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MULTI-AUTHOR REVIEW

Retrocyclins and their activity against HIV-1

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Abstract Primate theta-defensins are physically distinguished as the only known fully-cyclic peptides of animal origin. Humans do not produce theta-defensin peptides due to a premature stop codon present in the signal sequence of all six theta-defensin pseudogenes. Instead, since the putative coding regions of human theta-defensin pseudogenes have remained remarkably intact, their corresponding peptides, called "retrocyclins", have been recreated using solid-phase synthetic approaches. Retrocyclins exhibit an exceptional therapeutic index both as inhibitors of HIV-1 entry and as bactericidal agents, which makes retrocyclins promising candidates for further development as topical microbicides to prevent sexually transmitted diseases. This review presents the evolution, antiretroviral mechanism of action, and potential clinical applications of retrocyclins to prevent sexual transmission of HIV-1.

Keywords Retrocyclin · Defensin · HIV-1 · Host defense peptide · Antimicrobial peptide · Antiviral · Microbicide

Abbreviations

HDP	Host-defense peptide
RC	Retrocyclin
RTD	Rhesus theta-defensin
CXCR4	CXC chemokine receptor 4
CCR5	CC chemokine 5
HNP	Human neutrophil peptide
RTI	Reverse transcriptase inhibitor

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Introduction

Defensins are one of the most widely studied families of mammalian host-defense peptides (HDPs). They are similar to other HDPs by their cationic charge and amphipathic nature, but are structurally distinguished by the presence of six cysteines and beta-sheet structures [1]. Defensins are divided into alpha-, beta-, and theta- subfamilies with lengths of 29–35, 35–45, or 18 amino acid residues, respectively. In addition to their native antimicrobial activity against a wide spectrum of microbes and viruses, defensins have also been ascribed various other roles in host defense including the ability to affect cell functions through direct interaction with the cell membrane and/or its receptors. In this way, defensins modify immune cell activities such as cytokine release, chemotaxis, antigen presentation, wound healing and angiogenesis in order to repair epithelial damage and prevent spread of infection [2]. Alpha- and beta-defensins have been extensively reviewed [3–5], and are thus not detailed herein. Instead, this chapter is centered on the theta-defensin subfamily of defensins, with particular focus on the retrocyclin class of human theta-defensins and their potential as prophylactics or therapeutics to prevent or treat viral and microbial infections.

Theta-defensins were first discovered from the leukocytes of rhesus macaques as the macrocyclic product of a posttranslational head-to-tail ligation of two truncated alpha-defensins, and are the only known circular peptides of mammalian origin (Fig. 1; [6]). Since rhesus macaques

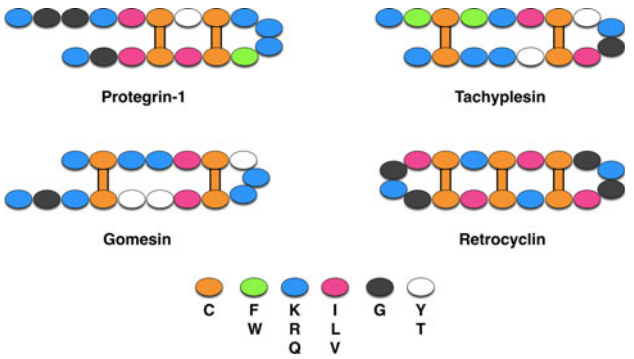

Fig. 1 Structural similarities of retrocyclin-like antimicrobial peptides across species. Retrocyclin was originally isolated when searching for other small molecules resembling protegrin-1, which was isolated from porcine leukocytes and possessed exceptional antibacterial activity. Other similar small HDPs include tachyplesin and gomesin, which were found in arthropods (tarantula spiders and horseshoe crabs, respectively)

express two different theta-defensin genes, there are three possible theta-defensins that can be formed: homodimeric products from two identical copies of either one or the other gene, or a heterodimeric product from one copy of each gene [7, 8]. While alpha- and beta-defensin peptides have been isolated and characterized from most mammals tested to date, the production of theta-defensin peptides is restricted to selected non-human primates [9]. Human theta-defensin genes are transcribed; however, a premature termination codon in the signal sequence precludes their translation and to date theta-defensin peptides have not been reported to occur naturally in human cells or tissues [10]. Since the human and rhesus macaque theta-defensin genes retain nearly 90% identity at the nucleotide level, and the coding region remained intact in the human genes, human theta-defensin peptides were recreated using synthetic approaches [11]. This class of theta-defensin peptides has been termed "retrocyclins"—"retro-" to signify their evolutionary past, and "-cyclin" to denote the circular nature of these peptide.

Retrocyclins and other theta-defensins share several attributes typical of many HDPs, including a net positive charge, an amphipathic structure, and broad spectrum activity against Gram-positive and Gram-negative bacteria, viruses, and fungi [11]. Micromolar concentrations of theta-defensins were shown to exert bactericidal activity against Gram-positive bacteria (*Listeria monocytogenes, Staphylococcus aureus*), Gram-negative bacteria (*E. coli*, *Salmonella typhimurium*), and fungi (*Candida albicans, Cryptococcus neoformans*) [6, 11]. Given their macrocyclic nature, retrocyclins are resistant to digestion by exopeptidases, which likely contributes to their increased physiological stability in biological fluids and tissues [12, 13]. In the sections that follow, we discuss diverse peptides that resemble the structure and function of retrocyclins, the evolution of retrocyclins and other theta-defensins, the mechanism of action of retrocyclins as viral entry inhibitors with emphasis on HIV-1, and future clinical applications of retrocyclins as preventatives to limit HIV-1 transmission.

