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RESEARCH PAPER

Pharmacodynamic Activity of Dapivirine and Maraviroc Single Entity and Combination Topical Gels for HIV-1 Prevention

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ABSTRACT

Purpose Dapivirine (DPV), a non-nucleoside reverse transcriptase inhibitor, and maraviroc (MVC), a CCR5 antagonist, were formulated into aqueous gels designed to prevent mucosal HIV transmission.

Methods 0.05% DPV, 0.1% MVC, 0.05% DPV/0.1% MVC and placebo gels were evaluated for pH, viscosity, osmolality, and *in vitro* release. *In vitro* assays and mucosal tissues were used to evaluate anti-HIV activity. Viability (*Lactobacilli* only) and epithelial integrity in cell lines and mucosal tissues defined safety.

Results The gels were acidic and viscous. DPV gel had an osmolality of 893 mOsm/kg while the other gels had an osmolality of <100 mOsm/kg. MVC release was similar from the single and combination gels (~5 $\mu g/cm^2/min^{1/2}$), while DPV release was 10-fold less from the single as compared to the combination gel (0.4331 $\mu g/cm^2/min^{1/2}$). Titrations of the gels showed 10-fold more drug was needed to protect ectocervical than colonic tissue. The combination gel showed ~10- and 100-fold improved activity as compared to DPV and MVC gel, respectively. All gels were safe.

Conclusions The DPV/MVC gel showed a benefit blocking HIV infection of mucosal tissue compared to the single entity gels. Combination products with drugs affecting unique steps in the viral replication cycle would be advantageous for HIV prevention.

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KEY WORDS Antiretroviral drugs · Drug combinations · HIV prevention · Pre-exposure prophylaxis · Rectal microbicides

ABBREVIATIONS

API	Active pharmaceutical ingredient
DPV	Dapivirine
HIV	Human immunodeficiency virus
IPM	International partnership for microbicides
MTN	Microbicide trials network
MVC	Maraviroc
NDRI	National disease research interchange
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleotide reverse transcriptase inhibitor
PrEP	Pre-exposure prophylaxis
TER	Trans-epithelial resistance
TFA	Trifluoroacetic acid
TFV	Tenofovir

INTRODUCTION

Since 2001, a slow but steady decline in newly acquired HIV infections globally has resulted from significant HIV prevention efforts that include HIV-infected people accessing treatment (1), the broad uptake of medical male circumcision (2), and the prevention of mother to child transmission (3). Despite this positive news, 2.3 million newly acquired HIV infections occurred last year, of which the majority was through sexual transmission (4). Efforts are underway to broaden the HIV prevention portfolio to include vaccines, pre-exposure prophylaxis (PrEP), and topical microbicides in the hope to halt the sexual transmission of HIV and achieve an AIDS-free generation. An array of different prevention methods is needed to provide options for persons wishing to incorporate the product into their stage of life. For example, while discordant HIV-infected couples have shown a benefit taking oral PrEP

to prevent the acquisition of HIV (5,6), high-risk young women have not (7,8). Further, high-risk women in CAPRISA 004 showed modest protection from HIV acquisition after using the 1% tenofovir (TFV) gel peri-coitally (9), but no benefit was seen when high-risk women in MTN-003 used the same topical gel daily (8). The lack of product efficacy may have been due to the poor product adherence. Thus, providing options for people at-risk for HIV acquisition will likely increase their use of the product.

The current paradigm for topical microbicide development has been focused on antiretroviral drugs as earlier work evaluating non-specific entry inhibitors showed no benefit to the participants in those clinical trials (10–12). The product furthest in development is 1% TFV gel. This gel has been evaluated in high-risk women in three phase 2b/3 clinical trials (8,9,13). The discrepant results between the CAPRISA 004 and the MTN-003 / FACT 001 trials could be due to adherence to study product, tissue drug levels attained, or a combination of both. While alternative dosage forms, such as intravaginal rings (IVRs), are being developed that should improve study product adherence, finding more potent molecules would be beneficial as well. TFV is a nucleotide reverse transcriptase inhibitor (NRTI) that requires cellular uptake and conversion to TFV-diphosphate (TFV-dp). TFV-dp is stable intracellularly with a half-life of up to 90 h in tissue (14) thus creating a cellular depot. The amount of TFV-dp in the tissue required for effectiveness is unknown, but women having greater than 1 μg/ml of TFV in the vaginal lumen fluid were more likely to be protected from HIV infection (15). This concentration is at the *in vitro* 50% effective dose (ED_{50}). More potent antiretroviral drugs may help reduce HIV acquisition by possibly decreasing the amount of drug needed and the frequency of dosing. Dapivirine (DPV) a non-nucleoside reverse transcriptase inhibitor (NNRTI), binding a hydrophobic pocket of the RT outside the catalytic site, and maraviroc (MVC) an entry inhibitor, blocking HIV attachment to the CCR5 receptor, are now being considered as topical microbicides. Both drugs have ED_{50}'s in the nM range; >100-fold lower than TFV. DPV and MVC have been formulated into hydrogels. We present the physiochemical characterization of these formulations and their pharmacodynamic activity and safety using our preclinical testing algorithm that includes human mucosal tissue (16).

MATERIALS AND METHODS

Products

0.05% DPV (formulation code 4759), 0.1% MVC (5225), 0.05% DPV/0.1% MVC (5158) and placebo (5159) gels were manufactured and provided by the International Partnership for Microbicides (IPM). The final concentrations of DPV and MVC in the formulations were determined by IPM. The 0.05% DPV gel contained purified water, hydroxyethylcellulose, polycarbophil, and propylene glycol, methylparaben and propylparaben. 0.1% MVC gel contained purified water, poloxamer 407, hydroxyethylcellulose, hydroxypropylcellulose, citric acid, sodium citrate and benzoic acid. The combination gel consisted of purified water, poloxamer 407, hydroxyethylcellulose, hydroxypropylcellulose, citrate buffer and benzoic acid (17). To determine if formulation differences affected the results, a DPV control gel (DPV-C) was formulated in the same base as the combination gel. The active pharmaceutical ingredients (APIs) were provided by IPM and working solutions were made in DMSO; aliquots stored at −20°C. Where appropriate, Gynol II (Ortho-McNeil-Janssen Pharmaceutical, Inc. Titusville, NJ) an over-the-counter 3% nonoxynol-9 (N9) gel was used as a control for cell and tissue toxicity.

Human Tissue

Normal human ectocervical (IRB # 0503103) and colorectal (IRB # 0602024) tissues were acquired from pre-menopausal women undergoing hysterectomy or persons undergoing colorectal surgery for non-inflammatory conditions, respectively. When the patients consent for surgery, they sign a general consent that tissue remainders can be used for research. Therefore, a study specific consent form was not deemed necessary. The IRB deemed this exempt because surgical tissue remainders that would otherwise be discarded are used for this research. No patient identifiers were provided and all tissues were collected through an Honest Broker de-linking patient ID to the investigators. Normal human ectocervical tissue was also purchased from National Disease Research Interchange (NDRI) (http://ndriresource.org/) through an approved protocol and shipped overnight on ice.

