ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXII, N 4, 10 SECTIO DDD 2009

Department of Pharmaceutical Chemistry, Medical University of Silesia

EWA BUSZMAN, DOROTA WRZEŚNIOK, TOMASZ HRYNIEWICZ, MAGDALENA GRZEGORCZYK

Effect of Cu^{2+} and Zn^{2+} ions on quinidine, disopyramide and metoprolol interaction with melanin in vitro

Wpływ jonów Cu^{2+} i Zn^{2+} na oddziaływanie chinidyny, dizopiramidu i metoprololu z melaniną *in vitro*

The pumping action of the heart involves three principle electrical events: the generation of a signal, the conduction or propagation of the signal and the fading away of the signal. When one or more of these events is disrupted, cardiac arrhythmias may arise [1].

Quinidine and disopyramide belong to class IA of antiarrythmic agents, metoprolol is a beta-blocker belonging to class II according to the Vaughan-Williams classification. The history of antiarrhythmic therapy reveals these agents to be associated with a high incidence of toxicity [4, 9]. Considering that these agents alter electrical conduction, it is not surprising that they also frequently affect the parasympathetic and sympathetic regulation of organ function. Toxicity may also arise from specific properties of the molecules, which block critical cellular function. Drug interactions arising from competition for the metabolic pathways of drug elimination, especially the P450 family of cytochromes, is another frequent mechanism of toxicity for these medications. The anticholinergic activity of class I agents affects the ciliary apparatus producing blurred vision, photophobia and aggravating glaucoma, and these agents have also been associated with optic neuritis. The application of the β-blocking agents may cause an oculomucocutaneous syndrome involving epidermalization of the conjunctival epithelium, with epitheliolysis and stromal ulceration of the cornea related to deficient tear secretion and the formation of an autoantibody to squamous epithelium [9].

Melanins are high-molecular-weight pigments that are ubiquitous in nature. Melanin is present in different regions within the human body: skin, hair, eye, inner ear and brain. The proposed functions of melanin are quite diverse, including photoprotection, photosensitization, metal ion chelation, thermoregulation, and free radical quenching [10]. The binding of drugs to melanin and proteins in ocular tissues is also an important factor in transscleral drug delivery because the chemical potential of unbound drug is the driving force in drug permeation and the bound drug acts as a reservoir. The ability of melanin biopolymers to bind chemicals such as various metal ions and drugs is one of the most characteristic features of the pigment. It has been postulated that the retention of these compounds is proportional to the degree of melanin pigmentation [7]. Quinidine, disopyramide and metoprolol are known to bind to melanin [3].

The aim of our study was to investigate whether metal ions (Cu^{2+} and Zn^{2+}), present in living organisms, may affect the antiarrythmic drugs (quinidine, disopyramide and metoprolol) interaction with melanin.

MATERIAL AND METHODS

Chemicals. L-3,4-dihydroxyphenylalanine (L-DOPA), quinidine (sulfate salt dihydrate), disopyramide (phosphate salt) and metoptrolol (tartrate salt) used in the studies were obtained from Sigma Chemical Co. 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 for 48h according to the method described by Binns et al [2].

Metal ion-melanin complex formation. Dry DOPA-melanin samples of 200mg each were mixed with 200ml of bidistilled water containing $1 \cdot 10^{-3}$M of Cu^{2+} or Zn^{2+} ions. The mixtures were incubated at room temperature for 24h 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 metal ion concentrations for method calibration were in the range from 0.320 to 5.080μg/ml (i.e. 5 to 80μM) for Cu^{2+} and from 0.065 to 0.981μg/ml (i.e. 1 to 15μM) for Zn^{2+}. The precision of the method characterized by RSDs was of 0.47–3.85% (average 2.54%) for copper ions and of 2.51–4.95% (average 4.14%) for zinc ions. The final metal ions-DOPA-melanin complexes contained 0.40μmol Cu^{2+}/mg mel or 0.29μmol Zn^{2+}/mg mel.

Drug-melanin complex formation. Binding of drugs to melanin was studied as follows: 5mg of melanin or metal ion-melanin complexes were placed in plastic test-tubes, where drug solutions were added to a final volume of 5ml. The initial concentration of drugs ranged from $4 \cdot 10^{-5}$M to $5 \cdot 10^{-3}$M. Control samples contained 5mg of melanin and 5ml of bidistilled water without drug. All samples were incubated for 24h at room temperature. The suspensions were filtered after incubation.

