JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 1127–1133

Received January 17, 2003
Accepted April 15, 2003
DOI 10.1211/0022357021594
ISSN 0022-3573

Binding characteristics of fluoroquinolones to synthetic levodopa melanin

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Abstract

To define the binding characteristics of fluoroquinolones to synthetic levodopa melanin, the binding of various drugs, including levofloxacin and ofloxacin, and positive controls (timolol and chloroquine), was investigated in-vitro. The affinity and capacity of the drug binding were calculated by Langmuir's adsorption isotherm. The affinity constant (K) and the binding capacity (r_{max}) of levofloxacin were similar to those of timolol and much lower than those of chloroquine. Racemic ofloxacin and its enantiomers showed similar K and r_{max}, suggesting that the binding lacked stereoselectivity. The binding experiment with levofloxacin derivatives indicated that the basic nitrogen atom at position 7 of the quinolone ring, but not carboxyl group at position 3, would play a critical role in the interaction of fluoroquinolones with melanin. The melanin–drug complexes of levofloxacin and chloroquine were washed with neutral phosphate buffer, ethanol and 1 M HCl solution to explain the nature of the interaction of melanin with the drugs. Electrostatic forces mainly participate in the formation of the chloroquine–melanin complex, whereas van der Waals' and hydrophobic interactions are involved in the levofloxacin–melanin complex in addition to electrostatic forces. The interactions of various fluoroquinolones such as norfloxacin, enoxacin, sparfloxacin, ciprofloxacin and lomefloxacin with melanin were also studied. The results showed that the relative K value was: chloroquine $\approx$ ciprofloxacin, sparfloxacin $\geq$ lomefloxacin $>$ timolol, levofloxacin $\approx$ enoxacin, norfloxacin, and that the relative r_{max} value was: norfloxacin, enoxacin $\geq$ chloroquine, sparfloxacin $>$ levofloxacin, ciprofloxacin, timolol, lomefloxacin. The fluoroquinolones vary in their affinity and capacity to bind with melanin, and ciprofloxacin and sparfloxacin showed a stronger interaction with melanin than the other fluoroquinolones studied.

Introduction

Fluoroquinolones represent a major class of antibacterials with huge therapeutic potential. The modification of the first-generation quinolones such as nalidixic acid has led to a considerable increase in their intrinsic antibacterial activity. The pharmacokinetic properties of these new fluoroquinolones are characterized by excellent oral bioavailability, extensive tissue penetration, low protein binding and a long elimination half-life. There are, however, significant differences between individual fluoroquinolones in their oral bioavailability, route of elimination, elimination half-life and drug interactions (Stein 1996).

Several tissues, such as iris, skin, hair, inner ear and substantia nigra, contain melanin. The observation that chronic administration of phenothiazines (Davidorf 1973) or long-term, high-dose chloroquine therapy (Hobbs et al 1959) produced chorioretinopathy led to the awareness of an association between the toxic effects of some drugs and their high affinity for the pigment melanin. Since these early observations, melanin binding of drugs has been implicated not only in ocular toxicity, but also in ototoxicity and pigment disturbances of the skin and hair (Ings 1984). Drug accumulation in these pigmented tissues is of considerable interest from both pharmacological and toxicological points of view.

Electrostatic forces would play an important role in the binding of drugs to melanin, however non-electrostatic contributions, including hydrophobic and van der Waals' interactions and charge transfer reactions, would also contribute to the

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binding for drugs such as chlorpromazine and chloroquine (Larsson & Tjälve 1979; Atlasik et al 1980; Stepien & Wilczok 1982). It has been reported that fluoroquinolones such as ofloxacin, levofloxacin, lomefloxacin and moxifloxacin showed high affinity for melanin and pigmented tissues (Fukuda & Sasaki 1989, 1990; Kurata et al 1991; Siefert et al 1999; Fukuda et al 2000). To date, however, the nature of the interactions between melanin and fluoroquinolones has not been fully characterized.

