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ENZYMATIC OXIDATION OF PHENOTHIAZINES BY LIPOXYGENASE/H_2O_2 SYSTEM

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Abstract—Lipoxygenase (LOX) (EC 1.13.11.12) oxidized a wide range of phenothiazine (Pt) tranquillizers to their corresponding radical cations in the presence of H_2O_2 by means of an enzymatic chemical second-order mechanism with substrate regeneration similar to that of horseradish peroxidase. The optimum pH of LOX for this hydroperoxidase activity was in the acid range (pH 3.0–4.0), as has been shown for other Pt oxidizing systems, such as peroxidase/H_2O_2 and haemoglobin. LOX showed Michaelis constants for Pt ranging from 1.4 to 8.5 mM and which, in some cases, e.g. trifluoperazine, displayed substrate inhibition. By contrast, it had a high affinity for H_2O_2 in the μM to mM range. A new, previously undescribed plot, which relates the enzymatic affinity and the apparent second-order decay of the cation radical, was developed to study the influence of the 2- and 10-substituents in the Pt ring. The implications of this new plot and the LOX-mediated Pt oxidation are also discussed.

Key words: lipoxygenase; phenothiazine; hydroperoxidase activity; xenobiotic

Pts† are among the most commonly used antipsychotic agents for the treatment of schizophrenia and manic phases of manic depressive illnesses [1]. The action mechanism of these compounds has been a subject of intense study since their use began in the early 1950s. There is significant evidence that the drugs interact with dopamine receptors, and strong correlation exists between drug potency and the strength of this interaction [2, 3]. Chemically, Pts have a three-ring structure in which two benzene rings are linked by a sulphur and a nitrogen atom (Table 1) and they exist as a wide range of structurally related compounds which vary in substitution at the 2- and 10-position [4].

The influence of the 2-position substituent and the structure of the 10-position side chain on the antipsychotic activity of Pts has been studied in detail [1]. At the 10-position, the most effective antipsychotic compounds are those with a piperazine group, followed by those with aliphatic side chains and those with a piperidine moiety in the side chain. Substitution by an electron-withdrawing group, such as halogens, at the 2-position increases the efficiency of Pt [5].

Pts have excellent electron-donating properties since their highest occupied orbital is anti-bonding [6], which leads to the formation of $Pt^{+\cdot}$ [4] during their *in vivo* enzymatic oxidative metabolism to $PtSO.^+$ It has been suggested that the oxidation of Pts to their corresponding free radicals is an essential transformation both for their pharmacological activity and their metabolism [4]. However, the relationship between the stability of the free radicals and the pharmacological action is not clear.

In addition, little is known about the substituent effect on the kinetic parameters (V_m and K_m) of the oxidative enzymes [7] and the knowledge is restricted to heme enzymes (cytochrome P450, HRP and Hb). To date, only the influence on the k_3 rate constant involved in the transformation of Pts from HRP compound II has been studied [7].

The aim of the present work was to extend the study of the Pt substituent effect to other enzymes with non-heme groups in the active centre and which have a wide extra-hepatic distribution, such as LOXs. These enzymes have been shown to be involved in the oxidation of xenobiotics [8, 9].

MATERIALS AND METHODS

Pts and electrophoretically pure soybean LOX (EC 1.13.11.12) Type-V (646.000 Sigma units/mg protein) [8] were purchased from Sigma (Madrid, Spain). Fluphenazine was from RBI (Natick, MA, U.S.A.) and methylpromazine and methoxypromazine were gifts from Rhône-Poulenc (Paris, France). All other chemicals used were of analytical grade.

The activity was followed spectrophotometrically in an Uvikon 940 (Kontron) at the absorption maximum of each Pt cation radical. Molar absorptivities, ε, of Pt radical cations were calculated according to the method of Vazquez *et al.* [10].

H_2O_2 and Pt solutions were freshly prepared every day. The H_2O_2 and LOX concentrations were calculated using $\varepsilon_{240} = 39.4\ M^{-1}cm^{-1}$[11] and $\varepsilon_{280} = 160{,}000\ M^{-1}\ cm^{-1}$ [12], respectively.

