Divalent Copper Complex as an Anti-Influenza Agent



Introduction

Influenza A is involved in thousands of deaths annually 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), respectively, exhibited success initially. Over time, these drugs began exhibiting mixed success as the virus began drug resistance. With the increasingly growing resistance to drugs there is a need to investigate novel compounds as antiinfluenza A agents.

Ala30, Ser31

His-box

Bridging cluster

Trp-basket

Exit cluster

Asp44, Arg45

M2 is a proton channel responsible for the acidification of the viral interior which initiates release of the viral RNA into the host. This protein has been demonstrated to be a feasible target for organic compounds including AMT.

M2 has recently been structurally investigated as a target for metal ions as drug candidates. Among various metal ions that were tested, copper

was demonstrated to best cause M2 inhibition. The copper ion reduced M2 activity by binding to the His37 tetrad located within the homotetramer (see figure), which confers protonselectivity to ion transport by the M2.

In this research, divalent copper compounds are tested against S13N (A/California/07/2009 H1N1) influenza A viral strains in viral mini-plaque assays as well as WT M2 residues 22-62 (A/Udorn/72 H3N2) and its site-specific variant S31N in reconstituted liposomes to assess their anti-viral activity. A novel generation of AMT-based Cu²⁺ complexes was

found to be effective as M2 target-specific blockers.

Methods & Materials

Liposome Assay

Liposomes were prepared as described in *Moffat et al.* and *Peterson et al.*, using *Escherichia* coli (*E.* coli) polar lipid (200 to 300 μL of 25 mg/mL stock in chloroform). The chloroform was dried using a rotary evaporator while blowing nitrogen until a thin lipid layer was formed. M2 residues 22-62 protein from a stock solution (0.603 mg/mL MeOH) was added to a test tube (10 μL/50 μL E. coli lipid) assigned to contain protein (+M2 and +M2+Drug), dried again to a thin film, and then resuspended in internal buffer (50 mM KCl, 50 mM K₂HPO₄, and 50 mM KH₂PO₄, pH 8.0) to yield the overall desired protein concentration (0.1 mg/20 mg lipid/1 mL solution). For controls (-M2-Drug and –M2+Drug), the same procedure was followed. Protein, M2 WT residues 22-62 (A/Udorn/72 H3N2 WT), with or without the S31N

M2 alteration, was provided by Timothy A. Cross and Huajun Qin (Florida State University). Liposomes were resuspended via sonication in internal buffer (240 to 360 µL per test tube) containing drug candidate in the same concentration as drug candidate in external buffer (20, 50, or 100 μM) or no drug candidate. Liposomes were then transferred to centrifuge tubes (1.5 μL) and underwent freeze, thaw, sonicate three times before being extruded through a filter (200 nm pore size, 21 passages) to produce uniformity in liposome diameters. Extruded liposome samples were then subjected to the time dependent treatment.

Viral Mini-Plaque Assay

MDCK cells (from 80-90% confluent 75 cm² flask) were split and 1 mL of trypsinized cells was added to 9 mL of DMEM with 5% FBS, which was further diluted by adding 1 mL of this solution to 29 mL growth medium for a final dilution (1:300) of medium with cells. Medium with cells (1 mL) from the final 1:300 dilution was added to each shell vial. The MDCK cells were allowed to grow to 70-80% confluency while being incubated at 37°C.

DMEM with 5% FBS was discarded from each shell vial and serum-free medium (1 mL) was added to each vial. The mixture was gently mixed before discarding the serum-free medium. Growth medium containing 1% Bovine Serum Albumin or BSA (1 mL) was added to each vial. Drug at various concentrations ranging from 2 to 50 μM was added to vials before gently mixing by swirling each shell vial.

The virus was activated by adding tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin (10 μg) to a solution containing virus (1 mL) and BSA growth medium (4 mL) and mixed gently before allowing the virus-containing trypsin mixture to sit for 30 minutes. This mixture (1 mL) was added to 19 mL of 1% BSA growth medium and gently mixed. The virus suspension was added (100 μ L) to the 1 mL of medium in each shell vial and incubated at 33°C for 16-18 h. The calculated titer for the A/California/07/2009 H1N1 M2 S31N strain used in this assay was 5 x 10⁵ plaque forming units or PFU/mL.

Each coverslip was then removed from the shell vial using forceps, placed on a 96-well plate, and allowed to dry for 30 min. Anti-influenza antibody (Ab) tagged with fluorescein isothiocyanate (FITC) was added (23-26 μL) to each coverslip. Antibody-treated coverslips were incubated at 37°C for 40 min. The mini-plaques of infected cells were then counted using fluorescence microscopy.

