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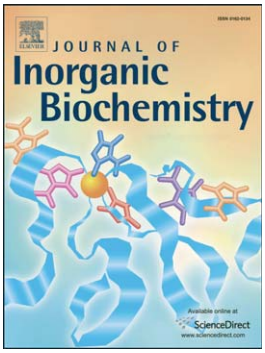

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Enhancement of antibiotic-activity through complexation with metal ions - combined ITC, NMR, enzymatic and biological studies

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Abstract

Alternative solutions need to be developed to overcome the growing problem of multi-drug resistant bacteria. This study explored the possibility of creating complexes of antibiotics with metal ions, thereby increasing their activity. Analytical techniques such as isothermal titration calorimetry and nuclear magnetic resonance were used to examine the structure and interactions between Cu(II), Ag(I) or Zn(II) and β-lactam antibiotics. The metal- β-lactam complexes were also tested for antimicrobial activity, by micro-broth dilution and disk diffusion methods, showing a synergistic increase in the activity of the drugs, and enzymatic inhibition assays confirming inhibition of β-lactamases responsible for resistance. The metal-antibiotic complex concept was proven to be successful with the activity of the drugs enhanced against β-lactamase-producing bacteria. The highest synergistic effects were observed for complexes formed with Ag(I).

1. Introduction

The treatment of infectious diseases with β-lactam antibiotics is threatened by the evolution of superbugs. Careless use of antibiotics has led to the evolution of multi-drug resistant (MDR) bacteria resulting in untreatable infections, and this rise and spread of resistant bacteria is a serious threat for health systems across the globe.[1] With the ongoing increase in drug resistance, the treatment of bacterial diseases by antibiotics is becoming less effective, and there are very few new antibiotics in the clinical pipeline,[2] necessitating the development of alternative approaches.[3] The possibility of modifying existing commercially available drugs to overcome MDR is a favorable approach, saving costs and time compared to the design and development of completely new drugs.[1, 4-6]

One way to selectively target bacteria in the human body is to focus on cellular components that are unique to bacteria. The mode of action of β-lactam antibiotics involves the inactivation of transpeptidases by inhibition of the crosslinking step essential for the synthesis of peptidoglycan. Formation of the cell wall is blocked, leading to bacterial death induced through osmotic pressure.[7-10] To prevent this destruction, bacteria have developed a variety of different resistance strategies, with mechanisms based on the degradation of the β-lactam antibiotic through hydrolysis of the lactam ring being the most dynamic approach.[9] Different types of β-lactamases have evolved to disable new generations of β-lactam antibiotics, with metallo-β-lactamases (MBLs) being one of the major causes of widespread antibacterial resistance towards the important carbapenem class of antibiotics.[11, 12] These metallohydrolases require at least one metal ion in the active site to coordinate to the nucleophile required for hydrolysis.[7-10, 13-17] In recent years, several groups have been

developing compounds that inhibit some of the MBLs, but to date no universally active MBL inhibitor has emerged.[18-24]

Since conventional approaches to develop MBL inhibitors have been unsuccessful so far, alternative methods are needed. One possible tactic to increase the activity of antibiotics involves their complexation with metal cations. Metal ions such as Cu(II), Ag(I) or silver nanoparticles are known both for their antimicrobial properties and for their ability to increase the activity of different antibiotics.[25-32] Coordination of drugs with certain transition metals is known to influence the drug´s antimicrobial properties and biological activity.[29, 30] Most of the reported antibiotic complexes bind the metal ions through electron donor groups.[25-29]

An understanding of the relationship between the structure and chemical compositions of active drugs can be crucial to decipher the mode of drug action. In this study, attempts to enhance the activity of β-lactam antibiotics through complexation with metal ions are described. Specifically, Zn(II) was used as it is an essential cofactor for MBLs, whereas Cu(II) and Ag(I) were selected due to their inherent antimicrobial properties. Isothermal titration calorimetry (ITC) and nuclear magnetic resonance (NMR) studies were applied to investigate binding modes between ampicillin and penicillin G with these metal ions; cefuroxime was included specifically to record reproducible *in vitro* inhibition data (Figure 1). These antibiotics were also selected because they are already severely affected in their effectiveness due to evolved resistance. Based on the determined molar ratio, the effects of complexation on the antimicrobial activity were then examined using micro-broth dilution assays against sensitive and lactam-resistant strains. Finally, kinetic enzymatic studies were carried out to demonstrate that an observed improvement in activity against resistant strains resulted from β-lactamase

inhibition, and indeed a decrease of β-lactamase activity towards cefuroxime in the presence of Ag(I) ions was observed.

2. Materials and Methods

2.1 Materials

All chemicals, including the antibiotics ampicillin, penicillin G, cefuroxime and meropenem as the respective sodium salts, were purchased from Sigma Aldrich unless otherwise stated and used without further purification. Deuterated water was purchased from Cambridge Isotope Laboratories. Metal salts selected were $AgNO_3$, $CuCl_2$, $ZnCl_2$, and $Zn(OAc)_2$. Nitrate was chosen as counter ion for silver due to its good solubility in the buffer employed, chloride and acetate for copper and zinc as they are biocompatible as drug salts. Water for all the aqueous solutions was sourced from a Milli-Q purification system. IMP-1, a class B zinc metallo-β-lactamase was expressed and purified by transforming competent BL21 (DE3) *Escherichia coli* cells with a pET47b-IMP-1 vector using the protocol developed by Vella et al.[33] For the disk diffusion, and micro-broth dilution antimicrobial assays the following bacteria strains were used: *Escherichia coli* ATCC 25922 FDA control strain, IMP-4 metallo-β-lactamase producing *E. coli* (CR48),[34, 35] NDM-4-producing *E. coli* (CR53),[34] CTX-M-15-producing *E. coli* (Ec71)[36] and *E. coli* TOP 10, *Klebsiella pneumoniae* ATCC 700603 MDR, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853, methicillin-resistant *Staphylococcus aureus* ATCC 43300. All ATCC strains were obtained from ATCC (American Type Culture Collection). All other *E. coli* were clinical strains *E. coli* which have been characterized for the antimicrobial resistance markers and sequence types.[34-36]

2.2 Methods

2.2.1 ITC measurements

ITC experiments were performed using a MicroCal Omega Auto iTC200 (GE Healthcare). The measurements were carried out at a temperature of 298.15 K under an atmosphere of nitrogen, with a stirring speed of 1000 rpm and a reference power of 10 µcal/s. The reference cell was filled with ultrapure water (250 µL), the sample cell (220 µL) and the syringe (40 µL) with the respective sample in 10 mM HEPES buffer solution (pH of 7.4), with injections repeated three times consecutively. Furthermore, water to water measurements were performed between each experiment. The association constant (K), the change in enthalpy (ΔH), entropy (ΔS) and

the stoichiometry (*N*) were fitted with a single site binding model. Calculations using the equation $\Delta G = -RT\ ln(K)$ (where *T* is the absolute temperature and *R* the universal gas constant) revealed Gibbs free energy (ΔG). As suggested by the device company, the first raw data point was removed to minimize additional effects during the first injection (0.4 µL over a period of 0.8 s). Control experiments including every binding participant and the buffer were carried out to determine dilution effects [injection setup: Volume (2 µL), duration (2 s), spacing (150 s), filter period (5 s)]. The setup for the binding measurements consisted of titrating solutions of metal salts with concentrations from 1.5 mM up to 4 mM into solutions of the antibiotic salts with a concentration of 0.4 mM. The experiments comprised either 20 injections (each 2 µL), 30 injections (each 1.3 µL) or 40 injections (each 1 µL) with a range of spacing from 300 s to 1200 s.

