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Mechanism of Toxicity of the Antimelanoma Drug 4-hydroxyanisole in Mouse Hepatocytes

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To elucidate the mechanism of the hepatotoxicity of 4-hydroxyanisole (4-HA), its effect on the viability of mouse hepatocytes in suspension was investigated. Cell viability was assessed by measurement of release of lactate dehydrogenase into the medium. 4-HA was cytotoxic in a concentration-dependent and time-dependent fashion with an IC_{50} of 0.26 mmol/l after 4 h incubation. Almost all cells were killed after exposure to 4-HA for 4 h at 0.5 mmol/l or for 2 h at 1.0 mmol/l. At 5 and 10 mmol/l, 4-HA caused less cytotoxicity than 1 mmol/l or below. On coincubation with the P450 inhibitor octylamine, 4-HA cytotoxicity was reduced, which suggests the involvement of cytochrome P450 in the hepatocytotoxicity of this drug. Induction of P450 isoenzymes IA, IIB and IIE1 by pretreatment of mice with phenobarbitone, 3-methylcholanthrene or acetone had no significant effect on the toxicity of 4-HA towards hepatocytes. Depletion of hepatic glutathione by pretreatment of mice with buthionine sulphoximine (1.6 g/kg, intraperitoneally) 4 h before cell isolation led to an increase in 4-HA cytotoxicity. Incubation with N-acetylcysteine (10 mmol/l) abolished the cytotoxicity of 4-HA (1 mmol/l). Both these results are consistent with the intermediacy of a reactive metabolite of 4-HA. Production of hydroquinone by oxidative demethylation of 4-HA as toxication mechanism can be excluded as formation of formaldehyde was not observed on incubation of 4-HA with mouse liver microsomes. 3,4-diacetoxyanisole, a prodrug of the known 4-HA metabolite 3,4-dihydroxyanisole, was not more cytotoxic towards hepatocytes than 4-HA. This result suggests that 3,4-dihydroxyanisole is unlikely to mediate the hepatotoxicy of 4-HA.
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INTRODUCTION

4-HYDROXYANISOLE (4-hydroxy-1-methoxybenzene, 4-HA) possesses remarkable depigmenting activity, which has caused interest in its use as a therapeutic agent against malignant melanoma. A number of studies have demonstrated the cytotoxicity of 4-HA towards melanocytes [1–3] and melanoma cells [4–6]. The antimelanotic activity of 4-HA is thought to depend upon its conversion to toxic oxidation products catalysed by the enzyme tyrosinase. However, this contention has recently been questioned as a relationship between the cytotoxicity of 4-HA and tyrosinase activity in cell lines with varying tyrosine levels has not been found [7–9].

4-HA possesses several properties which could be related to its toxic potential [10]. Prominent among them are an antioxidant effect, which may affect the cell surface, and the ability to inhibit DNA synthesis, causing S-phase specific toxicity. These actions of 4-HA are thought not to require metabolism [10].

The tyrosinase-catalysed oxidation of 4-HA generates quinones, probably orthoquinones [10]; but the chemical nature of oxidation products of 4-HA generated in the liver has yet to be established. In a recent preliminary clinical trial 4-HA was found to be hepatotoxic (G. Rustin, Mt Vernon Hospital, Northwood, UK). The only published report on the toxicity of 4-HA contains results of short-term LD_{50} determinations in mice and rats [11] obtained more than 40 years ago. In the light of the scarcity of data on the toxicity of 4-HA the hypothesis was tested here that hepatocytes in suspension are an appropriate model for evaluation of its hepatotoxicity. Another objective of this study was to elucidate the mechanism by which 4-HA causes this detrimental effect. In particular the hypothesis was tested that metabolism plays a role in the hepatocytotoxicity of 4-HA. To investigate this proposition the effect on 4-HA-induced cytotoxicity of modulators of hepatic levels of cytochrome P450 were investigated. Furthermore, the ability of hepatocytes to metabolise 4-HA was studied and related to its cytotoxic potential.

