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AMIKACIN, KANAMYCIN AND TOBRAMYCIN BINDING TO MELANIN IN THE PRESENCE OF Ca^{2+} AND Mg^{2+} IONS

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Abstract: The aim of the presented work was to examine the interaction of amikacin, kanamycin and tobramycin with melanin in the presence of Ca^{2+} and Mg^{2+} ions. It has been demonstrated that the analyzed aminoglycosides form complexes with melanin in the presence of metal ions and the amount of drugs bound to the polymer increases with increasing initial antibiotics concentration. It has been also shown that two classes of binding sites participate in the formation of amikacin, kanamycin and tobramycin complexes with melanin containing Ca^{2+} or Mg^{2+} ions: high affinity binding sites (n_1) with the association constant $K_1 \sim 10^4$–$10^5\ M^{-1}$ and low affinity binding sites (n_2) with $K_2 \sim 10^3\ M^{-1}$. It has been demonstrated that calcium and magnesium significantly decrease the number of total binding sites (n_{tot}) as compared with aminoglycoside-melanin complexes obtained in the absence of metal ions.

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

The aminoglycoside antibiotics, such as amikacin, kanamycin and tobramycin, have a wide spectrum of activity against some Gram-positive and many Gram-negative bacteria. They are not absorbed from the gut, and for systemic infections must be administered by injection. However, they can be administered orally to control intestinal flora (1). The widespread use of aminoglycoside antibiotics is limited by their nephrotoxicity, which results in impaired kidney function, and by their ototoxicity, which is a serious side-effect and can lead to irreversible loss of hearing. Aminoglycosides are thus reserved for treatment of serious infections where less toxic antibiotics have proved ineffective (2–4).

Pigmentation being the most visible and differentiating human trait is dependent on the type of melanin, the pigment produced in specialized organelles (melanosomes) within dendritic melanocytes. Melanin is present in different regions of the human body: skin, hair, eye, inner ear and brain (5, 6). It is generally accepted that there are two major types of melanin: eumelanin and pheomelanin. Eumelanin is a dark brown to black pigment composed of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid monomer units with 6–9% nitrogen and does not contain sulfur, while a mixed reddish brown pigment called pheomelanin is composed of benzothiazine monomer units with 8–11% nitrogen and a variable percentage of sulfur (7).

The ability of melanin biopolymers to bind chemicals such as various metal ions and drugs is one of the most characteristic features of this pigment. It has been postulated that the retention of melanin-bound compounds is proportional to the degree of tissue pigmentation (6). It has been earlier demonstrated that amikacin, kanamycin and tobramycin form stable complexes with model synthetic melanin *in vitro* (8, 9). It has been also shown that Cu^{2+} and Zn^{2+} ions modify the amikacin and tobramycin interaction with melanin (10). In this study, the effect of Ca^{2+} and Mg^{2+} ions on amikacin, kanamycin and tobramycin binding to DOPA-melanin was analyzed. Synthetic DOPA-melanin was used in the studies because of its similarity to natural eumelanin.

EXPERIMENTAL

Chemicals

L-3,4-dihydroxyphenylalanine (L-DOPA) and kanamycin (sulfate salt) used in the studies were

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Table 1. Binding parameters for amikacin-melanin, kanamycin-melanin and tobramycin-melanin complexes obtained in the presence or absence of metal ions.

