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Prochlorperazine interaction with melanin and melanocytes

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Prochlorperazine is a phenothiazine-class antipsychotic drug usually used to treat nausea, vomiting and schizophrenia. Phenothiazine derivatives have been known to cause serious side effects, like extrapyramidal symptoms, but also skin disorders which mechanism has not been fully established. The aim of this study was to examine the interaction between prochlorperazine and melanin as well as to estimate the effect of prochlorperazine on cell viability, melanogenesis and antioxidant defense system in normal human melanocytes. We have demonstrated that prochlorperazine forms stable complexes with melanin, characterized by two classes of independent binding sites with the association constants $K_1 \sim 10^6\ M^{-1}$ and $K_2 \sim 10^2\ M^{-1}$. It has been shown that prochlorperazine induces concentration-dependent loss in cell viability. The value of EC_{50} was calculated to be 18.49 μM. Prochlorperazine in a concentration of 0.001 μM stimulated melanogenesis, while in concentrations 1.0 and 10.0 μM melanization process was inhibited. Furthermore, the drug in concentrations of 0.1, 1.0 and 10.0 μM caused changes in cellular antioxidant defense system, what indicated the induction of oxidative stress. The observed changes in cell viability, melanization and antioxidant defense system in normal human melanocytes after prochlorperazine treatment may explain a potential role of melanin, oxidative stress and melanocytes in mechanisms of undesirable side effects after accumulation of this drug in pigmented tissues.

1. Introduction

Prochlorperazine belongs to the phenothiazine class of neuroleptic drugs (Tashiro et al. 2012) and is used as first line therapy of nausea, vomiting, acute vertigo as well as to treat migraine and schizophrenia (Caughey et al. 2010; Tashiro et al. 2012; Weiss et al. 2011). The adverse side effects of prochlorperazine therapy include high extrapyramidal symptoms (EPS), such as dystonia and/or akathisia (Caughey et al. 2010; Kelley and Tepper 2012) and also ocular (e.g. cataract, lenticular pigmentation) and skin disorders, like pruritus, urticaria, exfoliative dermatitis, cutaneous photosensitivity and abnormal skin pigmentation (Caughey et al. 2010; Lal et al. 1993; Lamer et al. 2010; Ruigómez et al. 2000; Tashiro et al. 2012). Fixed drug reactions (FDR) have been specifically found in patients taking prochlorperazine. FDR usually appear on the genitalia, in the perianal area and anywhere on the skin surface as solitary, erythematous, bright red or dusky red maculae that may evolve into an edematous plaque. Bullous-type lesions as well as residual grayish hyperpigmentation may be also present (Lamer et al. 2010). Prochlorperazine therapy may cause Steven Johnson syndrome (SJS), which is a mucocutaneous cell-mediated hypersensitivity reaction (Ferraro et al. 2014).

Melanins are pigments synthesized in melanosomes, in the cytoplasm of melanocytes, during multistep complex process – melanogenesis (Otręba et al. 2012; Rok et al. 2012; Videira et al. 2013). The key regulatory and rate-limiting specific to melanocytes enzyme – tyrosinase catalyzes the first two steps of melanogenesis: hydroxylation of l-tyrosine and subsequent oxidation of the intermediate L-3,4-dihydroxyphenylalanine (l-DOPA) to yield l-DOPAquinone (Hearing 2011; Otręba et al. 2012; Schallreuter et al. 2008). Melanogenesis is also regulated by the major transcription factor - microphthalmia-associated transcription factor (MITF), which is an essential regulator of life and differentiation of melanocytes (Chakraborty et al. 2009). MITF regulates melanin biosynthesis by activation of tyrosinase (TYR) and tyrosinase related proteins 1 and 2 (Otręba et al. 2012; Vachtenheim and Borovanský 2011), and is responsible for cellular proliferation, dendrite formation and induction of antiapoptotic genes expression (Otręba et al. 2012; Rok et al. 2012; Solano 2014). Disturbation of melanogenesis may determine different types of pigmentation defects, which are classified as hypo- or hyperpigmentation and may occur with or without an altered number of melanocytes (Otręba et al. 2013, 2014; Videira et al. 2013).

Melanocytes are specialized dendritic cells found in the skin, eyes, inner ear, brain, adipose tissue, heart and lungs where they play very important physiological role, such as pigmentation, photoprotection, trapping reactive oxygen species (ROS), chelating metal ions and binding a variety of drugs/xenobiotics (e.g. psychotropic agents or fluoroquinolones) (Beberok et al. 2010; Buszman and Różańska 2003; Buszman et al. 2008; Plonka et al. 2009; Rok et al. 2012). Interaction of a drug with melanin biopolymer protects the organism against undesirable drug side effects, but on the other hand may change its pharmacodynamic properties and lead to accumulation of the drug in melanin-containing tissues increasing risk of those cells damage (Mars and Larsson 1999; Rok et al. 2012).

The antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) serve as a primary line of defense against free radicals. During normal physiological processes living cells generate reactive oxygen species, while under pathophysiological conditions enhance the generation of ROS what leads to oxidative stress (Chakraborty et al. 2009). It has been demonstrated that melanin may act as a free radical scavenger as well as to possess superoxide dismutase activity, what may reduce ROS content and thus protect pigmented tissues against cellular damage induced by oxidative stress (Brenner and Hearing 2008; Otręba et al. 2012).

Recent studies have shown that other neuroleptics, such as chlorpromazine (Otręba et al. 2015a) and thioridazine (Otręba et al. 2015b) modulate melanin biosynthesis and induce oxidative stress in human epidermal melanocytes dark pigmented.

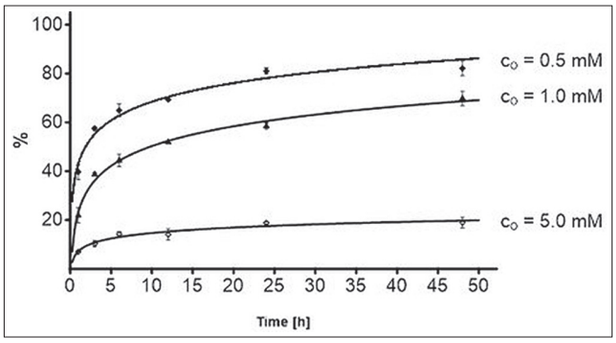

Fig. 1: The effect of incubation time and initial drug concentration (c_o) on the amount of prochlorperazine bound to DOPA-melanin (in %). Mean values ±SEM from three independent experiments are presented. Points without error bars indicate that SEM was less than the size of the symbol.

The aim of this study was to examine the interaction between prochlorperazine and melanin and to estimate the effect of prochlorperazine on cell viability, melanogenesis and antioxidant defense system in normal human melanocytes dark pigmented (HEMn-DP).

2. Investigations and results

2.1. Interaction of prochlorperazine with DOPA-melanin

The effect of the incubation time and initial drug concentration on the amount of prochlorperazine bound to melanin is presented in Fig. 1. It can be seen that the amount of prochlorperazine bound to melanin increases with the prolongation of incubation time and after 24 h it attains a plateau. It has been also shown that the amount of drug bound to melanin increases with the increasing initial drug concentration. Simultaneously, the decrease of complex formation efficiency (expressed in % as the ratio of the amount of drug bound to melanin to the initial amount of drug added to melanin) was observed with the increase of the initial drug concentration (Fig. 1). The dependence of the amount of prochlorperazine bound to melanin after 24 h of incubation as a function of the initial drug concentration is presented in Fig. 2A as binding isotherm. The amount of drug bound to a constant amount of melanin increases and reaches a plateau at about 0.8 µmol prochlorperazine per 1 mg melanin, which reflects the initial drug concentration of about 2.5 mM. The dependence of the amount of a drug bound to melanin (r) to the concentration of unbound drug (C_A), i.e., r/ C_A, versus r for prochlorperazine complexes with DOPA-melanin is presented in Fig. 2B as Scatchard plot. The use of the Scatchard method can provide information about the number and nature of binding sites in the analyzed complexes. The analysis of drug binding to melanin shows that the Scatchard plot is curvilinear with an upward concavity, indicating that at least two classes of independent binding sites must be implicated in drug-melanin complexes formation. The calculated binding parameters for the interaction of prochlorperazine with melanin are the following: strong binding sites (n_1=0.27 µmol drug/mg melanin) with the association constant $K_1 = 1.02 \cdot 10^6\ M^{-1}$ and weak binding sites (n_2=0.84 µmol drug/mg melanin) with the association constant $K_2 = 7.28 \cdot 10^2\ M^{-1}$.

2.2. Effect of prochlorperazine on cell viability

The cell viability was determined by the WST-1 test after 24-h incubation with prochlorperazine in a concentration range from 0.0001 µM to 100 µM. It has been demonstrated that the analyzed drug induces concentration-dependent loss in cell viability (Fig. 3). Melanocytes treated with 1.0, 10.0, 25.0, 50.0, 75.0 and 100.0 µM of prochlorperazine for 24 h lost 16.8 %, 37.8 %, 52.7 %, 83.8 %, 90.1 % and 97.2 % in cell viability, respectively. The value of EC_{50} (the concentration of a drug that produces loss in cell viability by 50%) was calculated to be 18.49 µM. At lower drug concentrations (0.0001, 0.001, 0.01 and 0.1 µM) the loss in melanocytes viability was not observed.

