Fluphenazine and perphenazine belong to the high-potency antipsychotic drugs with a piperazinyl structure, which are used for treatment of psychoses and positive symptoms of schizophrenia, such as hallucinations and delusions (1, 2). Perphenazine is also indicated in the manic phases of bipolar disorders (2). The phenothiazine treatment is associated with high extrapyramidal symptoms (EPS), such as dystonia and/or akathisia, as well as with other adverse side effects including movement disorders, akinesia, dyskinesia, dizziness, drowsiness, sedation, severe toxicity, tachycardia, hypotension, ocular effects (e.g., blurred vision, maculopathy and cataract) and skin disorders (e.g., rash, exfoliative dermatitis, drug hypersensitivity syndrome, cutaneous photosensitivity and abnormal skin pigmentation) (1-6).

Melanins are synthesized during multistep complex process called melanogenesis. The process is localized in specialized cytoplasmic organelles, melanosomes, in the cytoplasm of melanocytes and retinal pigment epithelial (RPE) cells. Disturbation of melanogenesis may determine hypopigmentation defects, which may occur with or without an altered number of melanocytes (7-10). Interestingly, fluphenazine therapy may rarely cause vitiligo depigmentation and chemical leukoderma, which are an acquired, hypopigmented dermatosis that result from repeated, cutaneous application of an agent that destroys epidermal melanocytes (11, 12).

Specialized pigment dendritic cells, melanocytes, protect organism from ultraviolet light, determine pigmentation of skin, hair and eyes.
reversibly bind metal ions, organic amines, cyclic compounds and drugs, e.g., psychotropic agents or fluoroquinolones, as well as protect from reactive oxygen species (ROS) and oxidative stress (9, 13-15). The binding of drugs to melanin probably protects organism against undesirable drug side effects, but also may lead to accumulation of the drug in melanin-containing tissues (especially in the eye, ear, skin and brain) causing degeneration of pigmented cells (9, 16).

Cutaneous melanin is synthesized in melanocytes, in specialized organelles called melanosomes during complex, multistage biochemical pathway leading to brownish-black eumelansins and/or reddish-yellow pheomelansins. The melanogenesis process is regulated by very important proteins like tyrosinase, tyrosinase related proteins TRP1 and TRP2 as well as transcription factor MITF, which is also responsible for proliferation, dendrite formation and induction of antipapoptotic genes expression. Synthesized melanin is moved from melanocytes to neighboring epidermal keratinocytes where is responsible for skin pigmentation, UV light absorption, scavenging free radicals, chelating metal ions and binding a variety of drugs and other xenobiotics (7, 9, 17).

To prevent the damage of melanocytes by ROS induced by endogenous and exogenous sources (e.g., melanogenesis, mitochondria, lipoxigenases, peroxisomes, ultraviolet radiation, toxins, chemicals, ionizing radiation and inflammation), cellular antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), are activated to restore normal redox state in cells (18). Melanin biosynthesis involves oxidation reactions as well as superoxide anion (O$_2^{-}$) and hydrogen peroxide (H$_2$O$_2$) generation, which subject melanocytes to oxidative stress (18). On the other hand, melanin acts as a free radicals scavenger and possesses superoxide dismutase activity, what may reduce ROS content and thus protect pigmented tissues against cellular damage induced by oxidative stress (7, 19).

Previously, we have documented that aminoglycoside antibiotics (20, 21) and fluoroquinolones (22, 23), induce inhibition of cell viability and melanogenesis in normal human epidermal melanocytes light pigmented (HEMn-LP). The aim of this study was to estimate the effect of fluphenazine and perphenazine on cell viability, melanogenesis and antioxidant defense system in normal human melanocytes dark pigmented (HEMn-DP).

