Detection of proton movement directly across viral membranes to identify

novel influenza M2 inhibitors

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ABSTRACT

The influenza M2 protein is a well-validated yet underexploited proton-selective ion channel essential for influenza virus infectivity. Because M2 is a toxic viral ion channel, existing M2 inhibitors have been discovered through live virus inhibition or medicinal chemistry rather than M2-targeted high-throughput screening (HTS), and direct measurement of its activity has been limited to live cells or reconstituted lipid bilayers. Here we describe a cell-free ion channel assay in which M2 ion channels are incorporated into virus-like particles (VLPs) and proton conductance is measured directly across the viral lipid bilayer, detecting changes in membrane potential, ion permeability, and ion channel function. Using this approach, we identified 19 M2-specific inhibitors, including two novel chemical scaffolds that inhibit both M2 function and influenza virus infectivity. Counter-screening for non-specific disruption of viral bilayer ion permeability also identified a broad-spectrum anti-viral compound that acts by disrupting the integrity of the viral membrane. In addition to its application to M2 and potentially other ion channels, this technology enables direct measurement of the electrochemical and biophysical characteristics of viral membranes.

INTRODUCTION

The M2 gene of influenza encodes a 97 amino acid, homo-tetrameric, proton-selective ion channel necessary for influenza virus infectivity (28, 29). Activation of M2 is triggered by low pH in host cell endosomes, resulting in an influx of protons into the virus and acidification that releases the viral RNA into the host cell (14, 22, 29). M2 also helps to package viral RNA (24, 19) and regulate pH in the Golgi of infected cells during viral assembly (32). Two FDA-approved adamantane-based drugs, amantadine and rimantadine (7, 29), are potent M2 inhibitors
that can neutralize influenza virus in humans and other animals. However, the emergence of widespread highly virulent adamantane-resistant strains such as avian and swine influenza strains (12, 36) has prompted the need for newer antivirals. Drug discovery efforts targeting M2 have been difficult due to the toxicity of M2 in cells (17), the difficulty of reconstituting M2 in liposomes for large-scale application (25, 45), and the labor intensive, low-throughput electrophysiological approaches typically used to study ion channels.

To address these challenges, we developed a rapid, cell-free assay to measure the movement of proton ions directly across the lipid bilayer of virus-like particles (VLPs). HTS of M2-VLPs using >100,000 compounds identified 19 M2-specific inhibitors, including two novel chemical scaffolds, that inhibited both M2 function and influenza virus infectivity. Counter-screening for non-specific disruption of FCCP protonophore activity also identified a broad-spectrum anti-viral compound that acts by disrupting the integrity of the viral membrane. In addition to its application to M2, FCCP, and potentially other ion channels, this technology enables measurement of the electrochemical and biophysical characteristics of viral membranes.

MATERIALS AND METHODS

Plasmid construction

Sequences for the M2 genes of influenza strains A/Udorn/307/1972 (J02167), A/PR/8/1934 (AAM75162), A/HK/156/1997 (AF084267), A/HK/1073/1999 (CAC04082), A/VN/1194/2004 (ABP35632), and B/Lee/40 (ABG85183) were obtained from the NCBI influenza database. The M2 gene for each strain was synthesized (BlueHeron, Seattle, WA) and subcloned into pcDNA3.1-V5-His (Invitrogen, CA). Mutations S31N or V27A were introduced
by site directed mutagenesis into strain Udorn/307/1972 (QuickChange, Clontech, CA) and mutations A27V and N31S were introduced into strain PR/8/1934 by gene synthesis (GENEART, Germany).

Chemical Libraries

Small molecule screening was carried out in-house and at the National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB) at the ICCB-Longwood Screening Facility, Harvard Medical School, Boston. Combinatorial libraries containing 107,572 compounds (Enamine, MayBridge, Life Chemicals, ChemDiv, Prestwick, Bionet, Microsource Discovery System, and TimTec) were used at final concentrations of 5-15 uM. For validation studies, a chemical compound library containing 71 known ion channel ligands (BIOMOL International, Farmingdale, NY) was used at a final concentration of 20 uM. All compounds were re-suspended in 100% DMSO.