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MATERIALS AND METHODS

Chemicals and animals

4-HA (Sigma or Aldrich, UK) was added to hepatocyte incubates dissolved in water. 3,4-diacetoxyanisole was synthesised [12] and dissolved in DMSO prior to addition to incubates. Collagenase was purchased from Boehringer Mannheim. 3-methylcholanthrene, octylamine, bovine serum albumin, sodium phenobarbitone, lubrol, N-acetylcysteine, D,L-buthionine-[S,R]-sulphoximine and biochemicals for the LDH assay were from Sigma. Horse serum and a concentrate of Hanks' buffered salt solution were obtained from Gibco. Male BALB/c mice were purchased from Bantin and Kingmans (UK) and studied when they weighed 18–23 g.

Preparation and incubation of isolated hepatocytes

Procedures which involved perfusion of murine liver with a buffer containing collagenase were identical to those described previously [13]. Cell preparations were discarded if initial viability was <85% as determined by trypan blue exclusion.

Pretreatments of animals

To induce hepatic levels of cytochrome P450, mice were treated intraperitoneally with either sodium phenobarbitone

(80 mg/kg in saline) for 4 days, or a single injection of 3-methylcholanthrene (80 mg/kg) 48 h before hepatocyte isolation. Acetone was administered in drinking water as a 1% solution for 7 days. In order to deplete livers of glutathione mice were pretreated with buthionine sulphoximine (1.6 g/kg intraperitoneally) 4 h prior to hepatocyte isolation.

Determination of cytochrome P450 content

Cells were solubilised by suspending 4 × 10^6 cells in a mixture of 4% lubrol (0.1 ml) and phosphate buffer (0.1 mol/l, pH 7.4, 3.9 ml) [14]. Cytochrome P450 was then determined according to the method of Omura and Sato [15]. The mean cytochrome P450 content of hepatocytes was as follows (given as nmol P450/10^6 cells): 0.27 (S.D. 0.03) in untreated control mice, 0.59 (0.10) after induction with phenobarbitone, 0.68 (0.07) after 3-methylcholanthrene pretreatment and 0.25 (0.06) after acetone pretreatment. Induction of P450IIE1 was determined using the p-nitrophenol assay [16]. Hepatocytes obtained from acetone-pretreated animals showed a 3-fold increase in the formation of p-nitrocatechol [13.7 (1.2) μmol/10^6 cells] in comparison to hepatocytes from untreated animals [4.8 (2.2) μmol/10^6 cells]. These values are the mean (S.D.) of four determinations.

Cytotoxicity assay

Cell viability was assessed by the determination of the release of lactate dehydrogenase (LDH) from cells into the medium according to Leathwood and Plummer [17]. Significance of differences between LDH values described in the legends to Figs 2–4 were established using Student's *t* test.

Determination of 4-HA by high performance liquid chromatography (HPLC)

To a sample (0.5 ml) of the incubation mixture perchloric acid (3 mol/l, 0.25 ml) was added. The precipitated protein was removed by centrifugation. The supernatant was filtered through a nylon filter (0.45 μm) and an aliquot (20 μl) was injected onto the HPLC column. HPLC analysis was performed on a Waters 600E multisolvent delivery system attached to a Waters UV detector set at 280 nm, a Waters 700 Satellite autoinjector and a Waters 745B data handling system. Separation was achieved on a Merck cartridge column (125 mm, 4 mm I.D.) and a precolumn, both packed with Merck LiChrospher RP select B material (particle size 7 μ). The mobile phase consisted of 0.5% aqueous acetic acid (solvent A) and methanol (solvent B). Column elution was isocratic for 2.5 min with 99% A and 1% B followed by a linear gradient over 40 min to 60% A and 40% B. The flow rate was 1.25 ml/min. Return to 99% A and 1% B was achieved by a reverse gradient for 10 min. 4-HA eluted at a retention time of 18.8 min. Residual 4-HA was determined by comparison with amount present at the beginning of the experiment (100%).

