Copper Complexes as Influenza Inhibitors

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ABSTRACT

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Influenza A is responsible for the death of an average of 85 000 people per year in the western hemisphere and the hospitalization of many more. Currently-available anti-influenza drugs were designed to target the M2 proton channel and disrupt the process of viral replication but widespread mutations to the M2 channel have rendered these drugs generally ineffective. In preparation for the event of a flu pandemic a new class of anti-influenza drugs is needed. Copper ions have been shown to successfully block the M2 channel and in this paper several novel compounds are complexed with copper and tested as potential M2 inhibitors via electrophysiology tests and miniplaque assays. The results show that several neutral compounds successfully block mutant and wild type viruses in oocyte studies. However, animal tests show no efficacy. Divalent compounds are shown to be ineffective in all cases. Monovalent compounds are hypothesized to be the best suited to M2 inhibition due to the channel's design, which is intended to transport monovalent protons. Future studies should include further testing of monovalent copper complexes.

Keywords: Influenza A; M2; S31N; Copper; Inhibitor

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I thank Dr. Roger Harrison for sponsoring me in his lab and mentoring me through the process of doing research and writing a capstone report. I also thank Johnny Lynch and Ethan Damron for their assistance and advice throughout my research and for being fantastic coworkers.

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1 Introduction

1.1 Motivation

Influenza A is a widespread virus which causes an average of 85,000 deaths per year in the western hemisphere.[1] Viral resistance to antiviral drugs is now too widespread to continue using the current options and new lines of drugs must be produced to counter the possibility of an epidemic.

1.2 Research Structure

This research is being done as a joint effort between Dr. David Busath of the BYU College Life Sciences and Dr. Roger Harrison of the BYU College of Physical and Mathematical Sciences. Dr. Harrison's lab is primarily responsible for synthesizing and isolating materials. Dr. Busath's lab performs the tests of those materials to determine stability, toxicity, and efficiency against both wild type and mutant strains of Influenza.

1.3 Viral Reproduction

The Influenza virus reproduces quickly and effectively by invading host cells and commandeering the cellular machinery. When a person is first exposed to influenza virus it is in the form of inactive virions which are incapable of autonomous movement. These virions flow

through the blood until they bind to a cell capable of hosting their reproduction. During the process of binding a signal is released which causes the cell to absorb the virion through the cell membrane and store it in an endosome, which is tasked with delivering molecules to destinations around the cell. In the process of normal endosomal function the pH inside the endosome decreases which activates the virion by means of the M2 proton channel.[2]

The M2 proton channel is critical to the insertion of viral RNA into the host cell's nucleus. When endosomal pH drops below a specific level, the channel is activated and protons are transported to the interior of the virion. The M2 channel accomplishes this function by a system of attraction and repulsion wherein the positively charged protons are attracted to negatively charged amino acid residues inside the channel. Once inside, the positive charge facilitates a conformational change which closes the outer portion and opens the inner portion of the channel. This movement effectively pumps the protons into the virion, acidifying it.[3]

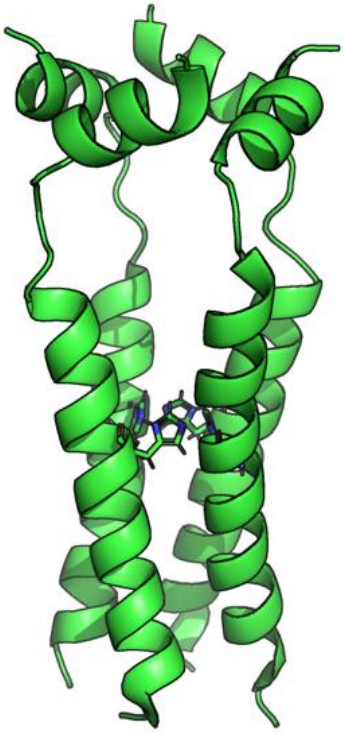

Figure 1.1 Structure of M2 proton channel. Created by Ethan Damron: used with permission.

