Governing Dynamics of Divalent Copper Binding by Influenza A Matrix Protein 2 His37 Imidazole

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Governing Dynamics of Divalent Copper Binding

by Influenza A Matrix Protein 2

His37 Imidazole

Kelly Lewis McGuire

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

Governing Dynamics of Divalent Copper Binding by Influenza A Matrix Protein 2
His37 Imidazole

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Doctor of Philosophy

Influenza A is involved in hundreds of thousands of deaths globally every year resulting from viral infection-related complications. Previous efforts to subdue the virus by preventing proper function of wild-type (WT) neuraminidase (N), and M2 proteins using oseltamivir and amantadine (AMT) or rimantadine (RMT), respectively, exhibited success initially. Over time, these drugs began exhibiting mixed success as the virus developed drug resistance. M2 is a proton channel responsible for the acidification of the viral interior which facilitates release of the viral RNA into the host. M2 has a His37-tetrad that is the selective filter for protons. This protein has been demonstrated to be a feasible target for organic compounds. However, due to a mutation from serine to asparagine at residue 31 of M2, which is found in the majority of influenza strains circulating in humans, AMT and RMT block is insufficient. From simulations, it is unclear whether the insensitivity results from weak binding or incomplete block. The question of how the S31N mutation caused AMT and RMT insensitivity in M2 is addressed here by analyzing the binding kinetics of AMT and RMT using the two-electrode voltage clamp electrophysiology method. The dissociation rate constant (k2) is dramatically increased compared to WT for both AMT and RMT, by 1500-fold and 17000-fold respectively. Testing of AMT at 10 mM demonstrates complete block, albeit weak, of the S31N M2 channel. At 10 mM, RMT does not reach complete block even though the binding site is saturated. When RMT is in the bound state, it is not blocking all the current, and is binding without block. These results motivated the development of novel M2 blockers using copper complexes focusing on the His37 complex in M2. I hypothesized that copper complexes would bind with the imidazole of a histidine in the His37 complex and prevent proton conductance. The His37 complex is highly conserved in the M2 channel and, therefore, would be important target for influenza therapeutics. By derivatizing the amines of known M2 blockers, AMT and cyclooctyalmine, to form the iminodiacetate or iminodiacetamide, we have synthesized Cu(II) containing complexes and characterized them by NMR, IR, MS, UV–vis, and inductively coupled plasma mass spectroscopy (ICP-MS). The copper complexes, but not the copper-free ligands, demonstrated H37-specific blocking of M2 channel currents and low micromolar anti-viral efficacies in both Amt-sensitive and Amt-resistant IAV strains with, for the best case, nearly 10-fold less cytotoxicity than CuCl2. Isothermal titration calorimetry was used to obtain enthalpies that showed the copper complexes bind to one imidazole and curve fitting to the electrophysiology data provided rate constants for binding in the M2 channel. Computational chemistry was used to obtain binding geometries and energies of the copper complexes to the His37-tetrad. The results show that the copper complexes do bind with the His37 complex and prevent proton conductance and influenza infection.

Keywords: influenza A virus (IAV), matrix protein 2 (M2), quantum mechanics (QM), copper complexes
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CHAPTER 1: Introduction

The influenza A virus (IAV) continues to pose a threat to both the health and economy of modern society. Influenza causes 291,000 – 645,000 deaths per year globally. The Spanish Flu of 1918 caused 50-100 million deaths. The annual cost in the U.S. to prevent and treat influenza is approximately 10$ billion and future pandemics are estimated to cost $3 trillion or more. One of the main targets for inhibiting influenza replication and, therefore, spread of the infection is by blocking the matrix protein 2 (M2). In 1985, the M2 protein was shown to be an integral membrane protein that is abundantly expressed at the plasma membrane of virus-infected cells (1.1). The M2 protein spans the membrane once, contains 97 residues with a single internal hydrophobic domain of 19 residues and is orientated such that it has 23 N-terminal extracellular residues, a 19-residue transmembrane (TM) domain, and a 54-residue cytoplasmic tail (1.19) (Figure 1.1).

All strains of influenza A encode the M2 protein, and the TM domain is the most conserved region of the M2 protein sequence. Analysis of the M2 protein on non-reducing gels and by chemical cross-linking showed that the native form of the M2 protein is a homotetramer consisting of either a pair of disulfide-linked dimers or a disulfide-linked tetramer (1.2,1.3,1.4). Studies using site-specific mutagenesis show that the M2 protein forms intermolecular disulfide-bonds at Cys17 and Cys19, is post-translationally modified by palmitoylation at Cys50, and is post-translationally modified by phosphorylation at Ser64 (1.2,1.3,1.5,1.6,1.7).
However, posttranslational modifications do not have a functional role in the M2 channel activity according to data that shows continued channel activity after site-specific altered forms of the M2 protein that are incapable of being post-translationally modified (1.5).

Influenza virus particles are internalized into cells by receptor-mediated endocytosis. Once the virions are exposed to the low pH environment in the endosomal compartment, the hemagglutinin (HA) undergoes a low-pH-induced conformational change which results in a protein refolding event that causes insertion of the hydrophobic fusion peptide into a target membrane and subsequent membrane fusion (1.8,1.9,1.10).

The result of this membrane fusion event is that the viral ribonucleoproteins (RNPs) are released into the cytoplasm and then the RNPs are transported to the nucleus to begin mRNA transcription. It is generally believed that once the virion particle has been endocytosed, the ion channel activity of the virion-associated M2 protein permits the flow of protons from the endosome into the virion interior to disrupt protein-protein interactions and free the RNPs from the influenza matrix protein 1 (M1) (1.11). Direct evidence that the M2 protein has ion channel activity was obtained by expression of the M2 protein in oocytes of *Xenopus* laevis and by measuring membrane currents (1.12). Because the intracellular sites of action of the M2 protein are the endosome and the trans-golgi network (TGN) compartments (both of which are acidic environments) it was hypothesized that M2 protein ion conductance would be regulated by changes in pH.

When tested, the M2 protein-associated ion channel currents increased monotonically with decreasing pH, and a change from pH 7.4 to pH 5.4 increased the inward current 7-10-fold (1.12). The only amino acid in the TM domain of the M2 protein capable of being protonated within this range of pH is histidine residue 37 (pKa ~5.7). To test this hypothesis, His37 was
replaced with either glutamate or glycine. It was found that these mutant proteins did form ion channels that were not activated by lowered pH (1.13). It is believed that the pore of the M2 channel is closed by high extravirion pH, because cells expressing the M2 protein develop large, inward H\(^+\) currents when they are bathed in media of low pH (1.12,1.14). It was proposed that the indole side chain of Trp41 is a gate that can retain H\(^+\) within the cell, and evidence of that was obtained (1.32). A model for activation of the M2 protein channel has been proposed. When pH\(_{\text{out}}\) is lowered, the His37 selectivity filter becomes protonated and as a result the indole of Trp41 rotates to permit H\(^+\) transport. This movement may occur due to cation-pi interactions as suggested from Raman spectroscopy (1.15,1.16,1.17). Returning to a higher pH\(_{\text{out}}\) discontinues the outward current flow because the deprotonation of the His37 selectivity filter in high pH medium causes the indole of Trp41 to return to it pore-blocking position. If Trp41 is mutated to have a smaller sized side chain, pore blockage does not occur. Thus, His37 acts as the detector of low pH\(_{\text{Out}}\) and Trp41 acts as the gate (1.18).

Amantadine has been a known influenza inhibitor since 1964, but the mechanism of block was not understood until 1982-91. During 1982-1991, it was found that in the presence of amantadine, the M1 protein fails to dissociate from the RNPs and the transport of the RNP complex to the nucleus does not occur. Viral mutants resistant to amantadine contain amino acid changes that map to the M2 TM domain which, at that time, suggested the amantadine target to be the M2 protein (1.19,1.20,1.21).

Electrophysiological methods supplied direct evidence for the mechanism of action of amantadine when the M2 protein was expressed in *Xenopus* laevis oocytes, and M2 ion channel activity was found to be blocked by amantadine (1.5,1.12,1.22,1.23).
The use of amantadine is associated with the rapid emergence of drug-resistant variants. According to a study which assessed more than 7,000 influenza A virus samples obtained from 1994 to 2005, drug resistance against amantadine and rimantadine had increased worldwide from 0.4% to 12.3%. Virus samples collected in 2004 from South Korea, Taiwan, Hong Kong, and China showed drug-resistance frequencies of 15%, 23%, 70%, and 74%, respectively. Recently, 109 out of 120 (91%) influenza A H3N2 viruses isolated from patients in the US contained an amino acid change at position 31 from serine to arginine (S31N) of the M2 protein, which confers resistance to amantadine and rimantadine. On the basis of these results, the Centre for Disease Control recommended that neither amantadine nor rimantadine be used for the treatment or prophylaxis of influenza A in the United States for the remainder of the 2005-06 influenza season (CDC 2006). The S31N amantadine-resistance mutation in the influenza A M2 sequence currently occurs more frequently in nature than the S31 wild-type (WT).

Developing novel antiviral therapies that overcome the resistance of the S31N mutation is the primary focus of M2 researchers. At this time, there are no FDA approved M2 blockers because of the drug resistance developed in the M2 channel. It has, therefore, become necessary to discover a new target that would inhibit M2 channel activation by use of drug therapies.

It is known that His-containing proteins are often found to coordinate transition elements. Therefore, these transition elements were screened for their ability to affect the function of the M2 protein and it was found that one element, Cu(II), inhibited the channel at low concentrations (2.5 µM) (1.24). This inhibition was slowly reversible and specific to Cu(II); Cu(I), Zn(II), Ni(II), Pt(II), Mg(II), and Mn(II) were ineffective. In order to confirm that the inhibition was due to coordination by Cu(II) with His37, Gandhi et al (1999) found that inhibition of either the M2-H37A or M2-H37G mutant protein by Cu(II) was incomplete and short-lived. It was also
found that extracellular application of a specific inhibitor of M2 channel function that interacts with residues external to His37, BL1743 (1.25), prevented the subsequent inhibition by extracellularly applied Cu²⁺. The effect of BL-1743 was reversible, and after the compound was removed, Cu²⁺ was once again able to inhibit the channel. Based on the results by Gandhi with Cu²⁺ against influenza A M2 WT, Nathan Gordon of the Busath lab decided to complex Cu²⁺ with a known M2 blocker (i.e. amantadine) to create a novel compound that would hopefully be a strong inhibitor of the M2 channel, stable in solution, and non-toxic. This compound (NAG 107) was prepared by adding amantadine to Cu²⁺-2,4-pentanedione (ACAC), which is shown in Figure 1.2. NAG 107 was tested using the liposome and mini-plaque assays (1.26). M2 peptides comprised of residues 22-62 of the M2 A/Udorn/72 (H3N2) conductance domain mutated at residue 31 from serine to asparagine (S31N) was used in a liposome assay. The controls used in this experiment were blank liposomes, liposomes with drug only, and liposomes with S31N M2 protein only.

The influenza A strain, A/California/07/2009, with the S31N M2 mutant was used in the mini-plaque assay with a titer of 5x10⁵ PFU/mL. EC⁵₀ values (concentration at which infection is reduced by 50%) were obtained by administering each drug individually in six different concentrations: 0 μM, 2 μM, 5 μM, 10 μM, 20 μM, and 50 μM. KaleidaGraph was used to calculate the EC⁵₀ values. The EC⁵₀ for NAG107 was shown to be 2.91 μM +/- 0.29 μM. Nathan Gordon’s successful inhibition of a critical drug-resistant viral strain containing the ubiquitous M2 mutation, S31N, suggests that AMT-derived Cu(II) complexes may serve as anti-influenza
agents against other influenza strains. Though Nathan speculated that Cu(II) and the AMT-
derived compound, NAG 107, binds to the histidine complex of the influenza M2 protein, this
was never definitively proven in his work. As well, NAG 107 was found to be unstable in
solution and, therefore, a more stable copper complex design is required.

Therefore, I hypothesized that the copper complexes bind to the His37 complex found in
TM domain of the M2 channel, which is conserved in all but 11 of 44,672 influenza A strains
sequenced to date, and block M2 by preventing the His37 complex from transporting protons
through M2, and consequentially inhibit viral infection. I approached this hypothesis from
multiple directions.

The first approach is to complex divalent copper with known M2 blockers so that the
copper complex is more stable in solution and test the compounds using electrophysiological
methods to know how effectively they block M2 current. These copper complexes are expected
to block the M2 S31N variant comparable to the block observed by amantadine against the WT
M2 channel and preliminary electrophysiology results with Cu\(^{2+}\) in M2 S31N.

The second approach is to estimate the compartment amplitudes and time constants (\(\tau\)), as
well as the steady state block levels, for M2 S31N channel and the copper complex blocking and
washout traces from experimental kinetic data obtained with electrophysiology using curve-
fitting with a theoretical kinetic models. This model is expected to determine the dynamics of
copper complex and imidazole interaction. Using the mathematical model of the M2-copper
complex interaction, I was be able to determine the concentrations of the kinetic species at all
times, the final equilibrium concentrations, and the rate constants for binding and dissociation for
the divalent copper complexes.
The third approach is to provide direct evidence that these compounds block the M2 channel by interacting with the histidine complex. This is tested by making a point mutation at residue 37 (H37A) and observing the difference in blocking efficacy compared to block of M2 S31N using the two-electrode voltage-clamp method. With the H37A mutation, the M2 block by the copper complexes is expected to be reduced or no longer present because the copper histidine interaction is eliminated by the replacement with alanines. As well, quantum chemical models were used to obtain evidence of the copper binding to the imidazole nitrogen on the histidine sidechain. By optimizing the copper complex structure in proximity to the imidazole nitrogen, I determined if a stable configuration exists with the copper binding to an imidazole nitrogen. I was also able to see how well the copper complex fits near the His37 cluster and if the copper can remain chelated to the ligand while interacting with the His37 cluster.

Beyond testing the blocking efficacy of these novel copper complexes against the M2 S31N variant, it is necessary to know the robustness of the complexes, which will help determine their viability as influenza therapeutics. The G34E mutation is a concern due to the glutamates’ potential of preventing the copper complexes from reaching and interacting with the His37 complex. A point-mutation in M2 and electrophysiology testing of the M2 G34E variant was used to determine whether there would be interference by the glutamates to block by the copper complexes. The G34E mutation was originally expected to reduce the copper complexes’ block of M2, but hopefully not eliminate the block completely.

The combination of these approaches show that the copper complexes block the M2 channel by interacting with the His37 complex and provide adequate information to determine the governing dynamics of copper complex binding to the influenza A M2 channel.
Section 1: Analysis of M2 Insensitivity to Amantadine and Rimantadine Conferred by S31N Mutation

The influenza A M2 channel is insensitive to AMT and RMT block due to the mutation of serine to asparagine at residue 31. The change in M2-AMT or M2-RMT interactions in the mutant channel have not been thoroughly studied. It is unclear whether the insensitivity is from weak binding or incomplete block. Chapter 2 (submitted to the Biophysical Journal) demonstrates that the mutation reduces binding for both, but when bound, AMT completely inhibits proton flow whereas RMT only inhibits 25%. This observation has significant implications for novel M2 blockers and should be considered during new drug design and development.
CHAPTER 2: Increased Dissociation of Adamantanamines in Influenza A M2 S31N with Partial Block by Rimantadine

K. McGuire, J. Hill, D. Busath
Biophysical Journal (Resubmitted Post Revisions)

Abstract

The ubiquitous mutation from serine (WT) to asparagine at residue 31 (S31N) in the influenza A M2 channel renders it insensitive to amantadine (AMT) and rimantadine (RMT) block, but it is unknown whether the inhibition results from weak binding or incomplete block. Two-electrode voltage clamp (TEVC) of transfected *Xenopus* oocytes revealed that the M2 S31N channel is essentially fully blocked by AMT at 10 mM, demonstrating that, albeit weak, AMT binding in a channel results in complete block of its proton current. In contrast, RMT achieves only a modest degree of block in the M2 S31N channel at 1 mM, with very little increase in block at 10 mM, indicating that the RMT binding site in the channel saturates with only modest block. From exponential curve fits to families of proton current wash-in and wash-out traces, the association rate constant ($k_1$) is somewhat decreased for both AMT and RMT in the S31N, but the dissociation rate constant ($k_2$) is dramatically increased compared to WT. The potentials of mean force (PMF) from Adaptive Biasing Force (ABF) molecular dynamics simulations predict that rate constants should be exquisitely sensitive to the charge state of the His37 selectivity filter of M2. With one exception out of eight cases, predictions from the simulations with 1 and 3 charged side chains bracket the experimental rate constants, as expected for the acidic bath used in the TEVC assay. From simulations, the weak binding can be accounted for by changes in the potentials of mean force, but the partial block by RMT remains unexplained.

Keywords: Amantadine (AMT), Rimantadine (RMT), two-electrode voltage clamp (TEVC), adaptive biasing force (ABF), potential of Mean force (PMF)
Introduction

The influenza A M2 channel is a proton channel responsible for the acidification of the viral interior required for effective release of the viral RNA into the host cytoplasm. The influenza virus is taken up by endocytosis. The M2 channel is activated when the pH drops in the late stage endosome. There are four His37 sidechains in the hometetrameric M2 channel. It is believed that three of the imidazoles need to be protonated before M2 is activated (2.1), and studies have investigated the pKa values for the M2 His37 protonation states (2.2). The late stage endosome reaches a pH low enough to protonate the three imidazoles required for M2 activation. Once activated, the His37 complex transports protons through one of two proposed models: the gating model (2.3,2.4,2.5) or the shuttle model (2.6,2.7). This acid activation is ideal for using the two-electrode voltage-clamp (TEVC) electrophysiology method to study the M2 channel.

The M2 channel has been demonstrated to be a feasible target for organic compounds including amantadine (AMT) and rimantadine (RMT). However, a mutation at residue 31 from serine to asparagine caused the M2 channel to become insensitive to AMT and RMT block. It is not well understood why this mutation caused the insensitivity. One important question that needs to be answered is whether there is AMT or RMT binding without block, or if the mutation reduces the binding rate constants of AMT and RMT.

Previous estimates of association (k1) and dissociation (k2) rate constants for AMT binding in WT M2 were based on curve fitting of the wash-in and wash-out trace at a selected bath drug concentration with data collected using TEVC (2.8). They estimated k1 to be 900 M$^{-1}$s$^{-1}$ and k2 to be 3x10$^{-4}$ s$^{-1}$.

Here we use same method but extended to a family of traces at different drug concentrations, to evaluate the kinetics of AMT or RMT binding to WT or M2 S31N. From these
traces, a global nonlinear least squares curve fit kinetic model, with one drug binding site having a variable degree of current block when occupied, was used to obtain $k_1$ and $k_2$ rate constants. Rate constants for AMT or RMT binding to WT or M2 S31N are compared. Most notably, $k_2$ values are dramatically increased in the M2 S31N, and there RMT binds with minimal block of proton transport.

Adaptive biasing force (ABF) molecular dynamics simulations (2.9) were used, along lines similar to those previously implemented (2.10), to elucidate AMT or RMT binding to M2 S31N. Potential of Mean Force (PMF) plots are used to show free-energy differences between AMT$^+$ or RMT$^+$ binding to WT or M2 S31N for three M2 His37 tetrad charge states, neutral, +1, and +3. From the PMFs, $k_2$ is estimated based on an expression from Kramer’s theory for diffusion over a barrier and $k_1$ based on an adaptation of Arrhenius theory with an estimated diffusion limited reaction rate constant as a prefactor. The selectivity filter charge is exquisitely impactful on the rate constants, probably because of electrostatic repulsion between the selectivity filter and the cationic drug. We report the sensitivity of these rate constant predictions to the drug type, channel type, and selectivity filter charge, identifying the probable selectivity filter charge as near +1 for the WT, but as yet indeterminate for the M2 S31N.

Materials and Methods

*A/Udorn/72 H3N2 M2 S31N mRNA Synthesis*

The A/Udorn/72 H3N2 plasmid, a gift from Dr. Larry Pinto, and its homolog with S31N produced by site-directed mutagenesis in the Busath lab, were transformed into chemically competent *E. coli* by standard methods. The plasmid was harvested using the Zymo Miniprep Kit (Zymo Research, Irvine, CA). To confirm that no mutations were introduced, the M2 DNA
segment was PCR amplified and Sanger sequenced (Figure 2.4). Following confirmation, the PCR product was transcribed using the mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Thermo Fisher Inc., Waltham, MA) to prepare mRNA for oocyte injections.

**Electrophysiology**

Oocytes from *Xenopus* laevis (Ecocyte, Austin, TX) were maintained in ND-96++ (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 2.5 mM sodium pyruvate, 5 mM HEPES-NaOH, pH 7.4) solution at 17° C until injection of ~40 ng of A/Udorn/72 H3N2 M2 or A/Udorn/72 H3N2 M2 S31N mRNA using a Nanoject II (Drummond Scientific, Broomall, PA). After injection, the oocytes were maintained at 4° C in ND96++ pH 7.4 until electrophysiological recording. 72 hrs after mRNA injection, whole-cell currents were recorded with a two-electrode voltage-clamp apparatus at Vm = -20 mV, room temperature, in Barth’s solution (0.3 mM NaNO3, 0.71 mM CaCl2, 0.82 mM MgSO4, 1.0 mM KCl, 2.4 mM NaHCO3, 88 mM NaCl, 15.0 mM HEPES, pH 7.5). Inward current was induced by perfusion with Barth’s pH 5.3 (15.0 mM MES instead of 15.0 mM HEPES). The oocytes were then perfused by Barth’s pH 5.3 with AMT or RMT hydrochloride at concentrations 10 μM, 100 μM, 500 μM, 1 mM, or 10 mM. A washout was done using Barth’s pH 5.3 without drug. Current traces were obtained from each of three oocytes for each of the concentrations.

Non-injected oocytes were also tested with the same acid perfusion protocols and concentrations of drug to assess the possible native acid-activated channel and AMT- or RMT-induced leak current in the oocytes.
Global Nonlinear Least Squares Curve Fit

Because the Hill coefficient for AMT-WT binding has been assessed as 0.91 (2.8), and considering the shape of the channel lumen, analysis of AMT or RMT block for each channel type was done using the one-site binding model shown here (Eq. 2.1):

\[
\begin{align*}
O & \overset{k_1}{\longleftarrow} C \\
\underset{k_2}{\longleftarrow} & C
\end{align*}
\]

(2.1)

The open state, O, is the unblocked M2 channel where proton conductance is unimpeded. The closed state, C, is the fully or partially blocked M2 proton current. The fractional leakage around the drug, i.e. channel current with drug bound / channel current without drug bound, is denoted f. For convenience in discussing the results, we will also refer to the fraction of proton current blocked by drug, 1-f.

It has been established (2.8) that Xenopus oocytes naturally have small amounts of acid-activated current without transfection. These native channels are not amantadine sensitive and are immediately active upon acid exposure without observable time-dependent gating. Furthermore, the application of hydrophobic amine drugs, especially at high concentrations, could transport current across the cell membrane in parallel with channel currents. Because each cell varies in the amount of native acid-activated current (plus drug-mediated current), which from our control experiments varied between -5 and -20 nA, generally <2.5% of the total acid-activated M2 current, a preliminary global fit was done using Eq. 2.2, where LC (leak current) was allowed to vary in that range independently for each cell, before normalizing the M2 current in wash-in and washout traces to range from 0 to 1.

In addition, for wash-in, the drug concentration near the channel mouth was treated as rising with time due to the diffusion of the drug in the bulk solution, measured to change around
the egg rapidly (on the ms timescale), through the unstirred layer. Knowing the diffusion coefficient of the drug in bulk, it was possible to estimate the thickness of the unstirred layer, \(L\), from the initial lag in current blocking by the drug. This is captured in Eq. 2.2 as a time dependence to the concentration of drug, \([D](t)\), elucidated by Eq. 2.4. No similar phenomenon was observable in the wash-out trace, and the unstirred layer effect was considered negligible for them.

Finally, the data traces for the three cells were averaged pointwise and Eqs. 2.2 and 2.3 were used simultaneously on all of the averaged, normalized wash-in (Eq. 2.2) and washout (Eq. 2.3) traces for the global Levenberg-Marquardt (2.9) curve fit with the electrophysiology data to obtain rate constants, \(k_1\) and \(k_2\) and the leakage parameter, \(f\). In this final fit, LC was set to 0.

\[
I_{\text{washin}} = (1 - LC) \cdot (f + (1 - f) \cdot \left(1 - \frac{k_2}{k_1[D](t)+k_2}\right) \cdot e^{-\frac{k_1[D](t)+k_2}{k_1[D](t)+k_2}} + \left(\frac{k_2}{k_1[D](t)+k_2}\right) + LC \tag{2.2}
\]

\[
I_{\text{washout}} = f + (1 - f) \cdot (1 - A \cdot e^{-k_2\cdot t}) \tag{2.3}
\]

\(A\) in Eq. 2.3 is the final probability of occupancy from the wash-in calculated during the global fit and incorporates the LC. \([D](t)\) is bulk drug concentration, \(D\) is the diffusion coefficient of the drug in the unstirred layer outside of the channel entry, and \(L\) is the thickness of the unstirred layer which was assumed to fill as a slab in contact with the bulk thus modulating the drug concentration at the channel entry (Eq. 2.4)(2.10):

\[
[D](t) = [D] - \left(\frac{4[D]}{\pi}\right) \cdot e^{-\frac{D\cdot\pi^2\cdot t}{4L^2}} \tag{2.4}
\]
where \([D]\) is the bulk drug concentration. A weighted reduced \(\chi^2\) (2.9) was calculated to
determine the quality of the curve fit. All curve fits and statistical analyses were done using
Matlab R2018a (2.52).

**Molecular Dynamics Simulations**

The simulations reported here were performed using NAMD (2.12) and the results were
analyzed using VMD (2.12). The CHARMM37b1 force field (2.13) was used for protein and
lipid molecules. The ligand parameters were developed using the CHARMM-GUI ligand reader
& modeler which uses CGENFF (2.13,2.14) and are shown in Table S3 and S4. The protein-bilayer system, consisting of the ssNMR 2KQT structure (2.15) centered in a DMPC bilayer (182
lipid molecules) orientated normal to the z-axis, was solvated with a tetragonal 60 Å x 60 Å x 90
Å water box ( z being the long axis) using 9,145 TIP3 water molecules. The total system charge
was neutralized with 98 Cl and 70 Na ions, representing 150 mM NaCl. The ions parameters
used are from the CHARMM37b1 force field. The non-bonded van der Waals interaction cutoff
was set to 12.0 Å. The particle mesh Ewald sums (PME) long-range electrostatics method was
used. The system was minimized for 1000 steps and equilibrated for 1 ns restraining the protein
center of mass (COM) to the COM of the DMPC bilayer harmonically. The temperature was
kept near 300 K with Langevin dynamics and the default NAMD relaxation rate parameter was
used. The equations of motion were integrated with a time step of 0.5 femtoseconds.

The ABF method was used to obtain a free-energy profile for AMT or RMT in the 2KQT
M2 model, which consists of a homotetramer of the transmembrane domain (residues 22-46)
having the same sequence as A/Udorn/72 WT. A homology model for the M2 S31N was created
from 2KQT with the VMD Mutator plugin. To explore the free-energy landscape of M2 with
AMT or RMT, the reaction coordinate, \(\xi\), was chosen as the distance N-ward along the channel
separating the COM of the M2 channel and the COM of AMT or RMT. 11 ABF simulation windows were used for each of the four cases of peptide and drug. The first ABF window placed the COM of AMT or RMT at the COM of 2KQT with the amine of AMT or RMT ~2 Å from the COM of the His37 cluster. Each window was 3 Å along the channel axis in width, with 100 kcal/mol·Å² constraints at each end of the window to keep the ligand from leaving. The other ten windows placed AMT or RMT at 3 Å intervals along the channel axis above (N-ward) from the first. The total reaction coordinate ranged from -0.09 Å below the COM of 2KQT to 35.91 Å above the COM of 2KQT, well into bulk water.

All restraints were removed for the ABF simulations. The protein-bilayer system was free to diffuse in the water box. The water box was large enough to allow for diffusion without interacting with the periodic boundaries. Each window simulation time was 100 ns. The FullSamples command was used to obtain an estimate of the bias force to avoid sudden jumps in the biasing force (2.16). The value 2000 was used for the FullSamples command, which is sufficient to obtain a reasonable estimate of the biasing force. A good estimate of the biasing force and millions of counts collected in the bins during the 1.1 µs simulations was sufficient to reach convergence. Counts were collected for each window in bins of size 0.1 angstroms, resulting in 30 bins per window. After a total of 1.1 µs simulation time, counts in all the bins were between 2x10⁶ and 3x10⁶, sufficient for adequate biasing force equilibration in each of the windows (19).

Rate Constants from the Simulated PMFs

The modified Arrhenius equation (Eq. 2.5) was used to calculate k₁, the second-order reaction rate constant for collision of a solute with a site, from the simulated PMFs. The diffusion-limited reaction rate constant, kₑ, was assigned the value of 10⁹/Mol·sec, a commonly
cited value for maximal association rate constants for small solutes diffusing in aqueous solution (2.17), with the assumption that if there is no energy barrier, then AMT$^+$ or RMT$^+$ would diffuse as in bulk water and collide with the channel entrance with that rate constant, $k_c$, times the bulk drug concentration. The Boltzmann factor associated with the entry barrier free energy, $\Delta G^i$, governs the fraction of collisions leading to successful channel entries.

$$k_1 = k_c e^{-\frac{\Delta G^i}{kT}} \quad (2.5)$$

An expression from Kramer’s theory for a first-order reaction involving diffusing over a barrier (Eq. 2.6) was used to extract exit rate constants, $k_2$, from the ABF free energy profiles.

$$k_2 = \frac{D \sqrt{\Phi A \Phi^i}}{\pi kT} e^{-\frac{\Delta G^i}{kT}} \quad (2.6)$$

The diffusion coefficient inside the channel, $D$, was obtained for each ABF window using the VMD Diffusion Coefficient Tool (2.18) and averaged for ABF windows 1-8 (M2 interior, Figure 2.7). The last 5000 frames of the simulations were used to calculate the diffusion coefficient in the VMD Diffusion Coefficient Tool because the simulations are converged after a microsecond of sampling. The free energy barrier height and shape was evaluated for Eq. 2.6 by manually fitting a parabola to the binding site energy well and an intersecting inverted parabola to the entry barrier (Figures 2.5 and 2.6). $\Delta G^i$ is the difference between the minimum of one parabola and the maximum of the other, $\Phi_A$ is the inverse width of the potential energy well, a parabola breadth parameter, and $\Phi^i$ is the inverse width of the barrier, an inverted parabola breadth parameter (2.19).
Results

*Global Nonlinear Least Squares Curve Fit*

Binding kinetics were evaluated for blocking and washout traces for M2 WT or S31N exposed to various concentrations of AMT (Figure 2.1). The normalized current traces are each point-wise averages of traces from three different cells. At 500 µM, 1 mM or 10 mM for AMT the M2 WT current is completely blocked by 200 seconds (3.5 minutes) (Figure 2.1a). No significant washout was observed for AMT in M2 WT by 190 seconds (Figure 2.1b). The rate constants from the global fit of these blocking and washout traces are 372 M⁻¹s⁻¹ for the association and 1.58x10⁻⁴ s⁻¹ for dissociation (Table 2.1), corresponding to an equilibrium constant for the dissociation reaction of $K_d = 0.42$ µM, similar to previous reports (2.20).

Unexpectedly, the M2 S31N current is more quickly blocked (Figure 2.1c) than the M2 WT, rapidly reaching an equilibrium block state at all concentrations tested. AMT block does not wash out appreciably from the WT channel (Figure 2.1b), but rapidly washes out in M2 S31N at all tested concentrations (Figure 2.1d). Surprisingly, the washout though rapid is incomplete; current levels never return to their original levels. It is unknown whether this is due to channel rundown, pH gradient rundown, a small amount of irreversible block by AMT in the S31N channel, or some other factor. Because each trace seemed to achieve a constant level, we chose to add a constant block parameter to the exponential washout model such that the theoretical prediction would not be forced to relax to 1.0 for the cases in Figure 1d. These allowed for better estimates of the washout relaxation time constant, which for the kinetic model must necessarily all be the same value, regardless of the starting (and ending) open state probability. It is notable that even though the theoretical curves (dashed lines) in Figure 2.1d appear to have different time constants because they cross each other, the crossovers are due to differing start amplitudes.
(represented in Eq. 2.3 by the parameter A) and ending amplitudes (due to the differing run-down/permanent block constants included in each of the fits).

These features of the AMT traces can be accounted for by changes in the kinetics of blocker entry into the binding site where the association rate constant, $k_1$, is higher in M2 S31N by nearly 10-fold (3150 vs. 372 M$^{-1}$s$^{-1}$) and the dissociation rate constant, $k_2$, is higher by 1500-fold (0.348 vs 1.58x10$^{-4}$ s$^{-1}$, Table 2.1). The calculated equilibrium constant for AMT in M2 S31N is above the therapeutic range: $K_d = 110 \mu$M, consistent with the observed insensitivity of modern influenza A to AMT. The potential for complete block at high doses (Figure 2.1a) and the cohesiveness of the global fit with just three parameters for each channel type confirms that the simple model is sufficient. In particular, the accurate prediction of intermediate equilibrium block levels consistent with the same pair of rate constants that predicts the equilibrium relaxation during wash-in (esp. Figure 2.1c) and the dissociation rate constant, which alone predicts the washout (Figures 1b and 1d), implies that the fractional block for the bound state ($1-f$) is nearly complete, 0.993 in M2 WT and 0.992 in M2 S31N (Table 2.1).

RMT blocks M2 WT (Figures 2.2a and 2.2b) with kinetics similar to that of AMT, with $k_1$ higher (659 vs 372 M$^{-1}$s$^{-1}$) and $k_2$ slightly higher (2.3x10$^{-4}$ vs 1.58x10$^{-4}$ s$^{-1}$), corresponding to a higher binding affinity ($K_d = 0.35 \mu$M), consistent with RMT’s commonly reported increased binding affinity and antiviral efficacy (e.g. 2.21). The washout for 10 µM RMT in Figure 2.2b is faster than the fitted $k_2$ would predict, perhaps suggesting that the washout has fast and slow stages hinting of a second binding site. However, the use of a single washout rate constant gives an adequate fit for the other washout traces and for the wash-ins where it comes into play in both the time constant and steady state occupancy.
Like AMT, RMT blocks (Figure 2.2c) and washes out (Figure 2.2d) more rapidly in M2 S31N than in M2 WT, essentially instantaneously on the timescale of the experiment. $k_1$ increases by 5.5-fold and $k_2$ increases by 17,000-fold (Table 2.1). But unlike AMT, block is incomplete at all concentrations tested. The fitted rate constants are higher for RMT than for AMT in M2 S31N, 3622 M$^{-1}$s$^{-1}$ and 2.73 s$^{-1}$, corresponding to $K_d = 754 \mu$M, too high to be therapeutically useful. From these results, we expect virions with M2 S31N to be rimantadine insensitive too (2.22). Despite applying saturating drug concentrations, i.e. concentrations of 1 and 10 mM – both higher than $K_d$, and despite rapid onset of block, RMT block is incomplete for M2 S31N at all drug concentrations (Figure 2.2c).

In Figure 2.2c inset, a plot of the expected probability of occupancy for the observed $K_d$, emphasizes the point that at the highest concentrations used, channel occupancy was apparently complete, so complete block was expected but not observed. Because of this observation, we included the third parameter in all the fits (f) and report the fractional block (1-f) for the occupied channels. Unlike the other three cases, where the block of the occupied channel is complete ($\geq 0.978$), the proton current block of the RMT-occupied M2 S31N channel is very low, just 25% (Table 2.1). This low fractional block cannot be RMT-induced leak current through the oocyte membrane, as controls were done to know how much leak current might be induced by high concentrations of drug, and amounts within the range observed in the control experiments were subtracted out as LC in the preliminary cell-wise fit before the final fit of the normalized, average traces.

Molecular Dynamics

Adaptive biasing force simulations were done to evaluate AMT$^+$ or RMT$^+$ binding to M2 WT or the homology model for M2 S31N (Figure 2.3). Each of the complete free-energy profiles
show an energy well N-ward to the H37 tetrad, between 2 and 8 Å, near the serine (or asparagine) in the M2 WT (or S31N). The energy barriers observed between 8 and 14 Å are nearest the Val27 luminal stricture. The flattening of the energy curve beyond 20 Å in the reaction coordinate represents AMT$^+$ or RMT$^+$ in bulk water.

As the M2 H37 tetrad charge is increased from neutral to +1 or +3, the energy well is increasingly shallower as expected from increasing electrostatic repulsion between the tetrad and the charged drug, except in one case - the neutral to +1 case for AMT$^+$ in the M2 S31N where the energy wells are unchanged, (Figure 2.3b, dotted and dashed lines). Generally, the energy well between 2 and 8 Å is deeper in the WT (Figure 2.3, left panels) than in S31N (right panels) - except in the case of AMT$^+$ in the +1 WT M2 (Figure 2.3a, dashed line) compared to AMT$^+$ in the +1 M2 S31N (Figure 2.3b, dashed line). Entry barriers are modestly higher for AMT$^+$ or RMT$^+$ in WT (Figure 2.3, left panels) than S31N (right panels). This corresponds to the higher rate of entry in M2 S31N seen in electrophysiology (Table 2.1).

Table 2.2 shows selected results of calculated rate constants from the PMFs using Eq. 2.5, the diffusion-limited rate constant reduced by the Boltzmann factor according to the entry barrier height (height of inverted parabola relative to bulk), for $k_1$; and, using Eq. 2.6, diffusion over the exit barrier according to the expression from Kramer’s theory, for $k_2$. The parabolas fitted to the PMF for use with the Eqs. 2.5 and 2.6 are shown in Figures 2.5 and 2.6.

The models with differing His37 charge states varied dramatically in predicted entry and exit rate constants (see Table 2.3 for complete results). Nevertheless, it is clear that the neutral tetrad would bind both drugs much too tightly with Eq. 2.6 $k_2$ exit rate constants 14-42 orders of magnitude too low compared to those measured here (Table 2.3), as suggested previously (2.22).

Table 2.6 is an example case for a parameter sensitivity test to show that the well and
barrier width parameters have a small effect on the k2 values, and the barrier height parameter has a significant effect on k2.

Clearly, the higher exit rate constants in the charged-tetrad models are more consistent with measured values, suggesting that electrostatic repulsion between the charged His37 tetrad and the charged drug plays an important role in facilitating drug exit. For WT M2, the His37 tetrad with a total charge of +1 produced k1 and k2 values very similar to the electrophysiology measured values for both AMT+ and RMT+ (Table 2.2). In contrast, none of the M2 S31N PMFs predicted k1 or k2 values similar to the measured values for the three tetrad charge states examined. The best case was the +1 charge state for the tetrad, which, with RMT+, predicted a k2 within a factor of 10 of the measured k2 value (Table 2.2), but the predicted k1 is too high because the entry barrier is too low. As noted above, the barrier to entry is uniformly low for all of the M2 S31N PMFs and the predicted k1s are all dramatically too high as a consequence (Table 2.3), suggesting that the model channel is too open at the Val27 stricture. As noticed previously (2.23), we found the M2 S31N homology model to draw more water into the channel than M2 WT. Perhaps the force field parameters for Asn, as they relate to TIP3 water parameters, over-exaggerate this effect to some degree. Finally, for the AMT+ exit rate constant, the observed k2 is bracketed by the predictions of the +1 and +3 tetrad charge states, suggesting that it might be possible to identify intermediate tetrad charge states for both AMT+ and RMT+ that would account for the two drug exit rate constants in M2 S31N, but we felt it unwise to explore these possibilities in view of the apparent inaccuracy of the entry barriers, which also impact the exit rate constants as they depend on the difference between the well depth and the entry barrier height. Examination of these results for a mechanism for partial block of M2 S31N by RMT+ will be discussed below.
Discussion

The kinetics of current-block and washout for WT and M2 S31N are presented for a broad range of AMT and RMT bath concentrations. The traces technically represent relaxations of the equilibrium binding state after abrupt alteration of the boundary condition (bath concentration) for the conditions that we assumed to be well described by a first-order ordinary differential equation. The onset of block has a time constant for the equilibrium relaxation equal to the inverse of the sum of the effective entry rate constant and the first order exit rate constant, where the effective entry rate constant is the second order entry reaction rate constant times the drug concentration. The final equilibrium occupancy state probability (after an infinitely long wash-in) is the exit rate constant times the relaxation time constant. The time constant for washout is the same as the time constant for onset of block, but with the bath drug concentration set to zero, making it the inverse of the exit rate constant for the first order chemical reaction. The global fit of the single site model with possible fractional block, with the parameters of association rate constant, dissociation rate constant, and fraction of current blocked in the drug-occupied channel state, where all of the features for the whole family of traces are modeled simultaneously, yielded secure estimate of the rate constants as well as the fractional occupied-state current parameter, f, for each of the four different cases in this two-drug, two-channel study.

The one-site binding model equation used includes parameters for diffusion of AMT or RMT through the unstirred layer that surrounds the oocyte. The model also includes a leak current subtraction for current that produced by the oocyte’s native current carrying channels. The use of a global fit (simultaneous optimization of the parameters for both washout and wash-in traces at each of several concentrations), allowing for partial block and corrected with the
unstirred layer parameters and leak current subtraction have not been included, traditionally, when extracting rate constants from the TEVC electrophysiology current traces.

New AMT or RMT binding insights were obtained from the curve fit. Both $k_1$ values for AMT and RMT in the M2 S31N channel were increased 10-fold suggesting a faster entry rate and binding to M2 S31N. But, both $k_2$ values increased even more drastically, by 1500-fold for AMT and 17000-fold for RMT. In the mutant channel, the two drugs spend less time in the bound state.

Also, a novel insight for RMT binding to M2 S31N was discovered. The fraction of block is very low, meaning that, as saturating test concentrations were used, RMT did not completely block proton transport. This is not true for AMT, where the fraction of block is high in the mutant channel and the current is completely blocked when saturating test concentrations are used. However, we were unable to identify the mechanism for partial block with molecular dynamics simulations.

ABF simulations for AMT$^+$ or RMT$^+$ in WT M2 using the ssNMR 2KQT structure for the model did allow us to obtain free-energy profiles for the binding reaction. The simulations were done with neutral, +1, or +3 M2 charge states. Rate constants were extracted from the profiles, projecting for the first time that the +1 His37 charge state agreed strongly with the measured rate constants. Because the diffusion-limited constant used to estimate $k_1$ is only an order-of-magnitude estimate, the predicted $k_1$ values are not to be considered accurate. However, a relative analysis between the $k_1$ values for different situations allows for ratiometric comparisons between them that would be due to differences in the entry barrier, insofar as the channel entryway, with missing N-terminal amino acids, is realistic. The charge state of the His37 tetrad, both in the absence (2.27) and presence (2.28) of AMT$^+$ has been the subject of many NMR and
simulation studies (2.29), including another study that used umbrella sampling to obtain PMFs for RMT in the +2 charge state of the 3LBW (WT) and 2LY0 (S31N) structures, but this is the first electrophysiology study attempting to relate AMT+ or RMT+ binding kinetics to interactions between the His37 tetrad charge and the drug charge. Throughout this report, the drugs are referred to as monovalent cations. This is the rational approach considering that they have a single protonation state with pKas (10.1 for AMT) well above the bath pH, even in the somewhat hydrophobic channel.

An S31N homology model was made from the WT 2KQT structure, and free-energy profiles were obtained for the neutral, +1, or +3 M2 charge states. Rate constants were extracted from the profiles. Neither of the $k_1$ values for the M2 S31N agreed with the measured $k_1$ values. We observed more water molecules in the M2 S31N channel homology model. The complexity of luminal water molecule configuration space may have created difficulty in adequate sampling of water configurations and drug orientation, leading to erroneous rate constant calculations from the free-energy profiles. However, the $k_2$ value for RMT+ in the +1 M2 S31N channel was within a factor of 10 of the experimentally measured $k_2$ value. Qualitatively, the free-energy profiles more closely matched expectations for changes in AMT+ or RMT+ binding to the +1 His37 of M2 S31N than to other charged states.

Naturally, we were most ambitious to use MD simulations to identify a mechanism explaining the enigma presented very clearly by the electrophysiology data, namely how RMT+ could cause only 25% block of the proton current when occupying the binding site of M2 S31N, whereas AMT+ completely blocks it. We started with the assumption that protons would essentially only move in association with water molecules, so that an intact or frequently reconnecting mobile water chain around the drug would be a prerequisite for proton current
leakage around a bound RMT$^+$. Although it is possible that protons could be shuttled by the drug itself rotating in the channel, this seems unlikely because the expected H$^+$ dissociation rate constant from an amine with pKa of 10.1 would be on the order of 1 s$^{-1}$, two orders of magnitude lower than the proton transport rate of the WT. If, as posited elsewhere for many related adamantanamines, RMT$^+$ and AMT$^+$ are rotated up in the S31N with the polar adduct projecting through the Val27 stricture and failing to form a tight seal, one would expect more of a seal with RMT$^+$ than AMT$^+$ because of its more bulky, hydrophobic adduct interacting with the Val27 side chains.

But that would be the opposite of what was observed with electrophysiology. If, on the other hand, either of the drugs point down but sit low in the channel, the strong water attraction of the Asn31 sidechains might draw water through the Val27 stricture and around the drug because of the breadth of the lumen, particularly around the Gly34 tetrad. But, in this scenario, it is difficult to imagine how that would be the case for RMT$^+$ but not AMT$^+$ unless somehow the more hydrophobic adduct on RMT$^+$ enhanced its tendency to sit low in the channel, disrupting the seal made by the adamantyl group with the Val27 tetrad, while the AMT$^+$ sits high and its seal remains intact. With MD simulations, we searched in vain for such a dichotomy. In our simulations with M2 S31N +1, AMT$^+$ primarily self-oriented with the amine up and RMT$^+$ amine-down. With S31N +3, both drugs self-oriented amine-up. In all cases, the seal between the adamantyl group and the Val27 side chains was weak and there was substantial mobility of water molecules around the drug, whether AMT$^+$ or RMT$^+$. The drug orientations were stable on the sub-µs timescale. Longer time-scale motions cannot be ruled out, but it seems unlikely that RMT$^+$ would rotate rapidly to account for the leak, while no-leak AMT$^+$ would not. Therefore,
we were unable to identify a specific configuration unique to RMT\textsuperscript{+} in M2 S31N that could account for leakage of proton current around the drug.

Conclusion

An accurate mathematical model using a family of traces was used to extract rate constants from TEVC experiments for AMT and RMT in WT or M2 S31N. The on and off-rate constants for both AMT and RMT are increased dramatically in M2 S31N compared to WT M2. The significant increase of $k_2$ is likely responsible for the AMT and RMT insensitivity in M2 S31N. The most intriguing result from the global fit shows that RMT binds without block in the M2 S31N, while AMT, albeit binding weakly, blocks all proton transport when in the binding site. Molecular dynamics simulations show that AMT\textsuperscript{+} can easily change binding conformation with its amine pointing up in M2 S31N instead of down as in the WT M2, whereas RMT\textsuperscript{+} most often remains in the binding site with its amine in the downward pointing conformation. Simulated potentials of mean force are most consistent with binding of monovalent cation drugs near a monovalent His37 tetrad selectivity filter.

