Research Paper

Bactericidal activity of manganese (IV) complex of 2-methylamino-pyridine against *Streptococcus pyogenes* and *Staphylococcus aureus*

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ABSTRACT

Objective: To investigate the bactericidal activity of manganese (IV) complex of 2-methylamino-pyridine against *Streptococcus pyogenes* (*S. pyogenes*) *and Staphylococcus aureus* (*S. aureus*).

Materials and Methods: The inhibitory effect of the complex was studied on the molecular level and by turbidity measurement. Treatment of bacteria was carried out using 5, 10, 25, 50 and 100 µmol of the complex per ml of culture media.

Results: The results showed that the growth of *S. pyogenes* rapidly decreased with increasing concentrations of the complex. In contrast, the complex caused no significant decrease in the growth rate of *S. aureus*. The molecular level studies showed that four protein bands, with apparent molecular weights of 19, 23, 30 and 54 Kda, respectively, increased in the protein pattern of the *S. pyogenes* extract after the complex treatment using silver stained polyacrylamide gels, under reducing condition. However, there was no detectable change in the protein pattern of the *S. aureus* extract after the complex treatment. No DNA damage was detected while using agarose gel electrophoresis and ethidium bromide staining in both types of bacteria.

Conclusion: Manganese (IV) complex of 2-methylamino-pyridine showed an apparent antibacterial inhibitory effect against *S. pyogenes,* but *S. aureus* was apparently resistant.

KEY WORDS: Free radicals, gram-positive cocci, metal complex, SOD.

Introduction

Gram-positive cocci are a heterogeneous collection of approximately 21 genera that colonise humans. Among these, Streptococcus and Staphylococcus are important pathogens in humans.[1] *S. pyogenes* has demonstrated the ability to develop drug resistance, particularly in patients with mixed infections that involve *S. aureus*. The drug resistance in staphylococci is due to penicillinase and acquisition of the mec A gene, which codes for a novel penicillin-binding protein, PBP2.[1,2]

Free radicals and, in particular, superoxide radical (O^-_2) cause cellular disruption due to peroxidation of membrane lipids. Superoxide dismutase (SOD) is believed to be involved in all oxygen-metabolising cells.[3] Four different types of SOD have been found, two of which have been found in eukaryotic cells. A copper and zinc containing form is located in the cytosol; and a manganese containing form is located in the mitochondria.[4] Its wide distribution among aerobic organisms[3] suggests that superoxide is formed inside all cells that grow in air and is toxic. Cells devoid of cytosolic SOD suffer enzyme inactivation, growth deficiencies and DNA damage. It has been suggested that the scant superoxide, generated by aerobic metabolism, harms even cells that contain abundant SOD. The vulnerability of bacteria to increased intracellular superoxide explains the widespread use of superoxide-producing drugs as bactericidal weapons.[5]

Extensive studies were conducted to address the antibacterial activity for many compounds and metal complexes that were found to have redox-cycling or pro-oxidative activity.[6-9] In a previous study, we found an antitumour activity of some metal complexes having SOD-like activity on Ehrlich ascites carcinoma cells.[10] The present study aims to examine the antibacterial activity of one of these complexes [Manganese (IV) complex of 2-methylamino-pyridine, $(MnL_2O)_2Cl_4.2H_2O$ where L is 2-methylamino-pyridine] on *S. aureus* and *S. pyogenes*. The inhibitory effect of this complex was studied on the molecular level and by turbidity measurement.

Materials and Methods

All chemicals used in this study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, Mo, USA), unless mentioned otherwise. Manganese (IV) complex of 2-methyl-aminopyridine was synthesised and supplied by Prof. AM Ramadan, Chemistry Department, Faculty of Education, Kafer El-Shiekh, Tanta University, Egypt. The SOD-like activity of the complex was ascertained by the method of Dechatelet, *et al.* [11] and has been reported earlier.[10]

Cultures

S. aureus ATCC25932 and *S. pyogens* ATCC4543 were grown in L-broth containing 10 g tryptone, 5 g yeast extract and 5 g/L NaCl at 37°C. [12] The cultures were grown, for at least three generations, to the late logarithmic phase (optical density of 0.8 at 600 nm).[13] The bacteria in the mid-exponential phase of growth were treated with the manganese (IV) complex in 5, 10, 25, 50 and 100 μmol concentrations. The change in the optical density was measured spectrophotometrically.

