TITLE: MIV-150 and zinc acetate combination provides potent and broad activity against HIV-1

SHORT TITLE: Anti-HIV-1 activity of the MIV-150/ZA combination

AUTHORS: Olga Mizenina[1§], Mayla Hsu[1§], Ninochka Jean-Pierre[1], Meropi Aravantinou[1], Keith Levendosky[1], Gabriela Paglini[2], Thomas M. Zydowsky[1], Melissa Robbiani[1*], José A. Fernández-Romero[1, 3*¶]

AFFILIATIONS:

[1]Center for Biomedical Research, Population Council, New York, NY, USA.

[2]Instituto de Virología J.M.Vanella-Facultad de Ciencias Médicas-Universidad Nacional de Córdoba, Argentina

[3]Science Department, Borough of Manhattan Community College, The City University of New York, New York, NY, USA

[§]equal first authors; *equal senior authors; [¶]corresponding author

KEYWORDS: HIV-1, Zinc, Antiviral, NNRTIs, Microbicides

ACKNOWLEDGMENTS:

We thank Dr. Jeffrey D. Lifson and Julian Bess at Leidos Biomedical Research, Inc. for providing the HIV-1_{BaL}, as well as Samantha Seidor and Ciby Abraham for preparation of ZA and control buffer solutions. This work was funded with the support of the United States Agency for International Development (USAID) Cooperative Agreement GPO-A-00-04-00019-00. This research is made possible by the generous support of the American people through the USAID. The contents of this manuscript are the sole responsibility of the Population Council and do not necessarily reflect the views or policies of USAID or of the U.S. government. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. None of the material in this article has been published or is under consideration elsewhere, including the internet. MR is a 2002 Elizabeth Glaser Scientist.

ABSTRACT

We previously showed that the combination of the non-nucleoside reverse transcriptase inhibitor (NNRTI) MIV-150 with zinc acetate (ZA) formulated in a carrageenan (CG; MZC) gel provided macaques significant protection against vaginal simian-human immunodeficiency virus-RT (SHIV-RT) challenge, better than either MIV-150/CG or ZA/CG. Herein, we used *in vitro* approaches to study the antiviral properties of ZA and the MIV-150/ZA combination, compared to other NNRTIs. Like other NNRTIs, MIV-150 has EC_{50} values in the sub-nM to nM range against wild type and NNRTI or RT resistant HIVs. While less potent than NNRTIs, ZA was shown to be active in primary cells against lab-adapted and primary HIV-1 isolates and HIV-1 isolates/clones with NNRTI and RT resistance mutations, with EC_{50} values between 20-110 μM. The MIV-150/ZA combination had additive antiviral effects in primary cells. *In vitro* resistance selection studies revealed that previously described NNRTI resistant mutations were selected by MIV-150. ZA-resistant virus retained susceptibility to MIV-150 (and other RTIs) and MIV-150-selected virus remained sensitive to ZA. Notably, resistant virus was not selected when cultured in the presence of both ZA and MIV-150. This underscores the potency and breadth of the MIV-150/ZA combination, supporting preclinical macaque studies and the advancement of MZC microbicides into clinical testing.

INTRODUCTION

We have tested microbicide products composed of MIV-150, a non-nucleoside reverse transcriptase inhibitor (NNRTI), and zinc acetate (ZA) formulated in carrageenan (CG). The MIV-150/ZA/CG (MZC) gel is highly efficacious at protecting rhesus macaques from vaginal (1-5) and rectal (6) SHIV-RT and mice from vaginal and rectal HSV-2 (7) and HPV pseudovirus (8) challenge. MZC gel is more protective than either MIV-150/CG or ZA/CG formulations (1-4) against SHIV-RT challenge, but the mechanistic basis for this greater potency is unclear.

Since MIV-150 is an NNRTI, it targets HIV RT with well-defined properties (9). However, it is unknown whether ZA boosts the host immune response and/or possesses antiviral properties itself to contribute to the antiviral effects *in vivo*. Biochemical assays have identified the formation of a stable, slowly polymerizing complex of zinc ions and RT (10), suggesting that ZA may also target RT.

