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PARACETAMOL, 3-MONOALKYL- AND 3,5-DIALKYL-SUBSTITUTED DERIVATIVES

ANTIOXIDANT ACTIVITY AND RELATIONSHIP BETWEEN LIPID PEROXIDATION AND CYTOTOXICITY

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Abstract—The analgesic drug paracetamol is known to cause lipid peroxidation and hepatotoxicity after overdosage. In this paper, the relationship between lipid peroxidation and toxicity in freshly isolated hepatocytes was studied using paracetamol and three 3-monoalkyl-substituted derivatives of paracetamol. Paracetamol was found to induce both toxicity and lipid peroxidation in the hepatocytes. 3-Monoalkyl substitution of paracetamol (R=CH_3, C_2H_5 and *iso*-C_3H_7) did not influence its cytotoxicity but, in contrast, inhibited the lipid peroxidation. This effect may be caused by the antioxidant activity of the substituted derivatives. Apart from 3-monoalkyl substitution, 3,5-dialkyl substitution of paracetamol was also found to potentiate the antioxidant activity of paracetamol. The antioxidant activity of paracetamol and its alkyl derivatives was found to be highly correlated to their lipophilicity.

The commonly used analgesic drug paracetamol is known to cause hepatotoxicity after overdosage [1–4]. The hepatotoxicity has been shown to be mediated by an electrophilic reactive metabolite which covalently binds to thiol groups of protein and glutathione. Covalent binding to proteins is generally believed to be involved in the initiation of cell death [5, 6]. More recently, cellular oxidative stress, resulting in cytotoxic processes such as protein thiol oxidation and lipid peroxidation, have been suggested too as possible alternatives accounting for the hepatocellular toxicity of paracetamol [7–10].

Recently, in toxicological studies on the effects of 3-monoalkyl and 3,5-dialkyl substitution of paracetamol (R=CH_3, C_2H_5 and *iso*-C_3H_7), we discovered that 3,5-dialkyl substitution prevented the toxicity due to paracetamol in freshly isolated rat hepatocytes [11] and the hepatotoxicity in mice [12]. Only in a tenfold higher concentration than paracetamol, 3,5-dimethyl-paracetamol was found to induce some toxicity in hepatocytes. This cytotoxicity, however, in contrast to that of paracetamol, occurred without induction of lipid peroxidation as we [13] and others found [14].

Like many phenols [15, 16], paracetamol has been shown to possess an antioxidant activity *in vitro*. Millimolar concentrations of paracetamol namely inhibit lipid peroxidation artificially induced in rat liver microsomes [8, 17]. The dissociation between lipid peroxidation and cytotoxicity of 3,5-dimethyl-paracetamol recently reported [13, 14], might therefore be explained by an enhanced antioxidant activity of this derivative when compared to paracetamol.

The aim of the present study was to further assess the relation between lipid peroxidation and toxicity in hepatocytes using paracetamol and the toxic 3-monoalkyl-substituted derivatives of paracetamol and, in addition, to determine quantitatively the antioxidant activity of paracetamol, and series of 3-monoalkyl- and 3,5-dialkyl-substituted derivatives using a microsomal lipid peroxidation system.

MATERIALS AND METHODS

Chemicals. The 3-monoalkyl- and 3,5-dialkyl-substituted derivatives (R=CH_3, C_2H_5, *iso*-C_3H_7, *tert*-C_4H_9) of paracetamol (4-hydroxyacetanilide), were synthesized from their corresponding phenols as described by Dearden and O'Hara [18]. All other chemicals used were of analytical grade.

Animals, isolation of hepatocytes and preparation of microsomes. Male albino Wistar rats (180–200 g) were used. They were treated once by intraperitoneal injection with 3-methylcholanthrene (40 mg/kg, dissolved in archides oil) 48 hr before isolation of hepatocytes. Hepatocytes were isolated by the collagenase perfusion method as described by Seglen [19], with few modifications [11]. Rat liver microsomes were prepared by homogenation and differential ultra centrifugation procedures as described previously [20].

