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INHIBITORY EFFECT OF PHENOTHIAZINE DERIVATIVES ON BONE *IN VIVO* AND OSTEOBLASTIC CELLS *IN VITRO*

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Abstract—We tested the inhibitory effects of phenothiazine derivatives on bone *in vivo* and osteoblastic cells *in vitro*. Chlorpromazine (CPZ) and trifluoperazine (TFPZ) dose-dependently decreased alkaline phosphatase activity in calvariae of rats: half-maximal inhibitory effects of CPZ and TFPZ were at 2.0 and 4.0 mg/kg, respectively. These effects were more specific for calvaria and ileum than for liver and duodenum. CPZ inhibited the proliferation of osteoblastic clone MC3T3-E1 cells to a greater extent than that of liver epithelial clone RLC-18(4) cells *in vitro*. CPZ, TFPZ and perphenazine (PNZ) also affected rather specifically alkaline phosphatase activity and collagen synthesis and were not cytotoxic. These *in vivo* and *in vitro* findings suggest inhibitory effects on osteoblastic cell function(s). However, promethazine (PMZ) had little effect *in vivo* and *in vitro*. In addition, increases in serum calcium and phosphate induced by CPZ indicate its possible involvement in bone resorption.

Chlorpromazine (CPZ)§, a phenothiazine derivative, is a potent tranquillizing agent for treatment of psychiatric disorders. However, CPZ is known to occasionally cause toxic reactions such as hepatitis with jaundice, hypoplastic anaemia and dermatitis [1–3]. Moreover, growth retardation and malformation by slow progress of ossification in the offspring of CPZ-treated pregnant animals have also been demonstrated [4–6]. However, the precise mechanism of CPZ action on skeletogenesis is not known.

Recently we demonstrated the inhibitory effect of CPZ on bone *in vivo* and *in vitro* [7]. CPZ decreased alkaline phosphatase activity in calvaria to a greater extent than that in liver and duodenum in rats. In addition, CPZ suppressed specifically alkaline phosphatase activity and collagen synthesis in osteoblastic clone MC3T3-E1 cells, a line retaining osteoblastic cell functions [8]. These *in vivo* and *in vitro* findings suggest that CPZ inhibits osteoblastic cell function, reflecting its mechanism of suppression of bone formation. However, CPZ is still used for treatment of emotionally disturbed subjects at doses which are sufficient to suppress osteoblastic cell function. In addition, many phenothiazine derivatives which have more potent therapeutic effects have now been synthesized and are used for treatment.

In this study, we therefore examined the inhibitory effects of phenothiazine derivatives on calvaria *in vivo* and osteoblastic cells *in vitro*, by assaying alkaline phosphatase activity and/or collagen synthesis.

MATERIALS AND METHODS

For *in vivo* experiments, 8-week-old rats of the Wistar strain, weighing about 200 g, were used throughout. Various doses of phenothiazine derivatives dissolved in 0.3% methyl alcohol was injected intraperitoneally. Tested phenothiazine derivatives were chlorpromazine·HCl (CPZ), trifluoperazine (TFPZ), promethazine (PMZ), and perphenazine (PNZ), all of which were purchased from Sigma Chemical Co., St. Louis, MO. After 17 hr of treatment, rats were killed and calvaria, liver, duodenum and ileum were quickly removed. These organs were weighed and homogenized in 5 vol. of 10 mM Tris–HCl (pH 8.4) containing 0.2 M lithium 3·5-diiodosalicylate, 0.2% Triton X-100, and 20% butanol at 4° for 2 min in a Waring blender. The homogenate was then centrifuged at 20,000 *g* for 20 min.