Here, we characterized the *in vitro* antiviral activities of ZA alone and in combination with MIV-150. We demonstrated the inhibitory activity of ZA in cells and tissues, and verified the breadth of activity of ZA and MIV-150 against a panel of primary isolates and multi-drug resistant (MDR) isolates/clones. ZA-resistant HIV remained sensitive to MIV-150 and other RTIs, and MIV-150-selected virus remained sensitive to ZA. Moreover, the combination of MIV-150 and ZA had additive inhibitory effects on HIV-1 and completely shut down HIV-1 replication. Taken together, these results suggest a mechanistic explanation of the efficient antiviral activity of the MIV-150/ZA combination observed in our *in vivo* microbicide testing**.**

MATERIALS AND METHODS

Compounds and solutions. MIV-150 was provided by Drs. Bo Öberg and Disa Böttiger at Medivir, DPV was provided by International Partnership for Microbicides and the other NNRTIs (EFV, ETR, RPV and NVP) and NRTIs (3TC) were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. Sodium acetate buffer (NA) was prepared by dissolving 1.3239 g sodium acetate trihydrate (Sigma-Aldrich, St. Louis, MO), 9 g sodium chloride (Sigma-Aldrich, St. Louis, MO), and 0.5 ml 1N glacial acetic acid (Sigma-Aldrich, St. Louis, MO) in 1L of water (Calbiochem, Darmstadt, Germany). The pH was adjusted to 6.22 with 1N sodium hydroxide (BDH, West Chester, PA), and the solution was mixed for 30 min at room temperature at 250 rpm. ZA was prepared by dissolving 0.676 g zinc acetate dihydrate (Spectrum, Gardena, CA) in 220 ml of NA buffer, and pH was adjusted to 6.2 by adding 1N glacial acetic acid. Osmolality for NA and ZA solutions were 304 mOsmol/kg and 317 mOsmol/kg, respectively. Solutions were filter-sterilized through 0.45 mm syringe filters before use in cell culture-based assays.

Cells. Human PBMCs were isolated from leukopacks (NY Blood Center, New York, NY) and were activated with 3X3 stimulation method (11). PBMC stimulation media consisted in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 20 U/ml rIL-2 (Roche Diagnostics, Indianapolis, IN) and antibiotics at a final concentration of 50 U/ml of penicillin and 50 µg/ml streptomycin (Life Technologies). TZM-bl cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH and were maintained in complete DMEM (Life Technologies) supplemented with 10% FBS.

HIV strains and clones. Online Resource 1 summarizes the HIV-1 laboratory strains, primary isolates and MDR isolates/clones used in our experiments. All HIV stocks were re-titered using 3x3 activated human PBMCs (11). SHIV-RT was titered in macaque PBMCs (8) and HIV-1_{BaL} was also titered in TZM-bl cells (12). $TCID_{50}$ values were calculated using the Reed and Muench formula. Aliquots of virus stocks were stored at -80°C.

Cytotoxicity assays.

*XTT assay***.** The day before the assay, 1.5 x 10^4 TZM-bl cells/well were plated in a 96-well flat-bottom microplate and incubated at 37ºC overnight. Media was shaken off cells, and the plate was blotted dry on paper towels. Varying dilutions of ZA were made in complete DMEM media, and were added to adherent cells for 1h at 37ºC. Media/ZA solutions were shaken off and the plate was blotted dry on paper towels, before addition of 100 ml/well of fresh DMEM/10% FBS. Cells were incubated for 3d at 37ºC. In PBMCs, varying concentrations of ZA were made in stimulation media and were incubated with activated cells at 37ºC overnight. The supernatant was replaced with fresh stimulation media containing the same ZA concentrations (or fresh stimulation media only for cell controls) on days 1 and 4 after starting the assay. Cell viability was estimated on day 7. For both cell types, XTT dye reduction was carried out as previously described (13).

CyQuant assay. Cytotoxicity in activated PBMCs was performed as described above with the only difference that the CyQuant Direct Cell Proliferation Assay (Life Technologies) was performed following the manufacturer's procedure.

Antiviral assays. HIV-1_{BaL} ($1x10^3$ $TCID_{50}$) was pre-incubated with varying doses of ZA, NA, or MIV-150 for 0-9h at 37ºC, before being added to TZM-bl cells for 1h at 37ºC. Supernatant was shaken out and replaced with fresh media. Duplicate wells were set up for each condition. After incubation for 3d at 37ºC, b-gal activity was measured, and percent inhibition of infection was determined relative to the NA control. The mean inhibition for ZA was determined by subtracting values for NA at the corresponding concentrations.

