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The entry inhibitor DS003 (BMS-599793), a BMS-806 analogue, provides superior activity as a pre-exposure prophylaxis candidate.

Carolina HERRERA^{#1}, Sarah HARMAN^{#2}, Yoann ALDON³, Paul ROGERS¹, Naomi ARMANASCO¹, Paul ZIPRIN⁴, Daniel STIEH⁵, Jeremy NUTTALL⁶, Robin J. SHATTOCK^{1,+}

(¹)Centre for Infection, Division of Cellular & Molecular Medicine, St George's University of London, UK. Current address: Section of Infectious Diseases, Faculty of Medicine, St Mary's Campus, Imperial College, UK.

(²)Centre for Infection, Division of Cellular & Molecular Medicine, St George's University of London, UK. Current address: London School of Hygiene and Tropical Medicine, London, UK.

(³)Section of Infectious Diseases, Faculty of Medicine, St Mary's Campus, Imperial College, UK. Current address: Amsterdam UMC, Netherlands.

(⁴)Department of Surgery and Cancer, St Mary's Hospital, Imperial College London, UK.

(⁵)Centre for Infection, Division of Cellular & Molecular Medicine, St George's University of London, UK. Current address: Janssen Vaccines & Prevention B.V. Leiden, Netherlands.

(⁶)International Partnership for Microbicides, Silver Spring, MD, USA.

These authors contributed equally to this work.

Abstract

Objective: Small molecule inhibitors able to bind to gp120 and prevent CD4-induced HIV-1 envelope conformational change provide an important class of inhibitors. Currently, only Fostemsavir is approved for HAART, which makes this class of inhibitors attractive candidates for prevention. We assessed the activity of DS003 (BMS-599793), an analogue of BMS-378806, in different mucosal tissues and elucidated its mechanism of action.

Design: Pre-clinical analysis was performed with human mucosal tissue models as surrogates of *in vivo* activity.

Methods: Antiviral efficacy of DS003 was assessed in mucosal tissue explants (ecto-cervical, penile and colorectal) and in trans-infection models (co-cultures of dendritic or mucosal migratory cells with CD4⁺T cells) with several dosing times (2 h, 24 h and sustained) and in combination with a fusion inhibitor. Binding of DS003 to gp120 was assessed by flow cytometry and bio-layer

⁺Corresponding author. Mailing address: Section of Infectious Diseases. Faculty of Medicine, Room 453 Wright-Fleming Institute, St Mary's Campus, Imperial College. Norfolk Place, London W2 1PG. United Kingdom. Tel: +44 (0) 207 594 5206; Fax: +44 (0) 207 594 2538; r.shattock@imperial.ac.uk.

AUTHOR CONTRIBUTIONS

Conceptualization: C.H., J.N. and R.J.S.; methodology: C.H., S.H., Y.A., P.R., N.A., P.Z., D.S.; writing original draft preparation: C.H., writing-review and editing by all the authors.

interferometry and further probed in competitive studies using soluble CD4 (sCD4) and an anti-CD4 induced antibody, 17b.

Results: In all models, the inhibitory activity of DS003 was increased with longer periods of exposure and by combination with a fusion inhibitor. Pre-exposure to sCD4 impeded DS003 binding to viral envelope (Env). In contrast, DS003 did not impact subsequent binding of sCD4. Furthermore, sCD4-induced epitope exposure as assessed by 17b binding was significantly reduced in the presence of DS003.

Conclusion: DS003 inhibits HIV-1 infection by binding to or near the CD4 binding site of gp120, preventing CD4-induced conformational change essential for viral fusion. These data highlight the potential of DS003 for development as a pre-exposure prophylaxis candidate.

Keywords

prevention; antiretroviral; entry; mucosal; explant; HIV-1

INTRODUCTION

A major target for potential HIV pre-exposure prophylaxis (PrEP) intervention is the binding of viral gp120 to CD4. Inhibiting this step can be achieved by blocking the CD4-binding site within gp120. Small proteins mimicking CD4 have shown high potency against HIV-1 *in vitro*^[1] and in animal studies^[2]. An alternative to inhibit effective gp120-CD4 binding is for a drug to bind to gp120 and block the conformational change in gp120 induced by CD4^[3–5]. The only drug with this mechanism of action currently approved for treatment is Fortemsavir and therefore this class of drugs provide an excellent option for prevention strategies to avoid transmission of resistant isolates. This type of inhibitors comprise a group of related small-molecules including BMS-378806, BMS-488043, BMS-626529, and BMS-663068. All of these compounds have been shown to be active against HIV-1 *in vitro*^[3, 4, 6, 7], *in vivo*^[8, 9], and BMS-378806 was shown to protect against vaginal challenge in non-human primates (NHPs)^[10]. DS003 (BMS-599793) is another related compound with a similar mechanism of action, developed for topical PrEP as a microbicide candidate by the International Partnership for Microbicides (IPM). DS003 has been advanced to a Phase I clinical trial, IPM042, (<https://clinicaltrials.gov/ct2/show/NCT02877979>) as a vaginal tablet showing a good safety profile and achieving concentrations in the female genital tract that were capable of inhibiting *ex vivo* HIV-1 infection of cervical biopsies^[11, 12]. However, no data are available on the inhibitory activity of DS003 in relevant cellular subsets or other mucosal portals of entry, including male genital tract and colorectum, and there is limited knowledge of its binding epitope to Env.

