INTERACTION OF AMIKACIN AND TOBRAMYCIN WITH MELANIN IN THE PRESENCE OF Cu^{2+} AND Zn^{2+} IONS

DOROTA WRZEŚNIOK, EWA BUSZMAN* and DOMINIK LAKOTA

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University of Silesia, Jagiellońska 4, 41-200 Sosnowiec, Poland

Abstract: Aminoglycoside antibiotics such as amikacin and tobramycin are the most commonly used treatment against Gram-negative bacterial infections. The widely used aminoglycosides have the unfortunate side-effect of targeting sensory hair cells of the inner ear, so that treatment often results in permanent hair cell loss. Because melanin can act as an antioxidant as well as drug and metal chelator, evidence for its role in protecting the stria and organ of Corti against noise, otootoxins, and aging has long been sought. Protective properties of melanin may derive from its ability to bind cations and metals and to scavenge free radical. The aim of the presented work was to examine the amikacin and tobramycin binding to melanin in the presence of Cu^{2+} and Zn^{2+} ions. It has been demonstrated that amikacin and tobramycin form stable complexes with melanin in the presence of metal ions and the amount of aminoglycoside antibiotics bound to melanin increases with the increasing of initial drugs concentration. For amikacin and tobramycin complexes with [melanin-Cu^{2+}] and [melanin-Zn^{2+}] one class of binding sites with the association constant K~10^{-3}M^{-1} has been found. It has been also shown that Cu^{2+} and Zn^{2+} ions administered to melanin before complexing with drugs decrease the amount of aminoglycosides bound to melanin, probably by blocking some active centers in the melanin molecule.

Keywords: amikacin, tobramycin, melanin, drug-melanin complexes

Aminoglycoside antibiotics, such as amikacin and tobramycin, are used in antibacterial therapy of severe Gram-negative infections. Being chemically similar, the aminoglycosides have many features in common, in particular their mechanism of antibacterial action, a broad antibacterial spectrum, partial or complete cross-resistance, bactericidal action in a slightly alkaline environment, poor absorption from gastrointestinal tract, elimination by glomerular filtration, nephro- and ototoxicity (1). The incidence of ototoxicity due to aminoglycosides varies in different studies, depending on the type of patients treated, the methods used to monitor ear function, and the aminoglycoside used (2). Although evidence clearly indicates that intracellular accumulation of aminoglycosides is associated with hair cell damage, the cellular and molecular mechanisms responsible for aminoglycoside-induced hair cell degeneration remain poorly elucidated (3, 4).

Melanin is a natural pigment that is synthesized in various animals and plant species. In humans, melanin is found in the skin, eyes, brain and ear (5). It has been suggested that its function is to protect the cochlea from various types of trauma, including the effects of ototoxic drugs, such as aminoglycoside antibiotics, and noise-induced sensorineural hearing loss (5). Many investigators have demonstrated the affinity of natural and synthetic melamins for various drugs by in vivo and in vitro studies. It is generally accepted that the ability of melanin-containing tissue to accumulate and retain these drugs is remarkable (6).

It is also known that metal ions, which potentially exist in living systems, may bind to melanin biopolymers and affect the drug-melanin interaction (7, 8). The melanin-metal ions binding has been ascribed to a cations exchange activity of melanin, which in turn may be related to the presence of free carboxyl groups in the melanin biopolymer. It was demonstrated that pigmented cells contain a rather high concentration of metal ions as compared with non-melanized homologous tissues (9).

Previous, it has been documented that amikacin and tobramycin form stable complexes

* Corresponding author: e-mail: ebuszman@sum.edu.pl
with model synthetic melanin in vitro (10). In the present study we have examined the effect of Cu\(^{2+}\) and Zn\(^{2+}\) ions on the interaction of amikacin and tobramycin with DOPA-melanin. Synthetic DOPA-melanin was used in the studies because of its similarity to natural eumelanin.