The objectives of this study were to define the binding characteristics of fluoroquinolones such as ofloxacin, levofloxacin, lomefloxacin, ciprofloxacin, sparfloxacin, enoxacin and norfloxacin, to synthetic melanin and to compare their affinity and capacity for binding with those of chloroquine and timolol, which are well known for their high affinity for melanin.

Materials and Methods

Chemicals and reagents

Melanin was synthesized in-vitro by a modification of a previously reported method (Shimada et al 1976). (−)-3,4-Dihydroxyphenylalanin (levodopa) (6.6 g) and mushroom tyrosinase (44 mg) were placed in 2 L of 0.1 M phosphate buffer (pH 7.4), and stirred at room temperature for 16 h with continual bubbling of oxygen gas. The resulting precipitates of melanin were collected after centrifuging at 19 000 *g* for 20 min, and washed by resuspending in distilled water. The washings, followed by centrifugation, were performed until a clear supernatant solution was obtained. The washed precipitate was freeze-dried. The yield of melanin was approximately 2.9 g.

Ofloxacin, levofloxacin [*S*-(−)-ofloxacin] hemihydrate, *R*-(+)-ofloxacin, demethyl levofloxacin, levofloxacin *N*-oxide, decarboxy levofloxacin and dimethylamino derivative of levofloxacin were synthesized by Daiichi Pharmaceutical Co., Ltd. Enoxacin sesquihydrate, sparfloxacin, norfloxacin, ciprofloxacin hydrochloride monohydrate and lomefloxacin hydrochloride were used. Mushroom tyrosinase, chloroquine diphosphate and timolol maleate were obtained from Sigma Chemical Co. (St Louis, MO, USA). Levodopa was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade and used without further purification. The chemical structures of test compounds are shown in Figure 1.

Melanin binding study

The binding of drugs to synthetic melanin was studied in 0.067 M phosphate buffer at pH 7.4. Melanin was suspended in phosphate buffer (400 μg mL^{-1}). The fluoroquinolones chloroquine and timolol were dissolved in water. The melanin suspension (0.5 mL) and fluoroquinolone solution (25–400 μM; 0.5 mL) were mixed and incubated for 16 h in a thermostated shaking incubator at 37 °C. At the end of the 16-h period, samples were centrifuged at 19 000 *g* for 30 min at 0 °C. The control samples contained drug solution and phosphate buffer. Drug concentrations in the supernatant were determined by high-performance liquid chromatography (HPLC). The amount of drug adsorbed by melanin was calculated by taking the difference between the amount initially added (concentration in the control sample) and the amount remaining in the supernatant. The average of three determinations was used for data analysis.

Treatment of binding data

The results obtained with the drugs suggested that the binding of the various fluoroquinolones, as well as chloroquine and timolol, to melanin could be analysed by assuming that the binding is analogous to the adsorption of a drug on a solid and follows a type I Langmuir isotherm. Specifically, the amount of drug bound per milligram of melanin, r, should be related to the concentration of free drug, $[D]_{free}$, by:

$$r = r_{max}K[D]_{free}/(1 + K[D]_{free}) \quad (1)$$

where r_{max} is the maximum moles bound per milligram of melanin, that is the amount needed to form a monolayer, and K is a constant related to the affinity or strength of the interaction. Rearrangement of Equation 1 to:

$$1/r = 1/r_{max} + 1/r_{max}K1/[D]_{free} \quad (2)$$

shows that a linear relation should be obtained when 1/r is plotted as a function of $1/[D]_{free}$. The extrapolated y-axis intercept is $1/r_{max}$, and the slope is $1/r_{max}K$. Thus, such treatment of the binding data would permit a calculation of r_{max} and K for each drug and provide a basis for comparisons of the binding of drugs to melanin (Shimada et al 1976).

