TOXICOLOGICAL STUDIES ON THE CHLOROQUINE-MELANIN AFFINITY IN VIVO AND IN VITRO IN RELATION TO THE CHLOROQUINE RETINOPATHY1,2

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Summary - Using whole body- and micro-radioautography it was found that radioactivity was specifically retained for more than a month following an i.v. injection of 14C-chloroquine in the retinal pigment epithelium of pigmented rats and guinea-pigs, but not in that of albino animals. By a long term oral administration of chloroquine the intensive accumulation of melanin granules in the pigment epithelium and also in the choroid of the pigmented guinea-pig was observed. Using synthetic melanin, chloroquine was found to be adsorbed by melanin in two modes; one operates in low chloroquine concentrations and is extremely strong and independent to pH, and the other operates in higher chloroquine concentrations and is pH-dependent and chloroquine can be released by washing.

Since Hobbs et al. (1959) reported the toxic retinopathy caused by chloroquine [7-chloro-4-(4'-diethylamino-1'-methyl-butylamino)-quinoline], a great number of works on this toxicity have been published. This retinopathy advances after the discontinuation of the drug (Meier-Ruge, 1973) and is irreversible in most cases (Hobbs et al., 1959). The strong affinity of chloroquine to melanin and resulting long lasting retention in the retina explain the etiology of the retinopathy, and which, at the same time, are the assumption of experimental studies on retinal toxicity of the drug, such as those on cytotoxic actions of chloroquine in the pigment epithelium recently reported by Gonasun and Potts (1974). Neither the precise site of retention of chloroquine in the retina nor the detailed kinetics of binding of chloroquine to melanin, however, is known. In this paper we provide, therefore, the results of histopathological investigations using whole body- and micro-radioautography and of closer investigations on the kinetics of the binding using synthetic melanin.

MATERIALS AND METHODS

Whole body radioautography with 14C-chloroquine in pigmented and albino rats. Pigmented male rats of the ACI strain and albino male rats of the Sprague-Dawley strain both weighing about 100 g were used. Chloroquine-3-14C (quinoline ring) with a specific activity of 2.36 mCi/mM was obtained from NEN, Boston, Massachusetts, U.S.A. and was dissolved as diphosphate in saline. Whole body radioautography was carried out according to the method described by Lindquist et al. (1972). 14C-Chloroquine, 5.2 μCi (10 mg/kg), was injected into the femoral vein. Twenty four hours and 1 month after injection, animals were anesthetized with ether and were killed by immersion into a mixture of solid CO2 and acetone. Radioautograms were made by apposition of the sections that were cut into 25 μ thick, to Sakura X-ray films

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2 Presented in part at the 1st meeting for the study of toxic effect in Tokyo, in February 1975.
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Microradioautography with $^{14}$C-chloroquine in the pigmented and the albino guinea-pig. Pigmented and albino male guinea-pigs, 200 g in body weight, were given 8.3 µCi of $^{14}$C-chloroquine (5.8 mg/kg) via the femoral vein. One month after injection, animals were anesthetized with ether and the eyes were enucleated and excised out. The specimens dissected from the eyes were freeze-dried and embedded in paraffin and sectioned 9 µ thick. Sections were stained with hematoxylin and eosin, and then put upon Sakura X-ray films. After exposure for a week, the films were once separated from the sections, then developed and fixed, and put upon the same position of the section as before. The silver granules were observed with light microscope.

Prolonged oral administration of chloroquine to the pigmented guinea-pigs. Pigmented male guinea-pigs, 250 g in body weight, were given chloroquine diphasate (Ono pharmaceutical Co.) dissolved in drinking water in doses of 26 ± 7 mg base/animal/day for 1 month or 3 months. Sections of the eyes for histopathological examination were prepared according to the method described above.

Binding reaction between chloroquine and synthetic melanin. Synthetic melanin was prepared from L-DOPA by the action of tyrosinase according to the method described by Potts (1964).

