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Towards a Coumarin Bibliography.

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[To be continuously expanded].

Coumarin has recently enjoyed mixed fortunes at the hands of 'expert' government department regulators, at least one of whom has had to be corrected publicly for (amongst other things) apparently failing to understand species differences relevant to coumarin metabolism. An account of the latter episode, the occurrence of coumarin in natural products, its' animal carcinogenicity and contentious sensitisation issues are explained in "Coumarin the Real Story", which can be found at <http://www.cropwatch.org/Coumarin%20-%20the%20real%20story%20update.pdf>

Contents:

- Coumarin: General**
- Coumarin: Toxicity**
- Coumarin: Allergenicity?**
- Coumarin: Non-allergenicity?**
- Coumarin: Hepatotoxicity / Carcinogenicity in various spp.**
- Coumarin: Metabolism**
- Coumarin: Percutaneous Absorption**
- Coumarin in Tobacco**
- Coumarin Analysis**
- [Early exposure to coumarins]***

*Off-topic, but included since many internet articles written by unqualified authors erroneously confuse coumarin with complex coumarins..

Coumarin: General

Appleton R.A. & Enzell C.R. (1971) "Triterpenoids & aromatic components of deer tongue leaf" *Phytochemistry* **10**, 447-449.

CIT (2001): CIT/Study No. 21214 TSS/RhodiascenTM Coumarine/Rhodia Services – RSP 13 Dec 2001

Clarke G.S. (1995) "Coumarin" *Perf & Flav.* **20**, (Nov/Dec 1995) 23-34.

Ehlers D. *et al.* (1996) "Reducing the coumarin content of tonka bean extracts using supercritical CO₂." *Int J. Food Sc & Techn* **31**, 93-95.

Marshall M.E., Mohler J.L., Edmonds K., Williams B., Butler K., Ryles M., Weiss L., Urban D., Bueschen A., Markiewicz M. & Cloud G. (1994) "An updated review of the clinical development of coumarin (1,2-benzopyrone) and 7-hydroxycoumarin." *J Cancer Res Clin Oncol* **120**, S39–S42

Montague-Jones (30th Oct 2007) "Controversial safety attack on Clarins' perfume" - see <http://www.cosmeticsdesign-europe.com/news/ng.asp?n=80996-clarins-fragrance-coumarin>

Montague-Jones G. (6th Dec 2007) "Clarins defends itself against interest group attacks". *Cosmetics-Design* see <http://www.cosmeticsdesign.com/news/ng.asp?id=81896-clarins-ntef-legal>

Montague-Jones G. 21st Dec 2007) "Research group raises alarm over coumarin" *Cosmetics-Design* – see <http://www.cosmeticsdesign-europe.com/news/ng.asp?n=82247-health-cosmetics-coumarin>

Coumarin: Toxicity

Carlton B. D., Aubrun J-C. & Simon G. S. (1996) "Effects of coumarin following perinatal and chronic exposure in Sprague-Dawley rats and CD-1 mice". *Fundamental and Applied Toxicology* **30**, 145-151.

Cohen A.J. (1979) "Critical Review of the toxicology of coumarin with special reference to interspecies differences in metabolism and hepatotoxic response & their significance to man" *Food Cosmet. Toxicol.* **17**, 277-289.

Endell W. & Seidel G. (1978) "Coumarin toxicity in different strains of mice." *Agents Action* **8**(3), 299-302. Abstract. The general toxic effects of coumarin, as well as coumarin hepatotoxicity were found to be less in DBA/2J mice than in CH3/HeJ mice. These results are discussed in the context of a strain-specific coumarin metabolism and indicate the need for suitable animal models to assess the toxicity of a given substance in man.

Evans J.G., Gaunt I.F. & Lake B.G. (1979) "Two year toxicity study on coumarin in the baboon." *Food & Cosmetics Toxicology* **17**, 187-193.

Evans J..G, Appleby E.C., Lake B.G.& Conning D/M.(1989) Studies on the induction of cholangiofibrosis by coumarin in the rat. *Toxicology*. **55**(1-2),207-24. Abstract. The histogenesis of coumarin-induced cholangiofibrosis in the rat has been determined. Proliferation of ductal structures was preceded by extensive damage to hepatocytes in the centrilobular region. Focal proliferation of ducts and fibrous tissue was present at 3 months and typical areas of cholangiofibrosis at 6 months. By 18 months the lesion was extensive and contained areas showing bizarre histological features suggestive of malignancy although no evidence of extra-hepatic metastasis was found. The lesion in animals returned to standard diet showed varying degrees of involution with extensive atrophy and fibrosis. A number of parameters of hepatic mixed function oxidase activity were reduced during the initial treatment period, at later times there was recovery of some microsomal enzyme activities. The activity of gamma-glutamyltransferase and the hepatic content of non-protein sulphhydryl groups, in contrast, were raised throughout the treatment period.

Hagan E.C. *et al* (1967) "Food flavourings & compounds of related structure II. Subacute & chronic toxicity." *Food & Cosmetics Toxicology* **5**, 141-157.

Hazleton L.W., Murer H.K., Thiessen R. Jr., Tusing TW & Zeitlin B.R.(1956) "Toxicity of coumarin." *J. Pharmacol & Exptl Therapeutics* **118**, 348-358.

Huwer T, Altmann H, Grunow W, Lenhardt S, Przybylski M & Eisenbrand G (1991) "Coumarin mercapturic acid isolated from rat urine indicates metabolic formation of coumarin 3,4-epoxide." *Chem Res Toxicol* **4**, 586-590.

Jenner P.M. *et al.* (1964) "Food flavourings & compounds of related structure. Acute oral Toxicity. *Food & Cosmetics Toxicology* **2**, 327-343.

Kienhuis A.S., Wortelboer H.M., Hoflack J.C., Moonen E.J., Kleinjans J.C., van Ommen B., van Delft J.H. & Stierum R.H.(2006) "Comparison of coumarin-induced toxicity between sandwich-cultured primary rat hepatocytes and rats in vivo: a toxicogenomics approach." *Drug Metab Dispos.* **34**(12), 2083-90. Abstract. Sandwich-cultured primary rat hepatocytes are often used as an in vitro model in toxicology and pharmacology. However, loss of liver-specific functions, in particular, the decline of cytochrome P450 (P450) enzyme activity, limits the value of this model for prediction of in vivo toxicity. In this study, we investigated whether a hepatic in vitro system with improved metabolic competence enhances the predictability for coumarin-induced in vivo toxicity by using a toxicogenomics approach. Therefore, primary rat hepatocytes were cultured in sandwich configuration in medium containing a mixture of low concentrations of P450 inducers, phenobarbital, dexamethasone, and beta-naphthoflavone. The toxicogenomics approach used enabled comparison of similar mechanistic endpoints at the molecular level between in vitro and in vivo conditions, namely, compound-induced changes in multiple genes and signaling pathways. Toxicant-induced cytotoxic effects and gene expression profiles observed in hepatocytes cultured in modified medium and hepatocytes cultured in standard medium (without inducers) were compared with results from a rat in vivo study. Coumarin

was used as a model compound because its toxicity depends on bioactivation by P450 enzymes. Metabolism of coumarin toward active metabolites, coumarin-induced cytotoxicity, and gene expression modulation were more pronounced in hepatocytes cultured in modified medium compared with hepatocytes cultured in standard medium. In addition, more genes and biological pathways were similarly affected by coumarin in hepatocytes cultured in modified medium and in vivo. In conclusion, these experiments showed that for coumarin-induced toxicity, sandwich-cultured hepatocytes maintained in modified medium better represent the situation in vivo compared with hepatocytes cultured in standard medium.

Lake B.G., Gray T.J., Evans J.G., Lewis D.F., Beamand J.A. & Hue K.L..(1989) "Studies on the mechanism of coumarin-induced toxicity in rat hepatocytes: comparison with dihydrocoumarin and other coumarin metabolites." *Toxicol Appl Pharmacol.* **97**(2):311-23. [Abstract](#). Single doses of coumarin (125 mg/kg, ip) produced a depletion of hepatic nonprotein sulfhydryl groups (mainly reduced glutathione; GSH) in young male Sprague-Dawley rats after 2 hr and increased liver weight and produced hepatic centrilobular necrosis after 24 hr. Coumarin also produced time- and dose-dependent toxic effects in primary rat hepatocyte cultures. A marked reduction of GSH levels was also observed in vitro and this was not due either to the formation of oxidized glutathione (GSSG) or to the leakage of GSH and/or GSSG from the hepatocytes. Coumarin-induced toxicity in rat hepatocytes could be inhibited by the cytochrome P450 inhibitors ellipticine and metyrapone and potentiated by depleting hepatocyte GSH levels with diethyl maleate. In contrast to coumarin, dihydrocoumarin--which lacks the 3,4-double bond--produced little toxicity in rat hepatocytes either in vivo (127 and 254 mg/kg, ip) or in vitro. Similarly, coumarin was more toxic to rat hepatocytes than a number of known coumarin metabolites including 3- and 7-hydroxycoumarin and o-hydroxyphenylacetic acid. The results of these studies demonstrate a good in vivo/in vitro correlation for the effects of coumarin and dihydrocoumarin in rat hepatocytes. Furthermore, the data suggest that coumarin hepatotoxicity in the rat is due to coumarin bioactivation by cytochrome P450-dependent enzymes to a toxic metabolite(s), which may be a coumarin 3,4-epoxide intermediate. GSH appears to protect against coumarin-induced toxicity possibly by the formation of conjugates with the toxic coumarin metabolite(s).

Lake B.G., Evans J.G., Lewis D.F. & Price R.J. (1994) "Studies on the acute effects of coumarin and some coumarin derivatives in the rat"..*Food Chem Toxicol.* **32**(4), 357-63. [Abstract](#). The mechanism of acute coumarin-induced hepatotoxicity in the rat has been investigated by comparing the effects of coumarin with those of a number of methyl-substituted coumarin derivatives. Male Sprague-Dawley rats were given single ip doses of corn oil (control), coumarin (0.86 and 1.71 mmol/kg body weight), 3,4-dimethylcoumarin (3,4-DMC, 1.71 and 2.57 mmol/kg), 3-, 4- and 6-methylcoumarins (3-MC, 4-MC and 6-MC, 1.71 mmol/kg) and 3- and 4-methyloctahydrocoumarins (3-MOHC and 4-MOHC, 2.57 mmol/kg) and hepatotoxicity assessed after 24 hr. Coumarin administration produced dose-related hepatic necrosis and a marked elevation of plasma alanine aminotransferase and aspartate aminotransferase activities. In contrast,

none of the coumarin derivatives examined produced either hepatic necrosis or elevated plasma transaminase activities. Treatment with coumarin reduced hepatic microsomal ethylmorphine N-demethylase and 7-ethoxycoumarin O-deethylase activities, whereas one or both mixed-function oxidases appeared to be induced by treatment with 3,4-DMC, 4-MC, 3-MOHC and 4-MOHC. These results provide further evidence that acute coumarin-induced hepatotoxicity in the rat is due to the formation of a coumarin 3,4-epoxide intermediate. That 3- and/or 4-methyl substitution (i.e. 3-MC, 4-MC and 3,4-DMC) leads to a reduction in coumarin-induced hepatotoxicity, due to diminished formation of 3,4-epoxide intermediates, was confirmed by the results of molecular orbital calculations.

Norman R.L. & Wood A. W. (1984) "o-Hydroxyphenylethanol, a novel lactone ring-opened metabolite of coumarin." *Drug Metabolism and Disposition* **12**, 543-549.

Opdyke D.L.J. (1974) "Monographs on fragrance raw materials: Coumarin." *Food Chem Toxicol* **12**, 358–388.

Price R.J., Mistry H., Wield P.T., Renwick A.B., Beaman J.A. & Lake B.G. (1996) "Comparison of the toxicity of allyl alcohol, coumarin and menadione in precision-cut rat, guinea-pig, cynomolgus monkey and human liver slices." *Arch Toxicol.* **71**(1-2), 107-11. [Abstract](#). The toxicity of allyl alcohol, coumarin and menadione has been studied in precision-cut liver slice cultures. Liver slices were prepared from male Sprague-Dawley rats, male Dunkin-Hartley guinea-pigs and from samples of Cynomolgus monkey and human liver using a Krumdieck tissue slicer. The liver slices were cultured with the test compounds for 24 h in a dynamic organ culture system. Toxicity was assessed by measurement of protein synthesis, potassium content and the MTT assay. At the concentrations examined, menadione produced marked toxicity in liver slices from all four species, whereas rat liver slices were less susceptible to allyl alcohol toxicity. Coumarin produced concentration-dependent toxic effects in rat and guinea-pig liver slices, whereas Cynomolgus monkey and human liver slices were relatively resistant, especially at low coumarin concentrations. At some concentrations of the test compounds examined, the MTT assay appeared to be a less sensitive indicator of toxicity than either protein synthesis or potassium content. These results demonstrate the usefulness of precision-cut liver slices for assessing species differences in xenobiotic-induced toxicity.

Coumarin: Allergenicity (?)

de Groot, A.C. *et al.* (1988) "Allergens in Cosmetics" *Arch. Dermatol.* **124**, 1525-1529.

Felter S.P., Vassallo J.D., Carlton B.D. & Daston G.P. (2006). "A safety assessment of coumarin taking into account species-specificity of toxicokinetics". *Food & Chem. Toxicol.* **44**, 462-475. [Abstract](#). Coumarin (1,2-benzopyrone) is a naturally occurring fragrant compound found in a variety of plants and spices. Exposure to the general public is through the diet and from its use as a perfume raw material in personal care products. High doses of coumarin by the oral route

are known to be associated with liver toxicity in rodents. Chronic oral bioassays conducted in the 1990s reported liver tumors in rats and mice and lung tumors in mice, raising concerns regarding the safety of coumarin. Since then, an extensive body of research has focused on understanding the etiology of these tumors. The data support a conclusion that coumarin is not DNA-reactive and that the induction of tumors at high doses in rodents is attributed to cytotoxicity and regenerative hyperplasia. The species-specific target organ toxicity is shown to be related to the pharmacokinetics of coumarin metabolism, with data showing rats to be particularly susceptible to liver effects and mice to be particularly susceptible to lung effects. A quantitative human health risk assessment that integrates both cancer and non-cancer effects is presented, confirming the safety of coumarin exposure from natural dietary sources as well as from its use as a perfume in personal care products.

Floc'h F. (2002) "Coumarin in plants and fruits: implications in perfumery." *Perf. & Flav.* **27** (Mar/Apr 2002), 32-36.

INSERM U503/Société Rhodia Services. "Evaluation du potentiel de sensibilisation cutanée des coumarines à l'aide du Local Lymph Node Assay murin." 10th Dec 2003.

Kunkeler A.C., Weijland J.W., Bruynzeel D.P. (1998) "The role of coumarin in patch testing." *Contact Dermatitis* **39**, 327–328.

Larsen W. *et al.* (1996) "Fragrance contact dermatitis. A worldwide multi-centre investigation (part 1)." *Am. J. of Contact Dermatitis* **7**, 77-83.

Malten K.E. *et al.* (1984) "Reactions in Selected Patients to 22 fragrance materials" *Contact Dermatitis* **11**, 1-10.

Malten *et al.* (1984); Larsen *et al.* (1992): Coumarin "known potent contact allergens" Larsen *et al.* (1992). **Cropwatch Comments:** Sloppy statement since several studies show zero reactions at 1-5% coumarin on human volunteers.