Analysis of drug binding to melanin. The concentrations of drugs remaining in each filtrate after incubation with melanin with respect to the control samples were determined spectrophotometrically according to the *Pharmacopoeia* method. All spectrophotometric measurements were performed by the use of JASCO model V-530, UV-VIS spectrophotometer, at wavelength 331nm for quinidine, 261nm for disopyramide and 274nm for metoprolol. The sensitivity of the spectrophotometric methods was investigated by measuring absorbance of different concentrations of drugs. All the data points produced strong (R^2>0.99) linear correlation. Linear regression analysis gave equation y=ax+b, where constant b was found to be insignificant. The calculated values of the molar absorption coefficient ($\varepsilon_{\lambda max}$) $9.63 \cdot 10^3$ for quinidine, $3.95 \cdot 10^3$ for disopyramide and $2.39 \cdot 10^3$ for metoprolol, were used to estimate the amount of drug bound to the polymer. The amount of quinidine, disopyramide and metoprolol bound to melanin, calculated as differences between the initial amounts of drug administered to melanin and the amounts of unbound drug (in filtrates after incubation), were expressed in μmol of bound drug per 1mg melanin.

The qualitative analysis of drug-melanin interaction was performed using the Scatchard plots of the experimental data according to Kalbitzer and Stehlik [6]. 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

Melanin is an important class of pigments that have attracted attention from a wide range of scientists. The structures of melanins are uncertain due to the amorphous, heterogeneous and

insoluble nature of these pigments, which precludes their structural solution given the currently available analytical tools. 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. Melanins possess numerous interesting physicochemical characteristics, including unusual optical, condensed phase electric, electron exchange, paramagnetic, ion exchange and electromagnetic radiation absorption properties [8].

These pigments may be obtained from a variety of precursors. In the present study, we examined synthetic melanin, obtained from L-DOPA, commonly used as a model for natural eumelanin pigment. Antiarrythmics-melanin interactions were characterized basing on binding isotherms and Scatchard plots.

The amounts of drugs bound to melanin in the presence of Cu^{2+} and Zn^{2+} ions are presented in Fig 1A as binding isotherms. The increase of the absolute amount of quinidine, disopyramide and metoprolol bound to melanin (in μmoles per mg melanin) with the increase of initial drug concentrations was indicated. The experimental data were also analyzed by constructing the Scatchard plots (Fig. 1B) to determine the binding sites and the number of relevant binding classes. For all the complexes antiarrythmics-[melanin-metal ion] the Scatchard plots are curvilinear, which indicates that at least two classes of independent binding sites are implicated in drugs-melanin complexes formation. The calculated binding parameters for the interaction of quinidine, disopyramide and metoprolol with melanin containing Cu^{2+} and Zn^{2+} ions and, for comparison, with melanin without metal ions [3] are shown in Table 1.

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

Analyzed complex	Quinidine		Disopyramide		Metoprolol	
	Association constants K [M^{-1}]	Number of binding sites n [μmol drug/ mg melanin]	Association constants K [M^{-1}]	Number of binding sites n [μmol drug/ mg melanin]	Association constants K [M^{-1}]	Number of binding sites n [μmol drug/ mg melanin]
Drug-melanin[a]	$K_1 = 3.00 \cdot 10^5$ $K_2 = 1.75 \cdot 10^3$	$n_1 = 0.154$ $n_2 = 0.371$ $n_{tot} = 0.545$	$K_1 = 1.12 \cdot 10^4$ $K_2 = 6.04 \cdot 10^2$	$n_1 = 0.212$ $n_2 = 0.281$ $n_{tot} = 0.493$	$K_1 = 1.42 \cdot 10^4$ $K_2 = 7.89 \cdot 10^2$	$n_1 = 0.167$ $n_2 = 0.220$ $n_{tot} = 0.387$
Drug-[melanin-Cu^{2+}]	$K_1 = 1.32 \cdot 10^4$ $K_2 = 5.63 \cdot 10^2$	$n_1 = 0.178$ $n_2 = 0.274$ $n_{tot} = 0.452$	$K_1 = 2.08 \cdot 10^4$ $K_2 = 6.29 \cdot 10^2$	$n_1 = 0.094$ $n_2 = 0.181$ $n_{tot} = 0.275$	$K_1 = 5.56 \cdot 10^4$ $K_2 = 7.76 \cdot 10^2$	$n_1 = 0.045$ $n_2 = 0.105$ $n_{tot} = 0.150$
Drug-[melanin-Zn^{2+}]	$K_1 = 1.71 \cdot 10^4$ $K_2 = 1.02 \cdot 10^3$	$n_1 = 0.185$ $n_2 = 0.318$ $n_{tot} = 0.503$	$K_1 = 3.40 \cdot 10^4$ $K_2 = 6.90 \cdot 10^2$	$n_1 = 0.129$ $n_2 = 0.251$ $n_{tot} = 0.380$	$K_1 = 1.83 \cdot 10^5$ $K_2 = 6.69 \cdot 10^2$	$n_1 = 0.093$ $n_2 = 0.167$ $n_{tot} = 0.260$

