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Lack of Prophylactic Efficacy of Oral Maraviroc in Macaques despite High Drug Concentrations in Rectal Tissues

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Maraviroc (MVC) is a potent CCR5 coreceptor antagonist that is in clinical testing for daily oral pre-exposure prophylaxis (PrEP) for HIV prevention. We used a macaque model consisting of weekly $SHIV_{162p3}$ exposures to evaluate the efficacy of oral MVC in preventing rectal SHIV transmission. MVC dosing was informed by the pharmacokinetic profile seen in blood and rectal tissues and consisted of a human-equivalent dose given 24 h before virus exposure, followed by a booster postexposure dose. In rectal secretions, MVC peaked at 24 h (10,242 ng/ml) with concentrations at 48 h that were about 40 times those required to block SHIV infection of peripheral blood mononuclear cells (PBMCs) *in vitro*. Median MVC concentrations in rectal tissues at 24 h (1,404 ng/g) were 30 and 10 times those achieved in vaginal or lymphoid tissues, respectively. MVC significantly reduced macrophage inflammatory protein 1β-induced CCR5 internalization in rectal mononuclear cells, an indication of efficient binding to CCR5 in rectal lymphocytes. The half-life of CCR5-bound MVC in PBMCs was 2.6 days. Despite this favorable profile, 5/6 treated macaques were infected during five rectal SHIV exposures as were 3/4 controls. MVC treatment was associated with a significant increase in the percentage of $CD3^+/CCR5^+$ cells in blood. We show that high and durable MVC concentrations in rectal tissues are not sufficient to prevent SHIV infection in macaques. The increases in $CD3^+/CCR5^+$ cells seen during MVC treatment point to unique immunological effects of CCR5 inhibition by MVC. The implications of these immunological effects on PrEP with MVC require further evaluation.

Daily pre-exposure prophylaxis (PrEP) with oral emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) is a novel HIV prevention strategy. Three human clinical trials with daily FTC/TDF among men who have sex with men and heterosexually active men and women have provided proof of concept that daily PrEP can prevent sexual HIV transmission (1–3). These results prompted the U.S. Food and Drug Administration to approve the use of FTC/TDF for PrEP to prevent HIV transmission in high-risk populations.

In the past few years, new classes of clinically approved drugs with different modes of action have gained considerable attention and are being currently considered for oral PrEP (4). Maraviroc (MVC) is a functional CCR5 coreceptor antagonist approved for the treatment of CCR5-tropic HIV infection and has many desirable characteristics for PrEP (5). Unlike FTC and TFV, which interfere with HIV replication after the virus has entered the cell, MVC prevents virus entry into the host cell by blocking the interaction between HIV-1 gp120 and CCR5. MVC is not currently recommended for first-line therapy and resistance is rare, as opposed to TFV and FTC, which are widely used clinically and result in the emergence and onward transmission of drug-resistant viruses (6). MVC is also rapidly absorbed and penetrates very efficiently into cervicovaginal and rectal tissues, achieving high and sustained concentrations (7, 8). A phase II, double-blind, randomized human clinical trial with daily MVC alone or in association with FTC or TDF is now assessing the safety and tolerability of MVC for PrEP in at-risk MSM (http://clinicaltrials.gov/).

Proof of concept that MVC could prevent HIV transmission was first provided in humanized RAG-hu mice that received oral (62 mg/kg) or topical (5 mM) MVC gel and were exposed vaginally to HIV (9, 10). Studies in macaques also showed efficacy of vaginal gels containing MVC at concentrations exceeding 1 mM. However, protection was found to be short-lived and efficacy was lost if gel was applied 6 to 12 h before virus challenge (11). These data and similar findings with other topically or orally administered CCR5 antagonists point to the potential of this class of drugs for HIV prevention (12, 13).

Predicting the efficacy of oral MVC against rectal transmission from vaginal efficacy studies is not possible because of physiological and pharmacological differences between rectal and vaginal tissues. The single-layer columnar epithelium of the rectum differs from the multilayered squamous epithelium that covers the vagina and has a unique immunological composition and CCR5 distribution that may influence early establishment and dissemination of infection (14–17). In humans, MVC exposure after oral dosing also differs in rectal and vaginal tissues, likely due to differences in tissue vascularization, drug trapping in mucus, and protein binding (7, 8). Therefore, it is important to define the prophylactic efficacy of oral MVC against rectal transmission using validated animal models.

Repeat low-dose macaque models of rectal and vaginal transmission have been developed and used to assess the efficacy of PrEP with FTC/TDF (18–21). These models closely mimic human transmission of HIV by using an R5-tropic $HIV\text{-}1_{SF162}$ envelope and lower and more physiologic virus doses. Virus exposures are repeated to mimic high-risk human exposures (19, 21, 22). The

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repeat low-dose macaque model of rectal SHIV transmission predicted the efficacy of oral FTC/TDF in humans and may also inform on the potential efficacy of oral MVC (18). Here, we modeled the efficacy of a peri-coital two-dose MVC regimen informed by the rectal and systemic pharmacokinetic profile of MVC after oral dosing. Despite high and durable MVC concentrations in secretions and high rectal tissue drug levels, MVC was not able to prevent SHIV infection in macaques. Interestingly, MVC treatment was associated with an increase in the percentage of $CD3^+$ cells expressing CCR5 in blood. This increase points to alterations in immune responses due to MVC and stress the need to fully define its potential impact on PrEP efficacy in humans.

MATERIALS AND METHODS

Ethics statement. All of the animal procedures performed in the present study were approved by the Institutional Centers for Disease Control and Prevention (CDC) Animal Care and Use Committee. Macaques were housed at the CDC under the full care of CDC veterinarians in accordance with the standards incorporated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council of the National Academies, 2010). SHIV-infected macaques were humanely euthanized in accordance with the American Veterinary Medical Association Guidelines on Euthanasia, June 2007. All procedures were performed under anesthesia using ketamine, and all efforts were made to minimize suffering, improve housing conditions, and provide enrichment opportunities.