MATERIALS AND METHODS

Cell and virus culture conditions

All cell cultures were maintained at 37°C in an atmosphere containing 5% CO₂. TZM-bl cells^[13–15] and HEK293T/17 cells (ATCC, UK) were grown in Dulbecco's Minimal Essential Medium (DMEM) (Sigma-Aldrich, Inc., St. Louis, MO) containing 10% fetal calf serum (FCS), 2mM L-glutamine and antibiotics (100 U of penicillin/ml, 100 µg of

streptomycin/ml). PM-1 cells^[16] (AIDS reagent project, National Institute for Biological Standards and Control, UK) were maintained in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine and antibiotics (100 U of penicillin/ml and 100 µg of streptomycin/ml). PBMCs were isolated from multi-donor buffy coats from healthy HIV-seronegative donors, by centrifugation onto Ficoll-Hypaque, mitogen stimulated as described previously^[17], and maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, antibiotics (100 U of penicillin/ml, 100 µg of streptomycin /ml), and 100 U of interleukin-2/ml. Immature dendritic cells (iDCs) were grown from PBMC-derived monocytes cultured for 6 days in complete RPMI medium supplemented with 1000 U/ml GM-CSF and 500 U/ml IL-4 (R&D Systems, Minneapolis, MN). Monocytes were isolated from PBMCs by autoMACS human CD14 Microbeads (Miltenyi Biotec, UK) following manufacturer's instructions. iDCs were phenotypically characterized by staining with anti-CD40, anti-CD80, anti-CD86, anti-CD83, anti-CD209, anti-CD123, and anti-CD11c (BD Pharmingen, UK). FACS analysis was performed with a BD FACSCanto II flow cytometry system using BD FACSDiva analysis software.

The laboratory-adapted isolate HIV-1_{BaL} was passaged through activated PBMCs for 11 days.

Reagents and plasmids

BMS-599793 (also known as DS003), BMS-378806 and L'644 (also known as C34-Chol or DS007) were provided by the International Partnership for Microbicides (IPM) (Silver Spring, MD). Stocks of DS003 were prepared in DMSO at 10 mM and serial dilutions in culture media where prepared at the required concentrations with good solubility. No precipitates were observed in the culture media during the assays.

HIV-1_{BaL}^[18] was obtained from the NIH AIDS Research & Reference Reagent Program (<http://www.aidsreagent.org/>). Transmitted founder clade B isolates, B17, B8, B14 and B3 were kindly provided by Prof. Eric Arts (Western University, Canada)^[19].

HIV-1_{BaL} gp160 *Env* gene was cloned into pcDNA3.1 (Invitrogen).

HIV-1 CN54 gp140, kindly provided by Polymun (Austria), was produced in CHO cells as an oligomer shown to be essentially trimeric with a projected mass of 420kDa as described previously^[20, 21]. Antigenicity of this protein has been shown in several clinical trials^[22–24].

Plasmids encoding for the genes of the heavy chain and light chain of the 17b human monoclonal antibody (mAb) were a gift from Dr James Robinson. 17b was produced in HEK293T/17 cells by transient co-transfection with polyethyleneimine (PEI) MAX 40,000 (Polysciences) of the heavy chain and light chain expression plasmids. Forty-eight hours following transfection, secreted mAb was purified using a HiTrap protein G HP column (GE Healthcare) following manufacturer's instructions.

Soluble *CD4* (*sCD4*) *D1D2 HIS* tagged gene was synthesized using GeneArt gene synthesis service and the protein produced in HEK293T/17 cells using PEI-transfection as for 17b production. *sCD4* protein was purified using a cOmplete® His-Tag purification column (Roche) following manufacturer's instructions.

Patients and tissue explants

Cervical tissue was obtained from patients undergoing planned therapeutic hysterectomy at St. Mary's Hospital, Imperial College Healthcare NHS Trust, St George's Hospital, and Kingston Hospital in London, UK. Penile tissue was obtained from patients undergoing gender reassignment at Charing Cross Hospital, London, UK, and having ceased hormonal therapy a minimum of 6 weeks prior to surgery. Surgically-resected specimens of colorectal tissue were collected at St George's Hospital and at St. Mary's Hospital, Imperial College Healthcare NHS Trust, London, UK. All tissues were collected after receiving signed informed consent from all patients under approved protocols by the Wandsworth Research Ethics Committee (01.31.3, 05/Q0803/86 & 99.4) and through the Imperial College Healthcare Tissue Bank approved by Research Ethics Committee Wales (IRAS 17/WA/0161). All patients were HIV negative. On arrival at the laboratory, a maximum of 1 h post-operation, resected tissue was cut into 2–3 mm³ explants comprising both epithelial and stromal tissue or muscularis mucosae, depending on the tissue, as described previously^[25–27]. Cervical and penile explants were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS), and antibiotics (100 U of penicillin/ml, 100 µg of streptomycin/ml). Colorectal explants were maintained with DMEM containing 10% fetal calf serum, 2mM L-glutamine and antibiotics (100 U of penicillin/ml, 100 µg of streptomycin/ml, 80 µg of gentamicin /ml). All tissues were incubated at 37°C in an atmosphere containing 5% CO₂.

Infectivity and inhibition assays

All inhibition assays in cellular and tissue explant models were performed using a standardized amount of virus culture supernatant normalized for infectivity. TZM-bl cells were incubated with serial dilutions of compounds for 1 h at 37°C, and then virus was added to cells and left for the duration of the experiment. The extent of virus replication was determined by luciferase quantitation of cell lysates after 48 h (Promega, Madison, WI) as previously described^[26].

To determine the activity of the compound against infection of iDCs, cells were exposed to compound just prior to addition of virus. After 2 h of incubation, cells were washed and kept in culture for a total of 7 days in the absence or presence of drug for 24 h or for the 7 days of culture. Inhibitory potency against transmission of cell-associated virus was measured with iDCs exposed to virus for 2 h, washed 3 times with PBS to remove unbound virus, and co-cultured with PM-1 cells at a 1:2 ratio of infected cells:PM-1 cells (equivalent to 1×10⁴ infected cells:2×10⁴ PM-1 cells) in the presence or absence of compound. Co-cultures were incubated with compound for different time periods: 2h, 24h or sustained (maintained throughout the culture). Cells were cultured for 14 days, with 50% media feeds every 2–3 days. The extent of virus replication was determined by measurement of p24 antigen levels in culture supernatants with the Innostest HIV antigen kit (Innogenetics, Belgium).

Cervical and penile explants were incubated with compound for 1 h before virus (10⁴ TCID₅₀/ml) was added for 2 h. Explants were then washed four times with PBS and cultured in complete medium in the presence (sustained) or absence (pulse) of drug for 24 h at 37°C. Tissue explants were then transferred to fresh microtiter plates and migratory cells left in the

original plate were washed twice with PBS and co-cultured with 4×10^4 PM-1 cells/well with or without compound in fresh 96-well plates. Tissue explants and cellular co-cultures were cultured for further 14 days in the presence or absence of compound. Approximately 50 % of the supernatants of explants and cellular cultures were harvested every 2 – 3 days, and both cultures were re-fed with fresh media in the presence or absence of compound.

