



# Cytotoxic effect of lomefloxacin in culture of human epidermal melanocytes

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## Abstract:

**Background:** Lomefloxacin is a potent bactericidal antibiotic. The use of this drug in treatment of various infections is accompanied by serious adverse effects on pigmented tissues. The exact mechanisms of lomefloxacin side effects have not been well established yet. The aim of this study was to characterize the interaction between lomefloxacin and melanin, and to examine how this interaction affects the cell viability and melanization in melanocytes.

**Methods:** Normal human epidermal melanocytes and the model DOPA-melanin were used. The binding parameters of lomefloxacin-melanin complexes as well as the antibiotic effect on cell viability and melanization in pigmented cells were investigated using a spectrophotometric method.

**Results:** Our results indicate that lomefloxacin forms stable complexes with melanin. The analysis of drug binding to melanin has shown that at least two classes of independent binding sites are involved in formation of these complexes. The WST-1 assay was used to detect the antibiotic cytotoxic effect. The value of ED<sub>50</sub> for lomefloxacin was about 0.75 mmol/l. It has been shown that lomefloxacin causes inhibition of tyrosinase activity, and reduces melanin content in human skin melanocytes in a dose-dependent manner.

**Conclusion:** The ability of the analyzed fluoroquinolone to form complexes with melanin, and the demonstrated inhibitory effect on a melanization process in melanocytes *in vitro* may explain a potential role of melanin biopolymer in the mechanisms of undesirable side effects of lomefloxacin *in vivo* resulting from its accumulation in pigmented tissues.

## Key words:

lomefloxacin, melanin, drug-melanin complexes, melanocytes, melanization

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## Introduction

Lomefloxacin is one of the fluoroquinolones, which are synthetically produced antibiotics active against a broad spectrum of pathogenic Gram-positive and Gram-negative bacteria including *Streptococcal* and *Staphylococcal* species. Lomefloxacin is widely used in clinical practice to treat infections of the respiratory or urinary tracts, as well as in ophthalmology and dermatology [35, 42]. Like other fluoroquinolones,

lomefloxacin acts by inhibiting DNA topoisomerases, of which DNA gyrase and topoisomerase IV are particularly important [28, 40]. Serious adverse phototoxic reactions on melanin containing tissues (e.g., allergy, toxic dermatitis, retinal degeneration) have been described for the use of lomefloxacin [27, 28, 36]. This fluoroquinolone belongs to the class of antibiotics known to exhibit severe phototoxicity [37]. Phototoxicity is postulated to occur as a result of fluoroquinolones photodegradation and the molecules

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ability to generate free oxygen radicals. In turn, these oxidative radicals may attack cellular lipid membranes, initiating inflammatory processes, and cause mitochondria or DNA damage [42]. The study conducted by Marrot et al. [26] on human skin cells, i.e., normal human fibroblasts, keratinocytes and Caucasian melanocytes, confirmed the ability of lomefloxacin to induce DNA damage such as strand breaks and pyrimidine dimers. The activation of the p53 pathway was also demonstrated. Another study has shown that fluoroquinolones, including lomefloxacin, absorb UV radiation which reaches the human lens epithelial cells [46]. Phototoxic damage leads to a loss of transparency of the human lens. Thus, fluoroquinolones taken systematically or injected intravitreally are potentially phototoxic to the eye and may contribute to early cataractogenesis. Halogenation (chlorine, fluorine) of position 8 in concert with fluorination of position 6 (the so-called double-halogenated quinolones) has demonstrated significant phototoxic potential [30]. Therefore, lomefloxacin and sparfloxacin have been reported to have relatively high phototoxic potential as compared with other fluoroquinolones, e.g., ciprofloxacin or norfloxacin [27, 42]. UV-induced defluorination at the 8-position generates a highly reactive aromatic carbene intermediate. In the presence of water and oxygen, carbene is converted to a reactive quinone-imine and hydrogen peroxide with subsequent formation of hydroxyl radicals from the latter *via* Fenton chemistry [5, 17, 42]. While free radical scavengers inhibit the formation of reactive oxygen species, they do not prevent against carbene or the quinone-imine-induced phototoxicity [5]. The exact mechanisms of fluoroquinolones side effects have not been well delineated. It has been demonstrated that fluoroquinolones bind well to melanin rich tissues [18, 29], but the relation between the affinity of these drugs to melanin and the skin or eye toxicity is not well documented.

Melanins are synthesized in melanocytes within specialized organelles, namely melanosomes. The precursors of melanocytes, i.e., melanoblasts, migrate, proliferate and differentiate en route for their eventual destinations in the basal epithelium of the epidermis and hair bulbs of the skin, the uveal tract of the eye, the stria vascularis, the vestibular organ and the endolymphatic sac of the ear and the leptomeninges of the brain [15].

Melanin is a polymeric dark pigment of an undefined structure. Despite the uncertainty of its precise

structure, melanin is described to exist in two principal forms: brown-black eumelanin and yellow-red pheomelanin, which differ not only in color but also in size, shape and packaging of their granules [15, 20]. In the body, melanin is present as a co-polymer of both types [24, 43].

