**Aspergillomarasmine A treatment for the inhibition of metallo-beta-lactamase antibiotic resistance**

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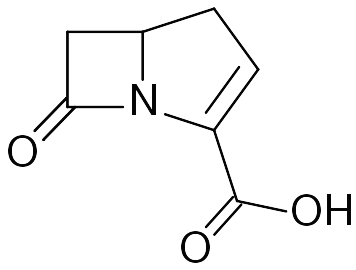
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**Literature Review**

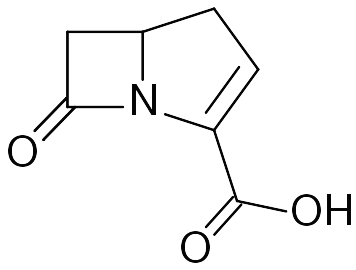
**Introduction**

Beta-lactamases are a class of enzymes produced by bacteria that allow resistance to beta-lactams, which are used widespread as key components of antibiotic drugs. These enzymes have posed many problems due to their prevalence in many deadly bacteria like *Salmonella*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Mycobacterium tuberculosis*. A large issue with treatment for these infections is that they are constantly mutation over time, and that the usefulness of antibiotics is diminishing. Even though antibiotics are usually very effective at killing bacteria, the sheer abundance of bacteria will ensure that some mutants will survive and replicate with this specific resistance (Hart 1998). Over time, most surviving strands of the species will carry the mutation, rendering the older treatment obsolete. Furthering the issue, bacteria can utilize gene transfer methods such as plasmids to pass genes horizontally. This can result in “blanket” multidrug resistances being utilized by a variety of strains. From the discovery of penicillin in the early 20th century, research has been focused on classifying beta-lactamases and mechanisms of action for beta-lactams (Kong et al. 2010). When given as drug treatments, beta-lactams such as penicillin act to inhibit cell wall metabolism (Zeng and Lin 2013) in gram-negative bacteria. Beta-lactams constitute over half of all commercially-available available antibiotics, therefore they constitute the primary method of eliminating disease-causing bacteria in the population.

The primary feature of antibiotic inhibition with beta-lactamases involves a four-membered ring common to all beta-lactams. Most mechanisms involve a nucleophilic attack on the carboxylate site of the ring, which can subsequently break down the cyclic structure.



**Figure 1**: Structure of carbapenems, a type of beta-lactam containing the four-membered amide cyclization1

The breakage of the amide bond in beta-lactams by beta-lactamase enzymes renders the beta-lactams functionally inactive, nullifying their role in killing the bacteria. Distinctions in classes of beta-lactamases are characterized by the molecules found in the active site of the bacteria and include classes A-D. **Metallo-beta-lactamases (MBLs)**, an example of class B beta-lactamases, are characterized by two metal ion binding sites containing histidine residues (Palzkill 2013). Unlike class A, C, or D beta-lactamases, which hydrolyze beta-lactams by forming a serine-based acyl intermediate, class B MBLs directly attack the active site with zinc ions. MBLs are also an example of carbapenemases, bacterial enzymes that specifically target carbapenems, a beta-lactam subset. Carbapenems, which are a subclass of beta-lactams, contain the recognizable four-membered amide ring found in all beta-lactams. They are known for having a much slower substrate binding rate than normal beta-lactams and for being present in a broad spectrum of antibiotics due to their potency (Papp-Wallace et al. 2011). Carbapenems are often used as last resort therapies for multidrug-resistant bacterial diseases like *Enterobacteriaceae* (Nordmann et al. 2012), making carbapenemases like MBLs especially problematic.

**New Delhi metallo-beta-lactamase (NDM-1)** was first discovered in 2008 from a Swedish patient returning from India with a Gram-negative bacterial infection (Johnson and Woodford 2013). Others claim that poor restrictions and overuse of antibiotics contribute to the spread of these diseases in the region (Ghafur 2010). While, bacteria containing NDM-1 are believed to originate from the Indian subcontinent, they have since been reported around the world (Manohar et al. 2018). Strains containing NDM-1 like *E. coli* or *Pseudomonas aeruginosa* are usually referred to as a “superbug” due to its strong multidrug resistance. In *E. coli*, the gene coding for NDM-1 is found on easily transferable plasmids, causing rapid spread of NDM-1 to other *E. coli* (Sun et al. 2014). NDM-1’s multidrug resistance comes from its high affinity to Zn2+ metal ions and its ability to hydrolyze virtually all forms of cyclic beta-lactams (Fast and Sutton 2013). Because no inhibitors are readily available for many NDM-1 genes (Spyrakis et al. 2018), newer and more permanent methods of inhibition are highly sought after.