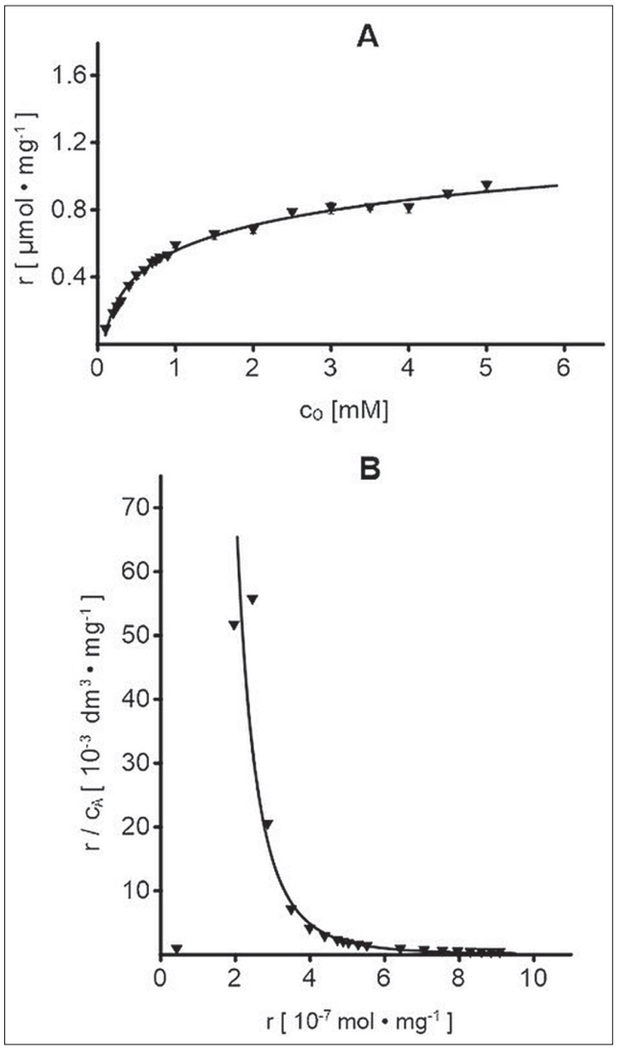

Fig. 2: Binding isotherm (A) and the Scatchard plot (B) for prochlorperazine-melanin complexes obtained after 24-h incubation; c_o- initial drug concentration, r- amount of drug bound to melanin, c_A-concentration of an unbound drug. Mean values ± SEM from three independent experiments are presented. Points without error bars indicate that SEM was less than the size of the symbol.

2.3. Effect of prochlorperazine on the melanization process

The effectiveness of the melanization process was estimated by measuring the melanin content, cellular tyrosinase activity and microphthalmia-associated transcription factor (MITF) content in melanocytes treated with 0.0001, 0.001, 0.01, 0.1, 1.0 and 10.0 µM of prochlorperazine for 24 h. After determining a calibration curve, the melanin content per cell was determined as 51.9 to 66.1 pg/cell for melanocytes treated with the analyzed drug and 62.4±0.62 pg/cell for a control sample. The obtained results, recalculated for culture ($1x10^5$ cells), were finally expressed as a percentage of the controls (Fig. 4). Treatment of HEMn-DP cells with 0.001 µM of a drug caused increase in melanin content by 6.1 %, while the concentrations of 1.0 and 10.0 µM decreased the pigment content by 4.1 % and 16.8 %, respectively. Prochlorperazine in concentrations 0.0001, 0.01 and 0.1 µM had no impact on melanin content in melanocytes. Tyrosinase activity in melanocytes treated with prochlorperazine increased in a manner correlating well with the effect on melanin production (Fig. 5). The enzyme activity was determined as 0.72 to 0.98 µmol/min/mg protein for melanocytes treated with prochlorperazine and 0.91±0.02 µmol/min/mg for a control sample. The tyrosinase activity was increased by 6.9 % for cells treated with a drug in concentration of 0.001 µM, while the concentrations of 1.0 and 10.0 µM

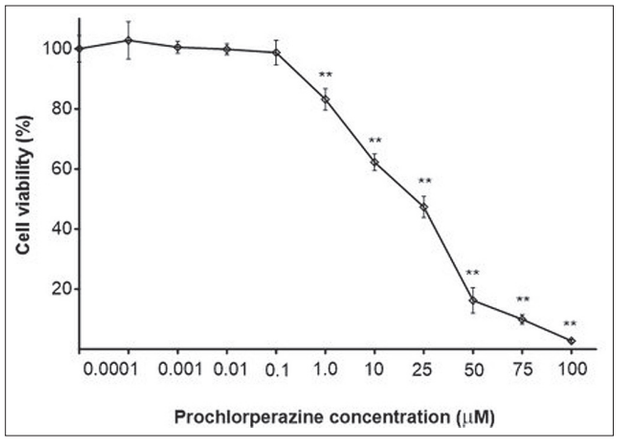

Fig. 3: The effect of prochlorperazine on viability of melanocytes. Cells were treated with various doses of prochlorperazine (0.0001—100 μM) and examined by WST-1 assay. Data are expressed as % of cell viability. Mean values ± SEM from three independent experiments performed in triplicate are presented. ** P < 0.01 vs. the control samples.