Melanins are a broad class of functional macromolecules found throughout nature. The biological function of melanin remains mostly obscure. Human melanocytes function as a pivotal protective barrier against ultraviolet (UV) radiation and oxidative stress by generating the radical-scavenging pigment melanin [21, 38]. By inhibiting or significantly restricting drug access to cell receptors, melanins protect organism against undesirable drug side effects. However, long-term exposure and slow release of drugs or their metabolites from bonds may increase the level of noxious substances stored on melanin, which may cause degeneration in the melanin-containing cells (e.g., in the eye and skin) and surrounding tissues [19, 23, 24].

Previously, we documented that a fluoroquinolone antibiotic, namely ciprofloxacin, demonstrated ability to form complexes with synthetic DOPA-melanin, and that this interaction affected the viability and melanization of melanocytes [2]. Ciprofloxacin induced evident concentration-dependent loss in melanocytes viability, as well as reduced melanin content and decreased tyrosinase activity, suggesting a mechanism for the drug-induced toxicity on pigmented tissues. Whether the same mechanism can be attributed to another fluoroquinolone derivative, i.e., lomefloxacin, with relatively high phototoxic potential, remains to be determined.

In the present study, we have examined the ability of lomefloxacin to form a complex with melanin, the stability constants of this complex as well as the effect of lomefloxacin on cell viability and melanization in human epidermal melanocytes.

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## Materials and Methods

### Materials

L-3,4-dihydroxyphenylalanine (L-DOPA), Triton X-100, mushroom tyrosinase, lomefloxacin hydrochloride and tyrosine-melanin (prepared by oxidation of tyrosine with hydrogen peroxide) were purchased from Sigma-Aldrich Inc. (USA). Penicillin was acquired from Polfa, Tarchomin (Poland). Growth medium

M-254, gentamicin-amphotericin B, 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).

### Preparation of DOPA-melanin

Model DOPA-melanin was obtained by oxidative polymerization of L-DOPA solution (1 mg/ml) in 0.067 mol/l phosphate buffer (pH 8.0) for 48 h, according to the method described earlier [11].

### Isolation of ocular melanin

The pig eyes were dissected to separate the iris and choroid with the retinal pigment epithelium (RPE). The procedure of melanin isolation was performed according to the Persad method [7, 32].

### Lomefloxacin-melanin complex formation

Drug-melanin complexes were obtained by suspending 5 mg of DOPA-melanin in 5 ml of lomefloxacin solution. Mixtures of melanin and drug solutions were incubated at room temperature and then filtered. Control samples, containing melanin suspended in distilled water (without a drug), were treated in the same manner.

### Determination of the amount of a drug bound to melanin

The UV spectrophotometric method was used for quantitative determination of the analyzed drug. Analytical wavelength ( $\lambda_{\max}$ ) for lomefloxacin was 284 nm. The calculated value of a molar absorption coefficient ( $\epsilon_{\lambda_{\max}}$ ):  $32978 \text{ dm}^3 \times \text{mol}^{-1} \times \text{cm}^{-1}$  for lomefloxacin was used to estimate the amount of a drug bound to the polymer. All spectrophotometric measurements were performed by a JASCO model V-630, UV-VIS spectrophotometer.

### Kinetics of drug-melanin complex formation

Kinetics of the formation of melanin complexes with lomefloxacin were evaluated on the basis of relationship between the amount of a drug bound to the polymer ( $\mu\text{mol}/\text{mg}$ ) and the time of complex formation. In

this study, the following drug initial concentrations were used: 0.1, 0.5, 1.0 and 5.0 mmol/l. Complex formation lasted for 0.5, 1, 3, 6, 12, 24 and 48 h.

### 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 lomefloxacin were calculated using the Scatchard plot, according to the method described earlier [11]. 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 0.05 to 5.0 mmol/l.

### Cell culture

The normal human epidermal melanocytes (HEMA-LP, Cascade Biologics) were grown according to the manufacturer's instruction. The cells were cultured in M-254 medium supplemented with HMGS-2, penicillin (100 U/ml), gentamicin (10  $\mu\text{g}/\text{ml}$ ) and amphotericin B (0.25  $\mu\text{g}/\text{ml}$ ) at 37°C in 5% CO<sub>2</sub>. All experiments were performed using cells in the passages 5–7.

### 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. WST-1 is a water-soluble tetrazolium salt, the rate of WST-1 cleavage by mitochondrial dehydrogenases correlates with the number of viable cells. In brief, 5000 cells per well were placed in a 96-well microplate in a supplemented M-254 growth medium and incubated at 37°C and 5% CO<sub>2</sub> for 48 h. Then, the medium was removed and cells were treated with lomefloxacin solutions in a concentration range from 0.001 to 1.0 mmol/l. After 21-h incubation, 10  $\mu\text{l}$  of WST-1 were added to 100  $\mu\text{l}$  of culture medium in each well, and the incubation was continued for three hours. The absorbance of the samples was measured at 440 nm with a reference wavelength of 650 nm, against the controls (the same cells but not treated with lomefloxacin) using a microplate reader UVM 340 (Biogenet). The controls were normalized to 100% for each assay and treatments were expressed as the percentage of the controls.