Masamoto Y. (2001) "Sensitisation & Cross-Reaction of Simple Coumarins." *Yagugaku Zasshi*, **121**, 97-103.

Mutterer V., Gimenez Arnau E., Lepoittevin J.P., *et al.* (1993) "Identification of coumarin as the sensitizer in a patient sensitive to her own perfume but negative to the fragrance mix." *Contact Dermatitis* **40**, 196–199.

Coumarin: Non-allergenicity?

Floc'h F. (2002) "Coumarin in Plants and Fruits: Implications in Perfumery." *Perf. & Flav.* **27** (Mar/Apr 2002), 32-36.

Schnuch A., Uter W., Geier J, Lessmann H. & Frosch P.J. (2007) "Sensitization to 26 fragrances to be labelled according to current European regulation. Results of the IVDK and review of the literature." *Contact Dermatitis* **57**(1), 1-10.

Vocanson M., Goujon C, Chabeau G, Castelaïne M, Valeyrie M., Floc'h F., Maliverney C., Gard A., Nicolas J.F. (2006) "The Skin Allergenic Properties of Chemicals May Depend on Contaminants - Evidence from Studies on Coumarin." *International Archives of Allergy and Immunology* **140**,231-238 Abstract.
Background/Aims: Positive patch tests are considered representative of a contact allergy to the tested chemical. However, contaminants and derivatives rather than the suspected chemical itself could be responsible for the allergic skin reactions. Here, we tested the importance of contaminants in the sensitizing and allergenic properties of coumarin in mice and humans. Coumarin, an ingredient in cosmetics and fragrances, was chosen as the reference chemical since conflicting results have been obtained regarding its ability to induce contact allergy. In some chemical preparations, this could be explained by the presence of coumarin derivatives endowed with allergenic properties. Methods: In mice, three different coumarin preparations were tested in the local lymph node assay. In humans, we assessed the irritant and allergenic properties of highly pure coumarin in nonallergic and fragrance-allergic patients. Results: Pure coumarin did not exhibit irritant or sensitizing properties in the local lymph node assay. In contrast, two other commercially available coumarins and three contaminants that were detected in these coumarin preparations were identified as weak and moderate sensitizers, respectively. In humans, pure coumarin was extremely well tolerated since only 1 out of 512 patients exhibited a positive patch test to the chemical. Conclusions: These results indicate that coumarin cannot be considered as a common contact allergen and further emphasize that purity of chemicals is mandatory for the assessment of their allergenicity.

Vocanson M., Valeyrie M., Rozières A., Hennino A., Floc'h F., Gard A. & Nicolas J.F. (2007) "Lack of evidence for allergenic properties of coumarin in a fragrance allergy mouse model." *Contact Dermatitis* **57**(6), 361-4. Abstract.
BACKGROUND: There is controversy as to whether coumarin, an ingredient in cosmetics and fragrances, is a contact allergen involved in fragrance allergy. We recently showed that the purity of coumarin is a critical parameter for its allergenicity because coumarin preparations containing trace amounts of contaminants induced cell proliferation in the local lymph node (LN) assay whereas pure coumarin did not. OBJECTIVE/METHOD: In the present study, we analyzed the sensitizing properties of coumarin (purity > 99.9) and of dihydrocoumarin (DHC), in a recently developed model of fragrance allergy in mice. RESULTS: DHC was able to prime T cells in LNs draining the sensitization skin site and to induce a typical allergic contact dermatitis (ACD) reaction upon challenge, confirming that DHC is endowed with moderate sensitizing properties. In contrast, no T-cell activation and no ACD responses were obtained following sensitization and challenge with coumarin. CONCLUSION: These results confirm that pure coumarin is endowed with very weak sensitizing capacities, if any, and suggest that the presence of contaminants in coumarin preparations may account for the previously reported allergenic properties of coumarin.

Wahlberg J.E. (1995) "Patch test guidelines" In: *Textbook of Dermatitis* 2nd edn Springer p241-268. **Cropwatch comments:** Coumarin applied at 5%; 1% negative in patch test with Fenn fragrances (0/100 positives)

Coumarin: Hepatotoxicity / Carcinogenicity in various spp.

Albert R.E. (1997) "Allergic contact sensitizing chemicals as environmental carcinogens." *Environ Health Perspect.*, **105**(9): 940–948.

Api A.M. (2001) "Lack of effect of coumarin on the formation of micronuclei in an in vivo mouse micronucleus assay." *Food & Chem Toxicol* **39**(8), 837-841 Abstract. Coumarin was tested for its potential to cause genotoxic effects in mouse bone marrow cells using an in vivo micronucleus assay. Male and female Swiss mice were administered a single oral dose of coumarin at 50, 100 or 200 mg/kg by gavage in corn oil vehicle. Control animals received only the vehicle. Groups of male mice were also administered mitomycin C at 0.75 mg/kg and served as positive controls. At 24 h after treatment, mice from all dose levels, and at 48 h after treatment, mice from the high dose level only were sacrificed. Bone marrow cells were collected and assayed for the presence of micronuclei. Coumarin did not cause any increase in the incidence of micronucleated polychromatic erythrocytes in male or female mice at any of the dose levels, the positive control mitomycin C produced a significant increase. There was no evidence of coumarin or mitomycin C treatment related cytotoxicity to bone marrow cells. The results of this study demonstrate that coumarin is negative in the mouse in vivo micronucleus assay.

Andréjak M. Gersberg M., Sgro C., Decocq G., Hamel J.D., Morin M. & Gras V. (1998) "French pharmacovigilance survey evaluating the hepatic toxicity of coumarin." *Pharmacoepidemiol Drug Saf.* **7** Suppl 1, S45-50 Abstract. Synthetic coumarin (benzopyrone) was launched in France in 1988 for the adjuvant therapy of lymphoedema of the upper limb following radiosurgical treatment of breast cancer. Further to the reporting of hepatic reactions, a national survey has been carried out. The survey dealt with 22 cases reported to the pharmacovigilance regional centres and 20 to Knoll France company (five duplicate cases) up to June 1996. Thirty-four cases corresponding to an elevation of ALT over 2N and/or alkaline phosphatase over 1.5N (criteria chosen for selection in this survey) had been taken into account. Among these cases, a causal relationship was considered likely or probable for 15 of them. Two positive rechallenges were reported. The hepatic reactions observed between 2 to 6 months of treatment in two-thirds of the cases (average dose: 90 mg/day; i.e. recommended dose) was essentially cytolytic in 85% of the cases, with jaundice in 14 cases and hyperbilirubinaemia reported in five other cases. In 23 cases (68%), the increase of ALT exceeded 10N. Of the patients 41% were hospitalized. Severe liver failure with encephalopathy justified liver transplantation once and likely led to encephalopathy and fatal evolution in two other cases. The evolution was favourable in the other cases. The drug was prescribed for other uses than the registered indication in more than 50% of these cases. No risk factors could be identified in the survey. This survey

provides a strong signal for potential hepatotoxicity of coumarin (likely due to the production of a reactive metabolite in some patients exhibiting a coumarin 7-hydroxylation deficiency).

Born S.L., Rodriguez P.A., Eddy C.L & Lehman-McKeeman L.D. (1997) Synthesis and reactivity of coumarin 3,4-epoxide. *Drug Metab Dispos.* **25**(11), 1318-24. Abstract. Coumarin is used widely as a fragrance constituent and is administered clinically in the treatment of certain lymphedemas and malignancies. Although toxicity occurs only rarely in humans treated clinically with high-dose coumarin, it is well established that coumarin is hepatotoxic in the rat. This species difference in susceptibility to toxicity reflects the disparate metabolic processes occurring in humans and rodents. In humans, coumarin is converted extensively via cytochrome P450 2A6 to the nontoxic 7-hydroxycoumarin metabolite. In contrast, coumarin 3,4-epoxidation is thought to predominate in rodent species, resulting in the formation of several potentially toxic metabolites. Coumarin epoxide is thought to be highly unstable and has not been isolated synthetically or as a microsomal product. To address this issue, coumarin 3,4-epoxide was synthesized, and its stability and fate have been determined. Coumarin 3,4-epoxide was prepared by reacting coumarin with dimethyldioxirane. The epoxide was stable in organic solvents and survived conditions required for analysis by gas chromatography. Its structure was confirmed via ¹H-NMR and gas chromatography-mass spectrometry-infrared spectroscopy (GC-MS-IR). In contrast, coumarin 3,4-epoxide was unstable in aqueous solution, converting within 20 sec to a ring-opened compound. Using GC-MS-IR analysis, the single coumarin 3,4-epoxide product was identified as o-hydroxyphenylacetaldehyde (o-HPA). Although other investigators have suggested that 3-hydroxycoumarin is an intermediate in o-HPA formation from coumarin 3,4-epoxide, we have demonstrated that 3-hydroxycoumarin, incubated in an aqueous system or with liver microsomal proteins, does not form o-HPA. Thus, the results of the present work establish that coumarin 3,4-epoxide can be synthesized and that o-HPA, which has previously been shown to be a prominent coumarin metabolite in rat liver microsomal incubations, is formed directly from coumarin 3,4-epoxide. These results suggest that both coumarin 3,4-epoxide and o-HPA may contribute to the hepatotoxicity of coumarin.

Born S.L., Fix A.S., Caudill D. & Lehman-McKeeman L.D. (1998) "Selective Clara cell injury in mouse lung following acute administration of coumarin." *Toxicol Appl Pharmacol.* **151**(1), 45-56. Abstract. Coumarin is a known hepatotoxicant in laboratory animals, particularly rats. However, the mouse lung was identified as a major target organ in a chronic bioassay, with an oral gavage dosage of 200 mg/kg coumarin increasing the incidence of alveolar/bronchiolar adenomas and carcinomas. The purpose of the present work was to determine whether coumarin was acutely toxic in the mouse and rat lung. Male and female B6C3F1 mice were dosed orally by gavage with coumarin at 0, 10, 20, 50, 100, 150, and 200 mg/kg and lung toxicity was determined 24 h later by histological evaluation. The results indicated that coumarin dosages ≥ 150 mg/kg caused selective injury to Clara cells in the distal bronchiolar epithelium. The time course of this

injury was studied from 6 h to 7 days after a single dosage of coumarin (200 mg/kg). At 12 h after dosing, Clara cell swelling was apparent along with the onset of necrosis and bronchiolar epithelial disorganization. At 24-48 h, necrotic Clara cells were observed sloughed into the lumens of the terminal bronchioles, with concomitant thinning of the epithelium and flattening of the remaining ciliated cells. By 72-96 h, there was epithelial hypertrophy and hyperplasia, and by 7 days after dosing, the Clara cells had regenerated and the bronchiolar epithelial architecture appeared nearly normal. Unlike the mouse, oral administration of coumarin (200 mg/kg) caused severe hepatotoxicity in male F344 rats, seen histologically as centrilobular necrosis and associated with increases, up to 140-fold, in serum ALT, AST, and SDH levels. Clara cell toxicity was not observed in the distal bronchioles of treated rats. However, in the upper airways, coumarin treatment produced generalized epithelial necrosis involving both ciliated and nonciliated cells. 3,4-Dihydrocoumarin (DHC), which is not a mouse lung carcinogen, did not cause Clara cell injury when dosed to mice at 800 mg/kg. This finding suggests, because DHC lacks a 3,4-double bond, that bioactivation of coumarin to a 3,4-epoxide intermediate may contribute to mouse lung Clara cell toxicity. Collectively, the results indicate that coumarin is a Clara cell toxicant and establish the mouse lung as a target organ for coumarin toxicity. These new findings lay the foundation for studies to determine the mechanisms of coumarin-induced toxicity and carcinogenicity and to define the relevance of these effects to humans.

Born S.L., Fix A.S., Caudill D. & Lehman-McKeeman LD. (1999) "Development of tolerance to Clara cell necrosis with repeat administration of coumarin." *Toxicol Sci.* **51**(2), 300-9. Abstract. Coumarin was identified as a mouse-lung carcinogen following oral gavage administration in a chronic bioassay, and was shown to cause the selective necrosis of terminal bronchiolar Clara (non-ciliated bronchiolar epithelial) cells in the mouse lung after acute administration. After oral gavage, a similar effect was not observed in the terminal bronchioles of rats, suggesting that coumarin-mediated Clara cell toxicity is a species-specific effect. Using coumarin dosages (50 and 200 mg/kg) and a dosing schedule modeled after the chronic bioassay, the current study examined the effects of repeated coumarin administration in mouse lung. A single dosage of coumarin (200 mg/kg) caused swelling of Clara cells and necrosis in mouse-lung terminal bronchioles. However, after 5 consecutive oral doses of coumarin (200 mg/kg), the mouse lung became tolerant to coumarin, and although areas of bronchiolar epithelial flattening and hyperplasia were noted, Clara cell necrosis was not observed. After 10 doses of coumarin, mouse lungs appeared nearly normal. Coumarin-mediated Clara cell injury is thought to result from the cytochrome P450-catalyzed formation of coumarin 3,4-epoxide and Western analysis of whole mouse lung microsomal P450 content indicated that, commensurate with Clara cell necrosis, many P450s were decreased. However, P450 levels appeared qualitatively normal in lung microsomes from tolerant mice. Similarly, coumarin epoxidation and 7-hydroxylation rates in whole lung microsomes from tolerant animals were similar to controls. To determine if animals tolerant to coumarin were tolerant to other Clara cell toxicants, a single toxic dose of naphthalene

(200 mg/kg) was administered to coumarin-tolerant mice. Coumarin pretreatment reduced naphthalene-mediated Clara cell toxicity, supporting the hypothesis that tolerance may result from general biochemical and molecular changes and not exclusively from alterations in chemical metabolism.

Born S.L., Caudill D., Smith B.J. & Lehman-McKeeman L.D. (2000). "In vitro kinetics of coumarin 3,4-epoxidation: application to species differences in toxicity and carcinogenicity." *Toxicol Sci.* **58**(1), 23-31. Abstract. Coumarin, a natural product and fragrance ingredient, is a well recognized rat liver toxicant, and dietary administration at toxic dosages increased the incidence of rat cholangiocarcinomas and parenchymal liver-cell tumors in a chronic bioassay. Hepatotoxicity in rats is site- and species-specific, and is thought to result from the formation of coumarin 3,4-epoxide and its rearrangement product, *o*-hydroxyphenylacetaldehyde (*o*-HPA). The goals of the current study were to describe the in vitro kinetics of the metabolic activation of coumarin, and determine whether species differences in susceptibility to liver injury correlate with coumarin bioactivation determined in vitro. Coumarin 3,4-epoxidation was quantified via the formation of *o*-HPA in pooled hepatic microsomes from female B6C3F1 mice, male F344 rats, and individual humans (n = 12 subjects), and the apparent kinetic constants for *o*-HPA production were calculated using nonlinear regression and fitting to either a one-enzyme or two-enzyme model. Eadie-Hofstee analyses indicated that *o*-HPA formation was biphasic in both rat and mouse liver. Although the apparent high affinity K_m in rat and mouse liver microsomes was 38.9 and 47.2 microM, respectively, the overall rate of *o*-HPA formation was far greater in mouse than in rat liver microsomes. Furthermore, the total clearance (CL_{int}) of coumarin via *o*-HPA formation in mouse liver microsomes was 4-fold greater than in rat liver microsomes. Since mice are relatively resistant to hepatotoxicity, the data indicated that rates of *o*-HPA formation in rat and mouse liver microsomes were not directly predictive of liver toxicity in vivo, and further suggested that *o*-HPA detoxification played a role in modulating coumarin-mediated toxicity. The current studies also indicated that coumarin 3,4-epoxidation in human hepatic microsomes was minimal. In human liver microsomes (n = 12), the kinetics of *o*-HPA formation were best described by a single enzyme model, with the K_m for *o*-HPA formation ranging from 1320-7420 microM. In the most active human sample, the intrinsic clearance of coumarin via the 3,4-epoxidation pathway was 1/9 and 1/38 that of the rat and mouse, respectively. The in vitro kinetics of *o*-HPA formation, and in particular, the large quantities of coumarin required for *o*-HPA production in human liver microsomes, strongly suggest that humans are unlikely to produce toxicologically relevant concentrations of this metabolite following low level coumarin exposures.

