

# Application of *in silico* methods for refining risk assessment of food-related substances

Takashi Yamada<sup>1</sup>, Norimasa Tamehiro<sup>2</sup> and  
Toshiyasu Teratani<sup>2</sup>

<sup>1</sup>National Institute of Health Sciences (NIHS),

<sup>2</sup>The Food Safety Commission of Japan (FSCJ)

*The views expressed are those of the speakers  
and not an official position of NIHS or FSCJ.*



# Agenda

- Food Safety Commission of Japan (FSCJ) and the research grant for establishing new methodologies for risk assessment
- Introduction of our work supported by FSCJ on *in silico* approaches to refine risk assessment of food-related substances
  - ✓ Case study1: **Exploring hepatotoxicity-related insights through analysis on animal test database** -How is animal toxicity data and the related data interpreted and used for NAM selection in future?-
  - ✓ Case study2: Combined risk assessment of food-derived coumarin with *in silico* approaches to support bridging experimental animals to humans



**The Food Safety Commission of Japan (FSCJ) is a risk assessment organization for science-based assessment of food safety risks to human health.**

**\* NAMs related Projects**

- **Encouraging Survey and Research Projects on Ensuring Food Safety**  
“Research projects on risk assessment methodology”
- **Guidance on the USE of the (Q)SAR Application in Risk Assessment of Genotoxicity by FSCJ (2021)**

FSCJ has invited proposals from the public for research projects on the assessment methodology to implement risk assessment appropriately from 2005.



[www.Asiaphotos.org](http://www.Asiaphotos.org)



**Akasaka  
Park  
Bld.  
(22F)**



## “Establishing new methodologies for risk assessment”

Year	Keywords	Title
FY2016-2017	QSAR・RAx (Database・ML)	Construction of the database of in vivo toxicity tests and its application to the in silico prediction and evaluation of in vivo toxicity (1602)
FY2018-2019	QSAR・RAx (Database・ML)	Development of new evaluation support technology: Examination of database utilization method for toxicity prediction (1801)
FY2019-2022	QSAR (Metabolites)	Study on risk assessment methods of metabolites from pesticide residues (1906)
FY2020-2021	QSAR/PBPK (NAMs)	<a href="#">Research for refinement of prediction approach of hepatotoxicity by introducing in silico methods(JPCAFSC 20202006)</a>
FY2021-2022	RAx (Hepatotoxicity)	Novel risk prediction and assessment methods for non-genotoxic carcinogenesis by chemicals (JPCAFSC 20222206)
FY2023-2024	RAx (NAMs)	Research for the application and reliability of the read-across assessment of food-related chemicals (JPCAFSC 20232301)

# Case study1: Extracting hepatotoxicity-related insights through analysis on animal test database

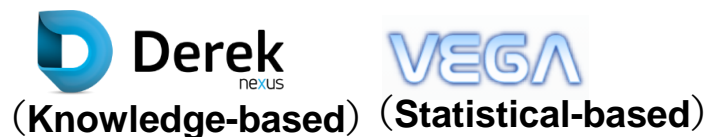


## ■ Purpose

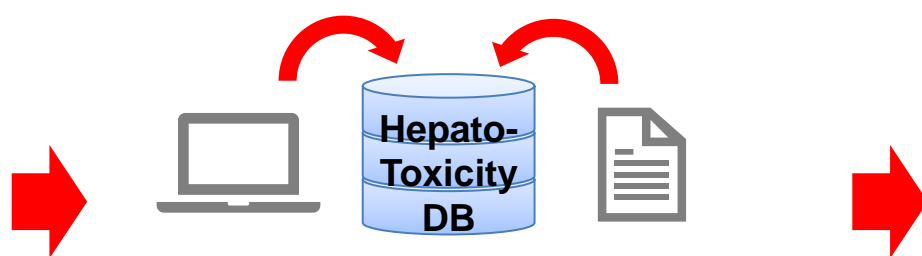
In this study, we evaluate existing hepatotoxicity prediction models and explore approaches and/or knowledges to improve them.

We collect and organize broad toxicological and other relevant knowledges on food-related substances to offer better insights on hepatotoxic risk to improve hepatotoxicity prediction and to refine risk assessment.