Physicochemical Testing

Rheological profile was determined using the CP52 spindle on a cone/plate Brookfield Model HADVIII+ viscometer (Brookfield Eng. Lab., Inc., Middleboro, MA). Data was collected using Rheocalc software (Brookfield Eng. Lab., Inc.). The sample was placed in the sample cup of the instrument and allowed to equilibrate to 25°C for 10 min. Viscosity was measured using a program where shear rate was increased from 0.2 to 10 s^{-1} with torque values between 10 and 90% and subsequently decreased to 0.2 s^{-1}. To compare data across samples, apparent viscosity values acquired at 0.4 rpm (equivalent to shear rate of 0.8 s^{-1}) were reported in the analysis. Plastic viscosity and yield stress was calculated by Rheocalc Software using the Casson equation which gave the best fit.

pH was determined using an Accumet AR20 pH meter (Fisher) with an AccuFet (Fisher) semisolid-state probe calibrated using three points, pH 4.0, 7.0 and 10.0.

Osmolality was determined using a freezing point osmometer (Model 3320, Advanced Instrument, Inc. Norwood, MA) calibrated with 50 and 850 mOsm/kg standards.

In vitro release studies were carried out using the Hanson Microette® system. Spectra/Por membrane discs with a molecular weight cut off of 6000–8000 (Spectrum Chemical Mfg. Corp., New Brunswick, NJ) were used as the inert membrane; the receptor medium was 1% cremophor. The membrane was placed on the Hanson Microette® system with 400 μL of the MVC, DPV, or DPV/MVC gel. Samples were taken from the receptor compartment at predetermined time intervals (0, 15, 30, 45, 60, 120, 180, 240, 300, 360 min) and assayed for drug content using a high pressure liquid chromatography method. A Waters (Waters Corp, Milford, MA) system equipped with Waters 600E pump, Waters W717 autosampler, Waters 2996 Photodiode Array Detector, and a computer with Empower Version 2 software (Waters) was used with a Alltech Alltima C18 5 μ 250× 4.6 mm column (GRACE, Columbia, MD). Separations were done by gradient elution. The initial step used 70% (*v*/*v*) of 0.1% TFA in water and 30%(*v*/*v*) of 0.05% TFA in acetonitrile, next the aqueous portion was decreased to 54% (*v*/*v*), and then the aqueous portion was gradually decreased to 20% (*v*/*v*), which then cycles back to the initial step. The wavelengths for detection of DPV and MVC were 290 and 210 nm, respectively.

Efficacy Testing

TZM-bl Assay

TZM-bl cells (18) (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were used to determine the 50% cytotoxic concentration (CC_{50}) and 50% effective dose (ED_{50}) of the APIs and gels using GraphPad Prism® (V5.02) software to calculate the therapeutic index. TZM-bl cells were plated and 100 μl of 10-fold serially diluted APIs or formulated product was applied. For toxicity testing, 100 μl of medium or 100 μl of medium containing final 6% whole semen (Lee BioSolutions, Inc., St. Louis, MO) was added to each well. The next day, 100 μl of medium was removed and replaced with 100 μl of CellTiter-Glo™ and the luminescence measured. Viability was determined based on deviations from the cell-only or the semen only controls and presented as the percentage viability ± SEM. For efficacy testing, 100 μl of medium containing HIV-1_{BaL} (CCR5-using HIV and representative of virus that is sexually transmitted) without or with 12% whole semen was added to each well. After 48 h, 100 μl of medium was removed and replaced with 100 μl of Bright-Glo™ (Promega Corp.), and the luminescence measured. The 48 h culture for efficacy improved the luminescence signal to noise. Inhibition was determined based on deviations from the HIV-1-only or HIV-1/semen controls and presented as the percentage inhibition ± SEM.

The benefit of DPV and MVC combination was assessed using the TZM-bl assay. Briefly, 2-fold dilutions encompassing the ED_{50} of each drug alone and in combination were added to the TZM-bl cells. After 48 h, half the volume was removed and replaced with Bright-Glo™ (Promega Corp.) and the luminescence measured. Assays were performed in triplicate and a minimum of 3 independent assays were performed. CalcuSyn software (Biosoft V2.1) was used to define the dose effect using the Chou-Talalay equation (19). The linear correlation coefficient was >0.90 to assure accuracy and conformity of the data. A combination index of <0.90 demonstrated drug synergy; 0.90 to 1.10 demonstrated additive activity; >1.10 demonstrated drug antagonism.

Explant Culture Testing

Normal human ectocervical and colorectal tissues were used. Polarized explant cultures were set-up as previously described (16). Briefly, the explant was placed with the luminal side up in a transwell. The edges around the explant were sealed with Matrigel™ (BD Biosciences, San Jose, CA). The explants were maintained with the luminal surface at the air-liquid interface. The lamina propria was immersed in medium for ectocervical explants or resting on medium-soaked gelfoam for colorectal explants. Cultures were maintained at 37°C in a 5% CO_2 atmosphere. The explants were prepared on day of surgery in duplicate and treated apically with titrations of unformulated DPV or MVC and HIV-1_{BaL} to determine the ability to prevent HIV-1 infection. To assess the efficacy and if formulation improved the drug activity, a 1:5 dilution of placebo, DPV, MVC, or combination gel was mixed with HIV-1_{BaL} and added to the apical side of the explants. Once the activity of the gels was confirmed, the potency of the DPV, MVC, and combination gels was determined by titrating them in culture medium and applying to the apical surface of mucosal tissue along with HIV-1_{BaL}. Regardless of the product tested, 18 h after application the explants were washed and fresh medium (without drug) was added to the basolateral compartment. Every 3 to 4 days over a 3 week period, supernatant was collected and stored at −80°C for HIV-1 p24gag analysis and fresh medium (without drug) was replenished. Immunohistochemistry (IHC) was performed only on ectocervical tissue for HIV-1 infected cells by staining for p24gag (16).

Safety Testing

Normal Vaginal Flora Testing

Lactobacillus species (*L. crispatus* ATCC 33197, *L. jensenii* ATCC 25258, and *L. jensenii* LBP 28Ab) were obtained from the American Type Culture Collection (Manassas, VA.). Bacterial suspensions were prepared by selecting isolated colonies from fresh, overnight culture plates and suspending the test organisms in saline to a density of a 2 McFarland standard. Suspensions were mixed 1:1 with each gel. After incubation at 35°C for 30 min, samples were plated onto the appropriate medium. Plates were incubated for 24 h and evaluated for killing of the test microorganisms by examination. Colony forming units were counted and the differences between the untreated and treated cultures were determined. Loss of viability ≥1 $\log_{10}$ for two species was considered a product failure.

Epithelial Cell Lines Testing

Epithelial cell lines were obtained from the American Type Culture Collection (Manassas, VA). Unless otherwise stated, culture reagents were purchased from Hyclone (Logan, UT). Caco-2 cells, a colorectal epithelial cell line, were grown in MEM alpha modified medium supplemented with 20% heat-inactivated fetal bovine serum (FBS; Gemini Bio-products, West Sacramento, CA), 100 μg/ml streptomycin, 100 U/ml penicillin, and 100 mM L-glutamine. HEC-1-A cells, an endometrial epithelial cell line, were grown in McCoy's 5A medium supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 100 mM L-glutamine.

For viability testing, dilutions (1:10, 1:20, 1:50, 1:80 and 1:100) were made of active and placebo gels in the appropriate cell culture medium to test for cell viability. Caco-2 or HEC-1-A cell lines were plated in triplicate in a 96-well plate for each treatment. Diluted active and placebo gels were added to the appropriate wells. Control wells with no treatment (cells only) and medium only were included for background luminescence. The plate was cultured for 24 h and then 100 μl of culture medium was replaced with CellTiter-Glo™ (Promega Corp., Madison, WI) to all the wells per the manufacturer's instructions and luminescence was measured using a Beckman DTX 880 plate reader. Viability was determined based on deviations from the cell only control and presented as% viability of control ± standard deviation.