Binding reactions were performed as follows. One ml of synthetic melanin suspension containing 5 mg of melanin, 0.5 ml of buffer (1/10 M acetate buffer, pH 3.8 and 5.0; 1/15 M phosphate buffer, pH 6.0; 1/30 M phosphate buffer, pH 7.0 and 8.0; 1/20 M Na$_2$B$_4$O$_7$-Na$_2$CO$_3$ buffer, pH 9.6 and 10.4; ionic strength was kept constant at 0.1), 1 ml of water, and 1 ml of each concentration of chloroquine solution were mixed and incubated for a given period at 37°C.

After incubation, mixtures were immediately centrifuged at 35,000 × g for 10 min and an appropriate aliquot of the supernatant was removed for determination of chloroquine.

For the measurement of releasing rate of chloroquine from melanin, they were resuspended in 3.5 ml of phosphate buffer, pH 6.0, and incubated for a given period, and the released chloroquine was measured as described above. This procedure was repeated 4 times for each melanin precipitate. Duration of incubation were 3 × 15 min and final 18 hr. Chloroquine was determined fluorimetrically with Hitachi spectrophotofluorometer model MPF-2A at fluorescence wave length of 375 mp and activation wave length of 330 mp.

RESULTS

Whole body radioautography of $^{14}$C-chloroquine in the pigmented and the albino rat. Thirty min after the injection of labeled chloroquine, strong radioactivities were observed in liver, kidneys, adrenals, lungs, intestine and Harder's glands of both pigmented and albino rats, and also in uveal tract of pigmented rats. In the pigmented rats, the radioactivity disappeared rapidly from the organs such as heart, lungs and kidneys within 24 hr after the injection, but high activities were recognized in intestine, liver, Harder's glands and the melanin bearing tissues such as hair follicles, interstitial tissues around the brain and uveal tracts of eyes (Fig. 1).

One month after the injection, a strong activity substantially and selectively persisted in melanin bearing tissues especially in uveal tracts of eyes (Fig. 1), whereas any activities were no longer detected in other organs. In albino rats, 24 hr after the injection, the intensity of the radioactivity in visceral organs was similar to that of pigmented rats, but the radioactivity in skin and uveal tract was very low, if any, in contrast to the pigmented rats (Fig. 2). Moreover, 1 month after the injection no radioactivity was detected in any organs and tissues.

Microradioautography with $^{14}$C-chloroquine in the eyes of the pigmented and the albino guinea-pig. One month after $^{14}$C-chloroquine injection, the radioactivity was observed mainly in the pigmented epithelium in the pigmented guinea-pig (Fig. 3), but was not recognized at all in any tissues of the eyes in the albino guinea-pig. It was noteworthy that much less activity was detected in choroid than in pigment epithelium, even when comparable amounts of melanin granules as in the pigment epithelium were seen in the choroid. No difference could be noticed in these situations between the tissues obtained 24 hr and 1 month after the injection.

Degeneration of the pigment epithelium and the choroid in the pigmented guinea-pig eye by prolonged oral administration of chloroquine. The degeneration observed in the guinea-pigs given
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After 24 hours.

After 1 month.

Fig. 1. Whole body radioautography after intravenous administration of \( ^{14}C \)-chloroquine in pigmented male rats.

After 24 hours.

Fig. 2. Whole body radioautography after intravenous administration of \( ^{14}C \)-chloroquine in albino male rats.

chloroquine for a month was the massive accumulation or intensive 'aggregation' of melanin granules in the pigment epithelium. In the animals given chloroquine for 3 months, the 'aggregation' was more pronounced in the choroid than in the pigment epithelium (Fig. 4), and a similar 'aggregation' was observed also in the epithelium of the iris. Degeneration was also seen in the cone-rod layer of the retina and in vessels in the choroid. In turn, no degeneration was observed in the cornea and the sclera which lack melanin granules.

