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PRELIMINARY *IN VITRO* INVESTIGATIONS ON ANTIMICROBIAL ACTIVITY OF THREE IRON COMPLEXES

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Summary

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The *in vitro* effect of three complexes of iron with ligands N,N'-bis(4-antipyrylmethyl)-piperazine (BAMP) and N,N'-tetra-(antipyryl-1-methyl)-1,2-diaminoethane (TAMEN) was examined on 22 Gram-positive and Gram-negative bacterial strains. Broad spectrum antibiotic thiamphenicol was used as a control. The experiments were performed by the routine agar-diffusion method of Bauer *et al.* and the method of minimum inhibitory concentrations (MICs). It was found that $Fe_2(TAMEN)Cl_6$ and $Fe_2(BAMP)Cl_6$ expressed antibacterial activity *in vitro*, especially against Gram-positive strains. The inhibitory effect of $Fe_2(TAMEN)Cl_6$ was more pronounced.

Key words: antibacterial activity, cobalt, iron, Mannich bases, polynuclear complexes, pyrazolone

INTRODUCTION

Nowadays, more and more strains of pathogenic microorganisms, including staphylococci, streptococci and enterobacteria, become resistant to many of available antibiotics and chemotherapeutic agents. For that reason the trials to request for new antibacterial means are very indispensable. There is a need of new antibacterial means that would circumvent bacterial resistance mechanisms (Stojiljkovic *et al.*, 1999).

The antimicrobial effect of iron has been previously established. The investigations of Berry *et al.* (1992) showed that seven examined metals including iron suppressed the growth of oral bacteria in dependence of the bacterial species and concentration, although the effect of iron was the weakest. Hvozdiak *et al.* (1996) ascertained that high-dispersed iron and silver have pronounced bactericidal effect unlike nondispersed preparations, which have weak or no antibacterial action. Donde *et al.* (2003) established enhanced antibacterial and antifungal activity of Fe (II) and Hg (II) complexes of hydrazone as compared to parent ligand.

Searching for such preparations, in this study we investigated the *in vitro* antibacterial activity of three iron complexes with ligands N,N'-bis(4-antipyrylmethyl)-piperazine (BAMP) and N,N'-tetra-(antipyryl-1-methyl)-1,2-diaminoethane (TAMEN). Antipyrine and its deriva-

tives exhibit antipyretic and analgetic activity. We decided to evaluate the antimicrobial properties of these new complexes because of two main reasons: 1) There are data published about the antibacterial effect of iron and different iron compounds (Diarra *et al.*, 1996; Gvozdyak *et al.*, 1996; Bacchi *et al.*, 1999; Donde *et al.*, 2003); 2) It has been found in our previous investigations that some copper (Popova *et al.*, 2004) and cobalt (Popova *et al.*, 2006, in press) complexes with BAMP and TAMEN exhibit antimicrobial properties.

MATERIALS AND METHODS

Compounds

The experiments were performed with two complexes of iron (III) and one complex of iron (II) with ligands containing antipyrine moieties like the Mannich-bases BAMP (Fig. 1) and TAMEN (Fig. 2): $Fe_2(BAMP)Cl_4$, $Fe_2(BAMP)Cl_6$ and $Fe_2(TAMEN)Cl_6$.

The compounds were obtained according to the methods described in previous works (Costisor *et al.*, 1994a,b). Iron complexes were initially dissolved in dimethyl sulfoxide (DMSO, Serva) and then

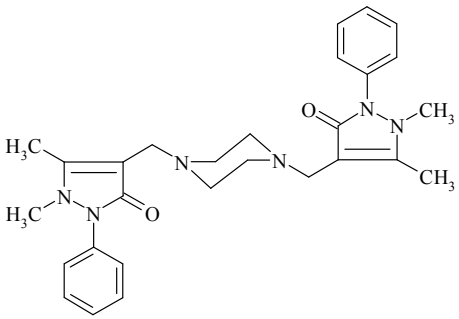

Fig. 1. N,N'-bis(4-antipyrylmethyl)-piperazine (BAMP).

