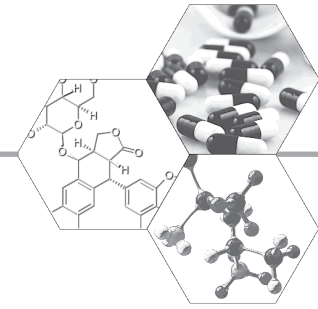

HIV microbicides: state-of-the-art and new perspectives on the development of entry inhibitors

Since the discovery of HIV at the beginning of the 1980s, numerous efforts have been devoted to the search of an efficient vaccine. There are at least 25 drugs available for HIV treatment, but no cure is available. The observation that therapy for HIV disease is life long and that these drugs are associated with a number of side effects underlines the need for approaches aimed at preventing rather than treating infection. Additionally, the economic burden of treatment for the HIV infection occupies an increasing share of healthcare expenditure, making current practices likely to become difficult to sustain in the long run. Unfortunately, no effective vaccine for this disease is foreseeable in the near future. Microbicides could be an alternate way to build preventative approaches to HIV infection. Strategies based on preventing the virus from reaching its target cells seem to have some room for development and application. In this review we explore the state-of-the-art of available microbicides, focusing on HIV entry inhibitors. In addition, we discuss new compounds that show anti-HIV activity, which could be effective candidates.

HIV infects more than 15,000 people every day. Most of these new infections are sexually transmitted and, so, compounds acting at a mucosal level and impeding infection would have a major epidemiological impact. Infections can be prevented by vaccines or by substances that block the penetration of pathogens in the body. In the first case we use adjuvanted antigens to elicit an immune response that will be able to avoid the insurgence of disease. It is important to notice that vaccines do not necessarily prevent infection (sterilizing immunity). Rather, these compounds impede disease: vaccine-stimulated immune responses modify the natural history of infection, avoiding the appearance of full-blown disease. Notably, with the exception of the results for the recent RV144 trial, the so-called 'Thai trial' [1,2], which showed a very modest possible preventative effect, no vaccine has worked to impede HIV infection, and no effective vaccine is foreseeable in the near future.

The second way to prevent infection is to block the penetration of a pathogen in the body. This modality does not imply active participation of the immune response; rather, physical and/or chemical means are used to impede the contact between pathogens and the host cell, and the subsequent penetration of the pathogen into such cells. In the case of HIV, the simplest preventative device is a condom. If properly used, condoms are highly effective in impeding infection. Nevertheless, possible ethical considerations aside, cultural factors often keep men and women from using condoms, or women from asking their partners to use such devices, in many parts of the world. Finally, it has been repeatedly shown that reduced risk of HIV transmission is only associated with consistent but not with occasional condom use. These considerations, the evidence of the ever-growing spread of new infections despite the availability of condoms and the objective difficulty in designing effective vaccines, led to the exploration of the ability of microbicides to prevent sexual transmission of HIV [3–7]. Microbicides can be classified into five different groups:

- Surfactants;
- Vaginal milieu protectors;
- Viral entry inhibitors;
- Agents that target viral replication (reverse transcriptase inhibitors);
- Other compounds, whose mechanisms are unknown [4].

Surfactants can disrupt membranes non-specifically and have been evaluated as topical microbicides. The protectors of vaginal milieu either operate as direct acidifying agents or as enhancers of *lactobacilli* production. Inhibitors of viral entry are a broad class of molecules, which can target either host cells or viral targets, and can block different steps of the pathways

Jose J Reina[1], Anna Bernardi[1], Mario Clerici[2,3] & Javier Rojo[†4]

[1]Universita' degli Studi di Milano, Dipartimento di Chimica Organica e Industriale and CISI, via Venezian 21, 20133 Milano, Italy
[2]Universita' degli Studi di Milano, Dipartimento di Scienze e Tecnologie Biomediche,via Flli Cervi 93, 20090 Segrate, Italy
[3]Don C. Gnocchi ONLUS Foundation IRCCS, Via Capecelatro 66, 20148 Milano, Italy
[4]Grupo de Carbohidratos, Instituto de Investigaciones Químicas, CSIC – Universidad de Sevilla, Av. Américo Vespucio 49, 41092 Sevilla, Spain
[†]Author for correspondence:
Tel.: +34 954 489 568
Fax: +34 954 460 565
E-mail: javier.rojo@iiq.csic.es

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used by HIV to cross mucosal barriers and to infect CD4+ lymphocytes, (e.g., the attachment of HIV to host cells, the fusion of virus and host cell membranes, or the entry of HIV into host cells).

More than 70 preclinical and 50 clinical trials have been performed using compounds that fall in each of the five classes. In the preclinical Phase, *in vitro* assays and cervical explants models are used; some clinical experimentation has proceeded to Phase III, the evaluation of possible efficacy. In brief, none of the compounds that reached Phase III has demonstrated any effect on the prevention of infection [8–10].

HIV entry process & potential molecular targets for antiviral therapy

HIV entry in CD4+ cells is a very complex process that proceeds through a series of steps **(Figure 1)**:

- gp120, expressed on the viral surface, binds to the CD4 molecule present on host cells;
- This process induces a conformational change in gp120 that allows it to bind either CCR5 or CXCR4, the main co-receptors for HIV;
- At this point, a rearrangement in a second viral protein, gp41, exposes on the surface of HIV a 'fusion peptide';
- The fusion peptide activates the complex fusion between the virus and the host cell membranes.

In 2000, it was suggested that gp120 recognition at the mucosal level by the dendritic cell membrane lectin, specific ICAM-3 grabbing nonintegrin (DC-SIGN) may contribute to infection by promoting viral transmission and/or by inducing a signaling cascade, which results in immunosuppression [11–17].

Based on previously described steps, molecules binding to the host cell receptors CD4, CCR5, CXCR4, or possibly to DC-SIGN, could act as inhibitors of viral entry and stop the initial events leading to productive infection. A similar effect can be obtained by targeting the viral envelope and blocking, for instance, gp120 or the 'fusion peptide'. All these are, as a consequence, very interesting approaches for the prevention of infection [18–23].

Indeed, as will be discussed in the following sections, much research has been dedicated to the discovery of entry inhibitors and many candidates have emerged for drug development. Negatively charged polyanions capable of binding to the surface of HIV were found to prevent infection *in vitro*. The fact that these compounds, besides being effective *in vitro*, are also easy to produce and economical, made them perfect potential candidates. Nevertheless, clinical trials of polyanions, including dextran sulfate and cellulose sulfate, failed to demonstrate any protection *in vivo* and, rather disappointingly, even increased plasma HIV viral load of infected individuals. Similarly, recent results from a Phase III clinical trial of the anionic inhibitor PRO-2000 have been disappointing [24].

Lectins targeting viral glycoproteins and potentially able to inhibit the binding process between receptors at the target cell membranes and the virus at the initial steps of infection, have been proven to be efficient *in vitro*, but their ability to modulate HIV infection *in vivo* has not yet undergone any clinical trial. Human and/or humanized monoclonal antibodies endowed with the ability to bind the CD4 binding domain and neutralize viral infectivity have been tested *in vitro* and in macaque models. In the animal model, these antibodies (e.g., B12, 2F5, 2G12 and 4E10) showed a promising, although not total, ability to prevent infection (clinical trials have not yet been started). CCR5 inhibitors, the newest class of antivirals used in clinical practice, are potentially very important, as viruses that utilize this co-receptor are associated with almost the totality of mucosally transmitted HIV infections. CCR5 inhibitors were tested in the macaque model and were shown to effectively block vaginal infection (clinical trials based on the utilization of these molecules as mucosal tools to prevent infection have not yet started). Notably, infection can also be prevented *in vitro* by the use of high doses of RANTES, the chemokine that binds CCR5. This observation led to the development of a number of RANTES analogues that are endowed with potent antiviral activity. Some of these analogues have been shown to protect macaques against vaginal infection. Finally, peptides designed to interfere with the processes resulting in the exposure of the fusion peptide on the surface of HIV (e.g., C52L) can convincingly prevent infection in the macaque model. Nevertheless, the effect is only observed in the presence of extremely elevated (100 mM) concentrations of drug.