2.2.3 NMR

Interactions between the antibiotics and silver ions were investigated by NMR spectroscopy using a Bruker AVANCE III 600 MHz instrument. The pH was adjusted to 7.4 by adding NaOH to a HEPES stock solution (10 mM with 5 v% D_2O). The antibiotics ampicillin and penicillin G were dissolved in the HEPES buffer and transferred into the NMR tube right before each measurement. The NMR tube was covered in aluminum foil (due to the light-sensitivity of Ag(I)) after adding the required volume of Ag(I)/HEPES solution. Nine different antibiotic to Ag(I) ratios were tested: 1:0.0; 1:0.25; 1:0.5; 1:0.75; 1:1.0; 1:1.5; 1:2.0; 1:3.0; 1:4.0 (details about samples preparation: Supporting information (SI), Table S1).

2.2.4 Antimicrobial micro-broth dilution assay

The different metal and antibiotic complexes were prepared immediately before the experiment by dissolving and mixing them in distilled water. All bacteria strains were cultured in Mueller Hinton (MH) broth at 37°C overnight with shaking (180 RPM). A sample of each culture was then diluted 40-fold in fresh MH-broth and incubated at 37°C for 2 - 3 h. The compounds were serially diluted two-fold across the wells, with concentrations ranging from 0.03 µg/mL to 256 µg/mL and plated in non-binding surface (NBS) plates (Corning, 3641). The bacteria cultures were diluted and 50 µL added to each well of the compound-containing 96-well plates to the

final cell concentration of 5×10^5 CFU/mL, and a final compound concentration range of 0.06 μg/mL to 128 μg/mL. All plates were covered and incubated at 37°C for 24 h. Minimum Inhibitory Concentrations (MICs) were determined visually, being defined as the lowest concentration showing no visible growth.

2.2.5 Disk-test of β-lactam antibiotics

The antimicrobial activity of several complexes of ampicillin and penicillin G with metal ions was also tested in a disk assay against *E. coli* strains that produce β-lactamase as a resistance mechanism against a wide spectrum of β-lactam antibiotics: four different *E. coli* strains (CR48, CR53, Ec71 and TOP10) were used for this purpose. Two of the multidrug-resistant *E. coli* strains (CR48 and CR53) produce NDM-4 (New Delhi metallo-β-lactamase) or IMP-4 metallo-β-lactamase, respectively. The third strain, Ec71, expresses an extended spectrum β-lactamase, CTX-M-15, which is able to cleave cefotaxime (third generation cephalosporin antibiotic). *E. coli* TOP10 is a susceptible *E. coli* strain without any resistance gene.

Colonies of the bacteria strains were added to different tubes with MH-broth until an optical density at 600 nm (OD600) of around 0.2 a.u. was reached. The bacteria suspension was then inoculated on to the MH agar plates in order to perform disk susceptibility testing. The appropriate disks were placed. Blank disks were also used by adding the penicillin G solution and its complexes on to these disks. Blank disks were also used to test the inhibitory activity of the metal compounds by themselves. For ampicillin and its complexes, standard disks containing 10 μg of ampicillin (Becton Dickinson) were used, with the metal ion solution added before placing on the agar plate. For penicillin G, blank disks were used and 10 μg of the antibiotic was added as a solution. Complexes of antibiotics and metal ions of different molar ratios were prepared: ampicillin /Ag(I) 1:1, 1:5 and 1:10 and for penicillin G/Ag(I) 1:0.5, 1:5 and 1:10. Furthermore, the solutions of the antibiotics and the metal solutions were added separately on a blank disk.

2.2.6 Enzymatic study

The β-lactamase activity of the purified enzyme IMP-1 metallo-β-lactamase towards cefuroxime was measured spectrophotometrically by monitoring the depletion of the absorbance at λ = 260 nm, a change induced through the cleavage of the β-lactam ring by the enzyme.[21] Reactions were performed for 60 s at 25°C in 20 mM HEPES (pH 7.4) with a final enzyme concentration of 16 nM IMP-1 metallo-β-lactamase. Michaelis-Menten curves were generated for assays conducted at different concentrations of $AgNO_3$ (0 µM, 2 µM and 5 µM). Reaction rates were determined from the initial linear portion of the reaction progress. The Michaelis-Menten parameters of each of the assays (k_{cat} (the maximum velocity of the enzyme expressed as the number of moles of substrate converted to product by one mole of enzyme in one second), K_M (the concentration of substrate needed to reach half maximum velocity) and k_{cat}/K_M) as well as the inhibition constant (K_i) and mode were determined using GraphPad Prism 7 software. All the measurements were carried out using a Varian Cary 50-BIO spectrophotometer connected to a Peltier Thermostat system. The reactions were carried out without the addition of extra zinc to the buffer.

3. Results and Discussion

3.1 Characterization of complexes between metal ions and antibiotics

3.1.1 ITC studies

The investigation of complex formation between antibiotics and metal ions by ITC revealed exothermic interactions between ampicillin, penicillin G and cefuroxime with $CuCl_2$ and $AgNO_3$ and endothermic interactions with $ZnCl_2$ and $Zn(OAc)_2$. No interactions could be found between $CaCl_2$ (at a concentration of 4 mM) and the examined antibiotics. The strongest interactions occurred with $AgNO_3$ (ΔG up to -7.5 kcal mol^{-1}), followed by $CuCl_2$ (ΔG around -7.0 kcal mol^{-1}) and $Zn(OAc)_2$ ($\Delta G \leq 6.7$ kcal mol^{-1}) (Table 1). The complexation of ampicillin and cefuroxime with $AgNO_3$ occurred with a strong enthalpically driven reaction (ΔH up to - 5.9 kcal mol^{-1}). The stability and purity of the resulting metal-antibiotic complexes were determined using LC-MS (SI, 2 LC-MS experiments). No cleavage of the lactam bond catalyzed through the presence of metal ions was observed on the timescale of the measurements by LC-MS.