Born S.L., Hu J.K. & Lehman-McKeeman L.D, (2000) "*o*-Hydroxyphenylacetaldehyde is a hepatotoxic metabolite of coumarin." *Drug Metab Dispos.* **28**(2), 218-23. 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-hydroxycoumarin (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.

Born S.L., Api A.M., Ford R.A., Lefever F.R. & Hawkins D.R. (2003) "Comparative metabolism and kinetics of coumarin in mice and rats." *Food Chem Toxicol.* **41**(2):247-58. Abstract. Coumarin, a well recognized rat hepatotoxicant, also causes acute, selective necrosis of terminal bronchiolar Clara cells in the mouse lung. Further, chronic oral gavage administration of coumarin at 200 mg/kg, a dose that causes Clara cell death, resulted in a statistically significant increased incidence of alveolar/bronchiolar adenomas and carcinomas in B6C3F1 mice. In contrast, mouse lung tumors were not observed at the 100 and 50 mg/kg dose levels in the oral gavage study, or in CD-1 mice following chronic intake of coumarin at levels equivalent to 276 mg/kg in diet. The current studies were designed to determine the impact of oral gavage vs dietary administration on the pharmacokinetics and metabolism of coumarin in CD-1 and B6C3F1 mice and F344 rats. Following the administration of 200 mg/kg ¹⁴C-coumarin via oral gavage, lung C(max) values (total ¹⁴C-associated radioactivity) were five- and 37-fold greater than those resulting from a 50 mg/kg oral gavage dose or 1000 ppm in diet, respectively. Coumarin (200 mg/kg) pharmacokinetics and metabolism was also examined in F344 rats following oral gavage dosing. Total ¹⁴C-coumarin associated radioactivity in plasma was 3.5-fold lower than in the mouse, and the plasma half-life in rats was five-times longer than in mice. Using non-radiolabeled compound (200 mg/kg), coumarin and products of the coumarin 3,4-epoxidation pathway were quantitated in plasma and urine after oral gavage administration to mice and rats. 7-Hydroxycoumarin (7-HC) was quantitated in mouse plasma and urine. o-Hydroxyphenylacetic acid (o-HPAA) reached a concentration of 37 microg/ml in plasma, and accounted for 41% of the dose in the urine, whereas the C(max) for 7-hydroxycoumarin was 3 microg/ml, and

represented 7% of the administered dose. In the rat, the plasma C(max) for o-HPA was 6 microg/ml, and accounted for 12% of the dose. The coumarin C(max) in rat plasma was comparable to that in mouse. Coumarin 3,4-epoxide (CE) and its rearrangement product o-hydroxyphenylacetaldehyde (o-HPA) and o-hydroxyphenylethanol (o-HPE), were not detected at any time point in plasma or urine. This analysis of coumarin and CE pharmacokinetics in rodents suggests that the differential tumor response in the mouse oral gavage and dietary bioassays is a function of the route of exposure, whereas species differences in lung toxicity between mice and rats result from heightened local bioactivation in the mouse lung.

Beamand J.A., Barton P.T., Price R.J. & Lake B.G. (1998) "Lack of effect of coumarin on unscheduled DNA synthesis in precision-cut human liver slices." *Food Chem Toxicol.* **36**(8), 647-53. [Abstract](#). In this study the effect of coumarin on unscheduled DNA synthesis (UDS) in precision-cut human liver slices has been examined. Liver slices from tissue samples from four donors were cultured for 24 hr in medium containing [3H]thymidine and 0-5.0 mM coumarin using a dynamic organ culture system and processed for autoradiographic evaluation of UDS. As positive controls liver slices were also cultured with three known genotoxic agents, namely 0.02 and 0.05 mM 2-acetylaminofluorene (2-AAF), 0.002 and 0.02 mM aflatoxin B1 (AFB1) and 0.005 and 0.05 mM 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP). UDS was quantified as the net grain count in centrilobular hepatocytes and as the percentage of centrilobular hepatocyte nuclei with more than five net grains. Compared with control liver slice cultures, treatment with 0.05-5.0 mM coumarin had no effect on UDS. In contrast, treatment with 0.02 and 0.05 mM 2-AAF, 0.002 and 0.02 mM AFB1 and 0.005 and 0.05 mM PhIP produced significant increases in the net grain counts of centrilobular hepatocytes. The greatest induction of UDS was observed in liver slices treated with 0.05 mM PhIP. Treatment with 2-AAF, AFB1 and PhIP also produced significant increases in the number of centrilobular hepatocyte nuclei with more than five net grains. At the concentrations examined neither coumarin, 2-AAF, AFB1 nor PhIP had any significant effect on replicative DNA synthesis in 24 hr cultured human liver slices. These results demonstrate that coumarin does not induce UDS in cultured human liver slices. However, all three positive control compounds produced marked significant increases in UDS, thus confirming the functional viability of the human liver slice preparations used in this study. The results of this study suggest that coumarin is not a genotoxic agent in human liver.

Cottrell S., Oliver K., Lake B.G. & Powell C.J. (1996) "Strain-specific enhancement or inhibition of coumarin hepatotoxicity in mice following pretreatment with two different liver enzyme-inducing agents." *Fundam Appl Toxicol.* **34**(1), 47-55. [Abstract](#). Human exposure to coumarin continues despite controversy over its hepatotoxic potential. Greater understanding of human reactions to coumarin may be achieved by studying murine interstrain differences. The metabolic basis of coumarin hepatotoxicity and its modulation by liver enzyme inducers, beta-naphthoflavone (beta NF) and aroclor 1254 (ARO),

were investigated in C3H/He and DBA/2 mice. Coumarin (200 mg/kg) was hepatotoxic to both strains, resulting in 2- to 15-fold plasma aminotransferase elevations, mild subcapsular linear hepatocyte necrosis after 24 hr, and, in some C3H/He mice, centrilobular necrosis. In this strain, beta NF pretreatment caused a 2- to 3-fold further increase in plasma aminotransferases and produced periportal necrosis. In contrast, ARO-pretreated C3H/He mice tended to exhibit lower plasma aminotransferases and occasional midzonal damage. Neither pretreatment significantly altered coumarin hepatotoxicity in DBA/2 mice. In C3H/He mice, hepatic microsomal metabolism of [3-14C]-coumarin via the 3-hydroxylation pathway doubled following both beta NF and ARO treatment. The contrasting nonresponsiveness of DBA/2 mice suggested that this pathway is linked to the Ah locus, which is defective in this strain. ARO treatment caused a maximal 5-fold increase in coumarin 7-hydroxylation in C3H/He mice, whereas DBA/2 mice were 30% less responsive. Potentiation of coumarin hepatotoxicity corresponded to an increase in the 3-:7-coumarin hydroxylation ratio. Pretreatment-dependent shifts in the location of hepatocyte damage may be related to changes in the translobular ratio of enzymes involved in activation and detoxication of coumarin. These data highlight how genetic background, individual variation, and xenobiotic-induced alterations in enzyme profiles, factors all relevant to human risk assessment, can influence the consequence of coumarin exposure.

Fentem J.H. & Fry J.R. (1993) "Species differences in the metabolism and hepatotoxicity of coumarin." *Comp Biochem Physiol C*. **104**(1), 1-8. Abstract. 1. Investigations of coumarin metabolism and hepatotoxicity have been reviewed. 2. Species differences in coumarin hepatotoxicity appear to be metabolism-mediated. 3. The rat, in which it is markedly hepatotoxic, primarily metabolises coumarin via 3-hydroxylation and cleavage of the heterocyclic ring. 4. Coumarin is less toxic in the baboon, gerbil and certain strains of mice, which resemble man in their extensive formation of the 7-hydroxy metabolite. 5. Liver toxicity in patients receiving relatively high daily doses of coumarin is very rare. 6. Recent studies indicate that coumarin 3,4-epoxide is the metabolic intermediate responsible for hepatotoxicity in the rat.

Fentem J. H. & Fry J. R. (1991) "Comparison of the effects of inducers of cytochrome P450 on Mongolian gerbil and rat hepatic microsomal monooxygenase activities." *Xenobiotica* **21**, 895-904.

Gu J, Walker VE, Lipinskas TW., Walker D.M. & Ding X (1997) "Intraperitoneal administration of coumarin causes tissue-selective depletion of cytochromes P450 and cytotoxicity in the olfactory mucosa." *Toxicol Appl Pharmacol* **146**:134-143.

Kaighen M. & Williams R.T. (1961) "The metabolism of 3-14C coumarin" *Journal of Med. Chem* **3**, 25-43.

Lake B.G., Collins M.A., Harris R.A., Phillips J.C., Evans J.G., Gangolli S.D..(1980) "Studies on the hepatotoxicity and metabolism of coumarin in the rat [proceedings]." *Biochem Soc Trans.* **8**(1),96-7.

Lake B.G. (1984) "Investigations into the mechanism of coumarin-induced hepatotoxicity in the rat." *Arch Toxicol Suppl* **7**, 16–29. [Abstract](#). The administration of single doses of coumarin to the rat was found to produce hepatic centrilobular necrosis and also to depress a number of hepatic enzyme activities within 24 h. Coumarin-induced liver damage was diminished by pretreatment with cobaltous chloride but potentiated by the administration of diethyl maleate. Hepatic reduced non-protein sulphhydryl levels were rapidly depleted following coumarin treatment whereas urinary mercapturic acid excretion was enhanced suggesting the formation of a coumarin metabolite or metabolites hitherto undetected in this species. In in vitro studies [3-14C]coumarin was converted by rat hepatic microsomes to reactive intermediates which became bound covalently to microsomal proteins. Additional studies established that the formation of reactive metabolites was a cytochrome P-450 dependent process and that macromolecular binding could be inhibited by sulphhydryl compounds (including reduced glutathione) and hepatic cytosol fractions. These results demonstrate that coumarin-induced hepatotoxicity in the rat is likely to be mediated via one or more reactive metabolites generated by cytochrome P-450 dependent enzymes and that reduced glutathione and other thiol agents constitute a detoxification pathway.

Lake B.G., Gaudin H., Price R.J. & Walters D.G. (1992) "Metabolism of [3-14C]coumarin to polar and covalently bound products by hepatic microsomes from the rat, Syrian hamster, gerbil and humans." *Food Chem Toxicol* **30**, 105–115. [Abstract](#). The metabolism of 0.19 and 2.0 mM-[3-14C]coumarin to polar products and covalently bound metabolites has been studied with hepatic microsomes from the rat, Syrian hamster, Mongolian gerbil and humans. [3-14C]Coumarin was metabolized by liver microsomes from all species to a number of polar products and to metabolite(s) that became covalently bound to microsomal proteins. The polar products included 3-, 5- and 7-hydroxycoumarins, o-hydroxyphenylacetaldehyde and o-hydroxyphenylacetic acid. Coumarin 7-hydroxylation was observed in all species except the rat. With 0.19 mM-[3-14C]coumarin, 7-hydroxycoumarin was the major metabolite in human liver microsomes, whereas in the other species with 0.19 mM substrate and in all species with 2.0 mM substrate o-hydroxyphenylacetaldehyde was the major metabolite. Of the three animal species studied the gerbil most resembled humans as this species also had a high coumarin 7-hydroxylase activity. The administration of Aroclor 1254 to the rat and Syrian hamster induced both microsomal cytochrome P-450 content and [3-14C]coumarin metabolism. With liver microsomes from all species a good correlation between rates of [3-14C]coumarin metabolism and covalent binding was observed at both substrate concentrations. However, in view of the known species difference between the rat and Syrian hamster in coumarin-induced hepatotoxicity, the present data are

not consistent with microsomal coumarin metabolite covalent binding being an indicator of potential liver damage.

Lake B.G. & Evans J.G. (1993) "Effect of pretreatment with some mixed-function oxidase enzyme inducers on the acute hepatotoxicity of coumarin in the rat." *Food Chem Toxicol.* **31**(12):963-70. [Abstract](#). Male Sprague-Dawley rats were pretreated with saline, corn oil, sodium phenobarbitone (PB) (100 mg/kg body weight/day), 20-methylcholanthrene (20 MC) (20 mg/kg body weight/day) or Aroclor 1254 (ARO) (100 mg/kg body weight/day) by daily ip injections for 5 days. Animals were then given single oral doses of either 250 or 500 mg coumarin/kg body weight and hepatotoxicity was assessed after 24 hr. Coumarin produced hepatotoxicity, which comprised hepatocyte necrosis and elevation of plasma alanine aminotransferase and aspartate aminotransferase activities, in all pretreated groups. Hepatic microsomal cytochrome P-450 levels were reduced after coumarin administration. In rats pretreated with saline, corn oil or PB, coumarin produced centrilobular hepatic necrosis, whereas in rats pretreated with 20 MC or ARO, coumarin produced periportal hepatic necrosis. These results demonstrate that mixed-function oxidase enzyme inducers can modulate acute coumarin-induced hepatotoxicity in the rat. As coumarin is known to be bioactivated by cytochrome P-450-dependent enzymes, the change in the lobular distribution of toxicity after pretreatment with 20 MC or ARO is presumably due to the induction of particular cytochrome P-450 isoenzymes in periportal hepatocytes.

Lake B.G., Evans J.G., Lewis D.F. & Price R.J. (1994) "Comparison of the hepatic effects of coumarin, 3,4-dimethylcoumarin, dihydrocoumarin and 6-methylcoumarin in the rat." *Food Chem Toxicol.* **32**(8), 743-51. [Abstract](#). The mechanism of coumarin-induced hepatotoxicity in the rat has been investigated by comparing the effects of coumarin with those of three coumarin derivatives, namely 3,4-dihydrocoumarin (DHC), 3,4-dimethylcoumarin (3,4-DMC) and 6-methylcoumarin (6-MC). Male Sprague-Dawley rats were fed either control diet or diets containing 0.5 or 0.75% coumarin, 0.76% DHC, 0.6 or 0.9% 3,4-DMC or 0.82% 6-MC for 13 wk. The dietary levels of 0.5% coumarin and 0.6% 3,4-DMC, were equimolar (3.43 mmol/100 g diet), as were the dietary levels of 0.75% coumarin, 0.76% DHC, 0.9% 3,4-DMC and 0.82% 6-MC (5.14 mmol/100 g diet). All treatments resulted in an increase in relative liver weight, but only coumarin increased plasma alanine aminotransferase and aspartate aminotransferase activities. Morphological examination of liver sections from coumarin treated rats revealed vacuolation of centrilobular hepatocytes and bile duct hyperplasia. Cholangiofibrosis was also observed, particularly in rats given 0.75% coumarin. Treatment with DHC produced no abnormalities, whereas a slight hypertrophy of centrilobular hepatocytes was observed in some 3,4-DMC treated animals and a slight vacuolation of individual hepatocytes was noted in some 6-MC treated rats. DHC, 6-MC and particularly 3,4-DMC treatment resulted in an induction of cytochrome P-450 dependent mixed function oxidase enzyme activities. All treatments induced hepatic GSHS-transferase and gamma-glutamyltransferase activities, induction being most marked in rats given coumarin and 6-MC. These

results provide further evidence that coumarin-induced hepatotoxicity in the rat is due to the formation of a 3,4-epoxide intermediate.