Acidification of the viral interior prepares viral ribonucleoproteins (vRNPs) for dissociation from their storage proteins and for release into the cell. At the same time, the virion binds to the membrane of the endosome and begins a process of fusing membranes, which results in the viral interior being released to the interior of the cell. From there, vRNPs are actively transported by cellular machinery into the nucleus where they serve as templates for the production of viral proteins and guide the construction of new virions.[2] At the end of the reproductive process, newly formed virions exit the cell by

budding and are released into the bloodstream to continue the cycle of infection and reproduction.

1.4 Influenza Inhibition

Wild-type strains of Influenza have been effectively stopped by adamantane-based drugs such as Amantadine and Rimantadine which block the function of the M2 channel.[4] (See Figure 1.2) These drugs interact with aspartate and leucine amino acid residues which are found lining the inside of the channel and hold the channel in the closed position. This prevents the pumping action from occurring and the interior of the virion is not acidified; consequently, replication is halted in all affected virions. If treatment doses are high enough, all virions are incapacitated and are eventually degraded by the lysosome.

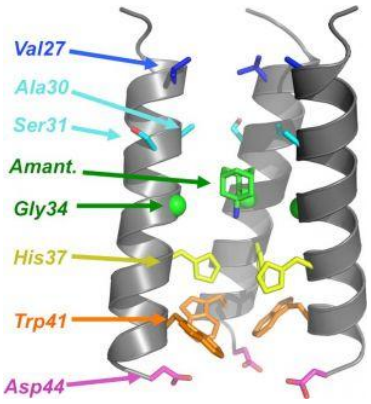

Figure 1.2 Amantadine blocking the M2 channel. Image from UCSF School of Pharmacy

As shown in Figure 1.3, new strains of Influenza including V27A, L26F, and S31N are resistant to antiviral drugs due to changes to the M2 channel.[5,6] These mutant strains have lost or replaced the targeted residues such that adamantane-based drugs are either unable to bind permanently in the channel or are unable to prevent the pumping action even while the drugs are bound. These mutations give near-complete resistance to the drugs in question. The success of

these mutant Influenza strains against treatment has helped them spread rapidly around the world.

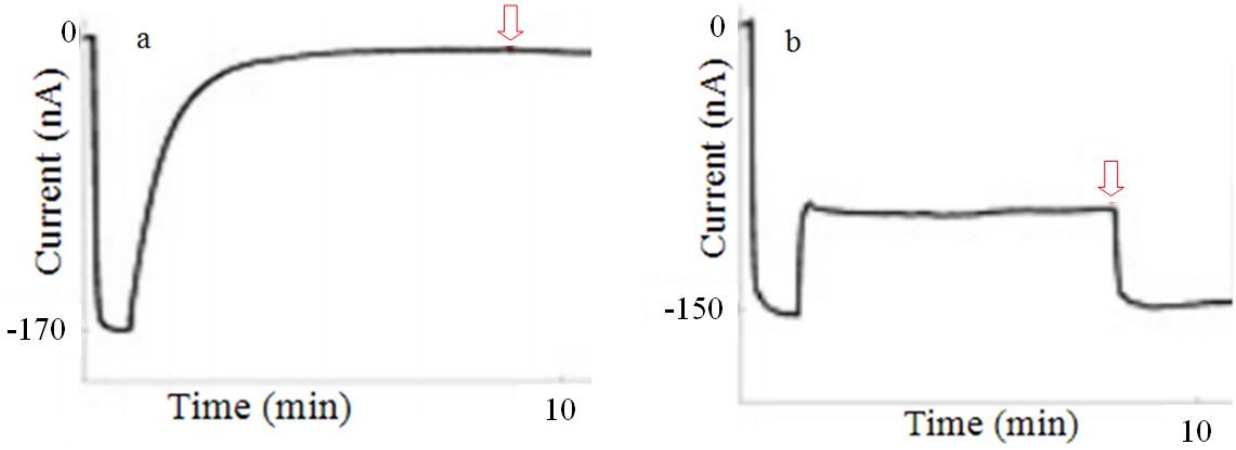

Figure 1.3 Amantadine vs. the wild-type (a) and the S31N mutant (b) M2 proton channel. A negative current indicates protons flowing through the channel. The red arrow indicates washout, where any excess amantadine was removed. A detailed explanation of these electrophysiology tests is given in Section 2.3. Images produced by Gordon et al.