Colorectal tissue explants were exposed to drug 1 h prior to virus exposure (10^4 TCID₅₀/ml). After 2 h of viral challenge, explants were washed four times with PBS before being transferred on gel foam rafts (Welbeck Pharmaceuticals, United Kingdom) and cultured for 15 days in the presence (sustained) or absence (pulse) of compound. Approximately 50 % of the supernatants were harvested every 2 – 3 days and explants were re-fed with fresh media with or without drug.

For all tissue explant models and migratory/PM-1 cells co-cultures, the extent of virus replication was determined by measuring the p24 antigen concentration in supernatants (HIV-1 p24 ELISA, AIDS Vaccine Program, National Cancer Institute, Frederick, MA)^[26].

Biolayer interferometry (BLI) binding analysis

Real time binding assays between purified IgG 17b and purified recombinant Env protein of HIV-1_{CN54}-gp140 in the presence/absence of purified recombinant soluble CD4 (sCD4) and DS003 were performed using BLI on an Octet K2 instrument (ForteBio, Fremont, CA). Briefly, anti-human IgG Fc capture biosensors were hydrated for 10 min in kinetics buffer (DPBS + 0.1% BSA + 0.02% Tween-20) immediately prior to use. CN54-gp140 alone or mixed with sCD4 and DS003 in 200 µl of kinetics buffer was loaded into black 96-well microplates.

Following optimization of biosensor coating density with 17b, a concentration of 10 µg/ml was chosen. Titration of HIV-1_{CN54} gp140 allowed us to select 100 µg/ml as the best concentration to obtain maximum full binding curve of sCD4-gp140 complex within the resolution of the instrument. Response of HIV-1_{CN54} gp140 + sCD4 complex to 17b was established by titration of sCD4 in the presence of HIV-1_{CN54} gp140 at 100 µg/ml, and plateau level was reached with 5 µg/ml of sCD4. Biosensors were loaded with 17b to half saturation and washed with kinetics buffer before being transferred into analyte, where the association rate constant (K_a) was measured. The biosensors were then transferred into kinetics buffer to measure the dissociation rate constant (K_d). Biosensors were regenerated using 0.1 M glycine, pH 1.5, before measuring subsequent samples. Values from a 17b-coated biosensor transferred into kinetics buffer alone were subtracted from all test values. All analyses were conducted at 22°C with a plate shaking speed of 1,000 r.p.m. Binding kinetics were calculated using the ForteBio Data Analysis software package fitting the observed dissociation curves to a 1:1 model to calculate the rate constants. Using a 2 to 1 binding model, representative of a trimeric Env, did not alter the goodness of fit.

Flow cytometry

HEK293T/17 cells were transfected with plasmid DNA expressing Env or with an empty pcDNA3 vector (Invitrogen) and surface expression of Env analyzed by flow cytometry. Cells were transfected with PEI MAX 40,000 (Polysciences) using a 1:3 DNA to PEI

ratio (w:w). Forty-eight hours post-transfection, cells were rinsed with PBS, resuspended in FACS buffer (2.5% FBS, 1 mM EDTA, 25 mM HEPES in 1X PBS) and stained with LIVE/DEAD fixable Aqua dye (1:400) (Life Technologies). Cells were then washed twice with FACS buffer prior to staining with 10 µg/ml of anti-Env 17b human IgG. DS003 and/or sCD4 were added to the cells before, during or after 17b mAb staining as required. Cells were washed twice with FACS buffer after each incubation. Following incubation with secondary F(ab')₂-goat anti-human IgG Fc PE Ab (Invitrogen), cells were fixed with 1.5% formaldehyde (Polysciences). Data were acquired on a LSRFortessa flow cytometer (BD) using FACSDiva (BD) and analyzed using FlowJo v.10.1 software (Treestar).

Statistical and mathematical analysis

50% inhibitory concentration (IC₅₀) values were calculated from sigmoid curve fitted (Prism, GraphPad Software, La Jolla, California, USA) fulfilling the criterion of $R^2 > 0.7$. *P* values were determined using a two-tailed Student *t* test (both paired and unpaired), and *P* ≤ 0.05 was considered statistically significant.

RESULTS

DS003 is more active than BMS-378806 against HIV-1 clade B isolates

Non-formulated DS003 demonstrated greater inhibitory activity than its analog BMS-378806 against HIV-1_{BaL} in TZM-bl cells with an IC₅₀ for DS003 of 1.09 ± 0.34 nM, compared to 191.45 ± 295.90 nM for BMS-378806 (Figure 1a).

The activity of DS003 was then tested against four transmitted founder clade B primary R5-tropic HIV-1 isolates from males infected either via heterosexual transmission (HIV-1_{B3} and HIV-1_{B8}) or unknown transmission mode (HIV-1_{B14} and HIV-1_{B17}). All four transmitted founder strains tested were inhibited by DS003 in the TZM-bl cell model, although strains HIV-1_{B8} and HIV-1_{B14} appeared to be more sensitive to DS003, with IC₅₀s of <0.004 and 0.031 nM, respectively (Figure 1b).

Inhibitory potency of DS003 in iDC cultures

Infection of iDCs was inhibited by a 2 h pulse exposure to DS003 with an IC₅₀ of 2.37 ± 0.46 nM. Longer exposure to DS003, for 24 h, increased the inhibitory potency (IC₅₀ = 0.35 ± 0.16 nM) to the same level as that observed with sustained exposure (IC₅₀ = 0.29 ± 0.21 nM) (Figure 1c).

Furthermore, DS003 was active against iDC *trans*-infection of CD4⁺ T cells with sustained exposure to compound during culture (IC₅₀ = 1.36 ± 0.54 nM) (Figure 1d); however, activity was significantly reduced with 2 h pulse exposures to compound as shown by an increase of the IC₅₀ to 509.61 ± 221.10 nM. Activity remained at the low nM level (IC₅₀ = 19.76 ± 6.00 nM) when the compound was maintained for the first 24 h of culture. Thus, DS003 is likely to be most active against this pathway if sustained for a minimum of 24 h following exposure to virus.