## ■ Method



Evaluate existing hepatotoxicity prediction models

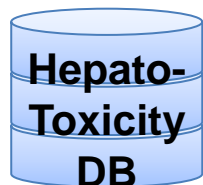
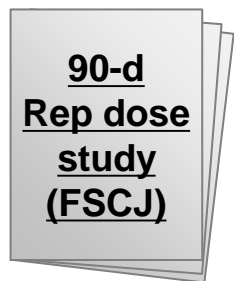


Extract hepatotoxicity-related information  
(FSCJ reports, PCP, ADME, structure, MoA)



Detection of hepatotoxicity using hepatotoxicity-related information

# Hepatotoxicity prediction performance of existing (Q)SAR models





Grouping by:

1. LOEL: Low / Medium / High
2. Severity of observed findings: Severe / Moderate / Mild

High-toxicity (**H**):  
75 substances

Low-toxicity (**L**):  
68 substances

213 substances  
(mainly pesticides)

Model	<i>In silico</i> prediction	<i>In vivo</i> hepatotoxicity	
		<b>H</b>	<b>L</b>
 Derek (Knowledge-based)	Positive	18	14
	Not positive	57	54
 VEGA (Statistical)	Positive	17	11
	Not positive	58	57
Derek or VEGA	Positive	27	22
	Not positive	48	46

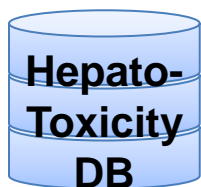
Predicted “Positive” **27** (36%) / 75 (tested positive)

Neither model predicted hepatotoxicity of 48 tested positive substances (false-negative).

- Training datasets may contain few food-related substances.
- Further consideration is needed:
  - ✓ accumulation of substances in the liver
  - ✓ metabolism and degradation
  - ✓ specific bioactivity

# Expanding database with hepatotoxicity related information

We expanded our database with the information possibly relevant to hepatotoxicity:



- ✓ Physicochemical properties (PCP)
- ✓ ADME (Absorption, Distribution, metabolism, excretion)
- ✓ Structural features
- ✓ Pesticide MoA



We highlighted following hepatotoxicity associated properties

- Prolonged exposure to the liver
    - ✓ Localization, accumulation, metabolic persistency, etc.
  - High reactivity which may cause liver damage
    - ✓ Reactive functional groups, potential formation of reactive metabolites
  - Specific interactions with biomolecules important for liver function
    - ✓ Targeting organelles or proteins abundant in liver
- to explore possible hepatotoxicity related factors in our database.