NDM-1 is classified as a carbapenemase due to the implications the enzyme has on the inhibition of many current antibacterial treatments. The main structure of NDM-1 contains an active site with two zinc atoms. These metal ions are coordinated tetrahedrally to water molecules and imidazole groups containing hydrophilic amino acids like histidine, aspartate, and cysteine (Green et al. 2011). The zinc ions can act as sequestering agents, allowing water molecules, in the form of hydroxide, to hydrolyze beta-lactams’ amide bond and inhibit their antibacterial effects.

Active site comparisons of K. pneumoniae apoNDM-1 (PDB id 3RKJ) (aqua) vs monozinc NDM-1 (PDB id 3SFP) (violet) vs di-zinc NDM-1 PDB id 3Q6X [49] (wheat).
Zn1â² and Zn2â² (wheat) are from the structure of di-zinc NDM-1 and Zn1â³ is from the structure of monozinc NDM-1. Conformations of residues coordinating Zn1 (His120, His122 and His189) and Zn2 (Asp124, Cys208 and His250) are shown for all three structures.

**Figure 2**:New Delhi metallo-beta-lactamase 1 active site structure2

Different treatments have been proposed for NDM-1. One such inhibitor, **aspergillomarasmine A (AMA)**, was discovered in 2014 to inhibit NDM-1. Derived from *Aspergillus* fungi, aspergillomarasmine A was shown to be effective in bacterial and mouse models against NDM-1 and a similar MBL, Verona integron-encoded metallo-β-lactamase (King et al. 2014). This review will overview various methods of novel NDM-1 inhibition, characterize AMA and its derivatives to find structural elements of inhibition. The status of AMA research and some of the issues hampering it will also be discussed.

**Discussion**

*Modes of inhibition for NDM-1*

NDM, like most beta-lactamases, attacks the active site to open beta-lactam substrates. In metallo-beta-lactamases like NDM, the critical feature of the active site are two zinc ions which nucleophilically hydrolyze the substrate and break the C-N bond of the cyclic structure. Spyrakis et al. (2018) tested 31 candidates for zinc-based inhibition, including molecules containing thiols, triazoles, and sulfonyl groups. Thiol-based inhibitors showed good targeting ability regarding the active site ligands. Derivatives of thiol-based compound captopril, a treatment for hypertension, showed inhibition of NDM and other MBLs. However, few synthetic studies have been performed on the effects of the molecular functional group, which are required to ensure the correct thiol compound can be produced (Büttner et al. 2017). Several drugs containing captopril have been considered, but no clinically-available drugs have shown the ability to treat NDM without significant compositional changes (Li et al. 2014) such as the derivatives used by Büttner. Likewise, characterization of many triazole compounds reveal levels of selectivity and effectiveness depending on the compound (Weide et al. 2010). A bismuth-based compound, colloidal bismuth subcitrate, was shown to replace zinc ions at the active site, showing effective treatment in mice (Wang et al. 2018). The common thread between them seems to be the removal of zinc metal ions found in the enzyme’s active site, reducing the inhibitory effects of NDM-1.

Instead of the chelation of metallic zinc ions, which tends to be too unselective and therefore not specific enough in drug treatments, a 2,6-dipicolinic acid derivative exhibited a different mechanism for inhibition (Chen et al. 2017). Instead of the chelation of zinc ions seen in most NDM-1 inhibitors, a ternary NMD-1:Zn(II):inhibitor complex was formed. When tested in *E.* coli and *K. pneumoniae* containing NDM-1, the interaction was shown to be extremely potent and active site-specific. This alternate mechanism of inhibition also bypasses some common issues like the inhibition of other Zn2+-dependent metalloenzymes *in vivo*. The cysteine proteinase inhibitors cystatin 9 and cystatin C are degraded proteins that have also shown to treat NDM-1 with a catalytic thiol acting as a nucleophile (Holloway et al. 2018). Although a few alternative modes of inhibition not involving the removal of zinc ions have been found to show some success, none have been developed far enough to be a definitive solution.

Many novel treatments for NDM-1 inhibition are synthesized or derived from other drugs, leading to issues over toxicity and safety. Development of a nontoxic and consistent treatment of infections containing NDM-1 is therefore at a high demand. Chandar et al. (2017) proposes the extraction of ethanol from a variety of medicinal plants, some of which showed enzyme inhibition and a degradation of cell wall structure in the bacteria. However, these extracts may be problematic if they contain multiple compounds, which can reduce selectivity and limit the inhibiting properties desired in these treatments. Likewise, AMA can be produced from natural products, spores of the fungus *aspergillus versicolor*.