In PBMCs, antiviral activity of ZA pre-incubated with virus was measured as follows: 1000 $TCID_{50}$ of HIV-1_{NL4-3} were pre-incubated at 37ºC with different ZA concentrations for 6h and added to activated PBMCs ($2x10^5$/well in U-bottom 96-well plates) before overnight incubation at 37ºC. The supernatant was replaced with fresh stimulation media on days 1 and 4 after starting the assay. For antiviral assays, dilutions of ZA, NNRTI or NRTI were prepared in stimulation media and $2x10^5$ activated PBMCs/well were incubated for 1h in U-bottom 96-well plates before adding 100 $TCID_{50}$ of HIV-1_{NL4-3} followed by an overnight incubation at 37ºC. The supernatant was replaced with fresh stimulation media on days 1 and 4 post- infection for ZA single application, NNRTIs and NRTI or fresh stimulation media containing the same ZA concentrations for ZA multiple applications. The supernatant p24 levels for all PBMCs antiviral assays were tested on day 7 after infection using the p24 ELISA (Zeptometrix, Buffalo, NY). The EC_{50} values were calculated using a dose-response-inhibition analysis on GraphPad Prism v5.0 software. All the ZA concentrations used in the cytotoxicity or antiviral assays were tested in triplicate.

Cell culture combination antiviral activity relationships. The antiviral activity of each compound (MIV-150 and ZA) or their combination was determined as described above in the PBMC assay. The assay was designed in a way that MIV-150 and ZA concentrations were combined based on their 50% effective concentration EC_{50}/EC_{50} ratios. Six different concentrations of MIV-150 (4.56, 2.28, 1.14, 0.57, 0.29, and 0.14 nM), ZA (228, 114, 57, 28.5, 14.2, and 7.1 μM) or their combination were prepared in PBMC stimulation media. The dilutions were added to $2x10^5$ activated PBMCs/well and were incubated for 1h in U-bottom 96-well plates before adding 100 $TCID_{50}$ of HIV-$1_{92BR014}$ followed by an overnight incubation at 37ºC. The supernatant was replaced with fresh stimulation media on days 1 and 4 post- infection for MIV-150 only treatment or fresh stimulation media containing the same ZA concentrations for ZA or ZA+MIV-150 treatments. The supernatant p24 levels were tested on day 7 after infection using the p24 ELISA (Zeptometrix). The percentage of virus inhibition was used to analyze the effect of the combination on the antiviral activity and to estimate the combination index (CI) values using Calcusyn for Windows software (Biosoft, Cambridge, United Kingdom) (14). All MIV-150, ZA, MIV-150+ZA concentrations or controls were tested in triplicate.

Selection for diminished ZA, MIV-150 or ZA+MIV-150 susceptibility. Activated PBMCs ($2x10^6$/ml) were pretreated with ZA, MIV-150 or their combination for 1h at 37ºC, then infected with an equal volume of ZA susceptible isolate HIV-$1_{92BR014}$ (100 $TCID_{50}$) for a final ZA and MIV-150 concentration of 90 mM and 0.5 nM respectively. With these conditions, about 80% inhibition of virus replication allows outgrowth of variants with diminished susceptibility to ZA. Cells were subcultured twice per week in the constant presence of ZA, MIV-150 or the their combination with one subculture containing fresh stimulation media and each compound or combination, and the second subculture containing fresh activated PBMCs pretreated with fresh stimulation media and each

compound or combination. Prior to each subculture, p24 was measured in the supernatants, and if elevated, the concentration of each compound or combination was gradually increased to an eventual final concentration of 180 mM for ZA and 800 nM for MIV-150 over the course of nine weeks. Culture supernatants were aliquotted and frozen at -80ºC. Additionally, MIV-150 only *in vitro* resistance development was also performed in two additional independent experiments using the same procedure described above using HIV-1_{MN} and MT-4 cells and virus replication was monitored with the TZM-bl assay instead of p24 ELISA.

RNA isolation, RT-PCR, cloning, sequencing. RNA was isolated from virus culture supernatants with the QIAamp UltraSens Virus Kit (Qiagen, Valencia, CA) and resuspended in ddH_2O. RNA was reverse-transcribed with the SuperScript III First Strand kit (Life Technologies) using random hexamer primers. PCR amplification of cDNA was carried out with Phusion Taq (New England Biolabs, Ipswich, MA) and Clade B *pol* gene primers Fwd: GGGAATTTTCTTCAGAGCAGACC and Rev: TCCCCTAGTGGGATGTGTACT, amplifying a product of 3,098 nt. PCR products were either purified with the PCR purification kit (Qiagen) and sent for sequencing, or were gel-purified (Qiagen) after separation on and excision from 0.5% agarose gels. Purified PCR products were cloned with Zero Blunt TOPO (Life Technologies). Transformed *E. coli* clones were DNA miniprepped (QIAprep Spin Miniprep). Sanger sequencing on PCR products and clones was carried out by primer extension sequencing by GENEWIZ, Inc. (South Plainfield, NJ) using Applied Biosystems BigDye version 3.1, followed by analysis on Applied Biosystem's 3730xl DNA Analyzer. DNA sequence analysis was carried out with DNASTAR Lasergene 10 software (Madison, WI).