Binding reaction between chloroquine and synthetic melanin. It was first examined the relationship between the amount of chloroquine bound to melanin and the initial chloroquine concentration in the incubation medium (Fig. 5). The binding ratio was almost 100 % up to 185 \( \gamma \)/ml of initial chloroquine concentration and over this concentration, it decreased gradually. It was next examined the time course of binding at two different chloroquine concentrations. As shown in Table 1, although moderately smaller amounts of chloroquine were found after 1 min incubation, significant difference was not found among those incubated for various durations more than 15 min. Then this binding reaction was examined
1 month after $^{14}$C-chloroquine injection.

**Fig. 3.** Micro radioautography after intravenous administration of $^{14}$C-chloroquine in the pigmented guinea-pig’s eye.

Above: The focus of the microscope is fitted on tissues. (× 70)
Below: The focus of the microscope is fitted on the radioactivity. (× 70)
Fig. 4. Degeneration of pigment epithelium and choroid in the pigmented guinea-pig's eye by oral administration of chloroquine.
chloroquine-melanin Affinity

![Graph showing chloroquine-melanin affinity](image)

**Fig. 5.** Isotherm for chloroquine on synthetic melanin

<table>
<thead>
<tr>
<th>Table 1. Time course of chloroquine (C.Q.) and melanin binding</th>
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<tr>
<td>(A) C.Q. initial concn. = 125 μg/ml</td>
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<td>Incubation Time</td>
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<td>1 min</td>
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<th>(B) C.Q. initial concn. = 620 μg/ml</th>
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<td>Incubation Time</td>
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according to the adsorption isotherm of Langmuir. As shown in Fig. 6, in lower equilibrium chloroquine concentrations a linear relationship was obtained (between C and C/V), but the relationship did not apply in higher concentrations.

It was also examined the effect of pH on chloroquine-melanin binding at three different initial chloroquine concentrations. As shown in Fig. 7, when melanin was incubated with 125 μg/ml of chloroquine, amounts of binding was scarcely influenced by pH of the medium, but when incubated with 370 μg/ml of chloroquine, the amounts increased markedly with increasing pH.

To examine the rate of dissociation of chloroquine from melanin, chloroquine-melanin complexes which had been formed in various chloroquine concentrations were incubated in a fresh medium. As shown in Table 2, from complexes formed at lower concentrations than 185 μg/ml, chloroquine was scarcely released, but from complexes formed at a higher concentration than 185 μg/ml it was more easily released.
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\[ C = \text{equilibrium C. Q. conc. ( } \gamma/\text{ml} \text{) } \]

\[ V = \text{bound C. Q. on melanin ( } \gamma/\text{mg melanin} \text{) } \]

Fig. 6. CV- C plot of chloroquine and melanin isotherm

\[ C = \text{Equilibrium chloroquine concentration ( } \gamma/\text{ml} \text{) } \]

Fig. 7. Effect of pH on chloroquine-melanin binding

DISCUSSION

It is known that chloroquine is accumulated and retained for a long time in the uveal tract of the pigmented animal (Bernstein et al., 1963; McChesney et al., 1965; Denker et al., 1973). The present studies in rats with whole body radioautography show that chloroquine is persistently retained in melanin

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Table 2. Release of chloroquine (C.Q.) from C.Q.-melanin complex by washing.

Melanin was incubated with various concentrations of C.Q. at pH 6.0, and was then washed four times with 3.5 ml of buffer, pH 6.0.

