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DRUG BIOCHEMISTRY

IMPACT OF METAL IONS ON NETILMICIN-MELANIN INTERACTION

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Abstract: Netilmicin, which is mainly used as the sulfate, is a semisynthetic, water soluble aminoglycoside antibiotic obtained by chemical modification of sisomicin. It is active against both Gram-positive and Gram-negative bacteria, including strains which are resistant to other aminoglycosides. Netilmicin form complexes with melanin. The aim of the presented work was to examine the effect of Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} on netilmicin binding to synthetic DOPA-melanin. It has been demonstrated that metal ions decrease the amount of antibiotic bound to melanin as compared with netilmicin-melanin complexes obtained in the absence of metals. It has been also shown that only one class of binding sites participates in netilmicin-[melanin-metal ion] complexes formation with the association constant $K \sim 10^3\ M^{-1}$. The obtained results demonstrate that Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} ions modify the interaction between netilmicin and melanin biopolymer. The blocking of some active centers in melanin molecules by metal ions, which potentially exist in living systems, may influence the clinical therapeutic efficiency as well as the undesirable side effects of netilmicin.

Keywords: netilmicin, melanin, drug-[melanin-metal ion] complexes, metal ions

Netilmicin, a semisynthetic aminoglycoside antibiotic, derivative of sisomicin, is a wide spectrum antibiotic even more effective than the other compounds of the same class such as tobramycin and gentamicin and is used as active substance in several ophthalmic and injectable products. Aminoglycosides inhibit protein synthesis in a variety of microorganisms by binding bacterial ribosomes. Netilmicin is particularly active against most of the Gram-negative bacteria and many Gram-positive bacteria including *Staphylococcus aureus* (1). The use of netilmicin, as other aminoglycosides, causes severe side-effects such as oto- and nephrotoxicity, which are major drawbacks to their utilization in critically-ill patients. The ototoxic effects can involve both cochlear and vestibular toxicity. The oto- and nephrotoxicity of netilmicin are substantially lower than those of other aminoglycoside antibiotics (2).

Melanins are negatively charged pigments of high molecular weight (3) that are composed of polymerized phenolic and/or indolic compounds (4). In man, melanins are present in the skin, hair, eyes, brain and inner ear (5, 6). Despite having been studied for so long, the exact structure of melanins has not been fully elucidated. The physico-chemical characteristics of melanins make them quite difficult to be studied by standard methods. The biological function of melanin remains mostly obscure. It is believed that in many pigment cells, melanin may act as a powerful antioxidant *via*, for example, metal sequestration. The carboxyl, phenolic, hydroxyl, and amine groups on melanin provide numerous potential binding/biosorption sites for drugs (5) as well as for metal ions (7). Netilmicin is known to bind to melanin (8).

Metal ions play an important role in the metabolism of all organisms, and this is reflected by the wide variety of chemical reactions in which they are involved. Metals can be cofactors of enzymes, catalyzing basic functions like electron transport, redox reactions and energy metabolism, and are essential for maintaining the osmotic pressure of the cell (9). The aim of the presented work was to examine the effect of Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} ions on netilmicin binding to synthetic DOPA-melanin. Although synthetic melanins are not a perfect model of natural melanin pigments, they have been successfully used in the studies of redox, paramagnetic and ion exchange properties of natural melanins (10).

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EXPERIMENTAL

Chemicals

L-3,4-dihydroxyphenylalanine (L-DOPA) used in the studies was obtained from Sigma Chemical Co. Netilmicin sulfate was obtained in the form of solution – Netromycine (100 mg/mL) from Schering-Plough, Belgium. The remaining chemicals were produced by POCH S.A., Poland.

Melanin synthesis

Model synthetic melanin was formed by oxidative polymerization of L-3,4-dihydroxyphenylalanine (L-DOPA) in 0.067 M phosphate buffer at pH 8.0 as described earlier (8, 11).

Melanin-metal ions complexes formation

Dry melanin samples of 200 mg each were mixed with 200 mL of bidistilled water containing $1 \cdot 10^{-3}$ M of Cu^{2+}, Zn^{2+}, Ca^{2+} or Mg^{2+} ions. Mixtures were incubated at room temperature for 24 h and then filtered. The amounts of metal ions bound to melanin were determined by the use of atomic absorption spectrophotometer type AAS 3 (Carl Zeiss, Jena). The final [melanin-metal ions] complexes contained 0.42 μM (26.69 μg) Cu^{2+}, 0.20 μM (13.08 μg) Zn^{2+}, 0.19 μM (7.61 μg) Ca^{2+} or 0.13 μM (3.16 μg) Mg^{2+} per 1 mg of melanin.