M2 VLP production

VLPs containing M2 (‘Lipoparticles’) were produced by co-transfection of HEK-293T cells with plasmids encoding the M2 gene and the retroviral (MLV) Gag protein, as previously described (15, 42). Null-VLPs (without M2) were produced by transfection of HEK-293T cells with the Gag plasmid alone. Mock particle preparations (M2 without Gag) were produced by transfection of HEK-293T cells with the M2 plasmid alone. 48 hours after transfection, supernatants containing VLPs were harvested, passed through a 0.45 um filter, centrifuged through 20% sucrose cushions or a 10-30% OptiPrep (Sigma, St. Louis, MO) density gradient, and resuspended in 10 mM HEPES pH 7.5. Each batch of VLPs produced was assayed for...
homogeneity by Dynamic Light Scattering (DLS) using a Proterion DynaPro DLS and for total protein concentration by BCA Protein Assay Reagent (Thermo Scientific, Waltham, MA). The incorporation of M2 proteins in VLPs was detected by Western blot using an antibody against a C-terminal V5 epitope tag (Life Technologies, Carlsbad, CA). M2-VLPs were imaged by transmission electron microscopy at the Fox Chase Cancer Center microscopy core facility (Philadelphia, PA).

M2-VLP screening

Compounds were screened against the HK/156/1997 strain of M2-VLPs and counter-screened against Null-VLPs treated with 5 uM FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) (Sigma, St. Louis, MO) in 384 well plates. VLPs were suspended in 10 mM HEPES pH 7.0, 150 mM NaCl buffer supplemented with 1% FMP-Blue dye (Molecular Devices, Sunnyvale, CA). 1 ug of M2-VLPs were dispensed into 384-well black clear bottom plates (Costar 3712, Corning, NY) and pre-incubated for 1 hour at room temperature with 100 nl of each compound delivered by pin tool (V&P Scientific, San Diego, CA). Baseline fluorescence measurements (ex530/em565) were acquired using a fluorescence plate reader (Molecular Devices FlexStation II or Perkin Elmer Envision II). 10 ul of 150 mM MES (2-(N-Morpholino)ethanesulfonic acid, 4-Morpholineethanesulfonic acid) (Sigma, St. Louis, MO), pH 4.5 (‘low pH buffer’) was injected into each well followed by fluorescence measurements. Using the Flexstation, fluorescence was measured every 2.5 sec for 150 sec. Using the Envision, single baseline and endpoint measurements were obtained. All screening values were baseline subtracted and the maximum signal was normalized to a no compound
control. Cherry picked compounds were re-screened prior to further characterization. Dose-response data was fit using non-linear regression in GraphPad Prism.

Cytotoxicity assay

Cells were passaged in Dulbecco’s modified Eagle’s medium (DMEM) containing glutamine, penicillin and streptomycin (100 ug/ml) supplemented with 10% fetal bovine serum (FBS). HEK-293T (5x10^4 cells/well), BHK (3x10^4 cells/well) and MDCK (1.5x10^4 cells/well) cells were seeded in 96 well black tissue culture treated plates (Costar 3603, Corning, NY). 24 hours later, cells were washed once with PBS and treated with dilutions of compounds up to 40 uM. Cells were incubated with compound for 48 hrs at 37°C, 5% CO2 followed by measurement of cell viability using CellTiter Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI). Luminescence values were measured using a Wallac Victor V plate reader. Data was normalized to untreated cells and expressed as percent viability.

Viral Plaque assay

Viral strain Udorn/307/1972 was purchased from ATCC and propagated either in chicken egg or Madin-Darby canine kidney (MDCK) cells. MDCK cells were seeded in 12 well tissue culture plates (5x10^5 cells/well) and incubated overnight at 37°C, 5% CO2. The next day, purified Udorn/307/1972 virus was diluted in DMEM supplemented with 1 ug/ml TPCK trypsin and 0.3% BSA to an MOI of 0.0001. Diluted virus was either added directly to cells followed immediately by the addition of compound or pre-incubated with compound at room temperature for 1 hour prior to infection. Cells were incubated with the virus and compound mixtures at 37°C for 1 hr. Subsequently, media containing virus and compound was removed, and cells were
washed once with PBS and layered with 1.8% agar either with or without compound in MEM and 2 ug/ml TPCK trypsin. Cells were incubated at 37°C for an additional 48 hrs to develop plaques. Cells were then stained with 0.1% crystal violet in 10% formaldehyde for 5 minutes and washed 3x with water. The total number of plaques was counted for each well and the activity of each compound was expressed as a percent of control in the absence of drug.

