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Antioxidant Action and Photosensitizing Effects of Three Different Chlorpromazines

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Abstract: Chlorpromazine inhibits by about 60% the lipid peroxidation stimulated by Fe^{2+}/ascorbate in liposomes and the lipid peroxidation stimulated by cumene hydroperoxide in microsomes. Under the same conditions, two new synthetic derivatives of chlorpromazine, i.e., a *N*-benzoyloxymethylchlorpromazine and a *N*-pivaloyloxymethylchlorpromazine, induce no more than a 20% inhibition. On the other hand, when the different chlorpromazines are entrapped in liposomes and subsequently irradiated with near-UV light, they act as photosensitizing agents giving rise to lipid peroxidation. The latter is quite extensive in the presence of chlorpromazine or *N*-pivaloyloxymethylchlorpromazine, whereas it is drastically lower in the presence of *N*-benzoyloxymethylchlorpromazine. The *N*-benzoyloxymethylchlorpromazine molecule, despite its low photodynamic effect, retains its neuroleptic properties. The possible mechanisms of the antioxidant and prooxidant actions of these compounds are discussed. **Key Words:** Chlorpromazine—Chlorpromazine derivatives—Lipid peroxidation—Malondialdehyde—Photosensitizing effect—Ultraviolet irradiation. **Bindoli A. et al.** Antioxidant action and photosensitizing effects of three different chlorpromazines. *J. Neurochem.* **50,** 138–141 (1988).

It is known that promethazines and other phenothiazines, at quite low concentrations, inhibit lipid peroxidation induced in microsomes by the NADPH/Fe^{3+}/ADP system. This inhibition has been related to both their antioxidant properties and chelating effect on iron ions. In particular, chlorpromazine (CPZ) has been found to prevent lipid peroxidation both in vitro (Slater, 1968; Slater and Sawyer, 1971; Cohen and Heikkila, 1974; Schaefer et al., 1975) and in vivo (Roy et al., 1984). In addition, an antiaging effect of CPZ (Samorajski and Rolsten, 1976) and a lipofuscinolytic activity similar to that of centrophenoxine (Roy et al., 1984) have been reported as well. When CPZ or other promazines are exposed to near-UV light they undergo photoionization, giving rise to the CPZ radical cation ($CPZ\cdot^{+}$) and hydrated electron (Naravatnan et al., 1978). The latter can produce the superoxide anion and, secondarily, hydrogen peroxide and hydroxyl radical, believed to be responsible for both the DNA strand breakage and lipid peroxidation (Lesko et al., 1980). Ocular opacities, described in patients under high doses of these drugs and exposed to sunlight, however, could be due to cross-linking of lens proteins (Merville et al., 1984). It has been observed in fact that in vitro, CPZ sensitizes the photodynamic oxidation of sulfhydryl groups of some biological molecules (Hoffman and Discher, 1968).

In the present work we have considered the effects of CPZ and two derivatives obtained by introducing two hydrophobic and sterically hindering groups, i.e., *N*-benzoyloxymethyl (CPZ-B) and *N*-pivaloyloxymethyl (CPZ-P), on the tertiary nitrogen which is thus quaternized (Fig. 1). We have compared the inhibitory action of CPZ, CPZ-B, and CPZ-P on lipid peroxidation induced by cumene hydroperoxide (CHP) and ascorbate/Fe^{2+} and their stimulatory effect on liposomal lipid peroxidation induced by near-UV light. We found that the addition of the two bulky groups on the tertiary nitrogen and the ensuing quaternization of the latter renders CPZ less effective as an antioxidant but also, at least in one case, less effective as a photosensitizing agent.

MATERIALS AND METHODS

Chemical synthesis

CPZ-B (MW 489.46; melting point 175°C) and CPZ-P (MW 469.47; melting point 188°C) are newly synthesized molecules with patent pending (Medea Research, Milano,