DS003 prevents infection and dissemination of HIV-1 in reproductive tract tissues

DS003 demonstrated high levels of inhibition in penile explants when left in for the duration of the assay ($IC_{50} = 7.29 \pm 5.69$ nM). The inhibitory potency of DS003 progressively decreased with reduction of dosing time ($IC_{50} = 67.63 \pm 84.93$ nM for 24 h drug exposure and $IC_{50} = 265.30 \pm 369.33$ nM for pulse dosing) (Figure 2a). In migratory cell co-cultures, complete inhibition within the range of concentrations tested was only observed with sustained exposure to DS003 ($IC_{50} < 1$ nM; $IC_{95} = 17.61 \pm 20.14$ nM) (Figure 2b).

Similarly, sustained exposure of cervical explants to DS003 was required for inhibition of *ex vivo* challenge with HIV-1_{BaL} ($IC_{50} < 1$ nM; $IC_{95} = 23.62 \pm 28.92$ nM) (Figure 2c). No inhibitory activity was observed with pulse or 24 h dosing within the range of DS003 concentrations tested (Figure 2c). Dose-response curves were obtained for DS003 against viral dissemination by migratory dendritic cells. Sustained exposure of migratory cells/T cells co-cultures to DS003 resulted in greater inhibitory potency ($IC_{50} = 5.95 \pm 8.27$ nM) than shorter dosing periods ($IC_{50} = 240.29 \pm 338.18$ nM for 24 h exposure; $IC_{50} = 216.99 \pm 304.27$ nM for pulse dosing) (Figure 2d).

Inhibitory activity of DS003 in colorectal tissue

Pulsed treatment of colorectal explants with DS003 during viral exposure (2 h) inhibited *ex vivo* infection of this mucosal tissue, with an IC_{50} value at day 15 post-infection of 88.91 ± 43.46 nM (Figure 2e). Sustained exposure to DS003 resulted in an increase of inhibitory activity with IC_{50} values below the range of concentrations tested and plateauing after the lowest concentration of DS003 tested ($IC_{95} = 701.65 \pm 72.36$ nM).

Binding of DS003 to HIV-1 Env

To better understand the mechanism of action of DS003, competition studies were performed to determine potential blockade of HIV Env binding to human CD4. We used an anti-gp120 monoclonal antibody, 17b, that recognizes an epitope exposed following binding of gp120 to CD4, known as CD4-induced epitope^[28]. As expected, using BLI technology, binding of 17b to HIV-1_{CN54}-gp140 was only observed in the presence of sCD4 and increased in a dose-response manner until plateauing at 5 μ g/ml (data not shown). The binding of 17b to HIV-1_{CN54}-gp140 pre-treated with sCD4, decreased with increasing concentrations of DS003 (Figure 3).

Previous studies^[29, 30] have shown that HIV-1_{BaL} Env has a partially open conformation that allows binding of 17b in the absence of sCD4. To assess if DS003 could inhibit the naturally occurring binding of 17b to HIV-1_{BaL} Env, we used 293T cells transfected with HIV-1_{BaL} gp160. As expected, staining with 17b was observed in the absence of sCD4 (Figure 4a). Treatment with increasing concentrations of DS003 prevented binding of 17b in a dose-dependent manner (Figure 4a), confirming that DS003 interferes with the exposure of the 17b epitope on Env. We then assessed the activity of DS003 in the presence of sCD4. When cells were treated with DS003 prior to addition of sCD4 or dosed simultaneously with DS003 and sCD4, 17b staining was reduced to the baseline level of 17b binding in the absence of sCD4 (Figure 4b). However, incubation with sCD4 prior to DS003 treatment,

did not decrease 17b binding (Figure 4b); indicating that DS003 prevents but cannot reverse sCD4-induced conformational changes of HIV-1 Env.

Effect of DS003 on a HIV-1 fusion inhibitor

We then hypothesized that DS003, thanks to its capacity to block CD4-induced conformational changes that lead to the formation of the gp41 six-helix bundle, would be a good candidate to combine with a fusion inhibitor. The activity of DS003 alone and in combination with a potent fusion inhibitor based on the C34 sequence of gp41 HR2, L'644^[31] was compared in several cellular and tissue models. In TZM-bl cells, an increase of activity was observed for L'644 when combined with DS003, which resulted in an IC₅₀ for L'644 41.05 ± 28.85 times lower than when tested alone (Figure 5a, f). However, in *ex vivo* models including PBMCs, co-cultures of ecto-cervical migratory cells with PM-1 CD4⁺T cells, and ecto-cervical and colorectal explants cultures (Figure 5b, c, d, e), the DS003-L'644 combination showed greater inhibitory potency than either of the two drugs tested individually (Figure 5f).

DISCUSSION

DS003 proved to be more potent against HIV-1 than the related compound BMS-378806 in the TZM-bl assay. BMS-378806 has previously been shown to be protective *in vivo* against simian–human immunodeficiency virus (SHIV-162P3) in macaques^[10], and therefore these data suggest that DS003 is also likely to be effective *in vivo*. DS003 was active against primary transmitted strains proving efficacy against biologically relevant isolates. In several models, DS003 was only active, or demonstrated improved activity, when the test system was exposed continuously to the drug. This is consistent with the presumed mechanism of action of DS003, which targets HIV-1 prior to entry into host cells and therefore, likely requires inhibitory concentrations to be present in the extracellular compartment. For example, activity against transmission of virus from dendritic cells to T cells was most potent under continuous exposure to drug but was still in the low nanomolar range when sustained for only 24 hours. DS003 showed good activity against HIV infection of penile, cervical and colorectal tissue explants demonstrating efficacy in relevant tissue models, although again, activity was most pronounced when compound was maintained throughout the culture period. The inhibition of viral dissemination by migratory cells indicates that DS003 may be active in preventing *trans*-infection via migratory DC populations from the localized site of infection to draining lymph nodes *in vivo*. Mirroring the data obtained with the DC *trans*-infection model, DS003 was more potent against *trans*-infection via migratory cells when maintained in culture. These data suggest DS003 would be most effective against HIV-1 transmission if delivered locally by sustained release technology.

