, Canada), is an anionic surfactant used as a thermoreversible gel, which means that the SDS solution becomes a gel at body temperature, providing a physical and chemical barrier [12]. This compound was shown to block HIV and other sexually transmitted diseases [71, 77], and positive results were also shown in clinical trials (Trottier *et al.*, 2002[1];

[1] Trottier, S.; Omar, R.F.; Desormeaux, A.; Drouin, J.; Gagnon, M.T.; Vezina, F.; Guilbert, E.; Mâsse, B.; Bergeron, M.G. Phase I clinical trial to evaluate the safety, tolerance and acceptability of the invisible condom when applied intravaginally to healthy female subjects. *XIV International AIDS Conference*, Barcelona, Spain, **2002**. Abstract LbPp2212.

Table 1. Microbicides in the Pipeline (in Clinical Trials)

Product	Study Sponsor*	Mechanism of Action	Phase	Trial Countries	Effects Shown
PRO2000	DFID, Indevus, MRC	Fusion/attachment inhibitor	Ongoing III	South Africa, Tanzania, Uganda, Zambia	Microbicide (HIV, HSV, *N.gonorrhoeae*, *C. trachomatis*); Spermicide
Tenofovir gel	CAPRISA, CONRAD, Gilead, FHI, South African Dept. of Science and Technology	Replication inhibitor	Ongoing IIB	South Africa	Microbicide (HIV)
VivaGel	NIAID, NIH, Starpharma	Fusion/attachment inhibitor (dendrimer)	Ongoing I/II	Australia	Microbicide (HIV, HSV, HPV); Spermicide
Dapivirine (TMC-120) gel	IPM	Replication inhibitor	Ongoing I	Belgium	Microbicide (HIV, HSV, HPV, *L. crispatus*, *N. gonorrhoeae*)
Ethanol in emmolient gel	NIAID	Surfactant	Ongoing I	Kenya	----
UC-781	NIAID, CONRAD, Thailand Ministry of Health, CDC, UCLA	Replication inhibitor	Ongoing I	USA, Thailand	Microbicide (HIV)
BufferGel	CDC, CONRAD, NIAID/NIH, USAID	Acidifying agent/Defense enhancer	Planned III	Madagascar	Microbicide (HIV, HSV, HPV, C. trachomatis, *N. gonorrhoeae*); Spermicide
Tenofovir	MRC/UVRI	Replication inhibitor	Planned III	Mozambique, South Africa, Tanzania, Uganda, Zambia	Microbicide (HIV)
Invisible Condom	----	Surfactant	Planned II/III	----	Microbicide (HIV, HSV, HPV, C. trachomatis, *N. gonorrhoeae*); Spermicide
CAP	New York Blood Center, NIAID	Fusion/attachment inhibitor	Planned I	United Kingdom	Microbicide (HIV, HSV, C. trachomatis, *N. gonorrhoeae*); Spermicide
PC-815	----	Replication inhibitor	Planned I	----	----

Source: Alliance for Microbicide Development website (www.microbicide.org), updated March 2009.
*DFID, Department for International Development; MRC, Medical Research Council; CAPRISA, Centre for the AIDS Programme of Research in South Africa; CONRAD, Contraceptive Research and Development; FHI, Family Health International; NIAID, National Institute of Allergy and Infectious Diseases; NIH, National Institutes of Health; IPM, International Partnership for Microbicides; CDC, Centers for Disease Control; UCLA, University of California, Los Angeles; USAID, United States Agency for International Development; UVRI, Uganda Virus Research Institute.

Omar, 2008[2]). Finally, the multi-function microbicide C31G or Savvy (Cellegy Pharmaceuticals, Quakertown, PA, USA), which consists in an equimolar mixture of two detergents (cetyl betaine and myristamine oxide), has also shown activity against sperm [78], HIV and other pathogenic agents [72, 79]. Some clinical trials were already performed [78], but a phase III, double-blind, randomized, placebo-controlled trial in Nigeria was recently rejected, since Savvy did not reduce the incidence of HIV infection [80].

[2] Omar, R.F. Phase I/II randomized, double-blinded, placebo controlled trials on the safety, tolerance and acceptability of the invisible condom, a physical and chemical barrier vaginal gel against HIV, in healthy volunteers in Cameroon. *Microbicides 2008 Conference*, New Dehli, India, **2008**. Abstract B01-86.

