OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT

# Proposition 65

## Evidence on the Carcinogenicity of Coumarin

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Reproductive and Cancer Hazard Assessment Branch
Office of Environmental Health Hazard Assessment
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The Office of Environmental Health Hazard Assessment's (OEHHA) Reproductive and Cancer Hazard Assessment Branch was responsible for the preparation of this document.

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## PREFACE

Proposition 65[1] requires the publication of a list of chemicals "known to the state" to cause cancer or reproductive toxicity. The Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency maintains this list in its role as lead agency for implementing Proposition 65. The Carcinogen Identification Committee (CIC) advises and assists OEHHA in compiling the list of chemicals that cause cancer as required by Health and Safety Code section 25249.8. The Committee serves as the state's qualified experts for determining whether a chemical has been clearly shown to cause cancer.

In 2011, OEHHA brought coumarin to the CIC for prioritization and ranking for future listing consideration. OEHHA subsequently selected coumarin for consideration for listing by the CIC. Upon selection, the public was given the opportunity to submit information relevant to the assessment of the evidence on their carcinogenicity. OEHHA reviewed and considered those submissions in preparing this document.

On November 2, 2017, the CIC is scheduled to deliberate on the carcinogenicity of coumarin. OEHHA developed this document with information on the evidence of carcinogenicity of coumarin to assist the CIC in its deliberations. The original papers discussed in the document will also be provided to the CIC as part of the hazard identification materials. Comments on this hazard identification document received during the public comment period also form part of the hazard identification materials, and are provided to the CIC members prior to their formal deliberations.

[1] The Safe Drinking Water and Toxic Enforcement Act of 1986 (California Health and Safety Code 25249.5 *et seq*.)

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## Acronyms and abbreviations

| | |
|---|---|
| AhR | Aryl hydrocarbon receptor |
| ALDH | Aldehyde dehydrogenase |
| ALP | Alkaline phosphatase |
| ALT | Alkaline aminotransferase |
| AST | Aspartate aminotransferase |
| AUC | Area under the curve |
| BP | Benzo[*a*]pyrene |
| CA | Chromosomal aberrations |
| *o*-CA | *Ortho*-coumaric acid |
| CE | Coumarin 3,4-epoxide |
| CE-SG | Coumarin glutathione conjugate |
| CHO | Chinese hamster ovary |
| $C_{max}$ | Maximum serum concentration |
| CTD | Comparative Toxicogenomics Database |
| CYP450 | Cytochrome P450 |
| DAVID | Database for Annotation, Visualization and Integrated Discovery |
| 3,4-DHC | 3,4-dihydrocoumarin |
| 6,7-DiHC | 6,7-dihydroxycoumarin |
| DMBA | Dimethylbenz[*a*]anthracene |
| EFSA | The European Food Safety Authority |
| ER | Estrogen receptor |
| GC | Gas chromatography |
| GSH | Reduced glutathione |
| GSSG | Glutathione disulfide |
| GO | Gene ontology |
| GPCR | G-protein-coupled receptor |
| 3-HC | 3-hydroxycoumarin |
| 4-HC | 4-hydroxycoumarin |
| 5-HC | 5-hydroxycoumarin |
| 6-HC | 6-hydroxycoumarin |
| 7-HC | 7-hydroxycoumarin |
| 7-HCG | 7-hydroxycoumarin glucuronide conjugate |
| *o*-HPA | *Ortho*-hydroxyphenylacetaldehyde |
| *o*-HPAA | *Ortho*-hydroxyphenylacetic acid |
| *o*-HPE | *Ortho*-hydroxyphenylethanol |
| *o*-HPLA | *Ortho*-hydroxyphenyllactic acid |

| | |
|---|---|
| *o*-HPPA | *Ortho*-hydroxyphenyl-propionic acid |
| *o*-HPPyA | *Ortho*-hydroxyphenylpyruvic acid |
| HPHCs | Harmful and Potentially Harmful Constituents |
| HPLC | High performance liquid chromatography |
| HTS | High-throughput screening |
| *i.v.* | Intravenous |
| $K_d$ | Dissociation constant |
| LFTs | Liver function tests |
| MAMA | Methylazoxymethanole acetate |
| MN | Micronuclei |
| MNNG | N-methyl-N-nitro-N-nitrosoguanidine |
| MS | Mass Spectrometry |
| NMR | Nuclear Magnetic Resonance Spectroscopy |
| PCA | Principal component analysis |
| PPAR | Peroxisome proliferator–activated receptor |
| ROS | Reactive oxygen species |
| SCE | Sister chromatid exchange |
| S-D | Sprague-Dawley |
| SLRL | Sex linked recessive lethal |
| SNP | Single nucleotide polymorphism |
| TDI | Tolerable Daily Intake |
| TG-GATEs | Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System |
| TLC | Thin-layer chromatography |
| $T_{max}$ | Time to reach $C_{max}$ |
| UDS | Unscheduled DNA synthesis |
| 3'-UTR | 3'-untranslated region |
| UV | Ultraviolet |

# 1. EXECUTIVE SUMMARY

Coumarin is a benzopyrone compound found in many plants, such as tonka beans, green tea leaves, and some fruits, herbs, and spices (*e.g.*, cinnamon) (Born *et al*., 2000; BfR, 2006). Coumarin can be extracted from plants or synthesized for commercial uses (IARC, 2000). Coumarin is used as a flavoring agent in tobacco products, as a fragrance enhancer in a number of personal care products, such as perfume, cosmetics, hair spray, detergents, and soaps, and as an industrial chemical to mask odors in plastic materials and paints (Lake, 1999; Boisde *et al.*, 2000). In the US, coumarin is not approved for use as a direct food additive, or as a drug.

No epidemiology studies were identified that investigated the risk of cancer associated with exposure to coumarin.

Long-term carcinogenicity studies of coumarin have been conducted in rats, mice, and hamsters. Tumors were observed in four studies in rats, four studies in mice, and one study in hamsters. These findings are as follows:

*Kidney tumors*

- In the 103-week continuous exposure groups in the male F344/N rat study by NTP (1993a), the incidences of rare renal tubule adenoma, and adenoma and carcinoma combined were statistically significant in the mid-dose group by pairwise comparison with controls. Two uncommon renal tubule oncocytomas were observed in the low-dose group.
- In the stop-exposure groups in the male F344/N rat study by NTP (1993a), a statistically significant increase in the incidence of rare renal tubule adenomas was observed at 103 weeks in the group exposed for 9-months. Two uncommon renal tubule oncocytomas were observed in the 15-month stop-exposure group at 103 weeks.
- In the female F344/N rat study by NTP (1993a), a significant dose-related trend in the incidence of rare renal tubule adenomas was observed. The incidence in the mid- and high-dose groups exceeded the incidence reported in NTP historical controls.

*Liver tumors*

- In the male Sprague-Dawley rat study by Carlton *et al.* (1996), the incidences of metastasizing cholangiocarcinomas, non-metastasizing cholangiocarcinomas, and hepatocellular adenomas and carcinomas combined were significantly

increased in the highest dose group by pairwise comparison with controls. Significant dose-related trends were observed for each of these tumor types.

- In the female Sprague-Dawley rat study by Carlton *et al.* (1996), the incidences of non-metastasizing cholangiocarcinoma and hepatocellular adenomas and carcinomas combined were significantly increased in the highest dose group by pairwise comparison with controls. Significant dose-related trends were observed for each of these tumor types. One metastasizing cholangiocarcinoma was observed in the highest dose group.
- In the female B6C3F$_1$ mouse study by NTP (1993a), the incidences of hepatocellular adenomas, and adenomas and carcinomas combined were significantly increased in the low- and mid-dose groups by pairwise comparison with controls. These increases exceeded the liver tumor incidence reported for NTP historical controls.
- In the female CD-1 mouse study by Carlton *et al.* (1996), the incidence of hepatocellular adenomas and carcinomas combined was significantly increased in the low-dose group by pairwise comparison with controls.

*Lung tumors*

- In the male B6C3F$_1$ mouse study by NTP (1993a), the incidences of alveolar/bronchiolar adenomas, and adenomas and carcinomas combined were significantly increased in the high-dose group by pairwise comparison with controls, with significant dose-related trends.
- In the male CD-1 mouse study by Carlton *et al.* (1996), the incidence of alveolar/bronchiolar carcinomas was significantly increased in the high-dose group by pairwise comparison with controls, with a significant dose-related trend.
- In the female B6C3F$_1$ mouse study by NTP (1993a), incidences of alveolar/bronchiolar adenomas, carcinomas, and adenomas and carcinomas combined were significantly increased in the high-dose group by pairwise comparison with controls, with significant dose-related trends.

*Forestomach tumors*

- In the male B6C3F$_1$ mouse study by NTP (1993a), the incidence of forestomach squamous cell papillomas and carcinomas combined was significantly increased in the low-dose group by pairwise comparison with controls. The NTP report (NTP, 1993a) concluded that the increase in forestomach papillomas in male mice may have been related to coumarin administration. One rare forestomach carcinoma was observed in the low-dose group, and two were observed in the mid-dose group.
- In the female B6C3F$_1$ mouse study by NTP (1993a), the incidences of forestomach squamous cell papillomas and carcinomas combined in each dose

group were increased, but the increases were not statistically significant by pairwise comparison with controls. The observed increases in forestomach squamous cell papillomas were within the range of NTP historical control incidence. The NTP report (NTP, 1993a) concluded that the increase in forestomach papillomas in female mice may have been related to coumarin administration. One rare forestomach squamous cell carcinoma was observed in each of the low- and mid-dose groups.

*Pancreatic tumors*

- In the female hamster study by Ueno and Hirono (1981), two uncommon pancreatic islet cell carcinomas were observed in the high-dose group, with none in the control or low-dose groups. The utility of this study for assessing the carcinogenicity of coumarin is limited by the small numbers of animals per group and poor survival in the control and treated groups.

Coumarin is metabolized to form a number of metabolic products, some of which have not yet been identified, through a number of different enzymatic pathways. Human and animal metabolism of coumarin is qualitatively similar. The two predominant pathways are 7-hydroxylation and 3,4-epoxidation. The 3,4-epoxidation pathway generates the reactive electrophilic metabolites coumarin 3,4-epoxide (CE) and *ortho*-hydroxyphenyl-acetaldehyde (*o*-HPA), which can bind covalently to cellular macromolecules.

The primary enzyme responsible for 7-hydroxylation of coumarin in humans is the highly polymorphic enzyme CYP2A6. Populations around the world carry certain allelic variants of CYP2A6 that are associated with either no enzyme function or reduced function. When coumarin 7-hydroxylation by CYP2A6 is compromised by genetic polymorphism or other non-genetic factors, this can lead to increased generation of CE and *o*-HPA. The kinetics of these reactions and subsequent detoxification reactions, including conjugation of CE with glutathione, and metabolism of *o*-HPA to either *ortho*-hydroxyphenylethanol (*o*-HPE) or *ortho*-hydroxyphenylacetic acid (*o*-HPAA), may determine the ultimate toxic effects of these metabolites.

A number of clinical trials and case reports indicate that coumarin causes hepatotoxicity in susceptible individuals.

Coumarin has tested positive for a number of genotoxicity endpoints.

- Base-pair substitution mutations in two strains of *Salmonella*
- Chromosome aberrations (CAs) in Chinese hamster ovary (CHO) and onion root tip cells

- Micronuclei (MN) formation in human lymphocytes and a human hepatoma cell line
- Sister chromatid exchange (SCE) in CHO and onion root tip cells
- Chromosome instability (*i.e.*, deletions) in *Aspergillus*
- Inhibition of DNA excision repair in *E. coli*
- Binding to single- and double-stranded calf thymus DNA

There is also some evidence for the genotoxicity of two coumarin metabolites, 7-HC and 3,4-dihydrocoumarin (3,4-DHC).

- 7-HC induced expression of the ada DNA repair gene in *E. coli*, was weakly positive in the induction of CA in CHO cells, formed DNA cycloadducts and DNA interstrand crosslinks in synthesized DNA after photoirradiation.
- 3,4-DHC induced SCE in CHO cells.

Coumarin may act via multiple mechanisms, including genotoxicity (as summarized above), formation of electrophilic metabolites, and oxidative stress induction.

- Coumarin is bioactivated by cytochrome P450 enzymes (CYPs) to form electrophilic metabolites, including the epoxide CE and the aldehyde *o*-HPA. The coumarin metabolites CE, *o*-HPA, and 7-HC bind covalently to microsomal proteins in rats and humans.

- Coumarin depletes cellular glutathione through formation of coumarin metabolite-derived glutathione conjugates. A reduction or depletion of the GSH pool may shift the cell's redox balance and impact the cell's overall ability to detoxify additional reactive species (*e.g.*, ROS), leading to oxidative stress.

- Coumarin and its metabolite 6,7-dihydroxycoumarin have been shown to increase cellular and mitochondrial ROS, respectively, in HeLa cells.

- *In vivo* and *in vitro* toxicogenomic studies indicate that coumarin exposure alters the expression of genes involved in the oxidative stress response and glutathione metabolism pathways. Additional analysis of toxicogenomic data by OEHHA identified several common cancer-related biological processes/pathways affected by coumarin in rat liver following *in vivo* exposure and in human primary hepatocytes exposed *in vitro*. These include up-regulated pathways related to nucleic acid binding and protein binding, and down-regulated pathways related to metabolism of xenobiotics by CYPs, oxidoreductase activity, and mitochondrial function.

# 2. INTRODUCTION

## 2.1 Identity of Coumarin

Coumarin is a crystalline solid that is freely soluble in ethanol, chloroform, and oils, and is slightly soluble in water (Lake, 1999). It has a pleasant sweet odor resembling that of vanilla beans or fresh cut grass. Coumarin is a lactone; and more specifically, it is a benzopyrone. Figure 1 shows the chemical structure of coumarin and the numbering of the individual carbon positions.

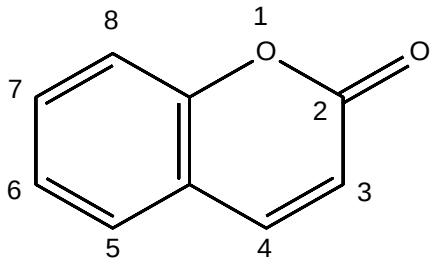



**Figure 1. Chemical structure of coumarin**

Some chemical and physical properties of coumarin are listed in Table 1.

**Table 1. Some chemical and physical properties of coumarin (IARC, 2000)**

| | |
|---|---|
| IUPAC Systematic Name | Coumarin |
| CAS Registry Number | 91-64-5 |
| Molecular Formula | $C_9H_6O_2$ |
| Molecular Weight | 146.15 g/mol |
| Melting Point | 71°C |
| Boiling Point | 301.7°C |
| Density | 0.935 g/cm$^3$ at 20°C |
| Water Solubility | 100 mg/L at 25°C |
| Vapor Pressure | 0.13 kPa at 106°C |
| Log P (Octanol-water) | 1.3 |
| Henry's Law Constant | 6.95 ×10$^{-6}$ (atm-m$^3$/mol at 25°C) |
| Synonyms | 1,2-benzopyrone; 5,6-benzo-2-pyrone; benzo-α-pyrone; *cis*-*o*-coumarinic acid lactone; coumarinic anhydride |

## 2.2 Occurrence, Use, and Exposure

Coumarin is a naturally occurring compound found in many plants, such as tonka beans, green tea leaves, some fruits (*e.g.*, strawberries, apricots), and some spices and herbs (*e.g.*, cinnamon, lavender, sweet woodruff, sage, dill, chamomile, peppermint) (Born *et al.*, 2000; BfR, 2006). Coumarin can be extracted from plants (*e.g.*, it was first isolated from tonka beans in 1822) or synthesized from *ortho*-cresol, phenol, and salicylaldehyde for commercial uses (IARC, 2000).

In the US, coumarin is used as a flavoring agent in tobacco products and as a fragrance enhancer in a variety of personal care products (*e.g.*, perfume, cosmetics, hair spray, detergents, soaps). In addition, coumarin is used as an industrial chemical to mask odors in plastic materials and paints (Lake, 1999; Boisde *et al.*, 2000). Coumarin has been identified in US cigarette tobacco and Indian bidi cigarette tobacco (Stanfill *et al.*, 2003), and is currently included on the US Food and Drug Administration (FDA) list of Harmful and Potentially Harmful Constituents (HPHCs) in tobacco products and tobacco smoke (FDA, 2012a). Due to its reported severe hepatotoxicity in animal studies, the FDA banned the use of coumarin as a direct food additive (in pure form, and as a constituent of tonka beans or tonka extracts) in 1954 (FDA, 2012b).

Coumarin is not approved for use as a drug in the US, although in the 1990s it was the subject of clinical trials as a potential cancer treatment (Marshall *et al.*, 1994). The term “coumarins” is often used in pharmaceutical research to refer to a group of coumarin derivatives that contain the basic coumarin/benzopyrone structure (*e.g.*, warfarin or coumarin sulfamate), but the term does not include coumarin itself (Musa *et al.*, 2008).

In the US, exposure to coumarin may result from consumption of coumarin-containing foods or tobacco products, use of personal care products (such as lotions or hair spray), and occupational exposures.

Two major types of cinnamon used in food are Ceylon and Cassia cinnamon. Wang *et al.* (2013) measured coumarin in cinnamon-flavored foods (*e.g.*, rolls, cereal) and cinnamon-based dietary supplements sold in the US, and reported coumarin levels in cinnamon. Ceylon cinnamon (“true” cinnamon) contains relatively low levels of coumarin (*i.e.*, 5 - 90 parts per million (ppm)) (Wang *et al.*, 2013). In contrast, Cassia cinnamon contains much higher levels of coumarin (*i.e.*, 85 - 9,300 ppm). Coumarin was detected in all 19 samples of cinnamon-flavored foods at concentrations ranging from 3 - 56 ppm. Coumarin levels measured in cinnamon-based dietary supplements ranged from 2,450 - 3,610 ppm.

Another potential dietary exposure to coumarin is through consumption of adulterated foods, such as fake vanilla extracts made from tonka beans. The FDA has issued warnings for coumarin found in some artificial vanilla extracts or flavorings purchased or imported from Mexico (FDA, 2009).

# 3. DATA ON CARCINOGENICITY

## 3.1 Carcinogenicity Studies in Humans

No cancer epidemiological studies on the effects of human exposure to coumarin were identified in a recent literature search conducted by OEHHA (Appendix A).

## 3.2 Carcinogenicity Studies in Animals

A review of the carcinogenicity studies of coumarin in experimental animals identified two gavage studies in Fischer 344/N (F344/N) rats, five dietary studies in rats (three in Sprague-Dawley (S-D), one in Osborne-Mendel, and one in an unspecified strain), two gavage studies in $B6C3F_1$ mice, two dietary studies in CD-1 mice, and two dietary studies in Syrian golden hamsters. A number of these studies were limited by small numbers of animals per group and inadequate reporting. Table 2 lists these studies by species, strain, and route of administration, and each is presented briefly below. Statistically significant or biologically important tumor findings are summarized in the text.

**Table 2. Overview of coumarin animal carcinogenicity studies**

| Study No. | Species | Strain | Sex (M, F) | Animals/ group | Route | Average Daily Dose (mg/kg-day) | Exposure Duration (weeks) | Reference |
|---|---|---|---|---|---|---|---|---|
| 1 | Rat | F344/N | M | 50-58 | gavage | 0, 18, 36, 71[1] | 103 | NTP (1993a) |
| 2 | | | F | 50 | | | | |
| 3 | | S-D | M | 65 | feed | 0, 13, 42, 87, 130, 234[2] | 104 | Carlton *et al*. (1996) |
| 4 | | | F | 65 | | 0, 16, 50, 107, 156, 283[2] | 110 | |
| 5 | | S-D | M | 5 | feed | 0, 317[3] | 4 – 78 | Evans *et al.* (1989) |
| 6 | | Albino (strain not specified) | M & F | 20, 25 or 32 | feed | M: 0, 4, 10, 12, 20[4] | 104 | Bär and Griepentrog (1967); Griepentrog (1973) |
| | | | | | | F: 0, 6, 14, 17, 29[4] | | |
| 7 | | Osborne-Mendel | M & F | 5-7 | feed | M: 0, 40, 100, 200[4] | 104 | Hagan *et al.* (1967) |
| | | | | | | F: 0, 50, 125, 250[4] | | |
| 8 | Mouse | $B6C3F_1$ | M | 50-51 | gavage | 0, 36, 71, 142[1] | 103 | NTP (1993a) |
| 9 | | | F | 50-52 | | | | |
| 10 | | CD-1 | M | 52 | feed | 0, 26, 86, 280[2] | 101 | Carlton *et al.* (1996) |
| 11 | | | F | 52 | | 0, 28, 91, 271[2] | 109 | |
| 12 | Hamster | Syrian golden | M | 11-12 | feed | 0, 92, 460[4] | 104 | Ueno and Hirono (1981) |
| 13 | | | F | 10-13 | | 0, 105, 523[4] | | |

[1] Calculated by OEHHA based on gavage dosing of 5 days per week
[2] As reported by study authors
[3] Average calculated by OEHHA using values reported by study authors
[4] Calculated by OEHHA using administered dose reported by study authors and standard body weight and food intake values from Gold and Zeiger (1997)

In addition, a less-than-lifetime study in baboons (Evans *et al.*, 1979) and four co-carcinogenicity studies with either the known carcinogens 7,12-dimethylbenz[*a*]anthracene (DMBA) or benzo[*a*]pyrene (BP), were identified. The co-carcinogenicity studies were in Wistar rats (Feuer *et al.*, 1976), Sprague-Dawley rats, ICR/Ha mice (Wattenberg *et al*., 1979), and Syrian golden hamsters (Baskaran *et al.*, 2012).

### *3.2.1 Studies in rats*

*3.2.1.1 103-week gavage studies in male and female F344/N rats (NTP, 1993a)*

Male F344/N rats (50, 50, 51 and 50 in the control, low-, mid- and high-dose group, respectively) and female F344/N rats (50/group) were administered coumarin (>97% purity) in corn oil by gavage at doses of 0, 25, 50, or 100 mg/kg body weight per day, 5 days per week for up to 103 weeks. Lifetime average daily doses in treated rats were calculated to be 18, 36, and 71 mg/kg-day. Males and females were 44 or 45 days old, respectively, at the start of the studies. An additional ten rats per sex per group were sacrificed at 15 months for interim evaluation, except for the 50 mg/kg group, where 9 additional male rats were sacrificed.

Additionally, a stop-exposure evaluation was conducted in male rats. In the 9-month group, 40 male rats were administered 100 mg/kg body weight per day coumarin in corn oil by gavage 5 days per week for 9 months (39-40 weeks). Twenty rats were necropsied and evaluated at this time, and the remainder received only the corn oil vehicle until the end of the 103-week study. In the 15-month group, 30 male rats were administered 100 mg/kg body weight per day coumarin in corn oil by gavage 5 days per week for 15 months (65 weeks). Ten rats were necropsied and evaluated at this time, and the remainder received only the corn oil vehicle until the end of the 103-week study. Control groups of 20 and 10 male rats were necropsied at 9 months and 15 months, respectively.

**Males**

***Continuous exposure for 103 weeks***

In the continuous exposure groups, survival of treated male rats was significantly lower than that of controls. Survival rates at week 77 were 43/50 (86%) in the control group, 42/50 (84%) in the low-dose group, 36/51 (71%) in the mid-dose group, and 29/50 (58%) in the high-dose group. Survival rates in the mid- and high-dose groups dropped precipitously as the study progressed. At week 89, survival rates were 35/50 (70%) in the control group, 32/50 (64%) in the low-dose group, 14/51 (28%) in the mid-dose group, and 10/50 (20%) in the high-dose group. Decreased survival of treated groups was attributed to treatment-related increases in severity of nephropathy (NTP, 1993a). Mean body weights of the mid- and high-dose groups were lower than those of controls. At 53 weeks, mean body weights in the mid- and high-dose groups were 6% and 14% lower, respectively, than controls, and 17% and 22% lower, respectively, at 89 weeks.

Kidney tumors were observed in male rats (Table 3). The kidneys in this study were initially examined by preparing a single section of each kidney. Since rare renal tubule adenomas were observed in all groups, additional step sections of the kidney were prepared, revealing additional kidney tumors. Renal tubule neoplasms are rare in male F344/N rats receiving corn oil by gavage in NTP historical controls (8/1,019; 0.8%) (NTP, 1993a). Increases in renal tubule adenomas were observed in all treatment groups, and a rare renal tubule carcinoma was seen in one rat in the low-dose group. The increases in the incidence of renal tubule adenoma and renal tubule adenoma and carcinoma combined were statistically significant in the mid-dose group compared to controls, but dose-related trends were not statistically significant at the $p < 0.05$ significance level. Two uncommon renal tubule oncocytomas, which are recognized as neoplasms distinct from renal tubule adenomas and carcinomas (Montgomery and Seely, 1990; Frazier *et al.*, 2012), were observed in two males in the low-dose group.

**Table 3. Kidney tumor incidence[1] in male F344/N rats administered coumarin via gavage 5 days/week for 103 weeks (NTP, 1993a)**

| Tumor type | Gavage Dose[2] (mg/kg) | | | | Trend test p-value[3] |
|---|---|---|---|---|---|
| | 0 | 25 | 50 | 100 | |
| **Renal tubule adenoma[4, 5] (r)**<br>Day of first tumor occurrence: 426 | 1/55 | 6/56 | 8/58* | 5/56 | NS |
| **Renal tubule carcinoma[4] (r)**<br>Day of first tumor occurrence: ≥613 | 0/37 | 1/35 | 0/19 | 0/13 | NS |
| **Combined renal tubule adenoma or carcinoma[4, 5] (r)**<br>Day of first tumor occurrence: 426 | 1/55 | 6/56 | 8/58* | 5/56 | NS |
| **Renal tubule oncocytoma[4]**<br>Day of first tumor occurrence: 565 | 0/43 | 2/42 | 0/30 | 0/24 | NS |

NS = not significant
[1] Number of tumor-bearing animals per number of animals alive at time of first occurrence of tumor.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls
[3] Exact trend test conducted by OEHHA
[4] Kidney lesions from single and step section evaluations combined
[5] Incidence includes animals in the 15-month interim sacrifice group
* $p < 0.05$. (r) denotes a rare tumor. See text for details.

*Non-neoplastic pathology findings*

Relative kidney weights of male rats were significantly increased in the high-dose group compared to controls at the 15-month interim sacrifice. While nephropathy was observed in all groups, even controls, the severity grade of nephropathy increased with dose. All treated groups were statistically significantly different from controls in severity (assessed by the Mann-Whitney U test as reported by NTP, 1993a). NTP (1993a) characterized nephropathy by "glomerulosclerosis, thickening of tubule basement

membrane, degeneration and atrophy of tubule epithelium, dilatation of tubule lumens by pale pink acellular material (hyaline casts), interstitial fibrosis, and chronic inflammation." Degenerative changes were often accompanied by regeneration of tubule epithelium, the extent of which reflected the overall severity of the degenerative changes. Statistically significant increases in incidences of renal tubule hyperplasia were observed in the low- and mid-dose groups, but not the high-dose group, by pairwise comparison with controls. The NTP considered the hyperplasia of the renal tubules observed in this study to be preneoplastic (NTP, 1993a).

Several non-neoplastic findings were observed in the livers of treated male rats. Absolute and relative liver weights were increased in the high-dose group compared to controls at the 15-month interim evaluation. At this evaluation, a statistically significant increase in the incidence of hepatocellular degeneration was observed in treated rats, most often located in the centrilobular region, and characterized by "the presence of multiple small, clear, intracytoplasmic vacuoles" or fewer larger vacuoles typical of fatty change. Minimal to mild necrosis often accompanied hepatocellular degeneration; in a few rats, moderate to marked necrosis was present. Cytologic alterations were characterized by the presence of enlarged hepatocytes in the peripheral regions of liver lobules, with increased cytoplasmic basophilia, enlarged vesicular nuclei, and an increase in the number of cells in mitosis. Statistically significant increases in coagulative necrosis and fibrosis were observed in the livers of male rats in treated groups at the end of the two-year continuous exposure study. Liver fibrosis, characterized by bands of connective tissue, was considered a consequence of necrosis (NTP, 1993a).

Bile duct hyperplasia increased in severity with increasing dose, and was significantly increased in the high-dose group compared to controls (NTP, 1993a). The bile duct hyperplasia observed in this study was characterized by "increased profiles of well-differentiated bile ductules in the portal areas," but "did not exhibit the mucus cell metaplasia or epithelial dysplasia typical of cholangiofibrosis" (NTP, 1993a). Bile duct hyperplasia, a common aging lesion in rats, does not often progress to neoplasia (Eustis *et al.*, 1990; Goodman *et al.*, 1994).

***Stop-exposure groups***

Stop-exposure groups were included in the NTP (1993a) male rat study. Groups of 40 and 30 male rats were administered 100 mg/kg coumarin via gavage 5 days/week for 9 and 15 months, when 20 and 10 rats were sacrificed, respectively. The remaining 20 rats in each group were administered corn oil via gavage until the end of the 103-week study.

Treatment-related effects on survival and body weight in the stop-exposure groups were consistent with those observed with 103-week continuous exposure. Survival was decreased in both the 9- and 15-month stop-exposure groups compared to controls. Nine of 20 males (45%) treated for 9 months and 2/20 males (10%) treated for 15 months survived until the end of the study. Decreased survival was attributed to treatment-related increases in the severity of renal nephropathy. Mean body weight of rats treated for 9 months was 16% less than controls at week 41 and 15% less at week 103, while mean body weight of rats treated for 15 months was 14% less than controls at week 41 and 22% less at week 103.

Tumor findings in the stop-exposure groups were also consistent with findings observed with 103-week continuous exposure (Table 4). A statistically significant increase in the incidence of renal tubule adenomas was observed in the 9-month stop-exposure group by pairwise comparison with controls at the end of the 103-week study. One renal tubule adenoma was observed at the 15-month interim evaluation, and two renal tubule adenomas were observed in the 15-month stop-exposure group at the end of the 103-week study. Two rats in the 15-month stop-exposure group also had uncommon renal tubule oncocytomas at the end of the 103-week study. Besides the single rare renal tubule adenoma observed at the 15-month interim sacrifice, no additional treatment-related tumors were observed at the 9- and 15-month interim evaluations.

**Table 4. Kidney tumor incidence[1] in male F344/N rats in 9- and 15-month stop exposure groups[2] [incidence from control and 103-week continuous exposure groups provided for comparison] (NTP, 1993a)**

| Tumor type | Gavage Dose[3] (mg/kg) | | | |
|---|---|---|---|---|
| | Stop Exposure (exposure period) | | | Continuous Exposure (exposure period) |
| | 0 | 100 (9-month exposure) | 100 (15-month exposure) | 100 (103-week exposure) |
| **Renal tubule adenoma[4, 5] (r)** | 1/55 (1.8%) | 4/18* (22%) | 2/36 (5.6%) | 5/56 (8.9%) |
| **Renal tubule oncocytoma[4]** | 0/43 (0%) | 0/17 (0%) | 2/14 (14%) | 0/24 (0%) |

[1] Number of tumor-bearing animals per number of animals alive at time of first occurrence of tumor.
[2] Administered 100 mg/kg/day coumarin via gavage 5 days/week for 9 or 15 months followed by corn oil for the remainder of the 2 years.
[3] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls.
[4] Kidney lesions from single and step section evaluations combined.
[5] Incidence includes animals in the 15-month interim sacrifice group.
* $p < 0.05$. (r) denotes a rare tumor. See text for details.

*Non-neoplastic pathology findings*

NTP considered the treatment-related liver and kidney lesions observed in the stop-exposure groups at the 9- and 15-month interim evaluations to be similar to those observed at the end of the 103-week continuous exposure study. The incidences and/or severity of the hepatic lesions observed in the stop-exposure groups returned to levels similar to controls following termination of coumarin exposure, indicating that the hepatocellular and biliary lesions were reversible. In contrast, the kidney lesions were largely irreversible in the stop-exposure groups and increased in severity with age. However, the severity of the nephropathy observed in the stop-exposure groups was less than that observed in the 103-week continuous exposure high dose group.

**Females (continuous exposure for 103 weeks)**

Mortality in dosed female rats was similar to that of controls. Mean body weights of the high-dose group were slightly lower than those of controls, but were not statistically significantly different.

Kidney tumors were observed only in treated female rats in this study (Table 5). The kidneys were examined by single and step sectioning of each kidney. No kidney tumors

were observed in the 15-month interim sacrifice groups. Renal tubule neoplasms are rare in female F344/N rats receiving corn oil by gavage in NTP historical controls (2/1,018; 0.2%) (NTP, 1993a). The incidence of renal tubule adenoma in the mid-dose (1/38, 2.6%) and high-dose (2/32, 6.25%) groups was greater than the incidence of these rare tumors in the NTP historical controls, and the increase with dose was statistically significant by the Exact trend test.

**Table 5. Kidney tumor incidence[1] in female F344/N rats administered coumarin via gavage 5 days/week for 103 weeks (NTP, 1993a)**

| Tumor type | Gavage Dose (mg/kg) | | | | Trend test p-value[2] |
|---|---|---|---|---|---|
| | 0 | 25 | 50 | 100 | |
| **Renal tubule adenoma[3] (r)** | 0/35 | 0/39 | 1/38 | 2/32 | 0.0489 |

[1] Number of tumor-bearing animals per number of animals alive at time of first occurrence of tumor (day 699).
[2] Exact trend test conducted by OEHHA.
[3] Kidney lesions from single and step section evaluations combined.
(r) denotes a rare tumor. See text for details.

*Non-neoplastic pathology findings*
Relative kidney weights of female rats were significantly increased in the high-dose group compared to controls at the 15-month interim evaluation (organ weights not reported for other time points). Statistically significant increases in the incidence of nephropathy was observed in all treatment groups by pairwise comparison with controls, in both the 15-month interim evaluation groups and the groups on test for 103-weeks. Additionally, severity grade of nephropathy increased with dose, and all treated groups were statistically significantly different from controls by the Mann-Whitney U test (as reported by NTP, 1993a). There was an increase in renal tubule hyperplasia in treated rats compared to controls, although it did not reach statistical significance.

In female rats, absolute and relative liver weights were increased in the high-dose group compared to controls at the 15-month interim evaluation. A dose-related increase in severity of hepatocellular degeneration was observed in treated rats in both the 15-month interim evaluation groups and the groups on test for 103-weeks. Incidences of coagulative necrosis, fibrosis, and cytologic alterations of the liver were statistically significantly increased in the high-dose group at 103 weeks compared to controls.

*3.2.1.2 Two-year feeding studies in male and female S-D rats (animals in the three lowest dose groups were also exposed in utero and via lactation) (Carlton et al., 1996)*

Male and female S-D rats (50/sex/group) were administered coumarin (>98% purity) in the diet at doses of 0, 333, 1000, 2000, 3000, or 5000 ppm. Rats receiving 333, 1000, and 2000 ppm coumarin were exposed *in utero*, during lactation, and following weaning

until sacrifice. Rats receiving 3000 and 5000 ppm were exposed after weaning, beginning at 21-28 days of age, until sacrifice. Male and female rats were sacrificed after 104 and 110 weeks of post-weaning exposure, respectively. Additional "satellite groups" of animals (15/group) were included in each study, and sacrificed at week 104. These satellite groups were evaluated for a subset of the parameters examined in the main studies (hematology, clinical chemistry, urinalysis, gross necropsy, organ weight determinations, and microscopic examination only of gross lesions). Achieved intakes of coumarin were reported by the study authors to be 13, 42, 87, 130, and 234 mg/kg-day in male rats and 16, 50, 107, 156, and 283 mg/kg-day in female rats. The lower doses (expressed as average daily dose) in these feeding studies are fairly comparable to the average daily doses in the NTP (1993a) gavage studies in Fischer rats, which were 18, 36, and 71 mg/kg-day.

**Males**

Survival at 104 weeks was below 50% in the controls and the groups dosed *in utero* and during lactation, and was significantly decreased in male rats in the 333 ppm group compared to controls. Survival was greater than controls in the two highest dose groups, in which dosing began post-weaning. Food consumption was significantly lower in the three highest dose groups compared to controls throughout the entire study, and dose-related reductions in body weight gain were observed in these same dose groups. Mean body weight in the high-dose group was approximately 43% lower than controls at 52 weeks and 35% lower at 104 weeks. These reductions in food consumption and body weight may have contributed to the greater survival rates observed in the highest two dose groups.

The study reported treatment-related increases in multiple types of liver tumors, as shown in Table 4. There were significant increases in incidences of metastasizing cholangiocarcinomas, non-metastasizing cholangiocarcinomas, and hepatocellular adenomas and carcinomas (combined; referred to as benign and malignant parenchymal tumors by the study authors) in the highest dose group by pairwise comparison with controls. Significant dose-response trends were observed for each of these tumor types. Satellite groups were examined only for gross lesions, which potentially resulted in under-ascertainment of the number of liver tumors in these animals.

**Table 6. Liver tumor incidence[1] in male S-D rats administered coumarin in feed for 104 weeks (with *in utero* and lactational exposure in the three lowest dose groups) (Carlton *et al.*, 1996)**

| Tumor type | Dose[2] (ppm) [Intake[3]] (mg/kg-day) | | | | | | Trend test p-value[6] |
|---|---|---|---|---|---|---|---|
| | 0 | 333[4] [13] | 1000[4] [42] | 2000[4] [87] | 3000[5] [130] | 5000[5] [234] | |
| **Cholangiocarcinoma (non-metastasizing)[7]** | 0/65 | 0/65 | 0/65 | 0/65 | 1/65 | 31/65*** | <0.001 |
| **Cholangiocarcinoma (metastasizing)[7]** | 0/65 | 0/65 | 0/65 | 0/65 | 0/65 | 6/65* | <0.01 |
| **Hepatocellular adenoma or carcinoma combined** | 2/65 | 2/65 | 1/65 | 1/65 | 6/65 | 29/65*** | <0.001 |

[1] Number of animals with lesion per number of animals with organ examined microscopically as reported by study authors. Animals in satellite groups were examined only for gross lesions, which may have resulted in under-ascertainment of tumors.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls [conducted by OEHHA].
[3] Achieved intake as reported by study authors.
[4] Exposed *in utero*, during lactation, and following weaning until sacrifice.
[5] Exposed after weaning, beginning at 21-28 days of age, until sacrifice.
[6] Exact trend test conducted by OEHHA in control, 3000 ppm, and 5000 ppm groups (groups with exposure *in utero* and during lactation were excluded).
[7] Non-metastasizing and metastasizing cholangiocarcinomas were reported separately by study authors. Individual animal data was not available.
* $p < 0.05$; *** $p < 0.001$.

Carlton *et al.* (1996) considered the liver tumors to be caused by exceedance of the maximum tolerated dose that led to hepatotoxicity, stating that “tumors were not metastatic and survival was significantly increased among rats in the two highest dose groups”. However, the increase in metastasizing cholangiocarcinomas in the high dose group was statistically significant ($p < 0.05$). Additionally, survival was significantly better compared to controls in the two highest dose groups. Body weight gain was decreased in the three highest dose groups in this study, this, however, is not by itself an indication of an excessive high dose. It is possible that a reduction in food consumption and consequent reduced body weight gain may have contributed to the greater survival rates observed in the highest two dose groups. Feed restriction studies have shown that reduced body weight is associated with increased survival and reductions in spontaneous liver tumor incidence (NTP, 1997). Yet in this study, increased incidences of treatment-related liver tumors were observed.

The US EPA Guidelines for Carcinogen Risk Assessment say:

> Animal studies are conducted at high doses in order to provide statistical power, the highest dose being one that is minimally toxic (maximum tolerated dose or MTD). Consequently, the question often arises of whether a carcinogenic effect at the highest dose may be a consequence of cell killing with compensatory cell replication or of general physiological disruption rather than inherent carcinogenicity of the tested agent... If adequate data demonstrate that the effects are solely the result of excessive toxicity rather than carcinogenicity of the tested agent *per se*, then the effects may be regarded as not appropriate to include in assessment of the potential for human carcinogenicity of the agent (US EPA, 2005).

*Non-neoplastic pathology and clinical chemistry findings*
Non-neoplastic liver effects reported in this study were an increase in relative liver weights in the two highest dose groups, an increased incidence of cholangiofibrosis (considered to be a pre-neoplastic lesion) in the highest dose group, and an increase in alkaline phosphatase (doses not specified).

Treatment-related anemia was observed from 6 weeks onward in the three highest dose groups. Treatment-related decreases in glucose and protein were seen in the highest dose group at 4 and 13 weeks, which was not as pronounced later in the study. Cholesterol levels of all treated groups except the highest dose group were elevated throughout the study. Plasma cholinesterase levels and red blood cell cholinesterase were not affected by treatment.

**Females**

Survival at 110 weeks was below 50% in the controls and the groups dosed *in utero* and during lactation. Survival was greater than controls in the two highest dose groups, in which dosing began post-weaning. Food consumption was significantly lower in the three highest dose groups compared to controls throughout the entire study, and dose-related reductions in body weight gain were observed in these same dose groups. Mean body weight in the high-dose group was approximately 44% lower than controls at 52 weeks and 46% lower at 110 weeks. These reductions in food consumption and body weight may have contributed to the greater survival rates observed in the highest two dose groups.

The study reported treatment-related increases in multiple types of liver tumors as shown in Table 7. There were significant increases in incidences of non-metastasizing cholangiocarcinoma and hepatocellular adenoma and carcinoma (combined; referred to

as benign and malignant parenchymal tumors by the study authors) in the highest dose group by pairwise comparison with controls. Significant dose-response trends were observed for each of these tumor types. One metastasizing cholangiocarcinoma was observed in the highest dose group. Satellite groups were examined only for gross lesions, which potentially resulted in under-ascertainment of the number of liver tumors in these animals.

**Table 7. Liver tumor incidence[1] in female S-D rats administered coumarin in feed for 104 or 110 weeks (with *in utero* and lactational exposure in the three lowest dose groups) (Carlton *et al.*, 1996)**

| Tumor type | Dose[2] (ppm) [Intake[3]] (mg/kg-day) | | | | | | Trend test p-value[6] |
|---|---|---|---|---|---|---|---|
| | 0 | 333[4] [16] | 1000[4] [50] | 2000[4] [107] | 3000[5] [156] | 5000[5] [283] | |
| **Cholangiocarcinoma (non-metastasizing)[7]** | 0/65 | 0/65 | 0/65 | 0/65 | 0/65 | 22/65*** | <0.001 |
| **Cholangiocarcinoma (metastasizing)[7]** | 0/65 | 0/65 | 0/65 | 0/65 | 0/65 | 1/65 | NS |
| **Hepatocellular adenoma or carcinoma** | 0/65 | 0/65 | 0/65 | 0/65 | 1/65 | 12/65*** | <0.001 |

NS = not significant; *** p < 0.001.

[1] Number of animals with lesion per number of animals with organ examined microscopically as reported by study authors. Animals in satellite groups were examined only for gross lesions, which may have resulted in under-ascertainment of tumors.

[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls [conducted by OEHHA].

[3] Achieved intake as reported by study authors.

[4] Exposed *in utero*, during lactation, and following weaning until sacrifice.

[5] Exposed after weaning, beginning at 21-28 days of age, until sacrifice.

[6] Exact trend test conducted by OEHHA in control, 3000 ppm, and 5000 ppm groups (groups with exposure *in utero* and during lactation were excluded).

[7] Non-metastasizing and metastasizing cholangiocarcinomas were reported separately by study authors.

Similar to the male rat study, the study authors imply that the maximum tolerated dose was exceeded in this study in the three highest dose groups, and cite the large body weight decrements in these groups. Increased liver weights were found in the four highest dose groups, increased incidences of cholangiofibrosis were observed in the highest dose group, and increases in blood potassium, alkaline phosphatase, and glutamic-pyruvic transaminanse were noted (doses not specified). However, female rats in the two highest dose groups had increased survival compared to controls. As explained above in the discussion of the male rat study, these observations do not support a conclusion that the liver tumors observed in the higher dose groups were the result of excessive toxicity.

*Non-neoplastic pathology and clinical chemistry findings*
Treatment-related anemia was observed from 6 weeks onward in the three highest dose groups, similar to the findings in the male rat study. White blood cell and lymphocyte counts were minimally elevated in the highest dose group from week 25 through the end of the study. Treatment-related decreases in glucose and protein were seen in the highest dose group at 4 and 13 weeks, which was not as pronounced later in the study. Plasma cholinesterase levels (but not red blood cell cholinesterase) were decreased in treated females.

*3.2.1.3 78-week feeding study in male S-D rats (Evans et al., 1989)*

Male S-D rats were given the control diet or administered coumarin in the diet at a dose of 5000 ppm. Groups of 5 control and 5 treated animals were sacrificed at 4, 12, 14, 18, 22, 26, 30, 52, and 78 weeks. There was no difference in survival between treated and control groups, but food intake and body weights were reduced in treated animals. No treatment-related tumors were reported. Cholangiofibrosis was observed in the livers of the majority of rats treated from 18 to 78 weeks. Evans *et al.* (1989) reported that cholangiofibrotic lesions "were particularly prominent in animals killed at 18 months (78 weeks) and were reminiscent of cholangiocarcinoma in other species although no evidence of local invasion or of metastasis was found" (Evans *et al.*, 1989). The utility of this study for assessing the carcinogenicity of coumarin is limited by a number of factors, including the less-than-lifetime study duration, numerous early interim sacrifices, small numbers of animals per interim sacrifice, administration of coumarin at a single dietary concentration, and inadequate reporting.

Although coumarin did not induce tumors in this study, coumarin did induce cholangiofibrosis, which is a potential precursor to biliary neoplasia in these male S-D rats. As described in Hailey *et al.* (2014), "the spectrum of lesions generally classified as cholangiofibrosis, cholangiofibroma, and cholangiocarcinoma in the rat is a morphological continuum, and identifying specific criteria for separation of the various categories is difficult and controversial with biological information lacking or conflicting".

*3.2.1.4 Two-year feeding study in male and female albino rats (Bär and Griepentrog, 1967; Griepentrog, 1973)*

Coumarin was administered to male and female albino rats (strain not specified) in feed containing 0, 1000, 2500, 5000, or 6000 ppm coumarin for up to two years (20 to 32 rats per group) (Bär and Griepentrog, 1967; Griepentrog, 1973). The lifetime average daily doses were estimated by OEHHA using standard body weight and food intake values to be 4, 10, 12, and 20 and 6, 14, 17, and 29 mg/kg-day in male and female rats,

respectively. Food consumption was reduced in the 6000 ppm group. The study reported the tumor incidences for male and female rats combined. The original study reported that bile duct carcinomas were observed in 12 rats in the 5000 ppm group and 5 rats in the 6000 ppm group. Reporting of histopathology was ambiguous and inconsistent between the two papers, information on other toxic endpoints and body weights is lacking, and there was no information on the purity of the coumarin administered.

Controversy surrounding the actual diagnosis of these lesions persists (Lake, 1999). In a re-evaluation of the slides by external pathologists, the bile duct carcinomas were reclassified as non-neoplastic cholangiofibrosis (Cohen, 1979). However, the distinction between the diagnosis of cholangiofibrosis, cholangiofibroma, or cholangiocarcinoma is not well-defined, and there are not specific criteria for making these determinations (Thoolen *et al.*, 2010; Hailey *et al.*, 2014).

*3.2.1.5 Two-year feeding study in male and female Osborne-Mendel rats (Hagan et al., 1967)*

Coumarin was administered in the diet at 0, 1000, 2500, or 5000 ppm to groups of 5 to 7 male and female Osborne-Mendel rats for two years. Reporting of the results for male and female rats were combined. No information on survival of rats was mentioned in the study. The study notes there was "growth retardation" and that food consumption, which was measured over the first year, was normal. However, the study publication does not provide the data on body weight or food consumption. No treatment-related tumors were reported (Hagan *et al.*, 1967).

Liver damage was observed as focal proliferation of bile ducts with cholangiofibrosis, fatty change, and focal necrosis in the 5000 ppm dose group. The study reported that the 2500 ppm dose group had minimal to slight proliferation of the bile ducts with fatty change and focal necrosis in the hepatic parenchyma, but cholangiofibrosis was not observed in this group. It reported that there was no effect in the 1000 ppm dose group. This study was limited by the small number of animals per group and inadequate reporting.

### *3.2.2 Studies in mice*

*3.2.2.1 103-week gavage studies in male and female B6C3F$_1$ mice (NTP, 1993a)*

Male B6C3F$_1$ mice (50, 50, 50 and 51 in the control, low-, mid- and high-dose group, respectively) and female B6C3F$_1$ mice (52, 50, 51, and 51 in the control, low-, mid- and high-dose group, respectively) were administered coumarin (> 97% purity) in corn oil by

gavage at doses of 0, 50, 100, and 200 mg/kg body weight per day, 5 days per week, for up to 103 weeks. Lifetime average daily doses in treated mice were calculated to be 36, 71, and 142 mg/kg-day. Males and females were 44 or 45 days old, respectively, at the start of the studies. Gross and microscopic examinations were performed on all major organs from animals found dead and on all animals sacrificed at the end of the two-year studies. At 15 months interim evaluation, an additional 20, 20, 20, and 19 male mice and 18, 20, 19, and 19 female mice from the control, low-, mid-, and high-dose groups were sacrificed, respectively. Not all the tissues of interest from animals in the 15-month interim evaluation groups were examined by histopathological analysis.