Contribution to Publication

First author, made the mRNA used in the electrophysiology experiments, designed the electrophysiology experiments and collected the data, derived the equation and wrote the Matlab program for the global fit, designed and performed the ABF simulations, all under the input and direction of Dr. Hill and Dr. Busath.
Figure 2.1: Global Nonlinear Least Squares Fit of AMT in M2. AMT wash-in traces (left) and washout traces (right) for M2 WT (upper) and S31N (lower). Each trace is the average of three normalized traces. For wash-in traces, the traces with more rapid and complete block were those at higher concentrations. The predictions of the global least square fits for the one-site partial-block model with different parameters for WT and S31N channels are the dashed curves. (a) M2 WT exposed with 100 µM, 500 µM, 1 mM, and 10 mM AMT. (b) Corresponding AMT washout traces in M2 WT. (c) M2 S31N exposed with 100 µM, 500 µM, 1 mM, and 10 mM AMT. (d) Corresponding AMT washouts in M2 S31N. The washout is incomplete for four of the five concentrations. Thus Equation 2 was adjusted to include a constant representing the observed incomplete return to the original acid-induced current level.
Figure 2.2: Global Nonlinear Least Squares Fit of AMT in M2. RMT wash-in traces (left) and washout traces (right) for M2 WT (upper) and S31N (lower). Each trace is the average of three normalized traces. The predictions of the global least square fits for the one-site partial-block model with different parameters for WT and S31N channels are the dashed curves. (a) M2 WT exposed with 10 µM, 100 µM, 500 µM, and 1 mM RMT. (b) Corresponding RMT washouts in M2 WT. (c) M2 S31N exposed with 100 µM, 500 µM, 1 mM, and 10 mM RMT. Inset: binding curve for RMT in M2 S31N. Solid curve is the probability of occupancy by RMT assuming $K_D = 754 \mu M$. Circles are positioned at points on the curve corresponding to concentrations tested in Figure 2c. M2 S31N binding is essentially saturated at 10 mM RMT. (d) Corresponding RMT washouts in M2 S31N.
Figure 2.3: Adaptive Biasing Force Simulation Results for AMT\textsuperscript{+} or RMT\textsuperscript{+} in M2 WT or S31N. Dotted lines represent neutral, dashed lines +1, and solid lines +3 charge state of M2. Each ABF plot represents 1.1 \(\mu\)s of total simulation time. (a) WT M2 with AMT\textsuperscript{+}. (b) M2 S31N with AMT\textsuperscript{+}. (c) WT M2 with RMT\textsuperscript{+}. (d) M2 S31N with RMT\textsuperscript{+}. 
Figure 2.4: Sanger Sequencing Chromatogram. M2 DNA sequence of transmembrane domain highlighting residues V27, N31, H37, and W41.

Figure 2.5: AMT+ ABF PMF Curve Fit. ABF PMFs (solid lines, re-zeroed to the PMF minimum) fit with parabolas for use with Eqs. 5 and 6. The intersecting upright (red) and inverted (blue) parabolas represent $\Phi^\dagger$ and $\Phi_A$ respectively. (a-c) AMT+ in WT M2 with neutral, +1, or +3 charge states respectively. (d-f) AMT+ in M2 S31N with neutral, +1, or +3 charge states respectively.
Figure 2.6: RMT$^+$ ABF PMF Curve Fit. ABF PMFs (solid lines, re-zeroed to the PMF minimum) fit with parabolas for use with Eqs. 5 and 6. The intersecting upright (red) and inverted (blue) parabolas represent $\Phi^+$ and $\Phi_A$ respectively. (a-c) RMT$^+$ in WT M2 with neutral, +1, or +3 charge states respectively. (d-f) RMT$^+$ in M2 S31N with neutral, +1, or +3 charge states respectively.

Figure 2.7: Diffusion Coefficient Estimates. Averaged diffusion coefficients from ABF windows 1-8 (M2 interior) compared to the average diffusion coefficient of AMT$^+$ or RMT$^+$ in bulk water (light blue) from ABF windows 9-11 (red). (a) The neutral WT M2 with AMT$^+$ has a lower diffusion coefficient compared to the other WT and M2 S31N charge states. (b) RMT$^+$ in WT or M2 S31N from ABF windows 1-8 (M2 interior) compared to the average diffusion coefficient of AMT$^+$ or RMT$^+$ in bulk water (light blue) from ABF windows 9-11.
Table 2.1: Association and Dissociation Rate Constants (k₁ and k₂) from Global Nonlinear Least Squares Fit. AMT or RMT in M2 WT or S31N. The fraction of drug-occupied channel proton-current block (1-f) is based on the third fitting parameter.

<table>
<thead>
<tr>
<th>Compound</th>
<th>k₁ (M⁻¹s⁻¹)</th>
<th>k₂ (s⁻¹)</th>
<th>Fraction of Block (1-f)</th>
<th>Weighted Reduced ( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMT (WT)</td>
<td>372 ± 1.07</td>
<td>1.6x10⁴ ± 2.5x10⁻⁶</td>
<td>0.993 ± 3.4x10⁻⁵</td>
<td>0.002</td>
</tr>
<tr>
<td>AMT (S31N)</td>
<td>3150 ± 49.5</td>
<td>0.35 ± 5.4x10⁻³</td>
<td>0.992 ± 2.2x10⁻⁴</td>
<td>0.23</td>
</tr>
<tr>
<td>RMT (WT)</td>
<td>659 ± 1.48</td>
<td>2.3x10⁻⁴ ± 4.2x10⁻⁶</td>
<td>0.978 ± 6.2x10⁻⁵</td>
<td>0.0123</td>
</tr>
<tr>
<td>RMT (S31N)</td>
<td>3622 ± 978</td>
<td>2.73 ± 0.74</td>
<td>0.25 ± 1.1x10⁻⁴</td>
<td>0.0132</td>
</tr>
</tbody>
</table>

Table 2.2: Association and Dissociation Rate Constants (k₁ and k₂) Calculated from ABF Simulations. AMT⁺ or RMT⁺ in WT or M2 S31N. Electrophysiologically measured rate constants (E.P.) are included for reference.

<table>
<thead>
<tr>
<th>ABF Rate Constant Results</th>
<th>k₁ (M⁻¹s⁻¹) = kₑ⋅e⁻( \Delta G )/kT</th>
<th>k₂ (s⁻¹)</th>
<th>E.P. k₁ (M⁻¹s⁻¹)</th>
<th>E.P. k₂ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT M2(+1) AMT⁺</td>
<td>380.9</td>
<td>1.11x10⁴</td>
<td>372</td>
<td>2.3x10⁴</td>
</tr>
<tr>
<td>WT M2(+1) RMT⁺</td>
<td>617.6</td>
<td>1.27x10⁴</td>
<td>659</td>
<td>1.58x10⁴</td>
</tr>
<tr>
<td>S31N M2(+1) RMT⁺</td>
<td>1.61x10⁵</td>
<td>24.9</td>
<td>3622</td>
<td>2.73</td>
</tr>
</tbody>
</table>

33
Table 2.3: Complete List of $k_1$ and $k_2$ Values Calculated from ABF Simulations. AMT$^+$ or RMT$^+$ in WT or M2 S31N.

<table>
<thead>
<tr>
<th></th>
<th><strong>ABF Rate Constant Results</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_1$ (M$^{-1}$s$^{-1}$) = $k_c e^{-\Delta G/kT}$</td>
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<tr>
<td><strong>WT</strong> M2(0) AMT$^+$</td>
<td>305.9</td>
</tr>
<tr>
<td><strong>WT</strong> M2(+1) AMT$^+$</td>
<td>380.9</td>
</tr>
<tr>
<td><strong>WT</strong> M2(+3) AMT$^+$</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>S31N</strong> M2(0) AMT$^+$</td>
<td>3.57x10$^7$</td>
</tr>
<tr>
<td><strong>S31N</strong> M2(+1) AMT$^+$</td>
<td>3.44x10$^8$</td>
</tr>
<tr>
<td><strong>S31N</strong> M2(+3) AMT$^+$</td>
<td>3.25x10$^5$</td>
</tr>
<tr>
<td><strong>WT</strong> M2(0) RMT$^+$</td>
<td>2670.3</td>
</tr>
<tr>
<td><strong>WT</strong> M2(+1) RMT$^+$</td>
<td>617.6</td>
</tr>
<tr>
<td><strong>WT</strong> M2(+3) RMT$^+$</td>
<td>109.1</td>
</tr>
<tr>
<td><strong>S31N</strong> M2(0) RMT$^+$</td>
<td>1.04x10$^5$</td>
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<td><strong>S31N</strong> M2(+1) RMT$^+$</td>
<td>1.61x10$^5$</td>
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<tr>
<td><strong>S31N</strong> M2(+3) RMT$^+$</td>
<td>4.54x10$^5$</td>
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Table 2.4: AMT⁺ CHARMM Parameters Used in ABF Simulations.

<table>
<thead>
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<th>AMT⁺ CHARMM Parameters</th>
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</thead>
<tbody>
<tr>
<td><strong>Bond</strong></td>
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<tr>
<td>CG301 - NG3P3</td>
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</table>

<table>
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<th><strong>Angle</strong></th>
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<tr>
<td>CG321 - CG301 - NG3P3</td>
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<tr>
<td>CG301 - CG321 - CG311</td>
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<tr>
<td>CG301 - NG3P3 - HGP2</td>
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<table>
<thead>
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<tr>
<td>NG3P3 - CG301 - CG321 - CG311</td>
</tr>
<tr>
<td>NG3P3 - CG301 - CG321 - HGA2</td>
</tr>
<tr>
<td>CG321 - CG301 - NG3P3 - HGP2</td>
</tr>
<tr>
<td>CG321 - CG311 - CG321 - CG301</td>
</tr>
<tr>
<td>HGA1 - CG311 - CG321 - CG301</td>
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Table 2.5: RMT+ CHARMM Parameters Used in ABF Simulations.

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<th>Bond</th>
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<th>$B_0$ (Å)</th>
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<td>CG301 - CG314</td>
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<td>1.55</td>
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<td>CG314 - NG3P3</td>
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<thead>
<tr>
<th>Angle</th>
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<th>$\theta_0$ (Degrees)</th>
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<tr>
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<td>CG301 - CG314 - HGA1</td>
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<td>110.1</td>
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<td>CG301 - CG321 - CG311</td>
<td>58.35</td>
<td>113.5</td>
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<table>
<thead>
<tr>
<th>Dihedral</th>
<th>$K_{\chi_i}$ (kcal/mol)</th>
<th>Multiplicity</th>
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<td>3</td>
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<tr>
<td>CG321 - CG301 - CG314 - NG3P3</td>
<td>0.2</td>
<td>3</td>
</tr>
<tr>
<td>CG321 - CG301 - CG314 - HGA1</td>
<td>0.195</td>
<td>3</td>
</tr>
<tr>
<td>CG314 - CG301 - CG321 - CG311</td>
<td>0.195</td>
<td>3</td>
</tr>
<tr>
<td>CG314 - CG301 - CG321 - HGA2</td>
<td>0.195</td>
<td>3</td>
</tr>
<tr>
<td>CG321 - CG301 - CG321 - CG311</td>
<td>0.2</td>
<td>3</td>
</tr>
<tr>
<td>CG321 - CG311 - CG321 - CG301</td>
<td>0.2</td>
<td>3</td>
</tr>
<tr>
<td>HGA1 - CG311 - CG321 - CG301</td>
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<td>3</td>
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<tr>
<td>CG301 - CG314 - CG331 - HGA3</td>
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<td>3</td>
</tr>
<tr>
<td>CG301 - CG314 - NG3P3 - HGP2</td>
<td>0.1</td>
<td>3</td>
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Table 2.6: PMF Curve Fit Parameter Sensitivity Test. Example case (WT M2(0) AMT⁺) of changing the well width, barrier width, or barrier height parameters to observe effect on \( k_2 \) values. The well and barrier width parameters do no show a significant effect on \( k_2 \). The barrier height parameter has the greatest effect on \( k_2 \).

<table>
<thead>
<tr>
<th>Width of Well</th>
<th>Width of Barrier</th>
<th>Height of Barrier</th>
<th>( k_2 )</th>
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<tbody>
<tr>
<td>2</td>
<td>0.7</td>
<td>44</td>
<td>2.5x10⁻²³</td>
</tr>
<tr>
<td>1.5</td>
<td>0.7</td>
<td>44</td>
<td>2.2x10⁻²³</td>
</tr>
<tr>
<td>1</td>
<td>0.7</td>
<td>44</td>
<td>1.8x10⁻²³</td>
</tr>
<tr>
<td>0.5</td>
<td>0.7</td>
<td>44</td>
<td>1.2x10⁻²³</td>
</tr>
<tr>
<td>1.5</td>
<td>1</td>
<td>44</td>
<td>2.6x10⁻²³</td>
</tr>
<tr>
<td>1.5</td>
<td>0.7</td>
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<td>2.2x10⁻²³</td>
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<td>1.5</td>
<td>0.4</td>
<td>44</td>
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<tr>
<td>1.5</td>
<td>0.1</td>
<td>44</td>
<td>8.1x10⁻²⁴</td>
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<tr>
<td>1.5</td>
<td>0.7</td>
<td>50</td>
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<tr>
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<td>0.7</td>
<td>44</td>
<td>2.2x10⁻²³</td>
</tr>
<tr>
<td>1.5</td>
<td>0.7</td>
<td>40</td>
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<tr>
<td>1.5</td>
<td>0.7</td>
<td>35</td>
<td>7.1x10⁻¹⁷</td>
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</tbody>
</table>
Section 2: Development and Testing of Novel Non-Metal M2 S31N Inhibitors

Section 2 contains chapters 3 (published in ACS Med. Chem. Lett.) and 4 (re-submitted to ACS Chemical Biology) and covers testing done with novel aminoadamantane derivative M2 blockers that target the same binding region in M2 as amantadine and rimantadine. Chapters 3 and 4 were a collaboration with Dr. Kolocouris from the University of Athens, Greece. In chapter 3, the aminoadamantane analogues showed low effect in blocking the M2 S31N variant. The TEVC data showed high exit rates for the drugs consistent with the high $K_d$ values from ITC and high $EC_{50}$ values from CPE. In chapter 4, the only compound 1 (aminoadamantane analogue) showed moderate block in TEVC against M2 S31N but with high exit rate and showed a moderate $EC_{50}$ value in the CPE assay against the M2 S31N variant. Compound 6 was had the best performance against the M2 S31N variant in TEVC and CPE assays, but that drug was developed by Dr. William DeGrado’s lab. Though our main focus turned to the M2 His37 complex as a therapeutic target for copper complexes, the possibility of discovering M2 S31N blockers that block the same region as amantadine and rimantadine was still an important focus. The studies in section 1 and 2 motivated my focus on the His37 complex in M2 as a better therapeutic target using copper complexes.
Abstract

Recently, the binding kinetics of a ligand–target interaction, such as the residence time of a small molecule on its protein target, are seen as increasingly important for drug efficacy. Here, we investigate these concepts to explain binding and proton blockage of rimantadine variants bearing progressively larger alkyl groups to influenza A virus M2 wild type (WT) and M2 S31N protein proton channel. We showed that resistance of M2 S31N to rimantadine analogues compared to M2 WT resulted from their higher k_{off} rates compared to the k_{on} rates according to electrophysiology (EP) measurements. This is due to the fact that, in M2 S31N, the loss of the V27 pocket for the adamantyl cage resulted in low residence time inside the M2 pore. Both rimantadine enantiomers have similar channel blockage and binding k_{on} and k_{off} against M2 WT. To compare the potency between the rimantadine variants against M2, we applied approaches using different mimicry of M2, i.e., isothermal titration calorimetry and molecular dynamics simulation, EP, and antiviral assays. It was also shown that a small change in an amino acid at site 28 of M2 WT, which does not line the pore, seriously affects M2 WT blockage kinetics.

Keywords: Two-electrode voltage clamp (TEVC), isothermal titration calorimetry (ITC), cytopathic effect (CPE)
Introduction

Novel approaches are necessary in early drug discovery for optimal drug design and improved therapy. Recently, the kinetics of a ligand–target interaction, such as the residence time of a small molecule on its protein target, are seen as increasingly important for in vivo efficacy and safety (3.1). The antiviral agents, amantadine 1 and rimantadine 2 (Figure 3.1), are well-established to be blockers of proton transport by the influenza A virus (IAV) (3.2,3.3). The primary binding site of 1 and 2 is the transmembrane domain lumen (TM, amino acids 22–46) in the four-helix bundle of tetrameric M2, which forms the proton transport path. Since 2008, high-resolution structures have become available for complexes of M2TM wild type (WT) with 1 or 2 (Figure 3.2) (3.4–3.9). Compounds 1 and 2 are effective prophylactics and therapeutics against IAVs, provided they contain the M2TM WT such as A/Udorn/72 H3N2 (Udorn) and A/Hong Kong/68 H3N2 (HK), but not those containing M2 S31N such as A/ WSN/33 H1N1 (WSN) (Figure 3.3). Since 2005, the amantadine 1-insensitive Ser-to-Asn mutation at position 31 in M2 (S31N) has become globally prevalent, abrogating the clinical usefulness of 1 (3.10). Compound 2 is ranked among the best binders to M2TM WT (3.11,3.12) and most potent anti-IAV agents among the aminoadamantane derivatives (3.13,3.14). Thus, the synthesis of symmetrical analogues of 2 with the addition of two methyl 3, ethyl 4, and n-propyl 5 groups on the carbon bridge was accomplished (Figure 3.1) aiming at filling progressively from 3 to 5 the extra space between the ligand and the walls in M2 WT or M2 S31N with a few alkyl groups. Binding affinities of 1, 2, 2-R, 2-S, and 3 - 5 were measured by ITC against the M2TM WT and its S31N variant in their closed form at pH 8. Furthermore, we measured the antiviral activity of the rimantadine analogues against IAV strains and the blocking effect of the compounds against full length Udorn M2, Udorn M2 S31N, and Udorn M2 V28I using electrophysiology (EP), and the
kinetics of binding were compared. Molecular dynamics (MD) simulations of ligand binding to M2TM WT and its S31N variant in their closed form were performed for investigation of the binding mode interactions.

Methods and Materials

Isothermal Titration Calorimetry

Binding affinities of aminoadamantane derivatives (Figure 3.1) for A/Udorn/72 M2TM were determined by ITC experiments for M2TM-ligand systems in DPC micelles at pH 8. All measurements were performed in triplicate with a TAM 2277 (TA Instruments) at pH 8 and 20°C in a buffer of 50 mM NaH₂PO₄ and 100 mM NaCl. The peptide and the aminoadamantane derivative were dissolved in a freshly prepared DPC solution with a concentration of 13 mM. Measurements were conducted using 2 mL of 125 µM peptide (corresponding to 31.25 µM M2TM tetramer). A ligand concentration of 1.1 mM was used for the titrant, of which 7.6 µL (equivalent to 8.4 nmol) were dispensed in the peptide/DPC solution with each injection. The time interval between two injections was set to at least 6 minutes, allowing sufficient time for relaxation of the equilibrium. Synthetic M2TM (residues 22-46) was reconstituted at a 1:57 monomer/lipid ratio - which guarantees the quantitative formation of M2TM tetramers in DPC micelles at pH 8 by dissolving and sonicating 225 nmol of M2TM with the 57-fold higher amount of DPC in the aforementioned buffer system. Solutions of ligands 1, 2-R, 2-S, and 3-5 in the buffer were titrated into the calorimetric cell at 20°C. The heat evolved was obtained from the integral of the calorimetric signal. The heat associated with the binding of the ligand to M2TM was obtained by subtracting the heat of dilution from the heat of reaction. 3,4 Data evaluation was carried out with Digitam for Windows v4.1. Affinity constants were calculated
by non-linear regression of the measured heat per injection using Origin 8.0.5 and are included in Table 3.1.

For the calculation, the concentration of the peptide was kept variable because the M2TM tetramer formation is not complete. Data of three independent measurements was used, whereby all measurements were performed with the same experimental conditions using one stock solution. Data evaluation was done by plotting the measured heat per amount of substance against the molar ratio of titrant to peptide tetramer. The resulting titration curve was fitted using a global fit including the data of the three independent measurements. ITC is a widely used method in drug discovery, especially in quantitative structure-activity relationship studies (81-83). However, there are some inherent limitations with respect to the overall applicability of this method. Since warmth released or consumed by an interaction is detected, the bigger the change of enthalpy during an interaction, the easier this interaction can be measured with ITC. A further limitation is set by the affinity of the interaction. The product of dissociation constant and receptor concentration (called Wiseman constant) determines the slope of the resulting titration curve and should be optimally between 10 and 100 (81-83). For very high affinity interactions, one needs to dilute the system. For low affinity interactions (i.e., $K_d$ values in the range of 10 $\mu$M) a very high quantity of the receptor is needed, and this is accompanied with difficulties due to limitations such as availability, cost, solubility, or stability. For the M2TM peptide investigated in this study, the solubility in the DPC micelles limits the possible concentration. Consequently, affinity constants of low affinity binders e.g. 5 against M2TM S31N possess relatively large errors.
**Cytopathic Effect Assay**

The cytopathic effect (CPE) inhibition assay was used (94) to compare the antiviral potency of 1–5 against HK, Udorn, WSN, and WSN M2 N31S (generated by reverse genetics from WSN) in MDCK cells (Table 3.2). The amino acid sequences of M2 WT in Udorn and HK are identical, not just in the TM region but in the full-length protein.

**Electrophysiology**

The inhibitors were tested with a two-electrode voltage clamp (TEVC) assay using *Xenopus laevis* frog oocytes microinjected with RNA expressing the M2 protein as in previous reports (88,92). The blocking effect of the aminoadamantane derivatives against M2 was investigated with EP experiments using Udorn M2 and Udorn M2 S31N. Because WSN has the V28I substitution in M2, Udorn M2 V28I was generated and studied in parallel to examine whether small changes in WSN in the side chains of amino acids that do not line the pore (Figure 3.3) affect aminoadamantane blocking properties. The blocking effect of the inhibitors was expressed as the inhibition percentage of the M2 current observed after 2, 5, and/or 10 min of incubation with 100 μM compound (Tables 3.3).

**Molecular Dynamics Simulations**

M2TM WT complexes were simulated using an experimental structure of M2TM WT (PDB ID 2KQT) (79,83) determined at pH 7.5 in the presence of 1 (86). No significant differences in measures were detected between trajectories with a production time of 80 ns (Table 3.4). To ensure that the measures were meaningful, the equilibration of the membranes was tested. To verify this, the average area per lipid headgroup was measured in the simulation.
of the different lipids and compared with experimental results. The calculated values approached the experimental ones of pure lipid bilayers (Figure 3.5).

Results and Discussion

Isothermal Titration Calorimetry

Table 1 includes thermodynamic parameters of binding against M2TM WT and M2TM S31N. Compound 1 has a $K_d$ of 2.17 μM. As depicted in Table 1, enantiomers 2-R and 2-S have the same $K_d$ values (92) against M2TM WT ($K_d = 0.34$ and 0.32 μM, respectively). Compound 3, having two methyl groups instead of one methyl group in 2, has the smallest $K_d = 0.13$ μM, i.e., the highest binding affinity of all studied aminoadamantane compounds, suggesting that polar and lipophilic characteristics are well balanced in its structure. The diethyl derivative 4 and dipropyl derivative 5 exhibit lower binding affinities against M2TM WT ($K_d = 4.59$ and 3.43 μM, respectively). A balance between enthalpy and entropy determines the free energy of binding as shown in Table 3.1.

The entropy presumably changes significantly from 1, 2 to 3, 4 on binding because the ordered clathrate water surrounding the ligand is dispersed as the ligand enters the water-poor channel cavity. This is more prominent for 3 and 4, as expected due to their larger hydrophobic surfaces. Presumably, it would have gone up even more for 5, but this clathrate effect was probably countered by a reduced ligand entropy in the channel due to restricted rotation inside the receptor binding area. Compounds 1–3 did not bind efficiently to M2TM S31N according to isothermal titration calorimetry (ITC) and previous surface plasmon resonance measurements for 1 (93), while 5, with a larger adduct connected to adamantane, binds weakly to M2TM S31N compared to M2TM WT according to ITC.
Cytopathic Effect Assay

There was no potency against the amantadine-resistant WSN with the compound concentrations used. All compounds showed low micromolar activity against Udorn, HK, and WSN M2 N31S with 3 being the most potent agent exhibiting submicromolar potency. Inhibition of replication of Udorn was further confirmed with plaque-reduction assay (results not shown). It is of note that 5 only reduced the plaque size but not the number of plaques. The cytotoxicity data (Table 3.2) showed that 1–4 are nontoxic with CC50 values >100 μM, but 5 is mildly toxic with CC50 ≈ 57 μM. The EC50 values for 1–5 (Table 3.2) prioritize the same derivative for M2 WT virus inhibition, i.e., 3, in agreement with the results from the Kd values from ITC experiments based on M2TM WT binding (Table 3.1). Compound 3 is almost equal in structure with rimantadine 2 without having a chiral center. Compound 3 has a promising selectivity index based on the in vitro cytotoxicity data.

Electrophysiology

After 5 min, 3 and 4 block Udorn M2 and Udorn M2 V28I as well as 1 (about 90% and 80%, respectively). Generally, after 2 and 5 min, the percentage of current inhibition was progressively increased for 3 and 4. It is noteworthy that 5 against Udorn M2 exhibited 27% blocking at 2 min, 38% at 5 min, and 61% at 10 min (Table 3.3). The IC50 values of 3 and 4 for Udorn M2 and Udorn M2 V28I were reduced from 2 to 5 min time points (Tables 3.3). These measurements at 2, 5, or 10 min are made prior to the establishment of equilibrium (96) due to very slow on- and off-rates for entry (see the kon and koff rate values in Tables 3 and 4), especially of the bulky ligands like 5, together with the difficulty of maintaining cells at low pH for extended periods. Thus, the very slow binding of 5 (Table 3.3) should not be viewed as inconsistent with the high antiviral potency (submicromolar EC50) against WT (V28; S31)
viruses (Table 3.2), the latter representing much longer exposure times than EP experiments.

In a very recent paper (95), the authors showed that when TEVC \( K_d = \frac{k_{off}}{k_{on}} \) was smaller than a threshold, an \textit{in vitro} antiviral activity was exhibited. For amantadine 1, \( \frac{k_{off}}{k_{on}} = 10^{-6} \) M (1 \( \mu \)M) was measured against M2 WT, which correlates with good \textit{in vitro} antiviral potency. When \( \frac{k_{off}}{k_{on}} \sim 100 \) \( \mu \)M or higher, antiviral potency was not observed even for quick binders. For example, 4-(2-adamantyl)piperidine (compound 3 in ref 95), although a quick blocker against Udorn M2 and the amantadine resistant Udorn M2 V27A, was ineffective against the corresponding influenza A strains. The authors also showed that 2-(1-adamantyl)piperidine (compound 8 in ref 95) was a slow binder against Udorn M2 (48% at 2 min, but 90% at 6 min) but still has good antiviral efficacy, possibly because, although \( k_{on} \) is low, \( k_{off} \) is really low. This is the case with compound 5 against Udorn M2 WT in the present study (see Table 3.3). It has a reduced onset of block compared to 1, 2, and 3, but also has a low dissociation rate constant, so it still has micromolar efficacy against infections of cell cultures by viral strains with M2 WT.

In studies focusing on the development of aminoadamantane ligands against IAV, derivatives are often initially tested in TEVC assays at 100 \( \mu \)M concentration at 2 min, and only the most potent compounds are then tested using whole cell assays (96). If the same procedure had been applied here, 5 would not have been tested, even though it proved to be a low micromolar inhibitor according to \( K_d \) values from ITC experiments with M2TM WT (Table 3.1) and CPE assay (Table 3.2) results. Thus, TEVC percent block for 100 \( \mu \)M at 2 min in MDCK cells underestimated the potential of 5.

Similarly, 4 would not have been tested based on percent block at 2 min in TEVC with Udorn M2 V28I (43 \( \pm \) 2%). Slow block could be associated with tight block, and this phenomenon should not be overlooked in short-lasting experiments. The results suggested that
TEVC results, when used for compound filtering, need careful interpretation for compounds having low association rate constant for binding to the full length M2, which also depends on the M2 pore. In this regard, ITC measurements represent an important additional tool for clarifying the binding energies of novel derivatives to M2TM given their capacity for sufficient relaxations of equilibrium between titration injections. Nevertheless, it is clear from ITC, CPE (all strains with M2 WT), that 5 (and 4 where tested) are ∼10-fold less active than 3.

For Udorn M2 V28I, the percentage of current inhibition is lower; compounds 3 and 4 inhibit Udorn M2 more rapidly than Udorn M2 V28I. A small change in an amino acid at site 28 (V28I) of M2, which does not line the pore, seriously affects M2 blockage kinetics. The inhibition of 3 and 4 on both Udorn M2 and Udorn M2 V28I are irreversible in our experimental time frame, as was also observed for 1 with both proteins (data not shown). After the aforementioned results highlighted the importance of k_{on}, k_{off} values on ligand behavior, we were intrigued to further investigate the block of rimantadine enantiomers against Udorn M2 WT protein in EP, considering the differences in resonances seen in ssNMR studies of 2-R or 2-S bound to the full-length Udorn M2 protein (97). We previously showed (92) that 2- R and 2-S showed similar channel blockage against Udorn M2 WT when tested in EP at 100 μM at the 2 or 5 min time point, and this result is consistent with that from ITC measurements (see ref 92 and Table 3.1) and antiviral assays (see ref 92 and Table 3.2).

In our present work we seek to further investigate the binding kinetics of 2-R, 2-S by measuring the respective k_{on}, k_{off}, K_d (TEVC) values. The EP measurements showed a k_{off} = 0.0013 s\(^{-1}\) for 2-R and a k_{off} = 0.0016 s\(^{-1}\) for 2-S (Table 3), i.e., the two enantiomers had very similar binding kinetics. Thus, 2-R has a bit longer residence time inside the receptor than 2-S, as reflected by its slightly lower k_{off} and K_d values (2.4 vs 3.2 μM). In ref 97, the first ssNMR study
of the full length M2 in complex with rimantadine enantiomers was published. Compound 2-R was argued to have a higher affinity than 2-S based on differences in peak intensities and position restrained MD simulations. The results published in ref 97 are in qualitative agreement with those reported here, but not in quantitative agreement, as here we see no statistically significant (ITC and EC50) or meaningful (EP) difference. Perhaps this is a consequence of the different methodologies applied, i.e., EP vs ssNMR spectroscopy.

Chemical shifts differences and peak intensities do not provide an accurate quantitative estimate of binding affinity values. The EP results, antiviral assays, and ITC results showed clearly that the two rimantadine enantiomers have similar binding free energies, channel blockage, $k_{on}$ and $k_{off}$ rate constants, and antiviral potencies. We conclude that they form equally stable complexes and have the same residence time inside M2 WT. The compounds did not bind to Udorn M2TM S31N according to ITC and did not exhibit antiviral potency against WSN virus, which contains both the S31N and the V28I mutations. We showed that a valuable parameter to explain the resistance of M2 S31N viruses to rimantadine analogues compared to M2 WT is a higher $k_{off}$ rate (i.e., a smaller residence time inside M2 S31N).

According to our previous results, this is due to the fact that, in M2 S31N, the loss of the V27 pocket for the adamantyl cage (86) resulted in low residence time inside M2TM and a lack of antiviral potency; but for 5, the sizable adducts resulted in a weak binding, which is albeit not sufficient for antiviral potency (86). It is the high dissociation rate constants that render aminoadamantanes useless against S31N viruses like WSN leading to $K_d$ (TEVC) in the millimolar range compared to the micromolar range for M2 WT binding.

In the S31N variants, TEVC (Table 3.5) shows very high exit rate constants, especially for 2 (0.9 s$^{-1}$) and 5 (0.14 s$^{-1}$), consistent with the unmeasurably high $K_d$ in ITC (Table 3.1) and
EC$_{50}$ in CPE (Table 3.2, WSN). Interestingly, in these two cases, 2 and 5 have low % block of inward currents at 2, 5, and 10 min in Udorn M2 S31N (Table 3.5), and somewhat similar $k_{on}$ rates to 1 and 3, albeit lower compared to M2 WT, (Table 3.3), demonstrating that mutations can have complex, ligand-dependent effects on entry and exit rates.

**Molecular Dynamics Simulations**

The center of mass between the four V27 residues and the adamantane cage of the ligand stabilized as explained in Figure 1 varies between 4.1 and 4.5 Å on average (Table 3.4). Hydrogen bond interactions for 2-R and 2-S and geometric measures, which reflect van der Waals contacts, were found to be similar for the two enantiomers, suggesting equal binding interactions as previously discussed (Figure 3.4) (92). In diethyl and di-n-propyl derivatives (4 and 5), the alkyl groups seem to better fill the space between the ligand and the pore walls; but in these cases, restricted motion and the resulting entropy cost of binding may be significant and decrease the binding affinities compared to 3. Configurations from the simulations of ligands 3 and 5 are depicted in Figure 3.6.

In all cases, in the region located above the adamantane core (i.e., toward the N-terminus) no water molecules were found, which is consistent with the proton blocking effect of the aminoadamantane derivatives (77,86,97,99). The MD simulations of the complex of 3 or 5 with M2TM S31N showed that the ligand cannot bind tightly to M2TM S31N because significant favorable van der Waals interactions are missing (Figure 3.5). The S31N mutation of M2TM results in a shift of the hydrophobic adamantyl ring toward the C-terminus, due to the enhanced repulsive forces of the asparagine amide side chains to the adamantyl ring and attraction to water molecules.
As a consequence, the stabilizing hydrophobic interactions of the V27 isopropyl groups with the adamantyl ring that are present in the M2TM WT are lost in M2TM S31N (86). The bulky Val27 and N31 side chains are oriented toward the N-terminus the latter forming hydrogen bonding interactions with water molecules; the ammonium group of the ligands are also turned toward the N-terminus, allowing significant hydrogen bonding interactions with the polar N31 side chains and the nearby water molecules.

Conclusion

In this work, we compared the potency between the rimantadine analogues against M2 using four different M2 mimicry methods, i.e., ITC, MD simulations, EP, and antiviral assays. We investigated the binding kinetics of rimantadine analogues with M2 WT and M2 S31N and how they influenced the outcome of potency. We provided a kinetic perspective to explain rimantadine variant binding, proton transport blockage, and antiviral potency against influenza M2 WT and M2 S31N. According to this study, aminoadamantane variants bearing a polar head should exhibit a kinetic profile of small $k_{off}$ rates (i.e., long residence time inside the M2 S31N protein channel pore) resulting in $K_d = k_{off}/k_{on}$ values at the low micromolar region, for them to exhibit inhibitory potency against M2 S31N protein.

Contribution to Publication

Collected the electrophysiology data in Table 3.3, wrote the electrophysiology methods and materials and results section, and provided input for the electrophysiology discussion.
Figure 3.1: Structures of Aminoadamantane Derivatives 1–5.

Figure 3.2: Structure and Amino Acid Sequence of M2TM WT. Cartoon representation of M2TM (PDB entry: 2KQT) with critical residues for binding: V27 (red), A30 (light blue), His37 (pink) depicted as sticks. Side view of M2TM; one monomer of the tetramer was removed for visualization purposes; the N-terminal end is at the top. The amino acid sequence of M2TM WT corresponding to 2KQT: SSDPLVVAASIIGILHLILWILDRL.
Figure 3.3: Transmembrane Domain Sequence for A/Udorn/72 (and A/Hong Kong/68). Compared to A/WSN/33, which differ at positions 28 and 31.
Figure 3.4: Synthetic Scheme for the Preparation of Compounds 3–5.
Figure 3.5: Representative Snapshots from the Simulations of Ligand 3(a) and 5 (b) Bound to M2TM S31N. Five and three water molecules are shown between ligand 3 and 5, respectively, and the H37 residues and ten and twelve water molecules, respectively, between N31 and the mouth of the pore. The ammonium group of the ligand is oriented towards the N-terminus, where it forms hydrogen bonds with water molecules or the carbonyl group of the N31 amide side chain. The loss of the V27 pocket for the adamantyl cage would be expected to lead to weak binding of aminoadamantane ligands.
Figure 3.6: Representative Snapshots from the Simulation of Ligands 3(a) and 5(b) Bound to M2TM WT. Nine and seven water molecules are shown between the ligand and H37 residues for 3 and 5, respectively. Three hydrogen bonds between the ammonium group of the ligand and three water molecules are shown. Hydrogen bonding with water molecules and van der Waals interactions of the adamantane core with V27 and A30 side chains stabilize the ligand inside the pore with its ammonium group oriented towards the C-terminus of the channel.
Table 3.1: Binding Constant, Free Energy, Enthalpy, and Entropy of Binding Derived from ITC Measurements. At 300 K for M2TM WT (from Udorn, Upper Table) and the M2TM S31N.  

<table>
<thead>
<tr>
<th>ligand</th>
<th>$K_d^{a}$</th>
<th>$\Delta G^{c}$</th>
<th>$\Delta H^{d}$</th>
<th>$-T\Delta S^{e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.17 ± 0.52</td>
<td>-7.77 ± 0.14</td>
<td>-6.66 ± 0.50</td>
<td>-1.11 ± 0.52</td>
</tr>
<tr>
<td>2</td>
<td>0.51 ± 0.26</td>
<td>-8.64 ± 0.30</td>
<td>-7.60 ± 0.28</td>
<td>-1.04 ± 0.41</td>
</tr>
<tr>
<td>2-R</td>
<td>0.32 ± 0.16</td>
<td>-8.97 ± 0.26</td>
<td>-7.54 ± 0.34</td>
<td>-1.42 ± 0.43</td>
</tr>
<tr>
<td>2-S</td>
<td>0.34 ± 0.12</td>
<td>-8.88 ± 0.21</td>
<td>-7.73 ± 0.28</td>
<td>-1.15 ± 0.35</td>
</tr>
<tr>
<td>3</td>
<td>0.13 ± 0.12</td>
<td>-9.30 ± 0.43</td>
<td>-4.19 ± 0.28</td>
<td>-5.12 ± 0.51</td>
</tr>
<tr>
<td>4</td>
<td>4.59 ± 2.21</td>
<td>-7.33 ± 0.28</td>
<td>-3.29 ± 0.62</td>
<td>-4.03 ± 0.68</td>
</tr>
<tr>
<td>5</td>
<td>3.43 ± 1.05</td>
<td>-7.50 ± 0.18</td>
<td>-6.23 ± 0.45</td>
<td>-1.27 ± 0.48</td>
</tr>
<tr>
<td>1–3</td>
<td>f</td>
<td>f</td>
<td>f</td>
<td>f</td>
</tr>
<tr>
<td>5</td>
<td>&gt;10</td>
<td>f</td>
<td>f</td>
<td>f</td>
</tr>
</tbody>
</table>

See Figure 3.1: $^b$Binding constant $K_d$ in $\mu$M.  
$^c$Free energy of binding in kcal mol$^{-1}$.  $^d$Binding enthalpy in kcal mol$^{-1}$.  
$^e$Entropy of binding in kcal mol$^{-1}$.  $^f$Values could not be determined reliably due to the limitations of the methods in the area of very weak binding.
Table 3.2: Cytotoxicity (CC50) and Antiviral Activity (EC50) of Compounds 1–5 Against IAVs HK, Udorn, WSN, and WSN M2 N31S. In Madin–Darby Canine Kidney Cells. \(^a\)Mean and standard deviations of the 50% inhibitory concentration (EC50) and the 50% cytotoxic concentration (CC50) of at least three independent assays. \(^b\)Inhibition of plaque size without reduction of plaque number. \(^c\)ND: Not determined.

<table>
<thead>
<tr>
<th>ligand</th>
<th>EC50 (μM)(^a)</th>
<th>CC50 (μM)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HK</td>
<td>Udorn</td>
</tr>
<tr>
<td></td>
<td>M2 (V28; S31)</td>
<td>M2 (V28; S31)</td>
</tr>
<tr>
<td>1</td>
<td>ND(^c)</td>
<td>0.78 ± 0.44</td>
</tr>
<tr>
<td>2</td>
<td>0.05 ± 0.04</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>2-R</td>
<td>ND(^c)</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>2-S</td>
<td>ND(^c)</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.012 ± 0.003</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>4</td>
<td>0.46 ± 0.25</td>
<td>0.41 ± 0.23</td>
</tr>
<tr>
<td>5</td>
<td>0.45 ± 0.34</td>
<td>1.07 ± 0.31(^b)</td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>0.002 ± 0.001</td>
<td>0.001</td>
</tr>
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Table 3.3: Block\(^a\) of Inward Currents in Oocytes\(^b\) Transfected with Full-Length Udorn M2 by Selected Compounds. \(^a\) For each compound, percent block of pH-dependent M2 current at listed concentrations (±SEM) and IC\(_{50}\) (μM) are shown. \(^b\) Three replicates were used for measurements at 100 μM. \(^c\) ND: Not determined. \(^d\) K\(_d\) (TEVC) = k\(_{off}\)/k\(_{on}\).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>% Block (2 min)</th>
<th>% Block (5 min)</th>
<th>% Block (10 min)</th>
<th>IC(_{50}) (2 min) (μM)</th>
<th>IC(_{50}) (5 min) (μM)</th>
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<tr>
<td>1</td>
<td>90 ± 2</td>
<td>95 ± 1</td>
<td>ND(^c)</td>
<td>12.5</td>
<td>4.7</td>
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<tr>
<td>2</td>
<td>96 ± 1</td>
<td>96 ± 1</td>
<td>ND(^c)</td>
<td>10.8</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>2-R</td>
<td>95 ± 1</td>
<td>96 ± 1</td>
<td>ND(^c)</td>
<td>ND(^c)</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>2-S</td>
<td>93 ± 1</td>
<td>95 ± 1</td>
<td>ND(^c)</td>
<td>ND(^c)</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>3</td>
<td>90 ± 2</td>
<td>96 ± 1</td>
<td>ND(^c)</td>
<td>9.3</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>78 ± 2</td>
<td>91 ± 1</td>
<td>ND(^c)</td>
<td>24.3</td>
<td>13.2</td>
</tr>
<tr>
<td>5</td>
<td>27 ± 1.2</td>
<td>38 ± 1.6</td>
<td>61 ± 2.3</td>
<td>ND(^c)</td>
<td>ND(^c)</td>
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</table>

<table>
<thead>
<tr>
<th>Ligand</th>
<th>k(_{on}) (M(^{-1}) s(^{-1}))</th>
<th>k(_{off}) (s(^{-1}))</th>
<th>K(_d) (μM)(^d)</th>
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<tbody>
<tr>
<td>1</td>
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<td>0.003</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>416</td>
<td>0.003</td>
<td>7</td>
</tr>
<tr>
<td>2-R</td>
<td>412</td>
<td>0.0013</td>
<td>3.2</td>
</tr>
<tr>
<td>2-S</td>
<td>407</td>
<td>0.0016</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>230</td>
<td>0.003</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>ND(^c)</td>
<td>ND(^c)</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>0.003</td>
<td>88</td>
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</table>
Table 3.4: Structural and Dynamic Measures from MD Trajectories of A/Udorn/72 M2TM-Ligand Complexes in DMPC Bilayer (4 ns).7
1See Figure 3.1; measures for 1 were added for comparison reasons.
2Maximum root-mean-square deviation (RMSD) for Cα atoms of M2TM relative to the initial structure (PDB entry: 2KQT) after root-mean-square fitting of Ca atoms of M2TM; in Å.
3Angle between the vector along the bond from the carbon atom of the adamantane core to the ligand nitrogen atom and the normal to the membrane; in degrees.
4Mean distance between center of mass of V27 and centers of mass of adamantane calculated using Gromacs tools; in Å.
5Mean number of H-bonds between ligand's ammonium group and waters.
6Mean distance in Å between the ligand N and the nearest Cl-.
7The axial position of the six compounds 1, 2, 2-R, 2-S, and 3-5 inside the pore differed only slightly, that is, 0-0.3 Å towards the C-end, relative to 1. The simulated M2TM-ligand complexes were stable, and in all cases the M2TM tetramer showed no large conformational changes in the course of the simulations, as demonstrated by RMSDs ≤ 1.8 Å for M2TM Cα-carbons with respect to the initial structure.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>RMSD (Cα)</th>
<th>Angle C-N vector</th>
<th>V27-Ad</th>
<th>H-bonds</th>
<th>Cl-N distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2 ± 0.2</td>
<td>9.8 ± 5.4</td>
<td>4.2 ± 0.3</td>
<td>2.7 ± 0.5</td>
<td>23.5 ± 9.3</td>
</tr>
<tr>
<td>2-R</td>
<td>1.8 ± 0.5</td>
<td>52.0 ± 6.7</td>
<td>4.2 ± 0.3</td>
<td>2.6 ± 0.5</td>
<td>34.2 ± 6.5</td>
</tr>
<tr>
<td>2-S</td>
<td>1.2 ± 0.2</td>
<td>50.9 ± 5.3</td>
<td>4.1 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>33.7 ± 7.4</td>
</tr>
<tr>
<td>3</td>
<td>1.2 ± 0.2</td>
<td>53.8 ± 8.3</td>
<td>4.1 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>32.7 ± 6.1</td>
</tr>
<tr>
<td>4</td>
<td>1.6 ± 0.5</td>
<td>54.3 ± 6.9</td>
<td>4.4 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>33.9 ± 7.7</td>
</tr>
<tr>
<td>5</td>
<td>1.0 ± 0.2</td>
<td>61.5 ± 7.1</td>
<td>4.5 ± 0.4</td>
<td>2.9 ± 0.3</td>
<td>32.4 ± 7.4</td>
</tr>
</tbody>
</table>
Table 3.5: Block of Full-Length Udorn M2 S31N-Dependent Current by Selected Compounds.

For each compound, percent block of pH-dependent M2 current at listed concentrations (±SEM) and IC$_{50}$ (μM) are shown. Three replicates were used for measurements at 100 μM. Racemic. ND: Not determined. $^aK_d = k_{off}/k_{on}$.

<table>
<thead>
<tr>
<th>ligand</th>
<th>% block after 2 min</th>
<th>% block after 5 min</th>
<th>% block after 10 min</th>
<th>$k_{off}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_d$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35 ± 2</td>
<td>36 ± 1</td>
<td>36.3 ± 1</td>
<td>143</td>
<td>0.03</td>
<td>210 μM</td>
</tr>
<tr>
<td>2$^c$</td>
<td>1.0 ± 0.2</td>
<td>1.5 ± 0.4</td>
<td>ND$^d$</td>
<td>22</td>
<td>0.9</td>
<td>&gt;10 mM</td>
</tr>
<tr>
<td>3</td>
<td>21 ± 2</td>
<td>30 ± 3</td>
<td>33 ± 1</td>
<td>18</td>
<td>0.008</td>
<td>444 μM</td>
</tr>
<tr>
<td>5</td>
<td>7.0 ± 0.4</td>
<td>7.6 ± 0.2</td>
<td>8.0 ± 0.4</td>
<td>79</td>
<td>0.14</td>
<td>1.8 mM</td>
</tr>
</tbody>
</table>
CHAPTER 4: Understanding Blocking of Influenza A M2 Channels Using Chemical Probes and Binding Kinetics


Abstract

Compounds consisting of amantadine linked to an aryl head group through a methylene bridge, are second generation amantadine variants that may block proton current mediated by the amantadine resistant M2 S31N and amantadine sensitive M2 WT channels. They may also be lysosomotropic, neutralizing late-stage endosomes and lysosomes as chloroquine is thought to inhibit SARS-CoV2 infection.

