AIDS

Maraviroc and reverse transcriptase inhibitors combinations as potential pre-exposure prophylaxis candidates

--Manuscript Draft--

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Abstract:	Objective: Receptive anal intercourse in both men and women is associated with the highest probability for sexual acquisition of HIV infection. As part of a programme to develop an effective prevention strategy, we performed an ex-vivo preclinical evaluation to determine the efficacy of multiple double combinations of maraviroc (MVC) and reverse transcriptase inhibitors (RTIs). Design: The entry inhibitor, MVC, a nucleotide RTI, tenofovir (TFV), and two nonnucleoside RTIs, UC781 and TMC120 (dapivirine, DPV), were used in double, combinations against a panel of CCR5-using clade B and clade C HIV-1 isolates and against MVC-escape variants. A gel-formulated version of MVC-DPV combination was also tested. Methods: Indicator cells, co-cultures of immature dendritic cells with CD4+T cells, and colorectal tissue explants were used to assess antiviral activity of drug combinations. Results: All dual MVC-RTI combinations tested inhibited MVC-sensitive and resistant isolates in cellular and colorectal explants models. All the combinations were positive with no reduction in the activity of MVC. In tissue explants, the combinations against all viral isolates tested produced an increase in the activity of MVC. An initial gel-formulation of MVC-DPV combination showed greater and prolonged anti-viral activity of MVC in mucosal tissue explants. Conclusion: This study demonstrates that combinations based on antiretroviral drugs inhibiting HIV transmission at viral entry and reverse transcription have potential as prevention strategies against colorectal transmission of HIV-1 including MVC-resistant isolates. Pre-clinical evaluation with colorectal tissue explants indicates that a gel-

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Dear Dr. Osbourne,

My co-authors and I found the reviewers' comments to be both helpful and constructive. We have made extensive modifications in response to each of the points as detailed below, with particular attention to discussion of the limitations of the explant assay and of the analysis of combinations. For your convenience we have highlighted in red the portions of the text that have been modified.

We believe that we have addressed all of the points raised by the reviewers and hope that our manuscript is now suitable for publication in AIDS.

Yours sincerely,

Robin Shattock

Reviewer #1:
- My major concern relates to how the separate activities of inhibitors in combination were extrapolated. The authors used a formula, IC50(drug A)/IC50(drug A + drug B), to calculate a ratio that formed the basis of their conclusion that the individual activity of MVC and RT inhibitors was enhanced when the two classes of inhibitors were combined. Conceptually I do not see how it is possible to draw a conclusion about the individual activity of an inhibitor when it is tested in combination with another inhibitor. While it might be true that the dual combinations were more potent than each of the individual constituents, the assay is not designed to delineate the interactions mediating this effect. A more conventional measure of combination drug effects uses the median effect equation, e.g., as described by Chou and Talaley, to determine whether two drugs are additive, synergistic or antagonistic. It would be helpful to see examples of some of the neutralization curves from the TZM-bl assays and to analyze the results in a more conventional manner.

As requested by the reviewer we have added a few examples of dose-response curves in Supplementary Figure 1. We have elected not to use Calcusyn due to its limitations: based on the equation of Chou-Talalay, the slopes of all the titration curves must be parallel and the activity of the drug must cover the full range between 0 and 100% of inhibition. When drugs with different mechanisms of action which results in dose-response curves with different slopes; in *ex vivo* models with the donor-dependent variation; and when resistant isolates are tested, the equation loses its validity. For this reason we have calculated, as a similar concept to the dose reduction values obtained with Calcusyn, the ratio of IC$_{50}$ for each drug alone/IC$_{50}$ of the same drug used in combination in order to prove an increase in inhibitory activity of a combination of drugs compared to each drug used alone against different HIV-1 isolates. We agree that this is a key issue and we have commented

on it in the discussion (lines 358-369).

Other points:
1. TZM-bl assay is not described.
We have added a description of the assay in the materials and methods section (Lines 147-153)

2. Lines 150-151: Normalized for infectivity in which assay?
We have clarified (lines 156-157) that the infectious inoculum was normalized in all infectivity assays, including those performed in cellular and tissue explant models; and have specified the titer used in each model (lines 158, 168)

3. Lines 164-165: Were the explants cultured for 14 or 15 days (both are stated)?
We kept the tissue cultures for 15 days and we have corrected this typo (lines 170-171).

4. Lines 181-183: The authors used an average IC50 across a panel of viruses to determine an equipotent ratio of drug concentrations to use in combination. What was the range of IC50 obtained? Is the average of this range really suitable to assume equipotency against all of the viruses?
We have defined the range of IC_{50} for the individual drugs against wild type isolates (lines 189-193). We have rephrased line 187-188 to clarify that for all viruses the combinations were set up maintaining a constant ratio of concentrations between the drugs, and this ratio was based on the ratio of average IC_{50} for each drug included in the combination. As a model for a real life microbicide, using the average IC_{50} allows us to detect differences of drug activity in a range of viruses including resistant isolates for which an IC_{50} cannot always be calculated when the drug tested is the target of the mutation.

5. Lines 198-199: When you say there was no significant difference, was the difference compared using statistical methods?
We have replaced the statement of "no significant" with "no apparent differences" because we did not perform a systematic statistical analysis.

6. Lines 240-242: What concentration of MVC was used in this 2 hr pulse assay? Does the negative result indicate a higher dose is needed or perhaps a longer incubation is needed?
We have clarified the range of concentrations tested (line 250). When the assay was performed with a 2h pulse despite the highest concentration tested (10 µM) being fully inhibitory, a high level of variability between samples was observed not allowing us to calculate robust dose-response parameters. We cannot exclude that doses >10 µM might have produced more reproducible inhibition, but these may have been difficult to reproduce within tissue in vivo, furthermore, longer time of exposure with the same range of concentrations resulted in lower variability of the dose-response curves.

7. Line 290: Define MTT.
We have defined the abbreviation in lines 301-302.

8. Table 1, second footnote: "For MVC-resistant isolates, where (typo?)…
We thank the reviewer for spotting this typo. This has been rectified.

Reviewer #3:

1. The statement in the Abstract, "All dual MVC-RTI combinations …, and produced, for at least one of the compounds, a change in the dose-response curve." Is not clear. Perhaps it can be reworded to specifically focus on the effects on MVC.
We have reworded the abstract (lines 38-41) paying attention to the effect of the combination on MVC

2. The Authors conclude that the combination DPV/MVC is an effective candidate microbicide. Given the focus of this research on colorectal tissues, this statement should be qualified by the inclusion of the word "rectal" (microbicide).
We have added this aspect in the abstract (line 48) reinforcing the discussion where it was also mentioned (line 398)

3. Furthermore, although the benefit of combining MVC with DPV is obvious based on the results, it is not so clear that DPV significantly benefits from the antiviral effect of MVC; at least not from the data shown. DPV decreases in IC50s are small, especially in tissues (Fig 2, Table 2, and Fig 3). Given that the doses typically delivered by these microbicidal gels are several orders of magnitude the IC50 of the active compound, what is the clinical relevance of 1-2 fold decrease in IC50?
We agree that the increase of activity observed for these drugs in combination is not the most important point. The reason for development combinations as prevention strategies is to prevent transmission of a broad range of isolates including ARV-resistant viruses. We have emphasized this aspect in the discussion (lines 391-393)

4. Fig 1 is large, contains a lot of data, and may be better placed in the supplemental material. Instead, it would be helpful to present actual IC50s for the individual compounds and combinations.
We have placed Fig 1 as the supplementary figure and have introduced a new table as requested by the reviewer with the IC_{50} values.

5. Legend for Fig. 1. Although presenting data on iDC to T infection transfer, the legend refers to normalizing to "explants."
We apologize for this error and have corrected in lines 618 and 619.