ACID-BUFFERING AGENTS

The non-specific mechanism of action for this class of compounds, also called vaginal defense enhancers, involves the maintenance of a protective vaginal pH, contributing to the development of a normal microflora [24]. Vaginal pH is usually acidic (3.5-4.5), due to the excreted products of commensal Lactobacilli, and HIV negatively affected by acidic environments. On the other hand, the ejaculate is a strong alkalinizer, neutralizing vaginal pH, and possibly leading to an increase in HIV infection and facilitated vaginal colonization by other opportunistic pathogens [81]. Thus, compounds that act by regulating vaginal pH may represent another possible simple strategy.

One such agent is BufferGel (ReProtect, Baltimore, MD, USA), made of the polymer carbopol, which acts by lowering the pH in the vaginal flora. This compound revealed spermicidal properties in terms of sperm immobilization and membrane damage, as well as antimicrobicidal activity against HIV, herpes simplex virus (HSV), human papilloma virus (HPV) and *C.trachomatis*, among others. These effects were obtained without damaging the reproductive epithelium or vaginal microflora [82-83]. AcidForm (Instead, Dallas, TX, USA), already approved as a sexual lubricant gel, was recently redirected as a potential multi-function microbicide due to its acid-buffering capabilities. The mixture of buffer salts and humectants was recently tested in a clinical setting, given that it is known to have anti-sperm activity and to also affect a range of STD pathogens (Cosgrove-Sweeney *et al.*, 2004[3]). However the results weren't very encouraging [84-85].

A more "probiotic" approach is also being considered. As mentioned above, a healthy vaginal microflora is a key element for protection and, consequently, for avoiding opportunistic infections. Some studies using N-9 revealed that this surfactant strongly attacks a range of Lactobacilli species, the protective organisms of the vaginal mucosa [74]. *Lactobacillus acidophilus* is particularly important in maintaining the acidic vaginal pH due to lactic acid production, and its destruction by N-9 leads to an increased vaginal pH, allowing colonization by STD pathogenic agents [74]. These studies, as well as others [49], revealed the importance of maintaining a correct genital pH. Based on this data new compounds have been developed, basically composed by commensal Lactobacilli that enhance the protective effect of the vaginal microflora. In a pilot trial involving nine women it was demonstrated that the treatment of the vaginal flora with these "living" compounds (in this case, *Lactobacillus crispatus*) resulted in 60% colonization rates [86]. Other authors are also trying to bioengineer lactobacilli so they will secrete HIV inhibitory proteins [87].

Lastly, the use of "household" substances should not be discarded [23]. Indeed, natural compounds, such as lemon/lime juice and vinegar, have been used for a long time in certain societies, and some tests have monitored their possible microbicide capacities. Unfortunately, recent clinical trials revealed that at concentrations effective against HIV lime juice is hazardous to the genital mucosa (Lackman-Smith *et al.*, 2006[4]) [88].

VIRAL CELL ENTRY/MEMBRANE FUSION INHIBITORS

Until now we have addressed non-specific microbicide strategies. We will now focus on viral entry inhibitors, thought to have a more specific mechanism of action. These candidate compounds can block pathogen attachment, membrane fusion or entry of virus into target cells. They can also have spermicidal effects, through the inhibition of sperm-oocyte attachment and fusion. Some examples of potential compounds are anionic polymers, CCR5 inhibitors and fusion inhibitors [66].

Anionic polymers are believed to interfere with CD_4-gp120 interaction, preventing host cell co-receptor activation [89]. The existing compounds include cellulose sulphate (CS), carrageenan or carraguard, PRO2000 (naphthalene sulphonate) and cellulose acetate phthalate (CAP). CS (Ushercell, Polydex, Toronto, Canada) binds to positively charged regions of gp120 and has shown *in vitro* activity against several STD pathogens [54, 90] as well as contraceptive effects [54, 91]. Although phase I safety data were very positive [92], two recent phase III clinical trials were halted in 2007, since the results suggested an increase in the risk of HIV transmission [93-94]. Carrageenan (Carraguard/R515, Population Council, NY, USA), a sulfated polysaccharide derived from the red seaweed *Gigartina skottsbergii*, besides preventing HIV transmission by binding to the viral envelope, also blocks the migration of HIV-infected macrophages from the vaginal epithelium to the lymph nodes [95], providing another valuable mechanism of action. It has important activity against HSV, HPV, *N. gonorrhoeae*, but not against sperm [96]. Several safety clinical trials on phase I, II and III have been carried out in South Africa and Thailand, with positive results (Johansson, 2008[5]) [97-98]. PRO2000 (Indevus Pharmaceuticals Inc., Lexington, MA, USA) is a synthetic ionic naphthalene sulfonate polymer that covers the vaginal epithelium and behaves as a fusion inhibitor, interfering with gp120-CD_4 binding. This compound showed *in vitro* activity against HIV, HSV, *N. gonorrhoeae* and *C. trachomatis* [99]. Phase I clinical results demonstrated that PRO2000 was well tolerated [100] except when the microbicide concentration was too high, leading to intermenstrual bleeding [101]. Further clinical research on safety and tolerability is still underway. Lastly, CAP, an anionic polymer belonging to the polycarboxylate group, blocks HSV and cell-free or cell-associated HIV infection [12, 102]. Preliminary data detected no inflammatory processes in cell culture systems or in a macaque model [103-104], which supports its use in human clinical trials. However, a recent Phase I trial

