Reduction of influenza virus envelope’s fusogenicity by viral fusion inhibitors

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Abstract

During cell entry of an enveloped virus, the viral membrane must be fused with the cellular membrane. The virus envelope has a unique structure consisting of viral proteins and a virus-specific lipid composition, whereas the host membrane has its own structure with host membrane proteins. Compound 136 was previously found to bind in close proximity to the viral envelope and inhibit influenza virus entry. We showed here that 136-treated influenza virus still caused hemolysis. When liposomes were used as the target membrane for 136-treated viruses, aberrant fusion occurred, few liposomes fused per virion and glycoproteins were not distributed evenly across fusion complexes. Additionally, large fusion aggregates did not form and in some instances neck-like structures were found. Based on previous results and hemolysis, fusion inhibition by 136 occurs post scission but prior to lipid mixing.
Keywords

lipid composition, liposome, fusogenicity, influenza virus, fusion inhibitor
Introduction

Membrane fusion is involved in a large number of biological processes in which contents are brought across the membrane. Membrane fusion is highly regulated by specific proteins. In addition, lipid composition is also a determining factor for membrane fusion. For infection by enveloped viruses, the membrane fusion step during virus entry must ensure that the virus genome is uncircuitously delivered to the right location within the host cell. For instance, the entry pathway of influenza virus, a negative strand RNA virus that replicates in the nucleus, was followed by live imaging. Binding of the viral host receptor recognition protein is the first step in the entry process. The attached virus particle is internalized in an endosome and trafficked to a location near the nucleus. There are three stages in the transport process. In the first stage, the virus-bearing endosome moves on the actin filaments, followed by dynein-directed translocation to the perinuclear region in the second stage. The third stage is an intermittent movement involving microtubule-based motilities in the perinuclear region where acidification of the endosomal interior occurs. Membrane fusion takes place in a short time frame at the end of the trafficking. Along the pathway, host proteins that interact with the virus-bearing endosomes, such as Rab5/Rab7 and SNAREs, are recruited to the endosome. Rab5 regulates the functions of early endosomes and Rab7 regulates the functions of late endosomes on which the entry of influenza virus is dependent. Before fusion takes place, SNARE complexes must be assembled on the endosomes. UV-radiation resistance-associated gene (UNRAG), an autophagic tumor suppressor, has been shown to be involved in the assembly of
the SNARE complexes to promote viral fusion with the later endosomes. It is clear that the fusion of the viral envelope membrane with the endosomal membrane during entry is highly regulated by host proteins associated with the endosomal membrane. The endocytic virus can program the endosomes to recruit specific cognate SNARE proteins onto the target membrane. This membrane fusion process is not between two lipid vesicles without regulatory proteins.

Changing the membrane structure is an effective way to inhibit viral fusion. The interferon-induced transmembrane proteins (IFITMs) have been shown to be membrane associated proteins and restrict virus infection. They assert their antiviral effects by changing the properties of the cellular membrane. IFITM3 was shown to be targeted to endosomes through its N-terminal region. IFITM3 is a type II transmembrane protein with a N-terminal intramembrane domain (IM1) and a C-terminal transmembrane domain (TM2) flanking a conserved intracellular loop (CIL). Overexpression of IFITM proteins increased the lipid order, making the membrane less fluidized, which could be reversed by addition of oleic acid that generates negative spontaneous curvature. IFITM proteins also promote accumulation of cholesterol in the late endosomes. The exact step at which IFITM3 inhibits membrane fusion was recently shown to be the pore expansion by altering the cytoplasmic leaflet. The accumulated data suggest that restriction of the fluidity of the cellular membrane by IFITM proteins
is an effective mechanism to block viral membrane fusion with the endosomal membrane.

Small molecule inhibitors have been shown to inhibit membrane fusion of influenza virus. A number of inhibitor compounds can block conformational changes of influenza virus hemagglutinin (HA), which is required for HA to induce viral membrane fusion\textsuperscript{13}. However, these inhibitors are HA subtype-specific. Some inhibitors worked on H1 (e.g. RO5464466), or H1-H2 (e.g. BYM-27709, CL61917 and Stachyflin), or H3 (e.g. TBHQ, and 4c), respectively. Other fusion inhibitors directly bind in the envelope and block viral fusion with cellular membranes\textsuperscript{14, 15}. These compounds appear to change the structure of the lipid envelope.