The development of compounds with the ability to prevent mucosal infection has also been slowed down by a number of other practical

issues. To summarize, an effective compound, besides being able to prevent infection, will also have to be:

- Safe and nontoxic;
- Devoid of inflammatory activity;
- Resistant to seminal fluid, to vaginal pH and body temperature;
- Easy and simple to use (e.g., nonoily and nonleaking);
- Economical;
- Simple to produce and store;
- Endowed with durable activity.

Finally, even if receptive anal intercourse (RAI) is less frequent than vaginal intercourse (RVI), the median estimate of transmission risk for RAI is approximately 20-fold higher than that observed for RVI. These considerations indicate that the design of compounds able to prevent sexual transmission of HIV altogether will need to take into account the necessity of protecting both the genital tract and the rectum. In the following sections we will discuss literature data concerning the discovery and development of HIV entry inhibitors. Molecules will be grouped according to the location of their target either on the host cell membrane or the viral envelope. Finally, recent reports exploring the potential for the combination of diverse entry inhibitors and of entry inhibitors with other microbicides will be discussed.

Host cell targets

■ CD4 inhibitors

CD4 is the main receptor for HIV-1 entry and, as a consequence, is a privileged target for strategies for preventing the infection of target cells. However, most antiviral compounds were targeted against the CD4 binding site of gp120 rather than against CD4 itself (gp120-targeting compounds are reviewed in the appropriate section). One of the few inhibitors targeting CD4 is an anti-CD4 humanized monoclonal antibody, known as ibalizumab or TNX-355 [25,26]. This molecule was developed as a potent *in vitro* inhibitor of HIV and has successfully completed Phase II clinical trials. Ibalizumab does not work by preventing gp120 binding to CD4, but appears to decrease the flexibility of CD4 and to hinder access of CD4-bound gp120 to the CCR5 and CXCR4 co-receptors. Recently, a bifunctional molecule comprising ibalizumab and two fusion inhibitor peptides has been described [27]. This novel bifunctional inhibitor was reported to display improved potency and favorable pharmacokinetic properties and may offer a novel approach to block both CCR5- and CXCR4-using viral variants.

■ Co-receptor inhibitors

The most important HIV-1 entry inhibitors targeting one of the cell membrane co-receptors (CXCR4 and CCR5) are shown in TABLE 1. Antagonists of the CCR5 co-receptor were

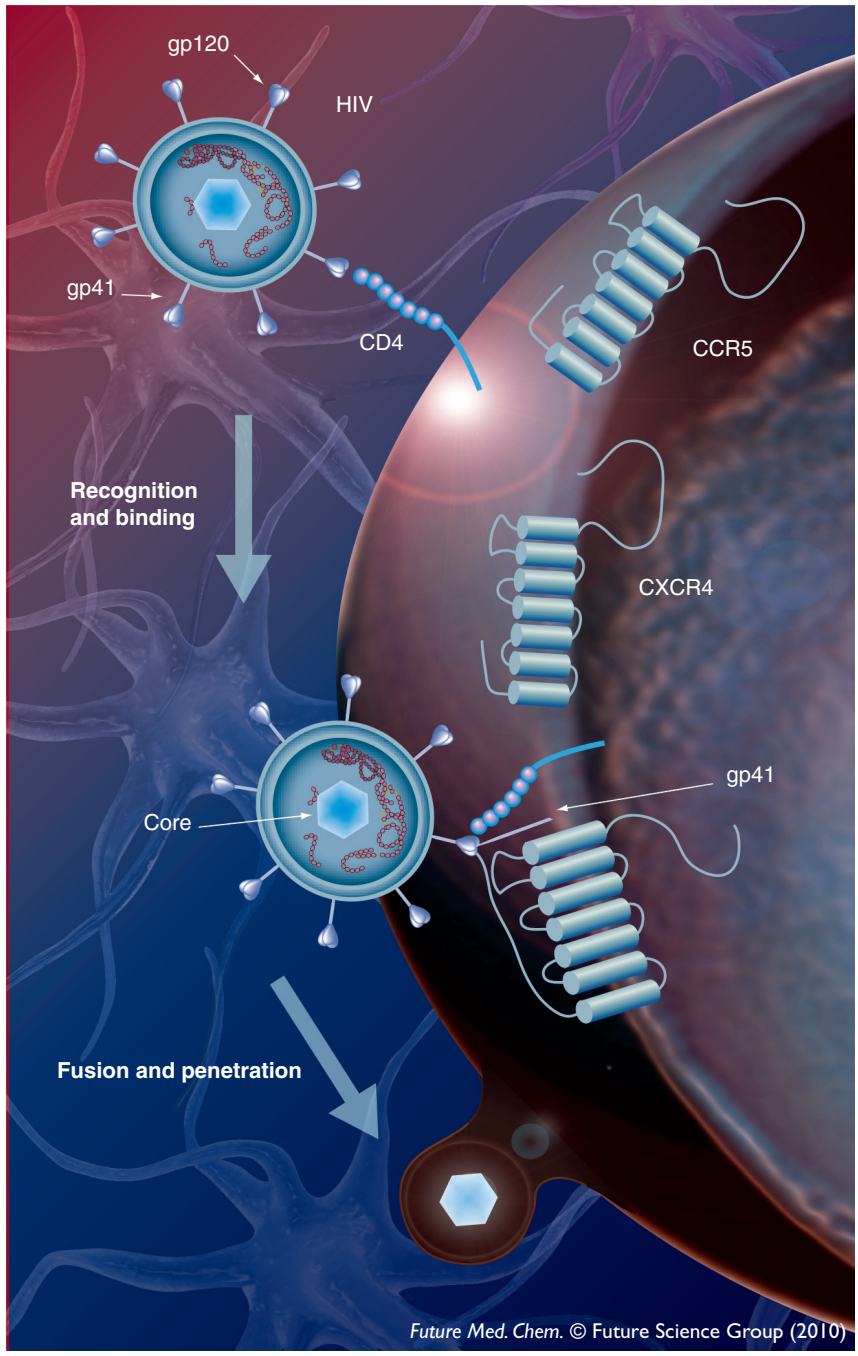


Figure 1. HIV-1 entry steps and some potential targets.

investigated more thoroughly because individuals carrying a natural deletion (Δ32) in the *CCR5* gene were found to be highly resistant to infection by CCR5-tropic strains and apparently healthy [28]. Furthermore, with few well-documented exceptions, primary infection is only supported by CCR5-directed strains of the virus. Tropism switch was found to occur in a high number of HIV infections and CXCR4-tropic variants may emerge when the CCR5-tropic virus is suppressed. This phenomenon, is nevertheless seen in the chronic phase of HIV infection only. In addition, systemic use of CXCR4 antagonists is likely to lead to adverse effects by suppressing the physiological functions of this receptor, which is involved with the migration and development of hematopoietic cells. Hence, research on HIV entry inhibitors has focused mostly on CCR5 antagonists.

Nonetheless, CXCR4 antagonists have also been investigated. In particular, two compounds have been described, AMD3100 (Plerixafor) [29] and KRH-1636 [30] (entries 1 & 2; **Table 1**, **Figure 2**), the most advanced of which, AMD3100, was found to possess little antiviral activity, but was approved by the US FDA (December 2008) for stem cell mobilization [31]. It was recently reported that the Cu^{2+} complex of AMD3100 shows improved potency relative to the starting compound, which was related to the increased residence time of the copper complex on CXCR4 [32]. Recent investigations focused on the optimization of a previous lead (AMD070; **Figure 2**) [33] have led to the identification of an orally bioavailable compound with subnanomolar anti-HIV-1activity **(1; Figure 2)** [34].

