Relationship Between Structure of Phenothiazines and *in Vitro* Cytotoxicity (35019)

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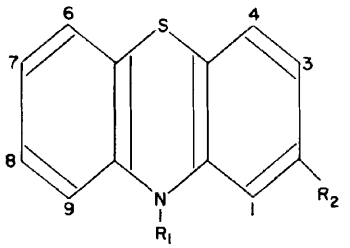
Previous studies in this laboratory have utilized *in vitro* systems to demonstrate the cytotoxicity of known and suspected hepatotoxic agents (1–4). Exposure of suspensions of cells, grown in tissue culture, to CCl_4 was found to lead to rapid loss of intracellular enzymes (1, 2). Chlorpromazine (CPZ), an agent known to produce jaundice or hepatic dysfunction in humans (5) also was found to lead to leakage of enzymes from such cell suspensions (4) and from rabbit liver slices (3); while promazine (PZ), an agent which rarely produces hepatic injury in man (5), led to a lesser (4) or no (3) effect. The two phenothiazines are, structurally, almost identical and differ only in that CPZ has a chlorine atom at position R_2 while PZ has a hydrogen atom at that position (Fig. 1). Accordingly, the present study directed at the relationship between the chemical structure of seven phenothiazine compounds and their cytotoxic effects are undertaken.

Material and Methods. Chang human liver cells grown as a monolayer in tissue culture were obtained from Microbiological Associates, Rockville, Md. The cells were processed within 24 hr of arrival during which time they were kept at 4°. Immediately before the experiment, the cells were centrifuged for 10 min at 180*g*, the original medium was discarded and the cells were resuspended in Hanks' base medium 119 (IX) free of antibiotics and tested to be free of activity of the enzymes assayed in this study (see below). For each experiment, 1 ml of suspended cells containing approximately 10^6 cells/ml was mixed with 1 ml of the medium used for suspension (controls) or 1 ml of the same medium containing 1 of the 7 phenothiazine compounds listed in Fig. 1 in different concentrations. (Several other phenothiazines considered for testing did not enter into satisfactory solution under the conditions of this experiment and could not be studied.) The final concentration of each compound ranged from 10^{-5} to 10^{-3} *M*. The pH of the medium was adjusted to 6.7 with 2.5 *N* HCl for all solutions of drugs (and controls) to fulfill the pH requirement for solution of these phenothiazines. Samples were incubated at 37° for 30 min, after which they were centrifuged for 5 min at 1000 rpm. The supernatant was decanted and the enzyme activity of cells and supernatant was measured. The activity of aspartate aminotransferase (glutamic oxalacetic transaminase, GOT), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) was measured according to previously reported methods (1) and reported as international units (IU) per liter or per 10^9 cells; IU is defined as micromoles of substrate altered per minute at 25°.

Results. Exposure of Chang cells to each of the phenothiazines led to leakage of LDH, MDH, and GOT (Fig. 2). A significant increase in the level of one or more of the enzymes of the medium was noted at a concentration of 5×10^{-5} or greater of trifluoperazine (TFPER), triflupromazine (TFPZ), thioridazine (THZ), fluphenazine (FPZ), and chlorpromazine (CPZ). Promethazine (PMZ) and promazine (PZ) required a concentration five times greater (5×10^{-4} *M*) to lead to significant leakage of LDH. Measurement of residual enzyme in the cells at the end of the period of incubation demonstrated a decrease that corresponded, in general, to the amount that had



Phenothiazine Nucleus

Drug	R_1	R_2
Trifluoperazine (TFPER)	$-CH_2-CH_2-CH_2-N\langle\rangle N-CH_3$	$-CF_3$
Triflupromazine (TFPZ)	$-CH_2-CH_2-CH_2-N(CH_3)_2$	$-CF_3$
Chlorpromazine (CPZ)	$-CH_2-CH_2-CH_2-N(CH_3)_2$	$-Cl$
Fluphenazine (FPZ)	$-CH_2-CH_2-CH_2-N\langle\rangle N-CH_2-CH_2OH$	$-CF_3$
Thioridazine (PMZ)	$-CH_2-CH_2-$	$-SCH_3$
Promazine (PZ)	$-CH_2-CH_2-CH_2-N(CH_3)_2$	H
Promethazine (THZ)	$-CH_2-CH(CH_3)-N(CH_3)_2$	H

FIG. 1. Structure of phenothiazine nucleus and description of substituents R_1 and R_2 in 7 phenothiazines tested.

"leaked" into the medium (Fig. 3). Leakage of MDH and GOT yielded somewhat similar patterns (Fig. 2).

The relative potency of the various phenothiazines with regard to their ability to induce enzyme leakage, was compared by examining the leakage at each concentration of the drug. The resulting values for enzyme leakage fell into three broad categories. The most potent effect seemed to be produced by TFPER, TFPZ, and THZ. CPZ and FPZ seemed to be of intermediate potency; while PMZ and PZ led to the least amount of enzyme leakage and required higher concentrations.

