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Attempted Synthesis & Antibacterial Properties of APT-6K Against NDM-1 K. Pneumoniae

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J. N. Andrews Honors Program

Andrews University

HONS 497

Honors Thesis

Attempted Synthesis & Antibacterial Properties of APT-6K Against NDM-1 *K. pneumoniae*

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March 31, 2022

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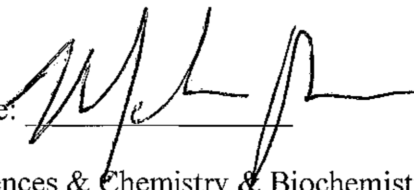
Primary Advisor Signature: 

Department: Medical Laboratory Sciences & Chemistry & Biochemistry

Abstract

NDM-1 *K. pneumoniae* is a highly resistant bacterial organism that is capable of causing debilitating nosocomial infections in immunocompromised patients. Only "last-resort" antibiotics—such as colistin—work against this organism. Therefore, new antibiotics are needed to help fight against these types of infections. APT-6K is a novel compound that was demonstrated to be effective against MRSA with nanomolar concentrations in a prior study. Novel methods of APT-6K synthesis and its testing for antibiotic effects against NDM-1 *K. pneumoniae* were attempted in this research. However, APT-6K synthesis was unsuccessful. Commercially-prepared APT-6K also did not demonstrate growth inhibition against NDM-1 *K. pneumoniae* nor against a wild-type *K. pneumoniae*. Suggestions for future research are discussed.

Introduction & Background

Antibiotic resistance by bacteria has been a growing problem for decades. According to Robert R. Redfield, a previous director of the CDC, we are now living in a post-antibiotic era (CDC, 2019). Antibiotics that were clinically useful in the past have now been rendered ineffective or inadequate by the growing number of antibiotic-resistant bacteria. Among these resistant bacteria of high concern are carbapenem-resistant *Enterobacteriaceae,* which the CDC classifies as an "urgent" threat level (CDC, 2019). These types of infections are extremely difficult to treat and cause great sickness to the patient.

Enterobacteriaceae are a family of gram-negative bacteria that are generally found ubiquitously throughout nature, such as in soil, animals, and the human digestive tract. However, one such carbapenem-resistant *Enterobacteriaceae* of growing concern is NDM-1-producing *Klebsiella pneumoniae. K. pneumoniae* is associated with a number of clinical conditions even without factoring in drug resistant mechanisms. One condition *K. pneumoniae* can cause is pneumonia, inspiring the bacteria's name. However, *K. pneumoniae* can also cause urinary-tract infections and blood-stream infections. *K. pneumoniae* is typically an opportunistic infection that targets immunocompromised people. When factoring in other species of the same genus, *Klebsiella* are the third leading cause of hospital-acquired infections in the US. Furthermore, mortality rates by pneumonia caused by *K. pneumoniae* are as high as 50% and bloodstream infection mortality is around 20-30% (Martin & Bachman, 2018). As a gram-negative bacterium, *K. pneumoniae* are intrinsically toxic due to the presence of endotoxin in the outer membrane, contributing to septic shock within an individual.

Bacteria are able to possess antibiotic resistance by having specific genes that give rise to the creation of molecules—beta-lactamases—that are able to neutralize antibiotics. For *K.*

pneumoniae, there are a variety of genes that could potentially confer antibiotic resistance if present in the bacterium. The gene that encodes the creation of NDM-1 is particularly worrisome. NDM-1, which stands for New Delhi metallo-beta-lactamase 1, is a beta-lactamase enzyme that is able to inactivate nearly all beta-lactam antimicrobials, including a powerful group of antibiotics called carbapenems. Furthermore, it is also able to transfer NDM-1 genes to other bacterium through the ability of some *K. pneumoniae* bacterium to initiate conjugation (Huang et al., 2018). NDM-1 *K. pneumoniae*'s antibiotic-resistant abilities are not to be taken lightly as treatment plans for patients with NDM-1 *K. pneumoniae* are extremely limited. Drugs like colistin are a viable option; however, this drug is not typically used and is reserved as a last-resort option because of the potential side effects, such as damage to the liver or nervous system (Martin & Bachman, 2018).