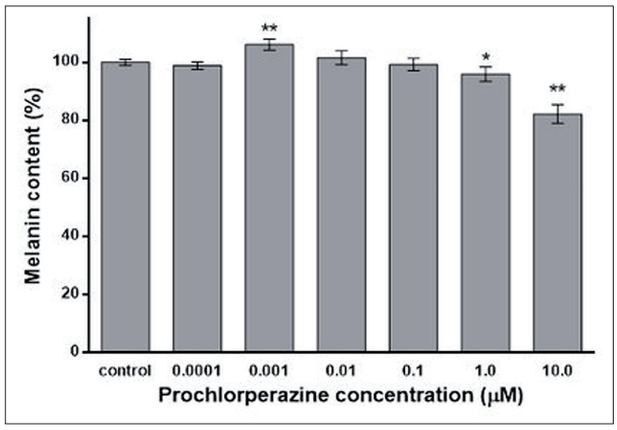

Fig. 4: The effect of prochlorperazine on melanin content in melanocytes. Cells were cultured with 0.0001, 0.001, 0.01, 0.1, 1.0 or 10.0 μM of prochlorperazine for 24 h and melanin content was measured as described in Materials and Methods. Results are expressed as percentages of the controls. Data are mean ± SEM of at least three independent experiments performed in triplicate. * P < 0.05 vs. the control samples; ** P < 0.01 vs. the control samples.

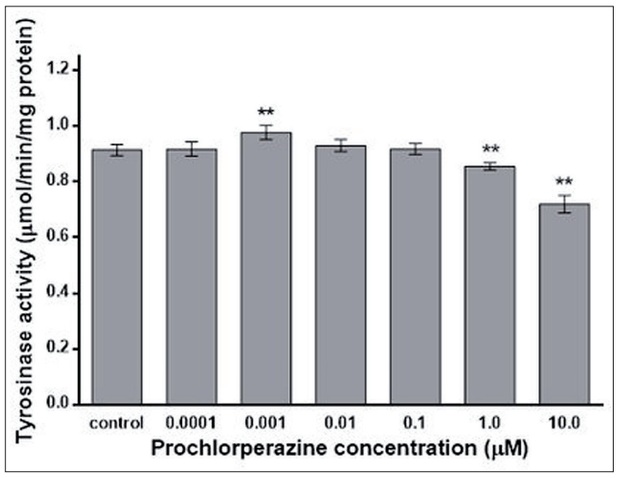

Fig. 5: The effect of prochlorperazine on tyrosinase activity in melanocytes. Cells were cultured with 0.0001, 0.001, 0.01, 0.1, 1.0 or 10.0 μM of prochlorperazine for 24 h and tyrosinase activity was measured as described in Materials and Methods. Data are mean ± SEM of at least three independent experiments performed in triplicate. ** P < 0.01 vs. the control samples.

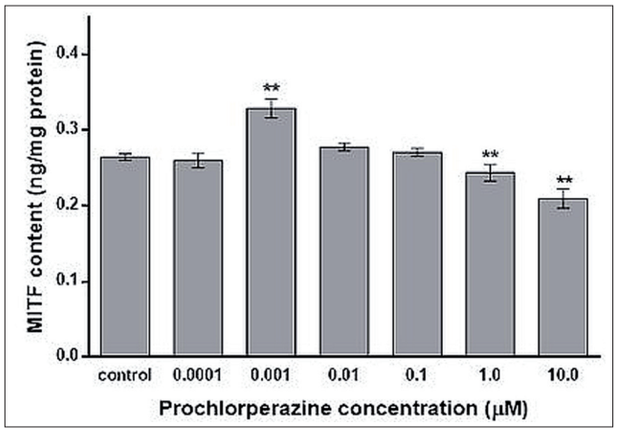

Fig. 6: Microphthalmia-associated transcription factor (MITF) content in HEMn-DP cells after 24-h incubation with 0.0001, 0.001, 0.01, 0.1, 1.0 or 10.0 μM of prochlorperazine. Data are mean ± SEM of at least three independent experiments performed in triplicate. ** P < 0.01 vs. the control samples.

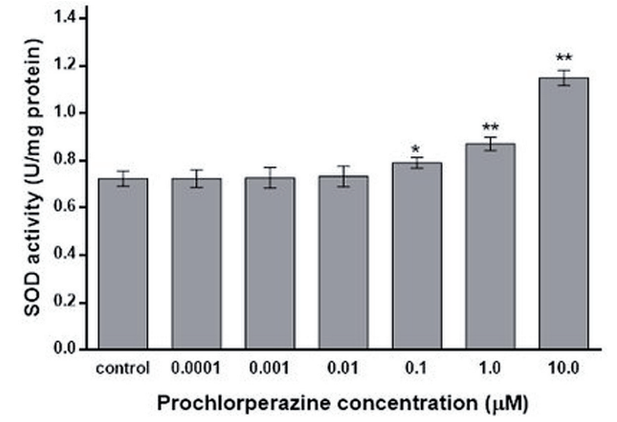

Fig. 7: Superoxide dismutase (SOD) activity in HEMn-DP cells after 24-h incubation with with 0.0001, 0.001, 0.01, 0.1, 1.0 or 10.0 μM of prochlorperazine. Data are mean ± SEM of at least three independent experiments performed in triplicate. * P < 0.05 vs. the control samples; ** P < 0.01 vs. the control samples.

decreased the enzyme activity by 6.4 % and 21.3 %, as compared with the controls. Prochlorperazine in concentrations 0.0001, 0.01 and 0.1 μM had no impact on cellular tyrosinase activity.

