Random Mutagenesis Analysis of the Influenza A M2 Proton Channel Reveals Novel Resistance Mutants

Paul Santner, † João Miguel da Silva Martins, † Caroline Kampmeyer, † Rasmus Hartmann-Petersen, † Jonas S. Laursen, † Amelie Stein, † Christian A. Olsen, †, §, ‡ Isaiah T. Arkin, † Jakob R. Winther, † Martin Willemoe, ⁄• Martin Willemoe, *† and Kresten Lindorff-Larsen*†

†Department of Biology, Section for Biomolecular Sciences, Lindnerstrom-Lang Centre for Protein Science, University of Copenhagen, Ole Maaloes Vej 5, 2200 Copenhagen N, Denmark
‡Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark
§Center for Biopharmaceuticals, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark
∥Department of Biological Chemistry, The Hebrew University of Jerusalem, Edmond J. Safra Campus, Givat-Ram, Jerusalem 91904, Israel

ABSTRACT: The influenza M2 proton channel is a major drug target, but unfortunately, the acquisition of resistance mutations greatly reduces the functional life span of a drug in influenza treatment. New M2 inhibitors that inhibit mutant M2 channels otherwise resistant to the early adamantane-based drugs have been reported, but it remains unclear whether and how easy resistance could arise to such inhibitors. We have combined a newly developed proton conduction assay with an established method for selection and screening, both Escherichia coli-based, to enable the study of M2 function and inhibition. Combining this platform with two groups of structurally different M2 inhibitors allowed us to isolate drug resistant M2 channels from a mutant library. Two groups of M2 variants emerged from this analysis. A first group appeared almost unaffected by the inhibitor, M_089 (N13I, I35L, and F47L) and M_272 (G16C and D44H), and the single-substitution variants derived from these (I35L, L43P, D44H, and L46P). Functionally, these resemble the known drug resistant M2 channels V27A, S31N, and swine flu. In addition, a second group of tested M2 variants were all still inhibited by drugs but to a lesser extent than wild type M2. Molecular dynamics simulations aided in distinguishing the two groups where drug binding to the wild type and the less resistant M2 group showed a stable positioning of the ligand in the canonical binding pose, as opposed to the drug resistant group in which the ligand rapidly dissociated from the complex during the simulations.

The M2 proton channel is an important player in the cycle of infection for the influenza virus, where it facilitates viral entry by equilibrating the acidification of the endosome with the virus interior. Proton conduction is, however, not the only role of the M2 protein, and M2 is involved in assembly, packaging, and budding of the virus. M2 is therefore an important target for fighting influenza, and efforts are being made to generate M2 inhibitors that cannot easily be circumvented by mutation. Previous investigations of the details of M2 inhibition have led to the current proposed mechanisms of M2 inhibition by adamantane-based compounds that range from allosteric inhibition and physical occlusion to electrostatic hindrance.

As an example, inhibitors of M2 variant S31N, the most prevalent resistance mutation, have been published. To evaluate the usefulness of a particular drug lead, it is crucial to be able to estimate how readily resistance of the virus is acquired by mutation. Therefore, methods for discovering previously unknown M2 variants can enable us to understand how inhibitor resistance in M2 is achieved and further aid in the development of new inhibitor candidates.

By combining random mutagenesis with a modified selection and screening method for M2 function, we isolated a number of mutant M2 channels with decreased sensitivity to known M2 inhibitors. A subset of 12 of these mutant M2 channels were analyzed in greater detail, including their proton conduction as determined with the pHlux assay. Finally, 11 single side chain substitution M2 variants, derived from the isolated M2 random variants, were constructed to elucidate the
specific role of these side chains in M2 inhibition by five different known M2 inhibitors.

**EXPERIMENTAL PROCEDURES**

**Chemicals.** Amantadine (Amt) and Rimantadine (Rim) were purchased from Sigma-Aldrich. 3-Aza-spiro[5.5]undecane (Aza) was purchased from Enamine. Spiro[5.5]undecan-3-ylmethanamine (Spm) and spiro[5.5]undecan-3-amine (Spa) were chemically synthesized from spiro[5.5]undecan-3-one, purchased from Atlantic SciTech Group, Inc., as previously reported. A structural overview of the inhibitors can be found in Table 1.

**Plasmids, Escherichia coli Strains, and Growth Media.** Vector pMal-p2X harboring the relevant M2-encoding allele in Table 1.