Elution study

The elution of levofloxacin and chloroquine from melanin was determined by washing (three times) the residue after the adsorption run where the mixtures of drug solutions (100 μM; 0.5 mL) and melanin suspension (400 μg mL^{-1}; 0.5 mL) were incubated with 0.067 M phosphate buffer (pH 7.4), 1 M HCl or EtOH (1 mL). The supernatant concentration of drugs eluted was determined by HPLC after centrifugation at 19 000 *g* for 30 min.

Drug assay

The concentrations of various fluoroquinolones, chloroquine and timolol in samples were determined by HPLC using a modification of the method previously reported (Tanaka et al 1993). The HPLC system (model 655A-11; Hitachi, Tokyo, Japan) was equipped with a variable-wavelength UV detector (model 655A). The wavelengths were set at 254 nm for chloroquine, at 280 nm for enoxacin, lomefloxacin, norfloxacin and decarboxy levofloxacin, at 286 nm for the dimethylamino derivative of levofloxacin, at 290 nm for sparfloxacin and ciprofloxacin, and at 295 nm for ofloxacin, levofloxacin, *R*-(+)-ofloxacin,

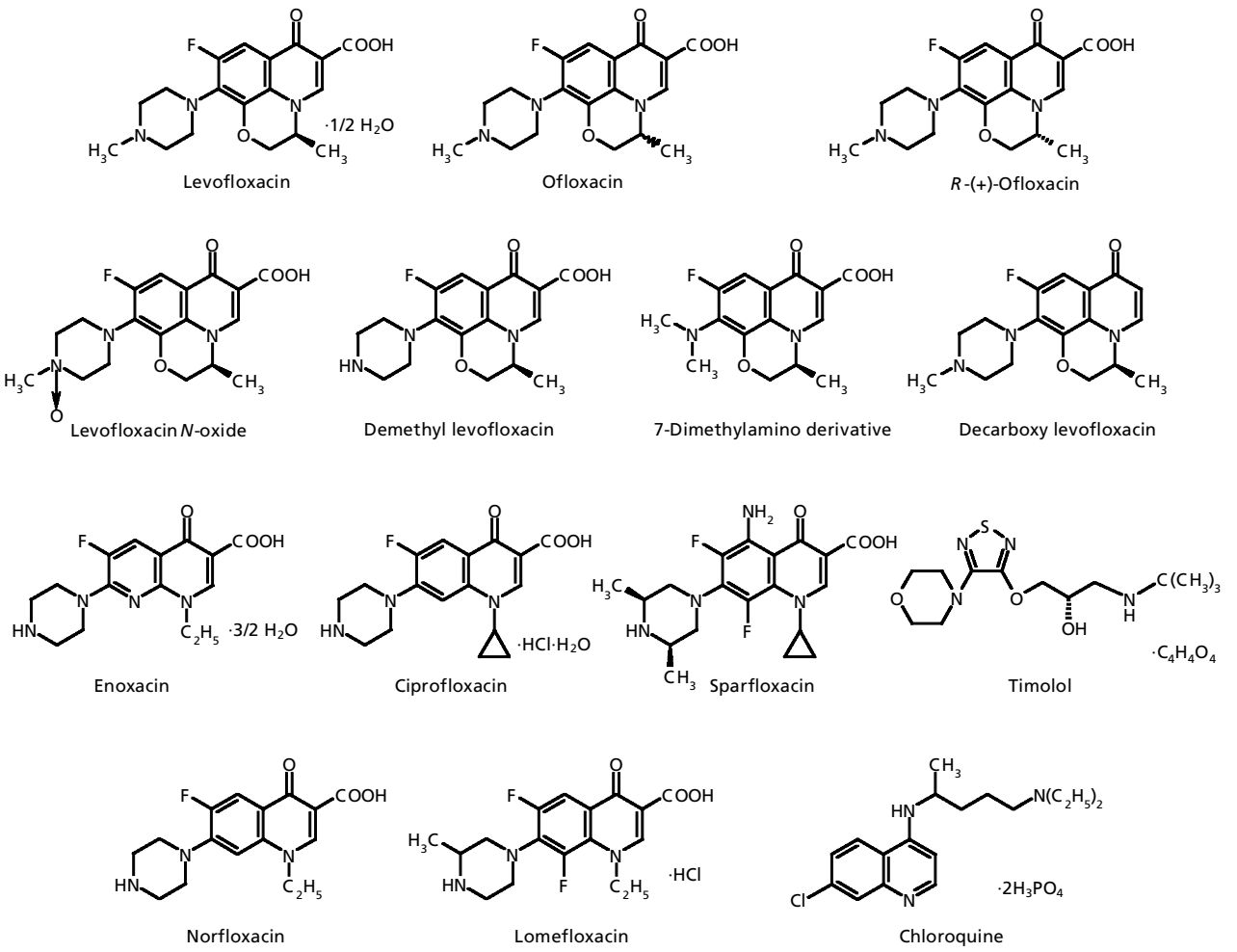

Figure 1 Chemical structures of the test compounds.

demethyl levofloxacin, levofloxacin *N*-oxide and timolol. Separation was achieved on a reversed-phase column (TSK-GEL ODS-80 TM, 5 μm, 150 × 4.6 mm i.d.; Tosoh, Tokyo, Japan) using a mobile phase consisting of tetrahydrofuran, 50 mM phosphate buffer (pH 2.0) and 1 M ammonium acetate in the following ratios (v/v): 4:96:1 for chloroquine and decarboxy levofloxacin; 8:92:1 for ofloxacin, levofloxacin, *R*-(+)-ofloxacin, demethyl levofloxacin, levofloxacin *N*-oxide and timolol; 11:89:1 for enoxacin, lomefloxacin and norfloxacin; 14:86:1 for sparfloxacin and ciprofloxacin; and 30:70:0 for the dimethylamino derivative of levofloxacin. The flow rate was 1.0 mL min^{-1}.