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Figure 1: Amantadine Compounds From left to right Amantadine, N-(1adamantly)-Iminomonoacetic Acid, and N-(1adamantyl)-Iminodiacetic Acid.

me Assay



ure 4: Calculated Proton Flux of Amt-IMA•Cu(II)•ACAC

n left to right Two negative control experiments used for this assay included blank omes (no Amt-IMA•Cu(II)•ACAC or M2 protein) labeled Blanks/-drug and liposomes n drug (100 μM Amt-IMA•Cu(II)•ACAC) but no M2 protein labeled Blanks/+drug. positive control had liposomes with M2 S31N protein (residues 22-62) labeled M2/-Liposomes with M2 S31N and drug (labeled M2/+drug 5 µM) is used to show that EC_{50} is less than 5 μ M. Liposomes with M2 S31N and drug at 100 μ M (labeled) +drug) shows significant reduction in proton flux (~ 84.1%).



are 6: EC₅₀ of Drug Candidates in Mini-Plaques *n left to right* Amantadine (Amt) has a high EC₅₀ of 64.3 μM. Amt-1 and Amt-2 e an EC₅₀ of ~ 41 μ M. CuCl₂ has an EC₅₀ that is comparable to Amt at 57.2 μ M. $Cu(II) \bullet Cl_2$ had a significantly lower EC_{50} than Amt at 8.7 μ M (~ 7.5 times lower). Cu(II)•en and DETA•Cu(II) did not have a significantly lower EC₅₀ than Amt. Amt-IDA•Cu(II) and Amt-IMA•Cu(II) each have an EC₅₀ approximately 2.7 times lower than Amt. Amt-IMA•Cu(II)•ACAC (novel compound) had the lowest EC₅₀ at $2.91 \,\mu\text{M}$ (22.1 times lower than Amt).

Structural Representations of Tested Compounds

Figure 2: Non-Amantadine Compounds *From left to right* Cu(en)Cl₂, Cu(en)₂Cl₂, and $Cu(DETA)Cl_2$.

Figure 5: EC₅₀ of Drug Candidates Against M2 S31N in Liposomes *From left to right* Amantadine (Amt) has a high EC_{50} of ~ 50 μ M. CuCl₂ has an EC₅₀ that is 8.1 times lower than Amt. en•Cu(II)•Cl₂, Amt-IDA•Cu(II), and Amt-IMA•Cu(II) each have an EC_{50} approximately 2.4 times lower than Amt. Amt-IMA•Cu(II)•ACAC (novel compound) had the lowest EC₅₀ at $4.5 \,\mu\text{M}$ (11 times lower than Amt).

Figure 3: Amantadine-based Compounds From left to right Amt-Iminomonoacetic Acid•Cu(II), Amt-Iminodiacetic Acid • Cu(II), and Amt-Iminomonoacetic Acid • Cu(II) • ACAC (novel complex).

Figure 7: Amt-IMA•Cu(II)•ACAC Binding to Histidine Complex The most exchangeable Cu(II) coordination sites may orient towards the four imidazole nitrogens from the His37 tetrad. The four imidazole nitrogens from His37 serve as strong ligands and should favorably bind to Cu(II). Cu(II) could bind to two His37 side chains in the four-fold symmetric channel to form a distorted octahedral complex.

Conclusions

Amantadine can potently block sensitive strains of influenza A, but a single particular mutation in the amantadine binding-site of M2 eliminates sensitivity. The strains of influenza A circulating in humans all carry this mutation. Several groups have tried and found difficulty in blocking strains that have this mutation. We have shown that copper-complexed amantadine blocks a strain carrying this mutation in cell culture experiments.

This is to be expected because copper is known to bind to the filtering region in the pore, which would block the M2 channel and prevent virus uncoating as well as protection of viral fusion proteins during expression. This filtering region is universally conserved: no virus can survive without it.

A novel anti-influenza A compound comprised of known M2 blockers (i.e. Amantadine), derivatized and complexed to the divalent metal ion Cu(II), has been examined for broad-spectrum activity against influenza A viral strains. Divalent copper has been the primary candidate for the metal complex.

Secondary ligands will be added as needed to stabilize the complex in animal/human fluids, to protect the complex from promiscuous associations, and to enhance blocking efficacy of the drug in the M2 channel. We propose to test a large set of such complexes to identify the optimal complex for blocking influenza A in humans.

We expect to find a complex that will block all sub-types of influenza A and hopefully influenza B, based on the metal binding to the universally shared filter.

References

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