**Reporter Virus Infectivity Assays**

Infections were carried out in a 96 well plate using HIV and VSV Envelope reporter viruses (pseudotyped onto an HIV reporter backbone), and Dengue reporter viruses (23), using target HEK-293T cells (5x10^4 cells/well) for HIV or VSV and BHK cells (3x10^4 cells/well) for Dengue virus infections. The compound was serially diluted and incubated with the respective viruses for 30 min at room temperature, added to each cell type, and incubated for 48 hrs at 37°C, 5% CO₂. For HIV pseudoviruses, target cells were transfected with CD4 and CXCR4 24 hours prior to infection. Viruses were detected by luminescence read out of renilla luciferase (Promega, Madison, WI) using a Wallac Victor V plate reader. Luminescence values were normalized to a no compound control and expressed as percent infection.

**RESULTS**

**Incorporation of M2 ion channels into VLPs**

Retroviral virus particles and virus-like particles (VLPs) are similarly formed by the budding of retroviral Gag through the plasma membrane of cells, and have been shown to incorporate complex membrane proteins without the use of detergents or protein purification (2, 10, 15, 42). Because membrane proteins incorporated into VLPs have proven structurally intact
when tested against conformational antibodies and ligands (2, 10, 15, 42), we reasoned that isolated ion channels such as M2 may also be functional if they could be incorporated and measured. VLPs were produced that contain high concentrations of the M2 ion channel protein. M2-VLPs contain a non-infectious retroviral (MLV) Gag core surrounded by a host cell-derived lipid membrane containing high concentrations of incorporated M2 protein. M2-VLPs, produced and purified as described previously (6, 15, 42) showed high purity and homogeneity, low polydispersity (4.9%), and a hydrodynamic diameter of ~250 nm, similar to other purified VLPs (Figure 1a). Purified VLPs imaged by electron microscopy (EM) were consistent with these measurements (Figure 1a, inset).

We produced VLPs containing M2 ion channels from diverse strains of influenza, including Udorn/307/1972, B/Lee/40, and HK/156/1997 (Figure 1b), as well as HK/1073/1999, PR/8/1934, and VN/1194/2004 (not shown). To confirm M2 incorporation, purified M2-VLPs were run on SDS-PAGE and probed with an antibody against a C-terminal V5 epitope tag on each M2 protein. A major band at ~15 kDa demonstrates the presence of monomeric M2 protein for each strain. Density gradient purification of M2-VLPs resulted in co-fractionation of virion particles, retroviral Gag, and M2 activity (Figure 1c and 1d). Taken together, these results demonstrate that full-length M2 from various strains of influenza can be successfully incorporated into highly purified preparations of VLPs.

**Activation of M2-VLPs**

Upon exposure to low pH in the endosome, M2 mediates an influx of protons that both depolarizes the influenza virus membrane and lowers the pH of the virion interior (28, 29). To detect a membrane potential change directly across the VLP membrane, we used a
potentiometric fluorescent dye (FMP, FLIPR membrane potential dye, Molecular Devices) (Figure 2a), which fluoresces more brightly upon membrane depolarization (3). Challenge of M2-VLPs with an acidic buffer (pH 4.5) resulted in increased M2-specific fluorescence (Figure 2b). No membrane depolarization was detected upon pH challenge of Null-VLPs (without M2), indicating the necessity of M2 for depolarization. Proton-driven membrane depolarization could also be mediated when the proton ionophore FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) (4) was added to Null-VLPs prior to pH challenge. Importantly, M2 inhibitors amantadine (100 μM) and rimantadine (10 μM) completely abrogated ion conductance in M2-VLPs but had no effect on FCCP-treated VLPs. M2-VLPs containing M2 proteins from diverse influenza strains demonstrated robust depolarization in response to pH challenge (Figure 2c). Mock VLP preparations from cells transfected with M2 but without Gag did not show activity, indicating that true VLPs rather than M2-induced membrane blebs (31) were responsible for the observed changes in membrane potential. Taken together, these data demonstrate the functionality of M2 ion channels in VLPs and validate their utility as a non-infectious, cell-free, in vitro biochemical platform capable of assessing M2 ion channel activity. The ability of VLPs to maintain a membrane potential also demonstrates for the first time that lipid viral membranes are structurally intact and impermeable to ions, a matter of previous uncertainty (26), and offers the possibility that membrane potential itself, rather than just pH changes, could play a role in some parts of the virus lifecycle (11, 21).