For *in vitro* experiments, 5×10^4 clone MC3T3-E1 cells were plated in 35-mm Flacon plastic dishes in 2 ml of α-minimum essential medium (α-MEM, Flow Laboratories, Rockville) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana), cultured for 3 days, and then transferred to medium containing 10% serum plus various concentrations of phenothiazine derivatives. After 2 days of cultivation, the cells were washed three times, scraped into 2 ml of 0.2% Nonidet P-40 containing 1 mM $MgCl_2$, and sonicated for 5 min with a sonifier cell disruptor (Model UCD-100, Tosho, Yokohama, Japan). The sonicates were centrifuged for 10 min at 3000 rpm, and the supernatants were used for the enzyme assay. Alkaline phosphatase activity in the

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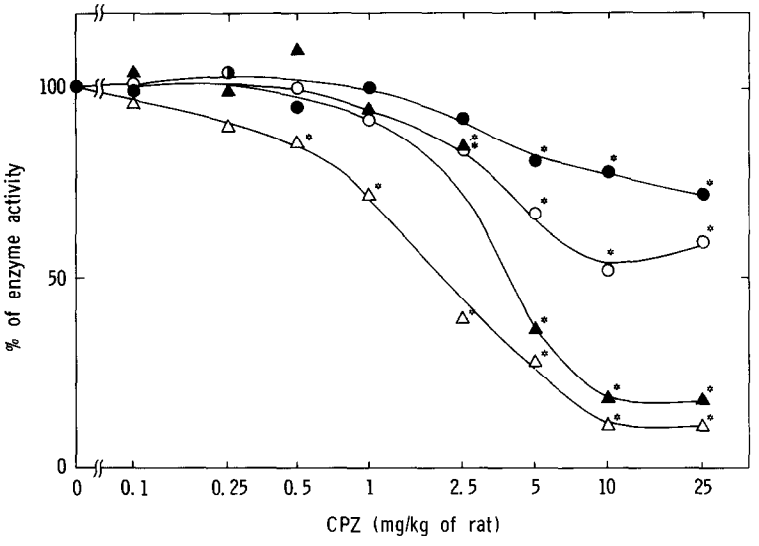
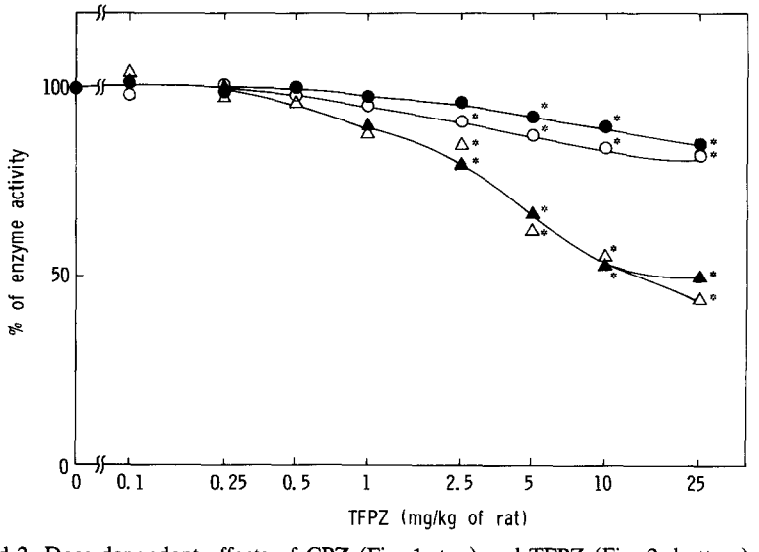
§ Abbreviations: CPZ, chlorpromazine; TEPZ, trifluoperazine; PNZ, perphenazine; PMZ, promethazine; α-MEM, α-minimum essential medium.

organs or the cells was assayed by the method of Lowry *et al.* [9], with *p*-nitrophenyl phosphate as substrate. DNA content was measured by a fluorometric method [10], and protein content was estimated by the method of Bradford [11].

Cells in culture were treated with various doses of the derivatives for 24 hr, and then media were replaced with 1 ml of α-MEM containing 50 μg each of ascorbic acid and β-aminopropionitrile. Labelling with 10 μCi of [^{3}H]-proline (L-3[3,4^{3}H], 20–30 Ci/m New England Nuclear Corp., Boston, MA) was then conducted for 3 hr. The cell suspension was treated with 10% trichloroacetic acid and 0.5% tannic acid (final concentrations). After centrifugation, the precipitates were washed three times with the same solution and twice with ice-cold acetone. Collagenase-digestible protein and non-collagen protein were determined according to the procedures of Peterkofsky and Diegelman [12].