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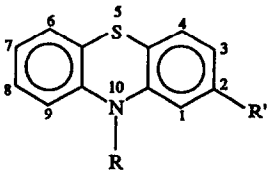
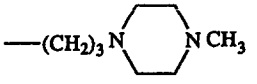
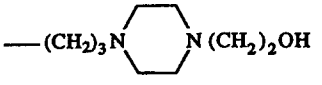
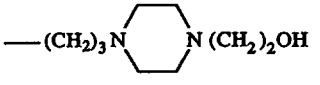
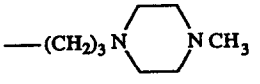
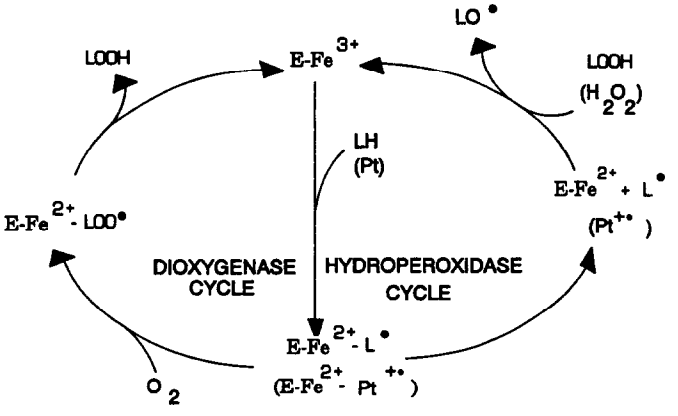
† Abbreviations: Pt, phenothiazine; $Pt^{+\cdot}$, phenothiazine radical cation; PtSO, phenothiazine sulphoxide; Hb, haemoglobin; HRP, horseradish peroxidase; K_m, Michaelis constant; LOX, lipoxygenase; PMN, polymorphonuclear leukocytes.

Table 1. Chemical structures of the phenothiazines studied



Phenothiazine	Substituents	
	2-position	10-position
Methylpromazine	$-CH_3$	$CH_2CH_2CH_2N(CH_3)_2$
Methoxypromazine	$-OCH_3$	$CH_2CH_2CH_2N(CH_3)_2$
Chlorpromazine	-Cl	$CH_2CH_2CH_2N(CH_3)_2$
Triflupromazine	$-CF_3$	$CH_2CH_2CH_2N(CH_3)_2$
Trifluoperazine	$-CF_3$	
Fluphenazine	$-CF_3$	
Perphenazine	-Cl	
Prochlorperazine	-Cl	
Trimeprazine	-H	$CH_2CH(CH_3)CH_2N(CH_3)_2$
Promazine	-H	$CH_2CH_2CH_2N(CH_3)_2$

Unless otherwise stated, activity measurements were carried out at 25° in a standard reaction medium containing 50 mM acetate buffer pH 3.5, 70 nM of soybean LOX, 1 mM H_2O_2 and concentrations of each Pt sufficient to produce the maximal experimental activity without any substrate inhibition, in a final volume of 1 mL. Perphenazine and prochlorperazine were added as a solution in ethanol and dimethyl sulphoxide, respectively. In both cases the assay solution was 1% in ethanol or



Scheme I. Catalytic cycle of soybean $_{LOX}$. In the dioxygenase cycle, both fatty acid (linoleic acid = LH) and O_2 were needed to render fatty acid hydroperoxide (LOOH) and to restore the enzyme to the active ferric form (E-Fe^{3+}). However, in the hydroperoxidase cycle, there was no need for O_2 and LH could be substituted by a reducing agent such as Pt. The ferrous inactive form produced was regenerated into the active form by using H_2O_2 or fatty acid hydroperoxide.

DMSO. This low level of water miscible organic solvent did not change the enzymatic activity and was routinely used to determine LOX activity [13].

RESULTS AND DISCUSSION

LOX (EC 1.13.11.12) has been extensively studied for its involvement in the biosynthesis of mediators (leukotrienes) in inflammatory and hypersensitive diseases [14]. The enzyme is a monomer with a single iron per active site, which is neither a heme nor an iron–sulphur centre. This iron is considered to be the oxidant that oxidizes the 1,4 diene of a polyunsaturated fatty acid to a pentadienyl radical intermediate (Scheme I). The dienyl radical is then trapped by oxygen to give a lipid hydroperoxide as a final product. This activity is known as dioxygenase activity. In addition, certain LOXs from plants [8, 9, 15, 16] and animals [17–19] exhibit a second peroxidative activity in the presence of a suitable hydrogen donor. In this reaction, lipid hydroperoxide or H_2O_2 was consumed to oxidize the donor (Scheme I), which was transformed into a radical. This hydroperoxidase activity was different from the classical heme peroxidase activity displayed by HRP and prostaglandin-H synthase, during which the hydroperoxide was reduced to the corresponding alcohol, thus providing its two oxidizing equivalents for substrate oxidation [19].