[a] Results from previous studies in the lab [3]

For the analyzed antiarrythmic drugs complexes with melanin strong binding sites (n_1) with the association constant $K_1 \sim 10^4$-10^5 M^{-1} and weak binding sites (n_2) with $K_2 \sim 10^2$-10^3 M^{-1} were stated. It was demonstrated that copper and zinc ions significantly decrease the number of total binding sites (n_{tot}) as compared with drugs-melanin complexes obtained in the absence of metal ions.

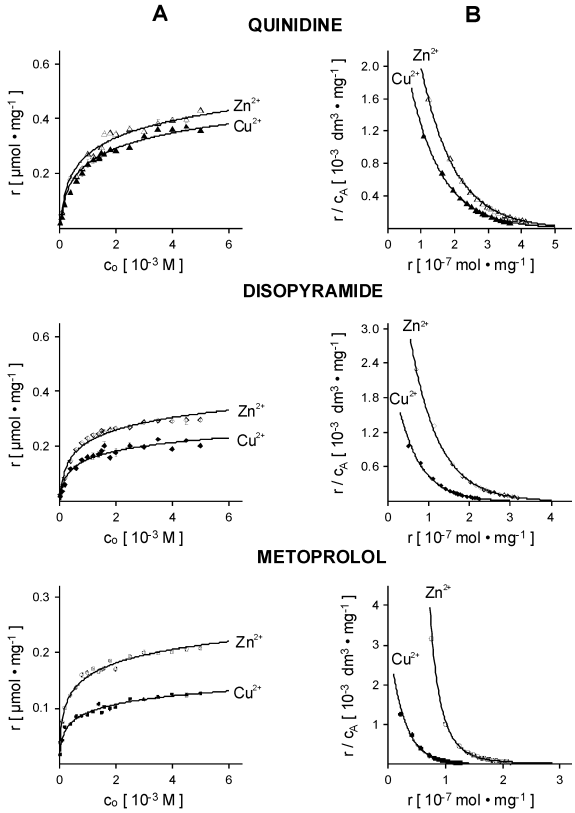

Fig. 1. Binding isotherms (A) and Scatchard plots (B) for quinidine, disopyramide and metoprolol complexes with DOPA-melanin containing copper or zinc ions; r – amount of drug bound to melanin, c_o – initial drug concentration, c_A – concentration of unbound drug. Mean values ±SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol

The accumulation of a drug in pigmented tissues may have serious physiological consequences as it could lead to potentially toxic effects. Despite several investigations into the nature of drug–melanin binding, the exact mechanism of the interaction remains unknown. Drug–melanin binding is a phenomenon that has been observed with structurally and pharmacologically unrelated drugs following administration by ocular and other routes. Of the drugs with known melanin affinity, many are positively charged at physiological pH and it is generally accepted that ionic interactions are a major contributor. Other factors involved in the reversible binding are the drug's lipophilicity, van der Waals forces and the ability to form charge transfer complexes [7].

Copper and zinc are essential metals for different physiological functions. Copper functions as the active centre of cuproenzymes such as cytochrome c oxidase, which is a component of the mitochondrial respiratory chain, and Cu,Zn-superoxide dismutase. Zinc plays an essential role in cell membrane integrity and is a component of more than 300 different enzymes that function in many aspects of cellular metabolism, involving metabolism of proteins, lipids and carbohydrates [5].

