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SHORT COMMUNICATION

In vitro melanogenesis inhibition by fluphenazine and prochlorperazine in normal human melanocytes lightly pigmented

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Abstract

Purpose Fluphenazine and prochlorperazine as phenothiazine-class antipsychotic drugs are widely used to treat schizophrenia, however their use is associated with significant side effects such as extrapyramidal symptoms, as well as ocular and skin disorders. Our goal was to determine the effect of fluphenazine and prochlorperazine on cell viability and melanogenesis in lightly pigmented normal human melanocytes.

Methods The viability of melanocytes was evaluated by the WST-1 colorimetric assay, while melanin content and tyrosinase activity were tested spectrophotometrically.

Results It has been shown that both phenothiazines induce the concentration-dependent loss in cell viability. The EC_{50} values were calculated to be 6.13 and 0.63 μM for fluphenazine and prochlorperazine, respectively. Fluphenazine in the concentration of 5.0 μM and prochlorperazine in concentrations of 0.5 and 0.75 μM decreased melanin content and tyrosinase activity. The observed inhibition of melanogenesis may be explained by the decrease of enzyme activity.

Conclusions The demonstrated changes in melanization process in lightly pigmented cells exposed to fluphenazine and prochlorperazine in vitro suggest a significant role of melanin and melanocytes in the mechanisms of undesirable side effects of these drugs in vivo.

Keywords Fluphenazine · Prochlorperazine · Melanocytes · Melanogenesis · Cell viability

Introduction

Fluphenazine and prochlorperazine belong to the phenothiazine antipsychotic drugs with piperazine-derived substituents used particularly the treatment of schizophrenia and bipolar [1]. Furthermore, these drugs possess a lot of new and very important biological properties like antibacterial, antiviral, anticancer, antiprotozoic and multidrug resistance reversal activity [2]. The most serious side effects of phenothiazines treatment are extrapyramidal symptoms (e.g. parkinsonism, dystonias, akathisia [3]), as well as ocular effects (e.g. blurred vision [4] and cataract [5]), and skin disorders (e.g. rash [4], fixed drug reactions [6]), cutaneous photosensitivity and abnormal skin pigmentation [4]) observed after fluphenazine and/or prochlorperazine therapy. Interestingly, hypopigmentation defects such as vitiligoid depigmentation and chemical leukoderma rarely occur during fluphenazine therapy [7, 8].

Brownish-black eumelanins and/or reddish-yellow pheomelanins are synthesized in melanosomes of pigment cells, such as melanocytes, during a multistep complex process called melanogenesis. Melanins are responsible for photoprotection, pigmentation of the skin, hair and eyes, free radical scavenging effects, chelating metal ions and reversibly binding a range of drugs, including psychotropic agents [9, 10]. It is worthwhile to note that fluphenazine and prochlorperazine form complexes with melanin. In the formation of these drug–melanin complexes at least two classes of

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independent binding sites participate – strong binding sites with the association constant (K_1) as well as weak binding sites with the association constant (K_2) [11, 12]. The binding of drugs to melanin may have both positive (protection against undesirable drug side effects) and negative (accumulation of the drug in melanin-containing tissues leading to pigmented cells degradation) effects [10].

Previously, we have shown that chlorpromazine, fluphenazine, perphenazine, prochlorperazine and thioridazine modulate melanogenesis and decrease cell viability in normal human epidermal melanocytes darkly pigmented (HEMn-DP) [12–15]. Among all the analyzed drugs, the highest and lowest cell viability was observed in fluphenazine and prochlorperazine, respectively. Thus, it was interesting to investigate the effect of fluphenazine and prochlorperazine on cell viability and melanogenesis in normal human epidermal melanocytes lightly pigmented (HEMn-LP).

Materials and methods

Chemicals

Fluphenazine dihydrochloride, 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).

Cell treatment

Lightly pigmented normal human epidermal melanocytes (HEMn-LP, Cascade Biologics) were cultured according to the method described earlier [13–15]. Cells in the passages 5–8 were used in all performed experiments.