Many CCR5 small-molecule antagonists have been reported [22,23,35–37]. Early attempts included Takeda's TAK-779 [38,39] and Merck's CMPD-167 (**Table 1**; entry 3&4) [40,41]. Three compounds, maraviroc [42,43], vicriviroc [44,45] and aplaviroc [46] have reached clinical trials (entries 5–7; **Table 1** & **Figure 2**). Aplaviroc belongs to a class of spirodiketopiperazine derivatives **(Figure 2)**. Its development was halted in

Table 1. HIV-1 entry inhibitors targeting co-receptors in $CD4^+$ cells.

Entry	Generic name	Experimental code	Company	Molecule type	Target	Notes	Ref.
1	Plerixafor	AMD3100		Small molecule	CXCR4	Little antiviral activity shown in clinical trials evolved into a stem cell-mobilizing agent.	[29,31]
2		KRH-1636	Kureha Chem Industry	Small molecule	CXCR4		[30]
3		TAK-779 TAK-220 TAK-652	Takeda Chemical Ind.	Small molecule	CCR5	TAK-779 currently not further developed. TAK-652 currently developed by Tobira Therapeutics as TBR-652.	[31,38,39,140]
4		CMPD167	Merck	Small molecule	CCR5		[40,41]
5	Maraviroc	UK-427,857	ViiV Healthcare Pfizer	Small molecule	CCR5	US FDA approved Aug 2007	[42,43]
6	Vicriviroc	SCH-D; SCH-417690	Schering-Plough Corporation (now Merck)	Small molecule	CCR5	Experimental Phase II ongoing. Possible association with increased risk for malignancies.	[31,44,45]
7	Aplaviroc		GlaxoSmithKline	Small molecule	CCR5	Discontinued for liver toxicity.	[46]
8		PRO 140	Progenics Pharmaceuticals	Monoclonal antibody	CCR5	Phase II trial concluded.	[60]
9		Modified RANTES (AOP-RANTES)		Modification of RANTES, a CCR5-binding chemokine	CCR5		[59]

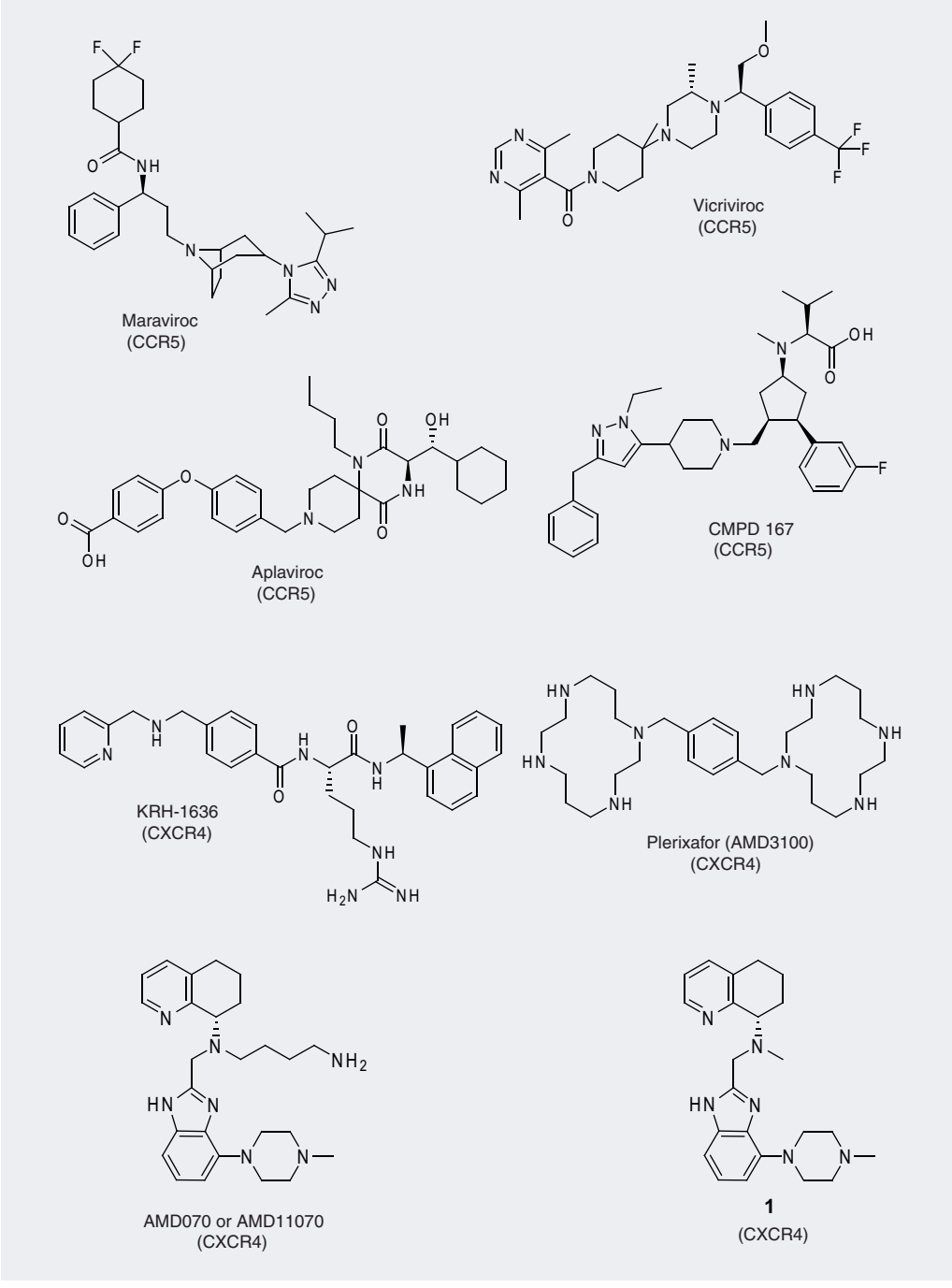


Figure 2. Small-molecule co-receptor antagonists as inhibitors of HIV entry in CD4+ cells.
The receptor targeted is in brackets.

2005 for dose-related liver toxicity problems. However, recent reports have concluded that co-administration of aplaviroc with the immunomodulatory drug rapamycin, which is able to reduce CCR5 density (receptors/cell), enhances the antiviral activity of aplaviroc, allowing a reduction of active concentration to nontoxic doses (as much as 25-fold *in vitro)* and could provide an effective means to control infection [47]. Optimization of aplaviroc's spirodiketopiperazine framework with improved pharmacokinetic profiles has been reported very recently by Ono Pharmaceuticals [48].

Vicriviroc contains a piperazine scaffold **(FIGURE 2)** and was developed by Schering-Plough starting from screening data; it exhibited

excellent selectivity for CCR5 receptors but clinical trials were recently interrupted, at least for the treatment of naive patients [201].

Maraviroc is the only CCR5 inhibitor currently approved by the FDA (Selzentry). It is based on a tropane scaffold **(FIGURE 2)** and its development, starting from high-throughput screening (HTS) leads, has been reviewed elsewhere [42,43]. The development and optimization of a new class of CCR5 antagonists replacing the tropane core of maraviroc by piperidine was recently described by Pfizer [49,50].

Other recent reports on the discovery and optimization of novel CCR5 antagonists as inhibitors of HIV-1 infection have uncovered selective and potent analogs, which include structural elements from the previous chemotypes [51–54] or novel, gem-disubstituted azacycles and one octahydro pyrrolo[3,4-c]pyrrole scaffold [55]. The different CCR5 antagonists, despite being rather diverse in shape and electronic properties, are all predicted to bind to a putative hydrophobic binding pocket located in the transmembrane domain of the receptor [56–58].

Macromolecules capable of antagonizing CCR5 include modifications of its natural ligand, the CCR5 chemokine also known as RANTES [59]. Notably, RANTES is not the only natural ligand of CCR5, as other chemokines, including MIP-1α, MIP-1β (its variant LD78β) and MCP2 can bind this receptor. Monoclonal antibodies have also been developed that recognize epitopes on CCR5 [60]. Their use in combination with small molecule antagonists has been recently reported as a promising new strategy for anti-HIV-1 therapy [61,62].