Results

Binding of levofloxacin and its derivatives

The binding of levofloxacin to synthetic melanin as a function of incubation time was investigated. It was found that the amount of bound levofloxacin reached almost a plateau within 16 h. Therefore, the incubation time was set at 16 h for each compound.

The binding characteristics of racemic ofloxacin, *S*-(−)-ofloxacin (levofloxacin), *R*-(+)-ofloxacin, demethyl levofloxacin, levofloxacin *N*-oxide, decarboxy levofloxacin and dimethylamino derivative of levofloxacin to synthetic levodopa melanin were investigated with increasing concentrations of drugs in a fixed volume of potassium phosphate buffer (pH 7.4) at 37 °C. The affinity and capacity of the drug binding were calculated by Langmuir's adsorption isotherm (Table 1; Figures 2 and 3). The K values of racemic ofloxacin and its enantiomers ranged from 2.05×10^4 to 3.64×10^4 M^{-1} and the r_{max} values for the compounds ranged from 2.81×10^{-7} to 4.47×10^{-7} mol mg^{-1}. These compounds showed similar affinity and capacity for synthetic melanin. Compared with levofloxacin, demethyl levofloxacin showed higher K values (1.03×10^5 M^{-1}) and similar r_{max} values (2.54×10^{-7} mol mg^{-1}). The K and r_{max} values of decarboxy levofloxacin were similar to those of levofloxacin.

Table 1 Linear regression parameters for the Langmuir treatment of binding of compounds to melanin.

