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Mechanism of inhibition of tumor necrosis factor production by chlorpromazine and its derivatives in mice

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Abstract

In previous work, we reported that chlorpromazine inhibits tumor necrosis factor (TNF) production in endotoxin lipopolysaccharide-treated mice, and protects against lipopolysaccharide toxicity. Chlorpromazine is used as an antipsychotic and has several effects on the central nervous system. It acts on different neurotransmitter receptors and has other biochemical activities some of which, like inhibition of phospholipase A_2, might be responsible for the inhibitory effect on TNF production. To investigate the role of these actions in the inhibition of TNF production by chlorpromazine, we have synthesized some chlorpromazine derivatives that do not have central activities. Some of these analogs have lost their affinity for various receptors and their phospholipase A_2 inhibitory activity, but still inhibit TNF production. No correlation was found between TNF inhibition and the ability to inhibit nitric oxide (NO) synthase, whereas a good correlation was evident between TNF inhibition and antioxidant activity.

Keywords: Chlorpromazine; TNF (tumor necrosis factor); Endotoxin

1. Introduction

As early as 1954, chlorpromazine was reported to protect against endotoxic shock (Chédid, 1954). In a series of studies, we have characterized this effect and observed that protection against lipopolysaccharide toxicity was associated with inhibition of tumor necrosis factor (TNF) production (Gadina et al., 1991; Mengozzi et al., 1994). Since TNF is a key mediator in the lethal action of lipopolysaccharide, as demonstrated by the protective effect of anti-TNF antibodies or inhibitors of TNF production like glucocorticoids (Bertini et al., 1989; Beutler and Cerami, 1986; Zimmerman et al., 1989), this inhibitory action of chlorpromazine might well be at the basis of its protective effect.

Our interest was to identify the mechanism of this inhibitory action. Chlorpromazine has a large number of pharmacological activities: it is a histamine receptor antagonist, 5-HT receptor antagonist, adrenoceptor antagonist, dopamine receptor antagonist, phospholipase A_2 inhibitor (Baldessarini, 1985; Vadas et al., 1986), and antioxidant (Slater, 1968; Jeding et al., 1995). Some of these actions might explain its inhibition of TNF production, and in fact, other 5-HT receptor antagonists, α- (not-β) adrenoceptor antagonists, histamine receptor antagonists and phospholipase A_2 inhibitors inhibit lipopolysaccharide-induced TNF production in vivo (Basedovsky and Del Rey, 1987). However, the relative importance of the sedative and psychotropic actions of chlorpromazine and, biochemically, of its neurotransmitter receptor binding, has not been clarified.

In the present study we used a different approach, synthesizing different analogs of chlorpromazine and analyzing their ability to inhibit TNF production. Some qua-

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ternary derivatives of chlorpromazine were synthesized in order to obtain molecules that could not pass the blood–brain barrier and therefore had no activity on the central nervous system (CNS). These chlorpromazine derivatives were synthesized on the basis of earlier reports or patents indicating that quaternary derivatives or sulphones have no pharmacological activity (Costentin et al., 1987). The compounds were then characterized with respect to some in vivo and in vitro activities to gain an idea which pharmacological activities of chlorpromazine are responsible for its inhibition of TNF production. The lack of central activity of some of these analogs was checked using a standard locomotor activity test to evaluate the sedative activity.

Their effect on blood pressure was also tested, to see whether they still had the hypotensive activity of chlorpromazine. For technical reasons, blood pressure was studied in rats. These studies were done to clarify whether the effect of chlorpromazine on TNF production was separate from its central and cardiovascular effects.

Since phospholipase A_2 inhibitors also inhibit TNF production (Spriggs et al., 1990), and chlorpromazine is a well-known inhibitor of this enzyme, chlorpromazine and its analogs were tested for their ability to inhibit purified phospholipase A_2 in vitro. Inhibition of nitric oxide synthase was also investigated, since some reports suggest nitric oxide (NO) is involved in lipopolysaccharide-induced TNF production (Moncada et al., 1991; Rojas et al., 1993). Finally, since other antioxidants have been shown to inhibit TNF production (Peristeris et al., 1992; Zhang et al., 1994), and chlorpromazine is described to be a scavenger of hydroxyl radicals (Jeding et al., 1995; Slater, 1968), we also investigated the antioxidant activity of chlorpromazine and its derivatives using an in vitro test for antioxidant activity (Halliwell et al., 1987; Aebi, 1974).