Here, we used the synthetic aminoadamantane-aryl head group conjugates 1-6, as sensitive probes for blocking M2 S31N and M2 WT channels as well as virus replication in cell culture. All of the compounds inhibit infection of cell cultures by influenza virus containing M2 S31N. Binding free energy calculations and binding kinetics experiments showed, correspondingly, that all compounds can bind to the M2 WT channel and have high association and low dissociation rate constants. In contrast, most have high dissociation rate constants for the M2 S31N channel. The blocking of the M2 S31N channel is very sensitive to the distance between adamantane and the aryl head, and even one additional isopropyl (dimethylmethylene) attenuates blocking. It is suggested that this is due to the smaller length along the M2 S31N pore to which a ligand can bind, i.e. from V27 to G34, compared with the longer length in the M2 WT channel, from V27 to H37. In M2 S31N, binding of the enlarged compounds causes deformations in the N-terminus according to MD simulations, enhancing dissociation rate. The aryl head composition plays a critical role in the balance between association rate and dissociation rate, since (4-methoxy-2-hydroxy)-benzyl
causes effective blockage against both M2 channels, while 3-(thiophenyl)isoxazolyl effected very strong blockage only against the M2 S31N channel. We conclude that only the latter complex blocks infection through the M2 block mechanism in the S31N variant, but that the other five block through the lysosomotropic effect, which is expected, because of their hydrophobic adducts, to be stronger than for amantadine.

Keywords: Madin-Darby canine kidney cells (MDCK), two-electrode voltage clamp (TEVC), cytopathic effect (CPE)
Introduction

SARS-COV2 is inhibited by chloroquine, probably because of endosome and/or trans-Golgi neutralization (4.1). The aminoadamantanes or amantadine variants are known to have these effects causing influenza A inhibition (4.2). Thus, aminoadamantanes, especially those with hydrophobic adducts, are lysosomotropic drugs (4.3,4.4) that can be accumulated through membrane permeation to lysosomes causing increased pH. Additionally, aminoadamantanes can block proton current mediated by the influenza A M2 channel(4.5-4.7), abrogating the low pH mediated release of viral ribonuclear proteins from the virus capsid at endosome expulsion and protection against premature hemagglutinin conversion in the trans Golgi network. These drugs are thought to block M2 current by binding to a high affinity site in the lumen of the homotetrameric channel, with the adamantyl group at the level of pore-lining residues 30 and 31 cluster of four residues on the channel axis. Outside of that site, four V27 residues form a hydrophobic ring lining the narrow channel entry. Deeper inside the channel, four H37 residues form the proton selectivity filter. In M2 WT, amantadine binds at this site with the amine projecting inwards towards the H37 cluster, with the adamantyl group contacting the hydrophobic gate to seal the channel to water-mediated proton flow.

Resistance of influenza A virus (IAV) to the proton channel drugs amantadine and rimantadine is associated with mutations in the M2 transmembrane domain (M2TM). The vast majority of resistant viruses (95%) bear the S31N substitution in M2 (4.8,4.9). The M2 S31N mutant, which is currently the main epidemic strain, is a naturally occurring amantadine- and rimantadine-resistant mutation that otherwise maintains channel function nearly identical to the M2 WT, referring to the strain A/Udorn/72 commonly used in electrophysiology (EP) studies.
DeGrado and Wang discovered potent compounds acting against M2 S31N in both EP and antiviral assays (4.10-4.17). These compounds are second generation amantadine-based drug molecules, which include aryl or heteroaryl rings linked with amantadine through a methylene bridge, as in compounds 1 and 6 in Figure 4.1. Extensive structure-activity relationships (SAR) investigations were performed through modifications of the adamantyl group and the aryl head group. DeGrado and Hong used MD simulations based on the solution state structure for M2TM S31N in micelles (PDB 2LY0) (4.11) or solid state (ss) NMR structure in lipid bilayers (4.11,4.17,4.18) to show that the aryl head of these compounds, represented by 6, is oriented outward with the aryl ring trapped between the V27 side chains. These experiments suggest that the isoxazolyl group forms hydrogen bonds with the N31 amide side chains and the thiophenyl group lies between V27 side chains. This orientation of 6 in the M2 S31N (4.17) channel is opposite from that of amantadine in the WT channel (4.7,4.19).

While, the binding of the commercial drugs amantadine, rimantadine and other amantadine variants to the M2 WT has been extensively studied by experimental structures and MD simulations (4.7,4.19-4.23), Busath, Cross, and Kolocouris, using MD simulations and ssNMR, suggested that amantadine is oriented outward in the M2TM S31N channel, losing its fit with V27 side chains and therefore its proton current blockage efficiency and antiviral potency (118,119). DeGrado and Wang also discovered that amantadine-aryl head dual channel-type inhibitors adopt a different orientation in the M2 S31N (4.17) from that of amantadine in the WT channel (4.7,119). For the dual blocker having an apolar 2-bromo-thiophenyl group connected with amantadine, NMR NOEs and MD simulations suggested that is oriented with the aryl head group outward in the M2TM S31N and inward in the M2TM WT pore (4.17).
Kinetic parameters of protein-ligand interactions are considered as critical for rational drug discovery. A targeted optimization of binding kinetics is not easy to achieve, and systematic studies are necessary to increase the understanding about molecular interaction involved (4.26,4.27). In this work, based on the activity of the dual inhibitor 1 and the specificity of 6 for blocking only M2 S31N, we synthesized compounds 2-5, which expand on the structural motifs in 1 (Figure 4.1). We added a linker between the adamantane and amino group, which is CMe2 in 2 or phenyl in 3. Additionally, we explored the impact of using a larger non-polar component, diamantyl in 4 and triamantyl in 5. Thus, compared to 1, in 2, 3, 4, and 5 the amantadine scaffold is replaced with the 2-(1-adamantyl)-propan-2-amine 8, 4-(1-adamantyl)-1-benzenamine 9, 4-aminodiadamantane 10, and 9-aminotriadamantane 11 respectively. Compounds 1-6 provide a set of comparable but distinctive ligands and are useful chemical probes for exploring the molecular features affecting the energetics, orientational trajectory, and kinetics of binding to the M2 WT and S31N channel lumen as well as the lysosomotropic behavior.

We used these chemical probe molecules to explore: (1) What is the orientation of the ligands when bound in M2 WT and M2 S31N? (2) Can the 14-carbon diamantane or the 18-carbon triamantane in 3 or 4, respectively, fit into the M2 channel? (3) Whether the binding efficiency of the ligands leads to M2 blockage. We explored (1)-(3) using MD simulations and binding free energy calculations, i.e., docking and Molecular Mechanics - Poisson Boltzmann Surface Area (MM-PBSA) calculations. (4) How the length of the linker between the non-polar moiety of aryl head group (like the methoxy group in 1 and the thiophenyl group in 6) affects M2 WT and M2 S31N blockage? (5) How kinetics affect M2 blocking efficiency and anti-viral potency? To address (4) and (5), we also investigated the potency and kinetics of binding (entry and exit process) against M2 WT and M2 S31N using anti-viral assays and electrophysiology (EP),
respectively. Then, we evaluated (6) the lysosomotropic efficacy using the assumption that any antiviral effects that were not mediated by direct M2 channel block are most likely due to endosome neutralization caused by deprotonated drug diffusion through the endosome membrane where, thereafter, proton buffering and charged drug trapping occurs.

This work adds to previous SAR examinations of the kinetics of binding of amantadine derivatives with aryl head group conjugates blocking M2 S31N (128), or aminoadamantane analogues blocking M2 WT and M2 V27A (4.29,4.30). We show that the low degree of block observed in EP and anti-viral efficacy is due to the kinetics of binding and in particular to the low association rate, which turns out to be critical for M2 channel blockers or any other channel blockers in general.

Methods and Materials

Cells and Viruses

Madin-Darby canine kidney (MDCK) cells (Cat.no. RIE 328, Friedrich-Loeffler Institute, Riems, Germany) were propagated as monolayer in Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate and 2 mM L-glutamine. Amantadine-sensitive A/Udorn/72, amantadine-resistant A/WSN/33-M2-WT (with an N31 in M2) and its variant with N31S amino acid substitution in the M2 ion channel were used in this study. Briefly for the generation of A/WSN/33-M2-N31S (4.31) the plasmid pHW187-M2-N31S was altered by site-directed mutagenesis PCR and afterwards used as part of a plasmid set for the recovery of A/WSN/33 virus (4.32). A/WSN/33-variants were propagated on MDCK cells in serum-free EMEM supplemented with 2 mM L-glutamine, 2 μg/mL trypsin to activate the virus, and 0.1% sodium bicarbonate (test medium). Virus-containing supernatant was harvested after about 48 h of incubation at 37 °C when cytopathic effect became
microscopically visible. Aliquots were stored at –80 °C until use. The M2 gene identity of all recombinant viruses was verified by sequencing.

**CPE Assay**

Cytotoxicity and CPE inhibition studies were performed on two-day-old confluent monolayers of MDCK cells grown in 96-well plates as published (4.48). Cytotoxicity was analyzed 72 h after compound addition. In the CPE inhibition assay, 50 μL of a serial half-log dilution of test compound or control compound (amantadine or oseltamivir) in test medium (maximum concentration 100 µM) was added to the cell-containing wells while maintaining a constant multiplicity of infection. Then, plates were incubated at 37 °C with 5% CO2 for 48 h. Crystal violet staining and determination of the 50% cytotoxic (CC50) and 50% inhibitory concentration (EC50) was performed as described before (4.48,4.49). At least three independent assays were conducted.

**Electrophysiology**

Using methods published previously (4.30,4.50), oocytes from *Xenopus laevis* (Ecocyte, Austin, TX) were maintained in ND-96++ solution at 17° C until injection of ~40 ng of A/Udorn/72 H3N2 M2 S31 (WT) or A/Udorn/72 H3N2 M2 S31N mRNA using a Nanoject II (Drummond Scientific, Broomall, PA). After injection, the oocytes were maintained at 4° C in ND-96++ pH 7.4 until electrophysiological recording. 72 h after mRNA injection, whole-cell currents were recorded with a two-electrode voltage-clamp apparatus at Vm = –20 mV, room temperature, in Barth’s solution, pH 7.5. Inward current was induced by perfusion with Barth’s pH 5.3. Current block was induced by perfusion of Barth’s pH 5.3 100-µM drug solution. Percentage block of the original inward current by test compound was measured just before washout, which was done 10 min after drug exposure for each compound. Percentage washout, relative to original inward current (i.e. if
40% block was achieved during wash-in and all of it washed out, the percentage washout is 40%),
was measured after 5 min of perfusion with Barth’s pH 5.3 (no drug). To obtain rate constants for
the relaxation to equilibrium after perfusion with test compound, a single exponential plus a
baseline was used to fit the first two minutes of perfusion or the first three minutes of washout.
From the inverse of the washout time constant, the dissociation rate constant, $k_{off}$, was obtained.
The inverse of the wash-in time constant was taken as the sum of the association and dissociation
rates, $[\text{drug}] \times k_{on} + k_{off}'$, where $k_{off}'$ was taken to be negligible here. $K_d$ was taken to be $k_{off}/k_{on}$.

_Molecular Docking Calculations_

The ligands in their ammonium forms were built by means of Maestro program
(4.51,4.52), were then minimized by means of Macromodel 9.6 and the MMFF94 force field
(4.53) implemented with Macromodel 9.6 using the CG method and a distance-dependent
dielectric constant of 4.0 until a convergence value of 0.0001 kJ Å$^{-1}$ mol$^{-1}$ was reached. The
ligands minimized in this manner were docked into the M2TM WT or M2TM S31N binding
area. The M2TM WT-amantadine complex structure of the homotetrameric protein structure for
A/Hong Kong/156/1997 M2 H5N1 (identical in TMD sequence to that of A/Udorn/72, which is
traditionally used for EP), derived from the solid-state NMR structure (PDB 2KQT) (4.21),
determined at pH 7.5, and keeping the structural waters between amantadine and H37 observed
in the high resolution x-ray structure of the complex (4.7), was used for the protein model for
M2TM WT with bound ligands. N- and C-termini of the M2TM model systems were capped by
acetyl and methylamino groups. After applying the protein preparation module of Maestro, all
hydrogens of the protein complex were minimized with the AMBER* force field by means of
Maestro/Macromodel 9.6 using a distance-dependent dielectric constant of 4.0. The molecular
mechanics minimizations were performed with a conjugate gradient (CG) method and a
threshold value of 0.0001 kJ Å$^{-1}$ mol$^{-1}$ as the convergence criterion. The structures of the protein and amantadine were saved separately and were used for the subsequent docking calculations. A model of M2TMs31N apo-protein was generated from M2TMWT apo-protein for PDB ID 2KQT by mutating amino acids S31 to N31 with Maestro and preparing the structure as described above. This M2TM S31N (22-46) structure was superimposed with that of M2TM S31N (18-60) determined by solution NMR spectroscopy in DPC micelles (PDB ID 2LY0) at alkaline pH (4.11), and deletion of M2 S31N (22-65) apo-protein and amantadine, in order to produce a complex between the M2TM S31N (22-46) and 6. Then the structures of the M2TM S31N and 6 were saved separately and were used for the subsequent docking calculations. Docking poses of compounds 1-6 inside the M2TM WT were generated with Glide (4.54,4.55) including five structural water molecules located between ammonium group of amantadine and H37 within the M2TMWT pore-binding site and applying with XP scoring function (156), implemented in the software. The same results favoring outward orientation of the aryl head group were also obtained using Glide and the induced-fit docking method (4.54,4.55) with XP scoring function. The highest-scored docking solutions for compound 1-6 in this amantadine-insensitive channel adopt an outward orientation of the aryl head group. The inward orientations of the compounds were also obtained, but as less favorable in docking score. Docking of compounds 1-6 inside M2TM S31N was performed as previously described. The docking poses of the ligand inside M2TM were visually inspected using the UCSF Chimera package (4.57). Two docking poses were kept for MD simulation, one with polar head group oriented outwards as in the experimental solution NMR structure of complex with 6 (PDB: 2LY0) and one with the polar head group oriented inwards.
For the MD simulations, the structure of amantadine in complex with the homotetrameric protein structure M2TM WT for A/Hong Kong/156/1997 M2 H5N1 was used as a template. The protein structure was modeled after the solid-state NMR structure (PDB ID 2KQT, based, in part, on constraints representing a previously determined structure) (4.21,4.58) determined at pH 7.5 in DMPC bilayers extending 10 Å beyond the proteins in two dimensions. The proteo-lipid structure was solvated using the TIP3P (4.59) water model, with Na\(^+\) and Cl\(^-\) ions placed in the water phase at the experimental salt concentration of 0.150 M NaCl with a disparity appropriate to neutralize the system using the “System Builder” utility of Desmond (4.52,4.60). The ionizable residue, histidine 37, was maintained at a neutral charge (HSE) corresponding to alkaline solution (pH >9). For Table 4.1 and Figure 4.2, compounds **1-6** were positioned in the highest-scored docking solution in the M2TM WT or M2TM S31N channels. The polar head group of the ligands (one of **1-6**) were positioned oriented inward (WT) or outward (S31N).

The MD simulation conditions were found to be well tuned from reference simulations with amantadine, the aminodiamantane, and aminotriamantane analogs (10 and 11) in complex with M2TM WT in fully hydrated DMPC bilayers (see Supporting Information). The complexes of each of the compounds **1-6** with M2TM WT (22-46) were embedded in fully hydrated DMPC bilayers and subjected to 80-ns MD simulations. The OPLS2005 force field (4.61,4.62,4.63) was used to model all protein and ligand interactions. The particle mesh Ewald method (PME) (4.64,4.65) was employed to calculate long-range electrostatic interactions with a grid spacing of 0.8 Å. Van der Waals and short range electrostatic interactions were smoothly truncated at 9.0 Å. Periodic boundary conditions were applied with a system size of approximately 50×50×80 Å\(^3\). The Nosé-Hoover thermostat (4.66) was utilized to maintain a constant temperature in all simulations,
and the Martyna-Tobias-Klein method (4.66) was used to control the pressure at 1 atm. The equations of motion were integrated using the multistep RESPA integrator with an inner time step of 2 fs for bonded interactions and non-bonded interactions within a cutoff of 9 Å. An outer time step of 6.0 fs was used for non-bonded interactions beyond the cut-off. Each system was equilibrated in MD simulations with a modification of the default protocol provided in Desmond, which consists of a series of restrained minimizations and MD simulations designed to relax the system, while not deviating substantially from the initial coordinates. First, two rounds of steepest descent minimization were performed with a maximum of 2000 steps and with harmonic restraints of 50 kcal mol\(^{-1}\) Å\(^{-2}\) applied on all solute atoms (non-water atoms), followed by 10000 steps of minimization without restraints. The first MD simulation was run for 200 ps at a temperature of 10 K in the NVT (constant number of particles, volume, and temperature) ensemble with solute heavy atoms restrained with a force constant of 50 kcal mol\(^{-1}\) Å\(^{-2}\). The temperature was then raised during a 200 ps MD simulation to 310 K in the NVT ensemble with the force constant retained. The temperature of 310 K was used in our MD simulations in order to ensure that the membrane state is above the melting temperature state of 297 K for DMPC lipids (4.68). The heating was followed by equilibration runs. First, two stages of NPT equilibration (constant number of particles, pressure, and temperature) were performed, one with the heavy atoms of the system restrained for 1 ns and one with the heavy atoms of solvent and lipids only restrained for 10 ns, with a force constant of 10 kcal mol\(^{-1}\) Å\(^{-2}\) for the harmonic constraints. An NPT simulation followed, with the C\(_\alpha\) atoms in the protein restrained for 1 ns with a force constant of 2 kcal mol\(^{-1}\) Å\(^{-2}\).
**MD Simulations with the Amber14 Software and ff14 Force Field**

System set-up was achieved as mentioned before for the simulations with Desmond. The MD simulations were also performed with Amber14 and each complex-bilayer system was processed by the LEaP module in AmberTools14 using the Amber14 software package (4.69). Amber ff14SB force field parameters (4.70) were applied to the protein, lipid14 to the lipids (4.71), GAFF to the ligands (4.72) and TIP3P (4.59) to the water molecules for the calculation of bonded, vdw parameters and electrostatic interactions. Atomic charges were computed according to the RESP procedure (4.73) using Gaussian03 (4.74) and antechamber of AmberTools14 (4.69). The systems were minimized by 2500 steps of steepest descent to remove bad contacts and 7500 steps of conjugated gradient minimization in the presence of a harmonic restraint with a force constant of 5 kcal mol\(^{-1}\) Å\(^{-2}\) on all atoms of protein and ligand and non-bonded cut-off of 8.0 Å. The next stage in MD simulation protocol is to allow the system to heat up from 0 K to 310 K. Langevin thermostat (dynamics) (4.75) as implemented in Amber14 (4.69). Temperature control employed a Langevin collision frequency of 2.0 ps\(^{-1}\). The system was heated in two consecutive steps to 310 K in the presence of a harmonic restraint with a force constant of 10 kcal mol\(^{-1}\) Å\(^{-2}\) on all membrane, protein, and ligand atoms. In the first step, systems were heated to 100 K in an NVT simulation of 50 ps length, where the adjustment of the density was realized using the Berendsen barostat (176) with a 2 ps coupling time. In the second step, the temperature was raised to 310 K in an NPT\(\gamma\) (with \(\gamma = 10 \text{ dyn cm}^{-1}\)) simulation of 500 ps length. Subsequently, the systems were equilibrated without restraints in a NPT\(\gamma\) simulation of 1 ns length with \(T = 310 \text{ K}\) and \(\gamma = 10 \text{ dyn cm}^{-1}\). The equilibration phase was followed by production simulation for 100 ns. In the NPT\(\gamma\) simulations pressure scaling to \(p = 1 \text{ bar}\) was applied using a pressure relaxation time of 1.0 ps.

For the treatment of long-range electrostatic interactions the Particle-mesh Ewald summation
method (4.64, 4.65) was used, and short-range non-bonding interactions were truncated with an 8 Å cutoff. Bonds involving hydrogen atoms were constrained by the SHAKE algorithm (4.77), and a time step of 2 fs was used for the integration of the equations of motion. Snapshots were recorded every 20 ps during the production. Properties and dynamics of the protein and ligand systems as well as of the membrane were analyzed with the ptraj and cpptraj modules of AmberTools12 (4.69).

The above-mentioned equilibration with Desmond or Amber14 was followed by an 80 ns NPT simulation without restraints. Measures in Tables 4.2 and 4.3 were obtained using these trajectories. Within this time, the total energy and the RMSD reached a plateau, and the systems were considered equilibrated.

**MD Simulations with NAMD Software and CHARMM36 Force Field**

For Figures 4.3 and 4.4, the homotetrameric protein structure for A/Hong Kong/156/1997 M2 H5N1, derived from the solid-state NMR structure (PDB 2KQT) and was used for the protein model. The ionizable residue, histidine 37, was maintained at a neutral charge (HSE), corresponding to alkaline solution. Residue 31 was mutated from serine to asparagine (N31). The protein was inserted into a DMPC lipid bilayer that was oriented perpendicular to the z-axis solvated by 0.15 M NaCl in TIP3P water (4.59). Amantadine was deleted from the protein structure and replaced by one of compounds 1-6. The ligands were each positioned with the adamantane amine approximately 2 Å in from the center-of-mass of residue 31 with the polar amine groups out. The ligands were parameterized using CGENFF (4.78). The systems were heated to 300 K and equilibrated for 10 ns with strong harmonic restraints on the protein backbone and ligand. All restraints were then removed for the MD simulation production runs. All of these simulations were performed using NAMD (4.79). For the study of average water
position, 100 ns simulations without restraints done with NAMD were applied for the systems with compounds 3-6. The average water, ligand, and protein positions were obtained using the VolMap Tool from VMD (180). Images of the average water position were made using VMD (4.80).

**MM-PBSA Calculations with Amber14 and ff14**

Relative binding free energies of aminoadamantane compounds were estimated by the 1-trajectory MM-PBSA (4.81) approach using MD simulation trajectories obtained with Amber14. Effective binding energies ($\Delta G_{\text{eff}}$) (4.82,4.83) were computed considering the gas phase energy and solvation free energy contributions to binding. For this, structural ensembles were extracted in intervals of 20 ps from the last 50 ns of the production simulations of the M2TM-ligand complex. Prior to the calculations all water molecules, ions, and lipids were removed, and the structures were positioned such that the geometric center of M2TM was located at the coordinate origin. Molecular mechanics energies and the non-polar contribution to the solvation free energy were calculated. The polar part of the solvation free energy was determined by Poisson-Boltzmann (PB) calculations (4.84-4.86). In these calculations, a dielectric constant of $\varepsilon_{\text{solute}} = 1$ was assigned to M2TM. Entropy effects were ignored assumed to be similar for the complexes of ligand 1-6 with the M2TM WT and S31N. The binding free energy for each complex was calculated using Eq. 4.1

$$\Delta G_{\text{eff}} = \Delta E_{\text{MM}} + \Delta G_{\text{sol}} \quad (4.1)$$

In Eq. 4.1 $\Delta G_{\text{eff}}$ is the binding free energy for each calculated complex neglecting the effect of entropic contributions or assuming them to be similar for the complexes studied. $\Delta E_{\text{MM}}$ defines the interaction energy between the protein and the ligand as calculated by molecular mechanics in
the gas phase. $\Delta G_{\text{sol}}$ is the desolvation free energy for transferring the ligand from water to the binding area calculated using the PBSA model. The terms for each complex $\Delta E_{\text{MM}}$ and $\Delta G_{\text{sol}}$ are calculated using Eqs. 4.2 and 4.3

$$\Delta E_{\text{MM}} = \Delta E_{\text{elec}} + \Delta E_{\text{vdW}} \quad (4.2)$$

$$\Delta G_{\text{sol}} = \Delta G_{\text{P}} + \Delta G_{\text{NP}} \quad (4.3)$$

In Eq. 4.2 $\Delta E_{\text{elec}}$ and $\Delta E_{\text{vdW}}$ are the electrostatic and the van der Waals interaction energies, respectively. In Eq. 4.3 $\Delta G_{\text{P}}$ is the electrostatic or polar contribution to free energy of solvation and the term $\Delta G_{\text{NP}}$ is the non-polar or hydrophobic contribution to solvation free energy. Molecular mechanics energies and the non-polar contribution to the solvation free energy for MM-PBSA calculations were computed with the mmpbsa.pl module (4.87) of Amber14 (4.88) that takes individual trajectory snapshots and calculates $\Delta G_{\text{eff}}$ and its energetic contributions.

**SMD Simulations with NAMD Software and CHARMM36 Force Field**

For Figures 4.5 and 4.6, steered MD was used to explore the ease of exit from M2 S31N. System set-up for these simulations was achieved as mentioned before for MD simulations with CHARMM36 force field. For SMD simulations, we were interested in the unbinding of the ligand from the protein. The transport coordinate was defined as the distance between the ligand center-of-mass and the N31 center-of-mass. For constant force simulations, the pulling force was set to 1.2 nN on the center-of-mass of each ligand. The force was applied such that the ligand was pulled out of the channel along the z-coordinate. Simulation time was sufficient (60 ps) to allow the ligands to dissociate from the protein and attain complete solvation. The average
of the transport coordinate positions from five independent simulations was plotted against the trajectory time. Constant velocity SMD simulations were carried out by applying a virtual spring (0.5 kcal mol\(^{-1}\) Å\(^{-2}\)) between a dummy atom and the ligand center-of-mass (1 kcal mol\(^{-1}\) ≡ 69.48 pN Å), with the dummy atom velocity = 1 Å ps\(^{-1}\). The dummy atom was moved out, i.e., in the +z direction. The average force from five randomly seeded simulations was deduced from spring stretch length using Hook’s law and plotted against the time in picoseconds (4.89).

Results

**In Vitro Testing**

We applied the cytopathic effect (CPE) inhibition assay (4.14,4.15) to compare the antiviral activity of 1-6 against the amantadine-resistant A/WSN/33 virus (with naturally occurring M2 N31) and A/WSN/33 M2 N31S virus, which was generated by reverse genetics from A/WSN/33. (4.31,4.32) Compound 1 was included as a dual-inhibitor reference 12 and compound 6 as inhibitor of Udorn M2 S31N but not Udorn (M2 S31) (4.11), and all were tested against A/WSN/33 and A/WSN/33 M2 N31S viruses.

Compounds 1-4 exhibit low micromolar or submicromolar potency (similar to amantadine or rimantadine, respectively (4.33)) against the amantadine-sensitive A/WSN/33 M2 N31S revertant virus (Table 4.4), while for 5 the anti-viral activity is reduced to a moderate level. Hence, 2-4 block viral replication at concentrations 0.13-0.66 μM compared to 1.13 μM for 1 and 8.10 μM for 5. Against amantadine-resistant A/WSN/33 (with M2 N31), only 6 has sub-μM potency. The oseltamivir controls demonstrate that the genetically engineered M2 mutant virus remains vulnerable to the potent neuraminidase inhibitor as expected. Compounds 1-4 are moderately potent, with 3 as effective as the dual-inhibitor 1, and therefore also classifiable as a dual inhibitor.

As will be shown below, 2-4 are all much more potent than would be expected from their
M2 S31N block potency, indicating that they block viral replication by the lysosomotropic mechanism. The moderate potency of 1 and high potency of 6 against the amantadine-resistant virus M2 S31N found here are consistent with previous results by Wang and DeGrado for these and similar compounds that inhibit M2 S31N virus by blocking of M2 S31N proton current (4.11,4.12).

**TEVC Experiments**

Using two-electrode voltage clamp (TEVC), we calculated %-block at 10 mins of 100 µM drug perfusion (Tables 4.5 and 4.6) to obtain more reliable results, as we recently suggested (4.28-4.30), compared to 2 min usually determined in studies dealing with testing of new drug molecules from synthesis (4.11-4.13,4.17,4.34). We found that molecules 1-5 strongly block the M2 WT channel-mediated proton current (Table 4.5), but that 2-4, which inhibit A/WSN/33 replication in cell culture at lower concentrations, do not effectively block proton currents through M2 S31N expressed in oocytes (Table 4.6). As found previously, 1 and 6 are produce high 10-min %-block of M2 S31N.

To quantify the kinetics of the block and unblock processes, we evaluated the association (k_on) and dissociation (k_off) rate constants from the exponential block and unblock rates associated with the equilibrium relaxation, and then calculated K_d from the ratio k_off/k_on. These rate constants allow one to examine the ease of exit and entry. EC_{50} values for the A/WSN/33-native (N31) and revertant N31S M2 constructs represented in Table 4.4 can be compared and contrasted with K_d values in the last columns of Tables 4.5 and 4.6, respectively. Compound 1 blocks M2 WT current well (Tables 4.5, 4.7), whereas 6 has almost no potency. Compounds 2-5 are much more potent M2 WT blockers than 1 or 6, with K_as from EP ranging between 1 and 10 µM, more similar to amantadine, which blocks M2 WT with an apparent K_i of 0.3 µM (4.5). Thus, compared to
compound 1, the increase in the length of linker between adamantane and the amino group in 2 and 3 is well accommodated by the M2TM WT pore. The 14-carbon diadamante or the 18-carbon tri-adamantane fit into the M2TM WT pore and block the M2 WT channel, with 5 being probably the bulkiest M2 WT channel blocker found yet. Evidently, the extension of the aryl head group and/or the expanded adamantane cluster in 2-5 facilitate both entry and dwell time in the WT channel. In particular, compounds 2-5 show significant block of inward current in the M2 WT channel (Table 4.5), with high percent block, low percent washout, high association rate $k_{on}$, and low dissociation rate $k_{off}$.

In the M2 S31N channel (Table 4.6), the percent inward current blockage by compounds 2-5 is significantly reduced (compared to WT, Table 4.5), with compound 2 showing no measurable block. In spite of 2 being over 30-fold more potent in blocking M2 WT current than 1, and moderately effective in vitro against the M2 S31N bearing influenza virus strain A/WSN/33, it is strikingly impotent against M2 S31N in the EP assay of molecular function, indicating that a simple isopropyl linker eliminates potency in this M2 S31N environment. The reduction in %-block is due to a 5- to 10-fold reduction in $k_{on}$ for compounds 3-5, and 10- to 30-fold increase in $k_{off}$ for compounds 3-5. Therefore, with low association rates and high dissociation rates, the dissociation constant $K_d$ calculated from the fitted rate constants is above 100 µM for compounds 3-5, a low binding affinity. The antiviral efficacy against M2 S31N virus (Table 4.4) is also moderate for 2-4 (8 – 21 µM), but much higher than that suggested by the $K_d$ computed from the EP rate constants. This may suggest that compounds 2-5 have other mechanism of inhibiting virus M2 S31N replication, observed previously by Scholtissek (4.2), Busath and Kolocouris (135), and by Naesens and Vazquez (4.36) such as endosome neutralization M2 S31N, perhaps due to an increased hydrophobicity-based membrane permeability (4.2,4.37).
As a contrasting case, we tested 6, a known blocker of M2 S31N. As found previously (103), 6 shows significant block of the S31N channel, with almost complete block of inward current and low percent washout. The percent-washout and \( k_{\text{off}} \) are similar to those of compounds 3 and 4, but the \( k_{\text{on}} \) is very high and complete (99.2%) block is attained, such that 6 has high potency in blocking M2 S31N, as well as in blocking the replication of virus strains containing this amantadine-insensitive mutation (4.3).

Indeed, perhaps the most intriguing result in the SAR is the remarkably high \( k_{\text{on}} \) for 6 in the S31N (Table 4.6). Although still well below the diffusion limited rate of \( \sim 10^{10} \text{ M}^{-1}\text{s}^{-1} \), the association rate constant is much higher than observed for 1 in S31N, or for 2-5 in WT (Table 4.5). The previously reported IC\(_{50}\) for 6 in TEVC with M2 S31N is 16 µM (4.3), similar to the K\(_d\) found here, 7.5 µM. At the same time, these authors found a much lower EC\(_{50}\) using the plaque reduction assay for the antiviral activity of this compound against A/WSN/33, ca 0.1 µM, similar to that found here in the cytopathic effect assay, 0.153 µM (Table 4.4). The difference of nearly two orders of magnitude between the anti-viral potency and the channel inhibition constant is not understood but may again suggest additional mechanisms of anti-viral activity for 6. In 6, an inhibitor of M2 S31N but not M2 WT, the aryl head group consists of the polar isoxazole ring linked with the hydrophobic thiophenyl ring, and in 1, which blocks both M2 isoforms (4.12,4.17), it consists of the apolar 4-methoxy group attached to 2-hydroxyl phenyl ring (Figure 4.1).

**MD Simulations**

Unrestrained 80-ns MD simulations of complexes of 1-6 with M2TM WT in hydrated DMPC bilayers MD simulations were performed with OPLS2005 force field (ff) implemented in Desmond, amberff14 implemented in amber14 and CHARMM36 implemented in NAMD (see Methods Section) with similar results, although M2TM \( \alpha \)-helices tend to unfold slightly with
OPLS2005. The converged MD simulations show that compounds 1-6 bind in M2 WT in the region spanning from V27 to H37 (Figure 4.2,A or 4.2,C), with the ammonium forming a hydrogen bonding interaction with waters and G34 carbonyls and adamantane accommodated by V27 side chains. The axial separation between the four-V27 center of mass (COM) position and the COM of the adamantyl cage in 1-6 varies between c.a. 4 Å and 5 Å (Table 4.2).

In the case of M2 S31N the ligands bind higher in the channel, in the region between V27 and G34, with the polar adduct facing outward (Figure 4.2, B and D). The distance from the four-V27 COM and the adamantyl cage in 1-6 is longer than for M2 WT complexes, varying between c.a. 6 Å and 7 Å (Table 4.3). The ammonium group and the polar part of the aryl head (2-hydroxyl group of 4-methoxy-phenyl in 1-5 or oxazole in 6) form hydrogen bonds with waters and N31 side chains, which may guide association, consistent with a lower desolvation penalty calculated in MM-PBSA calculations (Tables 4.8-4.11). The V27 side chain cluster is tightly packed around the 4-methoxy in 1 and the 4-methoxy-phenyl in 2-5 and thiophenyl ring in 6, while the adamantane is positioned deep to the N31 side chains between G34 and A30 (Figure 4.2, B and D).

Like WT, S31N has a V27 sphincter, but it is splayed upward; and, the N31 side chains are longer, more polar, more hydrophilic, and better hydrogen bond acceptors than the serine side chains. It appears that the additional waters near the entry of the S31N channel, as the MD simulations suggested (Figure 4.2), contribute significantly to the stabilization of the amphiphilic aryl head of the ligand, i.e. the hydroxyl in 1-5 or isoxazole in 6 in the outward-facing direction, whereas the inward configuration in WT channel appears to be stabilized by better LD dispersion forces. None of the simulations for either protein showed a water wire forming around the compounds, as would be needed for proton transport. Either the adamantyl or the aryl head forms a tight water seal (4.25,4.38,4.39) consistent with a proton blocking effect of aminoadamantane.
derivatives 1-6 (4.5, 4.24, 4.40, 4.41). This suggests that all compounds 1-6 are capable of completely blocking water-mediated proton transport through the M2 WT and S31N channels once they reach a “bound” configuration. A more in-depth analysis of the water occlusion produced by the ligands in the M2TM S31N using isosurfaces is shown representatively for compounds 3, 4, in Figure 4.4, showing waters positioned near the ammonium group.

*Calculation of Binding Free Energies*

To evaluate the preferred orientation of 1-6 in the channels, whether inwardly or outwardly projecting aryl group, the MM-PBSA binding free energies relative to bulk solution ($\Delta G_{\text{eff}}$) were evaluated (Table 4.1). As found experimentally for M2 S31N (4.11, 4.18) 6 binds outwardly facing, i.e. with a more negative $\Delta G_{\text{eff}}$ compared to the inwardly projecting orientation. The dual blocker, 1, binds to M2 WT with the experimentally observed inward orientation and to M2 S31N with the outward conformation (4.12), also consistent with predictions from the MM-PBSA free energies. For 2-5, the inward configuration is energetically preferred in the M2TM WT and the outward configuration is preferred in M2TM S31N. The same results were obtained with the cruder docking and scoring calculations using Glide (Table 4.12) (116, 128).

Because the accuracy of the method is only c.a. 4 kcal mol$^{-1}$, our MM-PBSA calculations cannot be used to rank the ligand affinities for the two channels, but taking the energies of 1-M2 S31N (outward), 1-M2 WT (inward), and 6-M2 S31N (outward) as a reference, the negative binding energies suggest that all six ligands can bind to M2, both WT and S31N. But by EP only 1-5 bind to and block the M2 WT channel and only 1 and 6 binds to and block the M2 S31N channel.
Interpretation of EP Kinetic Results

From observations made with the MD simulations, we suggest that an N-terminus deformation of M2TM S31N associated with binding of 2-5 may kinetically prevent ligand insertion into the channel and facilitate ligand exit, leading to reduced $k_{on}$ constants and increased $k_{off}$ (compared to 1 or 6), observed with TEVC (Table 4.6). The small isoxazole group of 6 (Figure 4.2D) is embedded in the four-V27 cluster in M2 S31N, whereas in 2 (Figure 4.2B) and similarly 3-5 (not shown) it is the larger phenyl group that is surrounded by V27 side chains. The elongation of the spacer between adamantane and aryl head in 2-5 causes distortions in the V27 cluster of M2 S31N, whereas no deformations are observed with 1 or 6, according to MD simulations. As is shown in Figure 4.2B for complexes with 2 (similarly for 3-5), the lumen of the M2 S31N channel opens such that V27 side chains are partially exposed to the outer solvent and the separation between Cγ atoms from opposite monomers increases to c.a. 9 Å, compared to c.a. 7 Å in the WT. No such deformation is observed in the MD simulations for Ph(OH)(OMe) in 1 or isoxazole thiophenyl in 6. Perhaps this deformation plays a role in the kinetics, particularly the rapid escape (high $k_{off}$) for 3-5 and presumably 2 in S31N (Table 4.6) compared to WT (Table 4.5).

Compounds 1-5 block M2TM WT effectively and are all fast to enter and slow to leave M2TM WT (Table 4.5) with similar high $k_{on}$, and low $k_{off}$ rates and similar proton blockage percentage. Compound 6 sterically fits inside the M2 WT channel, with minor conformational differences from 1-5, due to the longer part of the binding area being deeper in the M2 WT channel, from V27-H37 and the smaller isoxazole spanning the four-V27 side chain cluster. However, while 1-5 block M2 WT, 6 does not block M2 WT, but blocks M2 S31N with a remarkably high $k_{on}$, suggesting that the isoxazole thiophenyl aryl head in 6 confers special properties on its entry trajectory compared to Ph(OH)(OMe).
Conclusion

The kinetics of a ligand binding to its protein target are seen as increasingly important for \textit{in vivo} efficacy in drug discovery (4.26,4.42,4.43). Here, using a set of chemical probes, with amantadine linked to either a 4-methoxy-2-hydroxyl-phenyl ring or 3-(thiophenyl)isoxazolyl ring as reference compounds 1 or 6, respectively, we showed that neither the added length between the aryl head and adamantane (compounds 2 and 3), nor the adamantyl group length and girth (compounds 4 and 5) prevent binding. Our binding free energy calculations show that the compounds, if they get into the M2 WT or S31N channel, can bind fairly well and can sterically inhibit water entry. However, only compounds 1-5 significantly reduce average proton current through the M2 WT channel in TEVC, while only 1 and 6 block the M2 S31N channel current.

To explain these data, using EP in oocytes, we calculated the association (\(k_{on}\)) and dissociation (\(k_{off}\)) rate constants for this set of M2 proton current blockers using TEVC experiments. For one set of seven compounds that block M2 S31N efficiently, it was previously observed that \(k_{on}\) values range from 29 to 691 M\(^{-1}\)s\(^{-1}\) and \(k_{off}\) values range from 0.3-10.4 x 10\(^{-3}\) s\(^{-1}\) (128). The compounds were classified as fast on/slow off, slow on/slow off or fast on/fast off (114,128), with the kinetics of binding being very dependent on the adamantane scaffold (128). Here, we show that they are also very dependent on the linker between adamantane and the aryl head group.

The \(k_{on}\) values of 1-5 for M2 WT are in the range 84-381 M\(^{-1}\)s\(^{-1}\) and the \(k_{off}\) values are in the range 0.56-5.1 x 10\(^{-3}\) s\(^{-1}\) and thus, 1-5 are fast on / slow off or fast on / fast off. Compound 1 and its four variants 2-5, which all efficiently block the M2 WT channel, have high \(k_{on}\), with 2-5 higher than 1 by >4. The \(k_{on}\) of 1 for M2 S31N is 124 M\(^{-1}\)s\(^{-1}\) and \(k_{off}\) is 2.6 x 10\(^{-3}\) s\(^{-1}\). Thus, 1 is fast on / slow off while 6 is very fast on / fast off.
In contrast, compounds 3-5 do not block M2 S31N channel efficiently in TEVC, and 2 is completely inactive. The inactivity of 2-5 to block M2 S31N is due to the low $k_{on}$ and high $k_{off}$, i.e., the high energy barrier to get into the M2 channel and low energy barrier to exit. From simulations for M2 S31N, it appears that 2-5 have an equilibrium binding configuration with the phenyl group embedded in the V27 cluster – a position of incomplete entry, whereas 1 and 6 achieve a deeper binding position. The entry barriers for 2-5 may be higher due to the deformations in the N-terminus of the channel required for entry, and the exit barrier lower due to ligand-induced priming of the channel structure for release, even with a difference between inactive 2 and active 1 of only the isopropyl linker.

Interestingly, $k_{on}$ for 6 in blocking M2 S31N is dramatically higher, c.a. 10-fold higher, than others tested to date (4.10-4.17,4.30) (Table 4.1). The high association rate in 6 to the M2 S31N, is consistent with its high blocking percentage and IAV replication. Yet, the rate constant is still 6-7 orders of magnitude lower than expected for unhindered diffusion-limited binding (4.44,4.45), suggesting it still faces a smaller yet still substantial energetic barrier to entry. The entry rate of 6 into M2 WT is even faster (Table 4.5, footnote h), but the exit rate from M2 WT is dramatically faster still, resulting in non-efficient block. The propensity for 6 to assume an inward orientation in M2 WT, to enter faster than other related compounds and exit faster still, while exiting the M2 S31N much slower, are yet to be explained.

From their moderately high anti-viral efficacies against A/WSN/33 in MDCK cell culture in the face of inefficient M2 S31N block, it is clear that 2-4 can also block influenza virus by another mechanism. Although other possibilities, such as direct interactions with hemagglutinin could be possible (4.35), it is most likely that these compounds buffer late-stage endosomes to prevent acid-induced fusion of the virus envelope with the endosome membrane. Perhaps 5 is
less effective because its triadamantyl group is so hydrophobic that it is slow to exit the endosome membrane to enter the endosome and buffer the pH. Evidently, 6 is not effective by this mechanism or it would be a more effective antiviral against the revertant WT M2, A/WSN/33 M2 N31S. Such lysosomotropic agents are effective against many types of viruses in vitro, including the coronavirus SARS-COV2 (190). Although some of these agents may not be useful clinically because they inhibit inflammatory responses (4.91), it is possible that the compounds studied here for the M2 block kinetics will become a helpful addition in the search for clinically-optimized lysosomotropic antivirals.

Contribution to Publication

Co-first author, performed 100 ns MD simulation averaged water and averaged water position in Figure 4.4, performed constant force steered MD in Figure 4.5, performed constant velocity steered MD in Figure 4.6, and collected electrophysiology data in Tables 4.5 and 4.6.
Figure 4.1: Structures of Compounds 1-6. Used as chemical probes to investigate lysosomotropic effects and binding to M2 WT and M2 S31N channels. In the upper part is shown the reductive amination procedure applied for the synthesis of aminoadamantane-aryl head group conjugates 1-5 through imines 12-16 starting from lipophilic amines 7-11.
Figure 4.2: Screenshots of the Ligand-Protein Complexes in Hydrated DMPC. 80 ns MD simulations using the amber14sb force field (1 atm, 310 K). Compound 2 bound to (A) M2TM WT, or (B) M2TM S31N. Compound 6 bound to M2TM WT (C) or M2TM S31N (D) The N-terminal channel mouth expands, particularly in 1,D, to accommodate the thermal fluctuations of the ligand.
Figure 4.3: Screenshot from the 80 ns-MD Simulation. Compound 1 bound to M2TM WT (PDB ID 2KQT) in hydrated DMPC (1 atm, 310 K).
Figure 4.4: Average Water and Ligand Position Simulations. Aqueous access to the channel in the presence of compounds 3 (left), 4 (right) in the M2TM S31N (2KQT mutated with S31N) in a DMPC bilayer (1 atm, 300 K) averaged over an entire 100 ns MD simulation trajectory. Figure shows the average water and ligand positions displayed as isosurfaces, i.e. surfaces based on whether voxel occupancy over the entire simulation surpasses a given threshold (0.45). Average water positions (blue, QuickSurf blown glass), ligand positions (red, isosurface with isovalue 0.45 such that the molecular definition indicates the configurational stability), and protein positions (green, first and third segments of 2KQT structure aligned to the protein volume slice).
Figure 4.5: Constant Force Steered-Molecular Dynamics Simulations. Center of mass (COM) for each ligand with constant force (1.2 nN) SMD relative to the N31 COM position. The average of six independent simulation trajectories of 1-6 in complex with M2TM S31N (2KQT mutated with S31N) in hydrated DMPC bilayer using CHARMM36 force field V27 COM = 5 Å, D24 COM = 10 Å, and S22 COM = 13 Å. 1 (Black), 2 (Red), 3 (Yellow), 4 (Green), 5 (Purple), and 6 (Cyan).
Figure 4.6: Constant Velocity Steered-Molecular Dynamics Simulations. Average of 6 independent trajectories for each ligand COM with constant velocity (1 Å/ps) SMD simulation trajectories of 1-6 in complex with M2TM S31N (2KQT mutated with S31N) in hydrated DMPC bilayer using CHARMM36 force field, starting with ligand COM at N31 positions. The force applied by the dummy atom to the ligand COM is plotted against trajectory time and increases for each compound until the ligand comes free, at which point it decreases. 1 (Black), 2 (Red), 3 (Yellow), 4 (Green), 5 (Purple), and 6 (Cyan).
Table 4.1: Calculated Binding Free Energies (ΔG<sub>eff</sub>). In kcal·mol<sup>-1</sup> using the MM-PBSA method and the amber ff14 force field.