Preparations of microsomes and determination of microsomal O-dealkylation

Mouse livers were excised, minced and homogenised in ice-cold homogenisation buffer (0.25 mol/l sucrose with 0.05 mol/l Tris and 0.1 mol/l EDTA pH 7.4) to give a 15–20% homogenate. Hepatic microsomes were obtained by differential centrifugation of the homogenate first at 9000 *g* for 20 min and then at 100 000 *g* for 1 h in a Beckman L8-60M ultracentrifuge. The microsomal pellet was suspended in homogenisation buffer, recentrifuged at 100 000 *g*, and resuspended in 0.25 mol/l sucrose. The protein content of the microsomal suspension was determined according to Lowry *et al.* [18]. Incubations for 30 min at 37°C in Tris buffer (0.2 mol/l, pH 7.4) were performed in duplicate. Mixtures contained microsomes 1–1.5 mg protein/ml, NADPH 10 mmol/l and either 4-HA or aminopyrine (each 5 mmol/l) or buffer only in a final volume of 2 ml. After termination with trichloroacetic acid (12.5%) the protein-free supernatant was used for colorimetric determination of formaldehyde according to Nash [19].

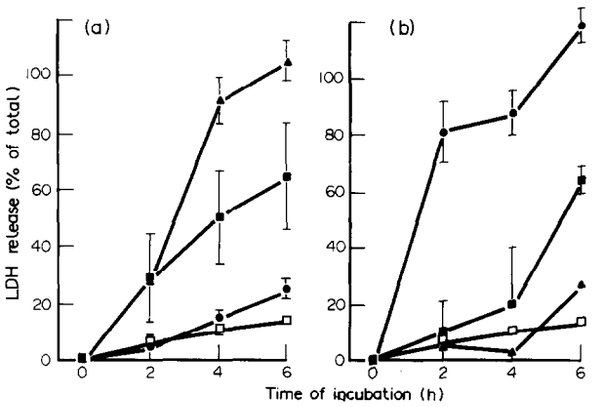

Fig. 1. Effect of 4-HA at (a) low or (b) high concentrations on viability of mouse hepatocytes. Cytotoxicity was assessed by measurement of LDH activity released into the medium. Values are the mean (S.D.) of 3 separate preparations of hepatocytes. (a) □ = control, ● = 0.10, ■ = 0.25, ▲ = 0.5 mmol/l; (b) □ = control, ● = 1, ■ = 5, ▲ = 10 mmol/l.

RESULTS

Hepatocytoxicity of 4-hydroxyanisole

On incubation with mouse hepatocytes 4-HA exhibited concentration-dependent and time-dependent cytotoxicity in the range 0.1 to 1.0 mmol/l as measured by the release of LDH into the medium (Fig. 1a). This measure of cytotoxicity is related to severe damage to the cell membrane leading to increased membrane permeability. After incubation for 4 h, 4-HA was innocuous at 0.1 mmol/l, slightly cytotoxic at 0.25 mmol/l and severely cytotoxic at 0.5 mmol/l. Exposure to 1 mmol/l 4-HA for 2 h was lethal to all cells. LC_{50} values were calculated as 0.26 mmol/ at 4 h and 0.21 mmol/l at 6 h. Surprisingly, 5 mmol/l and 10 mmol/l 4-HA were less cytotoxic than 1 mmol/l and at 10 mmol/l 4-HA gave the same cytotoxicity profile seen with 0.1 mmol/l (Fig. 1b). We considered the possibility that high concentrations of 4-HA might interfere with the LDH assay. This was, however, not the case: in a separate experiment LDH activity was unaffected by the presence of 4-HA (5 or 10 mmol/l) added to samples of cells from which LDH had previously been released by treatment with 1% Triton (data not shown).

4-HA is only poorly soluble in water. Therefore the formulation, which was used in the clinical trial during which hepatotoxicity was observed (G. Rustin, Mt Vernon Hospital, Northwood, UK), consisted of a solution made up of 4-HA (0.6 g/ml) in ethanol which was then diluted 1:300 with saline. To assess involvement of ethanol in the observed hepatotoxicity of 4-HA we investigated the effect on hepatocellular viability of 4-HA (0.1–0.5 mmol/l) added to the cell suspension in a 10% ethanol/water mixture. The presence of ethanol of up to 0.3% in the incubate neither potentiated nor ameliorated the cytotoxicity of 4-HA (data not shown).