Drug	Slope $\times 10^2$ ($mg\,L^{-1}$) (lower/upper limit)[a]	Intercept $\times 10^6$ ($mg\,mol^{-1}$) (lower/upper limit)[a]	r	r_{max} ($mol\,mg^{-1}$)	Affinity (K) (M^{-1})
Levofloxacin	1.09 (0.94/1.25)	2.24 (0.61/3.87)	0.997	4.47×10^{-7}	2.05×10^4
R-(+)-Ofloxacin	0.98 (0.82/1.13)	3.55 (1.86/5.25)	0.996	2.81×10^{-7}	3.64×10^4
Ofloxacin	1.13 (0.97/1.28)	2.67 (1.08/4.27)	0.997	3.74×10^{-7}	2.37×10^4
Demethyl levofloxacin	0.38 (0.31/0.46)	3.94 (2.38/5.51)	0.994	2.54×10^{-7}	1.03×10^5
Levofloxacin *N*-oxide	7.09 (4.69/9.48)	11.50 (−1.27/24.28)	0.983	0.87×10^{-7}	1.62×10^4
Decarboxy levofloxacin	0.61 (0.47/0.75)	2.07 (−0.15/4.29)	0.992	4.83×10^{-7}	3.37×10^4
Timolol	1.93 (1.40/2.46)	4.28 (0.21/8.35)	0.989	2.33×10^{-7}	2.22×10^4
Chloroquine	0.02 (0.019/0.022)	0.88 (0.75/1.01)	0.999	1.13×10^{-6}	4.26×10^5

[a]95% confidence intervals calculated at $P = 0.05$. r, correlation coefficient; r_{max}, maximum moles bound per milligram of melanin.

On the other hand, levofloxacin *N*-oxide showed lower K and r_{max} values compared with those of levofloxacin, and dimethylamino derivative of levofloxacin showed too low affinity to calculate the parameters. The results obtained indicated that the basic nitrogen atom at position 7 of the quinolone ring would play a critical role in the interaction of quinolones with melanin.

The K and r_{max} values of chloroquine were $4.26 \times 10^5\ M^{-1}$ and $1.13 \times 10^{-6}\ mol\,mg^{-1}$, respectively, and those for timolol were $2.22 \times 10^4\ M^{-1}$ and $2.33 \times 10^{-7}\ mol\,mg^{-1}$, respectively. Chloroquine showed much higher binding affinity and capacity than the other compounds tested.

Recovery of levofloxacin and chloroquine from melanin–drug complexes

The melanin–drug complexes were washed three times with various solutions in order to explain the nature of the interaction between melanin and the drugs. The elution profiles for melanin–levofloxacin and melanin–chloroquine complexes are shown in Figure 4. Approximately 38%, 34% and 47% of levofloxacin was released when drug-bound melanin was washed with neutral phosphate buffer, EtOH and 1 M HCl, respectively. Surprisingly, the chloroquine bound to melanin was completely eluted by washing with 1 M HCl, whereas only 2.2% and 15.6% of the drug was recovered after washing with neutral phosphate buffer and EtOH, respectively.

Binding of various quinolones

The in-vitro interaction of various fluoroquinolone antimicrobial agents such as norfloxacin, enoxacin, sparfloxacin, ciprofloxacin and lomefloxacin with melanin were studied. Timolol and chloroquine were used as positive controls. The results showed that the relative K value was: chloroquine ≈ ciprofloxacin, sparfloxacin ≥ lomefloxacin > timolol, levofloxacin ≈ enoxacin and norfloxacin; the relative r_{max}

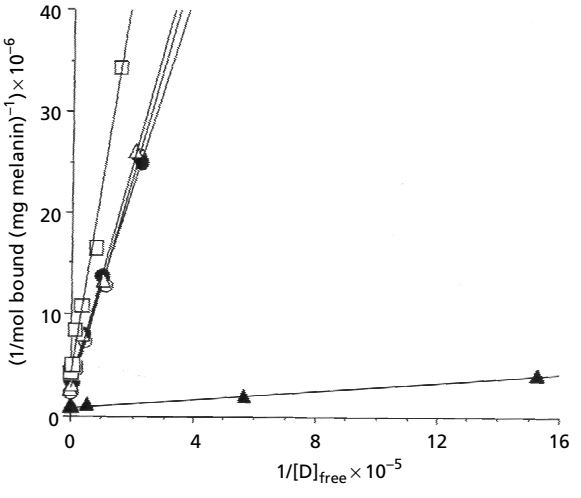

Figure 2 Langmuir plots: reciprocal of amount of drug bound versus reciprocal of molar free drug concentration for levofloxacin (○), *R*-(+)-ofloxacin (●), racemic ofloxacin (△), timolol (□) and chloroquine (▲).

