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Attachment and fusion inhibitors potently prevent dendritic cell-driven HIV infection

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Abstract

Dendritic cells (DCs) efficiently transfer captured (*trans*) or *de novo* produced (*cis*) virus to CD4 T cells. Using monocyte-derived DCs we evaluated entry inhibitors targeting HIV envelope (BMS-C, T-1249) or CCR5 (CMPD167) for their potency to prevent DC-infection, DC-driven infection in T cells in *trans* and *cis*, and direct infection of DC-T cell mixtures. Immature DC-T cultures with distinct mechanisms of viral transfer yielded similar levels of infection, and produced more proviral DNA compared to matched mature DC-T cultures or infected immature DCs. Although all compounds completely blocked HIV replication, 16 times more of each inhibitor (250 vs 15.6nM) was required to prevent low-level infection of DCs compared to the productive DC-T cell cocultures. Across all cell systems tested, BMS-C blocked infection most potently. BMS-C was significantly more effective than CMPD167 at preventing DC infection. In fact, low doses of CMPD167 significantly enhanced DC-infection. Elevated levels of CCL4 were observed when immature DCs were cultured with CMPD167. Viral entry inhibitors did not interfere with *Candida albicans*-specific DC cytokine/chemokine responses. These findings indicate that an envelope-binding small molecule is a promising tool for topical microbicide design to prevent the infection of early targets needed to establish and disseminate HIV infection.

Keywords

Dendritic cells; HIV transmission; HIV envelope-binding small-molecule inhibitor; CCR5 antagonist; fusion inhibitor

Introduction

HIV mucosal transmission represents the principal route of virus acquisition, particularly of R5-tropic virus^{1, 2}. In the initial phase of infection, HIV crosses the mucosal epithelial barrier likely within hours³ to access target cells in the submucosa including CD4 T cells, DCs and macrophages⁴. Small local founder populations of infected cells are generated^{5, 6} that then undergo local expansion to produce enough virus and infected cells that can migrate to proximal lymphoid tissue to establish a systemic infection^{5, 7-9}. More recently, sequencing viruses in heterosexual transmission pairs and in acute HIV infection provided evidence that a single virus (or infected cell) initiated productive infection in close to 80% of the individuals tested, with the remaining individuals infected by between two and five viruses^{10, 11}. This underscores the challenges faced in preventing HIV infection and spread. Productive infection of a cell is initiated through attachment of HIV envelope to CD4. This interaction triggers conformational changes in the viral envelope that enable HIV to bind to

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its coreceptor CCR5 or CXCR4¹²⁻¹⁴. Further conformational changes in the envelope lead to the formation of a fusion pore mediating infection of the target cell^{15, 16}.

The availability of CD4 and CCR5 molecules on immature DCs render them vulnerable to infection via fusion. While immature DCs produce much less virus compared to CD4 T cells¹⁷⁻¹⁹, limited amounts of *de novo* produced virions are efficiently transmitted (in *cis*) to resting CD4 T cells¹⁹, especially effector memory CD4 T cells abundant in mucosal tissues²⁰. Additionally, immature and mature DCs express C-type lectin receptors (CLRs) and may express undefined molecules that facilitate binding and internalization²¹⁻²⁵. Such captured virus can be rapidly (within hours) and efficiently transferred (in *trans*) to CD4 T cells upon cell-cell contact, referred to as infectious or virological synapses²⁶⁻²⁹, a process that is independent of productive DC infection^{25, 27}. Typically the efficiency of HIV transmission can be increased by maturation of DCs^{18, 27, 30, 31}, indicating that mature DCs in the local and draining lymphoid tissues might further facilitate HIV spread to CD4 T cells. Not surprisingly, numerous studies have highlighted an important role for DC-mediated HIV transmission during mucosal HIV infection, which likely involves the multiple modalities of DC-driven infection^{3, 6, 17, 32-38}. Thus, topical microbicides that contain agents targeting specific attachment and fusion events need to be extremely efficient in preventing these critical virus-cell interactions.