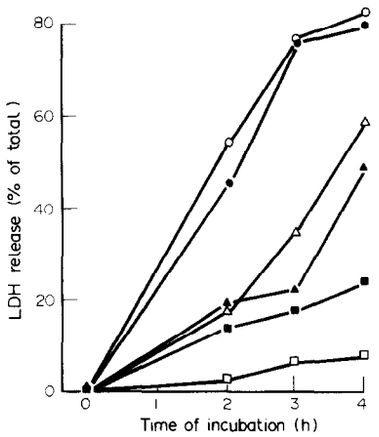

Fig. 2. Effect of octylamine on cytotoxicity of 4-HA and paracetamol towards mouse hepatocytes. Control incubates (□); incubates with either octylamine (0.5 mmol/l) (■), or 4-HA (0.5 mmol/l) (○), or paracetamol (2.5 mmol/l) (●); incubates with 4-HA and octylamine (△); incubates with paracetamol and octylamine (▲). Values represent the mean of three separate preparations of hepatocytes. S.D. values ranged from 6% (at 2 h) to 22% (at 4 h). Differences between values for 4-HA or paracetamol alone and 4-HA with ocytalmine or paracetamol with octylamine were statistically significant with $P < 0.005$ at the 2 and 3 h points for 4-HA, $P < 0.05$ at 2 h and $P < 0.001$ at 3 h for paracetamol, and $P < 0.05$ at 4 h for both.

Effect of modulation of cytochrome P450 activities on cytotoxicity

The hypothesis was tested that hepatic cytochrome P450-dependent mono-oxygenase enzymes are involved with the toxicity of 4-HA towards hepatocytes. To this end the effect of 4-HA on cell viability was tested in the presence of the specific cytochrome P450 inhibitor octylamine [20]. The analgesic drug paracetamol is known to require metabolic oxidation to its quinoneimine to exert hepatocytotoxicity [21]. Therefore, experiments in which the effect of P450 modulation on the cytotoxicity of 4-HA was examined included cellular incubates with paracetamol. Figure 2 demonstrates that 4-HA (0.5 mmol/l) and paracetamol (2.5 mmol/l) showed comparable cytotoxicity profiles towards mouse hepatocytes. The toxicity of both agents at these concentrations was inhibited significantly by 0.5 mmol/l octylamine.

In order to assess in a preliminary fashion which isoenzyme of cytochrome P450 might be involved with the toxification of 4-HA, it was incubated with hepatocytes obtained from mice which had received the P450 inducers phenobarbitone, 3-methylcholanthrene or acetone. Of these pretreatments only 3-methylcholanthrene had a marked effect on the cytotoxic potential of 4-HA (Fig. 3). Pretreatment with 3-methylcholanthrene abrogated completely the cytotoxicity of 0.25 mmol/l 4-HA which was observed in hepatocytes from untreated mice (cf. Fig 1a and Fig. 3b); even though at 0.5 mmol/l 4-HA was equally damaging towards cells from naive or pretreated animals.

Effect of modulation of cellular thiol status on cytotoxicity

Intrahepatocellular levels of soluble thiols were altered by either coincubation of cellular suspensions with N-acetylcysteine, or by pretreatment of mice with buthionine sulphoximine, which inhibits glutathione biosynthesis [22]. Depletion of hepatocytic glutathione caused a significant but only weak increase in cytotoxicity of 4-HA (0.1 mmol/, Fig. 4a). Incubation with N-acetylcysteine (10 mmol/l) for 2 h completely protected hepatocytes against the cytotoxic effects of 4-HA (1 mmol/, Fig. 4b). After incubation with N-acetylcysteine for 4 h the reduction in cytotoxicity compared to control incubations was still significant (result not shown).

