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ELECTRON SPIN RESONANCE STUDIES OF CHLOROQUINE–MELANIN COMPLEXES*

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Abstract—Electron spin resonance (ESR) studies of chloroquine complexes with synthetic DOPA-melanin, synthetic catechol-melanin, melanin isolated from bananas and humic acid were performed. Two chloroquine concentrations (5×10^{-5} M and 1×10^{-3} M) were used for chloroquine-melanin complex formation. ESR studies showed differences in free radicals concentration depending on melanin origin. It was demonstrated that addition of chloroquine to melanin results in broadening of the melanin ESR signal and is accompanied by changes in the integrated intensity of the analyzed melanin samples.

A great affinity of many drugs, e.g. chloroquine and its derivatives, to melanin containing tissues is commonly known [1]. However, not many data about the nature and interaction mechanisms exist in literature.

Potts [2, 3] suggested, that drug-binding to melanin was due to charge-transfer reaction. The free radicals in melanin act like electron traps for electrons from the highest filled orbital of the donor chloroquine molecule to the lowest empty orbital of melanin acceptor. Such transitions do not involve any structural changes in classical chemical terms. In chloroquine–melanin interaction both ionic bonds and van der Waals forces exist. However, the possibility of hydrophobic interaction in drug-binding to melanin was also considered [1].

More complete analysis of the chloroquine–melanin complex was presented by Stepień *et al.* [4–6], where it was shown that two classes of binding sites exist in such complexes: strong binding sites with the association constant $K_1 \sim 10^5\ M^{-1}$, and weak binding sites with $K_2 \sim 10^4\ M^{-1}$. These binding sites represent the hydrophobic interaction and electrostatic attraction between the protonated nitrogens of chloroquine and the anionic groups of melanins. The contribution of van der Waals forces at the conjunction of the aromatic rings of the drug and the aromatic indole-nuclei of the melanin was also postulated [6].

Our studies were designed to investigate chloroquine (Fig. 1) binding ability to melanin of various origin, showing different chemical and space structure and to characterize the obtained complexes by the use of ESR spectroscopy.

The following melanin samples have been used:

(a) Synthetic DOPA-melanin as a reference material, which drug binding mechanism was previously described by Stepień and Wilczok [6].

(b) Synthetic catechol-melanin free from carboxy groups, of partially determined chemical structure [7].

(c) Melanin from bananas as an example of natural melanin, which after deproteinization with hydrochloric acid contains some humic substances effecting the drug-binding ability.

(d) Humic acid with physicochemical properties similar to melanin for demonstration its influence on chloroquine binding to melanin isolated from natural sources.

MATERIALS AND METHODS

Materials

Synthetic DOPA-melanin was formed by oxidative polymerization of 3,4-dihydroxyphenylalanine

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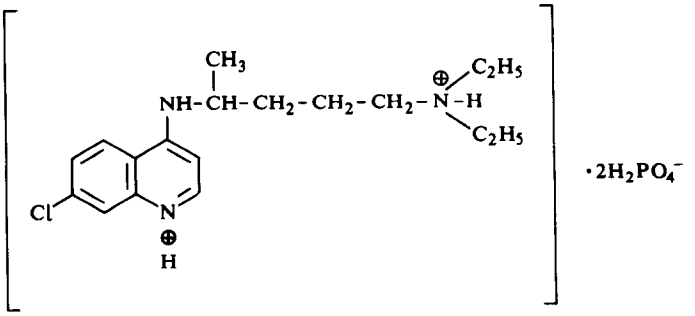

Fig. 1. Structural formula of chloroquine diphosphate.

(DOPA) at pH 8.0 according to the method described by Binns *et al.* [8].

Synthetic catechol-melanin was obtained from catechol in similar manner as DOPA-melanin.

Natural melanin from bananas was isolated from dried banana peels by treatment with concentrated hydrochloric acid at room temperature for two weeks. The insoluble residue was washed with water and then refluxed with 6 N HCl for 24 hr. The obtained dark brown pigment was washed with distilled water and dried over phosphorus pentoxide.

Humic acid was formed by refluxing saccharose with 6 N HCl for 48 hr. The obtained brown pigment was washed with distilled water and dried as above.

Chloroquine diphosphate was obtained from Polfa, Pabianice, Poland. The drug was dissolved in 0.067 M phosphate buffer solution at pH 7.0.

Chloroquine–melanin complex formation

Binding of chloroquine to various samples of melanin in 0.067 M phosphate buffer at pH 7.0 was studied. 20 mg of melanin were placed in Erlenmayer flasks, where buffer and chloroquine solutions were added to final volume of 40 ml. The initial concentration of drug was 5×10^{-5} M or 1×10^{-3} M. Control samples contained 20 mg of melanin and 40 ml of buffer without chloroquine. All samples were incubated at room temperature for 20 hr. The concentration of chloroquine and incubation time were chosen according to experiments previously described by Atasik *et al.* [4]. After 20 hr the suspensions were filtered and the absorbance of each filtrate measured spectrophotometrically with regard to the control sample.