A potent fusion inhibitor, (Z)-3-(bicyclo[2.2.1]heptan-2-yl)-5-((5-(4′-chlorophenyl)-3-(3-(piperazin-1-yl)pentyl)furan-2-yl)-methylene)-2-thioxothiazolidin-4-one, (named compound 136) was shown to block fusion of the viral envelope with the cellular membrane\textsuperscript{16}. In this study, we show that 136 reduces the fusogenicity of influenza virus envelope. 136 appears to alter the structure of the viral membrane so it could not fuse with the more rigid endosomal membrane as shown by electron microscopic images of lipid-influenza virus fusion, in contrast to IFITM proteins that restrict the fluidity of the cellular membrane to block fusion.

**Results**
3.1. Crystal structure

In previous studies, a potent fusion inhibitor, compound 136, was shown to inhibit influenza virus infection (X-31) with an EC$_{50}$ value of 50 picomolar and a selectivity index of 1x10$^6$. Because of the high potency and selectivity index of 136, further in vitro characterization was carried out. The crystal structure of a compound (7937) that represents the main 136 body indicates that compound 136 has a rigid configuration and has a similar shape as cholesterol, except for the flexible linker (Figure 1, Supplementary Figure S1, Supplemental Table S1).

3.2. Hemolysis

In the fusion model proposed by Lee, scission of the target membrane occurs prior to lipid mixing with influenza virus. Red blood cells (RBCs) have been used to study fusion of influenza virus with authentic plasma membranes. During the fusion process influenza virus breaks the RBC plasma membrane allowing hemoglobin to leak into the bulk solution. The RBCs can be spun down and the extent of hemoglobin leakage into the bulk solution can be quantitated by measuring the absorbance at 540 nm. Influenza virus treated with DMSO or the control compound 211 ((E)-endo/exo-1-(bicyclo[2.2.1]heptan-2-yl)-3-((5-(4-chlorophenyl)-3-(5-hydroxy-pentyl)-furan-2-yl)methylene)pyrrolidine-2,5-dione) caused hemolysis at a pH of 5.6 or less as anticipated (Figure 1E). Influenza virus treated with 136 also induced hemolysis, but to a slightly greater extent at pH 5.2-5.8 (Figure 1D). The exact cause for this increase is not clear. We speculate that HA molecules in 136-treated virions may be able to aggregate somewhat more to induce a slightly increased pore size. The same level of
hemolysis is achieved at pH 5.0 with DMSO, 211, and 136 treatment indicating that scission of the host cell target membrane is not inhibited. Based on a previous study\(^\text{16}\) finding 136 blocks lipid mixing with authentic cellular membranes, we conclude that 136 inhibits viral fusion post scission of the target membrane but prior to lipid mixing.

3.3. Electron microscopy

To directly visualize how 136-treated X-31 virus fused \textit{in vitro} to liposomes, negative stained electron microscopy was performed. As a control, 136-treated virus and liposomes were mixed at pH 7.5 as shown in Figure 2A. Clearly the virus is intact and appears identical to untreated, DMSO, or 211-treated virus (data not shown). Liposomes are visible as well. Same samples were also acidified to pH 5.0 to initiate fusion of virus to liposomes. Most DMSO or 211-treated virions fused with liposomes trapping unfused virions (Figure 2B) or fused extensively with many virions and liposomes (Figure 2C). The unfused viruses in these aggregates are not available to fuse with liposomes simply because of steric hindrance by the surrounding fused virus particles and liposomes. The portion of DMSO and 211-treated viruses that were not incorporated into aggregates fused to liposomes with an even distribution of virus glycoproteins in the liposome lipids. Figure 2I shows an example of evenly distributed viral glycoproteins in liposome lipids after fusion. Some virus particles treated with 136 were able to undergo fusion with liposomes, but the extent was greatly reduced (Figure 2D-G). Instead of one particle fusing with multiple liposomes in case of the wt virus particles, fusion of influenza virus treated with
usually occurred with just one liposome, and examples of large aggregates like those shown in Figure 2B and 2C were not found. In addition, these samples had an uneven distribution of viral glycoproteins on the surface of the fused virus and liposome membrane as shown in Figure 2D-G. The solid white lines indicate the regions where viral glycoproteins are present. Some 136-treated viruses were found occasionally to form a neck-like structure (white arrows) by bringing a liposomal membrane into close proximity to the viral membrane but did not appear to induce lipid mixing (Figure 2E and 2H, white arrows). Similar situations were not observed in DMSO or 211-treated samples although it is possible that such structures could be present in the large aggregates but are refractory to imaging. The neck-like structures between liposomes and 136-treated virus in Figure 2E and 2H may reflect how scission of the target membrane could occur but lipid mixing is blocked at cellular membranes.