After performing a calibration curve, the MITF content was determined as 0.21 to 0.33 ng/mg protein for melanocytes treated with a drug and 0.26±0.01 ng/mg protein for a control sample (Fig. 6). Treatment of HEMn-DP cells with prochlorperazine in a concentration of 0.001 μM increased MITF content by 24.5 %, while the drug in concentrations of 1.0 and 10.0 μM decreased MITF content by 7.8 % and 18.1 %, respectively. Prochlorperazine in concentrations 0.0001, 0.01 and 0.1 μM had no impact on the cellular MITF content in comparison to the control cells.

2.4. Effect of prochlorperazine on antioxidant defense system in melanocytes

To study the effect of prochlorperazine on reactive oxygen species metabolism in melanocytes, the activity of antioxidant enzymes and the content of hydrogen peroxide were determined. Cells were exposed to prochlorperazine in concentrations of 0.0001, 0.001, 0.01, 0.1, 1.0 and 10.0 μM for 24 h.

Prochlorperazine rised SOD activity (Fig. 7). After performing a calibration curve, the SOD activity was determined as 0.73 to 1.15 U/mg protein for melanocytes treated with a drug and 0.72±0.03 U/mg protein for a control sample. The treatment of cells with 0.1,

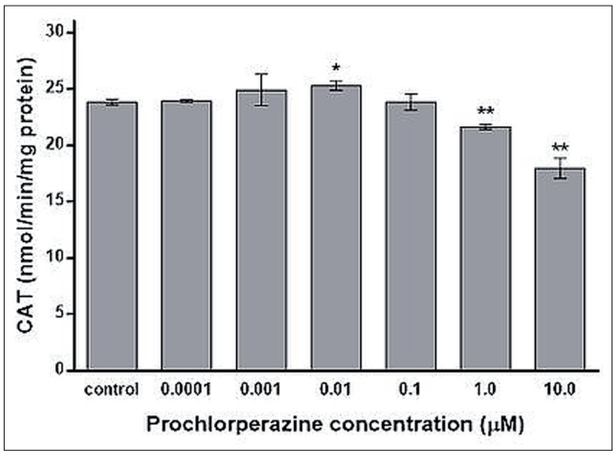

Fig. 8: Catalase (CAT) activity in HEMn-DP cells after 24-h incubation with 0.0001, 0.001, 0.01, 0.1, 1.0 or 10.0 μM of prochlorperazine. Data are mean ± SEM of at least three independent experiments performed in triplicate. * P < 0.05 vs. the control samples; ** P < 0.01 vs. the control samples.

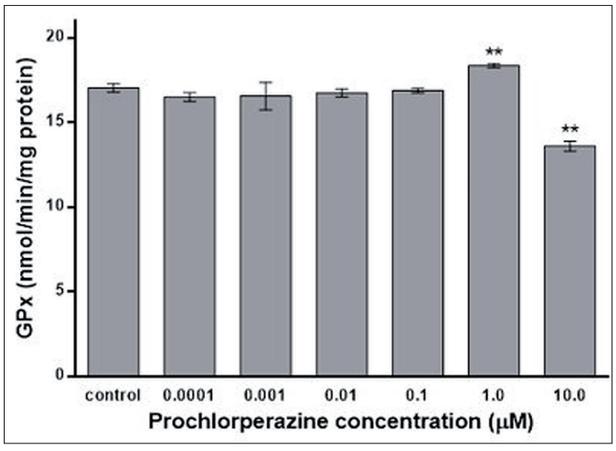

Fig. 9: Glutathione peroxidase (GPx) activity in HEMn-DP cells after 24-h incubation with 0.0001, 0.001, 0.01, 0.1, 1.0 or 10.0 μM of prochlorperazine. Data are mean ± SEM of at least three independent experiments performed in triplicate. ** P < 0.01 vs. the control samples.

1.0 and 10.0 μM of prochlorperazine increased the SOD activity by 9.1 %, 21.1 % and 59.2 %, respectively, as compared with the controls. The analyzed drug in concentration range from 0.0001 to 0.01 μM had no impact on SOD activity.