6. Within Statistical Analysis, the statement "IC50 data presented fulfill the criterion of R2 > 0.7." is not clear.
We have amended the sentence to clarify that the IC50 values were calculated from a curve fitted to the experimental data which fulfilled the criterion of $R^2>0.7$.

ABSTRACT

Objective: Receptive anal intercourse in both men and women is associated with the highest probability for sexual acquisition of HIV infection. As part of a programme to develop an effective prevention strategy, we performed an ex-vivo preclinical evaluation to determine the efficacy of multiple double combinations of maraviroc (MVC) and reverse transcriptase inhibitors (RTIs).

Design: The entry inhibitor, MVC, a nucleotide RTI, tenofovir (TFV), and two nonnucleoside RTIs, UC781 and TMC120 (dapivirine, DPV), were used in double, combinations against a panel of CCR5-using clade B and clade C HIV-1 isolates and against MVC-escape variants. A gel-formulated version of MVC-DPV combination was also tested.

Methods: Indicator cells, co-cultures of immature dendritic cells with CD4$^+$T cells, and colorectal tissue explants were used to assess antiviral activity of drug combinations.

Results: All dual MVC-RTI combinations tested inhibited MVC-sensitive and resistant isolates in cellular and colorectal explants models. All the combinations were positive with no reduction in the activity of MVC. In tissue explants, the combinations against all viral isolates tested produced an increase in the activity of MVC. An initial gel-formulation of MVC-DPV combination showed greater and prolonged anti-viral activity of MVC in mucosal tissue explants.

Conclusion: This study demonstrates that combinations based on antiretroviral drugs inhibiting HIV transmission at viral entry and reverse transcription have potential as prevention strategies against colorectal transmission of HIV-1 including MVC-resistant isolates. Pre-clinical evaluation with colorectal tissue explants indicates that a gel-formulation of MVC-DPV is an effective candidate colorectal microbicide.

1   **Maraviroc and reverse transcriptase inhibitors combinations as potential pre-**

2   **exposure prophylaxis candidates**

3

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6

7   Running Title: Combinations of entry and RT inhibitors as PrEP

8

9   Keywords: prevention; antiretrovirals; combination; mucosal; explant

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18

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24

ABSTRACT

Objective: Receptive anal intercourse in both men and women is associated with the highest probability for sexual acquisition of HIV infection. As part of a programme to develop an effective prevention strategy, we performed an ex-vivo preclinical evaluation to determine the efficacy of multiple double combinations of maraviroc (MVC) and reverse transcriptase inhibitors (RTIs).

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Conclusion: This study demonstrates that combinations based on antiretroviral drugs inhibiting HIV transmission at viral entry and reverse transcription have potential as prevention strategies against colorectal transmission of HIV-1 including MVC-resistant isolates. Pre-clinical evaluation with colorectal tissue explants indicates that a gel-

48   formulation of MVC-DPV is an effective candidate colorectal microbicide.

49   **INTRODUCTION**

50   Multiple drug combinations are used in conventional HIV-1 treatment, known as

51   highly active antiretroviral therapy (HAART) [1]. Combinations may also be more

52   effective than single drug formulations as prevention strategies against HIV-1 sexual

53   transmission, including transmission of resistant isolates, which are increasingly

54   prevalent [2, 3]. Receptive anal intercourse (RAI) between serodiscordant couples in both

55   men and women is associated with the highest probability of sexual HIV transmission [4-

56   7] partly due to the abundance of highly activated $CCR5^+$ cells in the colorectal mucosa

57   [8-10]. A limited number of topical prevention strategies, referred to as microbicides,

58   against colorectal transmission have been tested in clinical trials. Furthermore, all

59   completed phase I [11-15] and on going phase II trials [16] are based on a single

60   antiretroviral (ARV) drug, specifically an RTI.

61   The majority of HAART regimes combine ARVs targeting different steps of the

62   viral replication cycle. Taking into account the predominant transmission of R5-tropic

63   isolates compared to X4-viruses during sexual intercourse [17, 18], a good candidate for a

64   combination with an RTI is a CCR5 small molecule inhibitor. MVC is the first small-

65   molecule CCR5 inhibitor to have been included in HAART. Formulated for topical

66   application, MVC has shown promising pharmacological results in humans and non-

67   human primates (NHPs) [19, 20] and efficacy in NHPs when tested as a vaginal gel

68   microbicide [21, 22]. It has also been formulated as a vaginal ring in combination with an

69   RTI (DPV) and tested in a phase I clinical trial (IPM-026/MTN013) [23]. The DPV/MVC

70   rings were safe and well tolerated, however, very low levels of MVC were detected in

71 tissue and, therefore, MVC did not block ex vivo challenge of vaginal biopsies.

72   We have investigated the inhibitory activity of dual combinations of MVC with

73 an RTI, either a nucleotide RTI tenofovir (TFV) or non-nucleoside RTIs, UC-781 or

74 TMC120 (dapivirine, DPV); as potential candidates for prevention of colorectal

75 transmission, including topical prevention, with MVC and DPV gel-formulated as a rectal

76 microbicide candidate. The anti-viral potency of these compounds alone and in

77 combination was evaluated against chronic or transmitted/founder R5-isolates and against

78 MVC-resistant clones using cellular and colorectal tissue explant models.

79

MATERIALS AND METHODS

Reagents and plasmids

Base compounds: 9-[R-2-(phosphonylmethoxy)propyl] adenine monohydrate (PMPA, or tenofovir (TFV)) was donated by Gilead Sciences, Inc. (Foster City, CA), UC781 was donated by Biosyn, Inc. (Huntington Valley, PA), MVC (UK-427,857) and DPV (TMC120) were provided by the International Partnership for Microbicides (IPM) (Silver Spring, MD) and by Janssen ID & V (Beerse, Belgium).

MVC 0.10 % gel, DPV 0.05% gel, combination MVC 0.10%-DPV 0.05 % gel and placebo gel were manufactured by Particle Sciences (Bethlehem, PA) for IPM as hypo-osmolar gels (<100 mOsm/kg).

HIV-1 BaL [24] was provided by the NIH AIDS Research & Reference Reagent Program (http://www.aidsreagent.org/). Full-length, replication and infection-competent proviral HIV-1 clone, pYU.2 [25, 26] was provided by the NIH AIDS Research & Reference Reagent Program (http://www.aidsreagent.org/). Transmitted founder clade C isolates, CH042, CH198, CH067, CH162 and CH164 were kindly provided by C. Ochsenbauer and J. Kappes at University of Alabama (Birmingham, AL) [27]. The sequences encoding the MVC-sensitive (MVC-Sens), MVC-resistant (MVC-Res) and MVC-Sens with the V3 loop of MVC-Res (MVC-Sens(V3R)) Envs have been previously reported [28]. MVC-Sens and MVC-Res sequences come from the dominant circulating viruses isolated from a patient of the MOTIVATE study before and after MVC therapy, respectively [29]. The V3 loop of MVC-Res Env contains two changes (P308S and G310_P311InsAla) that confer high MVC resistance [28, 30, 31]. Chimeric viruses carrying the full length envelope from MVC-Sens, MVC-Res and MVC-Sens(V3R) were generated as

103 previously described [28]. Briefly, the three gp160 described above were digested with

104 KspI and NotI from parental constructions and cloned into the pNL-KspI/Env/NotI vector

105 derived from the HIV-1 proviral clone pNL4-3 to produce replication-competent viruses.

106 The KspI and NotI restriction sites were introduced at the nucleotide positions 6214 and

107 8796 respectively in pNL4-3 as previously described [28]. DNA sequences of the cloned

108 full-length Envs were confirmed by sequencing.