Comparison of the effects of TFPER with those of FPZ, in order to estimate the importance of the R_1 substituent, and of the effects of TFPZ with those of CPZ, in order to appraise the importance of the R_2 substituent, is shown in Table I. At concentrations ranging from 5×10^{-5} to 10^{-3} *M*, TFPER led to significantly greater degree of enzyme "leakage" than did FPZ. TFPZ seemed to lead to significantly ($p<0.01$) greater degrees of "leakage" than did CPZ at a concentration of 5×10^{4} but to equivocal or no significant differences at other concentrations.

Discussion. The demonstration in the present study as in previous studies (3, 4) of enzyme leakage from tissue culture cells of hepatic origin exposed to phenothiazines *in vitro*, is presumptive evidence that these drugs produce damage to the cells or alter the permeability of their membranes. Changes

TABLE I. Comparison of the Effects of TFPER with those of FPZ and of the Effects of TFPZ with those of CPZ as Reflected in Enzyme Levels of Medium.

Concentrates of drugs 5×10^{-4} *M*.

	TFPER		FPZ			TFPZ		CPZ		
Enzyme	No.[a]	Value	No.	Value	p <	No.[a]	Value	No.	Value	p <
LDH	11	617 ± 36	19	468 ± 26	0.005	17	521 ± 22	12	376 ± 35	0.01
MDH	11	182 ± 14	19	96 ± 11	0.005	16	174 ± 14	12	112 ± 10	0.01
GOT	7	47 ± 8	17	39 ± 5	NS	17	44 ± 3	12	39 ± 4	NS

[a] Number of experiments.

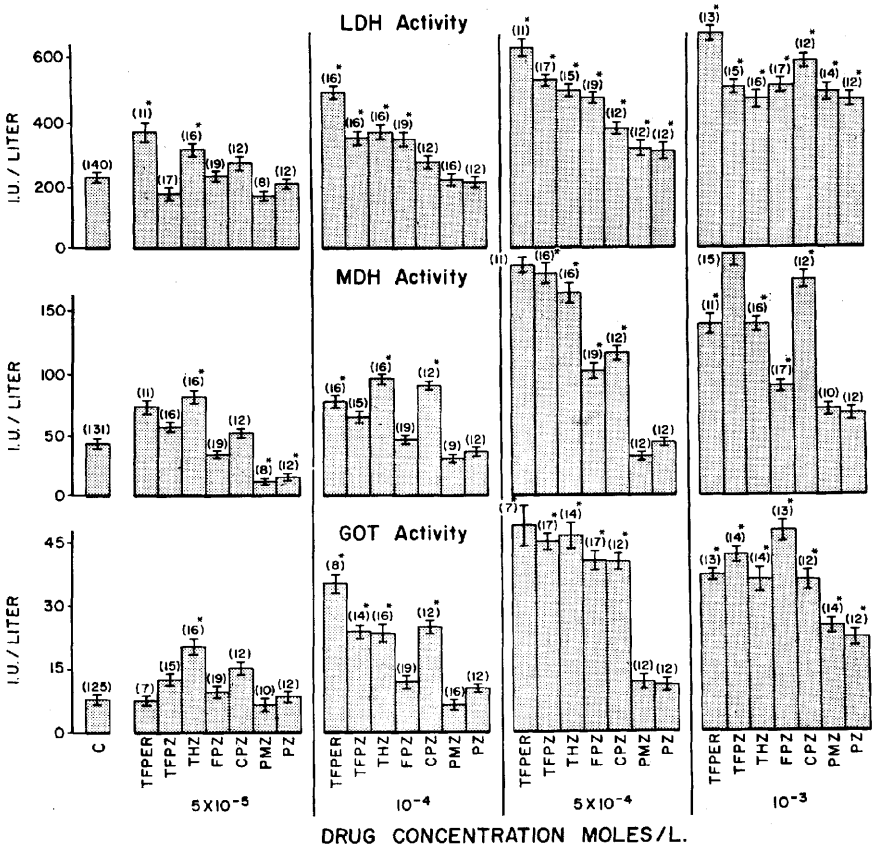


FIG. 2. Increase in LDH, MDH, and GOT content of medium after 30 min of exposure of cells to phenothiazines at concentrations indicated along abscissa. See Fig. 1 for meaning of abbreviation for drugs and text for meaning of international units (IU). Asterisks indicate values significantly different from control ($p<0.01$).

in apparent enzyme concentrations of the medium could be identified as due to leakage of enzymes from cells since the increase in level in the medium was accompanied by a decrease in content in the cells. The effect of the phenothiazines on the enzyme leakage was dependent on the concentration of the respective drug and varied with the different drugs.