Lake B.G. & Grasso P. (1996) "Comparison of the hepatotoxicity of coumarin in the rat, mouse, and Syrian hamster: A dose and time response study." *Fundam Appl. Toxicol* **34**,105–117. [Abstract](#). The effects of coumarin treatment have been compared in male Sprague-Dawley CD rats, male CD-1 mice, and male Syrian hamsters. Rats were fed 0-0.75% coumarin for 1 and 4 weeks and 0-0.5% coumarin for 13 weeks, whereas mice and Syrian hamsters were fed 0-0.5 and 0-1.0% coumarin, respectively, for periods of 1, 4, and 13 weeks. In the rat, coumarin produced dose-related hepatotoxic effects which included vacuolar degeneration, apoptosis, and bile duct proliferation. These effects were particularly marked at dose levels of 0.3 and 0.5%, where liver tumors have been observed in a chronic study. Coumarin administration to rats also increased serum bilirubin content and both serum and hepatic gamma-glutamyltransferase activity. While levels of hepatic total glutathione were increased by coumarin administration, microsomal cytochrome P450 content and ethylmorphine N-demethylase activity were reduced. Such effects were either less marked or absent in the mouse and Syrian hamster. Replicative DNA synthesis was studied by implanting osmotic pumps containing 5-bromo-2'-deoxyuridine during Study Weeks 0-1, 3-4, and 12-13. In the rat, coumarin administration for 4 and 13 weeks at dose levels of 0.3 and 0.5% produced a sustained stimulation of hepatocyte replicative DNA synthesis. No such effects were observed in the mouse and Syrian hamster. These results demonstrate marked species differences in coumarin-induced hepatotoxicity. While tumor formation in the rat appears due to chronic hepatotoxicity associated with a sustained regenerative hyperplasia, such effects were not observed in the CD-1 mouse and Syrian hamster. In assessing the hazard of coumarin to humans, account needs to be taken of both levels of exposure and species differences in response.

Lake B.G., Osborne D.J., Walters D.G. & Price R.J. (1992) "Identification of o-hydroxyphenylacetaldehyde as a major metabolite of coumarin in rat hepatic microsomes." *Food Chem Toxicol* **30**, 99–104. [Abstract](#). The metabolism of [3-14C]coumarin has been studied in hepatic microsomes from control (corn-oil treated) and Aroclor 1254-treated (100 mg/kg body weight/day, 5 days, ip) rats. [3-14C]Coumarin metabolites in incubate extracts were separated by HPLC and identified by comparison with the retention times of known coumarin metabolites. The major product produced by incubation of 0.25-2.5 mM-[3-14C]coumarin with both control and Aroclor 1254-induced hepatic microsomes was a novel coumarin metabolite. This novel metabolite was extracted from pooled microsomal incubations, purified by semi-preparative HPLC and identified by mass spectrometry as o-hydroxyphenylacetaldehyde (o-HPA). Some possible pathways for the formation of o-HPA from coumarin are proposed.

Lake B.G. (1999) "Coumarin metabolism, toxicity & carcinogenicity: relevance for human risk assessment." *Food & Chem. Toxicity* **37**(4), 423-453(31). [Abstract](#). The metabolism, toxicity and results of tests for carcinogenicity have been

reviewed with respect to the safety for humans of coumarin present in foodstuffs and from fragrance use in cosmetic products. Coumarin is a natural product which exhibits marked species differences in both metabolism and toxicity. The majority of tests for mutagenic and genotoxic potential suggest that coumarin is not a genotoxic agent. The target organs for toxicity and carcinogenicity in the rat and mouse are primarily the liver and lung. Moreover, the dose-response relationships for coumarin-induced toxicity and carcinogenicity are non-linear, with tumour formation only being observed at high doses which are associated with hepatic and pulmonary toxicity. Other species, including the Syrian hamster, are seemingly resistant to coumarin-induced toxicity. There are marked differences in coumarin metabolism between susceptible rodent species and other species including humans. It appears that the 7-hydroxylation pathway of coumarin metabolism, the major pathway in most human subjects but only a minor pathway in the rat and mouse, is a detoxification pathway. In contrast, the major route of coumarin metabolism in the rat and mouse is by a 3,4-epoxidation pathway resulting in the formation of toxic metabolites. The maximum daily human exposure to coumarin from dietary sources for a 60-kg consumer has been estimated to be 0.02 mg/kg/day. From fragrance use in cosmetic products, coumarin exposure has been estimated to be 0.04 mg/kg/day. The total daily human exposure from dietary sources together with fragrance use in cosmetic products is thus 0.06 mg/kg/day. No adverse effects of coumarin have been reported in susceptible species in response to doses which are more than 100 times the maximum human daily intake. The mechanism of coumarin-induced tumour formation in rodents is associated with metabolism-mediated, toxicity and it is concluded that exposure to coumarin from food and/or cosmetic products poses no health risk to humans.

Lake B.G. & Gray T.J.B. (1999) "Studies on the mechanism of coumarin-induced toxicity in rat hepatocytes: Comparison with dihydrocoumarin and other coumarin metabolites." *Toxicology and Applied Pharmacology* **97**(2), 311-323. Abstract. Single doses of coumarin (125 mg/kg, ip) produced a depletion of hepatic nonprotein sulfhydryl groups (mainly reduced glutathione; GSH) in young male Sprague-Dawley rats after 2 hr and increased liver weight and produced hepatic centrilobular necrosis after 24 hr. Coumarin also produced time- and dose-dependent toxic effects in primary rat hepatocyte cultures. A marked reduction of GSH levels was also observed in vitro and this was not due either to the formation of oxidized glutathione (GSSG) or to the leakage of GSH and/or GSSG from the hepatocytes. Coumarin-induced toxicity in rat hepatocytes could be inhibited by the cytochrome P450 inhibitors ellipticine and metyrapone and potentiated by depleting hepatocyte GSH levels with diethyl maleate. In contrast to coumarin, dihydrocoumarin—which lacks the 3,4-double bond—produced little toxicity in rat hepatocytes either in vivo (127 and 254 mg/kg, ip) or in vitro. Similarly, coumarin was more toxic to rat hepatocytes than a number of known coumarin metabolites including 3- and 7-hydroxycoumarin and o-hydroxyphenylacetic acid. The results of these studies demonstrate a good in vivo/in vitro correlation for the effects of coumarin and dihydrocoumarin in rat hepatocytes. Furthermore, the data suggest that coumarin hepatotoxicity in the

rat is due to coumarin bioactivation by cytochrome P450-dependent enzymes to a toxic metabolite(s), which may be a coumarin 3,4-epoxide intermediate. GSH appears to protect against coumarin-induced toxicity possibly by the formation of conjugates with the toxic coumarin metabolite(s).

Lake B.G., Evans J.G., Chapuis F., Walters D.G. & Price R.J..(2002) "Studies on the disposition, metabolism and hepatotoxicity of coumarin in the rat and Syrian hamster." *Food Chem Toxicol.* **40**(6), 809-23. [Abstract](#). The hepatotoxicity, metabolism and disposition of coumarin has been compared in male Sprague-Dawley rats and Syrian hamsters. The treatment of rats for 12, 24 and 42 weeks with diets containing 0.2 and 0.5% coumarin resulted in hepatotoxicity and increased relative liver weights. While levels of cytochrome P450 (CYP) and CYP-dependent enzymes were decreased, levels of reduced glutathione (GSH) and activities of UDP glucuronosyltransferase, gamma-glutamyltransferase and GSH S-transferase were increased. In contrast, coumarin produced few hepatic changes in the Syrian hamster. Following a single oral dose of 25 mg/kg [3-¹⁴C]coumarin, radioactivity was rapidly excreted by the rat and Syrian hamster with the urine containing 63.5 and 89.9%, respectively, and the faeces 38.0 and 12.4%, respectively, of the administered dose after 96 h. The biliary excretion of radioactivity was greater in the rat than in the Syrian hamster. Analysis of 0-24-h urine samples revealed that both species were poor 7-hydroxylators of coumarin. In the rat, treatment with 0.5% coumarin in the diet for 24 weeks was found to increase the urinary excretion of single oral gavage doses of 25 and 300 mg/kg [3-¹⁴C]coumarin. The marked species difference in hepatotoxicity between the rat and Syrian hamster observed in this study may be at least partially attributable to differences in coumarin disposition. However, additional studies are required to elucidate the metabolic pathways of coumarin in both species.

Loprinzi *et al.* (1997) "Coumarin-induced hepatotoxicity" *J Clin Oncol.* **15**,3167-3168

Mohler J.L., Williams B.T., Thompson I.M. & Marshall M.E. (1994) "Coumarin (1,2-benzopyrone) for the treatment of prostatic carcinoma.". *J Cancer Res Clin Oncol* **120**, S35–S38

National Toxicology Program. (1993) "NTP Toxicology and Carcinogenesis Studies of Coumarin (CAS No. 91-64-5) in F344/N Rats and B6C3F1 Mice (Gavage Studies)." *Natl Toxicol Program Tech Rep Ser.* **422**, 1-340. [Abstract](#). Coumarin is the basic structure of numerous naturally occurring compounds with important and diverse physiological activities. More than a thousand coumarin derivatives have been described, varying from simple coumarins containing alkyl and hydroxyl side chains to complex coumarins with benzoyl, furanoyl, pyranoyl, or alkylphosphorothionyl substituents. Coumarin and 3,4-dihydrocoumarin were nominated by the Food and Drug Administration and the National Cancer Institute for study because of the widespread use of coumarin in perfumes, cosmetics, and other products as a fragrance, continued interest in coumarin compounds as flavor-enhancing agents for foods, and the interest in structure-activity relationships of this important group of compounds. Coumarin is believed

to be metabolized to a 3,4-epoxide intermediate, which may be responsible for its toxic effects, while 3,4-dihydrocoumarin, which lacks the 3,4-double bond, is not considered likely to form an epoxide intermediate. Toxicity and carcinogenicity studies were conducted by administering coumarin (97% pure) in corn oil by gavage to groups of male and female F344/N rats and B6C3F1 mice for 16 days, 13 weeks, and 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium*, cultured Chinese hamster ovary cells, *Drosophila melanogaster*, and B6C3F1 mice. 16-DAY STUDY IN RATS: Groups of five male and five female rats received coumarin in corn oil by gavage at doses of 0, 25, 50, 100, 200, or 400 mg per kg body weight, 5 days a week for a total of 12 doses in a 16-day period. All female rats and four male rats receiving 400 mg/kg died. The mean body weight gains and final mean body weights of surviving dosed male and female rats were similar to those of the controls. There were no clinical signs of organ-specific toxicity, and there was no evidence of impaired blood coagulation from measurements of capillary clotting time or prothrombin and activated partial thromboplastin time. 16-DAY STUDY IN MICE: Groups of five male and five female mice received coumarin in corn oil by gavage at doses of 0, 40, 75, 150, 300, or 600 mg per kg body weight, 5 days a week for a total of 12 doses in a 16-day period. All mice receiving 600 mg/kg, two male mice receiving 300 mg/kg, and one male mouse receiving 75 mg/kg died. The mean body weight gains and final mean body weights of surviving dosed male and female mice were similar to those of the controls. Clinical findings of inactivity, excessive lacrimation, piloerection, bradypnea, ptosis, or ataxia were observed in some mice from the 300 and 600 mg/kg groups within the first several hours after dosing. Capillary clotting time and platelet counts of dosed mice were similar to those of controls. 13-WEEK STUDY IN RATS: Groups of 10 male and 10 female rats received coumarin in corn oil by gavage at doses of 0, 19, 38, 75, 150, or 300 mg per kg body weight. Three male and three female rats receiving 300 mg/kg died. The mean body weight gains and final mean body weights of male rats that received 150 and 300 mg/kg were significantly lower than those of the controls. There were no clinical signs related to specific organ toxicity. Male and female rats receiving coumarin exhibited dose-related decreases in mean erythrocyte volume and mean erythrocyte hemoglobin, and dose-related increases in erythrocyte counts. Serum levels of total bilirubin and one or more cytoplasmic enzymes including alanine aminotransferase, aspartate aminotransferase, ornithine carbamoyltransferase, and/or sorbitol dehydrogenase in males and females receiving 300 mg/kg were higher than those of controls. The absolute and relative liver weights of male and female rats that received 150 and 300 mg/kg were significantly greater than those of the controls. Centrilobular hepatocellular degeneration and necrosis, chronic active inflammation, and bile duct hyperplasia were observed in the liver of rats receiving 150 or 300 mg/kg. The high dose selected for the 2-year study was 100 mg/kg, which was just below the level at which mortality, lower final mean body weights, and treatment-related liver lesions were observed in the 13-week study. 13-WEEK STUDY IN MICE: Groups of 10 male and 10 female mice received coumarin in corn oil by gavage at doses of 0, 19, 38, 75, 150, or 300 mg per kg body weight.

Two male mice receiving 300 mg/kg died. The mean body weight gain and final mean body weight of surviving male mice that received 300 mg/kg were significantly lower than those of the controls. No clinical signs of toxicity were observed. Male and female mice receiving coumarin exhibited dose-related decreases in mean erythrocyte volume and mean erythrocyte hemoglobin. The absolute and relative liver weights of males and females that received 150 and 300 mg/kg were significantly greater than those of the controls. Centrilobular hepatocellular hypertrophy was observed in male and female mice receiving 300 mg/kg. The high dose selected for the 2-year study was 200 mg/kg, which was just below the level at which mortality and liver lesions were observed in the 13-week study.

2-YEAR STUDY IN RATS: Groups of 60 male and 60 female rats were administered coumarin in corn oil by gavage at doses of 0, 25, 50, or 100 mg per kg body weight. After 15 months, 10 animals from each group were evaluated.

Survival, Body Weights, and Clinical Findings: None of the male rats receiving 100 mg/kg and only two males receiving 50 mg/kg survived until the end of the study (vehicle control, 28/50; 25 mg/kg, 9/50; 50 mg/kg, 2/51; 100 mg/kg, 0/50). Survival of dosed female rats was similar to that of the controls (29/50, 38/50, 36/50, 30/50). The reduced survival in dosed male rats was primarily attributed to chemical-related exacerbation of spontaneously occurring renal disease. Final mean body weights of female rats that received 100 mg/kg and all dosed groups of male rats were lower than those of the controls. There were no clinical signs of toxicity in rats, other than nonspecific signs relating to debilitation as a result of renal or other spontaneous disease.

Hematology and Clinical Chemistry: At the 15-month interim evaluation, the values for one or more hematologic parameters including mean erythrocyte volume, mean erythrocyte hemoglobin in 50 and 100 mg/kg rats, and hematocrit or hemoglobin in 100 mg/kg rats were significantly lower than those of controls. Activated partial thromboplastin times were also significantly lower in 50 and 100 mg/kg males, while platelet counts were significantly higher. Activities of alanine aminotransferase, sorbitol dehydrogenase, or g-glutamyltransferase in 50 and 100 mg/kg male and 100 mg/kg female rats were significantly higher than those of the controls at the 15-month interim evaluation.