**Table 1. Overview of the M2 Inhibitors Used in This Study**

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** Purification of Taq Polymerase.** To ensure a supply of the original Taq polymerase for the error-prone polymerase chain reaction (PCR) protocol used for random mutagenesis as described below, we decided to produce and purify this polymerase ourselves. We do not exclude the use of commercial polymerases; however, sometimes these are optimized to decrease mutation frequencies, which is not desired in this particular case. DHTs was transformed with plasmid pTaq37 grown overnight in LB medium, and used for inoculation of 0.5 L of preheated ABLB medium. Growth was continued with vigorous agitation and hourly addition of ampicillin to a final concentration of 100 μg mL\(^{-1}\) for 7 h to reach an OD\(_{366}\) of 8.5. Then Taq polymerase synthesis was induced by adding IPTG to the culture to a final concentration of 0.5 mM, after which the culture was left to incubate overnight. The next day the culture had reached an OD\(_{366}\) of 22. The cells were harvested by centrifugation at 3000g for 10 min. The cell pellet was resuspended in 30 mL of 50 mM Tris-HCl (pH 8.0) (buffer A) and incubated with 120 mg of lysozyme for 15 min at room temperature with 50 mM Tris-HCl (pH 8.0) and 25 mM KCl added until the final volume reached 200 mL. The lysate was heated for 1 h at 75 °C in a water bath and centrifuged for 20 min at 25000g, and the heat treatment was repeated. Streptomycin sulfate was added to a final concentration of 1% (w/w) to the supernatant over 5 min while it was being stirred on ice. The supernatant was recovered by centrifugation as described above, and ammonium sulfate was added, while the mixture was being stirred on ice, to 80% saturation. The precipitate was collected by centrifugation as described above, resuspended in 5 mL of buffer A, and dialyzed at 4 °C against several changes of the same buffer. DNasel (15 units) treatment of the dialysate for 1 h at 37 °C was performed after addition of 2.25 mM MgCl\(_2\) and 0.25 mM CaCl\(_2\) and subsequently terminated by addition of EDTA (pH 8) to a final concentration of 3 mM and heating to 65 °C for 15 min. After that, the sample was cooled on ice and centrifuged at 25000g for 15 min. The supernatant was recovered by centrifugation as described above, and eluted over a 5 column volume gradient from 0 to 1 M NaCl in buffer A. The eluate was collected as fractions of 1 mL, and eluted fractions were analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Fractions containing Taq polymerase were pooled and dialyzed at 4 °C with several changes of 25 mM Tris-HCl (pH 8.0), 50% glycerol, and 1 mM dithiothreitol (DTT). The purified protein was stored at −20 °C with 20% glycerol and 0.02% (w/v) sodium azide. To check for DNA impurities, PCRs were performed with appropriate deoxyribo-oligonucleotides as primers that anneal to the pTaq vector, or the E. coli gene pyrG35 for analysis of chromosomal DNA. The concentration of polymerase in the final stock was adjusted to the lowest of a series of 10-fold dilutions that resulted in no visible decrease in the amount of PCR product in a standard PCR as evaluated by agarose gel electrophoresis and subsequent ethidium bromide staining.
Random Mutagenesis. Sublibraries of mutant M2 encoding DNA were created on the basis of a previously described error-prone PCR protocol. The error-prone PCR mixture contained Taq polymerase prepared as described above, with oligo-deoxyribonucleotides annealing to the DNA regions flanking the M2 reading frame of the template CAACCTCGGATCGAGGAGGTTCGAGATTTCCGAAATTC (M2-IL-fw) and CGAGTCCGCCGCAAGCTTC (M2-IL-rv), 50 ng of the pMal-p2X plasmid containing the M2 wild type, and a varying volume of mutagenic buffer as previously described. The mutagenic buffer was prepared fresh for each reaction from stocks containing the metal ions (11 mM MgCl₂ and 1 mM MnCl₂) and nucleoside triphosphates (1.6 mM dTTP and 1.6 mM dCTP) separately. Thermal cycling conditions were a 2 min initial denaturation at 95 °C, followed by 30 cycles of amplification (95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min), and finally 10 min at 72 °C. PCR products from several incubations were pooled and purified with the QIAquick PCR Puriﬁcation Kit (Qiagen). With this procedure, we obtained two mutant libraries: low- and high-mutation frequency libraries, both covering the entire M2 gene reading frame of the plasmid.