3.4. Lipid mixing

To gain further insight of how 136 blocks fusion to cellular membranes we tested lipid mixing of DiD labeled X-31 virus with liposomes by lowering the pH and monitoring the dequenching of DiD for 20 minutes. Samples treated with DMSO or 211 dequenched similarly (Figure 3). The initial lipid mixing took place faster then what could be measured by our instrument. Samples treated with 136 dequenched at a slower initial rate, but all the samples achieve the same extent of dequenching after 20 minutes. Importantly, 136 quenched the DiD baseline fluorescence to a further extent than when DMSO or 211 was used to treat viruses. The baseline at pH 7.5 with DMSO or 211 is about 305 fluorescence...
units, and with 136 the baseline shifts to about 230 fluorescence units. The quenching of DiD at the baseline suggests that 136 binds to the virion in close proximity to DiD in the viral membrane.

3.5. Content mixing

Based on 136-treated influenza virus mixing lipids with liposomes, we decided to quantitate content mixing in the liposome system using the same virus and liposome concentrations as Figure 2. To accurately quantitate the extent of content mixing, we encapsulated the liposomes with trypsin and initiated the fusion reaction (Figure 4). If trypsin inside the liposomes was able to mix with the contents of the virus then trypsin would degrade the M1 and NP proteins. If trypsin was to leak outside of the liposomes then it would degrade HA. Because trypsin is a 23-kDa protein, a large pore connecting the lumen of the liposome to the interior of the virus must be present if M1 and NP are degraded. We found that DMSO and 211-treated viruses allowed approximately half of the M1 and NP proteins to be degraded in the sample. We believe that the aggregation caused by extensive fusion between the liposomes and virus prevented further degradation of the M1 and NP proteins. Interestingly, with 136-treated virus we saw an increase (one way ANOVA, post-hoc Tukey test, P < 0.01) in trypsin digestion of M1 and NP but not of HA as compared to DMSO and 211-treated samples. Virus treated with 136 can undergo more content mixing during fusion to liposomes due to less aggregation in comparison with DMSO and 211-treated viruses, but fusion is arrested during fusion to cellular membranes. This suggests the composition of the target membrane is a critical factor for inhibition by 136.
Aggregation trapping unfused virions and liposomes explains why 211 and DMSO treated samples exhibited less content mixing (Figure 2C).

**Discussion**

Fusion inhibition by small molecules is a promising mechanism to target with antiviral agents\textsuperscript{18}. Compound 136 was previously shown by live imaging to be a potent inhibitor that prevents fusion of influenza virus with the cellular membrane\textsuperscript{16}. Trypsin-digestion studies further revealed that the inhibitors do not destroy the viral envelope, destabilize hemagglutinin (HA), or prevent the low pH-induced conformational change of HA.

The EM images of X-31 virus with and without treatment with 136 revealed two different modes of membrane fusion with liposomes. The virus treated with DMSO or 211 was robust in fusion with liposomes. One virus particle was shown to fuse with multiple liposomes to form large aggregates (Figure 2B). On the other hand, the virus treated with 136 appeared to have lost its high fusion potential even though fusion with liposomes was not completely blocked. This observation is consistent with the result from our studies on 136-treated viruses with cellular membranes in which fusion was reduced to 20% of untreated viruses\textsuperscript{16}. The 136-treated virus could not fuse with many liposomes to form large fusion aggregates like the 211-treated virus. In cases that aberrant fusion occurred, the glycoproteins were not distributed across the whole membrane (Figure 2D - 2G). Sometimes 136-treated virus fusion could not proceed to
completion (Figure 2E and 2H). This may reflect how the fusion process was arrested when authentic cellular membranes were used as target membranes\textsuperscript{16}.