# Possible hepatotoxicity-related factors (PCP)



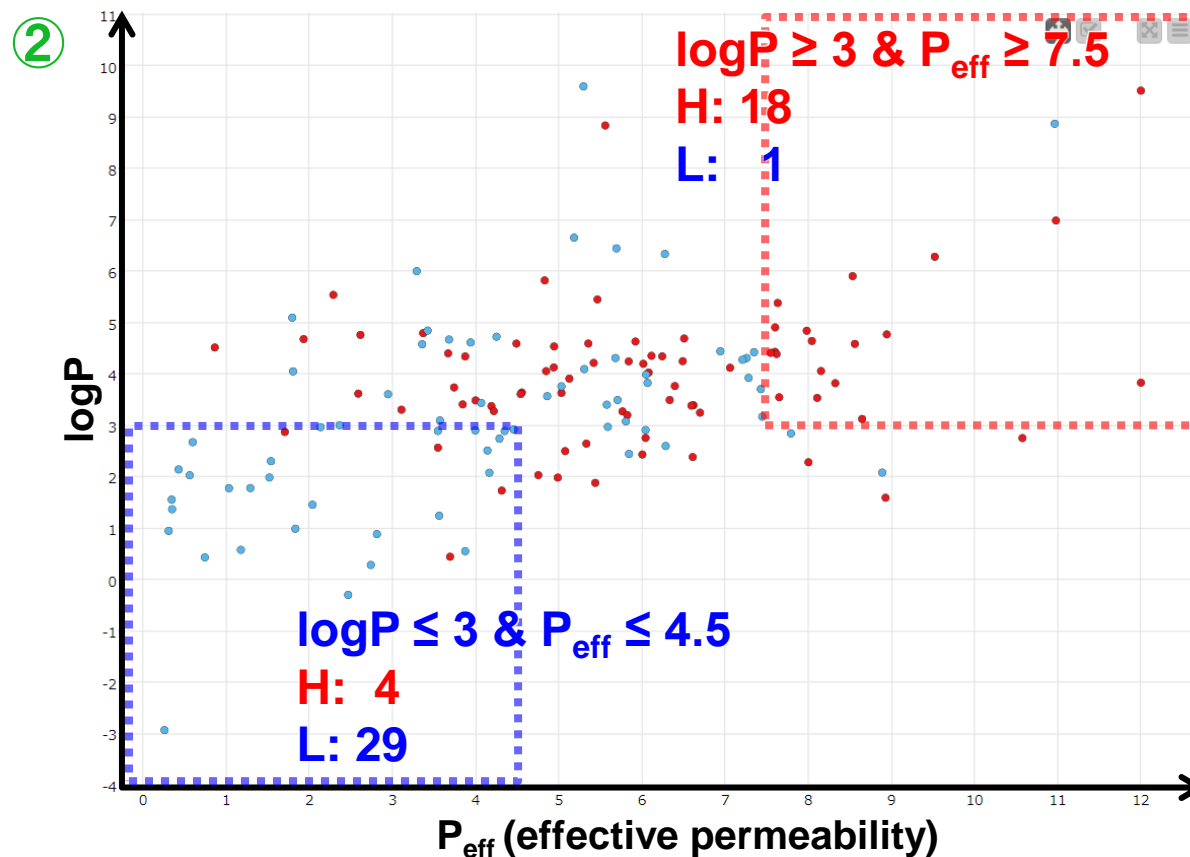
A range of the parameter values were defined in which “High toxicity” substances were selectively classified.

Category	Factor	Criterion	Prediction* or Measured
PCP	logP	3 / 6	Prediction
	Molecular Weight (MW)	350 / 500	Prediction
	fuP	5 / 10	Prediction
	P <sub>eff</sub>	-	Prediction
	P <sub>app</sub>	-	Prediction
	ECCS class	-	Prediction

\*ADMET Predictor ver.10.2

①

logP & MW	H	L
<b><math>3 \leq \log P \leq 6</math> &amp; <math>MW \geq 350</math></b>	<b>29</b>	<b>9</b>
Others	46	59
Total	75	68



- ① Substances meeting  $3 \leq \log P \leq 6$  &  $MW \geq 350$  may be more likely to be retained in the central compartment, including the liver.
- ② Combination of “logP and P<sub>eff</sub>” would be an alternative indicator to infer hepatotoxicity from magnitude of absorption.