RESULTS

We showed previously that repeated application of a ZA/CG gel protected macaques against vaginal SHIV-RT challenge. Additionally, the inclusion of MIV-150 in the gel (MZC) afforded significant protection (2, 4). We wanted to better understand the antiviral effects of the MIV-150/ZA combination in the MZC formulation. To first explore the antiviral effects of ZA that might be at work *in vivo*, we utilized TZM-bl cells as a rapid test to evaluate *in vitro* antiviral activity of ZA. Low-level time- and dose-dependent antiviral activity was demonstrated by pre-incubating HIV-1_{BaL} with ZA before the addition to TZM-bl cells (Fig. 1a). It was not possible to calculate an EC_{50} value, since inhibition did not achieve 100% using sub-cytotoxic ZA concentrations (CC_{50}=2mM). In contrast, high concentrations of MIV-150 (200 nM) readily achieved maximal inhibitory activity when pre-incubated for 0-9h. Greater activity was observed when ZA and MIV-150 were used together (Fig. 1b and c).

To verify the antiviral activity of ZA, we utilized the more biologically relevant primary cell model. Repeated application of non-toxic doses of ZA (cell viability was ~100% at the highest dose tested in the antiviral assay) inhibited HIV-1_{NL4-3} replication in PBMCs, where the EC_{50} was 62.2 μM. The appeal of the PBMC system is that it allowed us to evaluate the antiviral activity different HIV-1 clades and multi-drug resistant (MDR) HIVs (primary isolates and clones; Online Resource 1) and assess the combined activities of MIV-150 and ZA under non-toxic conditions. ZA had broad activity with EC_{50} values ranging from 20 to >228 μM, with ZA-sensitive viruses (EC_{50} values <228 μM) present among different clades and MDRs (Table 1). All but one (isolate OL-1/4(II)d4) of the ZA-

resistant viruses tested were sensitive to MIV-150 as well as other the NNRTIs Dapivirine (DPV), Etravirine (ETR) and EFV (Table 1). MIV-150 and the other NNRTIs showed potent and broad activity against many of the HIVs, except isolate OL-1/4(II)d4 and isolate W1023892-2 (Table 1). Building on the earlier observation in TZM-bl cells that combining MIV-150 and ZA resulted in increased anti-HIV activity (Fig. 1), we were able to examine MIV-150/ZA equipotential combinations (based on their individual EC_{50s}) in the PBMC assay, and determined that the MIV-150/ZA combination has additive antiviral effects (Table 2).

Development of drug resistance to single drugs is expected, but combining more than one antiviral agent could reduce the likelihood of this occurring. Although no drug-resistant virus has been detected in MZC-treated macaques that have become infected with SHIV-RT (1, 2, 4, 6, 8, 15), we set out to evaluate the development of drug resistance against the MIV-150/ZA combination under stringent *in vitro* conditions. Initial studies using MT-4 cells, HIV-1_{MN}, and MIV-150, verified the selection of MIV-150-resistant HIV (post-selection EC_{50} values >10 nM vs. pre-selection EC_{50} value of 0.2 nM, 95% CI 0.18 to 0.25) expressing the triple NNRTI mutations L100I, K103N, and Y181C or E138G, Y181C, and M230L. Not surprisingly, these triple mutations in RT also conferred resistance to the NNRTIs EFV, NVP, DPV and ETR with more than 10 fold increased EC_{50} values. Notably, both of these two viruses remained sensitive to ZA with EC_{50} values of 63.8 μM (95% CI, 35.7 to 113.9) and 38.8 μM (95% CI, 24.8 to 60.7), respectively. Selection studies were then performed in PBMCs using the HIV-$1_{92BR014}$ R5/X4 isolate with MIV-150, ZA, or the MIV-150/ZA combination. HIV-$1_{92BR014}$ has no known RTI mutations and is sensitive to both MIV-150 (EC_{50} 0.7 nM, CI 0.6 to 0.9) and ZA (EC_{50} 65.1 μM, CI 57.5 to 73.4). As in the MT-4 cells infected with HIV-1_{MN}, MIV-150 selected for virus with multiple NNRTI mutations (K103N, E138G, Y181N, and M230L) in PBMCs infected with HIV-$1_{92BR014}$, but the low titer of this virus precluded determination of the EC_{50} values. Importantly, ZA-selected HIV-$1_{92BR014}$ (ZA EC_{50} >228 μM) remained sensitive to MIV-150, as well as rilpivirine (RPV), ETR, EFV, and Lamivudine (3TC) that are used to treat HIV infection (Table 3). We explored the presence of potential mutations in *pol* gene that could be linked to ZA resistance but there was not a consistent mutation pattern to support this hypothesis. Supporting the *in vivo* observations using MZC gel (1, 2, 4, 6, 8, 15), no resistant virus could be selected in cultures treated with the MIV-150/ZA combination.