Drug preparation and administration. MVC tablets (Selzentry; 1,200 mg containing 300 mg of MVC) were ground to powder, dissolved in phosphate-buffered saline (PBS) at 17 mg/ml in a final pH of 7.0, and filtered through a 0.2-μm-pore filter (75-mm membrane; Thermo Fisher Scientific, Rochester, NY) to remove insoluble material. This filtration procedure (50 ml of MVC solution per filter) did not result in a significant loss of MVC as measured by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS; data not shown). MVC was given based on body weight at a dose of 44 mg/kg. This drug dose is higher than the human dose (300 mg or ~4.3 mg/kg) because it is adjusted for the species and size difference of macaques since smaller mammals eliminate drugs faster than larger mammals (23). MVC was given by gavage to anesthetized macaques via a gastric feeding tube to ensure full dosing (24).

Analysis of MVC concentrations in plasma, rectal and vaginal secretions, and tissues. The concentrations of MVC were measured after a single oral dose in 12 rhesus macaques (3 males and 9 females). Plasma and rectal and vaginal secretions were collected 2 h, 5 h, 24 h, 2 days, 4 days, and 7 days after dosing. Rectal and vaginal secretions were collected in sponges (Weck-Cel surgical spear; Medtronic Ophthalmic, Jacksonville, FL) using an established protocol (24, 25). MVC concentrations were expressed as ng of MVC per ml of plasma or secretions.

The distribution of MVC in tissues was investigated in a second group of four SHIV-infected macaques that received a single oral dose of MVC. MVC levels were measured 24 h after dosing in jejunum, ileum, cervicovaginal tissue, rectal tissue, and lymph nodes (mesenteric lymph nodes [MLN], axillary lymph nodes [ALN], and inguinal lymph nodes [ILN]) collected at necropsy. Cell suspensions comprising mostly mononuclear and epithelial cells were also prepared from rectal tissues using an enzyme cocktail containing collagenase type II, elastase, hyaluronidase, and DNase I as previously described (24). Rectal tissues were first rinsed with saline solution to remove feces. MVC concentrations were expressed as ng of MVC per g of tissue.

The concentrations of MVC in plasma, mucosal secretions, and tissues were measured by HPLC-MS/MS (Shimadzu Scientific, Columbia, MD; ABSciex, Foster City, CA). Briefly, MVC was extracted from plasma (100 μl), rectal or vaginal secretion eluates (25 μl), or tissue (~100 mg) by protein precipitation using 370 μl of methanol containing internal standard (isotopically [deuterium]-labeled MVC [MVC-d6]; Toronto Research Chemicals, Toronto, Canada). After a brief centrifugation and removal of protein precipitates, the supernatant was evaporated to near dryness in a vacuum centrifuge at 65°C for 45 min, and then resuspended in 150 μl of mobile phase A (5.9 mM ammonium hydroxide, 9.4 mM formic acid, and 9.9 mM acetic acid in water). A 10-μl portion of the final solution was injected into an Ascentis Phenyl column (2 by 100 mm; Sigma-Aldrich, St. Louis, MO) connected to the HPLC-MS/MS system. An aqueous-acetonitrile mobile-phase gradient was used to elute the MVC from the column and into the MS/MS system. Mass transitions (*m/z*) of 514.1/117.2 and 514.1/389.5 were monitored in positive MRM mode. MVC concentrations were estimated from a standard curve of MVC range of 1 to 2,000 ng/ml. The lower limit of quantification of this assay was 5 ng/ml.

MVC protein binding and stability in plasma and in rectal and vaginal secretions. MVC protein binding in plasma, rectal secretions, and vaginal secretions was determined by rapid equilibrium dialysis as previously described (rapid equilibrium dialysis device system; Thermo Scientific) (7). Briefly, a 100-μl volume of plasma or secretions from treated animals was incubated for 10 h at 37°C in dialysis cartridges, followed by protein precipitation and measurement of total and free MVC concentrations. These values were used to calculate the percentage of MVC bound to proteins according to the manufacturer's instructions. The stability of MVC at 37°C was evaluated by spiking a known concentration of MVC (400 ng/ml) in plasma, rectal and vaginal secretions, or PBS, followed by analysis of total and free MVC after 24 h.

Efficacy of MVC in preventing rectal SHIV transmission in macaques. The efficacy of MVC in preventing rectal virus transmission was investigated using a macaque model consisting of weekly exposures to a $SHIV_{162p3}$ chimeric virus that contains an R5-tropic HIV-1 envelope (26). Six macaques received weekly a 44-mg/kg MVC dose 24 h before each virus exposure and a second dose 2 h after (−24 h/+2 h regimen). Four untreated macaques were used as controls. Virus exposures were done under anesthesia by a nontraumatic inoculation of 1 ml of virus (10 $TCID_{50}$) into the rectal vault via a sterile gastric feeding tube of adjusted length (18). Anesthetized macaques remained recumbent for at least 15 min after each intrarectal inoculation. Exposures were stopped when a macaque became SHIV RNA positive. All MVC-treated macaques that become infected continued receiving the two weekly doses of MVC for 15 to 19 weeks after confirmed infection to monitor drug concentrations in rectal secretions and emergence of drug resistance.

Animals in the MVC arm were considered protected from systemic SHIV infection if they remained seronegative and negative for SHIV plasma RNA. SHIV RNA was quantified by a real-time reverse transcription-PCR assay (RT-PCR) with a sensitivity of 50 RNA copies per ml as previously described (21). Virus-specific serologic responses (immunoglobulins G and M) were measured by using a synthetic peptide enzyme immunoassay assay (Genetic Systems HIV-1/HIV-2; Bio-Rad, Redmond, WA).

Analysis of MVC binding to CCR5 by a MIP-1β internalization assay. The binding of MVC to CCR5 was determined *ex vivo* using a MIP-1β internalization assay previously described (27–29). In this assay, free CCR5 receptors in the cell surface are internalized upon stimulation with MIP-1β, which is a natural ligand of CCR5 (30). The binding of MVC to CCR5 prevents internalization of CCR5 by MIP-1β and allows the detection of CCR5 by flow cytometry. Thus, the degree of CCR5 internalization by MIP-1β provides an indirect measurement of MVC binding to CCR5. For this assay, cells were gated in the lymphocyte region according to size and granularity. We defined the maximum CCR5 internalization in the absence of MVC in a given sample as the ratio in the percentage of CCR5-positive ($CCR5^+$) cells seen in reactions done with or without MIP-1β (×100). The maximum CCR5 internalization by MIP-1β in rhesus lymphocytes ranged between 18.9 and 89.1% and, as with human CCR5, was not increased upon incubation of cells with 100-fold-higher (1,200 ng) concentrations of MIP-1β (31; data not shown). Maximum internalization values were used as a reference to estimate the binding of MVC to CCR5 (27, 28).