Diverse peptides that resemble retrocyclins

Other small hairpin peptides of 17–18 amino acids from evolutionarily diverse species share remarkable structural and functional similarity to retrocyclins. Three such peptides are gomesins, protegrins and tachyplesins/polyphemusins, which were isolated from spider hemocytes [14], porcine leukocytes [15], and horseshoe crab hemocytes [16], respectively. Gomesins consist of 18 amino acids with two internal disulfide bridges resulting in a beta-hairpin structure essential for optimal function. Studies have shown that gomesins exhibit activity against fungi, bacteria, and protozoan parasites (e.g., *P. falciparum*) as well as antitumor activity [17–19]. Protegrins are particularly known for their potent broad-spectrum antimicrobial activity that targets Gram-positive and Gram-negative bacteria, filamentous fungi, and yeast cells, as well as specific viruses such as HIV-1 [1, 20]. Tachyplesins are 17–18 amino acid peptides with a C-terminal alpha-amide group that forms a rigid 2-stranded anti-parallel beta-sheet structure connected by a beta-turn. These peptides are potently active against Gram-positive and Gram-negative bacteria, and fungi. Natural tachyplesins exhibit modest anti-HIV activity against X4 tropic viruses (HIV-1 that utilizes CXCR4 as a co-receptor for entry), but not R5 tropic viruses (CCR5 coreceptor). However, these HDPs have undesirable hemolytic activity against human erythrocytes and show more pronounced activity at high salt concentrations more typical of the sea water living conditions of horseshoe crabs. Manipulation of the tachyplesin-1 backbone via production of cyclic end-to-end peptides reduces the hemolytic activity while retaining a significant amount of antibacterial activity [1, 21]. Synthetic tachyplesins (e.g., T22) mimic portions of the HIV-1 protein gp41, can bind gp120 with relatively high affinity, and are quite active in preventing X4 tropic HIV-1 infection of T lymphocytes [22–26]. A comparison of the structural and functional similarities of retrocyclins to other vertebrate and invertebrate HDPs is provided in Fig. 1 and Table 1.

Only a small subset of HDPs are cyclic by nature, with the most diverse group of cyclic peptides, cyclotides, being produced by plants. Cyclotides were first identified and isolated from the Violaceae (violet), Rubiaceae (coffee) and Cucurbitaceae (cucurbit) families, but may also be present in other plant species [27]. Cyclotides are small ($\sim$30 amino acid) proteins that have three interlocking

Table 1 Structural and functional similarities of small host-defense peptides (HDPs) in animals

Peptide	Origin/isolated from	Structure	Function/activity	References[a]
Cyclotides	Plant-derived (violet, coffee and cucurbit families)	30 amino acids; head-to-tail cyclized backboned stabilized by 3 disulfide bonds with cystine knot motif	Anti-tumor activity Uterotonic, anti-HIV, antimicrobial, and insecticidal activities	[30, 76]
Gomesins	Spider hemocytes (*Acanthoscurria gomesiana*)	18-residue cysteine-rich HDP; beta-hairpin stabilized by 2 disulfide bridges	Activity against fungus, bacteria and yeast Activity against *P. falciparium* Antitumor activity	[14, 17, 18]
Tachyplesins and polyphemusins	Horseshoe crab hemocytes (*Tachypleus tridentatus* and *Limulus polyphemus*)	17 residue cysteine-rich HDP; beta-hairpin stabilized by 2 disulfide bridges	Antibacterial activity, including mutiresistant *P. aeruginosa* Anti- HIV-1 activity Anti-tumor activity	[16, 77–80]
Protegrins	Porcine leukocytes	Cysteine-rich, 18-residue beta-hairpin stabilized by 2 disulfide bridges	General antimicrobial activity Low MIC values (0.12–2.0 μg/mL) against various microorganisms Antimicrobial activity against MRSA, *N. gonorrhea*, and *M. tuberculosis*	[81–84]
Theta-defensins	Rhesus macaque leukocytes	Cysteine-rich, 18-residue circular peptide stabilized by an internal tridisulfide ladder making it tetracyclic	Antiviral activity against HIV-1, HSV-1, SARS	[8, 41, 85]

MIC Minimum inhibitory concentration, *MRSA* methicillin-resistant *Staphylococcus aureus*, *SARS* severe acute respiratory syndrome (coronavirus lung disease)

[a] The references included here are to provide the reader with just a starting point for learning more about these peptides and are not intended to be complete or comprehensive

disulfide bonds resulting in a cystine knot motif, which contributes significantly to protein stability [28], a characteristic that distinguishes them from many other proteins [29]. These peptides are stable at high temperatures, and are resistant to proteases and chemical chaotropes [30]. Cyclotides have a wide range of inherent biological activities including the following: uterotonic, anti-HIV, neurotensin antagonism, hemolysis, antimicrobial, cytotoxicity, insecticidal and anthelmintic [30]. Due to their significant stability, unique cyclotides can be synthesized which have a number of amino acid sequences replaced, giving them the capacity to be either modified to optimize their inherent activity or to be used as a scaffold for biologically active epitopes. Similar synthetic approaches have been used to create congeners of retrocyclin, which enhance their antiviral or antimicrobial activity or select for other attributes desirable towards developing retrocyclins as therapeutics or preventatives [31].