<table>
<thead>
<tr>
<th>C.Q. Conc. in Incubation Buffer ((\gamma/ml))</th>
<th>Binding % ((\gamma/mg\text{ Melanin}))</th>
<th>Bound C.Q. ((\gamma/mg\text{ Melanin}))</th>
<th>Released C.Q. from 1 mg Melanin ((\gamma/mg\text{ Melanin}))</th>
<th>Total C.Q. Released ((\gamma/mg\text{ Melanin}))</th>
<th>Remained C.Q. ((\gamma/mg\text{ Melanin}))</th>
</tr>
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<tbody>
<tr>
<td>60</td>
<td>98.9 ± 0.1</td>
<td>42.9 ± 0.4</td>
<td>0.40 ± 0.05, 0.03 ± 0.00, 0.13 ± 0.06, 0.00 ± 0.07</td>
<td>0.56</td>
<td>42.4</td>
</tr>
<tr>
<td>125</td>
<td>99.5 ± 0.2</td>
<td>86.3 ± 0.2</td>
<td>0.33 ± 0.03, 0.17 ± 0.07, 0.40 ± 0.00, 0.17 ± 0.07</td>
<td>1.07</td>
<td>85.3</td>
</tr>
<tr>
<td>185</td>
<td>98.9 ± 0.2</td>
<td>128.7 ± 0.3</td>
<td>1.29 ± 0.20, 0.79 ± 0.17, 2.77 ± 0.21, 0.33 ± 0.03</td>
<td>5.18</td>
<td>123.5</td>
</tr>
<tr>
<td>250</td>
<td>91.3 ± 0.7</td>
<td>158.4 ± 1.3</td>
<td>25.0 ± 0.7, 5.54 ± 0.6, 3.85 ± 0.26, 1.58 ± 0.15</td>
<td>36.0</td>
<td>122.4</td>
</tr>
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bearing tissues. The precise identification of the chloroquine retention in choroid was clearly indicated by microradiography to be in the pigment epithelium. The fact that little radioactivity was found in the pigment granules of the choroid makes it unlikely that the high activity in the pigment epithelium resulted solely from the high affinity of chloroquine to melanin. It might result from difference in intracellular concentration due to some difference in the nature of the membrane as a barrier to chloroquine, although possibilities cannot be ruled out that it is an artifact caused by fixation procedure, and that the pigment cells of choroid are deficient in melanin.

That the histopathological changes similar to human chloroquine-retnopathy were produced in guinea-pigs besides in cats and rabbits (Meier-Ruge, 1973) by long term administration of chloroquine suggests that the disease per se of the patient plays a minor role in the chloroquine-retnopathy. Denker et al. (1973) have recently reported that the antibiotics such as streptomycin and viomycin remained in the inner ear and that these drugs have also the affinity to melanin and assumed that one of the reasons of auditory impediment by these drugs consists in binding of them to melanin in the inner ear.

In vitro studies using synthetic melanin suggested that there may be at least two modes in the binding of chloroquine to melanin (Fig. 7 and Table 2), since in low concentrations the relationship between C and C/V was linear, but not in higher concentrations. The former and the latter were designated here as adsorption, and adsorption, respectively. The heat of adsorption calculated according to B. E. T. isotherm was about 7.5 Kcal/mole, which indicated a strong physical adsorption. In low concentrations, binding was independent to pH of the medium, but in a higher concentration, amount of chloroquine bound on melanin ran pararell with pH. Adsorption may be a binding of non-ionized form of molecules, while adsorption may independent to the form of molecules, ionized or neutral. The long lasting retention of chloroquine in the uveal tract may be explained by the adsorption.

Gonassan and Potts (1974) estimated the blood level of chloroquine in patients after a dose of 250 mg of diphasate to be 1.17 \times 10^{-6} M (about 30 \gamma/ml). If melanin reaches an equilibrium with this concentration of chloroquine, adsorption should mainly operate, but the level is close to the concentration at which adsorption begins to operate; the blood level may exceed the estimated value depending on the dose or the detoxication rate of the patient. PH dependency of adsorption may explain the accelerated excretion of chloroquine by ammonium chloride (Rubin, 1963).

Chloroquine bound on melanin may retard the decay of the intracellular chloroquine level during the interval of repeated doses, resulting selective intoxication of the pigment epithelium at lower doses than those which affect the other cells. It is rather unlikely that the persisting chloroquine on the melanin contributes to the further advance of the retinopathy after the discontinuation of the drug, for the limited amount of chloroquine on the melanin may not be able to maintain effective toxic concentration in the cell for weeks or months, unless some additional mechanism, such as a hypersensitivity to chloroquine, is postulated.

ACKNOWLEDGMENTS. We are grateful to Dr. Y. Fujita for his helpful advice on the adsorption studies.
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REFERENCES