$CuCl_2$ to ampicillin and penicillin G: The titration of 4 mM of $CuCl_2$ to a 0.4 mM solution of ampicillin [number of injection: 40, spacing time: 600 s, Volume per injection: 1 µL] revealed an interaction between one Cu(II) cation and two anionic ampicillin molecules. In the experiments consisting of 4 mM $CuCl_2$ titrated to 0.4 mM penicillin G [spacing time expanded to 1200 s] the stoichiometry was found to be one to one (SI, Figure S1). The values of ΔG were in the same range, only small differences could be found in the exothermic enthalpy (higher values for penicillin G with ΔH = -2.8 kcal mol^{-1} compared to -1.5 kcal mol^{-1} for ampicillin) and the entropy (higher increase for titrations involving ampicillin with $T \cdot \Delta S$ = 5.4 kcal mol^{-1}).

$AgNO_3$ to ampicillin and penicillin G: Interactions were determined by titrating 3 mM $AgNO_3$ to 0.4 mM of ampicillin [number of injection: 40, spacing time: 600 s, Volume per injection: 1 µL] and 1.5 mM $AgNO_3$ titrating to 0.4 mM penicillin G, subsequently (SI, Figure S1 and S2). The measurements revealed an equimolar ratio for Ag(I) cation and anionic ampicillin and a binding mode of one Ag (I) cation to two anionic penicillin G molecules. Compared to the interactions with Cu(II), the interactions showed a comparable Gibbs energy and an entropically driven reaction for the antibiotic penicillin G but an enthalpic interaction in case of ampicillin.

$AgNO_3$ to cefuroxime: In this case 2.5 mM $AgNO_3$ was titrated into 0.4 mM cefuroxime [number of injection: 30, spacing time: 600 s, Volume per injection: 1.3 μL]. It was found that Ag(I) complexed with two cefuroxime molecules showing an exothermic interaction (Figure 2). The value of the association constant (*K*) was found to be in between the value for the titration of Ag(I) to ampicillin and that for Ag(I) to penicillin G. The ΔG value was in the same range of the previous mentioned Ag(I) complexes. However, the binding of Ag(I) to cefuroxime (-5.9 kcal mol^{-1}) proceeded with a higher enthalpy when compared to Ag(I) binding to ampicillin and penicillin G (-4.6 kcal mol^{-1} and - 2.1 kcal mol^{-1} respectively).

$ZnCl_2$/ $Zn(OAc)_2$ to ampicillin and penicillin G: To check the influence of the counterion on the interactions with the antibiotics, both $ZnCl_2$ and $Zn(OAc)_2$ were examined (SI, Figure S1). Titrations of 4 mM $ZnCl_2$ and $Zn(OAc)_2$ to 0.4 mM ampicillin and penicillin G [number of injection: 20, spacing time: 300 s, Volume per injection: 2 μL] revealed interactions to be around a molar ratio of one Zn(II) cation to two anionic antibiotic molecules for zinc chloride, and one Zn(II) cation to five anionic antibiotic molecules for zinc acetate. The overall processes were constant with an endothermic enthalpy in both cases and therefore a strong entropically driven process. The thermodynamic data were influenced by the counterion: ΔG and ΔH increased by a factor about 1.1 and $T \cdot \Delta S$ by 1 kcal mol^{-1} using the acetate salt instead of chloride.

ITC summary: ITC experiments revealed different binding modes between the antibiotics and the metal ions. Depending on the metal cation and its oxidation state, the stoichiometries of the metal-β-lactam complexes were found to be either 1:2 or 1:1 in case of $AgNO_3$ and $CuCl_2$. Related to the antibiotics ampicillin and penicillin G the different values for the stoichiometry could be explained due to the presence of an extra amino group in ampicillin (Figure 1). NMR studies were performed to confirm our speculations on the involvement of the amino group in the complex formation.

3.1.2 NMR studies

To confirm the ITC observations, NMR experiments were performed to obtain structural information about the complexation of these antibiotics with silver ions. The NMR measurements showed moderate interactions between the antibiotics and Ag(I) ions based on concentration dependent proton shifts: The higher the concentration of Ag(I), the higher the observed shift of the proton signals in comparison to the starting material (Figure 3 and 4, Table S2 and S3, SI). The strongest interactions were detected for the protons H_9 in both cases (penicillin G and ampicillin, Figure 5 and 6), and for $H_{7\text{-}NH}$ in case of penicillin G. For ampicillin this particular proton was not visible. The results provide evidence that the Ag(I) is coordinated by the amide nitrogen N_7 and the sulfur atom in the thiethane ring on both cases. The protons H_8 and H_9 show two doublets around 5.5 ppm for penicillin G (Figure 3) but not for ampicillin (Figure 4) where only a singlet could be found. Interestingly, this singlet was found to split up into the expected two signals by the continuous addition of Ag(I). This provides evidence that Ag(I) stabilizes a rapidly isomerizing pair of structures, with a change of the three-dimensional orientation of the molecule in an aqueous solution mimicking physiological-like conditions. The NMR measurements, with observed linear proton shifting trend, are in a good agreement with the ITC results, displaying the Ag(I)/antibiotic ratio reaches 1:2 for the penicillin G complex (based on the proton $H_{7\text{-}NH}$), and 1:1 ratio for the ampicillin complex (based on overall proton shifts). Even though further increases of the silver concentrations were leading to slightly higher shifts, especially for the H_9 and H_{13} in the ampicillin complex, the trends were visible until reaching these ratios (Figure 5 and 6).

3.2 Influence of metal ions on the antibiotic activities

3.2.1 Antimicrobial micro-broth dilution assay

Broth microdilution assays to determine MICs (minimum inhibitory concentrations) were performed to investigate the influence of complexes between β-lactam-antibiotics and metals on bacterial growth. Both Gram-positive and Gram-negative bacteria strains were examined. All experiments were conducted with vancomycin as a positive inhibitor control for Gram-positive bacteria and colistin as a positive inhibitor control for Gram-negative bacteria. As expected,

experiments with penicillin G and ampicillin as examples of early generation β-lactam antibiotics showed antibacterial effects only against the control strain of *E. coli*, with almost no effect on other Gram-negative and MDR strains. Varying results were seen with the metal salts alone: $AgNO_3$ had an antibacterial effect against all examined strains in contrast to $CuCl_2$ and $Zn(OAc)_2$ where only a very low antimicrobial effect was visible.

For several complexes between antibiotics and metal ions synergistic effects were observed. Synergy occurs if the combined effect of two agents is greater than the sum of their individual effects. For the quantification of the synergistic effects the fractional inhibitory concentration index (FICI) was calculated with the following formula, whereas FICI values below ≤ 0.5 indicate synergistic effects[37] :

$$FICI = \frac{MIC_{antibiotic\ in\ the\ complex}}{MIC_{antibiotic\ alone}} + \frac{MIC_{metal\ in\ the\ complex}}{MIC_{metal\ alone}}$$

The complex of ampicillin with Ag(I) in the stoichiometry determined in the previous ITC experiments showed the lowest MIC values for its minimal inhibitory concentration with FICI values < 0.5 and therefore the best inhibition of bacterial growth. With the ratio of 1:1 for ampicillin and Ag(I) the highest synergistic effects could be observed (Table 2). Complexes with a 1:10 stoichiometry showed synergistic effects as well but in relation to the higher concentration of $AgNO_3$ the synergistic effects appeared less significant, though this was strain dependent: For *A. baumannii* and *P. aeruginosa* nearly 10-fold lower ampicillin concentrations were required for the 1:10 stoichiometry compared to the 1:1, with similar absolute Ag(I) concentrations. The same trend could be observed for the complexes of penicillin G with Ag(I) in the stoichiometry 1:0.5 and 1:5. In regards to the other metal salts, only complexes between ampicillin and $CuCl_2$ displayed antibacterial effects against the control strain of *E. coli*. Experiments involving Zn(II) revealed no activity against any of the bacteria strains. The complexes corresponding to the molar ratio determined in ITC experiments showed the best relation between activity and metal-ion concentration.