In summary, MIV-150 and ZA potentially target different sites in the HIV RT contributing to the potency of the MIV-150/ZA combination against different HIV-1 clades as well as MDR HIVs and reducing the likelihood of resistant viruses emerging. Together, these results underscore the potential of microbicide gels and intravaginal rings (IVRs) containing the MIV-150/ZA combination to limit HIV spread.

DISCUSSION

The MZC microbicide, comprised of the NNRTI MIV-150, ZA, and CG, has shown excellent safety and activity against HIV, HSV-2, and HPV in preclinical testing (1-8, 13, 15-17) and the first in-human safety testing of the MZC gel has been completed (18, 19). Additionally, we are developing sustained-release MZC-containing IVRs and recently observed that they significantly reduce SHIV-RT infection and HSV-2 shedding in macaques. Initial studies revealed that repeated treatment with a ZA/CG gel protected macaques against vaginal SHIV-RT challenge,

but that significant protection was observed when MIV-150 and ZA were used together (1-4, 7, 8). Herein we specifically investigated the *in vitro* antiviral activity of ZA and the attributes of the MIV-150/ZA combination, to further support the advancement of MZC microbicides.

NNRTIs are a class of drugs that shows promise for use in microbicides (9). Initial clinical studies on MIV-150 verified its safety after oral dosing (Bo Öberg, personal communication). MIV-150 is a particularly potent antiretroviral with subnanomolar EC_{50} values (8, 13, 16, 20), active against wild type HIVs of different clades and drug-resistant HIVs much like other NNRTIs. MIV-150 has a remarkable post washout effect in TZM-bl cells (not shown) similar to that seen in mucosal explant models for MIV-150 (21, 22) as well as other NNRTIs like UC781 (23) and DPV (24). Zinc is generally recognized as safe by the FDA and has a history as a component of topical agents such as sunscreens, diaper rash creams, and antiperspirants, with some previous investigation of its antiviral properties as a salt (25-28), ionophore (29-31) and in the context of other molecules, for example, the zinc-finger antiviral protein (ZAP) (32, 33). In addition, zinc salts have been proposed as safe, anti-HIV constituents of sexual lubricants (34). Herein, using cell lines and primary cells we demonstrated the *in vitro* antiviral activity of ZA, albeit of lower potency than the NNRTIs. Notably, the MIV-150/ZA combination was found to be additive *in vitro*, paralleling the earlier *in vivo* observations using the combined MZC (vs. MIV-150 or ZA) gel (2). Of note, we cannot rule out that immunomodulatory effects of ZA treatment also contributed to the antiviral effects observed *in vivo*. Strikingly, we demonstrated that virus was unable to grow in the continued presence of the MIV-150/ZA combination *in vitro*, suggesting that excessive selective pressure precluded virus replication.

NNRTIs represent one of the most potent molecules against HIV, but the downside of NNRTIs is the rapid development of viral resistance. While MIV-150 takes about twice as long to select HIV resistance *in vitro* compared to first and second generation NNRTIs (Bo Öberg, Medivir, personal communication), MIV-150-resistant viruses were isolated *in vitro*. MIV-150-resistant HIVs were shown to harbor three or four mutations that have been previously described in the literature for other NNRTIs (35). The triple or four mutations observed in our *in vitro* selection may confer high level cross-resistance against other NNRTIs, like EFV, ETR, and NVP (36). However, resistance selection after topical application of antiviral compounds remains as an area where more evidence needs to be accumulated. In fact, we have recently shown that while intramuscular administration of MIV-150 resulted in selection of NNRTI-associated mutations in SHIV-RT infected macaques, exposure to MIV-150 from a high-dose IVR resulted in limited emergence of NNRTI-associated mutations (20). Therefore, combining MIV-150 with other anti-HIV agents like ZA may result in even less resistance, which was supported by the lack of virus growth in MIV-150/ZA-treated cultures. This supports the *in vivo* findings where macaques that became infected with SHIV-RT in the presence of MZC microbicides did not carry NNRTI-resistant virus (1, 2, 4, 6, 8, 15). In fact, although *in vitro* selected MIV-150-resistant HIVs exhibited cross-resistance to other NNRTIs, they remained sensitive to ZA. Thus, the presence of ZA in a formulation provides a second line of attack against wild type and drug-resistant HIV.