\textit{In vitro} studies of 136-treated influenza virus suggests that the structure of the viral envelope was changed by binding of 136 to the virion. The baseline fluorescence of DiD labeled virions was further reduced by treatment with 136 but not 211 or DMSO, suggesting that 136 binds in close proximity to the membrane bound DiD (Figure 3). Lipid mixing of DMSO, 136, or 211-treated DiD labeled virus with liposomes occurred to the same extent although the initial rate of lipid mixing was slower in 136-treated samples (Figure 3). This observation can be explained by the pattern of the 136-treated virus with liposomes. Since no aggregates or trapped virions were present, the lipid mixing could be initiated simultaneously between a large number of virions and liposomes. The initiation of lipid mixing occurred more slowly at the beginning due to inhibition by 136, but eventually lipid mixing reached the same extent as 211-treated virus. The content exchange between the 136-treated virus and liposomes with encapsulated trypsin was also more complete because of the same reason (Figure 4).

During enveloped virus assembly, the virus buds at the host cell membrane. For viruses like influenza virus, the viral glycoproteins and other envelope proteins are concentrated at membrane microdomains\textsuperscript{20}. In the released virus progenies, the virus envelope has a lipid composition rich in sphingolipids and cholesterol, which is very different from the cellular membrane\textsuperscript{21}. It has been shown that the cholesterol content in the membrane has various effects on the fusion kinetics of enveloped viruses\textsuperscript{22}. It is also shown that the
transmembrane domains of viral glycoproteins play an important role in the fusogenicity of the viral envelope \(^{23}\). The unique structure of the viral envelope is constructed with virus-specific lipid composition and the transmembrane domains of viral glycoproteins \(^{24}\). Fusion inhibitors like compound 136 may bind in the viral envelope, reduce fusogenicity of the virus, and block fusion post scission but prior to lipid mixing with cellular membranes.

From previous studies we found that 136 blocks lipid mixing of influenza virus with the endosomal membrane of human lung epithelial cells \(^{16}\). Additionally, when 136-treated influenza virus was fused at the plasma membrane of human lung epithelial cells analogous to the liposome assays performed in this work, lipid mixing was blocked\(^{16}\). Here we have narrowed down the step of the fusion pathway blocked by 136 to post scission of the host cell membrane and prior to lipid mixing. *In vitro* liposome fusion assays revealed that 136-treated viruses lost their high fusion potential and exhibited aberrant fusion to liposomes with limited distribution of viral glycoproteins. In some instances the 136-treated virions did not complete fusion; instead a neck-like structure between the viral membrane and the liposomal membrane was present. This may reflect how fusion is arrested by 136 at the plasma membrane and endosomal membranes of human lung epithelial cells.

**Methods**

2.1. Crystallization.
A vial in a vial technique was used to crystallize compound 7937. The inner vial contained 0.5 mL of 25 mg/mL 7937 dissolved in chloroform. The outer vial contained 4.5 mL of pentane. The outer vial was sealed then left at room temperature for one week. Large single crystals appeared in the inner vial within 1 week. Crystal were shipped to the X-ray Crystallography Center at Emory University for structure determination.

2.2. Cells and viruses.

MDCK-2 cells were cultured in EMEM supplemented with 5% FBS and penicillin/streptomycin. All influenza viruses were grown in MDCK-2 cells. Influenza virus strain X-31 (H3N2) was amplified by infecting confluent MDCK-2 cells at an MOI of 0.001. Viruses were purified on a 20-50% sucrose gradient by centrifugation for 1.75 hours at 60,000 RCF.

2.3. Preparation of liposomes and fluorescently labeled virus.

Similar as in Schmidt et al. \(^{25}\), POPC, POPE, and cholesterol were dissolved in chloroform:methanol (2:1) to make stock solutions. Liposomes composed of POPC:POPE:cholesterol (1:1:2) were made by mixing aliquots of the POPC, POPE, and cholesterol stock solutions in a glass vial. The solvent was evaporated with a stream of nitrogen leaving a thin film of lipids on the bottom of the glass vial. Residual solvent was removed by leaving the samples under high vacuum (less than 10 µm mercury) overnight. To produce 100 nm unilamellar liposomes, samples were hydrated at 2 mg/mL in 10 mM HEPES, 100 mM NaCl, pH 7.5. The hydrated liposomes were subject to 5 cycles of
freeze-and-thaw using liquid nitrogen and a 37°C water bath, then extruded through a 100 nm diameter polycarbonate filter 21 times (Avanti Polar Lipids). X-31 virus was labeled with DiD by directly adding a 5 µL aliquot of DiD Vybrant solution to 500 µL of 2 mg/mL virus sample. Labeling was performed for 2 hours at 37°C with constant shaking. Unincorporated dye was removed by centrifugation at 60,000 RCF for 30 minutes and the pellet was resuspended in 10 mM HEPES, 100 mM NaCl, pH 7.5.