Determination of chloroquine bound to melanins

The molar extinction coefficient $\varepsilon = 18{,}050$ l/mole cm of chloroquine determined at 343 nm was used to calculate the amount of chloroquine in the chloroquine–melanin complexes. The amount of chloroquine remaining in filtrates after complex removing represents the amount of drug (in μmole) not bound to 20 mg of melanin.

For each sample the complex formation efficiency (in %) was determined as the ratio of chloroquine (μmole) bound to melanin, to the total amount of chloroquine (μmole) added to melanin, multiplied by 100.

For purposes of comparison the quantity of chloroquine bound to melanin was calculated for all analysed complexes as amount of μmoles of chloroquine per 1 mg of melanin.

All spectrophotometric measurements were made using the low range UV Varian spectrophotometer model Cary 118C.

ESR studies of chloroquine–melanin complexes

Chloroquine–melanin complexes dried over phosphorus pentoxide for at least 72 hr were placed in stoppered glass tubes (1 × 50 mm) and examined at room temperature using a Radiopan EPR spectrometer model SEX-201 (Radiopan, Poland) with resonant cavity type TE_{102} at X band at microwave power $P = 15$ mW.

For purposes of comparison the following values of ESR derivative spectra were determined:

(a) ΔH_{max} [mT] is the line width measured between points of maximum slope of first derivative ESR spectrum,

(b) $I'_{o\,rel}$ [%] is the relative intensity of ESR derivative signal calculated as:

$$I'_{o\,rel}[\%] = \frac{I'_{oMEL}[mm]}{I'_{oIS}[mm]} \times 100,$$

where I'_{oMEL} [mm] is the peak to peak height of ESR derivative spectrum of melanin, and I'_{oIS} [mm] is the peak to peak height of fourth hyperfine structure signal of intensity standard (IS) used (0.01 M $MnSO_4$).

(c) I_{INT} is the integrated intensity of ESR derivative spectrum of melanin calculated using a simplified

Table 1. Amounts of chloroquine (CHQ) bound to melanins of various origin

Examined chloroquine–melanin complex	Complex formation efficiency [%]	μmole of CHQ bound to 1 mg of melanin
DOPA-melanin + CHQ (5×10^{-5} M)	94.5	0.09
DOPA-melanin + CHQ (1×10^{-3} M)	38.0	0.76
Catechol-melanin + CHQ (5×10^{-5} M)*	95.0	0.10
Catechol-melanin + CHQ (1×10^{-3} M)	25.7	0.51
Banana-melanin + CHQ (5×10^{-5} M)	92.6	0.09
Banana-melanin + CHQ (1×10^{-3} M)	21.5	0.43
Humic acid + CHQ (5×10^{-5} M)	71.6	0.07
Humic acid + CHQ (1×10^{-3} M)	13.4	0.27

* Catechol-melanin shows some solubility in phosphate buffer solution and the examination of dark filtrate after complex removing was impossible. For complex formation catechol–melanins were kept in buffer solution for a period of two weeks. The data presented for chloroquine concentration 5×10^{-5} M concern the residue insoluble in buffer. At higher chloroquine concentration (1×10^{-3} M) the solubility of catechol-melanin significantly decreased.

Table 2. ESR data for chloroquine (CHQ)–DOPA-melanin complexes

Examined sample	Line width ΔH_{max} [mT] ± 0.02	Relative intensity $I'_{o\,rel}$ [%]
DOPA-melanin	0.64	242.9 ± 39.8
DOPA-melanin + buffer	0.60	360.0 ± 59.0
DOPA-melanin + CHQ (5×10^{-5} M)	0.63	195.7 ± 32.1
DOPA-melanin + CHQ (1×10^{-3} M)	0.63	240.0 ± 39.4

expression [9, 10]:

$$I_{INT} = (\Delta H_{max})^2 \times I'_{o\,rel}.$$

It has been demonstrated by Tollin *et al.* [10] that the line width square multiplied by the signal intensity (i.e. the integrated intensity) is proportional to free radical content in melanin.

RESULTS

Investigating the amount of chloroquine bound to each melanin sample the molar extinction coefficient of chloroquine in 0.067 M phosphate buffer at pH 7.0 was determined at wavelength 343 nm where chloroquine exhibits its double absorption peak. The obtained ε_{343} value chosen for all calculations was 18,050 l/mole cm.