We observed different levels of inhibition in the three mucosal tissue tested. Contrary to other entry inhibitors whose mechanism of action is based on the binding to CD4 or to the viral co-receptors, DS003 binds to gp120. Hence, the differential activity of DS003 in the tissue explant and migratory cell models is not only linked to the state of activation of the target cells, but to other factors such as the accessibility of the virus for the drug within the mucosal milieu. Different levels and viscosity of mucosal secretions^[32] as well as cellular

drug transporters^[33] could reduce the drug availability in certain mucosal compartments. Furthermore, the lack of inhibition observed in ecto-cervical tissue explants following pulse or 24 h dosing with DS003 (Figure 2c) correlates with the *in vivo* pharmacokinetic and pharmacodynamic profiles observed during the IPM042 trial^[11, 12]. First, the DS003 vaginal tablet tested in the trial, is not a sustained delivery formulation, and showed a time to peak concentration in vaginal fluid of 0.5–12h with rapid subsequent decrease of concentration. Second, no protection was observed with the 1 mg vaginal tablet, resulting in a maximum concentration (C_{max}) in vaginal fluid of 281µg/g which is significantly above the higher concentration used *ex vivo* in tissue explants of 1 µM (equivalent to 95,7 ng DS003/tissue explant well). Topical dosing with the 10 mg vaginal tablet (C_{max} in vaginal fluid of 1078 µg/g) provided limited protection against *ex vivo* challenge of cervical biopsies (6/9); thus, indicating that a sustain delivery formulation would be more desirable for this drug as observed in ecto-cervical explants.

Technically, among the infection control replicates for each specimen, 100% of explants were productively infected.

We evaluated the potential activity of DS003 and L'644 combinations. These drugs have different mechanism of action and currently are not in use for therapy, reducing the potential for transmission of circulating resistant isolates and making them good candidates for PrEP.

With the approval of prevention interventions, such as Truvada and Descovy, the inclusion of placebo arms in trials is becoming ethically questionable and the incidence of infection within communities will decrease. Furthermore, adherence has been an important factor affecting the progress of PrEP candidates in the product development pipeline, and sustained release formulations are currently being prioritized. We have previously shown that sustained release results in greater inhibitory potency of entry and fusion inhibitors such as maraviroc^[34] and L'644^[31]. Hence, pre-clinical models are becoming increasingly important tools to facilitate and accelerate prioritization of PrEP candidate and their formulations for clinical testing. This study further validates the importance of tissue explants as a pre-clinical model which reflects the tissue and dosing-dependent efficacy of DS003.

Mechanistic studies suggest the DS003 prevents CD4 induced viral fusion. However, it does not appear that DS003 blocks the binding of gp120 to CD4.

In conclusion, DS003 demonstrated potent activity against HIV-1 in a variety of models designed to evaluate its potential as a microbicide. The data support the development of this compound as a prophylactic to prevent sexual transmission of HIV and suggest that it may be most effective in sustained release formulations.

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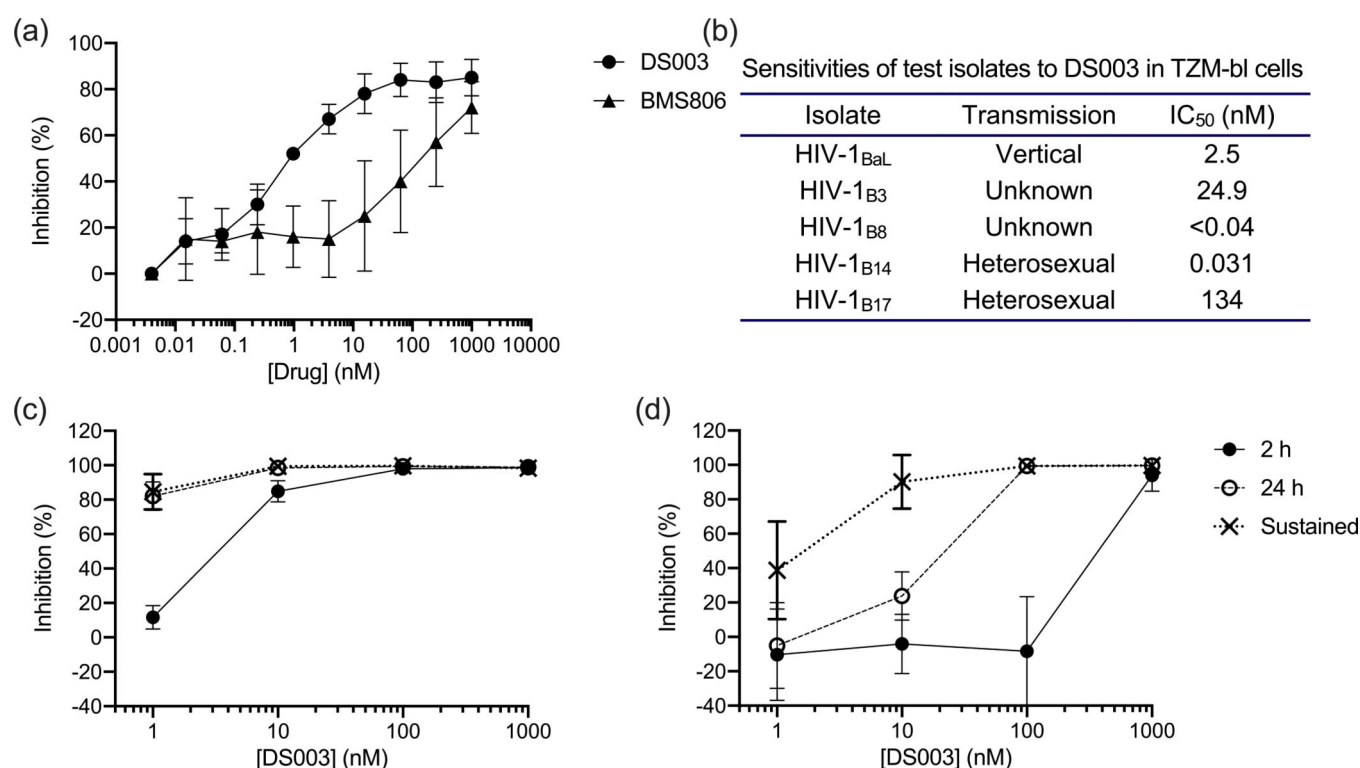


Figure 1. Anti-HIV-1 activity of DS003 in cellular models.