Within the limits defined by the *in vitro* cytotoxicity of ZA, we demonstrated the antiviral activity of ZA in 3 different systems *in vitro*. One limitation of this study is that the maximum ZA concentration defined by *in vitro* cytotoxicity is lower than the *in vivo* or *ex vivo* tolerated concentration. Tissue explants and animals exposed to neat

ZA-containing gels (ZA at 14 mM) for brief periods of time are not damaged (2, 4, 7, 21, 22). Because of the lower concentrations required for *in vitro* experimentation, we were not able to fully assess the antiviral activity at high concentrations that may be more physiologically realistic, and we may have underestimated the antiviral activity of ZA. Previous studies have shown that zinc inhibits HIV replication by diminishing the reverse transcriptase catalytic activity (10). Although we explored the potential presence of mutations in the *pol* gene in the ZA-resistant HIV viruses, we were not able to identify any mutation pattern to support this hypothesis.

Importantly, *in vitro* selected ZA-resistant HIV remained susceptible to MIV-150, as well as other RTIs. We were unable to perform additional studies examining the combinations of ZA with other NNRTIs due to limiting resources. However, these data suggest that other NNRTI/ZA combinations might similarly possess increased anti-HIV activity that could lessen the emergence of drug-resistant virus.

Together these data underscore that the MIV-150/ZA combination delivers a potent one-two punch against a broad range of HIV strains potentially by targeting different sites within RT and thereby limit the potential transmission and/or emergence of drug-resistant virus. MZC-containing on-demand gels for vaginal and rectal use as well as sustained release MZC-containing IVRs (37-41) represent exciting options for women and men to simultaneously provide potent, broad-acting activity against HIV, HSV-2 and HPV.

ETHICAL STANDARDS

All the experiments published in this manuscript comply with the current laws of the country in which they were performed. All institutional and national guidelines for the care and use of laboratory animals were followed.

O. Mizenina, M. Hsu, N. Jean-Pierre, M. Aravantinou, K. Levendosky, G. Paglini, T.M. Zydowsky, M. Robbiani and J. A. Fernández-Romero declare that they have no conflict of interest.

REFERENCES

1. Hsu M, Aravantinou M, Menon R, Seidor S, Goldman D, Kenney J, et al. A combination microbicide gel protects macaques against vaginal simian human immunodeficiency virus-reverse transcriptase infection, but only partially reduces herpes simplex virus-2 infection after a single high-dose cochallenge. AIDS Res Hum Retroviruses. 2014;30(2):174-83.
2. Kenney J, Aravantinou M, Singer R, Hsu M, Rodriguez A, Kizima L, et al. An antiretroviral/zinc combination gel provides 24 hours of complete protection against vaginal SHIV infection in macaques. PLoS One. 2011;6(1):e15835.
3. Kenney J, Rodriguez A, Kizima L, Seidor S, Menon R, Jean-Pierre N, et al. A modified zinc acetate gel, a potential nonantiretroviral microbicide, is safe and effective against simian-human immunodeficiency virus and herpes simplex virus 2 infection in vivo. Antimicrob Agents Chemother. 2013;57(8):4001-9.
4. Kenney J, Singer R, Derby N, Aravantinou M, Abraham CJ, Menon R, et al. A single dose of a MIV-150/Zinc acetate gel provides 24 h of protection against vaginal simian human immunodeficiency virus reverse

transcriptase infection, with more limited protection rectally 8-24 h after gel use. AIDS Res Hum Retroviruses. 2012;28(11):1476-84.

5. Singer R, Mawson P, Derby N, Rodriguez A, Kizima L, Menon R, et al. An intravaginal ring that releases the NNRTI MIV-150 reduces SHIV transmission in macaques. Sci Transl Med. 2012;4(150):150ra23.