Generally, the danger of *K. pneumoniae* is typically limited to immunocompromised people, such as those who are hospitalized. However, the development of hypervirulent, community-acquired strains have been recorded in the Asian Pacific Rim region (Martin & Bachman, 2018). These hypervirulent *K. pneumoniae* are present within people in the community, rather than immunocompromised people in the hospital. From the literature, these have been noted to cause pyogenic liver abscess (Martin & Bachman, 2018). Strains that have hypervirulent and antibiotic resistant genes are causes for concern, especially since *K. pneumoniae* bacterium are able to transfer genes between bacterium via conjugation (Huang et al., 2018). At present, a potentially devastating combination of both genes is not so far-fetched. In 2017, a hospital outbreak of an antibiotic resistant and hypervirulent *K. pneumoniae* isolate occurred in China (Martin & Bachman, 2018).

One strategy in fighting against growing resistance in bacteria are the development of new antibiotics. One potential type of antibiotic that could be utilized are copper-dependent inhibitors. Copper-dependent inhibitors are a class of compounds that confer antibacterial effects in the presence of copper. Alongside being discovered, these class of compounds have been found to be characteristic of many existing compounds. For example, an FDA-approved medication used to treat alcoholism, disulfiram, was found to have copper-dependent inhibition effects against bacteria (Crawford et al., 2020). Furthermore, the utilization of copper was no arbitrary choice. Copper has intrinsic antibacterial properties, but is also relevant to human physiological processes and helps to mediate some innate immune functions. For example, a mild copper deficiency is linked to decreased neutrophil functionality, one of our first-responders for bacterial infections (Djoko et al., 2015). Furthermore, when compared to the other transition metals, copper shows the highest stability when complexed with ligands (Dalecki et al., 2016). Therefore, due to the physiological relevance of copper and the stability of complexes it forms with ligands, copper-dependent inhibitors are feasible antibiotic drug candidates.

At the moment, the exact mechanism copper-dependent inhibitors utilize against bacteria is unknown. However, some studies have suggested the mechanism correlating to the inhibition of ATP generation. Vestergaard et al. (2017) demonstrated that impeding ATP synthase, and thus interfering with ATP generation, in *S. aureus* bacteria sensitized them to the antibiotic polymyxin B. This finding is particularly surprising because polymyxin B is typically used to treat gram-negative bacteria and are generally ineffective towards gram-positive bacteria like *S. aureus*. Crawford et al. (2020) also demonstrated that ATP inhibition may be a key factor in re-sensitizing drug-resistant *S. aureus* to the antibiotic ampicillin. These evidences suggest that copper-dependent inhibitors could be used synergistically with other drugs to fight against highly

drug-resistant bacteria and would have the benefit of utilizing existing antibiotics that are already being used in the clinical setting.

One copper-dependent inhibitor of interest is APT-6K. "APT" is an acronym that means adamantyl-bearing pyrazolyl-thioureas, which refers to its adamantyl, pyrazolyl, and thiourea groups (Figure 1). Adamantyl substituents are also of interest since they enhance the stability of drugs and increase plasma half-life (Dalecki et al., 2016). Thus, if "APT" molecules exhibit antimicrobial effects, then the increased stability could be a pharmacological bonus.