The oxidation of different Pts by LOX using H_2O_2 rendered a cation radical, whose spectrum could be ascribed to one of the five absorption spectra shown in Fig. 1, depending on the 2-substituent (Table 1). All radicals not substituted in the ring yielded a spectrum similar to promazine with an absorption maximum between 512 and 518 nm. Radicals with a 2-chloro substituent showed a maximum between 524 and 530 nm; 2-trifluoromethyl substituents yielded a spectral maximum at 500 nm. The remaining 2-methyl and 2-methoxyderivatives presented a maximum at 525 and 562 nm, respectively. No changes in the Pt spectra were found with or without boiled enzyme, nor with or without Fe^{3+} ions/H_2O_2 (data not shown).

The above radicals could suffer a series of reactions as dismutate, undergoing a further one-electron oxidation to an electrophilic two-electron oxidation product, or by undergoing a reaction with nucleophiles [20]. Thus, the enzymatic reaction was coupled to a non-enzymatic second-order reaction, causing the breakdown of the radical [21]. The overall reaction can be described as follows:

$$\mathrm{Pt} \xrightarrow[\mathrm{LOX}]{\mathrm{H_2O_2},\ v_0} \mathrm{Pt}^{+\cdot} \qquad (1)$$

$$2\mathrm{Pt}^{+\cdot} \xrightarrow[k_{app}]{} \mathrm{PtSO} + \mathrm{Pt} \qquad (2)$$

where v_0 is the enzymatic reaction rate and k_{app} the apparent second-order constant of radical breakdown.

A typical radical cation accumulation curve is shown in Fig. 2, it can be expressed mathematically as:

$$\frac{d[\mathrm{Pt}^{+\cdot}]}{dt} = v_0 - k'_{app}[\mathrm{Pt}^{+\cdot}]^2 \qquad (3)$$

where $k'_{app} = 2k_{app}$. The integrated form is:

$$[\mathrm{Pt}^{+\cdot}] = \sqrt{\frac{v_0}{k'_{app}}}\left(\frac{1 - e^{-2\sqrt{v_0 k'_{app}}t}}{1 + e^{-2\sqrt{v_0 k'_{app}}t}}\right) \qquad (4)$$

Using eqn 4, k'_{app} and v_0 could be evaluated by non-linear regression, using, as estimates, the tangent of the progress curve (v_0) and the maximum absorption value of the experimental curve which corresponded to the [Pt] at steady state (Fig. 2):

$$[\mathrm{Pt}^{+\cdot}]_{ss} = \sqrt{v_0/k'_{app}} \qquad (5)$$

The progress curves of different Pts depended on the enzyme concentration (Fig. 2) and the v_0 values

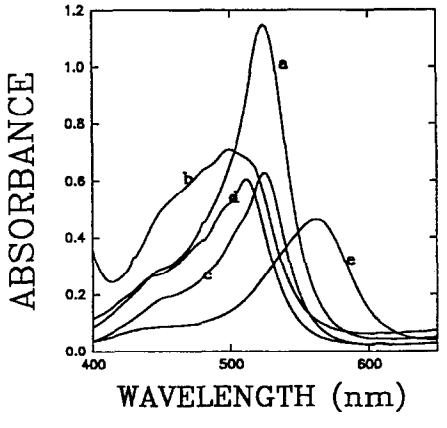


Fig. 1. Absorption spectra characteristic of different $Pt^{+\cdot}$s generated by LOX using H_2O_2 in the standard reaction medium, depending on the 2-substitution: (a) 2-acyl substituents (i.e. methylpromazine); (b) 2-trifluoromethyl substituents (i.e. fluphenazine); (c) 2-chloro substituents (i.e. perphenazine); (d) no substituent; (e) 2-methoxy substituents.

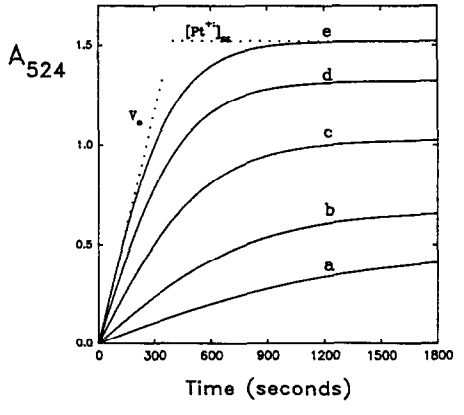


Fig. 2. Progress curves for perphenazine accumulation with different LOX concentrations (nM): (a) 16.6; (b) 33; (c) 66; (d) 99; (e) 133. The reaction medium contained 50 mM acetate buffer, 1.25 mM perphenazine and 1 mM H_2O_2. Data were analysed using Eqn 4, after graphic estimation of v_0 and k'_{app} from the tangent of the progress curve and from the maximum absorption value at 524 nm, respectively.