Validation of M2-VLP activity measurements

To further measure the native functionality of M2 in VLPs, dose-response curves were generated with amantadine and rimantadine, with full inhibition of the M2 signal observed with
high concentrations of drug (Figure 3a). Inhibition IC50 values of 0.5–2 uM for amantadine and 0.1–0.6 uM for rimantadine were calculated (depending on the experiment), comparable to published values of M2 ion channel inhibition by electrophysiology and influenza virus inhibition in tissue culture (33, 35). Inhibitory effects were specific to the M2 ion channel since even the highest drug concentrations tested did not abrogate signals mediated by the protonophore FCCP. Importantly, amantadine did not inhibit M2 variants derived from known amantadine-resistant strains PR/8/1934, VN/1194/2004, and B/Lee/40 (Figure 3b).

For many strains of influenza, sensitivity to adamantane compounds has been mapped to specific amino acids. Mutations S31N, and to a lesser extent V27A, are found in clinical strains of influenza which are insensitive to adamantanes (13, 29). To establish whether these mutations would cause a similar resistant phenotype in M2-VLPs, we introduced the S31N point mutation into M2 of the amantadine-sensitive strain Udorn/307/1972 and demonstrated that the mutation conferred complete drug resistance (Figure 3c). Conversely, the incorporation of the sensitizing mutations A27V and N31S were sufficient to confer amantadine sensitivity to M2-VLPs based on PR/8/1934, a normally resistant strain. The strict correlation between characterized influenza M2 genotypes and drug sensitivity phenotypes in M2-VLPs suggests that the in vitro M2-VLP assay can be used to monitor the inhibition of M2 function from diverse strains of influenza.

High-Throughput screening using M2-VLPs

We next adapted the M2-VLP assay to a 384-well microplate format to enable the screening of large compound libraries for inhibitory activity against M2. Upon pH challenge, we observed a clear separation of M2-dependent signal between control and amantadine inhibited signal with a Z’ >0.5 using seven different M2-VLP variants, indicating high sensitivity, low
variability, and suitability of this assay for high-throughput screening (Figure 4a and Table 1). We also developed a novel counter-screen using Null-VLPs incubated with the FCCP protonophore to identify compounds that non-specifically inhibit membrane potential across the viral lipid bilayer. Using these screens, amantadine and rimantadine were reliably identified as hits against M2-VLPs (Figure 4b). As a proof of concept, we screened a 71 compound library of known ion channel inhibitors against seven different M2 variants and against FCCP-treated Null-VLPs (Figure 4c and Table 1), and amantadine was again identified as a specific hit in all screens against adamantane-sensitive M2 strains but not in screens against resistant strains.

To identify novel inhibitors of influenza M2, we screened 107,572 compounds using M2-VLPs. We focused on the adamantane-sensitive strain HK/156/1997 for screening so that rimantadine could serve as an internal positive control. Each compound was screened at least once for activity against M2-VLPs and for lack of activity against FCCP-treated Null-VLPs. The top ~400 compounds showing the highest levels of inhibition against M2-VLPs and low inhibitory activity against FCCP-treated Null-VLPs were classified as primary hits and rescreened to confirm specific activity. Following confirmation and secondary screening, 19 compounds were classified as validated hits with potent in vitro IC\textsubscript{50} values (140 nM to ~13 uM), little or no toxicity at concentrations of 40 uM (~3-300-fold above their calculated in vitro IC\textsubscript{50} values), and activity against live influenza (Udorn/307/1972), with many compounds demonstrating potent anti-viral effects similar to amantadine and rimantadine (Table 2).