To introduce the error-prone PCR product into the vector in place of the nonmutated M2 reading frame, a primer extension reaction based on the Quickchange (Stratagene) protocol was performed with 50 ng of the pMal-p2X plasmid encoding wild type M2, 250 ng of the product from the error-prone PCR described above, 2% DMSO, 0.2 mM dNTP, 15 mM MgCl₂, 1.25–2.5 units of Long PCR Enzyme Mix (Fermentas), and 5 µL of the supplied buffer in a total volume of 50 µL. A two-step cycling protocol was executed, following the polymerase manufacturer’s recommendations, with preheating at 94 °C for 1 min, 10 amplification cycles at 94 °C for 20 s and at 68 °C for 7 min, and 25 amplification cycles with 20 s at 94 °C and 7 min, increasing 5 s each cycle, at 68 °C, and finally 10 min at 68 °C. The products from multiple reactions were pooled, treated with the restriction enzyme DpnI (Fermentas), and puriﬁed as described above.

The low- and high-mutation frequency sublibraries, generated as described above, and a commercially prepared third sublibrary with a medium mutation frequency (BaseClear) were transformed into MC1061, yielding 50000 individual clones with 1 ± 1 nucleotide changes, 22000 with 11 ± 3 nucleotide changes, and 100000 with 4 ± 2 nucleotide changes, respectively, with the mutation rates estimated from sequencing of 10 independent clones from each library. This step was done to allow for E. coli K12 host speciﬁc methylation of the synthetic DNA, thereby facilitating the use of the libraries in a HisΔR’ genetic background such as LR1 and LB650. All the clones from the transformations described above were pooled, and plasmid DNA was prepared and stored at −20 °C before being used in the selection procedure. This latter DNA preparation encompassing all the sublibraries described above constituted the mutant M2 library.

Selection and Screening of M2 Variants. The screening and selection procedures were adapted from a previously published study. The selection procedure is based on growth of potassium uptake deﬁcient E. coli strain LB650, which is complemented by M2 expression to facilitate potassium transport. LB650 cells were transformed with the mutant M2 library described above and grown on LB plates containing 100 µM inhibitor Rim or Axs. Plasmids from clones that grew in the presence of M2 inhibitors were isolated and transformed into LR1 cells.

Next, in the screening, we exploited the fact that high expression levels of functional M2 induced by IPTG hamper bacterial growth, presumably by permeabilizing the inner cell membrane, leading to a disruption of the proton motive force. This effect can be relieved by the addition of M2 inhibitors that block the channel. Bacteria that harbor resistant M2 mutant channels, which no longer are inhibited, will not be able to grow under these conditions. LR1 cells transformed with plasmids isolated from LB650 cells that grew in the presence of inhibitors, as described above, were plated under three different conditions: (1) LB medium, (2) LB medium with the addition of 100 µM IPTG, and (3) LB medium with 100 µM IPTG and 100 µM M2 inhibitor added. Cells that were able to grow neither in the presence of IPTG nor in the presence of both IPTG and an M2 inhibitor were concluded to encode a potentially functional and inhibitor resistant M2 channel. The plasmids of these variants were isolated and sequenced.

The single-amino acid substitution M2 channels constructed as described above were also tested following the principles of the selection procedure. Cultures of LB650 harboring plasmids encoding the relevant M2 variant were resuspended in sterile 0.9% NaCl to an OD₆₀₀ of 1, diluted, and subsequently spotted onto culture plates with or without the addition of an M2 inhibitor as outlined in Results and Discussion.

Western Blot Analysis of M2 Protein Levels in LR1 Cells. Levels of protein were assessed by Western blot analysis, using a procedure previously described in detail. In brief, LR1 cells transformed with the relevant plasmids were grown as described below in the preparation for the pHluorin assay. The cultures were harvested and resuspended in 0.9% NaCl until the OD₆₀₀ reached 2.5. One milliliter of those suspensions was centrifuged at 5000g for 5 min at 4 °C, and the cell pellets were held at −80 °C. Prior to being analyzed, cell pellets were dissolved in 1 mL of 0.9% NaCl, centrifuged for 5 min at 5000g, and resuspended in 100 µL of SDS-PAGE sample buffer and 2 µL of 1 M DTT. The samples were then heated to 95 °C for 10 min and centrifuged at 13000g for 3 min, and the supernatant was subjected to SDS-PAGE on 7 cm × 8 cm, 9% acrylamide gels. Proteins were transferred from the acrylamide gel to 0.2 µm nitrocellulose membranes and blocked in a phosphate-buffered saline (PBS) buffer solution, containing 5% fat-free milk powder and 0.1% Tween 20. Membranes were then probed with mouse monoclonal anti-penta-His (Qiagen) and polyclonal goat anti-mouse immunoglobulins (DAKO Cytomation) as the primary and secondary antibodies, respectively.