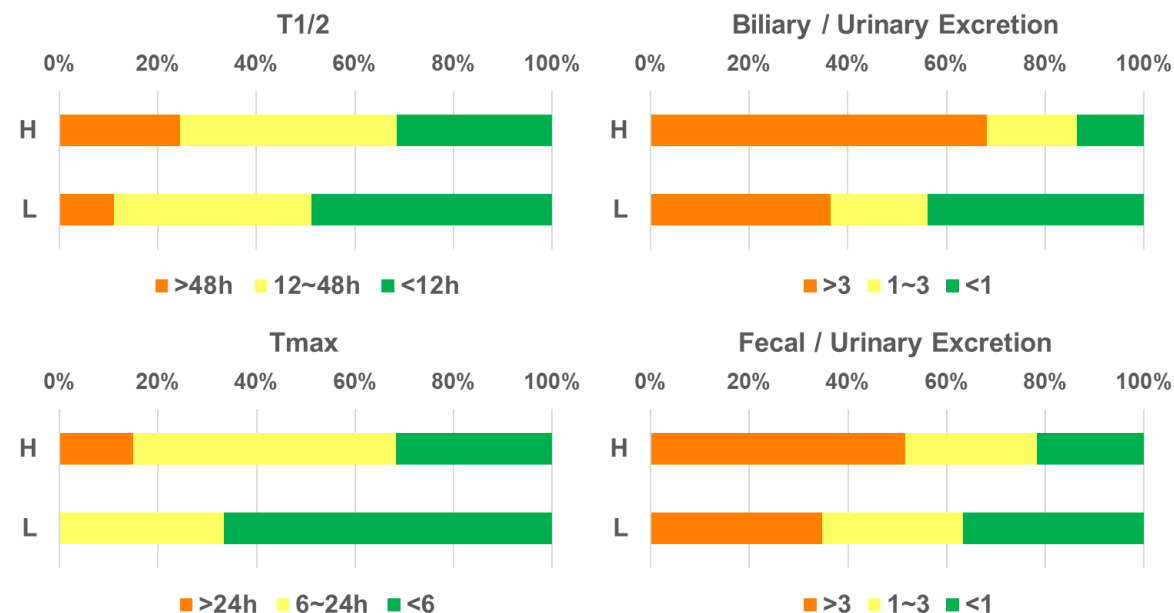


# Possible hepatotoxicity-related factors (ADME)

## The relationship between ADME parameters and hepatotoxicity

Category	Factor	Criterion	Prediction or Measured*
Blood conc. (Time)	$T_{1/2}$	12h / 48h	Measured
	$T_{max}$	6h / 24h	Measured
Distribution	Liver / Blood	3	Measured
	Fat tissue / Blood	3	Measured
Excretion	Biliary / Urinary	1 / 3	Measured
	Fecal / Urinary	1 / 3	Measured

\*from risk assessment report

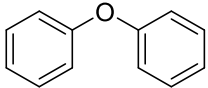



◆ ADME factors were suggested to be associated with hepatotoxicity (e.g. longer  $T_{1/2}$ ,  $T_{max}$  and biliary excretion).



- ✓ In addition, the following interpretations may be made:
- If the  $T_{1/2}$  is short but hepatotoxic, metabolites may be more involved in hepatotoxicity.
  - The results of this analysis of ADME data may be a useful resource for considering the toxicant (parent substance or metabolite) that causes hepatotoxicity.

# Possible hepatotoxicity-related factors (Structural features, MoA)

A couple of structural features relating to metabolic persistency were found to be associated with hepatotoxicity.

Structural feature	H	L
Diphenyl Ether 	7	2
Tri-Halogen (F, Cl, Br, I) 	19	10
≥3 Halogens / Molecule (F, Cl, Br, I)	4	2

Mitochondrial and CYP inhibitors, which target molecules or systems with important functions in the liver, were found to be associated with hepatotoxicity.

MoA	H	L
Mitochondria 	15	7
Cytochrome P450 	8	1

# Hepatotoxicity detection using extracted hepatotoxicity-related information

Hepatotoxicity detection by the hepatotoxicity-related information extracted through our analyses (structural features, PCP, MoA)

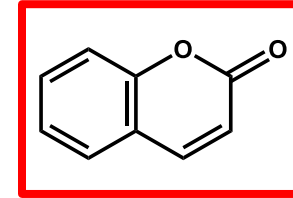
Methods	Prediction / Detection	<i>In vivo</i> hepatotoxicity		sensitivity	specificity
		H	L		
QSAR (Derek or VEGA)	Positive	27	22	<u>0.36</u>	<u>0.68</u>
	Not positive	48	46		
This study (structure, PCP, MoA)	Positive	52	23	<u>0.69</u>	<u>0.66</u>
	Not positive	23	45		
Total		75	68		

## Conclusion 1:

- The structural features, PCP and MoA proposed in this study provide a sign of hepatotoxicity. Food-related substances with the features should be noted for hepatotoxicity in risk assessment.

# Case study2: Combined risk assessment of food-derived coumarin with *in silico* approaches

- Coumarin is a naturally occurring organic chemical that is often taken in as part of foods that contain cinnamon.
- Coumarin intake from food is generally considered safe, but coumarin-induced liver damage has been reported to occur in humans.
- Other than food intake, a clinical trial of coumarin was conducted in lymphedema patients but was terminated due to the occurrence of liver damage. The US FDA has banned the use of coumarin as a food additive due to hepatotoxic risk.