TZM-bl cells were treated for 1 h in the presence or absence of DS003 or BMS-806, before addition of HIV-1_{BaL} (a, b) or clade B primary isolates (b). Luciferase expression (r.l.u. values) was determined after 48 h and the extent of inhibition by each drug was calculated. (c) iDCs grown from PBMC-derived monocytes were challenged with HIV-1_{BaL} for 2 h in the presence or absence of DS003. Cells were then washed and cultured in the absence or presence of drug for 24 h or for the remaining time of culture. Alternatively, (d) iDCs were exposed to HIV-1_{BaL} for 2 h, washed with PBS to remove unbound virus and then cocultured with PM-1 cells in the presence or absence of DS003 for different time periods: 2 h pulse, 24 h pulse or continuously. The concentrations of p24 in harvested supernatants were quantified by ELISA and the extent of inhibition was calculated. The percentage of inhibition in all models was normalized relative to the r.l.u. or the p24 values obtained for cells not exposed to virus (0% infectivity) and for cells infected with virus in the absence of compound (100% infectivity). Data for each model are means ($\pm$ s.d) from three independent experiments performed in triplicate.

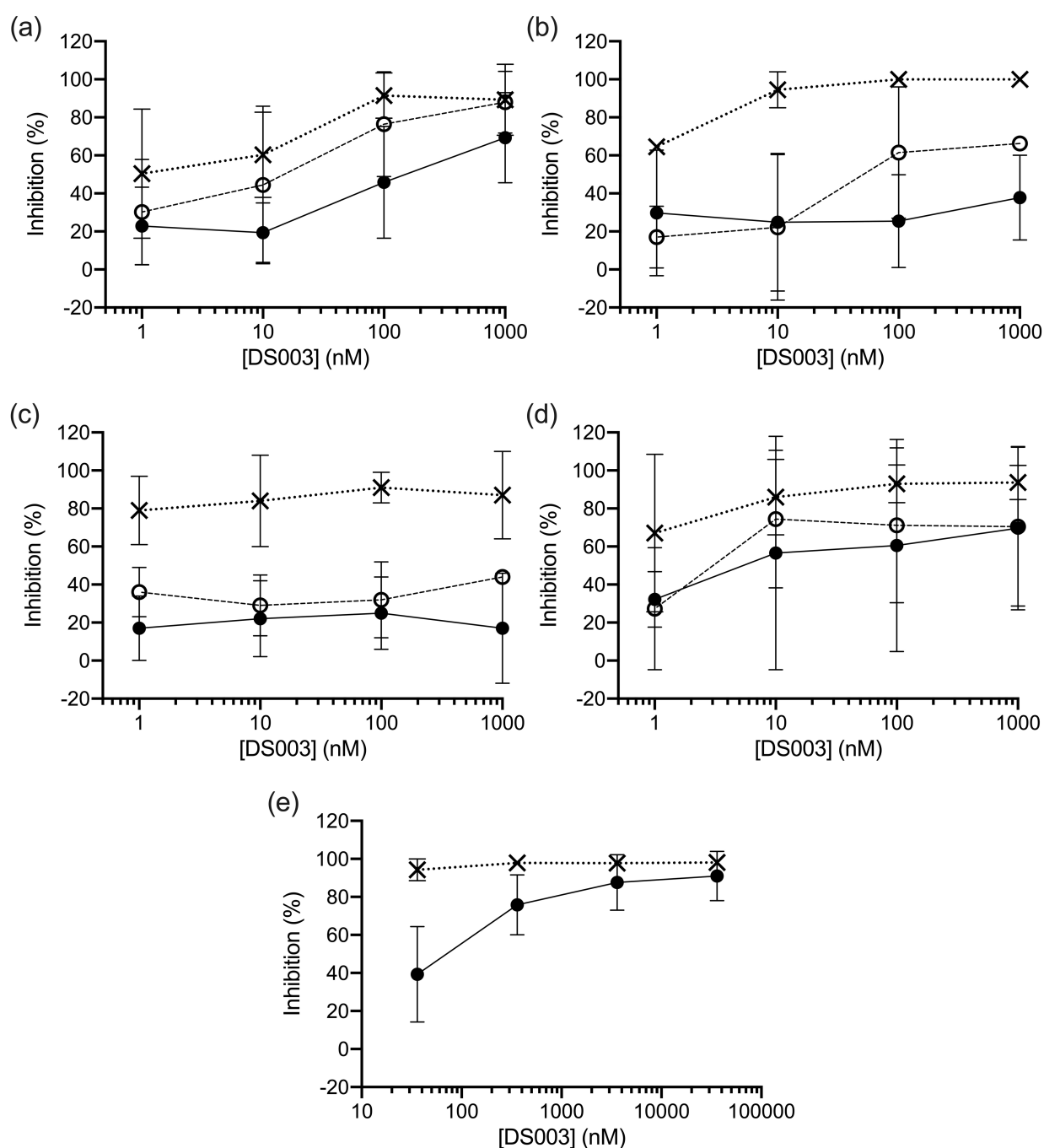
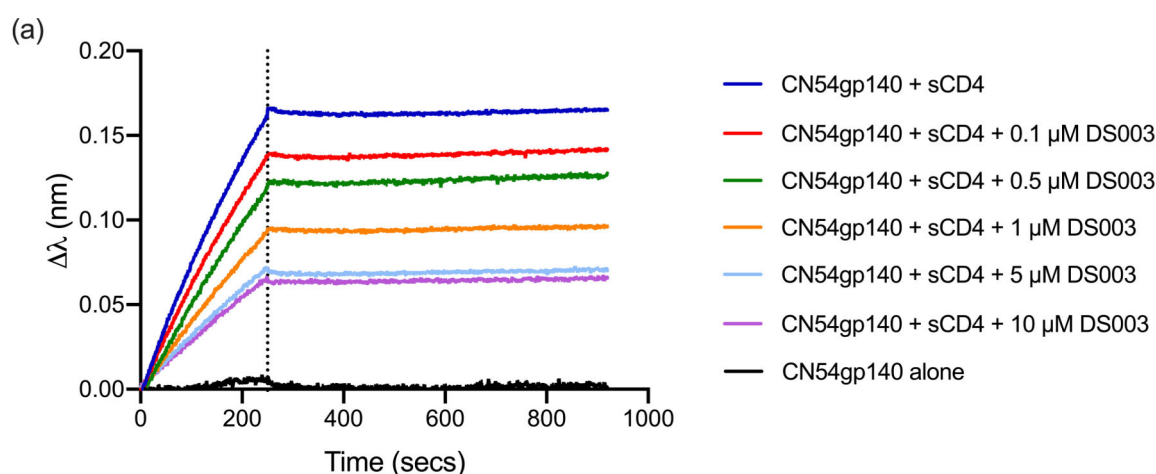