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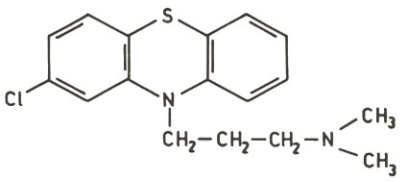
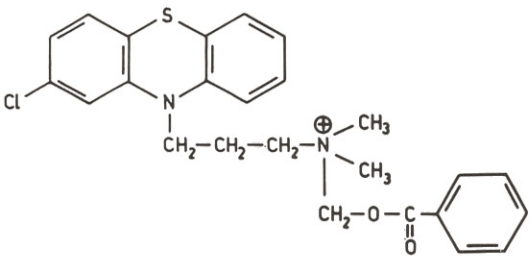
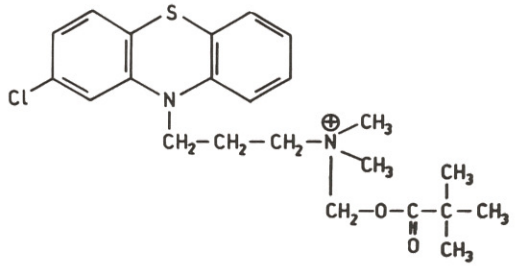
Abbreviations used: CHP, cumene hydroperoxide; CPZ, chlorpromazine; CPZ-B, *N*-benzoyloxymethylchlorpromazine; CPZ-P, *N*-pivaloyloxymethylchlorpromazine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MDA, malondialdehyde.



CHLORPROMAZINE (CPZ)



N-BENZOYL-OXYMETHYL-CHLORPROMAZINE (CPZ-B)



N-PIVALOYL-OXYMETHYL-CHLORPROMAZINE (CPZ-P)

FIG. 1. Structural formulas for CPZ, CPZ-B, and CPZ-P.

Italy). Equimolecular amounts of CPZ chloride and benzoyloxymethyl chloride (or pivaloyloxymethylchloride) were mixed and left overnight at 25°C. The precipitate was ground, resuspended in ethyl ether, and refluxed for 1 h. The final product was filtered and dried under vacuum. Final yields were 86% for CPZ-B and 91% for CPZ-P. The purity was checked by elemental analysis, TLC chromatography, and nuclear magnetic resonance (NMR) spectroscopy in $CDCl_3$ at 30°C. The chemical shifts (ppm) downfield from 3-(trimethylsilyl)-propanesulfonic acid · Na salt were those of the $^{+}N\text{-}CH_2-$ group (4.23), of the $-C(CH_3)_3$ group (1.14) for CPZ-P, and of the C_6H_5- group (7.76) for CPZ-B.

Methods

Liposomes were prepared with pentane-extracted phospholipids obtained from calf brain according to Gutteridge (1977). The phospholipids were suspended (20 mg/ml) in cold distilled water using a homogenizer with a Teflon pestle and sonicated twice for 1 min with a 60 W output at 0°C. Aliquots were diluted to 5 mg/ml with 0.125 *M* KCl, 15 m*M* Tris/7.5 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) and CPZ, CPZ-B, and CPZ-P at a final concentration of 0.5 m*M* were added. The three suspensions were again sonicated twice for 30 s.

Liver microsomes from Wistar male rats were prepared as described by Ernster and Nordenbrand (1967) and protein content was estimated by the biuret method (Gornall et al., 1949). CPZ, CPZ-B, and CPZ-P were incorporated in microsomes by sonication under the conditions used for the liposomes. UV irradiation was carried out with a near-UV radiation-emitting (320–400 nm) tube (Sylvania F6T5-BL) 20 cm long and placed at a distance of 5 cm from the sample.

Oxygen uptake was followed at 25°C with a platinum electrode assembly of the Clark type (Estabrook, 1967). Malondialdehyde (MDA) was determined by the thiobarbituric acid method as described by Buege and Aust (1978).

Supercoiled DNA of the plasmid pBR322 (MW 2.88 $\times 10^6$) from Boehringer (Mannheim, F.R.G.) was irradiated with near-UV light in the presence of the various phenothiazines. The separation of supercoiled and relaxed DNA was carried out by agarose gel electrophoresis in 0.08 *M* Tris-borate buffer at pH 8. The neuroleptic action of CPZ and its derivatives was assessed on the basis of the prolongation of the barbiturate-induced sleep with 36 Swiss male albino mice of 25 g fed with standard laboratory diet.

RESULTS

Table 1 shows the effect of CPZ and of the two derivatives, CPZ-B and CPZ-P, on MDA formation induced in liposomes and microsomes by Fe^{2+}/ascorbate. Calf brain liposomes undergo a time-dependent lipid peroxidation, measured as MDA formation, when incubated in the presence of Fe^{2+}/ascorbate. When liposomes, in which the various phenothiazines were previously incorporated, peroxidize in the presence of Fe^{2+}/ascorbate, a decrease in MDA formation is observed. CPZ inhibits lipid peroxidation by about 60%, whereas the inhibition induced by CPZ-B and CPZ-P does not exceed 20%, indicating that the two new derivatives are less efficient as lipid peroxidation inhibitors. Similar results were obtained with liver microsomes where CHP-induced MDA formation was measured; also in this case CPZ appears to be much more effective than CPZ-B and CPZ-P, which exhibit a lower inhibitory effect on lipid peroxidation (Table 1).