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### Measurement of melanin content

The melanocytes were seeded in a 35 mm dish at a density of  $1 \times 10^5$  cells per dish. Lomefloxacin treatment in concentrations of 0.0075, 0.0375, 0.075, 0.375 and 0.75 mmol/l, respectively, began 48 h after seeding. After 24 h of incubation, the cells were detached with trypsin-EDTA. Cell pellets were placed into Eppendorf tubes, dissolved in 100  $\mu$ l of 1 mol/l NaOH at 80°C for 1 h, and then centrifuged for 20 min at 16,000  $\times$  g. The supernatants were placed into a 96-well microplate, and absorbance was measured using a microplate reader at 405 nm – a wavelength at which melanin absorbs light [31]. A standard synthetic melanin curve (0 to 400  $\mu$ g/ml) was constructed in triplicate for each experiment. Melanin content in lomefloxacin treated cells was expressed as the percentage of the controls (untreated melanocytes).

### Tyrosinase activity assay

Tyrosinase activity in HEMa-LP cells was determined by measuring the rate of oxidation of L-DOPA to dopachrome according to the method described by Kim et al. [22] and Busca et al. [6], with a slight modification. The cells were cultured at a density of  $1 \times 10^5$  cells in a 35 mm dish for 48 h. After 24-h incubation with lomefloxacin (concentrations 0.0075, 0.0375, 0.075, 0.375 and 0.75 mmol/l) cells were lysed with phosphate buffer (pH 6.8) containing 0.1% Triton X-100, and lysates were clarified by centrifugation at 10,000  $\times$  g for 5 min. A tyrosinase substrate L-DOPA (2 mg/ml) was prepared in the same lysis phosphate buffer (without Triton). A hundred  $\mu$ l of each lysate were put in a 96-well plate, and the enzymatic assay was initiated by the addition of 40  $\mu$ l of L-DOPA solution at 37°C. Control wells contained 100  $\mu$ l of lysis buffer and 40  $\mu$ l of L-DOPA solution. Absorbance of dopachrome was measured every 10 min for at least 1.5 h at 475 nm using a microplate reader.

A cell-free assay system was used to test for direct effects on tyrosinase activity. Phosphate buffer (130  $\mu$ l) containing lomefloxacin in a concentration range from 0.0075 to 1.0 mmol/l was mixed with 20  $\mu$ l of mushroom tyrosinase (1000 units), and 100  $\mu$ l of L-DOPA solution (2 mg/ml) was added to each well. The assay mixtures were incubated at 37°C for 20 min, and absorbance of dopachrome was measured at 475 nm in a microplate reader. The mushroom tyrosinase activities were calculated in the relation to

the controls (samples without lomefloxacin). The value  $IC_{50}$  (the concentration of a drug that inhibits a standard response by 50%) was calculated on the basis of a dose-dependent inhibition curve, as described by Chung et al. [14].

Human tyrosinase activity was assayed spectrophotometrically by following the oxidation of L-DOPA to dopachrome at 475 nm. Melanocytes ( $2.5 \times 10^5$ ) were seeded in a 35 mm dish. After 24 h of incubation, the cells were detached with trypsin-EDTA and lysed with phosphate buffer (pH 6.8) containing 0.1% Triton X-100. A hundred thirty  $\mu$ l of phosphate buffer containing lomefloxacin in a concentration range from 0.0075 to 1.0 mmol/l were mixed with 20  $\mu$ l of each lysate, and 100  $\mu$ l of L-DOPA solution (2 mg/ml) was added to each well. Absorbance of dopachrome was measured every 1 min for at least 20 min at 475 nm in a microplate reader. The human tyrosinase activities were calculated in the relation to the controls (samples without lomefloxacin). The value  $IC_{50}$  was calculated as described for a mushroom tyrosinase assay.

### Statistical analysis

In all experiments, the mean values of at least three separate experiments performed in triplicate  $\pm$  standard deviations (SD) were calculated. The results were analyzed statistically with the Student's *t*-test. In all cases differences were considered as statistically significant when  $p < 0.05$ .

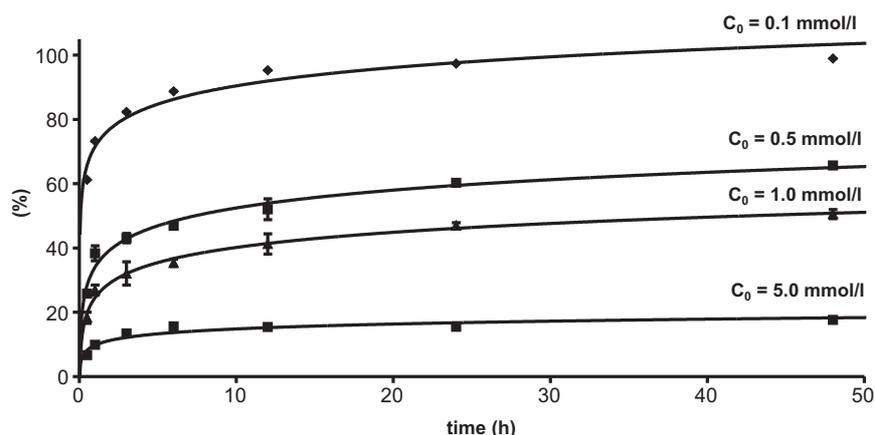
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## Results

