Protection of HIV Neutralizing Aptamers against Rectal and Vaginal Nucleases

IMPLICATIONS FOR RNA-BASED THERAPEUTICS*

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Michael D. Moore†, Jonathan Cookson‡, Veronica K. Coventry‡, Brian Sproat§, Lorna Rabe*, Ross D. Cranston*, Ian McGowan‡, and William James‡

From the †Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, United Kingdom, ‡Chemconsilium GCV, Jaarmarktstraat 48, 2221 Booishot, Belgium, and the §Magee-Womens Research Institute, Pittsburgh, Pennsylvania 15213

RNA-based drugs are an emerging class of therapeutics. They have the potential to regulate proteins, chromatin, as well as bind to specific proteins of interest in the form of aptamers. These aptamers are protected from nuclease attack by chemical modifications that enhance their stability for in vivo usage. However, nucleases are ubiquitous, and as we have yet to characterize the entire human microbiome it is likely that many nucleases are yet to be identified. Any novel, unusual enzymes present in vivo might reduce the efficacy of RNA-based therapeutics, even when they are chemically modified. We have previously identified an RNA-based aptamer capable of neutralizing a broad spectrum of clinical HIV-1 isolates and are developing it as a vaginal and rectal microbicidal candidate. As a first step we addressed aptamer stability in the milieu of proteins present in these environments. Here we uncover a number of different nucleases that are able to rapidly degrade 2′-F-modified RNA. We demonstrate that the aptamer can be protected from the nuclease(s) present in the vaginal setting, without affecting its antiviral activity, by replacement of key positions with 2′-O-Me-modified nucleotides. Finally, we show that the aptamer can be protected from all nucleases present in both vaginal and rectal compartments using Zn2+ cations. In conclusion we have derived a stable, antiviral RNA-based aptamer that could form the basis of a pre-exposure microbicide or be a valuable addition to the current tenofovir-based microbicidal candidate undergoing clinical trials.

Since the discovery of protein regulation by RNA interference (RNAi), RNA, as both a target and effector molecule has been widely researched for therapeutic purposes (1, 2). To the original exogenous small interfering RNAs (siRNA), microRNA, non-coding RNA, and long non-coding RNA have been added; all of which are capable of fine regulation of their target protein(s), and thereby cellular processes (3). This has opened up the possibility of managing both genetic and acquired diseases by modifying the levels of the important dis...
nal or rectal sex (14). It is hoped that these aptamers will be capable of inactivating virions before they come into contact with their target cells within the vaginal or rectal mucosa. With the intention to use the aptamer as a vaginal and/or rectal microbicide it was essential to ensure that it could survive in these two environments prior to commencement of in vivo studies. Thus, in this article we assessed the stability of the aptamer in both vaginal and rectal lavages and we demonstrate that multiple, unusual, and potent nucleases exist at these sites that could dampen the effectiveness of this type of therapeutic. However, we also present evidence that degradation can be circumvented by empirically identified chemical modifications and through product formulation design.

EXPERIMENTAL PROCEDURES

Lavage Acquisition—Lavage fluid (PBS wash) was recovered from the rectum (10 ml) or vagina (5 ml) and clarified through a 0.2-μm filter. The recovered liquid was aliquoted and stored at −80 °C. In all cases informed consent was obtained in writing from participants for biological sample collection, and the study had ethics committee approval.

Aptamer Synthesis—The 2′-F pyrimidine-modified, ribopurine aptamer UCLA005 is a derivative of the previously published synthetic aptamer UCLA1 (8). It differs from UCLA1 in that the 5′-end carries a terminal Cy5 dye followed by three modified nucleic acid thymidines instead of the 5′-DPTγ-C6-SS-C6 moiety. UCLA005 was synthesized by Integrated DNA Technologies, BVBA, Leuven, Belgium, by solid phase β-cyanoethylphosphoramidite chemistry and purified by HPLC. The synthetic protocol has been previously published (8).