<sup>a</sup> str. err. of mean. Bold: the lower of in and out configuration free energies.

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Table 4.2: Structural and Dynamic Measurements from MD Trajectories of M2TM WT Ligand (1-6) Complexes. In DMPC bilayer (mean values from the 80 ns trajectories).

a See Figure 4.1.
b Maximum root-mean-square deviation (RMSD) for Ca atoms of M2TM relative to the initial structure (PDB entry: 2KQT or 2KQT mutated with S31N) after root-mean-square fitting of Ca atoms of M2TM; in Å.
c Absolute values for the angle between the vector along the bond from the bridge-head carbon atom of the adamantyl core to the ligand nitrogen atom and the normal of the membrane; in degrees.
d Mean distance between COM of V27 and nitrogen atom of the ligand calculated using Gromacs tools; in Å.
e Mean distance between center of mass (COM) of V27 and centers of mass of adamantyl calculated using Gromacs tools; in Å.
f Mean number of hydrogen bonds between ligand's ammonium group and waters.
g Distance from the closest chlorine atom.
h Maximum root-mean-square fluctuation of the ligand inside the channel based on the starting structure.
i Mean number of hydrogen bonds between ligand's ammonium group and N31 amide chains.

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Table 4.3: Structural and Dynamic Measures from MD Trajectories of M2TM S31N Ligand (1-6) Complexes. In DMPC Bilayer (80 ns).

a See Figure 4.1.
b Maximum root-mean-square deviation (RMSD) for Ca atoms of M2TM relative to the initial structure (PDB entry: 2KQT or 2KQT mutated with S31N) after root-mean-square fitting of Ca atoms of M2TM; in Å.
c Absolute values for the angle between the vector along the bond from the bridge-head carbon atom of the adamantyl core to the ligand nitrogen atom and the normal of the membrane; in degrees.
d Mean distance between COM of V27 and nitrogen atom of the ligand calculated using Gromacs tools; in Å.
e Mean distance between center of mass (COM) of V27 and centers of mass of adamantyl calculated using Gromacs tools; in Å.
f Mean number of hydrogen bonds between ligand's ammonium group and waters.
g Distance from the closest chlorine atom.
h Maximum root-mean-square fluctuation of the ligand inside the channel based on the starting structure.
i Mean number of hydrogen bonds between ligand's ammonium group and N31 amide chains.

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<td>3</td>
<td>2.6 ± 0.4</td>
<td>-11.1 ± 4.5</td>
<td>4.0 ± 0.2</td>
<td>6.6 ± 0.6</td>
<td>3.7 ± 0.7</td>
<td>0.5 ± 0.6</td>
<td>32.4 ± 8.2</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>1.7 ± 0.3</td>
<td>-11.0 ± 6.9</td>
<td>3.0 ± 0.9</td>
<td>6.5 ± 0.4</td>
<td>1.8 ± 0.9</td>
<td>2.3 ± 0.8</td>
<td>31.1 ± 7.2</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>2.3 ± 0.5</td>
<td>-30.8 ± 5.6</td>
<td>2.7 ± 0.3</td>
<td>6.1 ± 0.4</td>
<td>1.1 ± 0.3</td>
<td>2.0 ± 0.8</td>
<td>33.3 ± 7.5</td>
<td>0.4</td>
</tr>
<tr>
<td>6</td>
<td>1.5 ± 0.5</td>
<td>-25.8 ± 2.6</td>
<td>2.9 ± 0.3</td>
<td>6.2 ± 0.4</td>
<td>2.5 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>33.0 ± 7.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Table 4.4: *In Vitro* Cytotoxicity (CC$_{50}$, µM) and Efficacy (EC$_{50}$, µM) of Compounds 1-6. Tested against initial cell infection (CPE inhibition assay) in MDCK cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC$_{50}$ ± SD [µM]</th>
<th>EC$_{50}$ ± SD [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDCK cells A/WSN/33 (M2 N31S)</td>
<td>A/WSN/33 (M2 N31)</td>
</tr>
<tr>
<td>1</td>
<td>49.86 ± 12.28</td>
<td>1.13 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>&gt;100</td>
<td>0.09 ± 0.005</td>
</tr>
<tr>
<td>3</td>
<td>&gt;100</td>
<td>0.66 ± 0.21</td>
</tr>
<tr>
<td>4</td>
<td>&gt;100</td>
<td>0.29 ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>27.13 ± 9.43</td>
<td>8.10 ± 2.72</td>
</tr>
<tr>
<td>6</td>
<td>not tested</td>
<td>not active</td>
</tr>
<tr>
<td>Amantadine</td>
<td>not tested</td>
<td>0.25 ± 0.07</td>
</tr>
<tr>
<td>Oseltamivir</td>
<td>not tested</td>
<td>0.03 ± 0.02</td>
</tr>
</tbody>
</table>
Table 4.5: Block of Full-Length Udorn M2 WT Current by Compounds 1-6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Sample Size</th>
<th>Percent Block (%)</th>
<th>Blockage Eliminated in Wash Out (%)</th>
<th>$k_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>$10^3$ x $k_{off}$ (s$^{-1}$)</th>
<th>$K_d$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 μM</td>
<td>N = 3</td>
<td>64 ± 1</td>
<td>48 ± 1</td>
<td>84.0 ± 9.9</td>
<td>5.1 ± 0.32</td>
<td>60.5 ± 10.4</td>
</tr>
<tr>
<td>2</td>
<td>100 μM</td>
<td>N = 3</td>
<td>77 ± 2</td>
<td>0.5 ± 0.1</td>
<td>332.6 ± 1.7</td>
<td>0.56 ± 0.03</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>100 μM</td>
<td>N = 3</td>
<td>80 ± 2</td>
<td>1.3 ± 0.2</td>
<td>381.7 ± 1.4</td>
<td>1.7 ± 0.2</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td>4</td>
<td>100 μM</td>
<td>N = 3</td>
<td>81 ± 2</td>
<td>2.1 ± 0.8</td>
<td>378.2 ± 1.1</td>
<td>2.1 ± 0.7</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>100 μM</td>
<td>N = 3</td>
<td>81 ± 2</td>
<td>3.1 ± 0.6</td>
<td>377.3 ± 2.1</td>
<td>3.8 ± 0.5</td>
<td>10.1 ± 1.2</td>
</tr>
<tr>
<td>6</td>
<td>100 μM</td>
<td>N = 3</td>
<td>11 ± 1</td>
<td>ND $^b$</td>
<td>ND</td>
<td>ND</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SEM calculated at 10 min of drug perfusion

$^b$ Mean ± SEM calculated at 5 min of washout; percent of original current that returned during washout

$^c$ Mean ± SEM of exponential fit rate constants obtained from the first two minutes of drug perfusion. This equilibrium relaxation rate constant divided by the compound concentration in the bath is actually an upper limit on the compound’s association rate constant (see methods section)

$^d$ Mean ± SEM of exponential fit rate constants obtained from the first three minutes of washout

$^e$ Calculated from the ratio, $k_{off}$/kon

$^f$ Blockage after 10 min of drug perfusion, blockage eliminated after 5 min of washout, kon is the relaxation rate constant from fit of the 3-min compound perfusion trace, Koff is the relaxation rate constant from fit of the 5-min compound washout trace

$^g$ Taken from ref. 3 as the mean ± SEM calculated at 2 min of drug perfusion

$^h$ Not determined. The small amount of block of M2 WT by 6 observed was so fast that it was limited by perfusion rate, judging by the rate of ungating upon return to alkaline solution, and the extent of block at 100 μM (11%) suggests a Kd ~ 900 μM and thus a koff > 1 s$^{-1}$. 

$^i$ Taken from ref. 3 as the mean ± SEM calculated at 2 min of drug perfusion
Table 4.6: TEVC Block of Full-Length Udorn M2 S31N Current by Compounds 1-6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Sample Size</th>
<th>Percent Block a</th>
<th>Blockage Eliminated in Wash Out(%) b</th>
<th>k_on (M⁻¹s⁻¹) c</th>
<th>10³ x k_off (s⁻¹) d</th>
<th>K_d (μM) e</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 μM</td>
<td>N = 3</td>
<td>59 ± 1 f</td>
<td>34 ± 1</td>
<td>124 ± 9</td>
<td>2.6 ± 0.2</td>
<td>21.3 ± 2.8</td>
</tr>
<tr>
<td>2</td>
<td>100 μM</td>
<td>N = 3</td>
<td>No Block</td>
<td>No Block</td>
<td>No Block</td>
<td>No Block</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>3</td>
<td>100 μM</td>
<td>N = 3</td>
<td>30 ± 2</td>
<td>4.8 ± 0.5</td>
<td>&lt;64</td>
<td>18 ± 2.2</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>4</td>
<td>100 μM</td>
<td>N = 3</td>
<td>25 ± 2</td>
<td>7.2 ± 0.8</td>
<td>&lt;43</td>
<td>56 ± 1.4</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>5</td>
<td>100 μM</td>
<td>N = 3</td>
<td>17 ± 1</td>
<td>1.4 ± 1.1</td>
<td>&lt;29</td>
<td>97 ± 3.6</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>6</td>
<td>100 μM</td>
<td>N = 3</td>
<td>99 ± 1</td>
<td>6.8 ± 1.2</td>
<td>2280 ± 150</td>
<td>17 ± 2</td>
<td>7.5 ± 0.3 f</td>
</tr>
</tbody>
</table>

a Mean ± SEM calculated at 10 min of drug perfusion (unmeasurable in 2)
b Mean ± SEM calculated at 5 min of washout; percent of original current that returned during washout
c Mean ± SEM of exponential fit rate constants obtained from the first two minutes of drug perfusion. This “equilibrium relaxation rate constant divided by the compound concentration in the bath” is actually an upper limit on the compound’s association rate constant (see methods section). Because the measured k_off is relatively high for 3-5, these cases, where the correction for k_off would substantially reduce the estimate of k_on, are denoted with <
d Mean ± SEM of exponential fit rate constants obtained from the first three minutes of washout
Calculation from the ratio, k_off/k_on
f Blockage after 10 min of drug perfusion, blockage eliminated after 5 min of washout, k_on is the relaxation rate constant from fit of the 3-min compound perfusion trace, k_off is the relaxation rate constant from fit of the 5-min compound washout trace
g 16 μM
Table 4.7: Kinetic Constants of Compound 1. Current block of Cali M2 WT and Udorn M2 WT.

<table>
<thead>
<tr>
<th>Kinetic data</th>
<th>Cali M2 WT</th>
<th>Udorn M2 WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{on} \text{ (M}^{-1}\text{s}^{-1})$</td>
<td>126.3 ± 18.8</td>
<td>91.0 ± 5.2</td>
</tr>
<tr>
<td>$10^3 \times k_{off} \text{ (s}^{-1})$</td>
<td>11.8 ± 1.1</td>
<td>8.2 ± 0.3</td>
</tr>
<tr>
<td>$K_D(\mu\text{M})$</td>
<td>93.7 ± 20.5</td>
<td>89.6 ± 7.8</td>
</tr>
</tbody>
</table>

Table 4.8: Calculated Binding Free Energies ($\Delta G_{\text{eff}}^a$) of Compounds 1-6 in M2 S31N “Out” Orientation. In kcal mol$^{-1}$ using the MM-PBSA method for compounds 1-6 in the M2 S31N “out” orientation.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{el}$</td>
<td>-135.44±0.41$^b$</td>
<td>-138.34±0.37</td>
<td>-133.14±0.48</td>
<td>-137.75±0.36</td>
<td>-128.21±0.31</td>
<td>-138.39±0.16</td>
</tr>
<tr>
<td>$E_{vdW}$</td>
<td>-43.15±0.18</td>
<td>-49.83±0.23</td>
<td>-47.81±0.18</td>
<td>-50.04±0.18</td>
<td>-53.49±0.22</td>
<td>-45.42±0.08</td>
</tr>
<tr>
<td>$\Delta G_{\text{solv}}$</td>
<td>134.85±0.45</td>
<td>144.90±0.27</td>
<td>143.79±0.42</td>
<td>139.76±0.25</td>
<td>133.21±0.39</td>
<td>140.58±0.14</td>
</tr>
<tr>
<td>$\Delta G_{\text{eff}}$</td>
<td>-42.29±0.42</td>
<td>-43.27±0.27</td>
<td>-37.16±0.20</td>
<td>-48.03±0.21</td>
<td>-50.48±0.24</td>
<td>-43.24±0.10</td>
</tr>
</tbody>
</table>

$^a$mean of 1000 frames at 20-ps intervals from the last 20 ns of each of two 100-ns trajectories run for each complex; $^b$std. err. of mean.
Table 4.9: Calculated Binding Free Energies ($\Delta G_{\text{eff}}$) of Compounds 1-6 in M2 S31N “In” Orientation. In kcal mol$^{-1}$ using the MM-PBSA method for compounds 1-6 in the M2 S31N “in” orientation.

\(^a\) mean of 1000 frames at 20-ps intervals from the last 20 ns of each of two 100-ns trajectories run for each complex; \(^b\) std. err. Of mean.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{el}}$</td>
<td>-136.72±0.46 (^b)</td>
<td>-132.68±0.29</td>
<td>-143.56±0.29</td>
<td>-126.91±0.29</td>
<td>-138.69±0.28</td>
<td>-139.34±0.20</td>
</tr>
<tr>
<td>$E_{\text{vdW}}$</td>
<td>-41.31±0.17</td>
<td>-48.78±0.20</td>
<td>-52.03±0.17</td>
<td>-53.23±0.16</td>
<td>-38.97±0.15</td>
<td>-45.46±0.08</td>
</tr>
<tr>
<td>$\Delta G_{\text{solv}}$</td>
<td>144.46±0.29</td>
<td>142.07±0.29</td>
<td>156.71±0.22</td>
<td>143.95±0.21</td>
<td>142.41±0.33</td>
<td>148.92±0.19</td>
</tr>
<tr>
<td>$\Delta G_{\text{eff}}$</td>
<td>-33.57±0.38</td>
<td>-39.39±0.21</td>
<td>-38.87±0.23</td>
<td>-36.19±0.26</td>
<td>-35.24±0.40</td>
<td>-35.88±0.12</td>
</tr>
</tbody>
</table>

Table 4.10: Calculated Binding Free Energies ($\Delta G_{\text{eff}}$) of Compounds 1-6 in M2 WT “Out” Orientation. In kcal mol$^{-1}$ using the MM-PBSA method for compounds 1-6 in the M2 WT “out” orientation.

\(^a\) mean of 1000 frames at 20-ps intervals from the last 20 ns of each of two 100-ns trajectories run for each complex; \(^b\) std. err. Of mean.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{el}}$</td>
<td>-64.42±0.05</td>
<td>-59.57±0.07</td>
<td>-52.91±0.09</td>
<td>-64.04±0.04</td>
<td>-67.85±0.05</td>
<td>-63.53±0.05</td>
</tr>
<tr>
<td>$E_{\text{vdW}}$</td>
<td>-41.31±0.03</td>
<td>-45.78±0.03</td>
<td>-45.23±0.03</td>
<td>-47.45±0.03</td>
<td>-59.32±0.03</td>
<td>-46.98±0.03</td>
</tr>
<tr>
<td>$\Delta G_{\text{solv}}$</td>
<td>80.21±0.04</td>
<td>71.15±0.05</td>
<td>67.64±0.08</td>
<td>81.92±0.06</td>
<td>88.27±0.04</td>
<td>83.93±0.06</td>
</tr>
<tr>
<td>$\Delta G_{\text{eff}}$</td>
<td>-25.52±0.03</td>
<td>-34.20±0.04</td>
<td>-30.49±0.04</td>
<td>-29.57±0.05</td>
<td>-39.01±0.04</td>
<td>-26.59±0.05</td>
</tr>
</tbody>
</table>
Table 4.11: Calculated Binding Free Energies ($\Delta G_{\text{eff}}$) of compounds 1-6 in M2 WT “In” Orientation. In kcal mol$^{-1}$ using the MM-PBSA method for compounds 1-6 in the M2 WT “in” orientation.

*mean of 1000 frames at 20-ps intervals from the last 20 ns of each of two 100-ns trajectories run for each complex; bstd. err. Of mean.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{el}}$</td>
<td>-63.11±0.04</td>
<td>-67.59±0.05</td>
<td>-76.17±0.06</td>
<td>-65.77±0.06</td>
<td>-67.16±0.07</td>
<td>-70.10±0.05</td>
</tr>
<tr>
<td>$E_{\text{vdW}}$</td>
<td>-49.73±0.02</td>
<td>-54.61±0.05</td>
<td>-58.99±0.03</td>
<td>-55.97±0.06</td>
<td>-57.91±0.02</td>
<td>-55.27±0.05</td>
</tr>
<tr>
<td>$\Delta G_{\text{solv}}$</td>
<td>80.79±0.04</td>
<td>84.62±0.05</td>
<td>97.83±0.06</td>
<td>81.72±0.05</td>
<td>84.58±0.06</td>
<td>88.76±0.06</td>
</tr>
<tr>
<td>$\Delta G_{\text{eff}}$</td>
<td>-32.05±0.03</td>
<td>-37.58±0.05</td>
<td>-37.34±0.05</td>
<td>-40.02±0.04</td>
<td>-40.59±0.04</td>
<td>-36.60±0.04</td>
</tr>
</tbody>
</table>

Table 4.12: Calculated Binding Free Energies ($\Delta G_{\text{bind}}$). In kcal mol$^{-1}$ using Glide and the XP-scoring function.

<table>
<thead>
<tr>
<th>Cmp /M2</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>S31N</td>
<td>$\Delta G_{\text{bind (out)}}$</td>
<td>-4.53</td>
<td>-4.87</td>
<td>-7.67</td>
<td>-5.28</td>
<td>-6.22</td>
</tr>
<tr>
<td></td>
<td>$\Delta G_{\text{bind (in)}}$</td>
<td>-3.44</td>
<td>a</td>
<td>a</td>
<td>-5.01</td>
<td>a</td>
</tr>
<tr>
<td>WT</td>
<td>$\Delta G_{\text{bind (out)}}$</td>
<td>a</td>
<td>-5.59</td>
<td>a</td>
<td>-4.15</td>
<td>-5.42</td>
</tr>
<tr>
<td></td>
<td>$\Delta G_{\text{bind (in)}}$</td>
<td>-6.68</td>
<td>-6.84</td>
<td>-8.58</td>
<td>-7.33</td>
<td>-6.76</td>
</tr>
</tbody>
</table>
Sections 1 and 2 provided the motivation to focus on the His37 complex as a more viable target for M2 blockers because the aminoadamantane drugs developed showed low efficacy in blocking the M2 S31N channel. Section 3 consists of chapters 5 (published in Antiviral Research), 6 (published in Engineered Nanomaterials – Health and Safety), and 7 (submitted to Biophysical Journal) and covers the synthesis and testing of the novel copper complexes, Cu(CO-IDA) and Cu(AMT-IDA). The hypothesis of this effort (chapters 5-7) was that the copper complexes bind to the His37 complex of the M2 channel and block M2 by preventing the His37 complex from transporting protons through M2, and therefore inhibit viral infection, making the copper complexes highly valuable M2 blockers, especially during a pandemic. It was important, also, to design copper complexes that are more stable in solution than NAG 107 so that they could be considered as an anti-influenza therapeutic. Chapters 5-7 show that the novel copper complexes, Cu(CO-IDA) and Cu(AMT-IDA), are very effective at blocking the M2 S31N channel and have low exit rates. The viral assays show low EC50 values against multiple strains of influenza A with the M2 S31N channel and the viruses do not develop resistance against the copper complexes. The zebrafish study shows significantly lower cytotoxicity for the copper complexes compared to free Cu²⁺. As well, the ITC binding study shows that the copper complexes have a strong affinity for binding imidazole, and the quantum chemical models show that the copper binds one imidazole in the channel while staying complexed to the CO-IDA or AMT-IDA ligands.
CHAPTER 5: Divalent Copper Complexes as Influenza A M2 Inhibitors

Nathan A. Gordon, Kelly L. McGuire, Spencer K. Wallentine, Gregory A. Mohl, Jonathan D. Lynch, Roger G. Harrison, David D. Busath
Antiviral Research. 2017;147,100-106.

Abstract

New M2 blockers effective against the ubiquitous amantadine resistant S31N M2 mutation in influenza A are needed. Six copper complexes, 2, 4, 6, 8, 9, and 10, were synthesized and found to block both wild type and S31N M2. Free Cu\(^{2+}\) also blocks M2 S31N but not S31N/H37A. The copper complexes do not block M2 H37A (either S31 or S31N). The complexes were effective against three influenza A strains in cell-culture assays, but less toxic to cells than CuCl\(_2\). For example, 4, Cu(cyclooctylamine-iminodiacetate), which was stable at pH>4 in the buffers used, had an EC\(_{50}\) against A/Calif/07/2009 H1N1 of 0.7 ± 0.1 µM with a CC\(_{50}\) of 147 µM (therapeutic index, averaged over three strains, 67.8). In contrast, CuCl\(_2\) had an EC\(_{50}\) of 3.8 ± 0.9 µM and CC\(_{50}\) of 19 µM. Because M2 H37 is highly conserved, these complexes show promise for further testing as drugs against all strains of influenza A.

Keywords: Cytotoxic concentration (CC\(_{50}\)), half maximal effect concentration (EC\(_{50}\)), simple electrolyte medium (SEM), two-electrode voltage clamp (TEVC), cytopathic effect (CPE), Madin-Darby canine kidney cells (MDCK)
Introduction

The release of ribonucleoproteins from M1 upon fusion of the influenza A envelope to the endosomal membrane (5.1) and protection of nascent hemagglutinin from premature acidification in the trans-Golgi of infected cells (5.2) are both mediated by the viral proton transporter, M2. Native (WT) M2 is a well-established antiviral target for the M2 channel blockers amantadine (5.3,5.4) and rimantadine (5.5,5.6), but the M2 S31N and other nearby mutations, (L26F, V27A/T/S, A30T, and G34E) are known to cause insensitivity to amantadine in vitro (5.7,5.8). Because the S31N mutation is now nearly universal in human infections (5.9), amantadine and rimantadine are no longer useful therapeutics (5.10). Several attempts have been made to identify variants of these drugs and others that could block the S31N mutation and/or other amantadine resistance mutations. For instance, isoxazole-adamantane (5.11) and pinanamine (5.12) compounds effectively block M2 S31N, spiranamine blocks M2 L26F, V27A, and WT (5.13), and benzyl substituted amantadines are active against both the WT and S31N mutant (5.14). However, they are all limited in the scope of M2 variants they block. This led us to explore an approach that could, in theory, target all functional forms of M2, perhaps in most or all amantadine-sensitive and -resistant strains.

The homotetrameric M2 channels are exquisitely selective for transport of H⁺ over other monovalent ions (5.6) due to a tightly-packed cluster of His37 side chains at the narrowest passage in the channel (5.15). This residue is highly conserved in influenza A (99.97% identical in all isolates found in GenBank).

The M2 current in transfected oocytes was found previously to be blocked by 250-500 μM Cu²⁺, with block eliminated by mutation of M2 His37 to Ala (5.16). Cu⁺ and other divalent cations Ni²⁺, Pt²⁺, Cd²⁺, Mg²⁺, Mn²⁺, and Zn²⁺ caused only partial, readily reversible block at 1-2
mM concentrations. Using triangulation of paramagnetic shielding effects in solid state NMR, Cu$^{2+}$ was found to bind in the core of the His37/Trp41 cluster located within the homotetramer (5.17).

Although divalent metals present the risk of toxicity through competition for functional sites in proteins and tight binding to phosphates, the use of metal complexes in medicine has been increasing. Platinum, ruthenium, and gold complexes are used to target DNA for chemotherapy, while manganese, copper and iron are potential superoxide dismutase mimics (5.18). Recently, copper complexes have been developed as anti-inflammatory, antibiotic, and chemotherapeutic agents (5.19,5.20,5.21,5.22,5.23,5.24,5.25,5.26). Although intracellular copper levels are tightly regulated (5.27,5.28), daily dietary intake limitations are modest (5.29), such that the bodily exposure to the copper content from moderate doses of such therapeutics by traditional routes can be expected to be well tolerated.

By derivatizing the amines of known M2 blockers, amantadine (Amt) and cyclooctyalmine, to form the iminodiacetate or iminodiacetamide, we have synthesized Cu$^{2+}$ containing complexes and characterized them by NMR, IR, MS, UV-vis, and inductively coupled plasma mass spectroscopy (ICP-MS). The copper complexes, but not the copper-free ligands, demonstrated H37-specific blocking of M2 channel currents and low micromolar antiviral efficacies in both Amt-sensitive and Amt-resistant IAV strains with, for the best case, nearly 10-fold less cytotoxicity than CuCl$_2$. These can be compared with other small molecule inhibitors of M2 S31N transport (5.30,5.31).
Methods and Materials

*Compound and Complex Synthesis*

Solvents and compounds were used as received by commercial suppliers. Purity of tested complexes was verified by $^1$H NMR, ICP and elemental analysis and found to be $\geq 95\%$. The copper complexes were usually accompanied by two water molecules, but in some cases the number of waters varied between batches and was quantified before running assays with the complexes. The IDAA complexes contained NaCl, which was quantified before doing assays. NaCl was verified by x-ray photoelectron spectroscopy. The hydrated complexes lost water during the melting point measurements but did not melt. See Supplementary Data for synthesis details.

*Stability Studies*

Solutions of the complexes were made by dissolving a few milligrams of complex in 3.0 mL of solvent to make 10 mM solutions. Complexes 2, 6, and 8 were dissolved in methanol and 4 was dissolved in water and the solutions were sonicated and gently warmed to aid dissolution. 100μl of each solution was then added to 9.90 mL of either water, Barth's or simple electrolyte medium (SEM; see Supplementary Data for compositions of solutions referenced here and below) to make 100 μM solutions and the solutions were capped. UV–vis spectra were taken at time increments over three days at room temperature and background subtraction of solvent was applied.
**Molecular Biology**

A pGEM3 vector containing the cDNA encoding full-length M2 from IAV/Udorn/72 H3N2 was kindly provided by L.H. Pinto (Evanston, Il). S31N and H37A mutants were developed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and verified through Sanger-method cycle sequencing (DNA Sequencing Center, BYU). Messenger RNA was produced from linearization of pGEM cDNA using the HindIII restriction site found downstream of the gene, and subsequent invitro transcription reactions of the linearized cDNA using mMESSAGE mMACHINE® T7Ultra Transcription Kit that included a Poly(A) tailing procedure (Ambion, Houston, TX).

**Electrophysiology**

Oocytes from Xenopus laevis (Ecocyte, Austin, TX) were maintained in ND-96++ solution at 17 °C until injection of ~40 ng of A/Udorn/72 H3N2, A/Udorn/72 H3N2 M2 S31N, or A/Udorn/72 H3N2 M2 S31/ H37A mRNA using a Nanoject II (Drummond Scientific, Broomall, PA). After injection, the oocytes were maintained at 4 °C in ND96++ pH 7.4 until electrophysiological recording. 72 hrs after mRNA injection, whole-cell currents were recorded with a two-electrode voltage-clamp apparatus at Vm = −20 mV, room temperature, in Barth's solution, pH 7.5. Inward current was induced by perfusion with Barth's pH 5.3. Percentage block of the original inward current by 100 μM test compound or complex was measured just before washout, which was done 8 min 50 s after drug exposure for each compound or complex, except where noted.
Cell Culture

Influenza-sensitive Madin-Darby canine kidney (MDCK, ATCC, Manassas, VA) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO). 1.1 g/L of sodium bicarbonate, 10 μM Hepes, 100 U/mL of Penicillin and 100 μg/mL of Streptomycin were added to the growth and cytotoxicity test media. Cell cultures were incubated at 37 °C in a humidified 5% CO2 incubator. Passaging was carried out twice weekly using 0.25% trypsin-EDTA (ThermoFischer Scientific, Waltham, MA). The growth medium was supplemented with 5% fetal bovine serum (FBS, Hyclone, Logan, UT).

Cytotoxicity Assay

MDCK cells were seeded into the 60 internal wells of a 96-well plate at a density of 2x10^4 cells/well in 5% FBS DMEM. After 48 h, growth media was removed, and replaced with DMEM supplemented with 1% FBS containing test complex at concentrations of 4 μM to 1 mM in 2-fold increments, six wells per concentration. Six wells without the test compound or complex served as controls. The cells were incubated with the test compound or complex for 72 h at 37 °C in a humidified 5% CO2 incubator. The crystal violet staining technique as described (5.32) was used to determine cytotoxic activity. After 72 h, the test medium was removed, and the cells were washed three times with 300 μL of PBS. The cells were next fixed and stained for 10 min with 50 μL of crystal violet solution (0.03% crystal violet (w/v) in 20% methanol). The cells were then washed 6 times with 300 μL of distilled water, before adding 100 μL of lysis buffer to the cells for 20 min. The optical density of each well was measured at 620 nm, averaged over the set of six wells for each concentration, and the 50% cytotoxic concentration, or the concentration of test compound or complex that killed half of the cells was calculated using a non-linear least squares fit in KaleidaGraph (Synergy Software, Reading, PA, USA), and are expressed as the
fitted $CC_{50} \pm SE(CC_{50})$, where the standard error of the parameter is derived from the parameter covariance matrix for the fit.

**Viral Infection Assay**

As described previously (Kolocouris et al., 2014), MDCK cells were seeded onto 12-mm glass coverslips at $2.4 \times 10^4$ cells/vial in 1 mL of DMEM with 5% FBS. The cells were incubated overnight at 37 °C in a humidified 5% CO$_2$ incubator. The culture was washed with serum-free medium to remove remaining FBS. SEM used in the miniplaque assay was selected for sustaining cell viability, allowing viral infection, and having minimal UV absorbance to allow easy determination of the complex stabilities. The EC$_{50}$ for Amt was $3.4 \pm 0.9$ μM in SEM and $2.8 \pm 0.3$ μM in DMEM against the Amt-sensitive A/Victoria/03/75 H3N2. Two to four replicates were done at each of five to six concentrations ranging from 1 to 50 μM, and four shell vials without any added test compound or complex served as controls. Stock virus was thawed, treated with tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin at 1 μg/mL, and then diluted with SEM to the appropriate dosage. The culture was inoculated with 100μL of the solution containing activated virus, either A/Calif/07/09 H1N1, A/WS/33 H1N1, or A/Victoria/03/75 H3N2, and incubated at 33 °C for 16 h. Coverslips were washed with phosphate-buffered saline (PBS), fixed with cold acetone (−80 °C), and then removed from the shell vials. After air-drying for 30 min, the coverslips were stained with anti-IAV FITC-labeled monoclonal antibody (Light Diagnostics #5017, EMD Millipore – now Millipore Sigma, Billerica, MA) and incubated at 37 °C for 30 min in a humidified chamber. Excess antibody was washed off with PBS containing 0.05% Tween20, 0.5 mM MgCl and 3.1 mM sodium azide and distilled water. Miniplaques, defined as one or a few adjacent cells that were antibody-labeled (considering mitotic events and neighbor infection events as single miniplaques) were then
counted with a fluorescent microscope (Figure 5.1). The 50%-effective concentrations (EC$_{50}$) were obtained by a non-linear least-squares fit using the Levenberg-Marquardt algorithm in KaleidaGraph and are expressed as the fitted EC$_{50}$ ± SE(EC$_{50}$), where the standard error of the parameter is derived from the parameter covariance matrix for the fit.

Results and Discussion

*Complex Synthesis and Structure*

Bromoacetic acid and bromoacetamide were added to Amt or cyclooctylamine to produce 1, 3, 5, and 7 (Figure 5.2). Only a weak acetate base was required to promote the coordination of Cu$^{2+}$ to 1 and 3. A strong base, NaH was needed to deprotonate the amides of 5 and 7 and promote Cu$^{2+}$ coordination (5.33). Upon Cu$^{2+}$ coordination to form 2, 4, 6, and 8, neutral complexes were formed due to the carboxylic acids and the amide nitrogens losing protons. Compounds 9 and 10, which were identical but without the scaffolds attached to the imino N, were formed from the amino diacetate or diacetamide. Crystals of 2 were grown and X-ray analysis of the crystals showed ligand coordinating in a meridional arrangement around Cu$^{2+}$ (Figure 5.3). Water molecules occupy an equatorial position and one of the axial positions. The equatorial bonds are shorter and close to 2.0 Å, while the axial bond is longer (2.4 Å) due to Jahn-Teller distortion. The water molecules are labile and will exchange with stronger ligands, such as ligands containing nitrogen (5.34). Except for a strong absorbance at around 200 nm, the Cu$^{2+}$-free compounds, 1,3,5,and 7,have very little absorbance (data not shown). Once Cu$^{2+}$ binding to the compounds occurs, a strong absorption band in the UV region at 250–280 nm is observed and absorption in the visible gives the complexes a blue coloration (see the legend of Figure 5.4 for extinction coefficients). UV absorption spectra of the complexes measured in water at therapy-like concentrations of 100 μM showed that 2, 4 and 8 were stable in water for three days, while 6
showed moderate decomposition (Figure 5.5). The d-d transition produces absorption, enhanced by complexation, at visible wavelengths that could provide similar information. However, the extinction coefficient is \(\sim\)100-fold lower than for the absorbance in the UV, so for assays at the therapeutically relevant concentrations, we focused on UV measurements. Stability studies of the complexes in biological electrolyte solutions showed a good degree of stability for most of the complexes. In the SEM used for miniplaque assays, 2 and 4 were again stable for three days, but 8 showed some reduction in absorbance and 6 fully decomposed (Figure 5.4), whereas in Barth's all four spectra were stable, but 6 and 8 showed different absorption profiles (Figure 5.6). Additional details about the pH-dependence of stability and 1H-NMR analysis (Figures 5.7 and 5.8) are provided in Supplementary Data.

**Electrophysiology**

Noting the stability of the complexes in aqueous solutions, viral protein blocking studies were performed. Copper(II) has been shown to block the oocyte current for the native A/Udorn/72 H3N2 M2, unless H37 in converted to G or A (5.15). Therefore, we predicted that Cu\(^{2+}\) would block the S31N mutant equally well because it contains H37. Indeed, the onset of block of Cu\(^{2+}\) and persistence during washout are similar to the block of the native channel by Amt (Figure 5.9a), demonstrating that the hydrogen bonding and hydration factors related to the N31 side chain in the S31N mutant do not limit Cu\(^{2+}\) binding. We therefore examined the blocking behaviors of the copper complexes, which have labile copper coordination sites to bind to residues in the M2 channel and compared them to that of Cu\(^{2+}\) (CuCl\(_2\)). The four metal complexes with adamantane (2 and 6) or cyclooctane (4 and 8) each produced an onset of binding that, like Cu\(^{2+}\), had fast and slow phases, and relief during washout similar to but distinctive from the Cu\(^{2+}\) solution (Figure 5.9b, d, f, and h). The complexes with iminodiacetate
(2 and 4) showed a smaller fast block followed by a prolonged slow block (Figure 5.9b and d; note the contracted time scales) compared to the Cu$^{2+}$, with similarly minor washout. In contrast, the complexes with iminodiamide (6 and 8) showed a much greater fast block than Cu$^{2+}$, different slow blocks (with the Amt being slower than Cu$^{2+}$, whereas the cyclooctyl is faster and followed by an enigmatic relief of block), and substantial relief of block during washout (Figure 5.9f and h). The inhibition can be compared to that of the 5-cyclopropyl and 5-cyclohexyl isoxazole extensions of N-methyl amantadine, which at 100 μM block 85% and 90% of the M2 S31N current, respectively (5.41). Although Cu$^{2+}$ and all four complexes proved successful at blocking S31N mutant, the current block and washout kinetics indicate that the complexes with iminodiacetate, albeit slower to initiate block, may ultimately block more thoroughly. The kinetics during wash-in for Cu$^{2+}$ (Figure 5.9a) are much different than for the complexes. For example, the Cu$^{2+}$ IDA complexes 2 (Figure 5.9b) and 4 (Figure 5.9d) block with a much slower rate. The IDAA complexes 6 (Figure 5.9f) and 8 (Figure 5.9h) also have distinctive wash-in kinetics and some wash-out while the IDA complexes and Cu$^{2+}$ do not. Furthermore, without the adamantyl or cyclooctyl scaffold, the blocking was nearly absent (9) or diminished (10) during onset (Figure 5.10), showing that the scaffolds both contribute favorably to the onset of block. These distinctions between the wash-in and wash-out equilibrium relaxation kinetics for different complexation structures suggest that the copper remains complexed to the ligands while they are in the channel. One might speculate that the biphasic time-course of block onset represents two-stage binding where the charge-neutral complexes (or Cu$^{2+}$) first enter the channel and inhibit proton flow partially or completely in the first stage, followed by complexation with His37 yielding complete proton block in the second stage. To determine whether Cu$^{2+}$ was key to the block, complexation ligands without Cu$^{2+}$ were tested. As was seen with Amt and the S31N
mutant (Figure 5.11), the scaffolds without Cu\(^{2+}\) caused minor block (Figure 5.9c, e, g, and i).

For Amt, the already low affinity of the S31N mutant was further reduced by derivatizing with iminodiacetic acid or iminodiacetamide. To confirm that the complexes interact with the His37 side chains, we measured the current-block produced by Cu\(^{2+}\), 4 and 8 with A/ Udorn/72 H3N2 M2 H37A. This construct, shown previously to have loss of high affinity block by Cu\(^{2+}\) (5.15), showed elimination of the slow phase of block (Figure 5.12), confirming the hypothesis that Cu\(^{2+}\), whether free or complexed, produces the slow block by binding to the imidazoles of the His37 side chains. Although it is not possible from the current experiments to determine the binding mode, we conjecture that because of the high stability constant for Cu\(^{2+}\) complexation to imidazole, log(K) 4.33 (5.35), that copper is binding to one or more members of the tetrad of H37 imidazoles probably while still attached to the scaffold. In the future, it should be possible to use solid state NMR (5.36,5.37) and crystal structure (5.38,5.39,5.40) information together with kinetic modeling of the current blockade during metal-complex wash-in and wash-out experiments, and with quantum mechanical and molecular dynamics simulations to evaluate the mechanism of binding and proton current block by these compounds. Table 5.1 summarizes, from Figure 5.9 and other data collected, the percentage of current block achieved in the initial fast stage (i.e. within <10s) and after prolonged exposure (8–60 min) in as low stage, as well as the percent of that block recovered during a 2 min washout. Amt exposure in the S31 WT M2 channel produced a maximal block of 94% and did not show a return of inward current from total percent block. In contrast, Amt exposure in the S31N mutant M2 channel showed a maximal block of only 30%, and had a complete return of inward current, consistent with Amt's lack of efficacy against influenza A strains bearing that mutation. Exposure of metal containing compounds (2-8) in S31N M2 showed block of oocyte current similar to the Amt control. For
comparison, 4 reached a maximal block of 90.5% after 30 min of drug exposure, and a minimal 1.2% return of inward current from the total percent block. 2 and 6 had similar results. Results for the same compounds without copper ligated (1,3,5, and 7) show at best a maximal block of 43% (3). No additional slow block was observed for the non-metal containing compounds. Exposure of the S31/H37A M2 variant to 4 and 8 show similar results to the S31N M2 Amt control. A reduction of maximal block is observed, and the additional slow block is not observed. Rapid and nearly complete recovery from block is seen for 4 and 8 in the S31/H37A M2. The results show that with Cu$^{2+}$, the complexes block in an additional slow phase, and with high affinity, whereas without Cu$^{2+}$ or without imidazoles at position 37 in the homotetramer, block is mild and rapidly reversible. In addition, we note that like Cu$^{2+}$, 10 bound more quickly and effectively than 9, but washed out more rapidly that either 9 or Cu$^{2+}$. These qualitatively differing results again indicate that the complex is binding and blocking, not dissociating to free Cu$^{2+}$ that then blocks.

**Antiviral Efficacy Assays and Cytotoxicity**

To evaluate the merits of the complexes in blocking infection of MDCK cells, we labeled infected cells with fluorescent antibody and counted the total number of infected cells in a fixed incubation period. We also investigated the importance of the adamantane and cyclooctyl moieties by testing the copper complexes of iminodiacetate and iminodiacetamide without scaffold. In this miniplaque assay, all of the metal complexes were effective at the low μM level against both the 1933 and 2009 H1N1 strains (both bearing M2 N31) as well as against the 1975 H3N2 strain (bearing M2 S31) (Table 5.2). 4 was the most effective complex in the miniplaque assay with an EC$_{50}$ of 0.7 μM in A/ Calif/07/09 H1N1, 2.1 μM in A/WS/33 H1N1, and 3.7 μM in A/Victoria/03/75 H3N2. The inhibition can be compared to that of the 5-cyclopropyl and 5-
cyclohexyl isoxazole extensions of N-methyl amantadine, which block 58% and 43% of plaque formation by A/WSN/33 at 1 μM, respectively (232). For other adamantane or cyclooctyl complexes, 2, 6, and 8, the EC50s were similarly potent, ranging from 2.3 to 11.6 μM. Although the low stabilities of 6 and 8 in SEM (Figure 5.5) on the 16-h time scale of the miniplaque assay allow the possibility of one of the dissociated components, e.g. Cu2+, to be the active component, it is also possible that the complexation with viral M2 takes place on the 6–8 min time-scale prior to viral uptake by the cell (Figure 5.9f and h) and is stable, which could explain the lower efficacy of the complexes than that of Cu2+ in this assay (Table 5.2). The complexes with iminodiacetate and iminodiacetamide, whether with scaffold (2 and 4, 6 and 8, respectively) or without scaffold (9, 10) were similar in efficacy. In some cases the scaffold helped to lower the EC50, such as with 4 vs. 9 in A/CA/07/09 and in other cases the scaffold had little effect, such as with 4 vs. 9 in A/Victoria/03/75. Importantly, the miniplaque anti-viral efficacy results are consistent with the electrophysiology results. In both assays, CuCl2 and the metal complexes were effective against both the S31N mutant and the native strains tested. Taken together, these results demonstrate that the metal complexes directly and uniquely cause the antiviral effect through block of M2 proton transport. The miniplaque data are most consistent with a mechanism of blockage of viral acidification during the endosomal stage of infection. Given the water solubility of the metal complexes, we do not expect them to be membrane permeable. Therefore, they probably bind to the virion M2 before or are entrapped with the virion during endocytosis. Exposure of cultured MDCK cells to different concentrations of complex for a three day period allowed assessment of their toxic effects at the cellular level. The cytotoxicity results indicate the copper complexes, with or without the scaffold, were considerably less toxic to the cells than copper chloride (Table 5.3). The cyclooctyl complexes were ∼3-fold less toxic than
the Amt complexes. The therapeutic index, taken as the EC$_{50}$ averaged over the three IAV strains tested, divided by the CC$_{50}$, was best for 4, 67.8. Except for 7, the compounds without Cu$^{2+}$ were not toxic in the range tested (Table 5.4). For example, 3 was nontoxic at 1 mM, while 4 caused 50% cell death at 147 $\mu$M, yet CuCl$_2$ was $>7$-fold more toxic and caused 50% cell death at 19 $\mu$M.

Conclusions

We confirmed that Cu$^{2+}$ blocks the native M2 channel and also find that it blocks M2 S31N in voltage-clamped transfected *Xenopus* oocytes. The washout shows that copper binds tightly in both M2 constructs. When Cu$^{2+}$ is complexed via IDA or IDAA with or without derivatives of known M2 blockers, Amt or cyclooctylamine, the complexes block M2 channels comparably to aquated Cu$^{2+}$. When the Cu$^{2+}$ is removed from the complex, the tight block is eliminated, which suggests Cu$^{2+}$ in the complex is adhering to the M2 channels. When the histidines are mutated to alanines (H37A), the slow block is also eliminated, indicating that the copper complexes bind to the histidines of the M2 channels during the slow phase. The copper complexes significantly reduce, with low cytotoxicity, infection of MDCK cells with three IAV strains, two Amt-insensitive and one Amt-sensitive, and have EC$_{50}$s in MDCK cell culture for the three strains of IAV of 0.7–11.6 $\mu$M. Complexation reduces the cytotoxicity of Cu$^{2+}$ approximately 10-fold. Although the adamantyl and cyclooctyl moieties don't affect the antiviral efficacy on the long time scale, it is clear that they enhance M2 blocking on the short time scale of oocyte experiments (2,4 > 9 and 6, 8 > 10). One complex in particular, 4, is potent at sub-micromolar concentrations against A/Calif/07/2009 H1N1, has a low cytotoxicity (average therapeutic index 67.8), and withstands decomposition in phosphate, sulfate, and nitrate containing buffers on the timescale of infection and experiments. Considering the human dietary
tolerance for copper and the measured EC$_{50}$s, we estimate that these complexes could be useful as novel treatments against multiple strains of IAV. They could be delivered either by inhalant, orally in acid-resistant encapsulation, or intravenously. Resistance testing and animal testing are needed to further explore these possibilities.