### Binding of lomefloxacin to melanin

Kinetics of the formation of lomefloxacin-melanin complexes shown as the relation between the amount of a drug bound to the polymer and the incubation time are presented in Figure 1 for four initial concentrations of the drug ( $c_0$ ). It has been demonstrated that prolongation of incubation time results in an increased amount of a drug bound to melanin. On the basis of the results it may be concluded that the maximum time to achieve the equilibrium state is 24 h. Complex formation efficiency (the ratio of the amount of a drug bound to melanin and the amount of

**Fig. 1.** The effect of incubation time and a drug initial concentration ( $c_0$ ) on the amount of lomefloxacin bound to DOPA-melanin (in %). Mean values  $\pm$  SD from three independent experiments are presented. Points without error bars indicate that SD value was lower than the size of the used symbol



**Tab. 1.** Amounts of lomefloxacin bound to various types of melanin after 24 h of incubation

Lomefloxacin concentration (mmol/l)	Amount of lomefloxacin bound to melanin $\pm$ SD ( $\mu\text{mol drug/mg melanin}$ )		
	Synthetic DOPA-melanin	Synthetic tyrosine-melanin	Natural pig eye-melanin
0.1	$0.086 \pm 0.003$	$0.083 \pm 0.001$	$0.077 \pm 0.014$
0.5	$0.328 \pm 0.003$	$0.321 \pm 0.008$	$0.243 \pm 0.020$
1.0	$0.513 \pm 0.013$	$0.505 \pm 0.011$	$0.346 \pm 0.002$
5.0	$0.747 \pm 0.014$	$0.702 \pm 0.020$	$0.649 \pm 0.021$

a drug added to form the complex, expressed as %) decreased along with an increased initial concentration of the drug.

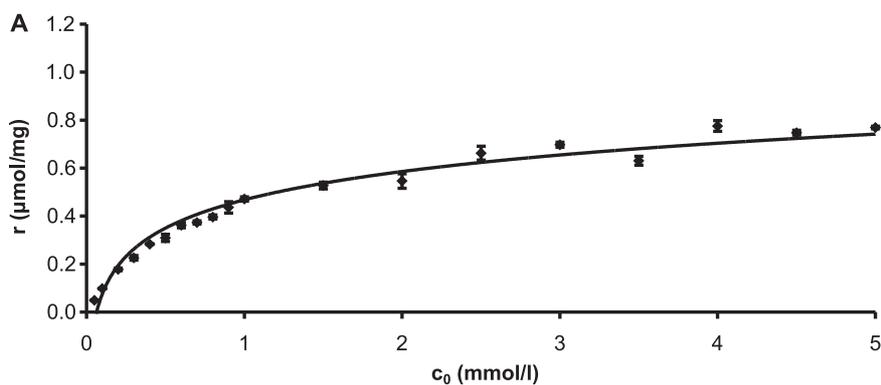
We repeated the experiment with natural pig eye-melanin and synthetic tyrosine-melanin. For the four given initial concentrations of the drug (0.1, 0.5, 1.0 and 5.0 mmol/l), respectively, the amounts of lomefloxacin bound to pig eye- and tyrosine-melanin (expressed as  $\mu\text{mol}$  of drug/mg melanin) are similar to the amount of the drug bound to synthetic DOPA-melanin (Tab. 1).

A relation between the amount of lomefloxacin bound to melanin after 24 h of incubation and the drug initial concentration is presented in Figure 2A as a binding isotherm. It can be seen from a binding curve that the amount of a drug bound to a constant amount of DOPA-melanin is increasing, and reaches a plateau at about  $0.6 \mu\text{mol}$  lomefloxacin per 1 mg melanin, which reflects the drug initial concentration of 3 mmol/l.