Coumarin



Cinnamon



Sakuramochi



Sweet clover extract

**We assessed the data of existing clinical and nonclinical studies as well as those of *in silico* models for humans in order to shed more light on the association of coumarin and liver damage.**

**Existing Data on coumarin**  
✓ Clinical and animal studies  
✓ ADME

**Data of *in silico* models**  
✓ Human PBPK model  
✓ FDA DILI score model

**Combined and assessed**

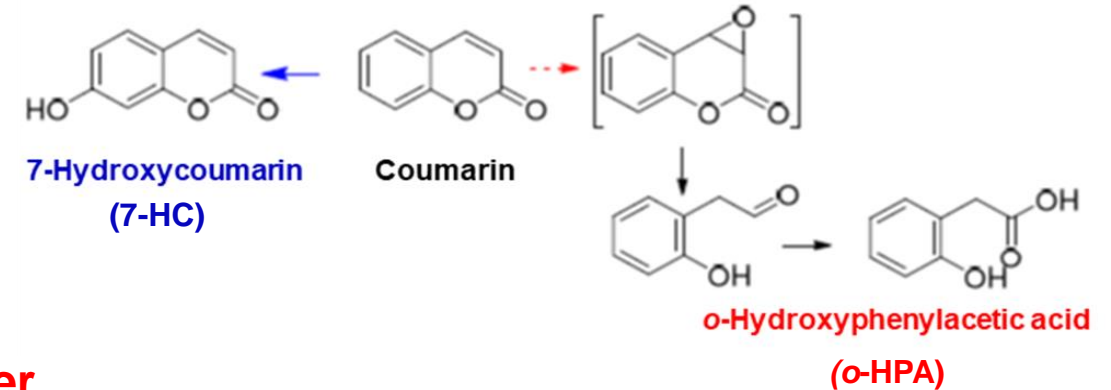
# Intake, ADME, toxicity of Coumarin

## Intake from food

- Average intake = 1-3 mg/day. 10-fold or more in the worst case (EFSA, 2004, Iwasaki et al., 2008, Abraham et al., 2010).

## ADME

- High absorption upon oral intake. High clearance. Little tissue accumulation.
- Metabolites (human and laboratory animals): **7-Hydroxycoumarin** and **o-Hydroxyphenylacetic acid**
- Human: **7-hydroxylation** > **3,4-epoxydation**  
(urinary excretion)
- Rat: **3,4-epoxidation** > **7-hydroxylation**  
(biliary excretion)



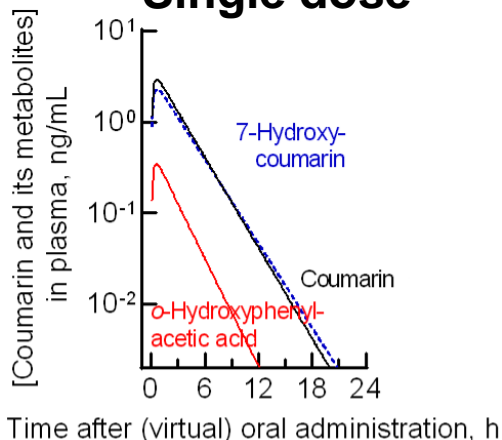
## Toxicity

- In animal testing: **histopathological changes in liver**
- TDI: 0.1 mg/kg/day (5 mg/day, based on NOAEL 10 mg/kg/day in dog and UF 100, EFSA, 2004)
- As for toxicity in **human** as a drug
  - ✓ **Elevated hepatic enzymes**
  - ✓ **25 mg/day** coumarin intake caused liver damage (German BfR, 2006)
  - ✓ NIH LiverTox Database: rate of liver toxicity by coumarin intake= 2 / 1000 person

# Predicted biokinetics of food-derived coumarin in human

Putative plasma level of coumarin upon **25 mg oral intake in human** (Simplified PBK model)