Metabolic removal of 4-HA from hepatocytes

In order to investigate whether mouse hepatocytes in suspension are capable of metabolising 4-HA, levels of parent drug were determined by HPLC after 6 h incubation. In incubates with 0.1 mmol/l 4-HA remaining drug could not be detected. At higher 4-HA concentrations residual drug was found. The percentage of initial drug which was metabolised within 6 h was 76 (S.D. 5)% at 0.25 mmol/l, 72 (13)% at 0.5 mmol/l and 29 (10)% at 1.0 mmol/l 4-HA ($n = 6$ in each case).

Hepatocytotoxicity of 3,4-diacetoxyanisole

3,4-dihydroxyanisole (Fig. 5) has been identified as the main human urinary metabolite of 4-HA [23]. We wanted to test the hypothesis that it is responsible for, or contributes to, the hepatotoxicity of 4-HA and investigated its cytotoxic potential

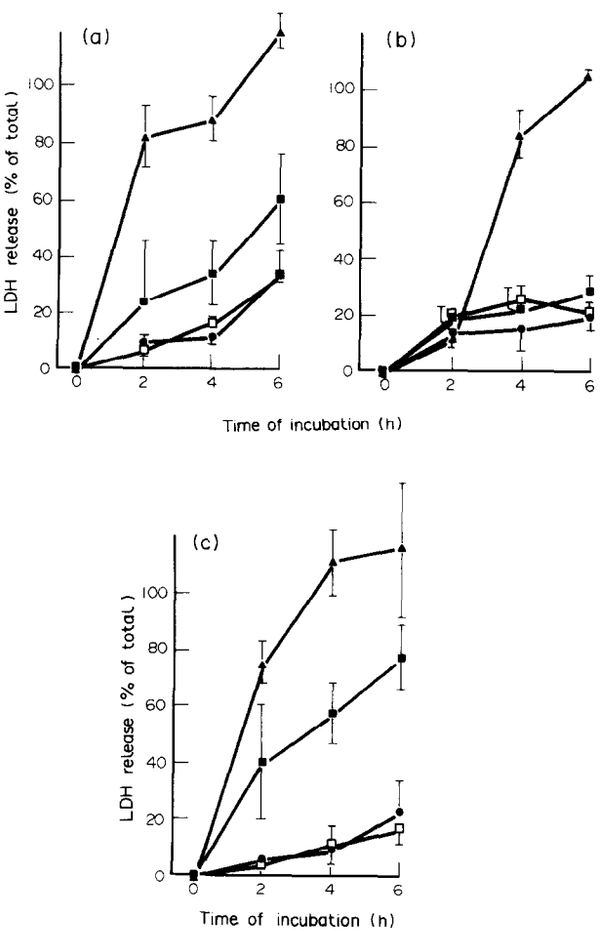

Fig. 3. Effect of inducers of cytochrome P450 on cytotoxicity of 4-HA towards mouse hepatocytes. Enzymes were induced by pretreatment of mice with (a) phenobarbitone to raise levels of P450IIB, (b) 3-methylcholanthrene to induce P450IA, and (c) acetone to elevate P450IIE1 levels. Concentrations of 4-HA were 0.1 mmol/l (●), 0.25 mmol/l (■) or 0.5 mmol/l (▲), control cells (□). Values represent the mean (S.D.) of four experiments with two separate preparations of hepatocytes. LDH values at the 4 h and 6 h points for cells from 3-methylcholanthrene-treated mice (b) incubated with 0.25 mmol/l 4-HA were significantly different ($P < 0.1$ at 2 h, $P < 0.05$ after 4 h and 6 h) from the equivalent values in incubates with cells from untreated mice (Fig. 4a).