Since CHP-induced lipid peroxidation in microsomes is completely independent of iron ions (O'Brien and Rahimtula, 1975; Bindoli et al., 1982), this result demonstrates that the effect of CPZ is not related to a chelating action but rather to a direct antioxidant action.

When a photosensitizing dye is entrapped in liposomes that are subsequently exposed to visible or UV light, according to the absorption wavelength of the entrapped dye, they undergo a lipid peroxidation measurable both as an oxygen uptake and MDA formation. As reported in Fig. 2, when CPZ is incorporated in liposomes, UV light induces a sudden increase in oxygen uptake that abruptly stops when the light is switched off. The rate of oxygen consumption in the presence of UV light is of 10.9 nmol/min/mg phospholipid. When CPZ-P, instead of CPZ, is incor-

TABLE 1. *MDA formation in liposomes and microsomes treated with CPZ and its derivatives*

	Liposomes				Microsomes	
	30 min	Percent	60 min	Percent	7 min	Percent
None	5.93 ± 1.64 (3)	100	7.67 ± 1.11 (3)	100	7.26 ± 1.27 (3)	100
CPZ	2.39 ± 1.65 (3)	40	2.99 ± 1.96 (3)	39	3.12 ± 0.60 (3)	43
CPZ-B	4.89 ± 1.16 (3)	82	6.52 ± 1.40 (3)	85	5.03 ± 0.76 (3)	70
CPZ-P	4.74 ± 0.95 (3)	80	6.52 ± 1.33 (3)	85	5.44 ± 0.68 (3)	75

Liposomes (5 mg/ml) and microsomes (1.2 mg protein/ml) were incubated in 0.125 *M* KCl, 15 m*M* Tris/7.5 m*M* HEPES buffer (pH 7.4) and, only with microsomes, 1 m*M* EDTA. CPZ, CPZ-B, and CPZ-P concentrations were 0.5 m*M*. Lipid peroxidation was initiated with 10 μM $FeSO_4$/0.1 m*M* ascorbate in liposomes and with 1 m*M* CHP in microsomes and carried out at 37°C for the indicated times. At the end of the incubation, MDA was determined on aliquots of 0.5 ml as indicated in Materials and Methods. Values, expressed as nanomoles MDA formed per milligram of phospholipid and nanomoles MDA formed per milligram of protein, respectively, for liposomes and microsomes, are means ± SD; the figures in parentheses show the number of experiments. The differences of CPZ-B and CPZ-P versus CPZ were statistically significant by Student's *t* test ($p < 0.01$).

porated in liposomes, the rate of oxygen consumption is almost the same (10.3 nmol/min/mg phospholipid), whereas when the liposomes are filled with CPZ-B, there is a dramatic decrease in the rate of oxygen consumption (2.5 nmol/min/mg phospholipid); in the latter case the UV light seems almost ineffective on the rate of oxygen consumption. Similar results were found when MDA formation, induced by the phenothiazines plus UV light, was measured at the end of the "on–off" experiment: a large amount of MDA was produced in the presence of CPZ and CPZ-P, whereas with CPZ-B the quantity of MDA formed was extremely low and very close to the control.

It has been shown that promazines, photoexcited with near-UV light and in the presence of oxygen, can produce free radicals responsible for DNA strand breakage (Decuyper et al., 1984). We have thus studied the relaxation of the supercoiled DNA of the plasmid pBR322 after irradiation with UV light in the presence of the various phenothiazines. We found that the supercoiling decreases when DNA is irradiated in the presence of CPZ or CPZ-P, whereas this decrease is lower when CPZ-B is used as a photosensitizing agent (data not reported). It is to be noted that CPZ-B, which does not exhibit considerable photosensitizing action, appears to be even more effective than CPZ when the length of the sleeping time in mice is measured (Table 2).