**EXPERIMENTAL**

**Chemicals**

Fluphenazine dihydrochloride, perphenazine, phosphated-buffered saline (PBS), 3,4-dihydroxy-L-phenylalanine (L-DOPA) and amphotericin B were purchased from Sigma-Aldrich Inc. (USA). Neomycin sulfate 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 Biologies (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**

The normal human epidermal melanocytes (HEMn-DP, Cascade Biologies) 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$_2$. Cells in the passages 6-9 were used in all performed experiments.

**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 disulfonate) colorimetric assay according to the method described earlier (22, 26).

**Measurement of melanin content**

Melanin content was measured in normal human melanocytes cultured in T-25 flasks at a density of 100,000 cells/flask. Cells were incubated for 48 h after seeding and then began 24-h fluphenazine or perphenazine treatment in a concentration range from 0.0001 to 1.0 µM and from 0.0001 to 3.0 µM, respectively. After the three times washings with PBS, cells were detached with trypsin-EDTA. Cell pellets were placed into Eppendorf tubes, dissolved in 100 µL of 1 M NaOH at 80°C for 1 h, and then centrifuged for 20 min at 16,000 × g. The supernatants were placed into a 96-well microplate, and absorbance was measured at 405 nm – a wavelength of...
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at which melanin absorbs light (27). A standard synthetic melanin curve (0 to 400 µg/mL) was performed in triplicate for each experiment. Melanin content in fluphenazine or perphenazine treated cells was expressed in pg/cell and finally as the percentage of the controls (untreated melanocytes).

**Tyrosinase activity assay**

Tyrosinase activity in melanocytes was determined by measuring the rate of oxidation of L-DOPA to DOPAchrome, according to the method described previously (22). The cells were cultured at a density of 100,000 cells/flask for 48 h and then, incubated with fluphenazine (concentration range from 0.0001 to 1.0 µM) or perphenazine (concentration range from 0.0001 to 3.0 µM) for 24 h. Before lysis and clarification (centrifugation at 10,000 × g for 5 min) cells were washed three times with PBS. Tyrosinase activity assay was performed in 96-well plate. A tyrosinase substrate L-DOPA (2 mg/mL) was prepared in the same lysis phosphate buffer. Hundred µL of each lysate and 40 µL of L-DOPA solution were pipetted into each well and incubated at 37°C for initiation of the enzymatic assay. Absorbance was measured every 10 min for at least 1.5 h at 475 nm using a microplate reader. Tyrosinase activity was expressed in µmol/min/mg protein.

**Microphthalmia-associated transcription factor (MITF) assay**

Microphthalmia-associated transcription factor (MITF) content was measured using ELISA an assay kit (USCN Life Science Inc, USA) according to the manufacturer’s instruction. This kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of MITF providing a 96-well microplate pre-coated with a biotin-conjugated antibody specific for MITF. The color change of the enzyme (horseradish peroxidase) – substrate (TMB) reaction was measured spectrophotometrically at 450 nm using a microplate reader. MITF content in the samples was expressed in ng/mg protein.

**Cellular antioxidant status assay**

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities and hydrogen peroxide (H₂O₂) content were measured using an assay kits (Cayman, MI, USA and Cell Biolabs, Inc., USA) according to the manufacturer’s instructions, after 24-h incubation of human melanocytes HEMn-DP with fluphenazine (in concentration range from 0.0001 to 1.0 µM) or perphenazine (in concentration range from 0.0001 to 3.0 µM). The SOD, CAT, GPx activity and H₂O₂ content were measured spectrophotometrically at 441, 540, 340 and 595 nm, respectively, using a microplate reader.

**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 established at value of p < 0.05 (*) or p < 0.01 (**), by comparing the data with those for control (cells without drug).