A derivative of UCLA005, called UCLA005v1, was synthesized as before except that the three LNA thymidines were replaced with three 2′-O-methyluridines. In addition, further derivatives of UCLA005v1 called v2 (2′-O-Me on A9), v3 (2′-O-Me on A10), v5 (phosphorothioate linkage between C8 and A9), v6 (phosphorothioate linkage between A9 and A10), v7 (phosphorothioate linkage between A10 and U11), v9 (2′-O-Me on A9 and A10), and v11 (2′-O-Me on A9, A10, G12, and A28) were prepared. Aptamers UCLA005 v1, v2, v3, v5, v6, v7, v9, and v11 were synthesized on small scale by ATDBio Ltd., Southampton, UK, and were purified by PAGE.

Aptamer Degradation Assay—The aptamers (2 μg) were incubated at 37 °C with 30 μl of lavage fluid in a total reaction volume of 50 μl. At various time points, from 5 min to 24 h, 5-μl samples of the reaction were removed and added to 5 μl of formamide loading buffer (10 mM EDTA, pH 8.0, 0.2% oranger G dye in formamide), heated at 95 °C for 3 min, and stored at 4 °C. A negative control, the equivalent to a 1:1 ratio of formamide loading buffer, was produced by adding 30 μl of PBS to the aptamer in a total of 50 μl and immediately adding a 1:1 ratio of formamide loading buffer, labeled Buffer in all figures). A size marker ladder of the UCLA005 aptamer was created by partial alkaline hydrolysis (incubation of 2 μg of aptamer in 50 mM NaHCO3, pH 9.2, at 95 °C for 13 min). Products of the degradation assay were separated by electrophoresis on an 18% polyacrylamide, 8 M urea gel. Bands were visualized using an Odyssey® (LI-COR) fluorescence scanner, and were quantified using the integrated intensities compensating for background using the median border method (LI-COR software).

Protein Purification—A number of lavages were buffer exchanged to remove any salt components. This was achieved using a PD-10 column equilibrated with PBS. To enhance the removal of any protein-bound cations, EDTA at a final concentration of 200 mM was added prior to the buffer exchange. The protein fraction from the PD10 column was then concentrated 10-fold through a 10-kDa MWCO Centricon filter (Millipore). This fraction was then separated according to size through a FPLC SD200 column equilibrated with PBS. Elution fractions were taken and assessed for the presence of nuclease activity in a reaction buffer (50 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 1 mM ATP, 10 mM dithiothreitol).

For both the zymogen gel and assessment of Zn2+ inhibition, the lavages were buffer exchanged through a PD-10 column equilibrated with 5 mM Tris-HCl (pH 8.0), 15 mM NaCl. The eluted proteins were then concentrated 10-fold by vacuum centrifugation, resulting in a final buffer concentration of 50 mM Tris-HCl, 150 mM NaCl.

Zymogen PAGE—A denaturing (0.5% SDS) 12% polyacrylamide gel was polymerized in the presence of 4 μg/ml of UCLA005 aptamer. Concentrated lavage proteins in Tris-buffered saline were mixed (2:1) with loading buffer (310 mM Tris-HCl, pH 6.8, 50% glycerol, 1% SDS, 0.05% bromphenol blue) prior to the proteins being separated by electrophoresis on the zymogen gel. The SDS was subsequently removed from the gel by incubation in unbuffered Triton (2.5% Triton X-100) at 30 °C for 1 h. The unbuffered Triton was removed and the gel was washed three times in dH2O. The gel was then incubated overnight at 30 °C in reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2) to renature the proteins and allow degradation of the aptamer to occur. Finally, the gel was washed three times in dH2O at 21 °C, then twice in dH2O at 4 °C. The gel was then scanned with an Odyssey (LI-COR) to detect the absence of fluorescence at nuclease protein bands.

Surface Plasmon Resonance—BIAcore surface plasmon resonance was performed to detect binding of aptamers to their gp120 target protein as previously described (15). Purified gp120 (AIDS reagent program) and an anti-CD4 antibody (QS4120, Santa Cruz Biotechnology) were immobilized onto different flow cells of a CM5 chip using amine coupling. Initially human CD4 was passed over all cells, including a negative cell, and was allowed to fully dissociate before increasing concentrations of each aptamer were added. A new CM5 chip was used for each aptamer due to the inability to fully regenerate the chip following aptamer binding to its target. Binding responses were all subtracted for the signal in the negative flow cell and then compared with the CD4 binding response as a measure of the functional protein (gp120 or anti-CD4 antibody) bound to the surface of the chip.