2. Materials and methods

2.1. Chlorpromazine derivatives

The quaternary ammonium compounds **1** and **2** were synthesized according to classical methods involving treatment of chlorpromazine with bromoalkanes. Nitric acid oxidation led to the formation of sulfoxide nitrates which, after neutralization, were quaternized to bromoalkanes to afford compounds **3** and **4**. Compound **5** was obtained from 4-chloro-2′-nitrodiphenylsulfide, which was reduced (by Zn/AcOH) and treated with (3-chloropropyl) dimethylamine under alkaline conditions. The structure of the compounds investigated is shown in Fig. 1. They include two quaternary derivatives, compound **1** (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] trymethyl-ammonium bromide) and compound **2** (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] butyl-dimethylammonium bromide); two sulfoxide-quaternary ammoniums, compound **3** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] butyl-dimethylammonium bromide) and compound **4** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] trimethylammonium bromide); and an open-ring derivative, compound **5** (*N*-2-(4-chlorophenyl) thio phenyl] *N,N′*-dimethyl propane 1,3-diammonium chloride).

2.2. Animals and treatments

Male CD-1 mice (25 g body weight) from Charles River (Calco, Como, Italy; Saint-Aubin, France) were used. Mice were housed five per cage and could feed ad libitum. Dexamethasone phosphate was from Istituto Farmacologico Milanese, Milan, Italy; lipopolysaccharide (phenol-extracted preparation from *Escherichia coli* O55:B5) was from Difco.

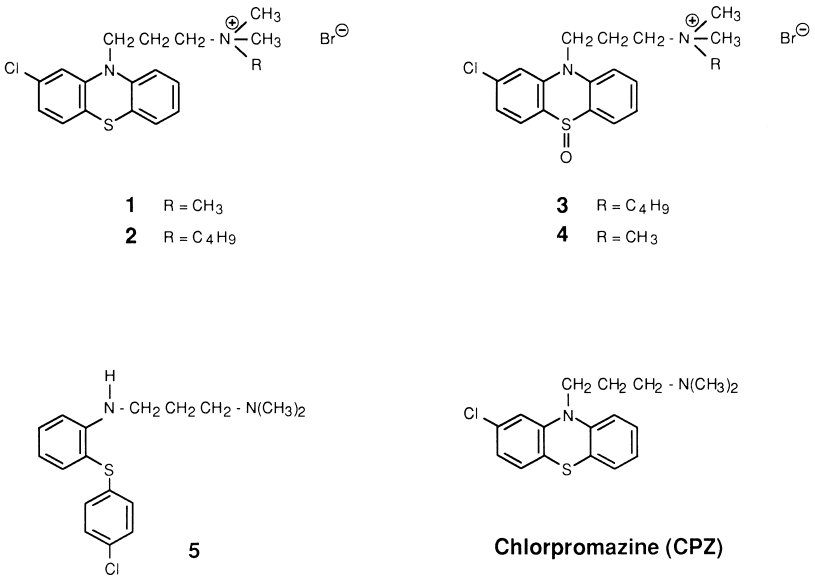

Fig. 1. Structure of chlorpromazine (CPZ) and of the synthetic CPZ derivatives.

Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana No. 40, February 18, 1992; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

For lipopolysaccharide toxicity studies, male, adult (22–24 g) BALB/c mice were given lipopolysaccharide as a single dose of 1 mg/mouse, i.p., with and without a 30-min pretreatment with the test compound, as previously described (Gadina et al., 1991). Survival was evaluated 72 h later.

2.3. Inhibition of TNF production

In some experiments mice were treated i.p. with chlorpromazine (4 mg/kg) or its derivatives (given at equimolar doses to 4 mg/kg chlorpromazine). Thirty minutes later, mice received an i.p. injection of 2.5 μg/mouse of lipopolysaccharide. Blood was obtained from the retro-orbital plexus under light ether anesthesia and serum was prepared. Serum TNF was measured in a cytotoxicity assay, using L929 cells in the presence of 1 μg/ml of actinomycin D, as previously described (Aggarwal et al., 1985), with human recombinant TNF as standard.

In another set of experiments, mice were given the test drugs orally, dissolved in water. Thirty minutes later, mice were injected i.v. with 250 μg of lipopolysaccharide in saline. One hour later, mice were anesthetized with isoflurane (Forene, Abbott Laboratories), and blood was obtained by cardiac puncture and collected on heparin (50 U/ml, Choay, France). Plasma TNF-α was assayed using a commercially available enzyme immunosorbent assay (ELISA) kit (Genzyme).