![Figure 1. The effect of fluphenazine and perphenazine on viability of melanocytes. Cells were treated with various drug concentrations (0.0001-10.0 µM) and examined by the WST-1 assay. Data are expressed as percent of the controls. Mean values ± SD from three independent experiments (n = 3) performed in triplicate are presented. * p < 0.05 vs. the control samples, ** p < 0.01 vs. the control samples](image-url)
RESULTS

The effect of fluphenazine and perphenazine on cell viability

The cell viability was determined by the WST-1 test after 24-h incubation with fluphenazine or perphenazine in concentrations range from 0.0001 µM to 10.0 µM. It has been demonstrated that the analyzed drugs induce concentration-dependent loss in cell viability (Fig. 1). Melanocytes treated with fluphenazine in concentrations from 0.001 µM to 10 µM lost 11.3% to 98% in cell viability. After incubation of cells with perphenazine in concentrations from 0.1 µM to 10 µM, the loss of cell viability was 19.1% to 98.5%. The value of EC50 (the concentration of a drug that produces loss in cell viability by 50%) was calculated to be 1.24 and 2.76 µM, respectively for fluphenazine and perphenazine. At lower concentrations of fluphenazine (0.0001 µM) and perphenazine (0.0001, 0.001 and 0.01 µM) the loss in melanocytes viability was not observed.

The effect of fluphenazine and perphenazine on melanization process in melanocytes

The effectiveness of melanization process was estimated by measuring the melanin content, cellular tyrosinase activity and microphthalmia-associated transcription factor (MITF) content in melanocytes treated with fluphenazine (concentration range from 0.0001 to 1.0 µM) or perphenazine (concentration range from 0.0001 to 3.0 µM) for 24 h. The melanin content per cell was determined as
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57.7 to 65.4 pg/cell and 53.9 to 63.3 pg/cell for melanocytes treated with fluphenazine and perphenazine, respectively, while the values determined for the control samples of both drugs were 62.5 ± 1.82 pg/cell and 61.3 ± 1.34 pg/cell, respectively. The obtained results, recalculated for culture (1 × 10^5 cells), were finally expressed as a percentage of the controls (Fig. 2A). Treatment of HEMn-DP cells with 1.0 µM of fluphenazine decreased melanin content by 7.7%, and perphenazine in concentrations of 1.0 and 3.0 µM also decreased the pigment content by 10.6 and 12.1%, respectively. Both analyzed drugs in concentrations from 0.0001 to 0.1 µM had no impact on melanin content in melanocytes.

Tyrosinase activity in melanocytes treated with fluphenazine or perphenazine decreased in a manner correlating well with the effect on melanin production (Fig. 2B). The enzyme activity was determined as 0.93 to 1.06 µmol/min/mg protein and 0.95 to 1.11 µmol/min/mg protein for melanocytes treated with fluphenazine and perphenazine, respectively,
while the values determined for the control samples of both drugs were 1.00 ± 0.03 nmol/min/mg protein and 1.07 ± 0.03 nmol/min/mg protein, respectively. The tyrosinase activity decreased by 6.7% for cells treated with fluphenazine in concentration of 1.0 µM, while perphenazine in concentrations of 1.0 and 3.0 µM decreased the enzyme activity by 10.1% and 11.9%, respectively, as compared with the controls. Fluphenazine and perphenazine in concentrations from 0.0001 to 0.1 µM had no impact on cellular tyrosinase activity.

The MITF content was determined as 0.14 to 0.33 ng/mg protein and 0.20 to 0.29 ng/mg protein for melanocytes treated with fluphenazine and perphenazine, respectively, while the values determined for the control samples of both drugs were 0.29 ± 0.02 ng/mg protein and 0.26 ± 0.02 ng/mg protein, respectively (Fig. 2C). Treatment of HEMn-DP cells with fluphenazine in concentration of 0.0001 µM increased MITF content by 15.6%, while the drug in concentrations of 0.01, 0.1 and 1.0 µM decreased this transcription factor content by 16.9, 29.9 and 52.9%. In contrast to fluphenazine, perphenazine only decreased the MITF content by 15.5 and 23.5% in concentrations of 1.0 and 3.0 µM, respectively. Fluphenazine in concentration of 0.001 µM and perphenazine in concentrations from 0.0001 to 0.1 µM had no impact on the cellular MITF content in comparison to the control cells.