Pathology Findings: The principal lesions associated with the administration of coumarin to rats for up to 2 years occurred in the liver, kidney, and forestomach. While the hepatic lesions were seen in all groups of males, they occurred only in the 50 and 100 mg/kg females. The lesions consisted of a spectrum of changes including hepatocellular necrosis, fibrosis, cytologic alteration, and increased severity of bile duct hyperplasia. The incidences of hepatocellular neoplasms were not increased in dosed rats. There was a chemical-related increase in the average severity of nephropathy in all groups of dosed male and female rats. There were corresponding increased incidences of parathyroid gland hyperplasia in all groups of dosed males, probably as a result of compromised renal function. In the standard evaluation of single kidney sections, a low incidence of renal adenomas was seen in all groups of males and in 100 mg/kg females (males: vehicle control, 1/49; 25 mg/kg, 2/50; 50 mg/kg, 2/51; 100 mg/kg, 1/50; females: 0/49, 0/50, 0/50, 2/49). An evaluation of step sections identified additional

individuals with renal tubule focal hyperplasia (males: 2/49, 12/50, 10/51, 6/50; females: 1/49, 0/50, 4/50, 2/49) and adenoma (males: 0/49, 4/50, 5/51, 4/50; females: 0/49, 0/50, 1/50, 1/49) in the dosed groups. The incidences of forestomach ulcers in all groups of dosed male rats and in 100 mg/kg female rats were significantly greater than those of the controls (males: 7/48, 24/50, 35/51, 34/50; females: 1/48, 1/49, 6/50, 9/48). STOP-EXPOSURE EVALUATION: A group of 40 male rats received 100 mg/kg coumarin in corn oil by gavage for 9 months, when 20 of the animals were necropsied and evaluated. The remainder of the male rats received only the corn oil vehicle during the 15-month recovery period. Similarly, a group of 30 male rats received 100 mg/kg coumarin in corn oil by gavage for 15 months, when 10 of the rats were necropsied and evaluated. The remaining 20 rats received only corn oil during the 9-month recovery period. A group of 20 vehicle control male rats were necropsied at 9 months, and another 10 vehicle control male rats were necropsied at 15 months. While chemical-related hepatic lesions were seen at both the 9- and 15-month interim evaluations, the incidences and severities of these lesions following the recovery period were generally similar to controls. Thus, the hepatic lesions produced by 9 or 15 months of exposure were reversible. In contrast to the liver lesions, the severity of nephropathy in male rats following the recovery period was significantly greater than that of males examined at the 9- and 15-month interim evaluations. This is not unexpected, since nephropathy is a progressive degenerative disease that naturally increases in severity with age. The incidence of renal tubule hyperplasia in the 15-month stop-exposure group (dosed for 15 months followed by the recovery period) and the incidence of renal tubule adenoma in the 9-month stop-exposure group were significantly greater than those of the control group. 2-YEAR STUDY IN MICE: Groups of 70 male and 70 female mice were administered coumarin in corn oil by gavage at doses of 0, 50, 100, or 200 mg per kg body weight for up to 2 years. After 15 months, 19 or 20 mice from each group were evaluated. Survival, Body Weights, and Clinical Findings: Survival of dosed male and female mice was similar to that of the controls (males: vehicle control, 43/50; 50 mg/kg, 47/50; 100 mg/kg, 42/50; 200 mg/kg, 37/51; females: 33/50, 40/50, 42/51, 28/51). The mean body weights of 200 mg/kg male and female mice were lower than those of controls throughout much of the study. There were no clinical findings related to chemical administration. Hematology and Clinical Chemistry: Mean erythrocyte volume, mean erythrocyte hemoglobin, and hematocrit of 200 mg/kg males and mean erythrocyte volume of 200 mg/kg females were significantly lower than those of the controls. Blood platelet counts of 200 mg/kg males and females were significantly higher than those of controls. There were no biologically significant differences in enzyme activities between dosed and control mice. Pathology Findings: The principal toxic lesions associated with the administration of coumarin to mice occurred in the liver. The incidences of centrilobular hypertrophy in 100 and 200 mg/kg males and 200 mg/kg females were significantly greater than those of controls. The incidences of syncytial alteration in all male dose groups and in 200 mg/kg females were also significantly greater than controls. The incidences of eosinophilic foci, a putative preneoplastic lesion,

and of hepatocellular adenoma were significantly greater in the 50 and 100 mg/kg females. Hepatocellular carcinomas occurred with low incidences in the dosed females, but none occurred in the controls. The overall incidence of hepatocellular neoplasms (benign and malignant combined) in the 50 and 100 mg/kg females (control, 8/50; 50 mg/kg, 27/49; 100 mg/kg, 31/51; 200 mg/kg, 13/50) exceeds the range in historical controls (range 2%-34%; 129/898, 14.4%) from recent NTP studies. The reason for a lack of liver response in 200 mg/kg female mice is not known, but may be due in part to the decrease in body weight. While the incidences of eosinophilic foci were marginally greater in dosed male mice, the incidences of hepatocellular neoplasms were similar among the dosed and control groups. The incidences of alveolar/bronchiolar adenomas were significantly greater in 200 mg/kg male and female mice than in the controls. Further, the incidence of alveolar/bronchiolar carcinoma in 200 mg/kg females was also significantly greater than in controls. The overall incidence of pulmonary neoplasms (benign and malignant combined) in the 200 mg/kg groups (males: 14/50, 9/50, 15/50, 25/51; females: 2/51, 5/49, 7/49, 27/51) exceeds the range in historical controls (males: range 6%-28%; 166/900, 18.4%; females: range 0%-14%; 58/899, 6.5%) from recent NTP studies. The incidence of squamous cell papilloma of the forestomach in 50 mg/kg males was greater than that of the controls (2/50, 8/50, 2/50, 0/51) and also exceeds the range of this neoplasm in control male mice from recent NTP studies (range 0%-14%; 27/902, 3.0%). The incidence of squamous cell papilloma of the forestomach in 50 mg/kg female mice was also slightly increased (1/52, 5/50, 2/51, 2/51); however, the incidence did not exceed the NTP historical range (27/901, 3%; range, 0%-10%).

GENETIC TOXICOLOGY: Coumarin induced gene mutations in *Salmonella typhimurium* strain TA100 in the presence, but not in the absence, of exogenous metabolic activation (S9); no mutations were induced in strains TA98, TA1535, or TA1537, with or without S9. In Chinese hamster ovary cells, coumarin induced sister chromatid exchanges in the absence of S9, and chromosomal aberrations in the presence of S9. Coumarin did not induce sex-linked recessive lethal mutations in germ cells of male *Drosophila melanogaster* treated either as adults by feeding or injection, or as larvae by feeding. No increase in the frequency of micronucleated erythrocytes was observed in peripheral blood of male and female B6C3F1 mice administered coumarin by gavage for 13 weeks.

CONCLUSIONS: Under the conditions of these 2-year gavage studies there was some evidence of carcinogenic activity of coumarin in male F344/N rats based on increased incidences of renal tubule adenomas. There was equivocal evidence of carcinogenic activity of coumarin in female F344/N rats based on a marginally increased incidence of renal tubule adenomas. There was some evidence of carcinogenic activity of coumarin in male B6C3F1 mice based on the increased incidence of alveolar/bronchiolar adenomas. There was clear evidence of carcinogenic activity of coumarin in female B6C3F1 mice based on increased incidences of alveolar/bronchiolar adenomas, alveolar/bronchiolar carcinomas, and hepatocellular adenomas. The marginally increased incidences of squamous cell papillomas of the forestomach

in male and female mice receiving 50 mg/kg may have been related to coumarin administration. The administration of coumarin to rats was also associated with an increased severity of nephropathy in the kidney and of bile duct hyperplasia in the liver, increased incidences of ulcers of the forestomach, and necrosis, fibrosis, and cytologic alteration of the liver. Administration of coumarin to mice was also associated with centrilobular hypertrophy, syncytial alteration, and eosinophilic focus in the liver. Synonyms: 5,6-benzo-alpha-pyrone, 2H-1-benzopyran-2-one, 2H-benzolbipyran-2-one, 1,2-oxo-1,2-benzopyran, 1,2-benzopyrone, cis-o-coumarinic acid lactone, coumarinic anhydride, coumarin, o-hydroxycinnamic acid lactone, kumarin, [2-propenoic acid, 3-(-2-hydroxyphenyl)-delta-lactone], Rattex, tonka bean camphor

National Toxicology Program. "NTP Toxicology and Carcinogenesis Studies of 3,4-Dihydrocoumarin (CAS No. 119-84-6) in F344/N Rats and B6C3F1 Mice (Gavage Studies)." *Natl Toxicol Program Tech Rep Ser.* **423**, 1-336.

Abstract. 3,4-Dihydrocoumarin was nominated by the Food and Drug Administration and the National Cancer Institute for study because of its widespread use as a flavoring agent in beverages, gelatins, puddings, candy, and other food items; as a fragrance in perfumes, creams, and cosmetics; and because of interest in the structure-activity relationships of the coumarin derivatives. Toxicity and carcinogenicity studies were conducted by administering 3,4-dihydrocoumarin (99% pure) in corn oil by gavage to groups of male and female F344/N rats and B6C3F1 mice for 16 days, 13 weeks, and 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium*, cultured Chinese hamster ovary cells, and peripheral blood cells of mice. 16-DAY STUDY IN RATS: Groups of five male and five female rats received 3,4-dihydrocoumarin in corn oil by gavage at doses of 0, 190, 375, 750, 1,500, or 3,000 mg/kg body weight 5 days per week for a total of 12 doses in a 16-day period. All male and female rats given 3,000 mg/kg, and four male rats and five female rats given 1,500 mg/kg died. Body weight gains and final mean body weights of rats receiving 190, 375, or 750 mg/kg were similar to those of the controls. There were no clinical findings of organ-specific toxicity or evidence of impaired blood coagulation. 16-DAY STUDY IN MICE: Groups of five male and five female mice received 3,4-dihydrocoumarin in corn oil by gavage at doses of 0, 140, 280, 560, 1,125, or 2,250 mg/kg body weight 5 days per week for a total of 12 doses in a 16-day period. All mice given 2,250 mg/kg died. Body weight gains and final mean body weights of mice receiving 140, 280, 560, and 1,125 mg/kg were similar to those of the controls. There were no clinical findings of organ-specific toxicity or evidence of impaired blood coagulation. 13-WEEK STUDY IN RATS: Groups of 10 male and 10 female rats received 3,4-dihydrocoumarin in corn oil by gavage at doses of 0, 75, 150, 300, 600, or 1,200 mg/kg body weight 5 days per week for 13 weeks. Two male rats and five female rats given 1,200 mg/kg died. The body weight gain and final mean body weight of male rats that received 1,200 mg/kg were significantly lower than those of the controls, but the final mean body weights of other dosed groups of male rats and all dosed groups of female rats were similar to or slightly greater than those of the controls. Platelet counts were significantly lower in males and females receiving 600 and 1,200

mg/kg and in females receiving 300 mg/kg. Hemoglobin and hematocrit values and erythrocyte counts were significantly lower in males that received 300 mg/kg or more. The absolute and relative liver and kidney weights of males and females receiving 600 and 1,200 mg/kg were significantly greater than those of the controls. Hepatocellular hypertrophy was observed in rats given 300, 600, and 1,200 mg/kg. The high dose selected for the 2-year study was 600 mg/kg, which was below the level at which mortality, lower final mean body weights, and treatment-related liver lesions were observed.

13-WEEK STUDY IN MICE: Groups of 10 male and 10 female mice received 3,4-dihydrocoumarin in corn oil by gavage at doses of 0, 100, 200, 400, 800, or 1,600 mg/kg body weight 5 days per week for 13 weeks. Eight male and five female mice receiving 1,600 mg/kg died. Deaths in other groups were attributed to dosing accidents. Final mean body weights of dosed male and female mice were similar to those of the controls, and there were no treatment-related changes in any hematologic parameters. The absolute and relative liver weights of males and females that received 1,600 mg/kg and the relative kidney weight of males that received 1,600 mg/kg were significantly greater than those of the controls. No treatment-related lesions were noted. The high dose selected for the 2-year study was 600 mg/kg, which was below the level at which mortality, lower final mean body weights, and treatment-related liver lesions were observed.

2-YEAR STUDY IN RATS: Groups of 60 male and 60 female rats received 3,4-dihydrocoumarin in corn oil by gavage at age at doses of 0, 150, 300, or 600 mg/kg body weight. After 15 months, up to 10 animals from each group were evaluated. **Survival, Body Weights, and Clinical Findings:** Survival rates of dosed male rats were lower than that of the controls (0 mg/kg, 28/51; 150 mg/kg, 12/50; 300 mg/kg, 8/50; 600 mg/kg, 2/50) but survival rates of dosed female rats were similar to that of the controls (31/50, 21/51, 26/50, 23/51). The decreased survival in dosed male rats was attributed to a chemical-related increase in the severity of nephropathy. The final mean body weight of male rats receiving 600 mg/kg was lower than that of the controls, but the final mean body weights of other dosed groups of male rats and all dosed groups of female rats were similar to those of the controls. No clinical findings related to chemical administration were observed. **Hematology and Clinical Chemistry:** At the 15-month interim evaluation, the hemoglobin concentrations, mean erythrocyte volumes, or mean erythrocyte hemoglobin concentrations in the 300 and 600 mg/kg female rats were slightly, but significantly, lower than those of the controls. In males, only the hemoglobin concentration in the 600 mg/kg group was significantly lower. Serum levels of alkaline phosphatase, alanine aminotransferase, sorbitol dehydrogenase, or g-glutamyltransferase in the 300 and 600 mg/kg male rats were significantly higher than those in the controls. In females, alkaline phosphatase and g-glutamyltransferase levels were significantly higher in the 600 mg/kg group. **Pathology Findings:** The principal lesions associated with the administration of 3,4-dihydrocoumarin to rats occurred in the kidney and forestomach. There was a chemical related increase in the severity of nephropathy in all dosed male rats and in 300 and 600 mg/kg female rats. There was a corresponding increased incidence of parathyroid gland hyperplasia, probably as a result of compromised

