**o-HYDROXYPHENYLACETALDEHYDE IS A HEPATOTOXIC METABOLITE OF COUMARIN**

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**ABSTRACT:**

o-Hydroxyphenylacetaldehyde (o-HPA), the product of coumarin 3,4-epoxide, was synthesized and its contribution to the hepatotoxic effects of coumarin in the rat was determined. The relative toxicity of coumarin and o-HPA were initially assessed in Chinese hamster ovary K1 (CHO K1) cells, a cell line that does not contain cytochrome P450. In CHO K1 cells, o-HPA-mediated toxicity greatly exceeded that of coumarin. CHO K1 cell viability, determined via the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), was decreased by 95 and 6% in cultures containing o-HPA and coumarin (4 mM), respectively. Coumarin and o-HPA were then incubated in metabolically competent primary rat hepatocyte cultures. Cell viability was determined via the reduction of MTT, and lactic dehydrogenase (LDH) release was used as a measure of cytotoxicity. Concentration-dependent decreases in cell viability and increased LDH release were observed using 0.2 to 0.8 mM o-HPA and coumarin, with coumarin being consistently less toxic than o-HPA. Cell viability was decreased by 11 and 50% at 0.5 mM coumarin or o-HPA, respectively. Hepatocyte LDH release increased 5-fold after a 6-h exposure to 0.8 mM o-HPA, corresponding to a greater than 90% loss of cell viability in these cultures. In contrast, 0.8 mM coumarin decreased cell viability by 60%, an effect likely due to the conversion of coumarin to coumarin epoxide and o-HPA. Furthermore, 3-hydroxy-coumarin (0.8 mM), which is not a product of coumarin epoxidation, had no effect on cell viability or hepatocellular LDH release. These studies demonstrate that metabolically active rat hepatocytes convert coumarin into toxic metabolites, and strongly suggest that o-HPA and coumarin 3,4-epoxide mediate the toxicity of coumarin in rodents in vivo.

Coumarin (cis-o-coumarinic acid lactone, 1,2-benzopyrone) is a natural product used extensively as a perfume ingredient. Coumarin is also used clinically in humans as a treatment for certain lymphedemas (Jamal and Casley-Smith, 1989; De Lafontan et al., 1991) and malignancies (Marshall et al., 1989; Pittman and Selby, 1994), and reports of adverse effects in humans are rare (Cox et al., 1989; Egan et al., 1990). In contrast, coumarin is toxic in laboratory animals and is a well recognized rat hepatotoxicant (Cohen, 1979; Lake, 1984).

Species differences in the hepatotoxic effects of coumarin are thought to be metabolism dependent (Cohen, 1979; Lake 1984; Fentem and Fry, 1993). In humans, coumarin is metabolized largely to 7-hydroxycoumarin via cytochrome P450 2A6 (Cohen, 1979), with 7-hydroxycoumarin and its glucuronide or sulfate conjugates (Mead et al., 1958), constituting 68 to 92% of human urinary coumarin metabolites after an oral dose (Shilling et al., 1969; Cholerton et al., 1992; Rautio et al., 1992). Due to the high capacity and high affinity of P450 2A6 for coumarin (Draper et al., 1997), and the negligible toxicity of the 7-hydroxylated metabolite (Lake et al., 1989a), this metabolic pathway has traditionally been viewed as a major route of detoxification. Coumarin metabolism in the rat differs significantly from that observed in humans, with coumarin 7-hydroxylation contributing little to the overall metabolism of this chemical (Kaighen and Williams, 1961; Cohen, 1979; Lake et al., 1989b). Rather, coumarin metabolism in rodents involves hydrolysis of the lactone moiety, forming ring-opened products. In rats, the major urinary metabolite of coumarin is o-hydroxyphenylacetic acid (o-HPAA) and o-hydroxyphenylacetalddehyde (o-HPA) (Fentem et al., 1991; Peters et al., 1991; Lake et al., 1992a,b). It has been postulated that o-HPAA and o-HPA form during a multi-step process involving the oxidation of coumarin to an unstable coumarin 3,4-epoxide, and the subsequent hydrolysis of the lactone ring and release of CO₂ (Kaighen and Williams, 1961; Lake, 1984).