The effect of fluphenazine and perphenazine on antioxidant defense system in melanocytes

To study the effect of analyzed drugs on reactive oxygen species metabolism in melanocytes, the activity of antioxidant enzymes and the content of hydrogen peroxide were determined. Cells were exposed to fluphenazine (concentration range from 0.0001 to 1.0 µM) or perphenazine (concentration range from 0.0001 to 3.0 µM) for 24 h.

Both of the analyzed drugs raised SOD activity (Fig. 3A). The SOD activity was determined as 0.88 to 1.26 U/mg protein and 0.84 to 1.08 U/mg protein for melanocytes treated with fluphenazine and perphenazine, respectively, while the values determined for the control samples of both drugs were 0.90 ± 0.06 U/mg protein and 0.83 ± 0.03 U/mg protein, respectively. The treatment of cells with 0.1 and 1.0 µM of fluphenazine or 1.0 and 3.0 µM of perphenazine increased the SOD activity by 21.1 and 40.1% or by 13.3 and 30.4%, respectively, as compared with the controls. Both analyzed drugs in concentration range from 0.0001 to 0.01 µM had no impact on SOD activity.

After 24-h incubation with fluphenazine or perphenazine the intracellular CAT activity decreased (Fig. 3B). The CAT activity was determined as 21.60 to 26.83 nmol/min/mg protein and 20.28 to 25.93 nmol/min/mg protein for melanocytes treated with fluphenazine and perphenazine, respectively, while the values determined for the control samples of both drugs were 26.75 ± 1.50 nmol/min/mg protein and 25.33 ± 1.91 nmol/min/mg protein, respectively. Treatment of HEMn-DP cells with 0.1 and 1.0 µM of fluphenazine or 1.0 and 3.0 µM of perphenazine decreased the CAT activity by 14.1 and 19.2% or by 13.9 and 19.9%, respectively, as compared with the controls. Both analyzed drugs in concentration range from 0.0001 to 0.01 µM had no impact on CAT activity.

Fluphenazine modified GPx activity, while perphenazine decreased the enzyme activity in melanocytes (Fig. 3C). The GPx activity was determined as 14.70 to 20.83 nmol/min/mg protein and 13.61 to 15.95 nmol/min/mg protein for cells treated with fluphenazine and perphenazine, respectively, while the values determined for the control samples of both drugs were 17.84 ± 0.47 nmol/min/mg protein and 15.24 ± 1.10 nmol/min/mg protein, respectively. Treatment of melanocytes with 0.1 µM of fluphenazine caused an increase in GPx activity by 16.8%, while the concentration 1.0 µM decreased the enzyme activity by 17.6%. Perphenazine in concentration of 3.0 µM decreased GPx activity by 10.7%. Both analyzed drugs in lower concentrations had no impact on GPx activity.

After 24-h incubation of melanocytes with the analyzed drugs, the hydrogen peroxide (H₂O₂) content increased in a concentration-dependent manner (Fig. 3D). The H₂O₂ content was determined as 158.81 to 276.98 µmol/ml/mg protein and 155.31 to 232.50 µmol/ml/mg protein for melanocytes treated with fluphenazine and perphenazine, respectively, while the values determined for the control samples of both drugs were 153.39 ± 2.09 µmol/mg protein and 156.41 ± 9.37 µmol/mg protein, respectively. The treatment of cells with fluphenazine in concentrations from 0.001 to 1.0 µM increased the H₂O₂ content by 9.2 to 80.6%, while perphenazine in concentrations from 0.01 to 3.0 µM increased the hydrogen peroxide content by 12.7 to 48.7%, as compared with the controls. Fluphenazine in concentration of 0.0001 µM and perphenazine in concentrations 0.0001 and 0.001 µM had no impact on H₂O₂ content in cells.
DISCUSSION

Like other phenothiazine derivatives, fluphenazine and perphenazine have been reported to exhibit a variety of new, important biological effects such as anticancer, antibacterial, antiprotoco- zic, antiviral and antimalarial drug resistance reversal activity. The biological activities of phenothiazine-based compounds are strongly dependent on their chemical structure, intrinsic physical properties and their ability to aggregate with themselves and solvent molecules (28, 29).