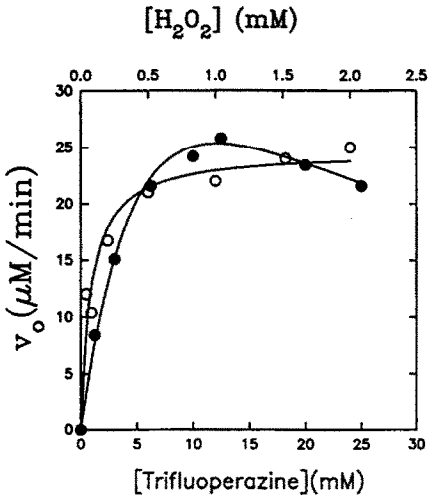


Fig. 3. Effect of substrate concentration in the oxidation of trifluoperazine by LOX in the standard reaction medium. (○) H_2O_2 was kept constant at 1.7 mM while trifluoperazine was raised from 1.25 to 25 mM. (●) Trifluoperazine was kept at 10 mM and H_2O_2 was varied from 0.04 to 2 mM.

being directly proportional to this concentration. However, v_0 found at a given enzyme concentration depended on the Pt used (data not shown).

The optimum pH found for the enzymatic step varied from one Pt to another, but was always in the acid range (pH 3.0–3.5) and had a bell-shaped profile with a clear decrease in activity at pH > 5.0 (data not shown). This optimum pH for Pt oxidation in the LOX/H_2O_2 system was similar to that found in the literature for other Pts oxidizing systems, such as HRP/H_2O_2 [7, 10, 21] and metHb/H_2O_2 [22]. The reasons for this acidic behaviour are not clear. Several factors were probably involved: the intrinsic pH dependence of the enzyme/H_2O_2 reaction, the higher stability of Pt radical cations at acidic pHs, the need for protonated monomeric forms that appeared at pH < 6.0 [23] and, in addition, the increase in Pt solubility at acidic pHs. *In vivo*, the increase in solubility and the existence of monomeric Pt molecules at physiological pH could be obtained by interaction with nucleotides [24], globulin proteins [25], organic polyphosphates [26] or biological membranes [27].

The effect of Pt and H_2O_2 concentration showed, in general, a hyperbolic dependence. However, some of the Pts studied displayed a decrease in enzymatic activity when the Pt concentration was raised, and showed a typical kinetic profile of substrate inhibition for one or for both of the substrates used for LOX (Fig. 3). The molecular reason for this type of inhibition is unknown, although it can be kinetically analysed by non-linear regression fitting [28] of the experimental points to the following equation:

$$v = \frac{V_{max}[S]}{\left(K_m + [S] + \frac{[S]^2}{K_{si}}\right)} \qquad (6)$$

Table 2. Kinetic parameters characterizing the oxidation of several Pts by LOX + H_2O_2

Name	V_m(mM/min/mg prot.)	K_m(mM)H_2O_2	K_{si}(mM)(H_2O_2)*	K_m(mM)Pt	K_{si}(mM)Pt*	log k'_{app}†
Methylpromazine	11.3	1.1	—	1.5	—	0.1
Methoxypromazine	21.7	1.1	9.0	1.4	—	1
Chlorpromazine	0.1	0.2	—	2.1	—	1.2
Triflupromazine	1.1	0.1	28.8	6.1	—	1.4
Promazine	32.3	1.2	7.1	8.0	10.3	0.5
Trimeprazine	2.2	0.2	—	4.5	—	1.4
Prochlorperazine	0.4	0.1	13.0	3.5	—	1.2
Perphenazine	0.8	0.05	—	3.4	—	1.2
Trifluoperazine	3.0	0.07	—	8.5	17.3	1.5
Fluphenazine	3.1	0.07	—	4.5	—	1.5

The values were obtained under standard reaction conditions, varying the concentration of the relevant substrate while keeping that of the second substrate at the concentration where the maximal experimental activity was obtained (see Materials and Methods).
* Calculated using Eqn 6.
† Calculated using Eqn 4.