Several studies have revealed that a defensin deficiency could have potential pathological significance for various diseases or conditions including Crohn's disease [32, 33], atopic dermatitis [34], and bacterial vaginosis [35, 36]. While speculative at best, susceptibility to HIV and progression to AIDS may be in part due to a genetic deficiency in retrocyclin expression that occurs in humans. Significantly, all tested strains of HIV-1 were highly susceptible to inhibition by retrocyclin [31, 37], while HIV-2 and SIV were less susceptible (unpublished observations). It has been speculated that this dichotomy between the two HIV serotypes may be a result of their evolution. HIV-2 evolved from SIV whose natural host is the Old World monkey, which still expresses theta-defensins. The majority of HIV-1 evolved from SIVcpz whose natural host is the chimpanzee, a primate that does not express theta-defensins. Consequently, HIV-2 may have evolved under selective pressure from theta-defensins while HIV-1 did not [38]. If this is the case, then HIV-1 and other viruses may be more susceptible in the presence of reawakened retrocyclins. A further analysis of the role of retrocyclin in protection against HIV-1 infection and spread is discussed below.

Evolution of retrocyclins

Naturally synthesized retrocyclin peptides have not been detected in human cells or tissues; however, the

retrocyclin-encoding mRNA transcript is present in bone marrow cells, spleen, thymus, testes, and skeletal muscle [9, 11]. Retrocyclin peptides are not detectable because of a premature termination codon that is present in the signal-peptide sequence, which prevents translation (Fig. 2). Analysis of the evolution of this premature termination codon from five prosimians, six New World monkeys, five Old World monkeys, one lesser ape, and four great apes revealed that the premature termination codon is present in humans along with their closest primate relatives (chimpanzee and gorilla), New World monkeys and prosimians, but not in Old World monkeys (e.g., rhesus macaque) from which theta-defensins were originally isolated ([9]; reviewed in [39]). Importantly, orangutans, from which humans evolved over 7 million years ago, have both intact and silenced copies of theta-defensin genes, suggesting that the premature termination codon mutation occurred approximately at this fork in evolution. Interestingly, while human cells do not make retrocyclin peptides naturally, certain cells have retained the ability to produce the peptide when coaxed with compounds that suppress the premature termination codon [40].

Although we may never fully comprehend the evolutionary importance for the introduction of a premature termination codon, determining the function of retrocyclins may help answer some important genetic questions about how the termination codon came to be and how it has remained conserved. Five of the six human theta-defensin genes cluster on chromosome 8q32, a genomic region of high diversity and where many other defensin genes are located, with the sole exception of the theta-defensin-encoding gene *DEFT6*, which is on chromosome 1. Due to the similarity in sequence, retrocyclin genes appear to have been derived from truncated alpha-defensin genes (Fig. 2). Indeed, the alpha-defensin HNP4 is the most closely related to theta-defensins. It is of particular note that, aside from the premature nonsense mutation, all six retrocyclin genes are remarkably intact. Given that most of the retrocyclin genes are located on a region of the 8th chromosome that is known for its high genetic diversity, it is puzzling why the retrocyclin genes remained intact over millions of years. There are several possibilities. Retrocyclin genes might have been lucky enough to escape further mutation, or our supposition about when the introduction of the premature nonsense mutation occurred during hominid evolution might be incorrect. Perhaps a more likely explanation is that retrocyclins have retained a contemporary function that has hitherto been unknown, which is supported by studies revealing that human cells have retained the complex machinery necessary to process retrocyclin peptides [40]. Under the right conditions, premature termination codons may in fact be a "yield" rather than a true "stop" of translation.

Similar to RTDs from rhesus monkeys, humans also have two different retrocyclin genes that could theoretically combine to create three different cyclic peptides: two homodimers [retrocyclin-1 (RC1) and RC3] and a heterodimer (RC2). These three different human retrocyclins were solid-phase synthesized, and tested for their antiviral activity against HIV-1 in several cell-based assays that measured viral release from lymphocytic cell lines or CD4-enriched PBMCs [11, 41]. Interesting, while RC1 (+4 net positive charge) and RC2 (+5 net positive charge) were active against all strains of HIV-1 tested, RC3 (+6 net positive charge) was not. RC2 proved to be the most active of these three against primary isolates of HIV-1 [42, 43,] and was thus used in most studies of retrocyclin structure. Moreover, a synthetic retrocyclin analog termed RC-106, which was engineered to have a +3 net positive charge, was likewise inactive against HIV-1. Together, these findings suggest that, similar to the mode of action of many HDPs against bacteria and fungi, electrostatic interactions might be important for the ability of retrocyclins to prevent HIV-1 infection.

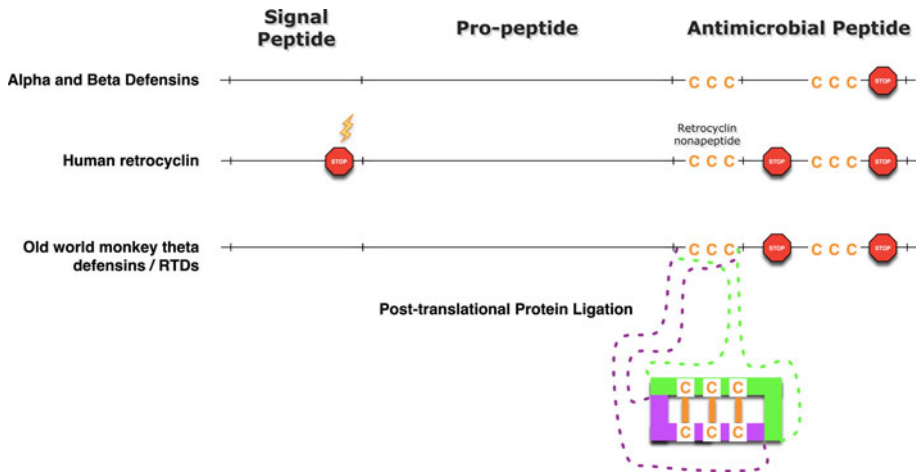

Fig. 2 Relationship of retrocyclin to other defensins. All defensins possess six cysteines. Approximately 7.5 million years ago, a single base pair nonsense mutation was sustained (lightning bolt) in the signal peptide-encoding region of the human retrocyclin gene. In other primates, theta-defensins are expressed after ligation of two nonapeptides to generate a functional cyclic 18 amino acid peptide. *RTD* Rhesus theta-defensin