For inhibitory affect of CPZ on cell proliferation, liver epithelial RLC-18(4) cells [13] and clone MC3T3-E1 cells were used. Clone RLC-18(4) cells were produced by the capillary method from line RLC-18 cells which had been obtained from the livers of normal JAR-2 strain rats [14]. Both these cells were plated in 35-mm plastic dishes and treated with 10 μg/ml of CPZ for 3 days. Cell number was estimated by removing the cells with 1 ml of a collagenase solution (0.1%) and counting a portion of the resulting cell suspension in a hemocytometer.





Figs. 1 and 2. Dose-dependent effects of CPZ (Fig. 1, top) and TFPZ (Fig. 2, bottom) on alkaline phosphatase activity in calvaria (△), liver (○), ileum (▲) and duodenum (●) from rats. Various doses of CPZ and TFPZ dissolved in 0.3% methyl alcohol were intraperitoneally injected into adult rats (about 200 g body wt). The enzyme activity was assayed at 17 hr after its injection. Each point represents the mean of five animals. Alkaline phosphatase activities in calvaria, liver, ileum and duodenum of control rats were 0.52, 0.038, 0.30 and 1.92 units/mg protein, respectively. * $P < 0.05$ compared to control animals.

Assays of serum calcium ion and inorganic phosphate contents were performed with an atomic absorption spectrophotometer (Model AA-660, Shimazu Instrument Co., Tokyo, Japan) by the method of Saith [15] and by colorimetry according to the method of Goldenberg and Fernandez [16], respectively.

RESULTS

First we tested the inhibitory effects of phenothiazine derivatives on bone tissues of rats *in vivo*. Of the derivatives tested CPZ and TFPZ had significant and dose-dependent inhibitory effects on alkaline phosphatase activity in calvaria, ileum, liver and duodenum at 17 hr of treatment (Figs. 1 and 2), while two other derivatives did not (data not shown). The effects of CPZ and TFPZ were more specific for calvaria and ileum than for liver and duodenum. Alkaline phosphatase activity in calvaria was significantly decreased by 0.5 mg/kg of CPZ and reached a minimum at 10 mg/kg, the half-maximal inhibitory effect being observed at 2.0 mg/kg (Fig. 1). TFPZ significantly decreased the enzyme activity at 2.5 mg/kg and suppressed the activity half-minimally at 4.0 mg/kg (Fig. 2). Moreover, their effects continued for a longer period in calvaria than in other organs.

The possibility of an effect of phenothiazine derivatives on osteoblastic cells was tested on clone MC3T3-E1 cells. Of the derivatives tested CPZ, TFPZ, and PNZ lowered dose-dependently the alkaline phosphatase activity (Table 1). However, TFPZ had a greater inhibitory effect on the enzyme activity, being twice as potent as CPZ contrary to the *in vivo* results. Moreover, PNZ affected the enzyme activity to a greater extent than did CPZ, although it had little effect on bone tissues *in vivo*. However, PMZ showed no effect (data not shown). The effects of the derivatives on protein and DNA contents were less than those on the enzyme activity. Moreover, we found that CPZ inhibited the proliferation of osteoblastic cells to a greater extent than that of liver epithelial RLC-18(4) cells *in vitro*. After 3 days of treatment with 10 μg/ml of CPZ, cell numbers ± S.D. of control and treated-liver epithelial or osteoblastic cells were 9.67 ± 1.04 and $8.99 \pm 0.40 \times 10^5$ ($P < 0.5$) or 6.11 ± 0.39 and $3.40 \pm 0.76 \times 10^5$ ($P < 0.01$), respectively.

Next, we examined the effects of the derivatives on collagen synthesis in clone MC3T3-E1 cells. The derivatives decreased collagen synthesis in the cells dose-dependently at 24 hr of treatment but CPZ and PNZ affected collagen synthesis to a greater extent than did TFPZ (Table 2). Non-collagen protein synthesis was also inhibited, but its inhibition rate was less than that of collagen synthesis: % of collagen synthesis in CPZ-treated cells became one-half of that in controls at 10 μg/ml of its addition (Table 2). However, CPZ and PNZ at 0.4 μg/ml increased collagen and non-collagen protein syntheses in the cells. Now we do not know the reason about it.