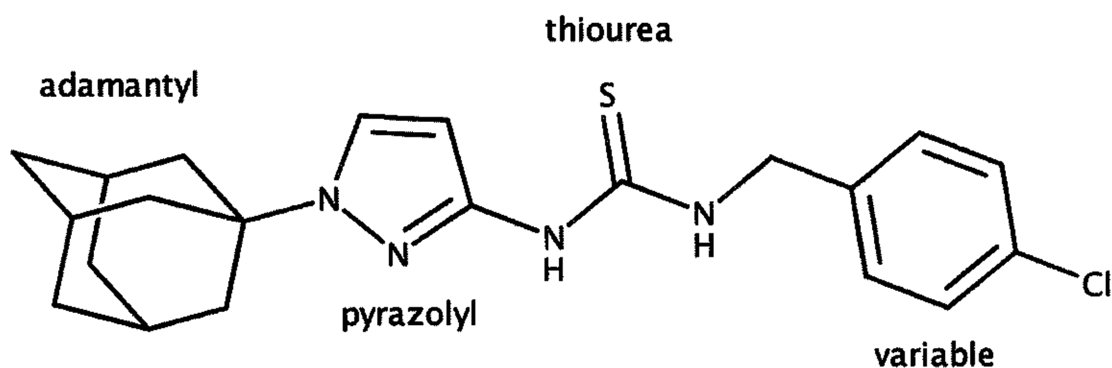


Figure 1. Structure of APT-6K. "APT" means adamantyl-bearing pyrazolyl-thiourea, referring to the substituents on APT-6K. There are a variety of "APT" molecules, each of which are differed by a distinct variable group (Dalecki et al., 2016), however the benzene ring with a chloride atom in para position is characteristic of APT-6K.

APT-6K was chosen to test against *K. pneumoniae* because it is novel and can inhibit the growth of multi-drug resistant *Staphylococcus aureus* (Crawford et al., 2020). At the time of this writing, the literature has shown that APT-6K has only been tested against the gram-positive *S. aureus*, which was performed by Crawford et al. (2020)'s team. Therefore, testing APT-6K against the gram-negative *K. pneumoniae* may provide some insight into the mechanism of action of APT-6K and the spectrum of organisms in which it can show efficacy.

Methodology

Synthesis of ATP-6K and Analogs

In order to synthesize APT-6K and analogs, the plan would be to first adamantylate the 1-N position of either 3-nitropyrazole or 3-aminopyrazole. If adamantylation of 3-nitropyrazole occurs, the nitro group would be reduced by using 10% palladium on carbon to create 1-(adamantan-1-yl)-1H-pyrazol-3-amine. Adamantylation of 3-aminopyrazole would yield 1-(adamantan-1-yl)-1H-pyrazol-3-amine, therefore no reduction step would be required. From there, 1-(adamantan-1-yl)-1H-pyrazol-3-amine would be reacted with isothiocyanate derivatives to create the thioureas APT-6K and analogous compounds. The reduction and thiourea synthesis steps are the easiest parts of APT-6K and analog synthesis. However, the adamanylation step is less characterized in the literature. Therefore, this study focused on the adamantylation of 3-nitropyrazole or 3-aminopyrazole. An example of an experiment that was attempted was inspired by Huang et al. (2017) on Figure 2. Here, if the reaction was successful, K_2CO_3 and DMSO work together to create a "super basic" medium that strips off the 1-H on the pyrazole ring, allowing electrons from the 1-N to nucleophilically attack the adamantyl cation once bromide is liberated from the adamantyl group.

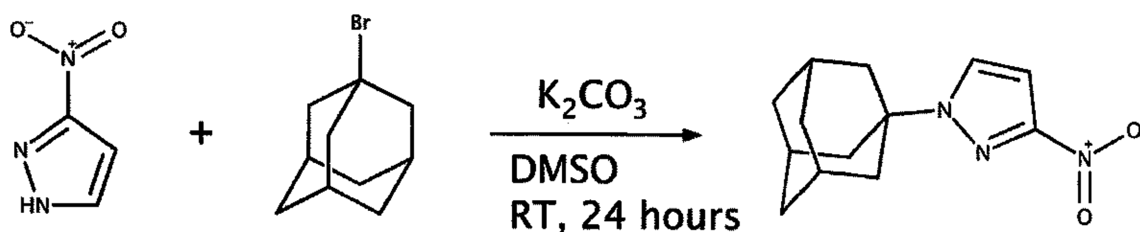


Figure 2. Reaction inspired by Huang et al. (2017). DMSO coordinates the potassium on K_2CO_3 making it more nucleophilic, stripping off the 1-H on the pyrazole ring. The 1-N electrons on 3-nitropyrazole nucleophilically attack the adamantyl cation after liberation of the bromide anion, theoretically forming 1-(adamantan-1-yl)-3-nitro-1H-pyrazole. However, the reaction was unsuccessful.

