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STUDIES ON THE BINDING OF CHLORPROMAZINE AND CHLOROQUINE TO MELANIN *IN VIVO*

HANS TJÄLVE, MONICA NILSSON and BENGT LARSSON
Department of Toxicology, University of Uppsala, Uppsala, Sweden

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Abstract—Recent experiments have shown that the complex-formation of chlorpromazine and chloroquine with melanin *in vitro* comprises an electrostatic binding of the positively charged drug molecules to negative groups of the melanin-polymer (probably carboxyl-groups), supplemented by additional forces, which may involve van der Waal's binding or charge-transfer complexes. The aim of the present study was to determine if a similar binding-mechanism may be present *in vivo*. Pigmented mice were injected with [^{35}S]chlorpromazine or [^{14}C]chloroquine. At 1 hr and 1 day the eyes were excised and the ability of cationic solutions to release the radioactivity from the pigment of eye-tissue preparations was determined by liquid scintillation counting. It was found that the cations were able to release the radioactivity from the pigment, with increasing effectiveness at increasing concentrations. The order of the ability of the cations to release the radioactivity was $H^+ > Ca^{2+} > Na^+$. A part of the radioactivity (for [^{35}S]chlorpromazine about 21 per cent, for [^{14}C]chloroquine about 8 per cent) was non-extractable. Similar results were obtained at the 1 hr and 1 day survival intervals. These results indicate that the binding-mechanism described above for the *in vitro* affinity is valid also for the situation *in vivo*.

It is known that many drugs are strongly taken up in melanin-containing tissues and that in these tissues toxic lesions may occur following long-term treatments with the drugs [1–3].

Recent experiments in our department with pigment from beef eyes have shown that electrostatic forces are important for the affinity to melanin [4]. Drugs such as chlorpromazine and chloroquine, which show a strong melanin-binding *in vivo*, are weak bases and present mainly as cations at physiological pH. Melanin is a polymer with an indole nucleus as the main building-stone [5]. The polymer is rich in free carboxyl groups, and presumably an ionic attraction occurs between these groups and the positively charged drug molecules [4]. The experiments with chlorpromazine and chloroquine also indicated that additional binding-forces are operative. These may consist of van der Waal's forces, occurring at the conjunction of the aromatic rings in the drugs and the aromatic rings of the melanin nuclei, or may be due to the formation of charge-transfer complexes, in which the melanin would act as an electron trap. An analysis of the binding to the beef-eye pigment of the bisquaternary ammonium compound paraquat (a herbicide), which is also bound to melanin *in vivo* [6], showed that for this substance the contribution of nonelectrostatic forces is smaller than for chlorpromazine and chloroquine [4]. In addition, results obtained by autoradiographic techniques indicated that ionic binding is involved in the affinity of paraquat to melanin *in vivo* [7]. No such *in vivo* data are as yet available for chlorpromazine and chloroquine. Therefore, in the present study, experiments have been performed to examine the possibility of showing that ionic forces are operative in the binding of these drugs to melanin *in vivo*. Pigmented mice were injected with [^{35}S]chlorpromazine or [^{14}C]chloroquine. The eyes were excised and the ability of various cationic solutions to release the radioactivity from the pigment of the eyes was then determined. Conceivably, the role for the affinity of electrostatic forces relative to other binding-forces might vary depending on the interval elapsed after the administration of the drugs. Therefore experiments have been performed at one short (1 hr) and one long (1 day) postinjection-interval.

MATERIAL AND METHODS

Isotopes. [^{35}S]Chlorpromazine chloride, sp. act. 97.1 μCi/mg, was obtained from the Radiochemical Centre (Amersham, U.K.). The radiochemical purity was about 99 per cent. [^{14}C]Chloroquine diphosphate (ring-3-^{14}C), sp. act. 58.8 μCi/mg, was obtained from New England Nuclear (Boston, MA). The radiochemical purity was about 95 per cent.