Infectivity Assay—TZM-bl cells (16, 17), containing a luciferase reporter gene under the control of a HIV-1 Tat responsive promoter, were plated at 1 × 104 cells per well in 96-well plates in 100 μl of complete media (DMEM, 10% FCS, 1% penicillin/streptomycin). The following day, aptamer, diluted in complete medium, was added in 50 μl and coincubated...
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with the cells for 30 min. HIV-1 pseudotyped with BaL or vesicular stomatitis virus-G envelope glycoproteins (50 μl) was added to the cell-aptamer mixture and after a 24-h co-incubation an additional 100 μl of complete medium was added. Infection by the single cycle competent NL4.3-based HIV-1 virus was quantified 48 h later using the Bright-Glo™ luciferase reagent (Promega) and measured with a luminometer.

RESULTS

Differential Activity in the Rectal and Vaginal Lavages—The chemically synthesized aptamer (UCLA005, Fig. 1A), containing a 5'-Cy5 label, was exposed to the rectal or vaginal lavages for increasing lengths of time, and analyzed by PAGE using the fluorescent dye Cy5 to visualize the RNA. Representative degradation plots for rectal and vaginal lavages, showing the successive cleavages of the aptamer, are shown in Fig. 1, B and C, respectively. The final detectable product corresponded to cleavage at either A9 or A10, which may be due to the amount of modification upstream of these two nucleotides (locked nucleic acids, 2'-O-dimethylallyl additions, and/or 2'-F), or to a size constraint of the nucleases. It is worth noting that in the absence of lavage no degradation of this 2'-F aptamer is seen at 37 °C over a 24-h time course (see Fig. 6A, Neg lane).

The two representative images are from a rectal and vaginal sample from the same individual, however, many more samples (n = 30 rectal and n = 17 vaginal) were assessed in the same way. Quantification of the residual full-length aptamer over time provided a measurement of the half-life of the aptamer in each lavage. The calculated half-lives demonstrate a large variability between samples, particularly from the rectal samples (from <1 min to >24 h, Fig. 1C), and no statistically significant difference between rectal or vaginal samples was revealed (mean 100 versus 187 min, p > 0.05, t test). Statistical analyses of the degradation half-lives were also unable to identify a consistent correlation between male and female or between rectal and vaginal samples from the same individual. The bacterial flora from the lavages, prior to filtration, was cultured in an attempt to correlate particular bacterial species to the presence of nuclease activity. Species identified included, but were not limited to Escherichia coli, Bacteroides fragilis group, Clostridium species, and Lactobacillus in the rectal samples, and Lactobacillus, Gardnerella vaginalis, and viridans Streptococcus in the vaginal samples. Although heterogeneity in the identified species was found between the lavages, no significant differences (t test, p > 0.05) from sample to sample were uncovered. The lack of correlation between the bacterial species and aptamer degradation could be due to the low number of samples analyzed, or the presence of bacterial species that could not be identified using standard culture techniques, and therefore were not detected.

Although there was no quantitative difference between the rectal and vaginal lavages, there were two major noteworthy qualitative differences between the two sample types in their substrate specificity (both seen in Fig. 1B). First, the final products of the rectal and vaginal lavages are different. The rectal lavages generally result in a product that corresponds to the aptamer cleaved between A9 and A10, whereas, the vaginal lavages result in the aptamer cleaved between A10 and U11. Also, the rectal lavages demonstrate less substrate specificity, both chemical and positional, compared with the vaginal lavages. The vaginal lavages preferentially cleave at only 4 positions, all of which are unmodified purine residues (A9, A10, G12, and A28). In contrast, the rectal lavages are able to cleave nearly all residues; most interestingly even at the 2'-F protected pyrimidines (see bands between A28 and A33).
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indicates that there may be more than one protein involved in the cleavage reactions and that the nuclease(s) involved are unusual in their target specificity.