If that was successful, synthesis of APT-6K and analogs would have been created by combining commercially-produced isothiocyanate derivatives and 1-(adamantan-1-yl)-1H-pyrazol-3-amine in order to create APT-6K and analogous compounds.

Calibration of Plate Reader

Our 96-well plate reader was calibrated in order to relate the number of colony-forming units to OD_{630}. This was performed by inoculating broth with *K. pneumoniae* and creating random optical densities per well. Depending on the optical density, dilutions with sterile saline of either 10^4, 10^5, 10^6, 10^7, and 10^8 were arbitrarily created. 100 µl of the diluted broth would then be inoculated onto Mueller-Hinton agar plates. The Mueller-Hinton agar plates were incubated at 37°C for 18-24 hours. After incubation, colony-forming units were then counted manually. Using this data, colony-forming units/mL were related to OD_{630}, allowing for quantitation of results in the APT-6K trials.

MIC Assay for Wild-type and NDM-1 K. pneumoniae

The MIC assay methodology was inspired by Crawford et al. (2020)'s methodology.

Preparation: A stock solution of 50 µM APT-6K in 2% DMSO was prepared and two-fold serial diluted onto a 96-well plate at concentrations corresponding to Figure 3. 10 µl of APT-6K solution remained in each test well. A stock solution of 1000 µM copper(ii) sulfate pentahydrate in sterile deionized H_2O was diluted to 800 µM. From there, two-fold serial dilutions of the 800 µM copper(ii) sulfate pentahydrate solution were created. 10 µl of each dilution were manually titrated in each test row in order to create concentrations corresponding to Figure 3. *K.*

pneumoniae was subsequently inoculated into Mueller-Hinton broth and diluted to a concentration of 5,555,555 CFU/mL. 180 µl of *K. pnemoniae* broth were manually titrated onto each well, correcting to a concentration of 5×10^6 CFU/mL. In total, 200 µl comprising of APT-6K solution, Cu^{2+} solution, and Mueller-Hinton broth were in each well. Growth and sterility control wells were also included. Each well plate was covered with parafilm to prevent evaporation of well solutions and incubated at 37°C for 18-24 hours. Triplicates were prepared.

