New Class of Broad-Spectrum Antivirals Improves Influenza Virus Vaccine Development

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Abstract

Enveloped viruses can cause devastating zoonotic diseases and are the most likely to cause global pandemics. We identified a new class of small-molecule sulfur-containing antiviral compounds (XM series) that broadly inhibit enveloped viruses. The antivirals' mechanism of action was explored via various multidisciplinary approaches, concluding that the XM antivirals alter membrane lipid chemical compositions, increase membrane order deep within the hydrophobic region of the bilayer, and increase membrane phase transition temperatures. Such effects cause inhibition of membrane fusion and viral entry, while leaving the viral glycoproteins and genomes largely unaffected. Consequently, we tested whether these features would lead to effective whole inactivated enveloped virus vaccines. As a proof-of-principle, we generated an inactivated influenza virus (IIV) vaccine using compound XM-01 (XM-01-IIV). We compared this new vaccine to traditional paraformaldehyde-inactivated, to control compound-inactivated, and to live virus vaccines, using a mouse model of disease. Excitingly, compared to a traditional IIV vaccine, XM-01-IIV vaccination improved neutralizing antibody responses against both hemagglutinin and neuraminidase, and decreased mouse morbidity and mortality following influenza virus challenge. Therefore, this study uncovers a novel class of broadly acting antivirals that enhances influenza virus vaccine development and offers great potential for broadly generating future highly-potent inactivated enveloped virus vaccines.

Introduction

The 2020 World Health Organization's (WHO) list of emerging pathogens most likely to cause major pandemics and require urgent research and development is comprised entirely of enveloped viruses, including Ebola, SARS-CoV-2, and Nipah (NiV) viruses [1]. Annually, enveloped viruses are responsible for millions of human deaths and trillions of dollars of economic cost [2-4]. While established vaccines reduce the human and economic cost of viral outbreaks, new vaccine candidates against emerging pathogens do not always generate adequate protection from illness [5]. Therefore, the ability to rapidly generate effective vaccines for emerging or established viruses is imperative.

Whole inactivated virus (WIV) vaccines have advantages over live attenuated virus (LAV) vaccines, including that WIV are replication-incompetent and thus typically safer, while still offering a broad repertoire of antigens presented in the context of viral particles as compared to sub-unit or genetic vaccines [6, 7]. However, WIV vaccines often induce insufficient immunity or even vaccine-enhanced disease often due to antigen conformational differences caused by viral inactivation [7] via chemical (typically formalin or β -propiolactone [BPL]) or physical (heat, UV light) methods. Attempts to generate new viral inactivation methods for WIV vaccines face many pitfalls, including the degradation of protein conformations and genetic components [8-16]. Thus, developing new methods to fully inactivate viruses while leaving their glycoproteins in their native and immunogenic conformations is highly desirable.

Current WIV vaccines for influenza viruses (IV), responsible yearly for 350,000-650,000 human deaths and up to a billion human infections worldwide, have rather low efficacies between 10% to 60%. [17, 18]. This

is partially due to a lack of heterologous immunity and abrogated immunogenicity due to the WIV preparation process [13, 16, 19], despite a vaccine including 3-4 IV strains. WIV vaccination shortcomings often point to the chemical methods of virus inactivation. For example, the properties that make formalin-inactivated vaccines ideal to inactivate a wide range of viruses, namely non-specific cross-linkers that react with amino acids, RNA, and/or DNA, can also cause destruction, alteration, or sub-optimal presentation of antigens to the immune system [11, 15, 16]. Indeed, in early clinical trials, formalin-based WIV vaccination of respiratory syncytial virus (RSV) resulted in enhancement of disease due to impaired glycoprotein structure [20, 21]. Therefore, maintaining the structure of viral glycoproteins is key to WIV vaccine development.

In the last decade, several potential broad-spectrum antivirals were identified which act specifically on the viral membrane, often via light activation, including LJ001, JL122, and the hydrogen sulfide (H₂S)-producing compound GYY4137. These compounds display inhibitory properties against multiple enveloped viruses including RSV, human metapneumovirus (hMPV), NiV, and others [22-24]. Targeting the viral membrane is an attractive option in preventing viral entry/infection; however, the abundance of host cellular membranes may limit the use of such compounds as therapeutics.

In this study, we identified a new class of antiviral compounds (XM series) that target the viral membrane while maintaining the integrity of viral surface glycoproteins, characteristics ideal for vaccine development. A multi-disciplinary characterization of the biological activity of XM compounds indicated that these light-independent persulfide-radical producing agents embed into viral membranes, alter their biophysical and chemical properties, and inhibit viral-cell membrane fusion, viral entry, and productive infection. To test whether these characteristics of XM compounds made them suitable for WIV vaccine development, we optimized the inactivation of a mouse-adapted influenza virus strain, and corroborated that XM treatment completely inactivated the virions without affecting viral glycoprotein functions. Further, mice vaccinated with XM-IV developed more highly neutralizing antibody responses to both hemagglutinin (HA) and neuraminidase (NA) glycoproteins, and upon viral challenge yielded reduced morbidity and mortality compared to other IIV approaches. Finally, we demonstrated that inflammation and immune responses in XM-WIV vaccinated mice at 5 days post challenge were comparable or improved relative to other vaccine groups. Thus, the new lipid-membrane targeting XM class of compounds constitute a breakthrough for the development of enveloped virus whole inactivated vaccines.