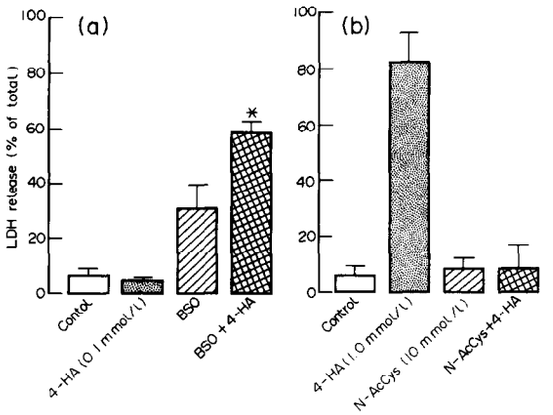

Fig. 4. Effect on cytotoxicity of 4-HA towards mouse hepatocytes of (a) depletion of intracellular glutathione levels by pretreatment of mice with buthionine sulphoximine (1.6 g/kg, intraperitoneally) or of (b) coincubation with N-acetylcysteine (10 mmol/l). Values represent the mean (S.D.) of three separate preparations of hepatocytes. * indicates significant difference ($P < 0.005$) between cells from buthionine sulphoximine-pretreated mice with and without 4-HA (0.1 mmol/l).

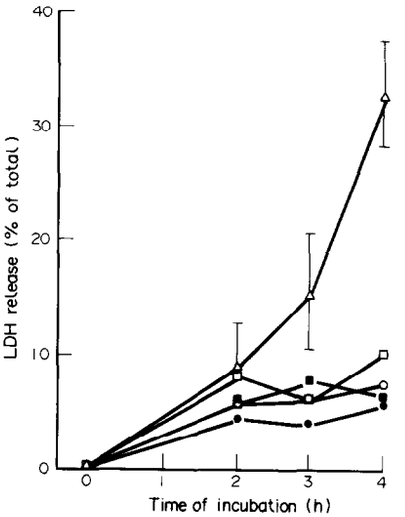

Fig. 6. Concentration dependence of hepatocytotoxicity of 3,4-diacetoxyanisole at either 0.025 mmol/l (■), 0.05 mmol/l (○), 0.1 mmol/l (●) or 0.25 mmol/l (△), control cells (□). Values represent the mean (S.D.) of three separate preparations of hepatocytes. S.D. for control incubations and incubations with 3,4-diacetoxyanisole at 0.1 mmol/l or lower concentrations were <6%.

towards mouse hepatocytes. Catechols are prone to autoxidise rapidly; furthermore, as polar molecules they may be incapable of entering cells to mimic their intracellular generation by metabolism [24]. Therefore 3,4-diacetoxyanisole was synthesised to serve as prodrug of 3,4-dihydroxyanisole. 3,4-diacetoxyanisole is more stable towards autoxidation than the catechol itself and it is probably more readily taken up into cells. Rapid generation of the catechol from the prodrug in the incubates was indicated by appearance of a red colour within 2 min, which is characteristic of orthoquinones. 3,4-diacetoxyanisole at concentrations of up to 0.1 mmol/l was not cytotoxic towards hepatocytes. At 0.25 mmol/l it was marginally cytotoxic, but slightly less so than equimolar concentrations of 4-HA (compare Fig. 6 with Fig. 1a).

3,4-diacetoxyanisole was added to the incubate dissolved in DMSO. In order to exclude the possibility that DMSO might have inhibitory effects on the cytotoxicity of 3,4-diacetoxyanisole, incubations were repeated with 3,4-diacetoxyanisole (0.1 and 0.4 mmol/l) dissolved in a mixture of acetone (10%) in water instead of DMSO. Significant toxicity was not observed (results not shown).

Microsomal O-demethylation of 4-HA

The above results indicate that 4-HA is activated by cytochrome P450, but that 3,4-dihydroxyanisole is not the species which mediates 4-HA hepatotoxicity. It is conceivable that 4-HA undergoes oxidative demethylation catalysed by cytochrome P450, releasing formaldehyde and the highly toxic hydroquinone (Fig. 5), which might be responsible for the observed cytotoxicity of 4-HA. This hypothesis was tested by incubating 4-HA (5 mmol/l) with mouse liver microsomes for 30 min at 37°C and assaying the samples for production of formaldehyde. In control incubations the microsomal N-demethylation of aminopyrine (5 mmol/l) was studied. Whereas the rate of production of formaldehyde from aminopyrine was 7.1 (S.D. 0.5) nmol/mg microsomal protein/min, its generation from 4-HA was negligible: 0.1 (0.2) nmol/mg protein/min ($n = 3$).