The formation and toxicity of coumarin 3,4-epoxide in rat liver have been evaluated using several indirect methods. Detection of a coumarin mercapturic acid from rat urine indicates that coumarin 3,4-epoxide is formed in vivo (Huwer et al., 1991). Furthermore, the lack of toxicity attributable to dihydrocoumarin (Lake et al., 1989a), which is saturated at the 3,4-position, suggests that metabolism at this position yields the toxic species. Thus, although coumarin 3,4-epoxide has not been identified in rat liver microsomal incubations containing

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1 Abbreviations used are: CHO K1, Chinese hamster ovary; o-HPA, o-hydroxyphenylacetaldehyde; o-HPAA, o-hydroxyphenylacetic acid; LDH, lactic dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide.

2 This work was presented in part at the 37th Annual Meeting of The Society for Toxicology, Seattle, WA, March 1998.
Coumarin, coumarin 3,4-epoxide has been putatively identified as the metabolite responsible for hepatic injury in rodents. To establish the involvement of coumarin 3,4-epoxide in rat liver necrosis, our laboratory successfully synthesized coumarin 3,4-epoxide, and confirmed its structure by NMR and gas chromatography-mass spectrometry-infrared spectroscopy analyses (Born et al., 1997). Although stable in an organic solvent, the epoxide rapidly and non-enzymatically rearranged to form \( \alpha \)-HPA in an aqueous medium. This unexpected result proved that \( \alpha \)-HPA was formed directly from coumarin 3,4-epoxide (see Fig. 1), and, contrary to earlier studies (Kaighen and Williams, 1961; Cohen, 1979; Lake, 1984), demonstrated that 3-hydroxycoumarin was not an intermediate in the formation of \( \alpha \)-HPA from coumarin epoxide. These data also suggested that coumarin 3,4-epoxide may exist only transiently in hepatic tissues, and that the more stable aldehyde metabolite may contribute to the toxic effects of coumarin in rat liver. The primary goal of the present study was to directly characterize the hepatotoxic potential of \( \alpha \)-HPA.

The direct toxicity of synthetic \( \alpha \)-HPA was examined using Chinese hamster ovary (CHO K1) cells, which do not contain cytochrome P450. Metabolically competent rat hepatocytes were then used to determine the toxicity of \( \alpha \)-HPA, coumarin, and 3-hydroxycoumarin in the relevant coumarin target organ.

**Materials and Methods**

**Chemicals.** Coumarin, \( \alpha \)-HPAA, \( \alpha \)-hydroxyphenylethanol, and 7-hydroxycoumarin were purchased from the Aldrich Chemical Company (Milwaukee, WI). CdCl\(_2\), 7-ethoxycoumarin, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO). 7-Hydroxycoumarin glucuronide and 7-hydroxycoumarin sulfate were purchased from GENTEST Corporation (Woburn, MA). \( \alpha \)-HPAA was synthesized according to the method of Bruce and Creed (1970). 3-Hydroxycoumarin was synthesized according to the method of Rajyalakshmi and Srinivasan (1978).

**Coomarin and Coumarin Metabolite-Mediated Toxicity in CHO K1 Cell Cultures.** CHO K1 cells do not contain cytochrome P450 enzymes and will not metabolize chemicals in culture (McGregor et al., 1991). Thus, CHO K1 cells provide a model in which the direct toxic effects of coumarin and its metabolites can be examined in the absence of biotransformation. CHO K1 cells were purchased from American Type Culture Collection (Rockville, MD) and cultured in Ham’s F-12 Nutrient Mixture (Life Technologies) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were subcultured twice weekly and were used before cell passage number 16. CHO K1 cells were subcultured in 96-well uncoated plastic culture plates at a density of \( 4.5 \times 10^4 \) cells/ml (0.2 ml/well) 24 h before experimental use. Cell viability was determined via trypan blue exclusion and exceeded 95% for all CHO K1 cell cultures.