The success of some compounds such as maraviroc, approved by the FDA for clinical use, validates viral entry inhibitors as a new class of antiretroviral drugs for clinical use. However, some problems remain. The HIV envelope glycoprotein is highly variable with diverse genotypes and, hence, the susceptibility of viral strains to different antagonists targeting the viral envelope glycoprotein may vary significantly. In addition, for the appropriate selection of treatment with co-receptor antagonists, HIV patients must initially be analyzed for the tropism of the virus they harbour. Moreover, because CCR5 and CXCR4 co-receptors are host cellular targets, the long-term effects and safety of drug use must also be closely monitored.

With increasing knowledge of the HIV entry process, other targets involved in different stages of viral entry have emerged and will be discussed in the following sections.

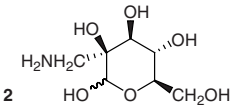
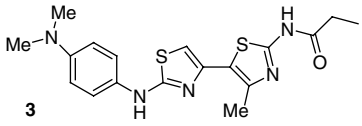
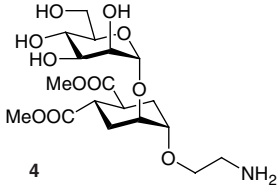
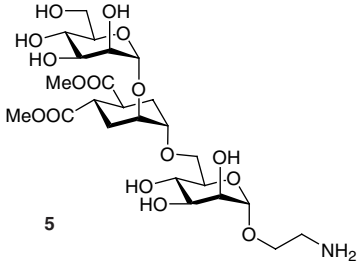
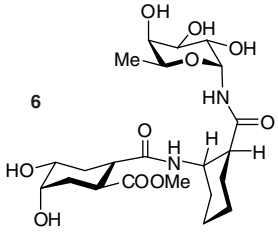
■ DC-SIGN inhibitors

A potential therapeutic target was recently identified in DC-SIGN, a receptor involved in the early stages of HIV infection [11]. DC-SIGN is a tetrameric calcium-dependent (C-type) lectin, expressed by dendritic cells (DC), which specifically recognizes highly glycosylated structures displayed at the surface of several pathogens [63–66]. Recognition by DC-SIGN was reported to play a key role in HIV transmission and is considered an interesting new target for the design of anti-viral agents [67–71].

DC-SIGN$^+$ immature DCs, located in vaginal, cervical and rectal mucosae are among the first cell types to encounter HIV during sexual transmission. DC-SIGN expressed by DC at mucosal tissues captures HIV at low titre by binding the envelope glycoprotein gp120. The lectin appears to act as an attachment factor, rather than an entry receptor, binding and concentrating HIV on the cell surface. Initial reports described two different pathways that may be involved in the transmission of DC-SIGN-bound HIV to T lymphocytes [72,73]. The first one, responsible for short term HIV transfer (24 h after HIV exposure), involves virion internalization into intracellular compartments where the virus is protected from degradation and retains a high infective capacity during DC migration to lymphoid tissues (the 'Trojan horse model') [74]. The second, involved in long-term HIV transfer (72 h after exposure), follows DC infection in *cis* by transfer of DC-SIGN-bound virus to canonical HIV entry receptor (CD4 and CCR5) resulting in infected DC with a continuous production of virus for the T-cells [73,75]. In both cases, upon arrival at lymphoid tissues DC efficiently transmit HIV to CD4+ T lymphocytes, a process called infection in *trans*. These models have been challenged [76] and other reports have shown that the majority of virus internalized by DC is regularly processed by major histocompatibility complex presentation [77,78]. On the other hand, HIV sequestration by and stimulation of DC-SIGN was reported to help HIV evade immune responses and spread to cells by alternative pathways, such as the triggering of activities required for HIV replication [79] or induction of immunosuppressive signals[12–17]. A timely and comprehensive discussion of the role of DC-SIGN in HIV infection has recently been published [80].

Despite uncertainties about the mechanism of action of this lectin, various groups are working to design molecules capable of blocking the interaction between DC-SIGN and HIV, with

Table 2. Known monovalent ligands of dendritic cell-specific ICAM-3 grabbing nonintegrin.

Entry	Compound	IC_{50} (mM)	Inhibition (%)	Assay	Ref.
1	 **2**	0.35†		SPR (gp120 chip)	[69]
2	Manα-1,2-Manα-1,2-Man		73‡	Microarrays	[141]
3	Manα-1,2-Manα-1,6-Manα-1,6-Man		73‡	Microarrays	[141]
4	 **3**	1.6 x 10^{-3}§		Competition with immobilized DC-SIGN	[68]
5	 **4**	0.62¶		Ebola *cis*-infection model	[67]
		0.6# 1.0††		SPR (inhibition of ManBSA binding)	[86]
6	 **5**	0.05# 0.125††		SPR (inhibition of ManBSA binding)	[86]
7	Lewis-x trisaccharide	0.8#,‡‡		SPR (inhibition of ManBSA binding)	[70]
8	 **6**	0.35#,‡‡		SPR (inhibition of ManBSA binding)	[70]

†*IC_{50} of mannose in the same assay 17 mM.*
‡*100% is Man_9 in the same assay.*
§*IC_{50} of ManNAc in the same assay 6.9 mM.*
¶*Manα-1,2-Manα-1-$OCH_2CH_2NH_2$ is 1.9 mM in the same assay.*
#*IC_{50} of mannose in the same assay 1.8 mM.*
††*IC_{50} of mannose in the same assay 2.5 mM.*
‡‡*IC_{50} of fucose in the same assay 1.2 mM.*
DC-SIGN: Dendritic cell-specific ICAM-3 grabbing nonintegrin; Man: Mannose; ManBSA: Mannose bovine serum albumin; SPR: Surface plasmon resonance.

the goal of disclosing a new strategy to prevent HIV transmission and infection of the host. A handful of compounds have been described so far **(Tables 2 & 3)**. The main natural ligands recognized by the carbohydrate recognition domain of DC-SIGN are the high mannose glycan, $(Man)_9(GlcNAc)_2$ also known as Man_9, and a group of fucosylated oligosaccharides bearing Lewis-type epitopes [74]. Highly mannosylated polyvalent compounds, structural analogues of Man_9 terminal di- or trisaccharides, and mimics of the Lewis-x trisaccharide have been investigated and found to be capable of antagonizing binding of gp120 to DC-SIGN. Although most of the work was concerned with mannose or mannose analogues, a fucose-based antagonist **(6)** was recently reported (entry 8; **Table 2**) [70]. The ligand is based on a α-fucosylamide anchor and inhibits DC-SIGN binding to mannosylated bovine serum albumin (BSA) with potency

similar to, or slightly better than, the natural ligand's Lewis-x. This molecule represents an interesting lead for further optimization.

Multivalent presentation of mannose using hyperbranched dendritic polymers of Boltorn type were shown to bind DC-SIGN and inhibit interaction with gp120-coated surface plasmon resonance (SPR) chips (entry 1; **Table 3**) [81–83]. The Wong group reported oligomannose dendrons that display complex oligomannoses in high density (entries 2 & 3; **Table 3**) and inhibit binding of gp120 to recombinant dimeric DC-SIGN with IC_{50} in the nanomolar range [84]. These systems are excellent models of DC-SIGN inhibition, but their practical application as therapeutic agents seems unlikely, given the complexity of the oligosaccharides used. Penadés, Alcami and their groups have reported that gold nanoparticles (GNP) displaying various linear and branched mannosyl oligosaccharides (Manno-GNPs, entries 4 & 5; **Table 3**) are potent inhibitors of DC-SIGN mediated HIV *trans*-infection of human activated peripheral blood mono-nuclear cells [85]. However, *in vivo* use of GNPs raises some concerns, mostly because of the potential toxicity produced by gold accumulation.