Figure 2. Inhibitory potency of DS003 in mucosal tissue explants and migratory cells.

Penile (a), ecto-cervical (c) and colorectal (e) tissue explants were either treated with drug for the duration of viral exposure (2 h pulse, ●) or for 24 h (24 h pulse, ○). Explants were then washed with PBS and cultured in the presence (sustained, ×) or absence of drug for 15 days. Following overnight culture, penile and ecto-cervical explants were transferred to fresh plates. Cells having migrated out of the tissue explants (penile (b) and ecto-cervical (d)) during the overnight culture, were collected and co-cultured with PM-1 cells. The concentrations of p24 in the harvested supernatants were quantified by ELISA and the

extent of inhibition was calculated. Data for each model are the mean ($\pm$ s.d) from three independent experiments performed in triplicates.



(b) Binding kinetics of HIV-1_{CN54} gp140 + sCD4 complex to 17b

Analyte	Response (nm)	K_D (M)	k_a (1/Ms)	k_d (1/s)	R_{max}
HIV-1 _{CN54} gp140 + sCD4	0.16 (0.004)	5.45e-10 (3.55e-10)	8286.57 (1520.50)	1.06e-5 (4.91e-6)	0.47 (0.05)
HIV-1 _{CN54} gp140 + sCD4 + 0.1 μ M DS003	0.13 (0.004)	9.19e-12 (1.33e-12)	6675.80 (844.21)	1.08e-7 (0.00)	0.44 (0.06)
HIV-1 _{CN54} gp140 + sCD4 + 0.5 μ M DS003	0.11 (0.007)	1.57e-11 (4.88e-12)	3409.50 (1062.50)	N/A	0.68 (0.17)
HIV-1 _{CN54} gp140 + sCD4 + 1 μ M DS003	0.09 (0.003)	1.30e-11 (3.16e-12)	5476.17 (1517.74)	N/A	0.47 (0.10)
HIV-1 _{CN54} gp140 + sCD4 + 5 μ M DS003	0.07 (0.002)	1.02e-10 (9.22e-11)	9436.83 (2868.36)	1.22e-5 (0.00)	0.26 (0.07)
HIV-1 _{CN54} gp140 + sCD4 + 10 μ M DS003	0.06 (0.001)	2.84e-9 (2.84e-9)	7065.40 (2494.56)	5.09e-5 (0.00)	0.25 (0.05)

[sCD4] = 5 μ g/ml; [HIV-1_{CN54} gp140] = 100 μ g/ml

K_D : equilibrium dissociation constant; k_a : association rate constant; k_d : dissociation rate constant; R_{max} : maximum binding level. Data are mean (SEM) of at least three replicate experiments.

Figure 3. Binding analysis of 17b to HIV-1_{CN54} gp140 + sCD4 complex in the presence of DS003 by biolayer interferometry.

Capture of HIV-1_{CN54} gp140 (100 μ g/ml) alone or complexed with sCD4 (5 μ g/ml) by 17b at 10 μ g/ml was assessed in the absence and with increasing concentrations of DS003.

(a) Change of wavelength ($\Delta\lambda$) is shown vs the length of the experiment. The dashed line represents the end of the association step (250 s). Curve fit data (a) and binding kinetics (b) were calculated by ForteBio Data analysis software and are the mean of up to six runs.