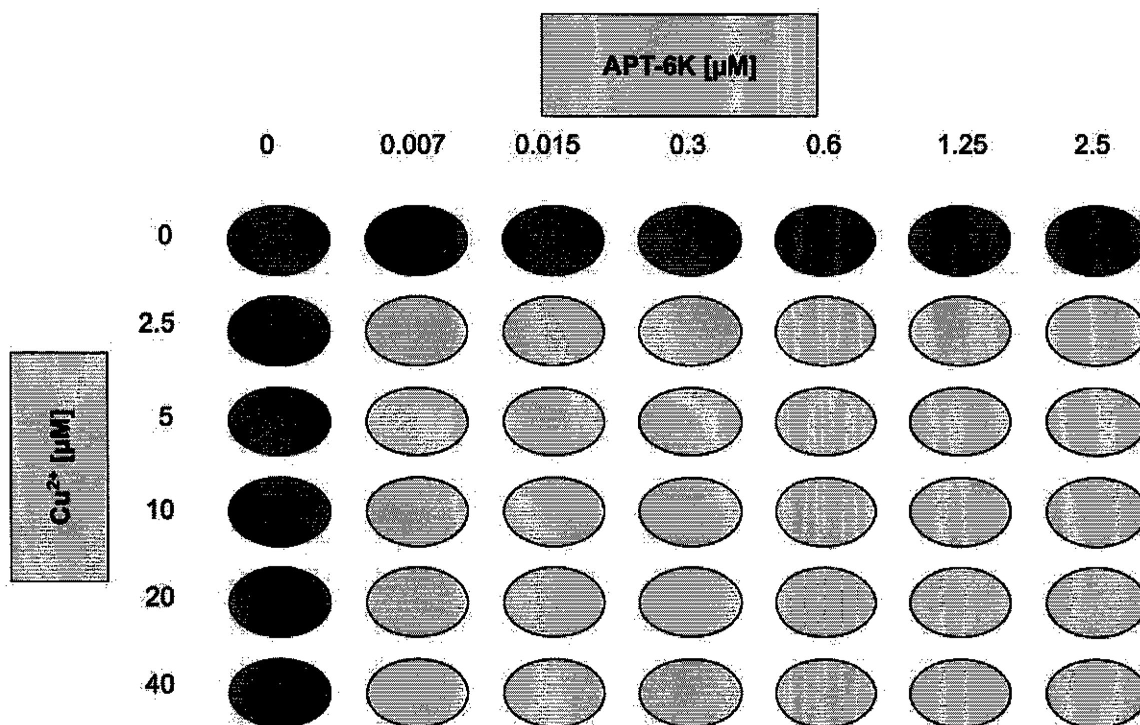


Figure 3. This figure demonstrates the final concentrations of each well in a 96-well plate. 180 µl of inoculated Mueller-Hinton broth, 10 µl of APT-6K solution, and 10 µl of Cu^{2+} solution were added to each well. Therefore, each well had a final volume of 200 µl of liquid. The pink wells represent control wells. The growth well is the well with no APT-6K nor Cu^{2+} solution and contained only reagent solution. A sterility well was also run, but is not shown on this figure.

Reading: After incubation, parafilm was removed from the well plate and read on our Accuris™ SmartReader 96 (model MR9600), our microplate absorbance reader. Each well was read using 630 nm wavelength of light, allowing us to get OD_{630} measurements in each well plate. From previous calibration, our OD_{630} to CFU/mL conversion equation was $8.85 \times 10^9(OD_{630}) + 3.84 \times 10^7$ with a linearity that ranged from 0.004 to 0.512. Therefore, any OD_{630} measurements that were above 0.512 were diluted by a forth. After the diluted value was converted to CFU/mL, the product was multiplied by the dilution factor 4 to derive the approximate CFU/mL in each well.

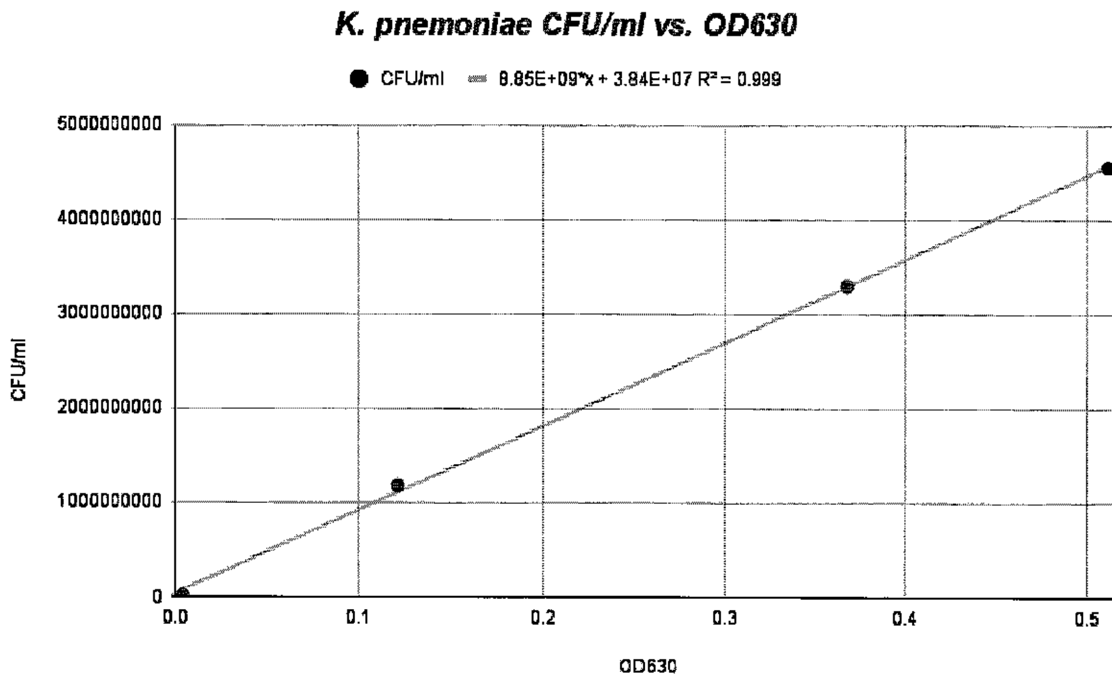


Figure 4. This figure demonstrates the calibration curve used to quantify the number *K. pneumoniae* per well using OD_{630}. The equation derived was $8.85 \times 10^9(OD_{630}) + 3.84 \times 10^7$.