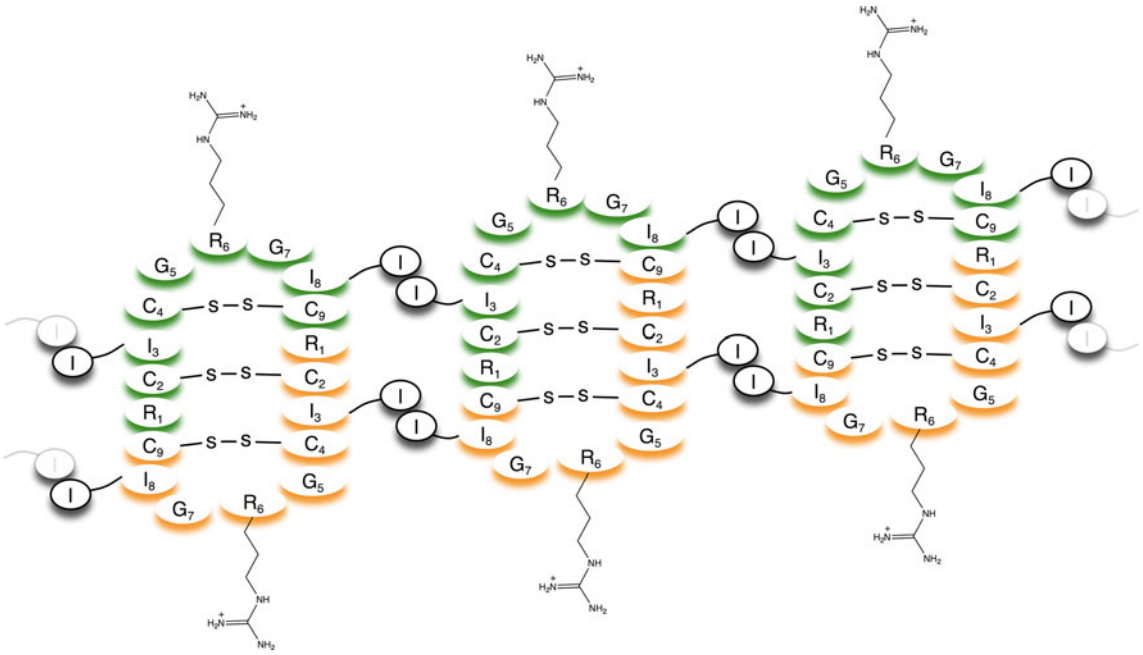

Fig. 3 Retrocyclin forms a trimer in solution. Isoleucines contribute to the formation of the trimeric structure due to hydrophobic intermolecular interactions. Glycines contribute to the hairpin turns while beta-pleated sheets extend vertically in this model. In the figure, the extreme left side of this molecule contacts the extreme right side to form a trimeric structure. For the retrocyclin congener RC-101, the cationic arginine shown at the *top* and the *bottom* of the structure is replaced with a cationic lysine

A few similarities between defensins and the envelope glycoproteins gp120 and gp41 of HIV deserve discussion. It was observed nearly two decades ago that defensins inhibit HIV-1 replication in vitro [44] and structurally resemble portions of the fusogenic HIV-1 protein gp41 [45]. Moreover, both gp41 peptides and defensins form trimeric antiparallel helical loops that can insert into membranes. Thus, defensins may exert some of their antiviral activity in part as competitive inhibitors of the gp120:gp41 membrane binding and fusion process that initiated HIV-1 entry. In solution, RC2 forms a trimeric structure in a concentration-dependent manner [46, 47] (Fig. 3). At lower concentrations, no particular structure is formed other than the beta-pleated sheet; however, at higher concentrations, the trimeric structure is formed. After binding to membranes, the prolate structure of each RC2 monomer is stabilized, which allows formation of the more stable trimer. By comparison with the rhesus macaque theta-defensin RTD-1, the more potent and higher binding affinity human RC2 possesses a more rigid structure and self-associates at a lower concentration than RC1. This trimeric retrocyclin structure is likely to increase dramatically the affinity of retrocyclin for the HIV-1 glycoproteins. Whether the structural similarities between gp41 and retrocyclins and other defensins is coincidental or a result of viral molecular mimicry remains unclear. Future studies to examine these relationships might reveal that portions of gp41 can mimic certain defensin-like actions in host immunity.