Structural analysis of validated M2 hits revealed compounds with varied chemical scaffolds. We identified two novel scaffolds based on a bicyclic structure (compounds 5 and 6) that inhibited both M2 in vitro and live influenza without cellular toxicity. Two additional compounds (compounds 3 and 4) contained a scaffold recently reported to have anti-influenza activity (16).
Other validated hits comprise a structurally-related family of compounds containing an adamantane ring (compounds 7-21). Although all of these compounds were active against the adamantane-sensitive strains HK/156/1997 and Udorn/307/1972, they did not exhibit activity against the adamantane-resistant strains VN/1194/2004, PR/8/1934, or B/Lee/40, or against an adamantane-resistant mutant, Udorn/307/1972 V27A, at concentrations up to 100 uM (Figure 5), consistent with the use of an adamantane-sensitive strain in our screening strategy. Using medicinal chemistry and computational predictions (although not high-throughput screening), ten non-adamantane scaffolds active against influenza M2 have been reported in recent years, none of which inhibit the ubiquitous S31N amantadine-resistance mutant (1, 9, 16, 38, 40, 44). Recently, however, amantadine-based compounds have been further derivatized to create variants that can inhibit wild type and S31N resistant strains (39, 41). Our ion channel VLP technology complements these efforts and our results suggest that future screening efforts using a resistant strain for primary screening should identify novel, non-adamantane-based compounds capable of inhibiting adamantane-resistant viruses.

Identification of broad spectrum membrane-active compounds

In addition to M2-specific inhibitors, our screening also identified a second group of ‘membrane active’ compounds which disrupted both M2 and FCCP-mediated viral membrane potential. Characterization of one of these compounds (Table 2, compound #22) revealed potent inhibition of M2-VLPs, FCCP-VLPs, and live influenza virus, with IC_{50} values between 1 and 5 uM (Figure 6). Inhibitory activity against FCCP treated VLPs suggests that this compound has a broad mechanism of action that disrupts the integrity of viral membranes. To test this, we assessed its inhibition against a panel of diverse reporter viruses, including HIV, dengue virus...
(DENV), and vesicular stomatitis virus (VSV). Remarkably, this compound inhibited the infectivity of each virus tested, with no significant toxicity on a number of target cell types. Together, these data suggest that this compound has a broad-spectrum mechanism of action that specifically disrupts viral membranes while sparing cellular membranes. During the course of our studies, a related compound was identified and characterized by others as a broad-spectrum compound that irreparably damages viral membranes (37, 43), corroborating our data and validating the utility of our counter-screening approach for discovering broad-spectrum viral inhibitors.

DISCUSSION

Influenza virus continues to be a major global disease that claims hundreds of thousands of lives each year and threatens to become a major global pandemic with the emergence of highly virulent and drug resistant strains. The M2-VLP assay described here provides a safe, rapid, and robust biochemical format for discovering novel influenza inhibitors using cell-free HTS campaigns. Our work also provides, to the best of our knowledge, the first measurements of membrane potential directly across viral membranes and the first evidence that viral membranes are impermeable to ions, so may enable a better understanding of the electrochemical and biophysical properties of viral membranes (6, 11, 18, 21), which have been under-explored areas of virology.

The technology described here may also provide a strategy for studying other viral and even cellular ion channels which may be toxic in live cells. Despite their biomedical significance, ion channels in general remain resistant to many traditional modes of manipulation and functional detection, in large part because their activity is often difficult to study outside of
living cells and in traditional high-throughput screening formats. Assays for ion channel activity usually require living cells because the cell membrane both maintains ion channel structure and forms the electrochemical barrier required for electrical signals. However, cells possess inherent limitations in size and environmental requirements, and many ion channels are inherently toxic or poorly expressed. As a result, traditional methods of discovering ion channel drugs have been very difficult. High-throughput patch-clamp techniques have accelerated electrophysiological studies of ion channels (5, 8), but remain expensive, require specialized machinery, and are not as fast as traditional fluorescent or luminescent microplate detection approaches.