Individual stock solutions of coumarin, coumarin metabolites, and CdCl\(_2\) were prepared in dimethyl sulfoxide (DMSO). The chemicals were diluted in warm F-12 medium, without serum, with the final DMSO concentration in the medium being less than 0.5%. After the aspiration of medium from the CHO K1 cell monolayers, 0.2 ml of DMSO- or chemical-containing medium was added to each well and the plates were incubated for 2 to 24 h. Medium was then removed from the wells via inversion of the culture plates, the cells were rinsed 1 time with 0.2 ml of complete F-12 medium, and 0.2 ml of complete medium (F-12 medium containing serum) was added to each well. MTT reduction was examined according to the methods of Mosmann (1983), with modification. Briefly, 0.02 ml of MTT (5 mg MTT/ml 0.9% sterile saline) was added to each culture well and the plates were incubated at 37°C for 2 h. The MTT solution was then aspirated, and 0.2 ml of acidified isopropanol (0.04 N HCL in isopropanol) was added to each well. The plates were covered and refrigerated for 7 to 10 h, after which the absorbance at 570 nm of each well (normalized to 650 nm) was determined using a Bio-Tek EL312 96-well plate reader (Bio-Tek Instruments, Winooski, VT). In a parallel experiment, treated cells were trypsinized, and CHO K1 cell viability and number were determined at 2 to 24 h via trypan blue exclusion and manual counting using a hemocytometer.

**Incubation of Rat Hepatocytes with Coumarin and its Metabolites.** Isolated male Sprague-Dawley rat hepatocytes were purchased from CEDRA.
Corporation (Austin, TX). The cells (5.25 × 10⁴ cells/well) were shipped plated on a collagen substratum in 96-well plastic dishes and topped with a solid nutrient medium matrix. Cell viability was determined before cell plating and was consistently above 95%, as determined by trypan blue exclusion. Likewise, cellular protein was quantitated in a cell suspension using the Micro BCA Protein Assay Reagent (Pierce, Rockford, IL). After receipt of the plated cells, 0.2 ml of warm culture medium (Waymouth MB 752/1 with 5% bovine serum buffered at pH 7.4 with 2.2 g/liter NaHCO₃ and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 ng/ml dexamethasone, without phenol red) was added to the top of each well and the plates were incubated at 37°C for 1 h. Once the solid medium matrix liquified, it was removed by inverting the culture plate. The hepatocytes were gently rinsed 2 times using 0.2 ml of warmed Hanks’ balanced salt solution, buffered with 25 mM HEPES, with the solution being removed via inversion of the culture plates. Complete medium (0.2 ml) was then added to each culture plate well and the hepatocytes were incubated for 1 h at 37°C in a CO₂ incubator.

To assess metabolic capacity of the hepatocytes, formation of the 7-hydroxycoumarin sulfate metabolite of [¹⁴C]-7-ethoxycoumarin (1 μCi/ml) was measured in four wells of each 96-well plate of hepatocytes. At 4 h, the medium was removed from 7-ethoxycoumarin-containing samples and stored at –80°C before the quantitation of metabolites. 7-Hydroxycoumarin sulfate was quantitated according to the method of Walsh et al. (1995), with modification (Wishnies et al., 1991).

Individual stock solutions of coumarin, coumarin metabolites, and CdCl₂ were prepared in DMSO. CdCl₂ (10 μM) (Bracken and Klaassen, 1987) served as a positive control for toxicity. All test chemicals were diluted in warm Waymouth MB 752/1 medium, without serum, with the final DMSO concentration in the medium being less than 0.5%. After the aspiration of media from the hepatocyte cultures, 0.2 ml of DMSO- or chemical-containing medium was added to each well and the plates were incubated for 2 to 24 h.