These observations suggest a relationship between the nature of substituents R_1 and R_2 of the phenothiazine molecule and the adverse effect on cell membranes. A trifluoromethyl group (TFPER, TFPZ, FPZ (or chlorine atom (CPZ) in position R_2 seems to confer a greater "cytotoxic" potential than the H atom (PZ, PMZ) in that position. Also it may be inferred that a $-CF_3$ group at the R_2 position enhances cytotoxicity more than does the –Cl substituent; since TFPZ and CPZ have identical R_1 chains but differ only in the R_2 substituent. Comparison of the effects of TFPER and FPZ, both of which have $-CF_3$ group at R_2, but differ slightly with regard to the R_1 chain, suggests that a terminal alcohol group (CH_2–CH_2OH) in that position (FPZ) yields a less cytotoxic molecule (in this model) than does a terminal methyl group (TFPER and TFPZ). Since both the R_1 and R_2 substituents of THZ differed from those of the other drugs, no inference can be drawn regarding the effects of these structural features, other than the deduction that one or both contributed to the relative cytotoxic effect.

These efforts to relate chemical structure

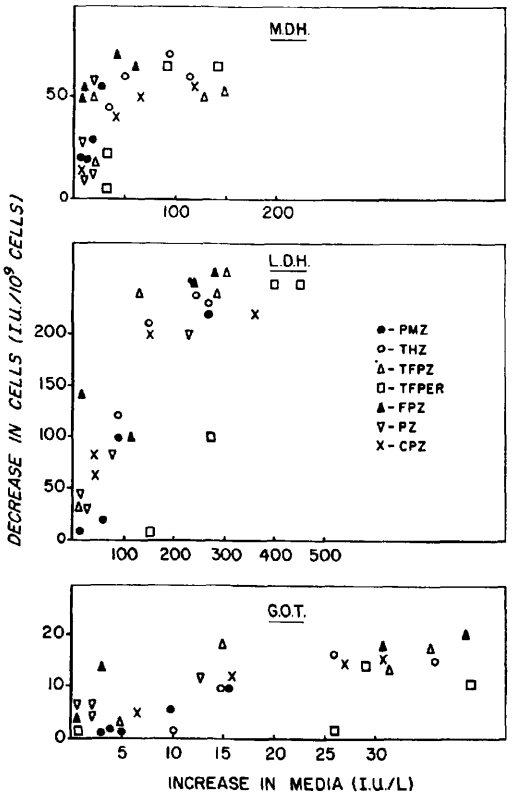


FIG. 3. Decrease in enzyme content of cells plotted against enzyme content of medium after 30 min of exposure of cells to phenothiazines at all concentrations studied. See Fig. 1 for meaning of abbreviations for drugs and text for meaning of international units (IU).

to *in vitro* cytotoxicity are based on studies with too few compounds to permit more than inferences. Nevertheless, the correlation between structure of the phenothiazine and adverse effects on Chang cells parallels generally the adverse effects in other *in vitro* models, *e.g.*, the relative potency of phenothiazines as inhibitors of motility of protozoa (*Tetrahymena pyriformis*) (6) and as hemolytic agents (7).

The correlation between the chemical structure and adverse effect on Chang cells also parallels the correlation between chemical structure and stability of the free radical intermediate formed during the oxidation of phenothiazines (8). Since the *in vivo* action of phenothiazines, and presumably their therapeutic effects, has been considered to be mediated by the respective free radical (9, 10), these parallels appear to validate the biologic significance of the *in vitro* model employed in the present study.

In previous studies, the greater potency of CPZ than of PZ in inducing enzyme leakage from Chang cells (4) and from rabbit liver slices (3) *in vitro* was considered of possible relevance to the effects of phenothiazines on the liver in humans; since PZ appears to produce jaundice or hepatic dysfunction in humans much less frequently than does CPZ (5). The possible relevance of these *in vitro* studies to hepatotoxic effects of other phenothiazines remains to be demonstrated. The alteration of membrane permeability that seems to be related to the structure of the individual compounds, however, provides another biological model for the study of phenothiazines and other drugs.

Summary. Suspensions of cultured human liver cells (Chang) were exposed to 1 of 7 phenothiazines at concentrations ranging from 10^{-5} to 10^{-3} *M*, for a period of 30 min. Identical preparations of cells without drugs served as the controls. The levels of three enzymes (lactate dehydrogenase, malate dehydrogenase and aspartate aminotransferase) were measured in the medium and cells. The differences between the controls and drug preparations were considered to be the result of cytotoxic effects of the drugs. In this model, the trifluoperazine, thioridazine, and triflupromazine showed the greatest cytotoxic potency; promazine and promethazine showed the least potent cytotoxic effect, and chlorpromazine and fluphenazine were of intermediate potency. The possible relationship between these effects of the respective phenothiazines and their chemical structure was considered.

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