Analyzed complex	AMIKACIN		KANAMYCIN		TOBRAMYCIN	
	Association constants K [M^{-1}]	Number of binding sites n [mmol drug/mg mel]	Association constants K [M^{-1}]	Number of binding sites n [mmol drug/mg mel]	Association constants K [M^{-1}]	Number of binding sites n [mmol drug/mg mel]
Aminoglycoside antibiotic -[melanin-Ca^{2+}]	$K_1 = 2.23 \times 10^4$ $K_2 = 2.22 \times 10^3$	$n_1 = 0.125$ $n_2 = 0.137$ $n_{tot} = 0.262$	$K_1 = 6.90 \times 10^4$ $K_2 = 3.04 \times 10^3$	$n_1 = 0.127$ $n_2 = 0.170$ $n_{tot} = 0.297$	$K_1 = 1.86 \times 10^4$ $K_2 = 2.13 \times 10^3$	$n_1 = 0.122$ $n_2 = 0.151$ $n_{tot} = 0.273$
Aminoglycoside antibiotic [melanin-Mg^{2+}]	$K_1 = 3.04 \times 10^4$ $K_2 = 2.44 \times 10^3$	$n_1 = 0.140$ $n_2 = 0.140$ $n_{tot} = 0.280$	$K_1 = 2.54 \times 10^5$ $K_2 = 2.97 \times 10^3$	$n_1 = 0.125$ $n_2 = 0.252$ $n_{tot} = 0.377$	$K_1 = 4.38 \times 10^4$ $K_2 = 2.73 \times 10^3$	$n_1 = 0.117$ $n_2 = 0.168$ $n_{tot} = 0.285$
Aminoglycoside antibiotic melanin*	$K_1 = 1.04 \times 10^5$ $K_2 = 1.01 \times 10^3$	$n_1 = 0.264$ $n_2 = 0.236$ $n_{tot} = 0.500$	$K_1 = 3.05 \times 10^5$ $K_2 = 4.34 \times 10^3$	$n_1 = 0.302$ $n_2 = 0.343$ $n_{tot} = 0.645$	$K_1 = 2.08 \times 10^5$ $K_2 = 4.94 \times 10^3$	$n_1 = 0.288$ $n_2 = 0.341$ $n_{tot} = 0.629$

*Results from previous studies in our lab (8, 9).

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 0.067M phosphate buffer at pH 8.0 as described earlier (11, 12).

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 Ca^{2+} or Mg^{2+} ions. The mixtures were incubated at room temperature for 24 h and then filtered. The amounts of calcium and magnesium bound to melanin were determined by the use of atomic absorption spectrophotometer type AAS 3 (Carl Zeiss, Jena, Germany). The final [melanin-metal ions] complexes contained 0.19 μmol Ca^{2+} or 0.13 μmol Mg^{2+} per 1 mg of melanin.

Drug-melanin complex formation

Binding of drugs to melanin was studied as follows: Five 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 1×10^{-4} M to 1×10^{-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 amount of aminoglycosides in each filtrate with respect to the control samples was determined spectrophotometrically using chloranil as colored reagent (13). All spectrophotometric measurements were performed by the use of JASCO model V-630, UV-VIS spectrophotometer, at 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 the amount of unbound drug (in filtrate after incubation), were expressed in μmoles of bound drug per 1 mg melanin. A qualitative analysis of antibiotic-melanin interaction was performed using Scatchard plots of the experimental data as described earlier (8, 11). 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

The aminoglycosides form an important group of antibiotic agents and are immediately recogniza-

