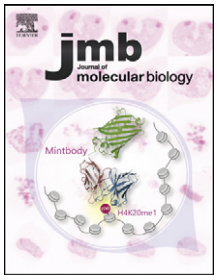

Mapping the Resistance Potential of Influenza's H^+ Channel against an Antiviral Blocker

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http://dx.doi.org/10.1016/j.jmb.2016.08.007
Edited by James Bowie

Abstract

The development of drug resistance has long plagued our efforts to curtail viral infections in general and influenza in particular. The problem is particularly challenging since the exact mode of resistance may be difficult to predict, without waiting for untreatable strains to evolve. Herein, a different approach is taken. Using a novel genetic screen, we map the resistance options of influenza's M2 channel against its aminoadamantane antiviral inhibitors. In the process, we could identify clinically known resistant mutations in a completely unbiased manner. Additionally, novel mutations were obtained, which, while known to exist in circulating viruses, were not previously classified as drug resistant. Finally, we demonstrated the approach against an anti-influenza drug that has not seen clinical use, identifying several resistance mutations in the process. In conclusion, we present and employ a method to predict the resistance options of influenza's M2 channel to antiviral agents ahead of clinical use and without medical hazard.

Introduction

The threat from influenza fully embodies Bacon's assertion about the importance of a continuous search for new remedies[†]. One of the main causes of this predicament is the notoriously low replication fidelity of the viral genetic information. For example, the error rates of influenza RNA polymerase is 10^{-4} [1], in comparison to replication errors of mammalian genomes that are 6 orders of magnitude lower. This leads to constant genetic drifts and shifts that change antigen epitopes and viral drug targets. In the former case, immunity is abolished, necessitating new vaccinations, while in the latter, cognate antiviral agents may become ineffective.

One of the challenges in combating the aforementioned evolutionary strategy of influenza is the need to predict its specifics and plan for it ahead of time. New vaccines cannot be generated without specific knowledge of what the new epitopes will be. Likewise, effective new antiviral drugs cannot be designed if the drug target keeps changing. In the meanwhile, the medical community can only wait until a new viral isolate is identified that is refractive to current therapy. Only then can the resistant virus be examined and the exact resistance mechanism (i.e., mutations) identified. We realize that one can accelerate evolutionary processes in laboratory experiments with the hopes of developing resistance [2,3]. However, such gain-of-function experiments may pose medical risk [4,5], if the mutated virus were to escape the laboratory confines.

In the current study, we decided to develop a different route to predict the resistance mechanisms of influenza against antiviral drugs. Of the two general classes of antiviral drugs against influenza—neuraminidase inhibitors and M2 blockers, we chose the latter. Our reasoning stemmed from the fact that amantadine was the first antiviral drug to be approved by the FDA against influenza A and was one of the first antiviral drugs in general [6,7]. Furthermore, resistance to aminoadamantanes is prevalent, providing ample positive controls for our strategy [8].

Amantadine (Symmetrel®) was developed and approved by the FDA in the 1960s in the context of containing the Asian flu (H2N2) pandemic outbreak [6,7]. Rimantadine (Flumadine®) was approved by the FDA in 1994. Medically, both drugs were used extensively as prophylactic agents against influenza

A, resulting in the appearance of widespread resistance [10]. For example, in recent epidemic and pandemic outbreaks, more than 90% of the isolates were resistant strains [11–14]. As a consequence, the drugs are no longer recommended as a clinical treatment for flu [15], although combination therapy trials seem promising [16–19].

The viral target of aminoadamantanes in influenza A is M2 [20], a 97-aa transmembrane protein. In its homotetrameric form [21], M2 functions as a pH-dependent H^+ channel [22], playing a crucial role in the virus' infection cycle [23,24].

Experimental and computational studies have identified the binding sites of the drugs to reside in the transmembrane segment of the protein around residues Ser31 and Gly34 [25–27]. This in turn is consistent with the fact that resistance mutations are located in that region of the protein [20].

Previous studies have examined the ion selectivity, amantadine resistance, and conductance of a selected set of M2 mutants [28–30]. While this work provides interesting insights on the mechanisms of H^+ conductance and drug resistance, its reliance on site-directed mutagenesis does not afford a comprehensive exploration of mutations in the channel that may confer resistance to aminoadamantanes.