Previous studies of M2 within virus particles have used indirect measurements of viral ion channel activity, such as fluorescent dye dissipation upon fusion (18). Using the technology described here, toxic ion channels can be tested, and the size of VLPs (~150 nm) permits ion channel activity screening to be miniaturized for microwell, microarray, or microfluidic applications that often cannot support live cells. Fluorescent probes that respond to changes in electrical membrane potential have been well described (reviewed in (3, 20, 30, 34)), but the dyes are typically toxic at high concentrations and partition into cell organelle structures that increase background and non-specific artifacts (27, 30, 46). Our use of membrane potential dyes within VLPs, however, avoids the limitations of living cells.
Figure 1. Production of VLPs containing M2 ion channels. (a) Dynamic Light Scattering (DLS) profile of purified M2-VLPs showed 95% purity and 4.9% polydispersity with a hydrodynamic diameter of 250 nm. An electron micrograph shows M2-VLPs (scale bar 500 nm), with one particle enlarged for clarity (inset). (b) Various strains of M2-VLPs, including Udorn/307/1972, B/Lee/40, and HK/156/1997, as well as HK/1073/1999, PR/8/1934, VN/1194/2004 (not shown), were analyzed by anti-V5 Western blot to detect epitope tags on M2. A major monomeric (~15 kDa) and minor dimeric (~30 kDa) species was observed. (c) M2-VLPs were purified using a 10-30% OptiPrep gradient and VLPs were detected in each fraction by DLS intensity. Particles co-fractionated with M2-mediated activity (inset). (d) M2-VLPs fractionated using a 10-30% OptiPrep gradient were analyzed by DLS intensity and Gag ELISA reactivity. Fractions 11-13 contained the majority of the DLS intensity and Gag protein. Co-localization of peaks confirmed that the DLS intensity profile represents true Gag-based VLPs.
Figure 2. Ion channel function within M2-VLPs (a) In the M2-VLP assay, pH challenge results in proton entry into VLPs via M2 (or FCCP). The incorporated dye fluoresces upon changes in membrane potential. (b) M2-VLPs were tested with or without inhibitor (100 uM amantadine). Null-VLPs and Null-FCCP VLPs served as controls. (c) M2 strains were tested in the M2-VLP assay. Null-VLPs without any ion channel and mock VLP preparations from cells transfected with M2 (HK/156/1997) but not Gag (‘M2 alone’) served as negative controls. The mean of triplicate values within an experiment are shown (b, c) and error bars represent s.d.

Figure 3. Validation of M2-VLP Function. (a) Established M2 inhibitors in the M2-VLP assay (HK/156/1997) demonstrated IC50 values of 1.3 uM (amantadine) and 0.18 uM (rimantadine). Rimantadine did not inhibit control FCCP-VLPs. (b) Correlation of M2 genotype and amantadine sensitivity in the M2-VLP assay using drug sensitive and resistant M2 variants. (c) Amantadine sensitive Udorn/307/1972 M2-VLPs were rendered resistant by an S31N mutation, and normally resistant PR/8/1934 M2-VLPs were rendered sensitive by A27V and N31S.
mutations. The mean of triplicate values within an experiment are shown (a-c) and error bars represent s.d.

Figure 4. Validation of M2-VLP assay for HTS. (a) M2-VLPs (HK/156/1997) pre-treated with (n=192) and without (n=192) 100 uM amantadine were tested for response to low pH buffer (pH 4.5) in a 384-well format. (b) M2-VLPs treated either with or without drug (1 to 100 uM amantadine or 0.1 to 10 um rimantadine) were challenged with low pH buffer and tested for ion conductance. In all but one drug treated well, the signal was attenuated to levels at least 3 standard deviations below the mean uninhibited signal. (c) Screening of M2-VLPs (HK/156/1997) with a small compound library (BioMol ion channel library). Fluorescence measurements are shown after treatment with each compound. The 20 uM amantadine sample included in the 71 compound set falls below 3 standard deviations of the mean signals. Cyclopiazonic acid (20 uM) was identified as a non-specific inhibitor that also inhibited Null-VLPs treated with FCCP.
Figure 5. Activity of inhibitory compounds against adamantane sensitive and resistant strains of influenza M2. The most potent compounds identified as having specific activity against M2 were tested for activity against adamantane-sensitive strains of M2 (Udorn/307/1972) and adamantane-resistant strains of influenza M2 (VN/1194/2004, PR/8/1934, B/Lee/40 and Udorn/307/1972(V27A)). Rimantadine was tested at 20 uM and all other compounds were tested at 100 uM. All compounds tested showed specific activity against only adamantane-sensitive M2. Data shown represents the mean ± s.d. of triplicate data points.
Figure 6. Disruption of viral membrane integrity by a broad-spectrum membrane active compound. (a) Membrane active Compound #22 abrogates the fluorescent response in both M2-VLP and Null-FCCP assays. (b) Dose-response curves using Compound #22 demonstrate IC50 values for viral membrane disruption of 1.3-3.7 uM. (c) Compound #22 inhibits the infectivity of a panel of viruses (live influenza virus (Udorn/307/972) and reporter viruses for dengue (DENV), HIV, and HIV-pseudotyped VSV Env) in a dose-dependent fashion. (d) Compound #22 was applied to various cell types for 48 hours, and cellular viability was assayed by assessment of cellular ATP levels. The mean of at least two duplicate values within an experiment are shown (a-d).
Table 1. Optimization of the M2-VLP assay for HTS