renal function. In the standard evaluation of single kidney sections, renal tubule adenomas were observed in one 150 and two 600 mg/kg males and one each in the control, 150, and 300 mg/kg females. Transitional cell carcinomas were also observed in two 600 mg/kg male rats. However, an extended evaluation of step sections identified significantly higher incidences of focal hyperplasia and adenoma in the 600 mg/kg males than in controls (hyperplasia: 0/50, 5/48, 6/47, 8/50; adenoma: 1/50, 1/48, 3/47, 6/50). The incidence of forestomach ulcers in all groups of dosed male rats was significantly greater than that of the controls (4/47, 14/48, 20/50, 16/46). STOP-EXPOSURE EVALUATION: A group of 40 male rats received 600 mg/kg 3,4-dihydrocoumarin in corn oil by gavage for 9 months, when 20 of the animals were necropsied and evaluated. The remainder of the male rats received only the corn oil vehicle until they died or until the end of the study. Similarly, a group of 30 male rats received 600 mg/kg 3,4-dihydrocoumarin in corn oil by gavage for 15 months, when 10 of the rats were necropsied and evaluated. The remaining 20 rats received only corn oil until the end of the study. A group of 20 vehicle control male rats was necropsied at 9 months, and another 10 vehicle control male rats were necropsied at 15 months. The severity of nephropathy in male rats of the stop-exposure groups was significantly greater than that of males examined at the 9- and 15-month interim evaluations. This was expected because nephropathy is a progressive degenerative disease that naturally increases in severity with age. 2-YEAR STUDY IN MICE: Groups of 70 male and 70 female mice received 3,4-dihydrocoumarin in corn oil by gavage at doses of 0, 200, 400, or 800 mg/kg body weight. After 15 months, five to 10 animals from each group were evaluated. Additional groups of 8 to 10 animals were evaluated for clinical pathology after 15 months. Survival, Body Weights, and Clinical Findings Survival rates of dosed male and female mice were similar to those of the controls (males: 0 mg/kg, 42/50; 200 mg/kg, 39/51; 400 mg/kg, 34/51; 800 mg/kg, 38/50; females: 36/51, 39/50, 41/50, 28/52). Final mean body weights of dosed male and female mice were similar to those of the controls. No clinical findings were noted that were related to chemical administration. Hematology and Clinical Chemistry: There were no differences in hematology or clinical chemistry parameters that were considered to be chemical related. Pathology Findings: The principal neoplasms associated with the administration of 3,4-dihydrocoumarin to mice occurred in the liver. There were significantly increased incidences of hepatocellular adenomas in all groups of dosed female mice. Further, the incidences of multiple hepatocellular adenomas in dosed female mice were greater than that of the controls (control, 0/51; 200 mg/kg, 6/50; 400 mg/kg, 9/50; 800 mg/kg, 9/52). However, there was no corresponding increased incidence of hepatocellular carcinoma in dosed female mice (3/51, 2/50, 4/50, 6/52), and the incidences of hepatocellular adenoma or carcinoma were similar between dosed and control male groups (adenoma: 29/50, 23/51, 36/51, 31/50; carcinoma: 11/50, 11/51, 11/51, 6/50). The incidence of alveolar/bronchiolar adenoma in the 200 and 400 mg/kg male mice was marginally greater than that of the controls (8/50, 15/50, 15/51, 10/50). However, these neoplasms were not considered chemical related because the increased incidence was slight and

there was no corresponding increased incidence in the 800 mg/kg group. The incidence of alveolar/bronchiolar neoplasms in female mice was similar between the dosed and control groups (adenoma: 2/51, 5/50, 1/48, 3/51; carcinoma: 0/51, 1/50, 0/48, 0/51). In the standard evaluation of single sections of kidney, focal hyperplasia and adenoma or carcinoma of the renal tubule were identified in several dosed male mice, but not in controls [adenoma or carcinoma (combined): 0/50,1/51, 2/51,1/49; hyperplasia: 2/50, 2/51, 5/51, 2/49]. In an extended evaluation of step sections, a few additional males with focal hyperplasia or renal tubule adenomas were identified in the dosed groups. However, the incidences of these lesions in dosed groups of male mice were not significantly greater than those of the controls, and did not increase with dose (hyperplasia: 0/50,1/51, 3/51, 1/49; renal tubule adenoma: 0/50, 0/51, 2/51, 1/49). Therefore, the low number of renal tubule neoplasms in male mice was not considered to be chemical related. GENETIC TOXICOLOGY: 3,4-Dihydrocoumarin did not induce gene mutations in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 with or without exogenous metabolic activation (S9). It induced sister chromatid exchanges but not chromosomal aberrations in cultured Chinese hamster ovary cells, with and without S9. No induction of micronuclei was noted in peripheral blood erythrocyte samples obtained from male and female B6C3F1 mice at the end of the 13-week toxicology study. CONCLUSIONS: Under the conditions of these 2-year gavage studies, there was some evidence of carcinogenic activity of 3,4-dihydrocoumarin in male F344/N rats based on increased incidences of renal tubule adenomas and focal hyperplasia. The transitional cell carcinomas in two 600 mg/kg males may also have been chemical related. There was no evidence of carcinogenic activity of 3,4-dihydrocoumarin in female F344/N rats receiving 150, 300, or 600 mg/kg. There was no evidence of carcinogenic activity of 3,4-dihydrocoumarin in male B6C3F1 mice receiving 200, 400, or 800 mg/kg. There was some evidence of carcinogenic activity in female B6C3F1 mice based on increased incidences of hepatocellular adenoma and hepatocellular adenoma or carcinoma (combined). 3,4-Dihydrocoumarin caused ulcers, hyperplasia, and inflammation of the forestomach, parathyroid gland hyperplasia, and increased severity of nephropathy in male rats. Synonyms: 1,2-benzodihydropyrone, 2H-1-benzopyran-2-one, 2-chromanone, 3,4-dihydro-2H-1-benzopyran-2-one, dihydrocoumarin, hydrocoumarin, o-hydroycinnamic acid, delta-lactone-hydrocinnamic acid, melilotin, melilotine, melilotol, 2-oxochroman

Vassallo J.D., Hicks S.M., Born S.L., & Daston GP. (2004) "Roles for epoxidation and detoxification of coumarin in determining species differences in clara cell toxicity." *Toxicol Sci.* **82**(1), 26-33. Abstract. Coumarin-induced mouse Clara cell toxicity is thought to result from the local formation of coumarin 3,4-epoxide (CE). However, this toxicity is not observed in the rat, indicating species differences in coumarin metabolism. The purpose of the present work was to characterize the in vitro kinetics of coumarin metabolism in mouse, rat, and human whole lung microsomes, and to determine whether species differences in coumarin-induced Clara cell toxicity correlate with coumarin epoxidation or detoxification. In B6C3F1 mouse lung microsomes, coumarin was metabolized to CE, which in the

absence of glutathione spontaneously rearranges to o-hydroxyphenylacetaldehyde (o-HPA). The $K(m)$ and $V(max)$ for o-HPA formation were 155 μM and 7.3 $nmol/min/mg$ protein, respectively. In contrast, the $K(m)$ and $V(max)$ were 2573 μM and 1.75 $nmol/min/mg$ protein, respectively, in F344 rat lung microsomes. Since the intrinsic clearance through the epoxidation pathway was 69 times higher in the mouse, the epoxidation rate was shown to correlate with species sensitivity to toxicity. To determine whether detoxification reactions contribute to species differences in toxicity, the fate of CE and o-HPA were examined. Detoxification of CE via conjugation with glutathione was evaluated in lung cytosol from mice and rats, and the $K(m)$ of this reaction was approximately 800 μM in both species, whereas the $V(max)$ was 3.5 and 6 $nmol/min/mg$ protein, respectively, indicating that conjugation is faster in the rat. Oxidation of o-HPA to o-hydroxyphenylacetic acid (o-HPAA) was examined in lung cytosol from mice and rats. The $K(m)$ of this reaction was approximately 1.5 μM in both species, whereas the $V(max)$ was 0.08 and 0.33 $nmol/min/mg$ protein in mice and rats, respectively, indicating that oxidation is faster in the rat. While the rate of epoxidation correlates with species sensitivity to coumarin, it is likely that Clara cell toxicity is modulated by CE and o-HPA detoxification. In contrast to rodent lung microsomes, bioactivation of coumarin to o-HPA did not occur in 16 different human lung microsomes, which suggests metabolism-dependent toxicity in the human lung is unlikely following low level coumarin exposure.

Vassallo J.D., Hicks S.M., Daston G.P., Lehman-McKeeman L.D. (2004) "Metabolic detoxification determines species differences in coumarin-induced hepatotoxicity." *Toxicol Sci.* **80**(2), 249-57. Abstract. Hepatotoxicity of coumarin is attributed to metabolic activation to an epoxide intermediate, coumarin 3,4-epoxide (CE). However, whereas rats are most susceptible to coumarin-induced hepatotoxicity, formation of CE is greatest in mouse liver microsomes, a species showing little evidence of hepatotoxicity. Therefore, the present work was designed to test the hypothesis that detoxification of CE is a major determinant of coumarin hepatotoxicity. CE can either rearrange spontaneously to o-hydroxyphenylacetaldehyde (o-HPA) or be conjugated with glutathione (GSH). o-HPA is hepatotoxic and is further detoxified by oxidation to o-hydroxyphenylacetic acid (o-HPAA). In vitro experiments were conducted using mouse liver microsomes to generate a constant amount of CE, and cytosols from F344 rats, B6C3F1 mice, and human liver were used to characterize CE detoxification. All metabolites were quantified by HPLC methods with UV detection. In rats and mice, GSH conjugation occurred non-enzymatically and through glutathione-S-transferases (GSTs), and the kinetics of GSH conjugation were similar in rats and mice. In rat liver cytosol, oxidation of o-HPA to o-HPAA was characterized with a high affinity $K(m)$ of approximately 12 μM , and a $V(max)$ of approximately 1.5 $nmol/min/mg$ protein. In contrast, the $K(m)$ and $V(max)$ for o-HPA oxidation in mouse liver cytosol were approximately 1.7 μM and 5 $nmol/min/mg$ protein, respectively, yielding a total intrinsic clearance through oxidation to o-HPAA that was 20 times higher in mouse than in rats. Human cytosols (two separate pools) detoxified CE through o-HPA

oxidation with an apparent $K(m)$ of 0.84 μM and a $V(\text{max})$ of 5.7 $\text{nmol}/\text{min}/\text{mg}$ protein, for a net intrinsic clearance that was more than 50 times higher than the rat. All species also reduced o-HPA to o-hydroxyphenylethanol (o-HPE), but this was only a major reaction in rats. In the presence of a metabolic reaction replete with all necessary cofactors, GSH conjugation accounted for nearly half of all CE metabolites in rat and mouse, whereas the GSH conjugate represented only 10% of the metabolites in human cytosol. In mouse, o-HPAA represented the major ring-opened metabolite, accounting for the remaining 50% of metabolites, and in human cytosol, o-HPAA was the major metabolite, representing nearly 90% of all CE metabolites. In contrast, no o-HPAA was detected in rats, whereas o-HPE represented a major metabolite. Collectively, these in vitro data implicate o-HPA detoxification through oxidation to o-HPAA as the major determinant of species differences in coumarin-induced hepatotoxicity.

Wattenberg L.W., Lam L.K.T & Fladmoe A.V. (1979) "Inhibition of chemical carcinogen-induced neoplasia by coumarins and α -angelicalactone." *Cancer Research* **39**, 1651-1654 Abstract. Coumarin, umbelliferone (7-hydroxycoumarin), scopoletin (7-hydroxy-6-methoxycoumarin), and limettin (5,7-dimethoxycoumarin), four naturally occurring plant constituents, were studied for their effects on 7,12-dimethylbenz(a)anthracene-induced neoplasia of the rat mammary gland. Coumarin was a moderately potent inhibitor, limettin was less effective, and scopoletin showed only marginal inhibitory activity. Umbelliferone did not inhibit. Coumarin and its three derivatives were also investigated for their effects on benzo(a)pyrene-induced neoplasia of the mouse forestomach. Coumarin inhibited, but the three derivatives did not. Coumaranone and phthalide, two related compounds, were inactive as were three substituted pyrones included in the study. Four five-membered ring lactones were also investigated. One of these, α -angelicalactone, inhibited benzo(a)pyrene-induced neoplasia of the mouse forestomach and was more potent in this regard than coumarin. The other three, γ -valerolactone, L-ascorbic acid, and isocitric lactone, were inactive. Three structure-activity relationships are evident from the present study. With the coumarins, increased polarity of substituents results in decreasing activity as inhibitors. For both the coumarins and the five-membered ring lactones studied, protic groups, such as hydroxy and carboxy groups, abolish the capacity to inhibit. While unsaturation in the lactone ring does not always lead to inhibitory activity, the presence of at least one double bond is essential. Thus, the property of inhibition of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene is not a general characteristic of all coumarins and alicyclic lactones but is restricted to those with specific structural features.

von Weymarn L.B. & Murphy S.E. (2001) "Coumarin metabolism by rat esophageal microsomes and cytochrome P450 2A3." *Chem Res Toxicol.* **14**(10), 1386-9. Abstract. The rat esophagus is strikingly sensitive to tumor induction by nitrosamines, and it has been hypothesized that this tissue contains cytochrome P450 enzymes (P450s) which catalyze the metabolic activation of these carcinogens. The metabolic capacity of the esophagus is not well characterized. In the study described here, the products of 14C-coumarin metabolism by rat

esophageal microsomes were identified and quantified. Metabolite characterization was by LC/MS/MS and GC/MS and comparison to standards, quantification was by radioflow HPLC. The coumarin metabolites formed by rat esophageal microsomes were compared to those formed by P450 2A3. The major metabolites formed by esophageal microsomes were 8-hydroxycoumarin, o-hydroxyphenylacetaldehyde (o-HPA), and o-hydroxyphenylacetic acid (o-HPAA). A smaller amount of 5-hydroxycoumarin, about one-third the 8-hydroxycoumarin, was also formed. o-HPA and o-HPAA are products of coumarin 3,4-epoxidation. The relative rates of coumarin 8-hydroxylation and 3,4-epoxidation were similar. Coumarin 8-hydroxylation has not previously been reported as a major pathway in any tissue, and no P450s have yet been reported to catalyze this reaction. P450 2A3 catalyzed both the 7-hydroxylation and 3,4-epoxidation of coumarin. P450 2A3 was previously characterized as a coumarin 7-hydroxylase, however, in this study, we report that it catalyzes the formation of o-HPA more efficiently. The K_m and V_{max} were $1.3 \pm 0.35 \mu\text{M}$ and $0.65 \pm 0.06 \text{ nmol/min/nmol P450}$ for coumarin 7-hydroxylation and $1.4 \pm 0.58 \mu\text{M}$ and $3.1 \pm 0.46 \text{ nmol/min/nmol P450}$ for o-HPA formation.

Coumarin: Metabolism

Booth A.N. *et al.* (1959). "Urinary metabolites of coumarin & o-coumaric acid." *J Biol Chem* **234**(4), 946-948.

Dominguez K., Fentem J. H., Garle M. J. and Fry J. R. (1990) "Comparison of Mongolian gerbil and rat hepatic microsomal mono-oxygenase activities: high coumarin 7-hydroxylase activity in the gerbil." *Biochemical Pharmacology* **39**, 1629-1631.

Donato M. T., Castell J. V. and Gomez-Lechon M. J. (1998) "The coumarin 7-hydroxylation assay in living hepatic cells in culture." *ATLA* **26**, 213-223.

Draper A. J., Madan A. and Parkinson A. (1997) "Inhibition of coumarin 7-hydroxylase activity in human liver microsomes." *Archives of Biochemistry and Biophysics* **341**, 47-61.