Interpretation: After each well had OD_{630} measurements, each OD_{630} value was converted to CFU/mL on Google Spreadsheets. Since triplicates were performed, the final OD_{630} value was derived by taking an average of all the runs. The averaged OD_{630} values were converted to

CFU/mL. The growth well was also converted to CFU/mL and represented the 100% mark of relative growth. A relative growth percentage was created by taking the CFU/mL of each test well and dividing it by the CFU/mL in the growth well. Any well that had a growth of less than 10% was considered to be inhibited. The MIC would have been the well plate with less than 10% growth and have the lowest concentration combination of APT-6K and Cu^{2+}.

Results

Attempted Synthesis of APT-6K and Analogs

Synthesis was unsuccessful due to the inability to adamantylate the 1-N position of the pyrazole ring. Therefore, APT-6K was purchased from a chemical supplier and used in MIC assays against wild-type and NDM-1 *K. pneumoniae*.

Effect of APT-6K and Cu^{2+} on Wild-type K. pneumoniae

Varying concentration combinations of APT-6K and Cu^{2+} did not seem to demonstrate any effect on the growth of wild-type *K. pneumoniae* in accordance to Figure 5. Each combination of APT-6K and Cu^{2+} seemed to keep the relative growth at around 100%, with the exception of an anomalous spike at 0.007 μM APT-6K and 40 μM Cu^{2+} demonstrating a relative growth of approximately 170%.