Copper ions have been shown to effectively inhibit the M2 channel, but at the concentration necessary for an efficient block the toxicity is too high for them to be used as treatment.[7] The ion flow test shown in Figure 1.4 confirms that Cu^{+2} ions will enter the M2 channel and bind irreversibly. This is especially significant because the primary binding site for the Cu^{+2} ions, made of several histidine amino acids, has been shown in past studies to be absolutely conserved (i.e. it is impossible for the virus to change or remove those amino acids and survive). In these initial tests the Cu^{+2} ions were delivered by treating infected cells with $CuCl_2$. The toxicity of $CuCl_2$ and free Cu^{+2} ions in the body makes it necessary to develop a line of molecules capable of bearing Cu^{+2} ions to the M2 channel without releasing them into the cells.

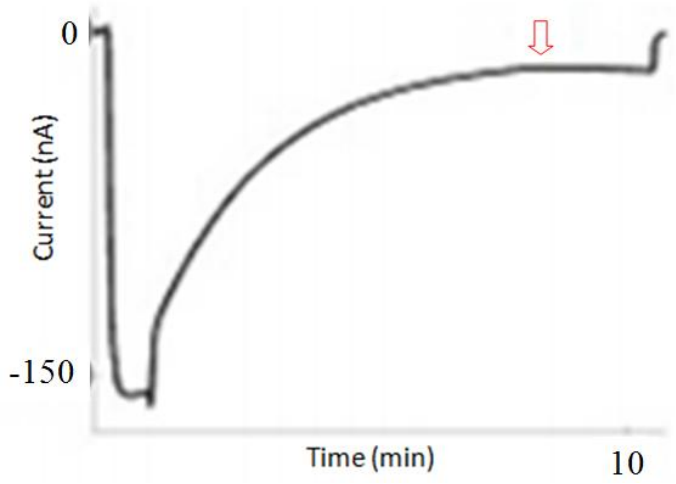

Figure 1.4 Free copper ions inhibiting the S31N mutant M2 proton channel. Image produced by Gordon et al.

We hypothesized that molecules designed to be similar to old antiviral drugs but also able to carry Cu^{+2} ions would be successful inhibitors of both mutant and wild-type strains of influenza. The adamantane-related sections of the molecule would help direct it into the M2 channel while other ligands (i.e. arms) would hold the Cu^{+2} ion safely in place. We further theorized that such copper-bearing molecules would be able to bind irreversibly in the M2 channel.

Several neutral copper complexes, such as those displayed in Figure 1.5, have had some success inhibiting the M2 channel but divalent complexes have been unsuccessful.[7] In ion flow testing of several neutral complexes we determined that they permanently and completely blocked the M2 channel, as demonstrated in Figure 1.6. However, the concentration necessary for efficacy was in the micromolar (μM) range, which is too high for commercially available drugs. Moreover, further testing against the virus in live animals has shown no effect whatsoever. Testing of divalent complexes (i.e. complexes with a +2 formal charge) had no effect even in the ion flow tests.

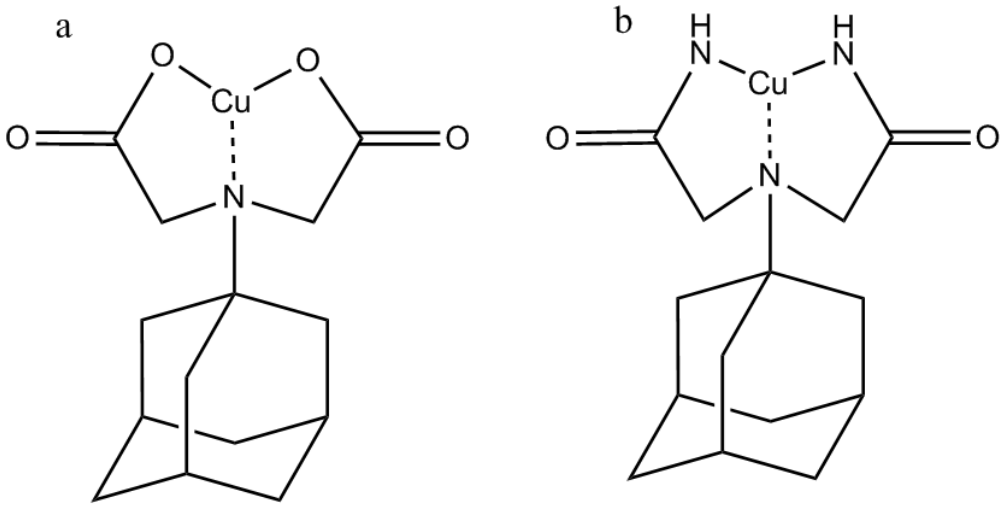

Figure 1.5 Structure of Cu[Amt-IDA] (a) and Cu[Amt-IDAA] (b).