To determine the effect of active and placebo gels on epithelial integrity, the transepithelial resistance (TER) was measured. HEC-1-A or Caco-2 cells were grown on transwell supports until a confluent, polarized monolayer developed as measured by a MilliCell-ERS resistance system (Millipore, Billerica, MA). At that time, a 1:20 dilution of products was added to the apical surface of the monolayer and resistance readings were measured as indicated. As controls, wells with cells alone, cells treated with N9, or no cells were used. The epithelial resistance was expressed as ($\Omega \times cm^2$) of the treated wells - ($\Omega \times cm^2$) of the no cell wells.

Explant Culture Testing

Explants were assembled as described above and prepared in duplicate. To ensure even spread of the gels and to allow it to be mixed with HIV-1 for the efficacy testing (below), a 1:5 dilution of active or placebo gels was applied to the apical side of the explants for 18 h. As controls, explants were untreated or a 1:5 dilution of 3% N9 gel was applied apically. The next day, explants were washed and viability was evaluated using the MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] assay and histology (16).

Statistical Analysis

In the tests of viability, the outcome of percent viability was compared on the log-transformed scale using an ANOVA model that controlled for experimental block, and the exponeniated coefficients were used as estimates. In the cell-line tests, each active/placebo gel and dilution combination was treated as a separate category. Statistical significance was determined using t-tests corrected for multiple comparisons using Bonferroni adjustments.

In the test of epithelial integrity, treated well TER minus no-cell well TER measurements were used as the outcome in an ANOVA model that controlled for baseline at time −1 and replication. For each gel or control, the estimate at each time point was compared to baseline using a *t*-test adjusted for multiple comparisons using the Bonferroni adjustment.

In the tests for API efficacy in tissue, linear mixed effects models with random intercepts were used to assess differences in p24 between active/placebo gels and controls at each time point. Statistical significance was determined using t-tests corrected for multiple comparisons using the Bonferroni adjustment.

RESULTS

DPV and MVC Drug Substance Testing

To initially characterize the drug activities, *in vitro* and *ex vivo* testing of the APIs was done. *In vitro* testing defined the ED_{50} of DPV and MVC APIs as 1.95 and 5.2 nM, respectively. The TI for DPV was 1620 and for MVC was 1938 indicating the efficacious dose exceeds the toxic dose by more than 1000-fold. These data are consistent with previously published findings (20,21). Testing in the presence of 6% whole semen had no impact on DPV or MVC activity with ED_{50} of 1.70 and 5.6 nM, respectively. To determine if combining both drugs

would be beneficial, titrations of DPV and MVC APIs applied at a 1:2 ratio showed the combination of both drugs to be synergistic (combination index <0.90) across combination index values at ED_{50}, ED_{75}, and ED_{90} (Table I).

API efficacy was also tested *ex vivo* using polarized ectocervical and colonic mucosal tissue explants. Unlike *in vitro* cell assays, mucosal tissue provides appropriate target cells for protection against HIV-1 infection (22). Initial testing was done by treating tissues apically with titrations of the individual APIs. DPV, the NNRTI, protected the polarized ectocervical tissue at 10 μM while polarized colonic tissue was protected down to 1 μM (Fig. 1; upper panels). In contrast, MVC, the CCR5 antagonist, required higher concentrations (100 μM) to completely protect the polarized ectocervical and colonic tissue (Fig. 1; lower panels). Partial protection of colonic tissue was shown for 10 μM of MVC. DPV and MVC were approximately 2- to 10-fold more potent in colonic tissue as compared to ectocervical tissue. However, approximately 5-fold more DPV and 20-fold more MVC were required for tissue protection as compared to the TZM-bl cell line. We next evaluated the formulated drugs to determine if this impacted the drug's potency.

DPV and MVC Formulation Physiochemical Characteristics

The gels were all aqueous based hydrogels. Due to the hydrophobicity of DPV, it was uniformly dispersed in the hydrogel. Both the single entity DPV and the combination DPV/MVC gels were opaque and viscous due to this dispersion. In contrast, the MVC and placebo gels were transparent and colorless since MVC was completely solubilized. These four products exhibited similar shear thinning and non-Newtonian pseudoplastic rheological behavior. In a shear thinning/pseudoplastic system, viscosity decreases as the applied shear to the product is increased. All four gels except for the DPV gel exhibited comparable apparent viscosities (Table II) ranging from 161,233 to 188,766 cps at 0.4 rpm. DPV gel was more viscous with an apparent viscosity of 446,490 cps at 0.4 rpm. All of the gels had similar pH ranging from 4.0 to 4.7. The DPV gel was hyperosmolar (893 mOsm/kg), while the other gels were hypoosmolar with an osmolality ranging from 84 to 91 mOsm/kg. The DPV-C gel formulated in the combination gel base was also hypoosmolar (80 mOsm/kg) and had comparable viscosity (183,805 cps at 0.4 rpm) to the three other gels made in the similar base (Table I). With respect to *in vitro* release, DPV showed biphasic *in vitro* release profile from either the combination or single entity gel with an initial phase of significantly lower release rate (<0.003 $\mu g/cm^2/min^{1/2}$) before 45 min. From second phase, 60 to 360 min, DPV release was slower from the single entity gel (0.046 $\mu g/cm^2/min^{1/2}$). The release rate obtained from the single entity gel was approximately 0.1-fold of the release rate from the combination gel (0.433 $\mu g/cm^2/min^{1/2}$) (Fig. 2). However, the DPV-C gel displayed a similar release for the second phase from 60 to 360 min as compared to the combination gel (0.383 $\mu g/cm^2/min^{1/2}$). MVC release rate was similar for both the single entity and combination gels (4.4425 $\mu g/cm^2/min^{1/2}$ and 4.8156 $\mu g/cm^2/min^{1/2}$, respectively).

DPV and MVC Formulation Testing

The ED_{50} of the DPV and MVC gels were 2 and 10.3 nM, which was consistent with the API ED_{50}. The DPV-C gel in the combination base formulation gel did not change the ED_{50}; 2 nM. Likewise, 6% whole semen did not impact the activity of the gels as shown by DPV and MVC gels similar ED_{50}, 1.7 and 11.25 nM. The combination gel showed a modest benefit compared to either single entity gel with an ED_{50} for DPV of 1.38 nM (with semen 0.82 nM) and for MVC of 1.73 nM (with semen 1.04 nM) with semen having no impact on the drug's effectiveness in the combination gel.