Drug-melanin complex formation

Drug-melanin complexes were obtained as follows: 5 mg of melanin-metal ion complexes were placed in plastic test-tubes, where netilmicin solutions were added to a final volume of 5 mL. The initial concentration of netilmicin ranged from $1 \cdot 10^{-4}$ M to $1 \cdot 10^{-3}$ M. Control samples contained 5 mg of melanin-metal ion complexes and 5 mL of bidistilled water without drug. All samples were incubated for 24 h at room temperature and then filtered.

Analysis of drug binding to melanin

The amounts of netilmicin in each filtrate with respect to the control samples were determined spectrophotometrically using chloranil as colored reagent (12). All spectrophotometric measurements were performed by the use of JASCO model V-530, UV-VIS spectrophotometer, at wavelength 350 nm. The amounts of netilmicin bound to melanin, calculated as the difference between the initial amount of drug administered to melanin and the amount of unbound drug (in filtrate after incubation), were expressed in mmoles of bound drug per 1 mg of melanin. A qualitative analysis of drug-melanin interaction was performed using Scatchard plots of the experimental data according to Kalbitzer and Stehlik (13). The number of binding sites (n) and the values of association constants (K) were calculated.

Statistical analysis

In all experiments, the mean values for three independent experiments ± standard deviation (SD) were calculated.

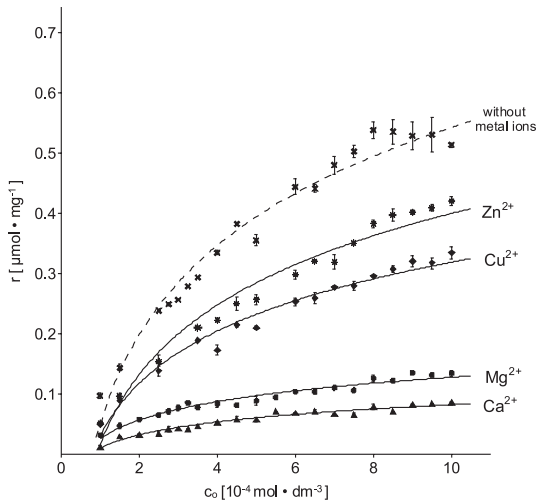

Figure 1. Binding isotherms for netilmicin complexes with melanin (8) and melanin containing Cu^{2+}, Zn^{2+}, Ca^{2+} or Mg^{2+} ions; r – amount of drug bound to melanin, c_o – initial drug concentration. The mean values ± SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

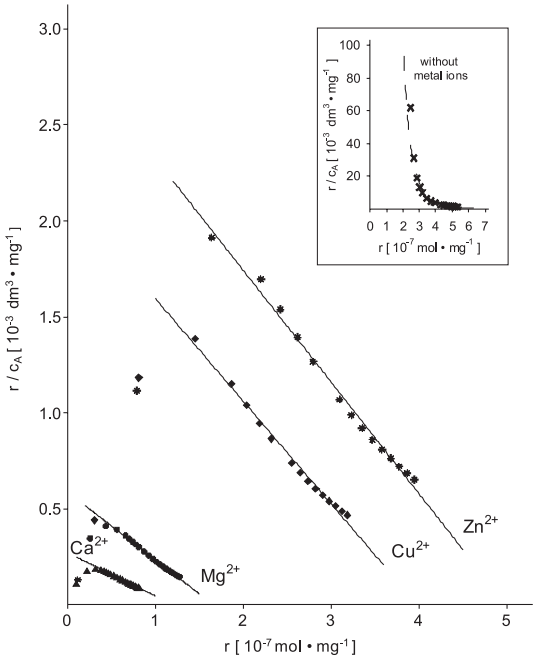

Figure 2. Scatchard plots for netilmicin complexes with melanin (8) and melanin containing Cu^{2+}, Zn^{2+}, Ca^{2+} or Mg^{2+} ions; r – amount of drug bound to melanin, c_A – concentration of unbound drug

Table 1. Binding parameters for netilmicin-melanin and netilmicin-[melanin-metal ions] complexes.

Analyzed complex	Association constants K [M^{-1}]	Number of binding sites n [µmol drug/mg mel]
Netilmicin-[melanin-Cu^{2+}]	$K = 5.34 \cdot 10^3$	n = 0.399
Netilmicin-[melanin-Zn^{2+}]	$K = 5.99 \cdot 10^3$	n = 0.495
Netilmicin-[melanin-Ca^{2+}]	$K = 2.16 \cdot 10^3$	n = 0.123
Netilmicin-[melanin-Mg^{2+}]	$K = 3.55 \cdot 10^3$	n = 0.166
Netilmicin-melanin*	$K_1 = 1.29 \cdot 10^6$ $K_2 = 7.12 \cdot 10^3$	$n_1 = 0.268$ $n_2 = 0.350$ $n_1 + n_2 = 0.618$