[3] Cosgrove-Sweeney, Y.; Butler, K.; Peterson, A.; Patton, D. Preclinical safety evaluation of Acidform in the macaque model. *Microbicides 2004 Conference*, London, England, **2004**. Abstract 02343-3.

[4] Lackman-Smith, C.; Snyder, B.A.; Luckenbaugh, K.A. *In vitro* assessment of efficacy and cytotoxicity of natural substances proposed as vaginal microbicides against sexual transmission of HIV. *13th Conference on Retroviruses and Opportunitic Infections*, Denver, USA, **2006**.

[5] Johansson, E. Results of phase III Carraguard trial. *Microbicides 2008 Conference*, New Delhi, India, **2008**.

was halted because of the occurrence of side-effects related to the hyperosmolarity of the formulation (Lacey, 2008[6]).

CCR5 inhibitors are other possible strategies to fight STD pathogens. The most famous drug in this group is PSC-RANTES, a synthetic CCR5 antagonist. Previous studies noticed that RANTES, a natural CCR5 ligand, was efficient in blocking HIV infection [105] and also that individuals with a polymorphism in the CCR5 HIV co-receptor have a slower progression of AIDS [106]. PSC-RANTES binds to CCR5 and blocks viral infection, interfering with the attachment of the virus to host cells, as shown primarily in animal models. In these studies a high and non-toxic protection against SHIV, a chimeric simian/human immunodeficiency virus was observed in rhesus macaques [107]. By inducing CCR5 internalization, these molecules were shown to be effective against all HIV clades and to block HIV infection of Langerhans cells in the vaginal epithelium as well [108-109].

Other promising molecules target the viral envelope. The most representative product of the group is Cyanovirin-N (CV-N), a carbohydrate-binding agent obtained and purified from Cyanobacteria. This compound binds to the numerous high-mannose glycans in the HIV gp120, causing deletions in those sites and, consequently, preventing viru-cells fusion [110]. CN-V efficiency was shown in experiments in monkeys, where the SHIV infection was prevented with an intravaginal or rectal topical use [111-112]. Some new bioengineering ideas, such as CN-V expression in other organisms (lactobacilli or plants), are being developed (Xu, 2008[7]) [113], and some formulations were already selected for clinical trials (Barnhart *et al*., 2006[8]).

REPLICATION INHIBITORS

Once inside the cell entry inhibitors can no longer block pathogens, and an appropriate strategy in this situation may be the inhibition of viral replication. Some therapeutic agents for chronic HIV infection have been recently exploited as potential multi-function microbicides, for HIV and unwanted pregnancy prevention. These antiretroviral drugs are considered highly specific due to the inhibition of viral reverse transcriptase (RT) activity, but are also known to negatively affect sperm and other STD pathogens [38]. The major disadvantage is the potential emergence of antiretroviral resistance [19].

The antiretroviral drugs being developed and redirected to multi-function action can be subdivided into nucleotide reverse transcriptase inhibitors (NRTI), such as Tenofovir; and non-nucleoside reverse transcriptase inhibitors (NNRTI), such as TMC-120 or UC-781. The first one to be evaluated as a microbicide, Tenofovir (PMPA, Gilead Sciences Inc.), an adenosine nucleoside monophosphate (nucleotide) analogue, was shown to be easily activated and to have a high resistance barrier (Wainberg, 2004[9]). This compound is activated via phosphorylation by intracellular nucleoside kinases, the activity of which is dependent on the cell status [114]. Current studies revealed that Tenofovir is efficient and safe in a variety of concentrations, both in non-human primates and in humans (Shattock, 2006[10]) [115]. Phase I and II clinical trials found a Tenofovir vaginal gel to be safe, well tolerated, acceptable to participants and without the ability to induce resistance mutations, at least for the time being (Hillier, 2008[11]; Schwartz *et al*., 2008[12]). Further clinical studies are being carried out.