Another notable feature of the digestion patterns was the appearance of larger, diffuse, fluorescent bands once the aptamer had been cleaved down to the A9 residue (Fig. 1B and highlighted by arrows in 1E). This feature was only associated with rectal samples (69 versus 0% for rectal and vaginal samples, respectively), although larger bands were seen with some of the vaginal samples when supplemented with additional divalent cations (Fig. 6B). These bands appear to be ligation products, however, no ATP or NAD was required for their appearance and a 3’-phosphate group was not required for their generation (data not shown). Thus, the appearance of larger, diffuse, fluorescent bands once the aptamer had been cleaved down to the A9 residue (Fig. 1A) indicates that there may be more than one protein involved in the cleavage reactions and that the nuclease(s) involved are unusual in their target specificity.

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Nuclease Inhibitors Do Not Prevent Cleavage—In an attempt to prevent the cleavage of the aptamer, the digestion reactions were performed in the presence of a broad spectrum nuclease inhibitor. SUPERase-IN™ (Ambion) inhibits RNase A, B, C, I, and T1. No reduction in degradation of UCLA005 by a rectal lavage was detected with the inhibitor, even after addition of EDTA, very high concentrations were required to have an effect, and even at the highest concentration assessed (100 mM), significant digestion was still apparent (Fig. 2B, lane 2 versus 5–7). These results suggest that either the enzyme(s) responsible do not depend on Mg²⁺, or there are very high levels of metal ions present within rectal lavages that require excessive amounts of EDTA to chelate them. The levels of EDTA sufficient to reduce the degradation of the aptamer would not be compatible with a topical microbicide due to the association of EDTA with toxic shock syndrome and its detrimental effect on the mucosa (19, 20).

Characterization of the Nucleases—To characterize the nucleases further, the proteins involved were separated by size exclusion chromatography. The lavages were treated with 200 mM EDTA to chelate divalent cations, the proteins were then separated from the electrolytes and small molecules present in the lavages by buffer exchange into PBS through a Sephadex G-25 PD-10 desalting column. The resulting proteins were separated according to size by FPLC, using a Superdex SD200 column eluted with PBS. The eluted fractions were subsequently assessed for their ability to perform nuclease digestion of the UCLA005 aptamer in a buffer containing Mg²⁺ (Fig. 3A). The two fractions that elicited the majority of the nuclease activity corresponded to between 20 and 50 kDa. These fractions (31 ± 32) were then pooled and tested for their metal ion dependence. In agreement with the inability to completely abolish cleavage using EDTA, the purified fractions were able, albeit with lower efficiency, to degrade the aptamer without the addition of any divalent cations (Fig. 3B, No Ions). However, the addition of Mg²⁺ enhanced the activity of the fraction, whereas Mn²⁺, Ca²⁺, and Zn²⁺ all appeared to prevent the small amount of cleavage detected without cations (Fig. 3B). Interestingly, the cleavage pattern without metal ions appeared to be restricted to the purine nucleotides, whereas in the presence of Mg²⁺ ions the 2’-F pyrimidines become attacked (highlighted by arrow in Fig. 3B), indicating the presence of more than one enzyme, of differential cation dependence, capable of digesting this type of nucleic acid.

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The potential for more than one enzyme to be present in the lavages was further explored using zymogen gels, which have the added benefit of providing better estimates of the molecular weights of any nucleases present. The zymogen gel technique relies on the co-polymerization of the substrate within the polyacrylamide gel and observing proteins of interest not by staining for the protein but by observing their enzymatic effect on the substrate within the gel. In our case, the UCLA005 aptamer (the substrate) was introduced into the SDS-polyacrylamide gel matrix and proteins within the concentrated lavage samples were separated on the zymogen gel by electrophoresis. Active nucleases capable of cleaving the Cy5-labeled UCLA005 aptamer appear on the gel as areas lacking fluorescence (white areas on a black background).