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* Screening metrics for the M2-VLP assay using seven different variants of M2 and FCCP-VLPs.

Each VLP variant was screened in at least two separate experiments against a small-scale library of 71 known ion channel inhibitors (BioMol ion channel library), along with 6 positive (uninhibited) controls and 4 negative controls. Average screening metrics for each strain are shown, including %CV of the positive controls, signal-to-noise ((mean signal-mean background)/(st. dev. of background)), signal-to-background (mean signal/mean background), and the estimated Z’-factor. The Z’-factor was calculated using 100 μM amantadine controls for adamantane-sensitive strains and Null-VLP controls for adamantane-resistant strains and FCCP-VLPs.

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**AUTHOR CONTRIBUTIONS**


**COMPETING INTERESTS STATEMENT**


**REFERENCES**


Table 2. Profiles of validated hits identified using the M2-VLP assay

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<tr>
<td>Non-adamantane M2 inhibitors</td>
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<td>3</td>
<td><img src="image.png" alt="6,6-dimethyl-4-bicyclo[3.1.1]heptanyl" /></td>
<td>(1R,4R,5R)-6,6-dimethyl-4-bicyclo[3.1.1]heptanyl</td>
<td>1.74</td>
<td>4</td>
<td>86</td>
<td>96</td>
</tr>
<tr>
<td>4</td>
<td><img src="image.png" alt="isopinocampheylamine" /></td>
<td>(15.25,35.5R)-+isopinocampheylamine</td>
<td>3.35</td>
<td>10</td>
<td>102</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td><img src="image.png" alt="4-bicyclo[2.2.1]heptan-3-amine" /></td>
<td>4-bicyclo[2.2.1]heptan-3-amine</td>
<td>4.54</td>
<td>24</td>
<td>94</td>
<td>115</td>
</tr>
<tr>
<td>6</td>
<td><img src="image.png" alt="4,7,7-trimethylbicyclo[3.1.1]heptan-3-amine" /></td>
<td>4,7,7-trimethylbicyclo[3.1.1]heptan-3-amine</td>
<td>4.87</td>
<td>56</td>
<td>95</td>
<td>88</td>
</tr>
<tr>
<td>Adamantane ring inhibitors</td>
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<tr>
<td>7</td>
<td><img src="image.png" alt="1-adamantylmethanamine" /></td>
<td>1-adamantylmethanamine</td>
<td>0.14</td>
<td>0</td>
<td>77</td>
<td>108</td>
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<tr>
<td>8</td>
<td><img src="image.png" alt="2-adamantylmethanamine" /></td>
<td>2-adamantylmethanamine</td>
<td>0.15</td>
<td>6</td>
<td>87</td>
<td>95</td>
</tr>
<tr>
<td>9</td>
<td><img src="image.png" alt="1-(1-adamantyl)-N-methylmethanamine" /></td>
<td>1-(1-adamantyl)-N-methylmethanamine</td>
<td>0.27</td>
<td>3</td>
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<td>107</td>
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<tr>
<td>10</td>
<td><img src="image.png" alt="2-(1-adamantyl)-2-aminoethanol" /></td>
<td>2-(1-adamantyl)-2-aminoethanol</td>
<td>0.35</td>
<td>10</td>
<td>102</td>
<td>88</td>
</tr>
<tr>
<td>11</td>
<td><img src="image.png" alt="N-1-(1-adamantyl)-4-methyl-5-(4-methylphenyl)-1,1-dioxo-1,2-thiazol-3-amine" /></td>
<td>N-1-(1-adamantyl)-4-methyl-5-(4-methylphenyl)-1,1-dioxo-1,2-thiazol-3-amine</td>
<td>0.40</td>
<td>4</td>
<td>94</td>
<td>96</td>
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<td>12</td>