Incubation procedures. Incubations of hepatocytes were performed at 37° in Krebs–Henseleit buffer (pH 7.4) as we described previously [11, 13]. Toxicity was determined by measuring the leakage of the cytosolic enzyme lactate dehydrogenase from the cells into the medium [21], and it was expressed as a percentage of the total lactate dehydrogenase activity present in cells. Lipid peroxidation was monitored by measuring spectrophotometrically the formation of membrane degradation products reacting with 2-thiobarbituric acid, essentially as described by Haenen and Bast [22].

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Incubations of rat liver microsomes were performed at 37° in 1-ml volumes of 100 mM Tris–HCl buffer (pH 7.4), containing microsomes (1 mg) and ascorbate (0.2 mM). Lipid peroxidation was initiated by the addition of a freshly prepared $FeSO_4$ solution up to a final concentration of 10 μM. After 30 min the reactions were stopped by the addition of a 2-thiobarbituric acid/trichloroacetic acid mixture [22]. After heating the mixtures for 20 min at 90°, followed by centrifugation for 15 min at 4000 rpm, the supernatants were used to determine the absorbance at 535 nm.

RESULTS AND DISCUSSION

Paracetamol is known to induce lipid peroxidation and hepatoxicity *in vivo*, and to be an antioxidant *in vitro*. Lipid peroxidation has been suggested to be the cause of the hepatotoxicity of the drug [7, 8], though the recently observed dissociation between cytotoxicity and lipid peroxidation in the case of high concentrations of 3,5-dimethyl paracetamol [13, 14] and the results of other studies [23, 24], cast doubt on this proposal. The absence of lipid peroxidation in the case of 3,5-dimethyl-paracetamol might suggest that alkyl-substituted derivatives of paracetamol are better antioxidants than paracetamol. In this study, firstly, a possible relationship between lipid peroxidation and toxicity was studied in hepatocytes using the toxic 3-monoalkyl-substituted derivatives of paracetamol (R=CH_3, C_2H_5, *iso*-C_3H_7 and *tert*-C_4H_9). These derivatives of paracetamol are known to undergo cytochrome P-450-mediated bioactivation in a rate comparable to that of paracetamol [11]. Secondly, the antioxidant activity of paracetamol and its 3-monoalkyl-and 3,5-dialkyl-substituted derivatives was determined in a microsomal lipid peroxidation system.

The possible relationship between lipid peroxidation and toxicity of paracetamol and analogues was studied in freshly isolated hepatocytes from 3-methylcholanthrene-treated rats using paracetamol and its 3-monoalkyl-substituted derivatives as substrates. Paracetamol was found to induce both lipid peroxidation, measured as the formation of 2-thiobarbituric acid reactive material, and toxicity, measured as the leakage of lactate dehydrogenase from cells (Fig. 1). Of the cytotoxic 3-monoalkyl derivatives of paracetamol, only 3-methyl-paracetamol was found to induce lipid peroxidation, though to a lesser extent than paracetamol (Fig. 1). The 3-*iso*-propyl and 3-*tert*-butyl derivatives did not induce any significant lipid peroxidation. In contrast, these compounds were even found to inhibit the spontaneous lipid peroxidation occurring in control incubations of hepatocytes (Fig. 1). This inhibition of lipid peroxidation may be caused by the fact that these derivatives are potent antioxidants. As far as the mechanism of toxicity of paracetamol and the 3-monoalkyl derivatives is concerned, these data clearly show that cytotoxicity and lipid peroxidation are unrelated.

The antioxidant activity of paracetamol and its 3-monoalkyl and 3,5-dialkyl derivatives were determined quantitatively by assessing the concentrations which inhibited 50% of the ascorbate/Fe^{2+}-induced microsomal lipid peroxidation (IC_{50}). As observed earlier [8, 17], paracetamol inhibited the microsomal lipid peroxidation, especially in concentrations in the millimolar range (Fig. 2, Table 1). As illustrated in Fig. 2, for 3-methyl-paracetamol and 3,5-dimethyl-paracetamol, both 3-monoalkyl and 3,5-dialkyl substitution enhanced the antioxidant activity of paracetamol. The IC_{50} values of all nine compounds investigated are listed in Table 1. The IC_{50} of the most potent antioxidant 3,5-di-*tert*-butyl-paracetamol was 0.92 μM, which is comparable to that of other frequently used antioxidants like, for instance, 2-*tert*-butyl-4-hydroxyanisole (0.20 μM) [25] and *n*-propylgallate (1.3 μM) [26]. The latter values were determined in a similar microsomal lipid peroxidation system.