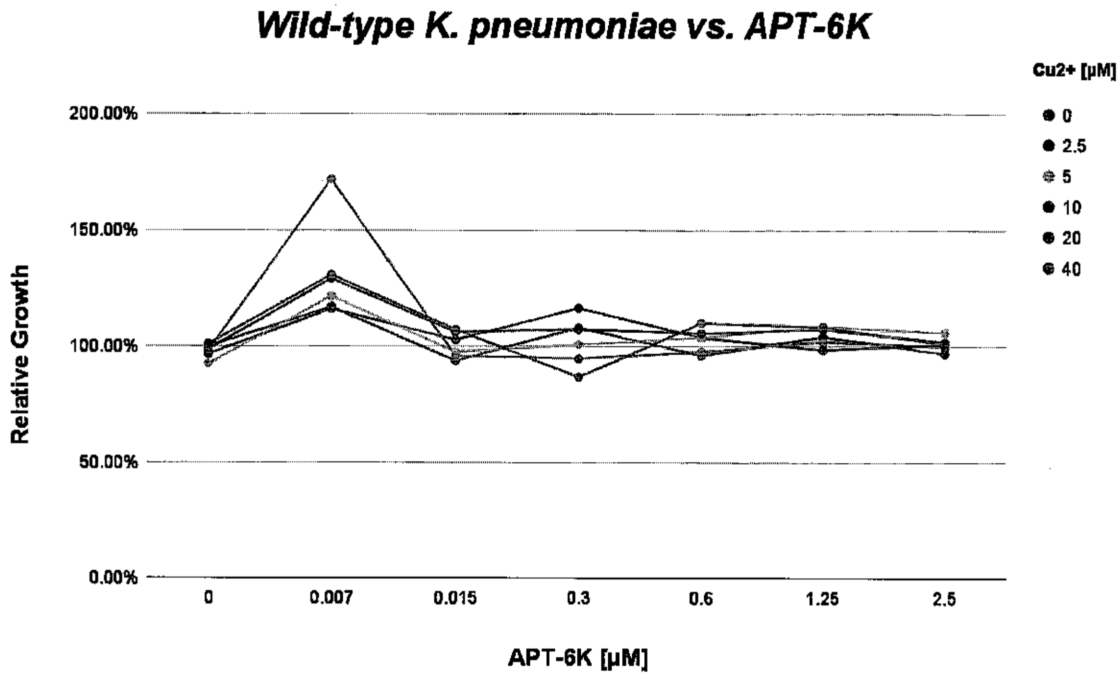


Figure 5. This figure shows the effects of differing concentration combinations of APT-6K and Cu^{2+} on the growth of wild-type *K. pneumoniae*. APT-6K concentrations (μM) were placed on the x-axis, while each Cu^{2+} concentration (μM) were differentiated by color. For Cu^{2+} concentrations, blue is 0; red is 2.5; yellow is 5; green is 10; orange is 20; and turquoise is 40.

Effect of APT-6K and Cu^{2+} on NDM-1 K. pneumoniae

According to Figure 6, varying concentration combinations of APT-6K and Cu^{2+} also did not seem to demonstrate any effect on the growth of NDM-1 *K. pneumoniae*. Each combination of APT-6K and Cu^{2+} seemed to keep the relative growth at around 100%

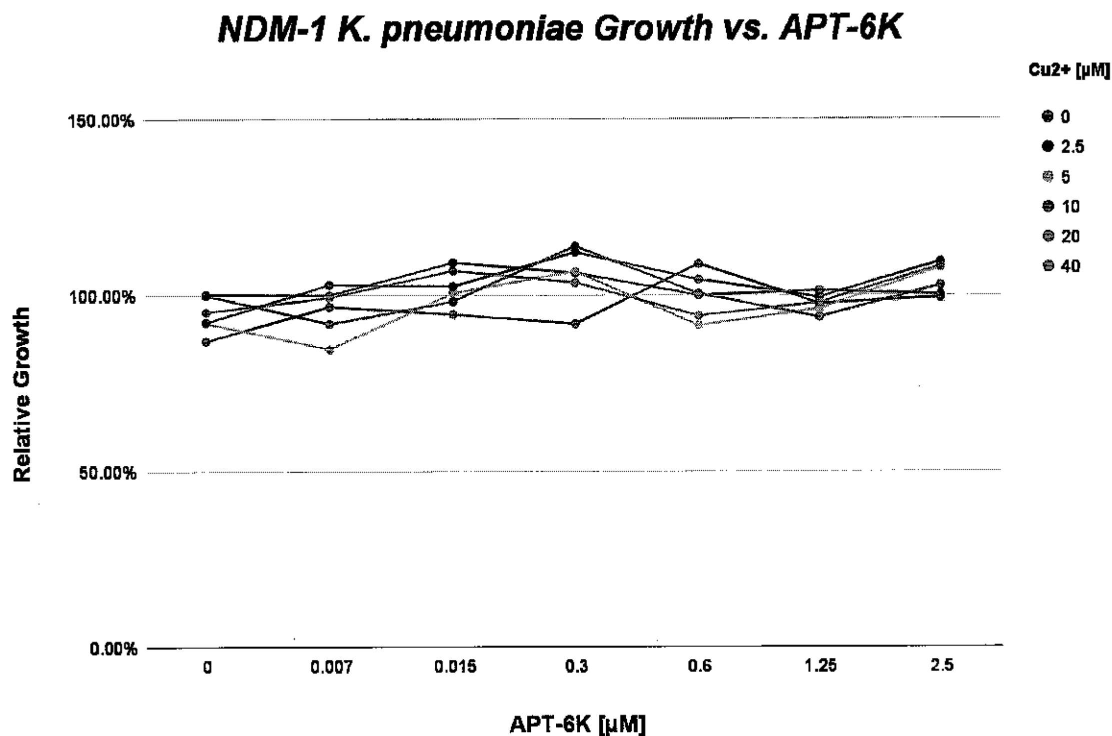


Figure 6. This figure shows the effects of differing concentration combinations of APT-6K and Cu^{2+} on the growth of NDM-1 *K. pneumoniae*. APT-6K concentrations (μM) were placed on the x-axis, while each Cu^{2+} concentration (μM) were differentiated by color. For Cu^{2+} concentrations, blue is 0; red is 2.5; yellow is 5; green is 10; orange is 20; and turquoise is 40.