After 24-h incubation with prochlorperazine the intracellular CAT activity decreased (Fig. 8). After performing a calibration curve, the CAT activity was determined as 17.99 to 25.32 nmol/min/mg protein for melanocytes treated with a drug and 23.84±0.24 nmol/min/mg protein for a control sample. Treatment of HEMn-DP cells with prochlorperazine in concentration of 0.01 μM increased catalase activity by 6.2 % , while the concentrations of 1.0 and 10.0 μM decreased the enzyme activity by 9.1 % and 24.7 %, respectively, as compared with the controls. Prochlorperazine in concentrations 0.0001, 0.001 and 0.1 μM had no impact on CAT activity.

The analyzed drug modified GPx activity in melanocytes (Fig. 9). After performing a calibration curve, the GPx activity was determined as 13.61 to 18.49 nmol/min/mg protein for cells treated with prochlorperazine and 17.04±0.25 nmol/min/mg protein for a control sample. Treatment of melanocytes with 1.0 μM of a drug increased GPx activity by 8.5 %, while the concentration 10.0 μM decreased the enzyme activity by 20.2 %. Prochlorperazine in concentration range from 0.0001 to 0.1 μM had no impact on GPx activity in comparison with the controls.

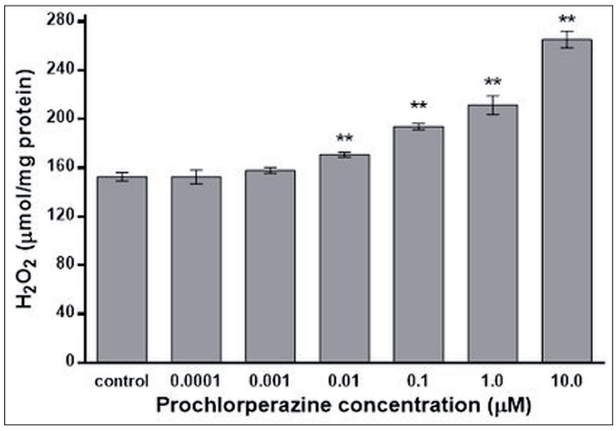

Fig. 10: Hydrogen peroxide (H_2O_2) content in HEMn-DP cells after 24-h incubation with 0.0001, 0.001, 0.01, 0.1, 1.0 or 10.0 μM of prochlorperazine. Data are mean ± SEM of at least three independent experiments performed in triplicate. ** P < 0.01 vs. the control samples.

After 24-h incubation of melanocytes with prochlorperazine the hydrogen peroxide (H_2O_2) content increased in a concentration-dependent manner (Fig. 10). The H_2O_2 content was determined as 153.68 to 265.21 μmol/mg protein for melanocytes treated with a drug and 152.70±3.45 μmol/mg protein for a control sample. The treatment of cells with 0.01, 0.1, 1.0 and 10.0 μM of prochlorperazine increased the H_2O_2 content by 12.7 %, 27.7 %, 39.5 % and 73.7 %, respectively, as compared with the controls. Prochlorperazine in concentrations of 0.0001 and 0.001 μM had no impact on H_2O_2 content in cells.

3. Discussion

In patients during and/or after prochlorperazine therapy FDR and SJS may occur, which are acute bullous disorders (Ferraro et al. 2014; Lamer et al. 2010). Prochlorperazine therapy is also associated with significant cutaneous side effects such as pigmentation changes, photosensitivity reactions and blue tongue (Ruigómez et al. 2000).

In the first step of our study synthetic DOPA-melanin was used as a model eumelanin. Synthetic melanins, prepared enzymatically or chemically from l-DOPA, contain more carboxyl groups than natural melanins (Ibrahim and Aubry 1995). However, the ion-exchange, redox and free radical properties established in synthetic melanin characterize also natural melanins. Both natural and synthetic melanin polymers were used in ligand-binding studies, and no significant differences in the affinity were observed (Buszman et al. 2008; Ibrahim and Aubry 1995).

Our previous studies showed the ability of ocular as well as synthetic melanin to form stable complexes with chlorpromazine, fluphenazine, trifluoperazine and thioridazine (Buszman and Różańska 2003; Buszman et al. 2008). The results presented in this study demonstrate that DOPA-melanin also forms complexes with prochlorperazine and the amount of a drug bound to melanin increases when initial drug concentration rises and incubation time is prolonged (Fig. 1). Simultaneously, a decrease in the complex formation efficiency (expressed as %) with increasing initial drug concentration was observed, what may be explained by saturation of the melanin binding sites. The Scatchard plot analysis of drug–melanin binding has shown that at least two classes of independent binding sites participate in the formation of the prochlorperazine–melanin complex – strong binding sites with the association constant $K_1 \sim 10^6\ M^{-1}$ and weak binding sites with $K_2 \sim 10^2\ M^{-1}$ (Fig. 2). The total number of binding sites (n_1+n_2) was 1.11 μmol prochlorperazine per 1 mg melanin. The affinity and capacity for binding of prochlorperazine to synthetic melanin are similar to those of chloroquine, which is well known for its high affinity for melanin (Larsson 1993).