2.4. Neurotransmitter receptor binding affinity

Binding assays were done on crude membrane preparations from rat brain areas, as described (Mennini et al., 1991; Testa et al., 1989). The type of receptors analyzed, the brain area used, radioligands and competitors used to define specific binding are summarized in Table 1. Binding in the presence of various concentrations of test compounds was expressed as a percentage of the specific binding without drug. The IC_{50} values were established by simultaneous nonlinear curve fitting according to the logistic equation reported by De Lean et al. (1978), using the 'Allfit' program. [^{3}H]Prazosin, [^{3}H]SCH 23390, [^{3}H]spiperone, [^{3}H]8 OH-DPAT, [^{3}H]5-HT, and [^{3}H]paroxetine were purchased from New England Nuclear.

2.5. Locomotor activity test

The sedative action of chlorpromazine and its derivatives was measured by recording the spontaneous locomotor activity of mice placed in a box representing a new environment, equipped with infra-red sensors to measure the number of movements (Activity Monitor, Digiscan-Omnitech Electronics, Sufraco, France). Test compounds were injected i.p. 30 min before the test, at the doses indicated, and locomotor activity was then evaluated for 15 min.

2.6. Inhibition of phospholipase A_2 in vitro

Porcine pancreatic phospholipase A_2 (Sigma) was incubated for 1 h at 37°C at a concentration of 5 ng/ml with 60 000–80 000 cpm/sample of *Escherichia coli* membranes radiolabelled with [^{3}H]oleic acid in a final volume of 0.4 ml, as previously described (Boraschi et al., 1991; Davidson et al., 1987). Test compounds were used at various concentrations; water-insoluble compounds were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 5%. Inhibition of enzyme activity was calculated in control samples containing equal amounts of DMSO.

2.7. Inhibition of NO synthase in vitro

Neuronal NO synthase activity was measured according to Bredt and Snyder (1989). Briefly, cerebella from Wistar rats (180–200 g) were rapidly removed, cleaned and minced in 20 volumes of ice-cold buffer (20 mM HEPES/Tris pH 7.2 containing 0.32 M sucrose, 0.5 mM

Table 1
Binding assays: summary of methods

Receptor	Localization	Radioligand (nM)	Competitor (nM)
α-1 NA	Cortex	[^{3}H]Prazosin (0.2)	Phentolamine (3)
DA-1	Striatum	[^{3}H]SCH23390 (0.4)	(−)*cis*-Flupentixol(10)
DA-2	Striatum	[^{3}H]Spiperone (0.2)	(−)Sulpiride (100)
5-HT-1A	Hippocampus	[^{3}H]8-OH-DPAT (1.0)	5-HT (1)
5-HT-1B	Striatum	[^{3}H]5-HT (2.0)	5-HT (10)
5-HT uptake	Cortex	[^{3}H]Paroxetine (0.4)	Fluoxetine (10)

5-HT, serotonin; DA-1, dopamine D_1 receptor; DA-2, dopamine D_2 receptor; NA, noradrenaline; 8-OH-DPAT, *N,N*-di[2,3(*n*)-3*H*-propylamino)-8-hydroxy-1,2,3,4-tetrahydronaphthalene.

EDTA and 1 mM dithiothreitol). After homogenization with a Dounce homogenizer, samples were centrifuged at $9000 \times g$ for 20 min and the supernatant was passed through a column of 1 ml of Dowex AG50WX8 (Na^+ form) to remove endogenous arginine. The assay was carried out in a total volume of 0.5 ml. The column eluate (0.4 ml) was supplemented with 2 mM NADPH, 0.45 mM $CaCl_2$, 100 μM arginine and [^{3}H]arginine (1 μCi/ml) as tracer, and the test drug at various concentrations. After 1 h at 30°C, two 0.2-ml samples of the incubation mixture were applied to a Dowex column (as above) to remove unreacted substrate, and eluted with 1 ml of water. NO synthase activity was determined by counting the radioactivity of the eluate (containing the radiolabeled citrulline formed), using 4 ml of Picofluor 40 (Packard), in a scintillation counter.

2.8. Hypotensive activity in rats

Systolic blood pressure was measured non-invasively, using a tail cuff, in adult male CD rats (Crl:CD(SD)BR, 215–240 g body weight, Charles River) up to 50 min after chlorpromazine or derivatives. The method is based on recording the arterial pulse from the caudal artery with a piezoelectric transducer applied to the tail (BP recorder 8005, Basile, Comerio, Italy). Rats were preconditioned for 10 min at 38°C to obtain a measurable pulse.

