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COMPARATIVE EFFECTS OF SUBSTITUTED PHENOTHIAZINES AND THEIR FREE RADICALS ON (Na^+,K^+)-ACTIVATED ADENOSINE TRIPHOSPHATASE*

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Abstract—The inhibitory effects of free radicals of various substituted phenothiazines on (Na^+,K^+)-activated ATPase [Mg^{2+}-dependent, (Na^+,K^+)-activated ATP phosphohydrolase; EC 3.6.1.3] were studied *in vitro*. Enzyme preparations were obtained from rat brain microsomal fractions after deoxycholic acid and NaI treatments. Free radicals were produced by either ultraviolet (253·7 nm) irradiation or enzymatic oxidation with peroxidase. Without enzymatic or photo-oxidation, phenothiazine derivatives failed to inhibit (Na^+,K^+)-activated ATPase activity significantly. Photo-oxidative intermediates of thioridazine, triflupromazine and trifluoperazine were potent inhibitors of (Na^+,K^+)-activated ATPase activity. Those from chlorpromazine, perphenazine and promazine were less potent. Peroxidase–hydrogen peroxide treatment of promazine, thioridazine, perphenazine and chlorpromazine produced free radical intermediates which significantly inhibited (Na^+,K^+)-activated ATPase activity. The same treatment of triflupromazine and trifluoperazine, however, failed to produce detectable amounts of free radical intermediates. Concomitantly, no inhibition of (Na^+,K^+)-activated ATPase activity was observed under these conditions. It was concluded that free radical intermediates of various substituted phenothiazines are differentially potent inhibitors of (Na^+,K^+)-activated ATPase, and that the formation of free radicals from substituted phenothiazines is dependent upon the oxidizing conditions and nature of substituent groups.

IT HAS BEEN reported from several laboratories that phenothiazine tranquilizers inhibit active transport of potassium across cytoplasmic membranes[1] and (Na^+,K^+)-activated ATPase activity *in vitro*.[1–5] Subsequently, the inhibition of (Na^+,K^+)-activated ATPase by chlorpromazine, one of the substituted phenothiazines, was found to depend on an intermediate oxidation product of the drug, namely a semiquinone free radical.[6] The original drug, chlorpromazine, and the final oxidation product, chlorpromazine sulfoxide, had only minimal inhibitory effects on the (Na^+,K^+)-activated ATPase.

It has been shown that free radical intermediates of chlorpromazine can be generated *in vivo*. These highly reactive forms may be produced *in vivo* during the oxidative metabolism of chlorpromazine,[7] by interaction of chlorpromazine with melanin[8,9] or by the exposure of the eye to light.[10] The present study indicates that free radicals of pharmacologically active phenothiazine derivatives are potent inhibitors of (Na^+,K^+)-

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activated ATPase activity *in vitro* and that the ability to form the free radical differs among the various phenothiazines depending upon the experimental conditions.

METHODS

(Na^+,K^+)-activated ATPase preparations were obtained from rat brain microsomal fractions after treatment with deoxycholic acid and NaI according to the method of Akera and Brody.[11] The ATPase activity was estimated from the amount of inorganic phosphate liberated from ATP during incubations at 37°.[11] Mg^{2+}-dependent ATPase (Mg^{2+}-dependent ATP phosphohydrolase; EC 3.6.1.3) activity, assayed in the presence of $MgCl_2$, was subtracted from the total ATPase activity, assayed in the presence of NaCl, KCl and $MgCl_2$, to calculate the (Na^+,K^+)-activated ATPase activity. (Na^+,K^+)-ATPase activity was approximately 160 μmoles Pi/mg protein/hr. Protein was estimated by the method of Lowry *et al.*[12] using bovine serum albumin as a standard.