Bacterial DNA isolation and agarose gel electrophoresis

DNA was isolated from bacterial cells using the DNA kit, according to the method described by the manufacturer (Invitrogen Co., Carlsbad, CA, USA). Electrophoertic studies were conducted on 0.8% agarose gel (50 V, 40 mA) as described previously.[14]

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out in 0.75 mm thick, 12% vertical slab gels (Bio-Rad, USA), according to the method described by Laemmli.[15] An equal protein content of the bacterial extracts (50 μg/lane) was applied to the gel wells, after mixing and boiling for 3 minutes with an equal volume of sample buffer [0.125 M trisma base, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) mercaptoethanol and 0.1% (w/v) bromophenol blue as a tracking dye]. A pre-stained molecular weight standard mixture (α-macroglobulin Mr 191.0 Kda, ß-galactosidase Mr 117 Kda, frcuctose-6-phosphate kinase Mr 91.8 Kda, pyruvate kinase Mr 72.7 Kda, fumarase Mr 57.8, lactic dehydrogenase Mr 40.8 Kda, and triosephosphate isomerase Mr 34.1Kda) was prepared in parallel. Electrophoresis was carried out with a constant of 200 V. The run was terminated when the bromophenol blue marker reached the bottom of the gel. The gel was silver stained, according to the method described by Morrissey, *et al*.[16]

Statistical analysis

Statistical analysis was performed using the Wilcoxon rank sum test for a comparison of non-parametric variables. $P<0.05$ was considered statistically significant.

Results

The results showed that there was a decrease in growth of *S. pyogenes* [Figure 1], but not *S. aureus* [Figure 2] after 1 and 3 h of the complex treatment.

The protein patterns of the *S. aureus* and *S. pyogenes* extracts were separated on silver stained polyacrylamide gels (12%), under reducing condition. The results showed no change in the protein profile of the *S. aureus* extract after the complex treatment. But in the *S. pyogenes* extract, there was a change in the protein profile at low molecular weights after the complex treatment. The intensity of four protein bands with apparent molecular weights of 19, 23, 30 and 54 Kda, respectively, increased as revealed by the silver stained SDS-PAGE gels after the complex treatment. [Figure 3]

No apparent DNA damage was produced upon treatment of *S. pyogenes* and *S. aureus* with different complex concentrations as revealed by agarose gel electrophoresis and ethidium bromide staining. [Figure 4]

Figure 1. Growth monitoring of *S. pyogenes* after treatment with different concentrations of the manganese (IV) complex at 1 and 3 h.

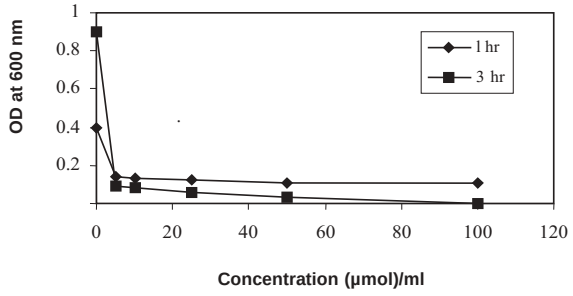
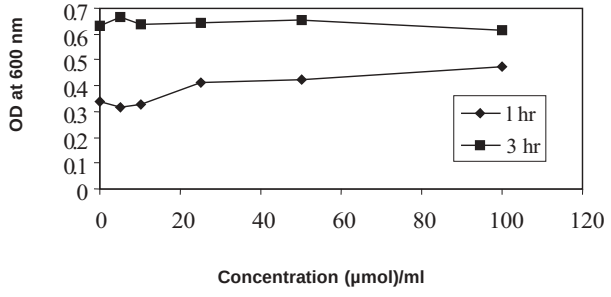


Figure 2. Growth monitoring of *S. aureus* after treatment with different concentrations of manganese (IV) complex at 1 and 3 h.



Discussion

Oxygen is known to form highly reactive free radicals (reactive oxygen species: ROS) such as superoxide ions, hydroxyl radicals and hydrogen peroxide in prokaryotic and eukaryotic cells. Free radicals, by the possession of unpaired electrons, make them very reactive as they urgently seek to gain or lose electrons in order to reach a more stable configuration.[17]

Most facultative and aerobic organisms contain a high concentration of superoxide dismutase enzyme (SOD). This enzyme converts the superoxide anion into ground state oxygen and hydrogen peroxide or both, to eliminate H_2O_2 and convert it into water and ground state oxygen.[18] We reported a pronounced antitumour effect of manganese (IV) complex of 2-methylamino-pyridine, with SOD-like activity due to the production of H_2O_2, earlier.[10] In this context, the antibacterial activity of this complex on *S. aureus* and *S. pyogenes* was examined and the possible mechanism of action was explained.

Figure 3. Silver stained polyacrylamide gel (12%) for the protein extracted from *S. pyogenes* before treatment (lane 1) and after treatment (lanes 2, 3, 4 and 5) with the manganese (IV) complex of 2-methylamino-pyridine in a concentration of 5, 10, 25 and 50 µmol/ml respectively.