Results

XM compounds inhibit enveloped virus infections. We screened for antiviral activity a library of sulfur-containing compounds in comparison to known hydrogen sulfide donors, NaHS and GYY4137 [25, 26], and to a light-dependent inhibitor of membrane fusion and viral entry, LJ001 (Figs. 1A, S1) [23, 27]. These compounds were first evaluated using a pseudotyped Nipah virus (NiV)/vesicular stomatitis virus (VSV)-based *Renilla* luciferase reporter infection system (pNiV) [28-30], in which the NiV attachment (G) and

fusion (F) glycoproteins replace the VSV surface glycoprotein G, and permit a single round of viral entry/infection. Pretreatment of pNiV with compounds at 10 μ M for 30 min prior to infection of Vero cells showed a significant decrease in virion infectivity for five XM compounds (Fig. 1A). Interestingly, all five active compounds (XM-01, -02, -03, -06, and -12) contain acyl disulfide moieties, potentially enabling persulfide radical formation (Fig. S1).

We then evaluated the cytotoxicity levels of these five inhibitory XM compounds in Vero cells using a CCK-8 cytotoxicity kit at 1, 3 and 10 μ M for 30 min to 24 h (Fig. 1B). Based on these results, we proceeded with XM-01 for further characterization. XM-01 cytotoxicity was then tested from 1 μ M - 1 mM in Vero cells (Fig. 1C) and 1-100 μ M in MDCK cells (Fig. S3) for 24 h, showing that XM-01 did not significantly affect cell viability at concentrations < 300 μ M. Further, XM-01 inhibited the enveloped pNiV virus, human herpes virus 1 (HSV-1), respiratory syncytial virus (RSV), human cytomegalovirus (HCMV), vesicular stomatitis virus (VSV), or influenza A/California/04/2009 (Ca09) virus (Fig. 1D) with IC_{50} s between 1 - 40 μ M, yielding selectivity indexes (CC_{50}/IC_{50}) between 1:25 and 1:1,000 depending on the virus. In contrast, entry of the non-enveloped rotavirus (Fig. 1D) or norovirus (data not shown) was not inhibited. Collectively, our results indicate that XM-01 broadly inhibits enveloped viruses regardless of viral genome type or viral family, suggesting that viral membranes may be the primary target of XM-01.

XM-01 perturbs viral membranes without affecting viral glycoproteins or RNA. To determine if viral infection is inhibited by XM-01 at viral entry or post-viral entry steps, we incubated Vero cells with XM-01 at different time points post- viral infection. Cells were infected with pNiV for 2, 4, 6, 8, 18, or 24 h, after which unbound pNiV was washed away from the cells, and 100 μ M XM-01 in media was added for the remainder of 24 h at 37 °C. pNiV infection was then measured via luciferase activity (Fig. 2A). No inhibition by XM-01 was observed compared to the DMSO vehicle control at any time point, indicating that once the virus has entered cells, XM-01 does not exert inhibitory activity, consistent with XM-01 inhibiting viral entry. We next incubated either Vero cells or pNiV for 30 min with XM-01 at various concentrations prior to viral infection (Fig. 2B). Viral infection assessed 24 hours post infection (h.p.i.) was decreased only when pNiV virions, but not when cells, was pre-incubated with XM-01, suggesting a direct effect of XM-01 on virions rather than on host cells.

Further, to narrow down whether viral membranes or viral glycoproteins were affected by XM-01, we tested whether XM-01 affected the conformations of NiV-F or -G. pNiV virions were incubated with 10 μ M XM-01 for 30 min, followed by measurement of binding of established conformational antibodies to NiV F or G via flow virometry, to determine potential structural changes in F or G [29, 31-33]. As a control we used LJ001, a compound known to affect viral membranes but not viral glycoprotein conformations [23, 27]. In both cases, no statistically significant changes were observed in conformational antibody binding to either glycoprotein (Fig. 2C), indicating that native glycoprotein conformations remained upon XM-01 treatment. To further test for potential effects in protein function, we used influenza virus (IV) Ca09. When IV Ca09 was treated with up to 1,000 mM XM-01 for 4 h, neither

neuraminidase (NA) nor hemagglutinin (HA) activity were significantly impacted as compared to the untreated control (Fig. 2D-E). These were the same conditions confirmed to yield fully-inactivated influenza virus (Fig. 2D), and used for vaccine development as further described below for Figs. 4, 5, and S6.

Further, to visually look for gross viral structural changes, we treated pNiV with 10 μ M XM-01 or LJ001 control compound for 30 min, then imaged particles via electron microscopy. Most viral particles treated with XM-01 or the LJ001 control displayed compromised membranes, altered morphologies, and frequent RNA spillage compared to the DMSO control, consistent with XM-01 and LJ001 affecting viral membranes (Fig. 2F) [34, 35]. Then to test the effect of XM-01 on viral RNA function, we *in vitro* transcribed CHIK reporter virus, which contains luciferase in the hypervariable domain of non-structural protein 3 (nsP3). RNA was treated with different compounds including XM-01 and decapping enzyme, and then electroporated in BHK cells. After 2 h, enough time for the CHIK reporter to initiate translation of non-structural protein and the luciferase to be produced, cells were lysed and luciferase activity measured. No effects of XM-01 were observed for XM-01 on viral RNA (Fig. 2G). Combined, these data indicate that XM-01 modulates viral entry by affecting viral membranes and not the viral glycoproteins or RNA.