Quantitative Analysis of M2 Proton Flux Inhibition. The pHlux assay, which we have described previously, measures the pH-induced change in ﬂuorescence of the intracellular GFP-based pH sensor pHluorin, constitutively expressed in LR1 cells, upon proton inlux when exposing them, expressing the M2 channel, to a rapid decrease in the extracellular pH of the cell suspension.

To prepare for the pHlux assay, cultures of LR1 clones harboring plasmids, encoding the relevant M2 variant, were grown in LB medium for 3 h before induction of protein synthesis by addition of 100 µM IPTG and continued incubation of the culture for 1 h. The cells were harvested and resuspended in assay buffer (McIlvaine buffer, consisting...
of 200 mM Na2HPO4 and 0.9% NaCl adjusted to pH 7.6 with 0.1 M citric acid) to give a cell density (OD600) of 0.25.

The cell suspension was then distributed into a 96-well plate containing different inhibitor concentrations and at time zero injected with 300 mM citric acid and 0.9% NaCl in a microplate reader using an injector module (Infinite F200 Pro, Tecan). The inhibitor sensitivity of mutant and wild type M2 channels was tested with the pHlux assay for five different drugs (Table 1) under different conditions: at a concentration equal to the Ki value of the inhibitor determined for wild type M2.24 (Rim, Ki = 6 nM; Amt, Ki = 63 nM; Azs, Ki = 189 nM; Spm, Ki = 304 nM; and Spa, Ki = 47 nM), at a concentration 20 times the Ki value for the inhibitor determined for wild type M2, and finally at constant values of 100 μM for all variants. Relative M2 proton conductance activities were calculated as the percentage of proton flux in the presence of the given inhibitor concentration compared to the proton flux of the same variant in its absence.

**Molecular Dynamics Simulations.** Simulations were performed for selected M2 variants and the same variants in the presence of the drugs tested in the experiments. All calculations were performed using Gromacs 5.0.6.40 The CHARMM27 force field was used for protein and water, with CHARMM36 force field parameters used for lipids. The protonation states of the drugs were predicted with ChemAxon’s pK value predictor, with major microspecies at pH 7 being parametrized using CGenFF.41 The original protein structure [Protein Data Bank (PDB) entry 2L0J] was mutated to have the same amino acid sequences as that of the structure (Figure 1 and Table 2) as outlined in Experimental Procedures. Fifty-four clones (30 in the group selected on Rim and 24 in the group selected on Azs) encoded previously undescribed mutations that lead to M2 channels that were either insensitive or less sensitive than wild type M2 to the tested inhibitors, whereas eight mutant clones (six in the group selected on Rim and two in the group selected on Azs) contained known resistance substitutions (e.g., S31N and V27A).

On the basis of the sequence of 12 inhibitor resistant mutant M2 channels isolated from the library, which each contained two or more amino acid substitutions, we synthesized and characterized 11 M2 mutant proteins with a single amino acid side chain substitution (Figure 1 and Table 2) as outlined in Experimental Procedures.

The choice of these individual side chain substitutions was made on the basis of a number of criteria. First, the residues are located in the transmembrane region (residues 25–46) but are not known to be important for proton conduction (residues 26, 27, 30, 31, 34, 37, 38, and 41) or are situated at positions previously identified to elicit inhibitor resistance.44–46 Second, positions for altered side chains were discarded as being important for inhibitor resistance on the basis of a comparison with a number of inhibitor sensitive mutant clones identified in the screening process (data not shown). In addition, positions 44–46 were also included as these positions are located at a previously suggested drug binding site situated far from the drug binding sites suggested from the most recent analyses.16,58,59 Furthermore, positions 45 and 46 are pore-lining residues, a feature shared by residues in known inhibitor resistant mutant M2. We chose these single-amino acid substitutions moreover to be as different as possible compared to the wild type chain with regard to size, polarity, and charge. Applying these criteria