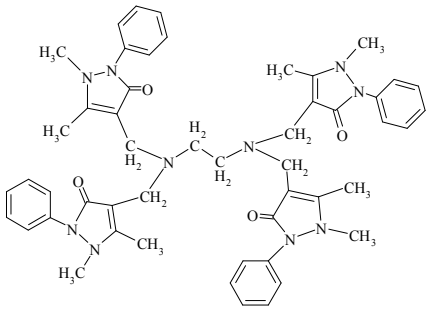

Fig. 2. N,N'-tetra-(antipyryl-1-methyl)-1,2-diaminoethane (TAMEN).

diluted in sterile phosphated buffered saline (PBS) with pH 7.2. The commercially available antibiotic thiamphenicol (Nikovet – Sofia) was also included in the experiments.

Bacterial strains

Pure cultures of 22 pathogenic bacterial strains, isolated from animals, as well as a control strain (*S. aureus* TSA MRSA) were used in the experiments. Eight of them were Gram-positive (4 *S. aureus* and 4 *S. pyogenes* strains) and the rest 14 were Gram-negative (4 *Escherichia* coli, 3 *Salmonella enterica ser. Enteritidis*, 3 *K. pneumoniae* and 4 *Pseudomonas aeruginosa* strains). The bacterial strains were isolated from patients with infections with different localization (skin, ears, conjunctiva, respiratory and urogenital tracts). The strains showed a high *in vitro* drug resistance (mainly to streptomycin, penicillin, oxacillin, ampicillin, and some of them also to amoxicillin) but were sensitive to amphenicols.

Antimicrobial tests

Studies were carried out by the classic agar-diffusion method of Bauer *et al.* (1966). Bacterial suspensions were inoculated at a dose of 2.10^6 cells/mL on Muel-

ler-Hinton's agar with pH 7,2 – 7,4 and 4 mm layer thickness in Petri dishes with diameter 9 mm. The compounds and controls were applied in PBS solutions by dropping of 0,1 mL in 9-mm holes in the agar at doses 50 μg/well for iron complexes and 30 μg/well for the antibiotic. Results were recorded by measuring the diameters of inhibitory zones in mm, including the hole diameter. Inhibitory effect of the salts was established at zones > 12 mm.

Establishment of the minimum inhibitory concentrations (MICs) was performed by the method of twofold serial dilutions on Mueller-Hinton's agar as per Ericsson and Sherris (1971). MIC_{50} were calculated mathematically depending on the number of inhibited colonies of the medium with the respective iron compound or antibiotic dilution compared to the control medium colonies without drugs.

Statistical analysis

Statistical analysis was performed by the standard method of Student – Fisher.

RESULTS AND DISCUSSION

The results from the studies performed by the method of Bauer *et al.* (1966) are presented in Table 1. Applied at the tested dose of 50 μg/mL $Fe_2(BAMP)Cl_4$ showed activity against two Gram-positive (50 % of *S. pyogenes)* and three Gram-negative bacteria (inhibitory zones 13 – 14 mm). $Fe_2(BAMP)Cl_6$ was effective against 38 % of the staphylococci and streptococci used in the experiments (13–15 mm zones) as well as against 36 % of the Gram-negative bacteria (inhibitory zones 14–18 mm). Administered at the same concentration $Fe_2(TAMEN)Cl_6$ was active against almost all tested Gram-positive bacteria: 75 % of *S. aureus* and 100 % of *S. pyogenes* (13–17 mm zones) but only against three Gram-negative bacteria (inhibitory zones 13–14 mm).

Most of bacterial strains used as test system in our investigations showed high susceptibility to the broad-spectrum antibiotic thiamphenicol. The inhibition zones caused by the antibiotic were usually larger then those of iron complexes examined. However in the case of *S. pyogenes* 1 and *E. coli* 045 $Fe_2(BAMP)Cl_6$ showed a more pronounced antibacterial effect than the antibiotic. In *S. Enteritidis* 3, *K. pneumoniae* 3, *P. aeruginosa* 1 and *P. aeruginosa* 44 the zones of growth inhibition caused by $Fe_2(BAMP)Cl_6$ were equal or similar to these of thiamphenicol.