2.9. Antioxidant activity

The deoxyribose assay for hydroxyl radicals was performed as already described (Halliwell et al., 1987). Briefly, the reaction was carried out in a total volume of 1 ml of potassium phosphate buffer (20 mM, pH 7.4) containing deoxyribose (5 mM), $FeCl_3$ (100 μM); EDTA (104 μM), H_2O_2 (1 mM), ascorbate (100 μM), and the test drugs at various doses. Reaction mixtures were incubated at 37°C for 1 h. Thiobarbituric acid (1 ml of 1% w/v) was then added, plus 1 ml 2.8% (w/v) trichloroacetic acid. The tubes were heated for 30 min at 100°C, cooled and the absorbance was measured at 532 nm against the appropriate blanks.

The possible effect of chlorpromazine and derivatives on the stability of hydrogen peroxide was evaluated spectrophotometrically by monitoring the H_2O_2 concentration at 240 nm (Aebi, 1974). Catalase (Sigma; 100 μg/ml) was used as a reference and caused complete degradation of H_2O_2 in this system.

3. Results

3.1. Inhibition of TNF production by chlorpromazine and its derivatives

Table 2 shows the effect of chlorpromazine and its derivatives on serum TNF production. The test compounds were administered i.p. 30 min before lipopolysaccharide (2.5 μg/mouse, i.p.) and serum TNF was measured 1 h after lipopolysaccharide administration, using the cytotoxicity assay. No TNF was detectable in the absence of lipopolysaccharide treatment (data not shown). The dose of chlorpromazine (4 mg/kg, equal to 12.6 μmol/kg) and the treatment schedule were chosen on the basis of previously published data (Gadina et al., 1991). The TNF production of control groups (lipopolysaccharide alone) obtained within the same experiment is reported for each compound. Chlorpromazine completely blocked TNF production (> 95% inhibition in three experiments), in agreement with previous reports. All the chlorpromazine deriva-

Table 2
Effect of intraperitoneal administration of chlorpromazine and its derivatives on TNF production in vivo

Compound	TNF (ng/ml)	
	Saline + lipopolysaccharide	Compound + lipopolysaccharide
1	102 ± 26	23 ± 4 [a] (−77%)
2	102 ± 26	11 ± 4 [a] (−89%)
3	44 ± 8	14 ± 7 [a] (−68%)
4	44 ± 8	20 ± 11 [a] (−54%)
5	102 ± 26	81 ± 15 (−20%)
Chlorpromazine	102 ± 26	< 0.5 [b] (> 95%)

1 (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] trymethyl-ammonium bromide); **2** (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] butyl-dimethylammonium bromide); **3** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] butyl-dimethylammonium bromide); **4** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] trimethylammonium bromide); **5** (*N*-2-(4-chlorophenyl) thio phenyl] *N*,*N*′-dimethyl propane 1,3-diammonium chloride). Drugs were administered i.p. in saline 30 min before lipopolysaccharide (2.5 μg/mouse, i.p.). TNF was measured by bioassay. Data (peak serum TNF levels) are means ± S.E. from 4–6 mice per group. Percent inhibition is given in parentheses. [a] $P < 0.05$, [b] $P < 0.01$ versus lipopolysaccharide alone by Dunnett's test.

Table 3
Effect of oral administration of chlorpromazine and its derivatives on TNF production in vivo

Compound	TNF (ng/ml)	% Inhibition
Vehicle (water)	60.9 ± 4.8	–
1	19.7 ± 6.7 [a]	68
2	16.9 ± 3.4 [a]	72
3	16.5 ± 8.5 [a]	73
4	24.3 ± 3.4 [a]	60
5	36.1 ± 10.2	41
Chlorpromazine	2.8 ± 0.4 [a]	95

1 (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] trymethyl-ammonium bromide); **2** (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] butyl-dimethylammonium bromide); **3** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] butyl-dimethylammonium bromide); **4** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] trimethylammonium bromide); **5** (*N*-2-(4-chlorophenyl) thio phenyl] *N*,*N*′-dimethyl propane 1,3-diammonium chloride). Drugs were administered orally in water 30 min before lipopolysaccharide (250 μg/mouse, i.p.). TNF was measured by ELISA. Data (peak serum TNF levels) are means ± S.E. from 4–6 mice per group. [a] $P < 0.01$ vs. control by Student's *t*-test.

tives, tested at doses equimolar to that of chlorpromazine, significantly inhibited TNF production, to varying extents.