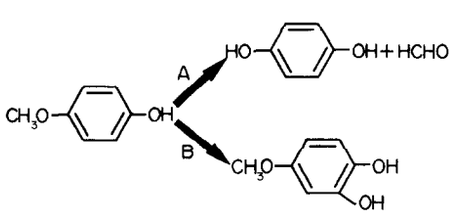

Fig. 5. Pathways of metabolic oxidation of 4-HA to hydroquinone (a) and 3,4-dihydroxyanisole (b).

DISCUSSION

4-HA exerted time-dependent and concentration-dependent cytotoxicity towards mouse hepatocytes in suspension. This result demonstrates clearly that suspensions of isolated hepatocytes are a suitable model for investigation of the mechanism of hepatotoxicity of 4-HA. 4-HA at 0.5 mmol/l was equicytotoxic with 2.5 mmol/l paracetamol. Although 4-HA is only a poor substrate for UDP-glucuronyltransferases the rate of glucuronidation of 4-HA is about three times the rate observed for paracetamol [25]. 4-HA is also nearly three times more soluble in lipid than paracetamol [25]. Therefore 4-HA might be able to enter cells and saturate detoxification pathways more efficiently and at lower extracellular concentrations than paracetamol. This explanation might account for the greater cytotoxic potential of 4-HA compared to that of paracetamol.

4-HA at 0.1 mmol/l, a non-toxic concentration, was completely metabolised within 6 hours' incubation. This finding is consistent with a report of the metabolism of 4-HA by rat hepatocytes maintained in primary culture [26]. A sample of the 6 hour incubate of 4-HA (0.1 mmol/l) with mouse hepatocytes was treated for 12 hours with a crude preparation of sulphatase and glucuronidase. Subsequent HPLC analysis afforded a peak which coeluted with 4-HA (result not shown). This finding suggests that sulphate and/or glucuronide conjugates are generated by hepatocytes as metabolites, probably the major ones, of 4-HA.

The surprising autocytoprotective effect of high concentrations (>1 mmol/l) of 4-HA might be related to its antioxidant properties [27], and this observation is consistent with an involvement of radical reactions in the toxification of 4-HA. Such an interpretation is supported by the findings that (i) coincubation with N-acetylcysteine protected hepatocytes against 4-HA toxicity, and (ii) depletion of hepatocytic GSH by pretreatment with buthionine sulphoximine exacerbated 4-HA-induced cytotoxicity. As an alternative to the possibility of a radical derived directly or indirectly from 4-HA as ultimate cytotoxicant, the drug might generate an electrophilic species or a precursor of an electrophile via oxidation catalysed by cytochrome P450. The contention that cytochrome P450 plays a role in the hepatocytotoxicity of 4-HA is strengthened by the observed reduction of cytotoxicity caused by inhibition of this enzyme using octylamine. The effect of modulation of intracellular thiol status on 4-HA cytotoxicity is also in accordance with P450-mediated generation of an electrophile as ultimate toxicant. The question of which cytochrome P450 isoenzyme might be involved with 4-HA toxication remains unresolved. We considered the three isoenzymes P450IA2, P450IIB and P450IIE1 to be the most likely to be implicated with the metabolism of 4-HA, as they are responsible for the oxidation of a variety of compounds with structural similarity to 4-HA. For example, the oxidative hepatotoxication of paracetamol is catalysed by P450IA2 and P450IIE1 [28].

P450IIE1 also catalyses the metablic hydroxylation and activation of benzene and phenol [29]. The microsomal formation of 4-bromocatechol via an initial 3,4-epoxide from bromobenzene or via a 2,3-epoxide from 4-bromophenol is catalysed by the P450IIB and P450IA subfamilies, respectively [30]. It would not be surprising if 3,4-dihydroxyanisole (4-methoxycatechol), the main metabolite of 4-HA *in vivo* [23], was generated by one of these P450 isoenzymes. Of the enzyme inducers used in this study, which affect levels of P450IA, P450IIB and P450IIE1, none increased 4-HA cytotoxicity. In contrast, P450IA seems to catalyse a pathway of 4-HA detoxication. In the interpretation of the results of these induction experiments it has to be borne in mind that the mouse hepatocyte model might not allow the discovery of involvement of specific P450 isozymes in lethal biosynthesis. Most P450 inducers also affect activities of other enzymes, such as glucuronyltransferases or epoxide hydrolases [31], and such an effect might counterbalance the consequence on toxicity of enhanced P450 activity. Alternatively, 4-HA might be metabolised to its ultimate toxicant by a P450 isoenzyme other than those investigated here.