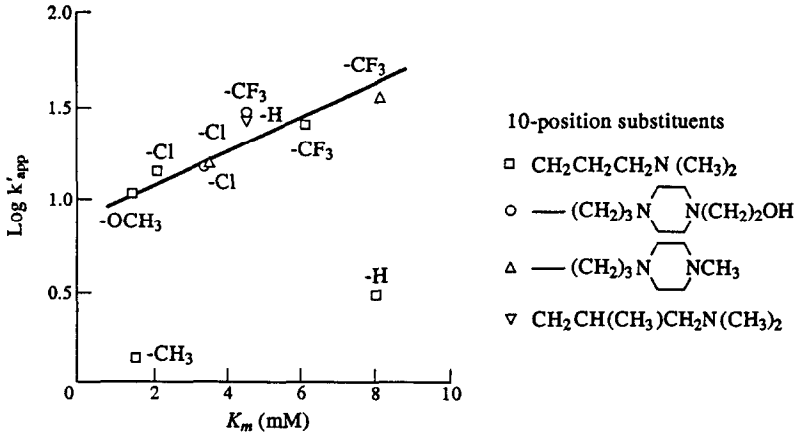


Fig. 4. Enzymatical–chemical plot. This plot represents the K_m values obtained with LOX (Table 2) for different Pts and the apparent second-order decay constant (k'_{app}) of the corresponding cation radical. This plot is a compendium of the two steps involved in the oxidation of Pt tranquillizers, one enzymatic and one chemical. Radicals in the graph represent the 2-position substituent.

where K_{si} is the substrate inhibition constant. The kinetic parameters obtained for all the Pts studied are presented in Table 2. The K_m for Pts were in the mM range, which implied that the LOX/H_2O_2 system had a similar affinity for Pts as previously reported oxidizing systems, such as HRP/H_2O_2 [21] and Hb/H_2O_2 [22]. In contrast, the K_m found for H_2O_2 was very low compared with the reported affinity for H_2O_2, which could reach values as high as 1.6 M for Hb/CPZ systems [22].

From Table 2, it was difficult to obtain any relation between the influence of the 2- and 10-substituents in the Pt structure and the affinity for LOX. However, a good linear relationship was found when the logarithm of the apparent second-order decay constant of each Pt, log k'_{app}, was plotted against its corresponding K_m (Fig. 4). The k'_{app} values used were experimentally determined in this work using eqn 4, and were in agreement with those reported by Levy *et al.* [4]. This plot, which may be called an enzymatical–chemical plot, and which has never before been described, is a compendium of both reactions involved in the oxidation of Pts, the enzymatic and the chemical. The K_m for each Pt seemed to be more related to the 10-substituent group than to the k'_{app} value. In fact, for the same 2-substituent, K_m increased as the length and volume of the 10-substituent increased. Two exceptions to this rule were the 2-methyl and 2-methoxy substituents, which showed the same K_m even when the volume and length of both groups were different. A particular case is presented for two non-2-substituted Pts, promazine and trimeprazine, which showed higher K_ms than expected. This might indicate that the presence of an electron withdrawing group was important for the catalysis, as described by Kelder *et al.* [29] who found an inverse relationship between the Pt conversion rate and the electron-withdrawing ability of the 2-substituent expressed as the Hammet σ_{para}-value.

The K_m values found for Pts were in agreement with those previously reported for HRP [21] and metHb [22]. This affinity for some xenobiotics, which differ structurally from their natural substrates, can be explained by the existence of hydrophobic binding clefts near the active centre. These hydrophobic bind sites have been found in HRP [30] and cytochrome *c* [31].

In conclusion, this paper shows that Pts could readily be oxidized not only by heme enzymes but also, as demonstrated here, by a non-heme enzyme, LOX, whose levels in some extrahepatic tissues were high enough to undergo rates of oxidation even higher than peroxidases. A physiological example of LOX-mediated oxidation would be the high rate of Pt oxidation found in stimulated human PMNs which cannot be readily explained by the release of mieloperoxidase or peroxidase [32]. The concentration of LOX and H_2O_2 in activated PMNs is enough to accomplish this intracellular oxidation of Pts, as suggested by Kelder *et al.* [32]. This action of LOX complements the activity of extracellular mieloperoxidase. This neutrophil-mediated metabolic activation of Pts might have important consequences for drug metabolism and drug induced toxicity under inflammatory conditions and in neutrophil-rich environments, such as bone marrow, where LOX levels are high [33].

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