**EXPERIMENTAL**

**Chemicals**

L-3,4-dihydroxyphenylalanine (L-DOPA) used in the studies was obtained from Sigma Chemical Co. Amikacin sulfate was obtained in the form of solution – Biodacyna (250 mg/2 mL) from Bioton, Poland and tobramycin sulfate as Brulamycin (80 mg/2 mL) from Biogal, Hungary. 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 for 48 h according to the method described by Binns et al. (11).

**Metal ion-melanin complex formation**

Dry DOPA-melanin samples of 200 mg each were mixed with 200 mL of bidistilled water containing 1×10^(-3) M of Cu\(^{2+}\) or Zn\(^{2+}\) ions. The mixtures

Figure 1. Binding isotherms (A) and Scatchard plots (B) for amikacin complexes with melanin containing Cu\(^{2+}\) and Zn\(^{2+}\) ions; r – amount of drug bound to melanin, c_0 – initial drug concentration, c_A – concentration of unbound drug. 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.
were incubated at room temperature for 24 h and then filtered. The amounts of copper and zinc bound to melanin were determined by the use of atomic absorption spectrophotometer type AAS 3 (Carl Zeiss, Jena). The final metal ions-DOPA-melanin complexes contained 0.40 mmol Cu\(^{2+}\)/mg mel or 0.29 mmol Zn\(^{2+}\)/mg mel.

**Drug-melanin complex formation**

Binding of drugs to melanin was studied as follows: 5 mg of melanin or metal ion-melanin complexes were placed in plastic test-tubes, where drug solutions were added to a final volume of 5 mL. The initial concentration of drugs ranged from $1\times10^{-4}$ M to $1\times10^{-3}$ M. Control samples contained 5 mg of melanin and 5 mL of bidistilled water without drug. All samples were incubated for 24 h at room temperature. The suspensions were filtered after incubation.

**Analysis of drug binding to melanin**

The amounts of amikacin and tobramycin 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 the wavelength of 350 nm. The amounts of drug bound to melanin, calculated as the difference between the initial amount of drug administered to melanin and

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Figure 2. Binding isotherms (A) and Scatchard plots (B) for tobramycin complexes with melanin containing Cu\(^{2+}\) and Zn\(^{2+}\) ions; $r$ – amount of drug bound to melanin, $c_0$ – initial drug concentration, $c_a$ – concentration of unbound drug. 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.
the amount of unbound drug (in filtrate after incubation), were expressed in mmoles of bound drug per 1 mg 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.

**RESULTS AND DISCUSSION**

Melanins are polymeric pigments formed from successive oxidations of tyrosine (14). Two general types are known, black eumelanins formed by polymerization of dihydroxyindole precursors and red/brown pheomelanins that are colored as a result of cysteine incorporation during oxidation. The chemical structure of these pigments is not well defined because of a variety of coupling modes and precursors available. The details of the polymeric structure and bonding patterns are difficult to characterize, and likely vary in subtle ways. Although often presented as separate forms, eumelanin and pheomelanin are rather qualitative descriptions of a wide variety of native melanins, likely co-polymers with both indolic (eu-) or benzothiazine (pheo-) sub-units (9, 15).

Eumelanin is the most prevalent and important form of melanin, and has been intensively studied (9, 16). Eumelanin oligomers comprise \(5,6\)-dihydroxyindolequinone, \(5,6\)-dihydroxyindole-2-carboxylic acid and their derived redox forms. These molecular units or monomers serve as the building blocks of eumelanin. How these units are put together to form the oligomers (also referred to as protomolecules) and eventually the pigment structure, has not yet been established (17).

Many drugs are known to be markedly accumulated and retained for a considerable time by pigmented tissues and the retention of these compounds is proportional to degree of melanin pigmentation (5). The ability of melanins to bind different drugs and transition metal ions is probably of the greatest biological importance (6).