The amounts of drug bound to melanin samples and complex formation efficiencies are presented in Table 1. All results are given for two different concentrations of added chloroquine: 5×10^{-5} M and 1×10^{-3} M.

The obtained results show that at lower chloroquine concentration the amounts of chloroquine (CHQ) bound to melanins were very similar (0.09–0.10 μmole CHQ per 1 mg melanin) for synthetic DOPA-melanin, catechol-melanin and for natural banana-melanin. A little smaller amount of chloroquine (0.07 μmole CHQ per 1 mg melanin) was bound to the humic acid sample.

A twenty-fold increase of the initial chloroquine concentration (1×10^{-3} M) shows differences in complex formation depending on melanin origin. The efficiency of chloroquine-melanin complex formation decreased in the order: DOPA-melanin > catechol-melanin > banana-melanin > humic acid. The above data also show that chloroquine binding to melanin depends on drug concentration but is not directly proportional. At low concentration of chloroquine the complex formation efficiency is high. At increased concentration the absolute amount of bound chloroquine is higher but the relative amount decreases. This may suggest that chloroquine is bound to melanin up to complete saturation of all binding sites present in the biopolymer. The amount of these binding sites depends on the melanin origin. The results are in good agreement with data described previously by Stepień and Wilczok [6] concerning the mechanism of chloroquine binding to synthetic DOPA-melanin.

ESR measurements were carried out for chloroquine-melanin complexes and also for untreated dry melanins and for control samples, where the suspension of analysed melanin in buffer solution without chloroquine was dried before measurements. ESR data of chloroquine–melanin complexes are presented in Tables 2–5 and in Fig. 2.

The presented results demonstrate that all analysed melanin samples were characterized by differ-

Table 3. ESR data for chloroquine (CHQ)–catechol-melanin complexes

Examined sample	Line width ΔH_{max} [mT] ± 0.02	Relative intensity $I'_{o\,rel}$ [%]
Catechol-melanin	0.48	137.4 ± 42.9
Catechol-melanin + buffer*	—	—
Catechol-melanin + CHQ (5×10^{-5} M)*	—	—
Catechol-melanin + CHQ (1×10^{-3} M)	0.59	80.8 ± 25.2

* ESR measurements of catechol-melanin suspended in buffer solution and chloroquine (5×10^{-5} M)–catechol-melanin complex in consequence of samples solubilization were impossible to carry out.

Table 4. ESR data for chloroquine (CHQ)–banana-melanin complexes

Examined sample	Line width ΔH_{max} [mT] ± 0.02	Relative intensity $I'_{o\,rel}$ [%]
Banana-melanin	0.73	6.1 ± 0.5
Banana-melanin + buffer	0.80	11.7 ± 0.9
Banana-melanin + CHQ (5×10^{-5} M)	0.83	10.4 ± 0.8
Banana-melanin + CHQ (1×10^{-3} M)	0.82	10.6 ± 0.8

Table 5. ESR data for chloroquine (CHQ)–humic acid complexes

Examined sample	Line width ΔH_{max} [mT] ± 0.02	Relative intensity $I_{0\,rel}$ [%]
Humic acid	0.73	2.6 ± 0.1
Humic acid + buffer	0.72	5.6 ± 0.2
Humic acid + CHQ (5×10^{-5} M)	0.76	6.9 ± 0.3
Humic acid + CHQ (1×10^{-3} M)	0.80	7.4 ± 0.3

ent values of ESR parameters. The free radical content which is proportional to integrated intensity (I_{INT}) of melanin signals varied significantly depending on melanin origin. The amount of free radicals in non-complexed melanin samples decreased in the same order as the above-mentioned efficiency of chloroquine–melanin complex formation. An increase in free radical content was observed when melanins treated with buffer solution were measured (control samples for complex formation).

For all chloroquine–melanin complexes the slight broadening of melanin signal line width (ΔH_{max}) was observed. The values of relative and integrated intensities of melanin signals also changed in the presence of chloroquine. The chloroquine complexes of DOPA- and catechol-melanins and less these of melanin from bananas show parameters of lower values in comparison to control samples, while humic acid complexes show increased integrated intensity after chloroquine binding.

All the results obtained using ESR spectroscopy show that the interaction of chloroquine, which takes place during chloroquine–melanin complex formation, changes the free radical content in melanin samples.

DISCUSSION

The results presented above show, that the ability of chloroquine binding to melanins originated from various sources depends on both melanin chemical structure and initial chloroquine concentration in complexing solutions. These results do not fully confirm the previous reports [4–6] where the important contribution of free carboxy groups in chloroquine binding to melanin was postulated. It was shown [6], that melanin methyl ester (obtained by the action of methanol saturated by gaseous HCl) has smaller ability for drug binding than nonmethylated polymers. The blocking of free carboxy acid groups by methanol decreases the number of weakly reacting sites [6].