In a second set of experiments, chlorpromazine and its derivatives were tested on lipopolysaccharide-induced TNF production, using a different procedure. Lipopolysaccharide was administered i.p. at the dose of 250 μg/mouse, the test compounds were administered orally, and TNF production was measured with an ELISA specific for TNF-α. Chlorpromazine and all its derivatives lowered serum TNF levels in lipopolysaccharide-treated mice (Table 3). Four selected compounds (chlorpromazine; *N*-[3-(2-chlorphenothiazin-10-yl) propyl] trymethyl-ammonium bromide (compound **1**); *N*-[3-(2-chlorphenothiazin-10-yl) propyl] butyl-dimethylammonium bromide (compound **2**); *N*-2-(4-chlorophenyl) thio phenyl] *N,N′*-dimethyl propane 1,3-diammonium chloride (compound **5**) were also tested for their ability to protect against the lethal effect of lipopolysaccharide (1 mg/mouse, i.p.) in BALB/c mice. Chlorpomazine (4 mg/kg), *N*-[3-(2-chlorphenothiazin-10-yl) propyl] trymethyl-ammonium bromide (compound **1**, 5 mg/kg), and *N*-[3-(2-chlorphenothiazin-10-yl) propyl] butyl-dimethylammonium bromide (compound **2**, 5 mg/kg) increased survival (77%, 80% and 40% respectively, compared to 0% survival in mice treated with lipopolysaccharide alone). *N*-2-(4-Chlorophenyl) thio phenyl] *N,N′*-dimethyl propane 1,3-diammonium chloride (compound **5**) at doses up to 100 mg/kg was not protective (data not shown).

3.2. Neurotransmitter receptor binding affinity

Table 4 reports the affinities of chlorpromazine and some of its derivatives on the most representative neurotransmitter receptors. Generally, the derivatives were less active than chlorpromazine. For dopamine receptors, chlorpromazine was about five times more active on dopamine