Our approach is based on creating a genetic screen in bacteria that may enable us to map the resistance potential of influenza's M2 drug target against its cognate antiviral drug. Since all of these experiments are conducted on an isolated viral protein in a bacterial host, they carry no medical risk whatsoever.

Armed with an appropriate genetic screen, we extensively map the resistance potential of the virus against aminoadamantanes. These results may guide future attempts to thwart influenza infectivity and provide a general route to understand and curb drug resistance in general.

Results

Genetic screen for viral protein activity

The purpose of this study was to map the resistance potential of influenza against M2 inhibitors (See Fig. 1). Toward this end, we constructed a genetic selection in which bacteria are unable to grow due to a defect that can be overcome by expression of M2 (see general scheme in Fig. 2).

Since the M2 protein is an ion channel [22], the bacterial strain that we chose is one that is defunct in this particular functionality, namely K^+ transport [31]. As shown in Fig. 3, the K^+-uptake-deficient bacteria are readily able to grow in K^+-rich media but are nonviable when incubated at low K^+ concentrations. In these limiting conditions (low $[K^+]$), expression of the M2 channel is able to revive bacterial growth

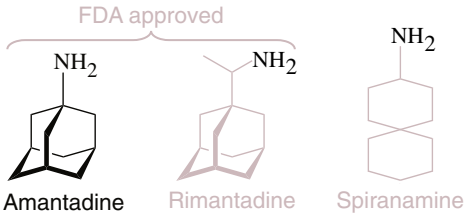

Fig. 1. Structures of the different compounds used in the study. On the left are the only two compounds approved for use by the FDA against the influenza A M2 channel. Spiranamine, shown on the right, was reported to inhibit some aminoadamantane-resistant flu strains [9]. Molecules are shown in their uncharged form.

(Fig. 3, middle plate). Addition of the antiviral, channel-blocker rimantadine abrogates the positive effect of the viral channel, and the bacteria are once more unable to grow under limiting conditions. However, the S31N mutant of M2 that is resistant to the channel blocker [29] is capable of sustaining bacterial growth under these limiting conditions,

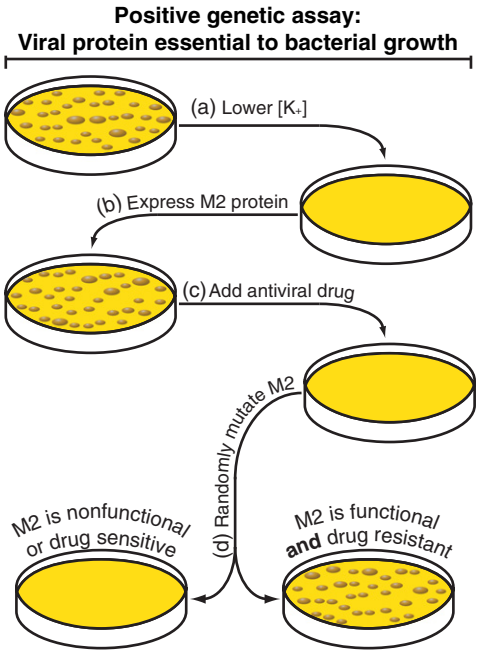

Fig. 2. The positive genetic selection used to map the resistance options of influenza's M2 channel against its cognate antiviral drug. (a) Lowering the $[K^+]$ renders the bacteria nonviable. (b) Expression of M2 enables the bacteria to grow under limiting conditions. (c) Addition of an antiviral drug that inhibits M2 results in bacterial death. (d) Random mutagenesis of M2 can identify drug resistance when the mutated protein is able to sustain bacterial growth even when the culture is exposed to the antiviral drug. Note that the specified actions may be performed either in successive manner (as illustrated) or in parallel.