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**RESULTS AND DISCUSSION**

We have performed a selection and screening of a mutant M2-encoding library to identify inhibitor resistant variants of the influenza M2 channel and have thereby gained insight into which mutations may lead to adaptation of the influenza virus to treatment with M2-directed drugs. A total of 213 mutant clones, 136 selected on Rim and 77 selected on Azs, were recovered in the selection for complementation of the potassium uptake requirement of LB650 with Rim representing the adamantane drug family and Azs that of spiro compounds (Table 1). All mutants were subsequently screened for decoupling of the proton gradient of LR1 cells as described in Experimental Procedures. Fifty-four clones (30 in the group selected on Rim and 24 in the group selected on Azs) encoded the single-amino acid side chain substitution (Figure 1 and Table 2).
resulted in the construction of the following single-amino acid substitution M2 variants (Table 2): I32M, I35L, and I42F, derived from mutant M2 proteins found to be resistant to inhibition by Rim in selection and screening; D44H, R45S, and L46P, derived from mutant M2 proteins resistant to inhibition by Azs; and A29T, I33S, and I39F, derived from mutants resistant to both inhibitors. The single-substitution L43P variant was both obtained from a selection on Rim and constructed as described in Experimental Procedures to verify that the obtained resistance or small amount of protein present in the LR1 cells was not caused by secondary mutations occurring outside the reading frame of the M2 fusion protein. The L40R variant was constructed to more fully cover the region of M2 between residues 39 and 44.

**M2 Protein Sequence Alignment.** From an alignment of 18445 full length influenza A M2 protein amino acid sequences within the Influenza Research Database (http://fludb.org/, April 13, 2016, data not shown), a canonical protein sequence was derived that differed at only four positions compared to the wild type M2 sequence used in this study: M1F, L54F, N82S, and S31N. The S31N variant of M2 is now prevalent with a 76% occurrence in the influenza A virus population. The following single-amino acid side chain substitutions covered in our study were found in the database and are listed along with their occurrence in parentheses among the 18445 compared sequences: V27A (93), A29T (21), S31N (14126), I32M (2), and L46P (8). Among the amino acid positions of the M2 sequence analyzed in this study, a minimum of 99% are generally conserved in all database sequences, except position 43, being only slightly more than 50% conserved. M2 protein sequence positions 28, 31, 36, and 43 that are part of the transmembrane region of M2 and could be expected to be highly conserved appear not to be so from analysis of the M2 sequence database. Changes in amino acid side chains at positions 28 and 36 were not obtained in our work (Figure 2); however, position 28 was altered in two M2 variants, and these were still sensitive to an inhibitor (V28M and V28L, data not shown).

**Expression Levels and Functionality.** We performed Western blotting of the investigated mutant M2 proteins (Figure 3) to establish if the proton conductance among the variants could be directly compared as a function of the M2 protein and not because of differences in expression levels. This Western blot analysis showed protein levels similar to that of the M2 wild type reference for all but one analyzed sample when the expression cultures were normalized to OD600; the exception is L46P that is found at a reduced level. Side chain position 46 is thought to be important

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<th>M2 protein variant</th>
<th>screening and selection inhibitor</th>
<th>amino acid side chain alterations with respect to wild type M2</th>
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<tr>
<td>M_018</td>
<td>Rim</td>
<td>W15C I39F S64I K78N Q80R S82R D85Y S89C</td>
</tr>
<tr>
<td>M_046</td>
<td>Rim</td>
<td>W15C I42F</td>
</tr>
<tr>
<td>M_060</td>
<td>Azs</td>
<td>V7L G16C L46P</td>
</tr>
<tr>
<td>M_067</td>
<td>Rim</td>
<td>G16C A29T I42F D85N</td>
</tr>
<tr>
<td>M_089</td>
<td>Rim</td>
<td>N13I I35L F47L</td>
</tr>
<tr>
<td>M_091</td>
<td>Rim</td>
<td>S2R P10Q I32M H90Q F91V</td>
</tr>
<tr>
<td>M_111</td>
<td>Azs</td>
<td>R18C I33F L46P E97G</td>
</tr>
<tr>
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<td>Rim and Azs</td>
<td>G16C I33S R77G S93T E97K</td>
</tr>
<tr>
<td>M_213</td>
<td>Azs</td>
<td>A29T I39F</td>
</tr>
<tr>
<td>M_250</td>
<td>Azs</td>
<td>S2C R45S V84E</td>
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<td>I1IT N13S G16E N20S V28I S31N L43T F54R H57Y R77Q K78Q A86V S89G S93N</td>
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| Data a | Amino acid substitutions constructed and evaluated as single-amino acid side chain M2 variants are shown in italics. Those mutations that appear more than once in the collection of resistant variants are shown in bold. The L43P substitution was isolated from the library as a single substitution.