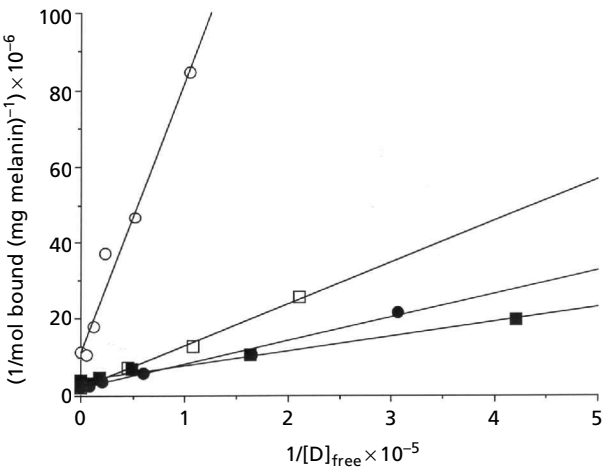

Figure 3 Langmuir plots: reciprocal of amount of drug bound versus reciprocal of molar free drug concentration for levofloxacin (□), demethyl levofloxacin (■), levofloxacin *N*-oxide (○) and decarboxy levofloxacin (●).

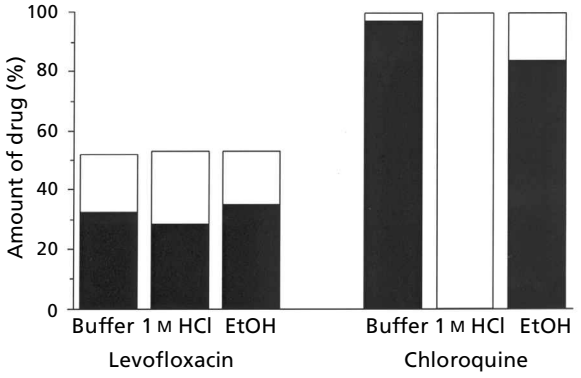

Figure 4 Elution of levofloxacin and chloroquine bound to melanin by washing with phosphate buffer (pH 7.4), 1 M HCl or EtOH. Drug concentration: 50 μM; melanin concentration: 200 μg mL^{-1}. Open bars, eluted fraction; closed bars, bound fraction.

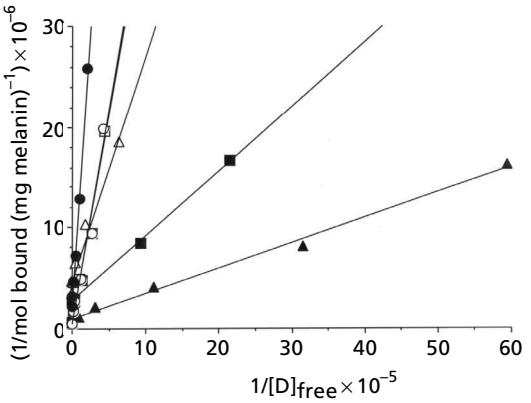

Figure 5 Langmuir plots: reciprocal of amount of drug bound versus reciprocal of molar free drug concentration for levofloxacin (●), norfloxacin (○), enoxacin (□), sparfloxacin (▲), ciprofloxacin (■) and lomefloxacin (△).

Table 2 Linear regression parameters for the Langmuir treatment of binding of fluoroquinolones to melanin.