Noncarbohydrate inhibitors with IC_{50} values in the low micromolar range, such as compound **3** (entry 4; **Table 2**), were identified by Kiessling and Borrok via HTS of approximately 36,000 compounds from commercial libraries [68]. Additional results from the Fleet group indicate that 2-C-substituted branched D-mannose analogues such as compound **2** (entry 1; **Table 2**) bind to DC-SIGN with significantly greater affinity than mannose [69]. We have described two glycomimetic compounds, **4** and **5** (entries 5 & 6 respectively; **Table 2**), which are structural mimics of linear di- and tri-mannosides and exhibit moderate anti-infective action against DC-SIGN-mediated infections by HIV and Ebola [67,86]. A tetravalent dendron containing four copies of the linear trimannoside mimic **5** (entry 7; **Table 3**) was found to inhibit DC-SIGN mediated HIV *trans*-infection of $CD4^+$ T lymphocytes at low micromolar range. The anti-infective action is exerted upstream of

Table 3. Affinity comparison for polyvalent ligands of dendritic cell-specific ICAM-3 grabbing nonintegrin.

Entry	Type of sugar	Support	Valency	IC_{50}	Rel. Pot.	Assay	Ref.
1	Man	3G Boltorn dendrimer	32	0.337 μM	118	Ebola *cis*-infection model	[82]
				0.1 μM		SPR (high-density DC-SIGN ECD)	[83]
				50 μM		SPR gp120 chip	[81]
2	Man_4 (Manα-1,2-Manα-1,2-Manα-1,3-Manα- R)	2G alkynyl dendrimer	9	0.020 μM†		gp120 –Fc-DC-SIGN ELISA	[84]
				0.16 μM		Competition with Man_4-coated array	
3	Man_9	2G alkynyl dendrimer	9	0.008 μM†		gp120 –Fc-DC-SIGN ELISA	[84]
				0.026 μM		Competition with Man_4-coated array	
4	Manα-1,2-Man	GNP	22	2‡–37§ nM		HIV *trans*-infection model	[85]
			25	0.12 μM 100% inhibition	2.9	SPR gp120 chip	[141]
5	Man_4 (Manα-1,2-Manα-1,2-Manα-1,3-Manα- R)	GNP	56	0.34‡–0.83§ nM		HIV *trans*-infection model	[85]
6	Man	Tetravalent boltorn dendron	4	50 μM		HIV *trans*-infection model	[86]
7	**5** (mimic of Manα-1,2-Manα-1,6-Manα)	Tetravalent boltorn dendron	4	5 μM		HIV *trans*-infection model	[86]

†Man is 8.5 mM in the same assay.
‡JR-Renilla viral strain (R5).
§NL-Renilla viral strain (X4).
DC-SIGN: Dendritic cell-specific ICAM-3 grabbing nonintegrin; ECD: Extracellular domain; GNP: Glyconanoparticle; Man: Mannose; SPR: Surface plasmon resonance.

cytokine involvement and, therefore, is independent of viral tropism, as shown by inhibition of a series of laboratory-adapted strains and primary isolates with different tropism. This tetravalent compound presents high solubility in physiological media, a neglectable cytotoxicity and a long-lasting effect and was found to be stable for at least 1 week at pH 5 (an acidic medium close to that found at the vaginal mucosa, pH 4). Time-course studies showed that the antiviral effect of this compound persists for hours, even after the B-THP/ DC-SIGN$^+$ cells used were exposed to HIV after washing out the antagonist. The mechanism of this inhibition after removal may be based on the persistency of the multivalent ligand on the receptor binding site (slow off-rate of the tetravalent compound from the protein). However, flow cytometry studies suggested that exposure of B-THP/DC-SIGN$^+$ cells to this compound may also alter the observed cell surface concentration of DC-SIGN, possibly by induced endocytosis. Depletion of receptor membrane concentration is an interesting feature of this compound and it could at least partially account for its antiviral activity.

Inhibition of DC-SIGN is not devoid of potential drawbacks. First and foremost, efficient inhibition of this lectin will prevent recognition of other pathogens besides HIV and, thus, may lead to a generalized reduction of the host immune response. This effect may be limited by topical, rather than systemic, use of inhibitors.

Virus targets

■ gp120 binders

Polyanions are one of the most popular groups of compounds to be formulated as microbicides due to their low cost and easy production. These compounds interact with the positively charged region of gp120, basically the conservative V3 loop, inhibiting the interaction with negative areas of the cell membrane surface. Different formulations of these compounds have reached clinical Phase trials **(TABLE 4)**. PRO 2000/5 or PRO-2000, a formaldehyde-sodium 2-naphthalenesulfonate polymer, was recently demonstrated to be ineffective in Phase III clinical trials [24]. SPL7013, a lysine-based dendrimer with naphthalene disulfonic acid surface groups, also known as VivaGel™, is in Phase I/II clinical trials [87]. Dextrin sulfate (DxS) is also currently in clinical trials [88]. Some of these polyanions, in contrast to their very good *in vitro* activity, have failed to demonstrate efficacy in preventing HIV transmission in Phase III clinical trials, as was the case of cellulose sulfate (CS), terminated prematurely in early 2007 [89], and Carraguard [90]. Although some of these polyanion compounds enhance HIV infection, apparently this effect is not related to the nature of the polyanion, but to the assay conditions and therefore cannot be extrapolated [91].

CD4 is considered to be the main receptor for the HIV-1. So, gp120-CD4 interaction has been one of the first targets against which anti-HIV drugs were developed. In 1990 it was reported that recombinant soluble CD4 (sCD4) protein was able to block HIV entry and subsequent infection *in vitro* [92]. However, Phase I/II clinical trials demonstrated that higher concentrations of this sCD4 were needed to inhibit infection *in vivo* using HIV primary isolates. Apparently, the binding of sCD4 to gp120 should produce conformational changes on the gp120 envelope protein, leading to the exposure of co-receptor binding sites. These changes could allow gp120 to bind the chemokine receptor at the cell surface, inducing virus–cell membrane fusion and virus entry [93]. This fact could explain the results found in the infection

Table 4. Current status of polyanions as microbicides.

Compound	Administration	Composition	Company	Current status	Ref.
PRO2000	Topical 0.5% gel	Formaldehyde-sodium 2-naphthalenesulfonate polymer	Indevus Pharmaceuticals	Failed in Phase III trials	[24]
SPL7013	VivaGel (3% SPL7013 gel)	Lysine dendrimer with naphtalene disulfonic acids	Starpharma	Phase I/II trials	[87]
Carraguard	Gel from carrageenan	Sulfated polysaccharide		Ineffective in Phase III trials	[90]
Dextrin sulfate	Oral or intravenous administration	Polysulfate dextran		Phase I/II trials	[88]
Cellulose sulfate	(Ushecell) Topical 6% gel	Polysulfate cellulose	Polydex Pharmaceuticals	Ineffective in Phase III trials	[89]

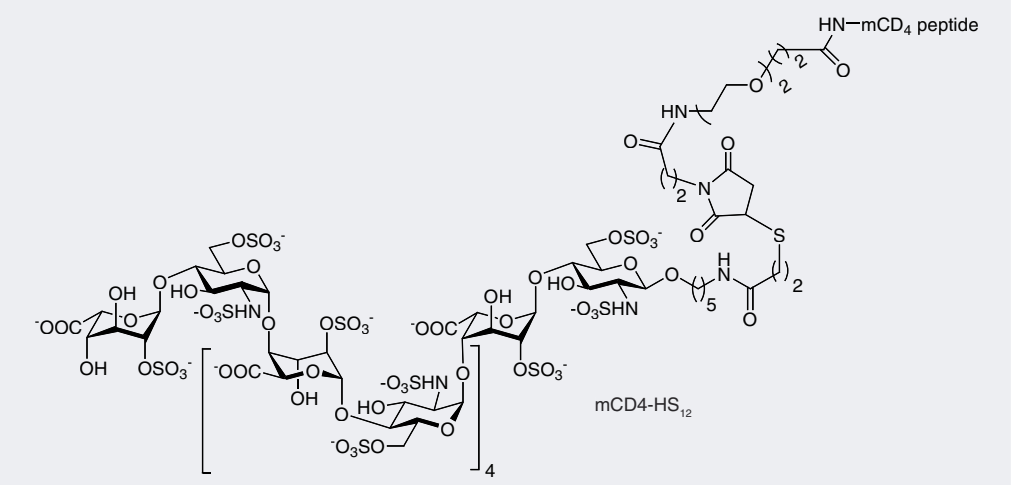


Figure 3. Heparan sulfate dodecasaccharide conjugated with CD4 peptide mimic (mCD4-HS_{12}).