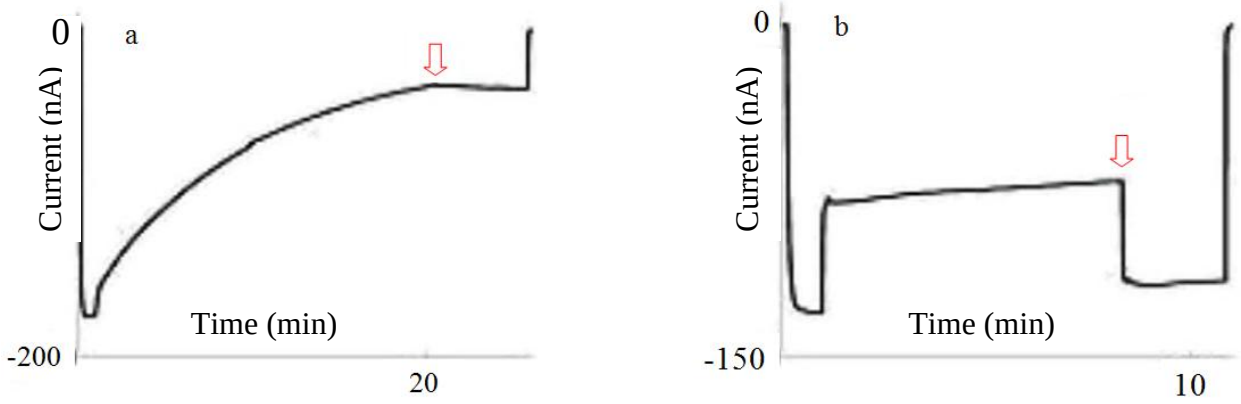

Figure 1.6 Amt-IDA with (a) or without (b) a Cu^{+2} ion attached inhibiting the S31N mutant M2 channel. At the red arrow, any unbound molecules were washed out. The copper-bound complex blocked proton flow more fully and more permanently than did the non-copper-bearing complex. Images produced by Gordon et al.

Our current synthesis and testing are focused on monovalent complexes which we believe may be more effective than both the neutral and the divalent complexes. The M2 channel is intended to receive protons, which have a +1 charge, and might be more receptive of the monovalent compounds.

1.5 Overview

This paper will discuss the synthesis and testing of several neutral and divalent compounds. The methods of synthesis and testing will be explained and the results will be analyzed. Data from the tests will be used to draw the conclusion that these compounds do not show sufficient potential to justify continued research due to their lack of efficiency, stability, or safety.

2 Methods

This chapter contains information regarding the identification, synthesis, and characterization of novel copper-bound complexes and the method of testing against wild-type and mutant influenza viruses.

2.1 Identification of Complexes

We identified several scaffold molecules and ligands which can be assembled into complexes which, we theorized, will transport copper safely through the body. The first scaffold of interest was 1,4,7-triazacyclononane (TACN) which is displayed as the central ring in Figure 2.1 (a) and Figure 2.1 (b). Ligands of interest were methyl-imidazole [at the bottom of Figure 2.1 (a and b) and the top right of Figure 2.1 (c)] and adamantane [at the top of Figure 2.1 (a and b) and the bottom of Figure 2.1 (c)]. Methyl-imidazole was chosen for its ability to help bind Cu^{2+} ions while adamantane is the primary structure of the old Influenza drugs and is known to interact strongly with the M2 channel.