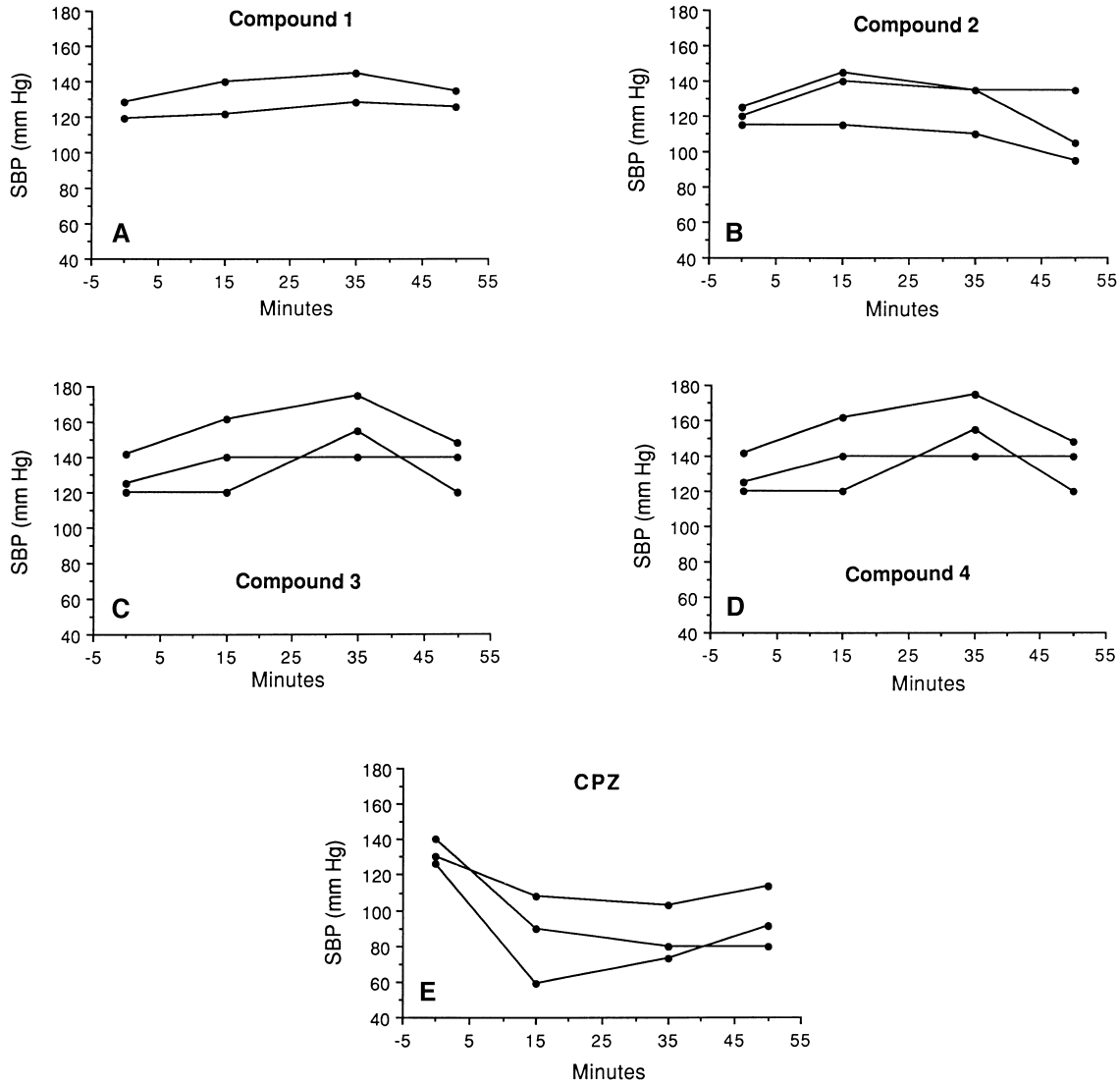

Fig. 2. Hypotensive effect of chlorpromazine in rats and comparison with its derivatives. Rats were injected i.p. with 16 mg/kg chlorpromazine (CPZ) or equimolar doses of its derivatives. Systolic blood pressure (SBP) was monitored up to 50 min. Each curve represents one rat.

Table 4
Neurotransmitter receptor binding affinity of chlorpromazine and its derivatives

Receptor	Chlorpromazine	Compound				
		1	**2**	**3**	**4**	**5**
α-1 NA	7.87	6.57	6.40	5.32	4.96	5.92
DA-1	6.91	4.69	4.93	n.a.	n.a.	5.17
DA-2	7.92	5.22	5.76	n.a.	n.a.	5.05
5-HT-1A	5.01	n.a.	n.a.	6.49	4.87	n.a.
5-HT-1B	5.38	n.a.	n.a.	n.a.	n.a.	5.20
5-HT uptake	7.02	n.a.	n.a.	n.a.	n.a.	n.a.

1 (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] trymethyl-ammonium bromide); **2** (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] butyl-dimethylammonium bromide); **3** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] butyl-dimethylammonium bromide); **4** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] trimethylammonium bromide); **5** (*N*-2-(4-chlorophenyl) thio phenyl] *N,N′*-dimethyl propane 1,3-diammonium chloride). 5-HT, serotonin; DA-1, dopamine D_1 receptor; DA-2, dopamine D_2 receptor; NA, noradrenaline. Data are expressed as pIC_{50} (M). Each drug was tested at 6–8 concentrations in triplicate (variation coefficients were $<10\%$). n.a. (not active) denotes an IC_{50} higher than 10^{-4} M.

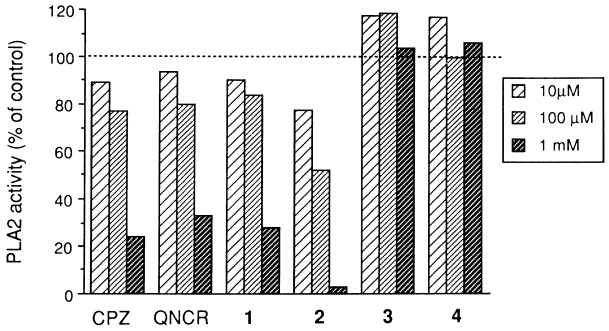

Fig. 3. Effect of chlorpromazine (CPZ) and its derivatives on phospholipase A_2 (PLA_2) in vitro. The activity of porcine pancreatic phospholipase A_2 was assayed by measuring the release of radioactivity from membranes of *E. coli* radiolabeled with [^{3}H]oleic acid. Data are means of triplicate samples (variability was $<10\%$). The percentage of control activity (no drug) is shown. Typically, 5 ng/ml phospholipase A_2 in control samples released 10 000–15 000 cpm of radioactivity from 60 000–80 000 cpm of membranes in 1-h incubation. Release of radioactivity in blank samples (no phospholipase A_2) was 500–1000 cpm. Quinacrine (QNCR) was used as a positive control.