2.4. Fusion assays with liposomes and fluorescently labeled virus.

X-31 virus was preincubated for 20 minutes with various concentrations of 136, 211, or DMSO only. Liposomes were added to the sample to a final concentration of 40 µg/ml X-31 virus and 1.5 mg/mL liposomes. To initiate fusion, the pH of the mixture was reduced to 5.0 using an aliquot of 10 mM HEPES, 100 mM NaCl, 50 mM Citrate, pH 3.0. Fusion progress was monitored by fluorescence measurements of DiD at $\lambda_{EX} = 644$ nm/$\lambda_{EM} = 665$nm. All fluorescence measurements were performed with continuous data collection for 20 minutes using a Cary Eclipse spectrofluorometer (Varian). The intensity of fluorescence was normalized by adding aliquots of Triton X-100 to each cuvette and recording the maximal fluorescence measurements. The formula for normalizing fluorescence measurements was $[F(t) - F(0)]/[F_{TX-100} - F(0)]$ where $F(t)$ is the fluorescence intensity at a time point, $F(0)$ is the initial fluorescence measurement, and $F_{TX-100}$ is the maximal fluorescence measurement after adding Triton X-100.
2.5. Hemolysis assay.

1 µL aliquots of inhibitor stocks were added to the wells of a 96 well plate. As a control 1 µL of DMSO only was added to wells. Next, 100 µL of X-31 virus at 10^8 pfu/mL was added to the wells and mixed. 100 µL of chicken red blood cells in DPBS was added to each well at a final concentration of 1% and incubated at 37°C for 10 minutes. The plate was subject to centrifugation at 3000 rpm for 2 minutes and the supernatant was removed. To initiate hemolysis, 250 µL of 138 mM NaCl 10 mM citrate, pH 5.0 – 6.0, was added to the appropriate wells. To establish the baseline level of hemolysis, 138 mM NaCl 10 mM HEPES, pH 7.5, was added to a well. The plate was placed in an incubator at 37°C for 10 minutes. Cells were pelleted at 3000 rpm and 200 µL of supernatant was transferred to a new 96 well plate. OD₅₄₀ was measured using a Biotek Synergy HT plate reader.

2.6. Negative stain electron microscopy.

Equal volume of 10 µg/mL X-31 virus and 250 µg/mL liposomes were mixed, acidified with an aliquot of 50 mM citrate pH 3.0, and incubated for 20 min at 37°C. Samples were reneutralized with an aliquot of 100 mM Tris pH 10.0 and incubated at 37°C for 1 hour. 7 µL of sample was applied to a glow discharged carbon coated grid for 30 seconds, blotted with filter paper, stained with 7 µL of 1% phosphotungstic acid pH 7.5 for 20 seconds, and blotted again. Samples were imaged with a FEI Tecnai 12 transmission electron microscope

2.7. Trypsin mixing assay.
Liposomes were prepared as above except 10 mg/mL trypsin was included during hydration and freeze thawing was omitted to preserve enzymatic activity. Excess trypsin that was not encapsulated into liposomes was removed by dialysis and trace amounts of trypsin were removed by passing the liposomes over a 2 mL STI-agarose conjugated column (GE healthcare) according to the manufactures instructions. Equal volumes of 10 µg/mL X-31 virus and 250 µg/mL liposomes were mixed, acidified with an aliquot of 50 mM citrate pH 3.0, and incubated for 20 min at 37°C. Samples were reneutralized with an aliquot of 100 mM Tris pH 10.0 and incubated at 37°C for 1 hour. The reaction was terminated by addition of 2 mM AEBSF for 20 minutes at 37°C. The samples were electrophoresed on a 10% nonreducing polyacrylamide gel. Gels were stained with SYPRO ruby, and imaged with a ccd based gel imager (Syngene G:box). ImageJ was used for quantitation of protein bands.