Drug	Slope × 10^2 (mg L^{-1}) (lower/upper limit)[a]	Intercept × 10^6 (mg mol^{-1}) (lower/upper limit)[a]	r	r_{max} (mol mg^{-1})	Affinity (K) (M^{-1})
Levofloxacin	1.09 (0.94/1.25)	2.24 (0.61/3.87)	0.997	4.47×10^{-7}	2.05×10^4
Sparfloxacin	0.025 (0.021/0.029)	0.96 (−0.14/2.07)	0.997	1.04×10^{-6}	3.84×10^5
Ciprofloxacin	0.06 (0.05/0.08)	2.78 (1.24/4.33)	0.992	3.59×10^{-7}	4.32×10^5
Lomefloxacin	0.22 (0.14/0.3)	4.59 (2.16/7.02)	0.981	2.18×10^{-7}	2.05×10^5
Norfloxacin	0.42 (0.23/0.61)	0.46 (−3.88/4.81)	0.971	2.16×10^{-6}	1.10×10^4
Enoxacin	0.41 (0.28/0.45)	0.59 (−2.40/3.59)	0.986	1.69×10^{-6}	1.44×10^4

[a]95% confidence intervals calculated at $P = 0.05$. r, correlation coefficient; r_{max}, maximum moles bound per milligram of melanin.

was: norfloxacin, enoxacin ≥ chloroquine, sparfloxacin > levofloxacin, ciprofloxacin, timolol, lomefloxacin (Figure 5; Tables 1 and 2). Ciprofloxacin and sparfloxacin showed stronger interaction with melanin than the other fluoroquinolones studied.

Discussion

The term melanin is a purely descriptive one with no chemical meaning. It only denotes a black pigment of biological origin. Melanin is synthesized by the oxidation of tyrosine in specialized cellular vesicles (melanosomes). Melanin is found in various tissues of animals and humans, including eye, inner ear, hair, hair follicles and brain. Whereas many compounds have an affinity for melanin (Potts 1964; Lindquist 1973), only a few of these substances, such as chloroquine, substituted phenothiazines, quinine and streptomycin, seem to cause toxicity. It is suggested that the presence of ocular pigment is a necessary, but not the sole, prerequisite for toxic activity (Ings 1984; Koneru et al 1986; Salazar-Bookaman et al 1994; Leblanc et al 1998). The question of association between drug accumulation and toxic action remains open.

Melanins are polyanions with a relatively high content of negatively charged carboxyl groups and *o*-semiquinones (Ings 1984; Larsson 1993). Electrostatic forces have been reported to play an important role in the binding of drugs to melanin, however non-electrostatic contributions, including hydrophobic and van der Waals' interactions and charge transfer reaction would also contribute to the binding for drugs such as chlorpromazine and chloroquine (Larsson & Tjälve 1979; Atlasik et al 1980; Stepien & Wilczok 1982; Ings 1984; Larsson 1993).

It has been reported that fluoroquinolones such as ofloxacin, levofloxacin, lomefloxacin and moxifloxacin show high affinity for melanin and pigmented tissues (Fukuda & Sasaki 1989, 1990; Kurata et al 1991; Siefert et al 1999; Fukuda et al 2000). However, the nature of the interaction between melanin and has not been fully characterized. In the present study, synthetic levodopa melanin was prepared and the binding affinity and capacity of fluoroquinolones were investigated.

The binding of levofloxacin and its derivatives to synthetic melanin was investigated to elucidate the nature of

the binding. The compounds tested had a varying capacity and affinity for binding to synthetic melanin. Racemic ofloxacin, *R*-(+)-ofloxacin and levofloxacin showed similar binding affinities and capacities, which suggested that the binding lacked stereoselectivity. The binding affinities and capacities of decarboxy levofloxacin were similar to those of levofloxacin, showing that the carboxyl group would not play an important role in the binding of fluoroquinolones to synthetic melanin. Demethyl levofloxacin showed higher affinity and similar capacity compared with levofloxacin. On the other hand, levofloxacin *N*-oxide showed lower affinity and capacity than levofloxacin, and the dimethylamino derivative of levofloxacin showed too low affinity to calculate the parameters. Both compounds lacked a basic aliphatic amino group at position 7 of the fluoroquinolone ring. The results obtained indicated that the basic nitrogen atom of the piperidine ring of levofloxacin would play a critical role in the interaction with melanin. This indicates that the binding involves electrostatic attraction between the protonated nitrogen atom of fluoroquinolone and carboxyl groups of melanin.