**Males**

No significant differences in survival were observed between control and treated male $B6C3F_1$ mice (survival rates in the control, 50, 100, and 200 mg/kg dose groups were 86, 94, 84, and 73 percent, respectively). The mean body weights of the high-dose group were slightly, but not statistically significantly, lower than those of controls from week 10 to 81 (3-10% lower), and were similar to controls at the end of the study.

Treatment-related tumor incidences observed in male mice are summarized in Table 8. Statistically significant increases in alveolar/bronchiolar adenomas and alveolar/bronchiolar adenomas and carcinomas combined were observed in the high-dose group compared to controls, with positive dose-response trends. In the 15-month interim evaluation groups, alveolar/bronchiolar adenomas were observed in two out of two low-dose animals examined, and three out of nine high-dose animals examined. Since not all lung tissues were examined histopathologically in the interim evaluation groups, these findings are not included in Table 8. In addition, the incidence of forestomach squamous cell papillomas and carcinomas combined was significantly increased ($p = 0.035$) in the low-dose group. Forestomach squamous cell papillomas occurred more frequently in the low-dose group than in controls, and slightly exceeded the range of NTP historical controls (27/902, 3%, range: 0-14%) (NTP, 1993a). The incidence in the mid-dose group was similar to controls, and none were observed in the high-dose group. One forestomach squamous cell carcinoma was observed in the low-dose group, two in the mid-dose group, and none in the high-dose group or in controls. Forestomach carcinomas are considered rare in untreated male $B6C3F_1$ mice (background incidence of 4/902; 0.4%, range 0-2%) (NTP, 1993a). No forestomach tumors were reported in the 15-month interim evaluation groups.

The NTP report (NTP, 1993a) concluded that the increase in forestomach papillomas in male mice may have been related to coumarin administration, noting that the incidence in the low-dose group was not significantly higher than the controls and there was not a corresponding increase over a fourfold dose range from 50 to 200 mg/kg. Applying a

pairwise comparison and using effective numbers results in a statistically significant increase in the incidence of combined papillomas and carcinomas in the low dose group.

**Table 8. Incidence[1] of treatment-related lesions in male B6C3F$_1$ mice administered coumarin via gavage 5 days/week for 103 weeks (NTP, 1993a)**

| Tumor Site and Type | | Gavage Dose[2] (mg/kg) | | | | Trend test p-value[3] |
|---|---|---|---|---|---|---|
| | | 0 | 50 | 100 | 200 | |
| Lung | **Alveolar/bronchiolar adenoma**<br>Day of first tumor occurrence: 558 | 14/48 | 8/49 | 14/46 | 24/45* | 0.001 |
| | **Alveolar/bronchiolar carcinoma**<br>Day of first tumor occurrence: 716 | 1/45 | 1/47 | 2/43 | 1/37 | NS |
| | **Combined**<br>Day of first tumor occurrence: 558 | 14/48 | 9/49 | 15/46 | 25/45** | <0.001 |
| Fore-stomach | **Squamous cell papilloma**<br>Day of first tumor occurrence: 729 | 2/43 | 8/47 | 2/42 | 0/37 | NS |
| | **Squamous cell carcinoma[4] (r)**<br>Day of first tumor occurrence: 1 | 0/48 | 1/49 | 2/49 | 0/47 | NS |
| | **Combined[4]**<br>Day of first tumor occurrence: 1 | 2/48 | 9/49* | 4/49 | 0/47 | NS |

NS = not significant; * $p < 0.05$; ** $p < 0.01$.
(r) denotes a rare tumor; see text for details.
[1] Number of tumor-bearing animals per number of animals alive at time of first occurrence of tumor.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls.
[3] Exact trend test conducted by OEHHA.
[4] One forestomach squamous cell carcinoma occurred in the 100 mg/kg group on Day 1 of the two-year study.

*Non-neoplastic pathology findings*

Several non-neoplastic findings were observed in the livers of treated male mice. A significant increase in liver syncytial alterations was observed in all dose groups compared to controls. Liver centrilobular hypertrophy was significantly increased in the mid- and high-dose groups compared to controls. In addition, a significant increase in

liver eosinophilic foci was observed at the end of the study in the low- and high-dose groups compared to controls.

**Females**

No significant differences in survival were observed between control and treated female $B6C3F_1$ mice (survival rates in the control, 50, 100, 200 mg/kg dose groups were 66, 80, 82, and 55%, respectively). The mean body weights of the high-dose group were slightly, but not statistically significantly, lower than those of controls from week 11 to 49 (3-18% lower), and were about 12% lower at the end of the study.

Treatment-related tumor incidences observed in female mice are summarized in Table 9. Statistically significant increases in alveolar/bronchiolar adenomas, carcinomas, and adenomas and carcinomas combined were observed in the high-dose group compared to controls, with positive dose-response trends. In the 15-month interim evaluation groups, alveolar/bronchiolar adenomas were observed in one out of one mid-dose animals examined, and two out of nine high-dose animals examined. Since not all lung tissues were examined histopathologically in the interim evaluation groups, these findings are not included in Table 9. There were significant increases in hepatocellular adenomas and adenomas and carcinomas combined in the low- and mid-dose groups (55% and 61%, respectively) compared to the controls (20%).

The incidences of liver tumors in the low- and mid-dose groups exceeded the NTP historical control incidence (14.4%; range 2-34%). According to NTP, the lower incidence of hepatocellular neoplasms in the high-dose group may be related to the reduced body weight of this group. In the 15-month interim evaluation groups, hepatocellular adenomas were observed in one out of eight control animals examined. Since not all liver tissues were examined histopathologically in the interim evaluation groups, these findings are not included in Table 9. There were increased incidences of forestomach squamous cell papillomas and carcinomas in each dose group, but these increases were not statistically significantly different from controls. The observed increases in forestomach squamous cell papillomas were within the range of NTP historical control incidence (27/901, 3%; range 0-10%; NTP, 1993a). One forestomach squamous cell carcinoma was observed in each of the low- and mid-dose groups. Forestomach squamous cell carcinomas are considered rare in female $B6C3F_1$ mice (background incidence 3/901, 0.3%; range 0-4%; NTP, 1993a). No forestomach tumors were reported in the 15-month interim evaluation groups.

The NTP report (NTP, 1993a) concluded that the increase in forestomach papillomas in female mice may have been related to coumarin administration, noting that the

incidences in the low-dose group was not significantly higher than the controls and there was no corresponding increase over a fourfold dose range from 50 to 200 mg/kg.

**Table 9. Incidence[1] of treatment-related lesions in female B6C3F$_1$ mice administered coumarin via gavage 5 days/week for 103 weeks (NTP, 1993a)**

| Tumor Site and Type | | Gavage Dose[2] (mg/kg) | | | | Trend test p-value[3] |
|---|---|---|---|---|---|---|
| | | 0 | 50 | 100 | 200 | |
| Lung | **Alveolar/ bronchiolar adenoma** Day of first tumor occurrence: 673 | 2/38 | 5/43 | 7/44 | 20/35*** | <0.001 |
| | **Alveolar/ bronchiolar carcinoma** Day of first tumor occurrence: 615 | 0/39 | 0/47 | 0/45 | 7/39** | <0.001 |
| | **Combined** Day of first tumor occurrence: 615 | 2/38 | 5/47 | 7/45 | 27/35*** | <0.001 |
| Liver | **Hepatocellular adenoma** Day of first tumor occurrence: 564 | 8/41 | 26/49*** | 29/48*** | 12/40 | NS |
| | **Hepatocellular carcinoma** Day of first tumor occurrence: 655 | 0/38 | 3/44 | 3/46 | 1/36 | NS |
| | **Combined** Day of first tumor occurrence: 564 | 8/41 | 27/49*** | 31/48*** | 13/40 | NS |
| Fore-stomach | **Squamous cell papilloma** Day of first tumor occurrence: 589 | 1/39 | 5/49 | 2/45 | 2/38 | NS |
| | **Squamous cell carcinoma (r)** Day of first tumor occurrence: 694 | 0/35 | 1/42 | 1/43 | 0/31 | NS |
| | **Combined** Day of first tumor occurrence: 589 | 1/39 | 6/49 | 3/45 | 2/38 | NS |

NS = not significant; ** $p < 0.01$; *** $p < 0.001$.
(r) denotes a rare tumor; see text for details.
[1] Number of tumor-bearing animals per number of animals alive at time of first occurrence of tumor.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls.
[3] Exact trend test conducted by OEHHA.

*Non-neoplastic pathology findings*
Similar to the study in male mice, several non-neoplastic findings were observed in the livers of treated female mice. Liver syncytial alterations and centrilobular hypertrophy were significantly increased in high-dose female mice compared to controls. In addition, a significant increase in liver eosinophilic foci was observed at the end of the study in the low- and mid-dose groups compared to controls.

*3.2.2.2 Two-year feeding studies in male and female CD-1 mice (Carlton et al., 1996)*

Male and female CD-1 mice (52/sex/group) were administered coumarin (>98% purity) in the diet at doses of 0, 300, 1000, and 3000 ppm. The achieved intakes of coumarin were reported by the study authors to be 26, 86, and 280 mg/kg-day and 28, 91, and 271 mg/kg-day for males and females, respectively. The two lower doses (expressed as average daily dose) in these feeding studies are fairly comparable to the average daily doses received by the low- and mid-dose groups in the NTP (1993a) gavage studies in $B6C3F_1$ mice, which were 36 and 71 mg/kg-day. The male and female studies were terminated at weeks 101 and 109, respectively. Gross necropsy with organ weight determinations was performed on all animals.

**Males**

Survival of treated male mice was similar to that of controls. Body weight gains in treated males were significantly reduced compared to controls, with an 18% reduction in the 3000 ppm group and a 10% reduction in the 1000 ppm group at week 52. Food intake in the 3000 ppm group was marginally lower than the controls.

Lung tumors were observed in male CD-1 mice (Table 10). The lung tumors observed in Carlton *et al*. (1996) were called pulmonary adenomas and adenocarcinomas, but these tumors are typically referred to as alveolar/bronchiolar adenomas and carcinomas. A statistically significant increase in alveolar/bronchiolar carcinomas was observed in the high-dose group (20/52; 38.5%) compared to controls (11/52; 21.2%), with a positive dose-response trend. The authors reported that the incidence of alveolar/bronchiolar carcinomas was within the laboratory historical control range for CD-1 male mice (range not reported).

**Table 10. Incidence[1] of treatment-related lesions in male CD-1 mice administered coumarin in feed for 101 weeks (Carlton *et al.*, 1996)**

| Tumor Site and Type | | Dose[2] (ppm) [Intake][3] (mg/kg-day) | | | | Trend test p-value[4] |
|---|---|---|---|---|---|---|
| | | 0 | 300 [26] | 1000 [86] | 3000 [280] | |
| Lung | Alveolar/bronchiolar adenoma | 0/52 | 1/52 | 2/52 | 0/52 | NS |
| | Alveolar/bronchiolar carcinoma | 11/52 | 12/52 | 10/52 | 20/52* | 0.014 |

NS = not significant; * $p < 0.05$.
[1] Number of animals with lesion per number of animals with organ examined microscopically as reported by study authors.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls [conducted by OEHHA].
[3] Achieved intake as reported by study authors.
[4] Exact trend test conducted by OEHHA.

*Non-neoplastic pathology and clinical chemistry findings*
No treatment-related changes in hematology or clinical chemistry were observed. There were no effects on liver weight, as reported by the study authors.

**Females**

Survival of treated female CD-1 mice was similar to that of controls, as were body weights. Food consumption was reported to be similar across all dose groups.

Liver tumors were observed in female CD-1 mice (Table 11). A statistically significant increase in benign and malignant parenchymal tumors combined (hepatocellular adenomas and carcinomas) was observed in low-dose female mice compared to controls. The increases in liver tumors observed in the mid- and high-dose groups did not reach statistical significance.

**Table 11. Incidence[1] of treatment-related lesions in female CD-1 mice administered coumarin in feed for 109 weeks (Carlton *et al.*, 1996)**

| Tumor Site and Type | | Dose[2] (ppm) [Intake[3]] (mg/kg-day) | | | | Trend test p-value[4] |
|---|---|---|---|---|---|---|
| | | 0 | 300 [28] | 1000 [91] | 3000 [271] | |
| **Liver** | **Hepatocellular adenoma or carcinoma** | 0/52 | 8/52* | 4/52 | 3/52 | NS |

NS = not significant; * $p < 0.05$.
[1] Number of animals with lesion per number of animals with organ examined microscopically as reported by study authors.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls [conducted by OEHHA].
[3] Achieved intakes as reported by study authors.
[4] Exact trend test conducted by OEHHA.

*Non-neoplastic pathology and clinical chemistry findings*
Absolute/relative liver weights in the high-dose group were significantly increased compared to controls. No treatment-related changes in hematology or clinical chemistry were observed.

### *3.2.3 Studies in hamsters*

#### *3.2.3.1 Two-year feeding studies in male and female Syrian golden hamsters (Ueno and Hirono, 1981)*

Coumarin was administered to 8-week old male and female Syrian golden hamsters via diet at levels of 0%, 0.1% and 0.5% for up to 2 years. OEHHA estimated the average daily doses to be 0, 92, and 460 mg/kg-day for the male hamster study, and 0, 105, and 523 mg/kg-day for the female hamster study, based on default body weights of 125 g for male and 110 g for female hamsters and food intake of 11.5 g per day for both males and females (Gold and Zeiger, 1997). The male hamster study had 12 controls and 11 animals each in the low- and high-dose groups. The female hamster study had 12 controls, 13 animals in the low-dose group, and 10 in the high-dose group.

In both the male and female studies a transient 20% reduction in food intake was observed in the coumarin-treated groups after one month. Food intakes returned to control levels by month 5 in both studies. No treatment-related effects on growth were observed in either study.

In the male hamster study, survival was poor in the low-dose treatment group. At 18 months, 7/11 (63%) of animals were alive in the low-dose group, whereas 100% in the

high-dose group and 10/12 (83%) in the control group were alive. At 22 months, only 2/11 (18%) survived in the low-dose group compared to 10/11 (90%) in the high-dose and 9/12 (75%) in the control group. No treatment-related tumors were observed. The utility of this study for assessing the carcinogenicity of coumarin is limited by the small numbers of animals per group, and poor survival in the low-dose group.

In the female hamster study, survival was poor in all groups, including the control group. At 18 months, survival was 2/13 (15%) in the low-dose group, 7/10 (70%) in the high-dose group, and 7/12 (58%) in the controls. Survival at 22 months was 0% in the low-dose group and 2/10 (20%) in the high-dose group; survival in the controls 5 was 3/12 (25%). No significant increases in tumors were observed in the treated groups; however, two pancreatic islet cell carcinomas were observed in the high-dose group, with none in the control or low-dose groups. Pancreatic islet cell tumors are uncommon in female hamsters (Schmidt and Hubbard, 1996). The utility of this study for assessing the carcinogenicity of coumarin is limited by the small numbers of animals per group and poor survival in the control and treated groups.

### *3.2.4 Less-than-lifetime study in baboons (Evans et al., 1979)*

Coumarin was administered in the diet to 34 male baboons, of 3 different species *(Papio anubis, Papio hamadras, Papio cynocephalus*), for up to 2 years. Groups of 8, 8, 8, 6, and 4 animals were fed diets containing 0, 2.5, 7.5, 22.5, or 67.5 mg/kg-day coumarin, respectively. The daily intake of one animal receiving 22.5 mg/kg-day was increased to 67.5 mg/kg/day at 18 months. One animal from each of the groups treated with 0, 22.5, and 67.5 mg/kg/day was sacrificed at 16 months. All animals in the 2.5 mg/kg-day group and 4 animals in the 7.5 mg/kg-day group were sacrificed at 18 months. All remaining animals were sacrificed at 24 months. The typical lifespan of these species of baboon ranges from 15 to 40 years. Thus, the length of this study was significantly less than lifetime.

No treatment-related tumors were reported. This is not unexpected given the short study duration, which, depending on the baboon species, ranged from approximately 5 to 13% of the animal's expected lifespan.

*Non-neoplastic pathology and clinical chemistry findings*
Relative liver weights were significantly higher in the 67.5 mg/kg-day group than the controls. Liver hypertrophy and dilatation of the endoplasmic reticulum were also observed in the 67.5 mg/kg-day group. There was no evidence of treatment-related biliary hyperplasia or fibrosis. Although there was not a statistically significant difference in biochemical or histochemical parameters between treated and control

groups, the authors characterized the effects on relative liver weight, liver hypertrophy, and dilatation of the endoplasmic reticulum as evidence of early cell damage in the liver.

## 3.2.5 Co-carcinogenicity studies

### *3.2.5.1 Co-carcinogenicity study in female Wistar rats (Feuer et al., 1976)*

This study investigated the effects of coumarin on mammary gland carcinogenesis induced by DMBA in female Wistar rats. Four groups of female Wistar rats (32/group) received DMBA (2 mg/injection) intravenously via the tail vein on days 50, 53, and 56 of life. Group 1 (DMBA only) received no further treatment. Group 2 (DMBA + coumarin in drinking water, days 44 - 61) received coumarin via drinking water (7.6 mg/100 mL tap water; calculated by study authors to be an average of 0.06–0.07 mM coumarin/kg body weight/day) from day 44 to 61. Group 3 (DMBA + coumarin via gavage, days 44 - 61) received coumarin in arachis oil via gavage from day 44 to 61 at a dose of 1 mM/kg body weight/5 mL oil. Group 4 (DMBA + coumarin via gavage, day 56 - 73) was administered coumarin after DMBA administration, from day 56 to 73, at a dose of 1 mM/kg. There were no significant differences in the mean body weights between groups. Six rats from each group were sacrificed on day 50 (after receiving the first DMBA injection) and 10 rats from each group were sacrificed on day 57. The remaining rats were sacrificed on day 198 and examined for mammary tumors.

Table 12 shows the mammary tumor incidences observed in the study. All animals in Group 1 (receiving DMBA only) had mammary gland adenocarcinomas and two animals also had mammary gland fibroadenomas. Administration of coumarin via drinking water on days 44 to 61 (before, during and after DMBA administration) (Group 2) resulted in slight decreases in mammary gland adenocarcinoma incidence and multiplicity, but no difference in size or growth rate of tumors compared to rats treated with DMBA only (Group 1). A higher dose of coumarin (Group 3) administered during the same time period via gavage resulted in a statistically significant reduction in the incidence of mammary gland adenocarcinomas compared to Group 1, as well as a reduction in tumor size and multiplicity. Administration of coumarin on days 56 to 73 (co-administered and after DMBA administration) (Group 4) did not affect mammary tumor incidence, size, or multiplicity. Results from this 28-week study suggest that co-administration of coumarin with DMBA may reduce the carcinogenic effect of DMBA. The authors suggest that these results are consistent with the possibility that there may be competition between the metabolism of coumarin and DMBA, and that a reduction in the bioactivation of DMBA to the active carcinogenic species resulted in a decrease in tumor incidence.

**Table 12. Mammary tumor incidence[1] in female Wistar rats administered DMBA (all groups) and coumarin (groups 2-4) (Feuer *et al.*, 1976)**

| Tumor Site and Type | | Group[2] | | | |
|---|---|---|---|---|---|
| | | 1 (DMBA only) | 2 (DMBA + coumarin in drinking water, day 44-61) | 3 (DMBA + coumarin via gavage, day 44-61) | 4 (DMBA + coumarin via gavage, day 56-73) |
| Mammary gland | Fibroadenoma | 2/16 | 4/13 | 2/16 | 1/12 |
| | Adenocarcinoma | 16/16 | 11/13 | 12/16* | 11/12 |
| | Fibroadenoma or adenocarcinoma | 16/16 | 11/13 | 12/16* | 11/12 |

[1] Number of animals with lesion per number of animals with organ examined microscopically as reported by study authors.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise. comparison with controls [conducted by OEHHA].
* $p < 0.05$

*3.2.5.2 Co-carcinogenicity study in female S-D rats (Wattenberg et al., 1979)*

This study investigated the effects of coumarin on mammary gland carcinogenesis induced by DMBA in female S-D rats. Six-week old female S-D rats (15/group) were administered coumarin in the diet at doses of 0, 0.034, or 0.068 mmol/g for 8 days. The average daily doses were estimated to be 248 and 497 mg/kg-day, respectively, as calculated by OEHHA. On the seventh day, rats were given 12 mg DMBA in 1 mL olive oil by gavage. The following day, the rats were placed on the control diet for the remainder of the experiment. All rats were sacrificed at 23 weeks of age. There were no significant differences in the mean body weight gains between groups.

Table 13 shows the mammary tumor incidences (tumor type not specified) observed in the study. Rats that received coumarin in feed for 7 days prior to administration of DMBA had statistically significantly fewer mammary tumors than rats administered only DMBA.

**Table 13. Mammary tumor incidence[1] in female S-D rats administered DMBA (all groups) and coumarin (groups 2 & 3) (Wattenberg *et al.*, 1979)**

| Tumor Site and Type | Group[2] | | |
|---|---|---|---|
| | 1 (DMBA only) | 2 (0.034 mmol/g coumarin in feed + DMBA) | 3 (0.068 mmol/g coumarin in feed + DMBA) |
| **Mammary gland tumors** | 22/24 (91.7%) | 4/13*** (30.7%) | 3/28*** (10.7%) |

[1] Number of animals with lesion per number of animals with organ examined microscopically as reported by study authors.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls [conducted by OEHHA].
*** $p < 0.001$

*3.2.5.3 Co-carcinogenicity study in female ICR/Ha mice (Wattenberg et al., 1979)*

This study investigated the effects of coumarin on forestomach tumor formation induced by benzo(*a*)pyrene (BP) in female ICR/Ha mice. Nine-week old female ICR/Ha mice (20/group) were administered coumarin in the diet at doses of 0, 0.034 or 0.068 mmol/g. The average daily doses were estimated to be 646 and 1292 mg/kg-day, respectively, as calculated by OEHHA. On the eighth day, mice were given 1 mg BP in 0.2 mL corn oil by gavage. Mice received a total of 8 doses of BP (2 times per week for 4 weeks). The mice were given diets containing coumarin throughout the period of BP administration until 3 days after the last dose, at which time they were given control diets. All mice were sacrificed at 30 weeks of age.

Table 14 shows the forestomach tumor incidences (tumor type not specified) observed in the study. Mice that received coumarin in feed had statistically significantly fewer forestomach tumors than mice administered only BP. Coumarin is metabolized by CYP2A5 (Zhuo *et al*., 1999), which is involved in bioactivation of BP (Shimada, 2017). Thus, it is possible that there may be competition between the metabolism of coumarin and BP that results in reduced bioactivation of coumarin to the active carcinogenic species.

**Table 14. Forestomach tumor incidence[1] in female ICR/Ha mice administered DMBA (all groups) and coumarin (groups 2 & 3) (Wattenberg *et al.*, 1979)**

| Tumor Site and Type | Group[2] | | |
|---|---|---|---|
| | 1 (DMBA only) | 2 (0.034 mmol/g coumarin in feed + DMBA) | 3 (0.068 mmol/g coumarin in feed + DMBA) |
| **Forestomach tumors** | 74/77 (96.1%) | 61/76** (80.2%) | 11/18*** (61.1%) |

[1] Number of animals with lesion per number of animals with organ examined microscopically as reported by study authors.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls [conducted by OEHHA].
** $p < 0.01$; *** $p < 0.001$.

*3.2.5.4 Co-carcinogenicity study in male Syrian golden hamsters (Baskaran et al., 2012)*

This study investigated the effect of coumarin on buccal pouch carcinogenesis induced by DMBA in male hamsters. Male hamsters were randomized into four groups (10 per group), and treatment was applied for a total of 14 weeks. Group I hamsters served as control and were painted with liquid paraffin three times a week for 14 weeks on their left buccal pouches. Groups II and III hamsters were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group II hamsters received no other treatment. Group III hamsters received oral administration of coumarin at a dose of 100 mg/kg bw/day, starting one week before exposure to the carcinogen and continuing on days alternate to DMBA painting, until study termination at week 16. Group IV hamsters received oral administration of coumarin (100 mg/kg bw) alone throughout the experimental period.

Histopathological examination of buccal mucosal tissues showed that DMBA induced epithelial tumors in 100 percent of the animals in Group II (DMBA only). No buccal mucosal tumors were observed in Group I (control), Group III (DMBA plus coumarin) or Group IV (coumarin). Results from this 16-week study indicate that co-administration of coumarin with DMBA reduces the carcinogenic effect of DMBA, and are consistent with the possibility that there may be competition between the metabolism of coumarin and DMBA that results in reduced bioactivation of coumarin to the active carcinogenic species.

### *3.2.6 Summary of animal carcinogenicity study findings*

Findings in the animal carcinogenicity studies of coumarin include:

- Rare renal tubule adenomas and adenomas and carcinomas combined in male F344 rats (mid-dose continuous 103-week exposure group), and renal tubule adenomas in male F344 rats (9-month stop-exposure group at 103 weeks) administered coumarin via gavage (NTP, 1993a)
- Rare renal tubule adenomas in female F344 rats administered coumarin via gavage (NTP, 1993a)
- Uncommon renal tubule oncocytomas in male F344 rats (two in the low-dose continuous 103-week exposure group; two in the 15-month stop-exposure group at 103 weeks) administered coumarin via gavage (NTP, 1993a)
- Cholangiocarcinomas in male and female S-D rats administered coumarin via diet (Carlton *et al.*, 1996)
- Hepatocellular adenomas and carcinomas combined in male and female S-D rats administered coumarin via diet (Carlton *et al.*, 1996)
- Hepatocellular adenomas and adenomas and carcinomas combined in female $B6C3F_1$ mice administered coumarin via gavage (NTP, 1993a)
- Hepatocellular adenomas and carcinomas combined in female CD-1 mice (low-dose group) administered coumarin via feed (Carlton *et al*., 1996)
- Alveolar/bronchiolar adenomas and adenomas and carcinomas combined in male and female $B6C3F_1$ mice, and alveolar/bronchiolar carcinomas in female mice administered coumarin via gavage (NTP, 1993a)
- Alveolar/bronchiolar carcinomas in male CD-1 mice administered coumarin via feed (Carlton *et al.*, 1996)
- Forestomach squamous cell papillomas and carcinomas combined in male $B6C3F_1$ mice (low-dose group) administered coumarin via gavage (NTP, 1993a). Forestomach squamous cell carcinomas are rare in male mice of this strain, and one was observed in the low-dose group, and two in the mid-dose group.
- Observations of rare forestomach squamous cell carcinomas in female $B6C3F_1$ mice (one each in low- and mid-dose groups) administered coumarin via gavage (NTP, 1993a)
- Two uncommon pancreatic islet cell carcinomas in female hamsters administered coumarin via feed (Ueno and Hirono, 1981)

No tumors were reported in limited studies conducted in rats (Evans *et al*., 1989; Bar and Griepentrog, 1967; Gripentrog, 1973; Hagan *et al*., 1967) and baboons (Evans *et al*., 1979). Findings in the co-carcinogenicity studies include a decrease in the number of mammary tumors induced by DMBA in female Wistar rats (Feuer *et al*., 1976) and

Sprague-Dawley rats (Wattenberg *et al*., 1979), a decrease in forestomach tumors induced by BP in female ICR/Ha mice (Wattenberg *et al*., 1979), and an absence of buccal mucosal tumors induced by DMBA in male Syrian golden hamsters (Baskaran *et al*., 2012) when animals also received coumarin.

## 3.3 Other Relevant Data

### *3.3.1 Pharmacokinetics and metabolism*

This section summarizes the absorption, distribution, metabolism and excretion (ADME) of coumarin in humans and animals. It first discusses key findings from studies in humans, followed by studies in animals, and then discusses genetic polymorphisms of CYP2A6, a key enzyme involved in human coumarin metabolism. Additional details on CYP2A6 polymorphisms are presented in Appendix B.

#### *3.3.1.1 Studies in humans*

The pharmacokinetics and metabolism of coumarin have been studied in humans *in vivo* and *in vitro*. Many of these studies have been reviewed previously (Lake, 1999; IARC, 2000; Felter *et al.*, 2006; EFSA, 2004). Briefly, absorption studies have been conducted *in vivo* by the oral and dermal routes and *in vitro* with a human skin absorption model (Ritschel *et al.*, 1977; Ritschel and Hoffmann, 1981; Beckley-Kartey *et al.*, 1997; Yourick and Bronaug, 1997; Ford *et al.*, 2001; Abraham *et al.*, 2010). Distribution studies were conducted in human volunteers by the *i.v.* and oral routes (Ritschel *et al.*, 1976; Ritschel *et al.*, 1977; Cohen, 1979). Studies of coumarin metabolism were conducted by oral, dermal and *i.v.* routes *in vivo* and were tested in human liver microsomal samples, human liver slice cultures, and recombinant human cytochrome P-450s *in vitro* (Shilling *et al.*, 1969; Ritschel *et al.*, 1977; Fentem *et al.*, 1991; Fentem and Fry, 1992; Lake *et al.*, 1992a; Steensma *et al.*, 1994; van Iersel *et al.*, 1994a; Born *et al.*, 1997; Hadidi *et al.*, 1997; Ford *et al.*, 2001; Born *et al.*, 2002; Vasallo *et al.*, 2003; Vassallo *et al.*, 2004b; Rietjens *et al.*, 2008; Meineke *et al.*, 1998; Sharifi *et al*., 1993). Excretion studies were conducted in human volunteers by the *i.v.*, oral and dermal routes (Shilling *et al.*, 1969; Cohen, 1979; Moran *et al.*, 1987; Rautio *et al.*, 1992; Iscan *et al.*, 1994; Egan and O'Kennedy, 1992; Sharifi *et al.*, 1993; Meineke *et al.*, 1998; Ford *et al.*, 2001; Abraham *et al.*, 2011).

Coumarin is quickly absorbed by the oral and dermal routes. Following oral administration, coumarin is rapidly and completely absorbed (Ritschel *et al.*, 1979; Abraham *et al*., 2011). In a dermal application study, 60% of the coumarin dose was absorbed within 6 hours (Ford *et al*., 2001). Similarly, *in vitro* studies with human skin

found that 66% of the applied dose was absorbed after 72 hours (Beckley-Kartey *et al*. 1997).

Coumarin and its metabolites are distributed throughout the body in humans (Ritschel *et al*., 1976; Cohen, 1979; Egan *et al.*, 1990; Pelkonen *et al.*, 1997). Coumarin is rapidly and extensively metabolized, and only a small amount of the parent compound (about 3% of the administered dose) is detected in the blood following oral administration (Ritschel *et al*., 1979). The half-life of coumarin in the blood is similar following administration via either the *i.v.* or oral routes, ranging from 1–1.5 hours (Ritschel *et al.,* 1977; Cohen, 1979). The plasma half-life following dermal exposure is 1.7 hours (Ford *et al*., 2001).

A number of coumarin metabolites have been identified in humans either *in vivo* from urine or blood samples or *in vitro* with human liver microsomes, liver slices, or recombinant cytochromes (Table 15). Metabolites identified in humans *in vivo* are 7-hydroxylation (7-HC), its conjugated glucuronides or sulfates, 3-hydroxylation (3-HC) and *o*-hydroxylphenylacetic acid (*o*-HPAA) (Sharifi *et al.*, 1993; Ford *et al*., 2001, Meineke *et al*., 1998; Hadidi *et al*., 1997; Ritschel *et al*., 1977). Additional metabolites that have been identified *in vitro* include 4-, 5-, 6-, and 8-HC, 6,7- dihydroxycoumarin (6,7- DiHC), *o*-coumaric acid (*o*-CA), *o*-hydroxyphenylpropionic acid (*o*-HPPA), *o*-hydroxyphenylethanol (*o*-HPE), and coumarin 3,4-epoxide glutathione conjugate (CE-SG) (van Iersel *et al*., 1994a; Fentem *et al*., 1991, Fentem and Fry 1992, Lake *et al*., 1992a, Rietjens *et al*., 2008, Born *et al*., 1997, Vassallo *et al*., 2004b; Steensma *et al*., 1994; Born *et al*., 2002). Other metabolic products of coumarin have been detected in humans, but their structures have not been identified (Steensma *et al.*, 1994; van Iersel *et al.*, 1994a; Ford *et al.*, 2001).

**Table 15. Summary of coumarin metabolites detected in humans *in vivo* and *in vitro***

| Biological Samples / Metabolites | Detected *in vivo* (Analytical methods)* | | Detected *in vitro* (Analytical methods)* | | |
|---|---|---|---|---|---|
| | Urine[a] | Blood[b] | Liver microsomes[c] | Liver slices[d] | Recombinant CYPs[e] |
| ***7-Hydroxylation Pathway*** | | | | | |
| **7-HC** | Yes (HPLC, GC) | Yes (HPLC) | Yes (HPLC) | Yes (HPLC) | Yes (GC) |
| **7-HCG** | Yes (HPLC) | Yes (HPLC) | — | Yes (HPLC) | — |
| **7-HC sulfate** | Yes (HPLC) | No | — | Yes (HPLC) | — |
| ***3,4-Epoxidation & 3-Hydroxylation Pathways*** | | | | | |
| **3-HC** | Yes (HPLC) | No | Yes (HPLC) | No | NA |
| **Coumarin 3,4-epoxide** | No | No | No | No | Yes** (GC) |
| ***o*-HPA** | No | No | Yes (HPLC) | Yes (HPLC) | Yes (GC) |
| ***o*-HPAA** | Yes (PC/TLC, HPLC, GC-MS) | No | Yes (HPLC) | Yes (HPLC) | NA |
| ***o*-HPE** | No | No | Yes (HPLC) | No | NA |
| **CE-SG** | No | No | Yes[f] (RP-HPLC, MS, NMR) | No | — |
| **Coumarin 3-mercapturic acid** | NA | NA | — | NA | — |
| ***Other Minor Pathways*** | | | | | |
| **3,4-DHC** | NA | NA | NA | NA | NA |
| ***o*-HPPA** | No | No | Yes (HPLC) | No | NA |
| ***o*-CA** | No | No | Yes (HPLC) | No | NA |
| **4-HC** | No | No | Yes (HPLC) | Yes (HPLC) | NA |
| **5-HC** | No | No | Yes (HPLC) | No | NA |
| **6-HC** | No | No | Yes (HPLC) | No | NA |
| **8-HC** | No | No | Yes (HPLC) | No | NA |
| **6,7-DiHC** | No | No | Yes (HPLC) | No | NA |

—: The phase II enzymes, such as β-glucuronidase, sulphatase and glutathione *S*-transferase, are absent in human liver microsomal and recombinant CYP preparations.
NA: Not assessed
* HPLC: High Performance Liquid Chromatography; GC: Gas Chromatography; PC/TLC: Paper Chromatography/ Thin Layer Chromatography; GC-MS: Gas Chromatography- Mass Spectrometry; RP-HPLC: Reversed-Phase High Performance Liquid Chromatography; MS: Mass Spectrometry; NMR: Nuclear Magnetic Resonance Spectroscopy
** Indirectly detected as *o*-HPA.
[a] data are from Shilling *et al*., 1969, Hadidi *et al*., 1997, Meineke *et al*., 1998, Ford *et al*., 2001
[b] data are from Ritschel *et al*., 1977
[c] data are from van Iersel *et al*., 1994a, Fentem *et al*., 1991, Fentem and Fry 1992, Lake *et al*., 1992a, Rietjens *et al*., 2008, Born *et al*., 1997, Vassallo *et al*., 2004b
[d] data are from Steensma *et al*., 1994
[e] data are from Born *et al*., 2002
[f] liver cytosol containing glutathione reductases and other phase II enzymes were added to the reactions, data are from Vassallo *et al*., 2004b.

As shown in Figure 2, coumarin is metabolized in humans via several different pathways. The primary pathways of coumarin metabolism are the 7-hydroxylation pathway and the 3,4-epoxidation pathway, although coumarin can be hydroxylated at other possible positions (*i.e.*, carbons 3, 4, 5, 6, and 8) and the opening of the lactone ring can yield various products (Figure 2). Multiple cytochrome P450 (CYP) enzymes can catalyze the 7-hydroxylation reaction to form 7-HC, including CYP2A6, CYP2A13, and CYP2B6, with CYP2A6 being the most active (Sai *et al.*, 1999). CYP2A6, also known as coumarin 7-hydroxylase, is a polymorphic enzyme. Multiple CYP enzymes can catalyze the 3,4-epoxidation reaction, including CYP1A1, CYP1A2, CYP2B6, CYP2A13, CYP3A4, and CYP2E1, with CYP1A1, CYP1A2, and CYP2E1 thought to be the most active (Lake *et al*., 1992; Hadidi *et al*., 1997; Zhou *et al*., 1999; Born *et al*., 2002; von Weymarn and Murphy, 2003).

The product of the 3,4-epoxidation pathway is coumarin 3,4-epoxide (CE). CE is an unstable highly reactive electrophile, and can react with glutathione to form a glutathione conjugate (CE-SG) or degrade spontaneously to form the reactive aldehyde *ortho*-hydroxyphenylacetaldehyde (*o*-HPA) via opening of the lactone ring and cleavage of $CO_2$ (Vassallo *et al*., 2003). *o*-HPA can be oxidized to *o*-HPAA by aldehyde dehydrogenases (ALDH) (IARC, 2000; Vassallo *et al.*, 2004b), or reduced to *o*-HPE. CE has a short half-life (4 seconds), and is generally measured indirectly as either *o*-HPA or *o*-HPAA (Born *et al*., 2002; Vassallo *et al*., 2003). Both CE and *o*-HPA are toxic electrophilic metabolites of coumarin that covalently bind to cellular macromolecules, and are associated with hepatotoxicity (Fentem *et al*., 1991; van Iersel *et al*., 1994a; Felter *et al.*, 2006; Born *et al.*, 2000a; Born *et al.*, 2000b; Vassallo *et al.*, 2004a; Vassallo *et al.*, 2004b).

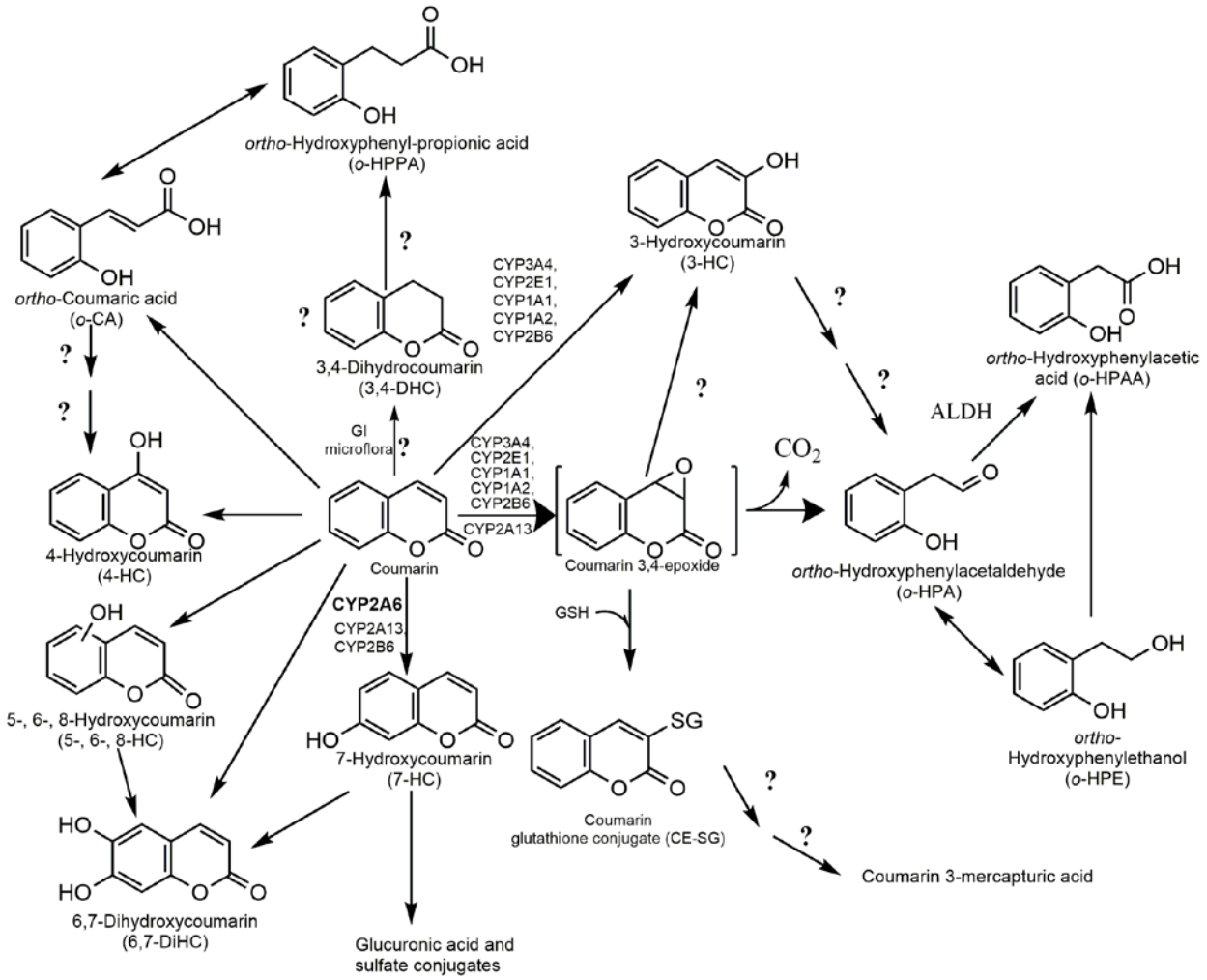



**Figure 2. Known and proposed metabolic pathways of coumarin in humans**
Adapted from Lake, 1999 with some modifications reported by NTP, 1993a and NTP, 1993b; Born *et al*., 2002 and Schlicht *et al.*, 2009. Question marks (?) denote proposed pathways and metabolites. ALDH: Aldehyde dehydrogenase.

It is unclear if 3-HC can be oxidized to *o*-HPA, with studies by Kaighen and Williams (1961) providing indirect support for this pathway with the finding of urinary *o*-HPAA in rabbits and rats administered 3-HC. *o*-HPAA is formed from *o*-HPA; however, it is also formed from *o*-hydroxyphenylpyruvic acid (*o*-HPPyA) and *o*-hydroxyphenyllactic acid (*o*-HPLA). On the other hand, studies by Born *et al*. (1997; 2000a) and Norman and Wood (1984) suggest that 3-HC is not converted to *o*-HPA.

The 7-hydroxylation pathway is often but not always the predominant pathway of coumarin metabolism in humans. As discussed in greater detail in Section 3.3.1.3 below, the relative importance of the 7-hydroxylation pathway versus the 3,4-epoxidation pathway in the metabolism of coumarin is determined primarily by an individual's CYP2A6 phenotype. The shift in the quantities of specific coumarin metabolites formed, either by decrease- or loss-of-function genetic polymorphisms of *CYP2A6* or non-genetic factors, can be significant. For example, 7-HC can constitute up to 92% of coumarin metabolites in some humans (Shilling *et al.*, 1969). However, in an individual homozygous for a loss-of-function *CYP2A6* variant allele, the amount of 7-HC measured in the urine represented less than 0.02% of the applied dose, while *o*-HPAA (a product of the 3,4-epoxidation pathway) accounted for up to 54.6% of the total urinary metabolites (Hadidi *et al*., 1997).

In humans, excretion of coumarin and its metabolites is rapid and proceeds primarily via urine. After oral administration, 95% was excreted within 4 hours in one study (Rautio *et al.*, 1992). Dermal application results in slower excretion, with 26% of the applied coumarin dose excreted in 2 hours, and 59% excreted in 120 hours (Ford *et al*., 2001). The primary urinary metabolites were conjugates of 7-HC, and small amounts of unconjugated 7-HC and *o*-HPAA. Fecal excretion has been measured only following dermal exposure, and amounted to 1 % of the applied dose in 120 hours (Ford *et al*., 2001). This finding suggests that very little biliary excretion of coumarin metabolites occurs in humans.

### *3.3.1.2 Studies in animals*

The pharmacokinetics and metabolism of coumarin have been studied in animals *in vivo* and *in vitro*, and many of these studies have been reviewed previously (IARC 2000; Lake, 1999; EFSA, 2004; Felter *et al.*, 2006). Briefly, *in vivo* studies have been conducted in several animal species, including rats (*i.p, i.v*., oral, dermal), mice (oral), rabbits (oral), dogs (*i.v*., oral), gerbils (*i.p.*), rhesus monkeys (*i.v*., oral), baboons (oral), squirrel monkeys (oral), ferrets (oral), guinea pigs (oral), pigs (oral), hamsters (oral), dogs (oral), and cats (oral) (IARC, 2000; Born *et al.*, 2003; Waller and Chasseaud, 1981; Mead *et al.*, 1958; Lake, 1999, Gangolli *et al.*, 1974; Ritschel *et al.*, 1988, Van Sumere and Teuchy, 1971; Ford *et al.*, 2001). *In vitro* studies were conducted with rat

and mouse skin (Beckley-Kartey *et al.*, 1997; Yourick and Bronaug, 1997); liver slices from rats, cynomolgus monkeys, mice, Syrian hamsters, rabbits. and baboons (Steensma *et al.* 1994); lung cytosolic fractions from rats and mice (Vassallo *et al.*, 2004a); recombinant cytochrome P450 enzymes (Born *et al*., 2002); and microsomal fractions from rats, mice, and other species (Fink and v. Kerekjarto, 1966; Gibbs *et al.*, 1971; Feuer, 1970; Kaipainen *et al.*, 1984; Pelkonen *et al.*, 1985; Fentem *et al.*, 1991; Peters *et al.*, 1991; Lake *et al.*, 1992a; Lake *et al.*, 1992b; van Iersel *et al.*, 1994b; Zhuo *et al.*, 1999; Born *et al.* 1997; 2000b; 2002; Vasallo *et al.*, 2003).

Coumarin is rapidly absorbed in rats and mice following oral or dermal administration, and is distributed throughout the body and extensively metabolized, with little excretion of the parent compound (Ritschel and Hussain, 1988; Ford *et al.*, 2001; Lake *et al.*, 2002; Born *et al.*, 2003; Waller and Chasseaud, 1981; Ford *et al.*, 2001). In mice, peak plasma concentrations can be reached within 10 minutes following oral gavage, and within 9 hours when coumarin is added to the diet (Born *et al.*, 2003). In baboons, 90% of the dose was absorbed within 45 minutes after oral administration (Waller and Chasseaud, 1981). When applied dermally to rats, absorption was considered complete within 6 hours (Ritschel and Hussain, 1988).

Following dermal or oral absorption, coumarin is rapidly distributed throughout the body and metabolized, as shown in studies conducted in baboons, rats, and gerbils (Piller, 1977; Ford *et al.*, 2001; Ritschel and Hardt, 1983; Ritschel and Hussain, 1988; Waller and Chasseaud, 1981). Following dermal application of radiolabeled coumarin to rats, the highest tissue concentrations of radioactivity were found in the small intestine, large intestine, and stomach, with lower concentrations observed in the kidney and liver (Ford *et al.*, 2001). These results are indicative of significant biliary excretion of coumarin in the rat (Ford *et al.*, 2001), and are supported by *i.p*. injection studies in rats (Piller, 1977). The plasma half-life of coumarin varies greatly by species, with values of 4 hours reported for mice and 5-20 hours for rats (Born *et al.*, 2003; Ford et al., 2001). By comparison, the plasma half-life in humans ranged from 1- 1.7 hours, depending on the route of exposure (Ritschel *et al*., 1977; Cohen, 1979; Ford *et al*., 2001).

Coumarin metabolism in animals is qualitatively similar to that in humans, although there are quantitative differences between humans and some species (*e.g.*, rats), as well as quantitative differences among some non-human species. For example, in baboons, 7-hydroxylation is the main pathway by which coumarin is metabolized, leading to 7-HC as the primary initial metabolite. In contrast, rats, hamsters, guinea pigs, and some strains of mice form little or no 7-HC and instead form CE and 3-HC (Kaighen and Williams, 1961; Cohen, 1979; Lake, 1999). Animal studies have identified some additional coumarin metabolites that have not been reported in human studies,

including *o*-hydroxyphenyllactic acid (*o*-HPLA), *o*-hydroxyphenylpyruvic acid, and N-acetyl-S-(3-coumarinyl) cysteine (Norman and Wood, 1984; Lake *et al.*, 2002; Kaighen and Williams, 1961; Huwer *et al.*, 1991). Other metabolic products of coumarin have been detected in animals, but the structures of these products have not yet been identified (Lake *et al*., 2002; Kaighen and Williams, 1961). Figure 3 below provides an overview of the known and putative metabolic pathways of coumarin in animals.