experiments. A detailed mechanistic study of the binding process of sCD4 (or a mimic) to the gp120 was carried out. Moreover, it was pointed out that the conformational changes caused by binding could expose neutralization epitopes on gp120, which were responsible for eliciting the generation of neutralizing antibodies as part of a strategy to develop vaccines [94,95]. There are some concerns with this approach, most of them refering to the problem of eliciting antibodies against the CD4 molecule. Crosslinked gp120-CD4 or similar strategies producing more stable complexes could avoid these problems.

In this context, a new strategy to inhibit HIV entry was very recently developed. This strategy was based on a neoglycoconjugate (mCD4-HS_{12}) that covalently combines a heparan sulfate dodecasaccharide (HS_{12}) and a CD4 peptide mimic (mCD4; **Figure 3**) [96]. The peptide was synthesized according to a solid-phase approach and a maleimido group was introduced on the Lys-5 side chain for sugar conjugation. The heparan sulfate dodecamer was prepared with a sulfydryl moiety at the reducing end allowing the conjugation to the maleimido-activate peptide. This conjugate simultaneously targets in a cooperative manner two highly conserved domains of gp120 that are critical for virus entry: the co-receptor and the CD4 binding sites. A clear synergistic effect was observed in comparison with the activity presented by the isolated moieties of the glycoconjugate. The most interesting feature of the binding mode resides on the first binding event of the mCD4 peptide to gp120, exposing the co-receptor binding site and allowing, in a second step, binding of the sugar moiety [96]. mCD4-HS_{12} binds to gp120 in a 1:1 molar ratio with a dissociation constant in the low nanomolar range, as demonstrated using surface plasmon resonance. Moreover, infection studies on blood mononuclear cells showed a dose-dependent inhibition effect independently of the viral strain (R5, X4 or dual tropism R5/X4). The inhibition activity was shown to be based on the blockage of the virus envelope [96].

Neutralizing antibodies are very attractive owing to their potential application as entry inhibitors of HIV as well as their importance for the development of vaccines against this virus [97,98]. Several monoclonal antibodies have been characterized showing different activities and selectivities. Those that present moderate to strong potency with a broad spectrum of recognition are the b12 antibody (interacting with the CD4 binding site on gp120) [99], the 2G12 antibody (which recognizes the carbohydrate epitope Manα1,2Man on gp120) [100] and 2F5 antibody (which interacts with the membrane proximal region on gp41) [101]. Active research in this area is ongoing and may open the door for the design and production of more effective and broad antibodies with therapeutic applications in HIV infection [102,103].

Carbohydrate-binding proteins (CBPs) are one of the most classical sources of compounds that exhibit antiviral activity through the interaction with glycans presented on gp120. These lectins can be isolated from different species such as plants, cyanobacteria and algae, and have recently been reviewed elsewhere [104,105]. Two of these CBPs have been studied more

extensively: cyanovirin N (CV-N) from cyanobacteria [106] and griffithsin (GRFT) from *Griffthsia sp* algae [107].

Cyanovirin N has been tested *in vivo* as a topical gel for vaginal [108] and rectal [109] application in macaques using the chimera SHIV as infective agent. In these studies, relevant antiviral activity was found demonstrating a promising application as microbicide. The potent antiviral activity has recently been evaluated in cellular and cervical explant models using different primary HIV-1 clinical isolates. *Ex vivo* experiments showed that CV-N was able to inhibit infection of cervical explants by HIV-1 (BaL strain) with an IC_{90} of 1 µM [110]. In addition, as preclinical evaluation, the antiviral activity was analyzed in the presence of semen, which produced a modest decrease in the antiviral activity of cyanovirin-N [110]. More significant were the safety concerns due to mitogenic properties of this lectin [111]. Low levels of T-cell proliferation were found after three days' exposure of cells to CV-N. This fact should be studied in more detail for the potential topical application of CV-N as a microbicide.

To date, GRFT isolated from marine red algae and characterized in 2005 is the most potent anti-HIV inhibitor described, with EC_{50} in the picomolar range [107]. The spectra of activity include HIV-1 clades A, B, and C. The mechanism of GRFT as an entry inhibitor centres on the binding, in a calcium-nondependent manner, to the sugars present on the envelope glycoprotein gp120, blocking the interaction of HIV to target cells. The high activity found for GRFT in comparison with other lectins such as CV-N has been explained based on the possibility of multivalent binding with gp120. This is possible because GRFT contains six carbohydrate-binding sites per homodimer. What makes this potential virucide especially interesting is the lack of toxicity and the extreme stability under a variety of physical conditions including low pH and temperatures up to 80°C [111]. Despite the high antiviral activity showed by these protein inhibitors, clinical trials have not yet been carried out. The main drawbacks of these potential drugs are the high production cost and the accessibility of the amounts of material required to perform clinical studies. In the particular case of GRFT, this problem has been successfully addressed by the production of recombinant GRFT produced in plants. The protein isolated from the plant (GRFT-P) showed the same antiviral activity as the natural protein. This approach allows the production of more than 1 g of protein (purity >99%) per kg of *Nicotiana benthamiana* leaf material, which is a very reasonable amount, suitable for clinical test formulations as a microbicide [112]. As stated in this publication, this technology will provide the necessary production of GRFT to proceed into clinical tests. Furthermore, it was demonstrated that this lectin had no toxic effects *in vitro* and *ex vivo* human cervical explants, and has not induced inflammatory response in vaginal epithelial tissues, which indicates a safety profile adequate for their human application. At this stage, griffithsin can be considered one of the most promising candidates for use as microbicide.

In general terms, the availability, synthetic cost, stability and immunogenic effects associated with peptides or proteins as drugs for clinical use, have led to the search for new strategies to design new carbohydrate-binding agents (CBAs) with nonpeptidic structures. Kiser and co-workers have developed an approach based on a polymer bearing several copies of *O*-hydroxymethylphenylboronic acid (benzoboroxole; **Figure 4**) [113]. At physiological pH, benzoboroxole interacts with the hydroxyl groups of manno- and galactopyranosides, carbohydrate units abundantly present on viral glycoproteins such as gp120. In these studies, it was demonstrated that these polymers exhibit strong antiviral activity inhibiting the infection of peripheral blood mononuclear cells by HIV of two different clades (B and C) and two co-receptor tropism (X4 and R5). The activity was related to the percentage of boronic acid present in the polymer, the most active being a polymer with a 75% functionalization (~ 450 benzoboroxoles) with an EC_{50} of 10 nM independently of the HIV strain used in the infection studies. These

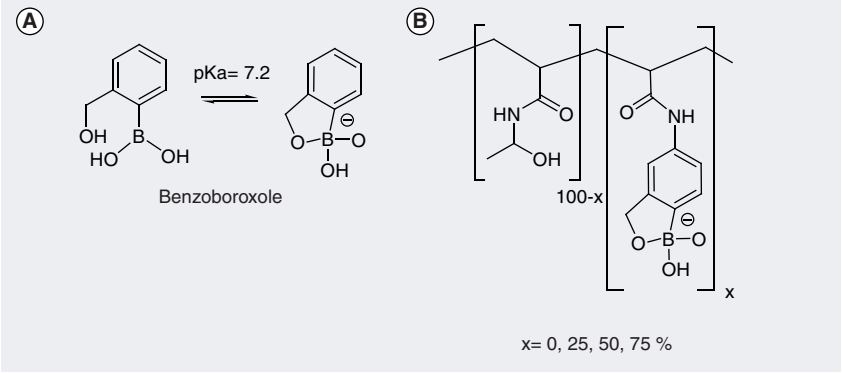


Figure 4. Benzoboroxole (A) equilibrium and (B) polymer derivative.