Cell viability assay

The viability of melanocytes was evaluated by the WST-1 colorimetric assay according to the method previously described [13–15]. Shortly, after a 48 h pre-incubation growth medium was removed and HEMn-LP cells were treated with 100 ul of fluphenazine and prochlorperazine solutions for next 24 h. Three hours before the end of incubation 10 μl of WST-1 were added. The controls were normalized to 100 % for each assay and treatments were expressed as the percentage of the controls.

Measurement of melanin content and tyrosinase activity

Measurements of melanin content and tyrosinase activity were performed spectrophotometrically, according to the methods described earlier [13–15]. Before lysis, as in our previous experiments, cells were washed three times with PBS to eliminate dead cells and to ensure that the observed decrease of melanin content and tyrosinase activity are not caused by cell damage. Than cells were detached and counted. Finally, the cell lysate contained appropriate cells amount. Melanin content results were normalized per number of cells [pg/cell], while tyrosinase activity results were normalized per total protein content [μmol/min/mg protein]. Finally, both measurements were expressed as the percentage of the controls.

Statistical analysis

In all experiments, mean values of at least three separate experiments ($n = 3$) performed in triplicate ± standard deviation (SD) 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 the untreated controls.

Results and discussion

In this study, we used normal human epidermal melanocytes lightly pigmented as an in vitro experimental model. We have demonstrated that the control samples of HEMn-LP cells contain from 33.7 ± 1.2 to 34.8 ± 2.0 pg melanin per cell, whereas in our earlier studies we have shown that darkly pigmented melanocytes contain from 62.4 ± 0.6 to 62.5 ± 1.8 pg melanin per cell [12, 15]. Our results are in agreement with Wakamatsu et al. (2006) and Dzierżęga-Lęcznar et al. (2017) who found that lightly pigmented melanocytes contain about two times less melanin than darkly pigmented cells [16, 17]. The possible explanation for this observation might be differences in the activity of tyrosinase – the key enzyme in melanogenesis regulation. In lightly pigmented melanocytes the enzyme activity was from 0.71 ± 0.01 to 0.79 ± 0.03 μmol/min per mg protein, while in darkly pigmented cells from 0.91 ± 0.02 to 1.00 ± 0.03 μmol/min per mg protein [12, 15].

Previously we have shown that fluphenazine [11] and prochlorperazine [12] form stable complexes with model synthetic and natural melanins. We have also demonstrated that both drugs modulate the melanogenesis process in HEMn-DP cells [12, 15]. Thus, it was still very interesting to study the effect of the analyzed drugs on cell viability, melanin content and tyrosinase activity in HEMn-LP cells.

It was observed for the first time that 24-h incubation of HEMn-LP cells with fluphenazine at concentrations 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 μM and prochlorperazine at concentrations 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, 10.0 μM caused the loss in cell viability in a concentration-dependent manner from 15.4 to 99.6% and from 17.3 to 99.5%, respectively (Fig. 1a, b). Based on the calculated EC_{50} values (the concentration of a drug that produces a loss in cell viability by 50%) it may be noticed that prochlorperazine ($EC_{50} = 0.63 \pm 0.018$ μM) is more cytotoxic for lightly pigmented melanocytes than fluphenazine ($EC_{50} = 6.13 \pm 0.57$ μM). In opposite to these results, we have previously found that the value of EC_{50} established for fluphenazine and prochlorperazine, in relation to darkly pigmented melanocytes was 1.24 and 18.49 μM, respectively [12, 15]. In comparison to darkly pigmented cells, HEMn-LP melanocytes are five times more resistant to fluphenazine treatment as well as thirty times more sensitive to prochlorperazine treatment. The obtained results may be explained by differences in the values of association constants and the total number of binding sites for both analyzed drug-melanin complexes. Total number of binding sites for fluphenazine and prochlorperazine was calculated to be 0.2546 μmol drug per 1 mg of melanin [11] and 1.11 μmol drug per 1 mg of melanin [12], respectively. The association constant for prochlorperazine were $K_1 = 1.0 \times 10^6\ M^{-1}$, $K_2 = 7.3 \times 10^2\ M^{-1}$ [12], while for fluphenazine were $K_1 = 2.6 \times 10^4\ M^{-1}$, $K_2 = 9.3 \times 10^2\ M^{-1}$ [11]. The affinity and capacity for binding of prochlorperazine to melanin is higher than fluphenazine [11, 12]. Thus, the concentration of unbound prochlorperazine in darkly pigmented cells may be much lower than in lightly pigmented melanocytes, what also explain the stronger cytotoxicity of prochlorperazine towards HEMn-LP cells. It indicates that the cytotoxicity of prochlorperazine depends on melanin content in melanocytes. In contrast, lower impact of fluphenazine on the viability of lightly pigmented melanocytes may be explained by the weaker affinity to melanin, which suggests that cytotoxic activity in this case is not related to melanin content, but may be caused by another mechanism of signalling pathways.