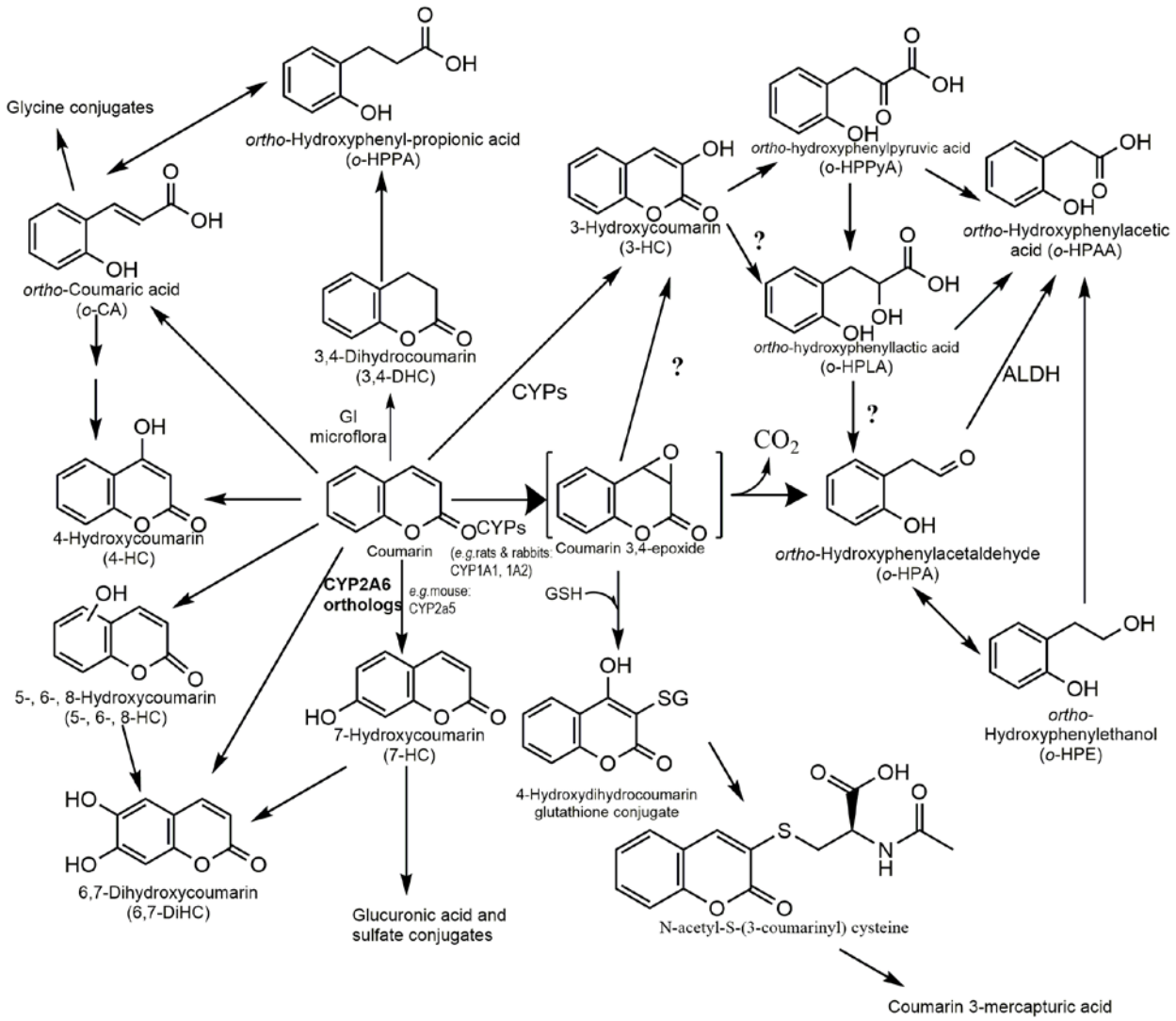



**Figure 3. Known and proposed metabolic pathways of coumarin in animals**
Adapted from Lake, 1999 with some modifications reported by NTP, 1993a and NTP, 1993b; Lake *et al*., 2002; Huwer *et al*., 1991; Feuer, 1974; Fentem and Fry, 1993. Question marks (?) denote proposed pathways. ALDH: Aldehyde dehydrogenase

Briefly, metabolism of coumarin in animals occurs primarily via either epoxidation or hydroxylation. These specific reactions are catalyzed by species- and tissue-specific CYP enzymes, and result in the formation of either the reactive intermediate CE (coumarin 3,4-epoxide) or various hydroxylated coumarins, with 7- and 3-HC being the most frequently formed hydroxylated metabolites (Mead *et al.*, 1958; Cohen, 1979; Fentem and Fry, 1993; Lake, 1999). As discussed previously, CE is reactive and unstable, and can spontaneously rearrange to form *o*-HPA, followed by further oxidation to *o*-HPAA or reduction to *o*-HPE (Born *et al*., 1997; Norman and Wood, 1984; IARC, 2000). CE may also form a glutathione conjugate and be excreted (Lake, 1999; Zhuo *et al*., 2009). Other metabolites resulting from ring-opening reactions include *o*-HPLA, *o*-HPPyA and *o*-hydroxyphenylpropionic acid (*o*-HPPA) (Lake, 1999; Lake *et al*., 2002; Kaighen and Williams, 1961; Huwer *et al.*, 1991). 3,4-Dihydrocoumarin (3,4-DHC) is another metabolite of coumarin which is formed in the intestine by microflora (NTP, 1993a;1993b). Scheline (1968) incubated coumarin with microflora from the caeca of rats and rabbits under anaerobic conditions and demonstrated the formation of 3,4-DHC and *o*-HPPA. Hydroxycoumarins and other coumarin metabolites can be conjugated with glucuronic acid or sulfate and excreted, and *o*-CA can be conjugated with glycine and excreted (Feuer, 1974; Fentem and Fry, 1993).

As discussed in the section on human metabolism, it is unclear if 3-HC can be metabolized to *o*-HPA, or if this reactive aldehyde (*i.e.*, *o*-HPA) is produced from coumarin solely through the 3,4-epoxidation pathway. The urinary excretion studies of Kaighen and Williams (1961) in rats and rabbits administered radiolabeled 3-HC provide some indirect support for the formation of *o*-HPA from 3-HC. These investigators looked for several possible coumarin metabolites, based on scientific understanding and analytical methods available at that time. *o*-HPA was not one of the analytes assessed; however, *o*-HPAA was assessed and it was detected in the urine of rats and rabbits dosed with 3-HC. *o*-HPAA is formed from *o*-HPA; however, it is also formed from *o*-HPPyA and *o*-HPLA, both of which were also detected in rats and rabbits administered 3-HC (Kaighen and Williams, 1961). Other studies conducted using various *in vitro* model systems suggest that 3-HC is not converted to *o*-HPA. These studies include those of Norman and Wood (1984), where the production of *o*-HPE, which is formed from *o*-HPA, was assessed in rat liver 10,000g supernatants incubated with either coumarin or 3-HC. While the production of *o*-HPE was observed in incubations with coumarin, no production of *o*-HPE was observed in incubations with 3-HC (Norman and Wood, 1984). Born *et al*. (1997) investigated the formation of *o*-HPA from either CE or 3-HC in an aqueous cell- and enzyme-free incubation system, and found that *o*-HPA was formed from CE, but not from 3-HC. These authors also studied *o*-HPA formation from CE and 3-HC in the presence of mouse liver microsomes, and found that *o*-HPA was formed in microsomes incubated with CE, but not with 3-HC

(Born *et al*., 1997). Comparative toxicity studies in which coumarin and *o*-HPA where found to be cytotoxic to rat hepatocytes, but 3-HC was not, provide additional, albeit indirect evidence that 3-HC is not converted to *o*-HPA (Born *et al*., 2000a; Lake *et al*., 1989).

Coumarin toxicity is associated with the formation of toxic and electrophilic metabolites that can bind covalently to cellular macromolecules, including CE and *o*-HPA (Fentem *et al*., 1991; Felter *et al.*, 2006; Born *et al.*, 2000a, b; Vasallo *et al*. 2004a). While CE and *o*-HPA are formed in all animal species studied, differences among species in the toxicity of coumarin may be explained not only by differences in metabolic activation, but also by differences in detoxification reactions. Mice appear to catalyze the oxidation of *o*-HPA to *o*-HPAA more efficiently than rats, as *o*-HPAA may account for up to 41% of the administered dose in mice and only 12% in rats (Born *et al.*, 2003). A faster clearance rate for the oxidation of *o*-HPA to *o*-HPAA in mice as compared to rats is supported by findings from studies with liver microsomal and cytosolic fractions. The total clearance of coumarin in microsomal incubations was 4-fold greater in mice than in rats (Born *et al*., 2000b); similarly, the total clearance of coumarin in cytosolic incubations was 20 times higher in mice compared to rats (Vasallo *et al*., 2004b). Both mice and rats reduced *o*-HPA to *o*-HPE; however, this is only a major reaction in rats. Vasallo *et al*. (2004b) suggest that a cycle of oxidation and reduction from *o*-HPA to *o*-HPE and back may contribute to slower hepatic clearance of the toxic aldehyde in the rat. The extent and kinetics of additional detoxification reactions such as conjugation with glutathione may also determine the extent to which electrophilic metabolites bind covalently with cellular macromolecules in a given tissue.

Excretion of coumarin and its metabolites occurs via expired air, urine and feces. About 30% of the radioactivity associated with a dose of [2-$^{14}$C] coumarin administered to rats was recovered in expired air as $CO_2$ (Van Sumere and Teuchty, 1971). Little or no radioactivity was found in expired air of rats (and other species) when [3-$^{14}$C or 4-$^{14}$C] coumarin was administered (Kaighen and Williams, 1961; Lake, 1999). These findings are consistent with loss of carbon number 2 on the lactone ring during coumarin metabolism to form *o*-HPA. Fecal excretion is attributed to biliary excretion of metabolites, including unidentified ring opened compounds, and is greater in many of the animal species studied than in humans (Ford *et al*., 2001; Piller, 1977; Van Sumere and Teuchy, 1971; Lake *et al*., 2002, Kaighen and Williams, 1961). The highest fecal excretion has been observed in rats (38%), followed by hamsters (12%), baboons (3.4%) and rabbits (1%) (Lake *et al*., 2002; Kaighen and Williams, 1961; Waller and Chassaud, 1981). Excretion of coumarin and its metabolites via urine and feces is rapid, with the majority occurring within 24 hours, and excretion being essentially complete within 96 hours.

*3.3.1.3 Genetic polymorphisms in CYP2A6, a key enzyme involved in human coumarin metabolism*

As discussed in Section 3.3.1.1, CYP2A6 is one of the key enzymes involved in the metabolism of coumarin in humans. CYP2A6 catalyzes the 7-hydroxylation of coumarin to form 7-HC. CYP2A6-mediated 7-hydroxylation is considered to be the major metabolic detoxification pathway for coumarin in humans, as 7-HC and its glucuronide and sulfate conjugates are rapidly excreted, primarily in the urine. *CYP2A6* is a highly polymorphic gene. Some polymorphisms affect CYP2A6 enzyme activity, with some resulting in increased activity, others resulting in reduced activity, and still others resulting in complete loss of enzyme activity (loss of function). Individuals with CYP2A6 polymorphisms that confer reduced enzyme function or loss of function may have increased susceptibility to coumarin toxicity.

Clinical trials with coumarin have shown that certain individuals are more susceptible to coumarin hepatotoxicity (Casley-Smith and Casley-Smith, 1995; Loprinzi *et al.*, 1999; Zhang and Chal, 2005; Farinola and Piller, 2007). Studies in humans or human tissues have also demonstrated inter-individual variability in coumarin metabolism to 7-HC. CYP2A6 polymorphisms are thought to be largely responsible for this variability. It has been hypothesized that reduced or loss-of-function CYP2A6 polymorphisms make individuals more susceptible to coumarin toxicity, including carcinogenicity, by increasing the metabolism of coumarin through the 3,4-epoxidation pathway, and increasing the formation of the reactive metabolites CE and *o*-HPA.

This shift in metabolism from 7-hydroxylation to the 3,4-epoxidation pathway and other pathways (*i.e.*, 3-hydroxylation) has been observed in microsomes prepared from some human liver samples, and *in vivo*, in an individual that lacked functional CYP2A6. In one study of coumarin metabolism using human liver microsomes prepared from 12 individuals, van Iersel *et al.* (1994a) demonstrated the presence of the following metabolites: 3-HC, 4-HC, 7-HC, 6,7-DiHC, *o*-CA, *o*-HPPA, *o*-HPA, *o*-HPE, and *o*-HPAA. For 11 of the 12 individuals, 7-HC was the major liver microsomal metabolite, accounting for 76 – 92% of the total polar products (*i.e.*, all metabolites except the ones covalently bound to the microsomal proteins). However, in microsomes prepared from the twelfth subject, 7-HC accounted for only 1.2% of the total polar products, while products of the 3,4-epoxidation and 3-hydroxylation pathways (*o*-HPA, *o*-HPE, *o*-HPAA, and 3-HC) accounted for 69% of the total polar products. In another study of coumarin metabolism using human liver microsomes prepared from four individuals from the UK, *o*-HPA was identified as the most abundant metabolite of coumarin (about 6 times that of 7-HC) in each of the four microsomal preparations (Fentem and Fry, 1992). This shift in coumarin metabolism to the 3,4-epoxidation and 3-hydroxylation pathways was also

evident *in vivo* in an individual with the *CYP2A6*2/*2* genotype (*i.e.*, no CYP2A6 coumarin 7-hydroxylase activity), in which 45.9-54.6% of an administered 2 mg dose of coumarin was excreted as *o*-HPAA, with less than 0.02% excreted as 7-HC (Hadidi *et al.*, 1997).

Consequently, it is of critical importance to identify the subgroups of the human population where this shift occurs, as these subgroups are likely to be more susceptible to coumarin toxicity. Besides genetic polymorphisms, the modulation of CYP2A6 activity by age, gender, and life style factors (such as drugs and dietary factors) could also lead to a shift of coumarin metabolism towards increased production of the reactive metabolites CE and *o*-HPA.

This section summarizes the available information on the coumarin 7-hydroxylase activity of CYP2A6 variants, the distribution of certain loss-of-function and decrease-of-function CYP2A6 variants in different ethnic populations, CYP2A6 genotype-phenotype correlation studies, and other factors that affect the CYP2A6 enzyme's ability to metabolize coumarin.

**CYP2A6 variants and associated coumarin 7-hydroxylase activities**

*CYP2A6* is a highly polymorphic gene. These genetic polymorphisms can affect the coumarin 7-hydroxylase activity of CYP2A6. To date, there are at least 45 identified allelic variants of CYP2A6, with many subgroups within some of the variants (Ingelman-Sundberg *et al.*, 2014). The complete list of nucleotide changes for each *CYP2A6* allele is available at http://www.cypalleles.ki.se/cyp2a6.htm.

Coumarin is one of the most well-studied substrates of CYP2A6, and measurement of 7-HC is commonly used to characterize CYP2A6-mediated 7-hydroxylase activity (Miles *et al.*, 1990; Yamano *et al.*, 1990; Zhuo *et al.*, 1999; Pelkonen *et al.*, 2000). While coumarin can also undergo 7-hydroxylation via CYP2A13, 7-HC formation is thought to occur primarily via CYP2A6 due to coumarin's high affinity as a substrate for CYP2A6. 7-HC is the primary metabolite of coumarin in most individuals. For example, in a small study of eight healthy volunteers about 79% of the total coumarin was excreted as 7-HC (the analytical method used did not distinguish between conjugated and unconjugated 7-HC), and about 4% was excreted as *o*-HPAA (Shilling *et al.*, 1969). Additional studies with human volunteers have demonstrated that considerable inter-individual variability in CYP2A6 coumarin 7-hydroxylase activity exists within the human population, and several studies have associated this variability with specific *CYP2A6* genetic polymorphisms (Hadidi *et al.*, 1997; Oscarson *et al.*, 1998; Oscarson *et al.*,

1999a; Oscarson *et al.*, 2002; Ujjin *et al.*, 2002; Xu *et al.*, 2002a; Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009).

Three model systems for measuring 7-hydroxylase activity of CYP2A6 variants are commonly used in research: the heterologous expression of recombinant protein in bacteria or cultured cell lines, the use of human liver microsomes, and the measurement of 7-HC in human subjects. Additional discussion of these methods is presented in Appendix B.

The genetic variations of *CYP2A6*, caused by either single nucleotide polymorphisms (SNPs) in coding or non-coding regions or gene conversions, duplications, or deletions, can result in an increase, decrease, or lack of coumarin 7-hydroxylase activity of the enzyme (Table 16).

**Table 16. Human CYP2A6 variants and their coumarin 7-hydroxylase activities[a]**

| Allele | Coumarin 7-hydroxylase activity | Types of Genetic Changes |
|---|---|---|
| ***1A (Wild-type)** | Fully functional | |
| ***1B, *14** | Increased activity | Gene conversion (**1B*); SNP (**14*) |
| ***15, *16, *21, *28, *31** | Similar activity to wild-type | Gene conversion and SNP (**28*); Mutation in the promoter region and SNP (**15*); SNP (**16, *21, *31*) |
| ***6, *7, *8, *9, *10, *11, *12, *13, *17, *18, *19, *22, *23, *24, *25, *35, *38, *39, *40, *41, *42, *43, *45** | Decreased activity | Gene conversion and SNP (**7, *8, *10, *12, *19, *24, *35*); Mutation in the promoter region (**9, *13*); SNP (**6, *11, *17, *18, *22, *23, *25, *38, *39, *40, *41, *42, *43, *45*) |
| ***2, *4, *5, *20, *26, *36, *37, *44** | No activity | Frameshift mutation (**20*); Gene conversion and SNP (**5, *36*); Gene deletion (**4*); SNP (**2, *26, *37, *44*) |
| ***1X2, *3, *27, *29, *30, *32, *33, *34** | No data on coumarin 7-hydroxylase activity[b] | Frameshift mutation (**27*); Gene conversion (**3, *34*); Gene duplication (**1X2*); To be released[c] (**29, *30, *32, *33*) |

[a] Activity was measured by heterologously expressed CYP2A6 in bacteria or cultured cell lines, human liver microsomes, or from human subjects.

[b] As of 2017, these CYP2A6 variants have not been tested for coumarin 7-hydroxylation. Some of these variants have been tested for nicotine metabolism, but nicotine c-oxidation activity and coumarin 7-hydroxylation activity of the same CYP2A6 variant do not always match. The coumarin 7-hydroxylation activity of CYP2A6*3 has not been tested, but studies have proposed that this allele results in an inactive enzyme because of the *CYP2A6* gene conversion with *CYP2A7* in exons 3, 6, and 8 (Fernandez-Salguero *et al.*, 1995; Chen *et al.*, 1999; Goodz and Tyndale, 2002; Hosono *et al.*, 2017). Similarly, Hosono *et al.* (2017) proposed that *27 and *34 would have no enzyme activity because of a frameshift mutation and gene conversion, respectively.

[c] According to the Human CYP Nomenclature Committee, these alleles have been identified by researchers but not yet published as of July, 2017. See http://www.cypalleles.ki.se/cyp2a6.htm

A detailed summary of the ability of individual CYP2A6 allele variants to catalyze 7-hydroxylation of coumarin is provided in Table B1 in Appendix B. Overall, there is consistency among studies reporting enzyme activity for specific CYP2A6 variants. Recently, Tanner *et al.* (2017) studied the correlation between several CYP2A6 allele variants (**2, *4, *7, *8, *9, *10, *12, *17, *20, *23, *25, *28, and *35*) and enzyme activity using human liver tissues from 360 donors of various ethnicities. They found a strong correlation between genotypes with one or more variant alleles and decreased CYP2A6 protein expression as well as coumarin 7-hydroxylation activity (Tanner *et al.*, 2017). This study also shows that, although the frequency of individual loss-of-function or

decrease-of-function alleles can be low, the proportion of variant allele carriers that are slow coumarin metabolizers can be significant.

Tanner *et al.* (2017) also observed a wide range of CYP2A6 mRNA expression, protein expression, and enzyme activity levels within the wild-type (**1/*1*) group. This variation in the coumarin 7-hydroxylation phenotype in *CYP2A6* wild-type individuals is consistent with observations from other studies. For example, in a group of Thai individuals, there were 17 poor coumarin 7-hydroxylators among the 55 wild-type (*CYP2A6*1A/*1A*) individuals (Ujjin *et al*., 2002). The variation of enzyme activity that was unaccounted for by the genotype was possibly due to unknown (or unassessed) genetic variations in this gene or upstream regulatory genes, or non-genetic factors. Overall, the collection of evidence shows that individuals with loss-of-function or decrease-of-function *CYP2A6* alleles are probably poor metabolizers for coumarin 7-hydroxylation, and in these individuals the metabolism of coumarin could shift towards increased production of the reactive metabolites CE and *o*-HPA.

**Distribution of *CYP2A6* alleles in different ethnicities and populations**

*CYP2A6* genetic variation directly alters the enzymatic activity of the protein and is therefore important for evaluating individual susceptibility to toxicants that are CYP2A6 substrates, such as coumarin. *CYP2A6* shows genotypic polymorphisms in populations across the world. Knowledge of the allele frequencies within different ethnic populations does not directly predict the genotype frequencies, but it does provide information on the potential for poor-metabolizer genotypes (homozygotes with two inactive alleles or heterozygotes with one inactive allele and one intermediate allele) to exist in those populations. We have summarized the findings on *CYP2A6* allele frequencies in different ethnicities and geographical areas (See Appendix B Table B2), focusing on variants with loss-of-function (*CYP2A6*2*, **4*, **5*, and **20*) and the three most studied decrease-of-function variants (*CYP2A6*7*, **9*, and **10*).

Table 17 summarizes the detailed findings from Appendix B Table B2 and presents them as ranges in each population/ethnicity. The results show that certain ethnic populations carry significant frequencies of some decrease-of-function or loss-of-function alleles.

The frequency of *CYP2A6*4*, an allele that results in an absence of functional enzyme in homozygous individuals, is elevated in East and Southeast Asian populations. The highest frequency of *CYP2A6*4* appears to be in the Japanese population (the frequency varies from 16% to 24.6%, based on 12 studies). The frequency of this allele is also high in Asian Americans (15.3% for a group of Asian Americans with unspecified

lineage, 6.6% in a group of Chinese Americans, and 22.2% in a group of Japanese Americans). One study showed that there is considerable variation among four different ethnic groups in China, with the frequency of *CYP2A6*4* ranging from 0 - 15% (Pang *et al.*, 2015). East or Southeast Asians also have elevated frequencies of the *CYP2A6*7, CYP2A6*9 and CYP2A6*10* alleles, which are decrease-of-function variants. These populations would be expected to be more susceptible to coumarin-induced toxicity because of the reduced capacity of the coumarin 7-hydroxylation detoxification pathway. In South Asian and Middle Eastern populations, *CYP2A6*4* is present, but at a lower frequency than in East Asians.

In general, Caucasians from Europe and North America carry relatively low levels of these seven loss-of-function and decrease-of-function alleles. *CYP2A6*9* seems to be present in most of the populations tested, and at higher frequencies in certain populations (*e.g.*, 16.4% in Mexicans and up to 23.6% in Asians). Lower frequencies of these four loss-of-function and three decrease-of-function polymorphisms were found in African populations, with no detection of *CYP2A6*5*, *CYP2A6*7*, or *CYP2A6*10*, and very low frequencies of *CYP2A6*2* reported.

The presence of other decrease-of-function alleles has been assessed in various ethnicities/populations. For example, a decrease-of-function allele, *CYP2A6*12,* was found in Caucasian Americans (1.9-2.4%), Iranians (1.3%), and Mexicans (3.5%) (Audrain-McGovern *et al.*, 2007; Emamghoreishi *et al.*, 2008; Bloom *et al.*, 2011; Borrego-Soto *et al.*, 2015). The decrease-of-function alleles *CYP2A6*17* and *CYP2A6*23* appeared to be unique to populations of African descent (Fukami *et al.*, 2004; Ho *et al.*, 2008; Adehin *et al.*, 2017), and the decrease-of-function allele *CYP2A6*35* appears to be more common in populations of African descent than other populations (Al Koudsi *et al.*, 2009).

**Table 17. Frequencies of several loss-of-function or decrease-of-function *CYP2A6* alleles in different ethnicities and populations[a]**

| Continent | Ethnicity/Population | Loss-of-function alleles | | | | Decrease-of-function alleles | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ***2*** | ***4*** | ***5*** | ***20*** | ***7*** | ***9*** | ***10*** | **others** |
| Africa | African[b] | 0-0.6% | 0-1.9% | 0 | | 0 | 2.8-11.4% | 0 | 12.5% (**17*) |
| Asia | East or Southeast Asian[c] | 0 | 0-24.6% | 0-14.6% | 0 | 0-19.6% | 10.4-23.6% | 1.0-4.3% | 0.4% (**6*), 1.45-5% (**8*), 0.6% (**11*), 0 (**24*), 0.5-0.8% (**35*) |
| | South Asian[d] | 0.3-1% | 1.4-11.3% | 0.7-0.9% | | 0 | | 0 | 0.9% (**8*) |
| | Middle Eastern[e] | 0-2.2% | 1.0-2.5% | | | | 6.9-12.4% | | 1.3% (**12*) |
| Europe | European[f] | 0.5-3.0% | 0.3-16.9% | 0-0.3% | | | 5.2-8.2% | | |
| North America | African North American | 0-1.1% | 0.5-2.7% | 0 | 1.1-1.7% | 0 | 7.1-9.6% | 0 | 7.3% (**17*), 2.0% (**23*), 0.7% (**24*), 2.5-2.9% (**35*) |
| | Indigenous Peoples in Alaska | 0.4% | 14.5% | | | 0 | 8.9% | 1.9% | |
| | Asian North American | 0 | 6.6-22.2% | | | 5.7-12.5% | | 3.2-4.3% | |
| | Indigenous Peoples in Canada | 0-0.9% | 1.0% | 0.5% | | 0 | 15.5% | 0 | |
| | Caucasian North American | 1.1-5.3% | 0-3.0% | 0-0.1% | 0 | 0-0.3% | 6.1-8.0% | 0 | 1.9-2.4% (**12*), 0 (**24*), 0 (**35*) |
| | Mexican | | | | | | 16.4% | | 3.5% (**12*) |
| South America | South American[g] | 1.7-2.0% | 0.5-7.1% | 0 | | | 5.7-10.3% | | |
| Oceania | New Zealand (Māori) | | 9.6% | | | 1.1% | 19.0% | | |

[a] The range of frequencies for each allele comes from a unique collection of studies on that allele. For more detailed information and references on the frequencies of the **2, *4, *5, *20, *7, *9,* and **10* alleles in different populations, see Appendix B Table B2. References for the other alleles listed in this table are: **6* (Kitagawa *et al.*, 2001), **8* (Nurfadhlina *et al.*, 2006), **11* (Fujieda *et al.*, 2004), **12* (Audrain-McGovern *et al.*, 2007;

Emamghoreishi *et al.*, 2008; Bloom *et al.*, 2011; Borrego-Soto *et al.*, 2015), **17* (Adehin *et al.*, 2017), **23* (Ho *et al.*, 2008), **24* (Al Koudsi *et al.*, 2009), and **35* (Al Koudsi *et al.*, 2009).

[b] Data for the African populations includes data from Ethiopian, Ghanaian, Namibian Ovambo, and Nigerian populations. The available data for each allele may come from a subset of these populations.

[c] Data for the East or Southeast Asian populations includes data from Chinese, Chinese Malaysian, Japanese, Korean, Malay, Taiwanese, Thai, and Vietnamese populations. The available data for each allele may come from a subset of these populations.

[d] Data for the South Asian populations includes data from Bangladeshi, Indian, Indian Malaysian, and Sri Lankan populations. The available data for each allele may come from a subset of these populations.

[e] Data for the Middle Eastern populations includes data from Iranian and Turkish populations. The available data for each allele may come from a subset of these populations.

[f] Data for the European populations includes data from British, Finnish, French, German, Russian (Tatar), Serbian, Spanish, and Swedish populations. The available data for each allele may come from a subset of these populations.

[g] Data for South American populations includes data from Brazilian, Chilean, and Mestizo Ecuadorian populations. The available data for each allele may come from a subset of these populations.

The allele frequencies mentioned above show that there is great variability of the *CYP2A6* genotype in populations throughout the world. The number of carriers of loss-of-function or decrease-of-function alleles can be significant, indicating that a significant number of individuals are poor metabolizers of coumarin (*i.e.*, poor 7-hydroxylators), and are thus more susceptible to hepatotoxicity from products of the coumarin 3,4-epoxidation pathway, such as CE and *o*-HPA.

### *In vivo* CYP2A6 genotype-phenotype correlation studies using coumarin as the substrate in the Thai population

Correlation studies are direct evidence of the impact of the *CYP2A6* genotype on an individual's 7-hydroxylation (considered the main detoxifying pathway) of coumarin. Three such studies, all in the Thai population, have been summarized here (Ujjin *et al.*, 2002; Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009). The findings on *CYP2A6* genetic polymorphisms and urinary excretion of 7-HC from these studies are summarized in Table 18.

These studies examined the amount of 7-HC (measured together with its glucuronide conjugate) excreted in the urine after an oral dose of coumarin. The coumarin doses used by Ujjin *et al.* (2002), Peamkrasatam *et al.* (2006), and Mahavorasirikul *et al.* (2009) were 15 mg, 15 mg, and 5 mg, respectively, and the urine samples were collected three hours, eight hours and two hours after dosing, respectively. In another study, coumarin metabolism was tested in 10 healthy volunteers with different doses and time intervals (Rautio *et al.*, 1992). With doses of 5, 10 and 30 mg coumarin, the amount of 7-HC (measured together with its glucuronide conjugate) recovered in the first two hours comprised 80.6%, 80.1%, and 79.2%, respectively, of the total 7-HC excretion in eight hours. Two tests within one to three months in the same individuals showed that the two-hour 7-HC excretion test is repeatable and a stable representation of total excretion. These data demonstrated that two hours is sufficient time for the coumarin metabolism assay, and that there is very little difference among different doses below 30 mg.

**Table 18. *In vivo* CYP2A6 genotype-phenotype correlation studies using coumarin as the substrate in the Thai population**

| *CYP2A6* Genotype | Ujjin *et al.*, 2002 | | Peamkrasatam *et al.*, 2006 | | Mahavorasirikul *et al.*, 2009 | |
|---|---|---|---|---|---|---|
| | Relative 7-HC Excretion[a] | N[b] | Relative 7-HC Excretion[a] | N[b] | Relative 7-HC Excretion[a,c] | N[b] |
| ***1A/*1A*** | 100% | 55 | 100% | 14 | 100% | 20 |
| ***1A/*1B*** | 119% | 80 | 144% | 22 | 94% | 37 |
| ***1B/*1B*** | 80% | 31 | 109% | 9 | 90% | 31 |
| ***1A/*4*** | 87% | 12 | 63% (**1/*4*) | 23 | 73% (**1A/*4C*) | 20 |
| ***1B/*4*** | 108% | 10 | | | 72% (**1B/*4C*) | 8 |
| ***1A/*7*** | NT | | 82% (**1/*7*) | 5 | 99% | 16 |
| ***1B/*7*** | | | | | 116% | 4 |
| ***1A/*8*** | | | - | 0 | 68% | 1 |
| ***1B/*8*** | | | - | 0 | 106% | 1 |
| ***1A/*9*** | | | 97% (**1/*9*) | 19 | 100% | 14 |
| ***1B/*9*** | | | | | 94% | 22 |
| ***1A/*10*** | | | 81% (**1/*10*) | 4 | 82% | 4 |
| ***1B/*10*** | | | | | 56% | 3 |
| ***4/*4*** | 1% | 4 | 7% | 4 | 15% (**4C/*4C*) | 1 |
| ***4/*7*** | NT | | 41% | 1 | - | 0 |
| ***4/*9*** | | | 46% | 2 | 83% (**4C/*9*) | 6 |
| ***7/*7*** | | | 55% | 3 | - | 0 |
| ***7/*9*** | | | - | 0 | 85% | 4 |
| ***7/*10*** | | | - | 0 | 16% | 1 |
| ***8/*8*** | | | - | 0 | - | 0 |
| ***9/*9*** | | | 80% | 14 | - | 0 |
| ***9/*10*** | | | - | 0 | 81% | 1 |

NT, not tested

[a] Average excretion of 7-HC and its glucuronide conjugate, compared to the average excretion in wild-type genotype (*CYP2A6*1A/1A*) individuals.

[b] N, number of subjects of a particular genotype

[c] Data extracted from Figure 2 of Mahavorasirikul *et al.* (2009) with GetData Graph Digitizer (version 2.26.0.20).

The three studies summarized in Table 18 show some similarities. Wild-type individuals, including **1A/*1A*, **1A/*1B*, and **1B/*1B*, were expected to be extensive metabolizers of coumarin. However, there was considerable variation among the wild-

type individuals. For example, among the 55 individuals of the **1A/*1A* genotype in Ujjin *et al.* (2012), only 12 were extensive or very extensive metabolizers, 26 were moderate metabolizers, and 17 were actually poor metabolizers. Heterozygous individuals with one wild-type allele and one variant allele, such as **1A/*4*, **1B/*4*, **1A/*7*, **1B/*7*, etc., showed variable levels of coumarin 7-hydroxylase activity, ranging from intermediate to extensive (56%-116%). Individuals with the deletion allele **4* and any of the **7*, **8*, or **9* alleles were generally poor metabolizers, with the exception of six **4C/*9* individuals showing 83% activity from the Mahavorasirikul *et al.* (2009) study. As shown in Table 18, eight individuals homozygous for **4* excreted 1-7% 7-HC relative to wild-type, while one person with **4C/*4C* excreted 15%. The individuals with two decrease-of-function *CYP2A6* alleles produced 7-HC with reduced activity, ranging from 16% to 85%.

Interpretation of these results is limited by small numbers of subjects for several of the variant *CYP2A6* genotypes, and by the fact that many other *CYP2A6* alleles were discovered after these studies were completed. The presence of unknown/assessed *CYP2A6* variant alleles could have confounded the results, and likely contributed to the inter-individual variation in coumarin 7-hydroxylase activity reported among the subjects categorized as “wild-type”. In spite of these limitations, the data showed large differences in CYP2A6 activity across the different *CYP2A6* genotypes. CYP2A6 enzyme activity among subjects who are heterozygous for one functional allele and one defective allele varied considerably (see Table 18 and Appendix B Table B1). Besides the genetic (known and unknown) and non-genetic factors, background exposures to coumarin (*e.g.*, from diet, tobacco or fragrances, see Section 2.2) and 7-HC (also known as umbelliferone, a sunscreen ingredient also present in some foods, such as carrots and golden apples) may also have contributed to the reported variation in phenotype within certain *CPY2A6* genotypes. One study measured 7-HC in “blank” urine samples from 14 healthy individuals from Norway not administered coumarin, and found that background levels of 7-HC in the urine averaged 254 nanograms per milliliter (ng/ml) with a range of 0 - 2799 ng/ml (Hadidi *et al.*, 1997).

While these results provide information on identifying genotypes associated with poor-metabolizers (*i.e.*, reduced or no coumarin 7-hydroxylase activity), more studies are needed regarding the distribution of *CYP2A6* alleles and genotypes within specific populations, as well as the identification and characterization of presently unknown *CYP2A6* genetic variants in order to better predict the susceptibility of different populations to coumarin toxicity.

**Other factors that influence coumarin 7-hydroxylase activity of CYP2A6**

Besides genetic polymorphisms, other factors can influence the activity of CYP2A6. These include age, gender, diet, and drugs. These factors, along with uncharacterized genetic polymorphisms, likely explain the variation in activity measured in humans with the same genotype.

*Age*

There are different results regarding the effect of age on CYP2A6 activity. One study with a group of 153 Spanish individuals aged 18-57 years concluded that age should be controlled for in epidemiological studies looking at CYP2A6 activity; older people in that study (>40 years of age) were shown to have significantly higher CYP2A6 metabolic activity, as measured using caffeine as the substrate (Sinues *et al.*, 2008). In another study, Sotaniemi *et al.* (1996) reported that coumarin metabolism is slower in elderly people (> 65 years old) compared with young adults (< 25 years old). The urinary excretion of 7-HC two hours after a 5 mg oral dose of coumarin was 65-68.1% in the young subjects, and 44.8-46.5% in the older subjects (Sotaniemi *et al.*, 1996). Yet in a study consisting of 100 Turkish individuals (ages 19-56), no correlation was observed between age and urinary excretion of 7-HC (Iscan *et al.*, 1994). In a recent US study, the age of liver donors (0-87 years old) was weakly positively correlated with CYP2A6 activity using coumarin as the substrate (Spearman $r = 0.13$, $p < 0.05$) (Tanner *et al.*, 2017). The effect of age is probably masked by genetic polymorphisms and environmental factors.

*Gender*

The effect of gender on CYP2A6 activity has been investigated in different populations.

The amount of 7-HC excreted in the first 4 hours after administration was higher in females than males in a study from Finland (Rautio *et al.*, 1992). In a study of 100 Turkish individuals, females had significantly higher urinary 7-HC excretion after two hours compared with males (Iscan *et al.*, 1994). A Thai study with 101 females and 101 males showed that the average amount of 7-HC excreted by females three hours after dosing was 17% higher than males, and more female (N = 8) than male (N = 3) “very extensive metabolizers” were observed in the study (Ujjin *et al.*, 2002). “Very extensive metabolizers” refers to those individuals who excreted more than 96% of the administered coumarin as 7-HC (Xu *et al.*, 2002b). In a group of African Canadians, the CYP2A6 activity (measured by nicotine metabolism) was significantly higher in females than males when smoking was controlled for (Mwenifumbo *et al.*, 2007).

Other studies have shown marginally higher activities in females or no difference between the two genders. In a Thai study of 120 subjects, females showed marginally higher excretion of 7-HC (Peamkrasatam *et al.*, 2006). In a study consisting of 120 Chinese subjects, the females showed higher CYP2A6 activity than the males, though the mean ratio of 7-HC: coumarin dose for men vs. women (0.637 vs. 0.699, respectively) was not statistically significantly different. In a study with 50 Turkish subjects, again, there was no significant difference between male and female subjects in the percentage of dose excreted as 7-HC in an eight-hour time period (Cok *et al.*, 2001). A recent *in vitro* study using human liver bank samples from the US reported that the formation of 7-HC in 139 female samples was higher than the 197 male samples, although the difference was not statistically significant (Tanner *et al.*, 2017).

The use of oral contraceptives in women has also been correlated with increased CYP2A6 activity. For example, the clearance of nicotine was higher in women than in men, and the use of oral contraceptives further accelerated nicotine clearance by induction of CYP2A6 (Benowitz *et al.*, 2004). In a study with 178 Spanish subjects using caffeine as a CYP2A6 substrate, 26 women who were taking oral contraceptives had significantly higher CYP2A6 activity (based on measurement of caffeine metabolites in the urine) than either women who were not taking oral contraceptives or men (Sinues *et al.*, 2008). The women who were not taking oral contraceptives had higher CYP2A6 activity than men, but the increase was not significant.

Taken together, these data suggest that CYP2A6 activity is higher in women than men. The ability of some studies to detect a gender difference may have been blunted by the distribution of genotypic polymorphisms in the test subjects (*e.g.*, uneven occurrence of defective alleles in male and female subjects in the samples tested). In studies where a gender effect was observed, life style factors such as the use of oral contraceptives might have contributed to the higher level of activity seen in females.

*Diet*

Compounds present in vegetables and fruits have been shown to inhibit CYP2A6 activity. Grapefruit juice, a potent inhibitor of CYP3A4, also inhibits CYP2A6-mediated nicotine-to-cotinine metabolism (Hukkanen *et al.*, 2006) and inhibits the formation of 7-HC in humans (Merkel *et al.*, 1994; Runkel *et al.*, 1997). Celery extract has been shown to irreversibly inhibit the coumarin 7-hydroxylation activity of human CYP2A6 (Deng *et al.*, 2016). Phenethyl isothiocyanate, a constituent of cruciferous vegetables, competitively inhibited coumarin 7-hydroxylase activity with a Ki value of 18.2 ± 2.5 µM (Nakajima *et al.*, 2001). Additionally, many other organosulfuric compounds have been

shown to be potent inhibitors of CYP2A6 (Fujita and Kamataki, 2001). Two such compounds are dialkyl sulfide and dialkyl disulfate, which are present in garlic oil. Of these two compounds, dialkyl disulfate is a stronger inhibitor of CYP2A6 than dialkyl sulfide and acts in a mixed competitive/noncompetitive manner, with a Ki value of 2.13 µM (Fujita and Kamataki, 2001).

On the other hand, a study with 21 Finnish subjects on a strict, uncooked (raw) vegan diet and 20 omnivorous controls found no significant effect of the raw vegan diet on coumarin 7-hydroxylase activity, and no difference in the incidence of phenotypically-defined poor metabolizers between the two groups (Rauma *et al.*, 1996).

Thus, while certain compounds present in the diet have been shown to affect the metabolism of coumarin by CYP2A6, the overall effect of diet on coumarin metabolism is unclear, as diet can also affect the activity of other cytochrome P450 enzymes, such as CYP3A4.

*Drugs*

Studies have shown that certain pharmaceutical agents can inhibit coumarin 7-hydroxylation by CYP2A6. Individuals taking these medications are more susceptible to coumarin toxicity. Isoniazid, a drug used in the treatment and prophylaxis of tuberculosis, inhibits CYP2A6 coumarin 7-hydroxylation *in vitro* in a time- and concentration-dependent manner (Wen *et al.*, 2002). Similarly, valproic acid, an anti-epilepsy drug, inhibits CYP2A6 coumarin 7-hydroxylation activity *in vitro* in a time- and concentration-dependent manner (Wen *et al.*, 2001). In another study, hepatotoxicity as measured by abnormal liver function tests in patients taking valproate (a salt of valproic acid) was significantly higher in *CYP2A6*4/*4* (loss-of-function) ($p = 0.006$) and *CYP2A6*1/*4* ($p=0.035$) individuals, compared to patients with wild-type *CYP2A6*1/*1* (Zhao *et al.*, 2017). The odds ratio (OR) for hepatotoxicity in *CYP2A6*4/*4* individuals was 20.27 (95% Confidence Interval [CI] 2.38-172.62), and the OR for hepatotoxicity in *CYP2A6*1/*4* individuals was 2.46 (95% CI 1.01-5.69). These results indicate that patients with reduced or no CYP2A6 activity are more susceptible to hepatotoxicity induced by the CYP2A6 inhibitor valproate. It is reasonable to hypothesize that poor 7-hydroxylators of coumarin who are taking drugs such as valproic acid and are simultaneously exposed to coumarin, are particularly susceptible to liver toxicity induced by both chemicals.

*3.3.1.4 Summary*

In summary, coumarin is rapidly absorbed, metabolized, and excreted in humans and in animals. Human and animal metabolism of coumarin is qualitatively similar. Coumarin is metabolized to form a number of metabolic products, some of which have not yet been identified, through a number of different enzymatic pathways. 7-Hydroxylation and 3,4-epoxidation are the predominant coumarin metabolic pathways in humans and animals. The primary enzyme responsible for 7-hydroxylation of coumarin in humans is CYP2A6. Populations around the world carry certain allelic variants of CYP2A6 that are associated with either no enzyme function or reduced function, and can result in poor 7-hydroxylator phenotypes. When coumarin 7-hydroxylation by CYP2A6 is compromised, this can lead to increased metabolism through the 3,4-epoxidation pathway, and increased generation of the reactive electrophilic metabolites CE and *o*-HPA. The kinetics of these reactions and subsequent detoxification reactions, including conjugation of CE with glutathione, and metabolism of *o*-HPA to either *o*-HPE or *o*-HPAA, may determine the ultimate toxic effects of these metabolites.

### *3.3.2 Human hepatotoxicity*

In the 1980s and 1990s, coumarin was evaluated for the treatment of lymphedema and various types of cancer. In many of these studies, a fraction of subjects treated orally with coumarin presented symptoms of hepatotoxicity and elevated liver function tests (LFTs). The severity of hepatotoxicity ranged from abnormal liver function as observed in blood tests to jaundice, pruritus, nausea and/or vomiting, to severe liver damage. In the mid-1990s the Adverse Drug Reactions Advisory Committee (ADRAC) of Australia estimated that the incidence of hepatotoxicity was at least 34 cases per 10,000 users (0.34%), which is greater than the incidence for other known hepatotoxins (*e.g.*, flucloxacillin, which has approximately 0.7 cases per 10,000 users) (ADRAC, 1995). Other clinical trials of coumarin saw even higher incidences of hepatotoxicity. Some smaller clinical trials reported that up to 6% of the participants treated with coumarin developed elevated aminotransferase levels (defined as greater than double the upper limit of normal). Following these reports of hepatotoxic effects attributed to treatment, coumarin was banned in Australia in 1996 and France in 1997 (Poage *et al.*, 2015). Coumarin is not approved to be used as a prescription drug in the US. Only one human study, conducted in Germany (Burian *et al.*, 2003), investigated the association between coumarin-induced hepatotoxicity and CYP2A6 genotype. This study was limited by the small number of CYP2A6 allelic variants assessed (wild-type [*1] and three loss-of-function alleles [*2, *3, *4]), by the absence of individuals studied that were homozygous for loss-of-function alleles, and by the small number of heterozygous individuals who carried one copy of the wild-type allele and one copy of a loss-of-function allele (n = 6).

The following summarizes clinical trials and case reports of hepatotoxicity involving orally-administered coumarin.

Cox *et al.* (1989) conducted a clinical trial of 2,173 patients taking coumarin for a variety of diagnoses (chronic brucellosis, breast cancer, melanoma, advanced renal cell carcinoma, glioma, chronic infections, and chronic fatigue syndrome) in Ireland. All patients underwent medical examinations and LFTs every 3 months throughout the study. The dose of coumarin ranged from 25 to 2,000 mg daily (the majority received 50 mg/day for 2 years). Five patients developed elevated LFTs within 1 to 4 months that returned to normal while still taking coumarin (50 or 100 mg/d), of which the authors could not determine the significance. Eight patients developed elevated LFTs (including elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and/or bilirubin) within 1 to 8 months, which was attributed to coumarin treatment (25 to 1600 mg/d). LFTs returned to normal after coumarin was stopped. Seven of these individuals were re-treated with coumarin and developed elevated LFTs a second time, which again returned to normal after treatment was stopped (Cox *et al.*, 1989). This study was the basis for the incidence of 0.34% cited by ADRAC (1995).

Subsequent clinical studies observed additional cases of hepatotoxicity. Fifty-four patients in the US with advanced malignancies were treated with 400 to 7000 mg coumarin daily in combination with cimetidine. One patient receiving a daily dose of 5000 mg coumarin developed hepatotoxicity and elevated LFTs (ALT, AST, GGT [gamma-glutamyl transferase]). Treatment was discontinued after 14 months, and LFTs returned to normal three weeks later (Marshall *et al.*, 1991). In a clinical trial for treatment of lymphedema in the US, 9 of 140 patients presented with elevated LFTs, including AST, ALT, GGT, ALP, and/or bilirubin, after 2 to 6 months of treatment with 400 mg coumarin daily (Loprinzi *et al.*, 1997; Loprinzi *et al.*, 1999). LFTs returned to normal after coumarin was withdrawn. In a study in Spain, two of 77 patients treated with 90 or 135 mg coumarin daily developed increased ALT after 6 months, which returned to normal without requiring treatment discontinuation (Burgos *et al.*, 1999). In a different study, nine of 114 patients in Germany treated with 90 mg coumarin daily in combination with troxerutin presented with elevated LFTs within 16 weeks (Schmeck-Lindenau *et al.*, 2003). Three of these cases were assessed as possibly due to coumarin treatment, and one was assessed as probable.

A number of case reports describe patients who presented with elevated LFTs and/or hepatotoxicity following treatment with coumarin. A woman in Australia developed elevated LFTs and jaundice after 7 weeks of treatment (dose not specified). Coumarin was withdrawn at 10 weeks and she improved slightly, but continued to have abnormal

LFTs at 15 weeks (Beinssen, 1994). Two women in France were treated for lymphedema with 90 mg coumarin daily for 5 months, and developed increased ALT, which resolved after withdrawing coumarin (Koch *et al.*, 1997). In Denmark, a woman was treated for lymphedema with 90 mg coumarin daily, and developed elevated bilirubin, ALP and AST (Faurschou, 1982). AST returned to normal 2 weeks after treatment withdrawal, and bilirubin and ALP returned to normal after 14 weeks. A male was treated with coumarin (dose not specified) for prostate cancer in the US, and presented with elevated AST, ALT, ALP and bilirubin and hepatic necrosis (Ghosh *et al.*, 1997). Withdrawal of coumarin resulted in the improvement of LFTs to normal after 9 weeks. A woman treated for lymphedema with 400 mg coumarin daily in the UK developed elevated bilirubin, ALT, and ALP (Morrison and Welsby, 1995). LFTs returned to normal 5 weeks after discontinuation of coumarin. A woman in Sri Lanka who was treated with coumarin (dose not specified) for lymphedema, presented with increased AST, ALT, ALP, and bilirubin. LFTs returned to normal 2 months after discontinuation of coumarin therapy (Satarasinghe and Jayawardana, 2009).

A few cases resulted in severe liver toxicity. A case report described a 65-year-old woman in Australia who presented with lethargy, anorexia, nausea and jaundice for three weeks. She had elevated bilirubin, ALT, and ALP. She had been taking 400 mg coumarin daily for 5 months and did not have risk factors for other causes of hepatotoxicity. She was admitted to the hospital in a coma, died 9 days later of hepatorenal failure, and showed massive hepatic necrosis on autopsy (Bassett and Dahlstrom, 1995). A survey of reported cases in France described 33 patients who had been taking 45 to 135 mg/d coumarin for an average of 1 year and presented with elevated ALT, bilirubin, jaundice, and/or severe pruritus (Andrejak *et al.*, 1998). Patients had been taking coumarin for 10 to 270 days before onset of hepatic injury. Twenty-nine of these patients fully recovered after withdrawal of coumarin. Andrejak *et al.* (1998) judged the effects to be likely or possibly due to coumarin treatment in 15 cases. Three of the cases developed hepatic damage that progressed to severe liver failure with encephalopathy. One of these had massive necrosis of the liver and required a liver transplant; the other two cases resulted in death. The dosage of one patient who died was 90 mg daily and unknown in the other two patients. Based on sales of coumarin, the authors estimated an incidence of 2 - 4 /10,000 for a mean duration of 12 months of treatment in France (Andrejak *et al.*, 1998).