<td><img src="image.png" alt="2-(1-adamantyl)ethanamine" /></td>
<td>2-(1-adamantyl)ethanamine</td>
<td>0.42</td>
<td>23</td>
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<td>112</td>
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<td>13</td>
<td><img src="image.png" alt="2-(1-adamantyl)pyrroldine" /></td>
<td>2-(1-adamantyl)pyrroldine</td>
<td>0.59</td>
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<td>91</td>
<td>98</td>
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<tr>
<td>14</td>
<td><img src="image.png" alt="1-adamantyl carbamimidothioate" /></td>
<td>1-adamantyl carbamimidothioate</td>
<td>0.80</td>
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<tr>
<td>15</td>
<td><img src="image.png" alt="N-ethyladamantan-1-amine" /></td>
<td>N-ethyladamantan-1-amine</td>
<td>2.43</td>
<td>36</td>
<td>102</td>
<td>113</td>
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<tr>
<td>16</td>
<td><img src="image.png" alt="2-[2-(1-adamantyl)ethanamido]benzoic acid" /></td>
<td>2-[2-(1-adamantyl)ethanamido]benzoic acid</td>
<td>2.82</td>
<td>19</td>
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<td>17</td>
<td><img src="image.png" alt="N-1-(1-adamantyl)carboxamide" /></td>
<td>N-1-(1-adamantyl)carboxamide</td>
<td>3.65</td>
<td>34</td>
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<td>82</td>
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<tr>
<td>18</td>
<td><img src="image.png" alt="N-1-(1-adamantyl)oxamidine" /></td>
<td>N-1-(1-adamantyl)oxamidine</td>
<td>4.71</td>
<td>9</td>
<td>92</td>
<td>95</td>
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<tr>
<td>19</td>
<td><img src="image.png" alt="N-1-(1-adamantyl)pyridinylamide" /></td>
<td>N-1-(1-adamantyl)pyridinylamide</td>
<td>5.15</td>
<td>51</td>
<td>89</td>
<td>92</td>
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<tr>
<td>20</td>
<td><img src="image.png" alt="N-(1-adamantyl)pyrazine-2-carboxamide" /></td>
<td>N-(1-adamantyl)pyrazine-2-carboxamide</td>
<td>7.71</td>
<td>38</td>
<td>101</td>
<td>81</td>
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<tr>
<td>21</td>
<td><img src="image.png" alt="N-(1-adamantyl)pyrazine-2-carboxamide" /></td>
<td>N-(1-adamantyl)pyrazine-2-carboxamide</td>
<td>12.60</td>
<td>50</td>
<td>89</td>
<td>89</td>
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<tr>
<td>Broad spectrum, membrane active inhibitor</td>
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<tr>
<td>22</td>
<td><img src="image.png" alt="2-chloro-5-(3-ethyl-4-oxo-2-thiazol-1,3-thiazolyl)-5-ydenylmethyl-2-furylbenzoic acid" /></td>
<td>2-chloro-5-(3-ethyl-4-oxo-2-thiazol-1,3-thiazolyl)-5-ydenylmethyl-2-furylbenzoic acid</td>
<td>1.26</td>
<td>24</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>

b Dose-response IC₅₀ data was generated using the in vitro M2-VLP assay. The infectivity of live influenza virus (Udorn/307/1972) by plaque reduction assay, cellular viability of MDCK cells, and in vitro inhibition of FCCP-VLP activity were determined at the indicated compound concentrations. All experimental values represent the average of at least two independent experiments.