Figure 2. Amino acid substitutions in M2 leading to a reduced affinity for adamantanes and spiro compounds. Results from selection and screening for mutant M2 clones performed as described in Experimental Procedures employing the inhibitors Rim and Azs (Table 1). Side chain substitutions occurring in M2 mutant proteins selected on Rim (top) and Azs (bottom). The bar height indicates how frequently a mutation was observed. The bar color indicates the amino acid; chemically similar amino acids have similar colors. Positions and wild type amino acids are indicated on the x-axis.
maintaining the transmembrane helical structure of M2, and the leucyl to prolyl substitution is likely to compromise this structure as it would for α-helices in general.

Via the complementation of potassium transport in LB650 by M2 following the screening principle described in Experimental Procedures, all LB650 cells expressing the constructed single-side chain substituted M2 variants grew like the wild type at low potassium concentrations (Figure 4), thus indicating normal potassium transport properties in the absence of channel inhibitors.

However, in the pHlux assay, variants S31N, I35L, and D44H showed reduced proton conduction levels similar to the

Figure 3. Expression levels of M2 variants. Western blot analysis of protein extracts from M2 mutants encoding single-amino acid side chain substitutions and subjected to SDS–PAGE. The extracts from cultures producing the mutant variants of M2 were separated by SDS–PAGE, blotted, and probed with anti-His, antibodies as described in Experimental Procedures. (A) Extracts of cultures producing the various M2 variants and adjusted to the same OD_{600}. (B) SDS–PAGE of the 40-fold concentrated extract of the L46P-producing culture from panel A compared to wild type M2.

Figure 4. Complementation of K⁺ transport by M2 variants in the potassium transport deficient strain, LB650. Spot test of cells transformed with plasmids encoding M2 variants. Experiments were performed as described in Experimental Procedures. LB650 cells harboring the relevant plasmids were spotted on LBK and LB medium as indicated. Wild type M2 (Wt) served as a positive control, and pMal-p2X (Vector) as a control, so that only cells with functional M2 channels can grow effectively at low potassium concentrations. The cell dilutions refer to cultures with an initial OD_{600} of 1.

Figure 5. Proton fluxes determined for mutant M2 proteins synthesized in LR1 and using the pHlux assay. Experiments were performed as described in Experimental Procedures. For comparison, results for cells synthesizing wild type M2 in the absence of inhibitor (Wt) and in the presence of 100 μM Rim (Wt inh) and cells harboring only pMal-p2X (Vector) are shown. Error bars indicate population standard deviations.
level of cells transformed with vector alone or the wild type M2 channel inhibited by Rim (Figure 5), demonstrating that potassium transport properties do not necessarily translate into those of proton conduction.23,35,60 This observation underscores the importance of analyzing the proton conductivity of M2 variants selected for inhibitor resistance using the potassium complementation selection system.

M2 protein side chain positions 44 and 45 (Figure 1) are highly conserved and are suggested to be important for proton exit and conduction as well as the stability of the protein.44,48,51 Nevertheless, we found that R45S is a fully functional M2 channel and the proton conduction displayed by D44H is greatly increased when combined with side chain substitution G16C in variant M_272 (Figure 6 and Table 2).

Sequence Context of Drug Resistant M2 Variants. In general, M2 variants with multiple-amino acid side chain substitutions (Table 2) and single-side chain substituted M2 variants analyzed in the pHlux assay were indeed less sensitive to inhibition by the inhibitor for which resistance originally was selected (compare Table 2 and Figure 6). Exceptions to this were A29T, I39F, R45S, and M_267 (Table 2 and Figures 6 and 7) that were selected in the presence of the less potent inhibitor Azs.