For initial testing of the formulated products in mucosal tissue, a 1:5 dilution of each gel, including the placebo gel, was applied to the tissue and HIV-1_{BaL} was added. After an overnight exposure, explants were washed and HIV-1 replication was monitored by release of p24gag antigen in the culture supernatant (Fig. 3). For the ectocervical and colonic tissues, there was a significant decrease from the control explants (no treatment; HIV-1 only) in those treated with DPV and MVC single entity gels and the combination gel by the end of culture ($P=0.002$). The placebo gel treated explants had similar HIV-1 replication and kinetics to the no treatment

Table I Dose Effect Analysis of Dapivirine and Maraviroc Active Pharmaceutical Ingredients by the TZM-bl Cell Line

Drug	CI values at			Dm	m	r
	ED_{50}	ED_{75}	ED_{90}			
Dapivirine	N/A	N/A	N/A	3.38230	2.54350	0.90071
Maraviroc	N/A	N/A	N/A	13.98388	2.02407	0.90969
DPV/MVC @ 1:2	0.51249	0.65382	0.83630	1.16825	1.54669	0.97191

CI combination index, *ED* effective dose, *Dm* median-effect dose (ED_{50}), *m* measurement of the sigmoidicity of the dose-effect curve, *r* linear correlation coefficient of the median-effect plot, *N/A* not applicable, *DPV* dapivirine, *MVC* maraviroc

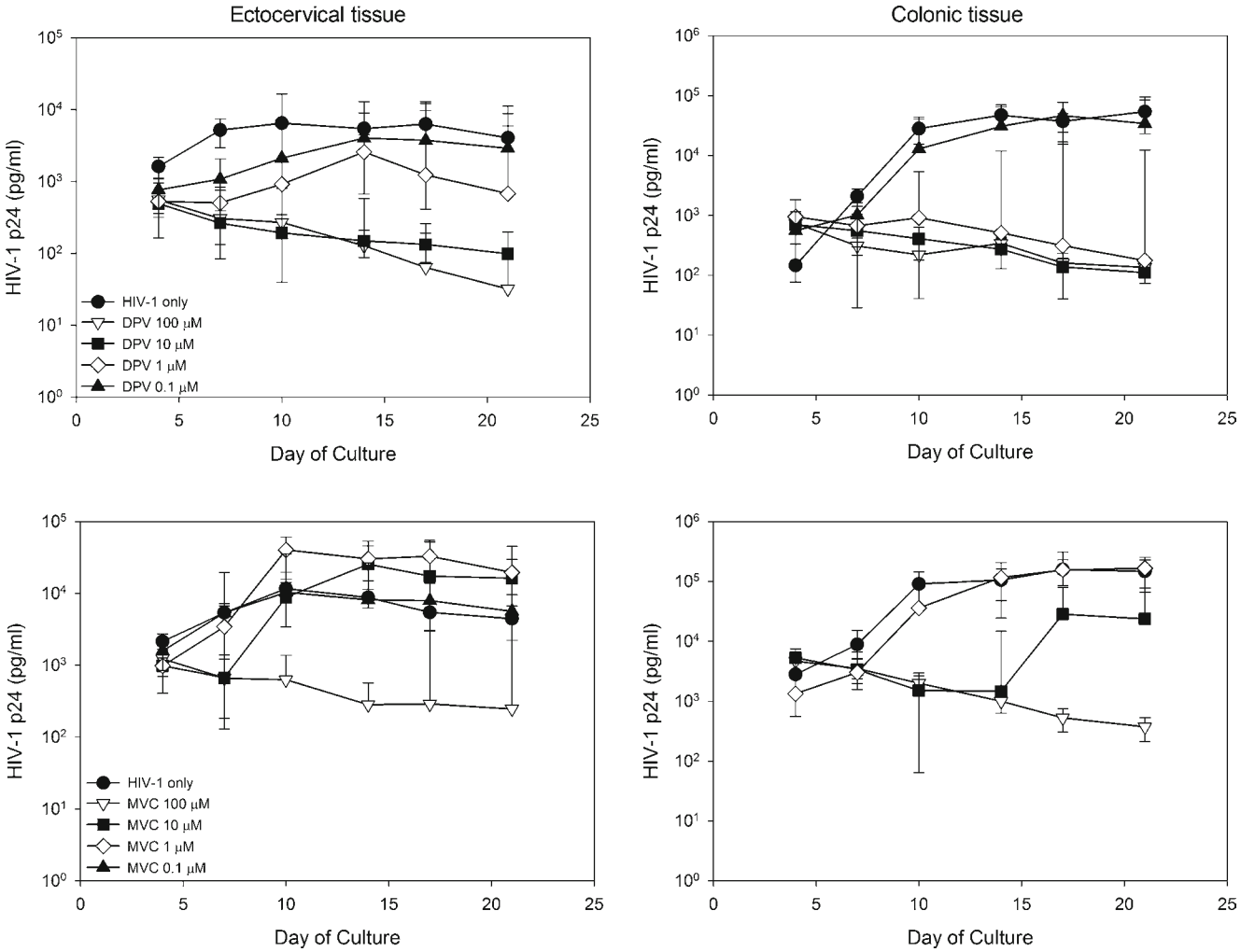


Fig. 1 Potency of dapivirine (DPV) and maraviroc (MVC) antiretroviral drugs in mucosal tissue. Ectocervical (*left panels*) and colonic (*right panels*) tissues were set-up in a polarized configuration and titrations of DPV (*upper panels*) or MVC (*lower panels*) were applied to the apical surface along with HIV-1_{BaL}. After an overnight culture, the tissues were washed and fresh basolateral medium was added. Every 3 to 4 days, basolateral supernatant was collected, stored, and replenished. HIV-1 replication was monitored in the basolateral supernatant by p24 ELISA. The data are presented as the median ± 95% confidence interval for 4 independent tissues tested in duplicate.

control explants showing the formulation had no intrinsic anti-HIV-1 activity. At study end, none of the ectocervical explants treated with DPV, MVC, or DPV/MVC showed any immunohistochemistry p24-positive cells while p24-positive cells were observed in the control and placebo-treated explants. These data show the anti-HIV activity was due to the drugs themselves and not the formulation as the placebo showed no protective benefit.

To define the potency of DPV and MVC gels and determine if formulation improved their activity, additional testing

Table II Physiochemical characterization of Dapivirine, Maraviroc, and Placebo Gel Formulations

Product	Appearance	Viscosity (cps) at 0.4 rpm, 25°C	Plastic viscosity (cps)	Yield Stress (D/cm^2)	pH	Osmolality (mOsm/kg)
Dapivirine Gel	Opaque, viscous gel	446,490	128,183	841	4.7	893
Maraviroc Gel	Transparent, colorless gel	161,233	32,126	313	4.1	84
Dapivirine/ Maraviroc Gel	Opaque, viscous gel	168,178	32,673	334	4.2	91
Placebo Gel	Transparent, colorless gel	188,766	51,413	262	4.2	86
Dapivirine Control Gel	Opaque, viscous gel	183,805	14,536	519	4.0	80

cps centipoise, *rpm* revolutions per minute, D/cm^2 dynes/square centimeter, *mOsm/kg* milliOsmoles/kilogram

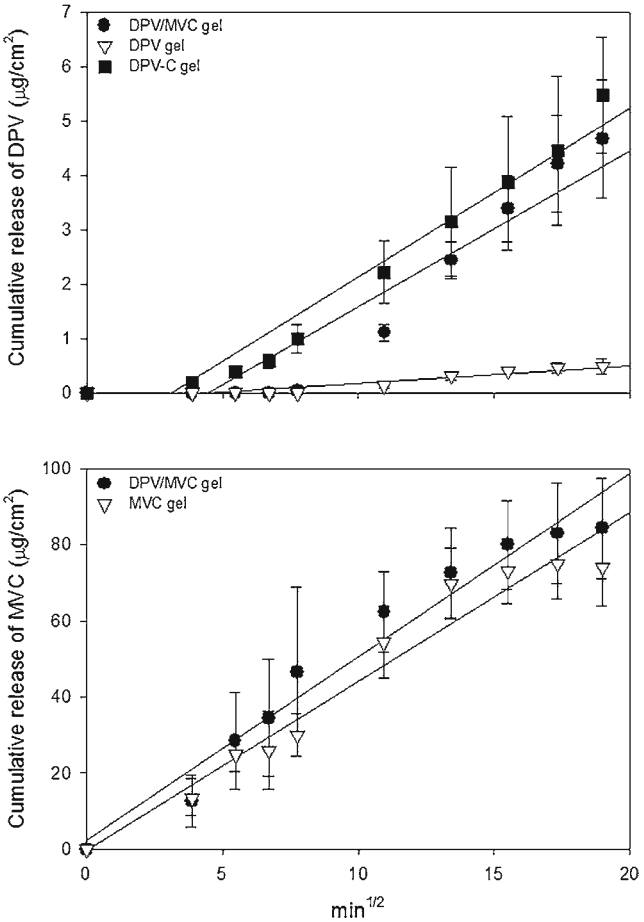


Fig. 2 *In vitro* release of dapivirine (DPV) and maraviroc (MVC) from the single entity and combination gels. Gels were placed in a Hanson Microette® system. Samples were taken from the receptor compartment at predetermined time intervals and assayed for drug content using high pressure liquid chromatography method.