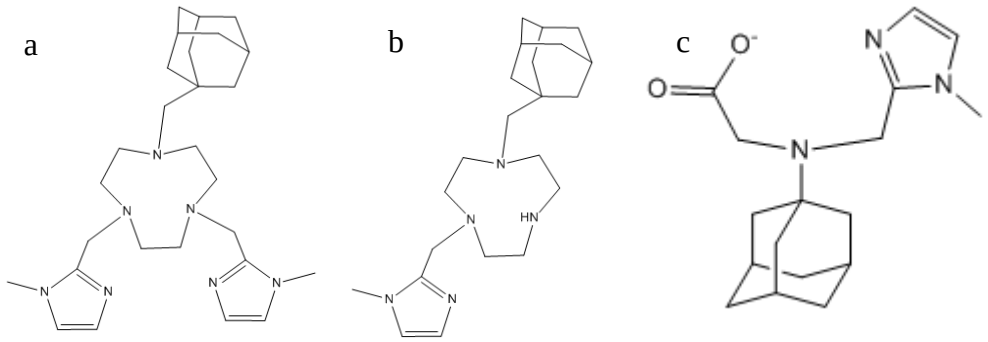

Figure 2.1 Structure of TACN-adamantyl-di-imidazole (a), TACN-adamantyl-imidazole (b), and Amantadine-imidazole-acetate (c).

I synthesized and isolated the molecules we had chosen and then characterized them using mass spectrometry (MS) and nuclear magnetic resonance (NMR). I then complexed the molecules with copper to form the copper compounds for testing. The final forms of the complexes are shown in Figure 2.2.

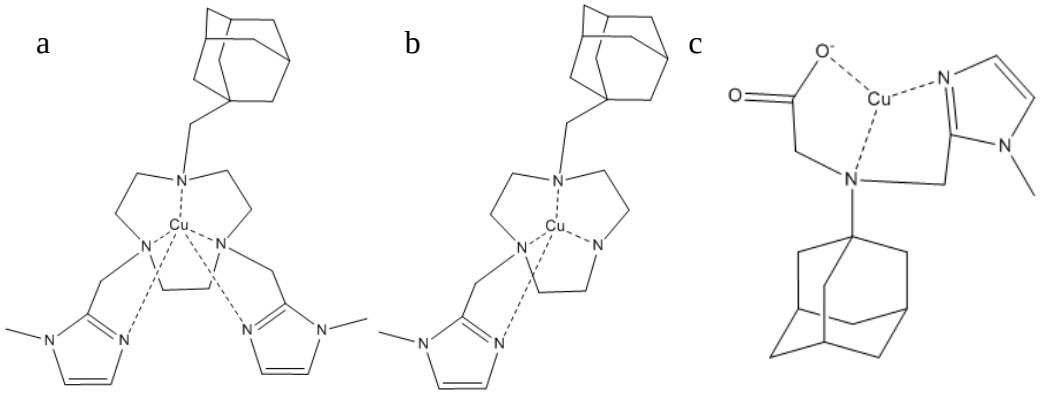

Figure 2.2 Structure of Copper TACN-adamantyl-di-imidazole (a), Copper TACN-adamantyl-imidazole (b), Copper amantadine-imidazole-acetate (c). Dashed lines indicate attraction between lone pair electrons from nitrogen and oxygen atoms with the positively charged copper ion.

2.2 Synthesis

The syntheses for the several compounds and copper complexes are as follows:

TACN[8]: A solution of tetrabutylammonium bromide was made by dissolving 1.04g in 4mL of dH_2O and set aside. In a 500mL round-bottom (rb) flask 50mL of dH_2O were combined with 16.2g K_2CO_3. The flask was placed in an oil bath heated to 90° C and a stir bar was added to agitate the mixture. Tri-tosyl diethylene triamine (19.82g) was added and allowed to dissolve. Solid NaOH (12.72g) was added slowly to prevent boiling. After complete dissolution of the NaOH the solution of tetrabutylammonium bromide was added followed by 160mL of toluene. Finally, 12mL of 1,2-dibromoethane were added dropwise and the mixture was heated and stirred for 16 hours. After 16 hours the mixture had formed a wispy, white solution and was allowed to cool to room temperature. The congealed substance was placed in a Buchner funnel with filter paper and washed with dH_2O. After pulling the water off by vacuum filtration, the product was dried by heating to 50° C in reduced pressure. Drying continued overnight.