## Single dose

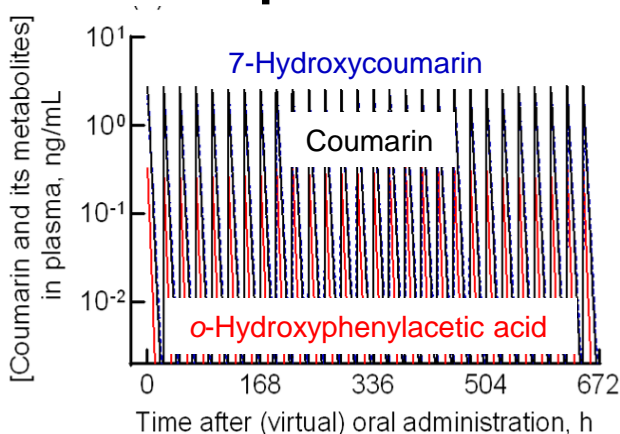


Coumarin is mainly metabolized to 7-HC, little to o-HPA in human.

Putative maximum plasma levels of coumarin and o-HPA in human are 19 nM and 2.0 nM, respectively. These are **obviously lower** than blood levels of those, 200  $\mu$ M and 80  $\mu$ M, respectively, when hepatotoxicity was observed in rat.



## 28-d repeated dose



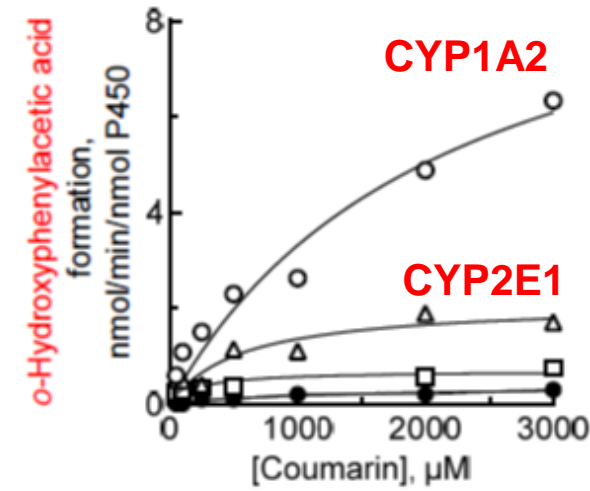
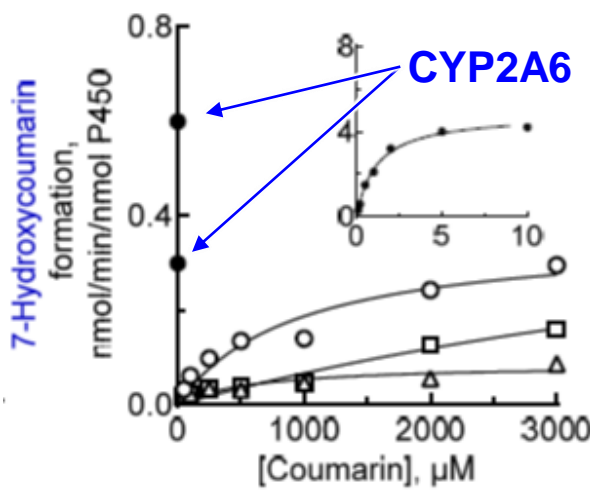
As long as metabolic activity of coumarin is at average level in human, **25 mg coumarin intake** is supposed to pose **low hepatotoxic concern**.

Nonetheless, case reports indicate that hepatotoxic concern upon >25 mg coumarin intake cannot be ruled out in human. This implies the presence of **highly sensitive subpopulation** to coumarin induced hepatotoxicity.