There is one case report of a woman in the US who developed hepatotoxicity following self-medication with a cinnamon supplement that may contain high levels of coumarin. She had been taking rosuvastatin for many months prior without any adverse effects. After one week of taking the cinnamon supplement she presented to the emergency department with elevated AST, ALT, ALP, and GGT. Both cinnamon and the statin

were withdrawn and LFTs returned to normal. Rosuvastatin was restarted without additional adverse effects on the liver (Brancheau *et al.*, 2015).

A number of clinical trials in the literature did not report hepatotoxic effects. Forty-five renal cell carcinoma patients receiving 100 mg coumarin daily in combination with cimetidine in the US reported no liver toxicity (Marshall *et al.*, 1987a). Subsequent trials with similar dosing regimens also did not report hepatotoxicity, including clinical trials of 24 non-small cell lung cancer patients in the US (Marshall *et al.*, 1987b), 22 and 17 malignant melanoma patients in the US and Denmark, respectively (Marshall *et al.*, 1989; Nolte *et al.*, 1987; Pedersen *et al.*, 1987), 14 prostate cancer patients in the US (Marshall *et al.*, 1990), and 31 and 50 advanced renal cell carcinoma patients in Germany and the US, respectively (Herrmann *et al.*, 1990; Dexeus *et al.*, 1990). Dexeus *et al.* (1990) reported a mild increase in ALP in 7 patients, but did not report other LFTs and did not consider this to be a significant adverse effect). Three studies of 52 patients in Australia (Casley-Smith *et al.*, 1993b), 64 patients in China (Casley-Smith *et al.*, 1993c), and 30 patients in China (Chang *et al.*, 1996) treated with 400 mg daily did not report hepatotoxic effects. A study of 47 patients treated with 400 mg coumarin daily and 45 patients taking 400 mg coumarin daily in combination with diethylcarbamazine did not report liver toxicity (Casley-Smith *et al.*, 1993a). Many of these studies were limited by small treated groups and incomplete reporting of side effects. The majority of these studies did not monitor patients for LFTs.

A retrospective study by Iwata *et al.* (2016) investigated the effects of Japanese herbal medications (Kampo medicines) that commonly contain Cassia cinnamon bark (which is known to contain high levels of coumarin). This study quantitatively determined the coumarin content of Kampo preparations and cinnamon bark using HPLC. Of 129 patients, the authors estimated an average intake of 0.113 mg/kg/d. Twenty-three cases had abnormal LFTs, but the authors concluded that none of the cases were related to intake of cinnamon bark. It is of note that the estimated doses of coumarin from the herbal medications studied were considerably less than what patients received in the clinical trials reported above.

It is clear that a portion of the population (as high as 6.4% in US studies (Loprinzi *et al.*, 1997; Loprinzi *et al.*, 1999) is at risk for hepatotoxicity from pharmaceutical coumarin exposure. The above-mentioned reports of adverse effects resulted in the withdrawal of coumarin for use as a pharmaceutical in Australia, France, and the US. Hepatotoxicity does not appear to be simply dose-related; some patients who were taking lower levels of coumarin presented with severe liver damage, while others who were taking high levels did not develop elevated LFTs. The hepatotoxicity appears to occur in susceptible subjects, and is possibly related to genetic polymorphisms of CYP2A6

which result in decreased coumarin-7-hydroxylase activity. Farinola and Piller (2007) proposed the use of pharmacogenomics to identify patients who are poor CYP2A6 metabolizers to reduce the incidence of toxicity in patients being treated for lymphedema.

The only study available in the literature that genotyped patients taking coumarin was Burian *et al.* (2003). This study is of limited relevance since it did not test for all allele variants, and it was conducted in a homogenous population with no homozygous individuals carrying two copies of the variant alleles. This study genotyped 104 German patients receiving coumarin (90 mg/d) and troxerutin (540 mg/d) from the clinical trial conducted by Schmeck-Lindenau *et al.* (2003) to determine if susceptibility to coumarin-induced hepatotoxicity is determined by a polymorphism in CYP2A6. The authors tested for three loss-of-function *CYP2A6* alleles, namely *CYP2A6*2*, *CYP2A6*3*, and *CYP2A6*4*. Of the 104 patients, 98 had the wild-type genotype *CYP2A6*1/*1,* and 6 had one copy of the variant allele and one copy of the wild-type allele. It is possible that the individuals marked as wild-type in this study actually carried decrease-of-function or loss-of-function alleles the authors did not test for. Eight of the nine patients who exhibited elevated LFTs carried the wild-type alleles. The ninth patient carried one copy of the *CYP2A6*2* allele. There was no statistically significant difference in incidence of hepatotoxicity between carriers of a single loss-of-function *CYP2A6* allele and those homozygous for the wild-type allele (Burian *et al.*, 2003).

In summary, a number of clinical trials and case reports indicate that coumarin causes hepatotoxicity in susceptible individuals. The majority of cases of hepatotoxicity occurred at doses that ranged from 45 to 400 mg/d (two occurred at doses of 1600 and 5000 mg/d). Given that hepatotoxicity and liver tumors have been observed in rats and mice treated with coumarin, it is important to consider the potential consequences of liver damage in humans following coumarin exposure. Hepatotoxicity caused by coumarin can lead to extensive liver damage, including necrosis, which has pro-inflammatory and tumor-promoting potential (Hanahan and Weinberg, 2011). However, additional factors may also play a role in potential carcinogenesis. Susceptible individuals who are poor 7-hydroxylators may form higher amounts of the electrophilic metabolites CE and *o*-HPA. These compounds may also conjugate with glutathione or bind covalently to cellular macromolecules resulting in hepatotoxicity (Fentem *et al*., 1991). A reduction or depletion of the reduced glutathione (GSH) pool may shift the redox balance and impact overall ability to detoxify additional reactive species (*e.g.*, reactive oxygen species [ROS]), leading to oxidative stress. Thus, multiple potential risk factors, including deficient CYP2A6 activity, liver damage, glutathione depletion, and covalent binding to cellular macromolecules could contribute to the development of cancer.

### *3.3.3 Genotoxicity*

The genotoxicity of coumarin has been studied in a variety of assay systems, including bacteria and fungi, *in vitro* systems of plant cells, mammalian cells, and human liver slices, cell-free systems, and *in vivo* in insects, mice, and rats. Endpoints assessed include gene mutations, chromosomal damage [micronuclei (MN), chromosomal aberrations (CA), sister chromatid exchange (SCE)], chromosome instability [*e.g.*, deletions], and DNA damage and other effects [DNA strand breaks, DNA repair, binding to DNA, unscheduled DNA synthesis (UDS)]. The findings from these assays are summarized in Tables 19-23.

The coumarin metabolites *o*-HPAA, 7-HC, 6,7-DiHC and 3,4-DHC have been studied in a limited number of genotoxicity assays for a limited number of endpoints. Specifically, both *o*-HPAA and 7-HC have been studied in bacteria (gene mutation) and in mammalian cells *in vitro* (CA, UDS); in addition, 7-HC has been studied in one *E. coli* DNA repair assay and one cell-free system for DNA adduct formation; 6,7-DiHC has been studied in bacteria (gene mutation), human cells *in vitro* (DNA strand breaks, MN), and mice *in vivo* (DNA strand breaks, MN), and 3,4-DHC has been studied in bacteria (gene mutation), in mammalian cells *in vitro* (CA, SCE), and mice *in vivo* (MN). The findings from these assays are summarized in Tables 24-26.

#### *3.3.2.1 Studies of coumarin*

As shown in Table 19, coumarin has been shown to be mutagenic in the presence of rat, mouse, or hamster liver S9 in the *Salmonella typhimurium* reverse mutation assay in strain TA100, which detects base-pair substitution mutations, in multiple studies (Stoltz and Scott, 1980; Norman and Wood, 1981; Haworth *et al.*, 1983). When tested in a series of *S. typhimurium* strains derived from TA100 that detect specific base pair substitutions, Gee *et al.* (1998) found that coumarin was positive in the presence of rat liver S9 in strain TA7002, which detects T:A —>A:T mutations. Coumarin was also found to inhibit DNA excision repair in two strains of *Escherichia coli* (Grigg, 1972), and to induce chromosome instability (deletions) in *Aspergillus nidulans* (Majerfeld and Roper, 1978). Coumarin did not induce reverse mutations in other strains of *S. typhimurium*, or DNA damage in the SOS chromotest in *E. coli* (see Table 19).

**Table 19. Genotoxicity studies of coumarin in *S. typhimurium, E. coli* and *A. nidulans***

| Strain | Concentration Tested | Activation System | Results[1] | | Reference |
|---|---|---|---|---|---|
| | | | -S9 | +S9 | |
| Gene Mutation in *S. typhimurium* | | | | | |
| TA98 | 3 µmol/plate | Rat liver S9, Aroclor 1254 or methylcholanthrene induced | - | - | Florin *et al.* (1980) |
| TA100 | | | - | - | |
| TA1535 | | | - | - | |
| TA1537 | | | - | - | |
| TA98 | 5 or 10 µm/plate | Rat liver homogenate, Aroclor 1254 induced | - | - | Stoltz and Scott (1980) [Abstract only] |
| TA100 | | | - | + | |
| TA1535 | | | - | - | |
| TA1537 | | | - | - | |
| TA1538 | | | - | - | |
| TA98 | 5, 50 or 500 µg/plate | Rat or mouse liver S9, Aroclor induced | - | - | Norman and Wood (1981) [Abstract only] |
| TA100 | | | - | + | |
| TA1535 | | | - | - | |
| TA1537 | | | - | - | |
| TA98 | 33-3333 µg/plate in pre-incubation assay | 10% rat liver S9, Aroclor 1254 induced | - | - | Haworth *et al.* (1983); NTP (1993a) |
| | | 10% hamster liver S9, Aroclor 1254 induced | - | - | |
| TA100 | | 10% rat liver S9, Aroclor 1254 induced | - | (+) | |
| | | 10% hamster liver S9, Aroclor 1254 induced | - | + | |
| TA1535 | | 10% rat liver S9, Aroclor 1254 induced | - | - | |
| | | 10% hamster liver S9, Aroclor 1254 induced | - | - | |
| TA1537 | | 10% rat liver S9, Aroclor 1254 induced | - | - | |
| | | 10% hamster liver S9, Aroclor 1254 induced | - | - | |
| TA98 | Up to 1000 µg/plate in a liquid fluctuation assay in microtiter plates | 4.9% rat liver S9, Aroclor 1254 induced | - | - | Gee *et al*. (1998) |
| TA1537 | | | - | - | |
| TA7001[2] | | | - | - | |
| TA7002[2,3] | | | - | + | |
| TA7003[2] | | | - | - | |
| TA7004[4] | | | - | - | |
| TA005[4] | | | - | - | |
| TA7006[4] | | | - | - | |
| Mix[5] | | | - | - | |
| TA98 | 1000, 3000, or 5000 µg/plate | Rat liver S9, Aroclor 1254 induced | - | - | Kanode *et al.* (2017) |
| TA1537 | | | - | - | |
| DNA Damage in *E. coli* | | | | | |
| PQ37[6] | 0-250 µg | Rat liver S9, Aroclor 1254 induced | - | - | Kevekordes *et al.* (1999) |
| Inhibition of DNA Excision Repair in *E. coli* | | | | | |
| Wp-2 | 0 - 4 mM | None | + | | Grigg (1972) |
| B | 0 - 4 mM | None | + | | |
| Chromosome instability (deletions) in *A. nidulans* | | | | | |
| A | 0 – 2 mM | None | + | | Majerfeld and Roper (1978) |
| B | | | + | | |

[1] +: positive; (+): weakly positive; -: negative
[2] Detects base changes at A:T base pairs
[3] Mutation detected: T:A → A:T
[4] Detects base changes at G:C base pairs
[5] Mix: mixture of the base specific strains (TA7001-TA7006)
[6] SOS chromotest

As shown in Table 20, coumarin causes DNA and chromosomal damage in human cells, other mammalian cells, and plant cells *in vitro*, and binds to calf thymus DNA (both single-stranded and double-stranded DNA).

Specifically, coumarin induced micronucleus formation (MN) in human lymphocytes in the presence of rat liver S9 (Kevekordes *et al.*, 2001), and in two studies with the Hep-G2 human hepatoma cell line (Sanyal *et al.*, 1997; Kevekordes *et al.,* 2001). In Chinese hamster ovary (CHO) cells, coumarin induced chromosomal aberrations (CA) in the presence of rat liver S9, and sister chromatid exchange (SCE) in the absence of rat liver S9 (Galloway *et al.*, 1987; NTP, 1993a). In studies with *Allium cepa* (onion) root tip cells, coumarin induced CA in two studies (Ostergren, 1948; Sharma *et al.*, 1963), and SCE in another (Quercioli *et al*., 1954 as reviewed by Grigg, 1977). Coumarin binds to calf thymus DNA (Grigg *et al.*, 1971; Sarwar *et al.*, 2015). In the studies by Grigg *et al*. (1971), coumarin was shown to bind to both single-stranded and double-stranded DNA. In the studies by Sarwar *et al*. (2015) the mode of binding of coumarin with calf thymus DNA was investigated through various biophysical techniques, including iodide-induced quenching, competitive binding assays with ethidium bromide, acridine orange, and Hoechst 33258, and *in silico* molecular docking studies. The authors concluded that coumarin possibly binds to the minor groove of DNA, likely by forming hydrogen bonds.

Coumarin did not induce unscheduled DNA synthesis (UDS) in human liver slices, gene mutations in CHO cells, or MN in primary rat hepatocytes (See Table 20).

**Table 20. *In vitro* genotoxicity studies of coumarin in mammalian cells, plant cells and cell-free systems**

| Test Endpoint/ System | Species/ Cell Type | Concentration Tested (LED or HID) | Activation system | Results | | Reference |
|---|---|---|---|---|---|---|
| | | | | -S9 | +S9 | |
| **Gene Mutation (mammalian cells)** | | | | | | |
| Mutation in *Hprt locus* | $K_1BH_4$ Chinese hamster ovary (CHO) cells | 500 µM | Chick embryo liver S9 | NT | - | Goeger *et al.* (1998) |
| | | 500 µM | Rat liver S9 | NT | - | |
| | | 500 µM | Human liver S9 | NT | - | Goeger *et al.* (1999) |
| Mutation in *Gpt locus* | AS52 CHO cells | 5 mM | Chick embryo liver S9 | NT | - | Goeger *et al.* (1998) |
| | | 5 mM | Rat liver S9 | NT | - | |
| **Unscheduled DNA Synthesis (mammalian cells)** | | | | | | |
| Unscheduled DNA synthesis (UDS) | Human liver slices | 5 mM | Endogenous | - | | Beamand *et al.* (1998) |
| **Chromosome Damage (mammalian cells)** | | | | | | |
| Micronucleus (MN) formation | Primary rat hepatocytes | 0.5 mM | Endogenous | - | | Muller-Tegethoff *et al*. (1995) |
| | Human hepatoma cell line Hep-G2 | 500 µg/ml | Endogenous | + | | Sanyal *et al*. (1997) |
| | Human lymphocytes | 400 µM (-S9); 50 µM (+S9) | Rat liver S9 | - | + | Kevekordes *et al.* (2001) |
| | Human hepatoma cell line Hep-G2 | 50 µM | Endogenous | + | | |
| Chromosomal aberrations (CA) | CHO cells | 500 µg/ml (-S9);1600 µg/ml (+S9) | Rat liver S9, Aroclor 1254 induced | - | + | NTP (1993a); Galloway *et al.* (1987) |
| Sister chromatid exchange (SCE) | Chinese hamster ovary cells | 100 µg/ml (-S9); 1600 µg/ml (+S9) | Rat liver S9, Aroclor 1254 induced | + | - | NTP (1993a); Galloway *et al*. (1987) |
| **Chromosome Damage (plant cells)** | | | | | | |
| CA | *Allium cepa* (onion) root tip cells | ≤ 1 mM | None | + | | Ostergren (1948) |
| | Healthy young onion root tip cells | 0.02% | None | + | | Sharma *et al.* (1963) |
| SCE | Onion root tip cells | ≤ 1 mM | None | + | | Quercioli *et al.* (1954), reviewed by Grigg (1977) |
| **DNA Binding (cell-free)** | | | | | | |
| Single strand DNA binding | Calf thymus DNA | 20 µM | None | + | | Grigg *et al.* (1971) |
| Double strand DNA Binding | Calf thymus DNA | 20 µM | None | + | | |
| DNA Binding | Calf thymus DNA | 50 µM | None | + | | Sarwar *et al*. (2015) |

*Hprt locus*: hypoxanthine-guanine phosphoribosyltransferase
*Gpt locus*: xanthine-guanine phosphoribosyltransferase
NT: Not tested
LED: lowest effective dose; HID: highest ineffective dose

Costa Rde *et al.* (2008) investigated the genotoxicity of an infusion (prepared in phosphate-buffered saline) and an ethanol extract (80% ethanol) of the South American medicinal plant *Mikania glomerata*, known as "guaco", which contains coumarin. As shown in Table 21 both the coumarin-containing infusion and the ethanol extract induced DNA strand breaks in the comet assay in rat hepatoma cells. The infusion also induced MN in rat hepatoma cells, but the ethanol extract did not.

**Table 21. *In vitro* genotoxicity studies of plant extracts containing coumarin in mammalian cells**

| Test Endpoint/ System | Species/ Cell Type | Concentration Tested (LED or HID) | Activation system | Results | Reference |
|---|---|---|---|---|---|
| DNA Damage | | | | | |
| DNA Strand Breaks (Comet Assay) | Rat hepatoma cells | Infusion of *Mikania glomerata* leaves (0.55 µg/ml coumarin) | Endogenous | + | Costa Rde *et al.* (2008) |
| | | Ethanol extract of *Mikania glomerata* leaves by simple maceration (15 µg/ml coumarin) | Endogenous | + | |
| Chromosome Damage | | | | | |
| MN formation | Rat hepatoma cells | Infusion of *Mikania glomerata* leaves (1.10 µg/ml coumarin) | Endogenous | + | Costa Rde *et al.* (2008) |
| | | Ethanol extract of *Mikania glomerata* leaves by simple maceration (30 µg/ml coumarin) | Endogenous | - | |

LED: lowest effective dose; HID: highest ineffective dose

As shown in Table 22, *in vivo* studies of coumarin have not observed genotoxicity. Specifically, coumarin did not induce sex-linked recessive lethal (SLRL) mutations in *Drosophila melanogaster* exposed to coumarin in feed as adults or larvae, or by injection as adults (Yoon *et al.*, 1985; NTP, 1993a). Coumarin did not increase MN formation in peripheral blood erythrocytes of male and female $B6C3F_1$ mice after 13 weeks of exposure (NTP, 1993a), bone marrow cells of male or female Swiss mice after a single gavage dose (Api, 2001), or bone marrow cells of male and female ICR mice after 6 daily gavage doses (Morris and Ward, 1992). Coumarin did not induce UDS in the hepatocytes of S-D rats following a single gavage dose (Edwards *et al.*, 2000), and covalent binding of [$^{14}$C] coumarin to DNA was not observed in the liver or kidney of S-D or Fischer 344 rats (Swenberg, 2003, as reviewed by EFSA, 2004).

**Table 22. *In vivo* genotoxicity studies of coumarin**

| Endpoint | Species/ Strain/Sex | Tissues Analyzed | Dose | Administration Route | Results | Reference |
|---|---|---|---|---|---|---|
| **Mutation** [sex-linked recessive lethal (SLRL) mutation] | *Drosophila melanogaster* (adults) | DNA in $F_2$[a] | 0, 70 ppm | Feeding | - | Yoon *et al.* (1985) ; NTP (1993a) |
| | *Drosophila melanogaster* (adults) | DNA in $F_2$[a] | 0, 500 µg/ml | Injection | - | |
| | *Drosophila melanogaster* (larvae) | DNA in $F_2$[a] | 0, 200, 194 ppm | Larval feeding | - | |
| **MN** | ICR mice, male & female | Bone marrow cells | 0, 65, 130 mg/kg/day, for 6 days | Gavage | - | Morris and Ward (1992) |
| | $B6C3F_1$ mice, male & female | Peripheral blood erythrocytes | 0-300 mg/kg, 5 days/week for 13 weeks | Gavage | - | NTP (1993a) |
| | Swiss mice, male & female | Bone marrow cells | 0, 50, 100, 200 mg/kg | Gavage (single dose) | - | Api (2001) |
| **UDS** | Sprague-Dawley rat, male | Hepatocytes | 0-320 mg/kg | Gavage (single dose) | - | Edwards *et al.* (2000) |
| **DNA covalent binding** | Sprague-Dawley rats, male | Liver and kidney | 0, 60, 120, 240 mg/kg | Gavage (single dose) | - | Swenberg (2003) as reviewed by EFSA (2004) |
| | Fischer 344 rats, male | Liver and kidney | 0, 25, 50, 100 mg/kg | Gavage (single dose) | - | |

[a] $F_2$: the second generation of the exposed parental generation ($F_0$).

### *3.3.3.2 Studies of coumarin metabolites*

As shown in Table 23, the coumarin metabolite 7-HC induced expression of ada, a gene associated with DNA repair, in *E. coli* (Kuo and Shankel, 1992). None of the four coumarin metabolites tested in the *S. typhimurium* reverse mutation assay (*i.e.*, *o*-HPAA, 7-HC, 6,7-DiHC, and 3,4-DHC) induced mutations in various *S. typhimurium* tester strains (with or without exogenous metabolic activation (S9)) (Microbiological Associates, 1993, as reviewed by Lake, 1999; Maistro *et al.*, 2015; NTP, 1993b).

**Table 23. Genotoxicity studies of coumarin metabolites in *S. typhimurium* and *E. coli***

| Strain | Concentration Tested | Activation System | Results | | Reference |
|---|---|---|---|---|---|
| | | | -S9 | +S9 | |
| *o*-HPAA | | | | | |
| Gene Mutation in *S. typhimurium* | | | | | |
| TA98 | 0-5000 µg/plate | Not reported | - | - | Microbiological Associates (1993), as reviewed by Lake, 1999 |
| TA100 | | | - | - | |
| TA1535 | | | - | - | |
| TA1537 | | | - | - | |
| TA1538 | | | - | - | |
| 7-Hydroxycoumarin (7-HC) | | | | | |
| Gene Mutation in *S. typhimurium* | | | | | |
| TA98 | 0-5000 µg/plate | Not reported | - | - | Microbiological Associates (1993), as reviewed by Lake, 1999 |
| TA100 | | | - | - | |
| TA1535 | | | - | - | |
| TA1537 | | | - | - | |
| TA1538 | | | - | - | |
| Induction of DNA Repair in *E. coli* | | | | | |
| K12 (fused to LacZ) | Not reported | Not reported | + | | Kuo and Shankel (1992) [Abstract only] |
| 6,7-Dihydroxycoumarin (6,7DiHC) | | | | | |
| Gene Mutation in *S. typhimurium* | | | | | |
| TA98 | 62.5, 125, 250, 500 and 750 µg/plate | 4% rat liver S9, Aroclor 1254 induced | - | - | Maistro *et al*. (2015) |
| TA100 | | | - | - | |
| TA102 | | | - | - | |
| TA97a | | | - | - | |
| 3,4-Dihydrocoumarin (3,4-DHC) | | | | | |
| Gene Mutation in *S. typhimurium* | | | | | |
| TA98 | 0-6,666 µg/plate | 10% rat liver S9, Aroclor 1254 induced | - | - | NTP (1993b) |
| TA98 | | 10% hamster liver S9, Aroclor 1254 induced | - | - | |
| TA100 | | 10% rat liver S9, Aroclor 1254 induced | - | - | |
| TA100 | | 10% hamster liver S9, Aroclor 1254 induced | - | - | |
| TA1535 | | 10% rat liver S9, Aroclor 1254 induced | - | - | |
| TA1535 | | 10% hamster liver S9, Aroclor 1254 induced | - | - | |
| TA1537 | | 10% rat liver S9, Aroclor 1254 induced | - | - | |
| TA1537 | | 10% hamster liver S9, Aroclor 1254 induced | - | - | |

As shown in Table 24, 7-HC was weakly positive in the absence of exogenous metablic activation in an assay for the induction of CA in CHO cells (Microbiological Associates, 1993, as reviewed by Lake *et al.*, 1999). Sun *et al.* (2016) found that incubation of 7-HC with oligodeoxyribonucleotides and photoirradiation at 350 nm results in the formation of

7-HC - DNA cycloadducts with thymine and cytosine, and DNA interstrand crosslinks. Cycloadduct formation was reversible with photoirradiation at 254 nm, however. The metabolite 3,4-DHC induced a dose-related increase in SCE in the absence of exogenous metabolic activation, as well as an increase in the presence of exogenous metabolic activation (which was dose-related in one of two replicate studies) (NTP, 1993b) (See Table 24).

Two *in vitro* mammalian genotoxicity studies have been reported to date for *o*-HPAA, and two for 3,4-DHC; each was negative. In addition, one negative *in vitro* mammalian genotoxicity study has been reported for 7-HC and 6,7-DiHC, respectively (See Table 24).

**Table 24. *In vitro* genotoxicity studies of coumarin metabolites in mammalian cells and cell-free systems**

| Test Endpoint/ System | Species/ Cell Type | Concentration Tested (LED or HID) | Activation system | Results[1] | | Reference |
|---|---|---|---|---|---|---|
| | | | | -S9 | +S9 | |
| *o*-HPAA | | | | | | |
| Chromosome Damage | | | | | | |
| CA | CHO cells | 5000 µg/ml | Not reported | - | - | Microbiological Associates (1993), as reviewed by Lake, 1999 |
| UDS | | | | | | |
| UDS | Rat hepatocytes | 150 µg/ml | Not reported | - | | Microbiological Associates (1993), as reviewed by Lake, 1999 |
| 7-HC | | | | | | |
| Chromosome Damage | | | | | | |
| CA | CHO cells | Not specified[2] | Not reported | (+) | - | Microbiological Associates (1993), as reviewed by Lake, 1999 |
| UDS | | | | | | |
| UDS | Rat hepatocytes | 150 µg/ml | Not reported | - | | Microbiological Associates (1993), as reviewed by Lake, 1999 |
| DNA Damage (cell-free) | | | | | | |
| DNA Cycloadducts/ Interstrand crosslinks | Oligodeoxy-ribonucleotides | 10 mM | Photo irradiation at 350 nm | + | | Sun *et al*. (2016) |
| 6,7-DiHC | | | | | | |
| DNA Damage | | | | | | |
| DNA Strand Breaks (Comet Assay) | Human peripheral blood lymphocytes | 32 µg/ml | None | - | | Maistro *et al.* (2015) |
| Chromosome Damage | | | | | | |
| MN formation | Human peripheral blood lymphocytes | 32 µg/ml | None | - | | Maistro *et al.* (2015) |
| 3,4-DHC | | | | | | |
| Chromosome Damage | | | | | | |
| CA | CHO cells | 500 µg/ml (-S9); 1600 µg/ml (+S9) | Rat liver S9, Aroclor 1254 induced | - | - | NTP (1993b) |
| SCE | CHO cells | 50 µg/ml (-S9); 1600 µg/ml (+S9) | Rat liver S9, Aroclor 1254 induced | + | + | NTP (1993b) |

LED: lowest effective dose; HID: highest ineffective dose

[1] +: positive; (+): weakly positive; -: negative
[2] "at high, toxic concentrations" (Lake, 1999)

As shown in Table 25, only two coumarin metabolites (6,7-DiHC and 3,4-DHC) have been tested for genotoxicity *in vivo*, and these studies have been negative. Specifically, 6,7-DiHC did not induce DNA strand breaks in Swiss albino mice in peripheral blood cells at 4 or 24 hours, or in liver, bone marrow or testicular cells at 24 hours after a single gavage dose, or MN in bone marrow cells at 24 or 48 hours after a single gavage dose (Marques *et al.*, 2015). 3,4-DHC did not induce MN in peripheral blood cells of B6C3F$_1$ mice following 13 weeks of exposure (NTP, 1993b).

**Table 25. *In vivo* genotoxicity studies of coumarin metabolites**

| Endpoint | Species /Strain/Sex | Tissues Analyzed | Dose | Administration Route | Results | Reference |
|---|---|---|---|---|---|---|
| **6, 7-DiHC** | | | | | | |
| **DNA Strand Breaks (Comet Assay)** | Swiss albino mice, male | Peripheral blood cells (4 hours) | 0, 25, 50 or 500 mg/kg bw | Gavage (single dose) | - | Marques *et al.* (2015) |
| | | Peripheral blood cells (24 hours) | | | - | |
| | | Liver (24 hours) | | | - | |
| | | Bone marrow (24 hours) | | | - | |
| | | Testicular (24 hours) | | | - | |
| **MN** | | Bone marrow (24 and 48 hours) | | | - | |
| **3,4-DHC** | | | | | | |
| **MN** | B6C3F$_1$ mice, male & female | Peripheral blood erythrocytes | 0, 400, 800 or 1600 mg/kg, 5 days/week for 13 weeks | Gavage | - | NTP (1993b) |

### *3.3.4 Cell transformation studies*

Cell transformation assays are designed to detect a change in the growth pattern of cells that is indicative of loss of contact inhibition, a phenotype that is characteristic of cancer cells.

Nashed and Brendel (1983) studied *in vitro* cell transformation of rat peritoneal cells from animals exposed to coumarin *in vivo*, using the Peritoneal Cell Test. This assay was developed as a short-term *in vivo-in vitro* alternative to rodent cancer bioassays, and the testing of coumarin was an early effort at assay validation. This test system was never widely used, and interpretation of results from use of this assay are uncertain. Coumarin was administered as a single gavage dose of 0, 7.5, 30 and 60 mg/kg bw to groups of ten male and female Wister rats. Six hours after dosing the

animals received 10 ml of a mitogenic medium by intraperitoneal (*i.p.*) injection to stimulate peritoneal cell proliferation. Animals received a second *i.p.* injection of mitogenic medium seven days after dosing. Two weeks after dosing, peritoneal cells were harvested and cultured for colony growth in soft agar for 7 days. Colony growth in soft agar is indicative of cell transformation (Macpherson, 1969; San *et al.*, 1979). Cultures were scored for colony size, colony frequency, and colony survival. Positive evidence of colony growth was observed in cultures from one out of 10 low- and high-dose males, one out of 10 mid-dose females and two out of 10 high-dose females, as compared to 2/10 male and 3/10 female positive controls (dimethylnitrosamine) and no evidence of colony growth in cultures from water controls of both sexes.

Milo *et al.* (1985) studied the inhibition of N-methyl-N-nitro-N-nitrosoguanidine (MNNG) or methylazoxymethanole acetate (MAMA) induced cell transformation of human fibroblasts by coumarin and other chemicals that inhibit the nuclear poly(ADP-ribose) polymerase system. Coumarin in combination with MNNG or MAMA inhibited the formation of transformed colonies at 69 µM (a non-toxic dose that inhibits transformation by 85-95%). A slight elevation in the number of transformed colonies per 50,000 cells (n = 13.0 ± 4) was observed with coumarin (69 µM) treatment alone.

### *3.3.5 Animal tumor pathology*

This section describes the relevant pathology details for the tumor types observed in the animal cancer bioassays of coumarin.

**Rats**

*Kidney*

Rare renal tubule tumors (adenomas and adenoma and carcinomas combined) were increased in male F344 rats treated with coumarin compared to controls, and three rare renal tubule adenomas were observed in female F344 rats treated with coumarin (NTP, 1993a). While renal tubule hyperplasia was observed in both control and treated male rats, the incidence of hyperplasia was statistically significantly increased in the low- and mid-dose groups compared to controls. Increases in hyperplasia were also observed in treated female rats, but the incidence was not statistically significantly different from controls. In discussing the renal tubule hyperplasia observed in treated male and female rats, NTP (1993a) states that it “was distinguished from the common regenerative epithelial changes commonly seen as a part of nephropathy and was considered a preneoplastic lesion. Hyperplasia, adenoma, and carcinoma were part of a morphological continuum and occurred in the cortex of the kidney.” NTP (1993a) defined the observed hyperplasia of the renal tubule epithelium as a single tubule filled

with normal or slightly enlarged epithelial cells. Renal tubule adenomas observed in these studies were characterized as “discrete, sometimes multinodular masses at least three times greater in diameter than an average tubule and composed of somewhat pleomorphic epithelial cells arranged in complex tubular structures and solid clusters” (NTP, 1993a). Renal tubule carcinomas were described as being larger than adenomas, with cellular pleomorphism, atypia, and central necrosis. Renal tubule adenomas and carcinomas are aggregated when evaluating study results (McConnell *et al.*, 1986).

Renal tubule oncocytomas were observed in male F344 rats treated with coumarin in both the continuous and the stop-exposure groups (NTP, 1993a). These lesions were morphologically distinct from renal tubule hyperplasia, adenoma, and carcinoma, but were also located in the cortex of the kidney (NTP, 1993a; Frazier *et al.*, 2012). The oncocytomas observed in coumarin-treated rats in this study were characterized as “small, discrete nodules of uniform cells with dense, hyperchromatic nuclei and granular eosinophilic cytoplasm” (NTP, 1993a). Oncocytomas are reported to arise in the collecting ducts from oncocytic hyperplasia, usually grow very slowly in rats, and are considered benign and uncommon in F344 rats (Bannasch, 1984; Nogueira and Bannasch, 1988; Montgomery and Seely, 1990). Oncocytomas are not observed (0/400) in NTP’s historical controls (NTP, 1999a).

*Liver*

Hepatocellular tumors (adenomas and carcinomas combined) were increased in male and female Sprague-Dawley rats treated with coumarin (Carlton *et al.*, 1996). Carlton *et al.* (1996) referred to these tumors as benign and malignant parenchymal tumors, which is an older term for hepatocellular tumors. Hepatocellular adenomas and carcinomas arise from the same cell type, and adenomas can progress to carcinomas. For this reason, these two tumor phenotypes are aggregated when evaluating study results (McConnell *et al.*, 1986).

Increased incidences of cholangiofibrosis were observed in coumarin-treated male S-D rats (Evans *et al.*, 1989; Carlton *et al.*, 1996), female S-D rats (Carlton *et al.*, 1996), male and female albino rats (Bär and Griepentrog, 1967; Griepentrog, 1973), and male and female Osborne-Mendel rats (Hagan *et al.*, 1967). Cholangiofibrosis is considered to be an early proliferative lesion on the continuum of proliferative lesions that progress to cholangiofibroma, and then to cholangiocarcinoma with time (Thoolen *et al.*, 2010; Hailey *et al.*, 2014). This lesion can be difficult to diagnose and is sometimes characterized as cholangiocarcinoma when there is not extensive involvement of the liver. Cholangiofibrosis is not considered to be a spontaneous lesion and is not typically seen in untreated rats (Thoolen *et al.*, 2010). Cholangiofibrosis was not observed in

Fischer rats in the NTP (1993a) studies; however, the average daily doses of coumarin were lower in the NTP Fischer rat studies than in several of the studies (all conducted in strains other than the Fischer rat) where coumarin induced cholangiofibrosis was observed (Evans *et al*., 1989, Carlton *et al*., 1996, Hagan *et al.*, 1967). Typically, fibrosis is observed in rats that have hepatotoxicity (Thoolen *et al.*, 2010).

Cholangiocarcinomas were observed in male and female Sprague-Dawley rats treated with coumarin (Carlton *et al.*, 1996). The authors did not report specific details on histopathology of these lesions. These bile duct tumors often metastasize and generally show invasive growth into blood vessels, lymph vessels, and connective tissue in the liver (Bannasch and Zerban, 1990). Cholangiocarcinomas are rare in Sprague-Dawley rats (Chandra *et al.*, 1992; McMartin *et al.*, 1992). These tumors usually develop after application of high doses of chemicals, cause marked necrosis in the liver parenchyma, are often associated with significant liver toxicity, and generally only occur in the presence of hepatocellular neoplasms (Hailey *et al.*, 2014). Carlton *et al.* (1996) does not report details on markers of liver toxicity or necrosis, but treated male and female Sprague-Dawley rats in the two highest dose groups did develop cholangiocarcinomas and hepatocellular tumors. Liver necrosis was evident in other studies in which rats were treated with coumarin (Evans *et al.*, 1989; NTP, 1993a).

**Mice**

*Lung*

Alveolar/bronchiolar adenomas and carcinomas were observed in male and female $B6C3F_1$ mice (NTP, 1993a) and male CD-1 mice (Carlton *et al*., 1996) treated with coumarin. The lung tumors observed in Carlton *et al*. (1996) were called pulmonary adenomas and adenocarcinomas, but they are typically referred to as alveolar/bronchiolar adenomas and carcinomas. According to the study authors, the increase was within the range of laboratory historical controls for age and strain of mice; however, numbers are not provided (Carlton *et al.*, 1996). These tumors may originate from alveolar Type II cells or Clara cells (Renne *et al.*, 2009). Alveolar/bronchiolar adenomas in mice are considered to have the potential to progress to carcinomas, and are aggregated when evaluating study results (McConnell *et al.*, 1986).

*Liver*

Hepatocellular tumors were observed in coumarin-treated female $B6C3F_1$ mice (adenomas and adenomas and carcinomas combined) (NTP, 1993a) and female CD-1 mice (adenomas and carcinomas combined) (Carlton *et al*., 1996). Carlton *et al.* (1996) referred to these tumors as benign and malignant parenchymal tumors, which is an older term for hepatocellular tumors (Harada *et al.*, 1999). In the NTP study, the

incidence of hepatocellular adenomas was increased in the low- and mid-dose B6C3F$_1$ females. An increased incidence of eosinophilic foci of the liver, which are morphologically similar to adenomas and are considered to be preneoplastic lesions in mice, was observed in low- and mid-dose females in the NTP study (NTP, 1993a). Hepatocellular adenomas arise from the same cell type as carcinomas and are considered to have the potential to progress to carcinomas. These two tumor phenotypes are aggregated when evaluating study results (McConnell *et al.*, 1986; Harada *et al.*, 1999).

*Forestomach*

Rare squamous cell carcinomas of the forestomach were observed in male and female mice treated with coumarin, and an increase in squamous cell papilloma and carcinoma combined occurred in low-dose males (NTP, 1993a). The squamous cell papillomas observed in these studies were described as consisting of thickened, folded epithelium with a fibrovascular core. Differentiation of the epithelium within the papillomas was normal and there were no atypical cellular changes. The squamous cell carcinomas consisted of cords of stratified squamous epithelium, which invaded the submucosa and muscularis (NTP, 1993a). Forestomach squamous cell papillomas are considered to have the potential to progress to carcinomas (McConnell *et al.*, 1986). In NTP studies conducted in B6C3F1 mice from 1984 to 1994, the spontaneous incidence of forestomach squamous cell carcinoma observed in controls receiving corn oil gavage was 3/464 (0.6%) in males and 0/463 (0%) in females (NTP, 1999b).

**Hamsters**

*Pancreas*

Pancreatic islet cell carcinomas were observed in two high-dose animals in the female Syrian golden hamster study by Ueno and Hirono (1981). Pancreatic islet cell tumors are uncommon in female hamsters (Schmidt and Hubbard, 1996).

### *3.3.6 Structure activity considerations*

Coumarin (2H-1-benzopyran-2-one; *cis*-*o*-coumarinic acid lactone; 1,2-benzopyrone; coumarinic anhydride) consists of an aromatic ring fused to a condensed lactone ring. OEHHA used Chemotyper (https://chemotyper.org/, accessed May 24, 2017), a tool available from the US EPA for searching and highlighting chemotypes (chemical structures or subgraphs), to identify chemicals that share structural similarities with coumarin. Eight structurally similar chemicals were chosen for structure activity comparison with coumarin, based on the following criteria: 1) presence of the basic

coumarin structure (1,2-benzopyrone), or a metabolite of coumarin; 2) no methoxy groups; 3) no halogen, nitrogen, or sulfur groups; 4) no additional aromatic rings; 5) testing for genotoxicity or animal carcinogenicity. The selected compounds were 3,4-DHC, 3-methylcoumarin, 4-methylcoumarin, 6-methylcoumarin, 6,7-DiHC, 7-HC, 7,8-dihydroxycoumarin, and 4-methyl-7-hydroxycoumarin. Information on the genotoxicity and carcinogenicity of each of the eight comparison chemicals is briefly described below and in Table 26. No human cancer epidemiology studies were identified for any of the eight comparison chemicals.

3,4-Dihydrocoumarin (3,4-DHC) is a metabolite of coumarin. It was tested in long-term carcinogenicity studies by NTP in male and female F344/N rats and $B6C3F_1$ mice (NTP, 1993b). In male rats, NTP concluded there was "some evidence" of carcinogenic activity based on increased incidences of renal tubule adenomas (1/44, 1/33, 3/43, and 6/35 in control, low-, mid-, and high-dose groups, respectively). The male rats were administered 3,4-DHC (>99% purity) in corn oil by gavage at doses of 0, 150, 300, or 600 mg/kg body weight per day, 5 days per week for up to 103 weeks. In female mice, NTP concluded there was "some evidence" of carcinogenic activity based on increased incidences of hepatocellular adenoma (10/48, 20/50, 22/50, 20/49) and hepatocellular adenoma or carcinoma (combined) (13/48, 21/50, 25/50, 24/49) (NTP, 1993b). The female mice were administered 3,4-DHC (>99% purity) in corn oil by gavage at doses of 0, 200, 400, or 800 mg/kg body weight per day, 5 days per week for up to 103 weeks. NTP concluded there was "no evidence" of carcinogenic activity observed in the studies in female rats and male mice.

3,4-DHC induced SCEs in CHO cells. It did not induce mutations in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 with and without metabolic activation (Haworth *et al.*, 1983), CAs in CHO cells, or MN in mice *in vivo* (NTP, 1993b).

3-Methylcoumarin and 4-methylcoumarin both induced CAs in *Allium cepa* (onion root tip) cells (D'Amato and D'Amato-Avanzi, 1954). No animal carcinogenicity studies were identified for either of these chemicals.

6-Methylcoumarin induced CAs in CHO cells (Kihlman *et al.*, 1971), CAs in *Allium cepa* cells in two studies (Ronchi and Arcara, 1967; Kihlman *et al.*, 1971), and was weakly mutagenic in *S. typhimurium* (HSDB, 2003). No tumors were observed following two years of dietary administration to male and female rats (Hagan *et al.*, 1967).

6,7-Dihydroxycoumarin (6,7-DiHC) is a metabolite of coumarin. It tested negative for mutagenicity in *S. typhimurium* strains TA98, TA100, and TA102, with and without rat liver S9 mix (Maistro *et al.*, 2015). It did not cause DNA damage in the comet assay in

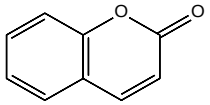
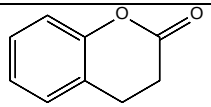
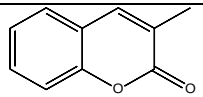
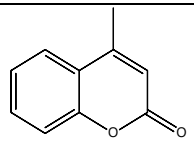
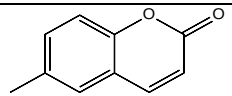
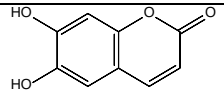
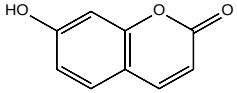
human peripheral blood lymphocytes or mouse cells *in vitro* and did not induce MN formation in human peripheral blood lymphocytes or mouse cells *in vitro* (NTP, 1993b; Maistro *et al.*, 2015; Marques *et al.*, 2015). No animal carcinogenicity studies were identified.

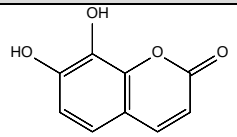
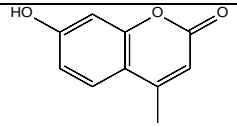
7-Hydroxycoumarin (umbelliferone; 7-HC) is a metabolite of coumarin. It induced the expression of the ada DNA repair gene in *E. coli* (Kuo and Shankel, 1992). 7-HC was weakly positive in the induction of CAs in CHO cells tested without S9 (Microbiological Associates, 1993b, as reported in Lake, 1999). It formed DNA cycloadducts with thymine and cytosine and DNA interstrand crosslinks after photoirradiation under cell-free conditions (Sun *et al.*, 2016). It did not induce UDS in rat hepatocytes *in vitro* or mutations in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 with or without metabolic activation (Microbiological Associates, 1993a, as reported in Lake, 1999). No animal carcinogenicity studies were identified.

7,8-Dihydroxycoumarin (daphnetin) has been demonstrated to bind to calf thymus DNA (Zhou *et al.*, 2015). No animal carcinogenicity studies were identified.

4-Methyl-7-hydroxycoumarin induced CAs in *Allium cepa* cells (D'Amato and D'Amato-Avanzi, 1954). It did not induce mutations in *S. typhimurium* (strains TA99, TA100, TA1535, and TA1537) with or without S9 activation (Haworth *et al.*, 1983). No animal carcinogenicity studies were identified.

**Table 26. Structure activity comparison between coumarin and eight structurally related chemicals**

| Chemical | Structure | Genotoxicity | | | Tumors in animal bioassays |
|---|---|---|---|---|---|
| | | Mutagenicity | Chromosomal effects | DNA damage/ DNA binding | |
| Coumarin |  | + *Salmonella* | + CA in CHO and onion root tip cells; MN in human cells; SCEs in CHO and onion root tip cells; Instability/deletions in *Aspergillus* | + Binding in calf thymus DNA; Inhibits excision repair | Kidney: male & female rats Liver: male & female rats & female mice Lung: male & female mice Forestomach: male mice[1] |
| 3,4-DHC (metabolite) |  | – | + SCEs in CHO cells | NT | Kidney: male rats Liver: female mice |
| 3-Methylcoumarin |  | NT | + CA in onion root tip cells | NT | NT |
| 4-Methylcoumarin |  | NT | + CA in onion root tip cells | NT | NT |
| 6-Methylcoumarin |  | + *Salmonella* | + CA in CHO and onion root tip cells | NT | –[2] |
| 6,7-DiHC (metabolite) |  | – | – | – | NT |
| 7-HC (metabolite) |  | – | (+) CA in CHO cells | + Induces DNA repair gene in *E. coli*; DNA cycloadducts/ interstrand crosslinks in cell free system | NT |

| Chemical | Structure | Genotoxicity | | | Tumors in animal bioassays |
|---|---|---|---|---|---|
| | | Mutagenicity | Chromosomal effects | DNA damage/ DNA binding | |
| 7,8-Dihydroxy-coumarin |  | NT | NT | + Binds to calf thymus DNA | NT |
| 4-Methyl-7-hydroxycoumarin |  | – | + CA in onion root tip cells | NT | NT |

+, positive; (+), weakly positive; –, negative; NT, not tested

[1] Observed in low-dose group

[2] Male and female

As summarized in Table 26, few of these chemicals have been tested for carcinogenicity in animals. Of the two chemicals that have been tested, 3,4-DHC induced tumors at similar sites to coumarin, including kidney tumors in rats and liver tumors in mice. One chemical induced mutations in *Salmonella*, several induced mutations in plant and mammalian cells, and two have the capability to bind to and/or damage DNA.

### *3.3.7 Toxicogenomic data*

This section summarizes the findings from studies that generated or analyzed toxicogenomic data on coumarin. These toxicogenomic data come from multiple research groups, and include gene expression data in mouse lung following exposure *in vivo* from one publication (Thomas *et al.*, 2007), in rat liver following exposure *in vivo* from five publications (Kienhuis *et al.*, 2006; Kiyosawa *et al.*, 2007; Uehara *et al.*, 2008a; Uehara *et al.*, 2008b; Eichner *et al.*, 2014), in rat primary hepatocytes exposed *in vitro* from two publications (Kienhuis *et al*., 2006; Uehara *et al*., 2008a), and in human primary hepatocytes exposed *in vitro* from two publications (Uehara *et al*., 2008a; Kienhuis *et al.*, 2009). An additional analysis by OEHHA of the *in vivo* rat liver toxicogenomic data from Uehara *et al*. (2008a) is also presented.

#### *3.3.7.1 Mouse lung in vivo (Thomas et al., 2007)*

Thomas *et al.* (2007) identified lung cancer biomarkers using microarray data from female $B6C3F_1$ mice. Thirteen diverse chemicals were chosen based on lung tumor findings in NTP carcinogenesis studies in female $B6C3F_1$ mice: seven lung carcinogens, including coumarin, and six non-carcinogens (*i.e.*, chemicals that did not induce lung tumors in female mice). The coumarin treatment group consisted of five female mice administered coumarin in corn oil by gavage at a dose of 200 mg/kg, 5 days per week for 13 weeks. The study also included appropriate vehicle controls. After 13 weeks, animals were sacrificed and the lung tissues collected for total RNA extraction and gene expression analysis with Affymetrix Mouse Genome 430 2.0 arrays.

A comparison of gene expression data from mice exposed to lung carcinogens and non-carcinogens revealed a total of 82 probe sets corresponding to 75 unique transcripts that were significantly altered (65 were up-regulated and 10 were down-regulated). A gene ontology (GO) analysis using DAVID (Database for Annotation, Visualization and Integrated Discovery) indicated that these gene expression changes are associated with several different biological processes categories (*e.g.*, glutathione metabolism and lipid metabolism) and molecular function categories (*e.g.*, glutathione transferase activity, oxidoreductase activity). The top six gene expression biomarkers identified as discriminating between lung carcinogens and non-carcinogens were from the following

genes: UDP-glucuronosyltransferase 1a (*Ugt1a*) family, carboxylesterase 1 (*Ces1*), fibroblast growth factor receptor 2 (*Fgfr2*), epoxide hydrolase 1, microsomal (*Ephx1*), glutathione S-transferase mu 1 (*Gstm1*), and an unannotated gene. Four gene products are enzymes involved in endogenous and xenobiotic metabolism. One gene product is a growth factor receptor involved in lung development. In validation studies, coumarin treatment was shown to result in significant changes in gene expression of two of these biomarkers (*Ces1* and *Ephx1*) in female mouse lung by qRT-PCR.