The dried white product of was placed in an oven at 120° C for 30 minutes for final drying. After cooling, 20.7g of product were weighed. This indicated roughly 100% yield. Concentrated H_2SO_4 (70mL, 18 molar) and a stir bar were added to a flat-bottomed round flask and placed in an oil bath heated to 120° C. The white product was added in increments of about 10g per 5 minutes. It rapidly dissolved into a black solution. The solution was left heated and stirred for 5 days. After heating, the solution was sealed and cooled overnight. A solution of cold absolute EtOH/Ethyl Ether (150mL/90mL) was prepared in an Erlenmeyer flask in an ice bath and the black solution was dripped in. The resulting solution was filtered by vacuum filtration using a

Buchner funnel and filter paper. As precipitate collected on the filter paper more of the EtOH/Ethyl Ether solution was used to wash it. A black, clay-like precipitate was left.

The black product was dissolved in dH_2O (just enough to dissolve all of it) and heated to 60° C with stirring for 2 hours. The solution was then cooled and filtered through a vacuum/celite/fritted filter. This produced a clear, amber-colored liquid. Concentrated HCl (20mL) was added with no result. Ethanol (20mL) was then added which caused a tan precipitate to form. The solution was cooled in the fridge overnight to aid precipitation. The precipitate was then gathered by gravity filtration and washed with cold EtOH and Ether. After washing, the precipitate was white.

The white product was dissolved in 50mL dH_2O and 3.55g NaOH were dissolved into the solution. At this point the solution should have been yellow or clear but it turned purple. We believe the purple color may have resulted from a stir bar which had not been cleaned properly. Over the course of several minutes, the color faded. Toluene (50mL) was added and the flask was attached to a Dean-Stark apparatus and refluxed at 110° C until boiling ceased. After the distillation removed the water from the solution we filtered it by gravity to remove any NaCl and washed it with toluene. The solution was then rotary evaporated, leaving a yellow oil which was immediately placed in the freezer. After several hours, large white crystals of TACN had formed from the oil and were placed in a sealed container in a desiccator. The TACN was weighed and found to be 1.2603g. From this we calculated a yield of 27.8%. The purity of the TACN was confirmed by mass spectrometry and by hNMR.

Copper TACN-di-imidazole[9]: All parts of this reaction were run under a nitrogen atmosphere. I prepared 0.2224g of TACN and 0.2392 g of chloroimidazole (CI). The TACN was

dissolved in a minimal amount of dry DMF in a dry, round-bottom flask with a stir bar. The flask was submerged in ice water up to the neck and the CI was added very slowly over 45 minutes. Some product formed immediately and the reaction was allowed to run at room temperature for 5 days. A clear, amber-colored liquid resulted with some solid adhering to the sides of the flask and to the stir bar. Ice water was used to cool the flask and promote final precipitation of product before the solution was filtered by gravity. The product was washed with 50/50 EtOH and Et_2O and then allowed to dry in open air for at least 24 hours. Mass spectrometry confirmed the presence of both the mono- and the di-substituted product

To separate the mixture of mono and di product a column chromatography was prepared using a 0.75 inch glass column and 9.5 inches of activated alumina gel. Over the drying period the product had oiled onto the filter paper. I collected the mixture by running 15mL of chloroform through the filter paper into a vial. The solution of products in chloroform was then added to the column. A strongly acidic mixture of HCl and dH_2O was used as the mobile phase after less polar mixtures failed to move the products through. Approximately 80mL of solution containing the mono product were collected followed by about 200mL of solution containing the di product. NaOH was used to neutralize the acid and the solution was rotary evaporated to dryness. A yellow-tinged salt was left.

The di product was extracted from the solid salt using acetone which dissolved the product while leaving the salt behind. The acetone was then removed by rotary evaporation leaving a pale yellow oil. The purity of the di product was confirmed by NMR. The final yield of the di product was 0.099g.

To complex the di-imidazole TACN with copper I dissolved 0.0208g in dH_2O in a 10mL round-bottom flask with a stir bar. Upon addition of 0.0104g of copper sulfate the solution turned clear blue. A condenser was added to the flask and the solution was left to reflux at 100°C overnight. The water was then removed by rotary evaporation leaving a dark blue solid in the flask. The product was dissolved in minimal methanol with sonication and produced a smoky blue solution. Adding 3mL of ethanol caused a dark gray precipitate to form. Mass spectrometry analysis of the precipitate and the solution revealed there was no product in the precipitate and had been left in the solution. The solution was then dried by rotary evaporation and the product, Copper di-imidazole TACN, was isolated and confirmed by mass spectrometry.