Taking into account that metal ions as well as the analyzed drugs 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 Cu^{2+} and Zn^{2+} ions, which potentially exist in living systems, may influence the clinical therapeutic efficiency as well as the undesirable side effects of quinidine, disopyramide and metoprolol *in vivo*.

CONCLUSIONS

1. Quinidine, disopyramide and metoprolol form stable complexes with melanin in the presence of Cu^{2+} and Zn^{2+} ions.

2. The Scatchard analysis of drugs binding to melanin showed that at least two classes of independent binding sites must be implicated in the antiarrythmics-[melanin-Cu^{2+}] and antiarrythmics-[melanin-Zn^{2+}] complexes formation.

3. It was demonstrated that Cu^{2+} and Zn^{2+} ions significantly decrease the amounts of quinidine, disopyramide and metoprolol bound to melanin as compared with drugs-melanin complexes obtained in the absence of metal ions.

4. 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 the analyzed antiarrythmic drugs.

Acknowledgements. This work was supported by the Medical University of Silesia, Katowice, Poland (Grant N° KNW-1-007/09)

REFERENCES

1. Abraham D. J.: Burger's Medicinal Chemistry and Drug Discowery. 6th Edition, Vol. 3: Cardiovascular Agents and Endocrines, John Wiley @ Sons, Inc., 2003.
2. Binns F., Chapman R. F., Robson N. C. et al.: Studies related to the chemistry of melanins. Part VIII. The pyrrolecarboxylic acids formed by oxidation or hydrolysis of melanins derived from 3,4-dihydroxyphenethylamine or (±)-3,4-dihydroxyphenylalanine. J. Chem. Soc. C, 1128, 1970.
3. Buszman E., Różańska R.: Interaction of quinidine, disopyramide and metoprolol with melanin *in vitro* in relation to drug-induced ocular toxicity. Pharmazie, 58, 507, 2003.
4. Cheong H. I., Johnson J., Cormier M., Hosseini K.: *In vitro* cytotoxicity of eight beta-blockers in human corneal epithelial and retinal pigment epithelial cell lines: comparison with epidermal keratinocytes and dermal fibroblasts. Toxicol. *In Vitro*, 22, 1070, 2008.
5. Formigari A., Irato P., Santon A.: Zinc, antioxidant systems and metallothionein in metal mediated-apoptosis: biochemical and cytochemical aspects. Comp. Biochem. Physiol. C, 146, 443, 2007.
6. Kalbitzer H. R., Stehlik D.: On the analysis of competitive binding of various ligands to cooperative and independent binding sites of macromolecules. Z. Naturforsch., 34c, 757, 1979.
7. Larsson B. S.: Interaction between chemicals and melanin. Pigment Cell Res., 6, 127, 1993.
8. Meredith P., Sarna T.: The physical and chemical properties of eumelanin, Pigment Cell Res., 19, 572, 2006.
9. Pollak P. T.: Clinical organ toxicity of antiarrhythmic compounds: ocular and pulmonary manifestations. Am. J. Cardiol., 84, 37R, 1999.
10. Prota G.: Melanins and Melanogenesis. Academic Press, London 1992.

SUMMARY

The binding of drugs to melanin may have significant pharmacologic consequences. In spite of many investigations into the nature of drug binding to melanin, the exact mechanism of this interaction remains unknown. The aim of the presented work was to examine the interaction of antiarrythmic drugs with melanin in the presence of copper and zinc ions. It was demonstrated that the analyzed drugs form complexes with melanin biopolymer. It was also shown that Cu^{2+} and Zn^{2+} ions administered to melanin before complexing with drugs decrease the total amount of quinidine, disopyramide and metoprolol bound to melanin.

STRESZCZENIE

Wiązanie leków do melaniny może mieć istotne farmakologiczne konsekwencje. Pomimo wielu badań dotyczących natury wiązania leków do melaniny dokładny mechanizm tego oddziaływania pozostaje nieznany. Celem pracy była analiza oddziaływania leków antyarytmicznych z melaniną w obecności jonów miedzi i cynku. Stwierdzono, że analizowane leki tworzą kompleksy z biopolimerem melaninowym. Ponadto wykazano, że jony Cu^{2+} i Zn^{2+} wbudowane do melaniny przed tworzeniem kompleksów z lekami zmniejszają całkowitą ilość chinidyny, dizopiramidu i metoprololu związanych z melaniną.