In the second part of this study we used the culture of normal human epidermal melanocytes as an *in vitro* experimental model system. It was observed that prochlorperazine in concentration range from 0.0001

to 0.1 μM had no significant effect on melanocytes viability. However, at higher drug concentrations (from 1.0 to 100.0 μM) the loss in cell viability was observed in a concentration-dependent manner (Fig. 3). In opposite to chlorpromazine (2.53 μM) (Otręba et al. 2015a) and thioridazine (2.24 μM) (Otręba et al. 2015b), the value of EC_{50} for prochlorperazine was calculated to be 18.49 μM. This indicates that the analyzed drug is less cytotoxic. The order of phenothiazine derivatives cytotoxicity directed to pigmented cells (melanocytes) would be as follows: thioridazine > chlorpromazine >> prochlorperazine.

The analysis of the melanogenesis process in cells cultured in the presence of prochlorperazine showed statistically significant increases in tyrosinase activity and melanin as well as MITF content at drug concentration 0.001 μM (Fig. 4, 5 and 6). The opposite effect was noticed at higher drug concentrations (1.0 and 10.0 μM), where inhibition of melanin biosynthesis was observed. This indicates that the modulatory effect of prochlorperazine on melanogenesis is probably due to its direct effect on the analyzed transcription factor content and/or tyrosinase activity. It is also suggested that long-term prochlorperazine therapy, even with low drug doses, may lead to hyperpigmentation disorders in skin and/or eye.

Human skin exposure to agents that produce oxidative stress such as: environmental pollutants, UV radiation, cosmetic products and xenobiotics/drugs generate ROS in excessive quantities that quickly overwhelm tissue antioxidants and other oxidant-degrading pathways. Furthermore they may be involved in the pathogenesis of multiple skin disorders, allergic reactions and neoplasms. Imbalance between free radical production and antioxidant defense can ultimately lead to oxidative stress and tissue injury. The endogenous antioxidants in melanocytes comprises enzymatic as well as non-enzimatic substances such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), ascorbic acid, glutathione or α-tocopherol. Antioxidant molecules in the skin interact with ROS or their by-products to either eliminate them and maintain an optimal redox balance or to prevent oxidative damage and minimize their deleterious effects (Bickers and Athar 2006; Diehl 2014). It is important to recognize that H_2O_2 can cause both activation and inactivation of enzymes (e.g. catalase) as well as proteins and peptides, depending on their low (micromolar) or high (milimolar) concentration (Otręba et al. 2013; Schallreuter et al. 2008).

In the present study, prochlorperazine in concentrations of 0.1, 1.0 and 10.0 μM significantly increased SOD activity (Fig. 7), which correlates well with the elevated level of H_2O_2 (Fig. 10). Prochlorperazine in a concentration of 0.01 μM increased the activity of CAT (Fig. 8), whereas at 1.0 μM increased CAT and decreased GPx activity, and at 10.0 μM drug concentration decreased both enzymes activity (Fig. 8, 9). The different effects on CAT and GPx activity may be connected with a redundant cellular H_2O_2 level that cannot be eliminated. It indicates that in high prochlorperazine concentrations antioxidant defense systems do not work properly, what induces oxidative stress in melanocytes. One of the reasons for this phenomenon may be generation of phenothiazine-derived free radicals. The phenothiazine-induced formation of phenothiazine cation radicals was stated by other authors (Eghbal et al. 2004; Gutiérrez-Correa et al. 2003; MacAllister et al. 2013). It was shown by Gutiérrez-Correa et al. (2003) that peroxidase/H_2O_2-activated promazine, trimeprazine, thioridazine, promethiazine, prochlorperazine, chlorpromazine, and perphenazine produce their corresponding cation radicals. Eghbal et al. (2004) demonstrated that peroxidases can catalyze the oxidation of phenothiazines and formation of cytotoxic phenothiazine free radicals at physiological pH, leading to increased cytotoxicity. Mac Allister et al. (2013) showed an increase in hepatocyte toxicity after thioridazine co-incubation with the nontoxic inflammatory model of rat hepatocytes and suggested that this cytotoxicity could be attributed to thioridazine oxidation to cation radicals.

Our present and previous studies demonstrate that higher concentrations of the phenothiazine derivatives prochlorperazine, chlorpromazine (Otręba et al. 2015a), and thioridazine (Otręba et al. 2015b) cause significant alterations of antioxidant enzymes activity in normal melanocytes. It may explain a potential role of a drug in the depletion of cellular antioxidant status leading to other adverse effects such as dystonia and/or akathisia, associated with the high-dose and/or long-term therapy, which may be related to production of excessive free radicals and oxidative stress (Tsang 2008).

The present work provides the first *in vitro* study on prochlorperazine binding to melanin as well as on mechanisms involved in prochlorperazine-induced disorders in HEMn-DP cells. The observed changes in cell viability, melanization and antioxidant defense system in normal human melanocytes after prochlorperazine treatment may explain a potential role of melanin, oxidative stress and melanocytes in mechanisms of undesirable side effects after accumulation of this drug in pigmented tissues. Our results also confirm that HEMn-DP melanocytes are a suitable cell model to study mechanisms regulating melanogenesis and antioxidant defense system in human pigmentation disorders.