During each stage of the sequential entry process HIV is vulnerable to blockade by compounds that bind to viral envelope or the cellular receptors CD4 and CCR5 or CXCR4. Recent *in vitro* studies showed that DC-mediated infection in *trans* or *cis* are sensitive to fusion inhibitors^{7, 28, 39}. The virological synapse is also sensitive to a CCR5 antagonist (TAK779), and several viral envelope-binding neutralizing antibodies^{28, 40}. Other small-molecule CCR5 ligands being tested in the macaque model show promise⁴¹⁻⁴⁴. An envelope-binding small molecule inhibitor (BMS-C) showed *in vitro* activity against HIV isolates from multiple genetic subtypes⁴⁵. Because of the central role of DCs in initiating HIV infection, we were interested to more extensively compare the efficiency of three inhibitors that antagonize distinct steps of virus entry, to prevent DC infection and DC-driven viral spread. T-1249, a gp41 peptide fusion inhibitor, is a 39-amino acid synthetic peptide and blocks viral fusion with the cell membrane by inhibiting late stage conformational changes within gp41⁴⁶. BMS-C is a small molecule attachment inhibitor that binds to gp120 to inhibit CD4-binding and subsequent conformational changes associated with co-receptor binding⁴⁷, while CMPD167 is a CCR5-specific receptor antagonist^{48, 49}. Applying these three viral entry inhibitors, we specifically compared distinct viral transfer mechanisms (*trans* vs *cis*), and a DC-T cell coculture setting that involves cell-free virus, allowing both infection mechanisms to occur simultaneously, along with direct infection of CD4 T cells. We demonstrate the greater potency of BMS-C in preventing DC and DC-driven infection, without perturbing innate DC function.

Materials and Methods

Entry Inhibitors

The HIV entry inhibitors T-1249⁴⁶, BMS-C⁴⁷, and CMPD167^{48, 49} were kindly provided by John P. Moore. For all inhibitors, working stocks at a maximum stock concentration of 0.1mM (CMPD167, in phosphate-buffered saline (PBS)), 1mM (BMS-C, in DMSO), and 503.6μM (T-1249, in PBS) were prepared and kept at -80°C until use. After thawing, aliquots were kept at 4°C for a maximum of four weeks.

Isolation and culture of primary leukocytes

Human monocyte-derived DCs were generated from highly enriched populations of CD14 monocytes, and autologous CD4 T cells isolated from CD14 negative cell fractions, as previously described, with the minor modification that PBS supplemented with 1% human serum (Sigma, St. Louis, MO, USA) and 2mM EDTA (Sigma) was used as the buffer²⁸. As needed, cells were activated on day 5 for 48hrs with the cocktail of PGE₂, IL-1, IL-6, and TNF- α as described⁵⁰. For immature DC infection, cells were used on day 5. When comparing immature and mature DCs in the cocultures, the cells were cultured in parallel and day 7 DCs used. Comparable levels of HIV infection are seen for day 5 or day 7 immature DCs. CD4 lymphocytes were sorted from CD14 negative cells by negative selection using the CD4 lymphocyte isolation kit (Miltenyi Biotec, Auburn, CA, USA). Cells were cultured in complete RPMI-1640 (R1) as described²⁸. R10 contained 10% fetal bovine serum (GIBCO-BRL Life Technologies, Grand Island, NY, USA).

Flow cytometric analysis

Phenotypic characterization of immature and mature DCs including purity controls of generated DCs and CD4 T cell populations (purity $\geq 97\%$ for DCs and T cells) were routinely monitored by 2-color flow cytometry as previously reported⁵¹. DCs were stained with a fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR against a panel of phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) characterizing an immature or mature DC phenotype: anti-CD25, anti-CD80, anti-CD86 (all from BD/Pharmingen, San Jose, CA, USA), and anti-CD83 (Immunotech-Beckman-Coulter, Marseille, France, EU). A PE-conjugated anti-CD3 mAb (BD/Pharmingen) was used to detect contaminating T cells in DC preparations. The purity of CD4 T cell populations was evaluated by stained cells with a FITC-conjugated anti-CD3 mAb against PE-conjugated mAbs anti-CD4 and anti-CD8 (all from BD/Pharmingen). Isotype controls IgG2a-FITC, IgG1-PE, and IgG2b-PE (all from BD/Pharmingen) were included in each experiment. Cell samples were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and data analysis was carried out using FlowJo software (Tree Star, Ashland, OR, USA).