*Results from previous studies in the lab (8)

RESULTS AND DISCUSSION

Melanins, the end-products of complex multi-step transformations of L-tyrosine, are polymorphous and multifunctional biopolymers, represented by eumelanin, pheomelanin, neuromelanin, and mixed melanin pigment (14). Melanin pigments have in common their arrangement of several units linked by carbon-carbon bonds (C-C), but differ from each other in chemical composition, as well as structural and physical properties. Thus eumelanins are polymorphous nitrogenous biopolymers (predominantly copolymers of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid), black to brown in color, insoluble in most solvents (15), and tightly associated with proteins through covalent bonds. Eumelanins behave like polyanions with the capability to reversibly bind cations and polyamines in reactions facilitated by their high carboxyl groups content (3).

The aminoglycosides are a family of bactericidal antibiotics which contain aminosugars in glycoside linkages. These molecules have a general capacity to bind to melanin, often by means of electrostatic interactions between the free carboxyl groups of melanin and the positively charged amino groups of the aminoglycosides. The Scatchard plot-type analysis of drug binding to melanin has demonstrated the participation of at least two classes of binding sites in gentamicin (16), kanamycin (17),

amikacin, neomycin, tobramycin (18) and netilmicin (8) complexes with melanin.

The abilities of melanin to bind netilmicin in the presence of metal ions are presented in Fig. 1 as binding isotherms. The obtained results demonstrate that the amounts of antibiotic bound to melanin increase with increasing initial drug concentration but significantly vary depending on the metal present in melanin molecule. The highest amounts of netilmicin bound to the polymer were observed in the presence of Zn^{2+} ions and the lowest ones – in the presence of Ca^{2+} ions.

The experimental data were analyzed by constructing the Scatchard plots (Fig. 2) to determine the binding sites and the number of relevant binding classes. For netilmicin-melanin complexes obtained in the absence of metal ions two classes of independent binding sites were found: strong binding sites (n_1) with the association constant $K_1 \sim 10^6\ M^{-1}$ and weak binding sites (n_2) with $K_2 \sim 10^3\ M^{-1}$ (8). The analysis of netilmicin-melanin binding in the presence of Cu^{2+}, Zn^{2+}, Ca^{2+} or Mg^{2+} ions has shown that Scatchard plots are linear functions, indicating that one class of binding sites must be implicated in these complexes formation. For all the analyzed complexes an upward convex part of the Scatchard plot at low netilmicin concentrations has been observed. The diminished melanin binding at low antibiotic concentrations is probably caused by the competition between exogenous cationic drug and endogenous metal ions present in the melanin polymer (19).

The calculated binding parameters for the interaction of netilmicin with melanin containing metal ions and, for comparison, with melanin without metal ions (8), are shown in Table 1. The values of association constant ($K \sim 10^3\ M^{-1}$) for antibiotic-melanin complexes obtained in the presence of Cu^{2+}, Zn^{2+}, Ca^{2+} or Mg^{2+} ions demonstrate that mainly weakly reacting sites exist in these complexes. Based on the values of association constants the following order of netilmicin-[melanin-metal ion] complexes stability has been stated: netilmicin-[melanin-Zn^{2+}] ≥ netilmicin-[melanin-Cu^{2+}] > netilmicin-[melanin-Mg^{2+}] > netilmicin-[melanin-Ca^{2+}]. It has been also found that metal ions cause the decrease of the total number of binding sites by about 20% (for Zn^{2+}) to 80% (for Ca^{2+}) in netilmicin-[melanin-metal ion] complexes as compared with drug-melanin complexes obtained in the absence of metal ions (Table 1).

Natural melanins contain a wide variety of bound metals *in vivo*, and they are unusual among the biopolymers in that they are crosslinked and heterogeneous polymers, whose bonding patterns likely vary in subtle ways (7). The interaction of melanins with metal ions is of great importance for the organisms and has always been of considerable interest. The binding of metals affects the ability of native melanins to protect cells from harmful redox active species, such as reactive oxygen species and photochemically generated radicals (14). It has been proposed that toxic effects in pigmented tissues are respectively higher or lower in the presence of Zn^{2+} or Cu^{2+} in melanin biopolymers. Diamagnetic zinc ions increase and paramagnetic copper ions decrease the free radical concentrations in melanin as well as in its complexes with netilmicin (11).

The obtained results indicate that Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} ions modify the interaction between netilmicin and melanin biopolymer. In the presence of metal ions the amount of netilmicin bound to melanin significantly decreases. The blocking of some active centers in melanin molecules by metal ions, which potentially exist in living systems, may influence the clinical therapeutic efficiency as well as the undesirable side effects of netilmicin and some other drugs with high affinity to melanin containing tissues.

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