Different trends were observed for the Gram-positive strain *S. aureus* (MRSA) (Table 2). The complexes with Cu(II) showed high synergistic effects with both penicillin G and ampicillin and even for the Zn(II) complexes synergistic effects were visible. The lowest MIC values were

obtained for the complexes of penicillin G with Cu(II) and Ag(I). Interestingly ampicillin and penicillin G alone showed higher potency against Gram-positive strains than Gram-negative strains, whereas Ag(I) showed a reduced activity. Furthermore, a preliminary MIC assay with NDM-4 and IMP-4 producing bacteria strains confirmed the synergistic effects of Ag(I) and the β-lactam antibiotics (Table S5, SI). The complexes used in this study should not cause toxicity, as most of the MIC values are below the LD50 values for these metal ions described in literature, with only one exception in case of the complex between penicillin G and Cu(II) (PenG/Cu 1:10 complex) where the Cu(II) concentration is higher than it's reported LD50, but this particular complex employed a large excess of copper and was less potent than PenG/Cu 1:1 (for details please see Table S6, SI). To confirm the results acquired within these MIC experiments, further investigations of the complexes were conducted as part of a disk diffusion susceptibility test (Table S7, SI, samples preparation).

3.2.2 **Disk Diffusion assay**

For this assay, zones free of bacterial growth (zone of inhibition) surrounding the disk containing antibiotic or antibiotic with metal ion were evaluated (Figure S3, Table 3). The diameter of these zones is proportional to the antimicrobial activity of the different compounds. The antimicrobial activity of ampicillin was tested together with different concentrations of Cu(II), Ag(I) and Zn(II) ions within the first trial. It was clearly visible that ampicillin, Cu(II) ions or Zn(II) ions alone had no activity against the NDM-4 and IMP-4-producing strains. Only Ag(I) showed a moderate antimicrobial activity against all strains tested (Table 3). Furthermore, for the complex of ampicillin and Ag(I) in an equimolar ratio, the diameter of the inhibition zone was the same as for Ag(I) alone. However, the concentration of Ag(I) in the complex is only half of the Ag(I) concentration of the disc with Ag(I) alone. The inhibition zone of the complex ampicillin and Ag(I) in the ratio 1:10 was larger than for the equimolar complex, resulting from the increased concentration of Ag(I) ions. This indicates that, for ampicillin and Ag(I) complexes, synergistic effects were observed, whereas the highest effect in respect of the metal concentration was obtained for the ratio determined by ITC. Penicillin was also tested in combination with Ag(I)

against the NDM-4 and IMP-4-producing strains, and also showed the highest synergistic effects at the ratio determined by ITC experiments.

Finally, the antimicrobial activity of penicillin G and ampicillin was tested against two additional *E. coli* strains (Ec71 resistant to 3rd generation cephalosporins and a susceptible *E. coli* TOP 10) to evaluate whether the observed synergistic effects are limited only to MBL-producing *E. coli* strains. The *E. coli* Top 10 strain was included as a negative control as it is not resistant to β-lactam antibiotics, whereas strain Ec71 produces a broad-spectrum β-lactamase that is not metal ion dependent. Due to the fact that the complexes with Cu(II) and Zn(II) showed no activity (data not shown), only the effect of complexes with Ag(I) were analyzed. While the growth of the TOP 10 strains appeared to be minimally affected by the presence of either the ampicillin or penicillin G complexes (the inhibition zone remained, more or less, constant at 10 mm) the growth of the Ec71 strain was closely related to that of the NDM-producing *E. coli* strain CR53. In the Ec71 system the complexes of penicillin G and ampicillin with Ag(I) showed synergistic effects; the strongest effect (relative to the concentration of Ag(I)) was observed for the ratio obtained by ITC (1:0.5). Thus, in summary, both the micro-broth dilution and the disk diffusion studies lead to the conclusion that the complexation of metal ions by β-lactam antibiotics can increase their antimicrobial activity through synergistic effects.

3.2.3 **Enzymatic study**

Kinetics assays were conducted to assess the ability of Ag(I) to inhibit the degradation of cefuroxime by IMP-1 metallo-β-lactamase (Table S8 to S10, SI, raw data). The activity of IMP-1 metallo-β-lactamase on cefuroxime was visibly inhibited upon addition of Ag(I) (Table 4, Figure 7). The assays were run using 0 µM, 2 µM and 5 µM of IMP-1 metallo-β-lactamase showing a K_i (the amount of inhibitor needed to reduce the activity of the enzyme by half) of 0.6 µM. These results, along with those from the disk diffusion test, indicate that Ag(I) can act as an effective inhibitor of β-lactamase activity, therefore enhancing the antimicrobial activity against resistant strains when used in combination with β-lactam antibiotics.

4. Conclusions

The observed synergism of the complexes between β-lactam antibiotics and some metal ions could be due to inhibition of β-lactamase activity. ITC and NMR experiments revealed interactions between Ag(I) or Cu(II) and β-lactam antibiotics, with these complexes showing an increase in activity of the antibiotic in the presence of the metal ions. However, Zn(II) complexes showed an endothermic binding by ITC, and negligible antimicrobial effect. Microbiological experiments revealed the highest synergistic effects of the complexes tended to match the molar ratio determined by ITC. Moreover, the activity of IMP-1 enzymes could be inhibited in the presence of complexes between Ag(I) and cefuroxime, demonstrating that at least some of the antimicrobial enhancement was due to prevention of lactam hydrolysis. These finding indicate that Ag(I) is synergistically working with β-lactam antibiotics by inhibiting and/or blocking the β-lactamase activity allowing the antibiotics to be active against bacteria and a potential lactam/lactamase inhibitor combination to be used in the clinic. This study also demonstrated that the counterion influenced the binding process, hence future plans also include testing of different metal salts, as well as different oxidation and physical states of metals including nanoparticles, organoparticles and colloidal forms.