**Abbreviations**

AEBSF, 4-(2-Aminoethyl)benzenesulfonfyl fluoride;

CIL, conserved intracellular loop;

DiD, 1,10-dioctadecyl-3,3,30,30-tetramethylindodicarbocyanine perchlorate;

HA, hemagglutinin;

IFITM, interferon-induced transmembrane protein;

IM1, intramembrane domain 1;

M1, matrix protein;
MDCK, Madin-Darby canine kidney;

NP, nucleoprotein;

POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine;

POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine;

RBC, red blood cell;

SNARE, Soluble N-ethylmaleimide-sensitive factor activating protein receptor;

STI, soybean trypsin inhibitor;

TM, transmembrane;

UNRAG, UV-radiation resistance-associated gene.
Author Information

All authors contributed to experiments. MR, YY, GXW and ML wrote the publication.

Acknowledgements

The work is supported in part by NIH grants [AI-080699] and [U54-AI-057157], and a grant from Shenzhen Municipal Government [ZDSY20130331145112855] to ML.

Supplementary Information

X-ray crystallographic information for compound 7937 is provided in the supplementary information section. This information is available free of charge via the Internet at http://pubs.acs.org/.

References


Figure Captions

Figure 1. Structure of compounds 136 and 211, and the crystal structure of a related compound, and hemolysis analysis. (A) and (B) Line drawings of the chemical structure of compounds 211 and 136, respectively. (C) Crystal structure of compound 7937, a compound analogous to 136. Due to the flexible aliphatic chain of 136 it was not successfully crystallized. (D) Hemolysis assays were conducted with 136 or (E) 211-treated viruses. Because hemolysis requires concentrated virus, 0.2 µM is the EC50, 0.6 µM is the EC90, and 1.2 µM is the EC99 for 136. 136 treatment at pH 5.8 caused significantly different hemolysis (P < 0.01) between all samples. Treatment with 136 caused significantly more hemolysis at pH 5.2 to 5.6 (one way ANOVA, post-hoc Tukey test, P < 0.05) as compared to DMSO. Hemolysis was not affected by treatment with 211. At pH 5.0 hemolysis is equivalent in all samples. Representative data shown of 2 independent experiments. Data points are the average of 2 replicates ± SD.

Figure 2. Fusion of 136-treated virus with liposomes results in less aggregation and uneven glycoprotein distribution.

136-treated virions and liposomes at pH 7.5 (A). All other samples had the same concentrations of virions and liposomes, and were acidified to pH 5.0. (B) Most 211-treated virions fused with liposomes trapping unfused virions or (C) fused extensively with many virions and liposomes. 136-treated virions fused to liposomes but uneven glycoprotein distribution was observed. (D-G, I) White
lines indicate continuous glycoproteins stretches on the surface of fused virions and liposomes. (E, H) Some 136-treated virions form a neck-like structure that was not observed in 211-treated samples. The white arrows point to the neck structures. (I) Complete fusion with 211-treated virions and liposomes show even glycoprotein mixing. Scale bar: 100 nm.

**Figure 3. 136 quenches DiD baseline fluorescence and affects the lipid mixing rate.**

DiD labeled X-31 virus was fused to liposomes by lowering the pH. The reaction progress was monitored as an increase in fluorescence intensity due to dequenching of DiD as it mixes with liposome lipids. As a negative control dequenching at pH 7.5 was measured. The baseline fluorescence at pH 7.5 is the same for DMSO and 211-treated samples but the fluorescence signal is significantly quenched by 136. The initial rate of dequenching was slowed by 136 treatment of virions. Final dequenching was nearly identical for all pH 5.0 samples. A 5 point moving average was applied to all samples to smooth the data.

**Figure 4. 136-treated virions mix contents with liposomes.** Inhibitor treated X-31 virus was fused to liposomes containing trypsin or empty liposomes as a control. The level of each protein was normalized to the control sample of X-31 virus and liposomes without trypsin (DMSO no tryp). DMSO and 125 nM 211-treated virions both showed that approximately 50% of NP and M1 were
degraded by trypsin. 125 nM 136-treated virions (125 nM was used to make sure 99% virions were treated by 136) showed apparently more degradation of NP and M1 by trypsin (one way ANOVA, post-hoc Tukey test, P < 0.01). When Triton X-100 was added to 136-treated samples complete degradation of all proteins occurred (TX).