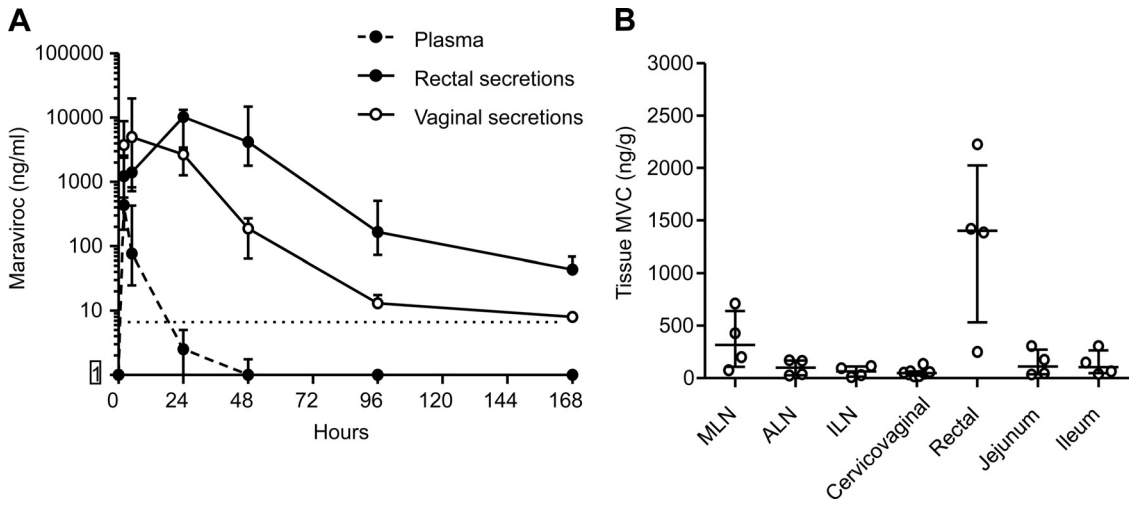

FIG 1 Pharmacokinetic profile of MVC in rhesus macaques. (A) Concentrations of MVC in plasma, vaginal secretions, and rectal secretions after a single oral dose of 44 mg/kg. The data are expressed as median and interquartile range. (B) Concentrations of MVC in tissues after a single oral dose of 44 mg/kg. Tissues were collected 24 h after drug dosing. The results are expressed as median and interquartile range. MLN, mesenteric lymph nodes; ALN, axillary lymph nodes; ILN, inguinal lymph nodes.

The binding of MVC to CCR5 was measured in longitudinal peripheral blood mononuclear cells (PBMCs) specimens collected from 6 macaques prior to and after (2 h, 5 h, 24 h, 48 h, 4 days, and 7 days) administration of a single oral dose of MVC. MVC binding to CCR5 was also measured in mononuclear cells from blood, rectal, and lymphoid tissues collected at necropsy in a second group of animals that received a single dose of MVC ($n = 4$) or no drug ($n = 10$). Briefly, 2×10^5 PMBCs or cells from rectal tissues were incubated with 12 ng of MIP-1β (recombinant human CCL4L1/MIP-1β isoform LAG-1; R&D Systems) or RPMI during 30 min at 37°C, followed by the addition of anti-CCR5-PE (3A9 monoclonal antibody; BD Biosciences), anti-CD4-FITC (FITC mouse anti-human CD4; BD Biosciences), and/or appropriate isotope controls. The mixture was incubated for 20 min at room temperature, fixed, and analyzed by flow cytometry. Cell populations were acquired within 24 h on a FACSCalibur using CellQuest Pro software (BD Biosciences), and analysis was performed using FlowJo software (v7.6.5; TreeStar). Cells were gated in the lymphocyte region according to size and granularity.

Changes in the percentage of $CCR5^+$, $CD4^+$, $CD3^+/CCR5^+$, or $CD3^-/CCR5^+$ cells during MVC treatment. We measured total $CCR5^+$ cells, total $CD4^+$ cells, and $CCR5^+/CD3^+$ or $CCR5^+/CD3^-$ subpopulations during two consecutive weeks in 6 macaques (3 treated and three untreated) from the MVC PrEP study. The following antibodies (clones) and reagents were used: L200 (CD4), 3A9 (CCR5), and SP34-2 (CD3) (BD Bioscience), L243 (HLA-DR) (BioLegend), and Fixable Live/Dead Aqua (Invitrogen). After staining and fixation, cells were run on an LSRII using FACS Diva Software (BD Biosciences) and analyzed using FlowJo. Lymphocytes were gated based on size/granularity, and monocytes and dead cells were excluded prior to gating lymphocyte subsets. The percentage of CCR5 expression was normalized from baseline stain, and the percent increase was compared in individual treated and untreated macaques.

Pharmacodynamic analysis of MVC in rhesus PBMCs *in vitro*. The relationship between MVC concentrations, binding of MVC to CCR5, and inhibition of virus replication was investigated *in vitro* using rhesus PBMCs. Briefly, CD8-depleted rhesus macaque PBMCs were exposed to several concentrations of MVC (500 to 0.0005 ng/ml) for 1 h, washed in complete media for PBMCs, and resuspended at 1.6×10^6 cells/ml. One aliquot of cells was used to measure CCR5 internalization and MVC binding as described above, and the remaining cells were exposed to $SHIV_{162p3}$ for 2 h at a multiplicity of infection of 0.0005. After the cells were washed three times in complete medium containing MVC, they were cultured with the appropriate concentration of MVC for 7 days. Cultures were monitored by measuring viral RNA in culture supernatants and cellular proviral DNA levels at days 4 and 7.