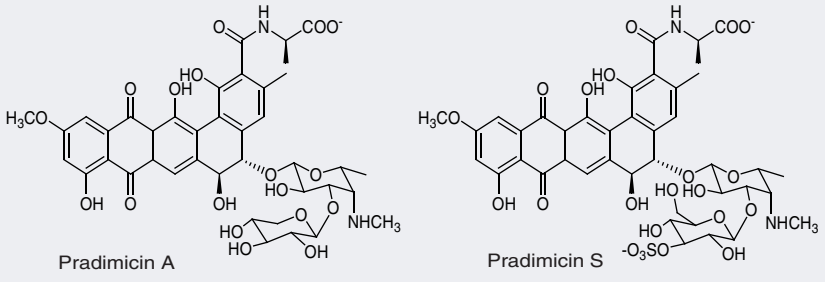


Figure 5. Pradimicin A and pradimicin S.

polymers exhibit low toxicity in the vaginal cell line used, at least for the highest concentration tested (two orders of magnitude > EC_{50}). The inhibition activity exhibited by these polymers are likely based on preventing binding of gp120 to CD4, the co-receptor, or both but this point was not clarified. The spectrum activity and the efficacy is comparable with CV-N but with the advantage of a more economically scalable production for application, which makes this compound very promising as microbicide, although *in vivo* activity still needs to be tested.

Among nonpeptidic compounds, benzonaphtacenequinone antibiotics pradimicin A (PRM-A) [114] and pradimicin S (PRM-S; **Figure 5**) [115] have a very interesting antiviral activity through interaction, in a calcium-dependent manner, with carbohydrates of the viral envelope glycoprotein gp120. PRMs were considered as CBA leads for HIV therapy with a novel therapeutic concept for a potential dual mechanism proposed for PRM derivatives. This dual mechanism consisted of a direct antiviral activity blocking HIV entry and a second action based on progressively inducing deletion of glycosylation sites on gp120, which induces neutralization through the activation of the immune system against previously hidden immunogenic epitopes. Their low toxicity, stability and broad spectrum of activity against HIV-1 (different clades), HIV-2 and SIV make these compounds good candidates for development as microbicides.

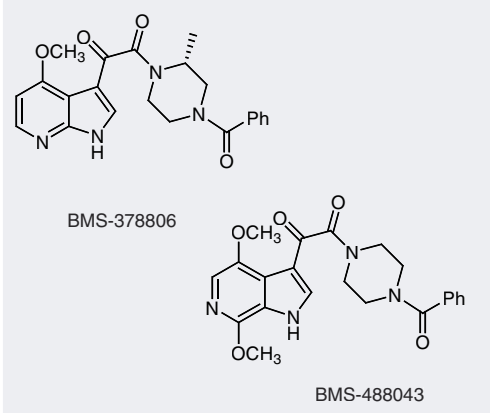


Figure 6. Azaindole derivatives BMS-378806 and BMS-488043.

In 2003, a small molecule based on a 4-methoxy-7-azaindole derivative named BMS-378806 **(Figure 6)** was described as a viral entry inhibitor of HIV [116]. This molecule showed a broad spectrum of activity against several HIV-1 laboratory strains and clinical isolates of the B subtype with an average EC_{50} of 40 nM [117]. For other virus subtypes, the antiviral activity of this compound decreases. This activity was independent of the HIV-1 co-receptor used (R5, X4 or R5/X4). The activity of this molecule resides on the interaction with the CD4 binding pocket of the viral envelope gp120 with a 1:1 stoichiometry. Initial toxicology test and pharmacokinetics assays demonstrated the potential application and the possibility to enter into clinical studies. Optimization of this molecule led to the identification of a 6-azaindole derivative BMS-488043 **(Figure 6)** [118]. This molecule has improved the pharmacokinetic profile of BMS-378806 but besides the oral bioavailability, drug dissolution in the gastrointestinal tract should be improved using additional formulation approaches.

BMS-378806, in combination with other inhibitors, CMPD167 (a CCR5 inhibitor) and the peptide C52L (a fusion inhibitor) acting at different stage of virus–cell attachment was tested *in vitro* and *in vivo* [40]. In this study, it was clearly demonstrated that the combination of the three compounds delivered vaginally protected macaques against infection by SHIV inoculated vaginally. This approach opens up the possibility of combining different available inhibitors in a synergistic manner, as a very powerful strategy against HIV infection.

■ gp41 binders (fusion inhibitors)

Fusion inhibitors are a class of entry inhibitors that interfere at some point with the helical regions domains (HR1 and 2) of gp41 involved in the 6-helix bundle formation, a step needed for membrane fusion, the process that proceeds to cell penetration. This type of HIV entry inhibitor has been reviewed very recently [119]. The discovery of the mechanism of HIV membrane fusion has allowed the

design of potential inhibitors interfering with the protein–protein interactions that trigger the fusion step. Peptides interfering with this protein–protein interaction were envisaged as potential therapeutic compounds inhibiting HIV entry. T20 (enfuvirtide), a 36-mer peptide, was the first generation of HIV fusion inhibitors. This peptide is, for the moment, the only one approved by the FDA (in 2003) for clinical use in the treatment of AIDS [120]. The appearance of resistant strains due to the application of enfuvirtide induced the development of new generations of fusion inhibitors such as T1249, a 39-mer peptide with higher activity than T20; C52L, a sequence-modified version of T20; T1144, a 38-mer peptide effective against T20-resistant HIV strains; sifurvitide, a 36-mer peptide with better activity than T20; C34, derived from the HR2 of gp41, all of which have inhibitory activities in the nanomolar range [121,122]. New strategies were suggested, including a combination of two or more peptide-fusion inhibitors looking for a synergic activity [122] or conjugation of cholesterol (as a lipid anchor on the cell membrane) to a peptide fusion inhibitor (C34-Chol), producing a conjugate which was 50-fold more potent than the corresponding peptide [123]. In all cases, the stability and production of these peptides in large quantities proved to be an important drawback of this approach. In this context, D-peptides constructed by D-amino acid were considered a very powerful strategy to avoid the stability problems affecting natural peptides [124]. In fact, a relevant example of the potent activity exhibited by these peptides has recently been reported with a D-peptide exhibiting an EC_{50} of 250 pM [125].

As a different approach for searching fusion inhibitors, nonpeptidic small molecules were considered. Natural triterpenes derivatives based on betulinic acid (BA; **FIGURE 7**) were described some time ago as potent antiviral compounds inhibiting HIV infection at an early stage of the infectious cycle, during the membrane fusion step [126]. These compounds were the first described with nonpeptidic nature that presented this activity. It has been reported that modification of the side chains can modulate the activity and the mechanism of action. Even more, depending on where these side chains (R_1, R_2 and R_3) are placed, the mechanism of action can change and the compounds can act as entry inhibitors or as a maturation inhibitors **(FIGURE 7)** [127]. One interesting example is bevirimat **(FIGURE 7)**, currently in Phase IIb clinical trials launched by Panacos Pharmaceuticals, Inc. and now developed by Myriad Genetics as a maturation inhibitor [128]. Compounds with no substitution in position C3 (R_3= H) interact with the V3 loop of HIV-1 inhibiting chemokine receptor binding. It seems that this inhibition stops the required conformational changes in gp41 needed for membrane fusion.