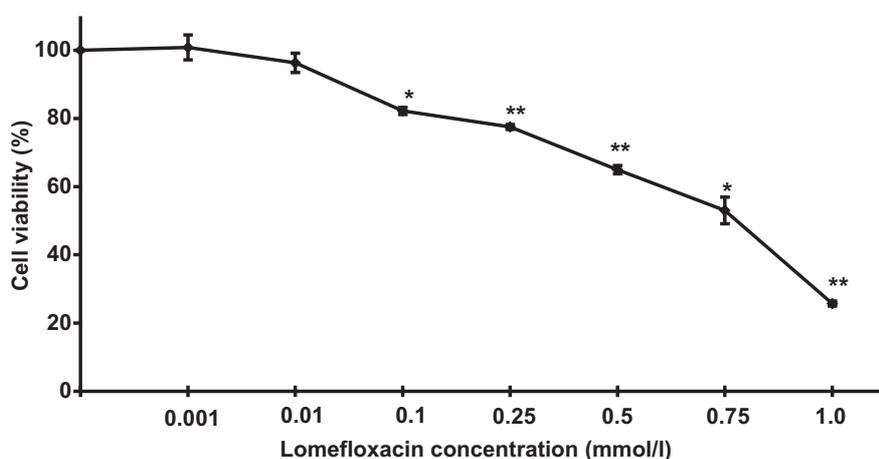
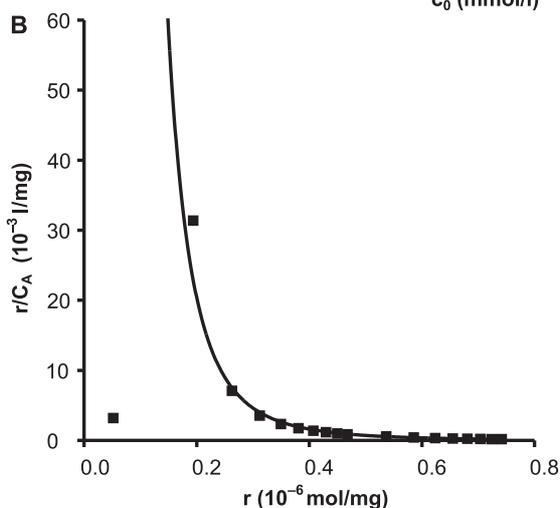
Relation of the amount of a drug bound to melanin ( $r$ ) to the concentration of an unbound drug ( $c_A$ ), i.e.,  $r/c_A$ , versus  $r$  for lomefloxacin complexes with DOPA-melanin is presented in Figure 2B as a Scatchard plot. The use of this method can provide information about the number and nature of binding sites in the analyzed complexes. The obtained plot is curvilinear with an upward concavity, indicating that at least two classes of independent binding sites must be implicated in lomefloxacin-melanin complex formation. The calculated values of binding parameters are as follows: the number of strong binding sites  $n_1 = 0.22 \mu\text{mol}$  lomefloxacin per 1 mg melanin, the association constant  $K_1 = 6.49 \times 10^5 \text{ M}^{-1}$ , the number of weak binding sites  $n_2 = 0.70 \mu\text{mol}$  lomefloxacin per 1 mg melanin, the association constant  $K_2 = 7.03 \times 10^2 \text{ M}^{-1}$ .

#### The effect of lomefloxacin on melanocytes viability

Cultured melanocytes were exposed to lomefloxacin in a range of concentrations from 0.001 to 1.0 mmol/l for 24 h. The effect induced by the antibiotic was determined by the WST-1 test assay, which measures the activity of mitochondrial dehydrogenases of living cells. The obtained results show that mitochondrial cytotoxicity is dependent on the drug concentration (Fig. 3). There was no significant difference in the cell viability (0–4%) of normal human epidermal melanocytes after 24-h exposure to relative low lomefloxacin concentration (0.001 or 0.01 mmol/l). Treatment of cells with 0.10, 0.25, 0.50, 0.75 and 1.0 mmol/l of lomefloxacin for 24 h leads to the loss of about 17.8, 22.5, 35.0, 47.0 and 74.3% in the cell viability, respectively. The value of  $\text{ED}_{50}$  (the amount of a drug that produces loss in cell viability by 50%) for lomefloxacin was found as about 0.75 mmol/l.



**Fig. 2.** A binding isotherm (A) and a Scatchard plot (B) for lomefloxacin-melanin complexes obtained after 24-h incubation;  $c_0$  – drug initial concentration,  $r$  – amount of a drug bound to melanin,  $c_A$  – concentration of an unbound drug. Mean values  $\pm$  SD from three independent experiments are presented. Points without error bars indicate that SD value was lower than the size of the used symbol



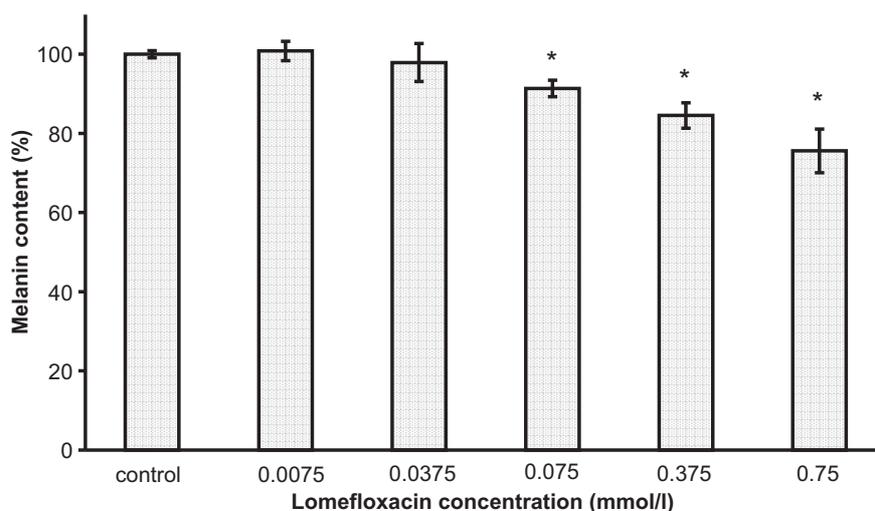
**Fig. 3.** The effect of lomefloxacin on viability of melanocytes. Cells were treated with various concentrations of lomefloxacin (0.001–1 mmol/l) and examined by the WST-1 assay. Data are expressed as % of cell viability. Mean values  $\pm$  SD from three independent experiments performed in triplicate are presented. \*  $p < 0.05$ , \*\*  $p < 0.01$  as compared with controls