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Tables and Figures

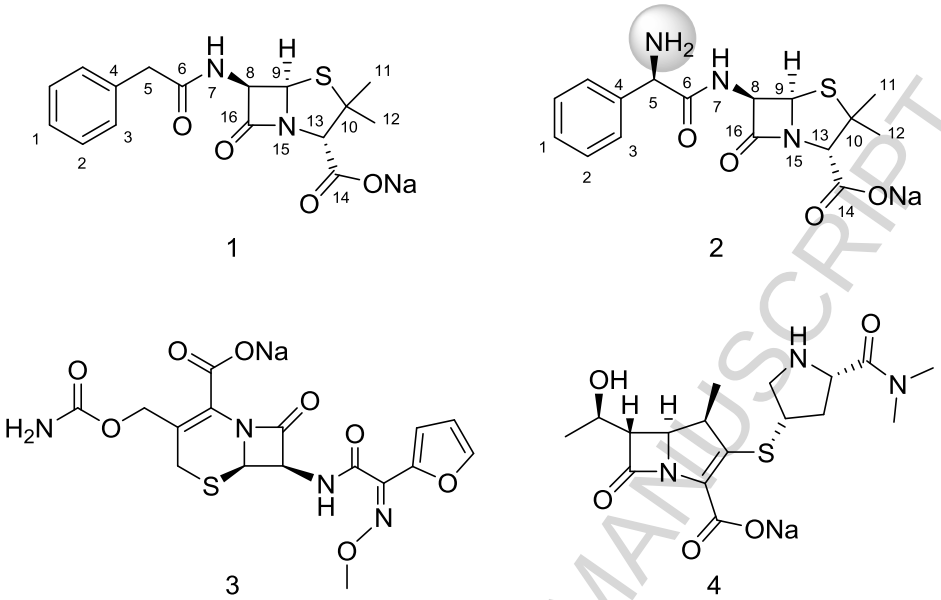


Figure 1: Chemical structure of the semisynthetic antibiotics penicillin G (compound **1**), ampicillin (compound **2**, with extra amine group highlighted), cefuroxime (compound **3**) and meropenem (compound **4**). These antibacterial agents are all β-lactam antibiotics and used for the treatment of a broad spectrum of infection.

Table 1: The thermodynamic data of interactions between Cu(II), Zn(II), Ag(I) with ampicillin, penicillin G or cefuroxime in 10 mM HEPES buffer (pH 7.4) at a constant temperature of 298 K. All experiments were performed at a concentration (conc.) of 0.4 mM for each antibiotic. T is the absolute Temperature, K is the association constant, ΔG is the change in Gibbs energy, ΔH is the change in enthalpy, ΔS is the change in entropy and N is the stoichiometry. Interactions determined by ITC experiments revealed Ag(I) as the most promising binding partner.

System	Conc. (metal salt) [mM]	K [10^5 M^{-1}]	ΔG [10^3 cal mol^{-1}]	ΔH [10^3 cal mol^{-1}]	T·ΔS [10^3 cal mol^{-1}]	N
$CuCl_2$ to ampicillin	4	1.3 ± 0.7	-7.0 ± 0.6	-1.5 ± 0.2	5.4 ± 0.2	0.51 ± 0.03
$CuCl_2$ to penicillin G	4	1.0 ± 0.3	-6.8 ± 0.6	-2.8 ± 0.2	4.0 ± 0.2	1.22 ± 0.04
$AgNO_3$ to ampicillin	3	2.6 ± 0.5	-7.5 ± 0.2	-4.6 ± 1.7	2.9 ± 1.5	1.05 ± 0.12
$AgNO_3$ to penicillin G	1.5	3.0 ± 1.2	-7.4 ± 0.2	-2.1 ± 0.2	5.3 ± 0.4	0.43± 0.21
$AgNO_3$ to cefuroxime	2.5	2.8 ± 0.1	-7.4 ± 0.1	-5.9 ± 0.2	1.6 ± 0.2	0.61 ± 0.01
$ZnCl_2$ to ampicillin	4	0.1 ± 0.1	-5.5 ± 0.7	0.7 ± 0.3	6.2 ± 0.4	0.58 ± 0.12
$ZnCl_2$ to penicillin G	4	0.1 ± 0.1	-5.4 ± 0.6	0.6 ± 0.1	6.1 ± 0.2	0.46 ± 0.07
$Zn(OAc)_2$ to ampicillin	4	0.3 ± 0.1	-6.2 ± 0.1	1.1 ± 0.2	7.2 ± 0.3	0.21 ± 0.08
$Zn(OAc)_2$ to penicillin G	4	0.9 ± 0.1	-6.7 ± 0.1	0.5 ± 0.1	7.2 ± 0.2	0.19 ± 0.04

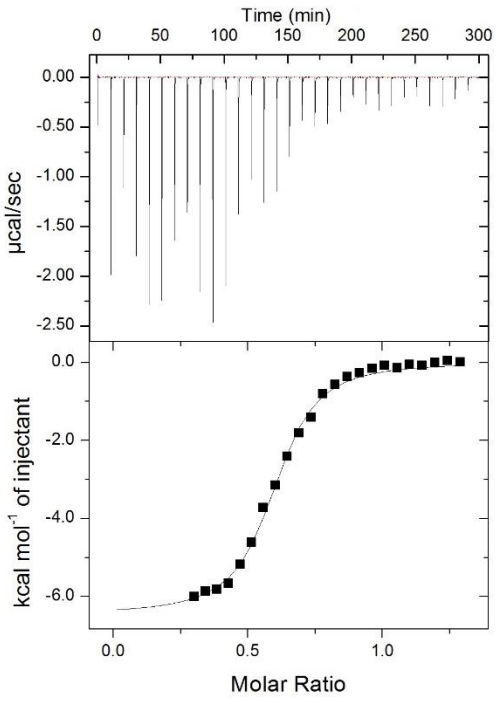


Figure 2: Results for the titration of 2.5 mM $AgNO_3$ to 0.4 mM cefuroxime in 10 mM HEPES buffer (pH 7.4) using 30 injections. The upper panel shows the heat response given by each injection and the lower panel shows the integrated heat curve points normalized per mole of injectant as a function of molar ratio. A One-site model was used to determine an N, K, ΔH and ΔS value of 0.601 ± 6.06 $x10^{-3}$, 2.74 x 10^5 ± 3.94 x 10^4 M, -6.15 x 10^3 ± 0.119 x 10^3 cal/mol and 4.25 cal/mol/K respectively. This shows an exothermic interaction between one silver ion and two cefuroxime molecules.