*3.3.7.2 Rat liver in vivo (Kienhuis et al., 2006; Kiyosawa et al., 2007; Uehara et al., 2008a; 2008b; Eichner et al., 2014)*

Kienhuis *et al.* (2006) reported the gene expression changes in 9- to 12-week-old male Wistar rats administered coumarin dissolved in corn oil by *i.p.* injection at 0, 17.5, 75, and 200 mg/kg (five animals per dose group). Animals were sacrificed 24 hours after dosing and liver samples were prepared for RNA extraction. RNA samples were extracted and labelled prior to hybridization for gene expression analysis with QIAGEN Operon oligonucleotide microarrays containing approximately 5800 different 70-mer oligonucleotide fragments. A total of 321 significantly altered genes and six pathways (*i.e.*, three metabolic-related pathways (methionine metabolism, tryptophan metabolism and fatty acid metabolism), γ-hexachlorocyclohexane degradation, complement and coagulation cascades and citrate cycle) were identified *in vivo*.

Kiyosawa *et al*. (2007) developed 161 glutathione depletion-responsive gene probe sets to identify chemicals that perturb glutathione homeostasis in rat liver. The authors grouped the probe sets into the following five categories: “antioxidant, phase II drug metabolizing enzymes and oxidative stress markers”, “transporter”, “metabolism”, “transcription factors and signal transduction-related and protein turnover-related genes”, and “miscellaneous”. The authors tested the usefulness of the glutathione depletion-responsive gene probe sets using several prototypical GSH depletors, including coumarin. Five six-week-old male Crj:CD(S-D)IGS rats were treated with a single dose of 150 mg/kg coumarin orally. Blood samples were collected at 3, 6, 9 and 24 hours after treatment to assay for markers of liver toxicity; no indicators of liver toxicity were observed in coumarin-treated animals. Animals were euthanized 24 hours after treatment, livers removed, and total RNA extracted for gene expression analysis with Affymetrix GeneChip RAE 230A probe arrays. Principal Component Analysis (PCA) was applied to the gene expression data using the glutathione depletion-responsive gene probe sets. Coumarin-treated rats showed the second most affected gene expression profile among the 15 chemicals studied (after bromobenzene, the most potent GSH-depletor). This is consistent with previous reports of reactive metabolites generated from coumarin oxidation in the liver being involved in coumarin-induced glutathione depletion (Lake, 1984; Lake *et al.*, 1989).

In the Uehara *et al.* (2008a) study, six-week-old male S-D rats were exposed to coumarin in corn oil by the oral route at 15, 50 or 150 mg/kg (five animals per dose) on days 1, 3, 7, 14, and 28. The rats were euthanized 24 hours after the last dose and liver samples were obtained immediately after sacrifice for total RNA extraction and gene expression analysis with Affymetrix GeneChip RAE 230A probe arrays. This study is part of the Genomics Assisted Toxicity Evaluation System, for the Toxicogenomics Project conducted in Japan (TG-GATEs).

Statistically differentially expressed genes were identified, including 136 up-regulated and 79 down-regulated probe sets related to glutathione metabolism and oxidative stress response. The most sensitive genes identified at the lowest dose were "aldo-keto reductase family 7, member A3" (*Akr7a3*), "NAD(P)H dehydrogenase, quinone 1 (*Nqo1*)", "glutathione reductase (*Gsr*)", "glutathione-S-transferase, pi 1/2 (*Gstp1/Gstp2*)", and "glutathione S-transferase Yc2 subunit. (*Gsta5*)". These five genes are involved in glutathione metabolism and cellular responses to oxidative stress.

Uehara *et al.* (2008b) applied a toxicogenomics approach to develop a Prediction Analysis of Microarray (PAM) classifier, consisting of 112 rat liver gene expression probe sets, to identify non-genotoxic hepatocarcinogens causing oxidative stress. Validation studies on the PAM classifier were conducted with 30 chemicals classified by the authors as either non-genotoxic rat liver carcinogens that cause oxidative stress, or non-carcinogens. Coumarin was included as one of the rat liver carcinogens. This study treated six-week-old male S-D rats orally with 150 mg/kg coumarin in corn oil using two dosing schemes: 1) a single dose administered, with sacrifices at either 3, 6, 9 or 24 hours after dosing; and 2) repeated doses administered daily for either 3, 7, 14 or 28 days with sacrifice 24 hours after the last dose (corresponding to days 4, 8, 15 and 29, respectively). Liver samples were obtained immediately after sacrifice for total RNA extraction and gene expression analysis with Affymetrix GeneChip RAE 230A probe arrays. The authors concluded that the PAM classifier correctly predicted coumarin to be a hepatocarcinogen causing oxidative stress, and noted that time-dependent increases in the PAM score were observed with coumarin treatment.

Eichner *et al.* (2014) developed two new approaches to select robust gene expression signatures to predict non-genotoxic carcinogens in rat liver using the TG-GATEs database (http://toxico.nibiohn.go.jp/english/). Rat liver gene expression data for two chemicals classified as genotoxic carcinogens, nine as non-genotoxic carcinogens (including coumarin), and 11 as non-carcinogens were analyzed using these approaches. Both approaches predicted that coumarin is a hepatocarcinogen. The top five genes incorporated into prediction models were phosphatidylinositol-3,4,5-

trisphosphate binding protein (*Phlda3*), cyclin-dependent protein kinase (*Cdkn1a*), NADP aldo-keto reductase (*Akr7a3*), Cyclin G1 (*Ccng1*) and ATP-binding cassette (*Abcb4*), all of which were altered by treatment with coumarin. These genes are related to either *p53* (a tumor suppressor) signaling, or to specific changes in anabolic processes or energy metabolism that are typically found in tumor cells. The NADP aldo-keto reductase gene *Akr7a3* was also among the affected genes *in vitro*, and among the five most sensitive genes identified at the lowest dose *in vivo* as having altered expression in the analysis by Uehara *et al.* (2008a).

*OEHHA's analysis of toxicogenomic data from Uehara et al. (2008a)*

In order to further characterize the effects of coumarin on cancer-associated biological processes and pathways, OEHHA conducted additional analyses of the toxicogenomic data of Uehara *et al*. (2008a), using DAVID (Huang da *et al.*, 2009) and the Comparative Toxicogenomics Database (CTD; http://ctdbase.org/, assessed June 14, 2017). Details of the methodology used for this analysis and the output from DAVID are presented in Appendix C.

Among the 136 up-regulated genes, 111 were recognized by DAVID and grouped into 17 annotation clusters of GO biological processes or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Among the 79 down-regulated genes, 69 were recognized by DAVID and grouped into 12 annotation clusters. In Table 27, findings with statistical significance as determined by a modified Fisher exact test ($p < 0.05$) are presented, consisting of ten annotation clusters of up-regulated genes and eight annotation pathways of down-regulated genes. The modified Fisher exact test was used to determine whether the number of up-regulated (or down-regulated) genes in a given annotation pathway were significantly different (or "enriched") (See Appendix C, Tables C1 – C4).

CTD was used to ascertain if any of the annotation clusters identified in the DAVID functional annotation clustering analysis are associated with cancer. The percentage of disease associations for a given GO or pathway cluster that were specifically related to cancer is shown in Table 27 as the CTD ratio of cancer to all diseases. The higher the CTD ratio of cancer to all diseases, the stronger the association of the annotation cluster with cancer.

The results of our analysis indicate that 10 of the 11 GO or pathway clusters identified as genes up-regulated by coumarin in rat liver are cancer-associated pathways or biological processes with CTD cancer association ratios ranging from 47% to 15% (Table 27). Ranked in order of highest association to lowest, these are: cell cycle, base

excision repair, DNA replication, aging, nucleotide-binding, metabolism of xenobiotics by cytochrome P450, negative regulation of apoptotic signaling pathway, response to oxidative stress, glutathione metabolic process, oxidation-reduction process, and antigen processing and presentation.

Five of the seven GOs or pathways identified as genes down-regulated by coumarin in rat liver have CTD cancer association ratios ranging from 100% to 17%. Ranked from highest association to lowest, these are: chemical carcinogenesis, two metabolic-related pathways ("secondary metabolites, biosynthesis, transport, and catabolism", and "drug metabolism – cytochrome P450"), oxidation-reduction process, and steroid hormone biosynthesis.

The ten key characteristics of carcinogens (Smith *et al.*, 2016) were also applied to assist in recognizing cancer-associated pathway clusters among those identified by the DAVID analysis (Table 27). Each of the ten key characteristics was associated with at least one annotation cluster of genes with significantly altered expression in rat liver following *in vivo* coumarin treatment.

In conclusion, our toxicogenomic data analysis provides additional evidence that administration of coumarin to rats *in vivo* induces changes in gene expression associated with cancer pathways and biological processes, and are consistent with the findings of Carlton *et al.* (1996) that coumarin induces liver tumors in male and female S-D rats. Our analysis identified multiple pathways that may be involved in coumarin-induced liver tumor formation in rats, including two major pathways of response to oxidative stress and the glutathione metabolic process that have also been identified by other researchers using either toxicogenomics (Uehara *et al*., 2008b; Kiyosawa *et al*., 2007) or traditional toxicological approaches (Lake *et al*., 1989; Lake *et al*., 1984; Fentem *et al.*, 1992; Fry *et al.*, 1993).

**Table 27. OEHHA's GO and KEGG pathway analysis of up- and down-regulated genes in the liver of rats administered 150 mg/kg coumarin by gavage 5 times over 28 days (day 1, 3, 7, 14, 28), and sacrificed 24 hours after the last dose (data from Uehara *et al*., 2008a)**

| GO/Pathway | No. of genes associated with pathway/no. of genes in tested gene set | *p*-value[d] | IARC ten key characteristics of carcinogens | CTD ratio of cancer to all diseases (%) |
|---|---|---|---|---|
| Chemical carcinogenesis | 4/69 (down[b]) | < 0.01 | All 10 key characteristics | 100.00 |
| Drug metabolism - cytochrome P450 | 4/69 (down) | < 0.01 | 1: Electrophilic | 27.13 |
| Metabolism of xenobiotics by cytochrome P450 | 4/111 (up[c]) | < 0.05 | 1: Electrophilic | 30.60 |
| Secondary metabolites biosynthesis, transport, and catabolism | 4/69 (down) | < 0.05 | 1: Electrophilic | 36.40 |
| Nucleotide-binding | 13/111 (up) | < 0.05 | 1: Electrophilic; 2: genotoxic | 30.64 |
| Base excision repair | 3/111 (up) | < 0.05 | 2: Genotoxic; 3: Alters DNA repair or causes genomic instability | 40.76 |
| DNA replication | 7/111 (up) | < 0.001 | 2: genotoxic; 3: genomic instability | 35.27 |
| Glutathione metabolic process | 6/111 (up) | < 0.001 | 5: Induces oxidative stress | 24.78 |
| Oxidation-reduction process | 15/111 (up) | < 0.001 | 5: Induces oxidative stress | 22.17 |
| | 12/69 (down) | < 0.001 | | |
| Response to oxidative stress | 7/111 (up) | < 0.001 | 5: Induces oxidative stress | 25.85 |
| Antigen processing and presentation | 6/111 (up) | < 0.001 | 6: Induces chronic inflammation; 7: Immunosuppressive | 14.68 |
| Calycin[a] | 3/69 (down) | < 0.05 | 6: Induces chronic inflammation; 8: Modulates receptor-mediated effects | 8.50 |
| Steroid hormone biosynthesis | 5/69 (down) | < 0.001 | 8: Modulates receptor-mediated effects | 16.78 |
| Cell cycle | 7/111 (up) | < 0.001 | 10: cell proliferation | 46.57 |
| Negative regulation of apoptotic signaling pathway | 4/111 (up) | < 0.01 | 10: cell proliferation, cell death | 26.45 |
| Pyridoxal phosphate | 4/69 (down) | < 0.001 | None | 10.00 |
| Protein processing in endoplasmic reticulum | 9/111 (up) | < 0.001 | None | 0 |

[a] Calycin is a GO term. Calycins are a large protein superfamily that share similar beta-barrel structures. The superfamily can be divided into families that include lipocalins, fatty acid binding proteins and thrombin inhibitor. The three down-regulated calycin genes encode retinol binding protein 1, major urinary protein 5, and alpha-2μ globulin.

[b] Down-regulation by coumarin treatment.
[c] Up-regulation by coumarin treatment.
[d] The p-values correspond to a more conservative version of the one-tailed Fisher Exact test that is commonly used for gene-enrichment analysis (DAVID user guide, https://david.ncifcrf.gov/helps/functional_annotation.html#).

### *3.3.7.3 Rat hepatocytes in vitro (Kienhuis et al., 2006 and Uehara et al., 2008a)*

Because of the expected differences in the extent to which coumarin is metabolically activated in hepatocytes *in vitro* versus in liver *in vivo*, the information reported in these *in vitro* studies may provide a limited picture of what happens in the *in vivo* system. In general, hepatocytes have less metabolic capacity *in vitro* than *in vivo*. Nevertheless, Kienhuis *et al.* (2006) and Uehara *et al.* (2008a) reported some common genes that were significantly modulated by coumarin exposure in both rat liver *in vivo* and rat hepatocytes *in vitro*.

In the Kienhuis *et al.* (2006) study, rat primary hepatocytes were prepared from three untreated nine- to twelve-week-old male Wistar rats. The primary rat hepatocytes were sandwich-cultured between two collagen layers in either a standard medium or an enhanced medium containing low concentrations of cytochrome P450 inducers (phenobarbitol, dexamethasone and β-naphthoflavone) for 72 hours, and then exposed to coumarin at doses of 0, 70, 200, and 600 µM for 24 hours. RNA samples were extracted and labelled prior to hybridization for gene expression analysis with QIAGEN Operon oligonucleotide microarrays containing approximately 5800 different 70-mer oligonucleotide fragments. *o*-HPAA, a metabolite of coumarin, was measured in the culture media after 24 hours of exposure to coumarin. Comparisons of significantly modulated genes and biological pathways were made among hepatocytes cultured with the standard versus the enhanced medium, and with the findings from the *in vivo* rat hepatic gene expression studies. Similar to the *in vivo* finding that *o*-HPAA was detected in rat urine, *o*-HPAA was only identified from hepatocytes cultured in the enhanced medium, but not the standard medium. The number of genes with significantly altered expression in response to coumarin treatment was 321 in liver *in vivo*, 13 in hepatocytes cultured with standard medium, and 92 in hepatocytes cultured with enhanced medium. Only one gene was altered in rat liver *in vivo* and in both *in vitro* hepatocyte systems, and an additional 23 were altered both *in vivo* and in the enhanced system. This demonstrates that the enhanced *in vitro* system with added metabolic activation capacity is better able to simulate the *in vivo* system, and indicates that metabolic capacity is an important factor to consider when evaluating and comparing results from *in vivo* and *in vitro* toxicogenomic studies. Biological pathway analysis of the genes with altered expression identified four biological pathways that were shared between the *in vivo* liver response and the hepatocyte response in the

enhanced *in vitro* system: methionine metabolism, fatty acid metabolism, γ-hexachlorocyclohexane degradation, and complement and coagulation cascades.

In the Uehara *et al.* (2008a) study, rat primary hepatocytes were prepared from livers of six-week-old male S-D rats following *i.p.* injection with 120 mg/kg sodium pentobarbital. The primary hepatocytes were exposed to coumarin at doses of 0, 12, 60, and 300 µM for 24 hours, and the total RNA extracted for gene expression analysis with Affymetrix GeneChip RAE 230A probe arrays. The authors compared the changes in gene expression observed in response to coumarin in rat liver *in vivo* with those in primary rat hepatocytes exposed *in vitro*. Fewer responsive genes were observed in the primary rat hepatocytes than in rat liver *in vivo*, and smaller fold changes were observed *in vitro* in those responsive gene probe sets, possibly due to limited metabolic activation in the *in vitro* system. The authors identified 37 up-regulated and 29 down-regulated gene probe sets as being differentially expressed in response to coumarin in both the rat liver *in vivo* and *in vitro* data sets. Many of these genes are involved in pathways related to the oxidative stress response or glutathione metabolism. For example, the up-regulated genes include “hypoxia upregulated 1 (*Hyou1*)”, “aldo-keto reductase family 7-member A3 (*Akr7a3*)”, “ischemia/reperfusion inducible protein (*Yrdc*)”, “glutathione reductase (*Gsr*)”, “glutamate-cysteine ligase-catalytic subunit (*Gclc*)”, “NAD(P)H dehydrogenase, quinone 1 (*Nqo1*)”, and “DNA-damage inducible transcript 4-like (*Ddit4l*)”.

*3.3.7.4 Human hepatocytes in vitro (Uehara et al., 2008a and Kienhuis et al., 2009)*

In Uehara *et al.* (2008a), frozen human hepatocytes were obtained from a commercial source. After thawing and plating, hepatocytes were exposed to coumarin at doses of 0, 12, 60, and 300 µM for 24 hours, and total RNA extracted for gene expression analysis with Affymetrix U133 Plus 2.0 arrays. Based on the differentially expressed probe sets identified in response to coumarin treatment as common in both rat liver *in vivo* and rat primary hepatocytes *in vitro*, human orthologs were identified for 14 up-regulated and 11 down-regulated probe sets. Many of these genes are involved in pathways related to the oxidative stress response or glutathione metabolism. Similar expression patterns were observed in these 14 up-regulated and 11 down-regulated probe sets in cultured human hepatocytes treated with coumarin as in rat primary hepatocytes *in vitro* and rat liver *in vivo*. Smaller fold changes were observed in response to coumarin in human hepatocytes than in rat hepatocytes; however, interpretation of this finding is limited in the absence of information on the relative metabolic competencies of the human and rat hepatocytes.

Using different methods, Kienhuis *et al.* (2009) obtained complete data sets of coumarin-induced gene expression profiles in primary human hepatocytes from five donors. The sandwich-cultured primary human hepatocytes were grown between two

collagen layers on collagen gel pre-coated plates. The human hepatocytes were then exposed to coumarin at two doses for 24 hours: 200 µM (equivalent to 100 mg/kg/day for a 70-kg person) and 600 µM (a proposed toxic dose). The labelled cRNA samples were hybridized on Agilent 22 K format 60-mer oligo microarrays (~20,000 probes, G4110B for human from Agilent Technologies, Palo Alto, CA); then gene expression data were analyzed. A total of 198 genes and 619 genes were significantly modulated at 200 and 600 µM coumarin, respectively, with an overlap of 135 differentially expressed genes. A clear dose-response relationship of differential gene expression was observed at the two doses. No cytotoxicity was observed in human hepatocytes at either of the doses.

The gene expression data were analyzed by T-profiler (http://www.t-profiler.org; Boorsma *et al*., 2005). At the 200 µM dose, down-regulated complement and coagulation cascades were observed. At 600 µM (Table 28), several pathways and processes were affected, including up-regulation of transcription and protein folding related pathways, and down-regulated complement and coagulation cascades, lipid metabolism pathways, oxidoreductase activity, metabolism of xenobiotics by CYPs, repression of energy-consuming biochemical pathways, and impairment of mitochondrial function. Oxidoreductase activity and metabolism of xenobiotics by CYPs are related to the ten key characteristics of carcinogens identified by an IARC working group (Smith *et al.*, 2016). Reprogramming energy metabolism was defined as one of the six cancer hallmarks in a review published by Hanahan and Weinberg (2011). Decreased mitochondrial membrane potential is observed in cancer cells *in vitro* and linked with cellular properties associated with cancer progression (Summerhayes *et al.*, 1982; Chen, 1988; Jellicoe *et al.*, 2008).

#### *3.3.7.5 Comparison of toxicogenomic data from rat liver and primary human hepatocytes*

Among the biological processes or biochemical pathways identified, several pathways identified in human hepatocytes by Kienhuis *et al.* (2009) are similar to OEHHA's analysis of Uehara *et al.* (2008a) rat data *in vivo* by DAVID, including oxido-reductase activity, metabolism of xenobiotics by CYPs, nuclear acid binding, and DNA binding (Table 27 and Table 28).

Table 28 summarizes the up- and down-regulated pathways and biological processes affected by coumarin in the sandwich-cultured primary human hepatocytes *in vitro* (Kienhuis *et al.*, 2009) and in liver tissues of S-D rats *in vivo* (data presented in Uehara *et al*., 2008a and analyzed by OEHHA; See Appendix C, Tables C3 and C4). Different from Table 27, the microarray data from rat livers *in vivo* (Uehara *et al*., 2008a) were analyzed by the general gene functional annotation approach, not the clustering

approach in DAVID to accommodate the annotation categories defined in Kienhuis *et al.* (2009). Several up-regulated cancer-associated pathways and biological processes induced by coumarin are similar in the livers of rats and humans, including nucleic acid binding and protein binding (Table 28). Several down-regulated cancer-associated pathways and biological processes induced by coumarin are similar in the livers of rats and humans, including metabolism of xenobiotics by CYPs, oxidoreductase activity and mitochondrial functions (Table 28). In addition, genes involved in lipid metabolism, including one that reprograms energy metabolism and is identified as a cancer hallmark by Hanahan and Weinberg (2011), were also down-regulated by coumarin in both rat and human livers.

**Table 28. Up- and down-regulated pathways and biological processes by coumarin in sandwich-cultured primary human hepatocytes *in vitro* (Kienhuis *et al.*, 2009) and liver tissues of S-D rats *in vivo* (data from Uehara *et al*., 2008a, analyzed by OEHHA)**

| Pathways and biological processes | Annotation categories | Direction of regulation | Human hepatocytes *in vitro* (extracted from Kienhuis *et al.*, 2009) | | S-D rat livers *in vivo* (data source: Uehara *et al*., 2008a) | |
|---|---|---|---|---|---|---|
| | | | Gene count | *p*-value[b] | No. of genes associated with pathway/no. of genes in tested gene set | *p*-value[c] |
| Nucleus | GO-cellular component | Up | 3041 | < 0.001 | 36/111 | < 0.05 |
| Nucleic acid binding | GO-molecular function | Up | 669 | < 0.001 | 4/111 | < 0.001 |
| Zinc ion binding | GO-molecular function | Up | 1714 | < 0.001 | 5/111 | 0.84 |
| Transcription | GO-biological process | Up | 1033 | < 0.001 | 3/111 | 0.93 |
| Response to unfolded protein | GO-biological process | Up | 38 | < 0.001 | None identified | |
| Regulation of transcription, DNA-dependent | GO-biological process | Up | 1435 | < 0.001 | 4/111 | 0.82 |
| Metal ion binding | GO-molecular function | Up | 1674 | 0.01 | 4/111 | 0.98 |
| DNA binding | GO-molecular function | Up | 912 | 0.01 | 10/111 | 0.14 |
| Protein folding | GO-biological process | Up | 188 | 0.03 | 4/111 | < 0.05 |
| Pores ion channels | KEGG | Up | 4 | 0.05 | None identified | |
| Metabolism of xenobiotics by cytochrome P450[a] | KEGG | Down | 56 | 0.01 | 3/69 | < 0.05 |
| Bile acid biosynthesis | KEGG | Down | 37 | < 0.001 | None identified | |
| Lyase activity | GO-molecular function | Down | 90 | 0.01 | 3/69 | < 0.05 |
| Peroxisome | GO-cellular component | Down | 56 | 0.01 | None identified | |
| Oxidoreductase activity[a] | GO-molecular function | Down | 420 | < 0.001 | 5/69 | 0.001 |
| Extracellular region | GO-cellular component | Down | 390 | < 0.001 | 4/69 | 0.45 |
| Metabolism[a] | GO-biological process | Down | 346 | < 0.001 | 17/69 | < 0.001 |
| Lipid transporter activity | GO-molecular function | Down | 33 | < 0.001 | None identified | |

| Pathways and biological processes | Annotation categories | Direction of regulation | Human hepatocytes *in vitro* (extracted from Kienhuis *et al.*, 2009) | | S-D rat livers *in vivo* (data source: Uehara *et al.*, 2008a) | |
|---|---|---|---|---|---|---|
| | | | Gene count | *p*-value[b] | No. of genes associated with pathway/no. of genes in tested gene set | *p*-value[c] |
| Complement and coagulation cascades | KEGG | Down | 65 | < 0.001 | None identified | |
| Lipid metabolism | GO-biological process | Down | 197 | < 0.001 | 7/69 | < 0.001 |
| Mitochondrion | GO-cellular component | Down | 543 | < 0.001 | 11/69 | < 0.05 |

[a] Oxidoreductase activity and metabolism of xenobiotics by CYPs are related to the ten key characteristics of carcinogens identified by an IARC working group (Smith *et al.*, 2016).
[b] Bonferroni-corrected *p*-value (Boorsma *et al.*, 2005).
[c] The p-values in this column correspond to a more conservative version of the one-tailed Fisher Exact test that is commonly used for gene-enrichment analysis (DAVID user guide, https://david.ncifcrf.gov/helps/functional_annotation.html#).

#### *3.3.7.6 Summary of toxicogenomic data*

In summary, several toxicogenomic studies and OEHHA's functional pathways analysis show that multiple biological processes/pathways could be involved in the hepato-carcinogenicity of coumarin, such as glutathione metabolism, and the oxidative stress response. In addition, as shown in Table 28, there are several common cancer-related biological processes/pathways altered by coumarin in rat liver and in human primary hepatocyes, including up-regulated pathways related to nucleic acid binding, and protein binding, and down-regulated pathways related to metabolism of xenobiotics by CYPs, oxidoreductase activity, and mitochondrial functions.

### *3.3.8 ToxCast high-throughput in vitro assays*

ToxCast™ is a chemical prioritization research program developed by the US EPA (Dix *et al.*, 2007). It is a multi-year project that launched in 2007. It utilizes various *in vitro* systems to identify chemical activity in a battery of high-throughput (HTS) screening assays. As of 2017, more than 9,000 chemicals have been tested and there have been more than 700 high-throughput assays that cover a range of high-level cell responses and approximately 300 signaling pathways in the ToxCast database.

This section highlights the ToxCast HTS assays in which coumarin and its metabolite 3,4-DHC were active. OEHHA has searched the ToxCast database via the Interactive

Chemical Safety for Sustainability (iCSS) Dashboard (http://actor.epa.gov/dashboard/, accessed on 5/19/2017), and identified chemical activity data on coumarin and one of its metabolites (3,4-DHC). There are 13 active ToxCast assays for coumarin, and one active assay for 3,4-DHC (Table 29).

**Table 29. Overview of ToxCast HTS assay activity for coumarin and 3,4-dihydrocoumarin (3,4-DHC)**

| Chemical | Coumarin | 3,4-DHC |
|---|---|---|
| Number of active assays / tested assays | 13/882 | 1/339 |
| Range of $AC_{50}$ values (µM) in active assays | 0.0113-163 | 1.72 |

Information on each of the active ToxCast HTS assays reported for coumarin and 3,4-DHC is presented in Table 30 and Table 31, respectively, with the assays sorted by lowest to highest $AC_{50}$ (the concentration that induces a half-maximal assay response).

**Table 30. Active ToxCast HTS assays for coumarin**

| Assay Endpoint | Target Gene Symbol | Biological Process / Intended Target Family | Cell line | $AC_{50}$ (μM) |
|---|---|---|---|---|
| BSK_CASM3C_IL6_down | *IL6* | Regulation of gene expression / cytokine | Human coronary artery smooth muscle cells | 0.0113 |
| BSK_CASM3C_SAA_up | *SAA1* | Regulation of gene expression / cell adhesion molecules | Human coronary artery smooth muscle cells | 0.0157 |
| BSK_hDFCGF_Proliferation_down | NA | Cell proliferation / cell cycle | Human primary foreskin fibroblast | 7.01 |
| BSK_LPS_PGE2_up | *PTGER2* | Regulation of gene expression / G-protein-coupled receptors | Human umbilical vein endothelium and peripheral blood mononuclear cells | 9.24 |
| BSK_LPS_MCP1_up | *CCL2* | Regulation of gene expression / cytokine | Human umbilical vein endothelium and peripheral blood mononuclear cells | 12.4 |
| NVS_ENZ_rMAOBC | *MAOB* | Regulation of catalytic activity / oxidoreductase | Cell-free assay with enzyme from rat brain tissue | 15.8 |
| NVS_ENZ_rMAOBP | *MAOB* | Regulation of catalytic activity / oxidoreductase | Cell-free assay with enzyme from rat brain tissue | 18.5 |
| NVS_ENZ_rMAOAP | *MAOA* | Regulation of catalytic activity / oxidoreductase | Cell-free assay with enzyme from rat liver mitochondrial membrane | 19.0 |
| TOX21_ERa_LUC_BG1_Agonist | *ESR1* | Regulation of transcription factor activity / nuclear receptor | BG1, a human ovarian cancer cell line | 21.8 |
| ATG_LXRa_TRANS_dn | NA | Regulation of transcription factor activity / nuclear receptor | HepG2, a human liver carcinoma cell line | 28.2 |
| ATG_RXRb_TRANS_dn | NA | Regulation of transcription factor activity / nuclear receptor | HepG2, a human liver carcinoma cell line | 31.1 |
| APR_HepG2_MitoMembPot_72h_dn | NA | Mitochondrial depolarization / cell morphology | HepG2, a human liver carcinoma cell line | 118 |
| ATG_TCF_b_cat_CIS_dn | NA | Regulation of transcription factor activity / DNA binding | HepG2, a human liver carcinoma cell line | 163 |

NA: not applicable

**Table 31. Active ToxCast HTS assays for 3,4-DHC**

| Assay Endpoint | Target Gene Symbol | Biological Process / Intended Target Family | Cell line | $AC_{50}$ (µM) |
|---|---|---|---|---|
| TOX21_ARE_BLA_agonist_viability | NA | Cell proliferation / Cytotoxicity | HepG2, a human liver carcinoma cell line | 1.72 |

Coumarin was active in assays related to several biological processes or intended target families, including cell cycle, nuclear receptor, cytokine and DNA binding in various human cell lines, and oxido-reductase regulation in rat cell-free assays. 3,4-DHC was active in an assay related to cytotoxicity in a human cell line.

### Genes involved in active coumarin ToxCast HTS assays

For each of the active ToxCast assays, we identified the genes, functions, and related pathways associated with the assay, as well as the curated associations of the gene with cancer that have been identified in the CTD (Comparative Toxicogenomics Database; http://ctdbase.org/; accessed on May 26, 2017). This information is summarized below for each identified gene (those with curated evidence are underlined). For coumarin, the following genes have curated associations with cancer, as compared with indirect or inferred[2] associations: *CCL2, MAOA, MAOB* and *ESR1*. The CTD ratios of cancer to all diseases[3] for each of the genes with curated associations were below 10%, ranging from 7.3-9.2%.

The gene descriptions provided below were adapted from CTD;

- ***CCL2*** (C-C motif chemokine ligand 2) is a gene that encodes CCL2, which works as a master regulator of monocyte or macrophage recruitment to tumor sites. Along with IL-6, CCL2 also promotes survival and differentiation of myeloid monocytes (Szebeni *et al.*, 2017). Several pathways were reported in CTD, including cytokine-cytokine receptor interaction, metabolism of protein, and the chemokine signaling pathway. Curated associations with cancer were reported

[2] In CTD, "curated" gene--disease relationships are extracted from the published literature by CTD curators or are derived from the OMIM database. In contrast, "inferred" relationships are established via CTD-curated chemical–gene interactions: gene A directly interacts with chemical B, and chemical B has a curated relationship with disease C, so gene A has an inferred relationship with disease C (via chemical B).

[3] The cancer to all diseases ratio is calculated as the number of associations with cancer for the specific gene divided by the number of associations with all diseases for the specific gene. The higher the cancer to all diseases ratio is, the more relevant the gene is to cancer.

for squamous cell carcinoma, multiple myeloma, endometrial neoplasms, thyroid neoplasms and mouth neoplasms.

- ***IL6*** (Interleukin 6) is a gene that encodes IL6, an interleukin that influences several key parameters of oncogenesis, such as increasing cell resistance to apoptosis, proliferation of cancer cells, angiogenesis, invasion, and malignancy (Dmitrieva *et al.*, 2016). Per CTD, this gene has many curated associations with cancers at various sites, such as lung, breast, liver (hepatocellular carcinoma), pancreas, stomach, prostate, multiple myeloma, squamous cell carcinoma, renal cell carcinoma, B-cell lymphocytic lymphoma, cholangiocarcinoma, Kaposi sarcoma, basal cell carcinoma, and malignant mesothelioma. Many pathways were associated with this gene, such as pathways related to cellular responses to stress, the immune system, a cytosolic DNA-sensing pathway, and pathways in cancer.
- ***PTGER2*** (prostaglandin E receptor 2) encodes a receptor for prostaglandin E2, a metabolite of arachidonic acid, which has different biologic activities in a wide range of tissues. Two pathways involved are neuroactive ligand-receptor interaction and signal transduction. GO annotations related to this gene include G-protein coupled receptor (GPCR) activity and prostaglandin E receptor activity.
- ***MAOA*** (monoamine oxidase A) encodes a mitochondrial enzyme that catalyzes the oxidative deamination of amines, such as dopamine, norepinephrine, and serotonin. Two subtypes of MAO have been identified, MAOA and MAOB. GO annotations related to this gene include oxidoreductase activity and primary amine oxidase activity. Per CTD, this gene has a curated association with pheochromocytoma.
- ***MAOB*** (monoamine oxidase B): Functions of MAO are presented above. Per CTD, this gene has curated associations with two types of cancer, colonic neoplasm and pheochromocytoma. Several pathways involving this gene include metabolic pathways and cytochrome P450 drug metabolism. GO annotations related to this gene include oxido-reductase activity and primary amine oxidase activity.
- ***SAA1*** (serum amyloid A1) encodes an apolipoprotein, which is a member of the serum amyloid A family. The encoded preproprotein is proteolytically processed to generate the mature protein. The protein is highly expressed in response to inflammation and tissue injury. High levels of this protein are associated with chronic inflammatory diseases including atherosclerosis and Alzheimer's disease. This protein may also be a potential biomarker for certain tumors.
- ***ESR1*** (estrogen receptor 1) encodes estrogen receptor α (a female hormone receptor) and also works as a ligand-activated transcription factor composed of several domains, which is important for hormone binding, DNA binding, and activation of transcription. Three pathways involving this gene were identified:

endocrine and other factors regulating calcium reabsorption, gene expression, and signal transduction. This gene has many curated associations with cancers, including breast, liver (hepatocellular carcinoma), prostatic, lung, urinary bladder, mammary neoplasms, and mesothelioma.

### *3.3.9 Other mechanistic studies*

#### *3.3.9.1 Effects on cell proliferation and cell cycle*

*Cell Proliferation and Apoptosis*

The effects of coumarin and its metabolites on cell proliferation and apoptosis have been studied in cultured rat hepatocytes and various human cancer cell lines. Coumarin was reported to increase cell proliferation in cultured rat hepatocytes, increasing the mitotic index 1.4-fold above controls (Muller-Tegethoff *et al.*, 1995). Other studies, however, have reported that coumarin and its metabolite 7-HC inhibited proliferation of various human cancer cell lines (Marshall *et al.*, 1994; Myers *et al.*, 1994; Jimenez-Orozco *et al.*, 1999; Lopez-Gonzalez *et al.*, 2004). The inhibition seemed to be concentration-dependent and the $IC_{50}$ for 7-HC tended to be lower than for coumarin. One study found that coumarin induced apoptosis in human cervical cancer HeLa cells (Chuang *et al.*, 2007). In this study, coumarin treatment led to cellular morphological changes and signs of cellular apoptosis, including internucleosomal DNA laddering fragmentation, and an increase in cells in the sub-$G_1$ phase of the cell cycle. On the molecular level, coumarin decreased the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xl, increased the expression of pro-apoptotic proteins such as P21, P53, Bax, cytochrome c, and the active forms of caspase-3 and caspase-9, increased intracellular $Ca^{2+}$ concentration and ROS production, and decreased mitochondrial membrane potential (Chuang *et al.*, 2007). The coumarin metabolite 7-HC has been shown to induce apoptosis in two studies. In one study in human promyelocytic leukemia HL-60 cells, 7-HC induced the appearance of DNA “ladder” patterns, a sign of apoptotic cell death (Marshall *et al.*, 1994). In the second study in human lung cancer A549 cells, treatment of 7-HC significantly increased Caspase-3 activity (Soto-Nunez *et al.*, 2015).

*Cell Cycle Regulation*

Coumarin and 7-HC have been shown to induce $G_1$ cell cycle arrest (thus blocking the $G_1$/S transition) in human cancer cell lines, such as lung carcinoma cell lines (Jimenez-Orozco *et al.*, 2001; Lopez-Gonzalez *et al.*, 2004) and HeLa cells (Chuang *et al.*, 2007), but not in cultured peripheral blood mononuclear cells (Lopez-Gonzalez *et al.*, 2004).

Induction of $G_1$ cell cycle arrest is consistent with the cytostatic effects of coumarin and 7-HC reported in these cell lines. The cell cycle arrest observed in HeLa cells was shown to be accompanied by decreased expression of cyclin D1, cdk2, and Cdc25A (Chuang *et al.*, 2007), while in human lung carcinoma A-427 cells, cell cycle arrest induced by 7-HC, but not coumarin, was accompanied by decreased expression of cyclin D1 (Jimenez-Orozco *et al.*, 2001).

*3.3.9.2 Effects on ROS production and glutathione depletion*

Multiple toxicogenomic studies, conducted in both *in vivo* and *in vitro* models, have reported that courmarin exposure alters the expression of genes involved in the oxidative stress response and glutathione metabolism pathways (see Section 3.3.7). Traditional toxicology studies of coumarin and its effects on reactive oxygen species (ROS) production and glutathione depletion are briefly summarized here.

When human cervical cancer HeLa cells were treated with coumarin at 0, 1, 5, 10, 25, 50 and 100 µm for 24 hours, there were significant increases in reactive oxygen species (ROS) at 25 µM or higher doses (Chuang *et al*., 2007). In another study treatment of HeLa cells with the metabolite 6,7-DiHC at 50 µM for 2, 4, and 6 hours induced significant increases in mitochondrial ROS (Yang *et al*., 2010). An increase in ROS is an upstream event that may lead to oxidative stress and DNA damage, as well as caspase activation and apoptosis (Chuang *et al*., 2007; Yang *et al.*, 2010).

Coumarin depletes glutathione in rat liver *in vivo* (Lake *et al*., 1989), in freshly isolated rat hepatocytes (den Besten *et al.*, 1990), and in rat primary hepatocyte cultures (Lake *et al*., 1989). These studies demonstrated that coumarin does not react directly with glutathione, coumarin metabolism by cytochrome P450 enzymes is required, and that GSH depletion results from the formation of metabolite-glutathione conjugates, rather than the oxidation of GSH to GSSG (glutathione disulfide). Formation of multiple coumarin metabolite-derived glutathione conjugates have been reported in other model systems, including in rat olfactory mucosal microsomes, rat liver microsomes, and human liver microsomes (Zhuo *et al.*, 2009).

# 4. MECHANISMS

Coumarin may act via multiple mechanisms, which can be grouped according to key characteristics of carcinogens described by Smith *et al.* (2016). These mechanisms include formation of electrophilic metabolites, oxidative stress induction, and genotoxicity (Table 32). There is some suggestion that alteration of cell proliferation and modulation of receptor–mediated effects may also play a role.

**Table 32. Ten key characteristics of carcinogens (taken from Smith *et al*., 2016)**

| Characteristic | Example of relevant evidence |
|---|---|
| 1. Is electrophilic or can be metabolically activated | Parent compound or metabolite with an electrophilic structure (*e.g.,* epoxide, quinone), formation of DNA and protein adducts |
| 2. Is genotoxic | DNA damage (DNA strand breaks, DNA–protein cross-links, UDS), intercalation, gene mutations, cytogenetic changes (*e.g.,* CAs, MN) |
| 3. Alters DNA repair or causes genomic instability | Alterations of DNA replication or repair (*e.g.,* topoisomerase II, base-excision or double-strand break repair) |
| 4. Induces epigenetic alterations | DNA methylation, histone modification, microRNA expression |
| 5. Induces oxidative stress | Oxygen radicals, oxidative stress, oxidative damage to macromolecules (*e.g.,* DNA, lipids) |
| 6. Induces chronic inflammation | Elevated white blood cells, myeloperoxidase activity, altered cytokine and/or chemokine production |
| 7. Is immunosuppressive | Decreased immunosurveillance, immune system dysfunction |
| 8. Modulates receptor-mediated effects | Receptor inactivation/activation (*e.g.,* ER, PPAR, AhR) or modulation of endogenous ligands (including hormones) |
| 9. Causes immortalization | Inhibition of senescence, cell transformation |
| 10. Alters cell proliferation, cell death, or nutrient supply | Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signaling pathways related to cellular replication or cell cycle control, angiogenesis |

AhR, aryl hydrocarbon receptor; ER, estrogen receptor; PPAR, peroxisome proliferator–activated Receptor. Any of the 10 characteristics in this table could interact with any other (e.g., oxidative stress, DNA damage, and chronic inflammation), which when combined provides stronger evidence for a cancer mechanism than would oxidative stress alone.

Coumarin metabolites are electrophilic. As described in Section 3.3.1, coumarin is bioactivated by CYPs to form electrophilic metabolites, including coumarin 3,4-epoxide (CE) and the aldehyde *o*-HPA. Electrophiles commonly form adducts with cellular macromolecules, including DNA and protein. The coumarin metabolites CE, *o*-HPA, and 7-HC have been shown to bind covalently to microsomal proteins in rats and humans (Lake, 1984; Peters *et al*., 1991, van Iersel *et al.*, 1994a).

Coumarin can induce oxidative stress. Many carcinogens are capable of influencing redox balance within target cells, thereby favoring formation of reactive oxygen and/or nitrogen species at the expense of their detoxification. Reactive oxygen species (ROS) and other free radicals may play key roles in many of the processes necessary for the conversion of normal cells to cancer cells (Smith *et al*., 2016). As discussed in Section 3.3.9.2, coumarin depletes cellular glutathione (GSH) as a result of the formation of coumarin metabolite-derived glutathione conjugates. A reduction or depletion of the GSH pool may shift the cell's redox balance and impact the cell's overall ability to detoxify additional reactive species (*e.g.*, ROS), leading to oxidative stress. In HeLa cells, coumarin and its metabolite 6,7-DiHC have been shown to induce significant increases in cellular ROS and mitochondrial ROS, respectively. In addition, as discussed in Section 3.3.7, *in vivo* and *in vitro* toxicogenomic studies have reported that coumarin exposure alters the expression of genes involved in the oxidative stress response and glutathione metabolism pathways.

As discussed in Section 3.3.3, coumarin is genotoxic, inducing base-pair substitution mutations in two strains of *Salmonella*, chromosome aberrations in CHO and onion root tip cells; micronuclei in human lymphocytes and a human hepatoma cell line; SCEs in CHO and onion root tip cells; and chromosome instability (*i.e.*, deletions) in *Aspergillus*. In addition, coumarin inhibits DNA excision repair in *E. coli* and binds to single- and double-stranded calf thymus DNA. There is also some evidence for the genotoxicity of two coumarin metabolites. Specifically, 3,4-dihydrocoumarin induced SCEs in CHO cells, and 7-HC was weakly positive in a CA assay in CHO cells, induced expression of a DNA repair gene in *E. coli*, and formed DNA cycloadducts with photoirradiation and DNA interstrand crosslinks in a cell free assay.

It is unclear what effects coumarin has on cell proliferation and cell death. As discussed in Section 3.3.9.1, while coumarin inhibits cell proliferation and induces apoptosis in some human cancer cell lines, it was observed to increase cell proliferation (as measured by an increase in the mitotic index) in cultured rat hepatocytes. A toxicogenomics study found that coumarin up-regulated expression of seven cell-cycle related genes in rat liver *in vivo* (see Section 3.3.7).

It is also unclear whether modulation of receptor-mediated effects plays a role in coumarin's mechanism of action. Coumarin was active in ToxCast assays (Section 3.3.8) related to prostaglandin E receptor 2, which may activate multiple GPCR-mediated downstream events (Jiang and Dingledine, 2013), and estrogen receptor α activities, indicating it may have receptor-mediated effects.

# 5. REVIEWS BY OTHER AGENCIES

Coumarin has been classified as to its potential carcinogenicity by the International Agency for Research on Cancer (IARC), but not by the US Environmental Protection Agency, the National Institute for Occupational Safety and Health, the NTP Report on Carcinogens, or the FDA.

IARC (1976) initially reviewed the carcinogenicity of coumarin in Volume 10 of the IARC Monographs series. In 1987, IARC classified coumarin in Group 3 (not classifiable as to its carcinogenicity to humans), based on *no adequate data* in humans, and *limited evidence* of carcinogenicity in experimental animals (IARC, 1987). In 2000 IARC re-evaluated coumarin and the chemical was again classified in Group 3, based on *no epidemiological data* and *limited evidence* in experimental animals.

The European Food Safety Authority (EFSA) of the European Union has also reviewed coumarin (EFSA, 2004; EFSA, 2008).

- In 1994, EFSA's predecessor, the European Commission's Scientific Committee for Food, concluded that "coumarin was a carcinogen in rats via the oral route and possibly in mice". EFSA further noted that "a key issue in assessing the risk of coumarin to humans was deciding whether or not coumarin was genotoxic and that particularly strong reassurance was needed that coumarin was not genotoxic *in vivo*, since, in addition to positive results from *in vitro* genotoxicity studies, an epoxide had been postulated as metabolic intermediate." EFSA further concluded that "the epoxide route could not be ruled out in humans and need only be a minor pathway for genotoxic/carcinogenic effects to be of concern."
- In 1999, EFSA stated that "new data on liver toxicity did not re-assure the Committee" and further concluded that the new data supported the 1994 conclusion.
- In 2004, EFSA concluded that the results of a study on DNA-adduct formation in kidney and liver of rats demonstrate that coumarin does not bind covalently to DNA, supporting a non-genotoxic mode of action for tumor induction. A Tolerable Daily Intake (TDI) of 0.1 mg/kg bw was derived.
- In 2008, EFSA considered toxicity studies and metabolism studies in humans with CYP2A6 polymorphism as well as clinical studies and re-affirmed the TDI.

# 6. SUMMARY AND CONCLUSIONS

## 6.1 Summary of Evidence

No epidemiology studies were identified that investigated the risk of cancer associated with exposure to coumarin.

Long-term carcinogenicity studies of coumarin have been conducted in rats, mice, and hamsters. Tumors were observed in four studies in rats, four studies in mice, and one study in hamsters. These findings are as follows:

*Kidney tumors*

- In the 103-week continuous exposure groups in the male F344/N rat study by NTP (1993a), the incidences of rare renal tubule adenoma, and adenoma and carcinoma combined were statistically significant in the mid-dose group by pairwise comparison with controls. Two uncommon renal tubule oncocytomas were observed in the low-dose group.
- In the stop-exposure groups in the male F344/N rat study by NTP (1993a), a statistically significant increase in the incidence of rare renal tubule adenomas was observed at 103 weeks in the group exposed for 9-months. Two uncommon renal tubule oncocytomas were observed in the 15-month stop-exposure group at 103 weeks.
- In the female F344/N rat study by NTP (1993a), a significant dose-related trend in the incidence of rare renal tubule adenomas was observed. The incidence in the mid- and high-dose groups exceeded the incidence reported in NTP historical controls.

*Liver tumors*

- In the male Sprague-Dawley rat study by Carlton *et al.* (1996), the incidences of metastasizing cholangiocarcinomas, non-metastasizing cholangiocarcinomas, and hepatocellular adenomas and carcinomas combined were significantly increased in the highest dose group by pairwise comparison with controls. Significant dose-related trends were observed for each of these tumor types.
- In the female Sprague-Dawley rat study by Carlton *et al.* (1996), the incidences of non-metastasizing cholangiocarcinoma and hepatocellular adenomas and carcinomas combined were significantly increased in the highest dose group by pairwise comparison with controls. Significant dose-related trends were observed for each of these tumor types. One metastasizing cholangiocarcinoma was observed in the highest dose group.

- In the female B6C3F$_1$ mouse study by NTP (1993a), the incidences of hepatocellular adenomas, and adenomas and carcinomas combined were significantly increased in the low- and mid-dose groups by pairwise comparison with controls. These increases exceeded the liver tumor incidence reported for NTP historical controls.
- In the female CD-1 mouse study by Carlton *et al.* (1996), the incidence of hepatocellular adenomas and carcinomas combined was significantly increased in the low-dose group by pairwise comparison with controls.

*Lung tumors*

- In the male B6C3F$_1$ mouse study by NTP (1993a), the incidences of alveolar/bronchiolar adenomas, and adenomas and carcinomas combined were significantly increased in the high-dose group by pairwise comparison with controls, with significant dose-related trends.
- In the male CD-1 mouse study by Carlton *et al.* (1996), the incidence of alveolar/bronchiolar carcinomas was significantly increased in the high-dose group by pairwise comparison with controls, with a significant dose-related trend.
- In the female B6C3F$_1$ mouse study by NTP (1993a), incidences of alveolar/bronchiolar adenomas, carcinomas, and adenomas and carcinomas combined were significantly increased in the high-dose group by pairwise comparison with controls, with significant dose-related trends.