Photo-oxidation of substituted phenothiazines by ultraviolet (253·7 nm) irradiation was performed as reported previously.[6] A mixture containing 0·04 mg of enzyme protein/ml, 10 μM substituted phenothiazine and 100 mM Tris–HCl buffer (pH 7·5) was exposed to ultraviolet light. After exposure, the mixture was diluted 2-fold with solutions containing the necessary ions and substrates. In all experiments, concentration of the substituted phenothiazine during the incubation for ATPase assay was 5 μM. To control the extraneous formation of free radical intermediates, exposure of phenothiazine solutions to light during the preparation and the incubation was minimized. The absorption spectra of each mixture, before and after photo-oxidation, were recorded with either a Beckman DB-GT or a Shimadzu model MPS-50L recording spectrophotometer. The amount of drug converted, a measure of the amount of free radical formed, was calculated from the decrease in peak ultraviolet absorbance and the respective molar extinction coefficient. This was possible, since extensive ultraviolet exposure of substituted phenothiazines at pH 7·5 abolished ultraviolet absorption without forming additional absorption peaks.

Enzymatic oxidation of substituted phenothiazines was performed by preincubating 10 μM of the drug, with 8 μg peroxidase (Sigma Chemical Company, Reinheitzahl 0·8) per ml, 40 μM hydrogen peroxide and 100 mM Tris–HCl buffer (pH 7·5) in the presence of 0·04 mg of (Na^+,K^+)-activated ATPase protein in a volume of 0·5 ml at 37° for 5 min. After the preincubation, necessary ions and substrate were added. Absorption spectra and ATPase activity of the mixture were assayed as described above. Statistical analysis was performed using either Tukey's procedure or Student's *t*-test.[13] Significance was at the $P < 0{\cdot}05$ level.

RESULTS

Irradiation at 253·7 nm was found to be suitable for the photo-oxidation of six substituted phenothiazines studied, since these agents had ultraviolet absorption maxima in the 253 to 262 nm range and molar extinction coefficients of 29–35 mM^{-1} cm^{-1} (Table 1). These values are in good agreement with those previously reported.[14] Percentages of these agents photo-oxidized after 4 min of irradiation were always greater than those after 2 min of irradiation. Among the six phenothiazine derivatives, the amount of photo-oxidized drug decreased as the distance between the irradiation wavelength (253·7 nm) and the respective absorption peaks increased. For

TABLE 1. PHOTO-OXIDATION OF SUBSTITUTED PHENOTHIAZINES*

	Absorption peak (nm)	Molar extinction coefficient ($mM^{-1}\ cm^{-1}$)	Drug converted 2-min irradiation (%)	Drug converted 4-min irradiation (%)
Promazine	253	33·5	22·1 ± 0·9 (8)†	38·7 ± 2·3 (8)†
Chlorpromazine	255	31·6	19·6 ± 1·1 (8)†	26·2 ± 0·6 (8)†
Perphenazine	255	29·8	15·4 ± 0·2 (8)†	24·4 ± 1·1 (8)†
Triflupromazine	256	32·4	8·5 ± 0·9 (8)†	11·5 ± 0·6 (8)†
Trifluoperazine	257	33·1	8·9 ± 0·4 (9)†	13·3 ± 1·9 (8)†
Thioridazine	262	34·8	5·8 ± 0·7 (9)†	12·0 ± 1·1 (6)†

* Absorption spectra were recorded before and after 2 or 4 min of ultraviolet (253·7 nm) irradiation. The amount of photo-oxidized phenothiazine derivatives was calculated from the decrease in absorption maximum, and their respective molar extinction coefficients. All samples contained 0·04 mg of (Na^+,K^+)-activated ATPase protein/ml, and 10 μM substituted phenothiazine in 100 mM Tris–HCl buffer (pH 7·5). The numbers in parentheses indicate numbers of experiments. The values given for per cent phenothiazine derivatives converted are means ± S.E.M.

† Significant amounts of photo-oxidative conversion occurred ($P < 0·05$ by paired t-test).

all experiments, the proportion of substituted phenothiazines photo-oxidized was from approximately 6 to 22 per cent for 2 min of ultraviolet irradiation, and 12 to 40 per cent for 4 min of ultraviolet irradiation (Table 1). The original phenothiazine derivative concentration was 10 nmole/ml (10 μM), and the amount of converted drug as calculated from decreases in ultraviolet absorption peaks was from 0·6 to 4·0 nmoles/ml.