Edwards AJ, Price RJ, Renwick AB, Lake BG. (2000) "Lack of effect of coumarin on unscheduled DNA synthesis in the in vivo rat hepatocyte DNA repair assay." *Food Chem Toxicol.* **38**(5), 403-9. [Abstract](#). The ability of coumarin to induce UDS in male Sprague-Dawley CD rat hepatocytes in vivo was assessed using the unscheduled DNA synthesis (UDS) assay. From a preliminary toxicity study the oral maximum tolerated dose (MTD) of coumarin was determined to be 320 mg/kg body weight. For the UDS studies, rats were treated with 0 (corn oil control), 32 (one-tenth the MTD), 107 (one-third the MTD) and 320 (MTD) mg/kg coumarin via oral gavage. Rats were also treated with 20mg/kg body weight dimethylnitrosamine (DMN) or 50mg/kg body weight 2-acetylaminofluorene (2-AAF) as positive controls for the 2-4 hr and 12-16 hr expression of UDS, respectively. Hepatocytes were isolated by liver perfusion either 2-4 hr or 12-16 hr after treatment and cultured in medium containing [methyl-(3)H]thymidine for 4 hr and assessed for UDS by grain counting of autoradiographs. Coumarin

treatment at doses of 32-320 mg/kg body weight had no statistically significant or dose-related effect on UDS in rat hepatocytes either 2-4 hr or 12-16 hr after dosing. In contrast, both DMN 2-4 hr after dosing and 2-AAF 12-16 hr after dosing produced significant increases in UDS assessed as the net nuclear grain count. Both genotoxins also increased the percentage of hepatocyte nuclei with greater than 5 net grains. Treatment with coumarin, DMN and 2-AAF had no statistically significant effect on the proportion of rat hepatocytes undergoing replicative DNA synthesis. In summary, this study demonstrates that coumarin does not induce UDS in hepatocytes of male Sprague-Dawley CD rats after oral administration at doses up to the MTD of 320 mg/kg. The responsiveness of the animals used in this study to genotoxic agents was demonstrated by the clear induction of DNA repair after treatment with DMN and 2-AAF.

Feuer G. (1974) "The metabolism & biological actions of coumarins." *Progress in Medicinal Chemistry* **10**, 85-158.

Fernandez-Salguero P., Ho□man S. M. G., Cholerton S., Mohrenweiser H., Raunio H., Rautio A., Pelkonen O., Huang J-D., Evans W. E., Idle J. R. & Gonzalez F. J. (1995) "A genetic polymorphism in coumarin 7-hydroxylation: sequence of the human CYP2A genes and identification of variant CYP2A6 alleles." *American Journal of Human Genetics* **57**, 651-660.

Fernyhough L., Kell S. W., Hammond A. H., Thomas N. W. & Fry J. R. (1994) "Comparison of in vivo and in vitro rat hepatic toxicity of coumarin and methyl analogues, and application of quantitative morphometry to toxicity in vivo." *Toxicology* **88**, 113-125.

Gangoli S.D. *et al.* (1974) "Studies on the metabolism & hepatotoxicity of coumarin in the baboon." *Biochem. Soc. Transactions* **2**, 310-312. *Archives Internationales de Physiologie et de Biochimie* **79**, 665-679.

Hadidi H., Zalsen K., Idle J.R. & Cholerton S. (1997) "A single amino acid substitution (Leu160His) in Cytochrome P450 CYP2A6. [Ref?](#)

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Huntingdon Life Sciences (1996c) "14C-Coumarin. Comparative metabolism and pharmacokinetics in the mouse after gavage and dietary administration." Project Number RIF 27/942434. Report to RIFM.

Kim D., Wu Z.L. & Guengerich F.P. (2005) "Analysis of coumarin 7-hydroxylation activity of cytochrome P450 2A6 using random mutagenesis." *J Biol Chem.* **280**(48), 40319-27. [Abstract](#). Cytochrome P450 (P450) 2A6 is an important human enzyme involved in the metabolism of many xenobiotic chemicals including coumarin, indole, nicotine, and carcinogenic nitrosamines. A combination of random mutagenesis and high-throughput screening was used in the analysis of P450 2A6, utilizing a fluorescent coumarin 7-hydroxylation assay.

The steady-state kinetic parameters (k_{cat} and K_m) for coumarin 7-hydroxylation by wild-type P450 2A6 and 35 selected mutants were measured and indicated that mutants throughout the coding region can have effects on activity. Five mutants showing decreased catalytic efficiency (k_{cat}/K_m) were further analyzed for substrate selectivity and binding affinities and showed reduced catalytic activities for 7-methoxycoumarin O-demethylation, tert-butyl methyl ether O-demethylation, and indole 3-hydroxylation. All mutants except one (K476E) showed decreased coumarin binding affinities (and also higher K_m values), indicating that this is a major basis for the decreased enzymatic activities. A recent x-ray crystal structure of P450 2A6 bound to coumarin (Yano, J. K., Hsu, M. H., Griffin, K. J., Stout, C. D., and Johnson, E. F. (2005) *Nat. Struct. Mol. Biol.* **12**, 822-823) indicates that the recovered A481T and N297S mutations appear to be close to coumarin, suggesting direct perturbation of substrate interaction. The decreased enzymatic activity of the K476E mutant was associated with decreases both in NADPH oxidation and the reduction rate of the ferric P450 2A6-coumarin complex. The attenuation is caused in part to lower binding affinity for NADPH-P450 reductase, but the K476E mutant did not achieve the wild-type coumarin 7-hydroxylation activity even at high reductase concentrations.

Lake B.G., Walters D.G. & Gangolli S.D. (1989) "Comparison of the metabolism and disposition of [3-14C]coumarin in the rat and marmoset (*Callithrix jacchus*)." *Toxicol Lett.* **45**(2-3):299-306. [Abstract](#). Male Sprague-Dawley rats and marmosets were given a single oral 25 mg/kg dose of [3-14C]coumarin and the excretion of radioactivity in the expired air, urine and faeces monitored up to 96 h. Excretion profiles were similar in both species with the bulk of the dose being excreted in the urine and faeces within 24 h. Chromatographic analysis of 0-48 h urine samples revealed similar metabolic profiles with only small amounts of unchanged coumarin and very little 7-hydroxycoumarin. Coumarin 7-hydroxylase activity was not detectable in hepatic microsomes from either species. These results demonstrate that the disposition of [3-14C]coumarin was similar in the rat and marmoset, a New World primate, and that both species, unlike man, are poor 7-hydroxylators of coumarin.

Lake B.G., Sauer M.J., Esclancon F., Beaman J.A., Price R.J. & Walters D.G.(1995) "Metabolism of coumarin by precision-cut calf liver slices and calf liver microsomes. *Xenobiotica.* **25**(2), 133-41. [Abstract](#). 1. The metabolism of 50 microM [3-14C]coumarin has been studied in precision-cut-calf liver slices. 2. The metabolism of 50 microM coumarin to 7-hydroxycoumarin has also been examined in calf, rat, Cynomolgus monkey and human liver microsomal preparations. 3. In precision-cut calf liver slices, [3-14C]coumarin was metabolized to various polar products and to metabolite(s) that bound covalently to calf liver slice proteins. The polar products included 7-hydroxycoumarin (which was extensively conjugated with D-glucuronic acid and/or sulphate), metabolites of the 3-hydroxylation pathway (mainly o-hydroxyphenylethanol and o-hydroxyphenylacetic acid), and unknown metabolites. 4. Coumarin 7-hydroxylase activity was readily detectable in calf, Cynomolgus monkey and human liver microsomes, but only barely detectable in rat liver microsomes. Enzyme activity

in calf, Cynomolgus monkey and human liver microsomes was inhibited by 8-methoxypsoralen (methoxsalen) with IC₅₀'s (concentration required to produce a 50% inhibition of enzyme activity) ranging from 0.3 to 2.8 microM. 5. These results and those of other studies demonstrate that precision-cut liver slices are a valuable in vitro model system for investigating species differences in xenobiotic metabolism. Coumarin is metabolized in calf liver by various pathways including both 3- and 7-hydroxylation. The inhibition of coumarin 7-hydroxylase activity by 8-methoxypsoralen suggests that calf liver microsomes contain P450A isoenzyme(s) similar to mouse 2A5 and human 2A6.

Lewis D.F. & Lake B.G. (2002) "Species differences in coumarin metabolism: a molecular modelling evaluation of CYP2A interactions. *Xenobiotica*. **32**(7),547-61. [Abstract](#). An account of the differences in coumarin metabolism between several mammalian species, including man, is reported. 2. The metabolism of coumarin via 7-hydroxylation in the human (CYP2A6) and mouse (CYP2A5) enzymes is explained in terms of molecular modelling of the active site interactions, whereas the rat orthologue (CYP2A1) exhibits 3,4-epoxidation of coumarin, which is also consistent with the modelled interaction between enzyme and substrate. 3. In addition, quantitative structure-activity relationships (QSARs) for coumarin 7-hydroxylation in wild-type and mutant CYP2A5 show the importance of amino acid residue properties for substrate binding, whereas QSARs for CYP2A6 substrates indicate the importance of hydrogen bonding and lipophilicity for favourable interactions with the enzyme.

Lewis D.F., Ito Y. & Lake B.G. (2006) "Metabolism of coumarin by human P450s: a molecular modelling study." *Toxicol In Vitro*. **20**(2), 256-64. [Abstract](#). The oxidative metabolism of coumarin via several human cytochrome P450 (CYP) enzymes from families CYP1, CYP2 and CYP3 is rationalized in terms of molecular modelling studies carried out on the key interactions with various amino acid residues in the relevant active sites. The findings from modelling by homology with the CYP2C5 crystallographic template are in agreement with the known metabolism of coumarin in human P450s from the CYP1, CYP2 and CYP3 families, which has been published recently, and with independently reported information from site-directed mutagenesis studies.

Lovell D.P., van Iersel M., Walters D.G., Price R.J. & Lake B.G. (1999) "Genetic variation in the metabolism of coumarin in mouse liver." *Pharmacogenetics* **9**(2), 239-50. [Abstract](#). The metabolism of 50 microM [3-¹⁴C] coumarin to polar products separated by high performance liquid chromatography (HPLC) and covalently bound metabolites in liver microsomes was compared in a series of inbred strains of mice. Coumarin metabolism to total polar products was higher in female than male mice. In all strains, the coumarin 3,4-epoxidation pathway was the major route of metabolism with o-hydroxyphenylacetaldehyde (o-HPA) as the major metabolite. However, in females, there was a major strain difference in the degree of metabolism to coumarin 7-hydroxylase with DBA/2 and 129 having high 7-hydroxycoumarin formation, CBA/Ca having intermediate levels and the other strains low levels. The differences between the strains was much less

pronounced in the male mice. There was also evidence for strain variation in metabolism in the quantities of a number of other coumarin metabolites as detected by HPLC analysis of incubate extracts. However, this variation was of a quantitative nature and relatively small. The metabolism of B6C3F1 hybrid mice, in which coumarin had been identified as carcinogenic in a long-term cancer bioassay, was qualitatively similar to that of the other genotypes. The DBA/2 mouse has been suggested as a model for the metabolism of coumarin in humans. The pattern of metabolism found in this strain is different from most other strains. However, the pattern found for all the mouse strains, including DBA/2, differed appreciably from the profiles for other species including humans in the extent of 7-hydroxylation.

Peters M.M., Walters D.G., van O.B., van B.J. & Lake B.G. (1991) "Effect of inducers of cytochrome P-450 on the metabolism of [3-14C]coumarin by rat hepatic microsomes." *Xenobiotica* **21**, 499–514. [Abstract](#). 1. The metabolism of [3-14C]coumarin has been studied in rat hepatic microsomes and with two purified cytochrome P-450 isoenzymes. 2. [3-14C]Coumarin was converted by liver microsomes to several polar products including 3- and/or 5-hydroxycoumarin, omicron-hydroxyphenylacetic acid and a major unidentified novel coumarin metabolite. 3. [3-14C]Coumarin was also converted to reactive metabolite(s) as indicated by covalent binding to proteins, and by the depletion of reduced glutathione added to the microsomal incubations. 4. [3-14C]Coumarin metabolism to polar and covalently bound metabolites by rat liver microsomes was induced by pretreatment with phenobarbitone, 3-methylcholanthrene, beta-naphthoflavone, Aroclor 1254 and isosafrole; but not by dexamethasone or nafenopin. 5. The profile of [3-14C]coumarin metabolism to polar products was similar in control and pretreated liver microsomes and in incubations with purified cytochrome P450 IA1 and P450 IIB1 isoenzymes. 6. The results indicate that coumarin is a substrate for isoenzymes of the cytochrome P450 IA and P450 IIB subfamilies. The bioactivation of coumarin by rat hepatic microsomes is postulated to result in the formation of a coumarin 3,4-epoxide intermediate which may rearrange to 3-hydroxycoumarin, be further metabolized to a coumarin 3,4-dihydrodiol, or form a glutathione conjugate.

Price R.J., Renwick A.B., Beaman J.A., Esclangon F., Wield P.T., Walters D.G., & Lake B.G. (1995) "Comparison of the metabolism of 7-ethoxycoumarin and coumarin in precision-cut rat liver and lung slices." *Food Chem Toxicol.* **33**(3), 233-7. [Abstract](#). The metabolism of 7-ethoxycoumarin and [3-(14)C]coumarin was compared in precision-cut rat liver and lung slices. The lung slices were prepared using an agarose gel instilling technique enabling the production of tissue cylinders followed by lung slices employing a Krumdieck tissue slicer. Both 50 microM 7-ethoxycoumarin and 50 microM [3-(14)C]coumarin were metabolized by rat liver and lung slices. 7-Ethoxycoumarin was converted to 7-hydroxycoumarin (7-HC) which was conjugated with both D-glucuronic acid and sulfate. 7-HC sulfate was the major metabolite formed by both liver and lung slices. [3-(14)C]Coumarin was metabolized by rat liver and lung slices to both polar products and to metabolite(s) that bound covalently to

tissue slice proteins. The polar products included unidentified metabolites and 3-hydroxylation pathway products, with only very small quantities of 7-HC being formed. These results demonstrate that precision-cut lung slices are a useful model in vitro system for studying the pulmonary metabolism of xenobiotics. Moreover, the precision-cut tissue slice technique may be employed for comparisons of hepatic and extrahepatic xenobiotic metabolism.

Ratanasavanh D, Lamiable D, Biour M, Guedes Y, Gersberg M, Leutenegger E & Riche C (1996) "Metabolism and toxicity of coumarin on cultured human, rat, mouse and rabbit hepatocytes." *Fundam Clin Pharmacol* **10**, 504–510.

van Iersel M., Walters D.G., Price R.J., Lovell D.P. & Lake B.G. (1994) "Sex and strain differences in mouse hepatic microsomal coumarin 7-hydroxylase activity." *Food Chem Toxicol.* **32**(4),387-90. [Abstract](#). Hepatic microsomal coumarin 7-hydroxylase activity has been determined in male and female mice of strains A/J, AKR, BALB/c, CBA/Ca, C3H/He, C57BL/6J, DBA/2 and 129. In males, coumarin 7-hydroxylase activity was highest in liver microsomes from DBA/2 mice and lowest in BALB/c mice. With female mice enzyme activity was highest in DBA/2 and 129 strains, intermediate in the CBA/Ca strain and comparatively low in the other five strains. Marked sex differences were observed in coumarin 7-hydroxylase activity with enzyme activity in female animals from strains DBA/2, 129 and CBA/Ca being 4.8-, 6.2- and 4.8-fold higher, respectively, than in male mice. In contrast, only minor sex and strain differences in levels of total microsomal cytochrome P-450 were observed. These results demonstrate marked sex and strain differences in mouse hepatic microsomal coumarin 7-hydroxylase activity. Such differences may be due to variations in particular cytochrome P-450 isoenzymes such as CYP2A5, not all of which can be explained by the known allelic difference in the Cyp2a-5 locus.

van Iersel M.L., Henderson C.J., Walters D.G., Price R.J., Wolf C.R. & Lake B.G. (1994) Metabolism of [3-14C] coumarin by human liver microsomes. *Xenobiotica.* **24**(8), 795-803. [Abstract](#). 1. The metabolism of 50 microM [3-14C] coumarin has been studied in a panel of 12 human liver microsomal samples of known P450 isoenzyme profile. 2. [3-14C] coumarin was metabolized by human liver microsomes to various polar products including 3-, 4- and 7-hydroxycoumarins (3-HC, 4-HC and 7-HC) 6,7-dihydroxycoumarin (6,7-DiHC), o-coumaric acid (o-CA), o-hydroxyphenyl-acetaldehyde (o-HPA), o-hydroxyphenylethanol (o-HPE), o-hydroxyphenylacetic acid (o-HPAA) and o-hydroxyphenylpropionic acid (o-HPPA) and to product(s) that bind covalently to microsomal proteins. 3. For all 12 subjects, mean rates of [3-14C] coumarin metabolism to total polar products (metabolism to all products except product(s) covalently bound to microsomal proteins), 7-HC, the 3-hydroxylation pathway (sum of 3-HC, o-HPA, o-HPE and o-HPAA), o-HPPA, 6,7-DiHC and covalent binding were 1420, 1230, 73.8, 52.5, 9.5 and 4.8 pmol/min/mg protein respectively. 4. Marked interindividual differences in [3-14C] coumarin metabolism to total polar products (30-fold variation) and 7-HC (2250-fold variation) were observed. 5. Good correlations were observed between [3-14C] coumarin metabolism and total polar products,

7-HC, o-HPPA and 6,7-DiHC, but not to 3-hydroxylation pathway products and levels of 2A6 and 2B6 in human liver microsomes. 6. [3-14C] coumarin metabolism to any polar products did not correlate with levels of 1A2, 2C8, 2C9, 2E1, 3A3/4 and 4A1 in human liver microsomes.