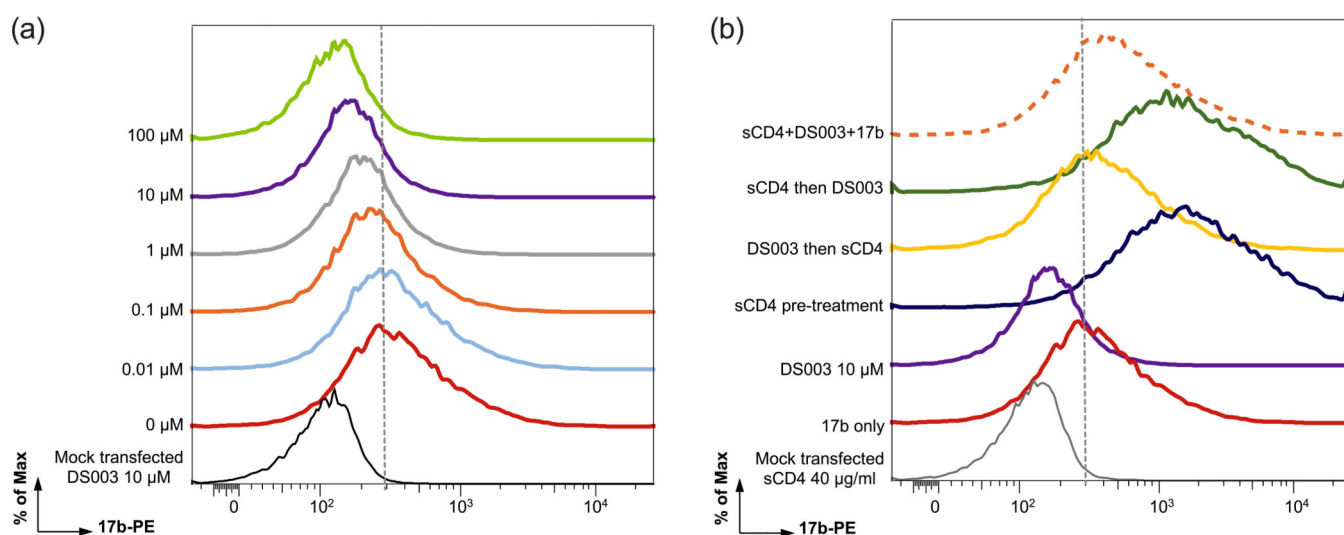


Figure 4. Effect of sCD4 and DS003 on 17b binding to HIV-1BaL gp160 expressed on 293T cells. HEK 293T cells (10⁶ cells/condition) transfected with HIV-1BaL gp160 were (a) treated with increasing concentrations of DS003 for 20 min at room temperature prior to immunostaining with 17b or (b) exposed simultaneously or sequentially to DS003 10 mM, sCD4 40 μg/ml and/or 17b 10 μg/ml. Non-specific fluorescence was determined with mock transfected cells.

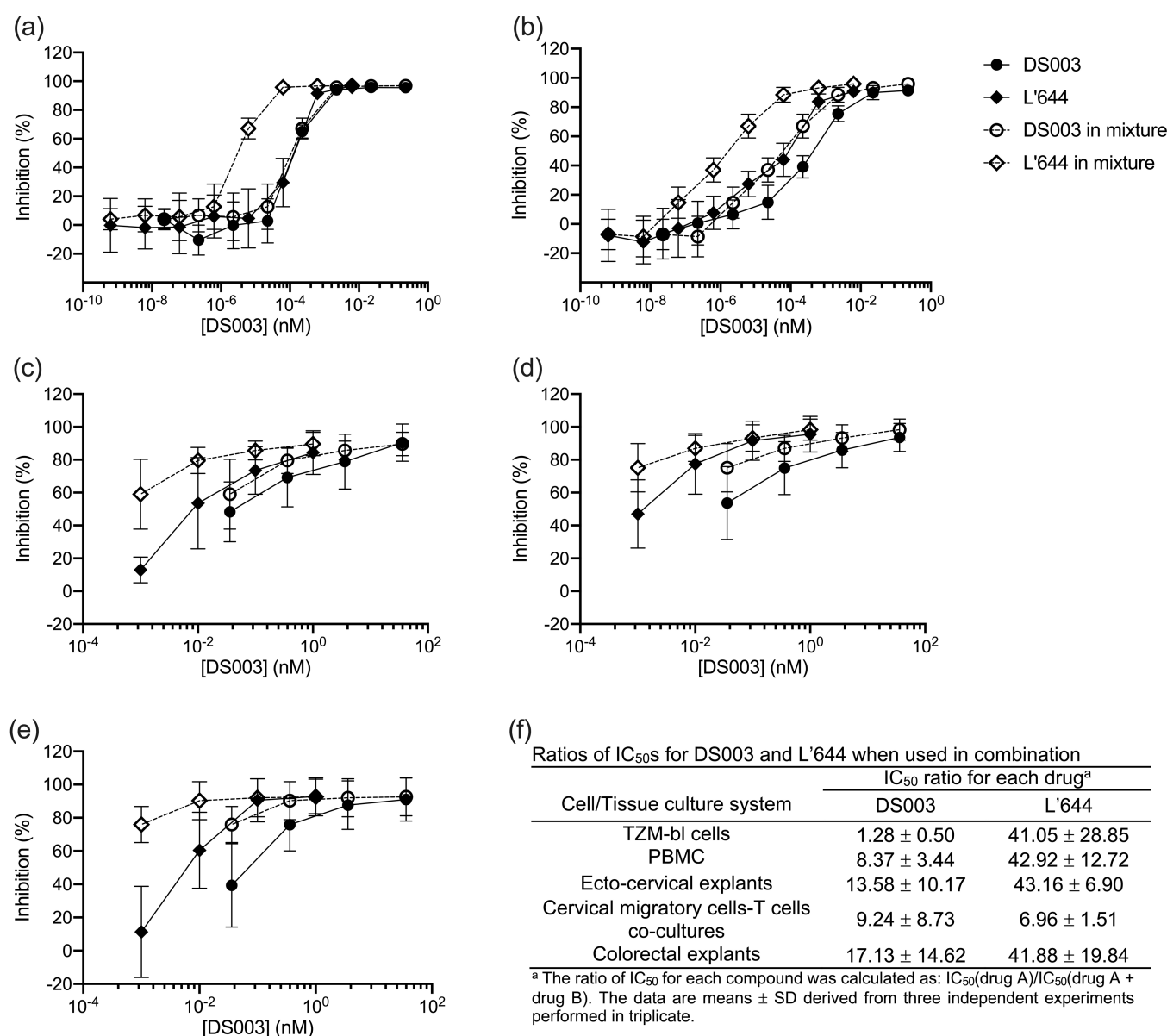


Figure 5. Activity of DS003 and L'644 in dual combinations against HIV-1_{BaL} in TZM-bl cells and colorectal explants.

TZM-bl cells (a) and PBMCs (b) were treated for 1h in the presence or absence of drugs alone and/or in combination. HIV-1_{BaL} was added to cells and left for the time of the experiment (48 h for TZM-bl cells and 7 days for PBMCs). Ecto-cervical (c) and colorectal explants (e) were treated for 1h in the presence or absence of drugs alone and/or in combination and then exposed to virus for 2 h before four washes with PBS. Colorectal explants were then transferred to gelfoam rafts. For ecto-cervical tissue, following overnight culture, explants were transferred to fresh plates and migratory cells co-cultured with PM-1 cells (d). Mucosal cultures were kept for 15 days. Infection was determined by measurement of luciferase expression in TZM-bl cells or of virion protein, p24 antigen, in the harvested supernatants from PBMCs and mucosal cultures. The extent of inhibition by each compound

or combination was calculated (e). Data for each model are means ($\pm$ sd) from three independent experiments performed in triplicate.