### The effect of lomefloxacin on melanization process

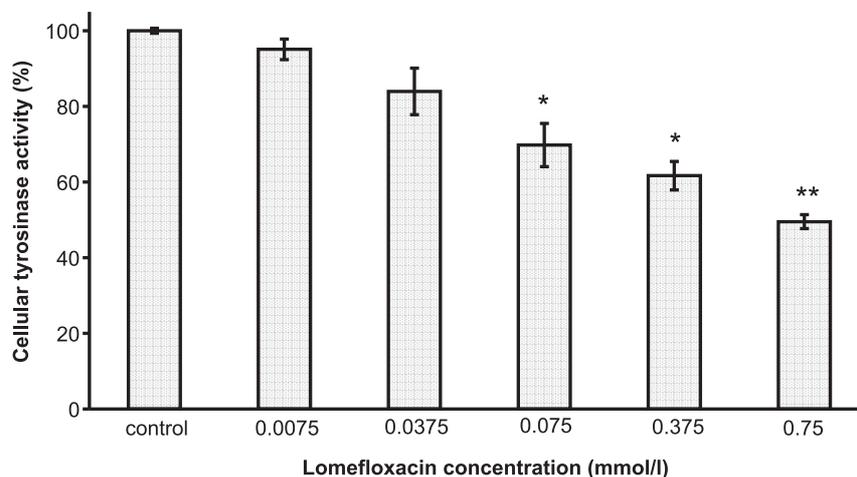
Melanin content and cellular tyrosinase activity were measured in melanocytes treated with lomefloxacin concentrations from 0.0075 to 0.75 mmol/l for 24 h. After constructing a calibration curve, the melanin content per cell was determined as 34.9, 33.1, 32.0,

28.2 and 25.5 pg/cell for lomefloxacin concentrations of 0.0075, 0.0375, 0.075, 0.375 and 0.75 mmol/l, respectively, and 37.1 pg/cell for a control sample. The obtained results, recalculated for the culture ( $1 \times 10^5$  cells), were finally expressed as a percentage of the controls (Fig. 4). It has been demonstrated that lomefloxacin dose-dependently reduces melanin content (Fig. 4) and decreases cellular tyrosinase activity (Fig. 5),

**Fig. 4.** The effect of lomefloxacin on melanin content in melanocytes. Cells were cultured with 0.0075, 0.0375, 0.075, 0.375 and 0.75 mmol/l of lomefloxacin, respectively, for 24 h and melanin content was measured as described in Materials and Methods. Results are expressed as percentages of the controls. Data are the mean  $\pm$  SD of at least three independent experiments performed in triplicate. \*  $p < 0.05$  as compared with controls



**Fig. 5.** The effect of lomefloxacin on tyrosinase activity in melanocytes. Cells were cultured with 0.0075, 0.0375, 0.075, 0.375 and 0.75 mmol/l of lomefloxacin, respectively, for 24 h and tyrosinase activities were measured as described in Materials and Methods. Results are expressed as percentages of the controls. Data are the mean  $\pm$  SD of at least three independent experiments performed in triplicate. \*  $p < 0.05$ , \*\*  $p < 0.01$  as compared with controls



a rate-limiting factor in melanin biosynthesis, in human skin melanocytes. Lomefloxacin in the concentration of 0.0075 mmol/l had no effect on tyrosinase activity and melanin content. The use of the analyzed antibiotic in the concentration of 0.0375 mmol/l suppressed tyrosinase activity to  $86.34 \pm 6.64\%$  but had no effect on melanin content in melanocytes as compared with the control cells. At concentrations of 0.075 and 0.375 mmol/l of lomefloxacin, most of cells were still viable, while cellular tyrosinase activity and melanin content decreased to  $69.76 \pm 5.71\%$ ,  $61.95 \pm 3.04\%$  and  $91.33 \pm 2.08\%$ ,  $83.46 \pm 3.15\%$  of control cells, respectively. The concentration of 0.75 mmol/l ( $ED_{50}$ ) caused the decrease of tyrosinase activity to  $49.51 \pm 1.84\%$  and reduction of melanin content in melanocytes to  $75.59 \pm 5.51\%$  as compared with the control samples. We observed a similar effect

caused by lomefloxacin in the concentration of 0.75 mmol/l ( $ED_{50}$ ) on both cellular and mushroom tyrosinase activities, as well as on the activity of human tyrosinase extracted from the cultured cells. The drug significantly decreased the mushroom and human tyrosinase activities (Tab. 2) in a dose-dependent manner. The concentration of lomefloxacin required for 50% inhibition of mushroom and human tyrosinase activities ( $IC_{50}$ ) was 0.83 and 1.23 mmol/l, respectively.