A comparison of multiple-side chain substituted M2 variants and those constructed to contain only one of these side chain substitutions revealed some interesting features. The amino acid substitutions, A29T and I42F, seemed to have synergistic effects on the reduced inhibitor sensitivity found for M_067 (Table 2), because the individual M2 variants harboring a single amino acid substitution were significantly more prone to inhibition (Figure 7) than M_067 (Figure 6). The three modeled structures of A29T, I42F, and M_067 did not provide clues about the observed differences in inhibition when compared to wild type M2, and only I42F displayed modest structural differences caused by the substitution with a bulkier side chain (data not shown). Analysis of M2 variant M_213 (Table 2) revealed a similar inhibitor desensitizing synergy between A29T and in this case I39F as M_213 was clearly less sensitive to inhibition by Rim (Figure 6) than the M2 variant carrying only the I39F substitution (Figure 7). Furthermore, M_046 (Table 2 and Figure 6) harboring the I42F and W15C substitutions was slightly less sensitive to inhibitors than was the M2 variant carrying the I42F substitution alone (Figure 7).

Figure 6. Relative activities of multiple-amino acid side chain substituted M2 variants. The relative proton flux (percentage of proton flux in the presence of an inhibitor compared to that in the absence of an inhibitor of the same variant) was determined with the pHlux assay at three different inhibitor concentrations: (first column) concentration corresponding to the K_I for wild type M2, (second column) 20 K_I, and (third column) 100 μM inhibitor as described in Experimental Procedures. Brackets indicate the inhibitor in which the presence of a particular resistant M2 variant was selected. Wild type M2 and swine flu M2 serve as controls. Error bars show the standard error, and asterisks indicate a significant, standard errors considered, higher relative activity compared to the wild type.
This observed reduced inhibitor sensitivity of M_046 compared to that of 142F must be linked to the additional side chain substitution, W15C, in M_046 even though the position of W15 outside of the transmembrane region makes it more difficult to interpret. It has previously been shown that the sequence context of single-amino acid substitutions has an influence on channel activity, stability, and resistance.45,47,54,65 For example, amino acid substitutions that confer resistance but destabilize the M2 protein and thus compromise virus progression may be accompanied by stabilizing substitutions and can revert to their original sequence in the absence of drug selection pressure. In conclusion, the influence of context seems to explain the observed differences in relative activity upon inhibition of the constructed single-amino acid side chain substituted M2 variants (Figure 7) when compared to their multiple-side chain substitution counterparts (Figure 6 and Table 1). A special case is the L46P substitution,66 discussed above (see also Figures 3–5), that shows greatly reduced sensitivity to inhibition when tested in the pHlux assay but also clearly has a dramatic effect on the stability and/or the expression level of the protein. However, when it is present in the multisubstitution variants M_60 and M_111 (Table 2 and Figures 6 and 7), the effect of reduced sensitivity to inhibitors is much less pronounced.

The M2 mutant proteins described in this work (Table 2 and Figures 6 and 7) can be split into two groups when comparing their resistance behavior. The first group appeared almost unaffected by the presence of an inhibitor (M_089, M_272, I35L, L43P, D44H, and L46P), as also reported for known drug resistant M2 variants V27A, S31N, and swine flu. Members of the second group, comprising the remaining tested M2 variants, were still inhibited, although to a significantly lesser extent than was wild type M2. In the (relatively short) 50 ns MD simulations of these variants with an inhibitor, we generally found stable positioning and preservation of many of the key interactions present in the canonical binding pose to the wild type protein (Figure 8). MD simulations involving other side chain substitutions giving rise to drug resistance in M2 variants (V27A, S31N, swine flu, M267, I32M, M067, A29T, and I42F) revealed an unstable positioning of inhibitors inside the pocket, leading to the ligand exiting from the pore within 50 ns of simulation.

**Drug Resistance and Structural Properties of M2 Inhibitors.** The type and position of amino acid substitutions...
in the analyzed M2 mutant proteins do not reveal a clear distinction with respect to what inhibitor was employed in the selection and screening process (Table 2 and Figure 2). This is somewhat surprising, considering that the chemical structure of spiro compounds Azs, Spa, and Spm differ substantially from that of the adamantane-based compounds, Amt and Rim (Table 1).

M2 variants found to be desensitized to inhibition by one spiro compound proved to be desensitized to all spiro compounds (Figures 6 and 7). In contrast, M2 mutant proteins A29T, I33S, M_046, M_060, and M_134 all revealed a significantly more prominent inhibition by Rim than by Amt (Table 2 and Figures 6 and 7) despite the common molecular traits that Amt and Rim share; for this reason, they are regarded as being similar in their mode of binding to M2.