SHIV 162p3 *env* sequencing. A 1.105-kb *env* fragment containing the V1-V5 region of gp 120 was amplified from plasma SHIV RNA as previously described and sequenced using an ABI3130xl automated sequencer (32). The Vector NTI Advance program (version 11.5.0) was used to analyze the data and to infer amino acid sequences. Tropism was evaluated using the geno2pheno coreceptor tool (version 2.0) available at http://coreceptor.bioinf.mpi-inf.mpg.de/index.php following the recommendations from the European Consensus Group.

Statistical analysis. Peak RNA viremias, CCR5 internalization by MIP-1β, and changes in $CCR5^+$ cells among MVC-treated and untreated controls were compared using a two-tailed Wilcoxon rank-sum test.

RESULTS

PK profile of MVC in rhesus macaques and tissue drug levels. The pharmacokinetic (PK) profile of maraviroc was evaluated following oral administration of a single 44-mg/kg dose in 12 rhesus macaques. Figure 1A shows the median concentrations of MVC seen in plasma, rectal secretions, and vaginal secretions over 7 days. MVC concentrations in plasma peaked at 2 h and were below the limit of quantification of the assay at 24 h. Median C_{max} (437 ng/ml) and AUC_{0-24} (1,685 ng·h/ml) values were within the range seen in humans receiving a single 300-mg dose (412 to 503 ng/ml and 1,680 to 1,950 ng·h/ml, respectively) (7, 8). In rectal secretions, MVC concentrations peaked at 24 h with C_{max} (10,242 ng/ml) and AUC_{0-24} (131,164 ng·h/ml) values that were 23 and 78 times as high as those seen in plasma, respectively. The AUC values in rectal secretions calculated at 48 h (AUC_{0-48}, 339,772 ng·h/ml) and 96 h (AUC_{0-96}, 660,596 ng·h/ml) increased by a factor of 2.6 and 5 relative to AUC_{0-24}, demonstrating sustained accumulation of MVC in rectal secretions during this time interval. In vaginal secretions, MVC concentrations peaked earlier ($t_{max} = 5$ h) with a C_{max} of 4,986 ng/ml (Fig. 1A). Taken together, these findings demonstrate that a 44-mg/kg MVC dose given to rhesus macaques approximates plasma MVC levels in humans and results in high MVC concentrations in both rectal and vaginal secretions.

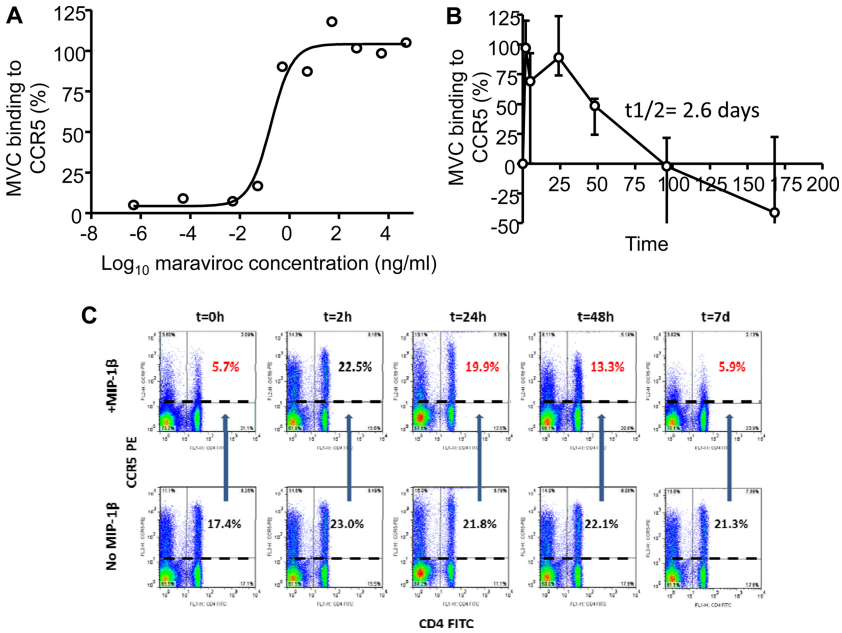

FIG 2 Kinetics of MVC binding to CCR5 in rhesus PBMCs *in vitro* and *in vivo*. (A) Rhesus macaque PBMCs were incubated *in vitro* with different concentrations of MVC, followed by analysis MVC binding to CCR5 by a MIP-1β internalization assay. (B) Six rhesus macaques received a single oral dose of MVC, followed by analysis of CCR5 occupancy and MVC binding to CCR5 in PBMCs collected over time. The results are expressed as percentage of MVC binding to CCR5 (median and interquartile range) relative to PBMCs collected from the same macaques prior to MVC treatment. (C) Representative results in rhesus PBMCs collected prior to and after a single oral dose of MVC. The addition of MIP-1β at $t = 0$ results in maximum internalization of CCR5 (from 17.4% of cells expressing CCR5 to 5.7%). The lack of CCR5 internalization at 2 h reflects the inability of MIP-1β to internalize CCR5 due to MVC binding to CCR5. The level of CCR5 internalization by MIP-1β increases overtime as MVC disassociates from CCR5 due to low plasma levels. The degree of CCR5 internalization by MIP-1β returns to baseline values at day 7.

We next investigated whether the high extracellular MVC levels in rectal secretions were associated with high tissue MVC concentrations. We administered a single oral dose of MVC to 4 SHIV-infected macaques, followed by necropsy to collect tissues 24 h later. Figure 1B shows the concentrations of MVC seen in rectal tissues compared to jejunum, ileum, cervicovaginal tissue, and mesenteric (MLN), axillary (ALN), and inguinal lymph nodes (ILN). The median concentration of MVC in rectal tissue (1,404 ng/g) was ~30 times as high as that seen in cervicovaginal tissues (48 ng/g), ~10 times as high as those seen in the jejunum and ileum (110 and 107 ng/g, respectively), and 4 to 22 times as high as those seen in lymphoid tissues (315 ng/g in MLN, 101 in ALN, and 63 ILN) (Fig. 1b). In these four macaques, MVC concentrations in rectal secretions were also very high (median, 3,745 ng/ml [range, 1,192 to 4,240 ng/ml]). Altogether, these results demonstrate that the high MVC concentrations seen in rectal secretions are associated with high tissue MVC levels and also show that MVC penetrates more efficiently into rectal tissues compared to cervicovaginal or lymphoid tissue.

