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Computational and experimental analysis of drug binding to the Influenza M2 channel ☆

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ABSTRACT

The Influenza Matrix 2 (M2) protein is the target of Amantadine and Rimantadine which block its H^+ channel activity. However, the potential of these aminoadamantyls to serve as anti-flu agents is marred by the rapid resistance that the virus develops against them. Herein, using a cell based assay that we developed, we identify two new aminoadamantyl derivatives that show increased activity against otherwise resistant M2 variants. In order to understand the distinguishing binding patterns of the different blockers, we computed the potential of mean force of the drug binding process. The results reveal that the new derivatives are less mobile and bind to a larger pocket in the channel. Finally, such analyses may prove useful in designing new, more effective M2 blockers as a means of curbing influenza. This article is part of a Special Issue entitled: Viral Membrane Proteins — Channels for Cellular Networking.

1. Introduction

The *Influenza A* virus is an RNA virus of the *Orthomyxoviridae* family, giving rise to widespread seasonal epidemics and, less frequently, to severe pandemics. While the seasonal epidemics have low mortality rate, past pandemic events caused heavy losses in human lives. Among these is the Spanish flu (1918), which killed 2–20% of those infected, with an infection rate as high as 50%. Later, smaller outbreaks such as the Asian flu (1957) and Hong Kong flu (1968) claimed the lives of millions, and in 2009, the H1N1 swine flu became a prominent illness causing agent in children and adults (ages 5–60) [1].

The Matrix 2 (M2) protein is a critical component of the *Influenza A* virus. Residing in the virus membrane, its 97 amino acid long sequence encompasses a 19-residue hydrophobic transmembrane domain (TM; residues 25 through 43) and undergoes tetramerization, forming a pore. The pore functions as an ion-channel, selectively allowing an influx of H^+s during cell infection and acidification of the virus lumen [2–4]. The M2 channel is a member of a group of viral proteins which enable membrane permeabilization and are involved in the viral pathogenicity, referred to as *Viroporins*. Other small hydrophobic proteins that are included in the group are HIV-1's Vpu, hepatitis C virus' p7 and HRSV's SH proteins [5,6].

Over the recent years, several structures of the M2 channel have been published, using NMR methods as well as X-ray crystallography, mostly by the groups of Chou, Cross and DeGrado (for example, see [7–10]). Although each structure is slightly different from one another, they all exhibit the same basic topology of a tetramer composed of tilted helices. Smaller details, including the tilt angle itself, vary from structure to structure, and an attempt to compare three of the structures has been done in the past [11].

The channel has been the subject of extensive research, as well as the target of anti-viral agents. The most prevalent drugs that target the channel's activity are Amantadine and Rimantadine, sold commercially as Symmetrel and Flumadine, respectively. However, use of these drugs had led to the development of resistant strains of the virus, and both drugs are currently not recommended as treatment options as anti-flu therapy [12,13].

Among the most prominent resistance-conferring mutations are the substitutions of Ser31 to Asn (S31N) [14] and Val27 to Ala (V27A), the former being present in the resistant swine flu strain. Additional mutations in other pore-lining residues, such as Ala30 and (rarely) Gly34 have also been reported as resistance conferring [15,16]. Previous works suggested that these mutations cause changes in the pore radius thus either interfering with the binding of the drug or rendering the binding futile [17].

In order to better understand the mechanism of H^+ conductance and resistance acquirement by the channel, various electrophysiological methods have been implemented, though some were difficult to carry out due to the acidic environment required to activate the channel [18,19]. Other studies made use of the consequence of the channel activity in order to indirectly measure it. In a method introduced by us in 2011 [20], the channel is heterologously expressed in bacteria, having

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a deleterious effect on the bacteria's growth. The growth inhibition is alleviated when an effective blocker of the channel is provided. A very similar system, differing principally by the choice of chimeric construct, was introduced by Inouye and co-workers in 2013 [21].

In the current study, we scanned a small library of candidate molecules using our bacterial cell-based assay, with the goal of finding an inhibitor to the resistant M2 S31N strain. Two molecules exhibiting such a property were found, and in this study we examine the mechanism of their inhibition by molecular dynamics (MD) simulations.

2. Results and discussion

2.1. Cell based assay for channel blockage

In pursuance of putative inhibitors of the M2 channel, a system for high throughput screening of molecules was established in our group in 2011, based on a bacterial assay [20]. A library of compounds was applied to channel-expressing *Escherichia coli*, while their growth was monitored by measuring $O.D._{600}$ over time.

When the channel protein is inserted into the plasma membrane, it facilitates an influx of H^+s through the cellular membrane and consequent growth retardation of the bacteria. Induction efficiently inhibits the growth, resulting in two-fold growth retardation, or lower (see Fig. 1). Accordingly, screening of potential channel blockers may be achieved by adding various compounds to the growth media and monitoring growth enhancement, as successful blockers of the channel should allow better bacterial growth.