By *in vivo* experiments, the derivatives were shown to strongly decrease alkaline phosphatase activity in ileum, suggesting that intestinal calcium transport could be lowered [17], resulting in a suppression of the serum calcium level. However, the serum calcium level was significantly increased by a

Table 1. Effect of phenothiazine derivatives on DNA and protein contents and alkaline phosphatase activity in clone MT3T3-E1 cells

The derivatives‡ (μg/ml)	DNA (μg/dish)	Protein (μg/dish)	Alkaline phosphatase activity (units/dish)
Expt. 1 CPZ			
None	36.4 ± 3.46	641 ± 22.7	54.9 ± 3.74 (100)
0.4	40.7 ± 3.14	706 ± 44.9	60.9 ± 3.29 (111)
2	43.2 ± 5.74	718 ± 51.3	58.9 ± 1.06 (107)
10	39.7 ± 3.84	567 ± 22.3†	25.1 ± 1.56† (46)
20	32.9 ± 3.07	467 ± 43.8†	19.8 ± 1.57† (36)
Expt. 2 PNZ			
None	38.0 ± 2.06	898 ± 49.3	70.2 ± 1.37 (100)
0.4	34.0 ± 2.29	883 ± 66.8	61.4 ± 6.47 (88)
2	37.5 ± 4.76	810 ± 43.4*	57.1 ± 1.33† (81)
10	26.8 ± 2.09†	507 ± 18.5†	14.7 ± 1.92† (21)
20	15.2 ± 3.10	163 ± 19.2†	16.9 ± 1.60† (24)
Expt. 3 TFPZ			
None	39.0 ± 2.63	802 ± 32.0	67.1 ± 9.29 (100)
0.4	41.3 ± 1.03	792 ± 81.6	57.2 ± 7.52 (85)
2	41.9 ± 1.24	833 ± 39.0	57.5 ± 4.54* (85)
10	34.9 ± 1.46*	575 ± 47.0†	15.2 ± 4.50† (23)
20	25.8 ± 0.63†	125 ± 6.82†	7.47 ± 1.27† (11)

At 48 hr after their addition DNA and protein contents and alkaline phosphatase activity were assayed. Values are means ± S.D. of four dishes. *$P < 0.05$; †$P < 0.01$ compared to controls.

‡ Therapeutic doses of CPZ, TFPZ, PNZ and PMZ are 1.0 ~ 4.0, 0.12 ~ 0.6, 0.16 ~ 0.64 and 0.20 ~ 1.0 mg/kg, respectively [18]. Values in parentheses show per cent for control.

Table 2. Effect of phenothiazine derivatives on collagen and non-collagen protein syntheses in clone MC3T3-E1 cells

The derivatives (μg/ml)	Collagen synthesis (dpm 10^{-4}/dish)	Non-collagen protein synthesis (dpm 10^{-4}/dish)	% of collagen*
Expt. 1 CPZ			
None	3.89 ± 0.57 (100)	5.08 ± 0.86 (100)	12.8 (100)
0.4	4.73 ± 0.77† (122)	6.69 ± 1.59† (132)	11.8 (92)
2	3.67 ± 0.57 (94)	5.30 ± 0.69 (104)	11.4 (89)
10	0.60 ± 0.11‡ (16)	1.90 ± 0.30‡ (37)	5.83‡ (46)
20	0.12 ± 0.01‡ (3)	0.78 ± 0.09‡ (15)	2.55‡ (20)
Expt. 2 PNZ			
None	1.95 ± 0.39 (100)	4.69 ± 0.43 (100)	7.11 (100)
0.4	3.52 ± 0.20‡ (181)	7.53 ± 0.46‡ (161)	7.76 (109)
2	1.96 ± 0.16 (100)	4.19 ± 0.26 (89)	8.22 (116)
10	0.08 ± 0.02‡ (4)	0.83 ± 0.10‡ (18)	2.00‡ (28)
20	0.01 ± 0.002‡ (1)	0.09 ± 0.02‡ (2)	1.39‡ (20)
Expt. 3 TFPZ			
None	6.96 ± 1.85 (100)	13.9 ± 4.96 (100)	8.49 (100)
0.4	6.35 ± 0.53 (91)	13.5 ± 1.61 (97)	8.00 (94)
2	6.32 ± 1.33 (91)	14.0 ± 6.46 (100)	7.82 (92)
10	5.23 ± 0.45† (75)	10.1 ± 3.87 (73)	8.75 (103)
20	1.14 ± 0.06‡ (16)	5.02 ± 0.24‡ (36)	4.04‡ (48)