The establishment of the minimum inhibitory concentrations (MICs) of antimicrobial means is a more precise method for determination of their effect. The MICs of metal complexes examined are presented in Table 2. Among the compounds tested $Fe_2(TAMEN)Cl_6$ was found to be most active (with the lowest MICs) against Gram-positive bacteria. The highest MICs were established for $Fe_2(BAMP)Cl_4$. The differences between the average MICs calculated for $Fe_2(TAMEN)Cl_6$ and $Fe_2(BAMP)Cl_4$ were found to be statistically significant ($P<0.05$). The sensitivity of Gram-positive bacteria to $Fe_2(BAMP)Cl_6$ were slightly higher as compared to $Fe_2(BAMP)Cl_4$ and lower than those to $Fe_2(TAMEN)Cl_6$, but the differences were not significant ($P>0.05$).

For most Gram-negative bacteria tested the MICs established for the three iron complexes were equal or similar. The differences between the average arithmetical MIC values were not statistically significant ($P>0.05$). For *S. Enteritidis* 3, *P. aeruginosa* 1 and *P. aeruginosa* 44 MICs of $Fe_2(BAMP)Cl_6$ were lower than

Table 1. Inhibitory effect of iron compounds on pathogenic bacteria in the agar-diffusion method*

Microorganisms	Inhibitory zones in mm			
	$Fe_2(BAMP)Cl_4$ 50 μg/well	$Fe_2(BAMP)Cl_6$ 50 μg/well	$Fe_2(TAMEN)Cl_6$ 50 μg/well	Thiamphenicol 30 μg/well
S. aureus TSA MRSA	10	13	15	30
S. aureus 207	10	15	13	25
S. aureus 208	12	12	14	30
S. aureus 216	12	12	12	24
S. pyogenes 1	11	12	14	13
S. pyogenes 2	14	11	16	27
S. pyogenes 4	14	14	17	28
S. pyogenes 13	10	10	13	33
E. coli 045	13	18	12	17
E. coli 0101	10	12	10	30
E. coli 075 18	10	11	12	20
E. coli 06 18B	10	10	10	14
S. Enteritidis 1	12	11	14	21
S. Enteritidis 2	10	10	10	23
S. Enteritidis 3	13	16	12	18
K. pneumoniae 1	10	10	10	17
K. pneumoniae 2	10	10	11	15
K. pneumoniae 3	10	14	13	15
P. aeruginosa 1	14	14	13	15
P. aeruginosa 44	10	14	12	14
P. aeruginosa 81	10	12	10	15
P. aeruginosa 83	10	11	12	22

*The data represent summarized results from three independent experiments.

those of the other two iron complexes. On the other hand, $Fe_2(TAMEN)Cl_6$ showed the highest activity against *E. coli* 075 18 and *P. aeruginosa* 81.

MICs values of thiamphenicol established for most bacterial strains used in the experiments were lower as compared to those of the metal complexes ($P<0.01$). The only exception was *P. aeruginosa* – the sensitivity of most strains of this bacterium to iron complexes examined was almost equal to that of the antibiotic.

The control solution PBS containing the same concentration of DMSO as samples examined, showed no antibacterial activity when tested independently by both methods.

The results obtained showed that the bacterial strains used in the experiments were sensitive to iron complexes investigated in spite of that their MICs were higher than these of thiamphenicol. It must be taken into consideration that the tested strains were isolated from patients treated with different antibiotics and most

Table 2. Minimum inhibitory concentration (MIC) of iron compounds against pathogenic Gram-positive and Gram-negative microorganisms*