Phenothiazine derivatives treatment may potentially lead to harmful toxic effects. Fluphenazine treatment is associated with extrapyramidal symptoms in 97% of patients, incident depression in 55% of patients, blurred vision, excess salivation as well as dose-related changes in prolactin levels. Similar side effects like acute extrapyramidal symptoms and hyperprolactinemia are observed during perphenazine therapy (30).

Interestingly, in the treatment of psychotic disorders, the daily dosage of fluphenazine up to 40 mg/day (31) is relatively lower in comparison to other phenothiazines: chlorpromazine more than 800 mg/day (32), perphenazine up to 64 mg/day (33) and thioridazine up to 800 mg/day (34). Because of the fact that long-term thioridazine therapy with a daily dose of 100 mg is required to trigger chorioretinal changes and the highest dose (800 mg/day) can induce a peripheral retinopathy, as well as that fluphenazine therapy 2 mg/day may lead to toxic retinopathy (maculopathy) – fluphenazine may be considered to be more toxic drug than other phenothiazines (5, 35).

Our previous studies showed the ability of phenothiazine derivatives: chlorpromazine, fluphenazine, trifluoperazine and thioridazine to form stable complexes with model synthetic as well as natural melanin (14, 36), which may be responsible for toxic effects of these drugs directed to pigmented tissues.

In this study, we have used the culture of normal human epidermal melanocytes as an in vitro experimental model system. It was observed that fluphenazine in the lowest concentration (0.0001 µM) and perphenazine in concentration range from 0.0001 to 0.01 µM did not have any significant effect on melanocytes viability. The treatment of cells with both drugs in higher concentrations resulted in the loss in cell viability in a concentration-dependent manner (Fig. 1). The value of EC50 was calculated to be 1.24 and 2.76 µM for fluphenazine and perphenazine, respectively. For the same cell line the value of EC50 established for chlorpromazine was 2.53 µM (26), what indicates that fluphenazine is more cytotoxic than chlorpromazine and perphenazine. Taking into account the value of EC50, the order of drugs cytotoxicity would be as follows: fluphenazine >> chlorpromazine > perphenazine.

The analysis of melanogenesis process in cells cultured in the presence of fluphenazine showed that the drug in concentration of 1.0 µM decreased tyrosinase activity and melanin content. Perphenazine in concentrations of 1.0 and 3.0 µM also decreased the enzyme activity, as well as melanin content (Fig. 2A and 2B). Our results demonstrate that both drugs at higher studied concentrations (= 1.0 µM) suppress melanogenesis in normal human melanocytes, probably due to their inhibitory effect on tyrosinase activity.

Because of the fact that MITF is a major transcriptional regulator of melanogenesis, cell survival and differentiation of melanocytes, we have examined the effect of fluphenazine and perphenazine on the content of this protein. Fluphenazine in concentration of 0.0001 µM increased the MITF content, while in the concentrations from 0.01 to 1.0 µM significantly decreased the content of this protein. Perphenazine in concentrations of 1.0 and 3.0 µM also decreased the content of MITF (Fig. 2C). The obtained results indicate that fluphenazine possesses greater inhibitory effect on MITF than perphenazine, what may explain that only during fluphenazine therapy hypopigmentation disorders, such as vitiligoid depigmentation and chemical leukoderma, occur. Moreover, the observed inhibition of melanogenesis process and decrease in cellular viability in the presence of fluphenazine and perphenazine is probably caused by the suppression of microphthalmia-associated transcription factor.