In contrast to anisole, which is metabolised *in vitro* to phenol [32], there is no evidence that 4-HA undergoes O-demethylation to hydroquinone (Fig. 5) in microsomes from mice as studied here or from rats as observed previously [33]. Therefore it is unlikely that the formation of hydroquinone is a major mechanism of 4-HA hepatotoxicity.

3,4-dihydroxyanisole has been characterised as the main metabolite of 4-HA in human urine [23] (for structure see Fig. 5). This short-lived catechol could conceivably be generated from 4-HA by tyrosinase and/or by cytochrome P450. Oxidation of 3,4-dihydroxyanisole would lead to a reactive orthoquinone which could react with intracellular bionucleophiles, perhaps protein thiols, thus precipitating events which culminate eventually in cell death. The finding that 3,4-diacetoxyanisole, a chemical prodrug of 3,4-dihyroxyanisole, showed no increased cytotoxicity over that observed with 4-HA indicates that 4-methoxycatechol is unlikely to be implicated in the hepatocytotoxicity caused by 4-HA. Nevertheless, this finding has to be interpreted with caution as we did not establish unequivocally that intracellular deacetylation of 3,4-diacetoxyanisole proceeds rapidly. In analogy to 3,4-dihydroxyanisole as metabolite of 4-HA, 3,4-dihydroxyacetanilide is a metabolic hydroxylation product of 3-hydroxyacetanilide, a non-toxic regioisomer of paracetamol. 3,4-dihydroxyacetanilide is considerably less toxic than paracetamol [34], just as we propose here that 3,4-dihyroxyanisole is less cytotoxic than 4-HA.

The search for the hepatotoxic metabolite of 4-HA conducted in this study has obviously not yielded unequivocally a culprit metabolite. We suggest that in contrast to the proposed mechanism of 4-HA cytotoxicity in melanoma cells an intermediate precursor of 3,4-dihydroxyanisole produced from 4-HA, for example an epoxide, is the toxic species responsible for 4-HA-induced liver damage.

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Co-purification of Calcium Transport-stimulating and DNA Synthesis-stimulating Agents with Parathormone-like Activity Isolated from the Hypercalcaemic Strain of the Walker 256 Tumour

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One of the strains of the Walker 256 carcinosarcoma induces in the rat a humoral hypercalcaemia of malignancy (HHM) syndrome which is similar to that reported in human patients. We have isolated from this tumour a chromatographic fraction which displays an adenylate cyclase stimulating activity in dog kidney cortical membranes, similar to that of a parathormone (PTH) related protein isolated from various HHM related tumours. In addition, this fraction stimulated initial calcium (Ca) uptake in confluent Madin-Darby canine kidney (MDCK) cell monolayers in a dose-dependent manner. Maximal stimulation of Ca uptake was associated with an enhanced Ca efflux from MDCK cells preloaded with the cation, and with an increased DNA synthesis in these cells. These activities might be involved in development of increased tubular calcium reabsorption in Walker 256 tumour-bearing rats.
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INTRODUCTION

HUMORAL HYPERCALCAEMIA of malignancy (HHM) is a syndrome which is now known to result from the elaboration by tumours of humoral factors that stimulate bone resorption and/or tubular calcium (Ca) reabsorption [1]. A parathormone (PTH) related protein (PTHrp), capable of interacting with PTH receptors in bone and kidney, has been suggested as a likely mediator for the HHM syndrome associated with human and animal tumours [2, 3]. However, tumour-derived growth factors have also been proposed as mediators for this syndrome [4]. The hypercalcaemic strain of the rat Walker 256 carcinosarcoma is one of the few known animal models for HHM [5]. The hypercalcaemia associated with this tumour appears to involve

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