Retrocyclin mechanism of action

In this section, we examine the molecular characteristics of retrocyclin binding to HIV-1, including binding affinity, HIV-1 mutants, and correlations with anti-HIV-1 activity, and the binding to the host CD4 receptor. Being only 18 residues in length, this apparent lack of complexity made retrocyclin particularly attractive for mutagenesis studies designed to identify retrocyclin congeners with greater biological activities, more favorable drug metabolism, and ultimately greater clinical potential. In an effort to investigate the antiviral role of each residue and to develop RC congeners with superior anti-HIV-1 potency, a tyrosine scan of each non-cysteine residue was performed for the entirety of RC1 [39]. While no superior tyrosine-containing congener was obtained, replacing any one of the four arginine residues abrogated activity against HIV-1. However, a charge-conservative substitution of one of the arginines with lysine resulted in a congener, RC-101, with increased anti-HIV-1 potency [31]. Ultimately, this indicated that certain modifications of the retrocyclin sequence have the potential for greater potency against HIV-1 than the parental molecule. RC-101 had low micromolar IC_{50} activity (1–5 μg/mL) for preventing HIV-1 infection of host cells [31, 42]. The sequences of RC1, RC2, RC3 and RC-101 are presented in Fig. 4.

Retrocyclin binds with high affinity to the HIV-1 glycoproteins gp120 and gp41, and the host cell glycoprotein

Retrocyclin-1 (RC1)	GICRCICGRGICRCICGR
Retrocyclin-2 (RC2)	GICRCICGR**R**ICRCICGR
Retrocyclin-3 (RC3)	**R**ICRCICGR**R**ICRCICGR
RC-101	GICRCICGRGICRCICG**K**

Fig. 4 Retrocyclin-1, -2 and -3 were synthesized based on the primary human genetic sequences encoding these peptides. RC-101 was synthesized with an arginine (*R*) to lysine (*K*) substitution which resulted in increased inhibition of HIV-1 infection

Table 2 The binding affinities from highest to lowest (top to bottom) between RC1 and interacting molecules measured by surface plasmon resonance

Interacting molecule	K_D (nM)
Galactosylceramide	24
CD4	31
gp120	35
Fetuin	42
gp41	68
BSA-galactosamide	242
dBSA-mannopyranose	1,050
Removal of both O & N-linked glycosyl groups from any of the above proteins	Negligible
RNase	Negligible
IgG	Negligible

CD4, which is due in part to the lectin-like properties of retrocyclins (Table 2; [31, 39, 42, 48]). Lectins represent vital extracellular carbohydrate-binding proteins involved in infectious disease, development, and immunological functions. Composed of just 18 amino acids, retrocyclin is one of the smallest known lectins; notably, it was the first described lectin to bind to both HIV-1 envelope glycoproteins. Other glycosylated proteins, including galactosylceramide, have been shown to potentially act as additional cell surface receptors for HIV-1 in cells that lack CD4 [49, 50]. RC-101 binds to gp120 with as much as 25-fold greater affinity than RC1, while binding to galactosylceramide with identical affinity (20–30 nM) [43]. Importantly, retrocyclin's potent antiviral activity has been observed against several viruses aside from HIV-1. In each case, retrocyclin disrupted the binding of viral glycoproteins to host cell glycoproteins while removal of viral glycosyl groups prevented retrocyclin binding [42, 43, 51, 52]. While retrocyclins can bind both HIV-1 and host cell glycoproteins, it does not prevent the virus from binding to the target host cell. In fact, the major anti-HIV-1 mechanism for retrocyclins involves inactivating gp41 in a non-lectin fashion [48]. This suggests that retrocyclin's ability to bind viral and host glycoproteins likely places them "in the right place at the right time" to exert subsequent anti-HIV-1 activity.

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. HIV glycoproteins comprise a heterotrimer consisting of three gp120 surface subunits and three gp41 transmembrane subunits. Once the trimer of gp120 binds to a trimer of host CD4 receptors present on macrophages, T lymphocytes or dendritic cells, a conformational change occurs in gp120, which enables it to bind either the CCR5 or CXCR4 coreceptor depending on the tropism of the virus (R5 tropic strains utilize CCR5 and X4 tropic strains utilize CXCR4). After HIV-1 gp120 binds to its receptor and coreceptor, the outer domain of the gp41 transmembrane protein undergoes a significant conformational change forming a membrane-penetrating fusogenic coiled-coil trimer consisting of six helices with three anti-parallel leucine/isoleucine zippers required for membrane fusion with the host cell. It is this step with which retrocyclins interfere, preventing viral entry by blocking 6 helix bundle formation [48]. In addition, retrocyclin binds to gp41 with high affinity, 68 nM K_D [48], likely aiding the process. The interaction of retrocyclin with gp41 occurs through two gp41 heptad repeat domains (HR1 and HR2). In silico and in vitro experimental data indicate that RC-101 prefers to bind to the HR2 domain of gp41 via ionic interaction between the positively charged cationic retrocyclin and the negatively charged HR2 domain [53]. Mutations in gp41 have a greater effect on retrocyclin's anti-HIV-1 binding activity than gp120 mutations [38]. Alpha-defensins HNP1-4 can all inhibit HIV-1 infection with HNP-4 having the greatest anti-HIV-1 potency [54]. Although HNP1-3 all bind to gp120 [43], HNP-4 does not bind to gp120. Given that retrocyclins are most similar to HNP-4, these collective observations suggest that retrocyclin binding to gp41 might be more significant for antiviral activity than binding to gp120.

The lectin characteristic of retrocyclin also plays a required role in its antiviral activity [42, 51]. HIV-1 gp120 protein has 20 N-glycosylation sites and carbohydrates account for approximately 55% of the weight of gp120 [55]. Given that retrocyclin binding to gp120 is dependent on the presence of glycosyl groups, there is potential that differences seen for RC-101 binding to different HIV-1 strains may be due to differences in glycosylation patterns of gp120 variants [42]. Removal of either the O-linked and N-linked sugars from immobilized or glycosylated CD4 resulted in a considerable reduction of RC binding activity while removal of both types of sugars nearly abolished RC binding activity.