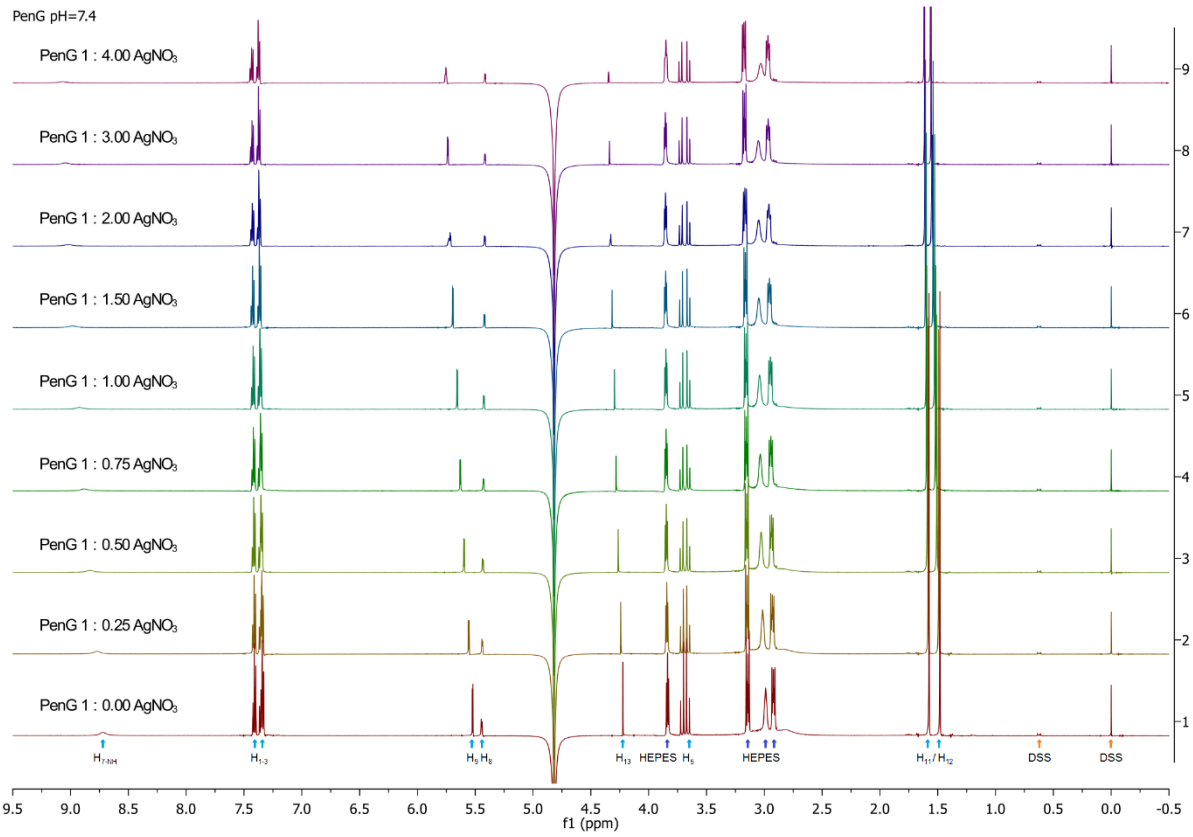


Figure 3: Comparison of the proton shifts (^{1}H-NMR) of penicillin G with nine different ratios of Ag(I) in HEPES buffer (pH 7.4). By increasing the Ag(I) concentration the proton signals get shifted more, especially H_9 at about 5.5 ppm and $H_{7\text{-NH}}$ at about 8.7 ppm.

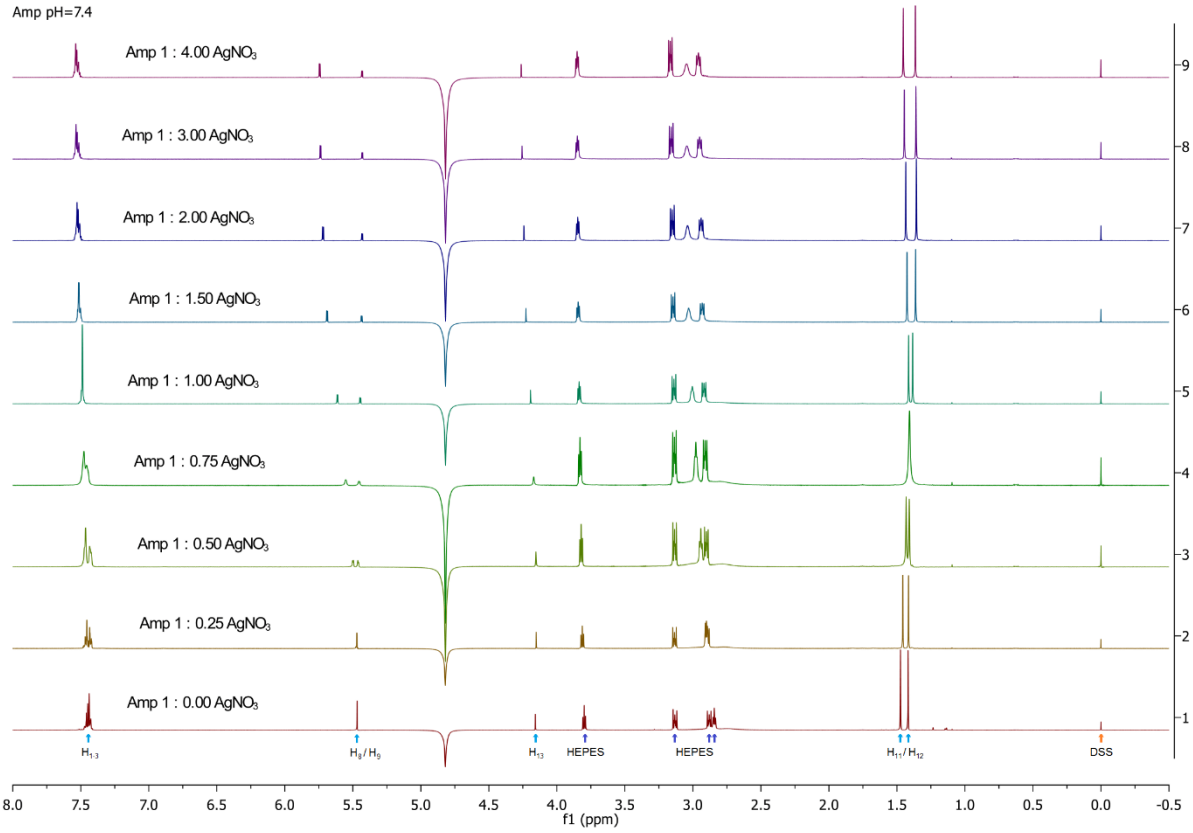


Figure 4: Comparison of the proton shifts (^{1}H-NMR) of ampicillin with nine different ratios of Ag(I) in HEPES buffer (pH 7.4). By increasing the Ag(I) concentration the proton signals get shifted more, especially H_9 at about 5.5 ppm, which becomes split into two signals.