*Forestomach tumors*

- In the male B6C3F$_1$ mouse study by NTP (1993a), the incidence of forestomach squamous cell papillomas and carcinomas combined was significantly increased in the low-dose group by pairwise comparison with controls. The NTP report (NTP, 1993a) concluded that the increase in forestomach papillomas in male mice may have been related to coumarin administration. One rare forestomach carcinoma was observed in the low-dose group, and two were observed in the mid-dose group.
- In the female B6C3F$_1$ mouse study by NTP (1993a), the incidences of forestomach squamous cell papillomas and carcinomas combined in each dose group were increased, but the increases were not statistically significant by pairwise comparison with controls. The observed increases in forestomach squamous cell papillomas were within the range of NTP historical control incidence. The NTP report (NTP, 1993a) concluded that the increase in forestomach papillomas in female mice may have been related to coumarin administration. One rare forestomach squamous cell carcinoma was observed in each of the low- and mid-dose groups.

*Pancreatic tumors*

- In the female hamster study by Ueno and Hirono (1981), two uncommon pancreatic islet cell carcinomas were observed in the high-dose group, with none in the control or low-dose groups. The utility of this study for assessing the carcinogenicity of coumarin is limited by the small numbers of animals per group and poor survival in the control and treated groups.

Metabolism studies in humans and animals indicate that coumarin is rapidly absorbed, metabolized, and excreted. Human and animal metabolism of coumarin is qualitatively similar. Coumarin is metabolized to form a number of metabolic products, some of which have not yet been identified, through a number of different enzymatic pathways. The predominant pathways of coumarin metabolism in humans and animals are the 7-hydroxylation pathway and the 3,4-epoxidation pathway, which produce 7-HC and CE (coumarin 3,4-epoxide) as initial metabolites, respectively. CE is unstable and can react with glutathione to form a glutathione conjugate (CE-SG) or degrade spontaneously to form *o*-HPA, *o*-HPAA and *o*-HPE. Both CE and *o*-HPA are electrophilic metabolites that covalently bind to cellular macromolecules, and are associated with toxicity, including hepatotoxicity.

Humans can be strong or weak 7-hydroxylators, forming varying amounts of 7-HC that can range from 92% in some individuals to non-detectable levels in others. 7-Hydroxylation of coumarin is catalyzed primarily by CYP2A6, and populations around the world carry certain allelic variants of CYP2A6 that are associated with either no enzyme function or reduced function, resulting in poor 7-hydroxylator phenotypes.

When coumarin 7-hydroxylation by CYP2A6 is compromised this can lead to increased metabolism through the 3,4-epoxidation pathway, and to increased generation of the reactive electrophilic metabolites CE and *o*-HPA.

The kinetics of these reactions and subsequent detoxification reactions, including conjugation of CE with glutathione, and metabolism of *o*-HPA to either *o*-HPE or *o*-HPAA, may determine the ultimate toxic effects of these metabolites.

A number of clinical trials and case reports indicate that coumarin causes hepatotoxicity in susceptible individuals. Hepatotoxicity caused by coumarin can lead to extensive liver damage, including necrosis, which has pro-inflammatory and tumor-promoting potential.

Coumarin has tested positive for a number of genotoxicity endpoints.

- Base-pair substitution mutations in two strains of *Salmonella*

- Chromosome aberrations (CAs) in CHO and onion root tip cells
- Micronuclei (MN) formation in human lymphocytes and a human hepatoma cell line
- Sister chromatid exchange (SCE) in CHO and onion root tip cells
- Chromosome instability (*i.e.*, deletions) in *Aspergillus*
- Inhibition of DNA excision repair in *E. coli*
- Binding to single- and double-stranded calf thymus DNA

There is also some evidence for the genotoxicity of two coumarin metabolites (7-HC and 3,4-DHC).

Specifically, 7-HC

- Induced expression of the ada DNA repair gene in *E. coli*
- Was weakly positive in the induction of CA in CHO cells
- Formed DNA cycloadducts with thymine and cytosine and DNA interstrand crosslinks in synthesized DNA after photoirradiation

3,4-DHC

- Induced SCE in CHO cells

The biological activity of coumarin was compared to eight structurally related compounds. Two of the comparison chemicals were tested in animal cancer bioassays, and one (the coumarin metabolite 3,4-DHC) induced tumors at similar sites to coumarin, including kidney tumors in male rats and liver tumors in female mice. Five of the comparison chemicals were tested for mutagenicity in *Salmonella*, and one (6-methylcoumarin) tested positive. Seven comparison chemicals were tested for chromosomal effects and six (3,4-DHC, 3-methylcoumarin, 4-methylcoumarin, 6-methylcoumarin, 7-HC, and 4-methyl-7-hydroxycoumarin) tested positive. Three comparison chemicals were tested for DNA damage/binding, and two (7-HC and 7,8-dihydroxycoumarin) tested positive.

Coumarin may act via multiple mechanisms, including genotoxicity (as summarized above), formation of electrophilic metabolites, and oxidative stress induction.

- Coumarin is bioactivated by CYPs to form electrophilic metabolites, including the epoxide CE and the aldehyde *o*-HPA. The coumarin metabolites CE, *o*-HPA, and 7-HC bind covalently to microsomal proteins in rats and humans.

- Coumarin depletes cellular glutathione through formation of coumarin metabolite-derived glutathione conjugates. A reduction or depletion of the GSH pool may

shift the cell's redox balance and impact the cell's overall ability to detoxify additional reactive species (*e.g.*, ROS), leading to oxidative stress.

- Coumarin and its metabolite 6,7-DiHC have been shown to increase cellular and mitochondrial ROS, respectively, in HeLa cells.

- *In vivo* and *in vitro* toxicogenomic studies indicate that coumarin exposure alters the expression of genes involved in the oxidative stress response and glutathione metabolism pathways. Additional analysis of toxicogenomic data by OEHHA identified several common cancer-related biological processes/pathways affected by coumarin in rat liver following *in vivo* exposure and in human primary hepatocytes exposed *in vitro*. These include up-regulated pathways related to nucleic acid binding and protein binding, and down-regulated pathways related to metabolism of xenobiotics by CYPs, oxidoreductase activity, and mitochondrial function.

## 6.2 Conclusions

The evidence for the carcinogenicity of coumarin comes from:

- Multiple studies in rats and mice
    - Renal tubule tumors in male and female F344/N rats
    - Hepatocellular tumors in male and female S-D rats, female $B6C3F_1$ mice, and female CD-1 mice (low-dose group)
    - Cholangiocarcinomas in male and female S-D rats
    - Lung alveolar/bronchiolar tumors in male and female $B6C3F_1$ mice, and in male CD-1 mice
    - Forestomach tumors in male $B6C3F_1$ mice (low-dose group)

- Studies of metabolism, human CYP2A6 polymorphism, and human hepatotoxicity
    - The two predominant pathways for coumarin metabolism operative in both humans and animals are 7-hydroxylation and 3,4-epoxidation. The 3,4-epoxidation pathway generates the reactive electrophilic metabolites CE and *o*-HPA, which can bind covalently to cellular macromolecules.
    - The primary enzyme responsible for 7-hydroxylation of coumarin in humans is the highly polymorphic enzyme CYP2A6. Populations around the world carry certain allelic variants of CYP2A6 that are associated with either no enzyme function or reduced function. When coumarin 7-hydroxylation by CYP2A6 is compromised by genetic polymorphism or

other non-genetic factors, this can lead to increased generation of CE and *o*-HPA.
    - A number of clinical trials and case reports indicate that coumarin causes hepatotoxicity in susceptible individuals.

- Observations from genotoxicity studies
    - Coumarin
        - Base-pair substitution mutations in two strains of *Salmonella*
        - CA in CHO and onion root tip cells
        - MN in human lymphocytes and a human hepatoma cell line
        - SCE in CHO and onion root tip cells
        - Chromosome instability (*i.e.*, deletions) in *Aspergillus*
        - Inhibition of DNA excision repair in *E. coli*
        - Binding to single- and double-stranded calf thymus DNA
    - Coumarin metabolites
        - 7-HC induced expression of the ada DNA repair gene in *E. coli*, was weakly positive in the induction of CA in CHO cells, formed DNA cycloadducts and DNA interstrand crosslinks in synthesized DNA after photoirradiation.
        - 3,4-DHC induced SCE in CHO cells.

- Additional mechanistic findings from traditional toxicological and toxicogenomic studies
    - Coumarin can deplete cellular GSH and increase ROS, which can lead to oxidative stress.
    - Several common cancer-related biological processes/pathways are affected by coumarin in rat liver following *in vivo* exposure and in human primary hepatocytes exposed *in vitro*.

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# Appendix A  Parameters for Literature Searches on the Carcinogenicity of Coumarin

General searches of the literature on the carcinogenicity of coumarin were conducted under a contract with the University of California, Berkeley (Charleen Kubota, M.L.I.S.). The goal was to identify peer-reviewed open source and proprietary journal articles, print and digital books, reports and gray literature that potentially reported relevant toxicological and epidemiological information on the carcinogenicity of this chemical. The search sought to specifically identify all literature relevant to the assessment of evidence on cancer.

**Databases**

The literature search utilized the following search platforms/database vendors:

- PubMed (National Library of Medicine)
- EMIC (National Library of Medicine)
- SciFinder®: CAS (Chemical Abstracts Service)
- TOXNET (National Library of Medicine): Toxicology Literature Online (TOXLINE), Genetic Toxicology Data Bank (GENE-TOX)
- Web of Knowledge: BIOSIS Previews®, Web of Science® (Thomson-Reuters, Inc.)

**Search Process**

Relevant subject terms were entered into the PubMed Search Builder to execute a search.

The following is a typical chemical search strategy used to search PubMed:

**(“*chemical name*” [MeSH] OR “CAS *registry number”*[RN]) AND ("bioassay"[MeSH] OR "carcinogenicity"[MeSH] OR "cancer"[MeSH] OR "tumor"[MeSH]) OR "neoplasm"[MeSH]) OR "genotoxicity"[MeSH]) OR "mutagenicity"[MeSH]) OR "metabolism"[MeSH]) OR "absorption"[MeSH]) OR "pharmacokinetics"[MeSH]) OR "structure activity relationship"[MeSH])**

ChemSpider (http://www.chemspider.com/) was used to search for synonyms of coumarin.

In PubMed, MeSH (Medical Subject Headings) terms at the top of hierarchical lists of subject headings are automatically “exploded” in a search to retrieve citations with more

specific MeSH terms. For example, the heading “carcinogenicity” includes broad conditions that are related to cancer induction in animals and humans.
Additional databases listed above were then searched. The search strategies were tailored according to the search features unique to each database. Web of Science, for example, was searched by entering chemical terms and refining the search by applying Web of Science categories Toxicology and/or Public, Environmental and Occupational Health. The search term used includes either the CAS registry number or the chemical name and its available synonyms. Sometimes other databases not listed here were searched as needed.

Additional focused searches were performed by OEHHA as needed. This search strategy is briefly described as follows:

Focused searches were conducted for coumarin. Relevant literature was also identified from citations in individual articles.

- ChemSpider was searched to gather synonyms, CAS registry number, MeSH terms and Chemical Abstracts Service headings before searching bibliographic databases.
- Databases and other resources used: Google search engine, PubMed & PubChem BioAssay (National Library of Medicine), TOXNET (National Library of Medicine), ChemoTyper (https://chemotyper.org/, Molecular Networks GmbH and Altamira LLC, 2013), Tox21 chemical structure database (ftp://ftp.epa.gov/dsstoxftp/DSSTox_Archive_20150930/TOX21S_DownloadFiles/), ChEBI chemical structure database (ftp://ftp.ebi.ac.uk/pub/databases/chebi/SDF/), iCSS Dashboard v2 (US EPA ToxCast Phase II data) , CTD (Comparative Toxicogenomics Database, http://ctdbase.org/), and DAVID (Database for Annotation, Visualization and Integrated Discovery) (https://david.ncifcrf.gov/home.jsp, assessed June 14, 2017).
- PubMed search strategy: relevant subject terms were entered into the PubMed Search Builder to execute a PubMed search.
- The following search strings, in whole or in part, was applied to the databases listed above, when applicable: ("coumarin" [MeSH] OR "1,2-benzopyrone” OR “91-64-5 [RN]”) AND (“Neoplasms” [MeSH] OR “Cancer” [MeSH] OR “Mutation” [MeSH] AND “Toxicity” [MeSH] OR “Mechanism” [MeSH] OR “CYP2A6” [MeSH] OR “Metabolism” [MeSH] OR “Polymorphism” [MeSH]).

In summary, more than 700 references, including government reports, peer-reviewed journal articles, and books, were identified through these search strategies. Among these, 337 references were cited in this document.

# Appendix B CYP2A6 Polymorphisms and Corresponding Coumarin 7-Hydroxylation Activities

## B1. Coumarin 7-Hydroxylase Activity of CYP2A6 Variants

There are three commonly used approaches to measuring the coumarin 7-hydroxylase activity of CYP2A6 variants. The first approach (referred to as the recombinant protein approach in Table B1) examines the heterologous expression of the gene variant in host cells, *e.g.*, *E. coli*, and measures 7-HC from the reaction mixture *in vitro*. 7-HC can be readily detected by High-Performance Liquid Chromatography (HPLC) (Soucek, 1999; Yamazaki *et al.*, 1999). The second approach uses prepared human liver microsomes and measures 7-hydroxylase activity *in vitro*. The third approach measures either plasma Area Under the Curve (AUC) or urinary excretion of 7-HC in humans administered known amounts of coumarin. In the latter two approaches, genotypes of the subjects are also identified.

The enzyme kinetic properties of CYP2A6 were measured and reported as $V_{max}$, $V_{max}/K_m$, or $k_{cat}/K_m$ values. $V_{max}$ is the maximum reaction rate in units of pmol 7-HC/min/mg protein or 7-HC/min/pmol protein. $K_m$ is the Michaelis-Menten constant, defined as the substrate concentration required to reach half $V_{max}$. The term $V_{max}/K_m$ stands for the apparent intrinsic clearance of the enzyme *in vitro,* and with scaling and extrapolation $V_{max}/K_m$ can predict the overall metabolic clearance *in vivo* (Davis and Rodrigues, 2008). $k_{cat}$ is the catalytic constant, which measures the maximum number of enzymatic reactions catalyzed per second. $k_{cat}/K_m$ measures enzyme catalytic efficiency. In the *in vivo* studies the level of the metabolite 7-HC was measured either in plasma or urine at certain time points (*e.g.*, two hours) after administration of coumarin. Rautio *et al.* (1992) found that urinary 7-HC recovered in the first two hours after dosing comprises about 80% of the total 7-HC excreted during an eight hour period in Finnish individuals; this finding has been confirmed by a study in the Turkish population (Iscan *et al.*, 1994). These data indicate that urine samples collected after two hours contain the majority (*i.e.*, consistently around 80%) of the 7-HC that will be excreted within eight hours. Thus even though some of the studies used collection times of up to 6 or 8 hours (Hadidi *et al.*, 1997; Xu *et al.*, 2002; Peamkrasatam *et al.*, 2006), studies using a two-hour collection time are also considered valid.

Each of the CYP2A6 allele variants that has been identified and its coumarin 7-hydroxylase activity characterized is briefly described below. A summary of the coumarin 7-hydroxylation activity of the CYP2A6 variants characterized to date is provided in Table B1. Data presented in Table B1 are expressed as percentages of the

values from wild-type alleles in studies conducted with recombinant protein, and as percentages of the arithmetic mean of the values from wild-type (*CYP2A6*1A/*1A*, or *CYP2A6*1/*1*, where the authors did not differentiate the **1* subtypes) subjects in studies conducted with human liver microsomes or in human subjects, except for Hadidi *et al.* (1997), which did not include wild-type subjects.

*CYP2A6*1B* carries a 58 base pair (bp) *CYP2A7* gene conversion in the 3'-untranslated region (3'-UTR) of *CYP2A6* (Ariyoshi *et al.*, 2000). In several studies, CYP2A6*1B showed higher activity than the wild-type CYP2A6*1A (Ujjin *et al.*, 2002; Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009). The gene conversion increases *CYP2A6*1B* mRNA stability and enzyme expression. The formation of 7-HC in the liver samples from *CYP2A6*1B/1B* individuals was significantly higher than that of the wild-type individuals (Wang *et al.*, 2006). CYP2A6*1B has been associated with faster *in vivo* nicotine metabolism as well (Mwenifumbo *et al.*, 2008b).

CYP2A6*2 is completely inactive for 7-hydroxylation of coumarin. The heterologously expressed CYP2A6*2 failed to incorporate heme, a critical cofactor of the enzyme, and exhibited no 7-hydroxylase activity (Yamano *et al.*, 1990; Hosono *et al.*, 2017). Two Caucasian individuals with the heterozygous genotype *CYP2A6*1/*2* showed much lower coumarin 7-hydroxylation activity (lower $V_{max}$ and higher $K_m$) by their liver microsomes, compared with the other 41 Caucasian subjects who were homozygous *CYP2A6*1/*1* (Inoue *et al.*, 2000). Individuals with homozygous *CYP2A6*2/*2* showed completely abolished 7-hydroxylation activity (Hadidi *et al.*, 1997; Oscarson *et al.*, 1998).

*CYP2A6*3* was first shown to be the product of gene conversion between *CYP2A6*1* and *CYP2A7* in the exons. It existed at frequencies up to 28%, and was proposed to be inactive (Fernandez-Salguero *et al.*, 1995). However, Oscarson *et al.* (1998) used primers that were designed to bind exons 1 and 4 instead of introns or flanking sequences and found the allele frequencies to be much lower in different populations (Oscarson *et al.*, 1998; Oscarson *et al.*, 1999a). There is still controversy over the detection methods and allele frequency, and the coumarin 7-hydroxylation activity of this allele is unknown (Kitagawa *et al.*, 2001; Goodz and Tyndale, 2002).

*CYP2A6*4* is a deleted allele. The deletion of *CYP2A6*4* is caused by the unequal crossover between *CYP2A6* and the neighboring gene *CYP2A7*. *CYP2A6*4* was discovered when Nunoya *et al.* (1998) found that 2.6 kb of the CYP2A6 gene was deleted in a Japanese individual, rendering the individual unable to metabolize coumarin to 7-HC. Individuals with the *CYP2A6*4/*4* genotype generally excreted little to no 7-HC. Individuals who were heterozygous for *CYP2A6*4* and a loss-of-function or

decrease-of-function variant allele (*e.g.*, genotype *CYP2A6*4/*7* or *CYP2A6*4/*10*) generally showed reduced coumarin 7-hydroxylation (Oscarson *et al.*, 1999b; Ujjin *et al.*, 2002; Xu *et al.*, 2002; Kiyotani *et al.*, 2003; Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009). Microsomal preparations from individuals with the *CYP2A6*4/*4* genotype produced no detectable 7-HC (Yoshida *et al.*, 2003); the $V_{max}$ values from the liver microsomes of heterozygous individuals carrying one copy of the *CYP2A6*4* allele (such as *CYP2A6*1/*4* and *CYP2A6*4/*9*) were lower than wild-type, while the $K_m$ values remained similar to wild-type values.

*CYP2A6*5* carries a SNP that translates into G479V as well as gene conversion in the 3'-UTR. Heterologous expression of CYP2A6*5 in *S. cerevisiae* resulted in very low levels of CPY2A6 protein expression; and coumarin 7-hydroxylase activity was not detectable (Oscarson *et al.*, 1999a). Also, an individual with the genotype *CYP2A6*4/*5* completely lacked coumarin 7-hydroxylation activity *in vivo* (Oscarson *et al.*, 1999a). The recombinant CYP2A6*5 protein was also undetectable in two other studies (Han *et al.*, 2012; Hosono *et al.*, 2017).

*CYP2A6*6* encodes a SNP that leads to the substitution R128Q. The coumarin 7-hydroxylase activity of heterologously expressed CYP2A6*6 was less than 2% of the wild-type in one study (Kitagawa *et al.*, 2001) and undetectable in another study (Hosono *et al.*, 2017). In the Kitagawa *et al.* (2001) study, the rate of the coumarin 7-hydroxylation reaction was only one-tenth that of the CYP2A6*1 enzyme, and the $K_m$ value of CYP2A6*6 was about 5-fold higher than CYP2A6*1. Spectrum analysis showed that CYP2A6*6 has reduced heme incorporation capacity.

CYP2A6*7 carries the substitution I471T resulting from a SNP in exon 9, and a *CYP2A7* gene conversion in the 3'-UTR. The I471 residue is located in the region that affects substrate specificity, though the effects of this mutation on the substrate specificity of the enzyme for coumarin are unclear. It is possible that individuals who are homozygous *CYP2A6*7/*7* or heterozygous with *CYP2A6*7* and an inactive allele may be poor metabolizers of coumarin. The coumarin 7-hydroxylation rate of the recombinant CYP2A6*7 expressed in *E. coli* was about 63% of that of the wild-type enzyme, while nicotine *C*-oxidation activity was completely abolished (Ariyoshi *et al.*, 2001). The recombinant CYP2A6*7 protein showed lower $V_{max}$, higher $K_m$, and much lower $V_{max}/K_m$ values (less than 10%) compared to CYP2A6*1 (Fukami *et al.*, 2005b). Iwasaki *et al.* (2004) examined the slope of the 7-HC velocity versus the substrate concentration and observed a $V_{max}/K_m$ value for CYP2A6*7 that was only 1.7% of wild-type. Other studies have also reported reduced 7-hydroxylase activity of CYP2A6*7, but to a different extent (Han *et al.*, 2012; Uno *et al.*, 2013; Yamamiya *et al.*, 2014; Hosono *et al.*, 2017). One study with human liver microsomes showed low activity with

the genotypes *CYP2A6*4/*7* and *CYP2A6*7/*10* (Yoshida et al., 2003). Two studies with human volunteers reported that individuals with the heterozygous *CYP2A6*1A/*7* genotype metabolized coumarin to a similar extent as the wild-type (Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009). Three homozygous *CYP2A6*7/*7* individuals showed reduced coumarin 7-hydroxylase activity (about 55% compared to wild-type individuals) (Peamkrasatam *et al.*, 2006). In a study of Japanese volunteers administered 50 mg of coumarin orally, one individual with the *CYP2A6*7/*7* genotype showed higher 7-hydroxylase activity than the individuals with wild-type *CYP2A6*1/*1*, and two individuals with the *CYP2A6*4/*7* genotype showed a slight decrease (Xu *et al.*, 2002). In another study, one individual with the *CYP2A6*4/*7* genotype showed reduced activity (about 40% compared to wild-type) (Peamkrasatam *et al.*, 2006). Overall, CYP2A6*7 is considered to have reduced coumarin 7-hydroxylase activity, compared to the wild-type allele.

CYP2A6*8 carries a single amino acid substitution R485L and a gene conversion in the 3' flanking region. The plasma AUC of 7-HC from an individual with the genotype *CYP2A6*1/*8* was slightly smaller than that from wild-type individuals (Xu *et al.*, 2002). The dissociation constant ($K_d$) of purified CYP2A6*8 for coumarin was much higher than that of the wild-type enzyme (Han *et al.*, 2012), and the $k_{cat}$ value for CYP2A6*8 was also higher than that of the wild-type enzyme. The catalytic efficiency ($k_{cat}/K_m$) of CYP2A6*8 was about 50% of CYP2A6*1. In three studies using heterologously expressed enzymes, CYP2A6*8 showed decreased coumarin 7-hydroxylase activity (Han *et al.*, 2012; Yamamiya *et al.*, 2014; Hosono *et al.*, 2017).

*CYP2A6*9* is different from other variants with SNPs in that it carries a mutation, -48T>G (upstream of the start codon), in the TATA box of the promoter of the gene (Pitarque *et al.*, 2001). Two other *CYP2A6* allele subtypes, **1D* and **1H*, also carry genetic polymorphisms in the promoter region that lead to reduced transcriptional activity *in vitro* (Haberl *et al.*, 2005). Because of the impaired promoter activity, the CYP2A6*9 protein expression level is about 50% of wild-type levels. The expression levels of CYP2A6 protein in *CYP2A6*1/*9* and *CYP2A6*4/*9* individuals were lower than that of wild-type individuals. The *in vitro* coumarin 7-hydroxylase activities of the liver microsomal preparations from individuals with genotypes **1/*9*, **4/*9*, or **9/*9* were lower than the individuals carrying the wild-type alleles (Kiyotani *et al.*, 2003; Yoshida *et al.*, 2003). Urinary excretion of 7-HC from Thai individuals carrying at least one copy of the **9* allele is slightly lower than the wild-type (Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009).

CYP2A6*10 carries both the CYP2A6*7 (I471T) and CYP2A6*8 (R485L) substitutions on the same allele. The homology model of CYP2A6, which is based on the crystal

structure of CYP2C5, showed that both I471 (as in CYP2A6*7) and R485 (as in CYP2A6*8) are not part of the active site (F480), but are in the neighboring hinge region that controls the conformation of one of the substrate recognition sites (Xu *et al.*, 2002). Heterologously expressed CYP2A6*10 showed very little or no detectable activity *in vitro* (Yamamiya *et al*., 2014; Hosono *et al*., 2017). In one study, an individual with the genotype *CYP2A6*4/*10* exhibited much lower 7-hydroxylation activity than the wild-type individuals (Xu *et al.*, 2002). This finding is consistent with the Nakajima *et al.* (2006) report that Korean and Japanese subjects carrying any two of the following four alleles had lower nicotine metabolic ratios: **4*, **7*, **9*, and **10*. In two other studies, *CYP2A6*1/*10* individuals exhibited greater than 80% of the 7-hydroxylase activity of wild-type individuals (Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009). In individuals with one wild-type allele and one defective allele, the effect of the defective allele on enzyme activity data is often masked or confounded by the presence of the functional allele.

*CYP2A6*11* was discovered by sequencing the *CYP2A6* alleles from a cancer patient who was not able to metabolize the drug Tegafur, and it carries a mutation that translates into the amino acid substitution S224P (Daigo *et al.*, 2002). The intrinsic clearance ($V_{max}/K_{m)}$ of recombinant CYP2A6*11 was lower than the wild-type enzyme in three studies (Daigo *et al.*, 2002; Yamamiya *et al.*, 2014; Hosono *et al.*, 2017).

*CYP2A6*12* is another allele that results from the unequal crossover between *CYP2A6* and *CYP2A7*. The CYP2A6*12 protein carries 10 amino acid substitutions. This allele was discovered in a Spanish population, from which three individuals from the same family were tested for coumarin 7-hydroxylase activity. The homozygous subject (**12/*12*) showed about 50% of 7-HC excretion compared to her wild-type sister; their heterozygous father (*CYP2A6*1/*12*) also showed about 50% activity. These results indicate decreased activity of CYP2A6*12 (Oscarson *et al.*, 2002). The *in vitro* coumarin 7-hydroxylation assays using recombinant protein showed a similar extent of reduction of the enzyme activity to the data from the three volunteers.

CYP2A6*13 (carrying the amino acid substitution G5R and -48T>G in the TATA box of the 5'-UTR) and CYP2A6*14 (S29N) have shown decreased and increased coumarin 7-hydroxylase activity compared to wild-type, respectively, when expressed heterologously (Hosono *et al.*, 2017).

Like *CYP2A6*9*, *CYP2A6*15* carries the mutation -48T>G; the enzyme CYP2A6*15 also carries the single amino acid substitution K194E. This residue is not located in any substrate recognition site (Kiyotani *et al.*, 2002). CYP2A6*15 showed higher $V_{max}$ but also higher $K_m$ values compared to the wild-type enzyme (Tiong *et al.*, 2010). The

intrinsic clearance ($V_{max}/K_m$) of CYP2A6*15 was about 85% of wild-type enzyme (CYP2A6*1) in one study (Tiong *et al.*, 2010). The activity of CYP2A6*15 was comparable to or higher than that of the wild-type enzyme in three other studies (Uno *et al.*, 2013; Tiong *et al.*, 2014; Hosono *et al.*, 2017).

CYP2A6*16 carries a SNP in exon 4, which translates into the substitution R203S (Kiyotani *et al.*, 2002). CYP2A6*16 did not show much difference in activity from the wild-type enzyme according to two studies that measured the 7-hydroxylase activity of the recombinant enzyme *in vitro* (Ho *et al.*, 2008; Tiong *et al.*, 2010). In another study, the activity of CYP2A6*16 was slightly lower than the wild-type enzyme (Hosono *et al.*, 2017).

The $K_m$ value of CYP2A6*17 was significantly higher than CYP2A6*1, whereas the $V_{max}$ values did not differ (Fukami *et al.*, 2004). The intrinsic clearance (Fukami *et al.*, 2004; Hosono *et al.*, 2017) as well as the $V_{max}$ (Ho *et al.*, 2008) for coumarin 7-hydroxylation were both reduced with this variant, as compared with the wild-type.

For both CYP2A6*18 and CYP2A6*19, there was considerable disagreement in the degree of change of CYP2A6 coumarin 7-hydroxylase kinetic properties. In one study of CYP2A6*18, the $V_{max}$ value was lower than that for CYP2A6*1, while the $K_m$ value was higher. The $V_{max}/K_m$ value was 50% of that of the wild-type enzyme (Fukami *et al.*, 2005b). Another study showed that the $V_{max}/K_m$ of recombinant CYP2A6*18 was similar to the wild-type (Hosono *et al.*, 2017). In a different study, the $k_{cat}/K_m$ value of CYP2A6*18 was around 77% of CYP2A6*1 (Han *et al.*, 2012).

CYP2A6*19 carries both substitutions from CYP2A6*7 (I471T) and CYP2A6*18 (Y392F), as well as gene conversion in the 3'-UTR (Fukami *et al.*, 2005b). Recombinant CYP2A6*19 showed higher $K_m$ and lower $V_{max}$, thus a lower $V_{max}/K_m$ (8% of the wild-type in one study and 15% in the other) (Fukami *et al.*, 2005b; Hosono *et al.*, 2017). Han *et al.* (2012) reported than CYP2A6*19 showed much lower $k_{cat}$ and slightly higher $K_m$. The $k_{cat}/K_m$ value was 66% compared with wild-type.

CYP2A6*20 is a truncated protein and has no enzyme activity (Fukami *et al.*, 2005a).

CYP2A6*21 carries a substitution K476R and exhibited 88-102% coumarin 7-hydroxylase activity compared to wild-type in three studies (Tiong *et al.*, 2010; Tiong *et al.*, 2014; Hosono *et al.*, 2017). The K476 residue is located in one of the substrate recognition sites, but substituting one strong basic residue, Lys, for another strong basic residue, Arg, did not seem to affect the enzyme function.

*CYP2A6*22* encodes an enzyme with the amino acid substitutions D158E and L160I, both located in the exterior of the putative active site. The purified recombinant CYP2A6*22 showed reduced activity compared to CYP2A6*1 (Tiong *et al.*, 2010; Tiong *et al.*, 2014; Hosono *et al.*, 2017).

*CYP2A6*23* carries a SNP in the gene that translates into R203C. In contrast to CYP2A6*16, which carries an amino acid change at the same residue (R203S) and retained most of the coumarin 7-hydroxylase activity *in vitro*, 7-HC formation with CYP2A6*23 was less than 30% (Ho *et al.*, 2008). Ho and colleagues believe that Arg203 is important in the orientation of Phe209, which is critical for coumarin binding. Another study using heterologously expressed protein showed that CYP2A6*23 retained 85% of activity compared to wild-type (Hosono *et al.*, 2017).

CYP2A6*24 carries two substitutions resulting from SNPs (V110L and N438Y) and gene conversion in the 3'-UTR. The heterologously expressed CYP2A6*24 enzyme showed 48% activity (measured by $V_{max}/K_m$) compared to wild-type (Hosono *et al.*, 2017).

CYP2A6*25 carries a single substitution F118L that led to decreased coumarin 7-hydroxylase activity as measured by two studies (Uno *et al.*, 2013; Uno *et al.*, 2015) and no detectable activity by one study (Hosono *et al.*, 2017).

CYP2A6*26 contains three amino acid substitutions, namely F118L, R128L, and S131A. Its coumarin 7-hydroxylase activity was not detectable (Hosono *et al.*, 2017).

CYP2A6*27 carries an amino acid substitution F118L, and a frame shift mutation at R203. The coumarin 7-hydroxylation activity of CYP2A6*27 has not been tested, but it was inactive when tested for trans-3'-hydroxycotinine/cotinine (3HC/COT) ratios (Mwenifumbo *et al.*, 2008a). Hosono *et al.* (2017) proposed that this enzyme would most likely be inactive.

CYP2A6*28 carries N418D and E419D, and has shown coumarin 7-hydroxylase activity that is similar to wild-type (Hosono *et al.*, 2017).

The next several alleles, *CYP2A6*29*, **30*, **32*, and **33*, have been identified by researchers but not published as of July 2017.

CYP2A6*31 carries a single substitution, M6L, and has shown coumarin 7-hydroxylase activity that is comparable to wild-type (Hosono *et al.*, 2017).

CYP2A6*34 is a CYP2A6 and CYP2A7 conversion allele, and carries multiple amino acid substitutions (di Iulio *et al.*, 2009). di Iulio and colleagues showed that one of the amino acid substitutions in CYP2A6*34, namely V117A, reduced coumarin 7-hydroxylase activity when the substitution is introduced to the wild-type CYP2A6 (He *et al.*, 2004). *CYP2A6*34* also carries ten SNPs that are also in *CYP2A6*12* and two SNPs that are also in *CYP2A6*26*; both CYP2A6*12 and CYP2A6*26 have decreased enzyme activity (Oscarson *et al.*, 2002; Mwenifumbo *et al.*, 2008a). There are no data on the coumarin 7-hydroxylase activity of CYP2A6*34, but it is reasonable to expect decreased, if not completely diminished, activity.

CYP2A6*35 carries a substitution (N438Y) as well as gene conversion in the 3'-UTR. Hosono *et al.* (2017) showed that CYP2A6*35 has decreased coumarin 7-hydroxylation activities. Al Koudsi *et al.* (2009) reported that the $V_{max}/K_m$ value of CYP2A6*35 was lower than the wild-type as examined by the nicotine C-oxidation assay, both *in vitro* and *in vivo*. The study showed a lower $K_m$ for CYP2A6*35. Another group reported decreased catalytic efficiency ($k_{cat}/K_m$) with CYP2A6*35, but not to a great extent, because both $k_{cat}$ and $K_m$ decreased (Han *et al.*, 2012).

CYP2A6*36 carries two SNP substitutions (N438Y and I471T) as well as gene conversion in the 3' UTR. CYP2A6*37 carries three SNP substitutions (N438Y, I471T, and R485L) as well as gene conversion in the 3'-UTR. Hosono *et al.* (2017) heterologously expressed these two variants in 293FT cells and assessed coumarin 7-hydroxylase activity. Both CYP2A6*36 and CYP2A6*37 showed no detectable activity.

CYP2A6*38, *39, *40, *41, *42, *43, *44, and *45 were tested for coumarin 7-hydroxylation *in vitro* by Hosono *et al.* (2017), and showed either no activity (*44) or decreased activity (*38, *39, *40, *41, *42, *43, and *45).

Among these newly discovered and characterized allele variants, *CYP2A6*41* carries a mutation that leads to the amino acid substitution R265Q. This substitution is located within a connection loop between two α-helices. A nearby alteration, I268T, is present in CYP2A6*42. When heterologously expressed, CYP2A6*41 was undetectable and CYP2A6*42 expression was very low (Piliguian *et al.*, 2014). The authors hypothesized that these two allele variants carry alterations in a region that is important for protein stability. Both variants had decreased coumarin 7-hydroxylation activities in a separate study (Hosono *et al.*, 2017).

**Table B1. Coumarin 7-hydroxylation activity of CYP2A6 variants[a]**

| CYP2A6 variant | Molecular variation | Functional consequence | Coumarin 7-hydroxylase activities of CYP2A6 | | | Parameters measured | Reference |
|---|---|---|---|---|---|---|---|
| | | | Recombinant protein | Human liver microsomes | Human subjects (*in vivo*) | | |
| CYP2A6*1A | None | Fully functional | 100% | 100% | | $V_{max}$ | Yamano *et al.* (1990) |
| CYP2A6*1B | 58bp conversion of CYP2A7 to the 3'-UTR of CYP2A6 | Increased activity (more enzyme) | | | 119% (**1A/*1B*, N=80), 80% (**1B/*1B*, N=31), 108% (**1B/*4*, N=10) | Urinary 7-HC excretion | Ujjin *et al.* (2002) |
| | | | | | 144% (**1A/*1B*, N=22), 109% (**1B/*1B*, N=9) | Urinary 7-HC excretion | Peamkrasatam *et al.* (2006) |
| | | | | | 94% (**1A/*1B*, N=37), 90% (**1B/*1B,* N=31), 72% (**1B/*4C*, N=8), 116% (**1B/*7*, N=4), 106% (**1B/*8*, N=1), 94% (**1B/*9*, N=22), 56% (**1B/*10*, N=3) | Urinary 7-HC excretion[b] | Mahavorasirikul *et al.* (2009) |
| CYP2A6*2 | L160H | No activity | ND | | | $V_{max}$ | Yamano *et al.* (1990) |
| | | | | | 74% (**1/*2*, N=3), <0.025% (**2/*2*, N=1) | Urinary 7-HC excretion | Hadidi *et al.* (1997) |
| | | | | | 79% (**1/*2*, N=3), 0 (**2/*2*, N=2) | Urinary 7-HC excretion | Oscarson *et al.* (1998) |
| | | | | 10% (**1/*2*, N=2) | | $V_{max}/K_m$ | Inoue *et al.* (2000) |
| | | | ND | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |

| CYP2A6 variant | Molecular variation | Functional consequence | Coumarin 7-hydroxylase activities of CYP2A6 | | | Parameters measured | Reference |
|---|---|---|---|---|---|---|---|
| | | | Recombinant protein | Human liver microsomes | Human subjects (*in vivo*) | | |
| CYP2A6*4 | Gene deleted | No activity (no enzyme) | | ND | | $V_{max}$ | Nunoya *et al.* (1998) |
| | | | | | 44% (**1/*4*, N=2), 0 (**4/*4*, N=1) | Urinary 7-HC excretion | Oscarson *et al.* (1999b) |
| | | | | | 87% (**1A/*4*, N=12), 108% (**1B/*4*, N=10), 1% (**4/*4*, N=4) | Urinary 7-HC excretion | Ujjin *et al.* (2002) |
| | | | | | 0 (**4/*4*, N=3), 88% (**4/*7*, N=2), 21% (**4/*10*, N=1) | 7-HC plasma AUC[b] | Xu *et al.* (2002) |
| | | | | 41% (**1/*4*, N=6), 0 (**4/*4*, N=2), 12% (**4/*9*, N=7) | | $V_{max}$ | Kiyotani *et al.* (2003) |
| | | | | 50% (**1/*4*, N=4), ND (**4/*4*, N=1), 4% (**4/*7*, N=1), 20% (**4/*9*, N=3) | | $V_{max}$ | Yoshida *et al.* (2003) |
| | | | | | 63% (**1/*4*, N=23), 7% (**4/*4*, N=4), 41% (**4/*7*, N=1), 46% (**4/*9*, N=2) | Urinary 7-HC excretion | Peamkrasatam *et al.* (2006) |
| | | | | | 73% (**1A/*4C*, N=20), 72% (**1B/*4C*, N=8), 15% (**4C/*4C*, N=1), 83% (**4C/*9*, N=6) | Urinary 7-HC excretion[b] | Mahavorasirikul *et al.* (2009) |

| CYP2A6 variant | Molecular variation | Functional consequence | Coumarin 7-hydroxylase activities of CYP2A6 | | | Parameters measured | Reference |
|---|---|---|---|---|---|---|---|
| | | | Recombinant protein | Human liver microsomes | Human subjects (*in vivo*) | | |
| CYP2A6*5 | G479V and gene conversion in the 3'-UTR | No activity (unstable enzyme) | ND | | | $V_{max}$ | Oscarson *et al.* (1999a) |
| | | | ND | | | $k_{cat}/K_m$ | Han *et al.* (2012) |
| | | | ND | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*6 | R128Q | Decreased activity | 2% | | | $V_{max}/K_m$ | Kitagawa *et al.* (2001) |
| | | | ND | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*7 | I471T and gene conversion in the 3'-UTR | Decreased activity | 63% | | | $V_{max}$ | Ariyoshi *et al.* (2001) |
| | | | | | 114% (**7/*7*, N=1), 88% (**4/*7*, N=2) | 7-HC plasma AUC[b] | Xu *et al.* (2002) |
| | | | | 4% (**4/*7*, N=1), 3% (**7/*10*, N=1) | | $V_{max}$ | Yoshida *et al.* (2003) |
| | | | 2% | | | $V_{max}/K_m$ | Iwasaki *et al.* (2004) |
| | | | 8% | | | $V_{max}/K_m$ | Fukami *et al.* (2005b) |
| | | | | | 82% (**1/*7*, N=5), 41% (**4/*7*, N=1), 55% (**7/*7*, N=3) | Urinary 7-HC excretion | Peamkrasatam *et al.* (2006) |
| | | | | | 99% (**1A/*7*, N=16), 117% (**1B/*7*, N=4), 85% (**7/*9*, N=4), 16% (**7/*10*, N=1) | Urinary 7-HC excretion[b] | Mahavorasirikul *et al.* (2009) |
| | | | 83% | | | $k_{cat}/K_m$ | Han *et al.* (2012) |

| CYP2A6 variant | Molecular variation | Functional consequence | Coumarin 7-hydroxylase activities of CYP2A6 | | | Parameters measured | Reference |
|---|---|---|---|---|---|---|---|
| | | | Recombinant protein | Human liver microsomes | Human subjects (*in vivo*) | | |
| | | | 31% | | | $V_{max}$ | Uno *et al.* (2013) |
| | | | 17% | | | $V_{max}/K_m$ | Yamamiya *et al.* (2014) |
| | | | 26% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*8 | R485L and gene conversion in the 3'-UTR | Decreased activity | | | 65% (**1/*8*, N=1) | 7-HC plasma AUC[b] | Xu *et al.* (2002) |
| | | | | | 68% (**1A/*8*, N=1), 107% (**1B/*8*, N=1) | Urinary 7-HC excretion[b] | Mahavorasirikul *et al.* (2009) |
| | | | 50% | | | $k_{cat}/K_m$ | Han *et al.* (2012) |
| | | | 51% | | | $V_{max}/K_m$ | Yamamiya *et al.* (2014) |
| | | | 23% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*9 | -48T>G in the TATA box | Decreased activity | | 71% (**1/*9*, N=9), 12% (**4/*9*, N=7) | | $V_{max}$ | Kiyotani *et al.* (2003) |
| | | | | 89% (**1/*9*, N=4), 20% (**4/*9*, N=3), 69% (**9/*9*, N=1) | | $V_{max}$ | Yoshida *et al.* (2003) |
| | | | | | 97% (**1/*9*, N=19), 80% (**9/*9*, N=14) | Urinary 7-HC excretion | Peamkrasatam *et al.* (2006) |

| CYP2A6 variant | Molecular variation | Functional consequence | Coumarin 7-hydroxylase activities of CYP2A6 | | | Parameters measured | Reference |
|---|---|---|---|---|---|---|---|
| | | | Recombinant protein | Human liver microsomes | Human subjects (*in vivo*) | | |
| | | | | | 100% (**1A/*9*, N=14), 93% (**1B/*9*, N=22), 81% (**9/*10*, N=1) | Urinary 7-HC excretion[b] | Mahavorasirikul *et al.* (2009) |
| CYP2A6*10 | I471T, R485L, and gene conversion in the 3'-UTR | Decreased activity | | | 21% (**4/*10*, N=1) | 7-HC plasma AUC[b] | Xu *et al.* (2002) |
| | | | | 3% (**7/*10*, N=1) | | $V_{max}$ | Yoshida *et al.* (2003) |
| | | | | | 81% (**1/*10*, N=4) | Urinary 7-HC excretion | Peamkrasatam *et al.* (2006) |
| | | | | | 82% (**1A/*10*, N=4), 56% (**1B/*10*, N=3), 16% (**7/*10*, N=1), 81% (**9/*10*, N=1) | Urinary 7-HC excretion[b] | Mahavorasirikul *et al.* (2009) |
| | | | 5% | | | $V_{max}/K_m$ | Yamamiya *et al.* (2014) |
| | | | ND | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*11 | S224P | Decreased activity | 59% | | | $V_{max}/K_m$ | Daigo *et al.* (2002) |
| | | | 37% | | | $V_{max}/K_m$ | Yamamiya *et al.* (2014) |
| | | | 26% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*12 | Hybrid of CYP2A6 and CYP2A7 with 10 amino acid substitutions | Decreased activity | 49% | | 51% (**1/*12*, N=1), 48% (**12/*12*, N=1) | *In vitro* data: amount of 7-HC formed; human data: coumarin index | Oscarson *et al.* (2002) |

| CYP2A6 variant | Molecular variation | Functional consequence | Coumarin 7-hydroxylase activities of CYP2A6 | | | Parameters measured | Reference |
|---|---|---|---|---|---|---|---|
| | | | Recombinant protein | Human liver microsomes | Human subjects (*in vivo*) | | |
| CYP2A6*13 | -48T>G in the TATA box and G5R | Decreased activity | 63% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*14 | S29N | Increased activity | 149% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*15 | -48T>G in the TATA box and K194E | Similar to wild-type or increased activity | 85% | | | $V_{max}/K_m$ | Tiong *et al.* (2010) |
| | | | 110% | | | $V_{max}$ | Uno *et al.* (2013) |
| | | | 236% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| | | | 108% | | | $V_{max}$[b] | Tiong *et al.* (2014) |
| CYP2A6*16 | R203S | Similar to wild-type | 100% | | | $V_{max}$[b,c] | Ho *et al.* (2008) |
| | | | 105% | | | $V_{max}/K_m$ | Tiong *et al.* (2010) |
| | | | 117% | | | $V_{max}$[b] | Tiong *et al.* (2014) |
| | | | 81% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*17 | V365M | Decreased activity | 58% | | | $V_{max}/K_m$ | Fukami *et al.* (2004) |
| | | | 83% | | | $V_{max}$[b,c] | Ho *et al.* (2008) |
| | | | 53% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*18 | Y392F | Decreased activity | 50% | | | $V_{max}/K_m$ | Fukami *et al.* (2005b) |
| | | | 77% | | | $K_{cat}/K_m$ | Han *et al.* (2012) |
| | | | 95% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |

| CYP2A6 variant | Molecular variation | Functional consequence | Coumarin 7-hydroxylase activities of CYP2A6 | | | Parameters measured | Reference |
|---|---|---|---|---|---|---|---|
| | | | Recombinant protein | Human liver microsomes | Human subjects (*in vivo*) | | |
| CYP2A6*19 | Y392F, I471T, and gene conversion in the 3'-UTR | Decreased activity | 8% | | | $V_{max}/K_m$ | Fukami *et al.* (2005b) |
| | | | 66% | | | $K_{cat}/K_m$ | Han *et al.* (2012) |
| | | | 15% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*20 | Frameshift mutation | No activity (truncated enzyme) | ND | | | $V_{max}$ | Fukami *et al.* (2005a) |
| CYP2A6*21 | K476R | Similar to wild-type | 91% | | | $V_{max}/K_m$ | Tiong *et al.* (2010) |
| | | | 102% | | | $V_{max}$[b] | Tiong *et al.* (2014) |
| | | | 88% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*22 | D158E, L160I | Decreased activity | 39% | | | $V_{max}/K_m$ | Tiong *et al.* (2010) |
| | | | 52% | | | $V_{max}$[b] | Tiong *et al.* (2014) |
| | | | 72% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*23 | R203C | Decreased activity | 28% | | | $V_{max}$[b,c] | Ho *et al.* (2008) |
| | | | 85% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*24 | V110L, N438Y, and gene conversion in the 3'-UTR | Decreased activity | 48% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*25 | F118L | Decreased activity | 41% | | | $V_{max}$ | Uno *et al.* (2013) |

| CYP2A6 variant | Molecular variation | Functional consequence | Coumarin 7-hydroxylase activities of CYP2A6 | | | Parameters measured | Reference |
|---|---|---|---|---|---|---|---|
| | | | Recombinant protein | Human liver microsomes | Human subjects (*in vivo*) | | |
| | | | 63% | | | $V_{max}$ | Uno *et al.* (2015) |
| | | | ND | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*26 | F118L, R128L, S131A | No activity | ND | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*28 | N418D, E419D, and gene conversion in the 3'-UTR | Similar to wild-type | 117% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*31 | M6L | Similar to wild-type | 115% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*35 | N438Y and gene conversion in the 3'-UTR | Decreased activity | 86% | | | $K_{cat}/K_m$ | Han *et al.* (2012) |
| | | | 61% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*36 | N438Y, I471T, and gene conversion in the 3'-UTR | No activity | ND | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*37 | N438Y, I471T, R485L | No activity | ND | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*38 | Y351H | Decreased activity | 45% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*39 | V68M | Decreased activity | 41% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*40 | I149M | Decreased activity | 33% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |

| CYP2A6 variant | Molecular variation | Functional consequence | Coumarin 7-hydroxylase activities of CYP2A6 | | | Parameters measured | Reference |
|---|---|---|---|---|---|---|---|
| | | | Recombinant protein | Human liver microsomes | Human subjects (*in vivo*) | | |
| CYP2A6*41 | R265Q | Decreased activity | 18% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*42 | I268T | Decreased activity | 6% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*43 | T303I | Decreased activity | 5% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*44 | E390K, N418D, E419D | No activity | ND | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |
| CYP2A6*45 | L462P | Decreased activity | 21% | | | $V_{max}/K_m$ | Hosono *et al.* (2017) |

[a] The coumarin 7-hydroxylase activity of the CYP2A6 variant is expressed as the percentage of the arithmetic mean of the reported wild-type (*i.e.*, CYP2A6*1) enzyme activity in the study.