109

110 *Cell and virus culture conditions*

111 All cell cultures were maintained at 37°C in an atmosphere containing 5% CO_2.

112 TZM-bl cells [32-34] were grown in Dulbecco's Minimal Essential Medium (DMEM)

113 (Sigma-Aldrich, Inc., St. Louis, MO) containing 10% fetal calf serum (FCS), 2mM L-

114 glutamine and antibiotics (100 U of penicillin/ml, 100 μg of streptomycin /ml). PM-1

115 cells [35] (AIDS reagent project, National Institute for Biological Standards and Control,

116 UK) were maintained in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine

117 and antibiotics (100 U of penicillin/ml and 100 μg of streptomycin/ml). PBMCs were

118 isolated from multi-donor buffy coats from healthy HIV-seronegative donors, by

119 centrifugation onto Ficoll-Hypaque, mitogen stimulated as previously described [36], and

120 maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, antibiotics

121 (100 U of penicillin/ml, 100 μg of streptomycin /ml), and 100 U of interleukin-2/ml.

122 Immature dendritic cells (iDCs) were grown from PBMC-derived monocytes cultured for

123 6 days in complete RPMI medium supplemented with 1000 U/ml GM-CSF and 500 U/ml

124 IL-4 (R&D Systems, Minneapolis, MN). Monocytes were isolated from PBMCs by

125 autoMACS human CD14 Microbeads (Miltenyi Biotec, UK) following manufacturers

126 instructions. iDCs were phenotypically characterized by staining with anti-CD40, anti-

127 CD80, anti-CD86, anti-CD83, anti-CD209, anti-CD123, and anti-CD11c (BD

128 Pharmingen, UK). FACS analysis was performed with a BD FACSCanto II flow

129 cytometry system using BD FACSDiva analysis software.

130   The laboratory-adapted isolates HIV-1 BaL and YU.2 were passaged through

131 activated PBMCs for 11 days.

132

133 *Patients and tissue explants*

134   Surgically-resected specimens of colorectal tissue were collected at St George's

135 Hospital, London and St Mary's Hospital, Imperial College London, UK. All tissues were

136 collected after receiving signed informed consent from all patients and under protocols

137 approved by the Local Research Ethics Committee. All patients were HIV negative. On

138 arrival in the laboratory, resected tissue was cut into 2-3 mm^3 explants comprising both

139 epithelial and muscularis mucosae as described previously [37]. Colorectal explants were

140 maintained with DMEM containing 10% fetal calf serum, 2mM L-glutamine and

141 antibiotics (100 U of penicillin/ml, 100 μg of streptomycin /ml, 80 μg of gentamicin /ml)

142 at 37ºC in an atmosphere containing 5% CO_2.

143

144 *Infectivity and inhibition assays*

145   The infectivity of virus preparations was estimated in TZM-bl cells (by luciferase

146 quantitation of cell lysates, Promega, Madison, WI) and PBMCs (by measure of p24

147 antigen content in cell culture supernatant). Briefly, TZM-bl cells were seeded at 3 x 10^3

148 cells/well 24 h prior to infection with HIV isolates. After incubation for 2 days, the cells

149 were washed with PBS and lysed with 100 µl of luciferase cell culture lysis reagent [38].

150 Fifty microliters were transferred to a white, opaque assay plate for luciferase

151 quantification in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments,

152 Inc. Burlington, VT), using 50 µl of luciferase assay reagent. The extent of luciferase

153 expression was recorded in relative light units (r.l.u). p24 content in supernatant was

154 measured with HIV-1 p24 ELISA (AIDS Vaccine Program, National Cancer Institute,

155 Frederick, MA) following manufacturer's instructions.

156   All inhibition assays in cellular and tissue explants models were performed using

157 a standardized amount of virus culture supernatant normalized for infectivity. Cells were

158 incubated with serial dilutions of drugs for 1 h at 37ºC, and then virus ($10^{3.3}$ $TCID_{50}$) was

159 added to cells and left for the time of the experiment. iDCs were exposed to virus for 2 h,

160 then washed 3 times with PBS to remove unbound virus. Infected iDCs were then co-

161 cultured with PM-1 cells at a 1:2 ratio of infected cells:PM-1 cells (equivalent to $1x10^4$

162 infected iDCs:$2x10^4$ PM-1 cells) in the presence or absence of drugs alone or in

163 combination. Cultures were maintained for 14 days, with 50% media feeds every 2-3

164 days. Drugs were kept in the co-culture for 2h, 24 h or continuously during the 14 days of

165 culture. HIV-1 infection was determined by measurement of p24 levels in culture

166 supernatants by ELISA (HIV-1 p24 ELISA, AIDS Vaccine Program, National Cancer

167 Institute, Frederick, MA). Alternatively, tissue explants were incubated with drug for 1 h

168 before virus (10^3 $TCID_{50}$) was added for 2 h. Explants were then washed 4 times with

169 PBS to remove unbound virus and drug. Colorectal explants were then transferred onto

170 gelfoam rafts (Welbeck Pharmaceuticals, UK) and cultured for 15 days as previously

171 described [39] in the presence or absence of drug. Explants were cultured for up to 15

172   days in the presence or absence of drug, and approximately 50 % of the supernatants

173   were harvested every 2 to 3 days and explants were re-fed with fresh media. The extent of

174   virus replication in tissue explants was determined by measuring the p24 antigen

175   concentration in supernatants (HIV-1 p24 ELISA, AIDS Vaccine Program, National

176   Cancer Institute, Frederick, MA).

177

178   *Statistical and mathematical analysis*

179   IC_{50} values were calculated from sigmoid curve fitted (Prism, GraphPad)

180   fulfilling the criterion of $R^2 > 0.7$.

181

182   **RESULTS**

183   **Double combinations of MVC and RTIs are more active than individual drugs in**

184   **TZM-bl cells.** To evaluate the potential of MVC, PMPA, UC781 and DPV as part of a

185   microbicide based on entry and RT inhibitors combination, we first tested the inhibitory

186   activity of each compound alone against a panel of clade B R5 isolates in order to design

187   combinations including concentrations of drugs based on a constant ratio of the average

188   IC_{50} for each individual ARV against wild type chronic clade B isolates included in the

189   mixture as previously described [37]. The average IC_{50} of PMPA (ranging from 2.3 to

190   6.7 μM) was approximately 352 fold higher than that of MVC (ranging from 2.35 to 6.99

191   nM); hence, to set up the dual combination, PMPA and MVC were combined at a ratio of

192   352:1. Similarly, MVC combinations with UC781 (IC_{50} between 5.86 and 10.21 nM) and

193   DPV (IC_{50} ranging from 0.15 to 0.84 nM) were titrated at set ratios of approximately 1:1

194   and 1:12, respectively. When establishing a ratio of the IC_{50} for each compound alone vs.

195   in combination, all combinations resulted in an increase of activity (examples in

196   Supplementary Fig. 1) with a decrease of IC_{50} for each compound included in the

197   combination (Table 1). For all three MVC-RTI combinations tested, the level of

198   reduction of IC_{50} values for MVC and RTIs was similar, with ratios IC_{50} drug A

199   alone/IC_{50} drug A combined of 2.36 ± 0.43 for MVC and 3.84 ± 1.90 for the three RTIs

200   in average.