At the appropriate time point, the medium was removed from the hepatocyte monolayers, and lactic dehydrogenase (LDH) released into the media was analyzed using a Hitachi 717 chemistry analyzer (Boehringer-Mannheim Corp., Indianapolis, IN). After rinsing the cells 1 time, 0.2 ml of complete Waymouth MB 752/1 medium and 0.02 ml of MTT (5 mg/ml) were added to each well, and the reduction of MTT was determined as described for CHO K1 cells.

Statistical Methods. Reducions in CHO K1 cell and hepatocyte viability resulting from the chemical treatments were analyzed for statistical significance (P < .05), by ANOVA followed by Dunnett’s multiple comparison test. The figure legends indicate the specific number of replicate samples for each experiment. The effects of each chemical were typically examined on multiple 96-well plates, with several chemical concentrations being examined on the same plate, n = 8 to 16 wells/concentration × plate). The time course of coumarin- and o-HPA-mediated toxicity in rat hepatocytes was determined using one 96-well plate per time point. For all other studies, values represent the mean and S.E. of data obtained on duplicate-triplicate plates. All analyses were conducted with Stat View statistical application (Abacus Concepts, Berkeley, CA).

Results

The use of CHO K1 cells provided an avenue to examine the toxicity of individual chemicals in the absence of metabolism. CHO K1 cells were relatively resistant to coumarin-mediated injury at concentrations ranging from 0.005 to 4 mM. In the absence of coumarin metabolism to coumarin epoxide and o-HPA, the parent coumarin was essentially nontoxic. As shown in Fig. 2, cell viability at 6 h (measured via the metabolism of MTT to a purple formazan dye) was not significantly reduced in cultures containing 4 mM coumarin, whereas 2 and 4 mM o-HPA reduced cell viability 39 and 95% versus control, respectively.

The toxic effects of coumarin and o-HPA were confirmed in CHO K1 cells using a separate measure of cell viability (Fig. 3). In a time course experiment, the number of live cells in cultures exposed to 2 mM coumarin or o-HPA was determined via trypan blue exclusion. Cell viability remained high, and cell number was relatively constant in 0- to 8-h cultures containing DMSO or 2 mM coumarin. In contrast, o-HPA rapidly caused cell death, with the number of live CHO K1 cells being decreased 4- and 14-fold at 4 and 6 h, respectively. No live cells were detected in 8-h o-HPA incubations.

Once the toxicity of o-HPA was established in CHO K1 cells, the next objective was to determine the effects of o-HPA in a relevant target organ, the rat liver. Recognizing that coumarin-mediated toxicity in rat liver is metabolism-dependent, 7-ethoxycoumarin-o-deethylase rates were determined at the onset of each hepatocyte experiment to confirm that the cells were metabolically competent. The observed rates of 7-hydroxycoumarin sulfate formation were 0.351 ± 0.086 nmol/(mg protein × h), (four wells per plate, n = 52 total wells) for the hepatocyte cultures used in these studies.

In a time course experiment (Fig. 4), the differential effects of

![Fig. 2. Cytotoxicity of coumarin and o-HPA in CHO K1 cells.](image)

In the absence of P450-mediated metabolism, coumarin was nontoxic to CHO K1 cells. In contrast, o-HPA was toxic to CHO K1 cells at all concentrations exceeding 0.8 mM, with 4 mM aldehyde reducing cell viability 95%. These results demonstrate that o-HPA is a toxic metabolite of coumarin. Results represent the mean ± S.E. of 48 culture wells from triplicate plates. Significantly different from control, *P < .05.

