The dual CCR5 and CCR2 inhibitor cenicriviroc does not redistribute HIV into extracellular space: implications for plasma viral load and intracellular DNA decline

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Objectives: Cenicriviroc is a potent antagonist of the chemokine coreceptors 5 and 2 (CCR5/CCR2) and blocks HIV-1 entry. The CCR5 inhibitor maraviroc has been shown in tissue culture to be able to repel cell-free virions from the cell surface into extracellular space. We hypothesized that cenicriviroc might exhibit a similar effect, and tested this using clinical samples from the Phase IIb study 652-2-202, by measuring rates of intracellular DNA decline. We also monitored viral RNA levels in culture fluids.

Methods: We infected PM-1 cells with CCR5-tropic HIV-1 BaL in the presence or absence of inhibitory concentrations of cenicriviroc (20 nM) or maraviroc (50 nM) or controls. Viral load levels and p24 were measured by ELISA, quantitative PCR and quantitative real-time reverse transcription PCR at 4 h post-infection. Frozen PBMC DNA samples from 30 patients with virological success in the Phase IIb study were studied, as were early and late reverse transcript levels. Docking studies compared binding between cenicriviroc/CCR5 and maraviroc/CCR5.

Results: Unlike maraviroc, cenicriviroc did not cause an increase in the amount of virus present in culture fluids at 4 h compared with baseline. The use of cenicriviroc did, however, result in lower levels of intracellular viral DNA after 4 h. Structural modelling indicates that cenicriviroc binds more deeply than maraviroc to the hydrophobic pocket of CCR5, providing an explanation for the absence of viral rebound with cenicriviroc.

Conclusions: In contrast to maraviroc, cenicriviroc does not repel virus back into extracellular space. Differences in results may be due to superior binding of cenicriviroc to CCR5 compared with maraviroc.

Keywords: antiretrovirals, entry inhibitors, antiviral

Introduction

The chemokine receptor type 5 (CCR5) is one of two coreceptors required for HIV-1 entry into target cells. HIV-1 envelope glycoprotein 120 (gp120) interacts with cellular CD4 at the surface of target cells, binds to CCR5 or chemokine (C-X-C motif) receptor 4 (CXCR4), and undergoes a conformational change to allow fusion between cellular and viral membranes (reviewed in Wilen et al.1). CCR5-tropic virus predominates during early and acute infection, as a result of preferential transmission (reviewed in Grivel et al.2). A tropism switch to CXCR4 or dual tropism generally manifests during later stages of infection and does not occur in all cases.

Currently, maraviroc is the only FDA-approved small-molecule antagonist targeting CCR5. Maraviroc was shown to effectively inhibit CCR5-tropic viral replication in treatment-experienced and treatment-naïve patients in the MOTIVATE and MERIT clinical trials, respectively.3,4 In MERIT, the number of patients who attained a viral load between 50 and 400 RNA copies/mL in the maraviroc arm was lower than in the efavirenz arm. Previous work from our group has shown that this higher proportion may be due, in part, to a phenomenon whereby free virions that are prevented from entering the target cell by maraviroc are able to return to plasma.5

Cenicriviroc is a novel, oral, once-daily, dual CCR5 and chemokine receptor type 2 (CCR2) antagonist that has completed Phase IIb development and possesses potent in vitro activity against CCR5-tropic virus.6 In vivo, cenicriviroc has shown efficacy during monotherapy of treatment-experienced individuals harbouring...
Cenicriviroc and viral load

In the Phase IIb clinical study (652-2-202; NCT01338883), cenicriviroc demonstrated similar efficacy at 24 weeks (primary analysis) to the NNRTI efavirenz, each administered in combination with emtricitabine and tenofovir, with favourable safety and tolerability. We hypothesized that the antiretroviral efficacy of cenicriviroc in Study 202 might have been underestimated as a result of the rebound phenomenon observed with maraviroc. Accordingly, we conducted an ex vivo sub-analysis of Study 202 by measuring intracellular HIV DNA declines in stored PBMCs from 28 patients who achieved virological success at week 24 of the study. We also performed in vitro assays to determine and compare the extent of any cell-free virion redistribution that cenicriviroc or maraviroc might cause.