MVC binding to CCR5 after oral dosing. We next evaluated whether high tissue concentration of MVC after oral dosing also resulted in effective binding of MVC to $CCR5^+$ target cells. The binding of MVC to CCR5 from rectal lymphocytes was investigated by measuring CCR5 internalization upon stimulation with MIP-1β. Before this analysis, we investigated *in vitro* the relationship between MVC concentrations and binding to CCR5 from PBMCs. Figure 2A shows that increases in the concentration of MVC were associated with increases in MVC binding to CCR5. At concentrations of MVC of 0.2 and 3.2 ng/ml, binding to CCR5 was close to 50 and 99%, respectively (Fig. 2A). Consistent with the *in vitro* data, MVC binding to CCR5 in PBMCs from the six treated macaques was highest within 24 h when MVC concentrations in plasma ranged between 437 ng/ml (2 h) and 2.5 ng/ml (24 h) (Fig. 2B). Based on the kinetics of MVC binding in PBMCs, we calculated a half-life of MVC bound to CCR5 of ~2 days. Figure 2C illustrates the changes in CCR5 internalization seen after MVC treatment in PBMCs from a representative animal.

The binding of MVC to CCR5 in rectal mononuclear cells was explored using cell suspensions prepared from rectal tissues collected from four SHIV-infected macaques 24 h after oral MVC dosing. Figure 3 shows that the degree of CCR5 internalization by MIP-1β in rectal tissues from these macaques was significantly reduced compared to untreated control animals ($P = 0.013$), demonstrating efficient binding of MVC to CCR5 in rectal tissues. As expected, MVC treatment also blocked CCR5 in mononuclear cells from blood and from axillary, mesenteric, and inguinal lymph nodes (Fig. 3).

Relationship between MVC concentrations, binding to CCR5, and inhibition of virus replication in rhesus PBMCs. We next investigated *in vitro* the relationship between MVC concentrations, CCR5 binding, and inhibition of virus replication. For this experiment, we incubated rhesus PBMCs with different concentrations of MVC, measured the degree of MVC binding to CCR5, and then exposed the cells to $SHIV_{162P3}$, followed by quantitative analysis of viral RNA and proviral DNA after 4 and 7 days of culture. The 50% inhibitory concentrations (IC_{50}s) for MVC were 0.45 ng/ml (day 4) and 0.92 ng/ml (day 7) based on RNA expression, and 1.96 ng/ml (day 4) and 2.6 ng/ml (day 7) based on

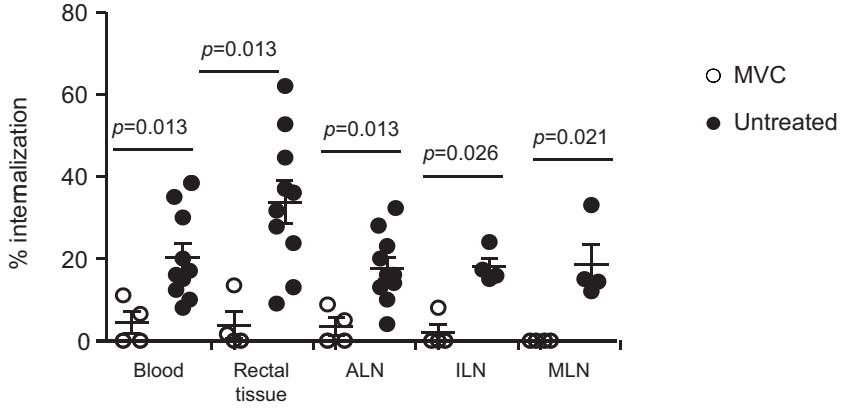

FIG 3 MVC binding to CCR5 in rectal and lymphoid tissues. Mononuclear cells from blood and rectal and lymphoid tissues collected at necropsy from MVC-treated (single oral dose; $n = 4$) or untreated ($n = 10$) macaques were exposed to MIP-1β or media. The results are expressed as the percentage of CCR5 internalization seen after incubation of cells with or without MIP-1β. The internalization of CCR5 by MIP-1β in mononuclear cells from blood, rectal tissues, and lymph nodes (axillary [ALN], mesenteric [MLN], and inguinal [ILN]) was significantly reduced in MVC-treated animals due to MVC binding to CCR5.

proviral DNA levels (Fig. 4). At a concentration near the IC_{50} (2 ng/ml), MVC binding was close to 95%, indicating that few unoccupied receptors are sufficient to sustain a productive infection *in vitro*. Figure 4 also shows that concentrations of MVC near the IC_{99} (~100 ng/ml) are associated with maximum MVC binding to CCR5. Thus, the levels of MVC seen at 48 h in rectal secretions from treated macaques (4,204 ng/ml, Fig. 1) are well above *in vitro* IC_{99} values for rhesus PBMCs.

Prophylactic efficacy of MVC against rectal transmission. To investigate the efficacy of MVC in preventing rectal SHIV infection, we administered to six macaques a 44-mg/kg dose of MVC 24 h before exposing them to SHIV, followed by a second dose 2 h after virus challenge (−24 h/+2 h schedule). Four untreated macaques were used as controls. Figure 5 shows the cumulative proportion of uninfected treated and untreated macaques as a function of the number of weekly rectal exposures. For comparison, results on 32 historical controls inoculated with the same virus stock and dose are also shown (33). Five of the six macaques receiving MVC were infected during the first 5 weekly virus challenges as did three of the four controls, demonstrating that this regimen was not able to prevent rectal SHIV infection. Peak viremias in MVC breakthrough animals (mean, 5.8 $\log_{10}$ RNA copies/ml; range, 4.6 to 7.2) were 0.7 $\log_{10}$ lower than in untreated controls (mean, 6.5 $\log_{10}$ RNA copies/ml; range, 6.3 to 6.7), although the differences were not statistically significant ($P = 0.25$). Post-peak viremias were also similar in both groups of animals ($P = 0.6$). Analysis of virus tropism in SHIV RNA sequences obtained at infection in all five macaques predicted R5 use in all isolates, indicating that infection of the macaques was not due to selection of low frequency X4 viruses that might be present in the