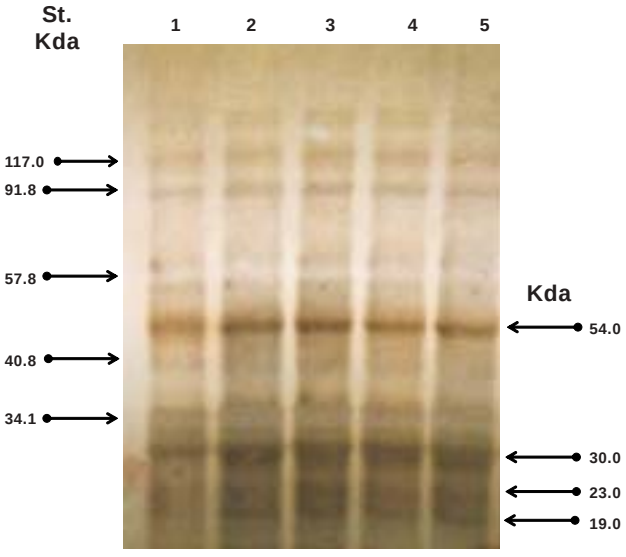
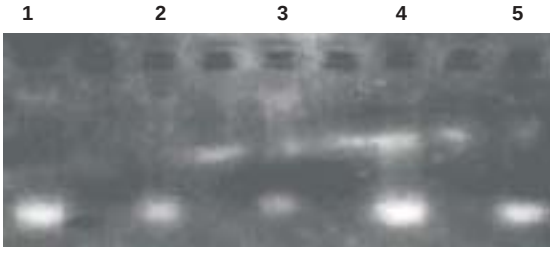


Figure 4. DNA pattern after treatment of *S. pyogenes* with the manganese (IV) complex of 2-methylamino-pyridine (lane 1, 2, 4 and 5) and lane 3 (control DNA).



The obtained data showed that a pronounced antibacterial activity of manganese (IV) complex of 2-methylamino-pyridine with different concentrations was found against *S. pyogenes.* Further, the antibacterial activity of the complex was not observed at any degree in the case of *S. aureus.* Moreover, in the *S. pyogenes* extract there was a change in the protein profile at low molecular weights after the complex treatment. The intensity of four protein bands with apparent molecular weights of 19, 23, 30 and 54 Kda, respectively, increased as revealed by the silver stained SDS-PAGE gels after the complex treatment. These bands may be related to the enzyme system of the bacteria that deal with free radicals utilisation.

Aronovitch, *et al* [13] reported that the bacterial activity of epinephrine-Cu (II) complex caused little killing of *E. coli*, but rapid killing was induced by the addition of 0.5 mM H_2O_2. The H_2O_2 forms a recycling redox system to damage the cytoplasmic membrane of *E. coli*, which is apparently the main reason for its bacterial activity. They suggested that with the addition of H_2O_2, the epinephrine-Cu (II) complex binds to the cell surface to induce oxidative membrane damage. Hoshino, *et al* [7] proposed that catechin-Cu (II) on the cell surface reacts locally with molecular oxygen to produce H_2O_2. The H_2O_2 generated on the surface can enter into the cell easily and cause damage to the cytoplasmic membrane. The apparently low concentrations of H_2O_2 observed in the bulk suspension, will not reflect the local concentration of H_2O_2 generated on the cell surface. Therefore, they proposed that Cu (I) and its redox reactions, involving catechins and H_2O_2 on the cell surface, must be involved in the killing of bacteria.

It was found that the DNA double strand did not break in the process of killing, indicating that the antibacterial activity of manganese (IV) complex of 2-methylamino-pyridine is derived from the damage of the cytoplasmic membrane. Hoshino, *et al* [7] treated the *E. coli* with catechin-copper (II) complexes and obtained the same result.

Gram-negative bacteria such as *E. coli* have negatively charged lipopolysaccharide on their cell surface. [12] Sonohara, *et al* [19] reported that gram-positive bacteria such as *S. aureus* have a positively charged surface and Tichy[20] reported that lipopolysaccharides had an ability to bind Cu (II). The binding of copper ions to *S. aureus* cells was found to decrease with an increase in the concentration of epigallocatechin (EGC). Indeed, the percentage of copper ions bound to *S. aureus* cells was only 2.4% in the presence of 100 µmol/L EGC. Therefore, it is possible that copper ions, complex with EGC and attracted by the negative charge of lipopolysaccharides of *E. coli*, bind to it tightly and generate H_2O_2 locally on the surface of *E. coli* cells. On the other hand, copper ions, complexed with EGC in the bulk buffer, cannot bind to the *S. aureus* cells. H_2O_2 was not generated on the surface of *S. aureus* cells but in the bulk buffer the EGC-Cu (II) complex does not generate active hydrogen species from H_2O_2. This may be the reason why the bactericidal activity of the catechin-Cu (II) complex is low against *S. aureus*. From studies on *S. aureus* and *E. coli*, it was concluded that the binding of copper ions to the cell surface plays an important role in the bactericidal activity of catechin Cu (II) complexes.[6]

The explanation of Hoshino, *et al* [6] is not applicable in our situation. Our study is concerned with two bacterial species which are both gram-positive cocci carrying the same charge. Although the antibacterial activity of the complex is apparent in *S. pyogenes,* it is not so in *S. aureus*. *S. pyogens* lacks the enzyme catalase, which utilises H_2O_2. However, *S. aureus* has this enzyme, which has the ability to convert H_2O_2 and protect the bacterial cells from the SOD-like activity of such a complex. The induction of endogenous catalase in the cells increased their ability to resist being killed by a combination of catechins and Cu (II). [7]

In conclusion, our results suggest that the metal complex (manganese (IV) complex of 2-methylamino-pyridine) with SOD-like activity exerts its antibacterial activity by increasing H_2O_2 production and possibly damaging the cytoplasmic membrane.

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