Four-min ultraviolet irradiation of the enzyme solution in the absence of drug produced a 7·4 per cent inhibition of (Na^+,K^+)-activated ATPase activity. Changes in microsomal (Na^+,K^+)-activated ATPase activity ranging from −2·9 to 9·8 per cent were observed with 5 μM substituted phenothiazines, when the experiments were performed avoiding exposure to light. These changes, however, were not significant. Significant inhibition was observed when a mixture containing 10 μM substituted phenothiazine and (Na^+,K^+)-activated ATPase preparation was irradiated for either 2 or 4 min (Fig. 1). Per cent inhibition of (Na^+,K^+)-activated ATPase by irradiated phenothiazines was calculated relative to the enzyme activity assayed with ultraviolet irradiation in the absence of the drug. In order to assess the relative potency of photo-oxidized phenothiazine derivatives, the inhibition of (Na^+,K^+)-activated ATPase activity was plotted against the amount of photo-oxidized drug. In the presence of substituted phenothiazines, the inhibition was always greater after 4 min of irradiation than after 2 min of irradiation (Fig. 1). The photo-oxidation products of perphenazine and chlorpromazine were shown to be more potent than that of promazine, since the amount converted by ultraviolet irradiation needed to achieve comparable inhibition of the enzyme activity was significantly smaller than that of promazine. Those of substituted phenothiazines such as thioridazine, trifluoperazine or triflupromazine required even smaller amounts of oxidation product and thus were even more potent.

Unlike photo-oxidation, enzymatic oxidation of phenothiazine derivatives resulted in an appearance of ultraviolet absorption peaks, as can be seen in Fig. 2 (left), which shows the absorption spectra of chlorpromazine before and after the incubation with

peroxidase and hydrogen peroxide. These peaks correspond to those reported for chlorpromazine free radical (270 and 278 nm) and chlorpromazine sulfoxide (240, 300 and 340 nm).[6,14] The superimposition of these peaks on the 255 nm chlorpromazine peak precluded the calculation of amount of oxidized drug from the ultraviolet spectrum. With promazine, thioridazine and perphenazine, qualitatively similar results were obtained, indicating the generation of the respective free radicals. The peroxidase system, however, failed to produce semiquinone free radicals from trifluo-

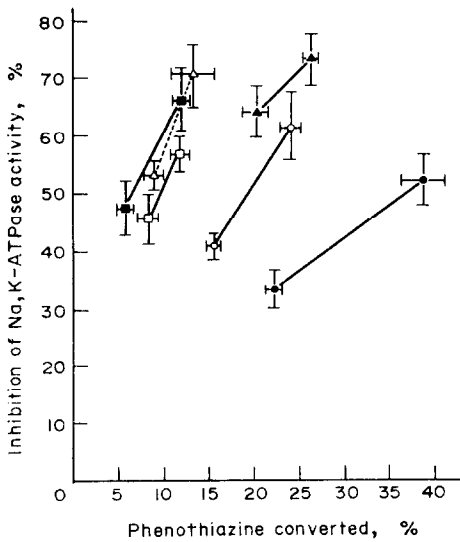

FIG. 1. Inhibition of (Na^+,K^+)-activated ATPase by photo-oxidative products of substituted phenothiazines. Mixtures containing enzyme preparation and 10 μM substituted phenothiazine were exposed to ultraviolet irradiation or kept in a dark place. After the exposure, cations and substrate were added and ATPase activity was assayed. Concentrations of substituted phenothiazines during the ATPase assay were 5 μM. Per cent inhibition of (Na^+,K^+)-activated ATPase by irradiated phenothiazines was calculated relative to the enzyme activity assayed after the ultraviolet irradiation in the absence of the drug. Each point represents the mean of eight experiments in duplicate, except for thioridazine and the 2-min (8·9 per cent drug conversion) irradiation of trifluoperazine where n = 9. Horizontal lines indicate S.E.M. for photo-oxidation of the drug, and vertical lines indicate S.E.M. for inhibition of the enzyme activity. Promazine (●), perphenazine (○), chlorpromazine (▲), trifluoperazine (△), triflupromazine (□) and thioridazine (■).