XM-01 inactivates viral membrane physical properties. We then investigated whether XM-01 affected the membrane's physical properties and whether XM-01's mechanism of action may involve radical formation, similarly to LJ001/JL122 [34, 35]. *We first performed electron spin resonance on large multilamellar vesicles treated with XM-01 using lipids with spin labels at various depths within the membrane. We detected spectral changes upon XM-01 binding to the spin-labeled lipid 1-palmitoyl-2-(16-doxyl stearoyl) phosphatidylcholine (16-PC) (Fig. 3A). At 25 °C, a shift of the high field peak upon XM-01 binding towards a lower frequency indicated that 16-PC was in a more hydrophobic environment (Fig. 3A left). An equivalent comparison at 20 °C (Fig. 3A right, arrow) showed a second component emerging upon XM-01 binding, indicating that XM-01 intercalates deep into the hydrophobic region of the membrane [36-43].*

To further understand the effect of XM-01 on membrane structure, we measured lipid order (ΔS_0). The ΔS_0 of liposomes with and without XM-01 binding was calculated for five XM-01:lipid Mol:Mol ratios, with a greater S_0 indicating a higher ordered lipid alignment. The lipid order in the headgroup region of dipalmitoyl phosphatidyl tempo (2,2,6,6-tetramethyl-1-oxy) choline (DPPTC) was unchanged upon XM-01 binding (Fig. 3B), but increased in the 16-PC region. We also observed that XM-01 increased the phase transition temperature of both pure POPC (~ 5.3 °C) and POPC/POPG (4:1) (~ 5.8 °C) membranes, consistent with XM-01 turning the liquid-ordered phase into a relatively more gel-ordered phase (Fig. 3C). Altogether, these data indicate that XM-01 intercalates deep in the hydrophobic region of the lipid bilayer, induces membrane ordering, and increases the phase transition temperature of the membrane, all consistent with a decrease in the capacity for membrane fusion.

Since the activity of control compound LJ001 involves light-dependent radical formation, we then investigated whether the activity of XM-01 would also be light-dependent, and possibly induce radical formation. Whereas compound LJ001 was not significantly active in the dark, XM-01 was (Fig. S2). Further, we next examined the chemical properties of XM-01. In the case of other H₂S donors, acyl groups on sulfur are transferred to nucleophiles and ultimately generate persulfides, which can be oxidized into perthiyl radicals [44]. We thus analyzed XM-01 and its reaction with butylamine in CH₂Cl₂, and recovered cysteine polysulfides, the decomposition products of persulfides (Fig. 3D and 3E). The formation of these products indicates the presence of a persulfide intermediate (RSSH) derived from XM-01. In cellular environments, the generation of persulfides is known to produce radicals [45], which suggests that XM-01 and its perthiyl radical products may induce chemical changes to the viral membrane by a radical-forming mechanism.

Since XM-01 intercalates within the lipid bilayers and is able to form persulfide radicals, we hypothesized that XM-01 may cause viral membrane lipid chemical changes. To test this, we performed lipidomic analyses on fully inactivated IV Ca09, using 1 mM XM-01, 0.02% paraformaldehyde (PFA), or 10% DMSO as a negative control, all treated for 4 h at room temperature (RT). The extracted membrane lipids were analyzed via liquid chromatography and tandem mass spectrometry (LC-MS/MS), and data analysis showed several classes of lipids to be markedly affected by XM-01 treatment (Fig. 3F-H), including changes in ratios of key lipids identified as crucial for initial steps in membrane fusion. These data suggest that XM-01 acts directly upon membrane lipids to change their relative distribution/presence, both at the lipid class and individual lipid structure levels (e.g., acyl chain length and degree of unsaturation). Strikingly, the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) lipids, required to be ~0.9-2:1 PC:PE for efficient membrane fusion (stalk formation), changed drastically in XM-01 treated membranes (Fig. 3G-H) [46]. The PC:PE ratio in XM-01 treated virus increased to 10.97:1 PC:PE, as the concentration of PC slightly increased and concentrations of PE fell sharply. This may be due to a significant portion of PE species in mammalian membranes being plasmalogens, which alleviate oxidative stress via free radical scavenging [47]. Overall, these changes are consistent with a decreasing amount of intrinsic negative curvature upon XM-01 treatment, making fusion stalk formation and thus membrane fusion energetically unfavorable.

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Vaccination with XM-01-inactivated virus improves neutralizing antibody responses and protection against live IV challenge. As a proof of principle, we sought to exploit the unique properties of XM-01 described in this study, namely being a membrane fusion antiviral that leaves viral glycoproteins largely unaffected, to produce an improved inactivated influenza virus (IIV) vaccine. We optimized complete inactivation of IV Ca09 with XM-01, JL-122 [23], and 0.02% PFA treatment (a traditional viral inactivation process). Treating 1x10⁵ PFU/mL Ca09 with 1 mM XM-01, 0.02% PFA, or 10 μM JL122 for 4h at RT completely inactivated Ca09, while DMSO vehicle treatment did not significantly inhibit virus viability. This was confirmed via plaque assays and 3 supernatant passages of treated virus in cell culture and in

chicken eggs (Fig. 2D, S5). Importantly, such XM-01 inactivation conditions preserved HA and NA functions (Figs. 2D-E).

We then vaccinated groups of 10 mice (5 males + 5 females) intramuscularly (IM) with XM-01-IIV, JL-122-IIV, PFA-IIV, live IV (Ca09), or phosphate buffer saline (PBS) as a mock control group, all mixed 1:1 with alum. Although live IV IM vaccination is not approved for human use, while intranasal (IN) IV challenge is lethal in mice, IM vaccination using live virus in mice does not lead to pathology/infection, and is a benchmark for ideal IV vaccines to achieve in mouse models, providing a robust and protective immune response [48]. Mice received 2 vaccinations, 2-weeks apart, and serum was collected 7-10 days post-vaccination to assess neutralizing antibodies (NAbs) using hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays (Fig. 4A-C) [49, 50]. Excitingly, the XM-01-IIV vaccinated mice generated statistically higher NAb titers against HA and NA compared to the PFA-IIV or JL-122-IIV vaccinated mice, and notably performed nearly as well (for NA NAb, Fig. 4B) or as well (for HA NAb, Fig. 4C) as the live-virus IM vaccinated mice (Fig. 4B-C). These results are particularly exciting, as most conventional IIV vaccines do not induce strong anti-NA NAb responses, [51] and NAb for the XM-01 treatment were statistically significantly improved over the PFA group at the higher Ab concentrations (Fig. 4B). Notably, in female mice after two vaccinations, XM-01-IIV NAb against both HA and NA were even superior to those generated by the live-virus vaccine (Fig S6A), and after a third dose the NAb for the live IM vaccine reached the levels of the XM-01 vaccination NAb (Fig. S6).