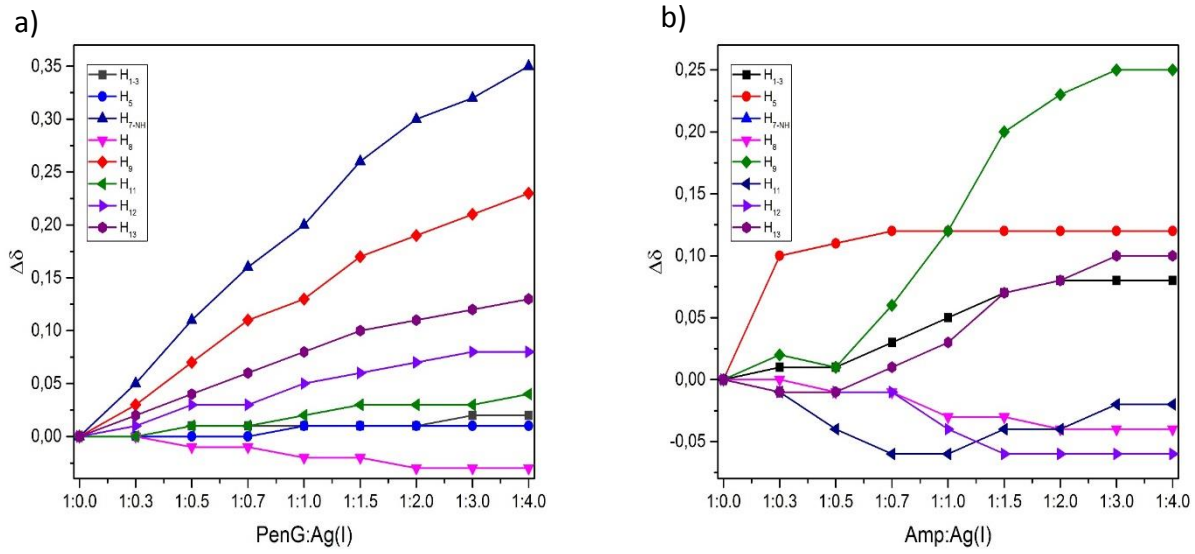


Figure 5: Difference between the shifts for the proton signals of the respective antibiotic and different ratios of Ag(I) in HEPES buffer (pH 7.4): a) penicillin G (PenG), b) ampicillin (Amp).

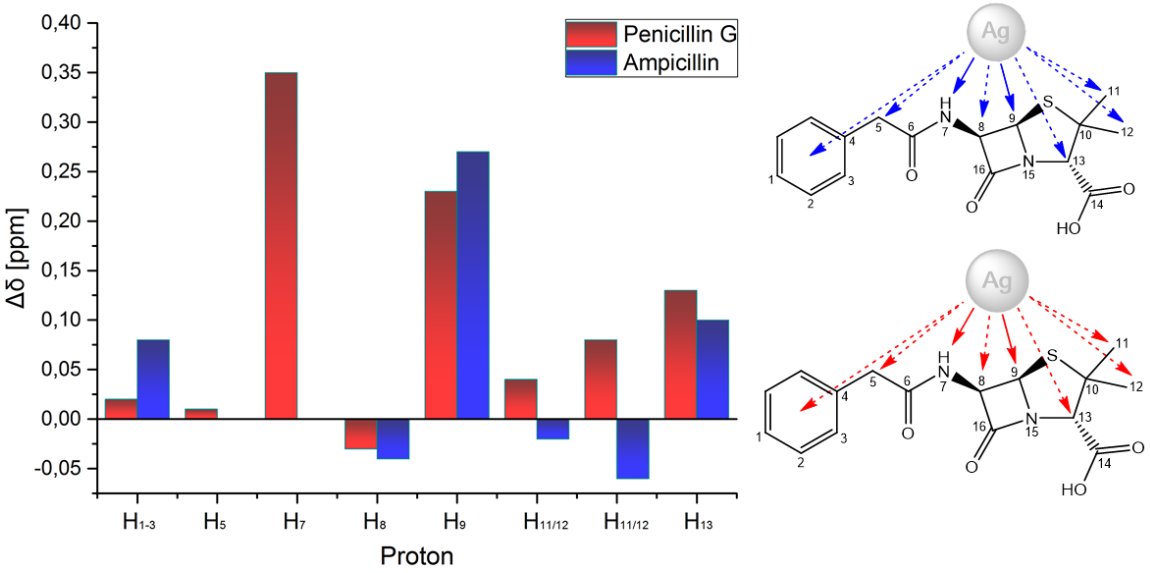


Figure 6: Comparison of the respective proton shifts (^{1}H-NMR) of ampicillin and penicillin G in HEPES buffer (pH 7.4) between the antibiotic shifts without silver salt and the observed shifts for the highest Ag(I) to antibiotic ratio (4:1).

Table 2: Micro-broth dilution results for the complexes between ampicillin and penicillin G with different metal ions. Complexes with Cu(II) and Zn(II) showed very low activity whereas the MIC (Minimum Inhibitory Concentration) values of the complex between $AgNO_3$ and ampicillin showed the most promising results against resistant bacteria strains. Fractional inhibitory concentration index (FICI) values were calculated for the most promising complexes. FICI values below ≤ 0.5 indicate synergistic effects, 0.5 < FICI < 4 define no interaction, FICI > 4 define antagonism. The FICI values <0.5 are written in bold. Duplicate values shown. The starting concentration of the respective antibiotics was 2.56 mg/mL. Shaded fields show those concentrations where the activity was better than either of the individual components.

Compound	Metal salt Starting Conc.	*E. coli*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*S. aureus*
		ATCC 25922	ATCC 700603	ATCC 19606	ATCC 27853	ATCC 43300
	[mg/mL]	FDA control	MDR	Type	QC strain	MRSA
		MIC [µg/mL]* (FICI)				
colistin		0.03/0.06	0.03/ 0.06	0.06	0.06/0.125	
vancomycin						1
ampicillin		4	>128	>128	>128	8/16
penicillin G		32/64	>128	>128	>128	8/16
$AgNO_3$	2.56	2	4	2	2/4	16
$CuCl_2$* 2 H_2O	2.56	>128	>128	>128	>128	>128
$Zn(OAc)_2$ * 2 H_2O	2.56	>128	>128	>128	>128	>128
Amp/Ag(I) 1:1	1.17	2 (0.96)	4 **(<0.48)**	2 **(0.47)**	2 **(<0.47/<0.24)**	2/4 **(0.31/0.36)**
Amp/Ag(I) 1:10	11.7	1/0.5 (2.5/1.3)	1 (<1.1)	0.125/0.25 **(<0.28**/<0.57)	0.25 (<0.57/**<0.28)**	1 **(0.41/0.12)**
PenG/Ag(I) 1:0.5	0.61	2/4 **(0.30**/0.53)	8 (<0.54)	2 **(<0.25)**	2/4 **(<0.25/<0.27)**	4 **(0.56/0.30)**
PenG/Ag(I) 1:5	3.05	2/4 (1.2/2.4)	4 (<1.22)	1 (<0.60)	1/2 (<0.60/<0.61)	2 **(0.40/0.27)**
Amp/Cu(II) 1:0.5	0.59	8/16 (<2.0/<4.0)	>128	>128	>128	32 (<4.1/<2.1)
Amp/Cu(II) 1:5	2.94	64/128 (<17/<33)	>128	>128	>128	2/4 **(<0.27/<0.29)**
PenG/Cu(II) 1:1	1.22	>128	>128	>128	>128	1 **(<0.13/<0.07)**
PenG/Cu(II) 1:10	12.2	>128	>128	>128	>128	4 (<0.65/**<0.27**)

Amp/Zn(II) 1:10	7.90	>128	>128	>128	>128	2/4 **(<0.3/<0.35)**
PenG/Zn(II) 1:10	7.50	>128	>128	>128	>128	16/32 (<2.4/<2.7)

*MIC values are provided for antibiotics, metal salts, and antibiotics in the complexes, while the MIC values of metals in the complexes, necessary to calculate the FICI, are provided in Table S4, SI.