4. Experimental

4.1. Materials

Prochlorperazine dimaleate, phosphated-buffered saline (PBS), 3,4-dihydroxy-l-phenylalanine (l-DOPA) and amphotericin B were purchased from Sigma-Aldrich Inc.(USA). Neomycin sulphate was obtained from Amara (Poland). Penicillin was acquired from Polfa Tarchomin (Poland). Growth medium M-254 and human melanocyte growth supplement-2 (HMGS-2) were obtained from Cascade Biologics (UK). Trypsin/EDTA was obtained from Cytogen (Poland). Cell Proliferation Reagent WST-1 was purchased from Roche GmbH (Germany). The remaining chemicals were produced by POCH S.A.(Poland).

4.2. Preparation of DOPA-melanin

Model DOPA-melanin was obtained by oxidative polymerization of l-DOPA solution (1mg/ml) in 0.067 M phosphate buffer (pH 8.0) for 48 h, according to the method described earlier (Buszman et al. 2008).

4.3. Prochlorperazine-melanin complex formation

Drug-melanin complexes were obtained by suspending 5 mg of DOPA-melanin in 5 ml of prochlorperazine solution. The initial concentration of drug ranged from 0.1 mM to 5.0 mM. Mixtures of melanin and drug solutions were incubated at room temperature and then filtered. Control samples, containing melanin suspended in distilled water, were treated in the same manner.

4.4. Determination of the amount of drug bound to melanin

The UV spectrophotometric method was used for quantitative determination of the analyzed drug. Analytical wavelength (λ_{max}) for prochlorperazine was 255 nm. The calculated value of molar absorption coefficient $\varepsilon_{\lambda max}$ = 29916 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ was used to estimate the amount of drug bound to the polymer. All spectrophotometric measurements were performed by a JASCO model V-630 UV-VIS spectrophotometer.

4.5. Kinetics of drug-melanin complex formation

Kinetics of the formation of melanin complexes with prochlorperazine were evaluated on the basis of relationship between the amount of a drug bound to the polymer (μmol/mg) and the time of complex formation. In this study, the following initial drug concentrations were used: 0.5 mM, 1.0 mM and 5.0 mM. Complex formation lasted for 1, 3, 6, 12, 24 and 48 hours.

4.6. Binding parameters of drug-melanin complexes

The number of strong (n_1) and weak (n_2) binding sites and the association constants (K) of the synthetic melanin complexes with prochlorperazine were calculated using the Scatchard plot according to the method described earlier (Buszman et al. 2007). Experimental binding isotherm was used to construct this plot. It shows the relationship between the amount of drug bound to melanin and its initial concentration after reaching an equilibrium state, i.e. after 24 h. A drug initial concentration ranged from 0.1 to 5.0 mM.

4.7. Cell culture

The normal human epidermal melanocytes (HEMn-DP, Cascade Biologics) were cultured in M-254 basal medium supplemented with HMGS-2, penicillin (100 U/ml), neomycin (10 μg/ml) and amphotericin B (0.25 μg/ml) at 37 °C in 5% CO_2.

Cell viability was evaluated after 24-h incubation of melanocytes with prochlorperazine solutions in a concentration range from 0.0001 to 100 μM, while cellular melanin biosynthesis and antioxidant status assays were performed in a concentration range from 0.0001 to 10.0 μM.

4.8. Cell viability assay

The viability of melanocytes was evaluated by the WST-1 (4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulphonate) colorimetric assay according to the method described earlier (Beberok et al. 2012; Otręba et al. 2015a,b) and was expressed as the percentage of the controls.

4.9. Cellular melanin biosynthesis assay

Measurements of melanin content and tyrosinase activity were perfomrmed spectrophotometrically, according to the method described earlier (Otręba et al. 2015a,b), and expressed as the percentage of the controls and in µmol/min/mg protein, respectively. MITF content was determined using ELISA an assay kit (USCN Life Science Inc, USA) (Otręba et al. 2015a,b) and expressed in ng/mg protein.

4.10. Cellular antioxidant status assay

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities and hydrogen peroxide (H_2O_2) content were measured spectrophotometrically using assay kits (Cayman, MI, USA and Cell Biolabs, Inc., USA), according to the methods described earlier (Otręba et al. 2015a,b).

4.11. Statistical analysis

In all experiments, mean values of at least three independent experiments (n=3) performed in triplicate±standard error of the mean (SEM) were calculated. Statistical analysis was performed with one-way ANOVA followed by Tukey post-hoc test using GraphPad Prism 6.01 Software. The significance level was estabilished at value of $p<0.05$ (*) or $p<0.01$ (**), by comparing the data with those for control (cells without prochlorperazine).

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