6. Singer R, Derby N, Rodriguez A, Kizima L, Kenney J, Aravantinou M, et al. The nonnucleoside reverse transcriptase inhibitor MIV-150 in carrageenan gel prevents rectal transmission of simian/human immunodeficiency virus infection in macaques. J Virol. 2011;85(11):5504-12.

7. Fernandez-Romero JA, Abraham CJ, Rodriguez A, Kizima L, Jean-Pierre N, Menon R, et al. Zinc acetate/carrageenan gels exhibit potent activity in vivo against high-dose herpes simplex virus 2 vaginal and rectal challenge. Antimicrob Agents Chemother. 2012;56(1):358-68.

8. Kizima L, Rodriguez A, Kenney J, Derby N, Mizenina O, Menon R, et al. A Potent Combination Microbicide that Targets SHIV-RT, HSV-2 and HPV. PLoS One. 2014;9(4):e94547.

9. D'Cruz OJ, Uckun FM. Dawn of non-nucleoside inhibitor-based anti-HIV microbicides. J Antimicrob Chemother. 2006;57(3):411-23.

10. Fenstermacher KJ, DeStefano JJ. Mechanism of HIV reverse transcriptase inhibition by zinc: formation of a highly stable enzyme-(primer-template) complex with profoundly diminished catalytic activity. J Biol Chem. 2011;286(47):40433-42.

11. Trkola A, Matthews J, Gordon C, Ketas T, Moore JP. A cell line-based neutralization assay for primary human immunodeficiency virus type 1 isolates that use either the CCR5 or the CXCR4 coreceptor. J Virol. 1999;73(11):8966-74.

12. Begay O, Jean-Pierre N, Abraham CJ, Chudolij A, Seidor S, Rodriguez A, et al. Identification of personal lubricants that can cause rectal epithelial cell damage and enhance HIV type 1 replication in vitro. AIDS Res Hum Retroviruses. 2011;27(9):1019-24.

13. Fernandez-Romero JA, Thorn M, Turville SG, Titchen K, Sudol K, Li J, et al. Carrageenan/MIV-150 (PC-815), a combination microbicide. Sex Transm Dis. 2007;34(1):9-14.

14. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev. 2006;58(3):621-81.

15. Kenney J, Aravantinou M, Ugaonkar S, Derby N, Wesenberg A, Wilk J, et al. A novel microbicide/contraceptive intravaginal ringprotects macaques against repeated SHIV-RT and HSV-2 challenge. Nature Medicine (submitted). 2014.

16. Ouattara LA, Barnable P, Mawson P, Seidor S, Zydowsky TM, Kizima L, et al. MIV-150-containing intravaginal rings protect macaque vaginal explants against SHIV-RT infection. Antimicrob Agents Chemother. 2014;58(5):2841-8.

17. Rodriguez A, Kleinbeck K, Mizenina O, Kizima L, Levendosky K, Jean-Pierre N, et al. In vitro and in vivo evaluation of two carrageenan-based formulations to prevent HPV acquisition. Antiviral Res. 2014;108:88-93.