Two active vaginal samples (V17 and V29), one inactive rectal (R4), and three active rectal samples (R6, R27, and R29) were tested using this assay. The gel (Fig. 3C) revealed specific cleavage of the aptamer in precise bands identifying multiple (up to 5 in R6) proteins of different sizes (from 20 to 130 kDa). The heterogeneity across the different donors suggests that these nucleases are not of human origin, but instead indicate the innate person-to-person heterogeneity in gut flora. Interestingly, a few of the bands appear to be present in more than one lavage, possibly indicating the presence of the same bacteria in these participants’ microflora. Although mass spectrometry of excised zymogen gel fragments did provide data on both human and bacterial proteins, the multitude of proteins, and low abundance of the active enzyme(s), as seen by the lack of silver-stained bands corresponding to the zymogen bands (Fig. 3D), made the identification of the nucleases responsible for aptamer cleavage impossible at this time. The silver-stained gel also shows that the heterogeneity in activity across lavages was not due to a difference in overall quantity of proteins, as R6 was one of the most potent degrading lavages (half-life <1 min), and yet very little protein could be detected on the gel compared with R4, which had almost no activity (half-life >10 h).

Protection from Degradation by Chemical Modification—Given the specificities of cleavage observed in the lavages, particularly the preferential cleavage between A9 and A10 in the rectal samples and A10 and U11 for the vaginal samples, targeted chemical modifications were made in the aptamer in an attempt to prevent cleavage at these sites. Two types of modifications were tested, 2′-O-methyl (2′-O-Me) and phosphorothioates (Fig. 4A), the former replaces the reactive 2′-OH group with a non-reactive 2′-O-Me group, whereas the latter replaces a non-bridging oxygen with sulfur in the phosphodiester bond, thereby increasing resistance to most nucleases, including snake venom V1 phosphodiesterase. Initially, single nucleotide modifications were incorporated into UCLA005 to observe their effect in isolation. UCLA005 v2 and v3 contained a single 2′-O-Me group on nucleotides A9 and A10, respectively, whereas UCLA005 v5, v6, and v7 contained a single phosphorothioate linkage between C8 and A9, A9 and A10, and A10 and U11, respectively. These were all compared with UCLA005v1, which only differs from the original UCLA005 by replacement of the three locked nucleic acid thymidine residues by three 2′-O-methyluridines (see Fig. 1A).
The new aptamers were exposed to both vaginal and rectal lavages and the extent of cleavage was evaluated for the first 80 min of exposure (Fig. 4, B and C). The vaginal lavages, which have previously been shown to only target the purine residues (pyrimidines being protected by 2'-F modifications), were prevented from cleaving the 2'-O-Me-modified nucleotide and had reduced efficiency in attacking the phosphorothioate linkage, when these modifications were present at the major site of vaginal cleavage, A10 (Fig. 4B, v3 and v7). Interestingly, when one nucleotide is protected by modification, other sites can become more avidly attacked (Fig. 4B, A9 in v3 compared with A10 in v1 in the vaginal lavage). Increased band intensities for products larger than the minimal detected band in the absence of chemical modifications does not necessarily demonstrate increased avidity for these sites, but may only indicate that they are intermediates, which can more readily be detected due to the protection of the more upstream cleavage event (as only the product containing the 5'-Cy5 can be detected), e.g. G12 and A28 bands in v3 and v7 compared with v1 in the vaginal lavage. Conversely, the rectal lavages, which have already been shown to cleave the aptamer at 2'-F-modified nucleotides (see Fig. 1B), were unaffected by the chemical modifications (Fig. 4C). No protection was afforded by either 2'-O-Me (v2, v3) or phosphorothioate linkages (v5, v6, and v7), showing that the nucleases within these lavages are both potent and unusual, not requiring a hydroxyl group at the 2'-position or a normal phosphodiester bond for cleavage.