Virus inhibition assays

Sucrose gradient purified infectious HIV BaL was kindly provided by the AIDS and Cancer Virus Program (SAIC Frederick, National Cancer Institute at Frederick, Frederick, MD, USA). HIV BaL (lot # P3953, P4022, P4126, P4143, P4237) was propagated (in SUPT1-CCR5 Cl.30 or Jurkat-TAT-CCR5 cells) and purified as described⁵². Aliquots of virus stocks were thawed and single use aliquots were stored at -80°C before the titer of virus was verified by titration on TZM-bl cells⁵³. Virus stocks were treated with 10 $\mu\text{g}/\text{ml}$ DNase (Roche Applied Science, Indianapolis, IN, USA) for 30min at 37°C prior to use. For the *trans* phase of viral transfer, immature and mature DCs were pulsed with HIV (8×10^3 50% tissue culture infective dose (TCID₅₀) per 1×10^5 DCs) for 2hrs at 37°C in a 15ml conical tube (pre-treated with R10 for 2min on ice) at a concentration of 10^6 DCs/100 μl (with a maximum of 1×10^7 cells/tube). During the last 30min of incubation staphylococcal enterotoxin B (SEB) peptide (Sigma; S4881) at a final concentration of 0.5 $\mu\text{g}/\text{ml}$ was added before cells were washed four times with ice-cold R1, the viable cells recounted by trypan blue exclusion and cell numbers adjusted to 2.5×10^6 cells/ml. For the *cis* phase of viral transfer virus-pulsed immature DCs were re-cultured at a concentration of 1×10^6 cells/ml in a total volume of 3ml per well in a 6-well plate (in R1 with IL-4/GM-CSF) for additional 48hrs, before virus-exposed DCs were collected, incubated with SEB peptide, washed and cell numbers adjusted as described for the *trans* phase. For viral replication in DC-T cell mixtures (Mix), immature and mature DCs were pre-treated with SEB peptide, washed, and cell numbers adjusted (as above). T cells (3×10^5 per well) were seeded in a 96-well flat bottom plate and the inhibitors (T-1249, BMS-C, or CMPD167; 0.06 to 250nM) added just

prior to addition of the virus/SEB-pulsed or SEB-pulsed DCs (1×10^5 cells/well). Virus (8×10^3 TCID₅₀) was added directly to the DC-T cell co-cultures that contained SEB-treated DCs. For immature DC infections, cells (3×10^5 per well) were seeded in a 96-well flat bottom plate and inhibitors (T-1249, BMS-C, or CMPD167; 0.06 to 250nM) added prior to addition of 2.4×10^4 TCID₅₀ virus per well. Samples were set up in duplicate. After 7 days of culture cells were harvested, washed, and lysed. Samples were stored at -80°C until quantitative PCR (qPCR) analyses.

Immature DC assays for cytokine/chemokine analysis

Candida albicans (strain SC5314, obtained from the American Type Culture Collection) was cultured and maintained as previously described⁵⁴. After overnight amplification in Sabouraud dextrose broth (Sigma) at 30°C *Candida* was washed $4 \times$ in PBS before viable *Candida* yeasts were counted by trypan blue exclusion and resuspended in R1. Immature DCs (3×10^5 /well of a 96-well flat bottom plate) were cultured in the presence and absence of 3×10^5 *Candida albicans* yeast. Amphotericin B ($5 \mu\text{g/ml}$, Sigma) was added to all conditions to limit *Candida* overgrowth. Viral entry inhibitors were added at a final concentration of 250nM/well. Cells were cultured at 37°C and supernatants harvested 24hrs or 7 days later. Harvested supernatants were spun and transferred to fresh plates to remove any cellular debris, and immediately frozen at -80°C until further analysis. Cytokines and chemokines were detected using a Becton Dickinson 24-Plex Detection System as previously described⁵⁴.

HIV gag qPCR

qPCR was performed as previously described²⁸ with the minor modification that HIV copies were normalized on cell numbers by using qPCR for albumin gene copy number. Albumin (Alb) forward (F) and reverse (R) primer/probe sequences were AlbF: TGC ATG AGA AAA CGC CAG TAA, AlbR: ATG GTC GCC TGT TCA CCA A, and AlbP: 5' FAM-TGA CAG AGT CAC CAA ATG CTG CAC AGA A-TAMRA 3'. Standards for quantification of viral and albumin copy numbers were set up by adding titrated quantities of the plasmid HIV AD8 NL43 DNA into a constant genomic background of SUPT1/CCR5 CL.30 cells. For albumin copies, known numbers of lysed uninfected SUPT1/CCR5 CL.30 cells were serially diluted in lysis buffer.