After these encouraging results, all mice were then challenged intranasally at 5 LD₅₀ (1,000 PFU/mouse) with IV Ca09. 100% of mice vaccinated with the XM-01-IIV or live-virus vaccines survived the challenge, whereas only 80% of PFA-IIV vaccinated mice survived, and all mock vaccinated mice succumbed to the infection within 6 days post-challenge (dpc) (Fig. 4D). Weight loss is a key predictor of IV morbidity in mice [52]. Importantly, mice vaccinated with XM-01-IIV and live-virus control suffered significantly lower morbidity, as shown by a lower amount of weight loss compared to all other IIV-vaccinated or control groups (Fig. 4E).

In a complementary experiment with fewer mice (female only) animals were euthanized at 5 dpc for histological analysis of various tissues, and flow cytometric analyses of immune cells (Fig. 5). Histological examination revealed peribronchiolar and perivascular inflammation with lymphocytes and granulocytes in all mice (Fig. 5A-J). Mice vaccinated with mock or JL-122-IIV vaccines developed more severe peribronchiolar and perivascular inflammation with degenerate neutrophils and cellular debris in airways (Fig. 5F, H). Mice vaccinated with PFA-IIV, XM-01-IIV, and live-virus had less inflammation and no signs of intraluminal degenerate neutrophils or debris in airways (Fig 5G, I, J). Flow cytometric analysis of immune cells corroborated the observation of reduced lung inflammation in the PFA-IIV, XM-01-IIV, and live-vaccinated groups when compared to JL-122-IIV and saline-vaccinated groups, with fewer lung neutrophils and CD8⁺ T cells (Fig. 5K-L). Additionally, the proportion of germinal center B cells was increased in the mediastinal lymph nodes of PFA-IIV, XM-01-IIV, and live-vaccinated groups compared to mock-vaccinated control (Fig. 5M, N), indicating a positive and specific response to vaccination.

Overall, viral inactivation using XM-01 generated a more effective IIV vaccine compared to traditional PFA treatment, by eliciting a relatively more protective neutralizing antibody immune response, correlating with reduced morbidity and mortality upon viral challenge.

Discussion

We report a new class of broad-spectrum antivirals with broad inhibitory properties against enveloped viruses. Compounds XM-01, 02, 03, 06, and 12 possess antiviral activity and display low levels of cytotoxicity. A series of mechanistic analyses demonstrated that XM-01 affects the virus membrane lipid bilayer chemically and physically, inhibiting viral entry, without significantly affecting the viral glycoproteins. Given these results, we explored the potential of XM-01 for improved WIV vaccine development, as one of the major reasons for the poor performance of many WIV vaccines is the damage of the viral glycoprotein antigens during the inactivation process [8, 9, 12, 15, 19, 53-59]. Excitingly, using a mouse IAV model, we demonstrated that XM-01 treatment improved the in vivo generation of NA and HA neutralizing antibodies, in turn lessening both morbidity and mortality upon viral infection challenge.

*We observed that XM-01 physically and chemically changes the lipid bilayer of viral membranes by intercalating into the lipid bilayer, increasing membrane order and rigidity deep within the membrane, and increasing the phase transition temperature, all consistent with making the negative curvature events required for membrane fusion energetically unfavorable. One exciting feature of XM-01 treatment is that the PC:PE ratios are drastically altered from the optimal range of 0.9-2.0 PC:PE [60] to the observed PC:PE ratio in XM-01 treated IAV of 10.97, mainly due to reduction in PE lipid species. This may be due to a significant portion of PE species in mammalian membranes being plasmalogens, which alleviate oxidative stress via the free radical scavenging capability of a vinyl-ether bond and *sn*-2 polyunsaturated fatty acid acyl chain [47]. Other global lipid changes included a global increase in lipid unsaturation and an increase in lysolipids, both of which can contribute to making the negative membrane curvature required during the membrane fusion process energetically unfavorable [61]. These chemical changes within the membrane may explain the observed changes in its physical properties. As with LJ001 and related compounds, XM compounds can exhibit effects against both cells and viruses, although at different concentrations, indicating no specificity in the type of membrane targeted. However, cells have an impressive capacity to repair their membranes while viruses lack lipid repair mechanisms, which may explain why the viral particles were significantly more sensitive than cells [62]. Importantly, we do not anticipate that XM or related compounds would be used as therapeutic agents unless they were specifically made to target viral membranes. This is because the abundant host cell membranes would likely sequester the XM compounds. However, all these XM characteristics, including the relative lack of changes in glycoprotein conformations or functions make the XM compound inactivation strategy ideal for WIV vaccine development.*