We now show that cenicriviroc does not trigger viral particle rebound. Indeed, comparable declines in intracellular DNA were seen in individuals treated with either cenicriviroc or efavirenz, suggesting that plasma viral load is an accurate measure of cenicriviroc treatment success. Structural modelling provides a potential explanation for differences between results obtained with maraviroc and cenicriviroc.

Methods

Cells

PM-1 cells that express CD4, CCR5 and CXCR4 were obtained through the AIDS Research and Reference Reagent Program (ARRRP) and were maintained in complete RPMI-1640 medium containing 10% FBS (R10 medium) at 37°C, 5% CO2. 293T cells used for transfection were maintained in complete DMEM containing 10% FBS (D10 medium) at 37°C, 5% CO2.

Virus stocks

HIV-1 Bal. virus was produced by transfecting 293T cells with the plasmid pWT/BaL. pWT/BaL was obtained through the ARRRP from Dr Bryan Cullen (Duke University, NC, USA). Lipofectamine 2000 (Invitrogen, CA, USA) was used as a transfection agent. Culture supernatants were collected at 48 h post-transfection, filtered through a 0.45 μm pore filter and treated with 50 U of benzonase per mL of virus stock for 20 min at 37°C to remove contaminating plasmid DNA. Virus stocks were frozen at −80°C to halt benzonase activity. Benzonate-treated virus stocks were propagated in cord blood mononuclear cells as previously described.

Infections

We exposed PM-1 cells to HIV-1 Bal. in the presence of inhibitory concentrations of cenicriviroc (20 nM) and maraviroc (50 nM), based on the in vitro IC50 of cenicriviroc and maraviroc versus HIV-1 Bal. being 1.9 and 9.8 nM, respectively. The inhibitory amounts were based on the IC50 for each compound, where cenicriviroc is more potent. Both drugs were incubated with PM-1 cells for 1 h at 37°C prior to the addition of virus. Five hundred nanograms of p24 antigen of HIV-1 Bal. was incubated per 5×10⁶ cells in 1 mL of R10 medium. Virus-only controls, described as ‘no cell’ in the text, were used to measure viral decay. Viral adsorption was measured in the no-drug controls, whereby 500 ng of p24 antigen of HIV-1 Bal. was added per 5×10⁵ PM-1 cells that were pre-incubated at 37°C for 1 h in the absence of drug treatment. Each drug treatment and control was performed in duplicate. Viral RNA was extracted from 140 μL of supernatant fluid using the QIAamp Viral RNA Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions. Samples were stored at −80°C until analysis. Supernatant viral loads were measured using quantitative real-time reverse transcription PCR (qRT–PCR) with the primers US1SSF (5′-AACTAGGGAACCCACTGCTTAA-3′), US1SSR (5′-TGAGGGATCTCTAGTACCAGAGTCA-3′) and US1SS probe (5′-(FAM) CCTCAATAAAGGCTTGTAGGTCCTCAA) and the Invirotogen (CA, USA) qRT–PCR Supermix Kit. All values are the result of replicate testing over two independent experiments using a Corbett Rotor-gene 6000 (Mortlake, NSW, Australia). RNA copy number was quantified by the use of 10-fold serial dilutions of pWT/Bal to generate standard curves for each assay and calibration against samples with known copy numbers from previous studies.

Supernatant p24 antigen levels were assessed using a Perkin-Elmer HIV-1 p24 antigen ELISA kit according to the manufacturer’s instructions.

Patient samples

PBMC samples were obtained from 28 patients (10, 11 and 7 on 100 mg of cenicriviroc, 200 mg of cenicriviroc and efavirenz, respectively) who achieved virological success at week 24 in Study 202 (652-2-202; NCT01338883).

Intracellular DNA quantitative PCR

Total DNA was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. Intracellular strong-stop DNA levels were quantified with the US1SS primer/probe set described above. Intracellular full-length DNA levels were quantified using the US1FL primer (Invitrogen)/probe (Biosearch Technologies) set (forward, 5′-ACTAGGGAACCCACTGCTTAA; reverse, 5′-CGAGTCTGGCTGAGAGAGA; probe, 5′-(FAM)-CCTCAATAAAGGCTTGTAGGTCCTCAA). Both DNA levels were multiplexed with a GAPDH primer/probe set (forward, 5′-ACCGGGA AGAAATGATAGG; reverse, 5′-GCAGGGACCCAGGTATGT; probe, 5′-(VIC)-ACCGGAGCTTCTAAGGCT) to normalize DNA inputs and verify sample integrity.