Discussion

The unsuccessful synthesis was perhaps due to the relatively tame conditions we placed our chemicals in. For example, in one experiment 3-nitropyrazole was mixed with 1-bromoadamantane for 24 hours at room temperature in a potassium carbonate and DMSO solution (Figure 2). In contrast, Cabildo et al. (1994) was able to use both high pressures and temperatures to successfully adamantylate the 1-N position of the pyrazole ring. However, replicating this was not possible due to lack of access to a high-pressure stainless-steel autoclave

for chemistry use. Had adamantylation of the 1-N of the pyrazole ring worked, perhaps synthesis of APT-6K and analogs may have worked. However, using tamer conditions to adamantylate the 1-N position of the pyrazole ring remains a gap in the literature and can be explored in further research.

The APT-6K MIC assay against *K. pneumoniae* strains also did not seem to demonstrate any effect against the bacteria. One reason this might have been was due to the membrane structure of *K. pneumoniae*. *K. pneumoniae* is a gram-negative bacterium, meaning it has two cell membranes with a peptidoglycan layer sandwiched in between. The experiment by Crawford et al. (2020) showed that APT-6K was potent against multi-drug resistant *Staphylococcus aureus*. Compared to *K. pneumoniae*, *S. aureus* has only one cell membrane with an outer peptidoglycan layer. Therefore, APT-6K may have had a more difficult time trying to penetrate both membranes of *K. pneumoniae*. Furthermore, the APT-6K molecule is quite bulky, which perhaps exacerbated the problem of getting APT-6K to penetrate the gram-negative membranes. The difficulty of getting bulky molecules to penetrate gram-negative membranes is not new, as the antibiotic vancomycin also has a difficult time penetrating gram-negative membranes because of its molecular size (Fernandes et al., 2017).

Suggestions for further research are to test higher concentration combinations of APT-6K and Cu^{2+}. It is possible that the concentrations of APT-6K and Cu^{2+} were just not high enough to produce an effect. A viability test could also be performed as this is able to give information on how much bacteria are alive and dead within a well. Spectrophotometric analysis only considers the optical density of solution, not the viability of bacterial organisms. Therefore, a viability test might suggest an effect if it was not demonstrated into spectrophotometric analysis. Furthermore, APT-6K should be tested on other gram-positive bacteria. APT-6K worked quite well against the

gram-positive *S. aureus*, but not the gram-negative *K. pneumoniae*. Thus, there may be more luck with using APT-6K against other gram-positive bacteria such as *Enterococcus* spp.

Some limitations of this study were the lack of statistical analysis involved. Although looking at Figure 1 and 2 visually did not demonstrate an effect, perhaps statistical analysis might suggest the opposite. Statistical analysis was not performed due to a lack of a reference curve associated with APT-6K since it is novel. Crawford et al. (2020)'s research showed that different strains of *S. aureus* with APT-6K may produce either sigmoidal or biphasic growth patterns. Therefore, performing a nonlinear multiple regression with current data may have not been very reliable. Furthermore, the storage degradation of reagent solutions was not accounted for as this information was not readily available. To minimize this possibility, the specimens were stored at -70°C between experimental runs to help eliminate this possibility. Solubility issues of APT-6K in our 2% DMSO solution also occurred. When first mixing the APT-6K in our 2% DMSO solution, it did not seem to fully solubilize at first. However, with time it did seem to dissolve. Whether it stayed solubilized after storage at -70°C however was unknown. To control for this as much as possible, the bottled solution was gently mixed before each MIC assay.

Conclusion

The synthesis of APT-6K and analogs was hindered by the difficulty in adamantylating the 1-N of the pyrazole ring. The use of Cu^{2+}-activated APT-6K was ineffective against both wild-type and NDM-1 *K. pneumoniae*. APT-6K likely did not work because either it was unable to penetrate the gram-negative membranes of *K. pneumoniae* or concentrations were not high enough to produce an effect.

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