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Impact of Cu^{2+} ions on the structure of colistin and cell-free system nucleic acid degradation

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ABSTRACT

Colistin and transition metal ions are commonly used as feed additives for livestock animals. This work presents the results of an analysis of combined potentiometric and spectroscopic (UV–vis, EPR, CD, NMR) data which lead to conclude that colistin is able to effectively chelate copper(II) ions. In cell-free system the oxidative activity of the complex manifests itself in the plasmid DNA destruction with simultaneous generation of reactive •OH species, when accompanied by hydrogen peroxide or ascorbic acid. The degradation of RNA occurs most likely via a hydrolytic mechanism not only for complexed compound but also colistin alone. Therefore, huge amounts of the used antibiotic for nontherapeutic purposes might have a potential influence on livestock health.

1. Introduction

Colistin (polymyxin E), a cyclic lipodecapeptide (Fig. 1), contains five positively charged amino groups at a fully protonated form (pH < 6). Its molecule is made up of a hydrophilic cyclopeptide ring and a tail — a tripeptide moiety with the hydrophobic acyl chain at the end [1]. The amino acid components are D-leucine, L-leucine, L-threonine, and L-α-γ-diaminobutyric acid (Dab). Dab-1 is linked to the fatty acid residue which has been identified as 6-methyloctanoic acid (colistin A) or 6-methyleptanoic acid (colistin B) [2]. Pharmaceutical preparations may contain different amounts of these two components [3,4]. This antibiotic is effective primarily against gram-negative bacteria, in particular those strains that are resistant to aminoglycosides, β-lactams and fluorochinolones [5]. Its target is the bacterial outer membrane where the antibiotic's amino groups bind to the acidic lipopolysaccharide molecules by displacing calcium and magnesium. This leads to permeability changes in the cell envelope, leakage of cell contents and finally to cell death [6,7].

Colistin was discovered as a non-ribosomally synthesized antibiotic of *Bacillus polymyxa* [8]. The intravenous formulations of colistin sulfate were gradually abandoned in the early 1980s because of the reported high incidence of neuro- and nephrotoxicity [9–11]. In veterinary medicine, colistin is still widely used in the treatment or prevention of infections caused mainly by *Escherichia coli*. For example, it was established that in France colistin accounted for one-third of pigs' exposure to antibiotics. This figure is even higher in case of poultry. Moreover, 90% of farms use colistin during the post-weaning period and 48% during maternity [12]. Studies carried out in other countries also show a high use of colistin [12–14].

This antibiotic is also used as a feed additive for growth promotion of animals [15]. The FDA (the Food and Drug Administration, U.S.A.) recently published a document which calls for the elimination of such practices. For over 50 years, antibiotics have widely been administered to food animals. The situation is very dangerous, since the antimicrobial drug residues found in the human diet may potentially alter the intestinal microbiota of consumers. They might change the equilibrium of the normal microbiota, encourage the overgrowth of resistant strains and allow potential pathogens to propagate. Upon long-term exposure colistin residues in food products may cause immediate adverse effects and lead to the occurrence of resistant microbes [15,16]. Studies have shown that the concentration of the drug in cow's milk may reach up to 50 μg/L [17], in meat up to 1000 μg/kg [18], while in offal even up to 2000 μg/kg [19].

Not only antibiotics, but also trace elements are added to livestock feeds in order to prevent diseases and enhance feed efficiency. It has been found that an average concentration of copper in chicken feeds amounts to approximately 22.6 mg/kg while in the case of pig feeds it is in excess of 105 mg/kg. Other studies have shown that a great excess of copper levels (around 125 ppm) improves the performance of meat birds [20]. This raises concerns regarding the trace elements accumulation

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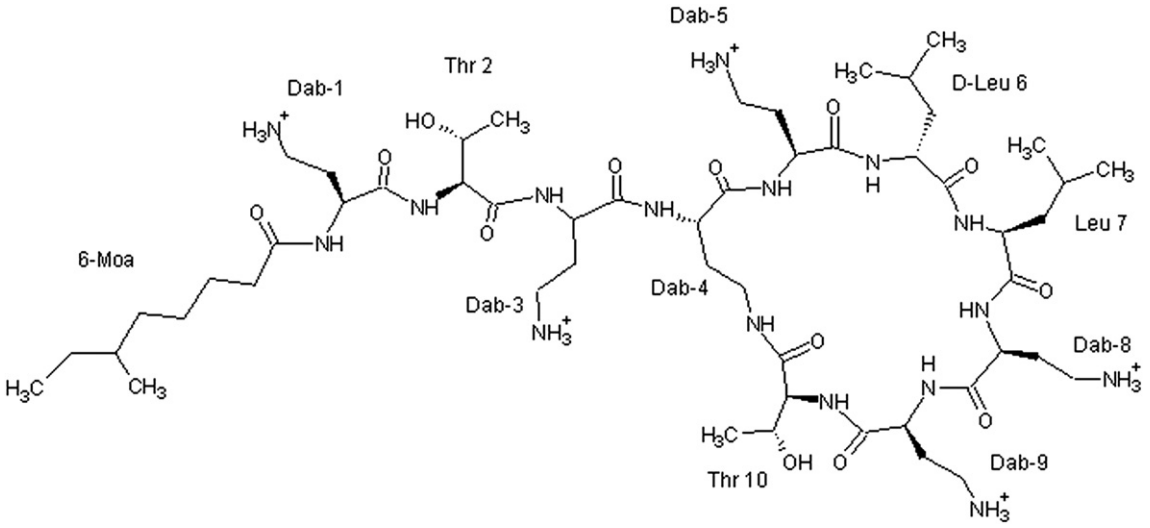

Fig. 1. Molecular formula of colistin, with the single residues highlighted by dashed lines. 6-Moa: 6-methyloctanoic acid; Dab: diaminobutyric acid.

in soils and animal organisms, and the consequences for the consumer's organism [21].