Statistical analysis

The Mann–Whitney test was used to analyse in vitro intracellular HIV DNA levels for all three treatment groups. All data were analysed using Prism 5 software (GraphPad, La Jolla, CA, USA).

Molecular docking of cenicriviroc in CCR5

The crystal structure of the CCR5 chemokine receptor [Protein Data Bank identification no. (PDB ID) 4MBS] was obtained through the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (http://www.rcsb.org/pdb/) and used as a docking target. The structure of cenicriviroc (formerly TAK-652/TBR-652) was obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov) and used as a ligand. Minimization of ligand-docked structures was facilitated by the use of a UCSF Chimera (http://www.cgl.ucsf.edu/chimera/),14 which prepared CCR5 and cenicriviroc as inputs for DOCK calculations, which predict the orientation of the ligand in the CCR5 seven-transmembrane (7TM) α-helix receptor cavity. Docking calculations were performed using AutoDock Vina15 within the virtual screening tool PyRx 0.8 (http://pyrx.scripps.edu). A maximum-sized grid box was used to include all possible docking sites in CCR5. The binding site consists of all residues <15 Å from the 7TM cavity (around residues Tyr108 and Glu283). Docking results were processed to identify intermolecular interactions using the structural visualization and image processing software PyMOL (Delano WL, The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrodinger, LLC.; Delano Scientific, San Carlos, CA, USA; http://www.pymol.org). The best nine poses were kept for further analysis. In order to validate the accuracy of the docking system, maraviroc was docked to CCR5 using the same method and its orientation with respect to the crystal structure was determined. The root mean square deviation (RMSD), calculated using PyMOL, between the observed crystal structure and the docked complex was 0.1 Å.
and the predicted conformation obtained from AutoDock Vina was 0.275 Å, indicating that the protocol was sound.

**Results**

First we quantified HIV intracellular DNA in order to validate measures of viral load that were obtained during the Study 202 clinical trial. Ex vivo analyses of full-length intracellular HIV DNA levels were similar across all groups (100 mg of cenicriviroc, 200 mg of cenicriviroc and 600 mg of efavirenz), with no statistically significant differences in the mean fold change between baseline and week 24 (Figure 1a).

Strong-stop intracellular HIV DNA levels were measured concomitantly with full-length levels at week 24 (Figure 1b). Differences in the mean fold change across groups were not statistically significant, though we observed a trend to larger fold changes when comparing both cenicriviroc groups with the efavirenz group.

In vitro experiments measuring extracellular viral levels following cenicriviroc and maraviroc exposure were also performed. Levels of virus in culture fluids were measured by qRT–PCR and p24 ELISA at 4 h following infection of entry-inhibitor-exposed cells. Culture fluids from the maraviroc-treated cells exhibited higher RNA levels compared with baseline than did cenicriviroc-treated cells, which did not change significantly (Figure 2a). Viral RNA declines for the no-cell and no-drug controls were similar (Figure 2a), p24 levels declined from baseline after 4 h with cenicriviroc treatment (Figure 2b), p24 levels of both the no-cell control and the no-drug control dropped similarly (Figure 2b).

These differences in extracellular virus levels following cenicriviroc and maraviroc treatment prompted us to examine intracellular strong-stop HIV DNA levels in PM-1 cells exposed to either cenicriviroc or maraviroc for 1 h before being infected with HIV-1 BaL. Total DNA was extracted from cell pellets after 4 h. We observed a relative DNA level of 0.02 in maraviroc-treated cells compared with the no-drug control, whereas cenicriviroc-treated cells exhibited a relative intracellular DNA level of only 0.1 (Figure 3). The difference between relative DNA levels of maraviroc and cenicriviroc-treated cells was significant.