was done on ectocervical and colonic mucosal tissue treated with 10-fold dilutions of the active gels. Dilutions of the active gels for ectocervical tissue began at 1:20 which resulted in initial DPV concentrations of ~75,900 nM and MVC concentrations of ~97,500 nM while dilutions for colonic tissue began at 1:2000, which was 100-fold more dilute than what was used for the ectocervical tissue. For ectocervical tissue (Fig. 4; left panels), 7,590 nM of DPV resulted in complete tissue protection while 97,500 nM of MVC was partially protective (6 of 8 explants showed no HIV replication). The combination gel diluted to 7,590 nM of DPV/9,750 nM of MVC completely protected the tissue, while 759 nM of DPV/975 nM of MVC was partially protective (6 of 8 explants showed no HIV replication). As mentioned, these drugs were more potent in colonic tissue, thus higher dilutions were tested (Fig. 4; right panel). DPV gel diluted to 759 nM completely protected the colonic explants, higher dilutions showed no protection. MVC gel diluted to 975 nM showed no substantial protection of the colonic tissue. The combination gel diluted to 759 nM of DPV/975 nM of MVC completely protected the colonic tissue while the 10-fold higher dilution was partially protective (6 of 8 explants showed no HIV replication). Testing the DPV-C gel showed similar activity to the DPV gel; 7,590 and 759 nM protected ectocervical and colonic tissue, respectively (Fig. 4). Formulation of either API showed consistent protection of tissue as compared to the unformulated API. The combination gel modestly improved efficacy in mucosal tissue compared to either single entity gel.

Product Safety

The gels were evaluated for several safety measures that include retention of vaginal flora, epithelial cell line viability and monolayer integrity, and mucosal tissue viability. Three *Lactobacillus* isolates were cultured with equal proportions of DPV, MVC, DPV/MVC and placebo gels. The gels showed <0.5 $\log_{10}$ change in viability (data not shown) indicating that they were safe and should not impact vaginal *Lactobacillus*.

Modest dilutions of the gels were made in tissue culture medium and exposed to epithelial cell lines which are representative of female genital tract (HEC-1-A) and colonic (Caco-2) epithelial cells (Fig. 5a). For both cell lines, the 1:10 dilution of DPV, MVC, DPV/MVC, and placebo gels showed the greatest loss of viability. As the gels were diluted further, viability improved such that cells treated with the 1:50 dilution of the gels were similar to the control (untreated) cells with the exception of both cell lines treated with the DPV/MVC gel. Further dilution to 1:100 of the combination gel was needed to return cell viability to the control cell levels. The Caco-2 cell line was more sensitive to DPV and DPV/MVC gels than the HEC-1-A cell line.

Another aspect of safety is the impact the gels may have on epithelial cell monolayers. This was tested by allowing the HEC-1-A and Caco-2 cell lines to develop a monolayer on transwell supports. Variation was noted after product was added to the monolayers, but none of the test gels decreased the resistance below the control (untreated) cell monolayers (Fig. 5b). By 24 h after the DPV, MVC, and DPV/MVC gel applications, there was a modest, but significant ($P=0.0014$) increase in the monolayer resistance in both cell lines. This increase in TER was not observed by the placebo gel. Conversely, application of an N9-containing gel showed significant ($P=0.0014$) reduction of the HEC-1-A monolayer by 2 h and the Caco-2 monolayer by 30 min. Monolayers treated with N9 showed no capacity to regenerate by 24 h.

To extend these *in vitro* findings to more relevant mucosal tissue, polarized ectocervical and colonic tissues were exposed to the DPV, MVC, DPV/MVC, and placebo gels for 24 h (Fig. 6). Viability was measured by the MTT assay. None of the test gels significantly affected the viability of the tissue and histology showed retention of the stratified squamous epithelium of the ectocervix tissue and of columnar epithelium of the