201   Transmitted/founder (T/F) isolates have been shown to have different CCR5

202   utilization than chronic viruses in the presence of MVC [40, 41]. We then tested the

203   activity of MVC and combinations with the three RTIs against a panel of T/F clade C

204   isolates. The IC_{50} of the T/Fs was in general a log lower (0.52 ± 0.01 nM for CH042; 0.66

205   ± 0.26 nM for CH198; 0.21 ± 0.04 nM for CH067; 0.62 ± 0.08 nM for CH164) than the

206   average IC_{50} for chronic virus (4.07 ± 1.67 nM); except for one T/F isolate, CH162,

207   whose IC_{50} was in the range of the chronic viruses (3.27 ± 0.55 nM). Despite the

208   difference in sensitivity to MVC between CH162 and the other T/F isolates, no apparent

209   differences were observed on the level of increase of drug activity when MVC was

210   combined with any of the three RTIs among all the T/Fs. Interestingly, the increase of

211   inhibitory activity for MVC and the RTIs was different between laboratory adapted clade

212   B viruses and clade C T/F isolates. Against clade C transmitted founder isolates, the

213   inhibitory activity of MVC was only slightly increased, with the IC_{50} for MVC 1.34 $\pm$

214   0.43 times lower in average when used in combination; however, a greater decrease of

215   IC_{50} values for the three RTIs was measured when used in combination with MVC

216   against clade C isolates (15.67 ± 13.84 times for TFV, 33.14 ± 36.83 for UC781 and

217   13.40 ± 10.03 for DPV) (Table 1).

218   The potential success of any microbicide may be dependent not only on its

219   activity against wild type isolates, but also against possible resistant isolates. A range of

220   mutations can emerge conferring resistance to MVC and prevalence may increase in

221   populations with the wider use of MVC in therapy. We studied the activity of MVC and

222   its combinations with the three RTIs against clonal viruses containing the MVC-resistant

223   Env derived from a patient who developed resistance (MVC-Res) in comparison to the

224   Env derived from the same subject prior to initiation of treatment including MVC (MVC-

225   Sens) [30, 31]. We also used a third MVC-resistant chimeric construct from the same

226   individual containing the V3 loop of the MVC-sensitive Env (MVC-Sens(V3R)) [30, 31].

227   In TZM-bl cells, an IC_{50} was not reached against MVC-Res within the range of MVC

228   concentrations tested ($IC_{50} \gg 142$ nM); however, with the chimeric construct MVC-

229   Sens(V3R), MVC reached a plateau of 50 % of inhibition at around 9 nM in contrast with

230   the MVC-sensitive isolate, against which a dose-response curve was measured for MVC

231   with average IC_{50} of 0.34 nM and IC_{95} of 10.02 nM. The different level of resistance to

232   MVC of MVC-Res and MVC-Sens(V3R) reflects the fact that mutations outside the V3

233   loop further contribute, although to a lesser extent, to the resistance profile. Interestingly,

234   the IC_{50} of MVC-Sens was in the sub-nM range similar to the T/F isolates. The three

235   RTI-MVC double combinations were then titrated against the MVC-sensitive and

236   resistant isolates. The three isolates were fully sensitive to the RTIs, therefore

237   combinations of MVC with TFV, UC 781 or DPV were able to inhibit infection in TZM-

238   bl cells with all the three isolates tested (Table 1). However, the dose-response curve of

239   the MVC-DPV combination was affected when titrated against MVC-Res, showing a

240   change in slope that resulted in an increase of IC_{50} for DPV when used in combination

241 with MVC. This resulted in a ratio of IC_{50}s of 0.6 ± 0.74 nM (Table 1), without affecting

242 the maximum level of inhibition reached by DPV alone or in combination at the highest

243 concentration tested (12 nM) (data not shown).

244

245 **Combinations are active against *trans*-infection between iDC and T cells.** The

246 inhibitory potency of MVC-RTI combinations were further evaluated in a cellular model

247 of co-culture of PM-1 $CD4^+$ T cells with infected iDC, mimicking the potential cell-

248 associated transmission of HIV-1 from virus-exposed iDC to uninfected $CD4^+$ T cells

249 that occurs during the local expansion of infection following establishment of the initial

250 foci of infection in mucosal tissues [42, 43]. Initial studies exposing the co-culture to

251 MVC in a range of concentrations between 10 and 0.0001 μM for a 2 h pulse did not

252 result in a robust inhibition of *trans*-infection (data not shown), indicating that the

253 effective level of MVC required to block local mucosal expansion should be higher.

254 Hence, we tested longer drug exposure times mimicking repeated dosing (24 h pulse) or

255 sustained release (continuous exposure). The three RTIs and MVC were able to inhibit

256 iDC-facilitated infection of PM-1 cells when added during a pulse of 24 h or kept

257 continuously during the 14 days of co-culture (as shown in Table 2 and Supplementary

258 Fig. 2). As expected, the plateau of maximum inhibition was reached at lower

259 concentrations with sustained exposure to compounds. The inhibitory activity of the

260 drugs was increased when titrated in MVC-RTI double combinations (Table 2 and

261 Supplementary Fig. 2) with a decrease of the IC_{50} for at least one of the compounds

262 included in all the MVC-RTI combinations tested.

263

264 **Inhibitory activity of MVC-RTI dual combination in tissue explants.** Based on the

265 results obtained with TZM-bl cells and iDC-PM-1 co-cultures, the double combinations

266 were also titrated against clade B HIV-1 BaL in colorectal tissue explants to assess the

267 potential of such combinations as colorectal microbicides. In colorectal explants we have

268 previously described that MVC reaches the maximum level of inhibition earlier than

269 RTIs, where after 11 days of culture the % of inhibition measured for MVC at the highest

270 concentration tested, 1 μM, decreased from ~ 85 % to less than 80 % at day 15 (Fletcher

271 et al., submitted). Hence, when assessing the effect of dual combinations on anti-viral

272 activity we compared days 11 and 15 of culture. A positive shift (to the left) in the dose-

273 response curve for all mixed compounds was seen at both days (Figure 1) with a

274 reduction of the IC_{50} of each drug when used in combination. For all combinations tested,

275 the IC_{50} values of all drugs combined showed a similar reduction at day 11 and 15 (Table

276 3). This is probably due to the slight loss of inhibitory potency between day 11 and day

277 15 observed for MVC. With the MVC-UC781 combination, both drugs had their activity

278 increased to similar proportions, however, when MVC was combined with TFV or DPV,

279 the % of reduction in the IC_{50} was greater for MVC than for the two RTIs. This reflects a

280 higher contribution by TFV and DPV to the activity of the dual combinations.

281 Based on the encouraging results obtained with the screening in TZM-bl against

282 the MVC-resistant isolates (MVC-Res and MVC-Sens(V3R)), we tested the three MVC-

283 RTI double combinations against MVC-Res in colorectal tissue explants. As expected,

284 the maximum level of inhibition reached at the highest concentration tested for MVC

285 against this isolate was significantly reduced, but an IC_{50} could be calculated within the

286 range of concentration tested (as shown in Supplementary Fig. 3). As in TZM-bl cells,

287 MVC- Res was sensitive to the three RTIs and the dual combinations with MVC resulted

288 in an increase of anti-viral potency. The ratios of $IC_{50}s$ for the three RTIs were similar to

289 those observed when the drugs alone and in combination were titrated against the MVC-

290 sensitive virus (Table 3). However, the reduction in IC_{50} for MVC against the MVC-

291 resistant isolate was, as predicted, greater than against wild type virus, resulting in higher

292 ratios of $IC_{50}s$ (Table 3) and reflecting, once more, the activity of the more potent drug in

293 the combination, in this case the RTIs (either TFV, UC 781 or DPV).

294

295 **Evaluation of a gel-formulated combination of MVC and DPV.** To optimize

296 formulation and taking into account the greater inhibitory potency of DPV, the MVC-

297 DPV combination gel was prepared with MVC at 0.10 % and DPV at 0.05 %, and not at

298 an equipotent ratio (based on the IC_{50} values of each drug). Hence, the activity of the gels

299 for each individual drug or the combination was first evaluated in TZM-bl cells against a

300 panel of clade B, clade C T/F and MVC-resistant isolates. The placebo gel had no

301 inhibitory activity *per se* (data not shown) and importantly, the gel-formulated MVC and

302 DPV alone and in combination showed no cytotoxic effect by a 3-(4,5-dimethyl-2-

303 thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assay (data not shown).