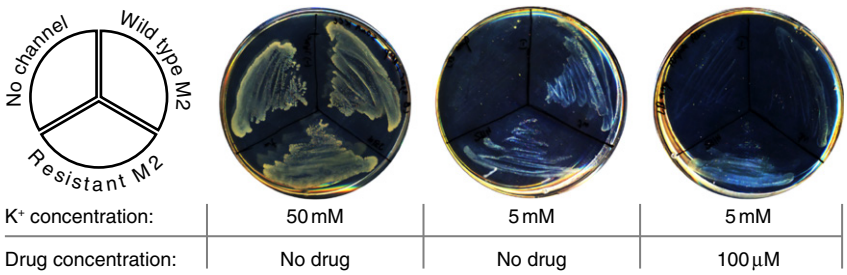

Fig. 3. M2 expression is able to recover the growth of a deficient bacterial strain. *Escherichia coli* LB650 [31] are able to grow on K^+-rich medium (left plate) but are unable to grow on K^+-poor media unless they express the M2 channel (middle plate). In the presence of an inhibitor (rimantadine), only a resistant strain of M2 (S31N mutant [29]) is able to rescue bacterial growth in K^+-poor media (right plate).

even in the presence of the antiviral drug (Fig. 3, right plate).

It is important to note that only a small amount of functional viral protein is needed to rescue the growth of K^+-transport-deficient *E. coli*. As shown in Fig. 4, when the channel was expressed at elevated levels, bacterial growth was retarded, most likely due to the deleterious effect of the channel's H^+ conductance [32]. This effect will be used for further examination below.

Evolution of resistance

As mentioned above, we have established a genetic selection in which the function of M2 is essential to bacterial viability. We can now utilize this system to map the resistance potential of the protein against an inhibitory antiviral drug (Fig. 2). First, a library of viral M2 protein mutants was created by error-prone PCR. Subsequently, the library of mutated viral genes was inserted into K^+-uptake deficient *E. coli*. Finally, the bacteria were grown on K^+-poor media in the presence of the inhibitor (Fig. 2d). The only bacteria that grew under such conditions were those that harbored an M2 protein that is both functional and resistant to the inhibitor.

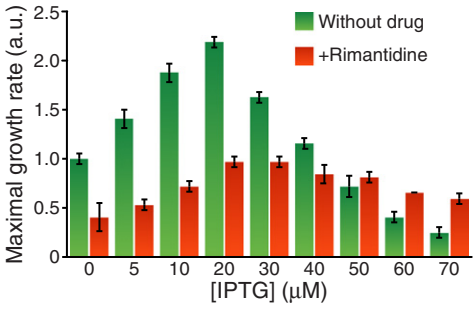

Fig. 4. Effect of channel concentration on the growth rate of K^+-transport-deficient bacteria. Maximal growth rates of LB650 *E. coli* expressing the wild-type M2 channel are plotted as a function of different inducer concentrations ([IPTG]), and as a function of 50 μM rimantadine. The growth rate was normalized to the rate without induction.

In several independent rounds of directed evolution, 44 different mutants were obtained by sequencing the plasmids of bacterial colonies that grew on low [K^+] agar plates. Since the binding site of the drug is known to be located in the transmembrane domain of the protein [25–27,29,33,34], we focused on mutations in that region (Ser22–Asp44). Additionally, mutations to and from cysteine residues could have unexpected consequences arising from the particularities of the bacterial expression system and were thus ommitted from subsequent analysis.

Finally, due to the random nature of the mutagenesis process, several of the resistance variants contained multiple mutations. In such instances, in order to analyze one mutation at a time, we reverted all other mutations to wild-type sequence.

The next step was to subject the individual resistant mutants to quantitative analysis. This was achieved by measuring the growth rates of bacteria that express the particular viral channel in liquid media and the impact of rimantadine thereupon. The results shown in Fig. 5a were compared to bacteria that expressed the wild-type viral channel, where addition of the antiviral drug lowered the growth rate substantially. In contrast, growth rates of bacteria that expressed resistant protein were less affected by the presence of the antiviral drug (compare green and red bars in Fig. 5a).

Assay reversal

One of the advantages of the particular genetic selection system that we have chosen is that it can be used in a negative mode (see schematic in Fig. 6). Expression of the viral channel at elevated levels and under non-limiting conditions is detrimental to bacterial growth, most likely due to excessive H^+ leakage of the host's membrane. Note that typical *E. coli* may be used in this assay. In this instance, addition of the antiviral drug will result in enhanced growth of the bacteria [32].