Within the series of both the 3-monoalkyl and 3,5-dialkyl substituted derivatives, the antioxidant

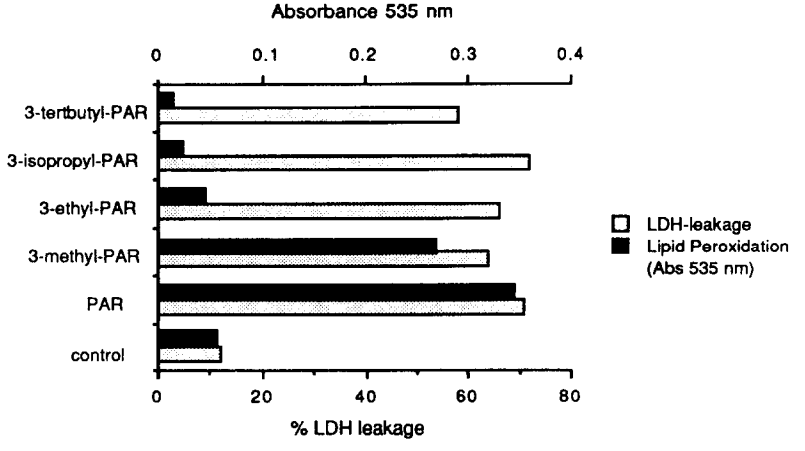

Fig. 1. Effects of 0.5 mM concentration of paracetamol and its 3-monoalkyl substituted derivatives on viability, expressed as % lactate dehydrogenase-leakage, and lipid peroxidation, expressed as the formation of 2-thiobarbituric acid reactive material absorbing at 535 nm, in hepatocytes freshly isolated from 3-methylcholanthrene-treated rats after 3 hr incubation.

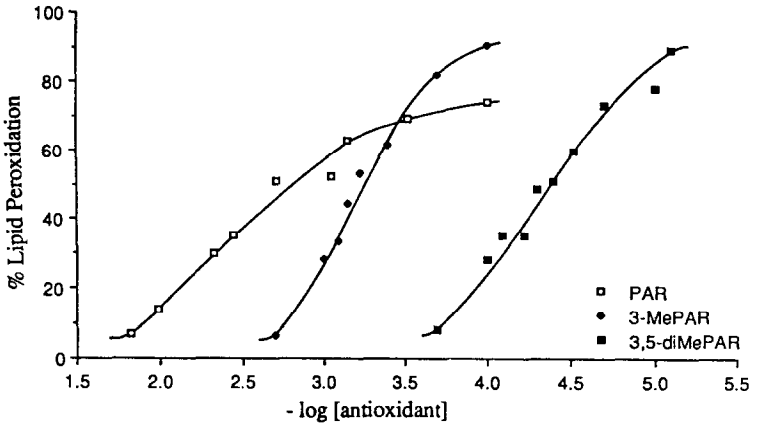

Fig. 2. Inhibitory effects of paracetamol, 3-methylparacetamol and 3,5-dimethylparacetamol on the ascorbate/Fe^{2+}-induced microsomal lipid peroxidation.

activity increased with increasing bulkiness, i.e. from methyl to *tert*-butyl. Supposing that the underlying mechanism of action is similar, this suggests a relation between the antioxidant activity and the nature of the substituent. Therefore, attempts were made to quantitatively correlate the antioxidant properties with structural characteristics of paracetamol and its derivatives. When calculated from the present data on paracetamol and its 3-monoalkyl- and 3,5-dialkyl derivatives, a strong correlation was observed between the antioxidant activity expressed as log $(1/IC_{50})$, and the lipophilicity expressed as log P (Fig. 3).