D_2 than on dopamine D_1 receptor binding. Compounds **1** and **2** showed the same proportion of binding, while compound **5** was about equipotent. However, they were all considerably less active (by at least two log units) than the parent compound. Compounds **3** and **4** did not show any dopamine D_1 or dopamine D_2 receptor binding up to the highest concentration tested (10^{-4} M).

For α-1 noradrenaline receptors, all the compounds showed some affinity, ranging from 20–1000 times lower than chlorpromazine, with the following rank order: chlorpromazine > compound **1** > compound **2** > compound **5** > compound **3** > compound **4**.

Chlorpromazine itself had no affinity for 5-HT_{1A} receptors whereas compound **3** showed affinity for this receptor subtype, higher than on the other receptors considered. For 5-HT_{1B} receptors compound **5**, like chlorpromazine, had micromolar affinity. None of the other compounds inhibited radioligand binding to 5-HT_{1A} or 5-HT_{1B} receptors even at a concentration as high as 10^{-4} M. Finally, except for chlorpromazine, none of the compounds tested inhibited [^{3}H]paroxetine binding.

3.3. Sedative action of chlorpromazine and its derivatives

To confirm the lack of central action of the quaternary derivatives, compounds **1** and **2** were tested in a spontaneous locomotor activity test. Unlike chlorpromazine, which at the dose of 2 mg/kg markedly depressed locomotor activity (-58%, $P<0.01$), compound **2** at the same dose had no such effect, a depressing effect being observed only at 5 mg/kg (-43%, $P<0.01$), and compound **1** was completely devoid of a sedative effect at doses up to 10 mg/kg (-9%, not significant, at this dose).

3.4. Hypotensive action of chlorpromazine and its derivatives in rats

As shown in Fig. 2, the dose of 16 mg/kg chlorpromazine reduced systemic blood pressure as early as 15 min after injection (data from three separate rats are shown). This dose was chosen on the basis of preliminary experiments showing that it had a reproducible hypotensive effect. Equimolar doses of chlorpromazine derivatives had no such effect.

3.5. Effect of chlorpromazine and its derivatives on phospholipase A_2 activity

The ability of chlorpromazine or its derivatives to inhibit phospholipase A_2 was tested in a completely cell-free assay with porcine phospholipase A_2. Quinacrine was tested as a reference phospholipase A_2 inhibitor. As shown

Table 5
Inhibition of rat cerebellar NO synthase

Compound	K_i (M)
1	>4
2	3.8×10^{-6}
3	2.4×10^{-5}
4	>4
5	4.2×10^{-6}
Chlorpromazine	3.8×10^{-6}
N-Nitro-L-arginine	1.1×10^{-7}

1 (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] trymethyl-ammonium bromide); **2** (*N*-[3-(2-chlorphenothiazin-10-yl) propyl] butyl-dimethylammonium bromide); **3** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] butyl-dimethylammonium bromide); **4** (*N*-[3-(2-chloro-5-oxide phenothiazin-10-yl) propyl] trimethylammonium bromide); **5** (*N*-2-(4-chlorophenyl) thio phenyl] *N,N′*-dimethyl propane 1,3-diammonium chloride).

in Fig. 3, chlorpromazine and quinacrine had comparable phospholipase A_2 inhibitory activity. Some of the test compounds had inhibitory activity comparable to or better than that of chlorpromazine (the quaternary derivatives compound **1** and compound **2**), while others were not inhibitory at all (the sulfoxide compounds **3** and **4**) even at the highest concentration tested (1 mM).

3.6. Effect of chlorpromazine and its derivatives on NO synthase activity

The ability of chlorpromazine or its derivatives to inhibit rat cerebellar NO synthase is shown in Table 5. Chlorpromazine and compounds **2** and **5** had a K_i in the micromolar range (about two times less than the reference inhibitor, *N*-nitro-L-arginine). Compound **3** was one log unit less active than chlorpromazine and compounds **1** and **4** were completely inactive ($K_i > 10^{-4}$ M).

3.7. Antioxidant ability of chlorpromazine and its derivatives

The antioxidant activity was tested as the scavenging of hydroxyl radicals generated by a mixture of ascorbic acid, H_2O_2, and ferric-EDTA. The sugar deoxyribose is degraded on exposure to hydroxyl radicals, setting off a series of reactions that eventually result in the formation of malondialdehyde, which is detected as a pink chromogen. Fig. 4 shows the antioxidant capability of chlorpromazine and its derivatives, measured as inhibition of deoxyribose degradation. It can be seen that all drugs tested in dose response (from 125 to 1000 μM) showed a similar scavenging activity, compared to that of the reference antioxidant, DMSO (10% v/v), with the only exception being compound **5**, which displayed very weak antioxidant activity.