Steensma A., Beaman D.G., Walters D.G. *et al.* (1994) "Metabolism of coumarin and 7-ethoxycoumarin by rat, mouse, guinea pig, Cynomolgus monkey & human precision-cut liver slices" *Xenobiotica* **24**, 893-907. [Abstract](#). 1. The metabolism of 50 microM 7-ethoxycoumarin and 50 microM [3-14C]coumarin has been studied in precision-cut liver slices from the male Sprague-Dawley rat, female DBA/2 mouse, male Dunkin-Hartley guinea pig, male Cynomolgus monkey and man. 2. In liver slices from all five species 7-ethoxycoumarin was metabolized to 7-hydroxycoumarin (7-HC), which was extensively conjugated with D-glucuronic acid and sulphate. In rat and mouse, 7-HC was preferentially conjugated with sulphate, whereas rates of glucuronidation and sulphation were similar in the other three species. 3. [3-14C]coumarin was metabolized by liver slices from all five species to various polar products and to metabolite(s) that bound covalently to liver slice proteins. In Cynomolgus monkey and both human subjects studied, 7-HC was the major metabolite that was conjugated with D-glucuronic acid and sulphate, whereas in rat the major metabolites were products of the 3-hydroxylation pathway and unknown metabolites. Major metabolites in mouse liver slices were 7-HC, 3-hydroxylation pathway products and unknown metabolites, and in guinea pig liver slices, 7-HC and unknown metabolites. 4. The metabolism of 7-ethoxycoumarin to free and conjugated 7-HC and [3-14C]coumarin to total polar products was greater in liver slices from mouse and Cynomolgus monkey than the other three species. 5. With liver slices from all five species there appeared to be little difference in the extent of metabolism of 7-ethoxycoumarin and [3-14C]coumarin to various products in either a complex tissue culture medium (RPMI 1640 plus foetal calf serum) or a simple balanced salt solution (Earle's balanced salt solution). 6. These results demonstrate that precision-cut liver slices are a valuable in vitro model system for investigating species differences in xenobiotic metabolism. Generally, the observed species differences in coumarin metabolism in vitro agree well with available in vivo data.

Waller A.R. & Chasseaud L.F. (1981) "The metabolic fate of [¹⁴C] coumarin in baboons." *Food & Chemical Toxicology* **19**, 1-6.

Wood A.W. & Taylor B.A. (1979) Genetic regulation of coumarin hydroxylase activity in mice. *J Biol Chem* **254**:5647–5651.

Coumarin: Percutaneous Absorption

Bekley_Kartey S.A.J. & Hotchkiss S.A.M. (1995) "The percutaneous absorption of coumarin through fresh human & rat skin in vitro." In: Brain K.R., Hadgraft J., James V.J., Walters K.A. (eds) *Prediction of percutaneous penetration: method, measurements & modelling* Vol 4b, STS, Cardiff pp299-302.

Hotcheiss S.A.M. (1999) "Absorbtion of Fragrance Ingredients Using in Vitro Models with human skin" in *Fragrances: Beneficial & Adverse Effects* ed. PJ Frosch, J.D. Johansen & I.R. White Springer-Verlag 1999 p132 [drawing on: Bekley_Kartey S.A.J., Hotchkiss S.A.M. (1995) above]. Quote: Hotcheiss (1999) "Coumarin: No skin metabolism in rat, mouse & humans. Coumarin Amount absorbed (%) in 72h: Rat 57% unoccluded 64% occluded. In humans 46% unoccluded.

Huntingdon Life Sciences (1996a) "14C-Coumarin. Dermal absorption in man. Project Number RIF 30/943257." Report to RIFM.

Huntingdon Life Sciences (1996b) "14C-Coumarin. Studies on the dermal absorption in the rat." Project Number RIF 28/943265. Report to RIFM.

Yourick J.J., Bronaugh R.L. "Percutaneous Absorption and Metabolism of Coumarin in Human and Rat Skin" *Journal of Applied Toxicology* **17**(3), pp153-158. Abstract. Coumarin is widely used in cosmetics, perfumes & soaps. The Food & Drug Administration banned coumarin use in food because of reports that coumarin produced hepatotoxicity in rodents. Concerns about coumarin's safety have also been raised by the National Toxicity Program. Therefore we initiated studies to measure the extent of coumarin absorption & metabolism in the skin. [14C] Coumarin (ca. 0.5 μ Ci per cell) absorption in skin was measured by using two vehicles: ethanol (15 μ l cm⁻²) and an oil-in-water emulsion (3mg cm⁻²). Absorption was determined for 24 h. by using flow-through diffusion cells (0.64cm², exposed skin). With a receptor fluid consisting of HEPES-buffered Hank's balanced salt solution (pH 7.4). Coumarin metabolism was determined by high-performance liquid chromatography. In rat skin (n=3) the percentages of applied dose absorbed after 24 h were 54.9 \pm 0.63 (mean \pm SEM) and 86.8 \pm 5.4 for the ethanol & emulsion vehicles, respectively, with ca. 5% remaining in skin. In human (n=2) the percentage of applied dose absorbed after 24 h. were 64.4 \pm 0.29 and 98.0 \pm 5.3 for the ethanol & emulsion vehicles, respectively, with ca. 1% remaining in the skin. The extent of skin absorption was greater from the emulsion vehicle in both human & rat skin. Coumarin rapidly penetrated both rat & human skin with >75% and 95%, respectively, of the absorbed dose found in the receptor fluid within 6h. No evidence of coumarin metabolism was found in either skin or receptor fluid fractions. These studies indicate that coumarin absorption is significant in the skin. Systematic coumarin absorption must be expected after dermal contact with coumarin-containing products.

Zhuo X., Gu J, Zhang Q-Y, David C. Spink D.C., Kaminsky L.S. & Ding X. (1999) "Biotransformation of coumarin by rodent and human cytochromes P-450: metabolic basis of tissue-selective toxicity in olfactory mucosa of rats and mice" *Pharmacology & Exptl Therapeutics* **288**(2), 463-471. Abstract. Coumarin was previously found to cause tissue-selective toxicity in the olfactory mucosa (OM) of rats and mice, with rats being the more sensitive species. The aim of this study was to explore the role of target tissue biotransformation in OM-selective toxicity and the metabolic basis of the species differences in coumarin toxicity. At least

six coumarin metabolites were detected in OM microsomal reactions, with o-hydroxyphenylacetaldehyde (o-HPA) being the most abundant. Formation of o-HPA was inhibited by reduced glutathione, confirming its origin from a reactive intermediate. There were significant differences in the rates and metabolite profiles of coumarin metabolism in the livers of Wistar rats and C57BL/6 mice. The rates of metabolic activation of coumarin, as indicated by the formation of o-HPA, were comparable in OM microsomes of the two species but about 25- and 3-fold higher in OM than in liver microsomes of rats and mice, respectively. Thus, target tissue activation seems to play an important role in the tissue-selective toxicity, whereas differences in the rates of hepatic metabolism may be responsible for the species difference in olfactory toxicity. Purified, heterologously expressed mouse CYP2A5 and CYP2G1 produced 7-hydroxycoumarin and o-HPA as the predominant products, respectively. Kinetic analysis and immunoinhibition studies indicated that the OM-specific CYP2G1 plays the major role in metabolic activation of coumarin. Furthermore, of 13 human cytochrome P-450s (P-450s) examined, five (CYP1A1, CYP1A2, CYP2B6, CYP2E1, and CYP3A4) were active in the metabolic activation of coumarin, suggesting a potential risk of coumarin toxicity in humans.

Coumarin in Tobacco

Givel M. (2003) "A comparison of US and Norwegian regulation of coumarin in tobacco products." *Tobacco Control* **12**, 401-405 Abstract. Objective: This paper examines policy processes regarding why the USA and Norway have not regulated coumarin in tobacco. Abstract. Design: A qualitative analysis of all tobacco industry documents regarding coumarin since the 1950s from the 1998 US Master Settlement Agreement and subsequent legal settlements. Additional data were collected from newspaper reports, general internet search engines, journal articles, scholarly reports, court cases, statutes, regulations, and informal correspondence with tobacco control experts in Norway. Main outcome measure: An overview, summary, and analysis of all documents related to coumarin. Results: In the USA from 1954 until 1985 when coumarin was reportedly removed from domestic cigarettes, but not from pipe tobacco until 1996, and not at all from imported Indian bidi cigarettes, regulatory efforts were stymied. In Norway, from 1973 to the present, the tobacco industry has never disclosed whether its tobacco products contain coumarin. In both the USA and Norway, the extreme delay and lack of vigorous evidence gathering and significant remedies were caused by tobacco industry assertions that revealing tobacco additives was a violation of trade secrets, and by weak regulatory authority and efforts to regulate coumarin. Conclusion: Vigorous and expeditious regulatory investigations and remedies for harmful additives in tobacco, such as coumarin, can protect the public health. Astute insider and outsider political advocacy by health advocates is required to hold elected officials and civil servants publicly accountable for failing to enact disclosure laws and to engage in effective regulatory efforts.

Polzin G.M., Stanfill S.B., Brown C.R., Ashley D.L. & Watson C.H. (2007) "Determination of eugenol, anethole, and coumarin in the mainstream cigarette

smoke of Indonesian clove cigarettes." *Food Chem Toxicol.* **45**(10), 1948-53. Abstract. Indonesian clove cigarettes (kreteks), typically have the appearance of a conventional domestic cigarette. The unique aspects of kreteks are that in addition to tobacco they contain dried clove buds (15-40%, by wt.), and are flavored with a proprietary "sauce". Whereas the clove buds contribute to generating high levels of eugenol in the smoke, the "sauce" may also contribute other potentially harmful constituents in addition to those associated with tobacco use. We measured levels of eugenol, *trans*-anethole (anethole), and coumarin in smoke from 33 brands of clove-flavored cigarettes (filtered and unfiltered) from five kretek manufacturers. In order to provide information for evaluating the delivery of these compounds under standard smoking conditions, a quantification method was developed for their measurement in mainstream cigarette smoke. The method allowed collection of mainstream cigarette smoke particulate matter on a Cambridge filter pad, extraction with methanol, sampling by automated headspace solid-phase microextraction, and subsequent analysis using gas chromatography/mass spectrometry. The presence of these compounds was confirmed in the smoke of kreteks using mass spectral library matching, high-resolution mass spectrometry (± 0.0002 amu), and agreement with a relative retention time index, and native standards. We found that when kreteks were smoked according to standardized machine smoke parameters as specified by the International Standards Organization, all 33 clove brands contained levels of eugenol ranging from 2,490 to 37,900 microg/cigarette (microg/cig). Anethole was detected in smoke from 13 brands at levels of 22.8-1,030 microg/cig, and coumarin was detected in 19 brands at levels ranging from 9.2 to 215 microg/cig. These detected levels are significantly higher than the levels found in commercial cigarette brands available in the United States.

Coumarin Analysis.

Egan D. A. & O'Kennedy R. (1992) "Rapid and sensitive determination of coumarin and 7-hydroxycoumarin and its glucuronide conjugate in urine and plasma by high-performance liquid chromatography." *Journal of Chromatography* **582**, 137-143.

Egan D., O'Kennedy R., Moran E., Cox D., Prosser E. and Thornes R. D. (1990) "The pharmacology, metabolism, analysis, and applications of coumarin and coumarin-related compounds." *Drug Metabolism Reviews* **22**, 503-529.

Rychlik M. (2008) "Quantification of Free Coumarin and Its Liberation from glucosylated precursors by stable isotope dilution assays based on liquid chromatography-tandem mass spectrometric detection." *J Agric Food Chem.* 2008 Jan 16 Abstract. A stable isotope dilution assay for the quantification of free coumarin and glucosylated coumarin precursors has been developed using [$(^{13}\text{C})_2$]-coumarin as the internal standard. The doubly labeled coumarin was synthesized by reacting [$(^{13}\text{C})_2$]-acetic anhydride with salicylic aldehyde and characterized by means of mass spectrometry and nuclear magnetic resonance (NMR) experiments. The specificity of liquid chromatography-tandem mass spectrometry enabled unequivocal determination and sensitive quantitation of the

odorant. Because of the very simple extraction procedure, free coumarin could be analyzed within 1h. For quantification of total coumarin, the odorant was liberated from its precursors by an incubation with hydrochloric acid or beta-glucosidase. In analyses of breakfast cereals, the intra-assay coefficient of variation was 9.9% (n = 5) for total coumarin. When coumarin was added to butter cookies at a level of 10 microg/kg, a recovery of 94.1% was found. Further addition studies revealed a detection limit of 2.9 microg/kg and a quantification limit of 8.6 microg/kg. Application of the stable isotope dilution assay to several plants, foods, and essential oils revealed high contents in cassia products and those foods in which cassia has been used as an ingredient. In contrast to this, Ceylon cinnamon contained much less coumarin. The odorant was also quantified in woodruff, clover seeds, and the essential oils of lavender, citron, and chamomile. Only trace amounts were detected in carrots and the essential oils of peppermint and dill, whereas in bilberries, black raspberries, and Angelica roots, coumarin was below detectable levels. In Ceylon cinnamon and cassia, the odorant occurred mainly in its free form, whereas in fenugreek seeds and woodruff, 68 and 88% of the total coumarin content was liberated from glucosylated precursors, respectively.

Walkers D.G., Lake B.G. & Cottrell R.C. (1980) "High performance liquid chromatography of coumarin and its metabolites." *Journal of Chromatography* **196**, 501-505.

[Early Exposure to Coumarins]

Wesseling J., Van Driel D., Heymans H.S., Van der Veer E., Sauer P.J., Touwen BC, Smrkovsky M. (2000) "Behavioural outcome of school-age children after prenatal exposure to coumarins." *Early Hum Dev.* **58**(3), 213-24.

Wesseling J., Van Driel D., Smrkovsky M., Van der Veer E., Geven-Boere LM, Sauer PJ, Touwen BC (2001). "Neurological outcome in school-age children after in utero exposure to coumarins." *Early Hum Dev.* **63**(2), 83-95.