[b] Data extracted from figures (bar graphs) with GetData Graph Digitizer (version 2.26.0.20).

[c] The percentages are the average of two experiments with 5 and 50 µM coumarin each.

Acronyms and parameters: $V_{max}$: Maximum Velocity; $K_m$: Michaelis-Menten Constant; $k_{cat}$: Catalytic Constant; ND: Not detected

## B2. Distribution of CYP2A6 allele variants in different populations and ethnicities

We have summarized the distribution of seven of the most well-studied and enzymatically consequential loss-of-function (**2, *4, *5,* and **20*) or decrease-of-function (**7, *9,* and **10*) *CYP2A6* alleles is summarized in Table B2, and discussed briefly in the text that follows the table. Information on the frequencies of other *CYP2A6* alleles has been reviewed by López-Flores *et al.* (2017).

**Table B2. Frequencies of seven *CYP2A6* loss-of-function and decrease-of-function alleles in different populations and ethnicities[4]**

| Geographic Continent | Ethnicity/ Population | Number of Subjects | Loss-of-function alleles (%) | | | | Decrease-of-function alleles (%) | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **2* | **4* | **5* | **20* | **7* | **9* | **10* | |
| Africa | Ethiopian | 172 (100 living in Ethiopia and 72 living in Sweden) | 0.6 | 0.6 | 0 | | | 2.8 | | Aklillu *et al.* (2014) |
| | Ghanaian | 105 | 0 | 1.9 | 0 | | 0 | 5.7 | 0 | Gyamfi *et al.* (2005) |
| | Namibian Ovambo | 150 | | 0 | | | | | | Takeshita *et al.* (2006) |
| | Nigerian | 180 | | | | | | 11.0 | | Adehin *et al.* (2017) |
| | Ugandan | 123 (HIV+ patients with Neuropsychiatric disorder) | | | | | | 11.4 | | Mukonzo *et al.* (2013) |
| | | 46 (HIV+ patients without neuropsychiatric disorder) | | | | | | 7.6 | | |
| Asia | Bangladeshi | 106 (patients with lung cancer) | | 4.7 | | | | | | Islam *et al.* (2013) |
| | | 116 (controls) | | 11.2 | | | | | | |
| | Chinese | 96 | 0 | 15.1 | 1.0 | | | | | Oscarson *et al.* (1999b); Oscarson *et al.* (1999a) |
| | | 102 | | | | | | 15.7 | | Pitarque *et al.* (2001) |
| | | 180 (patients with lung cancer) | | 6.4 | | | | | | Gu *et al.* (2005) |

[4] Ordered alphabetically by continent and ethnicity.

| Geographic Continent | Ethnicity/ Population | Number of Subjects | Loss-of-function alleles (%) | | | | Decrease-of-function alleles (%) | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *2 | *4 | *5 | *20 | *7 | *9 | *10 | |
| | | 224 (controls) | | 13.4 | | | | | | |
| | | 168 (non-smokers) | | 12.5 | | | | | | Tang *et al.* (2009) |
| | | 203 (smokers) | | 4.9 | | | | | | Tang *et al.* (2009) |
| | | 1328 (smokers) | | 8.5 | 1.2 | | 6.3 | 13.5 | 2.4 | Liu *et al.* (2011) |
| | | 77 (patients with digestive system cancer)[5] | | 12.3 | | | | | | Fang *et al.* (2012) |
| | | 100 (Bouyei Chinese) | | 0 | | | | | | Pang *et al.* (2015) |
| | | 120 (Han Chinese) | | 7.9 | | | | | | |
| | | 100 (Tibetan Chinese) | | 2.0 | | | | | | |
| | | 100 (Uighur Chinese) | | 15.0 | | | | | | |
| | | 325 (male patients with lung cancer) | | 4.2 | | | 11.1 | 22.0 | | Yuan *et al.* (2016) |
| | | 356 (male controls) | | 6.2 | | | 13.8 | 23.6 | | |
| | | 102 (Han Chinese with epilepsy) | | 8.3 | | | | | | Wang *et al.* (2017) |
| | | 279 (patients with epilepsy) | | 8.1 | | | | | | Zhao *et al.* (2017) |
| | Chinese Malaysian | 172 | 0 | 4.9 | 1.2 | | 7.0 | | 1.7 | Nurfadhlina *et al.* (2006) |
| | Indian | 350 (male patients with head-and-neck cancer) | | 3.9 | | | | | | Ruwali *et al.* (2009) |
| | | 350 (controls) | | 11.3 | | | | | | |
| | | 479 | 1.0 | 8.9 | 0.7 | | | | | Krishnakumar *et al.* (2012) |
| | Indian Malaysian | 174 | 0.3 | 1.4 | 0.9 | | 0 | | 0 | Nurfadhlina *et al.* (2006) |
| | Iranian | 250 | 2.2 | 1.0 | | | | 12.4 | | Emamghoreishi *et al.* (2008) |

[5] Allele frequencies were calculated based on genotype frequencies, and OEHHA's calculation results are different from what was reported by Fang *et al.* (2012) because Fang *et al.* (2012) did not calculate *CYP2A6*4/*4* as 2 alleles.

| Geographic Continent | Ethnicity/ Population | Number of Subjects | Loss-of-function alleles (%) | | | | Decrease-of-function alleles (%) | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **2* | **4* | **5* | **20* | **7* | **9* | **10* | |
| | | 200 (smokers) | 0 | 2.5 | | | | | | Heravi *et al.* (2010) |
| | Japanese | 894 | 0 | | | | | | | Kitagawa *et al.* (2001) |
| | | 63 | 0 | 22.2 | | | 6.3 | | 1.6 | Xu *et al.* (2002) |
| | | 1094 (male smokers with lung cancer) | | 16.0 | | | 9.7 | 19.7 | 2.5 | Fujieda *et al.* (2004) |
| | | 611 (male smokers as controls) | | 19.8 | | | 12.6 | 20.7 | 4.3 | |
| | | 184 | | | | 0 | 13 | | | Fukami *et al.* (2005a); Fukami *et al.* (2005b) |
| | | 200 (smokers) | | 16.5 | | | 11.0 | 20.3 | | Minematsu *et al.* (2006) |
| | | 92[6] | 0 | 19.0 | 0 | 0 | 9.8 | 19.0 | 2.2 | Nakajima *et al.* (2006) |
| | | 374 | | 20.3 | | | | | | Takeshita *et al.* (2006) |
| | | 46 (patients with non-small-cell lung cancer) | | 17.4 | | | 19.6 | 15.2 | | Kaida *et al.* (2008) |
| | | 57 (patients with advanced cancer) | | 21.1 | | | 19.3 | 18.4 | | Hirose *et al.* (2010) |
| | | 89 | | 21.4 | | | 14.6[7] | 21.4[8] | 2.3 | Kumasaka *et al.* (2012) |
| | | 163 (patients with bladder cancer, smokers) | | 18.7 | 0 | | 15.3 | 15.6 | 3.7 | Kumondai *et al.* (2016) |
| | | 116 (controls, smokers) | | 24.6 | 0.4 | | 8.6 | 22.8 | 3.0 | |

[6] It appears that the 92 Japanese individuals and 209 Korean individuals were tested earlier by the same laboratory and the allele frequencies were reported in previous studies by the same research group (Kwon *et al.*, 2001; Yoshida *et al.*, 2003; Fukami *et al.*, 2005a; Fukami *et al.*, 2005b). We are reporting the latest study results here.

[7] 14.6% is the sum of the frequencies of **7*, **36*, and **37*. These three alleles all have a SNP (substitution I471T) in Exon 9. The frequencies of **36* and **37* were assumed negligible since neither allele was found in 60 Japanese individuals (Al Koudsi *et al.*, 2009).

[8] 21.4% is the sum of the frequencies of **9* and **15*. These two alleles both have a SNP (-48T>G) in the TATA box. The frequency for **15* in Japanese individuals was 2.2% in another study (Nakajima *et al.*, 2006).

| Geographic Continent | Ethnicity/ Population | Number of Subjects | Loss-of-function alleles (%) | | | | Decrease-of-function alleles (%) | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *2 | *4 | *5 | *20 | *7 | *9 | *10 | |
| | Korean | 209[9] | 0 | 10.8 | 0.5 | 0 | 9.8 | 19.6 | 1.0 | Nakajima *et al.* (2006) |
| | | 108 (patients with metastatic gastric cancer) | | 13.4 | | | 6.9 | 19.9 | 2.8 | Park *et al.* (2011) |
| | | 144 | | 9.4 | 0 | | 11.1 | 21.9 | 4.2 | Djordjevic *et al.* (2012) |
| | Malay | 270 | 0 | 7.0 | 0.9 | | 4.3 | | 4.3 | Nurfadhlina *et al.* (2006) |
| | | 24 | 0 | 16.7 | | | 0 | 10.4 | | Yusof and Gan (2009) |
| | Sri Lankan | 286 (betel quid chewers with oral malignancies or premalignancies) | | 2.8 | | | | | | Topcu *et al.* (2002) |
| | | 135 (betel quid chewers, controls) | | 9.6 | | | | | | |
| | Taiwanese | 319 | | | | | 10.0 | | 4.1 | Mwenifumbo *et al.* (2005) |
| | Thai | 198 | | 7.8 | | | | | | Ujjin *et al.* (2002) |
| | | 120 | | 14.2 | | | 5.0 | 20.4 | 1.7 | Peamkrasatam *et al.* (2006) |
| | | 194 | | 9.3 | | | 6.4 | 12.1 | 2.4 | Mahavorasirikul *et al.* (2009) |
| | Turkish | 109 (or 132)[10] | | | | | | 6.9-7.2 | | Pitarque *et al.* (2001) |
| | | 185 | | 2.2 | | | | | | Takeshita *et al.* (2006) |
| | Vietnamese | 72 | | 11.8 | 14.6 | | | | | Veiga *et al.* (2009) |
| Europe | Caucasian British | 1372 | 2.6 | 0.3 | 0.3 | | | | | Huang *et al.* (2005) |
| | Caucasian French | 244 (patients with lung cancer) | 2.0 | 4.9 | | | | | | Loriot *et al.* (2001) |
| | | 250 (controls) | 2.6 | 3.8 | | | | | | |

[9] It appears that the 92 Japanese individuals and 209 Korean individuals were tested earlier by the same laboratory and the allele frequencies were reported in previous studies by the same research group (Kwon *et al.*, 2001; Yoshida *et al.*, 2003; Fukami *et al.*, 2005a; Fukami *et al.*, 2005b). We are reporting the latest study results here.

[10] The numbers of subjects were 132 for Pitarque *et al.* (2001) and 109 for von Richter *et al.* (2004). These two studies are discussed together because the human subjects analyzed were from the same participants of pharmacogenetics studies at University of Istanbul and could potentially be the same study populations/subjects.

| Geographic Continent | Ethnicity/ Population | Number of Subjects | Loss-of-function alleles (%) | | | | Decrease-of-function alleles (%) | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **2* | **4* | **5* | **20* | **7* | **9* | **10* | |
| | Finnish | 144 | 1.4 | | | | | | | Oscarson *et al.* (1998) |
| | | 100 | 3.0 | 1.0 | | | | | | Oscarson *et al.* (1999b) |
| | German | 216 | 2.3 | | | | | | | Bourian *et al.* (2000) |
| | Tatar Russian | 425 (patients with chronic obstructive pulmonary disease) | | 6.8 | | | | | | Korytina *et al.* (2014) |
| | | 457 (controls) | | 16.9 | | | | | | |
| | Serbian | 140 | | 2.9 | 0 | | | 8.2 | | Djordjevic *et al.* (2010) |
| | South Asian British | 96 adolescents | 0.5 | 0.5 | 0 | | | | | Huang *et al.* (2005) |
| | Spanish | 234 | | 4.0 | | | | 6.4 | | Soriano *et al.* (2011) |
| | Swedish | 90 | 1.1 | | | | | | | Oscarson *et al.* (1998) |
| | | 116 | | | | | | 5.2 | | Pitarque *et al.* (2001) |
| | | 190 | | 1.1 | 0 | | | 7.9 | | Djordjevic *et al.* (2012) |
| North America | African American | 305 | 0.3 | | | | | | | Paschke *et al.* (2001) |
| | | 94 | 0 | 0.5 | 0 | | 0 | 8 | 0 | Nakajima *et al.* (2004) |
| | | 175 (**7*) or 96 (**20*) | | | | 1.6 | 0 | | | Fukami *et al.* (2005a); Fukami *et al.* (2005b) |
| | | 176 | 0.3 | 0.9 | 0 | 1.7 | 0 | 8.5 | 0 | Nakajima *et al.* (2006) |
| | | 618 | 0.9 | 1.9 | | 1.5 | | 9.6 | | Ho *et al.* (2009) |
| | | 38 | | 1.3 | | | | | | Mwenifumbo *et al.* (2010) |
| | African American and African Canadian | 130 for **10* and 134 for all other alleles | 1.1 | 1.9 | 0 | | 0 | 7.1 | 0 | Schoedel *et al.* (2004) |
| | African Canadian | 113 | | | | | 0 | | 0 | Mwenifumbo *et al.* (2005) |

| Geographic Continent | Ethnicity/ Population | Number of Subjects | Loss-of-function alleles (%) | | | | Decrease-of-function alleles (%) | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **2* | **4* | **5* | **20* | **7* | **9* | **10* | |
| | | 281 (for **4*) and 280 (for **9* and **20*) | | 2.7 | | 1.1 | | 7.2 | | Mwenifumbo *et al.* (2008a); Mwenifumbo *et al.* (2010) |
| | Asian American | 49 | | 15.3 | | | | | | Mwenifumbo *et al.* (2010) |
| | Caucasian American | 145 (76 Americans and 69 Germans) | 1.4 | | | | | | | Paschke *et al.* (2001) |
| | | 165 | 1.2 | 3.0 | 0 | | 0 | 7.9 | 0 | Nakajima *et al.* (2004) |
| | | 187 | 1.1 | 0 | 0 | 0 | 0 | 8.0 | 0 | Nakajima *et al.* (2006) |
| | | 222 (adolescent smokers)[11] | 5.3 | 0.6 | | | | 6.1 | | Audrain-McGovern *et al.* (2007) |
| | | 83 | | 0 | | | | | | Mwenifumbo *et al.* (2010) |
| | | 189 | 3.2 | 1.6 | | | | 6.3 | | Bloom *et al.* (2011) |
| | Caucasian Canadian | 281 (smokers) | 3.4 | 0.2 | | | | 6.8 | | O'Loughlin *et al.* (2004) |
| | | (708-1168)[12] | 2.2 | 1.2 | 0.1 | | 0.3 | 7.1 | 0 | Schoedel *et al.* (2004) |
| | | 152 (smokers) | 1.6 | 1.0 | 0 | | 0.3 | 6.3 | 0 | Malaiyandi *et al.* (2006) |
| | | 301 (Xu *et al.*, 2002); 110 (Mwenifumbo *et al.*, 2005)[13] | 1.2 | 1.0 | | | 0 | | 0 | Xu *et al.* (2002); Mwenifumbo *et al.* (2005) |
| | Chinese American | 221 | | | | | 5.7 | | 4.3 | Mwenifumbo *et al.* (2005) |
| | Chinese Canadian | 112 | | | | | | | | Mwenifumbo *et al.* (2005) |

[11] The authors reported 222 study participants, but also noted that *CYP2A6*2*, **4*, and **9* were tested in 490, 478, and 494 alleles, respectively.

[12] Each allele was tested in different numbers of individuals. We are reporting the range of numbers of subjects here.

[13] As reported by Mwenifumbo *et al.* (2005), the *CYP2A6*7* genotyping methods used by Xu *et al.* (2002) "provided ambiguous results for the heterozygous genotypes, which resulted in their underestimation" for *CYP2A6*7*. *CYP2A6*7* and *CYP2A6*10* (**10* carries the same SNP and gene conversion as in **7*, and an additional SNP) frequencies were reassessed by Mwenifumbo *et al.* (2005) and the results for the Caucasian Canadians were the same as Xu *et al.* (2002).

| Geographic Continent | Ethnicity/ Population | Number of Subjects | Loss-of-function alleles (%) | | | | Decrease-of-function alleles (%) | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **2* | **4* | **5* | **20* | **7* | **9* | **10* | |
| | | 114 | 0 | 6.6 | | | 9.8[14] | | 4.0[15] | Xu *et al.* (2002) |
| | Indigenous Peoples in Alaska (Yupik) | 361 | 0.4 | 14.5 | | | 0 | 8.9 | 1.9 | Binnington *et al.* (2012) |
| | Indigenous Peoples in Canada | 108 | 0.9 | | | | | | | Nowak *et al.* (1998) |
| | | (97-101)[16] | 0 | 1.0 | 0.5 | | 0 | 15.5 | 0 | Schoedel *et al.* (2004) |
| | Japanese Canadian | 64 | | | | | 12.5[17] | | 3.2[18] | Mwenifumbo *et al.* (2005) |
| | | 63 | 0 | 22.2 | | | | | | Xu *et al.* (2002) |
| | Korean American | 207 | | | | | 9.4 | | 4.1 | Mwenifumbo *et al.* (2005) |
| | Mexican | 364 | | | | | | 16.4 | | Svyryd *et al.* (2016) |
| Oceania | Māori, New Zealand | 60 | | 9.6 | | | 1.1 | 19.0 | | Lea *et al.* (2008) |
| South America | Brazilian | 412 | 1.7 | 0.5 | | | | 5.7 | | Vasconcelos *et al.* (2005) |
| | | 289 (hospital outpatients with no cancer history) | 1.7 | | 0 | | | | | Rossini *et al.* (2006) |
| | Chilean | 54 | 1.9 | 3.7 | | | | | | Caceres *et al.* (2012) |
| | | 253 | 2.0 | 4 | | | | | | Roco *et al.* (2012) |
| | Mestizo Ecuadorian | 300 | | 7.1 | | | | 10.3 | | Soriano *et al.* (2011) |

[14] As reported by Mwenifumbo *et al.* (2005), the genotyping methods used by Xu *et al.* (2002) "provided ambiguous results for the heterozygous genotypes, which resulted in their underestimation" for *CYP2A6*7*. *CYP2A6*7* and *CYP2A6*10* (**10* carries the same SNP and gene conversion as in **7*, and an additional SNP) frequencies in Chinese Canadians and Japanese Canadians were reassessed by Mwenifumbo *et al.* (2005) and we are reporting the reassessed values here.

[15] *Ibid.*

[16] Each allele was tested in different numbers of individuals. We are reporting the range of numbers of subjects here.

[17] As reported by Mwenifumbo *et al.* (2005), the genotyping methods used by Xu *et al.* (2002) "provided ambiguous results for the heterozygous genotypes, which resulted in their underestimation" for *CYP2A6*7*. *CYP2A6*7* and *CYP2A6*10* (carrying the same SNP and gene conversion as in **7*, and an additional SNP) frequencies in Chinese Canadians and Japanese Canadians were reassessed by Mwenifumbo *et al.* (2005) and we are reporting the reassessed values here.

[18] *Ibid.*

***CYP2A6*2* (loss-of-function)**

The *CYP2A6*2* allele was observed at low frequencies, ranging from 0-5.3% among the studied populations.

Overall, the incidence of *CYP2A6*2* among people of African descent is low, with a maximum of 1.1%. This loss-of-function allele was not found in Ghanaian individuals (Gyamfi *et al.*, 2005), and was found at very low frequencies (0-1.1%) in Ethiopians, African Canadians, and African Americans (Paschke *et al.*, 2001; Nakajima *et al.*, 2004; Schoedel *et al.*, 2004; Nakajima *et al.*, 2006; Ho *et al.*, 2009; Aklillu *et al.*, 2014).

*CYP2A6*2* was not found in East Asian populations, such as Chinese, Chinese Canadians or Chinese Malaysians (Oscarson *et al.*, 1999b; Oscarson *et al.*, 1999a; Xu *et al.*, 2002; Nurfadhlina *et al.*, 2006), Japanese or Japanese Canadians (Kitagawa *et al.*, 2001; Xu *et al.*, 2002; Nakajima *et al.*, 2006), Koreans (Nakajima *et al.*, 2006), and Malays (Nurfadhlina *et al.*, 2006; Yusof and Gan, 2009). It was observed at low frequencies in Indians (1%) (Krishnakumar *et al.*, 2012) and Indian Malaysians (0.3%) (Nurfadhlina *et al.*, 2006). One study in Iranian men found that *CYP2A6*2* frequency was 2.2% (Emamghoreishi *et al.*, 2008), while another study in Iranian smokers did not identify any individuals with the *CYP2A6*2* allele (Heravi *et al.*, 2010).

In European populations, this allele is present at a range of 0.5-3%. Two studies in Finnish individuals showed a range of 1.4-3% (Oscarson *et al.*, 1998; Oscarson *et al.*, 1999b). The frequency in people of Swedish descent was 1.1% (Oscarson *et al.*, 1998). Caucasians in the UK, France, and Germany showed very similar frequencies of *CYP2A6*2*, between 2-2.6% (Bourian *et al.*, 2000; Loriot *et al.*, 2001; Huang *et al.*, 2005).

Similarly, *CYP2A6*2* has been detected in North American Caucasians, with frequencies in the range of 1.1-5.3% in Caucasians in the US (Paschke *et al.*, 2001; Nakajima *et al.*, 2004; Nakajima *et al.*, 2006; Audrain-McGovern *et al.*, 2007; Bloom *et al.*, 2011) and 1.2-3.4% in Caucasian Canadians (Xu *et al.*, 2002; O'Loughlin *et al.*, 2004; Schoedel *et al.*, 2004; Malaiyandi *et al.*, 2006). *CYP2A6*2* was not found in Indigenous Peoples in Canada in one study (Schoedel *et al.*, 2004), and was found at 0.9% in another (Nowak *et al.*, 1998). It was found at a low frequency (0.4%) in Indigenous Peoples in Alaska (Binnington *et al.*, 2012).

Reports from South American individuals showed that the frequency for *CYP2A6*2* was 1.7% in Brazilians (Vasconcelos *et al.*, 2005; Rossini *et al.*, 2006) and 1.9-2% in Chileans (Caceres *et al.*, 2012; Roco *et al.*, 2012).

***CYP2A6*4* (loss-of-function)**

The *CYP2A6*4* allele was observed at a range of 0-24.6% among the studied populations.

The frequency for *CYP2A6*4* is generally low in individuals of African descent, with the frequencies being zero in the Namibian Ovambo people (Takeshita *et al.*, 2006), 0.6% in Ethiopians (Aklillu *et al.*, 2014), 1.9% in Ghanaians (Gyamfi *et al.*, 2005), 0.5-1.9% in African Americans (Nakajima *et al.*, 2004; Nakajima *et al.*, 2006; Ho *et al.*, 2009; Mwenifumbo *et al.*, 2010) and 2.7% in African Canadians (Mwenifumbo *et al.*, 2010). A study of African Americans and African Canadians showed a frequency of 1.9% (Schoedel *et al.*, 2004), which falls in the range of reported frequencies in the populations of African descent.

The frequency for *CYP2A6*4* is high among East and Southeast Asian populations, with the highest incidence occurring in Japanese. The range for *CYP2A6*4* is between 16.0-24.6% in Japanese (Fujieda *et al.*, 2004; Minematsu *et al.*, 2006; Nakajima *et al.*, 2006; Takeshita *et al.*, 2006; Kaida *et al.*, 2008; Hirose *et al.*, 2010; Kumasaka *et al.*, 2012), 9.4-13.4% in Koreans (Nakajima *et al.*, 2006; Park *et al.*, 2011; Djordjevic *et al.*, 2012), and 0-15.1% in different ethnicities of Chinese (Oscarson *et al.*, 1999b; Oscarson *et al.*, 1999a; Pitarque *et al.*, 2001; Tang *et al.*, 2009; Liu *et al.*, 2011; Fang *et al.*, 2012; Pang *et al.*, 2015; Wang *et al.*, 2017; Zhao *et al.*, 2017). In Chinese Malaysians, the frequency was 4.9% (Nurfadhlina *et al.*, 2006). There is considerable difference of *CYP2A6*4* distributions across ethnicities in China, with the frequencies higher in Han (7.9%) and Uighur (15.0%) populations and much lower in Tibetan (2.0%) and Bouyei (0%) populations (Pang *et al.*, 2015). This allele has also been assessed in other Asian populations, with reported frequencies of 11.8% in Vietnamese (Veiga *et al.*, 2009) and 7.8-14.2% in Thais (Ujjin *et al.*, 2002; Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009). The frequency in Asian Americans was 15.3% (Mwenifumbo *et al.*, 2010), and Indigenous Peoples in Alaska had a frequency of 14.5% (Binnington *et al.*, 2012). Homozygotes of the **4* allele (*CYP2A6*4/*4*), who are completely devoid of enzyme activities, were found in 3.7% of 894 Japanese individuals studied (Kitagawa *et al.*, 2001).

The frequency of *CYP2A6*4* in Indians was between 3.9-11.3% (Ruwali *et al.*, 2009; Krishnakumar *et al.*, 2012). In Indian Malaysians, a frequency of 1.4% was reported

(Nurfadhlina *et al.*, 2006). In Sri Lanka, patients with oral lesions (also betel quid chewers) had a frequency of 2.8%, while a 9.6% frequency was observed in the controls (Topcu *et al.*, 2002). Iranians and Turkish individuals had frequencies of *CYP2A6*4* below 3% (Takeshita *et al.*, 2006; Emamghoreishi *et al.*, 2008; Heravi *et al.*, 2010).

*CYP2A6*4* was found in European populations at levels in the range of 0.3-16.9% (Oscarson *et al.*, 1998; Oscarson *et al.*, 1999b; Loriot *et al.*, 2001; Pitarque *et al.*, 2001; Huang *et al.*, 2005; Djordjevic *et al.*, 2010; Djordjevic *et al.*, 2012; Korytina *et al.*, 2014).

One study on the Tatar population from the Republic of Bashkortostan (part of the Russian Federation), reported *CYP2A6*4* was present in 6.8% of patients with chronic obstructive pulmonary disease and 16.9% in the controls (Korytina *et al.*, 2014).

The *CYP2A6*4* allele was not found in two studies of Caucasians in the US (Nakajima *et al.*, 2006; Mwenifumbo *et al.*, 2010), and was observed at low frequencies ranging from 0.6-3.0% in three other studies (Nakajima *et al.*, 2004; Audrain-McGovern *et al.*, 2007; Bloom *et al.*, 2011). Several studies in Caucasian Canadians showed low frequencies of *CYP2A6*4*, between 0.2-1.2% (Xu *et al.*, 2002; O'Loughlin *et al.*, 2004; Schoedel *et al.*, 2004; Mwenifumbo *et al.*, 2005; Malaiyandi *et al.*, 2006).

South American individuals carry *CYP2A6*4* at levels of 0.5% in Brazilians (Vasconcelos *et al.*, 2005), 7.1% in Mestizo Ecuadorians (Soriano *et al.*, 2011) and 3.7-4% in Chileans (Malaiyandi *et al.*, 2006; Caceres *et al.*, 2012).

One study in the Māori people in New Zealand found a frequency of 9.6% for *CYP2A6*4* (Lea *et al.*, 2008).

***CYP2A6*5* and *CYP2A6*20* (loss-of-function)**

Compared to *CYP2A6*2* and *CYP2A6*4*, there were fewer studies on the distributions of two other loss-of-function alleles, *CYP2A6*5* (encoding an unstable enzyme) and *CYP2A6*20* (encoding a truncated enzyme).

The *CYP2A6*5* allele was observed at a range of 0-1.2%, with an outlier of 14.5%, among the studied populations. *CYP2A6*5* was not found in the following populations: African Americans and African Canadians (Nakajima *et al.*, 2004; Schoedel *et al.*, 2004; Nakajima *et al.*, 2006), Ethiopians (Aklillu *et al.*, 2014), Ghanaians (Gyamfi *et al.*, 2005), Serbians (Djordjevic *et al.*, 2010), Swedes (Djordjevic *et al.*, 2012), Caucasian

Americans (Nakajima *et al.*, 2004; Nakajima *et al.*, 2006), Brazilians (Rossini *et al.*, 2006), or South Asian adolescents in the UK (Huang *et al.*, 2005).

In three populations discussed below, *CYP2A6*5* was not detected in some studies, and was found at low frequencies in others. *CYP2A6*5* was also not observed in a group of 152 Caucasian Canadian smokers (Malaiyandi *et al.*, 2006), and it was found with only 0.1% frequency in another group of 723 Caucasian Canadians (Schoedel *et al.*, 2004). In Japanese individuals, *CYP2A6*5* was not detected in one study (N=92) (Nakajima *et al.*, 2006), not found in another study in patients with bladder cancer (N=163) and present in one heterozygous individual (*CYP2A6*4/*5*) from the controls (N=116) (Kumondai *et al.*, 2016). Similar results were reported in Koreans: one study (N=144) did not find any individuals with *CYP2A6*5* (Djordjevic *et al.*, 2012), and in another study (N=209), the allele was found to be present at a low frequency (0.5%) (Nakajima *et al.*, 2006).

*CYP2A6*5* was also observed at frequencies less than 1% in Caucasian adolescents in the UK (0.3%) (Huang *et al.*, 2005), Indians (0.7%) (Krishnakumar *et al.*, 2012) and Indian Malaysians (0.9%) (Nurfadhlina *et al.*, 2006), Malays (0.9%) (Nurfadhlina *et al.*, 2006), and Indigenous Peoples in Canada (0.5%) (Schoedel *et al.*, 2004). The frequencies for *CYP2A6*5* in the Chinese population were 1-1.2% (Oscarson *et al.*, 1999b; Oscarson *et al.*, 1999a; Liu *et al.*, 2011), similar to the 1.2% frequency in Chinese Malaysians (Nurfadhlina *et al.*, 2006). In contrast to these results, one study in 72 Vietnamese individuals showed a high frequency of 14.6% for *CYP2A6*5* (Veiga *et al.*, 2009).

*CYP2A6*20* has only been assessed in a few populations, and was observed at low frequencies of 0-1.7% among the studied populations. It was not found in Caucasian Americans, Japanese, or Koreans (Fukami *et al.*, 2005a; Fukami *et al.*, 2005b; Nakajima *et al.*, 2006). The frequencies of this loss-of-function allele were between 1.5-1.7% in three groups of African North Americans (Nakajima *et al.*, 2006; Mwenifumbo *et al.*, 2008a; Ho *et al.*, 2009).

***CYP2A6*7* (decrease-of-function)**

The *CYP2A6*7* allele was observed at a range of 0-19.6% among the studied populations.

*CYP2A6*7* was found to occur most commonly among the Japanese, with frequencies ranging between 8.6-19.6% (Fujieda *et al.*, 2004; Fukami *et al.*, 2005a; Fukami *et al.*, 2005b; Minematsu *et al.*, 2006; Nakajima *et al.*, 2006; Kaida *et al.*, 2008; Hirose *et al.*,

2010; Kumondai *et al.*, 2016). One study found a combined frequency of 14.6% for *CYP2A6*7*, *CYP2A6*36*, and *CYP2A6*37* (Kumasaka *et al.*, 2012). Most of the 14.6% can probably be attributed to *CYP2A6*7* because the latter two alleles are uncommon in the Japanese; neither was found in any of 60 volunteers in a different study (Al Koudsi *et al.*, 2009). *CYP2A6*7* was present at 12.5% in a group of Japanese Canadians (Mwenifumbo *et al.*, 2005). Three studies of Japanese cancer patients showed high frequencies of 15.3% (Kumondai *et al.*, 2016), 19.3% (Hirose *et al.*, 2010) and 19.6% (Kaida *et al.*, 2008) of this allele, while a fourth study showed a lower frequency of 9.7% (Fujieda *et al.*, 2004). The frequency for *CYP2A6*7* in Koreans was between 6.9-11.1% (Nakajima *et al.*, 2006; Park *et al.*, 2011; Djordjevic *et al.*, 2012). The frequencies for *CYP2A6*7* in Chinese, Chinese Malaysians, Chinese Americans, and Chinese Canadians were 6.3%, 7.0%, 5.7%, and 9.8%, respectively (Mwenifumbo *et al.*, 2005; Nurfadhlina *et al.*, 2006; Liu *et al.*, 2011). One study in the Taiwanese showed a higher frequency of 10% (Mwenifumbo *et al.*, 2005). Thais (Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009) and Malays (Nurfadhlina *et al.*, 2006) showed similar frequencies, ranging from 4.3-6.4%. One study of the Malays failed to detect this allele, but the sample size was small (N = 24) (Yusof and Gan, 2009).

Caucasian populations have very low incidence *CYP2A6*7*, if any at all. This allele was not found in Caucasians in the US (Nakajima *et al.*, 2004; Fukami *et al.*, 2005a; Fukami *et al.*, 2005b; Nakajima *et al.*, 2006), and was observed only in 0.3% of studied Caucasian Canadians (Schoedel *et al.*, 2004; Malaiyandi *et al.*, 2006). The frequency of *CYP2A6*7* has not been examined in European populations.

A group of Māori people from New Zealand were tested for *CYP2A6*7,* and the allele frequency was 1.1% (Lea *et al.*, 2008).

*CYP2A6*7* has not been tested in Indians, but it was not found in Indian Malaysians (Nurfadhlina *et al.*, 2006). Neither was it found in Indigenous Peoples in Alaska (Binnington *et al.*, 2012) or Indigenous Peoples in Canada (Schoedel *et al.*, 2004). None of the tested individuals of African descent carried the *CYP2A6*7* allele, including Ghanaians (Gyamfi *et al.*, 2005), African Americans (Nakajima *et al.*, 2004; Fukami *et al.*, 2005a; Fukami *et al.*, 2005b; Nakajima *et al.*, 2006), African Canadians (Mwenifumbo *et al.*, 2005; Mwenifumbo *et al.*, 2010), and a group of African Americans and African Canadians (Schoedel *et al.*, 2004).

***CYP2A6*9* (decrease-of-function)**

*CYP2A6*9* is a relatively frequently occurring allele, ranging from 5.2-23.6% among all the populations tested except for one study of 172 Ethiopians, where the frequency was 2.8% (Aklillu *et al.*, 2014).

The frequencies of this allele in three other studies in Africans are 5.7% in Ghanaians (Gyamfi *et al.*, 2005), 11.0% in Nigerians (Takeshita *et al.*, 2006), and 7.6-11.4% in Ugandans (Mukonzo *et al.*, 2013). In studies of African Americans or African Canadians, the range was 7.1-8.5% (Nakajima *et al.*, 2004; Schoedel *et al.*, 2004; Nakajima *et al.*, 2006; Mwenifumbo *et al.*, 2008a).

*CYP2A6*9* occurs often in East Asian individuals, with frequencies ranging from 15.2-22.8% in Japanese (Fujieda *et al.*, 2004; Minematsu *et al.*, 2006; Nakajima *et al.*, 2006), 19.6-21.9% in Koreans (Nakajima *et al.*, 2006; Park *et al.*, 2011; Djordjevic *et al.*, 2012), and 13.5-23.6% in Chinese (Pitarque *et al.*, 2001; Liu *et al.*, 2011; Yuan *et al.*, 2016). The frequencies in Thai individuals were between 12.1-20.4% (Peamkrasatam *et al.*, 2006; Mahavorasirikul *et al.*, 2009). Other reports from Asia include 10.4% in Malays (Yusof and Gan, 2009), 6.9-7.2% in Turkish individuals (Pitarque *et al.*, 2001), and 12.4% in Iranian individuals (Emamghoreishi *et al.*, 2008).

Three European populations were tested for *CYP2A6*9* (Serbian, Spanish, and Swedish), and they carried this allele at frequencies of 5.2-8.2% (Pitarque *et al.*, 2001; Djordjevic *et al.*, 2010; Soriano *et al.*, 2011; Djordjevic *et al.*, 2012). Similarly, studies of Caucasians in North America showed consistent results, with the frequencies ranging from 6.1-8% (Nakajima *et al.*, 2004; O'Loughlin *et al.*, 2004; Schoedel *et al.*, 2004; Malaiyandi *et al.*, 2006; Nakajima *et al.*, 2006; Audrain-McGovern *et al.*, 2007; Bloom *et al.*, 2011).

In North America, Indigenous Peoples in Canada carry *CYP2A6*9* at a frequency of 15.5% (Schoedel *et al.*, 2004), Indigenous Peoples in Alaska carry this allele at a frequency of 8.9%, and Mexicans carry this allele at 16.4% (Svyryd *et al.*, 2016). In South America, this allele was reported at the following percentages: 5.7% in Brazilians (Vasconcelos *et al.*, 2005) and 10.3% in Mestizo Ecuadorians (Soriano *et al.*, 2011). In New Zealand, one study in the Māori population reported a high frequency of 19% (Lea *et al.*, 2008).

***CYP2A6*10* (decrease-of-function)**

All the populations tested for *CYP2A6*10* showed frequencies of less than 5%, with a range of 0-4.3%. The population-specific percentages for this allele are as follows: 2.2-4.3% in Japanese (Fujieda *et al.*, 2004; Nakajima *et al.*, 2006; Kumasaka *et al.*,

2012; Kumondai *et al.*, 2016), 1-4.2% in Koreans (Nakajima *et al.*, 2006; Djordjevic *et al.*, 2012), 1.7-2.4% in Chinese and Chinese Malaysians (Nurfadhlina *et al.*, 2006; Liu *et al.*, 2011), 4.1% in Taiwanese (Mwenifumbo *et al.*, 2005), 4.3% in Malays (Nurfadhlina *et al.*, 2006), and 1.9% in Indigenous Peoples in Alaska (Binnington *et al.*, 2012). Asians in North America showed similar frequencies of *CYP2A6*10* as Asians living in Asia, with a range of 3.2-4.3% (Mwenifumbo *et al.*, 2005).

*CYP2A6*10* was not found in the following populations: Ghanaians (Gyamfi *et al.*, 2005), African Americans and African Canadians (Nakajima *et al.*, 2004; Schoedel *et al.*, 2004; Mwenifumbo *et al.*, 2005; Nakajima *et al.*, 2006; Mwenifumbo *et al.*, 2010), Caucasians in North America (Xu *et al.*, 2002; Nakajima *et al.*, 2004; Schoedel *et al.*, 2004; Malaiyandi *et al.*, 2006; Nakajima *et al.*, 2006), Indigenous Peoples in Canada (Schoedel *et al.*, 2004), and Indian Malaysians (Nurfadhlina *et al.*, 2006).

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# Appendix C Methods Applied in Pathway Analysis of Uehara *et al*. (2008a) Toxicogenomic Data Generated from Rat Liver Following *in Vivo* Exposure to Coumarin

## C1. Pathway analysis using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway

Statistically significant up-regulated genes (136 probe sets) and down-regulated genes (79 probe sets) identified by Uehara *et al*. (2008a) from their study of altered gene expression in rat liver 24 hours after *in vivo* administration of coumarin (150 mg/kg) were analyzed using the National Institutes of Health-developed DAVID (Database for Annotation, Visualization and Integrated Discovery) (Huang *et al.*, 2009).

DAVID is a high-throughput and integrated data-mining environment for analyzing gene lists derived from high-throughput genomic experiments. It is an open-access web-based program with broad databases containing over 50 annotation categories from dozens of public databases, including gene ontology (GO) terms, PANTHER (**P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships) GO terms, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. We chose DAVID to perform GO and KEGG pathway analysis on these data because of its broad database coverage, free access, and wide recognition in the field.

Lists of the up-regulated and down-regulated genes identified by Uehara *et al*. (2008a) were uploaded into DAVID 6.8 analysis wizard (https://david.ncifcrf.gov/tools.jsp), along with each gene's unique *Affy* gene code (Affymetrix, Santa Clara, CA, USA). OEHHA analyzed these data using two different approaches selected from among those available in DAVID. In the first approach, we applied a functional annotation clustering tool to the gene lists. The DAVID functional annotation clustering tool uses a novel algorithm to measure relationships among the annotation terms based on the degrees of their co-association genes, in order to group similar annotation terms into annotation clusters. This clustering function groups similar annotations together and makes the interpretation of multiple pathways easier, as compared with the traditional chart report (see second approach below) that looks at the individual annotation terms.

In the second approach, we applied the traditional functional annotation chart to the gene lists. The DAVID traditional functional annotation chart is an annotation-term-focused view which lists annotation terms and their associated genes. All results of the Chart Reports have to pass the default threshold criteria: p value ≤ 0.1 and gene count ≥ 2.

For both of the above approaches we used all default options in DAVID and chose a medium level of clustering options and stringency.

In DAVID, a more conservative modified Fisher Exact test is used to measure gene-enrichment by annotation terms. A selected annotation pathway with a p-value of less than 0.1 (by default) means this pathway has more than a random gene-enrichment association compared to the background rat transcriptome. The p-values presented in Tables C1- C4 correspond to this more conservative version of the one-tailed Fisher Exact Test (online Functional Annotation Tool, https://david.ncifcrf.gov/helps/functional_annotation.html#).

Data output from the approach using functional annotation clustering is presented in Table C1 for the up-regulated genes (111 out of the 136 up-regulated genes from Uehara *et al*. (2008a) were recognized by DAVID), and Table C2 for the down-regulated genes (69 out of the 79 down-regulated genes from Uehara *et al*. (2008a) were recognized by DAVID).

Data output from the approach using the traditional functional annotation chart, presenting only the pathways with p-values less than 0.1; *i.e.*, DAVID default) is presented in Table C3 for the up-regulated genes and Table C4 for the down-regulated genes.

**Table C1. Data output of functional annotation clustering for 111 up-regulated genes from Uehara *et al.* (2008a)**

| Annotation Cluster 1 | Enrichment Score: 3.5788645275406865 | | | |
|---|---|---|---|---|
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Endoplasmic reticulum | 16 | 14.414 | 3.6E-07 |
| KEGG_PATHWAY | rno04141:Protein processing in endoplasmic reticulum | 9 | 8.108 | 3.5E-05 |
| GOTERM_CC_DIRECT | GO:0005783~endoplasmic reticulum | 15 | 13.514 | 1.2E-04 |
| UP_KEYWORDS | Signal | 28 | 25.225 | 6.8E-03 |
| UP_SEQ_FEATURE | signal peptide | 16 | 14.414 | 1.3E-01 |
| | | | | |
| Annotation Cluster 2 | Enrichment Score: 3.533490297685079 | | | |
| Category | Term | Count | % | P Value |
| GOTERM_BP_DIRECT | GO:0055114~oxidation-reduction process | 15 | 13.514 | 1.5E-05 |
| UP_KEYWORDS | NADP | 6 | 5.405 | 5.6E-04 |
| UP_KEYWORDS | Oxidoreductase | 9 | 8.108 | 3.0E-03 |
| | | | | |
| Annotation Cluster 3 | Enrichment Score: 3.3257844326100097 | | | |
| Category | Term | Count | % | P Value |
| GOTERM_CC_DIRECT | GO:0042555~MCM complex | 5 | 4.505 | 9.5E-08 |
| GOTERM_BP_DIRECT | GO:0006270~DNA replication initiation | 6 | 5.405 | 1.2E-07 |
| UP_KEYWORDS | DNA replication | 7 | 6.306 | 1.8E-07 |
| KEGG_PATHWAY | rno03030:DNA replication | 7 | 6.306 | 2.4E-07 |
| INTERPRO | IPR018525:Mini-chromosome maintenance, conserved site | 4 | 3.604 | 2.6E-06 |
| SMART | SM00350:MCM | 4 | 3.604 | 8.9E-06 |
| INTERPRO | IPR001208:Mini-chromosome maintenance, DNA-dependent ATPase | 4 | 3.604 | 1.5E-05 |
| GOTERM_MF_DIRECT | GO:0003678~DNA helicase activity | 4 | 3.604 | 1.7E-04 |
| KEGG_PATHWAY | rno04110:Cell cycle | 7 | 6.306 | 3.7E-04 |
| GOTERM_CC_DIRECT | GO:0000784~nuclear chromosome, telomeric region | 6 | 5.405 | 3.8E-04 |
| UP_KEYWORDS | Helicase | 4 | 3.604 | 4.6E-03 |
| INTERPRO | IPR012340:Nucleic acid-binding, OB-fold | 4 | 3.604 | 9.4E-03 |
| KEGG_PATHWAY | rno03410:Base excision repair | 3 | 2.703 | 3.2E-02 |
| UP_KEYWORDS | Nucleus | 18 | 16.216 | 7.9E-02 |
| GOTERM_MF_DIRECT | GO:0003677~DNA binding | 10 | 9.009 | 1.4E-01 |
| UP_KEYWORDS | DNA-binding | 8 | 7.207 | 1.7E-01 |
| GOTERM_CC_DIRECT | GO:0005654~nucleoplasm | 13 | 11.712 | 2.2E-01 |
| INTERPRO | IPR027417:P-loop containing nucleoside triphosphate hydrolase | 6 | 5.405 | 4.8E-01 |
| UP_KEYWORDS | Hydrolase | 7 | 6.306 | 5.7E-01 |

| Annotation Cluster 4 | Enrichment Score: 3.2384929782910743 | | | |
|---|---|---|---|---|
| Category | Term | Count | % | P Value |
| GOTERM_CC_DIRECT | GO:0034663~endoplasmic reticulum chaperone complex | 5 | 4.505 | 3.7E-07 |
| GOTERM_CC_DIRECT | GO:0005790~smooth endoplasmic reticulum | 5 | 4.505 | 1.6E-05 |
| GOTERM_CC_DIRECT | GO:0005788~endoplasmic reticulum lumen | 6 | 5.405 | 2.5E-05 |
| KEGG_PATHWAY | rno04141:Protein processing in endoplasmic reticulum | 9 | 8.108 | 3.5E-05 |
| GOTERM_BP_DIRECT | GO:0002931~response to ischemia | 5 | 4.505 | 1.4E-04 |
| GOTERM_BP_DIRECT | GO:0034976~response to endoplasmic reticulum stress | 5 | 4.505 | 1.1E-03 |
| UP_SEQ_FEATURE | short sequence motif:Prevents secretion from ER | 4 | 3.604 | 2.2E-03 |
| GOTERM_CC_DIRECT | GO:0042470~melanosome | 4 | 3.604 | 1.2E-02 |
| UP_KEYWORDS | Chaperone | 4 | 3.604 | 1.7E-02 |
| GOTERM_BP_DIRECT | GO:0001666~response to hypoxia | 6 | 5.405 | 1.8E-02 |
| GOTERM_CC_DIRECT | GO:0005925~focal adhesion | 4 | 3.604 | 3.6E-01 |
| | | | | |
| Annotation Cluster 5 | Enrichment Score: 3.143736398541981 | | | |
| Category | Term | Count | % | P Value |
| GOTERM_BP_DIRECT | GO:0006749~glutathione metabolic process | 6 | 5.405 | 1.0E-05 |
| KEGG_PATHWAY | rno00480:Glutathione metabolism | 6 | 5.405 | 7.1E-05 |
| GOTERM_BP_DIRECT | GO:0006979~response to oxidative stress | 7 | 6.306 | 1.7E-04 |
| GOTERM_BP_DIRECT | GO:0098869~cellular oxidant detoxification | 5 | 4.505 | 6.0E-04 |
| GOTERM_BP_DIRECT | GO:2001237~negative regulation of extrinsic apoptotic signaling pathway | 4 | 3.604 | 1.7E-03 |
| GOTERM_BP_DIRECT | GO:0007568~aging | 7 | 6.306 | 8.5E-03 |
| GOTERM_BP_DIRECT | GO:0014823~response to activity | 3 | 2.703 | 9.4E-02 |
| | | | | |
| Annotation Cluster 6 | Enrichment Score: 2.2938589256373807 | | | |
| Category | Term | Count | % | P Value |
| GOTERM_BP_DIRECT | GO:0006979~response to oxidative stress | 7 | 6.306 | 1.7E-04 |
| UP_KEYWORDS | NADP | 6 | 5.405 | 5.6E-04 |
| UP_KEYWORDS | FAD | 3 | 2.703 | 7.9E-02 |
| UP_KEYWORDS | Flavoprotein | 3 | 2.703 | 9.0E-02 |
| | | | | |
| Annotation Cluster 7 | Enrichment Score: 2.1337027168769036 | | | |
| Category | Term | Count | % | P Value |
| GOTERM_MF_DIRECT | GO:0004364~glutathione transferase activity | 4 | 3.604 | 1.7E-03 |
| INTERPRO | IPR012336:Thioredoxin-like fold | 5 | 4.505 | 5.8E-03 |
| INTERPRO | IPR004046:Glutathione S-transferase, C-terminal | 3 | 2.703 | 1.1E-02 |
| INTERPRO | IPR010987:Glutathione S-transferase, C-terminal-like | 3 | 2.703 | 2.7E-02 |
| | | | | |
| Annotation Cluster 8 | Enrichment Score: 2.106035485295958 | | | |
| Category | Term | Count | % | P Value |
| KEGG_PATHWAY | rno00480:Glutathione metabolism | 6 | 5.405 | 7.1E-05 |
| GOTERM_MF_DIRECT | GO:0004364~glutathione transferase activity | 4 | 3.604 | 1.7E-03 |
| KEGG_PATHWAY | rno00980:Metabolism of xenobiotics by cytochrome P450 | 4 | 3.604 | 1.6E-02 |

| | | | | |
|---|---|---|---|---|
| KEGG_PATHWAY | rno00982:Drug metabolism - cytochrome P450 | 3 | 2.703 | 1.0E-01 |
| KEGG_PATHWAY | rno05204:Chemical carcinogenesis | 3 | 2.703 | 1.5E-01 |
| | | | | |
| Annotation Cluster 9 | Enrichment Score: 1.62703299993579 | | | |
| Category | Term | Count | % | P Value |
| KEGG_PATHWAY | rno04110:Cell cycle | 7 | 6.306 | 3.7E-04 |
| UP_KEYWORDS | Cell division | 4 | 3.604 | 5.9E-02 |
| GOTERM_BP_DIRECT | GO:0051301~cell division | 4 | 3.604 | 7.9E-02 |
| UP_KEYWORDS | Cell cycle | 4 | 3.604 | 1.8E-01 |
| | | | | |
| Annotation Cluster 10 | Enrichment Score: 1.5245791605250545 | | | |
| Category | Term | Count | % | P Value |
| KEGG_PATHWAY | rno04612:Antigen processing and presentation | 6 | 5.405 | 8.0E-04 |
| KEGG_PATHWAY | rno05164:Influenza A | 4 | 3.604 | 1.4E-01 |
| KEGG_PATHWAY | rno05145:Toxoplasmosis | 3 | 2.703 | 2.4E-01 |
| | | | | |
| Annotation Cluster 11 | Enrichment Score: 1.1479668361800475 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | ATP-binding | 12 | 10.811 | 1.2E-02 |
| UP_KEYWORDS | Nucleotide-binding | 13 | 11.712 | 2.9E-02 |
| GOTERM_MF_DIRECT | GO:0005524~ATP binding | 12 | 10.811 | 1.5E-01 |
| INTERPRO | IPR027417:P-loop containing nucleoside triphosphate hydrolase | 6 | 5.405 | 4.8E-01 |
| | | | | |
| Annotation Cluster 12 | Enrichment Score: 1.1343272860465912 | | | |
| Category | Term | Count | % | P Value |
| KEGG_PATHWAY | rno04612:Antigen processing and presentation | 6 | 5.405 | 8.0E-04 |
| KEGG_PATHWAY | rno05332:Graft-versus-host disease | 4 | 3.604 | 1.4E-02 |
| KEGG_PATHWAY | rno05330:Allograft rejection | 4 | 3.604 | 1.6E-02 |
| KEGG_PATHWAY | rno04940:Type I diabetes mellitus | 4 | 3.604 | 2.0E-02 |
| KEGG_PATHWAY | rno05320:Autoimmune thyroid disease | 4 | 3.604 | 2.0E-02 |
| KEGG_PATHWAY | rno05416:Viral myocarditis | 4 | 3.604 | 3.1E-02 |
| SMART | SM00407:IGc1 | 3 | 2.703 | 4.9E-02 |
| INTERPRO | IPR011162:MHC classes I/II-like antigen recognition protein | 3 | 2.703 | 5.0E-02 |
| KEGG_PATHWAY | rno04145:Phagosome | 5 | 4.505 | 6.1E-02 |
| INTERPRO | IPR003006:Immunoglobulin/major histocompatibility complex, conserved site | 3 | 2.703 | 6.2E-02 |
| INTERPRO | IPR003597:Immunoglobulin C1-set | 3 | 2.703 | 6.6E-02 |
| KEGG_PATHWAY | rno05166:HTLV-I infection | 6 | 5.405 | 6.9E-02 |
| KEGG_PATHWAY | rno04514:Cell adhesion molecules (CAMs) | 4 | 3.604 | 1.4E-01 |
| GOTERM_BP_DIRECT | GO:0006955~immune response | 4 | 3.604 | 1.9E-01 |
| KEGG_PATHWAY | rno05168:Herpes simplex infection | 4 | 3.604 | 2.2E-01 |

| | | | | |
|---|---|---|---|---|
| UP_KEYWORDS | Immunity | 3 | 2.703 | 2.9E-01 |
| KEGG_PATHWAY | rno04144:Endocytosis | 4 | 3.604 | 3.7E-01 |
| KEGG_PATHWAY | rno05169:Epstein-Barr virus infection | 3 | 2.703 | 5.2E-01 |
| INTERPRO | IPR007110:Immunoglobulin-like domain | 3 | 2.703 | 7.8E-01 |
| INTERPRO | IPR013783:Immunoglobulin-like fold | 3 | 2.703 | 8.9E-01 |
| | | | | |
| Annotation Cluster 13 | Enrichment Score: 0.5558138120192609 | | | |
| Category | Term | Count | % | P Value |
| KEGG_PATHWAY | rno05215:Prostate cancer | 3 | 2.703 | 1.4E-01 |
| KEGG_PATHWAY | rno04151:PI3K-Akt signaling pathway | 5 | 4.505 | 2.6E-01 |
| KEGG_PATHWAY | rno05200:Pathways in cancer | 4 | 3.604 | 5.9E-01 |
| | | | | |
| Annotation Cluster 14 | Enrichment Score: 0.20910489892037354 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Repressor | 3 | 2.703 | 2.8E-01 |
| UP_KEYWORDS | Transcription regulation | 3 | 2.703 | 9.2E-01 |
| UP_KEYWORDS | Transcription | 3 | 2.703 | 9.3E-01 |
| | | | | |
| Annotation Cluster 15 | Enrichment Score: 0.12730057505293488 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Metal-binding | 11 | 9.910 | 6.0E-01 |
| UP_KEYWORDS | Zinc-finger | 4 | 3.604 | 7.9E-01 |
| UP_KEYWORDS | Zinc | 5 | 4.505 | 8.7E-01 |
| | | | | |
| Annotation Cluster 16 | Enrichment Score: 0.05355755420296627 | | | |
| Category | Term | Count | % | P Value |
| UP_SEQ_FEATURE | transmembrane region | 14 | 12.613 | 7.9E-01 |
| UP_SEQ_FEATURE | topological domain:Cytoplasmic | 8 | 7.207 | 9.4E-01 |
| UP_SEQ_FEATURE | topological domain:Extracellular | 6 | 5.405 | 9.4E-01 |
| | | | | |
| Annotation Cluster 17 | Enrichment Score: 0.032235260422429475 | | | |
| Category | Term | Count | % | P Value |
| UP_SEQ_FEATURE | transmembrane region | 14 | 12.613 | 7.9E-01 |
| UP_KEYWORDS | Membrane | 29 | 26.126 | 9.6E-01 |
| UP_KEYWORDS | Transmembrane helix | 24 | 21.622 | 9.6E-01 |
| UP_KEYWORDS | Transmembrane | 24 | 21.622 | 9.6E-01 |
| GOTERM_CC_DIRECT | GO:0016021~integral component of membrane | 21 | 18.919 | 9.9E-01 |