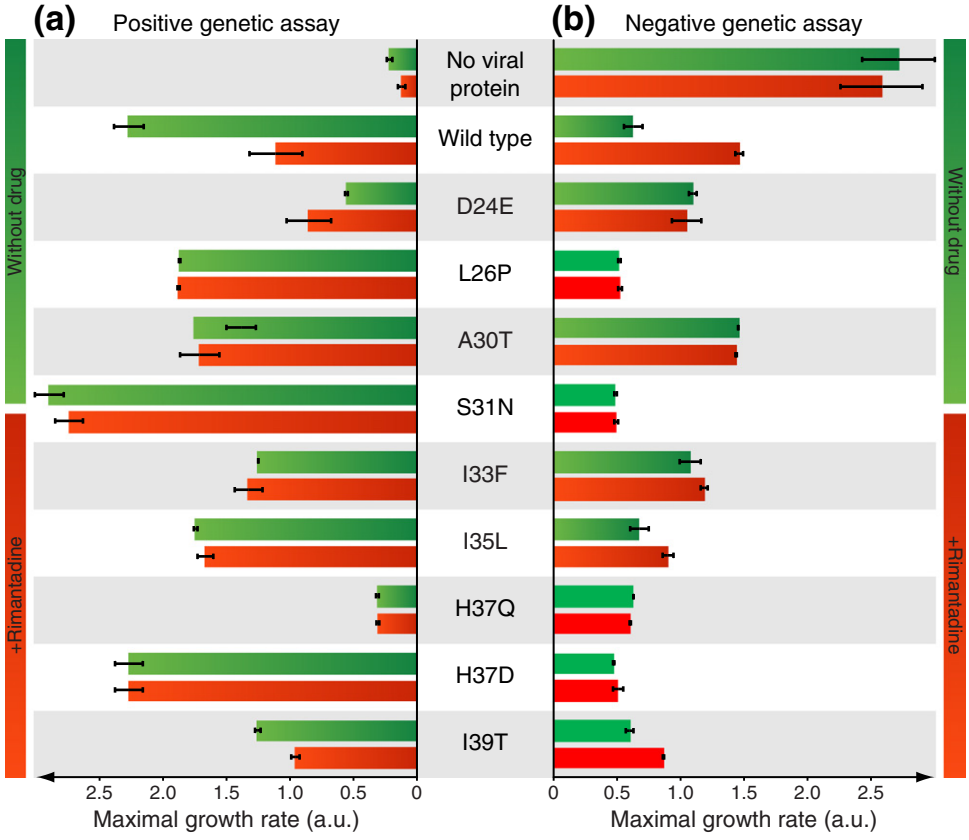

Fig. 5. Impact of drug-sensitive and -resistant viral proteins on the growth of K^+-transport-deficient bacteria (a) or regular *E. coli* (b). The concentration of the inducer IPTG was 5 μM or 50 μM in the positive (a) or the negative (b) genetic assays, respectively. The effect of rimantadine on the same bacterial cultures is shown in red. (a) In the positive assay, when the red bars are appreciably smaller than the green bars, it indicates that the channel is sensitive to rimantadine. (b) The opposite is true in the negative assay. For comparison, bacteria that do not express the viral protein are shown on top. Error bars represent the standard deviation of two or more experiments.

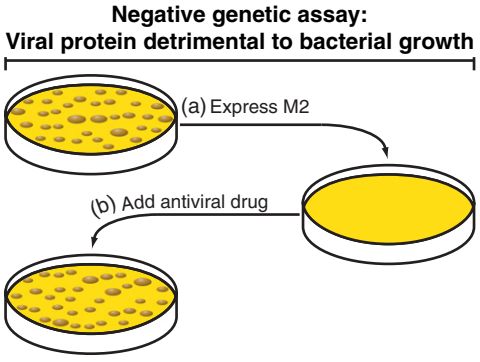

Fig. 6. The negative genetic assay used for verifying drug resistivity. (a) Expression of the viral channel at elevated levels and under non-limiting conditions is detrimental to bacterial growth. (b) Addition of an antiviral drug that inhibits the viral protein results in bacterial growth. Note that the specified actions may be performed either in successive manner (as illustrated) or in parallel.

Taken together, we have established two genetic assays: in the first assay (Fig. 2), M2 is essential to bacterial viability. Therefore, the "positive" genetic assay can be used to identify resistant mutations. In the second assay (Fig. 6), the viral protein is detrimental to the bacteria. Consequently, the "negative" genetic assay can be used to identify new antiviral drugs through screening [35]. Finally, the negative assay can provide additional verification of drug resistability of the mutants identified by the positive assay. Therefore, the resistant mutants that were analyzed in the positive assay were subjected to analysis in the negative assay, yielding reciprocal results (compare Fig. 5 panels a and b).