*AMA as a zinc-chelating inhibitor of NDM-1*

AMA was discovered in 1965 for its necrotic effects on plant leaves (King et al. 2014). Since then, it has been known as an inhibitor of angiotensin-converting enzyme for blood hormonal control and a chelator of metal ions. Additionally, both aspergillomarasmine A and B are potent inhibitors of endothelin-converting enzyme, an enzyme that processes endothelin peptides in the bloodstream (Arai et al. 1993). AMA may be derived from the fungus, which is found naturally in damp spaces and in mold. The King study originally found the molecule in samples of dirt sitting inside the lab’s fridge.

The method of which AMA uses to inhibit bacterial beta-lactamases comes from its ability to directly inactivate or remove Zn2+ from the active site of NDM-1 (Bergstrom et al. 2017). AMA can break the coordination of two zinc atoms coordinated to water and imidazole groups containing histidine, aspartate, and cysteine residues (Green et al. 2011). Specific to NDM-1, these two zinc atoms play a major role as chelators, and the water connecting them can directly perform a nucleophilic attack on the cyclic amide structure found in beta-lactam drugs. Although the mechanism AMA uses to disrupt this active site is not entirely known, AMA is a known to inhibit mammalian metalloproteinases through similar mechanisms (King et al. 2014) and act as a metal chelator in other metals such as Fe3+ (Barbier 1987). Whatever the mechanism, general AMA inhibition of NDM-1 has been present in enzyme assays (Zhang et al. 2017), NMR/MS chemical analysis (Bergstrom et al. 2017) and in mouse models (King et al. 2014).

Additionally, the spores of *pyrenophora teres* contain a toxin which can be excreted to AMA, which are known to causes disease in barley crops. A derivative found in this AMA, anhydro-aspergillomarasmine A, exhibits the defining cyclic structure of many beta-lactam drugs (Friis et al. 1991). AMA can also be synthesized through many synthetic pathways, including a total synthesis from an aldehyde, producing a synthetic molecule with four carboxylic acid groups. This synthetic process is very flexible, with reports showing up to seven analogous AMA compounds being produced (Zhang et al. 2017). Both *in vitro* and *in vivo* evaluations showed that these analogues are just as potent as the naturally occurring AMA products.

Since the identification of AMA as an inhibitor of NDM-1 and similar metallo-beta-lactamases, further study has been performed on the exact mechanism. Using primarily 1H NMR, Bergstrom et al. (2017) found that AMA removes Zn2+ from MBLs at approximately 2:1 molar ratio of AMA to the MBLs. Additionally, this process was deemed irreversible when they could not re-activate the enzyme with the addition of zinc. Unlike EDTA, AMA is also more selective at zinc binding sites. Similar results were found using cobalt-substituted MBLs, suggesting that AMA follows a consistent metal ion chelating mechanism in the active sites of NDM-1. However, AMA has only shown efficacy against MBLs due to this metal sequestration. Most of other beta-lactamase classes feature serine-based covalent attacks and show no response to AMA. Combined with meropenem, a widespread beta-lactam antibiotic, AMA was shown to provide carbapenem resistance that meropenem was lacking individually (Nussbaum and Schiffer 2014).

**Conclusion**

New Delhi metallo-beta-lactamase 1 (NDM-1) is a metallo-beta-lactam that has gained significant attention as a multidrug-resistant “superbug” that has spread all over the world. Most novel inhibitions methods follow a metal chelating mechanism at the carboxylate active site of the beta-lactamase. These treatments vary in both selectivity and effectiveness. Of them, AMA has been notable for its accessible synthesis routes. When combined with traditional beta-lactam antibiotics, AMA has shown to be successful in deactivating carbapenemases and in general enzyme inhibition. However, only bacterial and mouse models have been used, raising questions about toxicity and usefulness in humans. AMA research should be continued as a longer-term answer to bacterial infections containing multidrug resistance with enzymes such as NDM-1. To eradicate these diseases, there must be more permanent solutions to the ever-growing issue of transferable and evolving bacterial resistance.