Retrocyclins, like most HDPs, are cationic and bind to negatively charged residues. Exposing bacteria to cationic HDPs can stimulate bacteria to evolve resistance by selecting for strains that have undergone genetic mutation

in phospholipid/lipopolysaccharide synthesis, which alters their negative membrane charges [56–58]. Similarly to bacteria, HIV-1 can evolve in response to selective pressures by altering surface charge of its viral envelope glycoproteins [38]. New strains of the HIV-1 virus commonly evolve resistance to gp41/CXCR5/CCR4-targeted drugs within weeks after exposure [59–61]; for a review refer to [62]). Thus, anti-HIV-1 therapeutics including next-generation retrocyclins must be capable of persistently inhibiting HIV-1 by targeting the most critical amino acid residues required for infection.

Toward the goal of understanding the potential resistance of HIV-1 to retrocyclins, a survey of *env* sequences of hundreds of primary HIV-1 isolates obtained from the Los Alamos database suggested that most HIV-1 strains would likely be susceptible to inhibition by treatment of the retrocyclin RC-101 [53]. Subsequently, site-directed analyses of ten representative HIV-1 gp41 cationic-mutant isolates revealed only one HIV-1 gp41 mutant that became partially resistant to RC-101. To further examine the potential mechanism of RC-101-mediated resistance, HIV-infected human T lymphocytic cells (PM1) were serially passaged in the presence of RC-101 for over 100 days in 20 5-day rounds of infection. Even when infected cells were cultured in the presence of 5 μg/mL RC-101 (a concentration more than 50-fold lower than that tolerated by human cells), RC-101 inhibited infection by at least 88% for an additional nine rounds of infection [38]. Compared to data from other HIV entry inhibitors, which increased HIV-resistance 10,000- to 20,000-fold in similar passaging experiments [61, 63], RC-101 induced minimal HIV-1 resistance of only 5- to 10-fold. Surprisingly, these data suggested that mutations in HIV-1, which prevented RC-101 binding to HIV-1 proteins, also reduced HIV-1 infectivity [38, 53] thus imparting a viral fitness consequence to subverting retrocyclin action. Ultimately, even passaging beyond 140 days did not result in the evolution of a single fully RC-101 resistant strain of HIV-1 (A.M.C., unpublished results).

In a more physiological study, RC-101 also demonstrated anti-HIV-1 activity over a 9-day incubation period in cervicovaginal tissue even when mixed with vaginal fluid [12]. This is an important factor in testing therapeutics for vaginal delivery since the low pH and the proteolytic action within the vaginal mucosa could potentially inhibit the activity of various compounds. Furthermore, RC-101 did not induce inflammation or histotoxicity at concentrations many fold higher than required for antiviral activity in these organotypic tissues [12]. Most recently, RC-101 has been formulated as a quick-dissolve film, which was safe and remained antivirally active following repeated topical vaginal application in pigtailed macaques [13]. Collectively, these observations indicate that RC-101 can be highly effective against many evolving strains of HIV-1 and that it has the potential to succeed in the clinical setting where sexual transmission of HIV-1 is the primary mode of infection.

Potential clinical applications of retrocyclins in preventing HIV-infection

Over 33 million people are infected with HIV-1 worldwide, and women account for more than half of the estimated new infections occurring each year. Since unprotected heterosexual vaginal intercourse has become the main route of infection, therapeutic strategies that enable women to control their exposure to sexually transmitted infections (STIs) are critical. Therapies focused on vaginal delivery are considered of greatest value for local-acting drugs to prevent spread of STIs such as HIV. HIV-1 is known to cross the vaginal mucosa by an unknown mechanism, which may directly involve vaginal epithelial cells. Once the virus enters the lamina propria, it can directly infect T cells and macrophages and possibly dendritic cells, which then traffic to the lymph nodes, becoming factories for virus production [64, 65].

Due to their naturally broad antimicrobial activity, the development of retrocyclins as therapeutic agents for the prevention of STIs such as HIV has distinct advantages. Retrocyclins would likely have an immediate presence at the target mucosal site (e.g., vagina and cervix), lack cytotoxicity or inflammatory potential at bioactive concentrations, and exhibit strong activity against a broad range of R5- and X4-tropic strains of HIV. A topical vaginal microbicide may prevent HIV transmission by destroying or inhibiting the virus, augmenting natural immune defenses against the virus, blocking viral attachment or entry, or preventing transmigration of virus from the mucosa to regional lymph nodes. Moreover, vaginal therapies should not only be considered as local-acting therapies. Rather, the vast network of blood supply and large surface area of the vagina provide significant potential for systemic drug delivery while avoiding the problems of first-pass metabolism encountered by oral therapies [66]. It is important to note that vaginal structure and composition can also negatively affect drug delivery since one of the most common enzymes found in vaginal fluids belongs to the aminopeptidase family, which exhibits strong protein and peptide degradation activity. Furthermore, other enzymatic activities present in vaginal fluids have not yet been completely elucidated and the role of the vaginal mucosa in health and disease is still being uncovered. A more detailed discussion of vaginal physiology and its potential effects on the delivery and efficacy of drug treatment are discussed elsewhere [36].

Microbicide candidates currently in preclinical and clinical trials target the virus via different mechanisms of action, including: virucides, entry/fusion inhibitors, reverse-transcriptase inhibitors, integrase inhibitors or protease inhibitors [43]. Currently, there are over a dozen planned/funded trials to study microbicides and pre-exposure prophylaxis (www.avac.org/ht/a/GetDocumentAction/i/27266). Results from at least eight of the ongoing efficacy trials using microbicides in the treatment and prevention of AIDS and other STIs should be reported during the next 2 years. A summary of these trials is presented below and shown in Table 3.