The results of the negative genetic assay indeed demonstrate that the expression of wild-type M2 at elevated levels hinders bacterial growth appreciably. Similar growth retardation is obtained upon expression of the resistant viral proteins. However, the effect of the antiviral drug on the bacterial cultures

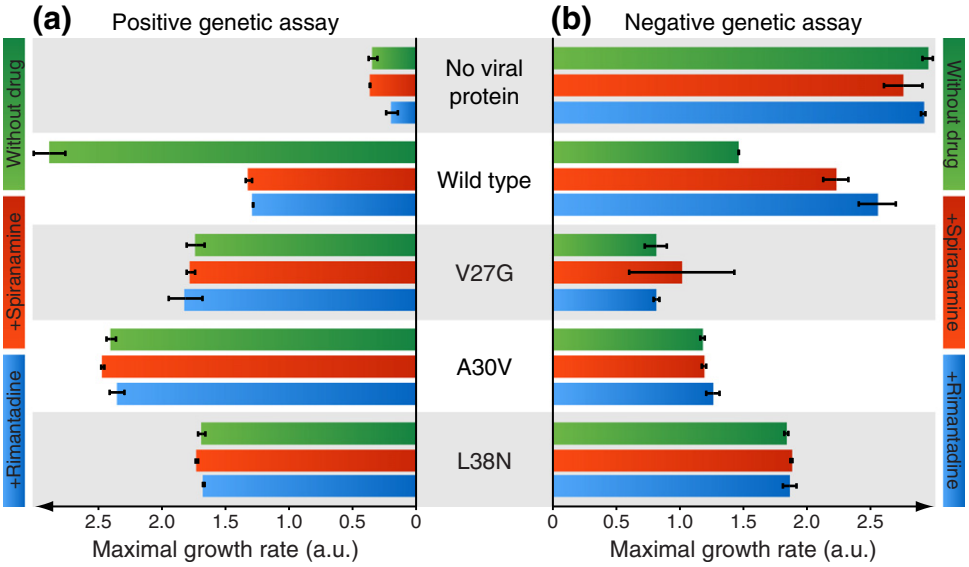

Fig. 7. Impact of drug-sensitive and -resistant viral proteins on the growth of K^+-transport-deficient bacteria (a) or regular *E. coli* (b). The concentration of the inducer IPTG was 5 μM or 50 μM in the positive (a) or the negative (b) genetic assays, respectively. The effect of spiranamine or rimantadine on the same bacterial cultures is shown in blue or red, respectively. (a) In the positive assay, when the red or blue bars are appreciably smaller than the green bars, it indicates that the channel is sensitive to the drug. (b) The opposite is true in the negative assay. For comparison, bacteria that do not express the viral protein are shown on top. Error bars represent standard deviation of two or more experiments.

readily distinguishes the resistant proteins. In bacteria expressing the wild-type viral protein, the addition of the antiviral drug allowed bacterial growth at more than double the growth rate without the drug, thus substantially overcoming the deleterious effect of the wild-type viral protein. In contrast, the antiviral drug has limited, if any, impact on the growth of bacteria that express resistant M2 variants.

Resistance to drugs that have yet to see clinical use

The underlying goal of this work was to map the resistance potential of influenza against M2 blockers drug before it emerges during clinical use. Since aminoadamantanes have already elicited considerable resistance during decades of medical use [10], we decided to examine a different compound. Toward that end, we attempted to identify resistance mutations against spiranamine, a drug that has yet to see clinical use and was reported to inhibit some aminoadamantane-resistant flu strains [9].

In few rounds of mutagenesis, three mutations that conferred resistance to spiranamine were identified between residues Val27 and Ile38. Subsequently, the mutations were subjected to quantitative analysis in liquid culture. Specifically, the impact of spiranamine upon the growth rates of bacteria that express the resistant mutants was examined and compared to bacteria that expressed drug-sensitive viral proteins. Both the positive and negative genetic assays were employed, yielding reciprocal results (Fig. 7) that are similar to those obtained with rimantadine-resistant mutants (Fig. 5). Finally, the resistivity of the mutants was examined against rimantadine as well, indicating that the two drugs exhibit the same profile (see Fig. 7).