**Proposal**

**Specific Aims**

The next step in **aspergillomarasmine A (AMA)** research in **New Delhi metallo-beta-lactamase 1 (NDM-1)** inhibition should focus on selecting the most potent structural arrangement for the molecule group and continuing research on the zinc removal mechanism. Additionally, the question of AMA toxicity should be further tested on mice. AMA’s benefits when combined with traditional drugs like meropenem, amoxicillin, or penicillin should be further expanded on. The proposal will combine elements of past research, including the incorporation of AMA to common beta-lactam drugs and the synthesis and characterization of AMA analogues. Using mouse models, this study will be able to determine which type of AMA is most effective at both integration with common treatments and inhibition of NDM-1 infections while remaining non-toxic to the model animals.

* Precursor step: replicate synthetic strategies of AMA developed by Zhang et al. 2017 and characterize via spectroscopic and kinetic analysis.
  + Predicted result: the synthesis of AMA compounds that are tested to be pure and easily distinguishable. Although their synthesis is not novel, differently-structured AMA derivatives will be evaluated based on their performance in NDM-1 inhibition. Three derivatives will be chosen for this comparison.
* *Aim 1* - perform preclinical trials on mouse models for survival data and bacterial enumeration after infection with NDM-1-containing bacteria and comparison to a control group without NDM-1.
  + Predicted result: AMA administration will result increased mouse survival and a decrease of NDM-containing bacteria compared to mice without AMA treatments.
* *Aim 2* - test the change in bacterial inhibition of AMA when combined with a common beta-lactam antibiotic like amoxicillin or meropenem in mice infected with NDM-1 bacteria.
  + Predicted result: AMA increases the effectiveness of antibiotic treatment compared to mouse models containing the antibiotic as a standalone.

**Research Strategy**

**Significance**

The current research of AMA in the inhibition of New Delhi metallo-beta-lactamase 1 (NDM-1) enzyme has progressed rapidly in the last five years. However, there are a few issues that have hindered the possibility of using this molecule as a treatment. Because of the novelty of AMA, synthetic pathways and extraction pathways are still being developed and tested for quality and selectivity (Zhang et al. 2017). The toxicity of AMA has also not been fully tested yet. Although reports of full NDM inhibition through zinc chelation without any toxic behavior do exist (Rotondo and Wright 2017), The molecule has been touted as more natural than alternative inhibitors due to its origins from fungi, but this does not ensure its safety. Most AMA studies are in vivo with bacteria like E. coli and Enterobacteriaceae. In mouse models (Nussbaum and Schiffer 2014), no issues were found with AMA. However, preclinical tests in mice do not necessarily translate with humans. Before treatments containing AMA can be developed, the molecule must undergo further study concerning its source and method of production.

Future testing is needed to bridge the gap between species and give the molecule a chance to be produced and used as a pharmaceutical treatment for NDM-1 inhibition in bacterial infections. This proposal does not propose human testing, but instead proposes further mouse treatments that could alleviate some of the issues that makes human testing inaccessible. This study could further knowledge of both NDM-1 inhibition and broader types of bacterial enzyme inhibitors, including metallo-beta lactamases and other classes beta lactamases. In addition, it can expand on fungal treatments for bacterial infections, and how they can be derived from natural sources.

**Innovation**

This study aims to combine previous studies and broaden knowledge on AMA derivatives. Up to seven analogous AMA compounds have been synthesized, but there have been no related mouse studies that expand on these compounds. By comparing these derivatives, a selection can be made based on quantifiable results. This can potentially give a more focused target for human clinical trials and eventual treatment development. Additionally, tests containing common beta-lactamase inhibitors like penicillin may aid in the development of drug combinations that are common in antibacterial pharmaceuticals.

**Approach**AMA can also be synthesized through many pathways, including a total synthesis from an aldehyde, producing a non-natural molecule with four carboxylic acid groups. This synthetic process is very flexible, with reports showing up to seven analogous AMA compounds being produced (Zhang et al. 2017). Both in vitro and in vivo evaluations showed that these analogues are just as potent as the naturally occurring AMA products.

The mice study will be almost identical to previous metallo-beta lactamase inhibition studies (King et al. 2014). Separate groups of mice will be infected with an identical Enterobacteriaceae expressing NDM beta-lactamase inhibition. After infection, the specific AMA derivative, one of the three derivatives chosen, will be given as treatment. The groups will be tested under the same conditions to see prognostic effects. Survival data and physical observations will be recorded, and key organs will be harvested after a four-day endpoint. The organs are homogenized, diluted, and plated on Brilliant Green agar (Oxoid) for cfu enumeration. Cfu enumeration provides a consistent method of quantifying survival rates of bacterial populations targeted by AMA treatment using fluorescence.