perazine and triflupromazine. The ultraviolet absorption spectra for these trifluomethyl substituted phenothiazines showed no peaks characteristic of free radical after treatment with peroxidase (Fig. 2, right). Promazine, thioridazine and perphenazine showed significant inhibition of (Na^+,K^+)-activated ATPase activity after enzymatic oxidation with peroxidase. Chlorpromazine also showed a significant inhibition of (Na^+,K^+)-activated ATPase activity (Table 2), although the magnitude of inhibition was significantly smaller. With triflupromazine and trifluoperazine, substituted phenothiazines which failed to form free radicals with peroxidase treatment, no inhibition of enzyme activity was observed. Chlorpromazine sulfoxide, promazine sulfoxide and trifluoperazine sulfoxide with peroxidase treatment did not inhibit the enzyme activity in concentrations of 5 μM.

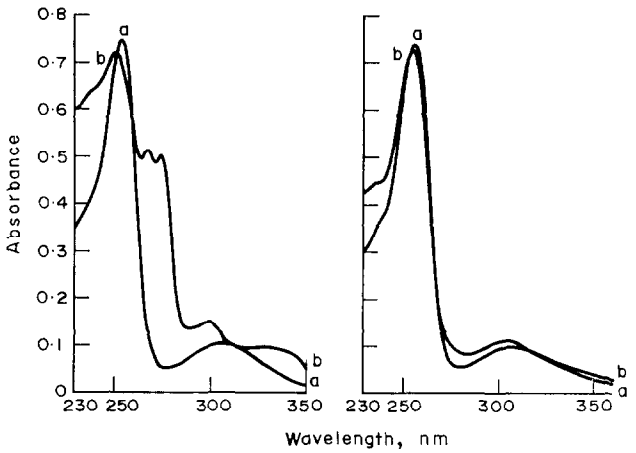

FIG. 2. Ultraviolet absorption spectra of chlorpromazine and triflupromazine with and without peroxidase–hydrogen peroxide treatment. Absorption spectra for chlorpromazine (left) and triflupromazine (right) are shown (a) before peroxidase–hydrogen peroxide treatment; and (b) after peroxidase–hydrogen peroxide treatment. Enzymatic oxidation of chlorpromazine (left) produced peaks in the ultraviolet absorption spectra corresponding to those reported[6] for chlorpromazine free radical (270 and 278 nm) and chlorpromazine sulfoxide (240, 300 and 340 nm). Promazine, thioridazine and perphenazine showed similar results, indicating the generation of free radicals. Peroxidase–hydrogen peroxide treatment failed to produce semiquinone free radicals from the trifluomethyl substituted phenothiazines tested. The ultraviolet absorption spectra for triflupromazine (right) showed no peaks characteristic of free radicals after such treatment.

TABLE 2. EFFECT OF PEROXIDASE TREATMENT ON THE INHIBITION OF (Na^+,K^+)-ACTIVATED ATPASE ACTIVITY BY VARIOUS SUBSTITUTED PHENOTHIAZINES*

Phenothiazine derivative	Formation of free radical	Inhibition of (Na^+,K^+)-activated ATPase (%)
Promazine	+	70·0 ± 2·2 (8)†
Thioridazine	+	63·5 ± 3·7 (8)†
Perphenazine	+	63·0 ± 5·0 (8)†
Chlorpromazine	+	37·2 ± 5·1 (8)†
Triflupromazine	−	4·8 ± 5·5 (9)
Trifluoperazine	−	−0·8 ± 7·6 (8)

* (Na^+,K^+)-activated ATPase activity was assayed at 37° after pre-incubation of a mixture containing enzyme preparation and substituted phenothiazine with peroxidase and hydrogen peroxide. Per cent inhibition of the enzyme activity was calculated relative to enzyme activities after peroxidase treatment in the absence of drugs. Free radical formation was determined from the ultraviolet absorption spectrum after peroxidase and hydrogen peroxide treatment. The values are means ± S.E.M. Numbers in parentheses indicate the number of experiments performed in duplicate.