Microorganisms	$MIC_{50,}$ μg/mL			
	$Fe_2(BAMP)Cl_4$	$Fe_2(BAMP)Cl_6$	$Fe_2(TAMEN)Cl_6$	Thiamphe-nicol
S. aureus TSA MRSA	32	32	32	4
S. aureus 207	32	16	16	4
S. aureus 208	32	16	32	4
S. aureus 216	16	16	8	4
S. pyogenes 1	32	32	16	4
S. pyogenes 2	32	32	16	4
S. pyogenes 4	32	16	16	4
S. pyogenes 13	32	16	16	4
E. coli 045	32	32	32	4
E. coli 0101	32	32	32	4
E. coli 075 18	64	64	32	16
E. coli 06 18B	64	64	64	32
S. Enteritidis 1	32	64	32	16
S. Enteritidis 2	32	32	32	16
S. Enteritidis 3	32	16	32	4
K. pneumoniae 1	32	32	32	8
K. pneumoniae 2	32	32	32	16
K. pneumoniae 3	32	32	32	4
P. aeruginosa 1	64	32	64	64
P. aeruginosa 44	32	16	32	16
P. aeruginosa 81	32	32	16	16
P. aeruginosa 83	32	32	32	4

*The data represent summarized results from three independent experiments.

likely had a high drug resistance. The imperfect solubility of the iron complexes probably exerted influence on the results as well. The metal complexes, especially $Fe_2(BAMP)Cl_6$ and $Fe_2(TAMEN)Cl_6$, expressed a more pronounced antibacterial activity against Gram-positive bacteria – the average arithmetical MIC values were lower than those for Gram-negative strains. The differences between MIC values for Gram-positive and Gram-negative bacteria were statistically significant for $Fe_2(BAMP)Cl_6$ and $Fe_2(TAMEN)Cl_6$ ($P<0.05$). As seen from Table 2, the examined Gram-positive bacteria were more sensitive to thiamphenicol too in comparison to the Gram-negative strains ($P<0.05$).

The examined Gram-positive and Gram-negative bacteria were more sensitive to the iron complexes with BAMP and TAMEN tested in these experiments than to some copper complexes with the same ligands (Popova *et al.*, 2004). Probably the bacteria assimilate better iron compounds than the copper ones due to use of haem uptake mechanisms. Al-

most all bacterial pathogens possess highly efficient haem uptake mechanisms that supply them with iron. These mechanisms of bacteria present a path for targeted drug delivery, going round the barrier functions of bacterial membranes (Stojiljkovic *et al.*, 1999).

Many pathogenic bacteria receive their essential iron by assimilation of iron-binding compounds through cell surface receptors (Diarra *et al.*, 1996). The iron complexes investigated in this study probably use one or several microbial iron transport pathways for assimilation through cell surface. It is thought that iron compounds use outer membrane iron-regulated proteins to enter into gram-negative bacteria. Some workers suppose the possibility to use these proteins to transport drugs into Gram-negative bacteria. It has been suggested that in Gram-negative bacteria resistant to iron compounds some of outher membrane iron-regulating proteins or specific receptors are absent or do not function (Diarra *et al.*, 1996; Miller & Malouin, 1993). According to Okamoto *et al.* (1990) the antimicrobial activity of such drugs increased in a low-iron environment and decreased in the presence of a high ferric ion concentration (concurrence for the sites of penetration – receptors). It is known that the penetration of some antibacterial agents such as beta-lactam antibiotics through the bacterial cell wall can be enhanced by utilizing various active transport systems like the active iron-transport system in bacteria (Mochizuki *et al.*, 1988; McKee *et al.*, 1991). It could be suggested that the penetration in bacterial cells of the iron compounds investigated in our study is delayed in environment with normal iron content because of competition in passing through the iron transport systems. Additional investigations on the antimicrobial effect of these complexes in a low-iron environment are needed to confirm such assumption.

CONCLUSIONS

In this study we report for the first time data about the antimicrobial activity of iron complexes with ligands containing the antipyrine moiety N,N'-bis(4-antipyrylmethyl)-piperazine (BAMP) and N,N'-tetra-(antipyryl-1-methyl)-1,2-diaminoethane (TAMEN). The examined Fe compounds manifest inhibitory activity against Gram-positive and Gram-negative bacterial strains used in the experiments. The complexes of Fe (III) with TAMEN – $Fe_2(TAMEN)Cl_6$ or BAMP – $Fe_2(BAMP)Cl_6$ are more active than Fe (II) complexes with BAMP – $Fe_2(BAMP)Cl_4$, especially against Gram-positive bacteria.

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