Collagen and non-collagen protein syntheses were assayed at 24 hr after their additions. Values are means ± S.D. of four dishes. *$P < 0.05$; ‡$P < 0.01$ compared to controls.

* Calculated assuming that collagen has an imino acid content 5.4 times higher than that of other protein [12].

Values in parentheses show per cent for control.

single injection of 10 mg/kg of CPZ: mean serum calcium levels ± S.D. in CPZ-treated and control animals were 5.7 ± 0.7 and 4.6 ± 0.4 mM, respectively ($P < 0.05$). The serum phosphate levers were slightly increased by CPZ: the means ± S.D. in treated and control animals were 3.9 ± 0.6 and 3.4 ± 0.7 mM, respectively ($P < 0.1$).

DISCUSSION

These *in vivo* and *in vitro* studies demonstrated that CPZ, TFPZ and PNZ of the phenotiazine derivatives have specific, inhibitory effects on osteoblastic cell function(s). The decreasing order of their effects was CPZ, TFPZ, PNA and PMZ: CPZ affected calvaria to a greater extent than TFPZ, with half-maximum inhibitory effects being observed at 2.0 mg/kg. These same doses are used for treatment of emotionally disturbed subjects (1.0 ~ 4.0 mg/kg), indicating caution in its application in man [18]. Therapeutic doses of TFPZ (0.12 ~ 0.60 mg/kg) are one-quarter of the minimum effective dose for affecting calvaria *in vivo* but are sufficient to affect the cells *in vitro*. PNZ (therapeutic doses, 0.16 ~ 0.64 mg/kg) affected osteoblastic cells to a greater extent than did TFPZ, although it was not effective *in vivo*. Thus, caution is also warranted in the application of TFPZ and PNZ in man, because animal species differences appear with regards to the side effects of drugs [19]. For example, side effects of vitamin A on mouse, house musk shrew, and man are observed at 200, 10 and one I.U./g, respectively. On the contrary, cytosine arabinoside affects mice at a 300-fold lower concentration than it does the house musk shrew.

CPZ, PNZ and TFPZ affected DNA and protein content to a lesser extent than the enzyme activity and collagen synthesis, suggesting that their inhibitory effects are probably directed toward osteoblastic cell function rather than being a reflection of general cytotoxicity. In general, substituents at the C_2 position of 3-ring structures increase the potency of the compound [18]. A CF_3 substituent at C_2 has the most antipsychotic and antiemetic potency, and its decreasing order is said to be F, CH_3, Cl and H substituents. In contrast, the derivatives with Cl substituent seem to have the strongest side effect. However, presently we do not know the mechanism of phenothiazine action on osteoblastic cells.

Phenothiazine derivatives also suppressed alkaline phosphatase activity in ileum as low as in calvaria of rats. Intestinal calcium transport could be lowered [17], resulting in a decrease in the serum calcium level in CPZ-treated rats, although we need a direct assay of the transport using everted duodenal sacs [20] to determine the derivatives' effect. However, we obtained unexpected results: the serum calcium level was significantly increased by a single injection of 10 μg/kg CPZ into hypercalcemic rats. Moreover, the phosphate content in serum was also elevated, and the ratio of Ca/P, 1.5, was similar to that of bone tissue [21]. Thus, these serum calcium and phosphate levels could be caused by their release from bone tissues resulting from stimulation of bone resorption. Their decrease in urinary excretion is also considered to be caused by the phenothiazine derivatives independently. Experiments are currently in progress to resolve these interesting possibilities.

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