DISCUSSION

Phenothiazines exert a protective action on lipid peroxidation probably because of their electron donor properties. In fact, after intercepting free radicals they are transformed into a resonance-stabilized free radical cation and, in this way, are capable of interrupting the free radical chain. The concentration

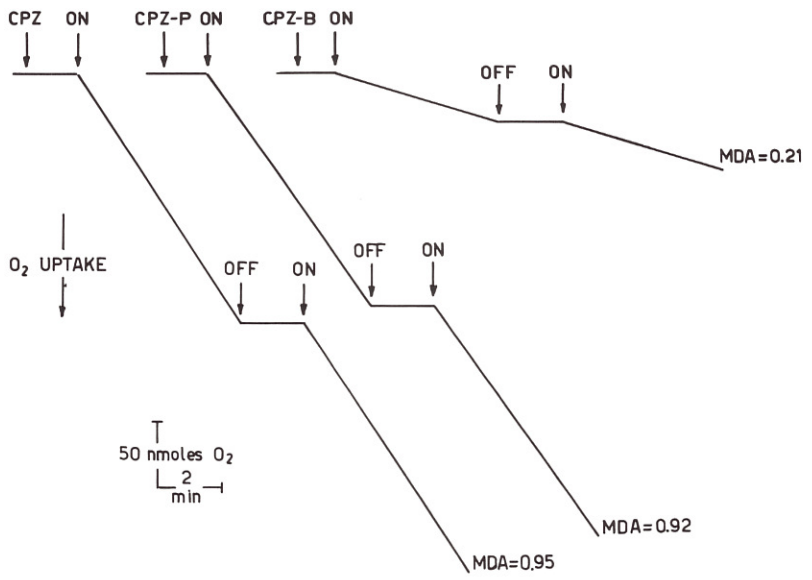


FIG. 2. Oxygen uptake by liposomes incorporated with CPZ, CPZ-B, and CPZ-P and irradiated with near-UV light. Liposomes (5 mg phospholipid/ml), in which 0.5 m*M* CPZ or CPZ-B or CPZ-P were incorporated, were irradiated in a medium formed by 0.125 *M* KCl, 15 m*M* Tris/7.5 m*M* HEPES (pH 7.40) for the indicated times with a near-UV radiation emitting (320–400 nm) tube at a distance of 5 cm. The oxygen uptake was initiated and stopped by respectively switching the light on and off. At the end of each curve the amount of MDA formed (nanomoles per milligram of phospholipid) is reported.

TABLE 2. *Action of CPZ and CPZ-B and CPZ-P on the length of sleep induced in the mice with hexobarbital*

	Sleeping time (h)
Control	1.15 ± 0.42 (9)
CPZ	6.98 ± 1.83 (9)
CPZ-B	7.36 ± 1.36 (9)
CPZ-P	7.08 ± 1.48 (9)

Albino swiss mice were first injected intraperitoneally with CPZ (5 mg/kg) or with CPZ-B and CPZ-P (6.8 mg/kg), and then, after 30 min, sleep was induced with hexobarbital (40 mg/kg) injected intravenously through the caudal vein. The sleeping time was measured as a duration of the sleep after the injection of hexobarbital. Values are means ± SD; figures in parentheses show the number of mice. The difference of CPZ-B versus CPZ was statistically significant by Student's t test ($p < 0.01$).

of the free radical cation of CPZ ($CPZ\cdot^+$), stoichiometric with the free radical produced, is probably low enough to prevent a dismutation that can give rise to new activated forms (Nakano et al., 1985). On the contrary, when CPZ is illuminated with UV long-wavelength radiations, a larger concentration of $CPZ\cdot^+$ free radical is formed. This radical can undergo dismutation, giving rise to an activated form of CPZ that can initiate lipid peroxidation. Another explanation of the photodynamic action of CPZ is that the photoejected electron can reduce the dioxygen with the production of superoxide anion, hydrogen peroxide, and secondarily hydroxyl radical. This second possibility is in agreement with the work of Decuyper et al. (1984), who did not find any singlet oxygen formation during the photoactivation of CPZ.

When the tertiary nitrogen of the side chain of CPZ is quaternized and substituted by two sterically hindering groups, the antioxidant properties of CPZ are strongly reduced and, at least in the case of CPZ-B, also its prooxidant (photodynamic) action is partially impaired. This behavior can be referred to a decreased stability of the radical cation formed after reaction with a free radical or after UV irradiation. The property is particularly relevant for the CPZ-B derivative in which the hydrophobic group bound to the nitrogen side chain of CPZ is also aromatic and whose neuroleptic properties are retained (Table 2). The decrease in the photodynamic action of CPZ-B is not restricted to the peroxidative action but it is also apparent by considering the nicking of supercoiled DNA.

In conclusion, from the reported results it appears that the introduction of a hydrophobic and sterically bulky substituent on the side-chain nitrogen causes a strong decrease on both the antioxidant and prooxidant action without affecting the neuroleptic properties. Considering the wide use of phenothiazines in psychiatry, a decrease in the phototoxic response could be an advantage for their clinical applications.

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