Statistical analysis

Data were analyzed using GraphPad Prism software version 5.03 (GraphPad InStat Inc, San Diego, CA, USA). Results of experiments have been summarized as the arithmetic mean and standard error of the mean (SEM). When two groups were compared, the null hypothesis of no group difference was evaluated with the non-parametric Mann-Whitney, the Wilcoxon Signed-Ranked, or the Student's *t* test. The 50% or 90% inhibitory concentration (IC₅₀ and IC₉₀) values of different compounds were modeled with non-linear regression employing a constant slope algorithm. The conventional measure of $p < 0.05$ was used to determine whether experimental differences were statistically significant.

Results

Establishing a sensitive infection assay to test inhibitors

In order to evaluate the efficacy of viral entry inhibitors to prevent HIV infection of immature DCs and DC-driven infection of CD4 T cells, it was necessary to establish a system with high reproducibility and sensitivity to detect low levels of viral replication. We used sensitive qPCR to monitor viral HIV late *gag* proviral DNA as a quantitative measure of productive infection^{28, 55}. Although qPCR was sensitive down to detecting one copy of

proviral DNA, immature DCs and co-cultures of immature DCs with resting CD4 T cells required a minimum of about 4000 (for DCs) or 2000 (for co-cultures) TCID₅₀ per 1×10^5 DCs for reliable detection of low copy numbers of viral DNA (data not shown). Therefore, we increased the viral inoculum to 8000 TCID₅₀ per 1×10^5 DCs for more consistent viral amplification across different donors. To increase levels of viral replication in cocultures, DCs were pre-exposed to an SEB peptide to uniformly activate the T cells. SEB-activation of DC-T cell cocultures has been used previously⁵⁶⁻⁵⁸. SEB did not impact the DC phenotype in the DC or DC-T cell cocultures (data not shown).

As previously reported^{18, 19, 59} immature DCs alone exhibited low-level infection with R5 HIV BaL, compared to the significantly greater replication seen in the presence of CD4 T cells (Fig. 1). The latter was true for both modes of DC-mediated transmission of virus to the T cells, as well as when virus was added directly to DC-T cell mixtures. All immature DC-T cell cocultures replicated virus to similar levels, independent of mode of infection (*trans*, *cis* or cell-free). Notably, under coculture conditions the immature DCs promoted more virus replication than their mature counterparts in *trans* infection systems and in the cell-free virus infected DC-T cell mixtures ($p < 0.02$).

BMS-C is most effective at blocking HIV infection in DC and DC-T cell cocultures

The ability of CMPD167, BMS-C, and T-1249 to block DC infection and the different modes of DC-driven infection in DC-T cell co-cultures was measured. No toxicity was detected at any of the inhibitor doses tested (data not shown). While each inhibitor effectively blocked HIV-infection across all settings, inhibitor- and cell system-dependent differences were observed. A complete block of infection in immature DCs required 250nM of each inhibitor, which was 16 fold greater than for the various DC-T cell co-cultures (Fig. 2). While all three compounds show comparable efficiency in the higher dose range (250nM to 15.6nM), the dose-response curve of the CCR5 inhibitor CMPD167 markedly deviated from the others at doses below 15.6nM. In fact, DC infection was significantly enhanced in the presence of low dose of CMPD167 (0.06nM; $p < 0.005$) (Fig. 2A). A similar effect was observed for concentrations lower than 0.06nM (data not shown). Furthermore, area under the curve analyses highlight significant differences between the activity of CMPD167 and T-1249 ($p < 0.04$) or BMS-C ($p < 0.02$) (Fig. 2B). On the other hand, the inhibitors exhibited similar levels of potency against HIV infection of the various DC-T cell environments (Fig. 2A), despite immature DCs driving more virus replication than mature DCs (Fig. 1).