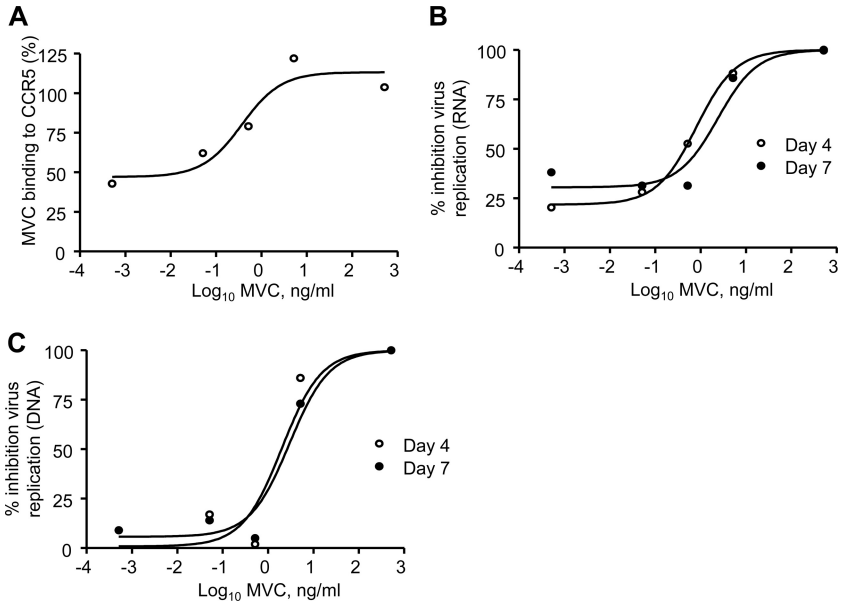

FIG 4 Relationship between MVC concentration, binding of MVC to CCR5, and inhibition of virus replication. (A) Rhesus PBMCs were incubated with different concentrations of MVC, followed by analysis of CCR5 occupancy by FACS. (B and C) Cells were then exposed to SHIV, followed by quantitative analysis of RNA (B) or proviral DNA (C) levels after 4 or 7 days of culture.

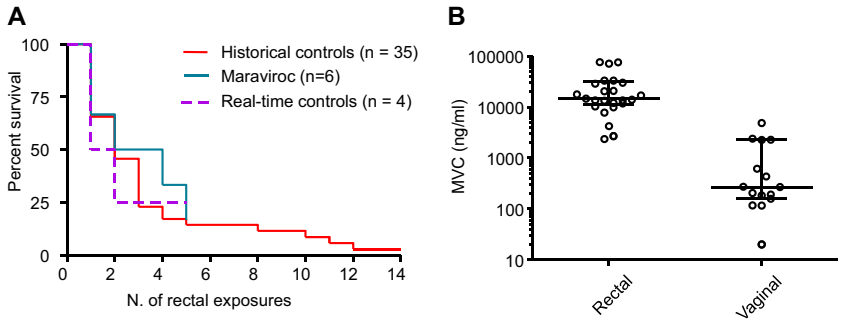

FIG 5 Prophylactic efficacy of pericoital oral MVC (−24 h/+2 h regimen). (A) Lack of protection against repeated rectal SHIV exposures. Each survival curve represents the cumulative percentage of uninfected macaques as a function of weekly rectal virus exposures. (B) MVC concentrations in rectal and vaginal secretions collected from the five macaques infected during prophylaxis with MVC. Animals were maintained on two weekly doses of MVC after infection to monitor drug concentrations in secretions. Each data point denotes the concentration of MVC obtained in rectal (5 weeks) or vaginal (3 weeks) secretions collected from each macaque. The levels (median and interquartile range) represent drug concentrations obtained 24 h after oral dosing.

$SHIV_{162P3}$ virus stock. SHIV *env* sequences obtained 7 to 17 weeks after infection in all five MVC-treated animals also predicted R5 use and showed absence of mutations associated with MVC resistance (data not shown).

To exclude suboptimal drug dosing or absorption as a possible explanation for infection outcome in the MVC-treated animals, we continued the MVC regimen after confirmed infection and measured drug concentrations in rectal and vaginal secretions. Figure 5 shows that the concentrations of MVC in rectal and vaginal secretions were both high (median, 14,637 and 269 ng/ml, respectively). We also assessed if the activity of MVC in secretions might be reduced because of high protein binding, thereby preventing it from binding to its receptor *in vivo*. Figure 6 shows that only about 15% of the MVC detected in rectal secretions was bound to proteins. The stability of MVC in rectal secretions was also evaluated by spiking MVC in secretions followed by measurement of free and protein-bound MVC at 24 h. Both free and protein-bound MVC were stable in rectal secretions during this period of time (Fig. 6). Taken together, these findings demonstrate that the failure of MVC to prevent rectal infection was not due to inadequate drug absorption or reduced free-drug exposures at the rectal mucosa.

Impact of MVC on CCR5. CCR5 blockage by MVC reduces the chemotactic activities of leukocytes *in vitro* and *ex vivo* (34, 35). To explore whether MVC treatment could have any impact in the $CCR5^+$ population in blood, we measured $CCR5^+$ cells during two consecutive weeks of MVC PrEP in 3 macaques and compared the levels with those seen prior to MVC treatment. We analyzed total $CCR5^+$ cells, as well as $CCR5^+$ subpopulations that also expressed CD3. Changes were compared to those seen in three in-