Table 3: Zone of inhibition measured for the disk diffusion tests for complexes between ampicillin and penicillin G with silver ions tested against *E. coli* expressing different β-lactamases. The best ratio of antimicrobial activity related to the Ag(I) concentration could be obtained for the complexes with the stoichiometry obtained by ITC experiments.

		E. coli	*E. coli*	*E. coli*	*E. coli*
	Metal salt conc. [µg/mL]	CR48 (IMP-4)	CR53 (NDM-4)	CTX-M-15 (Ec71 producing CTX-M-15)	TOP 10
Compound		Zone of inhibition (diameter) [mm]			
$AgNO_3$ only	1.68	10	7	8	10
Amp/Ag(I) 1:1	0.76	10	8	8.5	10
Amp/Ag(I) 1:5	3.8			8.5	10
Amp/Ag(I) 1:10	7.6	14	9	10	10
Amp only		0	0	0	10
PenG/Ag(I) 1:0.5	0.38	8	7	8	8
PenG/Ag(I) 1:5	1.9	11	7	9	10
PenG/Ag(I) 1:10	3.8	11.5	9	10	10
PenG only		0	0	0	0

Table 4: Kinetic parameters of the silver inhibition of the hydrolysis of cefuroxime by 16 nM IMP-1 metallo-β-lactamase using varying inhibitor concentrations. The catalytic rate k_{cat} (the maximum velocity of the enzyme expressed as the number of moles of substrate converted to product by one mole of enzyme in one second) of IMP-1 is reduced and the K_M (the concentration of substrate needed to reach half maximum velocity) is increased as the concentration of Ag(I) increases, resulting in a decrease in the catalytic efficiency expressed as k_{cat}/K_M (/sec/μM).

$[AgNO_3]$ [μM]	K_{cat} [/sec]	K_M [μM]	K_{cat}/K_M [/sec/μM]
0	16.6	5.92	2.80
2	15.3	24.1	0.635
5	9.88	27.6	0.358

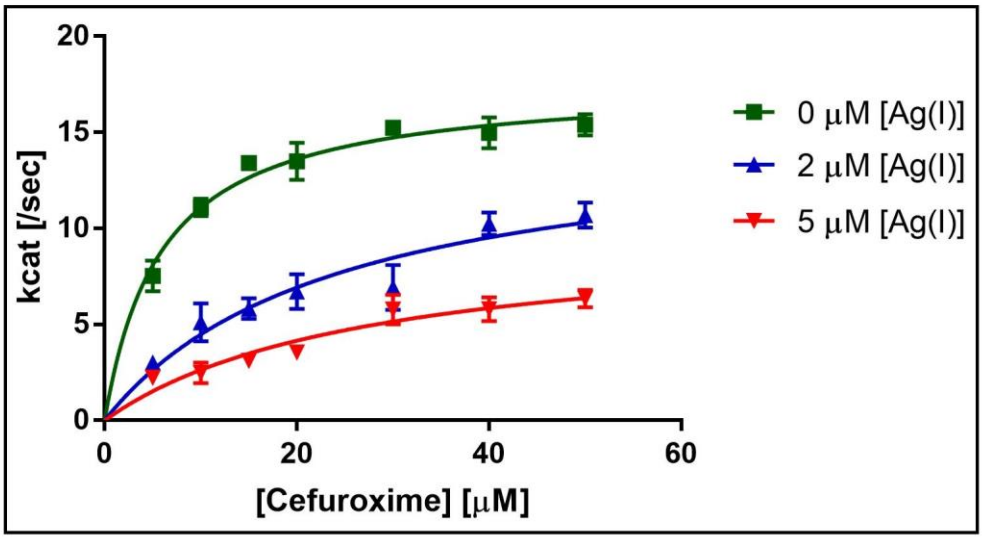
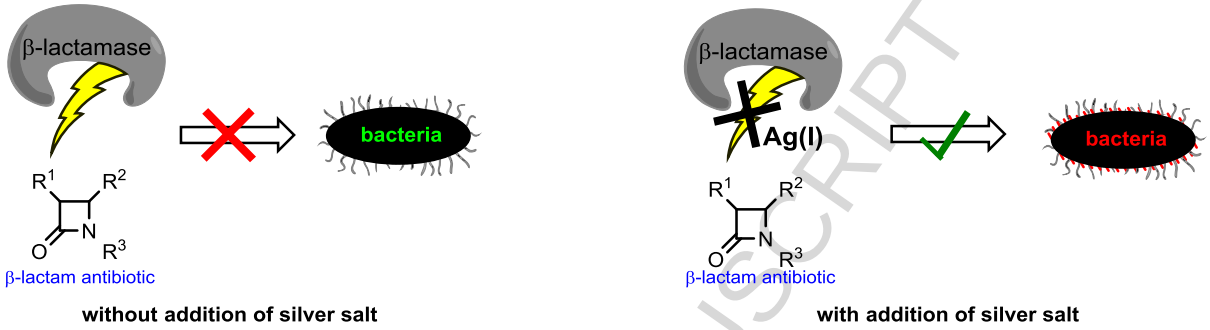


Figure 7: Michaelis-Menten curves for the hydrolysis of cefuroxime by IMP-1 metallo-β-lactamase with varying concentrations of $AgNO_3$ (0 μM, 2 μM and 5 μM). As the concentration of $AgNO_3$ increases the k_{cat} decreases (16.6 /sec, 15.3 /sec and 9.88 /sec for 0 μM, 2 μM and 5 μM respectively) and the K_M increases (5.92 μM, 24.1 μM and 27.6 μM for 0 μM, 2 μM and 5 μM respectively) resulting in a decrease in catalytic efficiency as the concentration of inhibitor increases (2.80 /sec/μM, 0.635 /sec/μM and 0.358 /sec/μM for 0 μM, 2 μM and 5 μM respectively).

Graphical abstract

Synergistic effect was observed in the complexes of β-lactam antibiotics formed with silver(I). Interactions between metal ions and antibiotics were explored by isothermal titration calorimetry.



Highlights

- Complex formation between β-lactam antibiotics and metal ions (Ag(I), Cu(II), Zn(II)) was investigated.
- Thermodynamics of complex formation were assessed by ITC.
- The activity of all metal:antibiotic complexes was tested in minimal inhibitory concentration (MIC) assays.
- The Ag(I):β-lactam antibiotic complex formation was confirmed by NMR, and potency was verified by enzymatic assay showing inhibition of β-lactamases responsible for resistance.