As a proof-of-principle, we utilized the unique properties of XM-01 to generate a WIV influenza vaccine. WIV vaccines are advantageous over subunit vaccines since WIV vaccines can present multiple viral

proteins and genetic material to the immune system in the stoichiometric context of the naturally immunogenic viral particle [63]. However, many viral inactivation strategies for the generation of WIVs, such as heat, UV light, or the traditional PFA and BPL methods commonly used for IIV generation, are known to often damage the viral glycoprotein antigens during the inactivation process, which in turn can lead to ineffective, or even deleterious (e.g. for RSV), immune responses to vaccination [8-11, 13, 15, 19, 64, 65]. Importantly, XM-01 fully inactivated IAV while leaving NA and HA enzymatic activity unaffected. Excitingly, XM-01-IIV vaccination induced high levels of NABs against both HA and NA, comparable to live-virus IM vaccination, performing better than the traditional PFA IIV vaccination. Although both anti-HA and anti-NA Nab responses can be protective, an increase in NABs against NA is exciting, as NA NABs are thought to provide relatively more broadly-protective responses against multiple influenza strains [51]. Indeed, previous studies showed that passive transfer of serum containing antibodies specific only to NA completely prevented death in lethally-challenged mice [51]. Further, traditional IIV human vaccination does not seem to yield high NA NAB responses, which may contribute to its poor performance [51]. Notably, female mice were found to have more effective anti-HA and anti-NA NAB responses following fewer vaccinations of XM-01-IIV than other vaccinations types, including that of *live-virus vaccination* (Fig. S4). These data suggest that XM-01 derived vaccines may allow faster seroconversion rates after a single boost. It has been previously reported that sex-associated immune responses for both influenza infection and vaccination are more robust in female mice, which may explain why this difference was more prominently noticed in this gender [66].

Histological analyses of various tissues and flow cytometric analyses of immune cells from vaccinated mice at 5 dpc supported the findings of enhanced induction of immunity by the XM-01 derived vaccine. Notably, histological examination of lung sections did not reveal quantitative differences between live-virus-vaccinated, XM-01-IIV, and PFA-IIV. Similarly, flow cytometric analysis of lung immune cells indicated reduced infiltration of neutrophils and CD8⁺ T cells, indicative of reduced lung inflammation upon challenge following vaccination with live-virus, XM-01-IIV, or PFA-IIV. However, the significant enhancement of NABs to both HA and NA of the XM-01-IIV compared to all other IIV-vaccine groups correlated with reduced morbidity and mortality. We speculate that these encouraging results may be due to both the *quantity* and *quality* of neutralizing antibodies, in turn due to increased glycoprotein preservation in the XM-01-IIV group, a major indicator of WIV vaccine potential [15]. Animal studies have shown that passive transfer of even non-NABs against IV challenge can be fully protective against mortality [67]. This may explain why PFA-IIV had lower NAB titers yet maintained similar histological and specific immune cell responses as compared to the live-vaccinated and XM-01-IIV groups. Ultimately, however, the PFA-IIV and JL-122-IIV groups did not perform as well as the live-vaccinated or the XM-01-IIV groups in overall morbidity (weight loss) and mortality, indicating that not only the overall strength of generated immune response impacts the outcome, but the specificity to key neutralizing antibody epitopes is likely critical for successful vaccination results.

In summary, our results demonstrate a first proof-of-principle study whereby membrane inactivators are shown useful for vaccine development. A new class of antivirals, represented by XM-01, effectively inactivates enveloped viruses by physically and chemically changing their membranes, while minimizing

perturbation of their glycoproteins. Excitingly, our data indicates that these characteristics of XM compounds make them useful to improve WIV vaccine development. Further mechanistic chemical studies, such as those that optimize the rate of persulfide radical formation, may lead to future derivatives that possess improved antiviral activity, perhaps further improving vaccine development. The exciting results of this proof-of-principle study may thus help us better prepare against current endemic as well as emerging enveloped viral pathogens and their potential pandemics, which pose a major threat to human and animal global health.

Methods

Small-molecule compounds tested. XM-01 and other sulfur-containing compounds tested were used in prior studies as H₂S donors. These compounds were synthesized using known protocols [68]. LJ001 and JL122 compounds were synthesized at the University of California, Los Angeles (UCLA) by Dr. Michael Jung's group [23, 27]. All stock solutions for these compounds were prepared in 100% DMSO, stored at -20 °C, and used within 6 months of reconstitution.

Cell culture. HEK293T (ATCC) and PK13 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) (Gibco, Life Technologies). Vero cells (ATCC) were cultured in minimal essential medium alpha with 10% FBS. Human lung epithelial cells (A549, ATCC), and Madin-Darby canine kidney epithelial cells (MDCK, ATCC) were grown in complete DMEM containing 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin (Gibco, Life Technologies). MA104 cells were obtained from ATCC and grown in Dulbecco's modified Eagle's medium with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin.

Pseudotyped NiV/VSV viral infection assays. pNiV virus particles were incubated for 30 min with or without the indicated amounts of the compound or the corresponding vehicle DMSO control. Vero cells were then infected with 10-fold dilutions of pseudotyped virus particles in infection buffer (PBS + 1% FBS) and incubated for 2 hours at 37 °C. After 2 h growth medium was added. 18-24 h post-infection, cells were lysed and an infinite M1000 microplate reader (Tecan Ltd) was used to measure luciferase activity. Three or more independent experiments were performed, error bars represent ±SD. Unpaired t-tests were performed using Graphpad PRISM software, with Bonferroni corrections.

Cytotoxicity assay. Vero cells were incubated with each compound for 30 min to 24 h, as indicated, at the specified concentrations. This was followed by incubation with a cell counting kit reagent (CCK-8) (Dojindo Molecular Technologies, Japan) for 1-2 h, absorbance was measured at 450 nm using an infinite M100 microplate reader (Tecan Ltd). The quantity of the formazan dye produced when WST-8 (Dojindo) is reduced by dehydrogenases is directly proportional to the number of living cells (i.e. cell viability).