2.2. Novel potential channel blockers

Among a limited number of compounds tested, two exhibited significant rescue of the bacterial growth, hinting that they might act as efficient inhibitors of the M2 channel (see Fig. 2c and d). While other compounds, applied at the same concentration as Rimantadine, either had no effect on growth (growth curves resemble those of *No drug*; data not shown), or had minor effect on the Rimantadine-sensitive Singapore *Influenza A* strain channel. The two new compounds alleviated growth to a higher extent than Rimantadine, in both the Singapore strain (Fig. 1a) and in the resistant swine flu's M2 channel (Fig. 1b), which contains the S31N mutation [22]. This implies that the H^+ flux into the bacterial cell has decreased appreciably due to the presence of the new compounds.

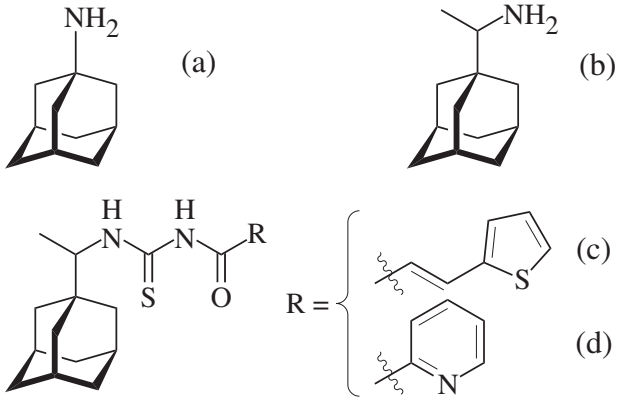

Fig. 2. Known and putative inhibitors of the M2 channel: (a) Amantadine, (b) Rimantadine, (c) Thiophene derivative, and (d) Pyridine derivative.

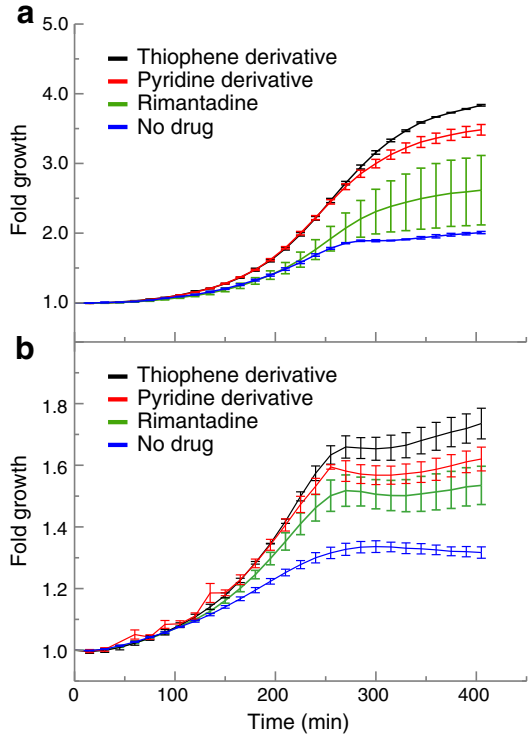

Fig. 1. Growth curves of transformed bacteria expressing the M2 channel from the Singapore strain (a) or the swine flu (b). The different curves correspond to treatments of different M2 blockers, as indicated. Growth was normalized in relation to the initial value.

2.3. Assessment of the compounds' EC50 and toxicity on bacteria

Based on the initial results from the chemical screening, we conducted an experiment to assess the effectiveness of the newly found compounds. Using the same expression system mentioned above, we added each compound in two-fold serial dilutions, varying from 0.8 μM to 50 μM final concentrations, to the growth media of the resistant swine flu M2 expressing bacteria. Additionally, we tested the compounds' toxicity upon the bacteria, by applying the same concentrations on un-induced bacteria. The compounds did not diminish the bacterial growth rate, as well as the final bacterial density (data not shown). Taken together we can conclude that the new compounds exhibited no toxicity towards the bacteria.

While Rimantadine could not rescue the growth of bacteria expressing the swine flu's M2 channel, both of the new compounds significantly

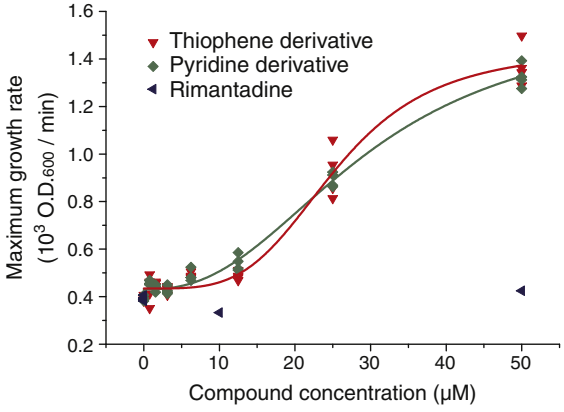

Fig. 3. Maximal growth rate of bacteria treated with the Pyridine derivative, the Thiophene derivative or Rimantadine. The smooth lines depict the fitted function for dose–response relationship of the Pyridine derivative or the Thiophene derivative.