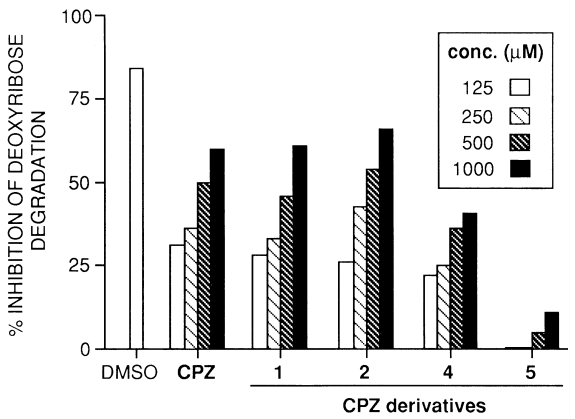

Fig. 4. Antioxidant activity of chlorpromazine (CPZ) and its derivatives in vitro. The antioxidant activity was assayed by measuring the release of thiobarbituric acid reactivity (A_{532}) from deoxyribose. Data are expressed as the percent inhibition of control activity (no drugs, 100% of degradation).

As a compound which degrades H_2O_2 would have also been active in this model, we investigated the direct effect of chlorpromazine and its derivatives on H_2O_2 degradation. No degradation was observed with any of the compounds at a concentration as high as 10 mM (data not shown).

4. Discussion

This paper shows that some derivatives of chlorpromazine, including quaternary salts and sulfoxides, maintain the parent compound's ability to inhibit TNF production when administered to mice before lipopolysaccharide. This activity correlates with their ability to protect mice from the effect of given a lethal dose of lipopolysaccharide. Interestingly, TNF production was inhibited when chlorpromazine or its derivatives were administered orally or i.p., which could be particularly important from the pharmacological point of view. Lower serum TNF levels were demonstrated by a TNF bioassay and ELISA, indicating that the effect of chlorpromazine was not merely attributable to interference in the TNF bioassay. The bioassay-confirmed activities of chlorpromazine were evident.

Inhibition of TNF production seemed maintained even in compounds that had lost the sedative activity of chlorpromazine and that, being quaternary salts, were unlikely to cross the blood–brain barrier. These data suggest that chlorpromazine does not require its central activity to exert its TNF inhibitory activity.

When the receptor activities of the chlorpromazine derivatives are taken into consideration, it can be seen that the various chemical modifications reduce the affinity for all the receptors considered by several orders of magnitude. It therefore seems reasonable to conclude that the antinoradrenergic, antidopaminergic and 5-HT receptor antagonist activities of chlorpromazine are not essential for its inhibitory effect on TNF production.

Most of the derivatives had lost the hypotensive action of chlorpromazine, which might be an undesirable effect in the prevention of endotoxic shock.

Finally, as regards the phospholipase A_2 inhibitory activity of chlorpromazine, compounds **3** and **4**, which were good TNF inhibitors, had no phospholipase A_2 inhibitory activity (unlike compounds **1** and **2** whose activity was comparable to that of chlorpromazine). A similar dissociation was observed between TNF inhibitory activity and the effect on NO synthase activity.

From the data reported here it seems that chlorpromazine's ability to bind to neurotransmitter receptors is not involved in its inhibition of TNF, nor is inhibition of phospholipase A_2 or NO synthase. The question therefore remains what pharmacological activities of chlorpromazine are involved. Since reactive oxygen intermediates have also been implicated in TNF induction, which is inhibited by antioxidants such as *N*-acetylcysteine (Peristeris et al.,

1992; Zhang et al., 1994), we evaluated the reported antioxidant activity of chlorpromazine (Slater, 1968; Jeding et al., 1995) compared to that of its derivatives. Our study shows a good correlation between antioxidant activity and inhibition of TNF production. In fact compound **5**, which had no antioxidant activity, only slightly inhibited TNF induction. The antioxidant activity seems therefore the characteristic of chlorpromazine that plays an important role in the inhibition of serum lipopolysaccharide-induced TNF. The antioxidant activity of chlorpromazine is at the level of that of hydroxyl radicals (this paper and Jeding et al. (1995)), but it does not scavenge superoxide radicals (Jeding et al., 1995) or hydrogen peroxide (this paper).

In conclusion, the present report indicates the utility of structural modification of chlorpromazine to investigate the mechanism of its inhibitory effect on TNF production.

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