Transmission electron microscopy (TEM) imaging. 5 μ L of concentrated VLPs or VSV-NiV were pipetted onto a 200-mesh Formvar-coated nickel grid and allowed to settle for 20 min at RT. Excess liquid was removed by wicking with filter paper before coating the deposited sample with 5 μ L of 1% uranyl acetate (UA) (Polysciences, Inc). After 2.5 min, excess UA was wicked off using filter paper and dried overnight in a desiccator. TEM micrographs of the samples were recorded under high vacuum with an electron beam strength set at 200 kV using the FEI Technai G2 20 Twin TEM (FEI Corp., Hillsboro, OR).

RNA Stability Assay. Briefly, we *in-vitro* transcribed CHIK reporter virus containing a luciferase gene in the hypervariable domain of nsP3. 20 μ g of the nsP3:luciferase construct was treated with decapping enzyme at 1, 3, 10, 30, and/or 100 μ M XM-01 concentrations for 1 h on ice, and then electroporated in BHK cells. After 2 h, enough time to initiate translation of nsP3 and luciferase, cells were lysed and luciferase activity measured.

Detection of protein conformations by flow virometry. Pseudotyped NiV (pNiV) virions were incubated for 30 min with XM-01 at 4 °C, then washed by ultracentrifugation with NTE buffer (150 mM NaCl, 40 mM Tris-HCl at pH 7.5, and 1 mM EDTA) at 110,000 x RCF for 2 h. The treated virus was resuspended in NTE buffer, then stained as previously described [69]. We used conformational anti-NiV F and/or anti-NiV G specific rabbit primary antibodies (Anti NiV F Ab 66, or anti-NiV G Ab 213) [29, 32, 33] at 1:100 dilution for 1 h, followed by a FACS buffer (1% FBS in PBS) wash and incubation with secondary Alexa 647 goat anti-rabbit antibodies (Life Technologies, NY) for 30 min followed by one more FACS buffer wash. We then measured the relative levels of antibody binding through flow virometry, using a Guava easyCyte8HT flow cytometer (EMD Millipore, MA) [69]. Background mean fluorescence intensity (MFI) was obtained by binding equal concentrations of primary and secondary reagents to mock pseudotyped VSV virus, then subtracted from the MFI of pseudotyped NiV/VSV virions.

Viral inactivation assay for measurement of infection. Specific viruses (IV, RSV, HSV-1, HCMV, or rotavirus) were serially diluted and then treated with either DMSO, XM-01, or JL-122 for 0.5 – 4 h at RT. Treated dilutions of the virus were titrated on appropriate cells and plaque-forming units or foci-forming units were counted 1-5 days post-infection [49, 70-72].

Lipids and Peptides. Lipids POPC, POPS, and the chain spin-labeled 5PC, 16PC and a head group spin-label dipalmitoylphosphatidyl-tempo-choline (DPPTC), all purchased from Avanti Polar Lipids (Alabaster, AL), and cholesterol, purchased from Sigma (St. Louis, MO), were used without further modification.

Electron Spin Resonance. Prepared MLVs (POPC, POPG and 0.5% (mol: mol) spin-labeled lipids), were resuspended and hydrated in a buffer consisting of 5 mM HEPES, 10 mM MES, 150 mM NaCl (pH 7) for 2 h at RT. Varying amounts of XM-01 (1 mg/mL in DMSO) were added to the MLV dispersion for 1 h at RT,

along with the vehicle control. ESR spectra of ultracentrifuged MLVs were collected on an ELEXSYS ESR spectrometer (Bruker Instruments, Billerica, MA) at X-band (9.5 GHz) using an N2 Temperature Controller (Bruker Instruments, Billerica, MA). The ESR spectra were analyzed by the NLLS fitting program based on the stochastic Liouville equation [41, 43] using the MOMD or Microscopic Order Macroscopic Disorder model, as in previous studies [37-39, 73, 74]. Each experiment (and subsequent fit) was repeated 2 or 3 times to check reproducibility and estimate experimental uncertainty.

XM-01 Decomposition. Butyl amine (47.4 mg, 0.65 mmol) was added to a solution of XM-01 (50 mg, 0.13 mmol) in CH₂Cl₂ (10 mL). The reaction mixture was stirred for 2 h at RT, then concentrated and subjected to column chromatography (30% Ethyl acetate/Hexane) to separate the products (33 mg) as a mixture of disulfide and polysulfides.

NMR Analysis:

Disulfide: ¹H NMR (300 MHz, Chloroform-d) δ 7.88 – 7.75 (m, 4H), 7.59 – 7.49 (m, 2H), 7.47 – 7.34 (m, 4H), 7.11 (d, J = 7.3 Hz, 2H), 5.07 (dt, J = 7.3, 5.1 Hz, 2H), 3.78 (s, 6H), 3.35 (d, J = 5.1 Hz, 4H); HRMS (ESI) m/z calcd. for C₂₂H₂₅N₂O₆S₂ [M+H]⁺ 477.1154, found 477.1148.

Polysulfides: ¹H NMR (300 MHz, Chloroform-d) δ 7.95 – 7.70 (m, 4H), 7.60 – 7.33 (m, 6H), 7.24 – 7.02 (m, 2H), 5.19 – 4.95 (m, 2H), 3.89 – 3.69 (m, 6H), 3.65 – 3.42 (m, 2H); HRMS (ESI) m/z calcd. for trisulfide C₂₂H₂₅N₂O₆S₃ [M+H]⁺ 509.0875, found 509.0868; tetrasulfide C₂₂H₂₅N₂O₆S₄ [M+H]⁺ 541.0595, found 541.0587; pentasulfide C₂₂H₂₅N₂O₆S₅ [M+H]⁺ 573.0326, found 573.0313; hexasulfide C₂₂H₂₅N₂O₆S₆ [M+H]⁺ 605.0037, found 605.0053.