18. Calenda G, Villegas G, Barnable P, Litterst C, Levendosky K, Gettie A, et al. MZC Gel Inhibits SHIV-RT and HSV-2 in Macaque Vaginal Mucosa and SHIV-RT in Rectal Mucosa. J Acquir Immune Defic Syndr. 2017;74(3):e67-e74.
19. Friedland BA, Hoesley CJ, Plagianos M, Hoskin E, Zhang S, Teleshova N, et al. First-in-Human Trial of MIV-150 and Zinc Acetate Coformulated in a Carrageenan Gel: Safety, Pharmacokinetics, Acceptability, Adherence, and Pharmacodynamics. J Acquir Immune Defic Syndr. 2016;73(5):489-96.
20. Hsu M, Keele BF, Aravantinou M, Krawczyk N, Seidor S, Abraham CJ, et al. Exposure to MIV-150 from a high-dose intravaginal ring results in limited emergence of drug resistance mutations in SHIV-RT infected rhesus macaques. PLoS One. 2014;9(2):e89300.
21. Barnable P, Calenda G, Bonnaire T, Menon R, Levendosky K, Gettie A, et al. MIV-150/zinc acetate gel inhibits cell-associated simian-human immunodeficiency virus reverse transcriptase infection in a macaque vaginal explant model. Antimicrob Agents Chemother. 2015;59(7):3829-37.
22. Barnable P, Calenda G, Ouattara L, Gettie A, Blanchard J, Jean-Pierre N, et al. A MIV-150/zinc acetate gel inhibits SHIV-RT infection in macaque vaginal explants. PLoS One. 2014;9(9):e108109.
23. Fletcher P, Kiselyeva Y, Wallace G, Romano J, Griffin G, Margolis L, et al. The nonnucleoside reverse transcriptase inhibitor UC-781 inhibits human immunodeficiency virus type 1 infection of human cervical tissue and dissemination by migratory cells. J Virol. 2005;79(17):11179-86.
24. Fletcher P, Harman S, Azijn H, Armanasco N, Manlow P, Perumal D, et al. Inhibition of human immunodeficiency virus type 1 infection by the candidate microbicide dapivirine, a nonnucleoside reverse transcriptase inhibitor. Antimicrob Agents Chemother. 2009;53(2):487-95.
25. Arens M, Travis S. Zinc salts inactivate clinical isolates of herpes simplex virus in vitro. J Clin Microbiol. 2000;38(5):1758-62.
26. Kumel G, Schrader S, Zentgraf H, Daus H, Brendel M. The mechanism of the antiherpetic activity of zinc sulphate. J Gen Virol. 1990;71 (Pt 12):2989-97.
27. Levinson W, Faras A, Woodson B, Jackson J, Bishop JM. Inhibition of RNA-dependent DNA polymerase of Rous sarcoma virus by thiosemicarbazones and several cations. Proc Natl Acad Sci U S A. 1973;70(1):164-8.
28. Liu CY, Kielian M. Identification of a specific region in the e1 fusion protein involved in zinc inhibition of semliki forest virus fusion. J Virol. 2012;86(7):3588-94.
29. Antoine TE, Mishra YK, Trigilio J, Tiwari V, Adelung R, Shukla D. Prophylactic, therapeutic and neutralizing effects of zinc oxide tetrapod structures against herpes simplex virus type-2 infection. Antiviral Res. 2012;96(3):363-75.
30. Krenn BM, Gaudernak E, Holzer B, Lanke K, Van Kuppeveld FJ, Seipelt J. Antiviral activity of the zinc ionophores pyrithione and hinokitiol against picornavirus infections. J Virol. 2009;83(1):58-64.
31. te Velthuis AJ, van den Worm SH, Sims AC, Baric RS, Snijder EJ, van Hemert MJ. Zn(2+) inhibits coronavirus and arterivirus RNA polymerase activity in vitro and zinc ionophores block the replication of these viruses in cell culture. PLoS Pathog. 2010;6(11):e1001176.

32. Karki S, Li MM, Schoggins JW, Tian S, Rice CM, MacDonald MR. Multiple interferon stimulated genes synergize with the zinc finger antiviral protein to mediate anti-alphavirus activity. PLoS One. 2012;7(5):e37398.
33. Wang X, Tu F, Zhu Y, Gao G. Zinc-finger antiviral protein inhibits XMRV infection. PLoS One. 2012;7(6):e39159.
34. Kelly P, inventorGenital lubricants containing zinc salts to reduce risk of HIV infection, U. Patent, Editor. USA1997.
35. de Bethune MP. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: a review of the last 20 years (1989-2009). Antiviral Res. 2010;85(1):75-90.
36. Liu TF, Shafer RW. Web resources for HIV type 1 genotypic-resistance test interpretation. Clin Infect Dis. 2006;42(11):1608-18.
37. Derby N, Zydowsky T, Robbiani M. In search of the optimal delivery method for anti-HIV microbicides: are intravaginal rings the way forward? Expert Rev Anti Infect Ther. 2013;11(1):5-8.
38. Fernandez-Romero JA, Deal C, Herold BC, Schiller J, Patton D, Zydowsky T, et al. Multipurpose prevention technologies: the future of HIV and STI protection. Trends in microbiology. 2015;23(7):429-36.
39. Fernández-Romero JA, Gil PI, Ré V, Robbiani M, Paglini G. Current status of microbicides for prevention of sexually transmitted infections and strategies for preclinical evaluation of new candidates. Rev Argent Microbiol. 2014;46(3):256-68.
40. Fernandez-Romero JA, Teleshova N, Zydowsky TM, Robbiani M. Preclinical assessments of vaginal microbicide candidate safety and efficacy. Advanced drug delivery reviews. 2015;92:27-38.
41. Ugaonkar SR, Wesenberg A, Wilk J, Seidor S, Mizenina O, Kizima L, et al. A novel intravaginal ring to prevent HIV-1, HSV-2, HPV, and unintended pregnancy. J Control Release. 2015;213:57-68.