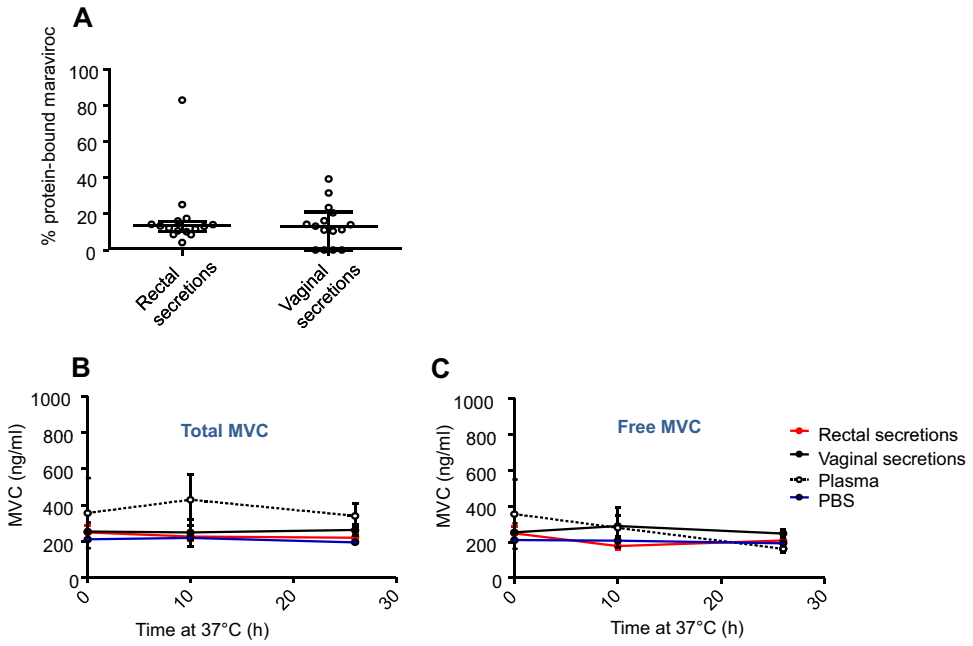

FIG 6 Protein binding and stability of MVC in rectal and vaginal secretions. (A) Percentage of protein-bound MVC measured in secretions by rapid equilibrium dialysis. Date reflects median and interquartile range. (B and C) Stability of MVC secretions. MVC (400 ng/ml) was spiked into PBS, plasma, and rectal and vaginal secretions and incubated for 24 h at 37°C. Total and free MVC concentrations were then measured by rapid equilibrium dialysis, followed by HPLC. The data reflect the median (interquartile range) values obtained in two separate experiments performed in duplicate.

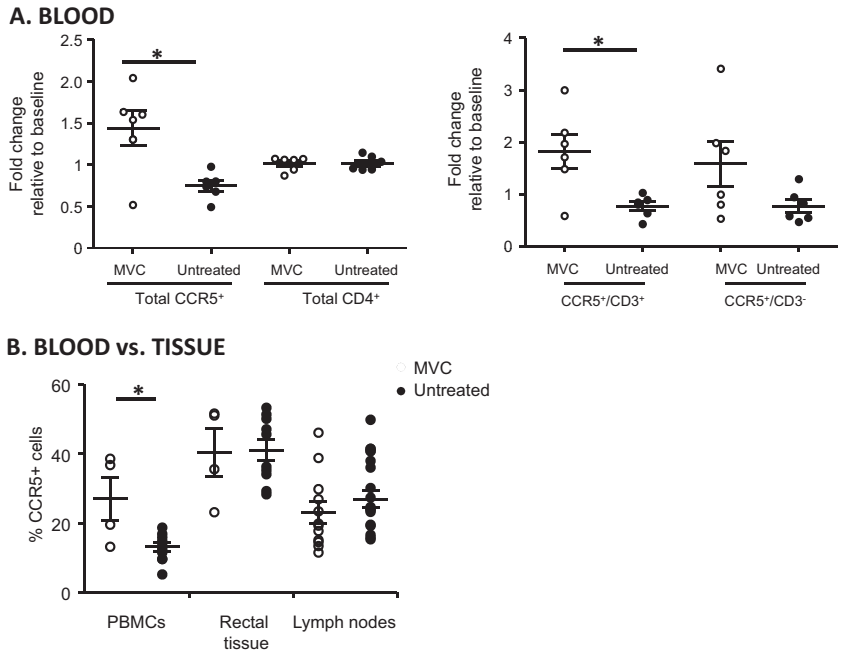

FIG 7 Impact of MVC on CCR5$^+$ cell populations in blood and tissues. (A) Impact of MVC on total CCR5$^+$, total CD4$^+$, CCR5$^+$/CD3$^+$, and CCR5$^+$/CD3$^-$ cells. Cell subpopulations were measured in blood specimens collected from three treated animals (24 h after MVC dosing) and from three untreated controls. For each macaque, we measured cell subpopulations for two consecutive weeks. The results are expressed as fold changes in the percentage of CCR5$^+$ or CD4$^+$ cells seen relative to the value seen prior to study initiation. The asterisk indicates a statistically significant increase in the MVC group compared to untreated controls. (B) Changes in the percentages of total CCR5$^+$ cells in tissues. Fourteen SHIV-infected macaques received MVC ($n = 4$) or no drug ($n = 10$) 24 h prior to necropsy to collect tissues. The results represent the percentage of CCR5$^+$ cells observed in the lymphocyte gate of cell suspensions from blood, rectal tissues, or lymph nodes (axillary, mesenteric, and inguinal). Horizontal lines denote means ± the standard errors of the mean.

fected untreated controls. Figure 7 shows that treatment with MVC was associated with a significant increase in the percentage of total CCR5$^+$ but not CD4$^+$ cells ($P = 0.041$ and 0.937, respectively). Such an increase was significant in the CD3$^+$ ($P = 0.0411$) but not in the CD3$^-$ population ($P = 0.1797$). Figure 7 also shows that the increase in the percentage of CCR5$^+$ cells was limited to blood and was not seen in rectal or lymphoid tissues.