## Discussion

The problem of the interaction of drugs with melamins, the natural brown/black pigments present in

**Tab. 2.** The inhibitory effect of lomefloxacin on mushroom and human tyrosinase activities

Lomefloxacin concentration (mmol/l)	Mushroom tyrosinase		Human tyrosinase	
	Inhibition $\pm$ SD (%)	IC <sub>50</sub> <sup>a)</sup> (mmol/l)	Inhibition $\pm$ SD (%)	IC <sub>50</sub> <sup>a)</sup> (mmol/l)
0.0075	24.77 $\pm$ 2.41		3.27 $\pm$ 1.15	
0.075	38.48 $\pm$ 1.79		22.17 $\pm$ 5.43	
0.75	46.66 $\pm$ 2.09	0.83	38.90 $\pm$ 3.63	1.23
1.0	54.34 $\pm$ 4.32		43.81 $\pm$ 4.66	

<sup>a)</sup> 50% inhibitory concentration

many different regions of the human and animal bodies, has attracted a considerable interest since 1962, the year of the publication of the first paper on the binding of phenothiazines to eye melanin [34]. Successively, a noticeable number of papers devoted to this topic appeared, and many drugs were studied with the aim to determine the possible toxic effects that their accumulation could cause at the level of pigmented tissues and organs [4].

Melanins are present in external and internal tissues (skin, hair, ear, eye and brain). Thus, their capacity to bind and release exogenous and endogenous substances in a dynamic fashion may result in various, possibly pathogenic effects on the organism involved. These conditions seem important in the pathogenesis of disease states associated with long-term therapy with number of drugs [23].

It is known that melanin acts as a biochemical dustbin, mopping up free radicals and other potentially toxic agents [24]. Such properties would be important in protecting pigment cells from natural toxins, oxygen, and reactive oxygen species [38]. Many drugs are known to be markedly accumulated and retained for considerable time by pigmented tissues, and the retention of these compounds is proportional to the degree of melanin pigmentation. Melanin binds many classes of drugs, e.g., aminoglycoside antibiotics [11, 12, 44], anesthetics [8], antineoplastic [41], antiarrhythmic [9, 10] and antipsychotic drugs [7]. Melanin binding has several pharmacological consequences. A lot of attention has been given to a possible link between melanin binding, drug accumulation and ocular toxicity, (e.g., phenothiazines) [25] or ototoxicity (e.g., aminoglycosides) [24].

The affinity of drugs to melanin is determined using *in vitro* binding studies. In these experiments, the

interaction between a drug and melanin may be characterized by determining the capacity and affinity of the binding process. These *in vitro* parameters may be correlated with the *in vivo* binding results or they can be used in pharmacokinetic simulation models [24, 33]. Synthetic melanin is widely used in binding studies, although its chemical composition and morphology are different from biological melanins [7, 33]. There are a few comparative studies in which binding parameters of drugs have been determined for synthetic and natural melanin. In our previous study, we demonstrated that antiarrhythmics bound both to natural and synthetic melanin [9]. Moreover, we found no differences between the melanin types and the binding parameters for three antipsychotic drugs, using synthetic and pig eye melanin [7]. The results described in this study reveal that the amounts of lomefloxacin bound to both synthetic DOPA- and tyrosine-melanins as well as to natural pig eye-melanin are similar (Tab. 1). Therefore, in this investigation the synthetic DOPA-melanin was used as a model eumelanin.

It has been shown that the absolute amount of lomefloxacin bound to melanin increases along with the rise in the drug initial concentration and prolongation of incubation time. Simultaneously, a decrease in the complex formation efficiency (expressed as %) was observed when the drug initial concentration increased, which may be explained by saturation of melanin binding sites. An analysis of drug binding to melanin by the use of a Scatchard plot has shown that more than one class of binding sites must be implicated in the lomefloxacin-melanin complex formation: strong binding sites with the association constant  $K_1 \sim 10^5 \text{ M}^{-1}$  and weak binding sites with the association constant  $K_2 \sim 10^2 \text{ M}^{-1}$ . The total number of bind-

ing sites ( $n_1 + n_2$ ) was 0.92  $\mu\text{mol}$  lomefloxacin per 1 mg melanin. The affinity and capacity for binding of lomefloxacin to synthetic melanin are comparable with those of norfloxacin, sparfloxacin [1] and ciprofloxacin [2], and also chloroquine [24, 29] which is well known for its high affinity for melanin.