Contribution to Publication

Co-first author by writing the stability and electrophysiology methods, results, and discussion, and helped write the complex synthesis and structure methods and results sections, managed lab group, collected electrophysiology data in Figure 5.4, 5.5, 5.6, and Table 5.1, and helped with UV-Vis and NMR data collection.
Figure 5.1: Miniplaque Fluorescence Image of Influenza A Infection. Fluorescence image of a typical field containing 5 miniplaques labeled with FITC-labeled antibody to the influenza A NP. In this case each miniplaque is a single infected cell, but occasionally neighboring cells are infected but are counted as a single miniplaque, on the assumption that mitosis had occurred after infection of the primary cell, or that the primary cell had been infected early in the incubation and had infected its neighbor. The multiplicity of infection was generally adjusted so that a coverslip would have about 100 miniplaques in the absence of anti-viral compounds. Counts were obtained by scanning the coverslip in a serpentine pattern for thorough, non-redundant coverage of about 100 such fields.
Figure 5.2: Synthetic Procedures for Compounds 2, 4, 6, and 8.
Figure 5.3: Crystal Structure (side and face views) for Cu(AMT-IDA).
Figure 5.4: UV-Vis 3 Day Stability Test in SEM. UV-Vis spectra of 100 uM solutions of (A) 2, (B) 4, (C) 6, and (D) 8 in SEM. A spectrum of SEM was subtracted as background. Spectra color: blue start, orange 4 hrs, green 12 hrs, purple 24 hrs, yellow 72 hrs. Spectral bands in the UV region upon Cu^{2+} binding: 252 nm (5300 abs M^{-1} cm^{-1}) for 2, 251 nm (3700 abs M^{-1} cm^{-1}) for 4, 286 nm (1600 abs M^{-1} cm^{-1}) for 6, and 285 nm (2800 abs M^{-1} cm^{-1}) for 8. Along with the UV band, there is a new band in the visible region: 705 nm (220 abs M^{-1} cm^{-1}) for 2, 717 nm (100 abs M^{-1} cm^{-1}) for 4, 650 nm (200 abs M^{-1} cm^{-1}) for 6, and 637 nm (170 abs M^{-1} cm^{-1}) for 8. The UV absorption with its intermediate molar absorptivity is proposed to be due to a ligand-to-metal charge transfer (LMCT) and the peak in the visible is due to a copper-based d-d transition.
Figure 5.5: UV-Vis 3 Day Stability Test in Water. UV-Vis spectra of 100 μM solutions of (A) 2, (B) 4, (C) 6, and (D) 8 in water. Spectra color: blue start, orange 4 h, green 12 h, purple 24h, yellow 72 h.
Figure 5.6: UV-Vis 3 Day Stability Test in Barth’s Solution. UV-Vis spectra of 100 uM solutions of (A) 2, (B) 4, (C) 6, and (D) 8 in Barth’s. A spectrum of Barth’s solution was subtracted as background. Spectra color: blue start, orange 4 hrs, green 12 hrs, purple 24 hrs, yellow 72 hrs.
Figure 5.7: $^1$H NMR (300 MHz) of Compound 2 in Acetonitrile Standard Before Acidification. In DMSO-d$_6$. Peak assignments (ppm): 0.00 TMS, 2.07 NCCH$_3$, 2.51 DMSO, 3.32 H$_2$O.
Figure 5.8: $^1$H NMR (300 MHz) of Compound 2 in Acetonitrile Standard After Acidification. With trifluoroacetic acid in DMSO-$d_6$. Peak assignments (ppm): 0.00 TMS, 1.63 adamantane CH$_2$, 1.97 adamantane CH$_2$, 2.07 NCCH$_3$, 2.17 adamantane CH, 2.57 DMSO, 3.94 H$_2$O, 4.18 IDA CH$_2$. 
Figure 5.9: Electrophysiology Current Traces for Cu(II) Complexes in M2 S31N. Using full-length A/Udorn/72 H3N2 M2 S31N. Transfected in *Xenopus* laevis oocytes. At $t = 0$, perfusion is switched to pH 5.3. At $t = 1$ min, the perfusion is switched to the same solution, but with 100 μM of test complex or compound. The washout (no drug), which lasted 1–3 min, is marked by the empty arrow. Finally, the perfusion is returned to Barth's pH 7.4 to check cell viability. a. CuCl$_2$, b. 2, c. 1, d. 4, e. 3, f. 6, g. 5, h. 8, and i. 7. Each trace is representative of 3 runs.
Figure 5.10: Electrophysiology Current Traces for Compounds a) 9 and b) 10 in M2 S31N. Using full-length A/Udorn/72 M2 S31N. At t=0, perfusion is switched to pH 5.3. At t=2.5 (a) or 1.2 (b) min, perfusion switches to same solution with 100 µM of test compound or complex. The washout (no drug), which lasted 1-3 minutes, is marked by the empty arrow. Finally, the perfusion is returned to Barth’s pH 7.4 to assess cell viability. Each trace is representative of 3 runs. The block by 9 was very small and didn’t wash out, whereas 10 produced substantial fast and slow phase block, but was fast to wash out with both fast and slow phases. In both cases, the block is not as substantial as when the ligand contains an adamantane or cyclooctylamine scaffold (Figure 3b,d,f,h), suggesting that the scaffolds enhance the block.
Figure 5.11: Electrophysiology Current Traces for AMT Block in WT or S31N M2. Block of inward current using full-length A/Udorn/72 M2 with a. WT- (S31) or b. S31N-M2 transfected Xenopus laevis oocytes. At t=0, perfusion is switched to pH 5.3. At t=1 min, the perfusion is switched to the same solution, but with 100 µM Amt. The washout (no drug), which lasted 1-3 minutes, is marked by the arrow. Finally, the perfusion is returned to Barth’s pH 7.4 to check cell viability. Each trace is representative of 3 runs.
Figure 5.12: Electrophysiology Current Traces for Cu$^{2+}$ and Cu(II) Complexes in M2 S31N-H37A. At $t=0$, perfusion is switched to pH 5.3. At $t=1$ min, perfusion switches to same solution with 100 µM of test compound or complex. The washout (no drug), which lasted 1-3 minutes, is marked by the empty arrow. Finally, the perfusion is returned to Barth’s pH 7.4 to assess cell viability. a. CuCl$_2$ b. 4 c. 8. Each trace is representative of 3 runs.
Table 5.1: Current-Blocking Characteristics in Electrophysiology.

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<tr>
<td>IDA</td>
<td>−34.3d (± 0.4, 3)</td>
<td>None</td>
<td>34.2 (± 0.3, 3, 2)</td>
</tr>
<tr>
<td>IDAA</td>
<td>23.2 (± 5.2, 3)</td>
<td>None</td>
<td>17.5 (± 0.1, 3, 2)</td>
</tr>
<tr>
<td>1</td>
<td>6.5 (± 1.5, 3)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>42.8 (± 0.6, 3)</td>
<td>None</td>
<td>33.4 (± 1.7, 3, 2)</td>
</tr>
<tr>
<td>5</td>
<td>9.6 (± 8.3, 3)</td>
<td>None</td>
<td>5.6 (± 3.7, 3, 2)</td>
</tr>
<tr>
<td>7</td>
<td>12.5 (± 2.5, 3)</td>
<td>None</td>
<td>6.8 (± 0.4, 3, 2)</td>
</tr>
<tr>
<td><strong>M2 H37A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuCl₂</td>
<td>19.8 (± 0.3, 3)</td>
<td>None</td>
<td>14.9 (± 0.2, 3, 2)</td>
</tr>
<tr>
<td>3</td>
<td>6.8 (± 0.4, 3)</td>
<td>None</td>
<td>4.5 (± 0.7, 3, 2)</td>
</tr>
<tr>
<td>4</td>
<td>12 (± 2.8, 3)</td>
<td>None</td>
<td>6 (± 1.4, 3, 2)</td>
</tr>
<tr>
<td>8</td>
<td>26 (± 10.9, 3)</td>
<td>None</td>
<td>24.6 (± 14.1, 3, 2)</td>
</tr>
</tbody>
</table>
Table 5.2: Calculated EC\textsubscript{50} Values (\(\mu\)M) for the Copper Complexes in the Miniplaque Assay.

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>CuCl\textsubscript{2}</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/CA/07/09</td>
<td>3.8 ± 0.9</td>
<td>6.9 ± 1.2</td>
<td>0.7 ± 0.1</td>
<td>4.9 ± 0.8</td>
<td>11.6 ± 1.1</td>
<td>8.2 ± 2.0</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>H1N1 M2 S31N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/WS/33</td>
<td>4.3 ± 1.0</td>
<td>2.4 ± 0.3</td>
<td>2.1 ± 0.7</td>
<td>4.1 ± 2.4</td>
<td>2.3 ± 0.2</td>
<td>4.7 ± 1.1</td>
<td>7.2 ± 5.3</td>
</tr>
<tr>
<td>H1N1 M2 S31N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/Victoria/03/75</td>
<td>1.1 ± 0.3</td>
<td>4.2 ± 1.7</td>
<td>3.7 ± 0.9</td>
<td>7.0 ± 1.2</td>
<td>8.4 ± 0.7</td>
<td>4.4 ± 2.2</td>
<td>16.3 ± 8.4</td>
</tr>
<tr>
<td>H1N2 M2 WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3: Calculated CC\textsubscript{50} Values (\(\mu\)M) for the Copper Compound and Complexes.

<table>
<thead>
<tr>
<th>CuCl\textsubscript{2}</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 ± 6</td>
<td>64 ± 7</td>
<td>147 ± 38</td>
<td>52 ± 5</td>
<td>180 ± 15</td>
<td>115 ± 9</td>
<td>64 ± 6</td>
</tr>
</tbody>
</table>

Table 5.4: Calculated CC\textsubscript{50} Values for the Copper-Free Compounds. Iminodiacetic acid: IDA; Iminodiacetamide: IDAA. (mM, unless indicated otherwise).

<table>
<thead>
<tr>
<th>IDA</th>
<th>IDAA</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 0.5</td>
<td>&gt; 1</td>
<td>&gt; 1</td>
<td>&gt; 1</td>
<td>&gt; 1</td>
<td>0.26 ± 0.02</td>
</tr>
</tbody>
</table>
CHAPTER 6: Copper Complexes as Influenza Antivirals: Reduced Zebrafish Toxicity


Abstract

Copper complexes have previously been developed to target His37 in influenza M2 and are effective blockers of both the wild type (WT) and the amantadine-resistant M2S31N. Here, we report that the complexes were much less toxic to zebrafish than CuCl2. In addition, we characterized albumin binding, mutagenicity, and virus resistance formation of these metal complexes, and employed steered molecular dynamics simulations to explore whether the complexes would fit in M2. We also examined their anti-viral efficacy in a multi-generation cell culture assay to extend the previous work with an initial-infection assay, discovering that this is complicated by cell culture medium components. The number of copper ions binding to bovine serum albumin (BSA) correlates well with the number of surface histidines and BSA binding affinity is low compared to M2. No mutagenicity of the complexes was observed when compared to sodium azide. After 10 passages of virus in MDCK culture, the EC50 was unchanged for each of the complexes, i.e. resistance did not develop. The simulations revealed that the compounds fit well in the M2 channel, much like amantadine.

Keywords: Bovine serum albumin (BSA), Madin-Darby canine kidney cells (MDCK), half maximal effective concentration (EC50), cyclooctylamine (CO), amantadine (AMT), iminodiacetamide (IDA), iminodiacetate (IDA), cytopathic effect (CPE), steered molecular dynamics (SMD)
Introduction

The influenza A M2 protein is a homotetrameric channel (6.1) that is particularly selective for protons (6.2) and is essential for uncoating of the virus (6.3). The proton selectivity is due to the cluster of His37 imidazole side chains in the channel (6.4,6.5). This channel has been a primary antiviral target. Amantadine (AMT) and rimantadine (RMT) were highly successful as M2 blockers (6.6,6.7,6.8), but they became ineffective in 2005 when a mutation from serine to asparagine at residue 31 (S31N) in M2 occurred (6.9,6.10).

Attempts have been made to develop variants of AMT, RMT and others that could block the V27A, L26F, or S31N mutations (6.11,6.12,6.13,6.14,6.15). We explored a different approach that could, in theory, target all functional forms of M2 (6.16).

Drawing from the observation that divalent cations, particularly copper, block M2 current (6.17) binding in the His37-Trp41 side chain quadruplex (249), divalent copper complexes of AMT were synthesized and found to be effective influenza A inhibitors with reduced cytotoxicity compared to CuCl₂ (6.16). Because Cu²⁺ binds strongly to imidazole, it was suggested that the Cu²⁺ complexes also block M2 through His37-imidazole binding. In addition, the His37 cluster is highly conserved in nature (6.18), making it a prime target in the M2 channel.

The copper ligands developed were based on AMT and the lesser-known, equally effective M2 WT blocker, cyclooctylamine (CO) (6.19,6.20), and extended via the amine with the functional groups iminodiacetate or iminodiacetamide. Six Cu²⁺ complexes (Figure 6.1) were synthesized and characterized using NMR, IR, MS, UV-Vis, and ICP-MS. The complexes demonstrated H37-specific block of M2 current in two electrode voltage-clamp (TEVC) studies.
with low μM potencies. The copper-free ligands did not show proton current block, demonstrating that the copper was key to the current-blocking process (6.16).

Because of the reduced toxicity to cultured cells found previously, we were interested to learn whether the six metal complexes were toxic to simple organisms. Zebrafish embryos were chosen because they have immune and nervous systems similar in many ways to more advanced organisms, because they are in an early, vulnerable stage of development, and because the compounds are readily administered at infection-relevant concentrations in their bathwater. We also explored and report additional properties of these copper complexes, including their efficacies in the cytopathic effect antiviral assay, their binding to albumin, mutagenicity testing in a bacterial assay, virus resistance development when passaged with cell culture in the presence of the compounds, and molecular dynamics simulations to explore how well the compounds fit in the M2 channel.

Methods and Materials

Cytopathic Effect Assay

Confluent MDCK cells were transferred into 60 wells of a 96-well plate in DMEM (Gibco Thermo Scientific Waltham, MA, 4.5 g/L D-Glucose) with 5% Fetal Bovine Serum (FBS, Hyclone, Logan, UT). The cells were washed with a diluted solution of 50% SEM/50% serumless DMEM. SEM (simple electrolyte medium) consists of 4.33 g NaCl, 0.244 g KCl, 0.103 g CaCl₂·2H₂O, MgCl₂·6H₂O, Na₂HPO₄·7H₂O, NaH₂PO₄·H₂O in 500 ml H₂O. The cells were incubated for an hour with activated A/WS/33 virus and then the media with virus was removed. The SEM/serumless DMEM with 100 μM metal complex was added to six wells. The complexes were then serial-diluted in two-fold increments six times. Six wells were used as positive controls with no complex or virus added. Six wells were used as negative controls with
only virus added and no complex. About 80 μM ribavirin (Sigma-Aldrich, St. Louis, MO) was added to six wells as a positive control. The plates were incubated for 48 h at 33°C.

The crystal violet staining technique described previously (6.21) was used to determine the fraction of cells that survived the exposure to the virus. After 48 h, the test medium was removed, and the cells were washed three times with 150 μl PBS. The cells were stained for 10 min with 50 μl crystal violet solution (0.03% crystal violet (w/v) in 20% methanol). The cells were then washed three times with 150 μl distilled water before adding 100 μl lysis buffer. After 20 min, the optical density (OD) of each well was measured at 590 or 620 nm and averaged over the set of six wells for each concentration.

Because viral dosing was sufficient to eliminate essentially all cells in treatment-free controls, their average OD was subtracted as baseline from the average of the treated well ODs. The result was divided by the average of the uninfected control well ODs to obtain a normalized vitality. Because the vitality can be affected by both reduction of virus cytopathic effect and increase of treatment toxicity as concentration is increased, we fitted the normalized concentration-dependent vitality, \( V(C) \), with a joint probability function (Eq. 6.1):

\[
V(C) = \frac{1}{1 + \left(\frac{EC_{50}}{C}\right)^{\frac{1}{n_1}}} \frac{1}{1 + \left(\frac{CC_{50}}{C}\right)^{n_2}}
\]  

(6.1)

Here, \( EC_{50} \) is the 50% effective dose of treatment that prevents viral cytopathic effect, \( CC_{50} \) is the 50% cytotoxic dose of the treatment, and \( n_1 \) and \( n_2 \) are their respective Hill coefficients. If the selectivity index, \( CC_{50}/EC_{50} \), and the Hill coefficients are sufficiently high, this function rises to unity at doses that are sufficient to prevent viral replication but below toxic levels. Non-linear least squares fitting weighted with standard errors of means was done with the
Marquardt algorithm in KaleidaGraph4 (Synergy Software, Reading, PA). In practice, it was necessary to fix the Hill coefficients to evaluate the effective doses, then manually adjust the Hill coefficients to improve the fit (due to low numbers of data points). Hence, the reported standard errors of the parameters obtained from the error matrix may be underestimated.

Protein Binding Assay

Each copper complex was dissolved in 25 ml of water to obtain a 1 mM and 800 μM solution. All water used in the protein binding assay was collected from a Millipore first-generation beige Milli-Q system. These solutions were sonicated until the crystals were fully dissolved. Four 1:2 serial dilutions were performed from the 800 μM solution to obtain 400, 200, 100, and 50 μM solutions, and a 1:5 dilution was performed from the 50 μM solution to obtain a 10 μM solution. 13.3 mg of BSA was then dissolved in 10 ml of each solution. The solutions were mixed thoroughly and allowed to stand at room temperature for approximately 20 min. Spin filtration was performed using a swinging bucket rotor at 4000 rpm for 6 min. The spin filters used were Amicon Ultra-15 centrifugal filters. The filtrates from each spin were collected to test for copper content in ICP-MS. Solutions for ICP-MS were prepared from both the original solutions and the filtrates. For each solution, 1 ml of solution was added to 1 ml of 4% HNO3 and 8 ml of 2% HNO3 to obtain a 1:10 dilution of each solution in 2% HNO3. Nitric acid used for ICP-MS analysis was OmniTrace trace-metal grade obtained from EMD Millipore Corporation. We used BSA to model copper binding histidine in solution and calculate relative dissociation constants (K_d) for each complex. Copper concentrations were obtained using ICP-MS. The data was fit to Eq. 6.2 to estimate K_d and the number of binding sites, n.

\[
\frac{[Cu]_t - [Cu]_f}{[P]_t} = \frac{n*[Cu]_f}{K_d + [Cu]_f} \tag{6.2}
\]
**Zebrafish Toxicity Test**

Following an approved BYU IACUC protocol, two AB wild-type male and female zebrafish were placed in an embryo media filled tank. The fish remained in a light and temperature-controlled facility until the following morning. Later that day, the fish were transferred into original tank. Embryos were moved into embryo media filled petri dishes (60 embryos/dish) and housed in an incubator for 2 days. Media was changed daily. Fish embryos were dechorionated at 48 hpf. In a multi-well plate, 10 embryos were selected and 5 were added to each of two wells for each concentration with fresh embryo media. Drug solution (0–200 μM) was then added to test toxicity and observed over 5 days. Drug solutions were changed daily. After 5 days, the fish were scored using a morbidity scale (Table 6.1) indicating response, spine shape, edema, equilibrium, and death. The average for each complex was normalized using the maximal morbidity score of 50/well. The fish were then euthanized.

**Ames Testing**

The Modified Ames ISO kit (Environmental Bio-Detection Products Inc., Mississauga, ON) was used with *S. typhimurium* TA100 (no S9 fraction). The complexes were compared against the mutagenicity of a positive control (NaN₃) and vehicle (water). The complexes were serially diluted 1:2 to compare the complexes’ mutagenic ability at each of six concentrations. TA100 was hydrated and incubated with histidine overnight at 37°C. Following the kit’s instructions, in 96-well plates’ exposure solution, diluted bacteria mix, and serial two-fold dilutions of complexes were combined with reversion media containing Bromocresol Purple, which serves as a pH indicator to identify infected wells. The 96 well plates were incubated for 6 days at 37°C without agitation. When a sample is mutagenic, it will revert the bacteria to WT, causing the media to turn slightly acidic and show a yellow color. The number of reverted wells
with complex was compared to the average number of reverted wells in the negative control. Significance was calculated using a one-tailed t-test.

**Simulations**

The 2KQT M2 structure was used and oriented in a DMPC lipid bilayer with a center-of-mass harmonic constraint. The copper complexes were oriented such that the copper was near (~2.0 Å) at least one of the four imidazole nitrogens. Water molecules within 2.2 Å of the complexes were deleted. The protein-bilayer system was solvated with a tetragonal 60 Å × 60 Å × 90 Å water box as shown in Figure 6.2. The system was minimized for 1000 steps of steepest descent and heated to 300 K. The M2 channel was equilibrated for 1 ns. The complexes were pulled using a constant force for 10 ps during the production runs. Frames were saved every 50 steps, which is every 50 fs, of production for a total of 200 frames. Standard CHARMM version 37b1 parameters were used. Copper dihedral parameters were created using a 20 kcal/mol/rad² energy penalty, which kept a conservatively rigid structure throughout the channel (Table 6.6). The distance between imidazole nitrogens and copper on the complexes was calculated using CHARMM’s CORREL subroutine for each frame. The time for each complex was recorded when the copper reached 30 Å away from the imidazole nitrogens. This distance was chosen to represent the complex leaving the mouth of the channel.

**Decisions Affecting Pulling Force**

The pulling force for each of the complexes was determined by normalizing the pulling forces to a 2.34 nN pulling force on AMT. The 2.34 nN force allowed comparisons to be made between compounds as they left the channel on the 10 ps timescale. These steered molecular dynamics (SMD) simulations were analyzed by computing the mean and standard deviation of
five independent. The five independent simulations were assigned random starting velocities and then analyzed to explore the time needed to pass the 30 Å threshold relative to the starting point from the copper atom on the complex. The analysis examined whether the pulling forces, copper ligation mechanism, or scaffold (CO, AMT, or neither), significantly affected the exit times relative to free Cu²⁺.

*Miniplaque Assays, Resistance Testing, and Sequencing*

MDCK cells were seeded into a six-well plate and grown in Dulbecco’s Modified Eagles Medium (DMEM, Sigma-Aldrich, St. Louis, MO) augmented by 5% with fetal bovine serum (FBS, Hyclone, Logan, UT) until confluent. After 48 h, the growth media was removed and replaced with DMEM. At this point the virus (A/CA/07/09) was introduced into the medium (200 pfu/ml) and allowed to adsorb for 1 h. The medium was then removed and replaced with fresh DMEM containing a specified concentration of complex and 5 ml of tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Thermo-Fischer Scientific, Waltham, MA, 1 mg/ml) was added to activate the virus. The plate was incubated at 33°C for 3 days. Then the medium was removed and centrifuged at 2000 rpm in order to remove cell debris. This virus-containing medium was then separated into 1-ml aliquots and frozen in Eppendorf tubes at −80°C. This process was repeated for each successive passage.

The concentration of virus was determined through an immunofluorescence assay (as previously described by (6.22)), which gave a multiplicity of infection (MOI) of 0.6. MDCK cells were seeded onto glass coverslips in vials containing 1 ml DMEM and trypsin in order to obtain 90% confluency after 24 h. The cells were allowed to grow overnight at 37°C, after which the growth medium was removed and replaced with DMEM. The sample of virus was then
diluted by factors of 10, and the various dilutions of virus were stirred into the vials with coverslips.

They grew at 33°C for 18 h. After this incubation period, the medium was removed, the cells were fixed with cold acetone (−80°C), and the coverslips were washed and stained with a fluorescein isothiocyanate labeled anti-IAV monoclonal antibody (Millipore Sigma, Burlington, MA, Cat. #5017). Excess antibody was washed off using a solution of 0.05% Tween20 in phosphate buffered saline and then again with distilled water. They were then viewed microscopically and individual infected cells (miniplaques) were counted.

This same process was followed in determining the new EC₅₀ against the specific complex of each resistant strain. Except, 100 pfu of virus was used in each vial. Several different concentrations of the complex with which it was passaged were introduced into the vials, with concentrations ranging from 2 to 70 μM. The cells were infected with the virus in a solution of SEM rather than DMEM. The EC₅₀ was calculated in KaleidaGraph using the Levenberg-Marquardt algorithm. The fitting parameters (sigmoidal function) were used to calculate the EC₅₀ and the standard error of the mean.

To sequence the genome, the viral sample was concentrated 10-fold using a spin filter (VWR North America, Radnor, PA, Cat. #82031-352). After that, viral RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD). The isolated RNA was stored at −20°C. RNA was then reverse-transcribed using Invitrogen’s Superscript III One-Step RT-PCR Platinum Taq HiFi kit (Thermo-Fischer Scientific, Waltham, MA).

The resulting isolated DNA was stored at −20°C. The DNA was then amplified with PCR using the Phusion High-Fidelity PCR kit (New England Biolabs, Ipswich, MA). The solution was purified using Qiagen’s QIAquick PCR Purification Kit (Qiagen, Germantown, MD). It was
sequenced using custom forward (TGTAAAACGACGGCCAGTACGAAAAGCAGGTAG) and reverse (CAGGAAACAGCTATGACCAGTAGAAACAAGGTAGT) primers for the segment of the new DNA that codes for the M2 protein.

Results and Discussion

_Cytopathic Effect Assay_

Although 1–4 had good potency against initial infections in the immunofluorescence (miniplaque) assay (6.16), the copper complexes had no effect in the cytopathic effect (CPE) assay with MDCK cells when dissolved in serumless DMEM. However, when the serumless DMEM was diluted with SEM, 1 (Figure 6.3) and to a lesser extent 3 (data not shown) exhibited cell protection. Using a dual-sigmoidal function curve fit, 1 has an EC$_{50}$ of 0.9 ± 0.08 $\mu$M and a CC$_{50}$ of 5.8 ± 0.37 $\mu$M. The submaximal efficacy is due to high cytotoxicity. The selectivity index for 1 is 6.44, given by the ratio of the CC$_{50}$ and EC$_{50}$. The low EC$_{50}$ compares favorably to the EC$_{50}$ in the miniplaque assay, 6.7 ± 1.2 $\mu$M. In contrast, 3 has an EC$_{50}$ greater than 100 $\mu$M, whereas its potency in the miniplaque assay was EC$_{50}$ = 0.7 ± 0.1 $\mu$M, and 2 and 4–6 showed no effect, indicating that other factors were involved. The fact that some efficacy is observed when the medium is diluted with amino-acid free SEM suggests that free amino acids in non-dilute DMEM interfere with the copper complex efficacy.

_Protein Binding Assay_

To illustrate the potential of the metal complexes to bind to proteins, binding to BSA was measured in which a protein solution was mixed with various concentrations of a CuCl$_2$ or copper complex solution. The copper content of the original sample was measured and compared to that of the filtrate. Taking the volume proportions into account, the “free copper
concentration” in the filtrate relative to the “total copper concentration” in the original sample was fitted to a model assuming that each protein molecule had n equivalent copper or copper complex binding sites. Table 6.2 shows the best fit K_d values, assuming that each albumin monomer has n equivalent binding sites. The two parameters interacted and were therefore poorly constrained in the optimization of the deviations squared, but Table 6.2 indicates that the number of binding sites is well above 10, consistent with the count of 13 surface histidines in monomeric albumin (Figure 6.6). Complexes 1, 3, and 5 have larger K_d values compared to that of CuCl_2 (59.1 μM). This indicates that the ligands on the metal complexes reduce the binding affinity for albumin binding sites, but also still allow for substantial binding. It is also consistent with the electrophysiology results for blocking through binding of copper complex to the His37 cluster in the M2 channel.

BSA has 13 surface histidines (Figure 6.6), however, all of the fits optimized n at >13 copper binding sites. This difference could suggest non-specific binding to other sites on BSA. The high K_d’s for the complexes relative to CuCl_2 indicate that the complexes remain intact during binding to BSA. The binding the copper complexes to BSA is very weak compared to that of the M_2S_3N (AMT resistant) channel, where block was ~80% for 1 and 3 after 57 and 27 min perfusion, respectively. This suggests that protein binding in vivo would be a minor concern. However, it is clear that binding by non-M2 proteins is detectable and, given their large quantity inside and outside the blood, they could limit access of the copper complexes to virus.

**Zebrafish Toxicity Test**

Toxicity was evaluated for zebrafish exposed to various concentrations of CuCl_2 or copper complex (1–5) added as methanolic solutions to the embryo bath medium starting 48 h post fertilization (Day 0) (Figure 6.4). At 200 μM copper complex on day 1, compounds 2,
4, and 5 show minimum toxicity effects, 1 and 3 show moderate toxicity including slow response to stimulation, slightly curved spine, and minor edema, whereas CuCl₂ causes major edema, strongly curved spine, no response to stimulation, and death. By day 2 at 200 μM, the toxicities of 1, 2, 4, and 5 have increased moderately but still only moderate spine curvature and minor edema, while 3 causes slow response to stimulation, strongly curved spine, and moderate to major edema. By days 3, 4, and 5 at 200 μM, all but 5 show low or no response, strongly curved spines, major edema, and some death. The MeOH vehicle controls showed statistically insignificant toxicity.

Compared to CuCl₂, the copper complexes show less toxicity, suggesting that the ligands are coordinating to the copper and helping to reduce its toxicity through day 2 of high dosage. All of the copper complexes produce some toxicity in the zebra fish for all experimental concentrations, but compound 5 does not increase in toxicity over time as much as the other complexes. This suggests further testing and modification of compound 5 could lead to a safe anti-influenza A therapeutic.

*Ames Testing*

The mutagenicity of the copper complexes was tested using the Ames test. Table 6.3 shows the percent reversion out of 48 wells of three complexes. They were tested for mutagenicity against *S. Typhimurium* TA100, which strain of bacteria allows a test for mutagenicity caused by base-pair substitution and oxidative damage. The percent of revertant wells (reversion rate) was compared against the negative control and found to be statistically insignificant (p > 0.01). The positive control (NaN₃) had an average 91.7% reversion rate compared to the negative control’s average rate of 43.8% (p < 0.0001).

Complexes 1, 3, and 4 did not show significant rate of reversion at any tested concentration
compared to NaN3. The copper complexes showed approximately the same reversion rates as the negative control after 6 days. Therefore, they do not cause mutagenicity due to base-pair substitution or oxidative damage.

\textit{Resistance Testing and Sequence}

Because the putative target for the metal complexes, the His37 quadruplex, is highly conserved in nature and functionally critical for vRNP uncoating, we explored the propensity for virus resistance formation with passaging in MDCK cell cultures. Because the incubation had to be done in DMEM, which is known to inhibit complex efficacy, we used higher concentrations of complexes for the incubations such that the efficacy of block was projected to be \(~50\%\), thus creating a concentration where mutation could occur. Ten passages (\~5 weeks) of incubated virus in DMEM dosed with increasing metal complex concentrations (ranging from 50 to 100 \(\mu\)M) was chosen as a rigorous test. Resistance would be identifiable by an increase in miniplaque EC\(_{50}\) after passaging relative to the original value. As shown in Table 6.4, the new EC\(_{50}\) (column 3) is comparable to the original EC\(_{50}\) (column 2). Because none of the copper complexes significantly increased the EC\(_{50}\) after 5 weeks of incubation, we conclude that resistance is slow to develop. This contrasts with rapid resistance development when passaging with AMT.

The vRNA M segment was extracted from the passaged virus exposed to 3, sequenced and compared to A/CA/07/2009 using a reverse-BLAST mechanism. The only base mutation discovered was G749A, which translates to the amino acid mutation G16E. This amino acid is positioned in the region of the channel entry that is outside of the membrane and unlikely to influence channel permeation. According to the results in the above table, this mutation did not confer resistance to this compound. We consider the occasionally observed natural M2 mutant
G34E to be likely to escape block by these complexes. Although we did not see resistance develop in our assays, a more direct assessment of the G34E site mutation using electrophysiology might be instructive about resistance potential for these compounds in future studies.

**MD Simulations**

Constant force steered molecular dynamics (MD) simulations were carried out to explore the steric limitations on metal complex exit from the M2 transmembrane domain AMT binding site. A 2.34 nN force was used to pull the complexes pass the 30 Å threshold and beyond the Val27 cluster within 10 ps. The 2.34 nN force gave a sufficient spread in leaving times to allow assessment of the ease of unbinding relative to AMT. For these simulations, the force was applied to the center-of-mass of the complex. Example trajectories for AMT (green) and 4 (yellow) are shown in Figure 6.5. The starting configurations (left) had the adamantyl groups of AMT and 4 superimposed with the copper atom of 4 oriented down, close to H37. This binding configuration was used for all of the metal complexes. V27 and H37 are shown as reference points along the channel.

Table 6.5 shows the average time to leave from five independent simulations (identical starting configurations, but randomly assigned atomic velocities) for each complex to pass the 30 Å threshold. All metal complexes took longer to leave the channel than AMT. AMT exited the channel in 2.77 ps. Complex 4 interacts with the V27 side chain and was the slowest compound to leave the channel, with its leaving time at 7.05 ps. By 4.4 and 6.75 ps, some distortion is seen on the protein subunit as 4 is pulled further out of the channel.
Conclusion

The copper complexes are relatively non-toxic in zebrafish embryos compared to CuCl₂ over a 5-day period. Also, they are efficacious in a 3-day assay (but with limitations due to serum protein binding and amino acid interference), are non-mutagenic compared to sodium azide, are slower to leave the M2 binding site compared to AMT, and, also compared to AMT, are not prone to resistance development. *In vivo* they would face competition with binding to other proteins and the therapeutic window is small. However, complexation of copper could be pharmacologically beneficial.

Further testing of these copper complexes should include isothermal titration calorimetry (ITC) experiments with influenza A M2 channel to obtain binding energies, two-electrode voltage clamp (TEVC) experiments to obtain rate constants of binding to M2 and testing in an animal model that more accurately represents the effects of the copper complexes on humans.

Contribution to Publication

First author, managed lab group, consulted on MD simulations, helped with data analysis.
Figure 6.1: Structures of Copper Complexes. The functional groups iminodiacetate or iminodiacetamide extended via the amine to either AMT or CO.
Figure 6.2: Solvated DMPC-2KQT System.
Figure 6.3: CPE Assay Results. Showing protective effect of 1 against A/WS/33 (M2S31N) infection of MDCK cells using dilute medium (50% DMEM, 50% SEM). MOI 0.6; 48-h incubation.
Figure 6.4: Zebrafish Toxicity of Copper Complexes. CuCl$_2$ (yellow), 1 (red), 2 (blue), 3 (green), 4 (magenta), 5 (black).
Figure 6.5: Exit Trajectories of AMT and Compound 4 Leaving M2 Channel. Three of the four M2TMD monomers from the simulation of 4 are displayed for reference. Valine 27 and Histidine 37 side chains are shown in grey, 4 is yellow and AMT is green. Both complexes are experiencing the same 2.34 nN pulling force.
Figure 6.6: Bovine Serum Albumin Complex (RCSB 3V03). Histidine side chains are colored yellow.
Table 6.1: Scoring Indicators Observed Daily for 5 Days. Five fish per group in each of two wells.

<table>
<thead>
<tr>
<th>Zebrafish scoring indicators</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Morbidity points</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Equilibrium</td>
<td>Upright position</td>
<td>Lying on side</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Response</td>
<td>Quick escape</td>
<td>Sluggish escape</td>
<td>No escape</td>
<td>NA</td>
</tr>
<tr>
<td>Spine shape</td>
<td>Straight</td>
<td>Slightly curved</td>
<td>Strongly curved</td>
<td>NA</td>
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<tr>
<td>Edema</td>
<td>None</td>
<td>1 place and minor</td>
<td>2 places or major</td>
<td>2 places and major</td>
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<tr>
<td>Death</td>
<td>=10</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Table 6.2: Binding to Bovine Serum Albumin Results. The value of $n$ was not well-constrained and was therefore fixed during the curve fit. $K_d$ values for representative compounds, 1, 3, and 5.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$K_d$ (µM)</th>
<th>Sites ($n$)</th>
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</thead>
<tbody>
<tr>
<td>CuCl$_2$</td>
<td>59.1</td>
<td>15</td>
</tr>
<tr>
<td>1</td>
<td>128.7</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>179.5</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>380</td>
<td>20$^+$</td>
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Table 6.3: Ames Mutagenicity Assay Results. Percent of reversion out of 48 wells for compounds 1, 3, and 4 for concentrations between 15 and 500 µM. Water with no complex is 0.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Complex</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
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<tr>
<td>500</td>
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<td>250</td>
<td>42</td>
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<td>125</td>
<td>31</td>
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<td>62.5</td>
<td>31</td>
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<td>31.25</td>
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<td>15.63</td>
<td>56</td>
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<tr>
<td>0</td>
<td>35</td>
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</table>
Table 6.4: Miniplaque Passaging Results. Before (original) and after 10 passages of virus in MDCK cells. DMEM was used for passage incubations and SEM for the miniplaque assays.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Original A/CA/09 (µM)</th>
<th>10 passages with complex (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.9 ± 1.2</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>4.9 ± 0.8</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>0.7 ± 0.1</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>11.6 ± 1.1</td>
<td>3.9 ± 6.8</td>
</tr>
<tr>
<td>5</td>
<td>8.2 ± 2.0</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>4.4 ± 0.6</td>
<td>2.9 ± 0.3</td>
</tr>
</tbody>
</table>
Table 6.5: SMD Results. Average time to leave (±standard deviation) the channel with a 2.34 nN force applied to the compound’s center-of-mass.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Average time to leave (ps)</th>
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</thead>
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<tr>
<td>1</td>
<td>4.60 ± 1.14</td>
</tr>
<tr>
<td>2</td>
<td>6.85 ± 1.01</td>
</tr>
<tr>
<td>3</td>
<td>4.67 ± 1.07</td>
</tr>
<tr>
<td>4</td>
<td>7.05 ± 0.53</td>
</tr>
<tr>
<td>5</td>
<td>3.75 ± 0.89</td>
</tr>
<tr>
<td>6</td>
<td>4.00 ± 1.06</td>
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<tr>
<td>AMT</td>
<td>2.77 ± 0.26</td>
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Table 6.6: CHARMM Copper Parameters.

<table>
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<th>$K_b$ (kcal/mol/Å$^2$)</th>
<th>$B_o$ (Å)</th>
<th>Angle</th>
<th>$K_b$ (kcal/mole/rad$^2$)</th>
<th>$\theta_o$ (degrees)</th>
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<td>123</td>
<td></td>
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<tr>
<td>CT$_2$-NH$_3$-CU</td>
<td>96.150</td>
<td>128.05</td>
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<tr>
<td>H-NPH-CU</td>
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<tr>
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<td>NPH-CU-NPH</td>
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<td>Dihedral</td>
<td>$\Phi$ (kcal/mole/rad$^2$)</td>
<td>Multiplicity</td>
<td>Delta (degrees)</td>
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<tr>
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<tr>
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<td>-58.6</td>
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<tr>
<td>H-NPH-CU-NH$_3$</td>
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<td>157.9</td>
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<tr>
<td>NPH-CU-NPH-H</td>
<td>20</td>
<td>2</td>
<td>161.2</td>
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<tr>
<td>H-NPH-CU-NH$_3$</td>
<td>20</td>
<td>2</td>
<td>157.9</td>
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<tr>
<td>C$_2$-NPH-CU-NPH</td>
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<td>2</td>
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<td>90.20</td>
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<tr>
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<tr>
<td>NPH-CU-NH$_3$-H</td>
<td>20</td>
<td>2</td>
<td>161.2</td>
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</table>
Abstract

Cu$^{2+}$ is known to bind in the influenza virus His37 cluster in the homotetrameric M2 proton channel and block the proton current needed for uncoating. It’s cytotoxicity and zebrafish-embryo toxicity are reduced by iminodiacetate complexation to amantadine and cyclooctylamine, and the complexes block the ubiquitous amantadine-insensitive M2 S31N variant in voltage-clamped oocytes, without resistance formation in vitro. The current block has fast and slow phases in contrast to the single phase found for amantadine block of WT M2. Here we evaluate the mechanism of block, addressing whether there is a reasonable binding configuration for each complex that would allow the Cu(II) to covalently bind to one or more of the His37 imidazoles. Isothermal titration calorimetry (ITC) for dissolved imidazole binding to the complexes indicates that a single imidazole binds effectively to the complexed Cu(II). Structural optimization using density functional theory (DFT) reveals that the complexes fit inside the channel structure and project the Cu(II) towards the His37 cluster allowing one imidazole to form a covalent bond. The current traces were fitted to parametrized differential equations. The energetics of the entry and covalent binding steps implied by the rate constants suggest that the second slow step in the current block is the covalent bond formation. Electrophysiology and DFT studies also show that the complexes block the G34E amantadine-resistant mutant in spite of some crowding in the binding site by the glutamates. Thus, the metal complexes overcome resistance formation by complexation of the Cu(II) to an imidazole in the obligate His37 cluster.
Introduction

The influenza A M2 channel is responsible for the acidification of the influenza virus interior, which leads to the separation of ribonuclear proteins from the virus, at which point replication begins. Protons are transported to the viral interior through the protonation of imidazole nitrogens in the His37 cluster (Figure 7.1). Amantadine and rimantadine block the M2 WT variant but they do not block the ubiquitous M2 S31N variant, nor several other naturally occurring variants including L26F, V27A, A30T, and G34E, and are no longer FDA-approved as influenza A anti-viral drugs. Numerous studies have been carried out in search of organic compounds to block these mutants, but none has been shown to be universally effective. The His37 cluster was previously shown to be a viable M2 target using copper complexes (7.1, 7.2) based on the knowledge that Cu$^{2+}$ binds with histidine (7.3, 7.4). The His37 cluster is an interesting target for an antiviral because it is almost perfectly conserved in nature, probably because of its key role in selecting protons for transport into virions after endocytosis. Previous work using the electrophysiology two-electrode voltage method (TEVC) showed that copper cyclooctylamine-iminodiacetate (Cu(CO-IDA)) and copper amantadine-iminodiacetate (Cu(AMT-IDA)) block the M2 S31N variant at low therapeutic concentrations similar to AMT block in M2 WT and in a two-phase manner. A fast phase was followed by a secondary slow phase block. The interaction of these copper complexes with M2 was shown to be strong by lack of significant washout of the copper complexes in drug-free perfusate.

This work also showed, by site-directed mutagenesis of the H37 to A37 (7.1), evidence that the copper complexes interact with the His37 cluster. The mutagenesis resulted in elimination of the secondary slow phase, leaving the initial fast phase of block followed by complete washout in drug-free perfusate.
In vitro, Cu(CO-IDA) was potent at sub-micromolar concentrations against A/Calif/07/2009 H1N1 and had low cytotoxicity. In an evaluation of M2 resistance development against these copper complexes, 10 passages were insufficient for resistance development in contrast to resistance to amantadine that develops after a few passages (7.5). However, there is one of the naturally occurring mutations, G34E, that seems, because of its proximity to the binding site and added bulk, to have the potential could to interfere with binding of the copper complexes. As we simulated the bound structure, it was apparent that the bulky, hydrophobic adamantyl or cyclooctyl group from the complex must be positioned in close contact with the glutamate side chain cluster in this variant. Steric hindrance and hydrophilicity presented by the glutamates would seem likely to disrupt copper complex binding. To explore why this mutation did not appear spontaneously during virus passaging, we thought it would be prudent to test the copper complexes in the M2 S31N-G34E variant using TEVC, and to explore possible copper complex binding configurations with DFT.

Evidence of low concentration potency, low cytotoxicity, and no resistance development suggest that these copper complexes might be viable anti-influenza A therapeutics. This research explores the mechanism by which Cu(CO-IDA) and Cu(AMT-IDA) block the M2 channel. We explore the binding kinetics and configurations of the copper complexes with M2 S31N. A global fit of electrophysiology data allowed the determination of rate constants, providing insight into the association, covalent binding, and dissociation processes. Isothermal titration calorimetry (ITC) is used to evaluate the number, enthalpy, and dissociation constant for the binding of imidazole to each of the copper complexes. Quantum chemical models help elucidate the configuration of the copper complexes in the covalently bound state.
Methods and Materials

A/Udorn/72 H3N2 M2 S31N-G34E mRNA Synthesis

A DNA fragment containing a G to E mutation to create the G34E allele was obtained from Twist Biosciences (San Francisco, CA). The fragment was digested using BamHI and HindIII restriction nucleases and cloned into A/Udorn/72 H3N2 S31N plasmid digested with the same restriction nuclease to create the A/Udorn/72 H3N2 S31N-G34E plasmid. The plasmid was transformed into chemically competent E. coli by standard methods. The plasmid was harvested using the Zymo Miniprep Kit (Zymo Research, Irvine, CA). To confirm that no mutations were introduced, the M2 DNA segment was PCR amplified and Sanger sequenced (Figure 7.2). Following confirmation, the PCR product was transcribed using the mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Thermo Fisher Inc., Waltham, MA) to prepare mRNA for oocyte injections.

Electrophysiology

Oocytes from Xenopus laevis (Ecocyte, Austin, TX) were maintained in ND-96++ (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 2.5 mM sodium pyruvate, 5 mM HEPES-NaOH, pH 7.4) solution at 17° C until injection of ~40 ng of A/Udorn/72 H3N2 M2 S31N or A/Udorn/72 H3N2 M2 S31N-G34E mRNA using a Nanoject II (Drummond Scientific, Broomall, PA). After injection, the oocytes were maintained at 4° C in ND96++ pH 7.4 until electrophysiological recording. 72 hrs after mRNA injection, whole-cell currents were recorded with a two-electrode voltage-clamp (TEVC) apparatus at Vm = -20 mV, room temperature, in Barth’s solution (0.3 mM NaNO3, 0.71 mM CaCl2, 0.82 mM MgSO4, 1.0 mM KCl, 2.4 mM NaHCO3, 88 mM NaCl, 15.0 mM HEPES, pH 7.5). Inward current was induced by perfusion with Barth’s pH 5.3 (15.0 mM MES instead of 15.0 mM HEPES). The oocytes were then
perfused by Barth’s pH 5.3 with CuCl₂, Cu(CO-IDA), or Cu(AMT-IDA) at concentrations 100 μM, 500 μM, or 1 mM. A washout was done using Barth’s pH 5.3 without drug. At the end of the experiment, Barth’s pH 7.4 was used to check the stability of the cell by observing the current return to baseline. Current traces were obtained from each of three oocytes for each of the concentrations.

Non-injected oocytes were also tested with the same acid perfusion protocols and concentrations of drug to assess the possible native acid-activated channel and CuCl₂, Cu(CO-IDA), or Cu(AMT-IDA)-induced leak current in the oocytes.

**Global Nonlinear Least Squares Curve Fit**

Considering the two-phase observation in the electrophysiology curves (i.e. fast and slow phase), analysis of CuCl₂, Cu(CO-IDA) or Cu(AMT-IDA) block was done using the two-site binding model shown here (Eq. 7.1):

\[
O \xrightleftharpoons{k_4}{k_1} C_1 \xrightleftharpoons{k_3}{k_2} C_2 
\]  

(7.1)

The open state, O, is the unblocked M2 channel where proton conductance is unimpeded. The partially closed state, C₁, the first binding site and fully closed state, and C₂, represents the second binding site and fully blocked M2 current where the copper complex binds to the His37 complex. The parameter, \( f \), in Eq. 7.2 and 7.3 is the fractional block. In the event the copper complex binds to the first or second site but partially blocks the current, this parameter takes that into account (7.5). To obtain the rate constants, \( k_1, k_2, k_3, \) and \( k_4 \), Eq. 7.2 and 7.3 were used to fit the electrophysiology data using the method found in (7.5). The derivation and variable definitions are shown in Appendix B.4.
To understand how strong the interaction is between M2 and the copper complexes, the effective equilibrium dissociation constant (dissociation from the second binding site to the open state, O, assuming the first binding site to be a small perturbation to the binding kinetics) was calculated using Eq. 7.4 (7.10):

\[
K_{\text{eff}} = \frac{k_3 k_4}{k_1 k_2}
\]

*(Isothermal Titration Calorimetry)*

Isothermal titration calorimetry (ITC) experiments were performed using a Nano-ITC low-volume calorimeter (TA Instruments, Layton, UT) equipped with reference and sample cells of 170 μL. All titration runs were carried out with a 50 μL injection syringe at 25 °C and a stirring rate of 250 rpm. Both the syringe and well solutions were adjusted to the desired pH of 6, 7 or 8 using HCl or NaOH. One or two μL volumes of imidazole solution were injected into copper complex solutions (Cu(Amt-IDA) and Cu(CO-IDA)). Resulting enthalpies were obtained using an independent model fit on the NanoAnalyze software (TA Instruments, New Castle, DE). The number of experiments, N, for each complex, which range from 2 to 6, were averaged. \(K_d\) values were dependent on the slope of the titration curve and optimal fits were obtained using the NanoAnalyze software.
Cu(Amt-IDA)·5H2O (418.93 g/mol) and Cu(CO-IDA)·3H2O (358.88 g/mol) were synthesized according to previously published procedures (7.4). Solutions of imidazole were prepared by adding imidazole to 8.0 mL of Milli-Q water, adding 1.0 mL of 20 mM Tris, adjusting the pH by adding either 10.0 or 1.0 M NaOH or HCl, and adding water to reach 10.0 mL of solution. Solutions of complexes were made by adding 41.5 mg Cu(Amt-IDA) or 27.3 mg Cu(CO-IDA) to 8 mL of Milli-Q water, adding 1.0 mL of 20 mM Tris, and sonicating and/or heating, until the complex dissolved (20-60 minutes). The pH was then adjusted to the target pH with 10.0 M or 1.0 M HCl or NaOH, and water added to reach 10 mL. Concentrations of copper complexes were verified by measuring UV absorbances of the complexes and using the Beer-Lambert law and by ICP analysis. The molar extinction coefficient for Cu(Amt-IDA) is 5300 M⁻¹cm⁻¹ and for Cu(CO-IDA) is 3700 M⁻¹cm⁻¹ at 252 nm (7.6).