Betulinic acid derivates exhibit a broad spectrum of activity against several HIV-1 laboratory strains and clinical isolates. Unfortunately, the clinical development of one of these derivatives (RPR 103611) by a pharmaceutical company was dropped because pharmacodynamic properties were found to be inadequate. Very recently, Lee and co-workers completed the synthesis of a large series of different BA derivatives with the aim of improving their properties and analyzing the effect of substituents on the inhibitory activity [129]. One of these compounds showed good solubility and the same range of activity as the previous best hit A43-D.

N-carboxyphenylpyrrole derivatives were found to be very promising molecules targeting gp41 [130]. In a hit-to-lead optimization

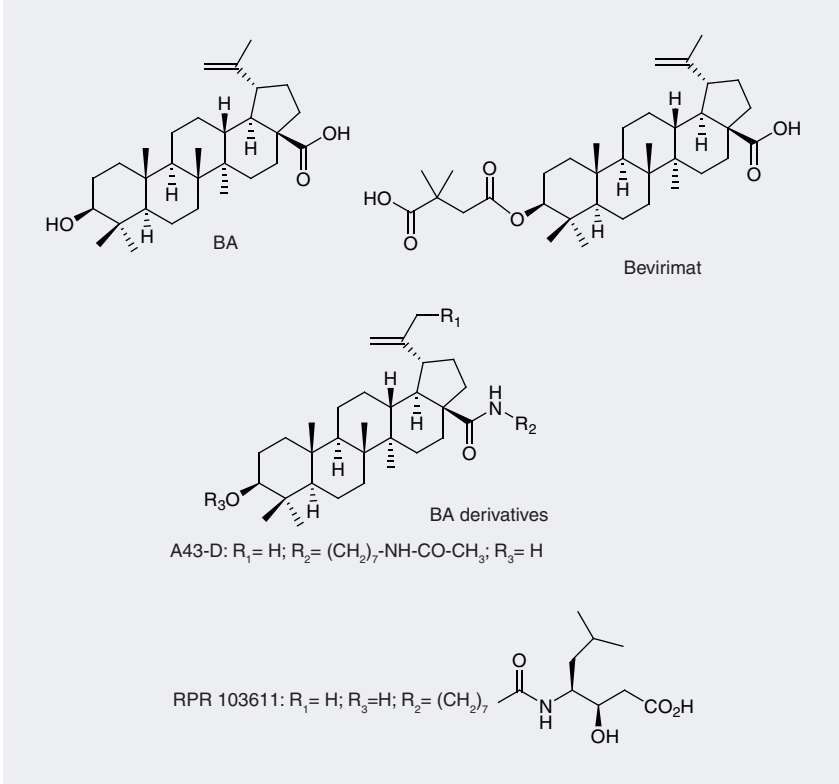


Figure 7. Betulinic acid and derivatives showing antiviral activity.
BA: Betulinic acid.

process, a molecule indicated as A12 **(FIGURE 8)** was selected as the best inhibitor able to inhibit the gp41 6-helical bundle formation, with an EC_{50} of 37.36 μM [131]. Using A12 as a starting point, a more potent candidate, GLS-22, was obtained using a computer-aided *de novo* drug-design methodology based on GeometryFit. This new *N*-carboxyphenylpyrrole derivative showed better activity (sixfold better than A12) as fusion inhibitor in HIV replication assay, low cytotoxicity (CC_{50}: 227–355 μM) and the highest selectivity index (CC_{50}/IC_{50}: 51.99) [132]. Very recently, a study to identify compounds as frequent hitters in biological high-throughput screens has been published. In this study, the structure of the A12 type compounds is described as one of the problematic leads, leading to questions regarding their applicability [133].

Another type of small molecule was identified as a nonpeptide entry inhibitor by NMR and docking experiments using a model of gp41 protein (Protein-1). From these studies, a series of molecules was selected, the best of which was **7 (FIGURE 9)** with an EC_{50} of 3 μM in a cell–cell fusion assay *in vitro* [134]. This value is in the same order of magnitude as the activity obtained with D-peptides, but still far from that of the C-peptide (EC_{50}: 4 nM). It is clear that this compound could be a potential hit for optimization.

Conclusion & future perspective

In the continuing absence of an effective vaccine, the use of a topical microbicide represents a credible alternative method for reducing the sexual transmission of HIV. However, so far, no compounds have been shown to prevent HIV transmission in efficacy trials. One currently emerging response to the failure of first-generation candidates is the use of multiple microbicides to block the different mechanisms

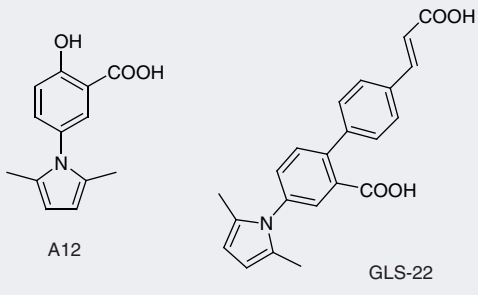


Figure 8. A12 and GLS-22.

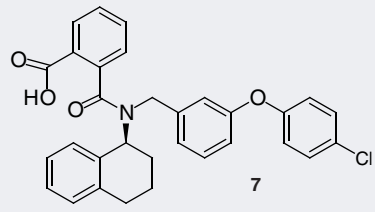


Figure 9. Bezamine derivative 7.

of HIV transmission [40,47,61,62,135,136]. The combination of microbicides with complementary mechanisms of action is expected to increase the potency of the formulation and to be one possible answer to the problems posed by HIV-1 diversity. Indeed, the combinations reported so far were all found to display synergistic activity in infection assays, so that, at lower concentrations, double and triple combinations were generally more effective than individual inhibitors [40,137,138]. The chances of a virus being simultaneously resistant to three compounds is less than to any single inhibitor, a principle well established from clinical experience with drug-based therapies for HIV-1 infection [139]. In addition, drug combination could decrease the risk of selection of antiretroviral drug-resistant strains and, finally, the combination of CXCR4- and CCR5-specific inhibitors may be useful for countering the transmission of dual-tropic viruses.

The spread of HIV infection will not slow down and, if anything, will accelerate. Actual therapeutic approaches to HIV infection are highly effective. These therapies, nevertheless, are life-long and are associated with potentially life-threatening side effects. Additionally, the economic burden of treatment for HIV infection occupies an increasing share of healthcare expenditure, making current practices likely to become hard to sustain in the long run. The need for effective ways to prevent HIV infection and the difficulties in designing an effective vaccine will drive a strong worldwide effort to develop effective microbicides. It is likely that glycans/lectins (compounds that are easy to make, economic and easy to store) will emerge as the best candidates. In 10 years from now, we will probably not have an effective vaccine, but we might have very strong and positive results from Phase III trials based on the prevention of HIV transmission using glycans/lectins or CCR5 inhibitor-based microbicides.

Financial & competing interests disclosure

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Executive summary

- HIV infection is still highly incident; no effective vaccine is foreseeable in the near future.
- HIV infection can be controlled but not cured by therapy, thus, prevention is indispensable.
- Mucosal HIV infection is a complex, multistep process that can be prevented by the use of condoms. Ethical and cultural problems can reduce the use of condoms.
- Microbicides capable of preventing HIV infection would have a major impact on the spread of disease; various compounds have been tested *in vitro* and/or *in vivo*, but so far, no effective compound has emerged.
- HIV entry inhibitors, targeting glycans/lectins, CD4, CCR5, or the 'fusion peptide' are highly attractive candidates for preventing mucosally transmitted HIV infection.
- A combination of microbicides targeting different steps of viral entry shows a synergistic effect and may result in more effective therapies and formulations.
- Effective microbicides must be safe and nontoxic; devoid of inflammatory activity; resistant to seminal fluid low pH and body temperature; easy and simple to use (e.g., nonoily and nonleaking); economical; simple to produce and store and endowed with durable activity.
- Effective microbicides must be able to protect against both receptive anal and vaginal intercourses.

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