In contrast to NRTIs, NNRTI compounds do not need to be metabolized for conversion into an active form, which is a crucial advantage. There are two major molecules designed as topical microbicides, TMC-120 (International Partnership for Microbicides) and UC-781 (CONRAD). TMC-120, a diarylpyrimidine with high activity against wild type and mutant HIV, showed promising results in a new mouse model [116]. Some Phase I and II trials are ongoing, and formulations such as slow drug release vaginal rings were assessed [117]. UC-781 is a thiocarboxanilide non-nucleoside HIV RT inhibitor that also produced promising data, given that the drug seems to be efficient in blocking cell-free and cell-associated HIV transmission and also in preventing dissemination by migratory cells [118-119]. A recent Phase I trial showed safety and efficiency six days following a one-dose exposure [120]. It was also shown that synergistic inhibition occurs when this compound is combined with CAP [121].

CONCLUSIONS/FUTURE DIRECTIONS

STDs, and particularly HIV, are among the major causes of mortality, especially in less developed countries. Furthermore, there is an imperative need for safe contraception that can be controlled by women (the most affected gender), mainly by those who do not have the economic or social power to influence their partners. Therefore, an appropriate strategy may be to develop dual-function partner-independent topical microbicides, simultaneously acting against STD pathogens and unwanted pregnancy. These types of compounds are, at the present time, biologically plausible, as shown by the several trials currently underway. However multiple challenges still remain.

Microbicide research is experiencing a stage of exponential progress. Yet, some critical steps need to be streamlined. One of them involves products used independently of the coital act, and replication inhibitors show great promise in this regard. Moreover, the development of vaginal microbicide delivery systems, such as gels, tablets or rings (the slowest delivery system described so far) will reduce the need for current several-times-a-day administration [122].

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The combination of prevention strategies is another issue to be taken into account. Combining agents with different mechanisms of action may be the best approach for the success of multi-function microbicides. Undoubtedly, the emerging reality is that no single method will be able to stop the spread of STDs on its own, given that pathogen transmission has redundant mechanisms. Therefore it is important to provide several lines of defense to guarantee effective protection [66].

The evaluation of microbicide citotoxicity is still one of the most critical issues. Given the N-9 case researchers have changed their views regarding the potential harm of a candidate compound. When N-9 was established as a microbicide, little or no *in vitro* or animal testing preceded its extensive *in vivo* use by women [40]. Currently it is accepted that this compound should not have progressed further than early *in vitro* evaluation, and new products need to pass through many *in vitro* tests and animal models (rabbit, rat, primates) before they are approved for human studies. These tests involve a variety of parameters, such as the presence of cervicovaginal pro-inflammatory molecules [5]. It will also be important to assess whether relevant biological fluids (namely seminal plasma and cervico-vaginal secretions) may influence, or be affected by, the activity of candidate microbicides [123]. It should be emphasized that drug doses required for protection against virus infection in animal models exceeded by far the concentrations sufficient for blocking virus replication *in vitro* [124]. Indeed, the activity of several candidate microbicides (mainly anionic polymers) was diminished in the presence of seminal plasma *in vitro*, possibly due to electrostatic interactions [125]. This suggests the need for more refined *in vitro* assays, more closely mimicking physiological conditions by taking biological fluids into account [123]. By including all those factors new *in vitro* tests will be more effective in detecting toxicity problems, prior to evaluation in animal models or clinical trials.

Still, we hope that, in the future, *in vitro* tests may be more rationally employed than they are now. Although animal models and pre-clinical studies offer more complete experimental conditions, *in vitro* experiments have the advantage of convenience, flexibility, speed and low cost. Easily systematized *in vitro* methodologies should be seen as a required pre-condition before more advanced tests, since they seem to provide crucial clues on the applicability and safety of a variety of compounds. Thus, it would be possible to perform sorting/screening of a large variety of molecules to decide which ones should continue to more complex, lengthy, stringent, ethically challenging and expensive experimental models [126].

In conclusion, the current lack of a compound that meets the full requirements described in this review should stimulate further efforts. New safer, effective and well-tolerated alternatives are constantly being mentioned. Several original products are in the development pipeline and hopefully, if financial resources allow, the recent setbacks will impel us to the required improvement and innovation, in order to invert the current state of affairs in terms of both STD and unwanted pregnancy pandemics.

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