† Significant inhibition of enzyme activity ($P < 0·05$).

DISCUSSION

The present data indicate that oxidation products of substituted phenothiazines were potent inhibitors of the (Na^+,K^+)-activated ATPase *in vitro* irrespective of the method by which they were produced. With effective photo-oxidation or enzymatic oxidation, significant inhibition of the enzyme activity was achieved with concentra-

tions of these compounds one or two orders of magnitude less than those reported by Davis and Brody.[5] The relative potencies of substituted phenothiazines, however, were similar in both studies, except for perphenazine which was, in contrast to previous data,[5] less potent than chlorpromazine after the controlled photo-oxidation. This discrepancy seems to be the result of uncontrolled photo-oxidation in the previous study.[5] Alternatively, the inhibition of enzyme activity in the previous study with high concentrations of substituted phenothiazines may be due to the parent compounds and the observed general correlation could be incidental. Peroxidase treatment did not result in the production of inhibitory species when a significant amount of the compound was not oxidized. Promazine sulfoxide, chlorpromazine sulfoxide and trifluoperazine sulfoxide in doses as high as 100 μM did not significantly inhibit (Na^+,K^+)-activated ATPase.[5] Chlorpromazine sulfoxide, promazine sulfoxide and trifluoperazine sulfoxide with peroxidase treatment also did not inhibit the enzyme activity, indicating that oxidation products beyond the sulfoxide forms were not active in inhibiting the (Na^+,K^+)-activated ATPase *in vitro*. Thus, the oxidation product formed which is intermediate between the parent phenothiazine derivatives and their sulfoxides must be the inhibitory species.

Phenothiazine derivatives can form semiquinone free radicals via one-electron oxidation mechanisms. The steady state concentration of such free radicals in solution depends on the rate of free radical production and the rate of spontaneous disproportionation. Tozer and Tuck[15] have shown that the rate of disproportionation of semiquinone free radicals of phenothiazine derivatives is markedly enhanced by 2-chloride substitution. Trifluoromethyl substitution increased the decay rate further.[15] Our data, however, indicate that this substitution increases the potency of free radicals to inhibit (Na^+,K^+)-activated ATPase. Therefore, it may be concluded that free radicals of 2-trifluoromethyl substituted phenothiazines, and to a lesser extent that of chlorpromazine, have higher reactivity or higher affinity for the enzyme.

Peroxidase treatment failed to produce free radicals from 2-trifluoromethyl substituted phenothiazines. This may be explained on the basis of the electron inductive withdrawing power of this substituent group. Substitution at position 2 differentially stabilizes the sulfur electrons in the phenothiazine resonance structure, making it less susceptible to conversion to free radical by coupled oxidation with peroxidase and hydrogen peroxide treatment. Thus, substituted phenothiazines which easily form free radicals by ultraviolet irradiation may not be oxidizable to free radicals by certain enzymatic mechanisms.

It may be concluded that free radical intermediates of various substituted phenothiazines are responsible for inhibition of rat brain (Na^+,K^+)-activated ATPase *in vitro*. Free radicals of different substituted phenothiazines had different potencies. Enzymatic oxidation produced free radicals from certain phenothiazine derivatives which were capable of inhibiting (Na^+,K^+)-activated ATPase, but failed to produce free radicals from other derivatives.

The differential oxidation of substituted phenothiazines by several oxidative mechanisms precluded a direct comparison between the clinical potency of phenothiazine tranquilizers and the potency of their free radicals to inhibit (Na^+,K^+)-activated ATPase *in vitro*. Some potent free radicals such as those of the trifluoromethyl substituted compounds may be produced by photo-oxidation, for example in the eye and skin, but are less likely to be generated by other oxidative mechanisms.

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