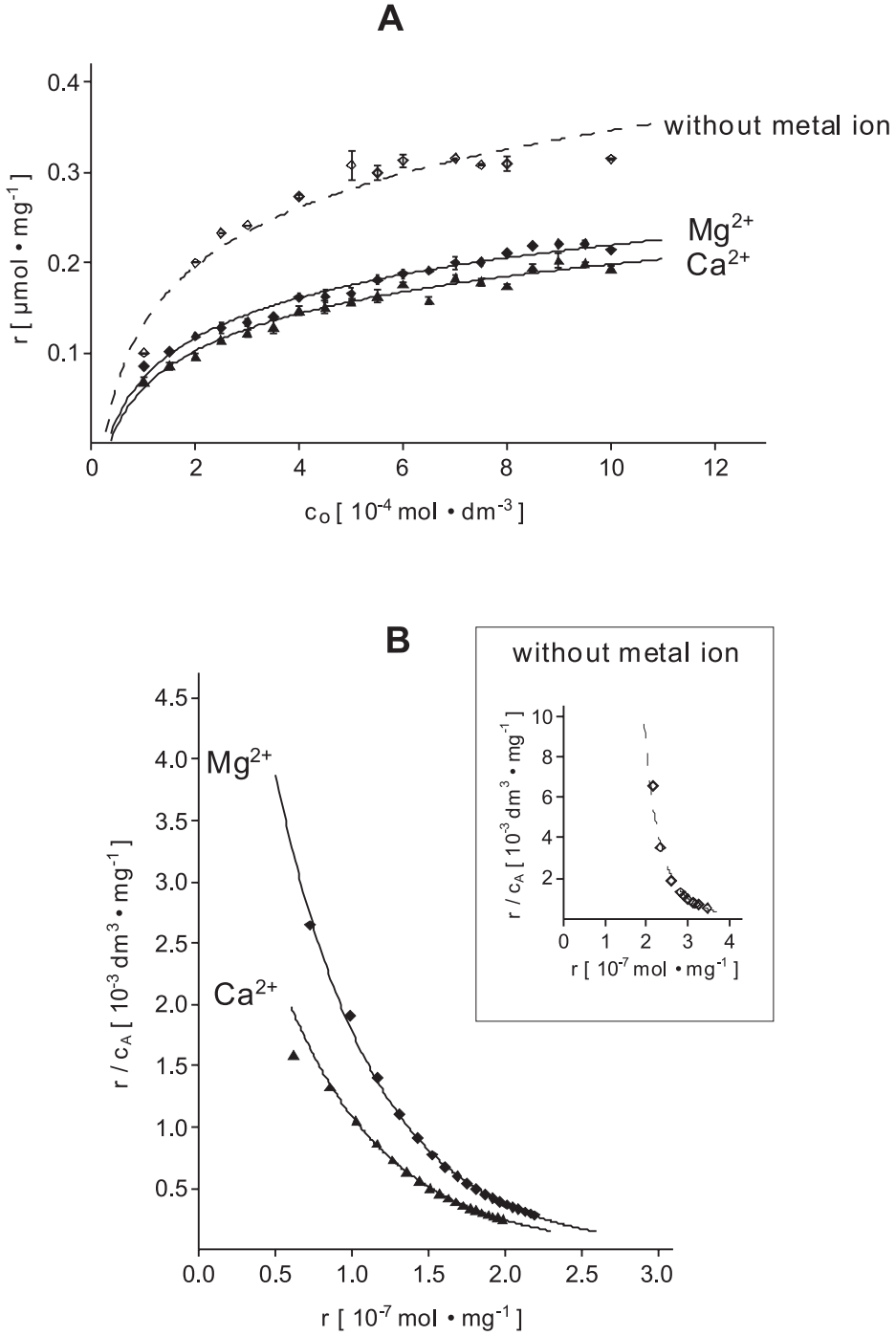

Figure 1. Binding isotherms (**A**) and Scatchard plots (**B**) for amikacin complexes with melanin (8) and melanin containing Ca^{2+} or Mg^{2+} ions; r – amount of drug bound to melanin, c_o – initial drug concentration, c_A – concentration of unbound drug. Mean values ± SD from three independent experiments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

ble as modified carbohydrate molecules. Typically, they have two or three uncommon sugars attached through glycoside linkages to an aminocyclitol, i.e., an amino-substituted cyclohexane system, which also has carbohydrate origin (1).

Kanamycin is an antibiotic produced by *Streptomyces kanamyceticus*. Amikacin is a semisynthetic derivative of kanamycin, with the 4-amino-2-hydroxybutyryl group helping to protect the antibiotic against enzymic deactivation at several positions.