Lipidomics Studies. 40 mL of ~1x10⁵ PFU/mL A/California/04/2009 was treated with either 0.02% PFA, 1mM XM-01, or 10% DMSO, for 4 h at RT under steady rocking using an Orbitron shaker. Samples were then cleared at 1200 RCF for 10 min at 4 °C, supernatant was taken, and virus particles were collected over a 20% sucrose gradient by ultracentrifugation at 110,000 x RCF for 90 min at 4 °C. Supernatant was removed and virus was resuspended in minimal NTE buffer before performing lipid extraction. Viral membrane lipids were extracted using a modified liquid-liquid extraction procedure as described previously [75]. Extracted lipids were analyzed by LC-MS/MS, as described previously [76].

Vaccine generation and vaccination. A/California/04/2009 (~2x10⁵ PFU/mL) was thawed on ice before being incubated for 4 h at RT with 1 mM of XM-01 in 10% DMSO, 10 μM JL-122 in 10% DMSO, 0.02% PFA,

and a mock control with vehicle only (10% DMSO). The solutions were then mixed 1:1 with alum for 30 min under light rocking before 100 µL was injected intramuscularly to mice.

Influenza virus (IV) propagation and IIV passage in egg culture. All infections were done in certified pathogen free eggs following standard protocols [49]. Briefly, eggs (10-12 days old) were surface decontaminated in a biosafety cabinet, then 100 µl of inactivated virus or a low dose ($\sim 1 \times 10^3$ PFU/mL) of A/California/04/2009 was injected into the amniotic fluid. The injection site was sealed with glue, and placed in an egg incubator for 72 h. Eggs were moved to 4 °C for 24 h before collecting amniotic fluid. Amniotic fluid was cleared by centrifugation (1000 x g for 5 min) and supernatant was aliquoted and snap-frozen in liquid nitrogen before being stored at -80 °C. Treated IV was passaged as described above, and amniotic fluid was assayed for infectious virus or passaged again in eggs up to three times to propagate any infectious particles.

Hemagglutination Inhibition (HI) Assay. Serum samples in HI buffer were added to a V-bottom 96-well plate, and diluted by 2-fold serial dilutions, then 8 HA units of Ca/04/2009 H1N1 in HI buffer was added. The plate was incubated for 30-45 min at RT, followed by the addition of 0.8% rooster blood in HI buffer. Results were read 30-45 min later and the HI titer value was read as the inverse of the lowest dilution of serum that completely inhibited hemagglutination [49]. Three or more independent experiments were performed, error bars represent $\pm$ SD. Unpaired t-tests were performed using Graphpad PRISM software, with Bonferroni corrections.

Neuraminidase Inhibition a(NI) ssay. NI assays were performed using a protocol adapted from Leang and Hurt [50]. Briefly, sera taken from mice was assayed using 2-fold serial dilutions, then incubated with A/California/04/2009 virus in flat-bottom plates in assay buffer for 30-45 min at RT before the addition of the MUNANA substrate. The sealed plate was incubated at 37 °C for 1 h before the addition of the stop solution. Results were read using a Tecan Spark plate reader set to 355 nm excitation measuring absorbance at 460 nm.

Histology. For histologic examination, lung tissues were collected directly after euthanasia and placed in 10% formalin for >72 h. Following paraffin embedding, 4 µm tissues sections were prepared and stained with hematoxylin and eosin (H&E).

Flow cytometry. Lymphocytes were extracted from lung, mediastinal lymph node, and spleen, as previously described [77]. Cells were stained in 30 μ L of 1:200 antibodies in PBS for 20 min at RT, washed, and then resuspended for flow cytometry. Fluorescent antibodies used for the study are listed as target (clone; catalog #). Antibodies against mouse antigens included CD16/32 (93; 14-0161-85) Fc block, CD3 (145-2C11; 45-0031-82), CD45 (30-F11; 56-0451-83), CD8 (53-6.7; 11-0081-82), CD19 (eBio1D3(1D3); 11-0193-82), CD23 (B3B4; 25-0232-82), GL-7 (GL-7(GL7); 48-5902-82) from Thermo Fisher Scientific. Ly6G (1A8; 565964), CD11b (M1/70; 563015) from BD Biosciences. Additionally, fixable viability dye (65-0866-14) was used to differentiate live/dead cells. Cells were analyzed on the BD Biosciences FACSymphony, and analyzed with FlowJo Software (BD).

Animal Care. All protocols were performed under BSL-2 conditions and approved by the Institutional Animal Care and Use Committee at Cornell University (IACUC # 2017-0108). Intranasal virus administration was performed under anesthesia, and all efforts were made to minimize animal suffering. Eight-week-old C57BL/6 mice were used for this study. Mice were intranasally inoculated with 1×10^3 PFU/animal. Following challenge, mice were monitored and weighed daily and euthanized at predetermined humane criteria following approved protocols, generally when weight loss reached 20% from day of challenge or mice became moribund with a clinical score >3 on a 5-point scale (58).

Declarations

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AUTHOR CONTRIBUTIONS

AP, IAM, DWB, ALL, QL, BC, SMP, AVN, YZ, BN, KS, EMH, GCY, JS, BI, MJ, SM, MS, YYY, AM, JLZ, NKS, SJ-B, SMP, CY, YZ, ZJM, CX, SD, MJ, GVdW, AA, JWJ, MH, SB, AN, MX, and HAC designed and/or performed experiments. AP, IAM, DWB, EMC, JWJ, and HAC wrote the manuscript and AP, IAM, DWB, and SM, JLZ, GVdW, AA, JWJ, MH, SB, MX, and HAC edited the manuscript.