To date, the only successful study of a topical vaginal microbicide has been the recent Centre for the AIDS Programme of Research in South Africa (CAPRISA) 004 Phase 2B trial [67]. The primary objective of this study was to assess safety and efficacy of 1% tenofovir [a nucleotide analogue reverse transcriptase inhibitor (nRTI)] vaginal microbicide gel to prevent HIV infection in approximately 900 South African women between the ages of 18 and 40. Secondary endpoints included assessment of tenofovir resistance in HIV seroconverters, viral load of women who may have become infected during the trial, evidence of deep epithelial disruption, renal toxicity, and any impact on pregnancy rates or outcomes. A 39% reduction in infectability was observed after tenofovir gel treatment, with the greatest reduction (54%, $P = 0.025$) observed in women who had a high adherence rate of >80% in applying the gel. Despite continued use of tenofovir gel, peak protection was seen at 12 months with steadily reduced effectiveness after 18 months. Reasons for reduced effectiveness are unknown at this time and may be due to a number of factors including reduced adherence. Initial review did not indicate tenofovir-related HIV resistance, but further studies are needed that include earlier time points as well as testing of both the genital and systemic tracts. There was no increased impact on renal, hepatic, pregnancy-related, or genital-related adverse events. Also, renal toxicity/hepatic flares were not observed which may have been due to low systemic absorption of the gel formulation. Together, these promising results set the stage for the next-generation of topical microbicides that are in late-stage pre-clinical studies or entering early clinical trials.

The MTN-003 (VOICE) Phase 2B trial is also exploring the use of tenofovir 1% gel, as well as tenofovir tablets either alone or in combination with emtricitabine, an nRTI, to estimate the effectiveness in preventing HIV acquisition in women in Africa (www.avac.org/ht/a/GetDocumentAction/i/27266). Secondary objectives include the rise of drug resistance in trial subjects and evaluating adherence and acceptability of the drug dosage forms (gel vs. tablet).

Table 3 Current microbicide and PrEP candidates

Candidate	Current status[a]/Phase
PRO 2000: gel	Completed
Truvada®: oral	Ongoing (FEM-PrEP study)—Phase 3
	Data analysis (iPrEX study)—Phase 3
	Data analysis—Phase 2
	Data analysis—Phase 1/2
Viread®: oral, truvada: oral	Ongoing—Phase 3
Viread®: oral	Planned—Phase 3
	Ongoing—Phase 2/3
	Data analysis—Phase 2
Tenofovir: gel	Planned—Phase 3
	Data analysis—Phase 2BData analysis—Phase 2
	Planned—Phase 1
Tenofovir: gel, viread: oral	Ongoing—Phase 2
Dapivirine: gel	Planned—Phase 3
	Ongoing—Phase 1/2
	Planned—Phase 1
VivaGel®: gel, viread: oral, truvada: oral	Ongoing—Phase 2B
Acidform: gel	Ongoing—Phase 1
Dapivirine: ring	Ongoing—Phase 1/2
	Data analysis—Phase 1
UC-781: gel	Planned—Phase 2
	Ongoing—Phase 1
BufferGel®: gel	Planned—Phase 3
Invisible condom®: gel	Planned—Phase 2/3
MIV-150 + gel: gel/ring	Planned—Phase 1
VivaGel®: gel	Data analysis—Phase 1
Zinc acetate: gel	Planned—Phase 1

PrEP Pre-exposure prophylaxis

Viread® and Truvada® are trade names for tenofovir formulations

[a] Includes multiple studies. A more detailed summary of these trials can be found at the AIDS Vaccine Advocacy Coalition (AVAC) website: http://www.avac.org/ht/a/GetDocumentAction/i/3109

This study is similar to the CAPRISA study described above, but VOICE focuses on daily dosing while CAPRISA focused on coitally-related dosing.

In February 2009, preliminary results were reported from the Phase 2 HPTN 035 trial, which studied the effectiveness of PRO 2000 gel (0.5%), a polyanionic naphthalene sulfonate polymer that can bind CD4 with nanomolar affinity preventing gp120 from binding [68]; BufferGel, a Carbopol 974P-based polyacrylic acidic buffering gel that enhances the natural protective action of the vagina to produce a broad-spectrum microbicidal environment; and placebo. An extensive Phase 3 placebo-controlled study (MDP 301) of PRO 2000 (0.5%) in 9,385

women at six centers in Africa failed to show a difference in the effectiveness of PRO 2000 compared to placebo despite reports from an earlier, smaller Phase 2 study that indicated borderline effectiveness of PRO 2000 (MDP 301 Trial Results, http://www.africacentre.ac.za/Default.aspx?tabid=204).

Dapivirine is a microbicide that is a non-nucleoside RTI that inhibits reverse transcription by binding to a conserved region of the transcriptase and preventing its progression. Dapivirine has been clinically tested for efficacy as a gel or delivered as a vaginal ring (Studies IPM 001, 003, 004, 005B and 008). The completed Phase 1 and 2 studies indicated that twice daily administration of the gel for 42 days was safe and well-tolerated (www.avac.org/ht/a/GetDocumentAction/i/27266). Additional trials are planned including a Phase 3 efficacy trial to be started in 2011 as well as trials to compare different formulations (tablet, soft-cell capsule and film) against placebo.