$$\log(1/IC_{50}) = 1.132(\pm 0.107)\log P + 2.489(\pm 0.205)$$
$$r = 0.970 \quad s = 0.281 \quad F = 111 \quad N = 9$$

This correlation tends to suggest that the antioxidant activity is governed primarily by the lipophilicity of the compounds. However, the partition coefficients of paracetamol and derivatives in the octanol/water system have been shown to be built up both from a lipophilic and a steric contribution of the substituents [18]. The steric contribution, which was found to be significant in the case of 3-*tert*-butyl- and 3,5-dialkyl substitution, was suggested to consist of shielding of the hydroxyl group by the substituents, an effect which is known to occur in ortho-substituted phenols [27]. Therefore, and in view of the above mentioned correlation, the antioxidant activity of the paracetamol derivatives may also be influenced by steric factors. In a study on the steric effects of ortho alkyl substitution on the rate of reaction of phenols with lipid-peroxy radicals, however, it was shown that mono-*tert*-butyl or dimethyl substitution is most optimal [15, 16]. Since no optimum was observed within the present series of paracetamol and the eight 3-monoalkyl and 3,5-dialkyl derivatives, we suggest that not the steric contribution, but rather the lipophilic contribution of the substituents is the most important factor for antioxidant activity.

In summary, paracetamol was found to induce toxicity and lipid peroxidation in freshly isolated hepatocytes. 3-Monoalkyl substitution of paracetamol did not influence its cytotoxicity but inhibited the lipid peroxidation in freshly isolated hepatocytes in a substituent-dependent manner. As far as the mechanism of toxicity of paracetamol is concerned, this observation indicates that lipid peroxidation apparently was not the cause of the toxicity induced by the 3-monoalkyl derivatives of paracetamol. Both 3-monoalkyl and 3,5-dialkyl substitution of paracetamol potentiated the antioxidant activity of the

Table 1. Antioxidant activities, expressed as IC_{50}, of paracetamol, its 3-monoalkyl and its 3,5-dialkyl substituted derivatives

Compound	IC_{50} (μM)	Log P*
Paracetamol	2460 ± 350	0.311
3-Methyl-paracetamol	621 ± 20	0.793
3-Ethyl-paracetamol	114 ± 26	1.306
3-*iso*-Propyl-paracetamol	40.7 ± 9.1	1.707
3-*tert*-Butyl-paracetamol	13.4 ± 1.9	2.357
3,5-Dimethyl-paracetamol	52.3 ± 8.1	1.108
3,5-Diethyl-paracetamol	14.7 ± 3.9	1.874
3,5-Di-*iso*-propyl-paracetamol	2.85 ± 0.52	2.671
3,5-Di-*tert*-butyl-paracetamol	0.92 ± 0.08	3.180

* Taken from Ref. 18.

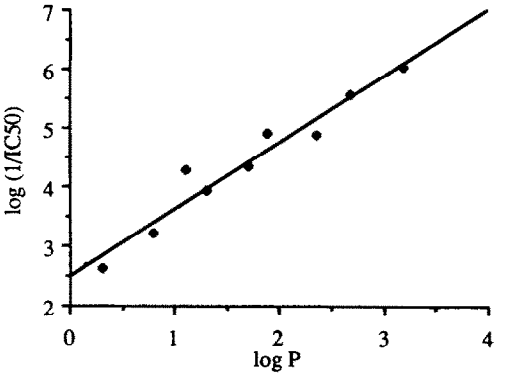

Fig. 3. Relationship between lipophilicity, log partition coefficient octanol–water (P), and antioxidant activity, log ($1/IC_{50}$), of paracetamol and its 3-monoalkyl- and 3,5-dialkyl derivatives.

drug as measured in a microsomal lipid peroxidation system. In the case of the 3,5-di-*tert*-butyl derivative, the antioxidant activity reached a level comparable to that of other frequently used antioxidants. The antioxidant activity of paracetamol and its alkyl substituted derivatives was highly correlated to lipophilicity.

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