Discussion

We present an approach to map the resistance potential of influenza against antiviral drugs that block its M2 channel. The method is based on a genetic screen in which the viral protein is essential to bacterial growth and therefore carries no medical risk. A complementary genetic screen in which the same viral protein is detrimental to bacterial growth is used to substantiate the results.

Resistance against biomedically tested drugs

We first used the approach on aminoadamantanes, which are antiviral inhibitors that have been used extensively in the clinic for decades. Two interesting findings arose:

- Upon examining the mutations found in the screen, it is striking to note that clinically relevant resistant mutations were identified [20]. For example, S31N, one of the mutations that was obtained, is the most common

resistant flu variety known. In a recent analysis, it was found in all 241 viral isolates that were sequenced [36]. Another example is the common mutation A30T [20]. Hence, our genetic selection was able to identify known resistant locations in a completely unbiased method. This finding demonstrates the relevance of our approach to *in vivo* settings.

- The second important result of our study is that we were able to uncover a considerable number of resistant mutations that were previously unknown in the literature [20]. However, a search in sequence databases was able to identify almost all of these mutations, although the sequences were not annotated or known to be from drug-resistant viruses (Pfam database [37]). Thus, all of the mutations that we identified were found in circulating viable viruses, which further substantiates our bacteria-based genetic selection scheme.

Resistance against novel drugs

Resistance against aminoadamantanes is exceptionally prevalent [10]. Therefore, it might not be a surprise that we were able to recreate resistance against them in our genetic screen, albeit much faster and without any medical risk. Therefore, the third important result of our study is that we were able to develop resistance against a drug, spiranamine, which has not seen any clinical use and is thought to be active against aminoadamantane-resistant flu strains [9].

In only a few rounds of mutagenesis cycles, we were able to generate three resistant mutations against spiranamine. Hence, our approach is indeed capable of predicting resistance mutations against drugs prior to clinical use.

Interestingly, when subsequently tested against aminoadamantanes, some of these mutations were found to be resistant as well (e.g., Ala30). Hence, the two drugs most likely bind and inhibit M2 in a similar fashion. This finding suggests that applying our method on prototype drugs before they are thoroughly tested in clinical use and even on currently circulating drugs could provide important clues on drug resistance.

Extension to other targets

The approach that we present to map the resistance potential against influenza's M2 inhibitors may be expanded to other pathogen transport proteins. The only requirement is that the proteins be expressed in functional form in *E. coli* and that the proteins promote K^+ transport. We note that the requirement for K^+ permeation is minimal at best. As an example, while the influenza M2 channel used in the current study is capable of K^+ transport [38], it is far more selective toward protons [34,39].

It is important to note that when the membrane transport drug target is a viral protein, the results may be medically relevant, as in the case of the current study. The reason being is that viral drug resistance is normally obtained via mutations in the target itself. This is exactly what our assay identifies. In non-viral systems, the results may not always be medically relevant, since drug resistance can also arise heterologously: emergence of enzymes that degrade the drug (e.g., β-lactamases in bacteria),or of transporters that excrete the drug (e.g., multidrug transporters in cancer cells and bacteria).

Finally, expansion of the approach to drug targets other than membrane transport proteins may be possible in principle. However, an entirely different genetic selection would be required, in which the particular drug target is able to revive bacterial growth due to its particular functionality.

Method limitation

An inherent feature of our approach is that it analyzes the viral drug target in isolation and not the virus as a whole. Hence, there is a possibility that some of the mutations that are detected in our approach will not be found in the clinic, since they will result in nonviable viruses. It is difficult to estimate the likelihood of this outcome, since in the analyses that we conducted with influenza, no such mutations were identified. In other words, all of the mutations that we uncovered were found in genomes of circulating viruses. Nonetheless, the aforementioned possibility cannot de discounted in its entirety.

Experimental Procedures

Strains and plasmids

Two strains of *E. coli* K12 were used: DH10B and LB650. LB650 carries three deletions in genes connected to K^+ uptake [31], *trkH*, *trkG*, and the *kdpABC5* system, and were a kind gift from Prof. K. Jung (Ludwig-Maximilians Universität München) and Prof. G.A. Berkowitz (University of Connecticut). *E. coli* DH10B cells were purchased from Invitrogen (Carlsbad, CA).