2.3 Testing

The isolated complexes were subjected to electrophysiology tests in oocytes (frog eggs) infected with wild-type or mutant Influenza strains. These tests are prepared by injecting viral mRNA into oocytes followed by chilling them for 72 hours. After incubation of the viral mRNA, probes placed on either side of the cell membrane measured whole-cell current as an indication of proton flow through the M2 channel. The test compound was administered to the infected oocytes and the change in current was recorded. Binding strength was qualitatively measured with a washout which removed any unbound compound and caused any weakly bound compounds to be flushed out of the channel. A persistent block after washout indicated a strongly-binding compound which could permanently block the channel.

Toxicity of the various test compounds was calculated using a cytotoxicity assay. Madin-Darby canine kidney (MDCK) cells were grown in culture and then exposed to varying concentrations of the test compounds or a control. After incubations for 72 hours a staining

technique was used to determine the percentage of living cells remaining in the culture. The data from multiple concentrations of a single test compound were used in a least-squares fit to calculate the concentration at which half of the cells died (CC_{50}).

2.4 Difficulties

The molecules we synthesized have never before been recorded in the literature, so we had to come up with new syntheses for them. We did this by searching for similar types of reactions and modifying the reactants to theoretically yield the proper product. The problem was that while the reactions often yielded the product without an issue they generally used purification techniques that would not work for our product. We often ended up using time-consuming separation columns which worked about half the time and significantly reduced the amount of product we could isolate. One way to respond to this issue was by using more reactants in an attempt to yield more product but the expense of the reactants was another barrier.

3 Results and Conclusions

This chapter presents the results of our tests and our conclusion that our line of complexes has expanded our understanding of how to inhibit the M2 channel but will not serve as useful inhibitors themselves. We also give several comments about possibilities for future research.

3.1 Results

The results of the electrophysiology study for two of the best neutral compounds are given in Figure 3.1. The results of those same compounds tested without a copper ion attached are given in Figure 3.2.

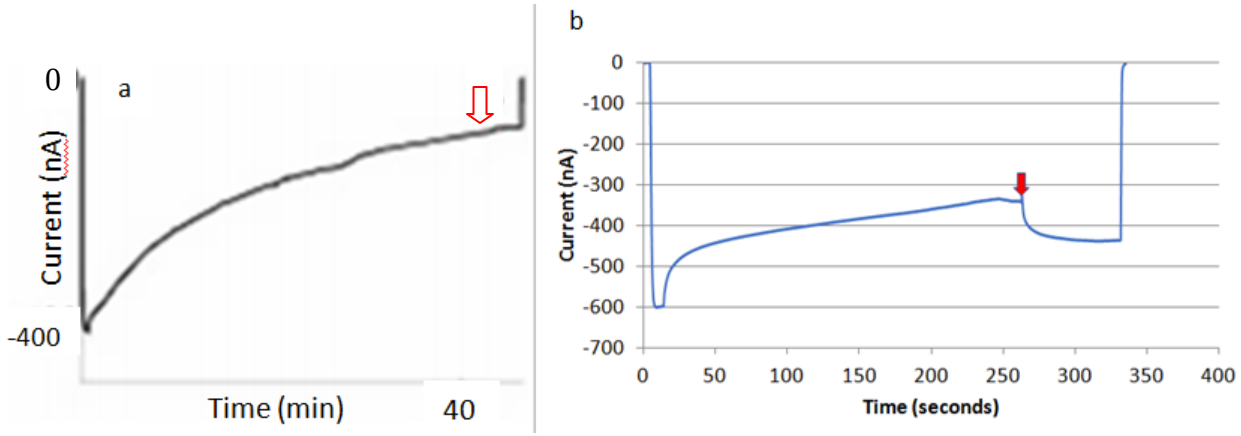

Figure 3.1 Blocking efficiency as measured by electrophysiology test for Cu(Amt-IDA) (a), and Cu(Amt-IDAA) (b). Figure 3.1 (a) used with permission from Gordon et al. Figure 3.1 (b) is the author's original.