304 The DPV gel and the MVC gel were active against all isolates tested, although, in

305 general, there was an increase of the $IC_{50}s$ of the DPV gel against all isolates tested

306 compared to the base compound. The effect of the combination on the activity of DPV

307 was positive against all isolates tested with an average reduction of IC_{50} against the clade

308 B MVC-sensitive viruses of 10.56 ± 6.59 times, of 2.30 ± 1.32 times for the MVC-

309 resistant clade B isolates and of 18.13 ± 12.20 times for clade C isolates (Table 1). MVC

310   was also more potent against MVC-sensitive clade B viruses when tested in the

311   combination gel, with IC_{50} values on average 1.88 ± 0.93 times lower than when titrated

312   alone. An increase in activity was also seen for MVC in combination against the clade C

313   isolates CH162 and CH164; however, a slight increase in the IC_{50} values for MVC was

314   seen against CH042, CH198 and CH067 resulting in ratio of $IC_{50}s < 1$ (Table 1). This

315   increase had no effect on the maximum level of inhibition reached by the combination gel

316   in comparison with the MVC gel at the highest concentration tested (142 nM) (data not

317   shown) and therefore reflected a slight change in the slope of the dose-response curve.

318   The activity of the gel-formulated drugs was also assessed against the two MVC-resistant

319   isolates. DPV gel was active against MVC-Res and MVC-Sens(V3R), however, an IC50

320   could not be calculated for MVC gel even at the highest concentration tested. The

321   combination gel was active against both isolates with an increase in activity reflected in

322   ratios of $IC_{50}s > 1$ for both drugs (Table 1).

323   The gels were next tested in the mucosal tissue explant model. In colorectal

324   explants, formulated MVC was more potent, reaching higher levels of inhibition, than

325   base compound against the clade B virus BaL. Furthermore, the MVC-DPV gel

326   combination was fully inhibitory at days 11 and 15 (Figure 2). Both drugs were more

327   potent in the combinatorial gel with a similar reduction in the IC_{50} values for MVC (IC_{50}

328   4.91 ± 0.66 times lower in combination than alone, as average of day 11 and 15) and for

329   DPV (IC_{50} values 2.61 ± 0.50 times lower in average at both time points) (Table 3). The

330   activity of the MVC gel against the MVC-resistant isolate MVC-Res reached a plateau at

331   around 50 % of inhibition at the highest concentration tested, but the MVC-DPV

332 combination gel was able to inhibit this isolate with a decrease in the value of IC_{50} for

333 MVC and for DPV, resulting in ratios of $IC_{50}s > 1$ for both drugs (Table 3).

334

335 **DISCUSSION**

336 To date, the majority of microbicide trials have tested the safety and/or efficacy of

337 single ARVs, and in the specific case of colorectal microbicides, all the trials have

338 assessed an RTI [44]. Only a phase I vaginal microbicide trial has assessed the

339 pharmacokinetics and safety of a combination of MVC with DPV formulated as a vaginal

340 ring [23]. Previous pre-clinical studies have shown that topical use of ARV combinations

341 could be effective against HIV-1 transmission during RAI [37, 45]. In this study we have

342 evaluated, side by side, the activity of MVC and three RTIs (TFV, UC781 and DPV)

343 alone or in combination in two cellular models and in colorectal explants. The order of

344 potency of the four ARVs was the same in all models; however, the IC_{50} of MVC was

345 affected by the level of expression of CCR5 on the cell surface of each model and by the

346 ability of the virus to recognize MVC-bound CCR5. Indeed, the average IC_{50} of MVC

347 against chronic R5-clade B isolates in TZM-bl was greater (4.07 ± 1.67 nM) (Table 1)

348 than that previously described in the literature when tested in PBMCs (1.2 nM) [46]. This

349 is due to the reported high levels of CCR5 in TZM-bl cells, which with stable

350 transfection, express > 2 logs more CCR5 than PBMC [47]. The differences of $IC_{50}s$ for

351 MVC in TZM-bl cells observed between T/F and laboratory adapted chronic isolates

352 (Table 1) have also previously been described in two consistent studies were T/F isolates

353 were more sensitive to MVC than chronic viruses on cells expressing high levels of

354 CCR5 [40, 41]. This is due to the capacity of R5-chronic isolates to infect cells despite

355 MVC being bound to CCR5. Among the T/F isolates tested one of them, CH162, had an

356 IC_{50} (3.27 ± 0.55 nM) in the range measured for chronic isolates (average IC_{50} of 4.07 ±

357 1.67 nM). The V3 loop of CH162 has greatest homology with BaL among the T/F tested

358 [27].

359 To assess the combinatorial activity (synergy / additivity / antagonism) of drugs it

360 was not possible to use the equation of Chou-Talalay [48] included in the analysis

361 software Calcusyn. To apply this equation correctly the slopes of all the titration curves

362 compared must be parallel and the activity of the drug must cover the full range between

363 0 and 100% of inhibition. However, donor-to-donor variation of the explant model,

364 assessment of ARVS with different mechanisms of action and use of RTI-resistant

365 isolates makes this impossible to achieve. Hence, to provide a quantitative indication of

366 the potential increase in activity we chose a similar concept to the 'dose reduction' [49]

367 and calculated for each drug the ratio of IC_{50} of drug alone versus IC_{50} of drug in

368 combination with another drug. While this does not provide a numerical indication

369 (combination index) of the combinatorial effects, it does allow classification of

370 combinations as being 'positive or negative combinations'.

371 In addition to determining the efficacy of individual ARVs and MVC-RTI dual

372 combinations against wild type isolates, assessment of their activity against resistant

373 strains is of critical importance. Different sets of mutations have been associated with

374 resistance to MVC [31, 50], in this study we chose a MVC-resistant isolate obtained from

375 a subject who commenced HAART including MVC in a phase III trial and experienced

376 virologic failure due to resistance to MVC [31]. The MVC-resistant and sensitive Envs

377 were isolated to obtain two viral clones, MVC-Res and MVC-Sens, respectively. A third

378   clone was prepared by replacing the V3 loop of MVC-Sens with the one of MVC-Res.

379   All three dual MVC-RTI combinations restored the activity against MVC-Res and MVC-

380   Sens(V3R). Interestingly, the dose-response curve of the MVC-DPV combination in

381   TZM-bl cells was affected when titrated against MVC-Res, showing a change in slope

382   that resulted in an increase in IC_{50} for DPV in the combination (Table 1). Importantly, the

383   increase in IC_{50} did not affect the maximum level of inhibition attained, reflecting a

384   change of slope in the dose-response curve. This highlights the importance of other

385   parameters in addition to the IC_{50} when evaluating the inhibitory profile of an ARV.

386   A first hypo-osmolar gel-formulated version of MVC-DPV was tested in this pre-

387   clinical study showing greater inhibitory activity than the gels containing MVC or DPV

388   alone against all isolates. In colorectal explants, two important effects were observed with

389   the MVC-gel formulation. A higher level of inhibition was obtained for MVC and the

390   activity was maintained between days 11 and 15 with the formulated drug (Figure 2).

391   This indicates that formulation of MVC as a gel for topical application could promote the

392   inhibitory potency of MVC in the mucosal environment. In addition, and more

393   importantly, this combination was able to fully inhibit MVC-resistant isolates in all the

394   models tested.