![Fig. 3. Coumarin and o-HPA toxicity time course in CHO K1 cell cultures.](image)

CHO K1 cells incubated with 2 mM coumarin or o-HPA exhibited different toxicity profiles over time. The number of viable cells excluding trypan blue remained relatively constant over 8 h in coumarin-containing cultures. In contrast, o-HPA (2 mM) rapidly caused cell death. At 4 h, the number of dye-excluding cells was reduced 77%, and at 8 h all cells were dead. These results indicate that cell viability measured via the reduction of MTT (Fig. 3) correlates well with the number of live cells in these cultures. Results represent the mean ± S.E. of 48 culture wells from triplicate 96-well plates. Significantly different from control, *P < .05.
Toxic effects of coumarin and o-HPA clearly exceeded that of coumarin, it should be noted that the toxic effects of o-HPA may have been underestimated in this cell system due to potential interactions with media components and cell surface proteins. Based on these time course data, the concentration-response of coumarin and o-HPA in rat hepatocytes was determined using 6-h incubations.

At low concentrations (0.05–0.1 mM), neither coumarin nor o-HPA were toxic to primary hepatocyte cultures. In cultures exposed to 0.2 to 0.8 mM coumarin or o-HPA, cell viability was decreased in a concentration-dependent manner and coumarin was consistently less toxic than o-HPA (Fig. 5). In these metabolically active cells, both 0.4 mM coumarin and o-HPA resulted in significant toxicity, decreasing cell viability to 68 and 54% of control, respectively. Hepatocyte LDH release, a measure of cellular toxicity, was inversely proportional to cell viability at each chemical concentration. LDH release was increased 5-fold after a 6-h exposure to 0.8 mM o-HPA, corresponding to a >90% loss of cell viability in these cultures. In contrast, 0.8 mM coumarin decreased hepatocyte viability 66%, and the LDH concentration in the medium was 42 U/liter, a 2-fold increase over DMSO controls.

3-Hydroxycoumarin was incubated with metabolically competent rat hepatocytes and its effects were compared with those of equimolar concentrations of coumarin and o-HPA (Fig. 6). At 6 h, coumarin (0.8 mM) had been biotransformed into toxic products, and cell viability was 35% of control. o-HPA (0.8 mM) and 10 μM CdCl₂ were equally toxic in hepatocyte cultures, reducing hepatocyte viability 90%. In contrast, as evidenced by the absence of toxicity in hepatocyte cultures incubated with 3-hydroxycoumarin, this metabolite was not directly toxic to the cells or metabolized to a toxic product in these target organ cells.

Discussion

Coumarin-mediated toxicity varies significantly between species, with the rat demonstrating enhanced susceptibility to hepatic injury. Early metabolism studies conducted by Kaighen and Williams (1961) concluded that coumarin was biotransformed predominately to ring-opened products in the rat, metabolites that were postulated to form via the epoxidation of the 3,4-bond and the subsequent hydrolysis of the coumarin lactone moiety. Efforts to identify toxic ring-opened coumarin metabolites, such as o-HPAA, have not been successful (Lake et al., 1989a), indicating that reactive intermediates likely mediate the necrotic effects of coumarin in rat liver.

Recent characterization of synthetic coumarin 3,4-epoxide confirmed the highly transient nature of this intermediate, and revealed that the epoxide rearranges quantitatively to form o-HPA. The direct conversion of coumarin 3,4-epoxide to o-HPA is consistent with o-HPA being the major coumarin metabolite formed in rat liver microsomes (Fentem et al., 1991; Peters et al., 1991; Lake et al.,
1992a,b). Furthermore, these data confirm that epoxidation is indeed the predominant route of coumarin metabolism in the rat liver.

The extensive formation of o-HPA in liver microsomes, and the relative stability of this aldehyde in comparison to coumarin 3,4-epoxide, suggested that both coumarin epoxide and o-HPA may contribute to coumarin-mediated toxicity. Aldehydes constitute a group of relatively reactive compounds, and the toxicity of these molecules is well established (Feron et al., 1991). Simple aldehydes, such as o-HPA, may conjugate with cellular macromolecules containing thiol or amino groups, and cross-linking of proteins with simple aldehydes has been reported (Feron et al., 1991). Although the molecular mechanisms of o-HPA-mediated toxicity have yet to be elucidated, this coumarin-derived aldehyde clearly causes concentration-dependent injury to isolated rat hepatocytes. These studies are the first to identify a toxic coumarin metabolite in vitro, and provide an important mechanistic link between coumarin metabolism and toxicity.