A crystal structure exists of the CCR5 7TM complexed with maraviroc (PDB ID 4MBS) and this was used to generate a model of CCR5 with cenicriviroc docked into the binding pocket. We predicted docked poses using AutoDock Vina, a method that was also assessed by re-docking maraviroc into CCR5; the top poses with the most favourable energies had the proper orientation and overlap with the conformation in the crystal structure (RMSD <0.3 Å). In silico CCR5 docking simulations indicated that cenicriviroc binds only at the hydrophobic pocket in the CCR5 structure, also known as the ligand-binding pocket (Figure 4). Only the top nine poses were kept for further analysis. There are three different conformations that cenicriviroc exhibits after docking into CCR5 and they are clustered into three sites (Figure 4a and b). The first site (site 1) spans deep into the hydrophobic pocket and fills a large volume (Figure 4a). The second site (site 2) is partially positioned in the middle of the pocket but also bulges outward from the CCR5 between TM1 and TM7 (Figure 4a). At the third site (site 3), few cenicriviroc poses are located near the entrance of the receptor cavity.

Site-directed mutagenesis of residues within the extracellular loops and transmembrane domain in CCR5 have identified key residues that are involved in gp120 binding; mutations at the different positions abolished, compromised or affected gp120 binding to CCR5. The 13 key residues that were identified to be important for gp120 binding within CCR5 are Tyr37, Trp86, Trp94, Leu104, Tyr108, Phe109, Phe112, Thr177, Ile198, Trp248, Tyr251, Leu255 and Glu283. Figure 5 shows a molecular surface representation of CCR5 with docked poses of cenicriviroc (left) and maraviroc (right) in the binding pocket. Maraviroc and cenicriviroc have molecular surface areas of ~1285 and 1790 Å² (calculated using PyMOL), respectively. Maraviroc occupies the middle of the binding pocket. All 13 residues that were determined to be important for gp120 binding are within 4 Å from maraviroc, as measured by PyMOL (cut-off distance used in this study for electrostatic and/or hydrophobic interactions). In contrast, the docked cenicriviroc poses occupy the same pocket but not at the centre, as seen for maraviroc (Figure 5). Rather, cenicriviroc shifts to one
side of the pocket (Figure 5) and a consensus of residues in CCR5 within 4 Å of cenicriviroc was determined. Even though cenicriviroc occupies a larger surface area than maraviroc, only 7 of the 13 residues that are important for gp120 binding are within 4 Å of cenicriviroc, i.e. Tyr37, Trp86, Tyr108, Phe109, Ile198, Leu255 and Glu283. Overall, these simulations suggest that cenicriviroc occupies a region similar but not identical to that of maraviroc in the binding pocket of CCR5.

Discussion

In this study we observed that cenicriviroc and maraviroc, both CCR5 antagonists preventing HIV entry, have different effects on extracellular virus levels.

We previously showed that maraviroc might artificially increase viral load, because cell-free virions can be repelled from the target cell following a failed attempt at entry in the presence of maraviroc. The current study was designed to address whether the same effect might occur for cenicriviroc and whether intracellular DNA measurements might be a more accurate representation of antiviral efficacy when comparing entry and reverse transcriptase inhibitors.

In fact, intracellular DNA levels across Study 202 treatment arms were similar at 24 weeks in selected samples, reflecting the trend observed during the intention-to-treat analysis. Full-length HIV DNA levels were also similar for all groups at week 24, suggesting similar antiviral efficacy for both cenicriviroc and efavirenz. Differences in strong-stop HIV DNA levels were observed between the cenicriviroc and efavirenz groups, whereby both cenicriviroc groups exhibited steeper declines in viral load compared with efavirenz. As strong-stop HIV DNA levels are directly impacted by entry inhibitors, it was expected. The similarities between efavirenz and cenicriviroc in terms of virological success and intracellular HIV DNA levels suggest that the antiviral potency of this dual CCR5 and CCR2 inhibitor is not masked by viral load measurements.

We also asked whether cenicriviroc can result in virus repulsion, as seen for maraviroc in vitro. Two separate virus quantifications showed that maraviroc treatment maintained extracellular viral levels up to 4 h post-infection. In contrast, treatment with cenicriviroc resulted in a decline in viral levels at 4 h comparable to that of the no-drug or no-cell controls.

A further examination of intracellular strong-stop DNA in vitro showed that cenicriviroc caused a slight, albeit significant, increase in levels compared with maraviroc. This may be due to the differential effect of both inhibitors on CCR5, which, in turn, affects the rate of dissociation between virus and receptor. This raises the possibility that gp120 may associate more durably with cenicriviroc-bound CCR5 compared with maraviroc.