**Table C2. Data output of functional annotation clustering for 69 down-regulated genes from Uehara *et al.* (2008a)**

| Annotation Cluster 1 | Enrichment Score: 3.01319611643087 | | | |
|---|---|---|---|---|
| Category | Term | Count | % | P Value |
| INTERPRO | IPR015424:Pyridoxal phosphate-dependent transferase | 4 | 5.797 | 4.32E-04 |
| INTERPRO | IPR015421:Pyridoxal phosphate-dependent transferase, major region, subdomain 1 | 4 | 5.797 | 4.32E-04 |
| UP_KEYWORDS | Pyridoxal phosphate | 4 | 5.797 | 5.62E-04 |
| GOTERM_MF_DIRECT | GO:0030170~pyridoxal phosphate binding | 4 | 5.797 | 1.08E-03 |
| INTERPRO | IPR015422:Pyridoxal phosphate-dependent transferase, major region, subdomain 2 | 3 | 4.348 | 7.57E-03 |
| | | | | |
| Annotation Cluster 2 | Enrichment Score: 2.7117194055635787 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Oxidoreductase | 11 | 15.942 | 3.86E-06 |
| GOTERM_BP_DIRECT | GO:0055114~oxidation-reduction process | 12 | 17.391 | 1.31E-05 |
| GOTERM_CC_DIRECT | GO:0031090~organelle membrane | 6 | 8.696 | 1.47E-05 |
| UP_KEYWORDS | Microsome | 6 | 8.696 | 4.27E-05 |
| UP_KEYWORDS | Monooxygenase | 5 | 7.246 | 4.40E-04 |
| KEGG_PATHWAY | rno00140:Steroid hormone biosynthesis | 5 | 7.246 | 4.47E-04 |
| UP_KEYWORDS | Lipid metabolism | 7 | 10.145 | 4.54E-04 |
| GOTERM_CC_DIRECT | GO:0043231~intracellular membrane-bounded organelle | 10 | 14.493 | 5.66E-04 |
| GOTERM_MF_DIRECT | GO:0005506~iron ion binding | 6 | 8.696 | 6.80E-04 |
| GOTERM_MF_DIRECT | GO:0016491~oxidoreductase activity | 5 | 7.246 | 9.96E-04 |
| UP_KEYWORDS | Iron | 6 | 8.696 | 2.15E-03 |
| INTERPRO | IPR017972:Cytochrome P450, conserved site | 4 | 5.797 | 2.73E-03 |
| GOTERM_CC_DIRECT | GO:0005789~endoplasmic reticulum membrane | 8 | 11.594 | 2.99E-03 |
| INTERPRO | IPR001128:Cytochrome P450 | 4 | 5.797 | 3.43E-03 |
| UP_KEYWORDS | Endoplasmic reticulum | 8 | 11.594 | 3.78E-03 |
| KEGG_PATHWAY | rno00982:Drug metabolism - cytochrome P450 | 4 | 5.797 | 3.87E-03 |
| GOTERM_BP_DIRECT | GO:0017144~drug metabolic process | 3 | 4.348 | 4.86E-03 |
| KEGG_PATHWAY | rno05204:Chemical carcinogenesis | 4 | 5.797 | 7.73E-03 |
| UP_KEYWORDS | NADP | 4 | 5.797 | 9.67E-03 |
| UP_KEYWORDS | Sterol metabolism | 3 | 4.348 | 1.05E-02 |
| COG_ONTOLOGY | Secondary metabolites biosynthesis, transport, and catabolism | 4 | 5.797 | 1.08E-02 |
| UP_KEYWORDS | Heme | 4 | 5.797 | 1.19E-02 |
| UP_KEYWORDS | Steroid metabolism | 3 | 4.348 | 1.66E-02 |
| UP_SEQ_FEATURE | metal ion-binding site:Iron (heme axial ligand) | 4 | 5.797 | 1.67E-02 |
| GOTERM_MF_DIRECT | GO:0020037~heme binding | 4 | 5.797 | 2.01E-02 |
| GOTERM_MF_DIRECT | GO:0004497~monooxygenase activity | 3 | 4.348 | 2.16E-02 |
| KEGG_PATHWAY | rno00980:Metabolism of xenobiotics by cytochrome P450 | 3 | 4.348 | 3.92E-02 |

| | | | | |
|---|---|---|---|---|
| KEGG_PATHWAY | rno00830:Retinol metabolism | 3 | 4.348 | 5.33E-02 |
| | | | | |
| Annotation Cluster 3 | Enrichment Score: 1.565916915682683 | | | |
| Category | Term | Count | % | P Value |
| INTERPRO | IPR000566:Lipocalin/cytosolic fatty-acid binding protein domain | 3 | 4.348 | 1.13E-02 |
| INTERPRO | IPR012674:Calycin | 3 | 4.348 | 1.27E-02 |
| INTERPRO | IPR011038:Calycin-like | 3 | 4.348 | 1.37E-02 |
| GOTERM_MF_DIRECT | GO:0005215~transporter activity | 3 | 4.348 | 7.44E-02 |
| GOTERM_BP_DIRECT | GO:0006810~transport | 3 | 4.348 | 1.01E-01 |
| | | | | |
| Annotation Cluster 4 | Enrichment Score: 0.9359287600313991 | | | |
| Category | Term | Count | % | P Value |
| SMART | SM00181:EGF | 3 | 4.348 | 7.14E-02 |
| INTERPRO | IPR013032:EGF-like, conserved site | 3 | 4.348 | 1.32E-01 |
| INTERPRO | IPR000742:Epidermal growth factor-like domain | 3 | 4.348 | 1.65E-01 |
| | | | | |
| Annotation Cluster 5 | Enrichment Score: 0.6822020922248954 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Lectin | 3 | 4.347826087 | 5.39E-02 |
| UP_KEYWORDS | Secreted | 6 | 8.695652174 | 2.46E-01 |
| GOTERM_CC_DIRECT | GO:0005615~extracellular space | 5 | 7.246376812 | 6.76E-01 |
| | | | | |
| Annotation Cluster 6 | Enrichment Score: 0.54810518040865 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Mitochondrion | 5 | 7.246376812 | 1.65E-01 |
| UP_KEYWORDS | Transit peptide | 3 | 4.347826087 | 2.78E-01 |
| UP_SEQ_FEATURE | transit peptide:Mitochondrion | 3 | 4.347826087 | 4.95E-01 |
| | | | | |
| Annotation Cluster 7 | Enrichment Score: 0.328883253144982 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Disulfide bond | 11 | 15.94202899 | 1.67E-01 |
| UP_KEYWORDS | Secreted | 6 | 8.695652174 | 2.46E-01 |
| UP_KEYWORDS | Signal | 13 | 18.84057971 | 4.23E-01 |
| GOTERM_CC_DIRECT | GO:0005576~extracellular region | 4 | 5.797101449 | 4.50E-01 |
| UP_SEQ_FEATURE | glycosylation site:N-linked (GlcNAc...) | 10 | 14.49275362 | 7.94E-01 |
| UP_SEQ_FEATURE | signal peptide | 8 | 11.5942029 | 8.37E-01 |
| UP_SEQ_FEATURE | disulfide bond | 5 | 7.246376812 | 9.61E-01 |
| | | | | |
| Annotation Cluster 8 | Enrichment Score: 0.2130930587661748 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Transcription regulation | 4 | 5.797101449 | 5.03E-01 |
| UP_KEYWORDS | Transcription | 4 | 5.797101449 | 5.42E-01 |

| | | | | |
|---|---|---|---|---|
| UP_KEYWORDS | Nucleus | 8 | 11.5942029 | 6.73E-01 |
| GOTERM_BP_DIRECT | GO:0006351~transcription, DNA-templated | 3 | 4.347826087 | 7.66E-01 |
| | | | | |
| Annotation Cluster 9 | Enrichment Score: 0.10752488186890709 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Nucleus | 8 | 11.5942029 | 6.73E-01 |
| UP_KEYWORDS | DNA-binding | 3 | 4.347826087 | 8.05E-01 |
| GOTERM_BP_DIRECT | GO:0045944~positive regulation of transcription from RNA polymerase II promoter | 3 | 4.347826087 | 8.79E-01 |
| Annotation Cluster 10 | Enrichment Score: 0.08769573194718908 | | | |
| Category | Term | Count | % | P Value |
| UP_SEQ_FEATURE | transmembrane region | 12 | 17.39130435 | 7.38E-01 |
| UP_KEYWORDS | Membrane | 22 | 31.88405797 | 7.74E-01 |
| UP_KEYWORDS | Transmembrane | 18 | 26.08695652 | 8.25E-01 |
| GOTERM_CC_DIRECT | GO:0016021~integral component of membrane | 17 | 24.63768116 | 8.76E-01 |
| UP_KEYWORDS | Transmembrane helix | 17 | 24.63768116 | 8.83E-01 |
| | | | | |
| Annotation Cluster 11 | Enrichment Score: 0.05963151809830033 | | | |
| Category | Term | Count | % | P Value |
| UP_KEYWORDS | Nucleotide-binding | 4 | 5.797101449 | 8.10E-01 |
| UP_KEYWORDS | ATP-binding | 3 | 4.347826087 | 8.51E-01 |
| GOTERM_MF_DIRECT | GO:0005524~ATP binding | 3 | 4.347826087 | 9.62E-01 |
| | | | | |
| Annotation Cluster 12 | Enrichment Score: 0.0013045622495406195 | | | |
| Category | Term | Count | % | P Value |
| UP_SEQ_FEATURE | topological domain:Extracellular | 3 | 4.347826087 | 9.95E-01 |
| UP_SEQ_FEATURE | topological domain:Cytoplasmic | 4 | 5.797101449 | 9.96E-01 |
| UP_KEYWORDS | Cell membrane | 3 | 4.347826087 | 1.00E+00 |

**Table C3. Data output of traditional functional annotation chart reports of 111 up-regulated genes from Uehara *et al.* (2008a)**

| Category | Term | Count | % | P Value |
|---|---|---|---|---|
| GOTERM_CC_DIRECT | GO:0042555~MCM complex | 5 | 4.505 | 9.48E-08 |
| GOTERM_BP_DIRECT | GO:0006270~DNA replication initiation | 6 | 5.405 | 1.20E-07 |
| UP_KEYWORDS | DNA replication | 7 | 6.306 | 1.81E-07 |
| KEGG_PATHWAY | rno03030:DNA replication | 7 | 6.306 | 2.39E-07 |
| UP_KEYWORDS | Endoplasmic reticulum | 16 | 14.414 | 3.58E-07 |
| GOTERM_CC_DIRECT | GO:0034663~endoplasmic reticulum chaperone complex | 5 | 4.505 | 3.68E-07 |
| INTERPRO | IPR018525:Mini-chromosome maintenance, conserved site | 4 | 3.604 | 2.56E-06 |
| SMART | SM00350:MCM | 4 | 3.604 | 8.92E-06 |
| GOTERM_BP_DIRECT | GO:0006749~glutathione metabolic process | 6 | 5.405 | 1.04E-05 |
| GOTERM_BP_DIRECT | GO:0055114~oxidation-reduction process | 15 | 13.514 | 1.50E-05 |
| INTERPRO | IPR001208:Mini-chromosome maintenance, DNA-dependent ATPase | 4 | 3.604 | 1.51E-05 |
| GOTERM_CC_DIRECT | GO:0005790~smooth endoplasmic reticulum | 5 | 4.505 | 1.65E-05 |
| GOTERM_BP_DIRECT | GO:0044752~response to human chorionic gonadotropin | 4 | 3.604 | 1.97E-05 |
| UP_KEYWORDS | Phosphoprotein | 39 | 35.135 | 2.12E-05 |
| GOTERM_CC_DIRECT | GO:0005788~endoplasmic reticulum lumen | 6 | 5.405 | 2.53E-05 |
| KEGG_PATHWAY | rno04141:Protein processing in endoplasmic reticulum | 9 | 8.108 | 3.47E-05 |
| KEGG_PATHWAY | rno00480:Glutathione metabolism | 6 | 5.405 | 7.11E-05 |
| GOTERM_BP_DIRECT | GO:0045471~response to ethanol | 8 | 7.207 | 1.04E-04 |
| GOTERM_CC_DIRECT | GO:0005783~endoplasmic reticulum | 15 | 13.514 | 1.16E-04 |
| GOTERM_BP_DIRECT | GO:0002931~response to ischemia | 5 | 4.505 | 1.36E-04 |
| GOTERM_BP_DIRECT | GO:0006979~response to oxidative stress | 7 | 6.306 | 1.69E-04 |
| GOTERM_MF_DIRECT | GO:0003678~DNA helicase activity | 4 | 3.604 | 1.74E-04 |
| KEGG_PATHWAY | rno04110:Cell cycle | 7 | 6.306 | 3.73E-04 |
| GOTERM_CC_DIRECT | GO:0000784~nuclear chromosome, telomeric region | 6 | 5.405 | 3.80E-04 |
| GOTERM_BP_DIRECT | GO:0045454~cell redox homeostasis | 5 | 4.505 | 4.51E-04 |
| UP_KEYWORDS | NADP | 6 | 5.405 | 5.57E-04 |
| GOTERM_BP_DIRECT | GO:0098869~cellular oxidant detoxification | 5 | 4.505 | 6.00E-04 |
| KEGG_PATHWAY | rno04612:Antigen processing and presentation | 6 | 5.405 | 7.99E-04 |
| UP_KEYWORDS | NAD | 6 | 5.405 | 8.17E-04 |
| UP_KEYWORDS | Redox-active center | 4 | 3.604 | 8.53E-04 |
| UP_KEYWORDS | Acetylation | 20 | 18.018 | 9.33E-04 |
| GOTERM_BP_DIRECT | GO:0007584~response to nutrient | 6 | 5.405 | 1.03E-03 |
| GOTERM_CC_DIRECT | GO:0016020~membrane | 24 | 21.622 | 1.12E-03 |
| GOTERM_BP_DIRECT | GO:0034976~response to endoplasmic reticulum stress | 5 | 4.505 | 1.15E-03 |
| GOTERM_BP_DIRECT | GO:0006260~DNA replication | 5 | 4.505 | 1.20E-03 |

| Category | Term | Count | % | P Value |
|---|---|---|---|---|
| GOTERM_BP_DIRECT | GO:0006750~glutathione biosynthetic process | 3 | 2.703 | 1.35E-03 |
| GOTERM_BP_DIRECT | GO:2001237~negative regulation of extrinsic apoptotic signaling pathway | 4 | 3.604 | 1.66E-03 |
| GOTERM_MF_DIRECT | GO:0004364~glutathione transferase activity | 4 | 3.604 | 1.72E-03 |
| UP_SEQ_FEATURE | short sequence motif:Prevents secretion from ER | 4 | 3.604 | 2.15E-03 |
| GOTERM_BP_DIRECT | GO:0043066~negative regulation of apoptotic process | 10 | 9.009 | 2.42E-03 |
| UP_KEYWORDS | Oxidoreductase | 9 | 8.108 | 2.99E-03 |
| GOTERM_MF_DIRECT | GO:0043295~glutathione binding | 3 | 2.703 | 3.89E-03 |
| UP_KEYWORDS | Helicase | 4 | 3.604 | 4.56E-03 |
| GOTERM_BP_DIRECT | GO:0032355~response to estradiol | 6 | 5.405 | 5.41E-03 |
| GOTERM_CC_DIRECT | GO:0070062~extracellular exosome | 25 | 22.523 | 5.67E-03 |
| INTERPRO | IPR012336:Thioredoxin-like fold | 5 | 4.505 | 5.78E-03 |
| INTERPRO | IPR023210:NADP-dependent oxidoreductase domain | 3 | 2.703 | 6.09E-03 |
| UP_KEYWORDS | Signal | 28 | 25.225 | 6.77E-03 |
| KEGG_PATHWAY | rno00240:Pyrimidine metabolism | 5 | 4.505 | 8.34E-03 |
| GOTERM_BP_DIRECT | GO:0007568~aging | 7 | 6.306 | 8.48E-03 |
| INTERPRO | IPR012340:Nucleic acid-binding, OB-fold | 4 | 3.604 | 9.43E-03 |
| GOTERM_CC_DIRECT | GO:0005789~endoplasmic reticulum membrane | 9 | 8.108 | 9.87E-03 |
| GOTERM_CC_DIRECT | GO:0005829~cytosol | 17 | 15.315 | 9.99E-03 |
| GOTERM_CC_DIRECT | GO:0017109~glutamate-cysteine ligase complex | 2 | 1.802 | 1.07E-02 |
| GOTERM_CC_DIRECT | GO:0005730~nucleolus | 11 | 9.910 | 1.08E-02 |
| UP_KEYWORDS | Isomerase | 4 | 3.604 | 1.08E-02 |
| INTERPRO | IPR004046:Glutathione S-transferase, C-terminal | 3 | 2.703 | 1.09E-02 |
| GOTERM_MF_DIRECT | GO:0004357~glutamate-cysteine ligase activity | 2 | 1.802 | 1.10E-02 |
| GOTERM_BP_DIRECT | GO:0046223~aflatoxin catabolic process | 2 | 1.802 | 1.11E-02 |
| GOTERM_BP_DIRECT | GO:1903334~positive regulation of protein folding | 2 | 1.802 | 1.11E-02 |
| GOTERM_BP_DIRECT | GO:0097069~cellular response to thyroxine stimulus | 2 | 1.802 | 1.11E-02 |
| UP_KEYWORDS | ATP-binding | 12 | 10.811 | 1.21E-02 |
| GOTERM_CC_DIRECT | GO:0042470~melanosome | 4 | 3.604 | 1.24E-02 |
| KEGG_PATHWAY | rno01100:Metabolic pathways | 18 | 16.216 | 1.32E-02 |
| KEGG_PATHWAY | rno05332:Graft-versus-host disease | 4 | 3.604 | 1.35E-02 |
| INTERPRO | IPR005792:Protein disulphide isomerase | 2 | 1.802 | 1.52E-02 |
| KEGG_PATHWAY | rno05330:Allograft rejection | 4 | 3.604 | 1.58E-02 |
| KEGG_PATHWAY | rno00980:Metabolism of xenobiotics by cytochrome P450 | 4 | 3.604 | 1.58E-02 |
| KEGG_PATHWAY | rno04976:Bile secretion | 4 | 3.604 | 1.64E-02 |
| GOTERM_BP_DIRECT | GO:0006534~cysteine metabolic process | 2 | 1.802 | 1.67E-02 |
| UP_KEYWORDS | Chaperone | 4 | 3.604 | 1.69E-02 |
| GOTERM_BP_DIRECT | GO:0001666~response to hypoxia | 6 | 5.405 | 1.78E-02 |
| INTERPRO | IPR016040:NAD(P)-binding domain | 5 | 4.505 | 1.81E-02 |
| GOTERM_BP_DIRECT | GO:0000723~telomere maintenance | 3 | 2.703 | 1.81E-02 |

| Category | Term | Count | % | P Value |
|---|---|---|---|---|
| KEGG_PATHWAY | rno04940:Type I diabetes mellitus | 4 | 3.604 | 1.97E-02 |
| UP_KEYWORDS | Disulfide bond | 19 | 17.117 | 2.03E-02 |
| KEGG_PATHWAY | rno05320:Autoimmune thyroid disease | 4 | 3.604 | 2.04E-02 |
| GOTERM_CC_DIRECT | GO:0043625~delta DNA polymerase complex | 2 | 1.802 | 2.12E-02 |
| GOTERM_BP_DIRECT | GO:0051900~regulation of mitochondrial depolarization | 2 | 1.802 | 2.22E-02 |
| GOTERM_BP_DIRECT | GO:1903298~negative regulation of hypoxia-induced intrinsic apoptotic signaling pathway | 2 | 1.802 | 2.22E-02 |
| GOTERM_BP_DIRECT | GO:0031667~response to nutrient levels | 4 | 3.604 | 2.49E-02 |
| GOTERM_BP_DIRECT | GO:0006457~protein folding | 4 | 3.604 | 2.49E-02 |
| INTERPRO | IPR005788:Disulphide isomerase | 2 | 1.802 | 2.53E-02 |
| UP_KEYWORDS | Calcium | 8 | 7.207 | 2.61E-02 |
| GOTERM_CC_DIRECT | GO:0005634~nucleus | 36 | 32.432 | 2.66E-02 |
| INTERPRO | IPR010987:Glutathione S-transferase, C-terminal-like | 3 | 2.703 | 2.70E-02 |
| GOTERM_BP_DIRECT | GO:0010664~negative regulation of striated muscle cell apoptotic process | 2 | 1.802 | 2.76E-02 |
| GOTERM_BP_DIRECT | GO:0051409~response to nitrosative stress | 2 | 1.802 | 2.76E-02 |
| GOTERM_BP_DIRECT | GO:0042493~response to drug | 8 | 7.207 | 2.85E-02 |
| UP_KEYWORDS | Metal-thiolate cluster | 2 | 1.802 | 2.91E-02 |
| UP_KEYWORDS | Nucleotide-binding | 13 | 11.712 | 2.91E-02 |
| GOTERM_BP_DIRECT | GO:0009636~response to toxic substance | 4 | 3.604 | 2.91E-02 |
| KEGG_PATHWAY | rno00040:Pentose and glucuronate interconversions | 3 | 2.703 | 3.02E-02 |
| INTERPRO | IPR003019:Metallothionein superfamily, eukaryotic | 2 | 1.802 | 3.03E-02 |
| INTERPRO | IPR000006:Metallothionein, vertebrate | 2 | 1.802 | 3.03E-02 |
| INTERPRO | IPR023587:Metallothionein domain, vertebrate | 2 | 1.802 | 3.03E-02 |
| INTERPRO | IPR017854:Metallothionein domain | 2 | 1.802 | 3.03E-02 |
| KEGG_PATHWAY | rno05416:Viral myocarditis | 4 | 3.604 | 3.06E-02 |
| KEGG_PATHWAY | rno03410:Base excision repair | 3 | 2.703 | 3.18E-02 |
| UP_KEYWORDS | Methylation | 7 | 6.306 | 3.23E-02 |
| GOTERM_MF_DIRECT | GO:0016491~oxidoreductase activity | 4 | 3.604 | 3.33E-02 |
| GOTERM_MF_DIRECT | GO:0042803~protein homodimerization activity | 10 | 9.009 | 3.36E-02 |
| UP_KEYWORDS | Glutathione biosynthesis | 2 | 1.802 | 3.38E-02 |
| INTERPRO | IPR004099:Pyridine nucleotide-disulphide oxidoreductase, dimerisation | 2 | 1.802 | 3.52E-02 |
| INTERPRO | IPR016156:FAD/NAD-linked reductase, dimerisation | 2 | 1.802 | 3.52E-02 |
| GOTERM_MF_DIRECT | GO:0005436~sodium:phosphate symporter activity | 2 | 1.802 | 3.81E-02 |
| UP_SEQ_FEATURE | domain:Thioredoxin 1 | 2 | 1.802 | 4.14E-02 |
| UP_SEQ_FEATURE | domain:Thioredoxin 2 | 2 | 1.802 | 4.14E-02 |
| GOTERM_BP_DIRECT | GO:0043200~response to amino acid | 3 | 2.703 | 4.17E-02 |
| GOTERM_MF_DIRECT | GO:0015321~sodium-dependent phosphate transmembrane transporter activity | 2 | 1.802 | 4.34E-02 |
| GOTERM_BP_DIRECT | GO:0044341~sodium-dependent phosphate transport | 2 | 1.802 | 4.39E-02 |

| Category | Term | Count | % | P Value |
|---|---|---|---|---|
| GOTERM_BP_DIRECT | GO:0070192~chromosome organization involved in meiotic cell cycle | 2 | 1.802 | 4.39E-02 |
| GOTERM_BP_DIRECT | GO:0006268~DNA unwinding involved in DNA replication | 2 | 1.802 | 4.39E-02 |
| GOTERM_CC_DIRECT | GO:0009986~cell surface | 8 | 7.207 | 4.57E-02 |
| GOTERM_BP_DIRECT | GO:0048545~response to steroid hormone | 3 | 2.703 | 4.57E-02 |
| GOTERM_CC_DIRECT | GO:0005737~cytoplasm | 37 | 33.333 | 4.77E-02 |
| UP_KEYWORDS | DNA-directed DNA polymerase | 2 | 1.802 | 4.80E-02 |
| GOTERM_MF_DIRECT | GO:0003688~DNA replication origin binding | 2 | 1.802 | 4.87E-02 |
| SMART | SM00407:IGc1 | 3 | 2.703 | 4.91E-02 |
| GOTERM_BP_DIRECT | GO:0015893~drug transport | 2 | 1.802 | 4.92E-02 |
| GOTERM_BP_DIRECT | GO:0046415~urate metabolic process | 2 | 1.802 | 4.92E-02 |
| INTERPRO | IPR011162:MHC classes I/II-like antigen recognition protein | 3 | 2.703 | 4.98E-02 |
| GOTERM_MF_DIRECT | GO:0050662~coenzyme binding | 2 | 1.802 | 5.39E-02 |
| GOTERM_MF_DIRECT | GO:0004032~alditol:NADP+ 1-oxidoreductase activity | 2 | 1.802 | 5.39E-02 |
| UP_KEYWORDS | Glycoprotein | 17 | 15.315 | 5.40E-02 |
| GOTERM_BP_DIRECT | GO:0010243~response to organonitrogen compound | 3 | 2.703 | 5.55E-02 |
| UP_KEYWORDS | Cell division | 4 | 3.604 | 5.89E-02 |
| GOTERM_BP_DIRECT | GO:1903827~regulation of cellular protein localization | 2 | 1.802 | 5.98E-02 |
| KEGG_PATHWAY | rno04145:Phagosome | 5 | 4.505 | 6.08E-02 |
| INTERPRO | IPR003006:Immunoglobulin/major histocompatibility complex, conserved site | 3 | 2.703 | 6.17E-02 |
| GOTERM_BP_DIRECT | GO:0050880~regulation of blood vessel size | 2 | 1.802 | 6.51E-02 |
| INTERPRO | IPR003597:Immunoglobulin C1-set | 3 | 2.703 | 6.59E-02 |
| GOTERM_MF_DIRECT | GO:0051082~unfolded protein binding | 3 | 2.703 | 6.62E-02 |
| GOTERM_BP_DIRECT | GO:0014070~response to organic cyclic compound | 5 | 4.505 | 6.62E-02 |
| GOTERM_MF_DIRECT | GO:0051087~chaperone binding | 3 | 2.703 | 6.77E-02 |
| KEGG_PATHWAY | rno05166:HTLV-I infection | 6 | 5.405 | 6.88E-02 |
| INTERPRO | IPR018181:Heat shock protein 70, conserved site | 2 | 1.802 | 6.92E-02 |
| INTERPRO | IPR008927:6-phosphogluconate dehydrogenase, C-terminal-like | 2 | 1.802 | 6.92E-02 |
| INTERPRO | IPR013126:Heat shock protein 70 family | 2 | 1.802 | 6.92E-02 |
| UP_KEYWORDS | Symport | 3 | 2.703 | 6.95E-02 |
| GOTERM_MF_DIRECT | GO:0051787~misfolded protein binding | 2 | 1.802 | 6.95E-02 |
| GOTERM_CC_DIRECT | GO:0042613~MHC class II protein complex | 2 | 1.802 | 7.23E-02 |
| GOTERM_MF_DIRECT | GO:0015238~drug transmembrane transporter activity | 2 | 1.802 | 7.47E-02 |
| SMART | SM01332:SM01332 | 2 | 1.802 | 7.53E-02 |
| GOTERM_BP_DIRECT | GO:0019886~antigen processing and presentation of exogenous peptide antigen via MHC class II | 2 | 1.802 | 7.55E-02 |

| Category | Term | Count | % | P Value |
|---|---|---|---|---|
| GOTERM_BP_DIRECT | GO:0006536~glutamate metabolic process | 2 | 1.802 | 7.55E-02 |
| GOTERM_CC_DIRECT | GO:0043231~intracellular membrane-bounded organelle | 8 | 7.207 | 7.55E-02 |
| COG_ONTOLOGY | Posttranslational modification, protein turnover, chaperones | 3 | 2.703 | 7.78E-02 |
| INTERPRO | IPR017937:Thioredoxin, conserved site | 2 | 1.802 | 7.87E-02 |
| UP_KEYWORDS | Nucleus | 18 | 16.216 | 7.88E-02 |
| GOTERM_BP_DIRECT | GO:0051301~cell division | 4 | 3.604 | 7.89E-02 |
| UP_KEYWORDS | FAD | 3 | 2.703 | 7.93E-02 |
| GOTERM_MF_DIRECT | GO:0016538~cyclin-dependent protein serine/threonine kinase regulator activity | 2 | 1.802 | 7.98E-02 |
| GOTERM_CC_DIRECT | GO:0005739~mitochondrion | 14 | 12.613 | 8.46E-02 |
| GOTERM_MF_DIRECT | GO:0070402~NADPH binding | 2 | 1.802 | 8.49E-02 |
| GOTERM_BP_DIRECT | GO:0030970~retrograde protein transport, ER to cytosol | 2 | 1.802 | 8.58E-02 |
| GOTERM_BP_DIRECT | GO:0006261~DNA-dependent DNA replication | 2 | 1.802 | 8.58E-02 |
| GOTERM_BP_DIRECT | GO:0000038~very long-chain fatty acid metabolic process | 2 | 1.802 | 8.58E-02 |
| GOTERM_CC_DIRECT | GO:0005576~extracellular region | 8 | 7.207 | 8.78E-02 |
| INTERPRO | IPR020471:Aldo/keto reductase subgroup | 2 | 1.802 | 8.81E-02 |
| INTERPRO | IPR004367:Cyclin, C-terminal domain | 2 | 1.802 | 8.81E-02 |
| GOTERM_BP_DIRECT | GO:0071333~cellular response to glucose stimulus | 3 | 2.703 | 8.86E-02 |
| UP_KEYWORDS | Flavoprotein | 3 | 2.703 | 8.96E-02 |
| GOTERM_BP_DIRECT | GO:0035729~cellular response to hepatocyte growth factor stimulus | 2 | 1.802 | 9.09E-02 |
| GOTERM_BP_DIRECT | GO:0031100~organ regeneration | 3 | 2.703 | 9.20E-02 |
| GOTERM_BP_DIRECT | GO:0014823~response to activity | 3 | 2.703 | 9.37E-02 |
| KEGG_PATHWAY | rno04918:Thyroid hormone synthesis | 3 | 2.703 | 9.38E-02 |
| GOTERM_BP_DIRECT | GO:0035435~phosphate ion transmembrane transport | 2 | 1.802 | 9.60E-02 |
| INTERPRO | IPR010579:MHC class I, alpha chain, C-terminal | 2 | 1.802 | 9.74E-02 |
| UP_KEYWORDS | MHC I | 2 | 1.802 | 9.82E-02 |
| GOTERM_CC_DIRECT | GO:0016323~basolateral plasma membrane | 4 | 3.604 | 9.94E-02 |

**Table C4. Data output of traditional functional annotation chart reports of 69 down-regulated genes from Uehara *et al.* (2008a)**

| Category | Term | Count | % | P Value |
|---|---|---|---|---|
| UP_KEYWORDS | Oxidoreductase | 11 | 15.942 | 3.86E-06 |
| GOTERM_BP_DIRECT | GO:0055114~oxidation-reduction process | 12 | 17.391 | 1.31E-05 |
| GOTERM_CC_DIRECT | GO:0031090~organelle membrane | 6 | 8.696 | 1.47E-05 |
| UP_KEYWORDS | Microsome | 6 | 8.696 | 4.27E-05 |
| KEGG_PATHWAY | rno01100:Metabolic pathways | 17 | 24.638 | 5.31E-05 |
| INTERPRO | IPR015421:Pyridoxal phosphate-dependent transferase, major region, subdomain 1 | 4 | 5.797 | 4.32E-04 |
| INTERPRO | IPR015424:Pyridoxal phosphate-dependent transferase | 4 | 5.797 | 4.32E-04 |
| UP_KEYWORDS | Monooxygenase | 5 | 7.246 | 4.40E-04 |
| KEGG_PATHWAY | rno00140:Steroid hormone biosynthesis | 5 | 7.246 | 4.47E-04 |
| UP_KEYWORDS | Lipid metabolism | 7 | 10.145 | 4.54E-04 |
| UP_KEYWORDS | Lipid biosynthesis | 5 | 7.246 | 5.20E-04 |
| UP_KEYWORDS | Pyridoxal phosphate | 4 | 5.797 | 5.62E-04 |
| GOTERM_CC_DIRECT | GO:0043231~intracellular membrane-bounded organelle | 10 | 14.493 | 5.66E-04 |
| GOTERM_MF_DIRECT | GO:0005506~iron ion binding | 6 | 8.696 | 6.80E-04 |
| GOTERM_MF_DIRECT | GO:0016491~oxidoreductase activity | 5 | 7.246 | 9.96E-04 |
| GOTERM_MF_DIRECT | GO:0030170~pyridoxal phosphate binding | 4 | 5.797 | 1.08E-03 |
| UP_KEYWORDS | Iron | 6 | 8.696 | 2.15E-03 |
| UP_KEYWORDS | Metal-binding | 16 | 23.188 | 2.71E-03 |
| GOTERM_CC_DIRECT | GO:0005829~cytosol | 14 | 20.290 | 2.72E-03 |
| INTERPRO | IPR017972:Cytochrome P450, conserved site | 4 | 5.797 | 2.73E-03 |
| KEGG_PATHWAY | rno01130:Biosynthesis of antibiotics | 6 | 8.696 | 2.75E-03 |
| GOTERM_CC_DIRECT | GO:0070062~extracellular exosome | 19 | 27.536 | 2.96E-03 |
| GOTERM_CC_DIRECT | GO:0005789~endoplasmic reticulum membrane | 8 | 11.594 | 2.99E-03 |
| INTERPRO | IPR001128:Cytochrome P450 | 4 | 5.797 | 3.43E-03 |
| UP_KEYWORDS | Cytoplasm | 17 | 24.638 | 3.52E-03 |
| UP_KEYWORDS | Endoplasmic reticulum | 8 | 11.594 | 3.78E-03 |
| KEGG_PATHWAY | rno00982:Drug metabolism - cytochrome P450 | 4 | 5.797 | 3.87E-03 |
| GOTERM_BP_DIRECT | GO:0017144~drug metabolic process | 3 | 4.348 | 4.86E-03 |
| INTERPRO | IPR015422:Pyridoxal phosphate-dependent transferase, major region, subdomain 2 | 3 | 4.348 | 7.57E-03 |
| KEGG_PATHWAY | rno05204:Chemical carcinogenesis | 4 | 5.797 | 7.73E-03 |
| UP_KEYWORDS | NADP | 4 | 5.797 | 9.67E-03 |
| GOTERM_BP_DIRECT | GO:0032787~monocarboxylic acid metabolic process | 2 | 2.899 | 1.04E-02 |
| GOTERM_MF_DIRECT | GO:0034875~caffeine oxidase activity | 2 | 2.899 | 1.04E-02 |
| UP_KEYWORDS | Sterol metabolism | 3 | 4.348 | 1.05E-02 |
| COG_ONTOLOGY | Secondary metabolites biosynthesis, transport, and catabolism | 4 | 5.797 | 1.08E-02 |
| INTERPRO | IPR000566:Lipocalin/cytosolic fatty-acid binding protein domain | 3 | 4.348 | 1.13E-02 |
| UP_KEYWORDS | Heme | 4 | 5.797 | 1.19E-02 |
| INTERPRO | IPR012674:Calycin | 3 | 4.348 | 1.27E-02 |
| INTERPRO | IPR011038:Calycin-like | 3 | 4.348 | 1.37E-02 |

| Category | Term | Count | % | P Value |
|---|---|---|---|---|
| GOTERM_BP_DIRECT | GO:0010033~response to organic substance | 4 | 5.797 | 1.59E-02 |
| UP_KEYWORDS | Steroid metabolism | 3 | 4.348 | 1.66E-02 |
| UP_SEQ_FEATURE | metal ion-binding site:Iron (heme axial ligand) | 4 | 5.797 | 1.67E-02 |
| GOTERM_BP_DIRECT | GO:0016098~monoterpenoid metabolic process | 2 | 2.899 | 1.73E-02 |
| GOTERM_BP_DIRECT | GO:0042737~drug catabolic process | 2 | 2.899 | 1.73E-02 |
| INTERPRO | IPR018485:Carbohydrate kinase, FGGY, C-terminal | 2 | 2.899 | 2.00E-02 |
| GOTERM_MF_DIRECT | GO:0020037~heme binding | 4 | 5.797 | 2.01E-02 |
| GOTERM_MF_DIRECT | GO:0004497~monooxygenase activity | 3 | 4.348 | 2.16E-02 |
| INTERPRO | IPR018484:Carbohydrate kinase, FGGY, N-terminal | 2 | 2.899 | 2.32E-02 |
| UP_KEYWORDS | Transferase | 10 | 14.493 | 2.48E-02 |
| GOTERM_BP_DIRECT | GO:0009404~toxin metabolic process | 2 | 2.899 | 3.09E-02 |
| GOTERM_BP_DIRECT | GO:0042738~exogenous drug catabolic process | 2 | 2.899 | 3.09E-02 |
| INTERPRO | IPR002971:Major urinary protein | 2 | 2.899 | 3.63E-02 |
| GOTERM_BP_DIRECT | GO:0070989~oxidative demethylation | 2 | 2.899 | 3.76E-02 |
| KEGG_PATHWAY | rno00980:Metabolism of xenobiotics by cytochrome P450 | 3 | 4.348 | 3.92E-02 |
| GOTERM_MF_DIRECT | GO:0005550~pheromone binding | 2 | 2.899 | 4.10E-02 |
| PIR_SUPERFAMILY | PIRSF000538:keto-sugar kinase | 2 | 2.899 | 4.16E-02 |
| INTERPRO | IPR005804:Fatty acid desaturase, type 1 | 2 | 2.899 | 4.27E-02 |
| INTERPRO | IPR002403:Cytochrome P450, E-class, group IV | 2 | 2.899 | 4.27E-02 |
| UP_SEQ_FEATURE | short sequence motif:Histidine box-1 | 2 | 2.899 | 4.52E-02 |
| UP_SEQ_FEATURE | short sequence motif:Histidine box-2 | 2 | 2.899 | 4.52E-02 |
| UP_SEQ_FEATURE | short sequence motif:Histidine box-3 | 2 | 2.899 | 4.52E-02 |
| KEGG_PATHWAY | rno03320:PPAR signaling pathway | 3 | 4.348 | 4.66E-02 |
| GOTERM_CC_DIRECT | GO:0005739~mitochondrion | 11 | 15.942 | 4.72E-02 |
| UP_KEYWORDS | Lyase | 3 | 4.348 | 4.75E-02 |
| GOTERM_BP_DIRECT | GO:0006636~unsaturated fatty acid biosynthetic process | 2 | 2.899 | 4.76E-02 |
| UP_KEYWORDS | Phosphoprotein | 20 | 28.986 | 5.04E-02 |
| KEGG_PATHWAY | rno00830:Retinol metabolism | 3 | 4.348 | 5.33E-02 |
| UP_KEYWORDS | Lectin | 3 | 4.348 | 5.39E-02 |
| INTERPRO | IPR002345:Lipocalin | 2 | 2.899 | 5.55E-02 |
| GOTERM_MF_DIRECT | GO:0036094~small molecule binding | 2 | 2.899 | 5.76E-02 |
| INTERPRO | IPR020471:Aldo/keto reductase subgroup | 2 | 2.899 | 5.87E-02 |
| GOTERM_CC_DIRECT | GO:0030018~Z disc | 3 | 4.348 | 6.19E-02 |
| INTERPRO | IPR018170:Aldo/keto reductase, conserved site | 2 | 2.899 | 6.50E-02 |
| UP_KEYWORDS | Laminin EGF-like domain | 2 | 2.899 | 6.56E-02 |
| UP_SEQ_FEATURE | chain:15.5 kDa fatty acid-binding protein | 2 | 2.899 | 6.71E-02 |
| UP_SEQ_FEATURE | chain:Major urinary protein | 2 | 2.899 | 6.71E-02 |
| INTERPRO | IPR001395:Aldo/keto reductase | 2 | 2.899 | 6.81E-02 |
| GOTERM_MF_DIRECT | GO:0004872~receptor activity | 3 | 4.348 | 6.94E-02 |
| GOTERM_BP_DIRECT | GO:0005975~carbohydrate metabolic process | 3 | 4.348 | 7.03E-02 |
| INTERPRO | IPR022272:Lipocalin conserved site | 2 | 2.899 | 7.13E-02 |
| SMART | SM00181:EGF | 3 | 4.348 | 7.14E-02 |

| Category | Term | Count | % | P Value |
|---|---|---|---|---|
| GOTERM_MF_DIRECT | GO:0005215~transporter activity | 3 | 4.348 | 7.44E-02 |
| INTERPRO | IPR023210:NADP-dependent oxidoreductase domain | 2 | 2.899 | 7.44E-02 |
| SMART | SM00180:EGF_Lam | 2 | 2.899 | 8.19E-02 |
| GOTERM_BP_DIRECT | GO:0007568~aging | 4 | 5.797 | 9.68E-02 |
| GOTERM_BP_DIRECT | GO:0006805~xenobiotic metabolic process | 2 | 2.899 | 9.93E-02 |
| GOTERM_BP_DIRECT | GO:0070207~protein homotrimerization | 2 | 2.899 | 9.93E-02 |

## C2. Application of the Comparative Toxicogenomics Database (CTD) to analyze cancer-associated pathways and biological processes

Comparative Toxicogenomics Database (CTD) was applied to the represented enriched pathways with p values less than 0.05 in selected clusters identified in Tables C1 and C2 to analyze the cancer-association of the pathways (Table 27).

CTD (http://ctdbase.org/) is a public database providing manually curated information about chemical-gene/protein interactions, and chemical-disease, gene-disease, gene ontology-gene, and pathway-gene associations. CTD uses text mining to sort literature, and each reference (abstract or full-text) is read by a biocurator to identify interactions and relationships, and all curated data is supported by its source citation (CTD, 2017). CTD also provides indirect "inferred**"** associations that are established via CTD-curated gene-disease, gene ontology-gene, and pathway-gene associations. CTD was used to ascertain if any of the annotation clusters identified in the DAVID functional annotation clustering analysis of coumarin are associated with cancer. The CTD ratio of cancer to (all) diseases (%) of a GO/pathway cluster represents its percentage of the associations with diseases that were specifically associated with cancer. For example, CTD inferred a cancer association with DNA replication. The number of inferred associations for DNA replication and all diseases is 1185, and the number of inferred associations for DNA replication and cancers is 418. Therefore, the cancer association ratio for DNA replication is 418 to 1185 and the value equal to 35.27% (shown in Table 27). The higher the CTD ratio of cancer to all disease, the stronger the cancer association.

### Appendix C References:

Huang DW, Sherman BT and Lempicki RA (2009). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37**(1): 1-13.

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