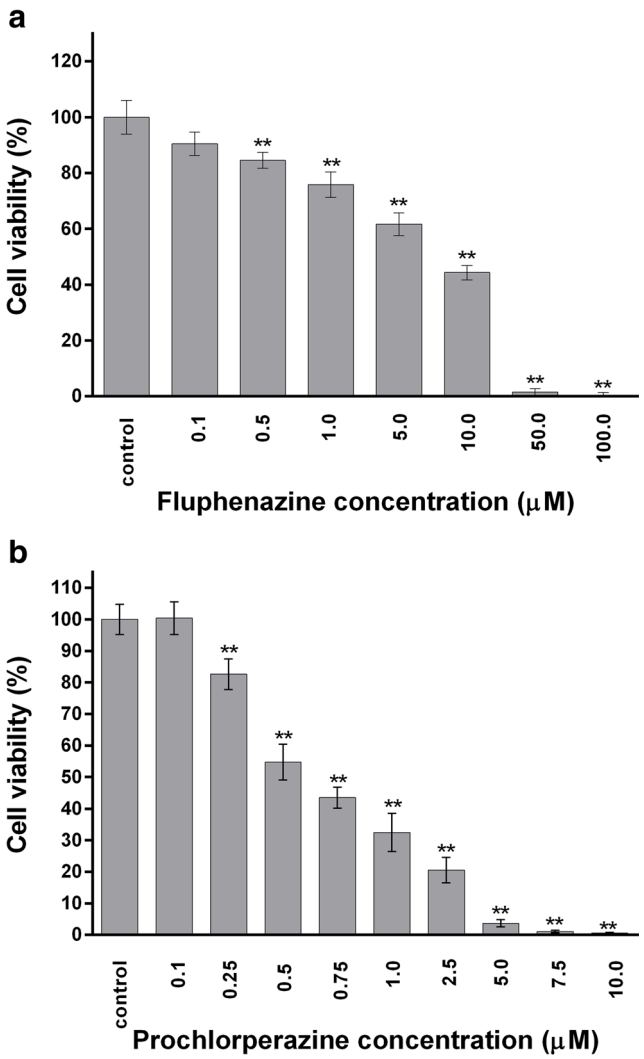

Fig. 1 The effect of fluphenazine (**a**) and prochlorperazine (**b**) on the viability of lightly pigmented melanocytes. Cells were treated with drugs in concentrations 0.1—100.0 μM and 0.1—10.0 μM, respectively, and examined by the WST-1 assay. Data are expressed as % of the controls. The results are expressed as percentages of control and data are mean ± SD from three independent experiments ($n = 3$) performed in triplicate are presented. ** $p < 0.01$ vs. the untreated control

In order to analyze the melanogenesis process in cells cultured in the presence of fluphenazine and prochlorperazine, we have measured melanin content and activity of tyrosinase. The enzyme catalyzes rate-limiting steps of the process – hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and oxidation of DOPA to dopaquinone [9, 10]. To avoid the death cell impact on tyrosinase activity, cells were washed by PBS before lysis. It has been shown that fluphenazine in the concentration of 5.0 μM decreases tyrosinase activity and melanin content by 9.8 and 10.9%, respectively (Figs. 3a and 2a). Prochlorperazine in concentrations of 0.5 and 0.75 μM more significantly decreases the enzyme activity, as well as melanin content by 15.7 and 23.5% as well as by 14.5 and 25.2%, respectively, as compared with the controls (Figs. 2b and 3b). The observed decrease was more significant after prochlorperazine than fluphenazine treatment. The obtained results may explain a potential role of both analyzed drugs in a decrease of tyrosinase activity leading to pigmentation disorders [4] observed during high dose and/or long-term therapy. Our earlier studies on HEMn-DP cells showed that tyrosinase activity and melanin content decreased by 6.7 and 7.7%, respectively for cells treated with fluphenazine only in the highest analyzed concentration (1.0 μM) [15]. In case of prochlorperazine in the concentrations of 1.0 and 10.0 μM the decrease of melanin content by 4.1 and 16.8%, as well as enzyme activity by 6.4 and 21.3% was observed. The statistically significant increase of melanin content (by 6.1%) and tyrosinase activity (by 6.9%) was caused by the treatment of