A high level of copper(II) ions and the presence of antibiotics which have appropriate donor binding groups may lead to the formation of complexes. Additionally, metal ion complexes usually have a higher positive charge than uncomplexed compounds. This means that they might interact more tightly with polyanionic DNA and RNA molecules. It has been shown that several metal ion complexes with antibiotics promote degradation of DNA [22–25]. Some antibiotics, such as bleomycin and amikacin, which form stable complexes with redox metal ions Fe^{2+} or Cu^{2+} also split the RNA chain via the free radicals mechanism [26,27]. On the other hand, uncomplexed neomycin B cleaves the HIV trans-activation response (TAR) RNA construct and $tRNA^{Phe}$ with an abasic site using the hydrolytic mechanism [28,29]. Recently, we have discovered that bacitracin promotes efficient degradation of RNA and less efficient of DNA in the absence of metal ions [30]. It seems to be interesting to determine whether colistin, another cyclic peptide antibiotic, is also able to induce the degradation of nucleic acids.

Since colistin and related molecules constitute an important therapeutic class in human and veterinary medicine it is worth examining how these compounds may influence the speciation of copper(II). In the present study, protonation equilibria and Cu^{2+} binding by colistin were analyzed using potentiometry, NMR, electronic absorption spectroscopy, circular dichroism and EPR. Moreover, we investigated whether the complex exhibits any oxidative properties against double-stranded DNA. In order to find out if colistin is able to interact with RNA, the impact of this antibiotic and its Cu^{2+}-complex on the catalytic activity of antigenomic hepatitis delta virus (HDV) ribozyme was analyzed. Possible nucleolytic properties of colistin were also assayed.

2. Results and discussion

2.1. Coordination of colistin towards Cu^{2+} ions

The presence of a large number of potential donor groups in the studied molecule implies that binding of metal ions may effectively occur. The positively charged amino groups are responsible for the therapeutic effect as well as for the toxicity of the antibiotic [4,31]. The comparison of acid dissociation constant values (Table 1) shows that all these functional groups deprotonate almost simultaneously. This fact is supported by the small differences among the pK_a values (less than 0.6 log units). Therefore, it becomes practically impossible to assign each calculated macroconstant to any individual deprotonation process [32]. Calculations based on potentiometric titrations were used to obtain the stoichiometry and overall stability constants of Cu^{2+}-colistin complexes. Six monomeric complexes are formed with the general formula CuH_nL, with n ranging from +3 to −2. The SUPERQUAD calculations did not offer the possibility of calculating the models with the CuL_2 or CuL_3 stoichiometry. On the other hand, titration done with an excess of the metal yielded precipitation of the copper hydroxide which excludes the Cu_2L or Cu_3L species.

The coordination process leads to the formation of the first species CuH_3L^{5+}. It is impossible to obtain the full spectroscopic characteristic for the complex form because of its low concentration and the coexistence of the CuH_2L^{4+} species (Fig. 2). Only the values of the EPR spectra parameters ($A_{||} = 160$ G, $g_{||} = 2.28$) indicate the involvement of two nitrogen donors in the binding process (Table 2) [33]. Most likely CuH_3L^{5+} binds copper(II) ions through the nitrogen atom of the amino anchoring group γ-NH_2 of Dab-3 residue and the neighboring ionized amide nitrogen (α-amide Dab-4).

When pH increases above 6.0, the CuH_2L^{4+} is formed with pK_a value equal to 6.36 ($CuH_3L^{5+} \rightarrow CuH_2L^{4+}$, Table 1). This value suggests the deprotonation and coordination of the next amide nitrogen [34]. It is supported by the spectroscopic data analysis. Spectral parameters of particular complex species calculated on the basis of species distribution plots (Fig. 2), together with spectral band assignments are presented in Table 2. The formation of the complex CuH_2L^{4+} is accompanied by a set of transitions. On the UV-visible (UV–vis) spectrum (Fig. S1,

Table 1
Potentiometric parameters for the colistin alone and its complex with Cu^{2+} ion.

Form	logβ[a]	pK_a[b]
H_5L^{5+}	44.64(1)	7.84
H_4L^{4+}	36.80(1)	8.63
H_3L^{3+}	28.17(1)	8.85
H_2L^{2+}	19.32(1)	9.42
HL^{+}	9.90(1)	9.90
CuH_3L^{5+}	34.93(6)	6.36
CuH_2L^{4+}	28.57(1)	7.16
$CuHL^{3+}$	21.41(3)	8.60
CuL^{2+}	12.81(4)	9.10
$CuH_{-1}L^{+}$	3.71(4)	9.71
$CuH_{-2}L$	−6.00(6)	–

[a] Overall stability constant (β) expressed by equations: for ligand $\beta(H_nL) = [H_nL] / ([L][H^+]^n)$; for complexes $\beta(CuH_nL) = [CuH_nL] / ([Cu][L][H^+]^n)$; statistical errors on the last digits of stability constant are given in parentheses.

[b] Deprotonation constant (pK_a) expressed by equations: for ligand $pK_a = \log\beta(H_nL) - \log\beta(H_{n-1}L)$; for complexes $pK_a = \log\beta(CuH_nL) - \log\beta(CuH_{n-1}L)$.