*Precursor steps: Replication of AMA Synthesis and Purification*

The synthetic pathways being used are identical to the ones reported by Zhang et al. 2017. Seven AMA derivatives, labeled as compounds 1-7, are synthesized under argon atmosphere. These can be prepared in under a day. Flash column chromatography is used to purify AMA residues. To characterize and confirm derivative structures, 1H NMR and 13C NMR are used. Mass spectrometry is used to compare molecular weight to the calculated weight based on chemical formula. Additionally, another AMA from a fungal extract (Aspergillus versicolor) can be purified to be used as a sample (King et al. 2014). Fractionation with HP20 and Sephadex LH-20 followed by recrystallization yields a white solid.

*Aim 1: Mouse studies for AMA effectiveness, selectivity, and toxicity*

2-month-old female mice will be injected intraperitoneally with Klebsiella pneumoniae containing NDM-1 bacteria at a concentration of 2\*106 cfu (King et al. 2014). The mice will be split into eight groups with a population of at least 10, based on the three AMA compounds. These include a control with no treatments and three groups with selected derivatives discussed in the first section. These three derivatives will be selected based on the quality of compounds synthesized. Ideally, all seven compounds described would be used, but for the sake of time, the three derivatives are selected based on the synthetic groups described by Zhang et al. 2017. Compounds **1-4**, **5**, and **6-7** are all distinct based on functional groups present, so one representative from each will be tested (**AMA1**, **AMA2**, **AMA3** in ***Table 1***). A control group will have no AMA derivative to interact with NDM-1 bacteria.

|  |  |  |  |
| --- | --- | --- | --- |
| **Group Name** | **Qualitative Observations** | **Survival Time** | **Bacterial Enumeration (cfu/g)** |
| **AMA1** |  |  |  |
| **AMA2** |  |  |  |
| **AMA3** |  |  |  |
| **Control** |  |  |  |

***Table 1***: Proposed model for *Aim 1*: comparison of AMA derivatives

Physical observations from the mice at the injected site will be recorded. The time of death, if any, will also be noted. Survival data analysis, such as Kaplan-Meier plot, can be used for each population to quantify survival time. After two weeks, the organs will be harvested with homogenization and dilution. Tissue from the spleen and liver can then be plated on Brilliant Green agar (Oxoid) for cfu enumeration. This will allow for quantification of NDM-1 bacteria and comparison of bacterial populations between treatments in post-mortem mouse tissue.

*Aim 2: use of AMA derivatives in tandem with common beta-lactam treatments*

Using the same controls, data determination, and mouse test subjects as the first section, the three derivatives can be tested for inhibition effects when added to a beta-lactam like meropenem (**Table 2**: **AMA1 + BL, AMA2 + BL, AMA3 + BL**, meropenem: ¼ of the minimum inhibitory concentration 0.125 μg/mL). A separate control group (**BL Control**) with only meropenem will also be used to compare effectiveness. With the same methods used as aim 1, the individual derivatives can also be compared to the results without meropenem addition (**Table 1**).

|  |  |  |  |
| --- | --- | --- | --- |
| **Group Name** | **Qualitative Observations** | **Survival Time** | **Bacterial Enumeration (cfu/g)** |
| **AMA1 + BL** |  |  |  |
| **AMA2 + BL** |  |  |  |
| **AMA3 + BL** |  |  |  |
| **BL Control** |  |  |  |

**Table 2**: Model for *aim 2*: addition of AMA to beta-lactam meropenem treatment

**Problems, alternative strategies, and hazards**

One major problem is that AMA derivatives may not be translatable to mice, due to either inability to physically deliver treatment or AMA having no effect on mice. Until the integration is tested there is no way of knowing whether this will occur. Should this happen, an alternative strategy could be to use a different method of characterization, either through kinetic or spectroscopic determination in vitro. Another issue is that AMA derivatives may not be able to be synthesized based on Zhang et al. 2017, due to difficulty of replication. In this case, the number of AMA derivatives may be reduced from three to however many compounds are able to be used.

NDM-1 is found in infectious bacteria, and should be used with care. Additionally, due to the hazardous nature of NDM-1, the bacteria should only be handled by trained individuals in a suitable testing environment. AMA is derived from fungal products which can potentially be hazardous in humans.

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**Image Credits**

1 http://insilicogenomics.in/lactabase-antibiotics-carbaphenems.asp

2 https://www.researchgate.net/figure/Active-site-comparisons-of-K-pneumoniae-apoNDM-1-PDB-id-3RKJ-aqua-vs-monozinc-NDM-1\_fig8\_51655804