We also aimed to understand how cenicriviroc inhibits HIV entry into target cells by examining the binding site of CCR5. An engineered human CCR5 construct has been previously crystalized in complex with maraviroc at a resolution of 2.7 Å. Although this is not a full-length crystal structure of CCR5, it was utilized to better understand cenicriviroc interactions with CCR5 in in silico docking assays. All purported docking models for cenicriviroc imply deep penetration of the drug into the 7TM cavity of CCR5, as is also seen for maraviroc. However, the
cenicriviroc docked poses were not in close proximity to extracellular loop 2 (ECL2); ECL2 remained accessible post-docking. Other groups have reported that the CCR5 N-terminus and ECL2 domains both play a critical role in the interaction of HIV-1 with CCR5. In addition, the stem region of the V3 loop of gp120 is reported to bind to the CCR5 N-terminus while the V3 crown interacts with ECL2 and with residues inside the binding pocket. Based on our model, we can assume that cenicriviroc

Figure 4. Multiple binding modes of cenicriviroc into CCR5. Coordinates of CCR5 were generated from the CCR5 crystal structure bound to maraviroc in the binding pocket (PDB ID: 4MBS). Cenicriviroc binding sites were examined after docking of cenicriviroc. Docked poses of cenicriviroc are displayed as coloured thin lines. The 7TM α-helices are represented by helices and numbered (1–7) according to the order of amino acid sequences. (a) Top view from the extracellular side of the receptor with three potential binding sites that are circled: site 1, white; site 2, black; site 3, light pink. (b) Side view in the CCR5 transmembrane cavity. ECL2 is labelled. Secondary structures are represented as cartoon structures. All images were processed using PyMOL software. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

Figure 5. Comparison of the ligand-binding pocket between CCR5/maraviroc and CCR5/cenicriviroc. Top view of CCR5 displaying docked poses (coloured thin lines) of cenicriviroc (left) and maraviroc (yellow sticks; right) in the ligand-binding pocket. CCR5 is shown as a molecular surface representation. Key residues: Tyr37, Trp86, Trp94, Leu104, Tyr108, Phe109, Phe112, Thr177, Ile198, Trp248, Tyr251, Leu255 and Glu283, which are involved in gp120 binding, are deep in the pocket and coloured red. ECL2 is labelled. CVC, cenicriviroc; MVC, maraviroc. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
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S. H. performed homology modelling. All authors edited and approved the final version of the manuscript.

References

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Author contributions
V. G. K., S. H., T. M., E. L. and M. A. W. designed the experiments, analysed the data and wrote the manuscript. S. P. C.-G. and M. O. performed tissue culture studies and p24 measurements. E. L. also provided patient information.

does not interfere directly with the gp120 V3 loop interaction with ECL2, since ECL2 appears to be exposed in the model.

It is conceivable that cenicriviroc can block CCR5 activation if CCR5 remains in an inactive state. Based on these studies, cenicriviroc receptor cavity while occupying the entirety of ECL2. However, mutagenesis studies that suggest that gp120 partly fills the binding site, and it may be that gp120 can still access water-mediated hydrogen bonds. In contrast, cenicriviroc occupies the binding site, and it may be that gp120 can still access some of the residues important for CCR5 binding even in the presence of docked cenicriviroc. In this setting, gp120 can still bind to CCR5, yet CCR5 is kept in an inactive state by cenicriviroc, preventing access to Trp248. This hypothesis is supported by site-directed mutagenesis studies that suggest that gp120 partly fills the receptor cavity while occupying the entirety of ECL2. However, the degree to which the V3 loop of gp120 penetrates the CCR5 7TM remains unknown. It has also been reported that dissociation rates of gp120 from CCR5 are accelerated in the presence of maraviroc, since the latter hinders the tight association between ECL2 and the V3 loop. Based on these studies, cenicriviroc may have a different effect on the ECL2/V3 interaction than does maraviroc. Dissociation and surface plasmon resonance studies as well as crystallization of CCR5 in complex with cenicriviroc will provide valuable information on this topic.

Site-directed mutagenesis and biochemical studies are required to elucidate the residues that are important for CCR5 interaction with cenicriviroc. Determining the proximal location of the N-terminus of CCR5 is also of interest.