Our study has demonstrated that amikacin and tobramycin form complexes with synthetic DOPA-melanin in the presence of \(\text{Cu}^{2+}\) and \(\text{Zn}^{2+}\) ions. A relation between the amount of aminoglycoside bound to melanin after 24 h of incubation and the initial drug concentration is presented in Figure 1A (for amikacin) and in Figure 2A (for tobramycin), as binding isotherms. It can be seen from the binding curves that the amount of antibiotics bound to melanin biopolymer increases with increasing initial drug concentration. The obtained data have been analyzed by the use of Scatchard method that can provide information about the number and nature of binding sites in the analyzed complexes. Dependencies of the amount of drugs bound to melanin (r) to the concentration of unbound drugs (c), i.e., \(r/c\) versus \(r\) for amikacin and tobramycin complexes with melanin containing \(\text{Cu}^{2+}\) and \(\text{Zn}^{2+}\) are presented in Figures 1B and 2B, respectively. For amikacin-[melanin-metal ion] and tobramycin-[melanin-metal ion] complexes, the Scatchard plots are linear, indicating that one class of binding sites participates in these complexes formation. For both amikacin and tobramycin, an upward convex part of the Scatchard plot at low drugs concentrations has been observed. The diminished melanin binding at low antibiotics concentrations may be caused by the competition between exogenous cationic drugs and endogenous metal ions present in the melanin polymer (18). The calculated binding parameters for the interaction of aminoglycosides with melanin and melanin-metal ion complexes are shown in Table 1. Analysis of the interaction of amikacin and tobramycin with melanin biopolymer in the absence of metal ions has shown that two classes of independent binding sites with the association constants \(K_{1}=10^5 \text{ M}^{-1}\) and \(K_{2}=10^3 \text{ M}^{-1}\) exist in such complexes (10). The values of association constant for the analyzed aminoglycoside antibiotics interaction with melanin containing zinc or copper (\(K=10^3 \text{ M}^{-1}\)) demonstrate that mainly weakly reacting sites exist in these complexes. In the presence of \(\text{Cu}^{2+}\) and \(\text{Zn}^{2+}\) ions an approximately two-fold decrease of the number of binding sites (n) was observed as compared with drug-melanin complexes obtained in the absence of metal ions.

Aminoglycosides are multifunctional hydrophilic sugars that possess several amino and hydroxyl groups. The amine moieties are mostly protonated in biological media and can be considered as polycationic species (19), and therefore they show a binding affinity for melanin polyanion. The nature of the drug-melanin interaction is still not well established but the existence of ionic bonds, non-electrostatic van der Waals interactions, hydrophobic forces or charge transfer reactions have been proposed. It has been suggested that for each drug even several classes of binding sites may be implicated (6).
Natural melanins contain various metal ions (20), and it is speculated that one of the functions of melanin is to trap excess of metal ions, thereby minimizing the toxic effects of redox active metal ions in the cell. Thus, a delicate balance is hypothesized between protective and deleterious roles of metal binding by melanin. A systematic study of metal cations binding by melanin may provide a foundation to understand these effects (20, 21). The results presented in this paper indicate that Cu" and Zn" ions modify the aminoglycoside antibiotics binding ability to melanin by blocking some active centers in the polymer molecule.

CONCLUSION

Amikacin and tobramycin form stable complexes with melanin in the presence of Cu" and Zn" ions. For amikacin and tobramycin complexes with [melanin-Cu+] and [melanin-Zn+] one class of binding sites with the association constant K~10^3 M^-1 has been shown. It has been demonstrated that Cu" and Zn" ions administered to DOPA-melanin before complexing with drugs significantly decrease the number of binding sites in aminoglycosides-melanin complexes. The obtained results demonstrate that copper and zinc ions modify the aminoglycosides binding ability to melanin biopolymer. The blocking of some active centers in melanin molecules by metal ions, which potentially exist in living systems, may change the clinical therapeutic efficiency of the analyzed drugs.

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REFERENCES


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