Due to the encouraging result seen with the chemical modifications in the vaginal lavages, particularly with the 2'-O-Me chemistry, we further modified the aptamer to include 2'-O-Me modifications on either both A9 and A10 (UCLA005v9) or on all four of the most susceptible cleavage sites for vaginal nucleases, namely A9, A10, G12, and A28 (UCLA005v11). These aptamers were then assessed in a vaginal lavage (Fig. 4D). Although some cleavage does occur, at sites other than those protected, it is clear that these events are less efficient. The double mutant, protected on A9 and A10, has similar stability in this lavage as the unmodified aptamer (half-life of 80 min for v1, 83 min for v9). This is due to the nucleases attacking G12 and A28 more avidly when the preferred A9 and A10 nucleotides are not available. Protection of these two additional nucleotides results in a 4-fold increase in aptamer stability (half-life of 242 min for v11).

An Ability to Bind Gp120 and Inhibit HIV Is Maintained in the Modified Aptamers—The activity of the anti-HIV-1 aptamer is reliant on specific intermolecular interactions (electrostatic, stacking, hydrophobic, etc.) between the aptamer and the target molecule, gp120. These interactions are dependent on the sequence of the aptamer and most importantly on its tertiary structure. The introduction of new chemical groups on the ribose (2'-O-Me) and swapping an oxygen atom for a sulfur atom in the phosphodiester linkage may either perturb the folding or remove essential interactions with gp120, thereby preventing high avidity binding and reducing the activity of the aptamer. To address this, the different aptamers were tested for their gp120-binding capacity and for activity against the HIV-1 BaL pseudotyped virus in a

**FIGURE 4. Protection of the aptamer through targeted chemical modifications.** A, common chemical modifications of normal RNA (middle) are shown. The 2'-O-Me (left) replaces the reactive 2'-OH by a 2'-O-methyl group, whereas the phosphorothioate linkage (S-bond, right) replaces a non-bridging oxygen atom for a sulfur atom, thus generating a phosphorothioate internucleoside linkage. B, single chemical modifications made to the major site of nuclease attack (A9 and A10) were assessed for their effects on the aptamer stability over time in either a vaginal, or C, a rectal lavage. Samples of the aptamer were taken at 5, 10, 20, 40, and 80 min. The modified aptamers included a 2'-O-Me on A9 (v2), a 2'-O-Me on A10 (v3), a phosphorothioate linkage between C8 and A9 (v5), a phosphorothioate linkage between A9 and A10 (v6), and a phosphorothioate linkage between A10 and U11 (v7). These were compared with their parental aptamer (v1), which only differs from UCLA005 by the replacement of the three locked nucleic acids with three 2'-O-Me uridines. D, multiple modifications were made to the aptamer to include 2'-O-Me modifications on A9 and A10 (v9) and on A9, A10, G12, and A28 (v11). These aptamers were then compared with the original, v1, in a vaginal lavage and the degradation observed at 5, 10, 20, 40, 80, 160, 320, and 1440 min is shown.
single-cycle luciferase reporter assay. The previously published, original minimal aptamer (UCLA1), from which the fluorescent version (UCLA005) was derived, UCLA005, and the quadruple-modified aptamer, UCLA005v11, were used at increasing concentrations in both SPR and infectivity assays. As a control for specificity of action, the aptamers were tested against an anti-CD4 antibody in the SPR assay and a vesicular stomatitis virus-G pseudotyped HIV-1 in the infectivity assay.

All three aptamers were able to bind gp120 (IIIb) immobilized to the surface of the BIACore chip (Fig. 5A), whereas no significant binding was detected on the control cell containing an anti-CD4 antibody (Fig. 5B). The response for each aptamer was compared with the saturation binding of human CD4 and normalized to the molecular mass difference between the aptamers and CD4. As with antibodies the aptamers demonstrated a very slow off-rate and, therefore, an accurate $K_D$ value is not possible to calculate. However, the nominal $K_D$ values were 634, 230, and 20 nM for UCLA1, UCLA005, and UCLA005v11, respectively.