Quantum Chemical Modeling of Cu(Amt-IDA) and Cu(CO-IDA)

As a quantum-chemical model for M2 channel binding, DFT calculations were used to examine the coordination enthalpy of pH 7 neutral imidazole to Cu(CO-IDA). All calculations were performed using Terachem v1.93P (PetaChem, LLC, Los Altos Hills, CA) using the ωB97X-D3 functional (7.7). Calculations were carried out with the default Terachem COSMO continuum solvent model (7.8). All structures were optimized to stationary points and confirmed as minima by vibrational frequency analysis using ωB97X-D3/6-31+G** with the LANL2DZ used for copper. A subsequent SCF energy evaluation was performed with ωB97X-D3/6-31+G(2d,p) with LANL2DZ used for copper so that final enthalpy values, which contain the DG of solvation estimate, are ωB97X-D3/6-31+G(2d,p)[LANL2DZ Cu]// ωB97X-D3/6-31+G**[LANL2DZ Cu]. The enthalpy was calculated using Eq. 7.5.
Quantum Chemical Modeling – Copper Complexes Binding His37 in S31N and S31N-G34E M2

Geometry optimization of the copper complexes binding to one histidine in the His37 cluster of the M2 S31N or S31N-G34E structure were done using Gaussian 16 (Gaussian, Inc., Wallingford, CT). The 2KQT NMR structure was used for the S31N or S31N-G34E model. The M2-copper complex system was optimized in gas phase. ONIOM (7.9) was set up with the high level QM region including all four histidines in the His37 cluster, Cu(AMT-IDA) or Cu(CO-IDA), and all four glycine or glutamate sidechains at positions 34. The low level region included all other regions of the M2 channel. The ωB97x-D3 DFT method and 6-31G(d,p) basis set was used to optimize the QM region, except for the copper atom where the LANL2DZ basis was applied. The PM6 semi-empirical method was used to optimize the low-level regions.

Results and Discussion

Electrophysiology – Global Nonlinear Least Squares Fit

Binding kinetics were determined from electrophysiology data for M2 S31N perfused with various concentrations of Cu2+(aq) (Figure 7.3). The normalized current traces are point-wise averages from three different *Xenopus* laevis cells with leak current subtracted from each trace. The simultaneous optimization in the global fit of the parameters from the block and washout electrophysiology data at varying concentrations constrained the parameters sufficiently to extract the rate constants (k1, k2, k3, and k4) and the fractional block, f, of the first binding site. The global fit using a two-site binding model provides more insight into the M2 current block by
the copper complexes previously reported (7.3) with the initial fast phase block and washout represented by $k_1$ and $k_4$, and the secondary slow block and washout represented by $k_2$ and $k_3$.

At 1.0 mM Cu$^{2+}$(aq) the M2 S31N current is almost completely blocked in 5 minutes (Figure 7.3a). No significant washout was observed for Cu$^{2+}$(aq) after 4 minutes (Figure 7.3b). The rate constants obtained in the global fit from the blocking and washout traces are 754 M$^{-1}$ s$^{-1}$ ($k_1$) and 0.0824 s$^{-1}$ ($k_2$) for the association with the first and second binding sites respectively, and 3.6x10$^{-4}$ s$^{-1}$ ($k_3$) and 0.97 s$^{-1}$ ($k_4$) for dissociation from the second and first binding sites respectively (Table 7.1). The corresponding effective equilibrium constant for the dissociation reaction, $K_{d_{\text{eff}}}$, of Cu$^{2+}$(aq) from M2 S31N is 56 nM.

Cu(AMT-IDA) blocks M2 S31N (Figure 7.4) with similar binding kinetics as Cu$^{2+}$(aq). Almost complete block was achieved for 0.50 and 1.0 mM by 60 minutes (Figure 7.4a) and no significant washout was observed by 5 minutes (Figure 7.4b). However, there are some significant differences with $k_1$ lower (594 vs 754 M$^{-1}$s$^{-1}$), $k_2$ lower (0.032 vs 0.0824 s$^{-1}$), $k_3$ higher (1.5x10$^{-5}$ vs 3.6x10$^{-6}$ s$^{-1}$), and $k_4$ lower (0.17 vs 0.97 s$^{-1}$) corresponding to a larger $K_{d_{\text{eff}}}$ equal to 134 nM.

Cu(CO-IDA) also had similar block and binding kinetics (Figure 7.5) as Cu$^{2+}$(aq) and Cu(AMT-IDA) with nearly complete block at 0.50 and 1.0 mM by 25 minutes (Figure 7.5a) and no significant washout after 5 minutes (Figure 7.5b). There is a large difference in $k_1$ from Cu$^{2+}$(aq) (2876 vs 754 M$^{-1}$s$^{-1}$), $k_2$ is lower (0.029 vs 0.0824 s$^{-1}$), $k_3$ is higher (2.9x10$^{-5}$ vs 0.36x10$^{-5}$ s$^{-1}$), and $k_4$ is lower (0.29 vs 0.97 s$^{-1}$), corresponding to a $K_{d_{\text{eff}}}$ equal to 101 nM, which is smaller than the $K_d$ for Cu(AMT-IDA) but larger than $K_d$ for Cu$^{2+}$(aq).

Cu$^{2+}$(aq) binds to M2 stronger the copper complexes. Perhaps Cu$^{2+}$(aq) can bind more than one imidazole. Cu$^{2+}$(aq) has four equatorial and two axial coordination locations, allowing
the tetrahedral binding configuration seen with Cu\(^{2+}\)(aq). If Cu\(^{2+}\)(aq) can bind more than one imidazole, this would explain the lower K\(_{\text{deff}}\) than that of Cu(AMT-IDA) and Cu(CO-IDA) (2- and 2.5-fold higher respectively). Because Cu\(^{2+}\)(aq) is smaller it can more easily reach the His37 imidazoles than the copper complexes as indicated by k\(_2\). Cu\(^{2+}\)(aq) and Cu(AMT-IDA) have similar entry rates into the M2 channel, though Cu(AMT-IDA) is slower perhaps due to the large adamantyl group and steric interactions with the sidechains. Cu(CO-IDA), however, is much faster than Cu\(^{2+}\)(aq) and Cu(AMT-IDA). It may be that Cu\(^{2+}\)(aq), due its charge, has interactions with other sidechains than slow it down even though it is an individual small atom, and Cu(AMT-IDA) is slower due to steric hindrance but would not have those charge interactions like Cu\(^{2+}\)(aq) because the copper complexes are neutral charge. However, the cyclooctyl group is more flexible than the adamantyl group, so entry into M2 is not as sterically hindered and no charge interactions with other sidechains would slow its entry. Overall, Cu(AMT-IDA) and Cu(CO-IDA) have similar binding strength Cu\(^{2+}\)(aq) to M2, which makes the copper complexes viable M2 blockers.

Electrophysiology – S31N-G34E

The S31N-G34E is concerning because the glutamates could, potentially, create steric hindrance preventing the copper complexes from reaching the His37 cluster. Blocking and washout traces for M2 S31N-G34E exposed to 100 \(\mu\)M of Cu\(^{2+}\)(aq), Cu(AMT-IDA), or Cu(CO-IDA) were obtained using TEVC (Figure 7.6). Cu\(^{2+}\)(aq) (Figure 7.6a) shows similar binding in the S31N-G34E M2 variant as in the S31N M2 (7.1). The initial fast phase block appears to reach a higher final block and is apparently slower than Cu\(^{2+}\)(aq) in the S31N M2. By the end of the slow phase block, the total block is 95% after 5 minutes of perfusion with Cu\(^{2+}\)(aq). The data in Figure 7.6a shows that Cu\(^{2+}\)(aq) does not have a significant washout indicated by the
slow return of inward current when perfusion is switched to Barth’s pH 5.3 minus Cu^{2+}(aq) (red arrow).

Cu(CO-IDA) blocks 86% of the M2 current after 12 minutes of perfusion (Figure 7.6b). The time to block M2 is more than double that of Cu^{2+}(aq). Cu(CO-IDA) shows more washout compared to Cu^{2+}(aq) (Figure 7.6a red arrow), though it appears more exaggerated due to the timescale being twice as long. Approximately 7% of the M2 current returns during the 3 minutes of perfusion with Barth’s pH 5.3 minus Cu(CO-IDA).

Cu(AMT-IDA) blocks 87% of the M2 current after 10 minutes of perfusion (Figure 7.6c). The time to block is also double that of Cu^{2+}(aq). The washout (Figure 7.6c red arrow) appears to have a fast phase, but levels off with approximately 3% return of the M2 current.

Figure 7.6d shows block by 100 μM AMT. Maximal block by AMT is reached quickly, with a total block of 30%. This result is similar to previous results with AMT in the S31N M2 variant (7.5). The washout (Figure 7.6d red arrow) is nearly complete after 3 minutes of perfusion with Barth’s pH 5.3 minus AMT.

These results show that Cu(CO-IDA) and Cu(AMT-IDA) can still block M2 current with the glutamates replacing the glycines. However, the kinetic analysis to obtain rate constants was not done. The data were not appropriate for quantitative analysis due to large unstirred layer effects appearing in the traces. But, the data shows qualitatively that the S31N-G34E AMT-resistant M2 construct is blockable. Other AMT-resistant constructs were not tested, but the S31N-G34E M2 construct is one of the more extreme examples tested, and this perhaps suggests that the other AMT-resistant M2 constructs are blockable. In the event the M2 S31N-G34E variant becomes ubiquitous in human circulating influenza A strains, these copper complexes are expected to function as M2 blockers.
Isothermal Titration Calorimetry

In order to evaluate the binding interaction of the copper complexes with the His37 cluster observed in electrophysiology, ITC measurements were done. Enthalpy values and dissociation concentrations are given in Table 7.2 and the fitted data traces are shown in Figures 7.13-7.15. The enthalpy values obtained at various pH levels for Cu(Amt-IDA) are similar to each other, with values of pH 6 = -5.5 ± 1.1 kcal/mol, pH 7 = -6.5 ± 1.1 kcal/mol, and pH 8 = -5.8 ± 0.3 kcal/mol. The enthalpy values of the Cu(CO-IDA) become more negative as pH increases, with values of -4.8 ± 0.5 kcal/mol (pH 6), -6.6 ± 0.4 kcal/mol (pH 7), and -8.4 ± 0.4 kcal/mol (pH 8). Both complexes are comparable at pH 6 and 7, the only significant difference between these two complexes appears at pH of 8, with the Cu(CO-IDA) having the most negative enthalpy of binding. Both copper complexes have Kd values of the same magnitude (10^-4) suggesting strong binding for both copper complexes to imidazole.

The ITC results show that Cu(AMT-IDA) and Cu(CO-IDA) bind imidazole comparable to other results (Arthur E. Martell 1971). However, there is an interesting difference in binding illuminated when the pH varies. At pH 6 and 7, Cu(AMT-IDA) and Cu(CO-IDA) have approximately the same enthalpy of binding with imidazole (-5.5 vs -4.8 and -6.5 vs -6.6). Yet, at pH 8 there is a difference of 2.6 kcal/mol between the copper complexes binding imidazole.

Quantum Chemical Modeling of Imidazole Coordination to Copper Complexes

Table 7.3 reports the ωB97X-D calculated imidazole coordination energies to Cu(CO-IDA)(OH2)2 and Cu(AMT-IDA)(OH2)2. This model represents the enthalpy estimate (with inclusion of solvation free energy) of coordination at pH 7 where the imidazole is de-protonated. The calculated ΔH value (at 298 K at 1 atm (no concentration correction was applied)) for Cu(CO-IDA) is -9.7 kcal/mol (Table 7.3), which is reasonably close to the measured enthalpy of
-6.6 kcal/mol (Table 7.2), especially considering only two explicit waters were used in the
calculation. The calculated Cu-imidazole nitrogen bond length is 1.99 Å, which is close to the
expected value of 2 Å (7.6,7.10,7.11).

The calculated imidazole coordination enthalpy for Cu(AMT-IDa) is -6.9 kcal/mol (Table
7.3), and this value is extremely close to the measured enthalpy value of -6.5 kcal/mol (Table
7.2). The calculated Cu-imidazole nitrogen bond length is 2.04 Å. These DFT coordination
energies suggest that the ITC values are indeed measuring unprotonated imidazole for water
substitution at Cu in the equatorial position and in the plane of the pincer-type ligand.
Importantly, these calculations provide the basis for a mechanism of copper complexes
coordination to a single His37 imidazole in the M2 channel.

Quantum Chemical Model – Copper Complexes Binding His37 In M2 S31N and G34E

The geometry optimization of Cu(CO-IDA) in the M2 S31N variant (Figure 7.9a and b)
shows the nitrogen is bonded in the open equatorial position on the copper while the copper
remains complexed with the CO-IDA. The copper remained chelated to the complex while
forming a bond with the imidazole nitrogen. The bond length between the copper and imidazole
nitrogen is 1.99 Å. The Cu-N\textsubscript{imid} bond directionality appears to be in the copper’s only
remaining equatorial position as expected.

Figure 7.10a and b shows the geometry optimization result for Cu(CO-IDA) in the M2
S31N-G34E variant. The glutamates at position 34 do not prevent Cu(CO-IDA) from reaching a
position to bind with a His37 imidazole which agrees with the electrophysiology results that
Cu(CO-IDA) still blocked M2 S31N-G34E current.

Figure 7.11a and b shows the geometry optimization result for Cu(AMT-IDa) in the M2
S31N variant. The result is similar to Cu(CO-IDA), though the adamantyl is not as flexible as
the cyclooctyl but still fits down near the His37 cluster allowing the copper to bind to the imidazole nitrogen. The imidazole nitrogen binds in the open equatorial position of the copper while the copper remains complexed with the AMT-IDA. The Cu-$N_{\text{imid}}$ bond length is 2 Å. The geometry optimization of Cu(AMT-IDA) in the M2 S31N-G34E variant showed the same binding result (Figure 7.12a and b) easily fitting near the His37 cluster and the copper able to form a bond with the imidazole nitrogen.

The computational chemistry simulations provide insight into the ability of the copper complexes to fit in the M2 channel near the His37 cluster and deliver the copper to the imidazole nitrogen. This evidence provides an explanation for the M2 current block in the electrophysiology results. While the copper is bound to an imidazole, the nitrogen cannot be protonated which prevents protons from passing through M2. While it is believed that M2 activation occurs with the protonation of three imidazoles (7.12), perhaps copper covalently binding one of the imidazoles combined with the steric block by the CO- or AMT-IDA group prevents further protonation of the other three imidazoles, thus shutting down proton transport. Explicit proton transport, however, was not explored in this study and further simulations are required. Because copper binding geometry is typically tetrahedral, there are two open axial positions available for binding. Two imidazoles binding to the copper (one equatorial the second axial) was attempted in these simulations. However, no proper starting geometries were achievable without extreme rotation of the copper complex and distortion of the M2 backbone. If more than one imidazole can bind the copper, it appears the copper would need to move away from the CO- or AMT-IDA group.
Conclusion

A two-site mathematical model used in a global fit with the electrophysiology traces varying in copper complex concentration provided rate constants for Cu(CO-IDA) and Cu(AMT-IDA) in M2 S31N. The copper complexes get in to the His37 binding site at nearly the same rate as Cu$^{2+}$(aq) (k$_2$ rate constant). k$_3$ shows the copper complexes are slow to leave the His37 binding site, on the same order as Cu$^{2+}$(aq), and because k$_2$ << k$_3$ the copper complex interaction in the His37 binding site is very strong. The complexed copper has increased residence time in the M2 channel (Cu$^{2+}$(aq) k$_4$ vs copper complex k$_4$). The independent fit from ITC shows that the copper complexes are capable of binding only one imidazole, though the axial positions are open for binding, which is agreement with the quantum chemical model. The quantum chemical model of the copper complexes in the channel predicts a stable configuration where the complexed copper can bind to one imidazole in either the S31N or S31N-G34E M2 variant.

Contribution to Publication

First author, made the mRNA used in the electrophysiology experiments, designed the electrophysiology experiments and collected the data, derived the equation and wrote the Matlab program for the global fit, designed and performed the quantum chemical model calculations, all under the input and direction of Dr. Hill, Dr. Harrison, Dr. Ess, and Dr. Busath.
Figure 7.1: M2 S31N Structure.
Figure 7.2: Sanger Sequence S31N-G34E M2.
Figure 7.3: TEVC Global Fit of CuCl₂ in S31N M2. Solid line is the average of three electrophysiology traces tested at 100 μM, 500 μM, and 1 mM. The dashed line is the theoretical fit to the data. The left image is the washin data and fit. The right image is the washout data and fit.
Figure 7.4: TEVC Global Fit of Cu(AMT-IDA) in S31N M2. Solid line is the average of three electrophysiology traces tested at 100 μM, 500 μM, and 1 mM. The dashed line is the theoretical fit to the data. The left image is the washin data and fit. The right image is the washout data and fit.
Figure 7.5: TEVC Global Fit of Cu(CO-IDA) in S31N M2. Solid line is the average of three electrophysiology traces tested at 100 μM, 500 μM, and 1 mM. The dashed line is the theoretical fit to the data. The left image is the washin data and fit. The right image is the washout data and fit.
Figure 7.6: TEVC Results in S31N-G34E M2. Each complex was tested at 100 μM. a) CuCl$_2$  b) Cu(CO-IDA)  c) Cu(AMT-IDA)  d) AMT.
Figure 7.7: Cu(AMT-IDA) Quantum Chemical Model. Reaction with unprotonated Imidazole in implicit water solvent.

Figure 7.8: Cu(CO-IDA) Quantum Chemical Model. Reaction with unprotonated Imidazole implicit water solvent.
Figure 7.9: Quantum Chemical Geometry Optimization of Cu(CO-IDA) in M2 S31N-G34E. The simulation was done in gas phase. The ribbons represent the backbone of M2. 

a) Face-on view. The front and back ribbon is not displayed for easier visualization of the imidazole binding with the copper complex, but the histidine is still connected to the backbone as shown in the side view. 

b) Side view. Magenta used to highlight imidazole ring.
Figure 7.10: Quantum Chemical Geometry Optimization of Cu(CO-IDA) in M2 S31N-G34. The simulation was done in gas phase. The ribbons represent the backbone of M2. a) Face-on view. The front and back ribbon is not displayed for easier visualization of the imidazole binding with the copper complex, but the histidine is still connected to the backbone as shown in the side view. a) Face-on view b) Side view. Atom selections are the same as in Figure 7.9.
Figure 7.11: Quantum Chemical Geometry Optimization of Cu(AMT-IDA) in M2 S31N-G34E. The simulation was done in gas phase. The ribbons represent the backbone of M2. a) Face-on view. The front and back ribbon is not displayed for easier visualization of the imidazole binding with the copper complex, but the histidine is still connected to the backbone as shown in the side view. a) Face-on view b) Side view. Atom selections are the same as in Figure 7.9.
Figure 7.12: Quantum Chemical Geometry Optimization of Cu(AMT-IDA) in M2 S31N-G34. The simulation was done in gas phase. The ribbons represent the backbone of M2. a) Face-on view. The front and back ribbon is not displayed for easier visualization of the imidazole binding with the copper complex, but the histidine is still connected to the backbone as shown in the side view.  

a) Face-on view  
b) Side view. Atom selections are the same as in Figure 7.9.
Figure 7.13: CuCl₂ ITC Results at pH 8. Raw heat measurements (left). Area under the curve (AUC) fit of data (right).
Figure 7.14: Cu(AMT-IDA) ITC Results. Raw heat measurements (left). Area under the curve (AUC) fit of data (right). a) pH 8 b) pH 7 c) pH 6.
Figure 7.15: Cu(CO-IDA) ITC Results. Raw heat measurements (left). Area under the curve (AUC) fit of data (right). a) pH 8  b) pH 7  c) pH 6.
Table 7.1: Global Nonlinear Least Squares Fit Results. Association and dissociation rate constants for the first binding site ($k_1$ and $k_4$) and the second binding site ($k_2$ and $k_3$) from the global nonlinear least squares fit for CuCl$_2$, Cu(CO-IDA), and Cu(AMT-IDA) in M2 S31N.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_1$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$k_3$ (s$^{-1}$)</th>
<th>$k_4$ (s$^{-1}$)</th>
<th>Weighted Reduced $\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuCl$_2$</td>
<td>754 ± 5.1x10$^{-6}$</td>
<td>0.0824 ± 1.2x10$^{-4}$</td>
<td>3.6x10$^4$ ± 7.7x10$^{-7}$</td>
<td>0.97 ± 8.7x10$^{-4}$</td>
<td>0.00174</td>
</tr>
<tr>
<td>Cu(CO-IDA)</td>
<td>2876 ± 3.4x10$^{-6}$</td>
<td>0.029 ± 4.1x10$^{-4}$</td>
<td>2.9x10$^5$ ± 4.4x10$^{-8}$</td>
<td>0.29 ± 0.011</td>
<td>0.00192</td>
</tr>
<tr>
<td>Cu(AMT-IDA)</td>
<td>594 ± 3.4x10$^{-7}$</td>
<td>0.032 ± 2.9x10$^{-7}$</td>
<td>1.5x10$^5$ ± 1.2x10$^{-8}$</td>
<td>0.17 ± 0.014</td>
<td>0.00104</td>
</tr>
</tbody>
</table>

Table 7.2: ITC Results. The heat of complexation ($\Delta H$) and binding constants ($K_a$) for Cu(AMT-IDA) and Cu(CO-IDA) at various pHs from fit of ITC data. The number of experiments, N, for each complex at each pH is not shown but ranged from 2 to 6.

<table>
<thead>
<tr>
<th>Copper Complex</th>
<th>pH</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta H$ Standard Dev.</th>
<th>$K_a$</th>
<th>$K_a$ Standard Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM Cu(Amt-IDA)</td>
<td>8</td>
<td>-5.8</td>
<td>0.3</td>
<td>0.87x10$^{-4}$</td>
<td>3.7x10$^{-5}$</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>-6.5</td>
<td>1.1</td>
<td>2.4x10$^{-4}$</td>
<td>8.3x10$^{-5}$</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-5.5</td>
<td>1.1</td>
<td>1.5x10$^{-4}$</td>
<td>3.5x10$^{-5}$</td>
</tr>
<tr>
<td>1 mM Cu(CO-IDA)</td>
<td>8</td>
<td>-8.4</td>
<td>0.4</td>
<td>1.8x10$^{-4}$</td>
<td>2.4x10$^{-5}$</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>-6.6</td>
<td>0.4</td>
<td>1.1x10$^{-4}$</td>
<td>1.2x10$^{-5}$</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-4.8</td>
<td>0.5</td>
<td>2.8x10$^{-4}$</td>
<td>1.9x10$^{-5}$</td>
</tr>
</tbody>
</table>
Table 7.3: Quantum Chemical Model Results. Copper Complexes Binding Imidazole N₆ in Implicit Water Solvent. Calculated enthalpy of reaction (difference between heat of formation of the reactants and products) using the wb97x DFT method and 6-31G** basis set in Terachem.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enthalpy of Reaction (kcal/mol)</th>
<th>Cu-Imidazole N₆ Bond Length (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazole N₆ + Cu-(COIDA)·2H₂O → Imidazole N₆·Cu-COIDA·H₂O + H₂O</td>
<td>-9.73</td>
<td>1.99</td>
</tr>
<tr>
<td>Imidazole N₆ + Cu(AMT-IDA)·2H₂O → Imidazole N₆·Cu-AMT-IDA·H₂O + H₂O</td>
<td>-6.85</td>
<td>2.04</td>
</tr>
</tbody>
</table>
CHAPTER 8: Conclusion

Influenza A has been a persistent threat throughout human history and continues to be a threat even with the advancement of society and medicine. The virus is responsible for the deaths of millions and each year, in the U.S. alone, it is responsible for tens of thousands of deaths. Though we are more prepared now than ever for an influenza epidemic or global pandemic, we have seen very recently how quickly such a pandemic can cause serious problems, not just to our health but to the world’s economy. At the time of this writing (July 24, 2020), the SARS-COV-2 global pandemic and the disease it inflicts (Covid-19) caused the death of approximately 634,000 people worldwide over a period of 6 months. The world’s economy was completely shut down, causing, as of now, unpredictable amounts of damage. Influenza A has the same potential and perhaps worse considering its history having killed 50 to 100 million in 1918.

Scientists around the world are trying to create better preventative measures and treatments for influenza. Influenza has three main targets for therapeutics so far. The hemagglutinin is a fusion peptide responsible for viral entry into the cell. The neuraminidase is an enzyme that releases the virus from the cell during viral budding. Oseltamivir, zanamivir, and peramivir target the virus at this stage. An additional target is to inhibit viral mRNA synthesis, which is the mechanism of Baloxavir. The viral interior is acidified in the late stage endosome after the virus has entered the cell. The acidification of the viral interior is made possible by the proton selectivity of the His37 cluster in M2. The acidification separates the ribonuclear proteins from the virus and replication begins. Previous M2 blockers, amantadine (AMT) and rimantadine (RMT), were an effective therapeutic until a mutation at residue 31 from serine to asparagine (S31N) rendered the M2 insensitive to AMT and RMT. This mutation occurred in 2005. During
the swine flu pandemic in 2009, and to this day, there are no FDA approved M2 blockers as therapeutics.

In chapter 2, I addressed the change in M2-AMT or M2-RMT interactions in the mutant channel which had not been thoroughly studied. It was unclear whether the insensitivity is from weak binding or incomplete block. My approach to better understand the reduced AMT and RMT M2 block was to develop a more accurate mathematical model and, using a family of electrophysiology traces, use the model to extract rate constants from TEVC experiments for AMT and RMT in WT or M2 S31N and analyze the binding kinetics.

The results demonstrate that the mutation reduces binding for both, but when bound, AMT completely inhibits M2 proton conductance, while RMT block is significantly reduced. The significant increase of the off-rate is likely responsible for the AMT and RMT insensitivity in M2 S31N. The most intriguing result showed that RMT binds without block in the M2 S31N, while AMT weakly blocks all proton transport when in the binding site.

The other approach to understanding change in AMT or RMT interaction with M2 was through the use of molecular dynamics simulations. Simulations helped to show that the S31N mutation can induce conformational changes in AMT and RMT binding and that more water can get into and around AMT and RMT allowing for proton conductance. These observations have significant implications for novel M2 blockers and needed to be considered during novel M2 drug design and development.

The development of novel M2 blockers is important if we want to maximize our options for treatments during a global pandemic. Development of a drug that blocks the M2 S31N has continued since the mutation in 2005, but researchers have struggled to find one comparable to AMT or RMT block in M2 WT.
This dissertation includes the work done in collaboration with Dr. Antonios Kolocouris who was attempting to develop M2 blockers that would bind in the same region as AMT and RMT. I tested these compounds (AK for Antonios Kolocouris compounds) using TEVC and analysis of the data to determine their potential as M2 blockers. The AK compounds did not show much potential in TEVC as viable M2 blockers. The binding kinetics study of AMT and RMT and the experiments with the AK compounds provided motivation to develop novel M2 blockers with a focus on the His37 complex in M2 as a better therapeutic target using copper complexes since histidine can interact strongly with copper.

Due to the struggle to find a drug comparable to AMT or RMT that blocks M2 at the same binding site, my attention turned to the His37 cluster as a target for M2 blocker development. The His37 cluster is an appealing target for M2 blocker development because it is highly conserved in nature. Histidines are known to bind divalent copper and preliminary data showed that Cu(II) blocks the M2 S31N channel. This idea was extended by chelating Cu(II) with AMT-IDA or CO-IDA ligands tested as M2 S31N blockers.

The main focus of my PhD dissertation interest was to develop novel copper complexes that could block the M2 S31N variant and other (potentially all) M2 variants, which would be highly valuable as a therapeutic, especially during a pandemic. Also copper complexes that were low in cytotoxicity and required a low concentration to be effective M2 blockers, and thus a low dosage high effective drug. Most importantly, the focus was to show that the mechanism of block by the copper complexes is through binding the M2 histidines.

I confirmed that Cu$^{2+}$ blocks M2 S31N in transfected *Xenopus* oocytes using TEVC. The data shows that copper binds tightly in both the WT and S31N M2 constructs and has a two-phase block, an initial fast phase followed by a slow phase block. When we chelated Cu$^{2+}$ via IDA or
IDAA with derivatives of known M2 blockers, AMT or cyclooctylamine (CO), the complexes block M2 channels comparably to aquated Cu$^{2+}$. When the Cu$^{2+}$ is removed from the complex, the tight block is eliminated, suggesting Cu$^{2+}$ in the complex is interacting with the M2 channel. When the histidines are mutated to alanines (H37A), the slow phase block is eliminated, indicating that the copper complex binds to the histidines of the M2 channels during the slow phase which is required to fully block M2 proton conductance.

The copper complexes were also tested in cell culture against the complete virus. The copper complexes significantly reduced infection of MDCK cells with three influenza A virus (IAV) strains, two AMT-insensitive and one AMT-sensitive, and only required low concentrations to be effective (EC$_{50}$ range 0.7–11.6 $\mu$M). Complexation reduced the cytotoxicity of Cu$^{2+}$ approximately 10-fold and helped withstand decomposition in phosphate, sulfate, and nitrate containing buffers on the timescale of infection and experiments. The complexes were tested in vivo as well. The complexes are relatively non-toxic in zebrafish embryos compared to CuCl$_2$. Also, they are efficacious in a 3-day assay, are non-mutagenic compared to sodium azide, are comparable to AMT (in M2 WT) in leaving the M2 S31N binding site and are not prone to resistance development. During a 10-passage resistance experiment, M2 did not develop resistance against the copper complexes. One mutation I was concerned with was the glycine at residue 34 going to glutamates which could prevent the copper complexes from reaching the His37 complex. But this mutation was not observed in the resistance experiments. We did find, however, in vivo the complexes would face competition with binding to other proteins (especially ones with histidines like albumin) and the therapeutic window is small.

I also took a similar approach as with AMT and RMT to binding kinetics analysis. In this case though, a two-site mathematical model was used in a global fit with the electrophysiology
traces varying in copper complex concentration to obtain rate constants for Cu(CO-IDA) and Cu(AMT-IDA) in M2 S31N. The results showed that the copper complexes had very low equilibrium dissociation constants ($K_d$ range 56nM to 130 nM), which suggests that the second binding site is a very strong interaction between the copper complex and M2. I believe this is due to the covalent bond between a His37 imidazole nitrogen and the copper. This agrees with the interaction shown with the H37A mutation in TEVC. TEVC experiments were also done on the M2 S31N-G34E variant as a test of robustness. Even though the viral assay resistance experiments did not show the mutation occurring, I made the mutation in M2 to confirm that the glutamates would not prevent the copper complexes from reaching the His37 complex. The results showed no impediment to M2 block by the copper complexes.

To obtain further confirmation of the interaction between the copper complexes and histidine, isothermal titration calorimetry (ITC) was used. We simplified the model down to free imidazole in a buffered solution instead of using the full histidine with the assumption that the rest of the sidechain wouldn’t play a major role in a bond formation between the copper and imidazole nitrogen. The ITC result shows that the copper complexes are comparable to the binding energy of Cu$^{2+}$(aq) and are capable of binding in the equatorial position, although the axial positions are open for coordination. This would be expected in the M2 as well considering the lower mobility of the full histidines constrained by the M2 backbone.

The final approach to studying the mechanism of block by the copper complexes utilized quantum chemical models to calculate the enthalpy of binding to free unprotonated imidazole in implicit solvent, and geometry optimizations of the copper complexes binding to one histidine in both M2 S31N and S31N-G34E variants. The S31N-G34E was included to show the complexes can still fit down near the histidines and bind without steric hindrance. The model from the
quantum chemical calculations suggest that one imidazole binds to the chelated copper and agrees with the ITC results. The quantum chemical models of the copper complexes in the channel show that the complexed copper can bind to one imidazole in either the S31N or S31N-G34E M2 variant. Additional imidazoles binding to the copper axial positions would be infeasible.

The conclusion of this work is that the His37 complex in M2 is a viable target for M2 blocker development using copper complexes and that the copper complexes block M2 by binding with the His37 complex. The M2 H37A electrophysiology data shows that the slow phase strong block by the copper complexes is reduced to only the fast weak block, and when the copper is not included in the CO-IDA or AMT-IDA complex then the slow phase strong block is also gone. This evidence indicates that the copper interacts with the His37 complex to prevent proton conductance. There is a balance in the design of the copper complexes, though, where the copper needs to be complexed to reduce toxicity of free copper but leave one coordination site open for one of the His37 imidazoles to bind. The next step with these copper complexes would be testing in an animal model that better represents humans.
Appendix A.1: Amantadine Copper(II) Chloride Conjugate with Possible Implementation in Influenza Virus Inhibition


This appendix is a collaboration paper with Dr. Kolocouris including the testing and results of additional copper complexes.

Abstract

1-Adamantanamine hydrochloride (AdNH3+Cl-) is an antiviral drug that is used in the prophylactic or symptomatic treatment of influenza. However, a mutation makes influenza virus, amantadine (AdNH2) resistant. Thus, a new formulation, which combines Cu(II) and amantadine (AdNH2) in one formula {[AdNH3+][CuCl3]-}, (CA) was developed. CA was characterized by m.p, conductivity measurements, FT-IR and UV-Vis spectroscopies, and X-ray crystallography. To the best of our knowledge the crystal and molecular structure of CA is the first one of amantadine with Cu(II) ions. CA was tested against the A/WSN/33 WT (M2 N31) virus and its mutations M2 S31N, M2 V27A, M2 L26F, M2 A30T and M2 G34E respectively. CA inhibits the wild virus A/WSN/33 wt-M2 N31, and its mutations -M2 S31N, -M2 L26F and -M2 A30T. The inhibition mechanism involves the increased membrane permeability of the CA, than free Cu(II) ions with simultaneous lower toxicity. CA acts by blocking the proton current of A/WSN/33 M2 S31N in a similar manner to Cu(II) ions. In silico studies confirmed that CA can effectively block proton current mediated by M2 S31N virus replication. Specifically, [CuCl3]- blocks the primary gate of the His37 tetrad which is essential for channel conductance and selectivity. The in vitro toxicity of CA was examined against normal human fetal lung fibroblast (MRC-5) cells. No toxic effect was detected when the MRC-5 cells were treated by CA with concentrations up to 30 μM. The absence of micronucleus (MN) in MRC-5 cells treated CA (30
μM) confirms the in vitro non genotoxic behavior of the compound. Moreover, no in vivo toxic effect was detected on *Artemia salina* upon their treatment with CA at a concentration up to 150 μM.

Keywords: Matrix protein 1 (M1), {[AdNH₃⁺][CuCl₃]} (CA), micronucleus (MN)
Introduction

Seasonal influenza infections are a major worldwide health concern. Influenza A virus contains eight RNA segments, viral glycoproteins (haemagglutinin which facilitates viral entry, and neuraminidase which facilitates viral release), viral nucleoprotein, matrix protein (M1) and membrane protein (M2), the nonstructural protein NS1 and nuclear export protein. Amantadine, 1-adamantylamine or 1-aminoadamantane is an antiviral drug that is used in the prophylactic or symptomatic treatment of influenza A. Its mechanism of action involves blocking of the influenza virus A M2 proton channel (wild type), which prevents viral replication and budding. However, amantadine-resistant mutations in the virus strains has occurred mainly at position 31 (A/WSN/33 M2 S31N) in more than 95% of the currently circulating viruses which deactivates the drug. Copper ions, on the other hand, have been widely used as antiviral agents. Their antiviral activity is extended to a variety of viruses such as influenza virus, herpes simplex virus etc.

The antiviral activity of copper ions is enhanced by their conjugation with reducing agents such as ascorbic acid. Copper complexes which block A/WSN/33 M2 S31N, are stable in pH > 4 and have an effective concentration (EC$_{50}$) against A/Calif/07/2009 H1N1 of 0.7 ± 0.1 μM with a cytotoxic concentration (CC$_{50}$) of 147 μM compared to CuCl$_2$ having an EC$_{50}$ of 3.8 ± 0.9 μM and CC$_{50}$ of 19 μM [7]. Moreover, influenza A virus effectiveness is significantly decreased upon its exposure onto copper surfaces even 6 h after. Therefore, the conjugation of amantadine with copper(II) might be of pharmacological importance and thus it is worthy to be investigated.

In the course of our studies for the design and development of new Conjugates of Drugs with Metal ions (CoMeDs) and understanding the principles which rule them, we explore here the antiviral properties of a new formula experimentally and theoretically, which is derived by
the combination of divalent copper and amantadine (AdNH₂) in one entity combined in one compound divalent copper and amantadine (Scheme 1) in the form of \([\text{AdNH}_3^+ \text{][CuCl}_3^-]\) (CA). The in vitro toxicity and genotoxicity of CA against MRC-5 cells is also studied. Moreover, the in vivo toxic effect of CA was detected on *Artemia salina*.

Materials and Methods

All solvents used were of reagent grade, while copper(II) chloride and and amantadine (Sigma-Aldrich, Merck) was used without further purification. Melting point was measured in open tubes with a Stuart Scientific apparatus and it is uncorrected. IR spectra in the region of 4000–370 cm⁻¹ were obtained from KBr discs, with a Perkin-Elmer Spectrum GX FT-IR spectrophotometer. A UV-1600 PC series spectrophotometer of VWR was used to obtain electronic absorption spectra. Conductivity measurement was carried out at 293 K in DMSO solution with a WTF LF-91 conductivity meter.

*Synthesis and Crystallization of \([\text{AdNH}_3^+ \text{][CuCl}_3^-]\)*

0.5 mmol of \([\text{AdNH}_3^+ \text{][Cl}^-]\) (0.094 g) in 10 mL CH₂Cl₂ was stirred for 4 h in ice bath. A 10 mL MeCN solution of 0.5 mmol CuCl (0.067 g) was added to the previous one under continues stirring for 1 h. The solution was filtered off. Crystals suitable for X-ray diffraction analysis were grown from the mother solutions. \([\text{AdNH}_3^+ \text{][CuCl}_3^-]\) (CA): White crystal, melting point: 198– 200 C; Elemental analysis found: C: 37.50; H: 5.42; N: 4.47%; calculated for C₁₀H₁₈Cl₃CuN: C: 37.28; H: 5.63, N: 4.35%. IR (cm⁻¹), (KBr): 3166 m, 2909vs, 2851 m, 1735 m, 1564 m, 1488 s, 1473 m, 1450 m, 1374 s, 1312 s, 1201 m, 1090 m, 457 m. Molar Conductivity in DMSO: 34.1 (initially) - 35.7 (24 h) Ω⁻¹ cm² mol⁻¹.
X-ray Structure Determination

Intensity data for the crystals of \([\text{[AdNH}_3^+ \text{][CuCl}_3^-]}\) were collected on an Oxford Diffraction CCD instrument, using graphite monochromated MoKa radiation (k = 0.71073 Å). Cell parameters were determined by least-squares refinement of the diffraction data from 25 reflections. All data were corrected for Lorentz-polarization effects and absorption. The structures were solved with direct methods with SHELXS97 and refined by full-matrix least-squares procedures on F2 with SHELXL97. All non-hydrogen atoms were refined anisotropically, hydrogen atoms were located at calculated positions and refined via the “riding model” with isotropic thermal parameters fixed at 1.2 (1.3 for CH₃ groups) times the Ueq value of the appropriate carrier atom. C₁₀H₁₈Cl₃CuN, MW = 322.16, Monoclinic, space group C2/m, a = 11.110(2), b = 6.6896(11), c = 17.463(3) Å, β = 108.327(9), V = 1232.0(4) Å³, Z = 4, T = 295 K, ρ (calc) = 1.737 g cm⁻³, λ = 2.389 mm⁻¹, F(0 0 0) = 660. 13,305 reflections measured, 1300 unique (Rint = 0.034). The final R1 = 0.0380 (for 986 reflections with I > 2 s(I)) and wR(F2) = 0.0714 (all data) S = 1.00. Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-1506901. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

Virus Inhibition Assays

Madin-Darby canine kidney (MDCK) cells (Cat.no. RIE 328, Friedrich-Loeffler Institute, Riems, Germany) were propagated as monolayer in Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate and 2 mM L-glutamine. Amantadine-sensitive A/Udorn/72, amantadine-
resistant A/WSN/33 M2 WT (with an N31 in M2) and its variant with S31N amino acid substitution in the M2 ion channel were used in this study. Briefly for the generation of A/WSN/33 M2-S31N [38] the plasmid pHW187 M2-S31N was altered by site-directed mutagenesis PCR and afterwards used as part of a plasmid set for the recovery of A/ WSN/33 virus. A/WSN/33-variants were propagated on MDCK cells in serum-free EMEM supplemented with 2 mM L-glutamine, 2 lg/mL trypsin, and 0.1% sodium bicarbonate (test medium). Virus-containing supernatant was harvested after about 48 h of incubation at 37 °C when cytopathic effect became microscopically visible. Aliquots were stored at -80 °C until use. The M2 gene identity of all recombinant viruses was verified by sequencing.

CPE Assay

Cytotoxicity and CPE inhibition studies were performed on two day-old confluent monolayers of MDCK cells grown in 96-well plates as published. Cytotoxicity was analyzed 72 hrs after compound addition. In the CPE inhibition assay, 50 μL of a serial half-log dilution of compound in test medium (maximum concentration 100 mM) was added to the cell-containing wells while maintaining at a constant multiplicity of infection. Then, plates were incubated at 37 °C with 5% CO₂ for 48 hrs. Crystal violet staining and determination of the 50% cytotoxic (CC₅₀) and 50% inhibitory concentration (IC₅₀) was performed as described before. At least three independent assays were conducted.

Electrophysiology

Oocytes from *Xenopus laevis* (Ecocyte, Austin, TX) were maintained in ND-96++ solution at 17 °C until injection of ~40 ng of A/ Udorn/72 H3N2 M2 S31 (WT) or A/Udorn/72 H3N2 M2 S31N mRNA using a Nanoject II (Drummond Scientific, Broomall, PA). After injection, the
oocytes were maintained at 4°C in ND-96++ pH 7.4 until electrophysiological recording. 72 hrs after mRNA injection, whole-cell currents were recorded with a two-electrode voltage-clamp (TEVC) apparatus at Vm = -20 mV, room temperature, in Barth’s solution, pH 7.5. Inward current was induced by perfusion with Barth’s pH 5.3. Current block was induced by perfusion of Barth’s pH 5.3 100 μM drug solution. Percentage block of the original inward current by test compound was measured just before washout, which was done 10 min after drug exposure for each compound. Percentage washout, relative to original inward current, was measured after 5 min of perfusion with Barth’s pH 5.3 (no drug). To obtain rate constants for the relaxation to equilibrium, a single exponential plus a baseline was used to fit the first two minutes of drug perfusion or the first three minutes of drug washout. From the inverse of the washout time constant, the dissociation rate constant, k_{off}, was obtained. The inverse of the wash-in time constant was taken as the sum of the association and dissociation rates, [drug] x k_{on} + k’_{off}, where k0_{off} was not utilized here. Simultaneously, with the assumption of complete block of occupied channels, the ratio [drug] x k_{on}/([drug] x k_{on} + k’_{off}) was obtained from the baseline percent-block. KD was taken to be k_{off}/k_{on}.

**In Silico Studies**

Molecular docking was carried out using YASARA structure (v19.9.17) software package. Global docking was performed with AutoDock VINA within a simulation covering the whole protein space using the default parameters exposed by the YASARA interface. Docked conformations were clustered with the tolerance of 5 Å RMSD and ranked by the lowest binding energy. Prior to molecular docking simulations the CuCl_3^- and amantadine were geometrically optimized using the PM6 Hamiltonian.
*In Vitro Toxicity Study Against MRC-5 Cells*

Biological experiments were carried in dimethyl sulfoxide Dulbecco’s Modified Eagle’s Medium solutions (DMEM) DMSO/DMEM for CA and the amantadine. Stock solutions of the CA and amantadine (0.01 M) in DMSO were freshly prepared and diluted in cell culture medium to the desired concentrations (0.05–30 μM). Results are expressed in terms of IC$_{50}$ values, which is the concentration of the compound required to inhibit cell growth by 50% compared to control, after of 48 hrs incubation of CA or amantadine towards MRC-5 cells. The cell viability was determined by SRB assay as previously described. The cells were seeded in 96-well flat-bottom microplates at a density of 2000 cells/well. Absorbance was read in a 96-well plate reader at 540 nm.

*In Vitro Genotoxicity Study Using MN Assay*

MRC-5 cells were seeded (at a density of 2x10$^4$ cells/well) in glass coverslips which were afterwards placed in six-well plates, with 3 mL of cell culture medium and incubate for 24 hrs. MRC-5 cells exposed with CA and amantadine at the concentration of 30 μM, for a period of 48 hrs. The percent of micronucleus was determined by micronucleus assay as previously described. The number of micronucleated cells per 1000 cells was determined in each compound.

*In Vivo Toxicity Against Fish Models*

Brine shrimp assay was performed by a method previously described. An aliquot (0.1 mL) containing about 10 to 15 nauplii was introduced to each well of 24-well plate and CA or amantadine was added in each well. The final volume of each well is 1 mL with NaCl 0.9%. The brine shrimps were observed after of 24 hrs, using a stereoscope. Larvae are considered dead if they do not exhibit any internal or external movement in 10 s of observation. The solution for the
growth of *Artemia salina* contains NaCl solution. The larvae are hatched in NaCl 3.4% w/v solution. Afterwards they were placed in a well in NaCl 0.9% w/v solution and they incubated for a period of 24 hrs with the agent (CA here) in order to avoid the influence of this high concentration of NaCl towards the tested agent. During this period, the lower concentration of NaCl solution has no effect in the larvae survival. Consequently the toxic effect of the agent is allowed to be determined. This is why the period of 24 hrs incubation of *Artemia salina* has been chosen.

