Inhibition of Aminoglycoside 6'-N-Acetyltransferase Type Ib-Mediated Amikacin Resistance in *Klebsiella pneumoniae* by Zinc and Copper Pyrithione

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The *in vitro* activity of the aminoglycoside 6'-N-acetyltransferase type Ib [AAC(6')-Ib] was inhibited by CuCl₂ with a 50% inhibitory concentration (IC₅₀) of 2.8 μM. The growth of an amikacin-resistant *Klebsiella pneumoniae* strain isolated from a neonate with meningitis was inhibited when amikacin was supplemented by the addition of Zn²⁺ or Cu²⁺ in complex with the ionophore pyrithione. Coordination complexes between cations and ionophores could be developed for their use, in combination with aminoglycosides, to treat resistant infections.

*Klebsiella pneumoniae* is part of the ESKAPE (Enterococcus faecium, Staphylococcus aureus, *Klebsiella pneumoniae*, Acinetobacter baumannii, *Pseudomonas aeruginosa*, and Enterobacter species) group of bacteria, which are responsible for the majority of U.S. hospital infections and received the name because they “escape” the effects of antibacterial drugs (1). The most common infections caused by *K. pneumoniae* are pneumonia, septicemias, urinary tract infections, meningitis, and soft tissue infections (2–4). Less common infections caused by this bacterium are septic arthritis (5), liver abscess and invasive syndrome (6), and generalized pustulosis (7). In addition, recent reports propose *K. pneumoniae* as a triggering factor for ankylosing spondylitis and Crohn’s disease (8).

Amikacin (AMK), alone or in combination, is an antibiotic of choice to treat certain *K. pneumoniae* infections: in particular, it is widely used in neonatal infections (4, 9, 10). However, in some parts of the world, AMK-resistant *K. pneumoniae* strains are becoming more common (11, 12). Resistance to AMK in *K. pneumoniae* is mostly caused by the aminoglycoside 6'-N-acetyltransferase type Ib [AAC(6')-Ib] enzyme, which is usually plasmid mediated (13, 14). *K. pneumoniae* JHCK1, a strain isolated from a neonate with meningitis, harbors 17 copies per cell of the plasmid pHCMW1, which includes the aac(6')-Ib gene (3, 15, 16). It is an adequate model to study *K. pneumoniae* virulence and resistance because its complete nucleotide sequence is known, and the resistance plasmid is one of the best characterized (15–17).

Recent efforts to isolate inhibitors of aminoglycoside-modifying enzymes and in particular AAC(6')-Ib led to the description of small molecules and metal ions complexed to ionophores, such as zinc pyrithione (ZnPT), that can reverse growth of AMK-resistant bacteria in the presence of the antibiotic (18–24). In this work, we show that Zn²⁺ and Cu²⁺ in complex with pyrithione induce a reduction in the AMK resistance levels of *K. pneumoniae* JHCK1.

*K. pneumoniae* JHCK1 (17) growth inhibition assays were carried out in Mueller-Hinton broth as described before (19). Zinc and copper pyrithione (ZnPT and CuPT, respectively) were purchased from Sigma-Aldrich (St. Louis, MO) and AK Scientific, Inc. (Union City, CA), respectively. Purification of AAC(6')-Ib for *in vitro* enzymatic assays was carried out after overexpression using *E. coli* XL10-Gold harboring a plasmid, pBADMW131, in which the aac(6')-Ib gene was placed under the control of the BAD promoter. Purification was done as described before (19) with the addition of a size exclusion fast-performance liquid chromatography (FPLC) step with a HiPrep 26/60 Sephacryl S-200 HR column under the conditions recommended by the supplier with a buffer containing 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, and 5% glycerol. Acetyltransferase activity was determined by monitoring the increase in absorbance at 412 nm when Ellman’s reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reacts with the coenzyme A (CoA)-SH released from acetyl-CoA after acetylation of the substrate (23).

We recently showed that Zn²⁺ significantly interferes with the acetylation of aminoglycosides catalyzed by AAC(6')-Ib (19). Assessment of other diveral cations showed that Cu²⁺ is also a robust inhibitor of the acetylation reaction with a 50% inhibitory concentration (IC₅₀) of 2 μM (Fig. 1A and B). This result agrees with that recently observed in a similar study (22). We showed before that the chloride salt of Zn²⁺ in combination with AMK induced inhibition of growth in *Escherichia coli* and Acinetobacter baumannii strains harboring aac(6')-Ib; however, very high concentrations were required (19). Instead, when in complex with the ionophore pyrithione, the concentrations needed to inhibit growth were about 1,000-fold lower. The enhancement of the inhibitory effect was attributed to the known stimulation of internalization of Zn²⁺ by the ionophore (19, 25). On the basis of these results, we tested the effect of ZnPT or CuPT on the resistance to AMK of *K. pneumoniae* JHCK1. Figure 2A and B show that addition of 5 μM ZnPT to cultures containing 16 μg/ml AMK resulted in full growth inhibition. It can also be observed that ZnPT at this
concentration produced an extension of the lag phase in the growth curve. However, the doubling time and maximum optical density at 600 nm (OD600) were unchanged with respect to cells growing in plain broth. This effect of pyrithione on \textit{K. pneumoniae} cells has been observed before: a concentration-dependent lag phase was followed by normal growth, and the cells became resistant to the levels of pyrithione used during the first culture (26). However, the tolerance was lost after successive cultures in the absence of pyrithione (26). Interestingly, CuPT showed lower levels of toxicity: at 10 μM, the lag had a similar duration to that observed at 5 μM ZnPT (compare Fig. 2A and C). Figure 2D shows that CuPT inhibited the resistance to AMK, but the level of inhibition was not as high as that observed when ZnPT was tested. This was an interesting observation since Cu$^{2+}$ was a more effective inhibitor of the acetylation reaction than Zn$^{2+}$ \textit{in vitro}. We do not know if the observed correlation between toxicity of the pyrithione complex and strength of inhibition of AMK resistance is a general property of metal-ionophore complexes. The mechanism of cation inhibition of enzymatic acetylation of aminoglycosides will be the subject of future studies. Inhibition could occur through binding to the enzyme in a competitive, noncompetitive, or uncompetitive manner, or it could occur through titration of the substrate aminoglycoside by formation of coordination complexes, a process that has been described for several metal ions, including Cu$^{2+}$ and Zn$^{2+}$ (27). Future studies will also test the action of ZnPT and CuPT on the proteins coded for by numerous existing alleles of \textit{aac(6’)-Ib} (14). While many of the proteins differ at the N terminus and have similar characteristics, others show variations as small as one or two amino acids at key positions that have profound effects on the substrate profiles of the enzymes (14).

We conclude that coordination complexes between Cu$^{2+}$ or
Zn$^{2+}$ and ionophores could be developed to act as adjuvants in combination with AMK or other aminoglycosides to treat infections caused by resistant pathogens.

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REFERENCES