395   The results with resistant-isolates demonstrate the importance of considering

396   combinations of compounds with different inhibitory mechanisms and/or targeting

397   different steps of the viral replication cycle in the design of microbicides. Furthermore, an

398   initial gel-formulation of MVC-DPV shows encouraging results for further development

399   of this combination as a colorectal microbicide. This study further validates the use of

400   multiple pre-clinical models including mucosal tissue explants tailored to the ARVs

considered and that reflect the multiple aspects affecting the potential *in vivo* efficacy of the candidate ARV-based microbicide. The results obtained with these chosen efficacy models (including IC_{50}, slope of the dose-response curve, maximum percentage of inhibition) will have to be correlated with safety and pharmacokinetic studies in humans to better predict the *in vivo* potential of any colorectal microbicide candidate.

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586

587   **FIGURE LEGENDS**

588

589   **Figure 1.** **Dual combinations of MVC, TFV, UC781 and/or TMC120 in colorectal explants are more active against HIV-1 BaL than individual drugs.** Colorectal explants were treated for 1h in the presence or absence of: MVC and/or TFV (a, d), MVC and/or UC781 (b, e), MVC and/or TMC120 (c, f). BaL was added for 2 h, and then the explants were washed four times with PBS and transferred to gelfoam rafts. Explants were kept in culture for 15 days. The concentrations of p24 in the harvested supernatants were quantified by ELISA at days 11 and 15 of culture, and the extent of inhibition by each compound or combination at each time point was calculated. The percentage of inhibition was normalized relative to the p24 values obtained for explants not exposed to virus (0% infectivity) and for explants infected with virus in the absence of compound (100% infectivity). Data are means (± standard deviations) from three independent experiments performed in triplicate.

601   **Figure 2. Inhibitory potency of gel-formulated MVC and/or TMC120 in colorectal**

602   **explants against HIV-1 BaL and HIV-1 MVC-Res.** Colorectal explants were treated

603   for 1h in the presence or absence of: MVC gel, TMC120 gel of MVC-TMC120

604   combination gel. BaL (a, b) or MVC-Res (c, d) was added for 2 h, and then the explants

605   were washed four times with PBS and transferred to gelfoam rafts. Explants were kept in

606   culture for 15 days. The concentrations of p24 in the harvested supernatants were

607   quantified by ELISA at days 11 and 15 of culture, and the extent of inhibition by each

608   compound or combination at each time point was calculated. The percentage of inhibition

609   was normalized relative to the p24 values obtained for explants not exposed to virus (0%

610   infectivity) and for explants infected with virus in the absence of compound (100%

611   infectivity). Data are means (± standard deviations) from three independent experiments

612   performed in triplicate.

613

614   **Supplementary Figure 1. Examples of dose-response curves of individual drugs and**

615   **dual combinations against HIV-1 BaL and YU.2 in TZM-bl cells.** TZM-bl cells were

616   treated for 1 h in the presence or absence of a, d) MVC and/or PMPA, b, e) MVC and/or

617   UC781, or c, f) MVC and/or TMC120. The cells were then exposed to BaL (a, b and c) or

618   YU.2 (d, e and f). Luciferase expression (r.l.u. values) was determined after 48 h and the

619   extent of inhibition by each drug was calculated. The percentage of inhibition was

620   normalized relative to the r.l.u values obtained for cells grown in the absence of virus

621   (0% infectivity) and for cells infected with virus in the absence of drug (100%

622   infectivity). Data are the mean (± SD) of three independent assays performed in triplicate.

623

624   **Supplementary Figure 2**. **Activity of MVC, TFV, UC781 and TMC120 in dual**

625   **combinations against *trans*-infection from iDC to T cells with HIV-1 BaL.** iDCs were

626   exposed to virus for 2 h, washed 3 times and then co-cultured with PM-1 cells in the

627   presence or absence of MVC and/or TFV (a, d), MVC and/or UC781 (b, e), MVC and/or

628   TMC120 (c, f), for 14 days. Drugs were maintained in culture for 24 h (24h pulse) or 14

629   days (sustained). The concentrations of p24 in the harvested supernatants were quantified

630   by ELISA, and the extent of inhibition by each compound or combination was calculated.

631   The percentage of inhibition was normalized relative to the p24 values obtained for co-

632   cultures not exposed to virus (0% infectivity) and for co-cultures infected with virus in

633   the absence of compound (100% infectivity). Data are means (± standard deviations)

634   from three independent experiments performed in triplicate.

635

636   **Supplementary Figure 3**. **Inhibitory activity of dual combinations of MVC, TFV,**

637   **UC781 and/or TMC120 in colorectal explants against HIV-1 MVC-Res.** Colorectal

638   explants were treated for 1h in the presence or absence of: MVC and/or TFV (a, d), MVC

639   and/or UC781 (b, e), MVC and/or TMC120 (c, f). MVC-Res was added for 2 h, and then

640   the explants were washed four times with PBS and transferred to gelfoam rafts. Explants

641   were kept in culture for 15 days. The concentrations of p24 in the harvested supernatants

642   were quantified by ELISA at days 11 and 15 of culture, and the extent of inhibition by

643   each compound or combination at each time point was calculated. The percentage of

644   inhibition was normalized relative to the p24 values obtained for explants not exposed to

645   virus (0% infectivity) and for explants infected with virus in the absence of compound

646 (100% infectivity). Data are means (± standard deviations) from three independent

647 experiments performed in triplicate.

648

649

650 **Authors contribution:** The project was conceptualized and supervised by C.H., J. N and

651 R.J.S. The study was designed by C.H. as well as the data analyzed and interpreted. The

652 resistant isolates were prepared by J.G.P and J.A. Human gastrointestinal surgeries were

653 performed by P.Z. The experiments were performed by C.H., N.A and N.O.