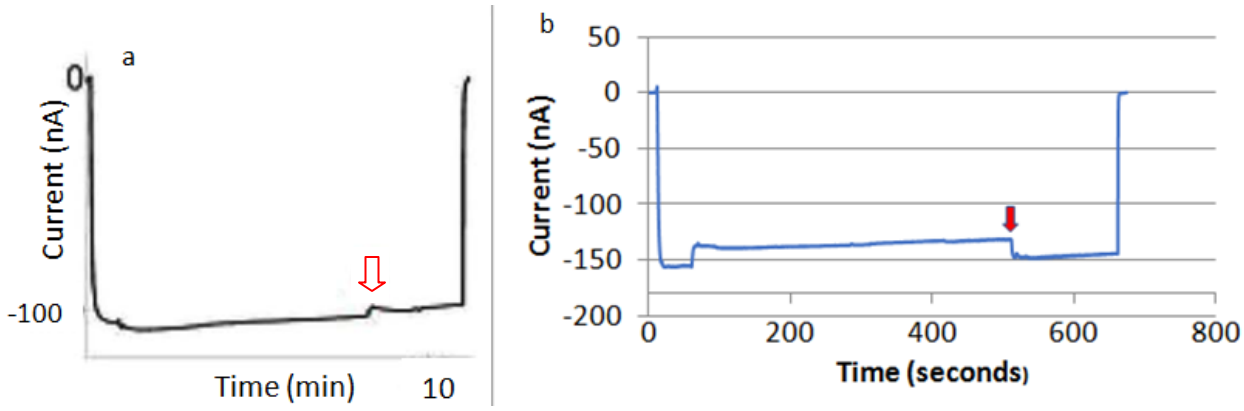

Figure 3.2 Blocking efficiency as measured by electrophysiology test for Amt-IDA (a) and Amt-IDAA (b). Figure 3.2 (a) used with permission from Gordon et al. Figure 3.2 (b) is the author's original.

3.2 Analysis

As shown in Figure 3.1 (a), the best of the neutral complexes completely and permanently blocked the M2 channel (both mutant and wild-type) during electrophysiology tests in oocytes.[7] However, none of the neutral complexes were found to be effective against the mutant channel in rodent tests. Therefore, the neutral complexes provided useful examples of structures with the capacity to block the channel but would be unable to serve as anti-influenza drugs themselves.

Divalent compounds were unable to block the channel efficiently in any case. We suspect this is because the channel, which is structured to bind and transport monovalent protons, is unfit to bind any divalent charge. This would explain why some divalent compounds were able to give a partial block of the channel but were washed out with the rest of the solution. Given these results, divalent compounds have no practical use as M2 inhibitors.

In both cases the copper-bound complexes demonstrated a more efficient blocking power than the compound without copper. Evidence for this conclusion is seen by comparing Figure 3.1 with Figure 3.2. This reinforces the assumption that the copper makes a significant difference in the ability of a molecule to inhibit proton flow through the M2 channel.

3.3 Conclusion

Our goal was to find a copper complex which has potential as an M2 inhibitor. We reaffirmed that free copper ions completely and permanently block both the mutant and wild-type M2 channel. However, the concentration of ions necessary for an efficient block is too toxic to be considered as a drug option. Through testing of novel compounds with TACN and Amantadine as scaffolds we found evidence that using a molecule as a shuttle for copper ions reduces the toxicity and may increase the efficiency of the blocking power. However, due to issues such as lack of permanent binding or non-viability in animal studies, none of the complexes we tested show potential as anti-influenza drugs.

3.4 Future Work

The area of copper-based M2 inhibitors does show some promise. Our results indicate that monovalent complexes may be the best suited of the copper complexes to enter and bind the M2 channel. Therefore, future research should include the synthesis and testing of movovalent complexes. Such research could also benefit from studying the efficacy of the backbone molecules and ligands. These future tests need to determine what type of molecule would be most effective against the mutated M2 channel in animals and then humans. Any new complexes should be developed in conjunction with further study and understanding of the M2 channel to create the best possible match for it.

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