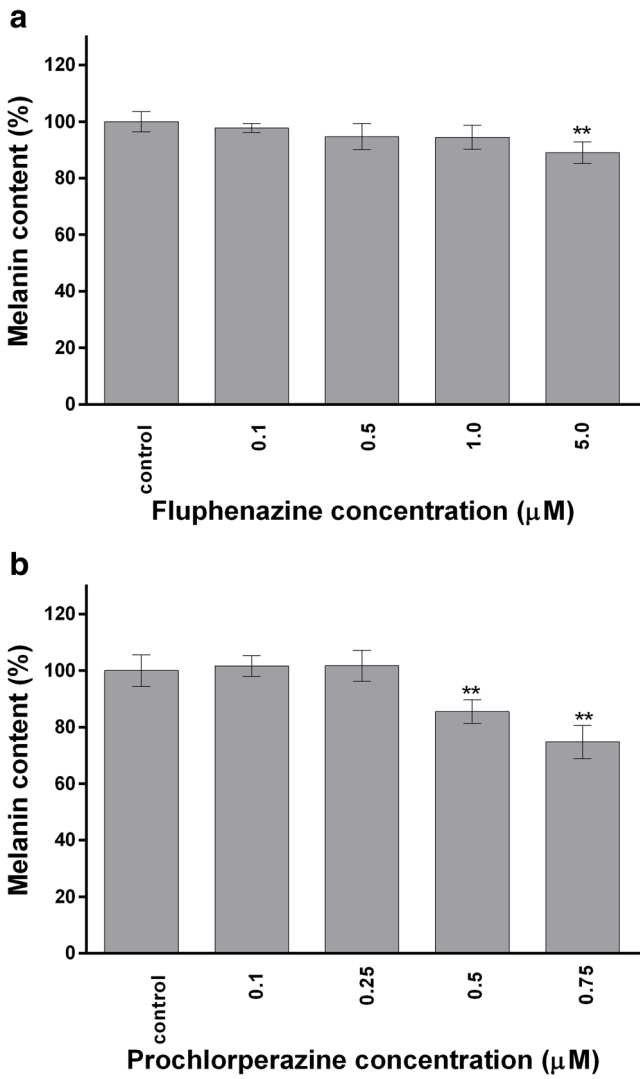

Fig. 2 The effect of fluphenazine (**a**) and prochlorperazine (**b**) on melanin content in lightly pigmented melanocytes. Cells were cultured in concentrations 0.1—5.0 μM and 0.1—0.75 μM, respectively for 24 h. The results are expressed as percentages of control and data are mean ± SD of at least three independent experiments (n = 3) performed in triplicate. ** $p < 0.01$ vs. the untreated control