Figure 1

Figure 1

Day 11

Day 15

Figure 2

Figure 2

Day 11

a

Day 15

b

c

d

- ● MVC gel alone
- ○ MVC gel in mixture
- ■ TMC120 gel alone
- □ TMC120 gel in mixture
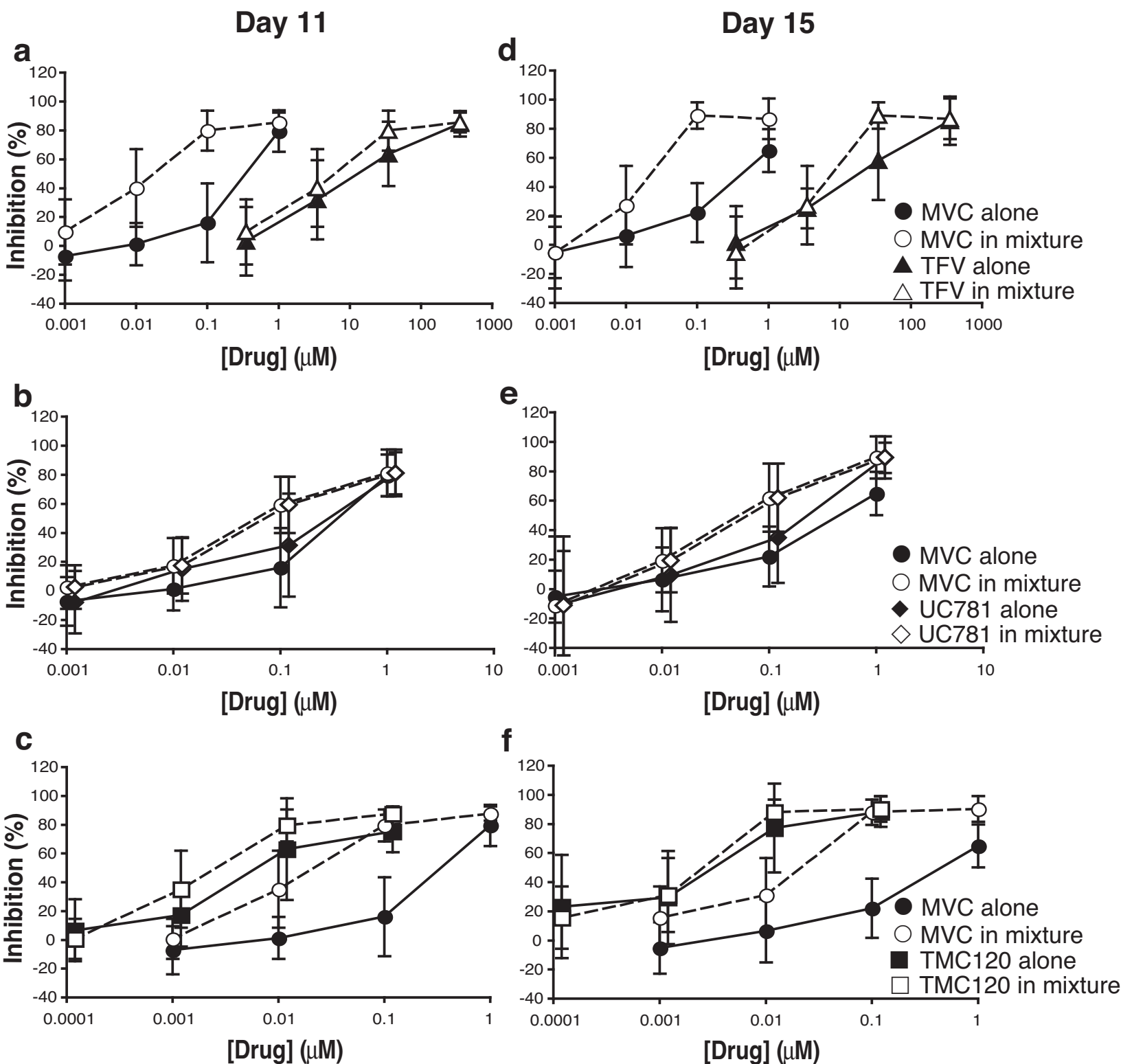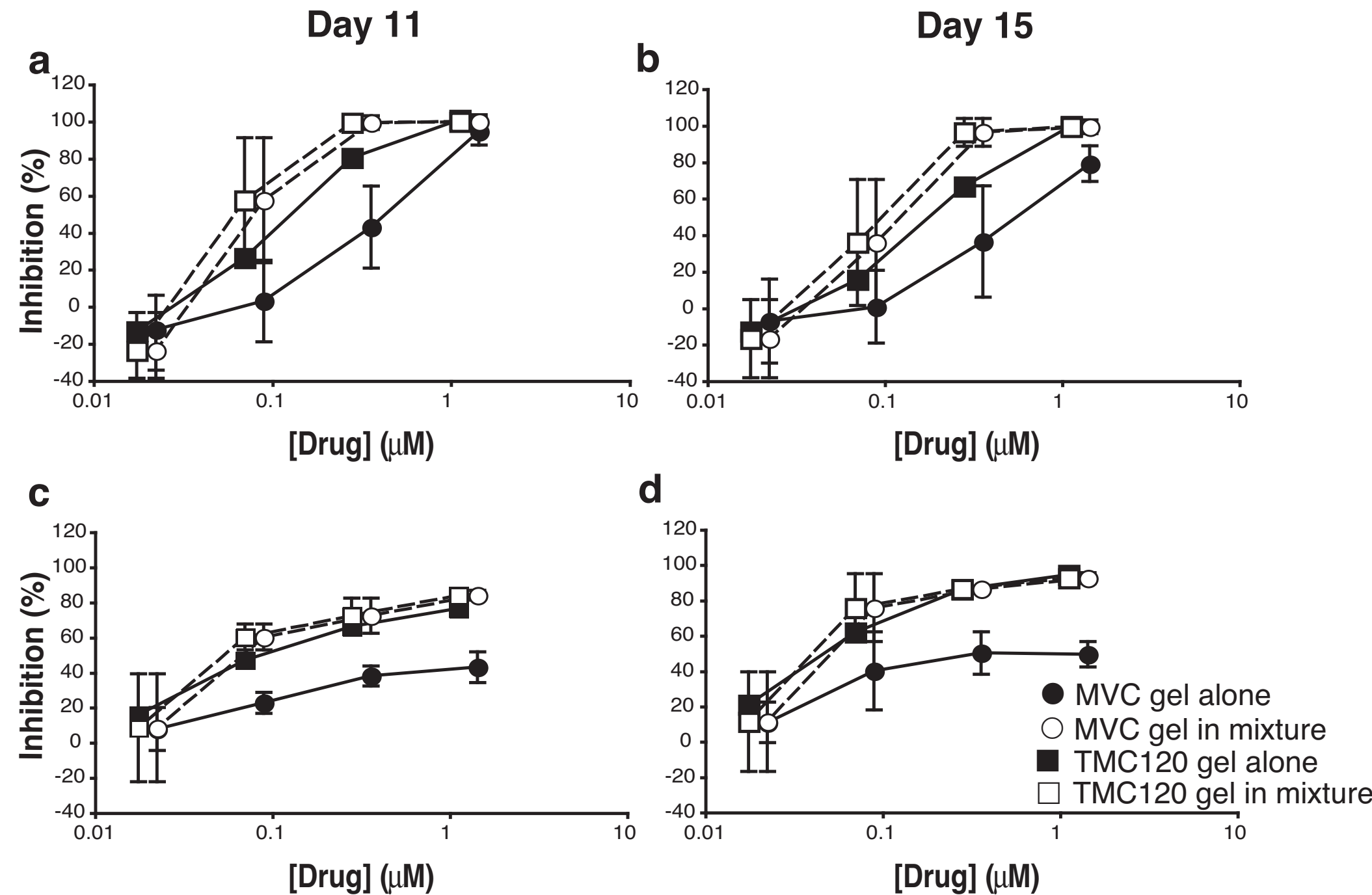
Table 1

TABLE 1. Ratios of IC$_{50}$s of drugs used in combination in TZM-bl cells

Combination and isolate		IC$_{50}$ ratio for each drug[a]					
		MVC	TFV	UC781	DPV	MVC gel	DPV gel
MVC-PMPA	BaL	3.18 ± 0.93	2.77 ± 0.88				
	YU.2	2.49 ± 0.90	4.97 ± 1.87				
	MVC-Sens	1.33 ± 0.34	17.54 ± 6.35				
	MVC-Res	> 142/1.60*	3.52 ± 1.69				
	MVC-SensV3Res	3.44 ± 5.97	5.80 ± 2.74				
	CH042	1.05 ± 0.22	9.57 ± 9.01				
	CH198	1.51 ± 0.08	15.05 ± 4.15				
	CH067	0.90 ± 0.02	33.76 ± 22.12				
	CH162	1.90 ± 0.56	5.12 ± 2.56				
	CH164	1.35 ± 0.38	14.86 ± 5.62				
MVC-UC781	BaL	2.80 ± 2.35		2.18 ± 0.90			
	YU.2	2.43 ± 0.96		4.82 ± 1.64			
	MVC-Sens	0.91 ± 0.22		7.56 ± 9.65			
	MVC-Res	> 142/2.07*		2.12 ± 2.81			
	MVC-SensV3Res	2.80 ± 4.85		3.67 ± 2.54			
	CH042	1.42 ± 0.12		65.36 ± 23.17			
	CH198	1.96 ± 0.44		80.74 ± 26.81			
	CH067	0.86 ± 0.08		9.30 ± 5.73			
	CH162	1.38 ± 0.38		1.55 ± 0.21			
	CH164	1.62 ± 0.36		8.73 ± 2.50			
MVC-DPV	BaL	2.04 ± 0.77			2.32 ± 1.34	1.50 ± 0.56	14.13 ± 6.80
	YU.2	1.92 ± 0.45			7.03 ± 1.73	2.56 ± 0.43	9.56 ± 8.05
	MVC-Sens	0.95 ± 0.25			8.83 ± 13.97	1.57 ± 1.39	7.99 ± 5.68
	MVC-Res	> 142/3.08*			0.60 ± 0.74	> 142/3.53*	2.11 ± 1.62
	MVC-SensV3Res	2.47 ± 4.27			1.89 ± 2.59	> 142/1.18*	2.49 ± 1.27
	CH042	1.47 ± 0.09			19.18 ± 2.49	0.85 ± 0.32	22.93 ± 6.61
	CH198	1.74 ± 0.55			22.40 ± 2.36	0.68 ± 0.12	30.77 ± 11.93
	CH067	1.12 ± 0.24			21.02 ± 7.25	0.84 ± 0.03	7.06 ± 2.01
	CH162	1.11 ± 0.26			2.80 ± 0.65	4.15 ± 1.03	5.27 ± 2.65
	CH164	2.31 ± 2.51			1.58 ± 1.52	2.72 ± 1.19	24.63 ± 8.66

[a] The ratio of IC$_{50}$ for each compound was calculated as: IC$_{50}$(drug A)/IC$_{50}$(drug A + drug B). The data are means ± SD derived from three independent experiments performed in triplicate.