DISCUSSION

To our knowledge, this study is the first to explore the prophylactic efficacy of oral MVC against rectal HIV or SIV transmission. We selected a dose of MVC that resulted in plasma drug levels in macaques that were within the range of those seen in humans receiving 300 mg (7, 8). Rectal SHIV challenges were performed 24 h after dosing to coincide with peak MVC concentrations in rectal secretions. We also administered a postchallenge dose to further increase and extend mucosal and systemic MVC exposure and maximize efficacy. With this dose of MVC, drug concentrations in rectal secretions over 24 h were about 80 times as high as in plasma and remained above the EC_{90} for up to 4 days. About 25% of the MVC dose in humans is eliminated through feces, resulting in high MVC concentration in rectal secretions (7). Fecal elimination of MVC may have also contributed to the high MVC concentrations seen in rhesus macaques in our study. We show that MVC in secretions was mostly unbound to proteins, which facilitates tissue dosing and further explain the high MVC concentrations seen in rectal tissues that exceeded those seen in vaginal or lymphoid tissue by 10 to 30 times. Despite this favorable pharmacokinetic profile, MVC was not protective, with most of the animals becoming infected within the first weeks of the study.

The lack of rectal protection raises questions about the concentration of MVC that is required to prevent transmission. The gastrointestinal mucosa has a high density of CCR5$^+$ cells and contains mostly activated, memory CD4$^+$ T cells that are highly susceptible to HIV infection, which all suggest the need for high MVC concentrations (36). Studies of vaginal gels in rhesus macaques also identified a half-maximal protection by a vaginal MVC gel of 0.5 mM MVC, which is ~25 times as high as the peak MVC concentration seen here (0.02 mM) (11, 37). This observation, although in a vaginal infection model, demonstrates the efficacy of topically applied MVC but points to a high mucosal MVC exposure needed for protection, which clearly was not achieved by the oral dose regimen that we used. In humans, daily MVC results in a 5-fold drug accumulation in rectal tissues (7). It is not known whether MVC accumulation in tissues and continuous systemic MVC exposure associated with daily dosing would have been sufficient to cross the drug protection threshold and prevent infection.

It is also notable that because of its unique mechanism of action through CCR5 binding, MVC may have immunologic effects that are independent of its antiviral activity. In a randomized, placebo-controlled MVC intensification trial in HIV-infected persons, MVC treatment was found to increase by 2-fold the levels of T cell activation and the percentage of CD4$^+$/CCR5$^+$ cells in rectal tissue (38). The MVC-mediated increase in activated CD4$^+$ T cells in rectal tissue, if confirmed in HIV-uninfected persons,

might have implications for PrEP efficacy by MVC because it might increase the risk of HIV acquisition and reduce the ability of MVC to prevent rectal infection. Although we did not measure the effect of MVC on T cell activation in rectal tissues, we did show that MVC increased the percentage of $CCR5^+/CD3^+$ cells in blood in macaques, further demonstrating the immunological effects of CCR5 inhibition by MVC in our model. These findings highlight the need to better define the impact of these immunological effects on the PrEP potential of MVC.

Although we found that MVC efficiently binds to CCR5 in rectal lymphocytes, we also documented virus replication *in vitro* when MVC binding to this coreceptor was close to 95%. This observation is consistent with clinical studies showing that almost complete CCR5 occupancy is required to significantly reduce plasma viremia (29) and suggests that only a few unoccupied receptors may be sufficient to initiate and sustain a spreading infection. However, as with human CCR5, rhesus CCR5 was not fully internalized by MIP-1β in PBMCs or rectal leukocytes in the *in vitro* assay, thus limiting our analysis to the proportion of CCR5 that was downregulated by MIP-1β *ex vivo* (31). Also, our analysis of MVC binding to rectal CCR5 was performed in SHIV-infected macaques, which might have overestimated occupancy since the number of high CCR5-expressing activated memory $CD4^+$ T cells are expected to be significantly reduced with SHIV infection (39). It will be important to perform this analysis in uninfected animals using improved assays that better define MVC binding to CCR5 in rectal mononuclear cells.

The demonstration that some HIV isolates can utilize MVC-bound conformations of CCR5 to initiate an infection *in vitro* also raises questions about the use of MVC as a single drug for PrEP (40, 41). In studies using a CD4/CCR5 dual-inducible (Affinofile) cell line, chronic isolates frequently exhibited resistance to MVC particularly when surface CCR5 expression was very high (40, 41). This phenotypic trait suggests that the ability of MVC to block infection may be variable among isolates and at different surface CCR5 densities.

The approach we used to model PrEP with MVC against rectal infection was similar to our previous studies assessing the efficacy of FTC and TDF. We first recapitulated the clinical MVC exposures in macaques and then conducted challenge studies using a similar repeat low-dose rectal transmission model. We have previously shown that this rectal model predicted the prophylactic efficacy of daily FTC/TDF combination seen in iPrEX, and that FTC/TDF was more protective than either TDF or FTC alone (2, 18, 21). We note that this rectal model does not inform vaginal efficacy of the FTC/TDF combination or TDF alone given the differences in drug penetration and susceptibility to infection between rectal and vaginal tissues (42, 43). Therefore, separate studies are needed to assess vaginal efficacy in macaques and to determine whether oral TDF alone prevents vaginal transmission, as was found in humans (1). Although our results here suggest that MVC may not be a good candidate for oral PrEP, they also need to be interpreted with caution. First, findings with a pericoital MVC regimen may not fully reflect the prophylactic efficacy of daily MVC, especially since daily dosing results in tissue drug accumulation and maintains systemic drug concentrations, which may both be important for protection (7). Second, efficacy of MVC might be influenced by biochemical differences between human and rhesus CCR5. Although MVC binds with similar affinity to both coreceptors, it disassociates faster from macaque CCR5 (44). It is unclear whether a faster disassociation from CCR5 is playing a role in our results since MVC concentrations in rectal secretions were high and sustained for 2 to 4 days, thus providing a continuous source of MVC for tissues and CCR5 blockage. Also, the R5-tropic HIV_{SF162} envelope contained in our SHIV isolate was fully susceptible to MVC, and the MVC EC_{50} in rhesus PBMCs was within the range of that seen with human PBMCs (45, 46).

In conclusion, we show that the high concentrations of MVC achieved in rectal tissues during a pericoital −24 h/+2 h MVC regimen are not sufficient to prevent rectal SHIV transmission in macaques. The lack of prophylactic efficacy may reflect drug concentrations below the threshold required to block a dense population of $CCR5^+$ in the rectal mucosa, biochemical differences between human and macaque CCR5, or other mechanisms associated with MVC treatment, including modulation of immune responses and susceptibility to infection.

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