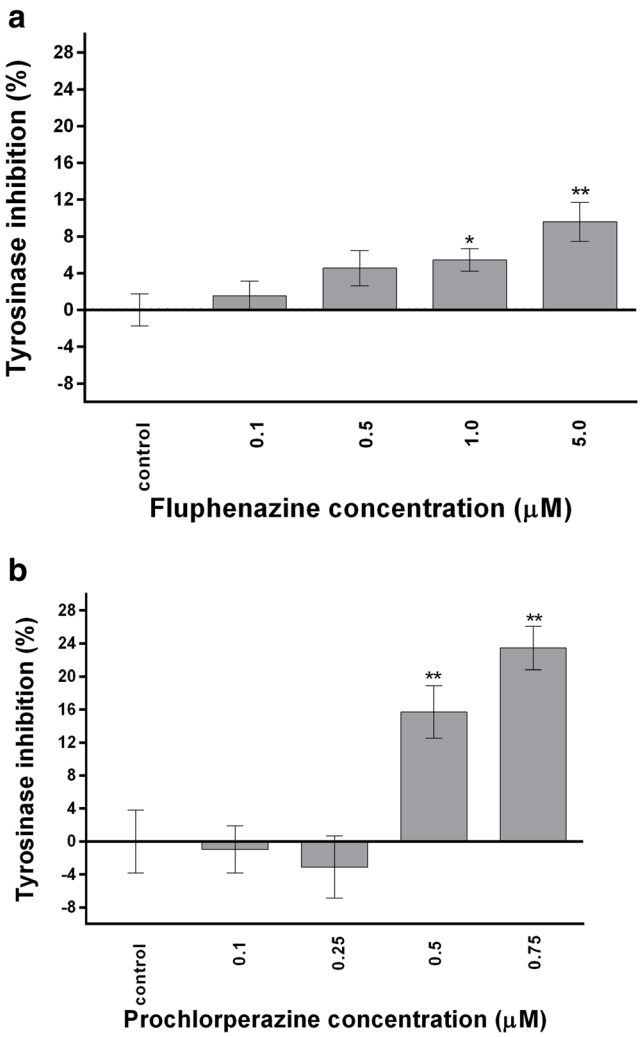

Fig. 3 The inhibition effect of fluphenazine (**a**) and prochlorperazine (**b**) on cellular tyrosinase activity in lightly pigmented melanocytes. Cells were cultured in concentrations 0.1—5.0 μM and 0.1—0.75 μM, respectively for 24 h. The results are expressed as percentages of control and data are mean ± SD of at least three independent experiments (n = 3) performed in triplicate. * $p < 0.05$ vs. the control samples, ** $p < 0.01$ vs. the untreated control

HEMn-DP cells with 0.001 μM of prochlorperazine. It suggests that the observed previously modulatory effect of the analyzed drug on melanogenesis in darkly pigmented melanocytes takes place probably due to its direct effect on tyrosinase activity [12]. The comparison of our present and previous results indicates and confirm that fluphenazine and prochlorperazine suppress melanogenesis in normal human melanocytes lightly and darkly pigmented, probably due to their inhibitory effect on tyrosinase activity.

The observed changes in HEMn-LP cells viability, melanin content and tyrosinase activity were observed in fluphenazine concentration from 0.5 to 100 μM, which is higher than toxic concentrations in human plasma, which range from 0.05 to 0.1 μg/ml (i.e. 0.098–0.196 μM) [18, 19]. Such a high concentration may be possible to observe, since phenothiazine derivatives are used in a high dose, associated with a higher rate of adverse effects [20] and/or long-term therapy (what has been the norm for treatment of patients with schizophrenia or other psychotic disorders [21]). Moreover, fluphenazine has high affinity and capacity to bind to melanin. In the case of prochlorperazine most of the analyzed concentration range (0.25–10 μM) is related to the toxic concentrations in human plasma, which range from 0.2 to 1 μg/ml (i.e. 0.33–1.65 μM) [18, 19].

Conclusion

Since phenothiazine derivatives are still a subject of new research, it is essential to explain molecular mechanisms

underlying these drugs toxic effects. We have demonstrated for the first time the decrease of cellular viability of HEMn-LP cell line after fluphenazine and prochlorperazine treatment. Values presented in the EC_{50} study show a ten times higher reduction of viability for prochlorperazine than fluphenazine, which may be explained by differences in the ability of these drugs to form complexes with melanin. The decrease of melanin content in normal human melanocytes can be explained by the inhibitory effect of analyzed drugs on tyrosinase activity. The observed changes in melanization process in lightly pigmented cells exposed to fluphenazine and prochlorperazine in vitro suggest a significant role of melanin and melanocytes in the mechanisms of undesirable side effects of these drugs in vivo, such as abnormal skin pigmentation, chemical leukoderma and vitiligoid depigmentation.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no conflict of interest. The manuscript entitled: "In vitro melanogenesis inhibition by fluphenazine and prochlorperazine in normal human melanocytes lightly pigmented", which all the listed authors have read and approved of, has not been submitted elsewhere for publication, in whole or in part, and is permitted to be published in this journal.

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