A Phase 3 trial (FEM-PrEP) is underway to evaluate the safety and efficacy of Truvada® in preventing HIV infection in 3,900 HIV-negative women from Kenya, Malawi, Tanzania, South Africa and Zambia who are at risk to become infected through sexual intercourse. Truvada is a fixed dose of emtricitabine (200 mg) and tenofovir disoproxil fumarate (300 mg). Primary outcome measures will determine the combined incidence of HIV-1 and HIV-2 infection as well as liver toxicity and frequency of adverse events associated with drug administration (www.avac.org/ht/a/GetDocumentAction/i/27266).

Vivagel® is a dendrimer (a class of polyanion macromolecules) developed as a vaginal microbicide which can prevent virus–cell interactions and thus inhibit infection. Results from the first clinical study were reported in 2010 [69], which studied safety and pharmacokinetics in healthy women. The gel was shown to be safe and well-tolerated after seven consecutive daily doses of escalating dose levels (1, 3, and 5%). Cervicovaginal fluid samples were taken from study participants at 1, 3, 12 and 24 h after dosing. These samples were then tested in the laboratory for their ability to prevent HIV and genital herpes (HSV-2) infection of susceptible cells. At each time point post-dose, the fluid samples were able to prevent in vitro infection of HIV-susceptible cells with >90% efficacy. Further trials are needed to determine clinical efficacy in study participants to confirm the in vitro data.

ACIDFORM gel was developed to provide a long-lasting coating of vaginal and cervical areas in order to maintain a spermicidal and potentially microbicidal vaginal pH. Initial Phase 1 feasibility study of a diaphragm coated with ACIDFORM gel indicated that women were willing and able to use the device for 6 months [70], but the benefit of protecting against HIV infection is to be determined in future trials.

Currently, only Phase 1 trials have been reported for UC781 and the Invisible Condom®. A Phase I trial is planned for MIV-150. UC-781 is a microbicidal non-nucleotide RTI administered as a gel. The Invisible Condom is a gel formulation that contains sodium lauryl sulfate—a polymer that can trap and hinder viruses, preventing their attachment and subsequent entry into susceptible cells. In vitro, sodium lauryl sulfate was shown to prevent HIV attachment to cells and inhibit cell entry by HSV [71]. MIV-150 is a non-nucleoside RTI that has demonstrated anti-HIV activity in vitro and anti- RT-SHIV (SIVmac239 bearing HIV reverse transcriptase) in vivo (macaques).

Since antiretroviral products are being developed for use primarily in developing countries, there are societal and cultural issues to be addressed, which can greatly impact drug use and efficacy, such as the use of condoms or other coitally-related products. Additionally, many gel formulations are often found by study participants to be leaky or messy, which greatly affects protocol adherence; however, some formulations were found to be acceptable and in some cases were found to enhance the experience (e.g., BufferGel and PRO 2000 trial data). Although first-generation microbicides, which are currently in Phase 2B/3 clinical development, are all gel formulations, newer products being developed are exploring other dosage forms (films, foams, oral tablets, vaginal suppositories, or intravaginal rings) in addition to gels. Some of these products may be coitally-independent and may be systemically active for several weeks or possibly months after dosage. Multi-regimen therapies are also being explored which allow for HIV-1 prevention with one drug and prevention of other STIs or conception with a second drug/therapy [72].

Summary and perspectives

Retrocyclins were identified as a potential new class of small molecules that could inhibit HIV-1 entry and fusion [11, 41]. Significantly, these synthetic peptides had little to no hemolytic activity or cytotoxicity in common experimental cell lines (H9 T cells and ME-180 cervical carcinoma cells) at concentrations up to 500 μg/mL (the highest amount tested in these studies), a far greater concentration than the 1–5 μg/mL range shown to provide protection against most HIV-1 strains [12, 31, 53]. Serial passaging of HIV-infected PM1 cells in the presence of RC-101 at 5 μg/mL (a concentration more than 50-fold lower than the tolerated limit of human cells) resulted in >88% inhibition of viral infection over multiple passages. Significantly, the HIV-1 mutants replicating in response to this selective pressure had reduced infectivity.

Furthermore, retrocyclins were shown to effectively 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 [31]; and prevent organotypic cervicovaginal tissues from HIV-1 infection [12]. Importantly, lack of inflammation and the ability to retain potent anti-HIV activity in the presence of vaginal fluids and tissues are factors critical to the clinical development of retrocyclins as microbicides to prevent HIV-1 transmission. As studies continued to look at this class of HDPs, it was observed that amino acid substitutions could be introduced in the retrocyclin backbone while maintaining structural integrity and improving targeted activity against HIV-1.

The microbicide clinical trial results obtained so far include RTIs and polyanionic compounds. However, microbicides that function as fusion/entry inhibitors are currently considered as having the greatest clinical potential because they have shown consistent and vigorous protection against vaginal HIV transmission in macaques [73, 74]. Additionally, this class of microbicides also tends to present greater effectiveness against drug-resistant viruses than microbicides that target other points in the viral replication cycle such as reverse transcription [75]. Success and overall protection against HIV infection and/or spread may best be achieved through the combined effects of HDPs with other HIV-1 inhibitors that exhibit different mechanisms of action. The use of microbicides might have their greatest effect at the initial stages of infection, which could reduce initial viremia. This reduction could subsequently lead to a smaller and more manageable initial impact on the immune system, shifting the balance toward immune control. Although HDPs and their target microbes and viruses have existed together through evolution, HDPs including retrocyclins remain highly effective against these targets and may be our best approach for a safe and effective microbicide in the near future.

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