While all inhibitors completely blocked HIV infection in different cell culture settings, the inhibitory doses varied within and across cultures. The most dramatic differences between inhibitors were observed in blocking the low level infection in immature DCs, while in contrast, dose-response curves of all three inhibitors were similar for the DC-mediated transfer in *cis* (Fig. 2A). To examine this more closely, we calculated the IC₅₀ and IC₉₀ values of the inhibitors in each biological system (Fig. 3). Significantly more T-1249 (p values from 0.008 to 0.02 for 50% and p values from 0.008 to 0.04 for 90% inhibition) and CMPD167 (p values from 0.0002 to 0.07 for 50% and p values from 0.001 to 0.03 for 90% inhibition) was needed to block immature DC infection compared to all other DC-T cell systems. In contrast, comparable amounts of BMS-C were required to block infection of immature DCs and the immature DC-T cell co-cultures. However, ~10 fold less BMS-C was necessary to prevent *trans*-mediated infection of mature DC-T co-cultures and mature DC-T cell mixtures ($p < 0.03$; IC₅₀=0.19nM and 0.14nM, respectively) compared to that required to inhibit immature DC infection (IC₅₀=1.58nM). Thus, while similar amounts of the inhibitors were needed to block the different modes of infection in the various DC-T cell systems, BMS-C was more efficient in preventing immature DC infection.

Accumulation of CCL4 in CMPD167-exposed immature DC cultures

As sentinels of the immune system, DCs produce cytokines and chemokines that mediate direct effector and immunoregulatory functions. Although entry antagonists have significant potential to prevent HIV entry into permissive target cells, inhibitors must not trigger the release of inflammatory factors that might exacerbate HIV infection or impede responses to other pathogens. We therefore investigated whether these viral entry inhibitors affected DC biology. Exposure of immature DCs to CMPD167, BMS-C, or T-1249 for up to 7 days did not alter their typical pattern of cell surface receptor expression (data not shown) including CD4, which is the main viral entry receptor that mediates HIV infection. However, when supernatants of such cultures were tested for the presence of different cytokines and chemokines, CCL4 levels were significantly increased after 7 days of culture with CMPD167 ($p < 0.02$) (Fig. 4A, left panel). The increased level of CCL4 after 24 hours of culture with CMPD167 was not significant. Minor increases in the levels of CCL5 after culture with CMPD167 were not significantly different from the control (Fig. 4A, right panel). No other cytokines or chemokines were elevated after culture with any of the inhibitors at these time points (data not shown for IL-1 β , IL-1RA, IL-2, IL-2R, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-17, TNF- α , IFN- α , IFN- γ , CCL2, CCL3, CXCL8, CXCL9, CXCL10).

To determine if the inhibitors might impair innate DC responses to common pathogens, we measured the impact of each inhibitor on the DC cytokine and chemokine production induced by *Candida albicans*. Immature DCs produce IL-1 β , IL-1RA, IL-2R, IL-6, IL-10, IL-12, TNF- α , CCL2, CCL3, CCL4, CXCL8, and CXCL10 in response to *Candida* yeast (Supplementary Fig. 1)⁵⁴. After 7 days, there was an increase in the production of CCL4 in the presence of CMPD167 (Fig. 4B, left panel), but this was not significant and a marginal increase of CCL5 in the presence of all three compounds (Fig. 4B, right panel). There was no impact on any of the other cytokine and chemokine responses induced by *Candida* yeasts (data not shown). Thus, apart from the higher levels of CCL4 detected in the presence of CMPD167, the inhibitors had minimal impact on innate DC cytokine/chemokine responses.

Discussion

Dendritic cells are believed to play a central role in the onset of HIV infection at the body surfaces through a variety of mechanisms^{2, 17, 37}. Immature and mature DCs can capture and internalize HIV that is then rapidly disseminated to CD4 T cells on DC-T cell contact^{27-29, 60}. Upon productive infection immature DCs produce much smaller amounts of virus compared to CD4 T cells, but they are extremely efficient in transmitting progeny virions to CD4 T cells^{32, 61}. Additionally, DCs readily trigger virus growth upon contacting virus-carrying CD4 T cells^{62, 63}. It is proposed that the role of immature DCs is more restricted to local HIV replication at the mucosal surface, while mature DCs might drive *trans*-infection in the lymph nodes⁶⁴. Activation of DCs triggered by local pathogens may also facilitate *trans*-infection at body surfaces.