All three aptamers also inhibited HIV-1 infection to a similar extent (IC$_{50}$: UCLA1, 202 nM; UCLA005, 380 nM; UCLA005v11, 289 nM; analysis of variance, $p > 0.05$) when the virus was pseudotyped with the BaL envelope (Fig. 5A), but had no activity against vesicular stomatitis virus-G-pseudotyped virions (Fig. 5B). These results indicate that the modifications made in UCLA005v11 were either not in residues critical for the binding to gp120 or did not affect any interactions between the aptamer and protein. Thus, the quadruple-modified aptamer, UCLA005v11, is not only stable in the vaginal environment but is also active against HIV-1. Therefore, UCLA005v11 represents a potential vaginal microbicide and is now suitable for efficacy studies in explant models.

Nucleases Are Sensitive to Inhibition by Zn$^{2+}$ Ions—Although the 2′-O-Me modifications at sites of nuclease attack increased the stability of the aptamer within the vaginal lavages, within rectal lavages the methyl groups had no effect. Thus an alternative way of increasing the stability of this type of molecule or preventing the nuclease activity will be required for the use of RNA-based therapeutics in the rectal setting. We have shown earlier that different divalent cations can have either a positive or negative effect on the overall activity of nucleases (Fig. 3B). Therefore, we set out to find an inhibitory divalent cation that could be included in a microbicide preparation as an excipient that would allow the stability of the aptamer to be enhanced sufficiently to maintain its activity over an acceptable time frame.

To this end, we performed degradation assays using the UCLA005 aptamer, with the addition of extra divalent cations, namely Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$, and Zn$^{2+}$, to the lavages. Consistently, and not surprisingly given our earlier demonstration that desalted lavages were dependent on Mg$^{2+}$ for full activity (Fig. 3B), Mg$^{2+}$ promoted cleavage of UCLA005 in the rectal lavage (Fig. 6A). In agreement with the results obtained with the desalted lavages (Fig. 3B), degradation of UCLA005 by the rectal lavage was inhibited by both Mn$^{2+}$ and Zn$^{2+}$. In contrast, Mg$^{2+}$ and Mn$^{2+}$ had no effect on the cleavage induced by the vaginal lavage (Fig. 6B). However, Ca$^{2+}$ altered the site specificity of the vaginal nucleases (highlighted by arrow in Fig. 6B). Encouragingly, Zn$^{2+}$ was also inhibitory to the nucleases present in the vaginal lavage.
To measure the efficacy of Zn$^{2+}$-mediated inhibition of degradation, a detailed time course was performed using a Tris buffer-exchanged lavage. The activity of this lavage was addressed either without metal ions or in the presence of either Mg$^{2+}$ alone, or with both Mg$^{2+}$ and Zn$^{2+}$ (Fig. 6C). Again the nuclease(s) were able to function without the addition of metal ions (aptamer half-life of 160 min), and upon addition of Mg$^{2+}$ the nuclease were more efficient (aptamer half-life of 9 min). However, with both Mg$^{2+}$ and Zn$^{2+}$ present, the half-life is extended to almost 4 h.

The cleavages that did appear in the presence of Zn$^{2+}$ ions were not those associated with lavage nucleases; hence, the effect of differing concentrations of Zn$^{2+}$ ions on the aptamer, either in the presence or absence of the lavage, was assayed. In this setting, it is clear to see that the Zn$^{2+}$ ions themselves are able to destabilize the aptamer; however, there is an optimum concentration at which Zn$^{2+}$ ions induced degradation is at a minimum and nuclease inhibition is still maintained (3 mM, Fig. 6D, compare lanes 6 and 12). Thus the addition of 3 mM Zn$^{2+}$ to an RNA-based microbicidal formulation would provide protection from the resident vaginal and rectal nucleases.

Conclusions—Since the sequencing of the human genome and the discovery that humans have fewer genes than predicted (21), the role of RNA in shaping the phenotype has been increasingly appreciated. It is no longer simply a mesomRNA that has been used to target HIV-infected cells with siRNA-based inhibitors (13). Due to the high safety rating of RNA-based molecules (23), this would provide a highly advantageous platform for a pre-exposure microbicidal product. The aptamer would bind to the incoming virus, preventing it from entering its target cell, and would be a potential additive to any microbicidal preparation, such as the tenofovir gel that recently provided the microbicidal field with its first clinical trial to result in a significant reduction in HIV acquisition (24).