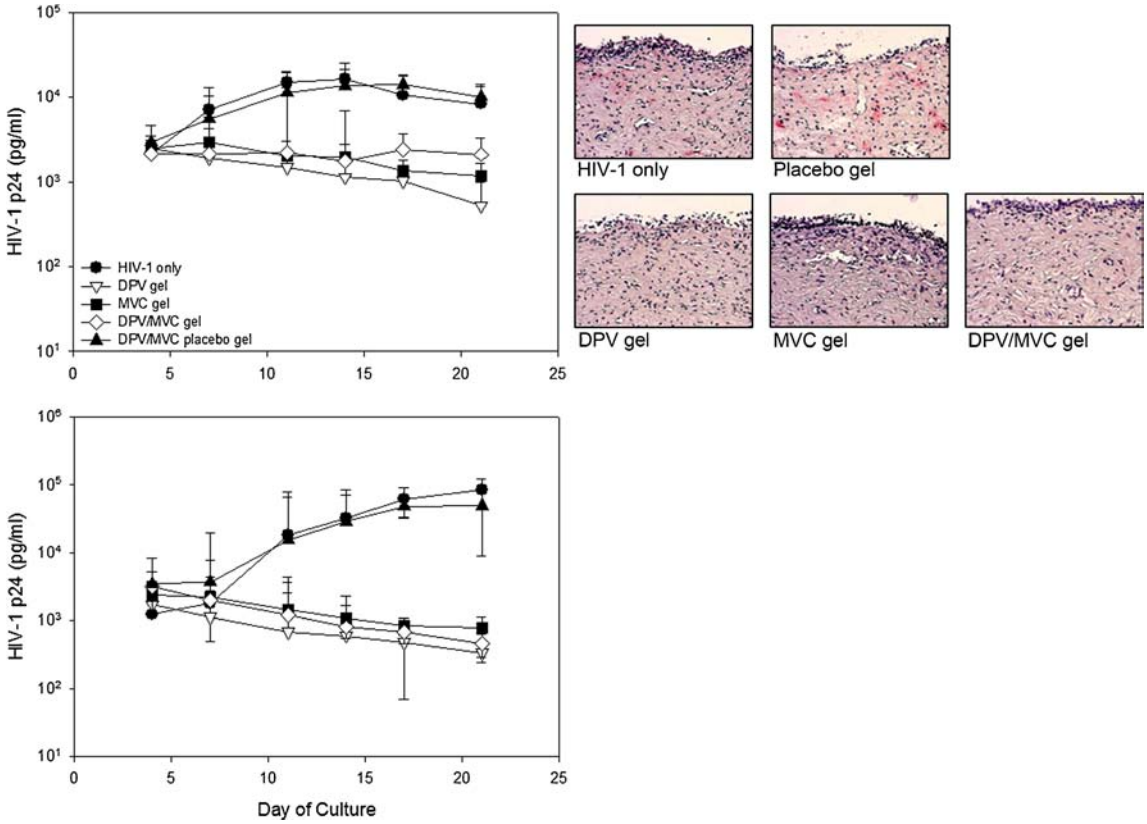


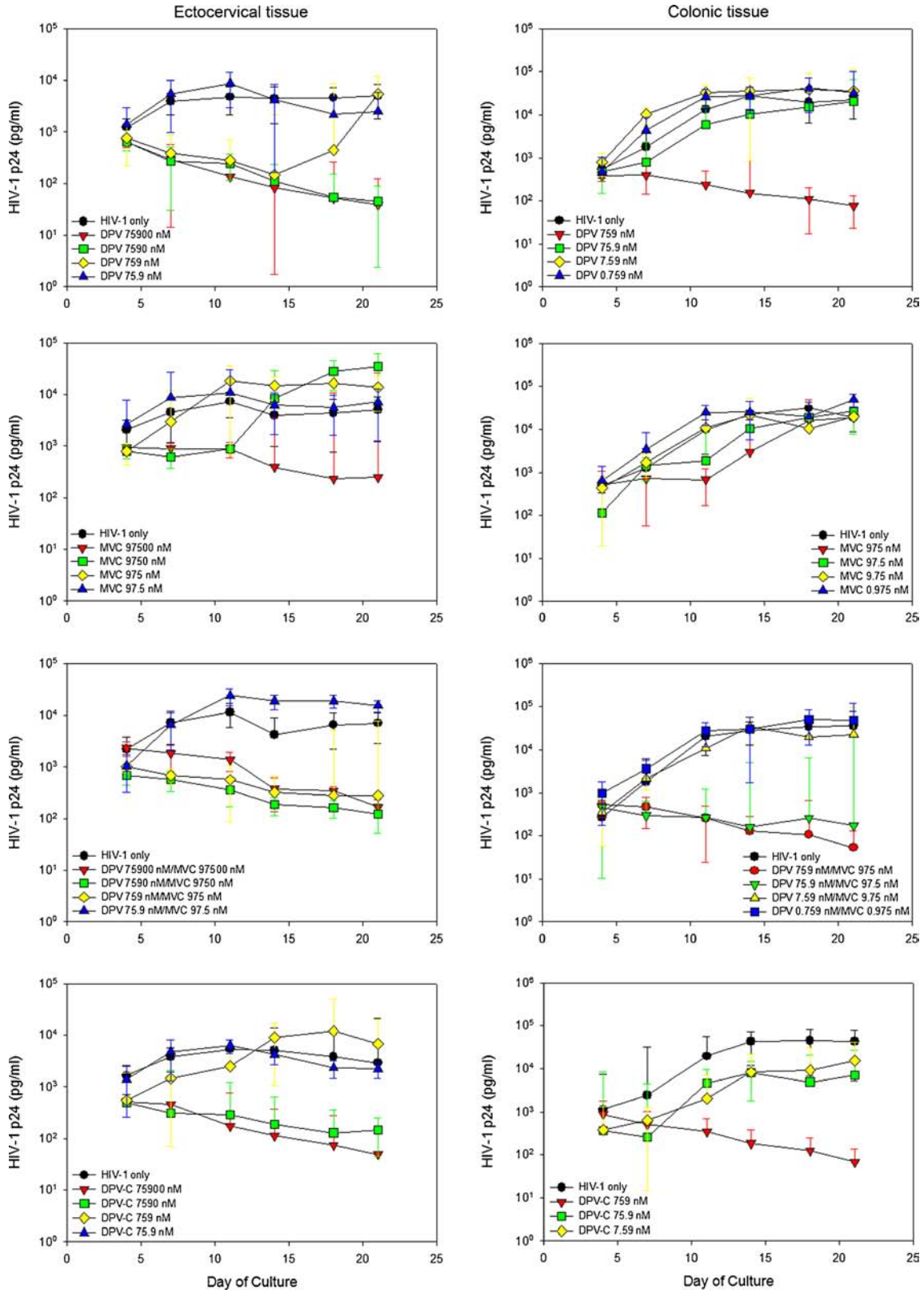
Fig. 3 Efficacy of formulated dapivirine (DPV), maraviroc (MVC), and combination gels against HIV-1. Ectocervical and colonic explants were placed in a polarized configuration and a 1:5 dilution of DPV, MVC, DPV/MVC, or placebo gel was applied to the apical surface along with HIV-1_{BaL}. Control explants only received HIV-1_{BaL}. After an overnight culture, the explants were washed and fresh basolateral medium was added. Every 3 to 4 days, basolateral supernatant was collected, stored, and replenished. HIV-1 replication was monitored in the basolateral supernatant by p24 ELISA. Ectocervical explant HIV growth curves are shown in the *upper panel* and colonic explant HIV growth curves are shown in the *lower panel*. The data are presented as the median ± 95% confidence interval for 5 independent tissues tested in duplicate. The immunohistochemistry sections were stained for HIV-1 p24gag antigen (*red color*). The data shown are representative from 1 of the 5 tissues.

colonic tissue. Further, these data show the differences in osmolality of the gels (Table II) did not impact tissue viability or histology. N9-containing gel significantly reduced tissue viability ($P=0.01$) and denuded (colonic) or fractured (ectocervical) epithelium with pyknotic nuclei.

DISCUSSION

The paradigm for microbicide development has been a single entity product designed for vaginal use. Combination products, much like the use of highly active antiretroviral therapy (HAART) for treatment (23), could help in further reducing the likelihood of becoming infected with HIV. Ideally, drugs that target different steps in the viral replication cycle would be beneficial especially with virus that may harbor drug resistance mutations to one of the drugs in the formulation (24,25). Here we evaluated DPV, an NNRTI, and MVC, a CCR5 antagonist, alone and in combination for gel product attributes, efficacy, and safety. The DPV gel (4759) has been evaluated in clinical trials for safety and drug distribution after vaginal (IPM 014A and IPM 020) and penile (MTN012/IPM 010) use. Plans are underway to evaluate this gel for the safety and pharmacokinetics following rectal administration (MTN-026). Both DPV and MVC drugs are greater than 100-fold more potent than the TFV gel that has been tested in clinical trials (8,9) and formulating them into hydrogels retained their potency. It should be noted that the DPV single entity gel (4759) was formulated in a different base than all other gels tested. This resulted in different gel physicochemical attributes including higher osmolality, approximately 2-fold higher viscosity, and much slower *in vitro* release of DPV from

Fig. 4 Potency of formulated dapivirine (DPV) and maraviroc (MVC), alone and in combination, in mucosal tissue. Ectocervical and colonic explants were placed in a polarized configuration and dilutions of DPV, MVC, DPV/MVC, or DPV control (DPV-C) gel were applied to the apical surface along with HIV-1_{BaL}. Control explants only received HIV-1_{BaL}. After an overnight culture, the explants were washed and fresh basolateral medium was added. Every 3 to 4 days, basolateral supernatant was collected, stored, and replenished. HIV-1 replication was monitored in the basolateral supernatant by p24 ELISA. The data are presented as the median ± 95% confidence interval for 4 to 5 independent tissues tested in duplicate. ▶



the product as compared to other gels. To eliminate potential effects due to formulation differences, efficacy and safety testing included a control DPV gel with identical excipient base composition as that of the combination gel. This DPV-C gel possessed comparable physicochemical parameters to the other gels tested (Table II and Fig. 2). However, the formulation