However in our experiments synthetic catechol-melanins which, like melanin methyl esters, are free from carboxy groups [7] envinced similar chloroquine binding ability like synthetic DOPA-melanin at drug concentrations 5×10^{-5} M (Table 1). At that concentration the melanin from banana probably containing certain humic substances as well as the humic acid sample show drug binding abilities similar to synthetic melanins. In such samples phenol and

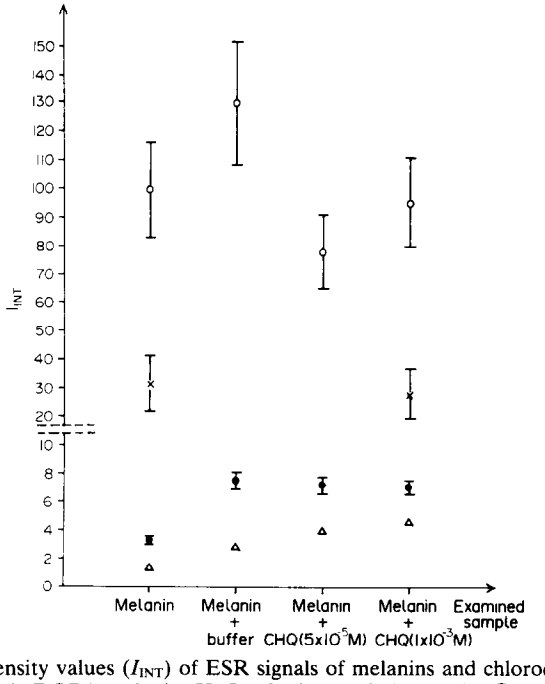

Fig. 2. Integrated intensity values (I_{INT}) of ESR signals of melanins and chloroquine (CHQ)–melanin complexes. ○, Synthetic DOPA-melanin; X, Synthetic catechol-melanin; ●, natural banana-melanin, △, humic acid.

quinoid hydroxy groups and also carboxy groups are present [7].

In previous works [5, 6] has been shown that at higher chloroquine concentration (1×10^{-3} M) an almost complete drug saturation of DOPA-melanin was achieved. For dopamine-melanin and melanin isolated from bovine eyes this saturation has got at lower chloroquine concentration [5].

Our studies confirm the fact, that depending on melanin origin, the saturation of all binding sites occurred at various chloroquine concentrations, what suggests that the analyzed melanins differ in structure and composition. The chloroquine amount bound to the analyzed samples decreases in the order: synthetic DOPA-melanin (0.76 μmole/mg) > synthetic catechol-melanin (0.51 μmole/mg) > melanin from banana (0.13 μmole/mg) > humic acid (0.27 μmole/mg) (Table 1).

ESR measurements were performed at X band what caused necessity of dry powder samples use. At this band frequencies a great nonresonant microwave absorption by water occurs [9]. As it was shown, the ESR parameters values of chloroquine–melanin complexes alter depending on melanin origin. The values of integrated intensity corresponding to the free radical content in melanin samples [10] are higher for melanins treated with buffer solution, i.e. for samples used as controls during chloroquine–melanin interaction. It could be supposed that this is connected with melanin ion-exchange properties [11, 12]. Generally, the ability of H^+ and OH^- ions exchange for Na^+, K^+ and $H_2PO_4^-$ ions, results from sodium and potassium phosphate dissociation when added into the neutral medium (pH 7.0) but such explanation does not take into account that both DOPA- and catechol-melanins were synthetized in phosphate buffer. The sole difference was only the neutral pH value (pH 7.0) at complexation with chloroquine as compared with pH 8.0 during melanin synthesis.

The slightly broadenings of melanin ESR signals and changes of intensities values obtained after chloroquine administration are the evidence for free radical content changes in comparison to control samples. Such changes exhibit the melanin free radicals equilibrium disturbance in the presence of the analyzed drug.

The obtained data suggest that the most important chloroquine binding sites in melanin are free ortho-semiquinone hydroxy groups responsible for the free radical content in melanin [7, 13]. Participation of the semiquinone hydroxy groups in drug–melanin complex formation in light of free radical content changes seems to be unquestionable. These units mutual arrangement and their relations to other monomeric groups of the polymer determine the binding sites accessibility. In these complexes certain state of equilibrium and drug saturation is probably fixed as postulated previously [5, 6].

Summarizing, the presented data demonstrate that except hydrophobic, ionic and van der Waals interactions described earlier [6], also free radicals of hydroxy semiquinone groups in melanin, i.e. charge-transfer reactions participate in chloroquine-melanin complex formation.

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