In this paper we set out to study the stability of the aptamer in the presence of proteins from the vaginal and rectal mucosa. Both compartments contain human proteins as well as commensal bacteria, and their secreted products. It was, therefore, important to determine the stability of the aptamer in these settings. Previous studies, using modified RNA, have described the RNA stability, but only in the presence of human serum (25, 26). However, the complexity of the microflora within both the vagina, and the gut, necessitates a more rigorous investigation into aptamer stability in the face of a large number of potentially deleterious proteins.

We took lavages from a wide group of both male and female participants, filtered out the bacteria, and exposed our HIV-neutralizing aptamer to the resulting fluid. The results clearly show that both the rectum and vagina contain proteins capable of degrading 2'-F pyrimidine-modified RNA mole-
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cules. Although there is much quantitative heterogeneity in the activity, it is noteworthy that 30% of rectal and 6% of the vaginal lavages were capable of fully degrading the aptamer in less than 5 min.

Subsequent investigations into the cause of the degradation indicated that many proteins, of a variety of sizes, isolated from multiple different donors, were active against the aptamer. This led us to conclude that the nuclease activity was secreted from the microflora and was not of human origin. We also demonstrated that most of these proteins required Mg$^{2+}$ to function, and that importantly for therapeutic purposes we were able to inhibit the nuclease activity using Zn$^{2+}$. Finally, we modified the aptamer by including targeted 2'-O-Me purine ribonucleosides and showed partial resistance to degradation by the RNases present in the vaginal lavages but not from those in the rectal lavages, and showed that the modifications did not affect gp120 binding or HIV neutralization. Thus, using a combination of targeted modifications and/or Zn$^{2+}$, RNA-based therapeutics still have the potential to be used in hostile environments such as the vagina and rectum.

Although the nuclease activities detected were too many, and at too low abundance among the multitude of other proteins present in both the vaginal and rectal lavages to be identified by MS, we conclude that they were not standard nucleases for a number of reasons. These enzymes targeted the purine residues within the 2'-F pyrimidine-substituted RNA, something the ubiquitous RNase A is unable to achieve. Although RNase I, secreted by E. coli, is able to cleave at ribopurines it is irreversibly inactivated by SDS. The zymogen gel contains SDS and as RNase I could not be observed on this type of gel (data not shown) it was not responsible for the majority of the cleavages detected. Conversely, the lavage nucleases were detected by the zymogen gel and were therefore resistant to SDS denaturation. Finally, some of the rectal lavage nucleases were able to cleave the RNA at 2'-F protected sites and were completely unaffected when 2'-O-Me modifications or phosphorothioate linkages were introduced at the cleavage site. The ability to cleave 2'-modified RNA can be found in the unusual RNase V1, discovered in cobra venom, which utilizes H$_2$O to perform the nucleophilic attack on the phosphodiester bond, instead of the usual 2'-OH group; however, even RNase V1 is unable to cleave phosphorothioates. Although the identities of the novel nucleases present in the rectal and vaginal lavages remain unknown, the continuing microbiome project may help us uncover their origins in the future (27).

The recent trial into the application of tenofovir gel, a microbicide, showed partial protection from HIV-1 acquisition (24). This is an exciting result for both microbicide development and “at risk” populations. However, it is interesting to note that even at the high levels of drug used (>3000 times the IC$_{50}$ for HIV-1 in vitro), the inhibition of HIV-1 infection achieved was only 54% in high adherence women. To boost this effect, combination therapy is likely to be the next step. We believe that the addition of a virus entry inhibitor, such as the aptamer used in this study, along with Zn$^{2+}$, would potentially enhance the antiviral effect and improve the likelihood of the success of the microbicide.

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Protection of HIV Neutralizing Aptamers against Rectal and Vaginal Nucleases: IMPLICATIONS FOR RNA-BASED THERAPEUTICS
Michael D. Moore, Jonathan Cookson, Veronica K. Coventry, Brian Sproat, Lorna Rabe, Ross D. Cranston, Ian McGowan and William James

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