Experiments. C57Bl-mice (b.w. 20 g) were injected i.v. with 2 μCi [^{35}S]-chlorpromazine (1.0 mg/kg b.w.) or 2 μCi [^{14}C]chloroquine (1.7 mg/kg b.w.) and were killed by exposure to gaseous carbon dioxide after 1 hr or 1 day. The eyes were excised and sliced open. The cornea, the lens, the aqueous and vitreous humors and the orbital fatty tissue were removed. The remaining parts of the eyes were homogenized manually for 3 min in Tenbroeck tissue grinders (Kontes Glass Co., Vineland, N. J., U.S.A.) in 10 ml distilled water or 10 ml distilled water to which various concentrations of NaCl, $CaCl_2$ or HCl had been added. The homogenized eyes were then transferred together with the grinding solutions to plastic centrifuge tubes and shaken at room-temperature for 1 hr in a shaking-apparatus (Gerhardt, Bonn, West Germany) using about 150 strokes/min. The tubes were then centrifuged at 35,000 *g* for 10 min in an MSE High Speed

Centrifuge. The supernatants were removed and aliquots were taken and the radioactivity determined by liquid scintillation counting in a Packard Tricarb model 2405 liquid scintillation spectrometer using Instagel® (Packard) as scintillation fluid. The pellets were resuspended in 10 ml distilled water and recentrifuged at 35,000 *g* for 10 min. The pellets were then collected and freeze-dried over-night. One ml Soluene-100® (Packard) was added and the material was sonicated for 1 hr in a Bransonic 12 sonifier (Bo Philip AB, Stockholm, Sweden). 0.2 ml 35 per cent H_2O_2 and 0.2 ml isopropanol were then added and the sonication was repeated for 30 min. After that, 15 ml Instagel® was added and the radioactivity was determined by liquid scintillation counting. An external standard was used for quenching corrections. The part of the total radioactivity in the eye material which was present in the pellet was then calculated.

In order to estimate the part of the radioactivity in the eye preparations which was not associated with the melanin-containing cells, albino NMRI mice (b.w. 20 g) were injected i.v. with 2 μCi of [^{35}S]chlorpromazine or [^{14}C]chloroquine. At 1 hr and 1 day, eyes were excised and the eye tissue was prepared in the same way as for the pigmented mice. The tissues were dissolved in 1 ml Soluene 350® (Packard) and the radioactivity determined by liquid scintillation counting after the addition of 10 ml of a scintillation fluid consisting of 4.9 g PPO and 0.1 g dimethyl POPOP/l toluene.

Expression of results. The results are given as means ± S.D. For statistical analysis, Student's *t*-test was used.

RESULTS

The amounts of radioactivity after the administration of [^{35}S]chlorpromazine and [^{14}C]chloroquine in the prepared eye tissues of the albino mice were less than 5 per cent at 1 hr and less than 2 per cent at 1 day of the amounts which were found at the same intervals in the eye tissues of the pigmented mice. In the following text, we therefore designate the radioactivity of the eye tissue preparations of the pigmented mice as pertaining to the melanin-containing cells.

The results obtained from the eye treatments are collected in Table 1. It can be seen that slightly more than 20 per cent of the [^{35}S]chlorpromazine radioactivity was released by the water treatment, whereas for [^{14}C]chloroquine less than 5 per cent was released by this treatment. Similar values were observed at 1 hr and 1 day.

Table 1. The ability of Na^+, Ca^{2+} and H^+ to release radioactivity from eye-pigment of mice injected with [^{35}S]chlorpromazine and [^{14}C]chloroquine*

Survival interval	Treatment	Concentration (M)	Per cent radioactivity bound to melanin after treatment [^{35}S]chlorpromazine	[^{14}C]chloroquine
1 hr	H_2O	—	78.3 ± 4.4	95.8 ± 0.8
1 hr	Na^+	0.01	77.4 ± 3.8	85.5 ± 8.6†
		0.1	70.0 ± 6.0	84.7 ± 3.2§
		1.0	51.1 ± 5.8§ §§	63.0 ± 8.2§ §§
1 hr	Ca^{2+}	0.01	66.6 ± 2.2‡‖	63.0 ± 6.4§‖
		0.1	64.4 ± 2.8‡	57.9 ± 2.8§¶
		1.0	46.9 ± 3.4§‖ ‖‖	41.0 ± 2.0§‖ ‖‖
1 hr	H^+	0.01	31.0 ± 2.4§¶ ††	45.1 ± 2.6§¶ **
		0.1	24.9 ± 2.0§¶ ††§§	20.6 ± 0.8§¶ ††‖‖
		1.0	20.8 ± 2.6§¶ ††‡‡	7.9 ± 0.6§¶ ††‖‖
1 day	H_2O	—	77.0 ± 2.2	96.8 ± 0.4
1 day	H^+	0.01	33.4 ± 5.8§	37.7 ± 1.8§
		0.1	24.4 ± 4.6§‡‡	15.3 ± 0.4§‖‖
		1.0	21.1 ± 2.4§	8.9 ± 0.8§‖‖

* C57Bl-mice were injected i.v. with [^{35}S]chlorpromazine (2 μCi; 1.0 mg/kg b.w.) or [^{14}C]chloroquine (2 μCi; 1.7 mg/kg b.w.) and were killed after 1 hr or 1 day. Eyes were excised. The tissues containing the pigment were isolated, homogenized and treated with distilled water or distilled water containing the indicated concentrations of NaCl, $CaCl_2$, or HCl. After centrifugation, the part of the radioactivity which remained associated with the pigment was determined. Means ± S.D. (n = 4) are given together with the statistical significance obtained by the treatments, as indicated in the footnotes.