**Toxicity and Neurotoxicity Testing**

Acute toxicity was measured at three different doses, 30, 100 and 300 mg/kg body weight, administered intraperitoneally (i.p). In addition to assessing acute lethality, mice were observed using a modified Functional Observational Battery (FOB) at 30, 60 and 120 min after i.p. administration. This neurotoxicity battery measures 22 different parameters and gives an approximation of behavioral effects and neurotoxicological damage. Mice were monitored for 48 hrs after administration to assess lethality. Early studies of lethality of amantadine indicated an LD$_{50}$ near to 700 mg/kg in mice, but our studies suggested a lower value in CD1 mice. In general, the maximum neurological effect of drug administration was observed within a few minutes, and death occurred within 24 hrs. Thus the absorption and distribution of the drug after i.p. administration are rapid. The FOB is designed to detect gross functional deficits in young adult rodents exposed to chemicals. It consists in the study of rodent behavior after drug administration on three different environments: home cage, handheld and in the open field, (with or without manipulation), a large surface where the animal can be observed exploring a new milieu. Each item of the FOB is ranked according to the scale below, which shows, in
parentheses, the range of and, in bold, the normal value for the rank. In addition, the numbers of rearing, climbing, grooming, defecation and urination were also recorded.

Results and Discussion

Crystals of the \(\{[\text{AdNH}_3^+] [\text{CuCl}_3^-]\}\) (CA) were grown from the filtrate of the solution resulted from mixing a dichloromethane solution of \(\{[\text{AdNH}_3^+]\text{Cl}^-\}\) with an acetonitrile one of CuCl\(_2\) (Figure A.1.1). The ionic nature of CA in DMSO solution (10\(^{-3}\) M) was confirmed by the molar conductance (Km) value (34.1 (0 h) and 35.7 (24 h) \(\Omega^{-1}\) cm\(^2\) mol\(^{-1}\)). This values suggests 2 ionic species in DMSO solution (30–40 \(\Omega^{-1}\) cm\(^2\) mol\(^{-1}\)) which is expected by the dissociation of CA in to a cationic \([\text{AdNH}_3]^+\) specie and its counter anion \([\text{CuCl}_3]^-\). Moreover, no further dissociation to ionic species of the initial ions was detected, during a period of 24 hrs.

Crystal and Molecular Structure of \(\{[\text{AdNH}_3^+] [\text{CuCl}_3^-]\}\)

A molecular diagram of the compound and selected bond distances and angles are shown in Figure A.1.2. The structure of the compound consists of two residues, one cationic \([\text{AdNH}_3]^+\), and one \([\text{CuCl}_3]^-\) counter anion (Figure A.1.2). The anionic part constructs a 3D network. Three short Cu-Cl bond distances (Cu1-Cl = 2.2845(16), Cu1-Cl2 = 2.2347(17) and Cu1-Cl3 = 2.2555(17) Å) constitute the anionic part. CuCl bridges (Cu1-Cl1i = 2.3562(17) Å), along the plane forms a ribbon assembly which is extend to a 3D architecture by two longer CuCl bonding interactions (Cu1-Cl3i = 2.858(2), Cu1-Cl3ii = 2.858 (2) Å). Taken into consideration these secondary interactions the geometry around the copper ion is disorder octahedron (Cl1-Cu1-Cl2 = 170.97(6), Cl1-Cu1-Cl3 = 94.86(7), Cl2-Cu1-Cl3 = 93.92(7) and Cl1i -Cu1-Cl3 = 166.47(5)). A similar conformation is also observed in the mixed ligand ionic salt \(\{(\text{C}_{10}\text{H}_{18}\text{N})^2^- (\text{Cu}_2\text{Cl}_6)^2^- 2 (\text{C}_{12}\text{H}_{24}\text{O}_6)\}\) of amantadine (C\(_{10}\)H\(_{18}\)N) and the crown ether, 18-crown-6 (C\(_{12}\)H\(_{24}\)O\(_6\)). To the best
of our knowledge the crystal and molecular structure of $\{[\text{AdNH}_3^+]\text{[CuCl}_3^-]\}$ is the first one of amantadine with copper ions.

**Vibrational Spectroscopy**

The FT-IR spectrum of the $\{[\text{AdNH}_3^+]\text{Cl}^-\}$ (Figure A.1.3) is dominated by a broad vibration band at 3038 cm$^{-1}$ due to the stretching vibration mode of the $\text{–NH}_3^+$ group and by the anti-symmetric and symmetric stretching vibrations of the $\text{–CH}_2^-$ group at 2926 and 2854 cm$^{-1}$ respectively. The bands at 1600, 1494 and 1366 cm$^{-1}$ are attributed to the deformation of the $\text{–NH}_3^+$ group, while the bands at 1452 and 1314 cm$^{-1}$ are assigned to the $\text{–CH}_2^-$ group. These bands are observed at 3014 cm$^{-1}$ mstretched($\text{–NH}_3^+$), 2912 cm$^{-1}$ mantisym($\text{–CH}_2^-$ 2850 cm$^{-1}$ msym($\text{–CH}_2^-$), at 1629, 1384 cm$^{-1}$ mdef($\text{–NH}_3^+$) and at 1586, 1310 cm$^{-1}$ mdef($\text{–CH}_2^-$) in the FT-IR spectrum of CA (Figure A.1.4). Therefore, significant variations are occurred on going from $\{[\text{AdNH}_3^+]\text{Cl}^-\}$ to CA in all at bands, while the mstretched($\text{–NH}_3^+$) shows the higher shift (24 cm$^{-1}$) confirming the replacement of the anionic part (Cl$^-$) by the $\{\text{CuCl}_3^-\}$ one.

**Stability of CA in DMSO Solution**

CA is dissociated in DMSO solution to $[\text{AdNH}_3^+]$ and $[\text{CuCl}_2^-]$. The retention of the formulae of the counter ions in solution is confirmed by UV–Vis studies (Figure A.1.5). No changes were observed between the initial UV spectrum and the corresponding one after 48 hrs (Figure A.1.5).

**In Vitro Testing Against Influenza Virus A**

The cytopathic effect (CPE) inhibition assay was used to compare the antiviral activity of the compound against the amantadine-resistant virus A/WSN/33-M2 N31 and A/WSN/33-M2 S31N, A/WSN/33-M2 S31N/V27A, A/WSN/33-M2 S31N/L26F, A/ WSN/33-M2 S31N/A30T, A/
A/WSN/33-M2 S31N/G34E, generated by reverse genetics from A/WSN/33. While no other adamantane derivative inhibits the A/WSN/33 virus bearing M2 N31 or M2 A30T mutations (Table A.1.1), the copper complex CA is the only one which inhibits both strains having EC$_{50}$ values 21.95 and 11.58 μM respectively (Table A.1.1). Moreover the antiviral effect of CA is 1.5 and 3.2 fold stronger than that of amantadine itself against the adamantanes sensitive strains A/WSN/33-M2 S31N and M2 L26F, respectively. However the virus A/WSN/33 bearing the mutation M2 V27A and M2 G34E are significantly less susceptible to CA.

Two-Electrode Voltage Clamp (TEVC)

TEVC is a conventional electrophysiological technique used to artificially control the membrane potential (Vm) of large cells to study the properties of electrogenic membrane proteins, especially ion channels. TEVC experiments showed that the M2 S31N channel is the protein target of the copper complex (Table A.1.2), where the percent inward current block is significantly reduced, with amantadine showing no measurable block. The percent washout was small showing the very high block which was attained. Therefore, CA acts by blocking the proton current of A/WSN/33-M2 S31N in a similar manner as free Cu(II) ions do.

In Silico Studies

The M2 protein of influenza virus A is a tetrameric transmembrane protein that form pH-dependent proton-selective channels. Proton conductance is required for viral replication and therefore, the M2 proton channel of influenza virus was the target protein of the FDA approved drugs amantadine and rimantadine which bind directly to the pore of the channel. Nevertheless, the use of those drugs has been discontinued due to drug-resistant mutations in M2 and the amantadine-insensitive Ser31-to-Asn31 mutation has become the predominant form in
M2(S31N). The M2 protein consists of a 96-residue monomer that forms a tetrameric channel allowing a relatively small binding cavity available for the potential drugs. The S31N mutation increases both the bulk and polarity of the residues, reducing even more the available interaction space. The resistance to amantadine mechanism due to the S31N mutation has been described by Thomaston and De Grado. Briefly, a network of hydrogen bonding between the repeated Asn31 residues within the channel prevents the binding of the inhibitor due to steric hindrance. Moreover, Asn imposes a hydrophilic character to the site that normally interacts with the hydrophobic adamantane cage. However, amantadine derivatives can still be important drug candidates because M2 is a proven drug target and essential for influenza virus replication. Recently adamantyl bromothiophene ((N-[5-bromothiophen-2yl] methyl) amandatan-1-amine) was reported as active against both the wild type and the S31N mutant [24]. Here, the complex CA, consisting of the amantadine and the [CuCl3]⁺ ion was in silico tested, via molecular docking studies, as a potent M2(S31N) channel blocker. The ionic complex dissociates in water and therefore the two ions were docked sequentially to the apo form of M2(S31N) structure (PDB ID: 2LY0). Global docking was performed covering the whole search space of the M2 channel in order not to favor any binding site. Results were clustered by RMSD and ranked in order of decreasing binding energy. Docking calculations showed that the [CuCl3]⁺ binding site is distinct from the binding site of the hydrophobic drug amantadine and about 4 Å from each His37 residue (Fig. A.1.6). Cation-Pi interactions are evident with average binding energy of approximately -2.4 kcal/mol. On the other hand, amantadine exhibits two different binding modes into the M2 pore with similar energy (approximately -5.2 to -5.0 kcal/mol). The first one is adjacent to [CuCl3]⁺ towards the N-terminus stabilized by hydrophobic forces and probably pushed there by the hydrophilic CONH₂ moiety of Asn31. This outcome corroborates with
molecular dynamics (MD) simulations performed on the complex amantadine-M2 (S31N) which showed that the ligand spontaneously moves towards the C-terminus into the central cavity of the channel. The second pose (of higher energy) was stabilized by hydrophobic interactions with Val27 and Ala30. Docking simulations are not inherently able to replicate recent studies carried out with MD simulations which suggested that the amino group of amantadine oppositely faces up toward the N-terminus and does not have specific binding residues in the channel pore of M2(S31N) allowing for proton conduction. Our docking studies confirmed that compound CA can effectively block proton current mediated by M2(S31N) virus replication. Specifically [CuCl3]− blocks the primary gate of the His37 tetrad which is essential for channel conductance and selectivity.

*In Vitro Toxicity Study Against MRC-5 Cells*

The normal human fetal lung fibroblast cells (MRC-5) are used in the human vaccines development from pharmaceutical industries, for long time period. They are used as the safest cell substrates for the production of human viral vaccines. This is why MRC-5 cells were chosen for the in vitro toxicity study of CA and amantadine by sulforhodamine B (SRB) assay. Both CA and amantadine are causing no cells inhibition when they are treated with CA or amantadine at the concentration of 30 μM.

*In Vitro Genotoxicity Study Using MN Assay*

In order to test the in vitro genotoxicity of CA, the micronucleus assay was performed. The micronucleus assay controls the genetic damage in normal human cells as it is a sensitive tool for toxicity screening, and it is capable in reducing the use of animals in toxicological testing. The presence of micronucleus (MN) can be a biomarker of mutagenic, genotoxic, or
teratogenic agent influence upon the treatment of the normal cells towards a tested drug. The in vitro genotoxicity caused by CA or amantadine at the concentration of 30 μM is evaluated towards MRC-5 cells (Fig. A.1.7). The % MN frequencies of the untreated MRC-5 cells are (0.8 ± 0.02)%.

The corresponding one of the treated cells by CA or amantadine is (1.67 ± 0.20)% and (1.75 ± 0.40)% respectively. The in vitro genotoxicity of CA is similar with that of amantadine, indicating that CA is considered as a no-mutagenic, no-genotoxic, or no-teratogenic agent.

In Vivo Toxicity Against Fish Models

The antiviral drug amantadine has received approval from U. S. Food and Drug Administration (F.D.A.) against influenza A long time ago. Since the CA displays no in vitro toxicity or genotoxicity against MRC-5 cells, the in vivo toxicity of CA was proceeded using brine shrimp *Artemia salina* assay. The brine shrimp *Artemia salina* assay is a preliminary toxicity test since the pharmacological activities of a bioactive compound can be evaluated with it. The assay is characterized by short life cycle, non-selective grazing and sensitivity to toxic substances. Toxicity to brine shrimp coincides with toxicity to mammalian cells in many cases. However, there is no correlation in the degree of toxicity between the two systems. The percentage of survival of *Artemia salina* larvae without or with CA at the tested concentrations 30, 60, 90, 120 and 150 μM for 24 h are shown in Fig. A.1.8.

The percentage of survival was determined in terms of alive larvae. The survival rate of brine shrimp larvae was found (85.7 ± 16.2) % in the case of the untreated larvae. The percentage of survival of brine shrimp larvae when were incubated with CA at 30, 60, 90, 120 and 150 μM are (78.3 ± 10.2), (85.4 ± 6.5), (87.9 ± 9.6), (82.6 ± 10.8) and (76.9 ± 11.9) %, respectively. The survival rates of brine shrimp larvae when they were incubated with CA up to 150 μM, are similar with that of the non-treated larvae, suggesting its non-toxic behavior.
Neurotoxicity Study

There was no evidence of cytotoxicity (inhibition of cell growth or cell morphological changes) for the compound at dosage \( \leq 30 \text{ mM} \). The compounds were injected i.p (10, 30 or 60 mg/Kg) in C57BL6 female and male mice weighing 25–30 g and 35–45 g respectively (Table A.1.3). Intraperitoneal doses were non-lethal at 30 mg/kg. Amantadine was lethal at 300 mg/kg while the CA was lethal at a dose of 60 mg/Kg in 4 out 5 mice (2 female and 2 male). At doses 10 and 30 mg/Kg the drug was not lethal within 48 hrs following drug administration. At 10 mg/Kg CA slightly decreased the arousal of the animals for 2 hrs following injection, and then the mobility returned to normal. At a dose of 30 mg/Kg CA caused a moderate decrease in arousal, moderately abnormal gate which was associated with partial extension of their hind limbs when held from their tails and mild lacrimation. These effects did not ameliorate during the 48 hrs of the experiment. The dose of 60 mg/Kg induced a severe decrease in arousal and gait. The animals were flattened and had rigid paralysis of their hind limbs. In none of the doses did tonic or clonic movements occur.

Conclusion

The conjugation of AdNH\(_2\) with CuCl\(_2\) results to the polymeric binary salt of formula \{[AdNH\(_3^+\)][CuCl\(_3^-\)]\} (CA). The activity of CA involves dissociation to its counter ions [AdNH\(_3^+\)] and [CuCl\(_3^-\)] in aqueous solution. CA acts by blocking the proton current of A/WSN/33-M2 S31N in a similar manner as free Cu(II) ions. The washout shows that CA binds tightly in both M2 constructs. While no other adamantane derivative inhibits the A/WSN/33 virus bearing M2 S31N or M2 A30T mutations (Table A.1.1), CA is the only one which inhibits both strains. Moreover the antiviral effect of CA is stronger than that of amantadine itself against the amantadine sensitive strains A/WSN/33-M2 S31N and M2 L26F, respectively. In silico
studies revealed that the \([\text{CuCl}_3]^-\) binds in a different site than the drug amantadine and about 4 Å from each His37 residue (Fig. A.1.6). Consequently \([\text{CuCl}_3]^-\) blocks the primary gate of the His37 tetrad which is essential for channel conductance and selectivity. As a result CA can effectively block proton current mediated by M2(S31N) virus replication. The in vitro toxicity and genotoxicity of the CA in cells and a fish model in vivo was negligible, while the neurotoxicity of CA is lower than 60 lg/Kg in a mouse model. Therefore, CA provides an alternative pharmacological target for M2(S31N) mutants.
Figure A.1.1: Molecular Drawing of Amantadine Used in Conjugation to Cu(II). CA preparation scheme.
Figure A.1.2: Molecular Diagram. (CA) Selected bond lengths [Å] and angles: Cu1-Cl1 = 2.2845, Cu1-Cl2 = 2.2347, Cu1-Cl3 = 2.2555, N1-C1 = 1.480[Å], Cu1-Cl1i = 2.3562, Cl1-Cu1-Cl2 = 170.97, Cl1-Cu1-Cl3 = 94.86[Å], Cl2-Cu1-Cl3 = 93.92[Å], Cl1i-Cu1-Cl3 = 166.47.
Figure A.1.3: FT-IR Spectrum of Amantadine.
Figure A.1.4: FT-IR Spectrum of CA.
Figure A.1.5: UV-Vis Spectrum of CA Complex in DMSO.
Figure A.1.6: Top Ranked Docking Poses of [CuCl$_3$]$^-$ and Amantadine. In M2 S31N channel protein. Each pose consists of two molecules for improved visualization.
Figure A.1.7: Snapshot of MRC-5 Cells. (A) un-incubated or incubated (48 hrs) with CA (B) or amantadine (C) (30 μM). Arrows indicate the micronucleus in MRC-5 cells.
Figure A.1.8: Artemia Salina Survival (%) Diagram. Non-treated larvae and treated with CA at 30, 60, 90, 120, and 150 μM for 24 hrs.
Table A.1.1: In Vitro Efficacy [EC$_{50}$, μM] of Compounds. Tested against initial cell infection (CPE inhibition assay).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(AdNH)$_2$ Cl$^-$</td>
<td>0.58 ± 0.29</td>
<td>n.a.</td>
<td>n.a.</td>
<td>55.78 ± 6.8</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CA</td>
<td>0.39 ± 0.09</td>
<td>21.95 ± 2.11</td>
<td>&gt; 31.8$^b$</td>
<td>17.4 ± 3.38</td>
<td>11.58</td>
<td>&gt; 31.6$^b$</td>
</tr>
</tbody>
</table>

Table A.1.2: Block of Full-Length Udorn M2 S31N Current.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Sample Size</th>
<th>Percent Block [%] 5 min$^a$</th>
<th>Percent Block [%] 10 min$^a$</th>
<th>Percent Washout [%]$^b$</th>
<th>Percent Washout [%]$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(AdNH)$_2$ Cl$^-$</td>
<td>100 μM</td>
<td>N = 3</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CA</td>
<td>100 μM</td>
<td>N = 3</td>
<td>61.3 ± 1.5</td>
<td>73.0 ± 1.4</td>
<td>18 ± 0.08</td>
<td>4.0 ± 0.4</td>
</tr>
</tbody>
</table>
Table A.1.3: In Vivo Toxicity Test in Mice.

\(^a\)Number of deaths within 48 h [number of males injected: number of females injected].
\(^b\)Slightly abnormal gate. Some quivers. Hyper-responsive to touch or noise.
\(^c\)Moderate trembling or quivers.
\(^d\)Ataxic gait. Moderately abnormal gait. Slightly or somewhat impaired mobility. Mild tremors. Motor in coordination.

<table>
<thead>
<tr>
<th>Dose [mg/kg]</th>
<th>(AdNH(_3) Cl(^-))</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0 [2M:2F] (^a)</td>
<td>0 [3M:2F]</td>
</tr>
<tr>
<td>30</td>
<td>0 [2M:2F] (^a)</td>
<td>0 [3M:3F] (^b)</td>
</tr>
<tr>
<td>60</td>
<td>N.D</td>
<td>4 [2F, 2M] [3M:2F] (^d)</td>
</tr>
<tr>
<td>100</td>
<td>0 [2M:1F] (^c)</td>
<td>N.D</td>
</tr>
<tr>
<td>300</td>
<td>3 [2M:1F]</td>
<td>N.D.</td>
</tr>
</tbody>
</table>
Appendix B.1: Molecular Dynamics Simulation Program

This appendix includes the code for running a Molecular Dynamics Simulation (Minimization, Annealing, and Equilibration)

Minimization Program

cutoff 12.0
pairlistdist 14.0
switching on
switchdist 10.0
PME on
PMEGridspacing 1
wrapAll on
wrapWater on
parameters topppar_all36_na_nad_ppi_gdp_gtp_qwikmd.str

---------------------------------------------------------------------------------------------
#cr
#cr          (C) Copyright 1995-2009 The Board of Trustees of the
#cr          University of Illinois
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#cr
---------------------------------------------------------------------------------------------

# RCS INFORMATION:
#
# $RCSfile: Minimization.conf,v $  
# $Author: johns $  $Locker: $  $State: Exp $  
# $Revision: 1.1 $  $Date: 2016/07/13 19:14:19 $  
#
#START HERE##
##Simulation Template##
# Simulation conditions
coordinates Simulation_QwikMD.pdb
structure Simulation_QwikMD.psf

#binCoordinates qwikmdTemp.restart.coor
#binVelocities qwikmdTemp.restart.coor
#extendedSystem Minimization.xsc

# Simulation conditions
temperature 0
# Harmonic constraints

constraints on
consref Minimization_restraints.pdb
conskfile Minimization_restraints.pdb
constraintScaling 2
conssxp 2
conskcool B

# Output Parameters

binaryoutput no
outputname Minimization
outputenergies 40
outputtiming 40
outputpressure 40
binaryrestart yes
dcdfile Minimization.dcd
dcdfreq 1000
XSTFreq 1000
restartfreq 1000
restartname Minimization.restart

# Thermostat Parameters

langevin on
langevintemp 0
langevinHydrogen off
langevindamping 1

# Barostat Parameters

usegrouppressure yes
useflexiblecell no
useConstantArea no
langevinpiston on
langevinpistontarget 1.01325
langevinpistonperiod 200
langevinpistondecay 100
langevinpistontemp 273
# Integrator Parameters
timestep 1
firstTimestep 0
fullElectFrequency 2
nonbondedfreq 1

# Force Field Parameters
paratypecharmm on
parameters toppar_water_ions_namd.str
parameters toppar_all36_carb_glycopeptide.str
parameters par_all36_lipid.prm
parameters par_all36_na.prm
parameters par_all36_prot.prm
parameters par_all36_carb.prm
parameters par_all36_cgenff.prm
parameters RMT_quikmd.str
exclude scaled1-4
1-4scaling 1.0
rigidbonds all

# Periodic Boundary Conditions
if [1] {
cellBasisVector1 78. 0. 0.
cellBasisVector2 0. 78. 0.
cellBasisVector3 0. 0. 240.
cellOrigin 0.2963975410461426 0.2530790017970668 -0.1195036843419075
}

# Implicit Solvent Parameters
gbs off
alphaCutoff 14.0
ionConcentration 0.15
# Script

minimize 3000

set file [open Minimization.check w+]
set done 1
if {[[file exists Minimization.restart.coor] != 1 ||
[file exists Minimization.restart.vol] != 1 ||
[file exists Minimization.restart.xsc] != 1 } {
    set done 0
}
if {$done == 1} {
    puts $file "DONE"
    flush $file
    close $file
} else {
    puts $file "One or more files failed to be written"
    flush $file
    close $file
}
Annealing Program

cutoff 12.0
pairlistdist 14.0
switching on
switchdist 10.0
PME on
PMERadiusSpacing 1
wrapAll on
wrapWater on
margin 2.0

parameters toppar_all36_na_nad_ppi_gdp_gtp_qwikmd.str

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#

# RCS INFORMATION:
#
#
# $RCSfile: Annealing.conf,v $
# $Author: johns $ $Locker: $ $State: Exp $
# $Revision: 1.1 $ $Date: 2016/07/13 19:14:19 $
#

##START HERE##
##Simulation Template##
# Simulation conditions
coordinates Simulation_QwikMD.pdb
structure Simulation_QwikMD.psf

binCoordinates Minimization.restart.coor
binVelocities Minimization.restart.vel
extendedSystem Minimization.restart.xsc

# Simulation conditions
#temperature 60
# Harmonic constraints

constraints on
consref Minimization_restraints.pdb
conskfile Minimization_restraints.pdb
constraintScaling 2
consexp 2
conskcool B

# Output Parameters

binaryoutput no
outputname Annealing
outputenergies 40
outputtiming 40
outputpressure 40
binaryrestart yes
dcdfile Annealing.dcd
dcdfreq 1000
XSTFreq 1000
restartfreq 1000
restartname Annealing.restart

# Thermostat Parameters
langevin on
langevintemp 60
langevinHydrogen off
langevindamping 1

# Barostat Parameters
langevinpiston on
usegrouppressure yes
useflexiblecell no
useConstantArea no
langevinpistonTarget 1.01325
langevinpistonPeriod 200
langevinpistonDecay 100
langevinpistonTemp 60
# Integrator parameters

timestep 2
firstTimestep 0
fullElectFrequency 2
nonbondedfreq 1

# Force Field Parameters

paratypecharm on
parameters toppar_water_ions_namd.str
parameters toppar_all36_carb_glycopeptide.str
parameters par_all36_lipid.prm
parameters par_all36_na.prm
parameters par_all36_prot.prm
parameters par_all36_carb.prm
parameters par_all36_ogenff.prm
parameters AMT_qwikmd.str
exclude scaled1-4
1-4scaling 1.0
rigidbonds all

# Implicit Solvent Parameters

gbs off
alphaCutoff 14.0
ionConcentration 0.15
# Script
set Temp 300
set barostat 1
set nSteps 600
for {set t 60} {$t <= $Temp} {incr t}
{run $nSteps;langevintemp $t;if {$barostat}
langevinpistontemp $t}}

set file [open Annealing.check w+]
set done 1
    {set done 0}
if {$done == 1} {
puts $file "DONE"
flush $file
close $file
} else {
puts $file "One or more files failed to be written"
flush $file
close $file
}
Equilibration Program

cutoff 12.0
pairlistdist 14.0
switching on
switchdist 10.0
PME on
PMESpacing 1
wrapAll on
wrapWater on
parameters toppar_all36_na_nad_ppi_qdp_qtp_qwikmd.str

#cr
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#cr
#cr

# RCS INFORMATION:
#
#    $RCSfile: Equilibration.conf,v $  
#    $Author: johns $     $Locker: $     $State: Exp $  
#    $Revision: 1.1 $     $Date: 2016/07/13 19:14:19 $  
#
#START HERE##
##Simulation Template##
# Simulation conditions
coordinates Simulation_QwikMD.pdb
structure Simulation_QwikMD.psf

binCoordinates Annealing.restart.coor
binVelocities Annealing.restart.vel
extendedSystem Annealing.restart.xsc

# Simulation conditions
#temperature 60
# Harmonic constraints

constraints on  
consref Minimization_restraints.pdb  
conskfile Minimization_restraints.pdb  
constraintScaling 2  
consexp 2  
conskcol B  

# Output Parameters  

binaryoutput no  
outputname Equilibration  
outputenergies 40  
outputtiming 40  
outputpressure 40  
binaryrestart yes  
dcdfile Equilibration.dcd  
dcdfreq 1000  
XSTFreq 1000  
restartfreq 1000  
restartname Equilibration restart  

# Thermostat Parameters  
langevin on  
langevintemp 300  
langevinHydrogen off  
langevindamping 1  

# Barostat Parameters  

langevinpiston on  
usegrouppressure yes  
useflexiblecell yes  
useConstantArea yes  
langevinpistonTarget 1.01325  
langevinpistonperiod 200  
langevinpistonDecay 100  
langevinpistonTemp 300
# Integrator Parameters

timestep 1
firstTimestep 0
fullElectFrequency 2
nonbondedfreq 1

# Force Field Parameters

cparamcharmm on
cparameters toppr_water_ions_namd.str
cparameters toppar_all36_carb_glycopeptide.str
cparameters par_all36_lipid.prm
cparameters par_all36_na.prm
cparameters par_all36_prot.prm
cparameters par_all36_carb.prm
cparameters par_all36_cgonff.prm
cparameters RMT_qwikmd.str
exclude scaled1-4
1-4scaling 1.0
rigidbonds all

# Implicit Solvent Parameters

gbsi off
calphaCutoff 14.0
cionConcentration 0.15

run 1000000

set file [open Equilibration.check w+]
set done 1
    set done 0
}
if {$done == 1} {
    puts $file "DONE"
    flush $file
    close $file
} else {
    puts $file "One or more files failed to be written"
    flush $file
    close $file
}
Appendix B.2: Adaptive Biasing Force Program

This appendix includes the code for running an Adaptive Biasing Force simulation and merging the output files which creates a PMF.

---

```plaintext
Window Colvars Input

colvarsTrajFrequency 2000
colvarsRestartFrequency 20000

colvar {
  name AtomDistance

  width 0.1

  lowerboundary -0.09
  upperboundary 2.91

  lowerwallconstant 100.0
  upperwallconstant 100.0

  distance2 {
    main {
      atommembers { 51898 51899 51900 51901 51902 51903 51904 51905 51906 51907 51908 51909 51910 51911 51913 51914 51915 51916 51917 51918 51919 51920 51921 51922 51923 51924 51925 51926 }
    }

    ref {

  axis {0, 0, 1} }

} 

abf {
  colvars AtomDistance
  fullSamples 5000
  hideJacobian
}
```

---

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### MD SECTION

set basenames $::env(namel)
set basenames2 $::env(namel)$::env(n)

# TOPOLOGY
structure Simulation_QwikMD.psf

# INPUT FILES
coordinates Simulation_QwikMD.pdb
#temperature 300.0

#binCoordinates Equilibration.restart.coor
#binVelocities Equilibration.restart.vel
#extendedSystem Equilibration.restart.xsc

#ABF Restart

colvarsInput $basenames1-1.colvars.state
binCoordinates $basenames1-1.coor
binVelocities $basenames1-1.vel
extendedSystem $basenames1-1.xsc

# Harmonic constraints
constraints off
#consref Minimization_restraints.pdb
#consfile Minimization_restraints.pdb
#constraintScaling 2
#consexp 2
#consKcol B

# NUMBER OF MD-STEPS

numsteps 45000000
# FORCE FIELD

paraTypeCharmm on
parameters toppar_water_ions_namd.str
parameters toppar_all36_carb_glycopeptide.str
parameters par_all36_lipid.prm
parameters par_all36_na.prm
parameters par_all36_prot.prm
parameters par_all36_carb.prm
parameters par_all36_cgenff.prm
parameters AMT_qwikmd.str

# 1-4 TERMS

exclude               scaled1-4
1-4scaling            1.0

# OUTPUT FILES

binaryoutput         no
binaryrestart        yes

outputname           ../Merge/$basename2
restartname          $basename2

# DCD FILE

dcdFile              $basename2.dcd

# FREQUENCY FOR DUMPING OUTPUT DATA

outputenergies       1000
outputtiming         1000
outputpressure       1000
restartfreq          1000
XSTFreq              1000
dcdFreq              1000

# CUT-OFFS

hgroupcutoff         2.8
switching            on
switchdist           10.0
cutoff               12.0
pairlistdist         14.0
PME on
PMEGridspacing 1
wrapAll on
wrapWater on
# Thermostat Parameters

langevin on
langevintemp 300.0
langevindamping 1.0

# Barostat Parameters

langevinpiston on
usegrouppressure yes
useflexiblecell no
useConstantArea no
langevinpistontarget 1.01325
langevinpistonperiod 200
langevinpistondelay 100
langevinpistontemp 300

# MULTIPLE TIME-STEP PROPAGATOR

timestep 0.5

# SHAKE/RATTLE

rigidbonds none

# PARALLELISM

stepspercyle 16
splitpatch hydrogen
margin 2.0

# ABF SECTION

colvars on
colvarsConfig win1.in
Merge Input File

colvarsTrajFrequency  2000
colvarsRestartFrequency  20000

colvar {
    name AtomDistance
    width 0.1
    lowerboundary -0.09
    upperboundary  35.91
    lowerwallconstant 100.0
    upperwallconstant 100.0
}

distanceZ {
    main {
        atomnumbers { 51898 51899 51900 51901 51902 51903 51904 51905 51906 51907 51908
                       51909 51910 51911 51912 51913 51914 51915 51916 51917 51918 51919 51920 51921
                       51922 51923 51924 51925 51926 }
    }
}

ref {
    atomnumbers { 1 5 12 13 14 16 23 24 25 27 35 36 37 41 49 50 51 53 68 69 70 72
                      84 85 86 88 100 101 102 104 110 111 112 114 120 121 122 124 131 132 133 135 150
                      151 152 154 169 170 171 173 176 177 178 180 195 196 197 199 214 215 216 218 231
                      232 233 235 250 251 252 254 269 270 271 273 288 289 290 292 312 331 314 316 331
                      332 333 335 350 351 352 354 362 363 364 366 386 387 388 390 405 406 407 408 412
                      419 420 421 423 430 431 432 434 442 443 444 448 456 457 458 460 475 476 477 479
                      491 492 493 495 507 508 509 511 517 518 519 521 527 528 529 531 534 538 539 540 542
                      557 558 559 561 576 577 578 580 583 584 585 587 602 603 604 606 621 622 623 625
                      638 639 640 642 657 658 659 661 676 677 678 680 695 696 697 699 719 720 721 723
                      738 739 740 742 757 758 759 761 769 770 771 773 793 794 795 797 812 813 814 815
                      819 826 827 828 830 837 838 839 841 849 850 851 855 863 864 865 867 882 883 884
                      886 898 899 900 902 914 915 916 918 924 925 926 928 934 935 936 938 945 946 947
                      949 964 965 966 968 983 984 985 987 990 991 992 994 1009 1010 1011 1013 1028 1029
                      1030 1032 1045 1046 1047 1049 1064 1065 1066 1068 1083 1084 1085 1087 1102 1103
                      1104 1106 1126 1127 1128 1130 1145 1146 1147 1149 1164 1165 1166 1169 1176 1177
                      1178 1180 1200 1201 1202 1204 1219 1222 1221 1222 1225 1233 1234 1235 1237 1244
                      1245 1246 1248 1256 1257 1258 1262 1267 1271 1272 1274 1289 1290 1291 1293 1305
                      1306 1307 1309 1321 1322 1323 1325 1331 1332 1333 1335 1341 1342 1343 1345 1352
                      1353 1354 1356 1371 1372 1373 1375 1390 1391 1392 1394 1397 1399 1401 1416
                      1417 1418 1420 1435 1436 1437 1439 1452 1453 1454 1456 1471 1472 1473 1475 1490
                      1491 1492 1494 1509 1510 1511 1513 1533 1534 1535 1537 1552 1553 1554 1556 1571
                      1572 1573 1575 1583 1584 1585 1587 1607 1608 1609 1611 1626 1627 1628 }
}

axis (0, 0, 1)

}

abf {
    colvars AtomDistance
    fullSamples 5000
    hideJacobian
    inputprefix win1-2 win2-2 win3-2 win4-2 win5-2 win6-2 win7-2 win8-2 win9-2 win10-2 win11-2
}
# ABF calculation
# 2KQT and Amt
# Merge Configuration File
#
# MD SECTION
#
# TOPOLOGY
structure Simulation_QwikMD.psf

# INPUT FILES
coordinates Simulation_QwikMD.pdb
temperature 300.0

extendedSystem Equilibration.restart.xsc

# Harmonic constraints

constraints on consref Minimization_restraints.pdb
conskfile Minimization_restraints.pdb
constraintScaling 2 = conexp 2
conskcol B

# FORCE FIELD

paratypeCharmm on
parameters toppar_water_ions_namd.str
parameters toppar_all136_carb_glycopeptide.str
parameters par_all136_lipid.prm
parameters par_all136_na.prm
parameters par_all136_prot.prm
parameters par_all136_carb.prm
parameters par_all136_cgenff.prm
parameters AMT_qwikmd.str
# 1-4 TERMS

exclude          scaled1-4
1-4scaling       1.0

# OUTPUT FILES

binaryoutput    no
binaryrestart   yes
#outputname      abf_00.0
restartname     abf_00

# DCD FILE

dcdFile       abf_00.dcd

# FREQUENCY FOR DUMPING OUTPUT DATA

outputenergies  1000
outputtiming    1000
outputpressure  1000
restartfreq     1000
XSTFreq         1000
dcdFreq         1000
outputName      merge

# CUT-OFFS

hgroupcutoff    2.8
switching       on
switchdist      10.0
cutoff          12.0
pairlistdist    14.0
PME on
PMEGridspacing  1
wrapAll on
wrapWater on

# Thermostat Parameters

langevin on
langevintemp    300.0
langevindamping 1.0
# Barostat Parameters

langevinpiston on
usegrouppressure yes
useflexiblecell no
useConstantArea no
langevinpistontarget 1.01325
langevinpistonperiod 200
langevinpistondecay 100
langevinpistontemp 300

# MULTIPLE TIME-STEP PROPAGATOR

timestep 0.5

# SHAKE/RATTLE

rigidbonds none

# PARALLELISM

stepspercycle 16
splitpatch hydrogen
margin 2.0

# ABF SECTION

colvars on
colvarsConfig merge.in

# NUMBER OF MD-STEPs

run 0
Appendix B.3: One-Site Global Nonlinear Least Squares Curve Fit Program

This appendix shows the Matlab program that was used to perform the global nonlinear least squares fit using the one-site model equation in chapter 2 along with the function to extract the rate constants.

```matlab
% Appendix B.3: One-Site Global Nonlinear Least Squares Curve Fit Program

This appendix shows the Matlab program that was used to perform the global nonlinear least squares fit using the one-site model equation in chapter 2 along with the function to extract the rate constants.

```
function [ PredCurrFun ] = GlobChiSqGdLC(p,T,Cq,Diff,xdata)

%Open file that writes predcurrfun output
fid = fopen('predcurrfun.txt', 'w+');

%This equation has four parameters: p1, p2, p3, and p4. p1 is k1 (on rate
%constant), p2 is k2 (off rate constant), p3 is the length of the unstimulated
%layer (L), p4 is f.

A = zeros(1,15);
B = zeros(1,1384);
C = zeros(1,1384);
D = zeros(1,15);

%Concentration vector used in PredCurrFun and i in the for loop
[b,y] = size(C);

n = 1; %Used in PredCurrFun to input values in the array correctly

%Fitting Wash-in
for i = 1:n
    t = xdata(T(i)+1);
    %Ex. T(2)+1 = 1 to T(10) = 386610, T(2)+1 = 386611 to T(2)+1 = T(3) = 556681, etc...
    K = (1-p(2))./p(1).*(C(1)-(4.*C(1))./p(1)).*exp(-Diff.*p(2).*t.)/(4.*(3.*t^2)+)+p(2));
    L = exp(-p(4)).*(C(1)-(4.*C(1))./p(1)).*exp(-Diff.*p(2).*t.)/(4.*(3.*t^2)+)+p(2).*t;
    M = (p(2))./(p(1)).*(C(1)-(4.*C(1))./p(1)).*exp(-Diff.*p(2).*t.)/(4.*(3.*t^2)+)+p(2);
    PredCurrFun(i) = (1-p(i+3)).*((p(3)+(1-p(3))).*(K.*L*M)+p(i+3));

K = (((1-p(2))./p(1)).*(C(1)-(4.*C(1))./p(1)).*exp(-Diff.*p(2).*t.)/(4.*(3.*t^2)+)+p(2)));
L = exp(-p(4)).*(C(1)-(4.*C(1))./p(1)).*exp(-Diff.*p(2).*t.)/(4.*(3.*t^2)+)+p(2).*t;
M = (p(2))./(p(1)).*(C(1)-(4.*C(1))./p(1)).*exp(-Diff.*p(2).*t.)/(4.*(3.*t^2)+)+p(2);
PredCurrFun(i) = (p(3)+(1-p(3))).*(K.*L*M);

%Probability with LC to pass onto washout equation
Prob(i) = (K.*L*M);

B(i) = Prob(i);
D(i) = B(i);
\% $D(19) = B(1677556);$
\% $D(20) = B(1773495);$
\% $D(21) = B(1868570);$
\% $D(22) = B(1945435);$
\% $D(23) = B(1980660);$
\% $D(24) = D(2358210);$
\% $D(25) = B(2471043);$

\% Probability without LC for Prob vs Conc plot - to check
\% Isomoodicity of result
\% \textbf{Prob2}(n) = K \cdot t \cdot M;

\texttt{C}(n) = \texttt{Prob2}(n);
\texttt{F}(14) = C(262);
\texttt{F}(15) = C(557);
\texttt{F}(16) = C(632);
\texttt{F}(17) = C(680);
\% \texttt{F}(18) = C(585);
\% \texttt{F}(19) = C(1677556);
\% \texttt{F}(20) = C(1773495);
\% \texttt{F}(21) = C(1868570);
\% \texttt{F}(22) = C(1945435);
\% \texttt{F}(23) = C(1980660);
\% \texttt{F}(24) = C(2358210);
\% \texttt{F}(25) = C(2471043);

\texttt{t} = \texttt{t} + 1;
\texttt{n} = \texttt{n} + 1;
\% end

A(5) = 1-D(14); \% Convert probability of unoccupied to probability of occupied with LC
A(6) = 1-D(15); \% A is used for the washout equation so that the washout starts where the washin ended
A(7) = 1-D(16);
A(8) = 1-D(17);
\% A(10) = 1-D(18);
\% A(19) = 1-D(19);
\% A(20) = 1-D(20);
\% A(21) = 1-D(21);
\% A(22) = 1-D(22);
\% A(23) = 1-D(23);
\% A(24) = 1-D(24);
\% A(25) = 1-D(25);

G(5) = 1-F(14); \% Convert probability of unoccupied to probability of occupied without LC
G(6) = 1-F(15); \% A is used for the washout equation so that the washout starts where the washin ended
G(7) = 1-F(16);
G(8) = 1-F(17);
\% G(10) = 1-F(18);
\% G(19) = 1-F(19);
\% G(20) = 1-F(20);
\% G(21) = 1-F(21);
\% G(22) = 1-F(22);
\% G(23) = 1-F(23);
\% G(24) = 1-F(24);
\% G(25) = 1-F(25);
%Fitting Wash-out

for k = 3:8
    %(k)/1 = (7/2)/1=0.5 = 4 to 7

    t = 0;

    for z = T(k)+1:T(k)+2
        %Ex. T(4)+1 = 945665+1 = 945666 to T(4)+4 = T(5) = 973319
        Pr0currRun(z) = p(z)+i=p(i)+1
        Vr0currRun(z) = p(z)+i=p(i)+1
        %h is probability of occupied, it decays to all unoccupied
        t = t + 1
        n = n + 1
    end

end

%write predCurrent to file
fprintf(files3, '\%h\n', predCurrent);
fclose(files3);

fprintf('A1 = %g\n', A(5));
fprintf('A2 = %g\n', A(6));
fprintf('A3 = %g\n', A(7));
fprintf('A4 = %g\n', A(8));
fprintf('A5 = %g\n', A(9));
fprintf('A6 = %g\n', A(10));
fprintf('A7 = %g\n', A(11));
fprintf('A8 = %g\n', A(12));
fprintf('A9 = %g\n', A(13));
fprintf('A10 = %g\n', A(14));
fprintf('A11 = %g\n', A(15));
fprintf('A12 = %g\n', A(16));
fprintf('A13 = %g\n', A(17));
fprintf('A14 = %g\n', A(18));
fprintf('A15 = %g\n', A(19));
fprintf('A16 = %g\n', A(20));
fprintf('A17 = %g\n', A(21));
fprintf('A18 = %g\n', A(22));
fprintf('A19 = %g\n', A(23));
fprintf('A20 = %g\n', A(24));
fprintf('A21 = %g\n', A(25));

fprintf('G1 = %g\n', G(5));
fprintf('G2 = %g\n', G(6));
fprintf('G3 = %g\n', G(7));
fprintf('G4 = %g\n', G(8));
fprintf('G5 = %g\n', G(9));
fprintf('G6 = %g\n', G(10));
fprintf('G7 = %g\n', G(11));
fprintf('G8 = %g\n', G(12));
fprintf('G9 = %g\n', G(13));
fprintf('G10 = %g\n', G(14));
fprintf('G11 = %g\n', G(15));
fprintf('G12 = %g\n', G(16));
fprintf('G13 = %g\n', G(17));
fprintf('G14 = %g\n', G(18));
fprintf('G15 = %g\n', G(19));
fprintf('G16 = %g\n', G(20));
fprintf('G17 = %g\n', G(21));
fprintf('G18 = %g\n', G(22));
fprintf('G19 = %g\n', G(23));
fprintf('G20 = %g\n', G(24));
fprintf('G21 = %g\n', G(25));

end
Appendix B.4: Two-site Mathematical Model Derivation

This appendix shows the derivation of the two-site model used in chapter 7 for the global fit of the electrophysiology data to extract rate constants for binding kinetics analysis.

Variable Definitions:

$P_o =$ time-dependent probability of the channel having no drug and is the same as $O$ in Eq. 7.1.

$P_{Cp} =$ time-dependent probability of the channel having a drug in the first binding site (partially blocked state) and is the same as $C_1$ in Eq. 7.1.

$P_C =$ time-dependent probability of the channel having a drug in the second binding site (fully blocked state) and is the same as $C_2$ in Eq. 7.1.

$P_{o eq} =$ fraction of channel without drug when an equilibrium has been reached. Initial value of $P_o$ different from equilibrium value relaxes to equilibrium value exponentially.

$P_{c eq} =$ fraction of channel fully blocked by drug when an equilibrium has been reached. Initial value of $P_C$ different from equilibrium value relaxes to equilibrium value exponentially.

$\lambda_1$ and $\lambda_2 =$ exponential decay constants; always negative and real for positive values of the rate constants $k_1$, $k_2$, $k_3$, and $k_4$.