The pMAL-p2X was used to construct a chimera between the maltose-binding protein and M2 (New England Biolabs, Ipswich, MA). Maltose-binding protein is a periplasmic protein and was fused to M2 to ensure that M2 will be inserted into the bacterial inner membrane in the right topology [32]. Protein expression was induced by IPTG [40] to the desired

levels as previously shown [32]. The Singapore M2 sequence was synthesized by GenScript (Piscataway, NJ) according to the A/Singapore/l/57 (H2N2; NCBI accession number P10920.1) [20] and was inserted into pMAL-p2X plasmid as described in Ref. [32]. Plasmids that contain M2 as described will be referred to as pMAL-M2 (strain), for example, pMAL-M2 wild-type (*wt*) or S31N.

Chemicals

IPTG was purchased from Biochemika-Fluka (Buchs, Switzerland). 1-Aminoadamantane (amantadine), racemic 1-(1-adamantyl)-ethanamine (rimantadine), and all other chemicals were purchased from Sigma-Aldrich laboratories (Rehovot, Israel).

Growth media

Lysogeny Broth (LB) was used for all bacterial growth [41] unless noted otherwise. LB-K was similar to LB, except that KCl replaces NaCl at 10 g/L. All media contained ampicillin at 100 μg/ml.

Bacterial growth

E. coli DH10B bacteria bearing or lacking (as a reference) the viral genes were grown overnight in LB at 37 ° C. Thereafter, the growth culture was diluted and the bacteria were grown until their OD600 reached 0.07–0.1. Bacteria were then divided into 96-well flat-bottomed plates (Nunc, Roskilde, Denmark) containing the different treatments. The growth volume in each well was 100 μl. Unless stated otherwise, IPTG was added to the cells to a final concentration of 60 μM. D-glucose was added to a concentration of 1%. 96-well plates were incubated for 16 h at 30 ° C in a Synergy 2 multi-detection microplate reader from Biotek (Winooski, VT) or in Infinite 200 from Tecan Group (Männedorf, Switzerland) at a constant high shaking rate. OD600 readings were recorded every 15 min. For every measurement, duplicates or triplicates were conducted.

For the *E. coli* LB650 bacteria, the same protocol was used, except that growth was done in LBK overnight. Thereafter, the growth medium was replaced to LB and the bacteria were diluted and grown until their OD600 reached 0.07–0.1; unless stated otherwise, IPTG was added to the LB650 bacteria to a final concentration of 10 μM.

Mutagenesis

Site-directed mutagenesis was performed with the Quick Change Lightning Kit (Agilent Technologies, CA). Random mutagenesis of the *wt* M2 gene was performed with the Genemorph II Random Mutagenesis Kit (Stratagene California, La Jolla, CA). After the mutagenic PCR was finished, the DNA fragments were electrophorated in 1% agarose gel, cut out of the gel, and extracted using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan). The segments were then used as megaprimers according to the Quick Change Lightning Kit's protocol (with pMAL-p2X-M2 *wt* as a template), thereby inserting them into the plasmid. The purification of the plasmids containing mutated M2 genes was continued according to the Quick Change Lightning Kit's protocol.

Acknowledgments

This work was supported in part by grants from the the Binational Science Foundation, the Israeli Science Foundation (175/13), and The Rudin Fellowship Trust (to R.A.). I.T.A. is the Arthur Lejwa Professor of Structural Biochemistry at the Hebrew University of Jerusalem.

Author contributions: **D.A., R.A., M.K., and I.T.A. designed the experiments and analyzed the resulting data. D.A. and M.K. performed the experiments. R.A. performed the sequence analyses. D.A., R.A., and I.T.A. wrote the manuscript.**

Received 30 May 2016;
Received in revised form 2 August 2016;
Accepted 6 August 2016
Available online 11 August 2016

Keywords:
influenza;
drug resistance;
channel blocker;
antiviral

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†"He that will not apply new remedies must expect new evils for time is the greatest innovator". Sir Francis Bacon, 'Of Innovations', Essays, 24 (1625).

Abbreviations used:
LB, Lysogeny broth; wt, wild-type.

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