† $P < 0.05$ compared with the water treatment.
‡ $P < 0.01$ compared with the water treatment.
§ $P < 0.001$ compared with the water treatment.
‖ $P < 0.01$ compared with the treatment with the same concentration of NaCl.
¶ $P < 0.001$ compared with the treatment with the same concentration of NaCl.
** $P < 0.01$ compared with the treatment with the same concentration of $CaCl_2$.
†† $P < 0.001$ compared with the treatment with the same concentration of $CaCl_2$.
‡‡ $P < 0.05$ compared with the treatment with the nearest lower concentration of the same salt or HCl.
§§ $P < 0.01$ compared with the treatment with the nearest lower concentration of the same salt or HCl.
‖‖ $P < 0.001$ compared with the treatment with the nearest lower concentration of the same salt or HCl.

The cations present in the incubation solutions were able to release the radioactivity from the pigment. Increased release was observed at increased concentrations of the cations. H^+ was more effective in releasing the radioactivity than Ca^{2+}, which in turn was more effective than Na^+. The differences observed between the different treatments were in most instances statistically significant.

Similar results were obtained at the 1 hr and 1 day survival intervals at the HCl treatments. It can be seen that a larger part of the [^{35}S]chlorpromazine radioactivity (about 21 per cent) is non-releasable by the highest H^+ concentration than is the case for the [^{14}C]chloroquine radioactivity (about 8 per cent).

DISCUSSION

The present observations that cations are able to release the [^{35}S]chlorpromazine and the [^{14}C]chloroquine radioactivity from the eye melanin—the ability increasing with increasing concentrations of the cations—indicate that in concordance with the situation *in vitro* [4], electrostatic forces are important for the complex-formation between these drugs and melanin *in vivo*. Both chlorpromazine and chloroquine undergo appreciable degradation in the body [8, 9]. No attempts were made to identify the material bound to the melanin in the present study, but since some metabolites like the parent compounds have the character of weak bases and will be present mainly as cations at physiological pH, parts of the radioactivity associated with the pigment may represent metabolites. As for the *in vitro* affinity [4], the ability of the inorganic cations to influence the *in vivo* binding follows a pattern characteristic for the attraction to a weak cation exchanger. In this type of interaction, there is a strong affinity for H^+ and an increasing affinity for the cations with an increasing valence [10]. This correlates with the present observations that the order of the effectiveness of the cations to release the radioactivity was $H^+ > Ca^{2+} > Na^+$. The binding of inorganic cations to melanin has been shown to follow the cation exchange pattern [11, 12]. It appears that both for organic substances which are positively charged at physiological pH, and for inorganic cations, the melanin polymer may function as an endogeneous cation-exchange material. It is probable that this property is due mainly to the free carboxyl groups, which are present in the melanin.

As with the binding *in vitro* [4], non-electrostatic forces also appear to be of importance for the affinity *in vivo*: Part of the radioactivity remained attached to the melanin even at the highest concentration of HCl. It is possible that the non-electrostatic contribution is provided by van der Waal's forces occurring at the apposition of the aromatic rings of the drugs and the aromatic rings of the indole monomers. Another possibility is that this interaction may involve the formation of a charge-transfer complex. Drugs which are bound are generally good electron donors and the melanin would act as the electron acceptor. We have recently shown that the free-radical derivative of chlorpromazine, which is formed by a single-electron-transfer process as the first oxidation step of the drug, has a high melanin affinity [4]. The present study showed that the part of the radioactivity which was non-displacable was higher for [^{35}S]chlorpromazine than for [^{14}C]chloroquine. Similar results were obtained *in vitro* [4].

A part of the radioactivity was released by the incubation in water—the proportion released being small for [^{14}C]chloroquine and somewhat larger for [^{35}S]chlorpromazine. The loosely bound pool may be attached to the melanin by very weak ionic bonds. Another possibility is that the radioactivity may be present in the melanocytes without being attached to the melanin granules.

The amounts of radioactivity which remained attached to the pigment after the HCl treatments were about equal at 1 hr and 1 day. This indicates that the relative participation of electrostatic and non-electrostatic forces in the binding does not change markedly with time.

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