*For MVC-resistant isolates where the IC$_{50}$ of MVC could not be calculated, the ratio is greater than the highest concentration of MVC (in nM) when titrated alone divided by the average IC$_{50}$ (nM) of MVC in combination.

Table 2

TABLE 2. IC$_{50}$s of drugs used alone and in combination in iDC – PM-1 T cells co-cultures

Treatment and exposure time		IC$_{50}$ for each drug (nM)[a]			
		MVC	TFV	UC781	DPV
Single drug	24 h Pulse	10.30 ± 9.50	241.64 ±199.62	4.75 ± 4.01	1.34 ± 1.29
	Sustained	0.14 ± 0.21	199.53 ± 3.01	3.45 ± 2.80	0.08 ± 0.06
MVC-PMPA	24 h Pulse	0.04 ± 0.03	14.37 ± 12.45		
	Sustained	0.01 ± 0.004	4.13 ± 1.55		
MVC-UC781	24 h Pulse	0.38 ± 0.30		4.57 ± 3.60	
	Sustained	0.02 ± 0.008		0.25 ± 0.10	
MVC-DPV	24 h Pulse	0.61 ± 0.78			0.30 ± 0.39
	Sustained	0.08 ± 0.06			0.04 ± 0.03

[a] The data are means ± SD derived from three independent experiments performed in triplicate.

Table 3

TABLE 3. Ratios of IC_{50}s of drugs used in combination in colorectal explants

Combination, isolate and day of culture		IC_{50} ratio for each drug[a]					
		MVC	TFV	UC781	DPV	MVC gel	DPV gel
MVC-PMPA	BaL						
	Day 11	20.37 ± 16.43	2.85 ± 1.18				
	Day 15	11.44 ± 3.13	4.70 ± 3.14				
	MVC-Res						
	Day 11	101.08 ± 121.63	4.93 ± 1.88				
	Day 15	383.01 ± 107.21	3.10 ± 0.88				
MVC-UC781	BaL						
	Day 11	7.32 ± 5.66		4.03 ± 3.52			
	Day 15	10.01 ± 8.56		4.29 ± 4.45			
	MVC-Res						
	Day 11	20.66 ± 18.99		2.23 ± 0.69			
	Day 15	31.56 ± 39.78		3.38 ± 0.93			
MVC-DPV	BaL						
	Day 11	35.72 ± 38.81			2.41 ± 1.82	5.88 ± 3.21	2.09 ± 0.58
	Day 15	27.22 ± 16.33			1.71 ± 0.47	4.28 ± 1.33	2.47 ± 1.13
	MVC-Res						
	Day 11	73.13 ± 86.68			2.73 ± 1.43	49.31 ± 66.11	1.93 ± 1.36
	Day 15	212.32 ± 290.87			1.54 ± 0.18	4.67 ± 6.28	1.29 ± 0.50

[a] The ratio of IC_{50} for each compound was calculated as: IC_{50}(drug A)/IC_{50}(drug A + drug B). The data are means ± SD derived from three independent experiments performed in triplicate.

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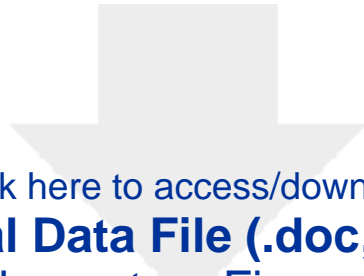
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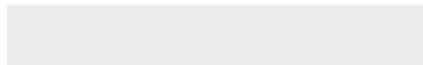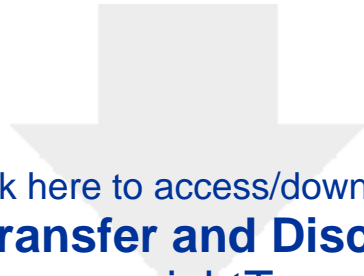
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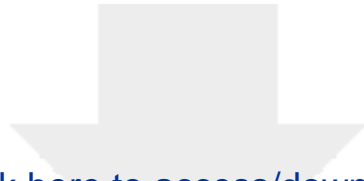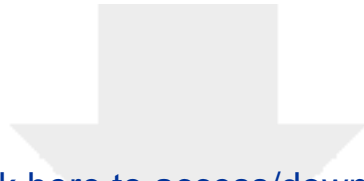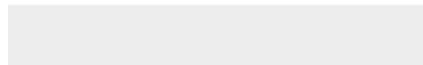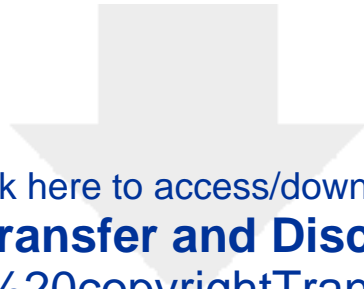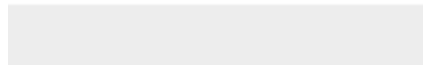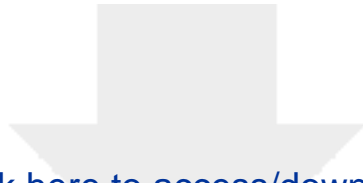Herrera copyrightTransfer.pdf
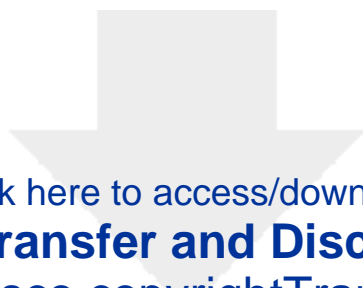
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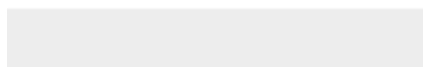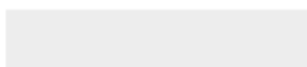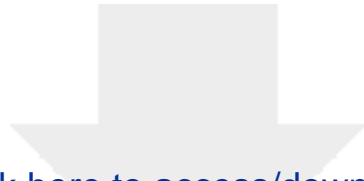
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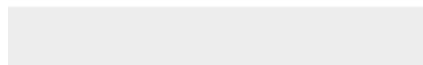
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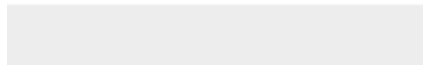**Copyright Transfer and Disclosure Form**
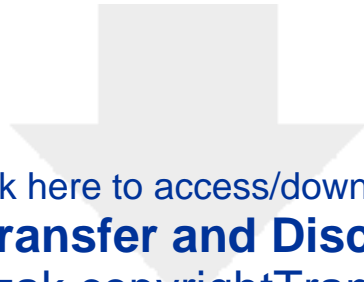Nuttall%20copyrightTransfer.pdf

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