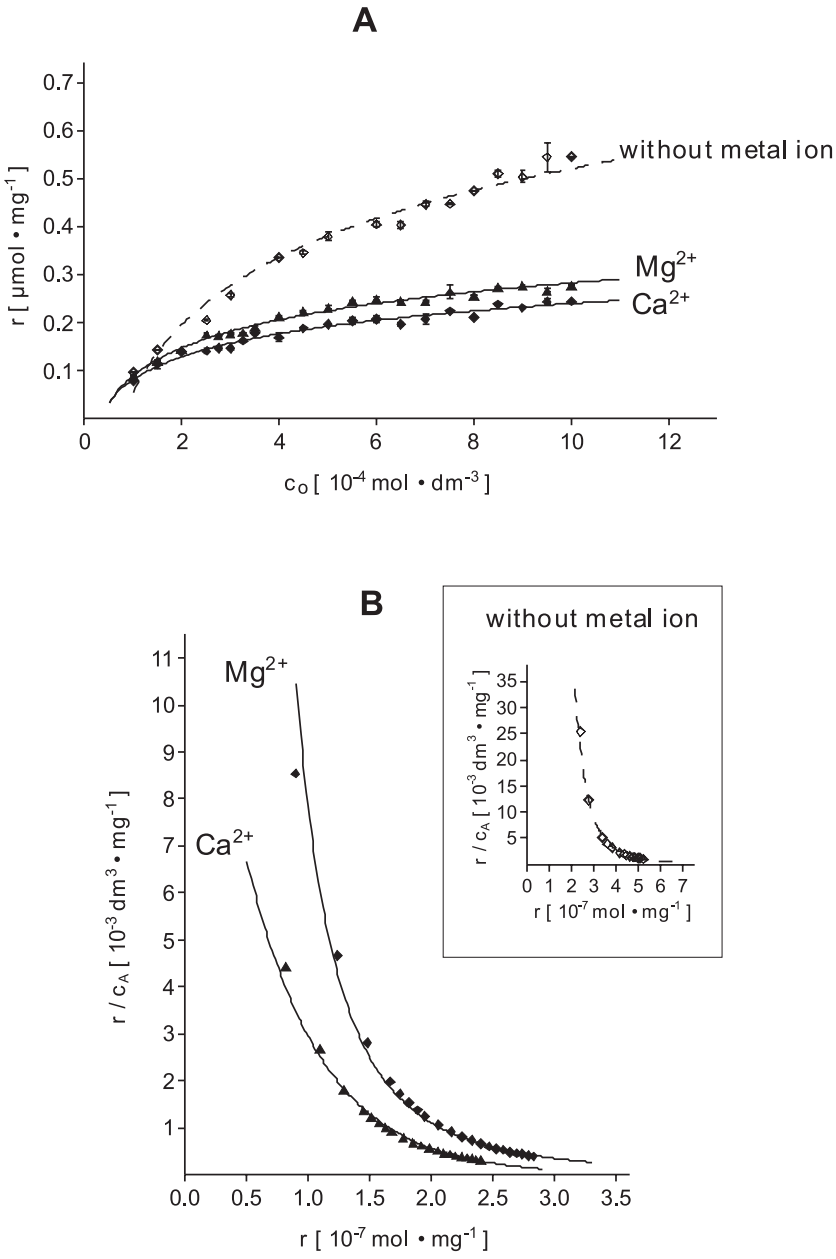

Figure 2. Binding isotherms (**A**) and Scatchard plots (**B**) for kanamycin complexes with melanin (9) and melanin containing Ca^{2+} or Mg^{2+} ions; r – amount of drug bound to melanin, c_o – initial drug concentration, c_A – concentration of unbound drug. Mean values ± SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

Tobramycin is an analogue of kanamycin isolated from *Streptomyces tenebrarius* (1, 2).

Melanin is synthesized and contained within organelles, so-called melanosomes that are transferred *via* dendrites. One melanocyte supplies melanosomes to roughly 30 keratinocytes through formation of 'epidermal melanin unit'. In a modified concept, the melanin unit has also been suggested to include Langerhans cells. Skin pigmentation depends on the number, size, cellular distribution