$a_1$, $a_2$, $a_3$, and $a_4 =$ coefficients of the specific solution to the general two exponential washin equation obtained using $t = 0$ and $[D] = 0$ initial conditions

$a_5$, $a_6$, $a_7$, and $a_8 =$ coefficients of the specific solution to the general two exponential washout equation obtained using $t = 0$ and $[D] = 0$ initial conditions

$P_{o'}$, $P_{c p'}$, and $P_{c'} =$ same as defined above but the specific solution to these time-dependent differential equations have different initial conditions.
Washin Derivation:

Get Equation For: $P_o$

Equations:

(1) \[ \frac{dP_o}{dt} = -k_1 D P_o + k_4 P_C \]

(2) \[ \frac{dP_C}{dt} = k_1 D P_o - k_2 P_C + k_3 P_C - k_4 P_C \]

(3) \[ \frac{dP_C}{dt} = k_2 P_C - k_3 P_C \]

(4) \[ 1 - P_o + P_C = P_C \]

Use equation (4), plug into (1), solve for $P_C$:

\[ 1 - P_o = P_C \]

\[ \frac{dP_o}{dt} = -k_1 D P_o + k_4 \left( 1 - P_o - P_C \right) \]

(5) \[ P_C = \frac{\left( \frac{dP_o}{dt} + k_1 D P_o - k_4 P_C \right)}{-k_4} \]

Solve equation (1) for $P_C$ and take derivative:

(6) \[ P_C = \frac{\frac{dP_o}{dt} + k_1 D P_o}{k_4} \]

(7) \[ \frac{dP_C}{dt} = \frac{\left( \frac{d^2P_o}{dt^2} + k_1 D \frac{dP_o}{dt} \right)}{k_4} \]

Now, plug equations (5) - (7) into equation (2) (*note: needed $\frac{k_1}{k_4}$ to get common denominator):

\[ \frac{\left( \frac{d^2P_o}{dt^2} + k_1 D \frac{dP_o}{dt} \right)}{k_4} = k_1 D P_o \left( \frac{k_4}{k_4} \right) - k_2 \left( \frac{\frac{dP_o}{dt} + k_2 D P_o}{k_4} \right) + k_3 \left( \frac{\frac{dP_o}{dt} + k_4 D P_o - k_4 + k_4 P_C}{-k_4} \right) - k_4 \left( \frac{\frac{dP_o}{dt} + k_1 D P_o}{k_4} \right) \]

Combine like terms and set equation to zero:

\[ \frac{d^2P_o}{dt^2} + \left( k_1 D + k_2 + k_3 + k_4 \right) \frac{dP_o}{dt} + \left( k_1 D + k_2 \right) P_o - k_4 P_o = 0 \]
Use change of variable to get rid of constant term:

\[ P_o' = P_o - \frac{k_1 k_4}{(k_1 k_2 D + k_1 k_3 D + k_2 k_4)} \]

\[ \frac{d^2 P_o'}{dt^2} + (k_1 D + k_2 + k_3 + k_4) \cdot \frac{dP_o'}{dt} + (k_1 k_2 D + k_1 k_3 D + k_2 k_4) \cdot P_o' = 0 \]

**Trial function and quadratic form:**

Trial function \( \dot{e}^{\lambda t} \), where \( \lambda \) is a negative number

\[ \frac{d^2 \dot{e}^{\lambda t}}{dt^2} + (k_1 D + k_2 + k_3 + k_4) \cdot \frac{d\dot{e}^{\lambda t}}{dt} + (k_1 k_2 D + k_1 k_3 D + k_2 k_4) \cdot \dot{e}^{\lambda t} = 0 \]

\[ \lambda^2 \dot{e}^{\lambda t} + (k_1 D + k_2 + k_3 + k_4) \cdot \lambda \cdot \dot{e}^{\lambda t} + (k_1 k_2 D + k_1 k_3 D + k_2 k_4) \cdot e^{\lambda t} = 0 \]

\[ \lambda^2 + (k_1 D + k_2 + k_3 + k_4) \cdot \lambda + (k_1 k_2 D + k_1 k_3 D + k_2 k_4) = 0 \]

**Solve quadratic equation for \( \lambda \):**

\[ \lambda = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \]

\[ \lambda_1 = \frac{(k_1 D + k_2 + k_3 + k_4) + \sqrt{(k_1 D + k_2 + k_3 + k_4)^2 - 4 \cdot (1) \cdot (k_1 k_2 D + k_1 k_3 D + k_2 k_4)}}{2} \]

\[ \lambda_2 = \frac{(k_1 D + k_2 + k_3 + k_4) - \sqrt{(k_1 D + k_2 + k_3 + k_4)^2 - 4 \cdot (1) \cdot (k_1 k_2 D + k_1 k_3 D + k_2 k_4)}}{2} \]

**General form:**

\[ P_o = -a_1 e^{\lambda_1 t} + a_2 e^{\lambda_2 t} \]

Recall \( P_o' = P_o - \frac{k_2 k_4}{(k_1 k_2 D + k_1 k_3 D + k_2 k_4)} \), so when \( P_o' \) decays to zero, \( P_{o eq} = -\frac{k_2 k_4}{(k_1 k_2 D + k_1 k_3 D + k_2 k_4)} \). This is the equilibrium condition.
Full general solution:

\[
P_o = a_1 \cdot e^{\left(\frac{(k_j \cdot D + k_j \cdot D + k_j \cdot D + k_j \cdot D)}{2}\right) t} + a_2 \cdot e^{\left(\frac{(k_j \cdot D + k_j \cdot D + k_j \cdot D + k_j \cdot D)}{2}\right) t} + \left(\frac{k_j \cdot k_d}{k_j \cdot k_j \cdot D + k_j \cdot k_j \cdot D + k_j \cdot k_j \cdot D + k_j \cdot k_j \cdot D}\right)
\]

Use initial conditions to get \( a1 \) and \( a2 \):

At \( t = 0 \): \( P_o = 1, P_{CP} = 0, P_C = 0 \)

Equation 1:

\[1 - a_1 + a_2 + P_{eq}\]

Where \( P_{eq} = \frac{k_j \cdot k_d}{k_j \cdot k_j \cdot D + k_j \cdot k_j \cdot D + k_j \cdot k_j \cdot D + k_j \cdot k_j \cdot D}\)

Equation 2:

\[k_j \cdot P_C = \left(\frac{dP}{dt} + k_j \cdot D \cdot P_o\right) = k_j \cdot D \cdot \left(\alpha_1 \cdot e^{\lambda_1 \cdot t} + \alpha_2 \cdot e^{\lambda_2 \cdot t} + P_{eq}\right) + \left(\alpha_1 \cdot \lambda_1 \cdot e^{\lambda_1 \cdot t} + \alpha_2 \cdot \lambda_2 \cdot e^{\lambda_2 \cdot t}\right)
\]

\[P_{CP} = 0 \text{ at } t = 0:
\]

\[0 = \left(\frac{dP}{dt} + k_j \cdot D \cdot P_o\right) \rightarrow 0 = k_j \cdot D \cdot a_1 + k_j \cdot D \cdot a_2 + k_j \cdot D \cdot P_{eq} + \alpha_1 \cdot \lambda_1 + \alpha_2 \cdot \lambda_2
\]

Solve for \( a1 \) and \( a2 \):

\[a_1:
\]

\[0 = k_j \cdot D \cdot a_1 + k_j \cdot D \cdot \left(1 - a_1 - P_{eq}\right) + k_j \cdot D \cdot P_{eq} + \alpha_1 \cdot \lambda_1 + \lambda_2 \cdot (1 - a_1 - P_{eq})
\]

\[0 = a_1 \cdot k_j \cdot D + \alpha_1 \cdot k_j \cdot D + \lambda_2 \cdot (1 - P_{eq}) - \lambda_2 \cdot \alpha_1 \cdot k_j \cdot D + a_1 \cdot \lambda_1 + \alpha_1 \cdot k_j \cdot D - \lambda_2 \cdot \alpha_1 \cdot k_j \cdot D + a_1 \cdot \lambda_2 + \alpha_1 \cdot \lambda_1 - \lambda_2 \cdot \alpha_1 \cdot k_j \cdot D - a_1 \cdot \lambda_2
\]

\[a_1 = \frac{-k_j \cdot D - \lambda_2 \cdot (1 - P_{eq})}{\lambda_1 - \lambda_2}
\]

\[a_2:
\]

\[a_2 = \frac{\lambda_2 \cdot \lambda_2}{\lambda_1 - \lambda_2} \cdot \frac{-k_j \cdot D - \lambda_2 \cdot (1 - P_{eq})}{\lambda_1 - \lambda_2} - \frac{P_{eq} \cdot (\lambda_2 - \lambda_2)}{\lambda_1 - \lambda_2}
\]

\[a_2 = \frac{k_j \cdot D + \lambda_1 \cdot (1 - P_{eq})}{\lambda_1 - \lambda_2}
\]
Final $P_o$ solution:

$$
P_o = \frac{-k_1 \cdot D - \lambda_2 \cdot (1 - P_{eq})}{\lambda_1 - \lambda_2} \cdot e^{-\left(\frac{(k_2 \cdot D + k_3 + k_4)^2 + \sqrt{(k_2 \cdot D + k_3 + k_4)^2 - 4 \cdot (k_2 \cdot D + k_3 + k_4)}}{2}\right)} + \frac{k_1 \cdot D + \lambda_2 \cdot (1 - P_{eq})}{\lambda_3 - \lambda_2} \cdot e^{\left(\frac{(k_2 \cdot D + k_3 + k_4)^2 - 4 \cdot (k_2 \cdot D + k_3 + k_4)}}{2}\right) + \frac{k_3 \cdot k_4}{(k_2 \cdot k_3 \cdot D + k_3 \cdot k_3 \cdot D + k_3 \cdot k_3)}
$$

Get Equation For: $P_{C_p}$

Equations:

1. \( \frac{dP_o}{dt} = -k_1 \cdot D \cdot P_o + k_4 \cdot P_{C_p} \)

2. \( \frac{dP_{C_p}}{dt} = k_1 \cdot D \cdot P_o - k_2 \cdot P_{C_p} + k_3 \cdot P_C - k_4 \cdot P_{C_p} \)

3. \( \frac{dP_C}{dt} = k_2 \cdot P_{C_p} - k_2 \cdot P_C \)

4. \( 1 = P_o + P_{C_p} + P_C \)

Use equation (4), plug into (2), solve for $P_C$:

\[ 1 = P_{C_p} - P_C \rightarrow P_o \]

\[ \frac{dP_{C_p}}{dt} = -k_1 \cdot D \cdot (1 - P_{C_p} - P_C) - k_2 \cdot P_{C_p} + k_3 \cdot P_C - k_4 \cdot P_{C_p} \]

\[ \frac{dP_{C_p}}{dt} = -k_1 \cdot D + k_1 \cdot D \cdot P_{C_p} + k_2 \cdot P_{C_p} + k_4 \cdot P_{C_p} = -k_1 \cdot D \cdot P_C + k_2 \cdot P_C \]

\[ P_C = \frac{\left( \frac{dP_{C_p}}{dt} - k_1 \cdot D + k_1 \cdot D \cdot P_{C_p} + k_2 \cdot P_{C_p} + k_4 \cdot P_{C_p} \right)}{(k_3 - k_1 \cdot D)} \]

Take derivative:

\[ \frac{dP_C}{dt} = \left( \frac{\frac{d^2P_{C_p}}{dt^2} + (k_1 \cdot D + k_2 \cdot D \cdot \frac{dP_{C_p}}{dt})}{(k_3 - k_1 \cdot D)} \right) \]
Plug $P_C$ and $\frac{dP_C}{dt}$ into equation (4):

$$\frac{d^2 P_C}{dt^2} + \left( k_1 \cdot D + k_2 + k_4 \right) \cdot \frac{dP_C}{dt} \cdot \frac{dP_C}{dt} \cdot \frac{k_3 - k_4 \cdot D}{(k_3 - k_4 \cdot D)} - k_2 \cdot P_C = k_3 \left( \frac{\frac{dP_C}{dt} - k_1 \cdot D + k_2 \cdot P_C + k_3 \cdot P_C + k_4 \cdot P_C}{(k_3 - k_4 \cdot D)} \right)$$

Combine like terms and set equation to zero (*note $\frac{k_3 - k_4 \cdot D}{(k_3 - k_4 \cdot D)}$ is used to get a common denominator):

$$\frac{d^2 P_C}{dt^2} + \left( k_1 \cdot D + k_2 + k_4 \right) \cdot \frac{dP_C}{dt} \cdot \frac{k_3 - k_4 \cdot D}{(k_3 - k_4 \cdot D)} = k_2 \cdot P_C \left( \frac{k_3 - k_4 \cdot D}{(k_3 - k_4 \cdot D)} \right) - k_4 \left( \frac{\frac{dP_C}{dt} - k_1 \cdot D + k_2 \cdot P_C + k_3 \cdot P_C + k_4 \cdot P_C}{(k_3 - k_4 \cdot D)} \right)$$

$$- k_3 \cdot k_2 \cdot P_C - k_1 \cdot k_3 \cdot D - k_3 \cdot P_C = k_2 \cdot k_3 \cdot P_C - k_3 \cdot k_4 \cdot P_C$$

$$\frac{d^2 P_C}{dt^2} + \left( k_1 \cdot D + k_2 + k_4 \right) \cdot \frac{dP_C}{dt} \cdot \frac{k_3 - k_4 \cdot D}{(k_3 - k_4 \cdot D)} = - k_1 \cdot D \cdot k_2 \cdot P_C \cdot \frac{k_3 - k_4 \cdot D}{(k_3 - k_4 \cdot D)} + \left( \frac{- k_3 \cdot \frac{dP_C}{dt} + k_1 \cdot k_3 \cdot D - k_3 \cdot k_4 \cdot P_C - k_3 \cdot k_4 \cdot P_C}{(k_3 - k_4 \cdot D)} \right)$$

$$\frac{d^2 P_C}{dt^2} + \left( k_1 \cdot D + k_2 + k_4 \right) \cdot \frac{dP_C}{dt} \cdot \frac{k_3 - k_4 \cdot D}{(k_3 - k_4 \cdot D)} + k_1 \cdot k_2 \cdot P_C \cdot \frac{k_3 - k_4 \cdot D}{(k_3 - k_4 \cdot D)} - \left( \frac{- k_3 \cdot \frac{dP_C}{dt} + k_1 \cdot k_3 \cdot D - k_3 \cdot k_4 \cdot P_C - k_3 \cdot k_4 \cdot P_C}{(k_3 - k_4 \cdot D)} \right) = 0$$

$$\frac{d^2 P_C}{dt^2} + \left( k_1 \cdot D + k_2 + k_4 \right) \cdot \frac{dP_C}{dt} + \left( k_1 \cdot D \cdot k_2 + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4 \right) \cdot P_C = k_1 \cdot k_2 \cdot D = 0$$

Use change of variable to get rid of constant term:

$$P'_C = P_C - \frac{k_1 \cdot k_3 \cdot D}{(k_1 \cdot D \cdot k_2 + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4)}$$

$$\frac{d^2 P'_C}{dt^2} + \left( k_1 \cdot D + k_2 + k_4 \right) \cdot \frac{dP'_C}{dt} + \left( k_1 \cdot D \cdot k_2 + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4 \right) \cdot P'_C = 0$$
Trial function and quadratic form:

Trial function = $e^{\lambda t}$, where $\lambda$ is a negative number

$$\frac{d^2 e^{\lambda t}}{dt^2} + \left( k_1 \cdot D + k_2 + k_3 + k_4 \right) \frac{de^{\lambda t}}{dt} + \left( k_1 \cdot k_2 \cdot D + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4 \right) e^{\lambda t} = 0$$

$$\lambda^2 + \left( k_1 \cdot D + k_2 + k_3 + k_4 \right) \lambda + \left( k_1 \cdot k_2 \cdot D + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4 \right) = 0$$

Solve quadratic equation for $\lambda$:

$$\lambda = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

$$\lambda_1 = \frac{-\left( k_1 \cdot D + k_2 + k_3 + k_4 \right) + \sqrt{\left( k_1 \cdot D + k_2 + k_3 + k_4 \right)^2 - 4 \cdot \left( k_1 \cdot k_2 \cdot D + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4 \right)}}{2}$$

$$\lambda_2 = \frac{-\left( k_1 \cdot D + k_2 + k_3 + k_4 \right) - \sqrt{\left( k_1 \cdot D + k_2 + k_3 + k_4 \right)^2 - 4 \cdot \left( k_1 \cdot k_2 \cdot D + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4 \right)}}{2}$$

General form:

$$P_o = \alpha_3 \cdot e^{\left( k_1 \cdot D + k_2 + k_3 + k_4 \right) + \sqrt{\left( k_1 \cdot D + k_2 + k_3 + k_4 \right)^2 - 4 \cdot \left( k_1 \cdot k_2 \cdot D + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4 \right)}} \right) t + \alpha_4$$

Recall $P_{Cp} = \frac{k_1 \cdot k_2 \cdot D}{\left( k_1 \cdot k_2 \cdot D + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4 \right)}$, so when $P_{Cp}$ decays to zero, $P_{Cp} = \frac{k_1 \cdot k_3 \cdot D}{\left( k_1 \cdot k_2 \cdot D + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4 \right)}$
Full general solution:

\[
P_{Cp} = a_2 \cdot e^{\left(\frac{(k_4 \cdot D + k_2 + k_3 + k_4)}{2} \cdot t\right)} + a_4 \cdot e^{\left(\frac{(k_4 \cdot D + k_2 + k_3 + k_4)}{2} \cdot t\right)} + \frac{k_1 \cdot k_3 \cdot D}{(k_1 \cdot k_2 \cdot D + k_1 \cdot k_3 \cdot D + k_3 \cdot k_4)}
\]

Use initial conditions to get a3 and a4:

At \(t = 0\): \(P_{Cp} = 0\)

Equation 1:

\[0 = a_2 + a_4 + P_{Cp eq}\]

Equation 2:

\(P_c = 0\) at \(t = 0\):

\[
\left(k_3 - k_4 \cdot D\right) P_c = \left(\frac{dP_{Cp}}{dt} - k_1 \cdot D + k_2 \cdot D \cdot P_{Cp} + k_2 \cdot P_{Cp} + k_3 \cdot P_{Cp}\right)
\]

\[0 = \lambda_1 \cdot a_2 \cdot e^{\lambda_1 \cdot t} + \lambda_2 \cdot a_4 \cdot e^{\lambda_2 \cdot t} - k_1 \cdot D + \left(k_2 \cdot D + k_2 + k_3\right) \left(a_2 \cdot e^{\lambda_1 \cdot t} + a_4 \cdot e^{\lambda_2 \cdot t} + P_{Cp eq}\right)
\]

At \(t = 0\), the exponentials are 1:

\[0 = \lambda_1 \cdot a_2 + \lambda_2 \cdot a_4 - k_1 \cdot D + k_2 \cdot D \cdot a_2 + k_2 \cdot a_2 + k_3 \cdot a_3 + k_1 \cdot D \cdot a_4 + k_2 \cdot a_4 + k_3 \cdot a_3 + k_1 \cdot D \cdot P_{Cp eq} + k_2 \cdot P_{Cp eq} + k_3 \cdot P_{Cp eq}\]
Solve for $a_3$ and $a_4$:

**a3:**

\[
a_3 = -a_2 - P_{C_{eq}}
\]

\[
0 = \lambda_1 a_2 + \lambda_2 a_3 + \lambda_3 a_2 - \frac{a_3}{k_1 P_{C_{eq}}} - k_2 P_{C_{eq}} + k_3 P_{C_{eq}} + k_4 P_{C_{eq}} + k_5 D + k_6 D + k_7 D + k_8 D + k_9 D
\]

\[
0 = \lambda_1 a_3 + \lambda_2 a_3 - \lambda_3 a_3 - \frac{a_3}{k_1 P_{C_{eq}}} - k_2 a_3 + k_3 a_3 + k_4 a_3 - k_5 a_3 - k_6 P_{C_{eq}} - k_7 P_{C_{eq}} + k_8 P_{C_{eq}} + k_9 P_{C_{eq}} - k_1 D
\]

\[
0 = \lambda_1 a_3 + \lambda_2 a_3 - \lambda_3 a_3 + \lambda_4 a_3 - \frac{a_3}{k_1 P_{C_{eq}}} + k_2 a_3 + k_3 a_3 + k_4 a_3 - k_5 a_3 + k_6 P_{C_{eq}} + k_7 P_{C_{eq}} + k_8 P_{C_{eq}} + k_9 P_{C_{eq}} - k_1 D
\]

\[
a_3 = \frac{(k_1 D + \lambda_2 P_{C_{eq}})}{(\lambda_1 - \lambda_2)}
\]

**a4:**

\[
a_4 = -a_2 - P_{C_{eq}} - \frac{a_4}{\lambda_1 - \lambda_2}
\]

\[
a_4 = \frac{-k_1 D - \lambda_2 a_3 + \lambda_1 P_{C_{eq}} + \lambda_2 P_{C_{eq}}}{\lambda_1 - \lambda_2}
\]

\[
a_4 = \frac{-\lambda_1 P_{C_{eq}} - k_1 D}{\lambda_1 - \lambda_2}
\]

**Final $P_{C_{eq}}$ solution:**

\[
P_{C_{eq}} = \frac{(k_1 D + \lambda_2 P_{C_{eq}})}{(\lambda_1 - \lambda_2)} - \frac{(k_2 D + k_3 + k_4 + k_5)}{\frac{\left(\frac{k_1 D + k_2 + k_3 + k_4}{2}\right)^2 - 4\left(1\right)\left(\frac{k_1 k_2 D + k_1 k_3 D + k_1 k_4}{2}\right)}{\left(\frac{k_1 - \lambda_2}{\lambda_1 - \lambda_2}\right)}} + \frac{-\lambda_1 P_{C_{eq}} - k_1 D}{\lambda_1 - \lambda_2} + \frac{k_1 k_2 D}{\left(\frac{k_1 k_2 D + k_1 k_2 D + k_1 k_4}{2}\right)}
\]
Total Current Equation (Washin):

\[ I_{\text{washin}} = i \cdot N \cdot P_o + i \cdot N \cdot f \cdot P_{Cp} = i \cdot N \left( P_o + f \cdot P_{Cp} \right) \]

\[ I_{\text{washin}} = i \cdot N \left( \left( \frac{-k_1 \cdot D - \lambda_2 \cdot (1 - P_{o_{eq}})}{\lambda_1 - \lambda_2} \cdot e^{\lambda_1 \cdot t} + \frac{k_2 \cdot D + \lambda_1 \cdot (1 - P_{o_{eq}})}{\lambda_1 - \lambda_2} \cdot e^{\lambda_2 \cdot t} + \frac{k_3 \cdot k_4}{(k_2 \cdot k_3 \cdot D + k_2 \cdot k_4)} \right) + f \left( \frac{k_1 \cdot D + \lambda_2 \cdot P_{o_{eq}}}{(\lambda_1 - \lambda_2)} \cdot e^{\lambda_1 \cdot t} \right) \right) \]

Washout Derivation:

The washout equation has the same general form as the washin equation:

\[ I = i \cdot N \cdot P_o + i \cdot N \cdot f \cdot P_{Cp} = i \cdot N \left( P_o + f \cdot P_{Cp} \right) \]

However, \( P_{o_{eq}} \) is now zero because the drug concentration, \( D \), is now zero, and \( P_{Cp_{eq}} \) is now one because the drug concentration is only found in the denominator and is now zero, leaving \( \frac{k_3 \cdot k_4}{k_3 \cdot k_4} \).

We also have new boundary conditions, where \( t \) is now called \( t' \) and equals a new zero point, and the new starting point is at the end of the washin. \( P_o \) does not equal one, but some number between zero and one, closer to zero since we should be near equilibrium and if a strong binder, then almost complete block. So, now \( P_o = P_{o'} \). Likewise, \( P_{Cp} \) no longer equals zero, because it has been occupied by drug during the washin.

So now, \( P_{Cp} = P_{Cp'} \).

So, here is the general form of the washout equation:

\[ I_{\text{washout}} = i \cdot N \left( \left( a_5 \cdot e^{\lambda_1 \cdot t'} + a_6 \cdot e^{\lambda_2 \cdot t'} + 1 \right) + f \left( a_7 \cdot e^{\lambda_1 \cdot t'} + a_8 \cdot e^{\lambda_2 \cdot t'} + 0 \right) \right) \]

or \( P_{Cp} = \left( a_5 \cdot e^{\lambda_1 \cdot t'} + a_6 \cdot e^{\lambda_2 \cdot t'} + 1 \right) + \left( a_7 \cdot e^{\lambda_1 \cdot t'} + a_8 \cdot e^{\lambda_2 \cdot t'} + 0 \right) \)
We have new relaxation coefficients (a5, a6, a7, and a8) to obtain:

Solve for a5 and a6:

To get a5, use the $P_o^{t'}$ equation, but with $P_{o_{eq}}^{t'}$ equal to one:

Boundary conditions:

At $t'=0$, $P_{o_{eq}} = 1, P_o = P_o^{t'}, P_{cp} = P_{cp}^{t'}$

\[ P_o^{t'} = a_5 e^{\lambda_1 t'} + a_6 e^{\lambda_2 t'} + 1 \]

Also from equation (6):

\[ k_j \cdot P_{cp} = \left( \frac{dP_o^{t'}}{dt} + k_j \cdot D \cdot P_o^{t'} \right) \Rightarrow k_j \cdot P_{cp} = k_j \cdot D \left( a_5 e^{\lambda_1 t'} + a_6 e^{\lambda_2 t'} + 1 \right) + \left( \lambda_1 a_5 e^{\lambda_1 t'} + \lambda_2 a_6 e^{\lambda_2 t'} \right) \]

At $t'=0$

\[ k_j \cdot P_{cp} = k_j \cdot D (a_5 + a_6 + 1) + (\lambda_1 a_5 + \lambda_2 a_6) \]

and $P_o^{t'} = a_5 + a_6 + 1$ to solve for and plug into a6:

\[ P_o^{t'} - a_5 - 1 = a_6 \]

\[ k_j \cdot P_{cp} = k_j \cdot D (a_5 + (P_o^{t'} - a_5 - 1) + 1) + (\lambda_1 a_5 + \lambda_2 (P_o^{t'} - a_5 - 1)) \]

D is equal to zero, so the first term goes away:

\[ k_j \cdot P_{cp} = (\lambda_1 a_5 + \lambda_2 (P_o^{t'} - a_5 - 1)) \]

\[ k_j \cdot P_{cp} = \lambda_1 a_5 + \lambda_2 (P_o^{t'} - a_5 - 1) \]

\[ k_j \cdot P_{cp} - a_5 (\lambda_1 - \lambda_2) + \lambda_2 (P_o^{t'} - 1) \]

\[ \frac{k_j \cdot P_{cp} - a_5 (P_o^{t'} - 1)}{\lambda_1 - \lambda_2} = a_5 \]

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To get a6, plug a5 into \( P_o' = a_2 + a_6 + 1 \):

\[
P_o' = \frac{k_2 \cdot p_{c_p}' - \lambda_2 (P_o' - 1)}{\lambda_1 - \lambda_2} + a_6 + 1
\]

\[
P_o' - \frac{k_2 \cdot p_{c_p}' - \lambda_2 (P_o' - 1)}{\lambda_1 - \lambda_2} - 1 = a_6
\]

\[
\frac{P_o' \cdot (\lambda_1 - \lambda_2) - k_2 \cdot p_{c_p}' + \lambda_2 (P_o' - 1) - (\lambda_1 - \lambda_2)}{\lambda_1 - \lambda_2} = a_6
\]

\[
-\frac{k_2 \cdot p_{c_p}' + \lambda_1 (P_o' - 1)}{\lambda_1 - \lambda_2} = a_6
\]

Use initial conditions to get a7 and a8:

At \( t' = 0 \): \( p_{c_p} = p_{c_p}' \)

\( p_{c_p}' = 0 \)

Equation 1:

\( p_{c_p}' = a_7 + a_8 + 0 \)

Equation 2:

\( p_{c_p} - p_{c_p}' \) at \( t' = 0 \):

\[
(k_3 - k_2 \cdot D) \cdot p_{c_p}' = \left( \frac{dp_{c_p}}{dt} - k_2 \cdot D + k_2 \cdot D \cdot p_{c_p} + k_2 \cdot p_{c_p} + k_4 \cdot p_{c_p} \right)
\]

At \( t' = 0 \), the exponentials are 1:

\[
(k_3 - k_2 \cdot D) \cdot p_{c_p}' = \lambda_1 \cdot a_7 + \lambda_2 \cdot a_8 - k_2 \cdot D + (k_2 \cdot D + k_2 + k_3) \cdot (a_7 + a_8)
\]

All k1D are zero at this new starting (i.e. the end of washin):

\[
(k_3) \cdot p_{c_p}' = \lambda_1 \cdot a_7 + \lambda_2 \cdot a_8 + (k_2 + k_3) \cdot (a_7 + a_8)
\]
Solve for \( a_7 \) and \( a_8 \):

\( a_7: \)
\[
P_{C_p} \cdot a_7 = a_8 \\
(k_3) \cdot P_{C} = \lambda_1 \cdot a_7 + \lambda_2 \cdot a_8 + k_2 \cdot a_7 + k_2 \cdot a_8 + k_3 \cdot a_7 + k_3 \cdot a_8 \\
(k_3) \cdot P_{C} = \lambda_1 \cdot a_7 + \lambda_2 \cdot (P_{C_p} \cdot a_7) + k_2 \cdot a_7 + k_2 \cdot (P_{C_p} \cdot a_7) + k_3 \cdot a_7 + k_3 \cdot (P_{C_p} \cdot a_7) \\
P_{C_p} \cdot k_3 = a_7 (\lambda_1 - \lambda_2) + P_{C_p} \cdot (\lambda_2 + k_2 + k_3) \\
\frac{P_{C_p} \cdot k_3 - P_{C_p} \cdot (\lambda_2 + k_2 + k_3)}{\lambda_1 - \lambda_2} = a_7
\]

\( a_8: \)
\[
P_{C_p} \cdot a_8 = a_8 \\
P_{C_p} \cdot k_3 = \frac{P_{C_p} \cdot (\lambda_2 + k_2 + k_3)}{\lambda_1 - \lambda_2} = a_8 \\
\frac{P_{C_p} \cdot (\lambda_1 - \lambda_2) - P_{C_p} \cdot k_3 + P_{C_p} \cdot (\lambda_2 + k_2 + k_3)}{\lambda_1 - \lambda_2} = a_8 \\
\frac{-P_{C_p} \cdot k_2 + P_{C_p} \cdot (\lambda_1 + k_2 + k_3)}{\lambda_1 - \lambda_2} = a_8
\]

**Total Current Equation (Washout):**

Remove all terms with \( D \):

\[
\lambda_3 = -\frac{(k_2 + k_2 + k_3) + \sqrt{(k_2 + k_2 + k_3)^2 - 4 \cdot (1 \cdot k_2 k_2)}}{2}
\]

\[
\lambda_4 = -\frac{(k_2 + k_2 + k_3) - \sqrt{(k_2 + k_2 + k_3)^2 - 4 \cdot (1 \cdot k_2 k_2)}}{2}
\]

\[
I_{\text{washout}} = i \cdot N \left( \left[ 1 + \frac{k_4 \cdot P_{C_p} \cdot (P_{o}^{-1} \cdot 1)}{\lambda_3 - \lambda_4} \cdot \lambda_3 \cdot \phi + \frac{-k_4 \cdot P_{C_p} \cdot (P_{o}^{-1} \cdot 1)}{\lambda_3 - \lambda_4} \cdot \lambda_4 \cdot \phi \right] + \frac{P_{C_p} \cdot k_3 - P_{C_p} \cdot (\lambda_4 + k_2 + k_3) \cdot \lambda_4 \cdot \phi}{\lambda_3 - \lambda_4} \right)
\]

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Appendix B.5: Global Nonlinear Least Squares Curve Fit Program

This appendix shows the Matlab program that was used to perform the global nonlinear least squares fit using the two-site model equation in chapter 7 and Appendix B.4 along with the function to extract the rate constants.

```matlab
Vtranspose t (time) and ydata (current) into row vectors. Use column vectors
% when input is only 1 trace. Use numeric matrix with multiple
% electrophysiology traces.

%The results are shown in the output window as p =
% number, number, number, and number. Those numbers in order from left to right
% are p(1), p(2), p(3), and p(4), and this translates to k1, k2, k3, and k4.2
% k1 is the on rate, k2 is the off rate, k3 is the length of the unstirred
% layer, and k4 is f, the fraction of current through the blocked channel
% (if any).

T = traceLength; Vtrace length row vector used for the for loop iteration count, starting from 0 i.e. 0, end of trace 1, 
% end of trace 2, etc.

xdata = time; Vtime vector in milliseconds

ydata = current; %Current traces concatenated end to end, beginning with washin traces (lowest concentration to highest),
% and then washout traces added to end of washin traces (lowest conc. to highest)

Diff = 2; %Diffusion coefficient in um^2/ ms

Co = concentration; %Concentrations in um. Take concentration into row vector i.e. 10 10 100 100 100 500 500 500 etc.,
% in same concentration order as current row vector

%Non-linear least squares fit of data using model function
%format long

options = optimset('disp','iter', 'LargeScale','off', 'TolFun', 0.0001, 'MaxIter', 100000, 'MaxFunEvals', 10000000);
lb = [2E-3, 2.5E-6, 3E-6, 2E-5, 0]; % k1 is in um^-1 m^-1, k2 is in um^-1, L is in um, and fractional block f.
ub = [1E-3, 2.5E-4, 3E-6, 2E-4, 1];

startingVals = [1E-3, 2.5E-4, 3E-6, 2E-4, 0.5]; % k1 is in um^-1, k2 is in um^-1, L is in um, and fractional block f.

% p, residual, J, Cov, MSE, ErrorModelInfo = multistart(xdata, ydata, @(p, xdata) GlobCh3GMoT3Number(p, T, Co, Diff, xdata, startingVal);

ReducedChiSquare = resnorm / (length(ydata) - length(p));
cl = nparci(p, residual, 'jacobsen', 3);
pCovarianceMatrix = inv((p - p0) * p0) * error(residual);

fprintf('k1 is in the range of [%.2f %.2f] at the 95% confidence level \n', cl(1,:));
fprintf('k2 is in the range of [%.2f %.2f] at the 95% confidence level \n', cl(2,:));

format long
fprintf('k1 = %f um^-1, p(1)*1000000*100000
');
fprintf('k2 = %f um^-1, p(2)*1000000
');
fprintf('k3 = %f um^-1, p(3)*1000000
');
fprintf('k4 = %f um^-1, p(4)*1000000
');
fprintf('k5 = %f um^-1, p(5)
');
fprintf('k6 = %f um^-1, p(6)
');
fprintf('k7 = %f um^-1, p(7)
');
fprintf('k8 = %f um^-1, p(8)
');
fprintf('k9 = %f um^-1, p(9)
');
fprintf('k10 = %f um^-1, p(10)
');
fprintf('k11 = %f um^-1, p(11)
');
fprintf('k12 = %f um^-1, p(12)
');
fprintf('k13 = %f um^-1, p(13)
');
fprintf('k14 = %f um^-1, p(14)
');
fprintf('k15 = %f um^-1, p(15)
');

fprintf('Reduced ChiSquare = %f um^-1, ReducedChiSquare);%

format long
SE = diag(sqrt(pCovarianceMatrix));
```
function [ FredCurrFun ] = GlobChiSqNu3ThresState(p,T,Co,eff,xdata)

%open file that writes FredCurrFun output
%titleID = fopen('fredcurrfun.txt', 'w');

%This equation has four parameters: p1, p2, p3, and p4. p1 is k1 (on rate constant), p2 is k2 (off rate constant), p3 is the length of the unstirred layer (L), p4 is f.

A = zeros(1,3);
B = zeros(1,121210); %This must be used for the prob. (B array) to work, column number of last washin end point.
C = zeros(1,121210);
G = zeros(1,121210);

%Concentration vector used in FredCurrFun and i in the for loop
[r,b] = size(Co);

n = 1; %Used in FredCurrFun to input values in the array correctly

%Fitting Mesh-in
for i = 1:b 
  n = ndata(T(i)+1);
  t = ndata(T(i)+1);
  for j = T(i)+1:T(i+1) 
    R = T(i)+1 to T(i+1) = T(2) = 386610, T(2)+1 = 386611 to T(2+1) = T(3) = 556611, etc...
    L1 = -(p(1),.*Co(i)+p(3).*p(3).*p(4)) + sqrt((p(1),).*Co(i)+p(3).*p(3).*p(4)).^2 - 4.*p(1),.*p(2),.*Co(i)+p(3).*p(3).*p(4))/2;
    L2 = -(p(1),.*Co(i)+p(3).*p(3).*p(4)) - sqrt((p(1),).*Co(i)+p(3).*p(3).*p(4)).^2 - 4.*p(1),.*p(2),.*Co(i)+p(3).*p(3).*p(4))/2;
    P1 = (p(3),.*p(4))./(p(1),.*p(2),.*Co(i)+p(1),.*p(3),.*Co(i)+p(3),.*p(4));
    P2 = (p(1),).*p(3),.*Co(i),.(p(1),).*p(2),.*Co(i)+p(1),.*p(3),.*Co(i)+p(3),.*p(4));
    a1 = -(p(3),.*Co(i)+L2.*(1-p1))/((L1-L2));
    a2 = (p(1),).*Co(i)+L1.*(1-p1))/((L1-L2));
    L3 = (p(1),).*Co(i)+L2.*p2)/((L1-L2));
    L4 = (-L1.*p2-p(1),).*Co(i))/((L1-L2));
    K = a1.*exp(L1.*t) + a2.*exp(L2.*t) + P1;
    L = p(5),.*L3.*exp(L1.*t)+t4.*exp(L2.*t)+P2);
$\text{FredCurfRun}(n) = (K+L)$;

\[ \text{Prob1}(n) = K; \]  % This calculates the probability of being in the open state (i.e. unoccupied channel)

\[ M = L3.*\exp(L1.*t)+L4.*\exp(L2.*t)+p2; \]

\[ \text{Prob2}(n) = M; \]

\[ \text{Prob3}(n) = 1-K-L; \]

\[ B(n) = \text{Prob1}(n); \]  % This takes the prob. of being in the unoccupied channel state and moves it to an array, B

\[ D(1) = B(121210); \]  % This takes the last point of the washin and assigns it to D; if there are multiple

\[ D(2) = B(626449); \]  % Washin traces, then need the prob. for the

\[ D(3) = B(707825); \]  % end of each washin. Example, the B(248450)

\[ D(17) = B(990); \]  % the last point of the first washin and is

\[ D(18) = B(1555277); \]  % assigned to D(1), where (1) represents

\[ D(19) = B(1677555); \]  % the first washin (can be any number).

\[ D(20) = B(1773495); \]

\[ D(21) = B(1869570); \]

\[ D(22) = B(1945435); \]

\[ D(23) = B(4989666); \]

\[ D(24) = B(2353210); \]

\[ D(25) = B(247144); \]

\[ C(n) = \text{Prob2}(n); \]

\[ F(1) = C(121210); \]

\[ F(2) = C(626449); \]

\[ F(3) = C(707825); \]

\[ G(n) = \text{Prob3}(n); \]

\[ H(1) = G(121210); \]

\[ H(2) = G(626449); \]

\[ H(3) = G(707825); \]

\[ t = t + 1; \]

\[ n = n + 1; \]

end
end
A(2) = D(1);
A(3) = D(2);
A(6) = D(3);   % Convert probability of unoccupied to probability of occupied with LC included in prob. calc.
A(15) = 1-D(15);   % A is used for the washout equation so that the washout starts where the washin ended
A(16) = 1-D(16);
A(17) = 1-D(17);
A(18) = 1-D(18);
A(19) = 1-D(19);
A(20) = 1-D(20);
A(21) = 1-D(21);
A(22) = 1-D(22);
A(23) = 1-D(23);
A(24) = 1-D(24);
A(25) = 1-D(25);

X(2) = F(1);   % prob. of Pc
X(5) = F(2);
X(6) = F(3);

Y(2) = H(1);   % prob. of Pc
Y(5) = H(2);
Y(6) = H(3);

% The # in A(##) needs to be a value where k below can start with the column number of the last washin trace. For example, if there are 3 washin traces and 3 washout traces (remember, the trace length starts with 0 in column 1, so washins go from column 2-4, not 1-3 and washouts go 5-7 in this example), then the first A(##) needs to have # = 4, the second # = 5, and the third # = 6. Then, make k below be k = 4:6. This will make k run through T(4)+1:T(##) which is column 4 (final washin) + 1 through column 5 (through the first washout). Then T(5)+1:T(##), which is column 5 + 1 through column 6 (the second washout). Then T(6)+1:T(##), which is column 6 + 1 through column 7 (the third washout), and so on. But, remember A(##), A(##), and A(##) need to be assigned the prob. of washin endpoints. So, make sure the B(#)'s get the washin endpoints, and then call D(#) -> D(1), D(2), D(3), and so on the last washin. This will represent washin 1, 2, 3, etc...
%Fitting Wash-out
)

for k = 2
% Needs to be the first washout column number to the last column number, because A(k)
% is assigned those numbers which is used in the washout.
% And, so that m is assigned the proper

end

% for m = T(k)+1:T(k+1) % Ex. T(4)+1 = 945665+1 = 945666 to T(1+4) = T(5) = 973519

L3 = -(p(2)*p(3)+p(4)) + sqrt((p(2)+p(3)+p(4)).*2 - 4.*p(3).*p(4)))./2;
L4 = -(p(2)+p(3)+p(4)) - sqrt((p(2)+p(3)+p(4)).*2 - 4.*p(3).*p(4)))./2;

a5 = (p(4).*X(k)-L4.*A(k))./(L3-L4);
a6 = (-p(4).*X(k)+L3.*A(k))./(L3-L4);
a7 = (Y(k).*p(3)-X(k).*L4+p(2)+p(3))/.(L3-L4);
a8 = -(Y(k).*p(3)+X(k).*L3+p(2)+p(3))/.(L3-L4);

PredCurrFun(n) = (1+(a5.*exp(L3.*t))+a6.*exp(L4.*t))+p(5).*((a7.*exp(L3.*t))+(a8.*exp(L4.*t)));

end

%Write PredCurrFun to file
fprintf(fileID,'%d\n',PredCurrFun);
fclose(fileID);

fprintf('A1 = %g\n', A(2));
fprintf('A2 = %g\n', A(3));
fprintf('A3 = %g\n', A(4));
fprintf('A4 = %g\n', A(17));
fprintf('A5 = %g\n', A(18));
fprintf('A6 = %g\n', A(19));
fprintf('A7 = %g\n', A(20));
fprintf('A8 = %g\n', A(21));
fprintf('A9 = %g\n', A(22));
fprintf('A10 = %g\n', A(23));
fprintf('A11 = %g\n', A(24));
fprintf('A12 = %g\n', A(25));

fprintf('X1 = %g\n', X(2));
fprintf('X2 = %g\n', X(3));
fprintf('X3 = %g\n', X(4));

fprintf('Y1 = %g\n', Y(2));
fprintf('Y2 = %g\n', Y(3));
fprintf('Y3 = %g\n', Y(4));

end
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M2 Transmembrane Domain in Lipid Bilayers and Dodecylphosphocholine Micelles


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Education
Brigham Young University – Provo, UT
Ph.D. Department of Physiology and Developmental Biology (PDBio) – Biophysics, received July 2020. Thesis: Governing Dynamics of Divalent Copper Binding by Influenza A Matrix Protein 2 His37-Imidazole.

Brigham Young University – Idaho – Rexburg, ID
Bachelor of Science, Physics (minor: biology), July 2012. Honors thesis: Surface Distribution (Topography) and X-Ray Emission from Scotch Tape (Triboluminescence).

Funding
Brigham Young University – Department of PDBio – Provo, UT
Scholarship - full tuition (fall 2013-summer 2020)
Research Assistantship (fall 2013-summer 2020)
Teaching Assistantship (winter 2014-winter 2020)

Awards
“Superior Oral Presentation”, 1st place, BYU Graduate Presentation Competition, December 2016

Research Experience
BYU Department of PDBio – Biophysics – Provo, UT
Advisor: David Busath
Project 1: (Experimental): Developed novel copper complexes as influenza A M2 S31N variant blockers. Explored the copper complexes’ binding properties through the two-electrode voltage clamp electrophysiology (TEVC) method, miniplaque assay, and cytopathic effect assay. Results from this work show significant proton block by binding to the His37 imidazoles cluster which
forms the proton selective filter. Performed site-directed mutagenesis on Udorn/72 M2 plasmid to test S31N, G34E, and H37 M2 variants. Further analysis of copper complex – M2 binding properties were done using isothermal titration calorimetry (ITC), and nonlinear least squares curve fitting with parameters from a two-site binding model using electrophysiology data to obtain binding rate constants. To confirm copper complex purity and structure, characterization was done using uv-vis, NMR, and x-ray crystallography. (June 2013 - present)

Project 1: (Computational Chemistry and Biophysics): Parameterized novel copper complexes for QM/MM simulations using VMD and Gaussian. Quantum mechanics – molecular mechanics hybrid simulations (QM/MM) to obtain free energy profiles of copper complex binding to M2, QM simulations using Gaussian to obtain enthalpy of binding of copper complexes to histidine sidechains. (June 2013 - present)

Project 2: (Experimental): Explored partial block of amantadine (AMT) and rimantadine (RMT) in influenza A M2 S31N through TEVC electrophysiology and molecular dynamics simulations. Analysis of partial block by AMT and RMT was done using nonlinear least squares curve fitting with parameters from a one-site binding model using electrophysiology data to obtain binding rate constants. Results from this work show no partial block by AMT, but significant partial block by RMT in the S31N variant. (June 2014 – present)

Project 2: (Computational Chemistry and Biophysics): Used adaptive biasing force molecular dynamics simulation method to obtain potential of mean force (PMF) plots and analyze drug binding thermodynamics to M2. Kramer’s electro-diffusion equation was used to predict rate constants from the PMFs, which were compared with the electrophysiology rate constants to connect the simulation results to electrophysiology results. (June 2014 – present)

Project 3: (Experimental): Collaboration with Antonios Kolokouris (University of Athens, Greece). Analyzed adamantane derivative M2 blockers using TEVC electrophysiology to obtain rate constants. (June 2018 – present)
Project 3: (Computational Chemistry and Biophysics): Parameterized adamantane derivatives using VMD and Gaussian. Used steered-molecular dynamics to observe leaving times (unbinding) of drugs from M2.

Huntsman Cancer Institute Salt Lake City, UT
Integrated evaluation bioinformatics method development for analysis and eventual classification of unclassified sequences variants in breast cancer susceptibility genes BRCA1 and BRCA2. Homology modeling and mutation analysis of the BRCA2 gene. (June 2012 – May 2013)

BYU-I Department of Physics and Biology Rexburg, ID
Advisors: Todd Lines (physics) and Clair Eckersell (biology)
Project 1: Analysis of triboluminescent properties of Scotch Tape by examination of surface topographical characteristics using white-light interferometry with the Olympus BX61 microscope and 3D optical metrology methods. Further analysis of triboluminescence by surface charge distribution and velocity dependent x-ray counts. (June 2010 – June 2012)

Project 2: Development of a predictive model for myocardial infarction using chaos Theory. (June 2011 - June 2012)

Teaching Experience
BYU Department of PDBio – Biophysics Provo, UT
Busath Laboratory Group Leader and Manager. Managed teams of undergraduate students in their research and publications. Managed the laboratory including instrumentation, supplies, and budget.

Biophysics. Teaching Assistant. Helped write and grade problem sets and exams. Assisted students individually with problems and material they found difficult to understand. (Fall semesters: 2013 – 2019)

Molecular Dynamics. Teaching Assistant. Helped students with simulation setup and simulation analysis and instructed on principles of molecular dynamics. (Spring terms: 2014 – 2018)

BYU-I Department of Physics and Biology                    Rexburg, ID

Electricity and Magnetism. Teaching Assistant. Prepared problem sets and exams. Helped instruct students on electromagnetic theory.

Anatomy & Physiology. Teaching Assistant. Helped students understand the principles of anatomy and physiology. Helped prepare exams and run anatomy and physiology review sessions and labs.

Publications


Conference Presentations


Meeting of the Northwest Section of the APS in Corvallis, OR, October 2011.

**Invited Talks**

“The Graduate Student Experience and Biophysics Research”, BYU-Idaho Department of Physics colloquium, 2018.

**Professional Society Memberships**

Biophysical Society (2013 - present)

American Association for the Advancement of Sciences, AAAS (2016 - present)

**Skills**

Gel electrophoresis, mRNA synthesis, site-directed mutagenesis, molecular biology techniques (plasmid cloning, transformation, digest, and linearization), TEVC electrophysiology, NMR, mass spectrometry, uv-vis, electron microscopy

Programming: Linux, Python, C/C++, Matlab, Maple

Computational Biophysics and Chemistry: Terachem, Gaussian, ORCA, MOPAC, CHARMM, NAMD, VMD

**Languages**

English (native), Portuguese (proficient)

**Activities**

Especially For Youth (EFY) counselor, BYU-Idaho Get Connected I-Team Mentor and Volunteer and Council Member, BYU-Idaho Fencing Club instructor, BYU Ultimate Frisbee Work team captain, Aikido martial arts assistant instructor, flying Cessna 170 and 180 aircraft