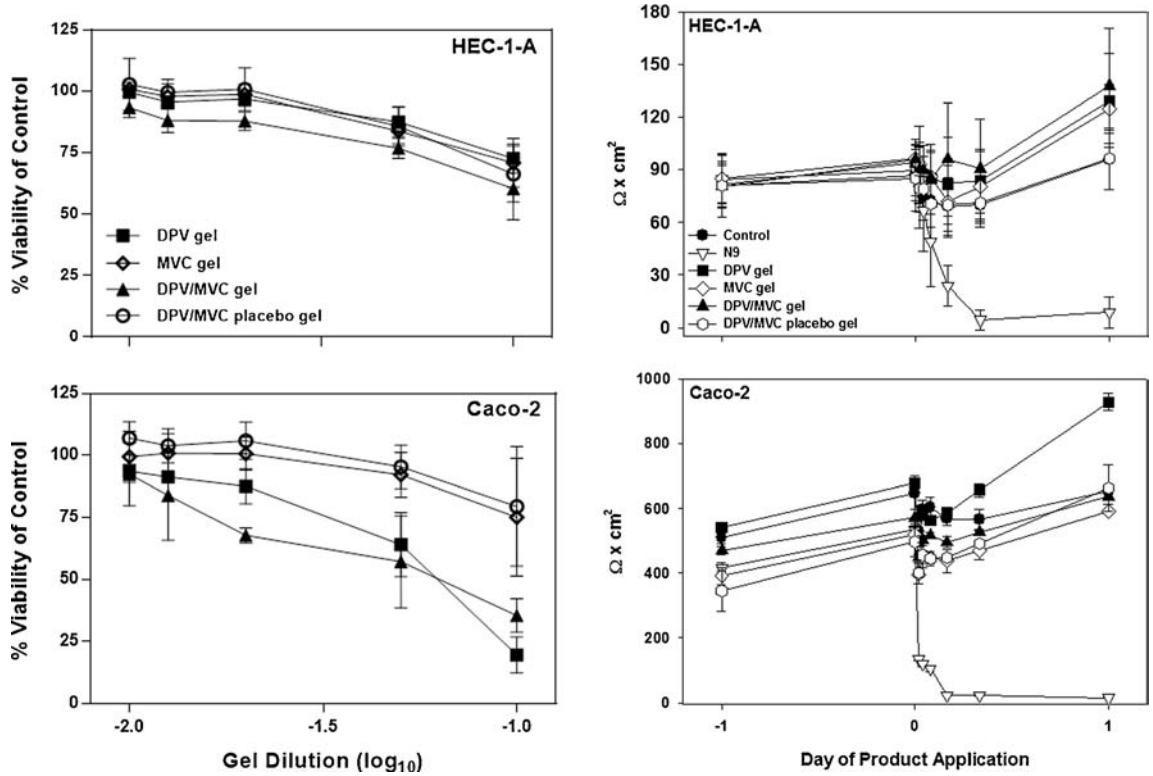


Fig. 5 Impact of formulated dapivirine (DPV), maraviroc (MVC), and placebo products on epithelial viability and monolayer integrity. (**a**) Modest dilutions (1:10, 1:20, 1:50, 1:80, and 1:100) of the gels were made in tissue culture medium and applied to HEC-1-A or Caco-2 epithelial cells for 24 h. Cell viability was measured using CellTiter-Glo® according to the manufacturer's instructions. The data shown represent the mean ± standard deviation of 5 independent experiments performed in triplicate. (**b**) HEC-1-A and Caco-2 cells were grown on transwell supports until they formed stable monolayers. A 1:20 dilution of each gel was added to the apical chamber at t = 0 and resistance readings were measured at 30 min, 1, 2, 4, 8, and 24 h. As a control, a 1:50 dilution (0.6 mg/ml) of nonoxynol-9 (N9) gel was added to the indicated apical chambers and resistance readings were measured. The data shown represent the mean ± standard deviation of 5 independent experiments performed in duplicate.

difference between DPV-C and DPV gels had no perceptible impact on efficacy results obtained in *in vitro* and *ex vivo* assays despite the DPV-C gel exhibiting much faster *in vitro* drug release. The dilution of the formulations for testing could have impacted the DPV release rates. It should be noted as well that *in vitro* release testing was conducted over 6 h whereas efficacy testing was conducted over 48 h to 21 days. *In vitro* release testing was done as a product quality control measure for the gel product and not intended to mimic efficacy study experimental design. Overall, DPV was ≥10-fold more potent than MVC when evaluated in ectocervical and colonic tissues as single entities. Of interest, we observed a >1000-fold and >100-fold difference between ectocervical and colonic tissue, respectively, in the activity of DPV and MVC compared to the ED_{50} determined from the TZM-bl assay and is similar to the findings of Nicol and colleagues (26). The difference in drug activity between the two models likely reflects the availability of target cells and drug uptake. The antiviral activities of DPV and MVC were synergistic when combined, even in the presence of semen. With respect to safety, in cell-based integrity studies although it appears that the undiluted DPV gel product resulted in increased epithelial cell resistance in Caco-2 cell line studies (Fig. 4b), this result is more than likely due to the increased viscosity (~2-fold) of the DPV gel product. While primarily designed for vaginal use due to the lower pH, the gels were safe toward *ex vivo* colonic tissue in addition to ectocervical tissue suggesting use as dual-compartment gels. Collectively, these data show successful formulation of DPV and MVC into hydrogels that could be used either vaginally or rectally to prevent the sexual transmission of HIV.

DPV, a prototypical diarylpyrimidine NNRTI, has broad and potent anti-HIV activity including toward drug resistant variants likely due to the flexibility in binding to the NNTRI pocket (20). The tight-binding to the RT and high hydrophobicity allows DPV to inactivate cell-free HIV as well as cell-associated HIV (27). While not pursued for therapeutic use for treatment of HIV, DPV is being tested for HIV prevention with the development of topical gels and IVRs and vaginal use shows it penetrates the cervical and vaginal tissues, but has low systemic absorption (17,28). While topical administration shows DPV has a terminal half-life in cervicovaginal fluid of ~16 h (17), little information is available understanding drug uptake, efflux and metabolism, likely because it was not in the therapeutic pipeline. Recently, DPV was shown to be differentially metabolized in mucosal tissues that will be exposed to a topical product. In vaginal and colonic tissue, DPV