The affinities of chloroquine and timolol were approximately 21-fold greater than, and almost equal to, that of levofloxacin, respectively. Thus, levofloxacin showed much lower affinity compared with chloroquine, and similar affinity to timolol.

In the present study, recovery of chloroquine and levofloxacin from melanin–drug complexes proved that these bindings were reversible. The washing of the melanin–drug complexes with various solvents allowed the nature of the binding between the drugs and melanin to be explained. The elution of the drug by neutral buffer solution and EtOH would suggest participation of van der Waals' and hydrophobic interactions, respectively. Recovery of the drugs by strongly acidic 1 M HCl solution would indicate that electrostatic force could play an important role in the complex formation. The chloroquine bound to melanin was almost completely eluted by washing with 1 M HCl, whereas only a small amount of the drug was recovered after washing with neutral phosphate buffer and EtOH. These results indicate that electrostatic interactions between the protonated amino group of chloroquine and the carboxyl groups of melanin would play a critical role in the drug–melanin complex formation.

The binding characteristics of levofloxacin were found to be different from those of chloroquine. Approximately 38%, 34% and 47% of levofloxacin bound to melanin was released when the drug-bound melanin was washed with neutral phosphate buffer, EtOH or 1 M HCl, respectively. These results suggest that hydrophobic and van der Waals' interactions also operate to form the drug–melanin complex in addition to electrostatic interactions. Hydrophobic and van der Waals' interactions are much weaker than electrochemical interactions. Compared with chloroquine, levofloxacin bound to melanin was released to a much greater extent by washing with neutral phosphate buffer, which is close to physiological conditions. This suggests that the levels of levofloxacin in melanin-containing tissues would decline more rapidly compared with chloroquine in-vivo.

Among the fluoroquinolones tested, ciprofloxacin and sparfloxacin showed relatively stronger interactions with melanin. The turnover of ocular melanin is very low (Ings 1984). Therefore, it is possible that ciprofloxacin and sparfloxacin might accumulate in melanin-containing ocular tissues after multiple dosing to a greater extent than other fluoroquinoles. However, to our knowledge, no report has been published on ocular tissue distribution after repeated administration of ^{14}C-labelled fluoroquinolones in pigmented animals.

It has been reported that prolonged high dosage of chloroquine (0.25 g or more daily for several years) is usually necessary before ocular toxicity develops (Böke et al 1967). The low dosage associated with antimalarial treatment does not normally produce noticeable ocular changes (Goldman & Preston 1957; Hobbs et al 1959). It has been reported that drug-related toxic effects on the retina described in humans and animals were unrelated to melanin binding, and that melanin binding and retinal toxicity were two separate entities, the latter being related to the intrinsic toxicity of the compound rather than its ability to bind (Leblanc et al 1998). It would be unlikely that levofloxacin would cause toxicity because of its weaker interaction with melanin and shorter duration of therapy compared with chloroquine.

Conclusions

The interactions of fluoroquinolones, including levofloxacin, and positive controls (timolol and chloroquine), with synthetic levodopa melanin were investigated in-vitro. Levofloxacin showed much lower affinity compared with chloroquine and similar affinity to timolol. The binding characteristics of levofloxacin were different from those of chloroquine. Electrostatic interactions between the protonated amino group of chloroquine and the carboxyl groups of melanin would play a critical role in the drug–melanin complex formation. On the other hand, weak hydrophobic and van der Waals' interactions would also operate to form the levofloxacin–melanin complex as well as electrostatic interactions, suggesting that the accumulation of levofloxacin in melanin-containing tissues would be lower than chloroquine in-vivo.

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