The molecular nature of drug binding to melanin is rather complex. Several parameters, such as ionic and aromatic interactions, van der Waals attraction or the formation of charge-transfer complexes, determine the affinity of substances to melanin [23]. In most cases, the binding is reversible. It may be suggested that an electrostatic interaction between the protonated nitrogen atom of lomefloxacin and carboxyl groups of melanin would play a critical role in the drug-melanin complex formation [1, 29]. The results published by Fukuda et al. [16] showed that hydroxyl groups in the melanin structure were involved in the binding of fluoroquinolones to melanin, and that this binding was reversible, namely the drugs may be released from the drug-melanin complexes. Moreover, the demonstrated elution of the drug with a metal ion solution suggests that metal ions affect the binding of fluoroquinolones to melanin. The authors indicate that such relations among drugs, melanin and metal ions may occur similarly in a living body. Moreover, one has to take into consideration that, as we previously demonstrated, free radicals play also an important role in fluoroquinolones (ciprofloxacin, lomefloxacin, norfloxacin and sparfloxacin) binding to melanin [3].

Many advances have been made in the understanding of physiology and pathophysiology of pigmentation process [39]. Melanogenesis has been the interest of intense research for many decades. Melanocytes are unique cells that produce melanosomes, specific melanin-containing intracellular organelles [45]. In our study, the effect of lomefloxacin on melanocytes viability was analyzed. We have found that lomefloxacin in the concentration of 0.001–0.01 mmol/l does not demonstrate any significant influence on melanocytes proliferation. Higher drug concentrations (from 0.10 to 1.0 mmol/l) have led to a loss of cell viability in a dose-dependent manner. The value of  $\text{ED}_{50}$  was determined as about 0.75 mmol/l. For the same cell line, the value of  $\text{ED}_{50}$  established for ciprofloxacin [2] was 1.5-fold lower (0.5 mmol/l), which indicates that lomefloxacin is less cytotoxic.

The synthesis of melanin is a multistep process, catalyzed by at least one or several enzymes, the most prominent of which are tyrosinase, tyrosinase-related

protein 1 (TRP-1) and dopachrome tautomerase (TRP-2). Melanogenesis is initiated by the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and its subsequent oxidation to dopaquinone. Dopaquinone undergoes nonenzymatic intramolecular cyclization generating cyclodopa (leucodopachrome) and then, as a result of subsequent reactions catalyzed by tyrosinase and two related enzymes, TRP-1 and TRP-2, it turns into eumelanin, or into pheomelanin when combined with cysteine or glutathione [15, 39, 43].

In this study, we have used the culture of normal human epidermal melanocytes as an *in vitro* experimental model system for the study of lomefloxacin effect on melanogenesis. Since tyrosinase is a major regulator of melanin synthesis, we have examined the direct inhibitory effect of lomefloxacin on the activity of tyrosinase in melanocytes. The cellular tyrosinase activity was reduced by about 5, 14 and 30% in non-cytotoxic drug concentrations (0.0075, 0.0375 and 0.075 mmol/l) or by about 38% and 50% in the concentrations of 0.375 and 0.75 mmol/l ( $\text{ED}_{50}$ ), respectively, as compared with control cells. The results of our previous study [2] and those described in this study reveal that lomefloxacin demonstrates a greater inhibitory effect (by about 10%) on cellular tyrosinase activity than ciprofloxacin.

We repeated the experiment with commonly used mushroom tyrosinase as well as with human tyrosinase extracted from the cultured cells, and obtained similar results. The commercial availability of mushroom tyrosinase plays a critical role in tyrosinase inhibitory studies, and most research has been conducted with this enzyme, which is well studied and easily purified from *A. Bisporus*, the common mushroom [13]. We observed that lomefloxacin in concentrations of 0.0075 to 1.0 mmol/l decreased both the mushroom and human tyrosinase activities by about 25 to 54% and 3 to 44%, respectively, in a dose-dependent manner.

To study the effect of lomefloxacin on the effectiveness of melanin synthesis in normal human epidermal melanocytes, the melanin content in cells cultured in the presence or absence of a drug was measured. Melanin production in cells treated with lomefloxacin in concentrations from 0.075 to 0.75 mmol/l was reduced by 9 to 25%, respectively, as compared with untreated cells. Lomefloxacin in lower concentrations (0.0075 or 0.0375 mmol/l) had no effect on melanin content. For lomefloxacin concentration  $\text{ED}_{50}$ , the melanin content

in melanocytes does not correlate with the number of living cells, but the melanin content was determined for all cells in the analyzed culture.

Lomefloxacin is a potent bactericidal antibiotic. The use of this drug in treatment of various infections is accompanied by serious adverse effects on pigmented tissues. The exact mechanisms of lomefloxacin side effects have not been well established yet.

Our results indicate that the ability of the analyzed fluoroquinolone, i.e., lomefloxacin, to form stable complexes with melanin, and the demonstrated inhibitory effect on cell viability and a melanization process in melanocytes *in vitro* may explain a potential mechanism for the undesirable side effects of lomefloxacin *in vivo*, which result from accumulation of this drug in pigmented tissues. In addition, we have demonstrated that the inhibitory effect of lomefloxacin on melanogenesis is probably due to its direct inhibition of tyrosinase activity.

The ability of lomefloxacin to form complexes with melanin and its greater inhibitory effect on melanogenesis in normal human melanocytes in comparison to ciprofloxacin [2] may be an explanation for various phototoxic activities of these two fluoroquinolones derivatives *in vivo*.

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