Two in vitro systems, CHO K1 cells and rat hepatocytes, were used to demonstrate the role of o-HPA in coumarin-mediated toxicity. Synthetic o-HPA was highly toxic to CHO K1 cells and isolated hepatocytes, indicating that o-HPA itself, and not a metabolite of o-HPA, was the toxic species. In contrast, the effects of coumarin were manifest only in hepatocytes, a result consistent with bioactivation being a requisite event in coumarin toxicity in the rat. Although the short half-life of synthetic coumarin 3,4-epoxide in aqueous solution precluded the direct assessment of its toxicity in vitro, hepatocyte injury in cultures incubated with coumarin may be attributed to the formation of both coumarin 3,4-epoxide and o-HPA. In contrast, o-HPAA, the oxidation product of o-HPA and the major ring-opened coumarin metabolite formed in vivo, was not toxic to isolated rat hepatocytes (Lake et al., 1989a). Thus, the oxidation of o-HPA to o-HPAA by microsomal and cytosolic enzymes (Fentem et al., 1991) should be considered a route of detoxification in the rat liver.

3-Hydroxycoumarin, which is not a product of coumarin 3,4-epoxide or a precursor to o-HPA, was also examined for its ability to induce toxicity in isolated rat hepatocytes. Consistent with previous studies in rat hepatocyte cultures (Lake et al., 1989a), 3-hydroxycoumarin and/or its metabolites did not reduce hepatocyte viability. These data confirm that 3-hydroxycoumarin is not converted to o-HPA, and demonstrate unequivocally that 3-hydroxycoumarin, and any subsequent product of the 3-hydroxylation pathway, does not play a role in coumarin-mediated rat liver necrosis. Furthermore, the apparent lack of toxicity attributable to 3-hydroxycoumarin suggests that, like 7-hydroxycoumarin (Lake et al., 1989), this metabolite may function in the detoxification of coumarin.

The current studies identify o-HPA as a toxic coumarin metabolite that, in conjunction with coumarin 3,4-epoxide, likely mediates the classic hepatotoxic effects of coumarin in the rat. However, the data also suggest that relatively high concentrations of aldehyde (greater than 100 μM) may be required to elicit hepatic injury in the rat in vivo. Although the rat appears most susceptible to coumarin-mediated hepatotoxicity, coumarin epoxidation is not exclusive to the rat liver, with o-HPA being detected in hepatic microsomes from mice, gerbils, hamsters, and humans (Fentem and Fry, 1992; Lake et al., 1992). With regard to coumarin bioactivation in humans, o-HPA formation in rat liver microsomes is 3-fold higher than that observed in human liver microsomes (Fentem and Fry, 1992). Furthermore, coumarin detoxification via 7-hydroxylation predominates over epoxidation in human liver microsomes incubated with coumarin concentrations less than 100 μM (Fentem and Fry, 1992), a result consistent with the available clinical data, indicating that liver injury occurs rarely in humans treated with coumarin (Cox et al., 1989; Egan et al., 1990). Although specific data on coumarin epoxide and o-HPA detoxification is limited, the extensive body of coumarin literature suggests that species differences in coumarin toxicity are likely dictated by multiple factors, such as the rate of epoxidation, the extent of coumarin epoxide and o-HPA detoxification, and the rate of coumarin 7-hydroxylation in the target organ. Additional studies of coumarin metabolism in rodents and humans are ongoing, focusing on the kinetics of o-HPA formation in hepatic microsomes, and the subsequent detoxification of o-HPA in hepatic S9 fractions. A thorough characterization of both of these factors will be required to better understand species differences in coumarin metabolism and to define the relationship between coumarin epoxidation and toxicity.

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References