In contrast to fluphenazine and perphenazine, chlorpromazine used in lower concentrations (0.0001, 0.001 and 0.01 µM) increased melanin and MITF content as well as tyrosinase activity in HEMn-DP cells, without any impact on melanogenesis in higher drug concentrations (0.1 and 1.0 µM) (26). This may explain the occurrence of photodistributed hyperpigmentation in patients during chlorpromazine therapy.

Reactive oxygen species are produced during normal cellular metabolic processes, under pathologic conditions as well as by many exogenous factors including UV radiation, xenobiotics and drugs. The overproduction of pro-oxidant species in cells and/or reduction of cellular antioxidant capacity leads to oxidative stress and results in damage of nucleic acids, lipids, and proteins causing cell muta-
genesis or death (18, 37, 38). The human body has a complex antioxidant defense system which contains small-molecule antioxidants (e.g., glutathione, uric acid, vitamin C, vitamin E) as well as antioxidant enzymes such as SOD, GPx and CAT (37).

In the present study, we have examined the effect of fluphenazine and perphenazine on the activity of antioxidant enzymes. Fluphenazine in concentrations of 0.1 and 1.0 µM and perphenazine in concentrations of 1.0 and 3.0 µM significantly increased SOD activity, decreased CAT activity and exerted different effect on GPx activity (Fig. 3A, 3B and 3C). The observed changes in antioxidant enzymes activity after exposure of melanocytes to the analyzed drugs might be the main reason for overproduction of superoxide anion and elevated level of H$_2$O$_2$ (Fig. 3D) that cannot be eliminated. This indicates that antioxidant defense system does not work properly. The obtained results also indicate that fluphenazine possesses greater stimulatory effect on SOD formation, produces higher levels of hydrogen peroxide and therefore induces oxidative stress in melanocytes, what confirms that fluphenazine is more toxic than perphenazine.

The fluphenazine and perphenazine induced changes in SOD and GPx activities were also stated by other authors. Abdalla et al. (39) showed the increased SOD and decreased GPx activity as well as an accumulation of H$_2$O$_2$ in red blood cells in schizophrenic patients treated with fluphenazine and chlorpromazine in combination with 8 different antipsychotic drugs. Michel et al. (40) demonstrated the increase of SOD activity in the brain of patients with schizophrenic psychosis treated with fluphenazine and Zhang et al. (41) suggested that increased SOD level in schizophrenic patients, treated with fluphenazine, perphenazine, trifluperazine and chlorpromazine in combination with 3 different antipsychotic drugs, may be a response to increased oxidative stress associated with neuroleptic treatment (40, 41).

One has to take into consideration that fluphenazine and perphenazine concentrations found to have a modulatory effect on melanogenesis and antioxidant defense system in normal human melanocytes are about 10- to 100-fold higher for fluphenazine and 20- to 60-fold higher for perphenazine than the concentrations normally observed in blood (42). However, we have previously demonstrated that phenothiazine derivatives form complexes with melanin, what may lead to the accumulation of these drugs in melanin reach tissues (14, 36). Such accumulation increases the drug concentration and may induce some toxic effects on melanin containing cells and surrounding tissues. Thus, it is possible that fluphenazine and perphenazine concentrations in melanocytes may be significantly higher than that in blood and therefore the reduction of melanin content, the inhibition of tyrosinase activity as well as the depletion of cellular antioxidant status in the presence of these drugs could be observed in vivo.

CONCLUSION

Due to the fact that phenothiazine derivatives are the subject of new researches, it is important to explain molecular mechanisms underlying these drugs toxic effects. The present work provides the first in vitro study on mechanisms involved in fluphenazine and perphenazine induced disorders in HEMn-DP cells. The observed changes in melanization process and antioxidant defense system in pigmented cells exposed to the analyzed drugs in vitro suggest a significant role of melanin and melanocytes in the mechanisms of undesirable side effects of these drugs in vivo. The presented differences in modulation of biochemical processes in melanocytes may be an explanation for various toxic activity of the analyzed phenothiazine derivatives in vivo.

Declaration of interest

All authors declare that there are no conflicts of interest.

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