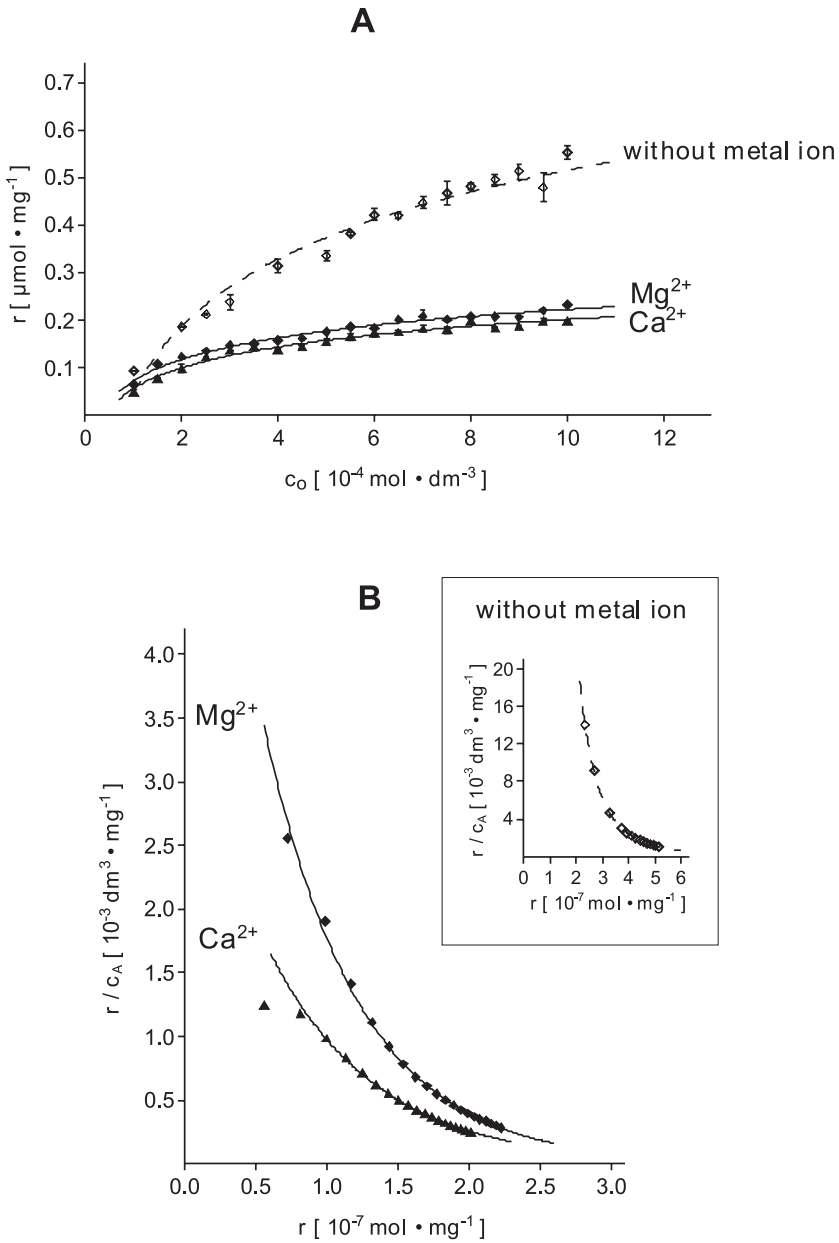

Figure 3. Binding isotherms (**A**) and Scatchard plots (**B**) for tobramycin complexes with melanin (8) and melanin containing Ca^{2+} or Mg^{2+} ions; r – amount of drug bound to melanin, c_o – initial drug concentration, c_A – concentration of unbound drug. Mean values ± SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

and type of melanosomes rather than on the number of melanocytes (14).

Melanocytes are not only found in the skin but also in the stria vascularis of the cochlea (15). The stria vascularis forms the lateral wall of the scala media, the endolymph-filled chamber that houses the organ of Corti, which is the principal sound-detecting component of the inner ear . The melanocytes of the stria vascularis are termed intermediate cells and provide functions that are necessary for normal hearing. Deafness results from loss of these cells *via* trauma, infection, or perhaps noise (16). Ototoxic agents, such as aminoglycoside antibiotics, may damage intermediate cells, resulting in loss of hearing (1, 2). The range of functions provided by intermediate cells has not been characterized extensively but it is known that the stria vascularis is responsible for endolymph maintenance (16).

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 (8). The ability of melanins to bind different drugs and transition metal ions is probably of the greatest biological importance (17).