CONFLICTS OF INTEREST

HAC, IAM, DWB, are inventors on patent applications on XM-01 antivirals and XM-01 generated vaccines filed by Cornell University. HAC, IAM and DWB certify no potential conflicts of interest. All authors have submitted Disclosures of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

MATERIALS AND CORRESPONDENCE.

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Figures

Figure 1

XM-01 broadly inhibits enveloped viruses without significantly decreasing cell viability. Upon screening of a short S-containing compound library, (A) Five XM compounds at 10 μ M concentrations inhibit viral infectivity of pseudotyped NiV/VSV virions (pNiV) in Vero cells; (B) Five active XM antiviral compounds exert little to no effects on cell viability, determined measuring dehydrogenase activity of live cells; (C) XM-01 low cytotoxicity at 1 μ M – 1 mM in Vero cells; (D) XM-01 broadly inhibited infection by many enveloped viruses, but not by a non-enveloped rotavirus. Statistical analyses were performed with one-

ANOVA and Dunnett's multiple comparisons test (N = 3; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

Figure 2

XM-01 inhibits viral entry while preserving glycoprotein conformations and RNA integrity. (A) XM-01 shows no post-infection inhibitory effects. (B) Time of addition of XM-01 shows inhibition at the viral, and not at the cellular level. (C) XM-01 did not significantly affect conformations of Nipah virus glycoproteins G or F. (D) XM-01 inhibition of influenza virus A/California/04/09 (Ca09) strain did not significantly affect neuraminidase (NA) enzymatic activity. (E) XM-01 treatment of influenza Ca09 virions did not affect the hemagglutination activity of its hemagglutinin (HA) glycoproteins. (F) Electron microscopy images of XM-01 treated virions show effects on viral membranes. (G) nsp3:luciferase RNA assay shows that XM-01 treatment does not affect RNA functional integrity. Each experiment was performed a minimum of 3 times and statistical significance determined using t-tests in comparison to control.

Figure 3

XM-01 mechanism of action involves induction of changes in viral membranes. (A) XM-01 causes an increase in membrane order at various XM01:lipid ratios. (B-C) XM-01 increases the phase transition temperature of viral membranes ($P < 0.005$). (D) XM-01 creates disulfide and polysulfide molecules, subsequently forming perthiyl radicals. (E) Mass spectrum reveals formation of polysulfides, indicating the presence of persulfide intermediates (RSSH) from XM-01. (F) LC-MS/MS lipid analysis displaying top 50 most differentially expressed lipids upon XM-01 treatment compared to PFA or untreated influenza virus. $N = 4$ per group, data were sum normalized, log transformed, and mean centered. Volcano plot highlighting features that had a $*P < 0.05$ (orange), $**P < 0.01$ (blue), and $***P < 0.001$ (red) when comparing untreated and XM-01. The x-axis is $\log_2(\text{FC})$ (FC= fold change) and the y-axis is $-\log_{10}(p)$ (p = p-value based on t-test). (G) LC-MS/MS lipid statistical analyses showing percentages of total lipid abundance for XM-01 vs control groups, and for (H) specific lipid class abundances for these groups. $N = 4$ per group and values are represented as mean $\pm$ SEM (standard error of the mean).

Figure 4

XM-01-inactivated influenza virions constitute an effective vaccine. (A) Timeline of mouse study. Compared to control PFA-inactivated vaccine group, as well as to other control groups, sera from XM-01 IIV vaccinated mice yield improved (B) anti-NA neutralizing antibody titers (*, $P < 0.05$ compared to PFA-IIV) and (C) anti-HA neutralizing antibody titers (**). XM-01 IIV vaccinated animals display (D) decreased mortality and (E) decreased morbidity shown as weight loss XM-01 IIV shows significantly decreased morbidity (* or **) compared to PFA-IIV upon challenge with IAV Ca09 strain. $N = 10$ mice (5 males and 5 females) per group. Significance assessed using standard t-tests (* $P < 0.05$, ** $P < 0.01$).

Figure 5

Histological analysis and specific immune responses post vaccination and challenge. Representative lung histology of female mice vaccinated with PBS, PFA-IIV, JL-122-IIB, XM-01-IIV, or live virus on day 5 following infection with live influenza virus. (A-J) Representative lung histology of female mice vaccinated with PBS, PFA-IIV, JL-122-IIV, XM-01-IIV, or live virus on day 5 following infection with live influenza virus. Peribronchiolar and perivascular inflammation (arrowhead) was present in all mice (N = 3 male, 3 female per group) with more severe inflammation in mice vaccinated with PBS and JL-122-IIV (arrows) and reduced inflammation in mice vaccinated with PFA-IIV, XM-01-IIV, and live-virus. Regions of interstitial pneumonia (A, asterisk) were only detected in mice vaccinated with PBS. (A-E, H&E). Scale bars: 1 mm (A-E) and 50 μ m. (F-J) Degenerate neutrophils within bronchiolar lumina (asterisk) were present in all female mice vaccinated with PBS or JL-122-IIV and absent in mice vaccinated with PFA-IIV, XM-01-IIV, and live-virus. (K-N) Immune cells detected by flow cytometry. (K) Infiltrating lung neutrophils (CD11b⁺Ly6G⁺); (L) CD8 T cells (TCRb⁺CD8⁺); (M) Total lymph node B cells (CD19⁺) were quantified; and (N) percent germinal center B cells (CD19⁺CD23⁺GL7⁺). Statistical analyses were performed with one-ANOVA and Dunnett's multiple comparisons test, which compared each IIV type to the mock control vaccinated group (N = 3; *P < 0.05, **P < 0.01, ***P < 0.001).

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