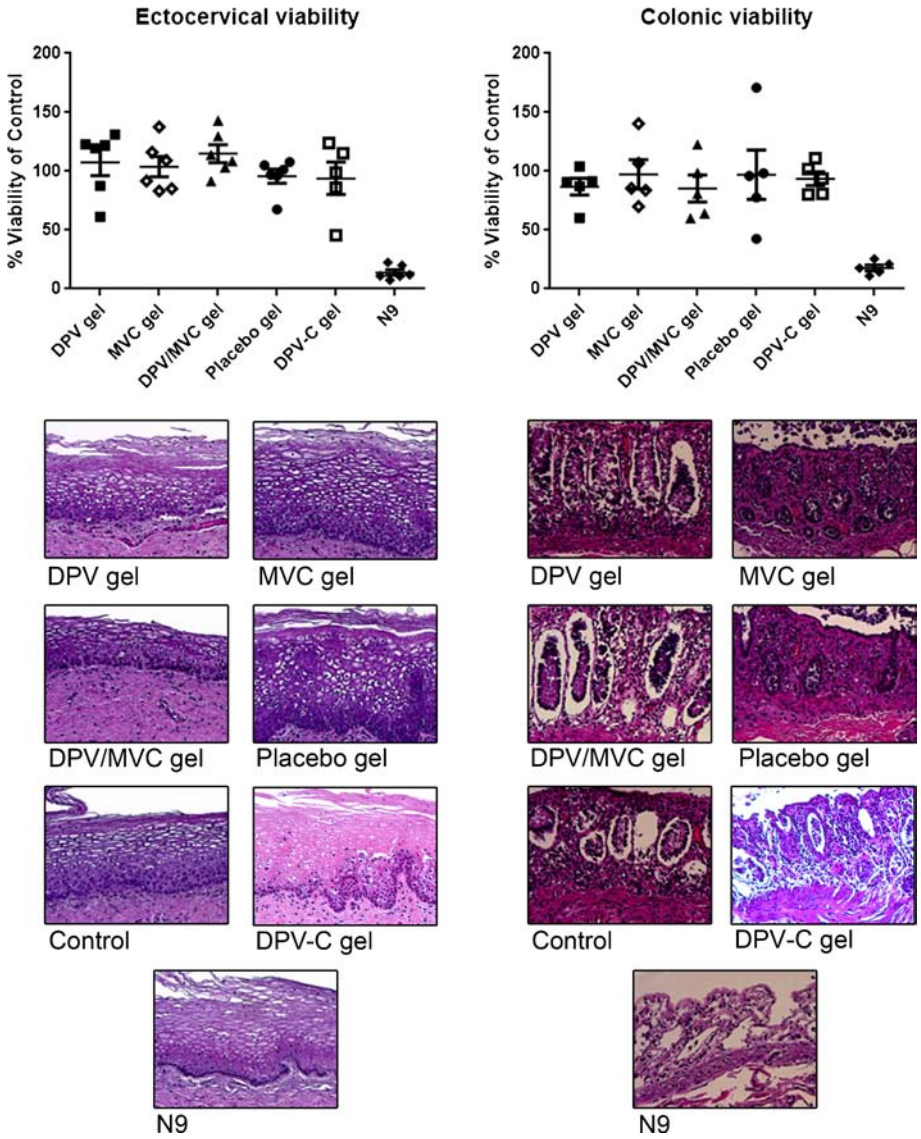


Fig. 6 Ectocervical and colonic tissue viability after a 24 h exposure to formulated dapivirine (DPV), maraviroc (MVC), and placebo products. Ectocervical and colonic explants were polarized and each product was diluted 1:5 in the appropriate culture medium and applied to the apical surface. A 1:5 dilution of nonoxynol-9 (N9) was applied apically to the explants at the same time as the other products for a toxicity control. Untreated explants were the control tissues. The explants were cultured for 24 h, washed five times, and placed in either medium containing 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) to assess tissue viability by measuring mitochondrial activity or formalin to fix the tissue for hematoxylin-eosin staining for histology. Explants from five tissue donors were evaluated for viability after exposure to topical gels. The% viability was determined by dividing the corrected optical density of the treated explant by the corrected optical density of the control explant. Histology shown is representative of the 5 tissues evaluated.

metabolism can be attributed to cytochrome P450 (CYP), while in colonic tissue, only UDP-glucuronosyltransferase was also shown to metabolize DPV (29). The activity of the metabolites is unknown, but they may be involved in the enhanced activity observed in colonic tissue presented here. Currently, no information is available for DPV efflux or uptake transporters. Additional work is needed to fully understand DPV distribution and activity in mucosal tissue.

MVC, one of the first licensed CCR5 antagonists, is primarily used as second-line therapy for individuals who have failed first-line therapy. Using MVC for HIV prevention has gained interest because it is not first-line therapy and blocking entry of HIV eliminates the possibility of a cryptic infection. Oral delivery of MVC shows it penetrates human vaginal and rectal tissue (30,31). However, oral MVC failed to protect nonhuman primates rectally exposed to repeat, low dose SHIV challenges despite demonstrating high tissue and blood levels of the drug (32). The lack of protection could be due to slightly lower affinity of MVC for the nonhuman primate CCR5 receptor (33) and/or lack of saturation of the CCR5 receptor in the animals (32). Further, MVC is actively transported out of mucosal tissue *via* the ATP-binding cassette (ABC) superfamily of transporters (34) and it's metabolized by CYP in colonic tissue (29) suggesting MVC is quickly cleared from the tissue likely reducing drug efficacy. Topical dosing of MVC, such as using a gel or vaginal ring, may overcome the transporter / metabolizing concerns because it would provide several $\log_{10}$ more drug than what could be achieved with oral medication. This was noted for topical delivery of TFV which provides several $\log_{10}$ higher levels than oral delivery to the mucosa (14). Indeed, topical application (vaginal and rectal) of MVC prevented SHIV infection (35,36).

DPV and MVC gels were effective at blocking HIV infection in both ectocervical and colonic explant tissues.

Formulation of DPV and MVC into hydrogels had negligible improvement on their activity in either ectocervical or colonic tissue. Of interest, ~10-fold less DPV or MVC was needed to protect colonic tissue than was needed for ectocervical tissue. The role of drug transporters and metabolizing enzymes may differentially influence the activity of both drugs (29), but it is not clear at this time as more work is needed to fully understand the drugs pharmacogenomics. There is a great deal of variability in expression of these enzymes and transporters even within regions of the vaginal tract (29,37). The expression along the rectum/colon is currently not known. The better performance in colonic tissue *ex vivo* may be due to the drugs saturating the tissue and reaching the target cells more easily because the tissue begins to autolyze soon after removal from the patient while ectocervical tissue remains intact through several weeks of culture (38,39). The increased topical concentrations and/or access to target cells may allow the drugs to out compete the virus in the polarized colonic model better than the polarized ectocervical model.

There is a benefit for combination therapy in terms of morbidity and mortality of HIV infected persons on effective treatment (23). While DPV and MVC showed synergy against HIV-1_{BaL} in our models, the benefit of the combination gel product as compared to either single entity gel product when used clinically remains to be determined. The DPV / MVC combination may have a greater observable benefit against drug resistant HIV. DPV with TFV (NRTI) and/or UC781 (NNRTI) showed synergy against wildtype and NNRTI-drug resistant clade B and C viruses (24,25). Clade B and C viruses are representative of viruses from regions that are highly affected by HIV – western Europe/Americas and sub-Saharan Africa. This is important as women and men interested in enrolling into HIV prevention clinical trials may live in communities with circulating drug resistant virus. A recent study from Durban South Africa showed that of 1073 women who were screened for a large HIV prevention trial evaluating TFV-based drugs, 400 were infected with HIV; 352 of which had viral drug resistance testing (40). Twenty six women (7.4%) harbored drug resistant virus with 62% having NNRTI resistance and 19% having NRTI/NNRTI resistance. With the predicted resistance to NNRTIs to likely increase in South Africa due to use in first-line and prevention of mother-to-child transmission therapies (41), having a combination microbicide that works against more than one step in the viral replication cycle could circumvent the possibility of acquiring drug-resistant virus. While drug resistance to NNRTIs is common, it is rare with MVC (21), but recent data suggests that virus could evolve to overcome MVC by utilizing the occupied receptor (42). While our study did not test the gels against drug-resistant virus, we are working with primary HIV isolates that harbor NNRTI mutations to determine this benefit.

CONCLUSION

Providing potent, highly effective microbicide products for HIV prevention should complement other HIV prevention modalities such as PrEP, medical male circumcision, and vaccines when available to work toward an AIDS-free generation. DPV and MVC hydrogels were safe toward ectocervical and colonic mucosal tissue and effective at preventing HIV infection *ex vivo*. Combining both drugs demonstrated synergy. Collectively, these data provide a rationale for further testing of these products as dual compartment microbicides.

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