However, Rim contains a chiral center and as such appears as a racemate in selections, screenings, and analyses, and this mix of stereoisomers has until recently been vastly ignored. The occurrence of two stereochemically different forms of Rim in the racemate may hold clues about the observed difference in inhibition between Rim and Amt with mutant M2 proteins in this study.

**M2 Protein Structural Features in the Observed Drug Resistance.** Two distinct mechanisms for achieving reduced inhibitor sensitivity of mutant M2 proteins could be identified on the basis of MD simulations, when compared to our experimental results. Figure 9 shows the M2 channel pore profiles for the last 10 ns of the MD simulation performed for the wild type and the V27A and S31N mutants, for which the pore entrance accessibility varied dramatically, as also discussed previously.

It was also previously proposed that there may be a secondary gating mechanism coordinated by V27, restricting access of water and inhibitors to the protein pore. Wild type M2 clearly displayed this secondary gating mechanism, which limited access to the channel and was further blocked by the main histidine gate formed by H37. The secondary gate was greatly diminished in the V27A variant, leaving a channel with a radius the size of a water molecule that allowed for direct access to the main gate. We observed almost the opposite situation for the M2 mutant variant S31N, in which pore channel access appeared to be more restricted than for wild type M2, combined with a reduced size pocket for inhibitor binding. Interestingly, our experimental data indicate that proton conductivity is unaffected by removal of the secondary gate in V27A (Figure 5) despite an increased pore radius around this site (Figure 9). The MD simulation of the S31N M2 variant indicated that the inhibitor binding pocket is occupied by the asparagine side chains in this variant, resulting in steric hindrance of the inhibitor with respect to its binding site.

As indicated by this work, drug resistance of influenza A when mediated through side chain substitutions in the M2 protein not only targets the upper, N-terminal, extraviral transmembrane region but also seemingly points toward alternative hot spots. This observation may be of use when designing new inhibitors of M2 aimed at overcoming the ease by which influenza A appears to escape drug treatment directed toward M2 function.

**CONCLUSIONS**

The pHlux assay used in this work for the characterization of mutant M2 channels is based on steady state measurements of proton flux through M2 in the absence of, or when present after, pre-equilibration with an inhibitor at neutral pH. Ultimately, this enables us to determine the dissociation constant for the complex between M2 and inhibitors, as demonstrated in ref 24. Electrophysiological experiments on M2, on the other hand, are typically analyzed in terms of the kinetics of binding of an inhibitor to channels already opened at a decreased pH focusing mostly on on-rates, though recently both on- and off-rates were determined for a set of M2 inhibitors. Interestingly, the kinetic experiments reveal that changes in affinity stem from changes in both on- and off-rates when altering inhibitor chemistry or studying mutant channels. The two different techniques therefore complement each other.
when studying the relative potency of an inhibitor for binding to M2 or binding of the inhibitor to mutant channels.

In this work, we have combined a genetic selection and screening system with biochemical assays and computational modeling to study inhibitor resistance in the influenza M2 proton channel. The selection system was validated by finding several known resistance mutants, revealed cross-resistance to several inhibitors, and suggested that our approach can be used to find potential resistance mutations during drug development efforts. When studies expand to larger numbers of mutants and drugs, this type of analysis will also prove useful in developing predictive methods for drug resistance.

### AUTHOR INFORMATION

**Corresponding Authors**
*E-mail: lindorff@bio.ku.dk.*  
*E-mail: willemoes@bio.ku.dk.*

**ORCID**
Christian A. Olsen: 0000-0002-2953-8942  
Jakob R. Winther: 0000-0001-6995-9154  
Martin Willemoes: 0000-0003-1689-2712  
Kresten Lindorff-Larsen: 0000-0002-4750-6039

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**Notes**
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### ABBREVIATIONS

Amt, Amantadine; Azs, 3-aza-spiro[5.5]undecane; DTT, dithiothreitol; pHlux assay, fluorescence-based M2 proton conduction assay; MD, molecular dynamics; Rim, Rimantadine; Spa, spiro[5.5]undecan-3-amine; Spm, spiro[5.5]:undecan-3-ylmethanamine.

### REFERENCES