We examined the ability of three viral entry inhibitors that are being explored as potential microbicide candidates to prevent cell-free infection of immature DCs and DC-T cell mixtures, as well as DC-mediated infection of CD4 T cells, using the blood monocyte-derived DC model. This system provides sufficient cell numbers to carry out side-by-side analyses of the three inhibitors. Unlike in an earlier study⁶¹, the levels of virus replication after *trans* vs *cis* transfer by DCs were comparable. This might be due to methodology differences, especially the inclusion of the SEB peptide herein to activate the recipient T cells to allow more robust replication of virus in both settings. Similarly, the use of SEB peptide-pulsed immature DCs here likely overcame the greater efficiency in DC-T cell communication and conjugate formation by mature DCs, facilitating relatively greater

replication in the presence of immature DCs than previously observed^{28, 65}. Augmented DC-T cell communication in the presence of SEB peptide may also provide signals to increase replication in the immature DCs^{66, 67} that would further amplify virus directly, as well as provide additional new virus to spread to T cells.

The compounds used in this study represent the three main classes of HIV entry inhibitors. All three inhibitors prevented R5 HIV replication in the different DC systems, impeding the distinct stages of the sequential cascade of viral fusion. This is in agreement with previous findings by us and others, where fusion peptides^{28, 68-70}, CCR5 antagonists^{69, 70}, or other potential entry inhibitors⁶¹ readily blocked DC-mediated infection in *trans* or *cis*. Recent reports suggest that even neutralizing Abs are more efficient at blocking HIV transfer than originally thought^{67, 71-74}. Similarly, cell-free or cell-to-cell spread between T cells were recently shown to be equally sensitive to viral entry inhibition⁴⁰. Inhibition of DC-driven *trans* and *cis* infection further suggests that the recently described formation of nanotubes^{75, 76} or cytoplasmic extensions by immature DCs that envelop CD4 T cells⁷⁷ and are enriched with immature virus particles, are freely accessible to viral entry inhibitors. Therefore, even in the presence of virological synapse formation each step of the viral fusion cascade is vulnerable to the inherent antiviral potency of the different viral entry inhibitors. Immature and mature DCs (i) sequester entrapped virions in unique compartments, (ii) exhibit differences in DC-T cell conjugate formation, and (iii) release entrapped particles and/or produce new viruses at CD4 T cell-DC junctions²⁸. These features may contribute to differences in the sensitivity of immature or mature DC-driven, and the *trans* or *cis* DC-driven virus spread to inhibition.

Another main finding in this study is the enhancement of DC infection at low doses of CMPD167. This possibly relates to increased levels of CCL4 induced by CMPD167 and likely parallels what CCL5 does in macrophages^{69, 70, 78}. An increase in CCL4 levels in DC cultures could also explain previous observations where only partial inhibition of immature DC infection was achieved using CCL5, a natural ligand for CCR5, to block infection. Additionally, CCL4 could encourage the recruitment of additional target cells to augment HIV amplification⁷⁹ *in vivo*.

The remarkable low efficiency of CCR5 and fusion inhibitors to block DC-infection compared to the various DC-T cell cocultures is in agreement with previous findings, where immature DC infection was more resistant to complete inhibition by 20 or 36 fold for a CCR5 (SCH-C) vs 6 or 10 fold for a fusion peptide inhibitor than PBMCs or *trans*-mediated infection^{69, 70}. This effect possibly relates to the common sensitivity of CCR5 and fusion inhibitors to the level of cell surface expression of CCR5. While the cooperation of multiple CCR5 coreceptors is required for infection by HIV79, high or low levels of CCR5 affect the efficiency of a CCR5 antagonist, and can impact the speed of fusion pore formation and therefore the efficiency of a fusion peptide inhibitor⁸⁰. Unlike in DC infection, HIV receptors CD4 and CCR5 (or CXCR4) on the T cell are recruited to the DC-T cell contact sites thereby enhancing productive infection events, and possibly the inhibition of such events by CCR5 or fusion inhibitors. On the other hand, direct binding to the virus, thereby preventing virus attachment to its target cell, likely explains the high potency of BMS-C to prevent cell-free and DC-driven infection to comparable levels. Although herein inhibitors were only tested against one R5-virus and variations in the potency of the three chosen inhibitors across several R5-viruses cannot be excluded, a recent study showed no substantive, virus-specific pattern to the actions of the CMPD167, BMS-C, or a fusion peptide against a panel of R5 viruses⁴⁵. This is consistent with earlier reports on other entry inhibitors and with the subtype-independent nature of the HIV fusion process^{81, 82}.