Our study has demonstrated that the analyzed aminoglycoside antibiotics form complexes with synthetic DOPA-melanin in the presence of Ca^{2+} and Mg^{2+} ions. The amounts of drugs bound to melanin in the presence of metal ions are presented in Fig. 1A, 2A and 3A, as binding isotherms, for amikacin, kanamycin and tobramycin, respectively. The increase of the absolute amount of aminoglycoside bound to melanin (in μmoles per 1 mg of melanin) with the increase of initial drug concentration has been demonstrated. The obtained results 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_A), i.e., r/c_A, versus r for amikacin, kanamycin and tobramycin complexes with melanin containing Ca^{2+} and Mg^{2+} are presented in Fig. 1B, 2B and 3B, respectively. For all the analyzed aminoglycoside antibiotic-[melanin-metal ion] complexes the Scatchard plots are curvilinear, which indicates that at least two classes of independent binding sites are implicated in drug-melanin complexes formation. The calculated binding parameters for the interaction of amikacin, kanamycin and tobramycin with melanin containing Ca^{2+} and Mg^{2+} ions and, for comparison, with melanin without metal ions (8, 9), are shown in Table 1. For the analyzed aminoglycoside antibiotics-melanin complexes high affinity binding sites (n_1) with the association constant K_1 ~10^4–10^5 M^{-1} and low affinity binding sites (n_2) with K_2 ~10^3 M^{-1} were stated. It has been demonstrated that calcium or magnesium ions significantly decrease the number of total binding sites (n_{tot}) as compared with drugs-melanin complexes obtained in the absence of metal ions.

The nature of drug-melanin interaction is still not well established but the existence of ionic bonds, non-electrostatic van der Waals and hydrophobic interactions or charge transfer reactions have been proposed (6).

Taking into account that metal ions as well as the analyzed antibiotics exist in the form of cations at physiological pH, probably the same active centers in melanin polyanion are responsible for these ligands binding. The observed *in vitro* blocking of some active centers in melanin molecules by Ca^{2+} and Mg^{2+} ions, which potentially exist in living systems, may influence the clinical therapeutic efficiency as well as the undesirable side effects of aminoglycoside antibiotics *in vivo*.

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REFERENCES

1. Buszman E., Wrześniok D., Grzegorczyk A., Matusiński B., Molęda K.: Farm. Przegl. Nauk. 2, 2 (2007).
2. Durante-Mangoni E., Grammatikos A., Utili R., Falagas M.F.: Int. J. Antimicrob. Agents 33, 201 (2009).
3. Selimoglu E.: Curr. Pharm. Des. 13, 119 (2007).
4. Guthrie O.W.: Toxicology 249, 91 (2008).
5. Plonka PM., Passeron T., Brenner M., Tobin D.J., Shibahara S., Thomas A., Slominski A. et al.: Exp. Dermatol. 18, 799 (2009).
6. Larsson B.S.: Pigment Cell Res. 6, 127 (1993).
7. Meredith P., Sarna T.: Pigment Cell Res. 19, 572 (2006).
8. Buszman E., Wrześniok D., Trzcionka J.: Pharmazie 62, 210 (2007).
9. Wrześniok D., Buszman E., Karna E., Pałka J.: Pharmazie 60, 439 (2005).
10. Wrześniok D., Buszman E., Lakota D.: Acta Pol. Pharm. Drug Res. 68, 493 (2011).

11. Buszman E., Wrześniok D., Surażyński A., Pałka J., Molęda K.: Bioorg. Med. Chem. 14, 8155 (2006).
12. Buszman E., Pilawa B., Zdybel M., Wrześniok D., Grzegorczyk A., Wilczok T.: Chem. Phys. Lett. 403, 22 (2005).
13. Rizk M., Younis F.: Anal. Lett. 17, 1803 (1984).
14. Scherer D., Kumar R.: Mutat. Res. 705, 141 (2010).
15. Hayashi H., Sone M., Schachern P.A., Wakamatsu K., Paparella M.M., Nakashima T.: Arch. Otolaryngol. Head Neck Surg. 133, 151 (2007).
16. Tolleson W.H.: J. Environ. Sci. Health C 23, 105 (2005).
17. Hong L., Simon J.D.: J. Phys. Chem. B 111, 7938 (2007).

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