Several approaches utilizing inhibitors of virus-cell interactions are being explored for topical microbicides^{28, 40, 44, 83}, particularly CCR5 inhibitors^{42, 84, 85}. Although CMPD167 has been shown to inhibit vaginal transmission of an R5 SHIV to macaques^{42, 85}, there might be concern that residual low levels of drug might ultimately enhance infection if animals were exposed to virus at later time points when drug would become limiting. Alternatively, small molecule inhibitors like BMS-C that directly target viral envelope may have considerable advantages compared to CCR5 or fusion peptide inhibitors due to their superior ability to efficiently prevent DC-driven HIV infection and spread, coincident with their limited impact on innate immune function. Despite a preferential transmission of R5 HIV during mucosal infection future studies are needed to evaluate the potency of entry inhibitors against X4- and dual-tropic HIV to advance our understanding for the development of a potent topical prevention strategy. Overall, combination strategies using inhibitors targeting multiple stages of the infection process will likely be most effective against HIV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Virus replication levels in the various DC systems

HIV infection levels were measured in immature DCs (Imm DC) and the various DC-T cell systems; immature and mature (Mat) DC-mediated transfer in *trans* (Trans) or immature DC-mediated transfer in *cis* (Cis) and the direct infection of immature or mature DC-T cell mixtures (Mix). After 7 days, infection was measured by qPCR for HIV *gag* DNA and cell copy numbers were measured by qPCR for albumin. The data are expressed as HIV DNA copies per cell. Immature DC-T cell cultures of the *trans* (Trans; $p < 0.02$) and mixed (Mix; $p < 0.02$) settings carried significantly more HIV DNA copies per cell compared to matched mature DC-T cultures. Results are representative of 11 (Imm DC), 14 (Imm DC-T Trans), 9 (Mat DC-T Trans), 10 (Imm DC-T Mix), 8 (Mat DC-T Mix), and 4 (Imm DC-T Cis) independent experiments. Error bars represent standard error of the mean ($\pm$ SEM).

Figure 2. Dose titrations of inhibitors against DC-driven HIV replication

(A) Titrated doses of each inhibitor were used to block infection in the indicated DC systems described in Figure 1. Inhibitors were added to the immature DCs or the DC-T cell mixtures prior to the addition of virus, while inhibitors were added just prior to adding virus-loaded DCs to the T cells in the *trans* and *cis* settings. Infection was measured after 7 days by qPCR and the data are expressed as the percent infection relative to the no drug control (set as 100%). Each data point represents the mean ($\pm$ SEM) of 5-8 independent experiments. (B) The area under the curve (AUC) was calculated between 0 and 15.63nM of each drug for the immature DC infection. Statistical analysis was carried out using the non-parametric Mann-Whitney test. The asterisk indicates a significant increase of the AUC of CMPD167 compared to the AUC of T-1249 ($p < 0.04$) or BMS-C ($p < 0.02$).

Figure 3. BMS-C most potently blocks DC-driven HIV replication

The IC₅₀ (A) and IC₉₀ (B) values of several inhibitor titration series including the data shown in Figure 2 were determined, modeled with non-linear regression employing a constant slope. Results represent the mean IC₅₀ or IC₉₀ ($\pm$ SEM) of 4-12 independent experiments. Statistical analyses were carried out using then non-parametric Mann-Whitney test.

Figure 4. CMPD167-dependent accumulation of CCL4 in immature DC cultures

Immature DCs were cultured with medium (A) or *Candida* yeasts (1:1; B) in the presence or absence of the inhibitors (250nM) for 24hrs or 7 days. CCL4 and CCL5 levels were measured in the supernatants by Luminex. Mean ng/ml ($\pm$ SEM, 5 donors) are shown. Statistical analysis was carried out using the Wilcoxon Signed-Rank test. The asterisk shows a significant increase of CCL4 compared to the control (set as 1; $p < 0.02$).