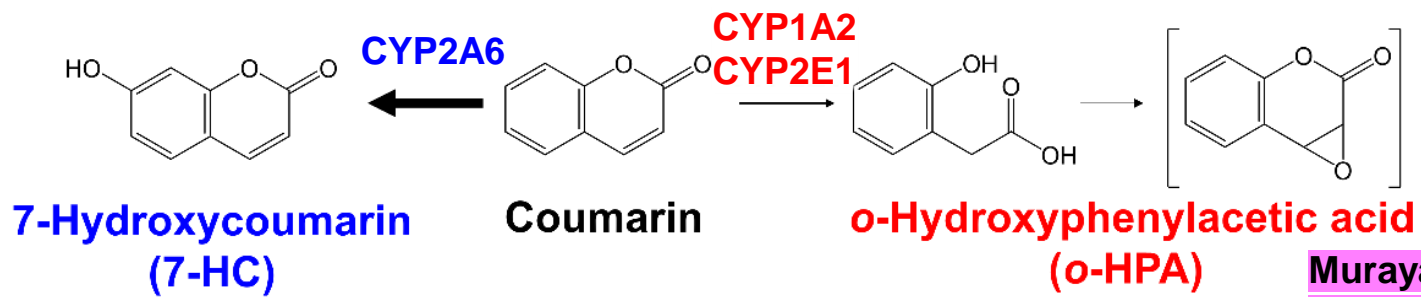


# CYP species involved in coumarin metabolism in human

## Coumarin metabolism study by human liver microsomes with / without the CYP inhibitors



Human liver microsomes	Inhibitor of P450 form	Product formation, pmol/min/mg microsomal protein		Formation ratio of 7-hydroxycoumarin to that of <i>o</i> -hydroxyphenylacetic acid	7-HC/ <i>o</i> -HPA
		7-Hydroxycoumarin	<i>o</i> -Hydroxyphenylacetic acid		
Experiment I (Individual microsomes)					
Individual liver microsomes HH2 <sup>a</sup>		36	3.8	9.5	The ratio varies greatly among individuals
Individual liver microsomes HH31 <sup>b</sup>		430	6.8	63	
Pooled human liver microsomes H150		560 (100)	4.4 (100)	130	
+ 10 μM furafylline	1A2	480 (86)	0.66 (15) *	730	
+ 30 μM α-naphthoflavone	1A2	430 (77)	2.4 (55) *	180	
+ 1.0 μM 8-methoxypsoralen	2A6	98 (18) *	3.2 (73)	31	
+ 30 μM 4-methylpyrazole	2E1	550 (98)	2.6 (59) *	210	
+ 1.0 μM ketoconazole	3A4	450 (80)	3.2 (73)	140	
Experiment II (pooled microsomes)					
Non-inactivated control		470 (100)	2.3 (100)	200	
Selectively inactivated by mechanism-based P450 1A2 inhibitor	1A2	360 (77)	0.80 (35) *	450	
Selectively inactivated by mechanism-based P450 2A6 inhibitor	2A6	68 (15) *	1.7 (74)	40	
Selectively inactivated by mechanism-based P450 3A4 inhibitor	3A4	490 (105)	3.0 (117)	160	



Murayama et al., J. Toxicol. Sci. 2021

Overall, detoxification involved CYP2A6, and metabolic activation involved CYP1A2 and 2E1.

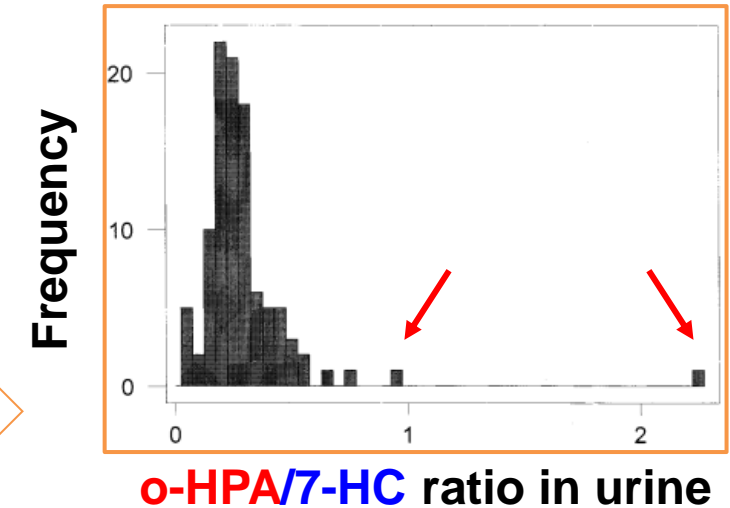


# Individual variability of coumarin metabolism

Further examination on literatures:

Some literatures reported “**7-HC** < **o-HPA**” cases in human.

- *In vitro* metabolites of 1 mM coumarin with human liver microsome (Fentem et al., 1992)
- Urine of a few individuals upon 2 mg coumarin administration ([Hadidi et al., 1998](#))

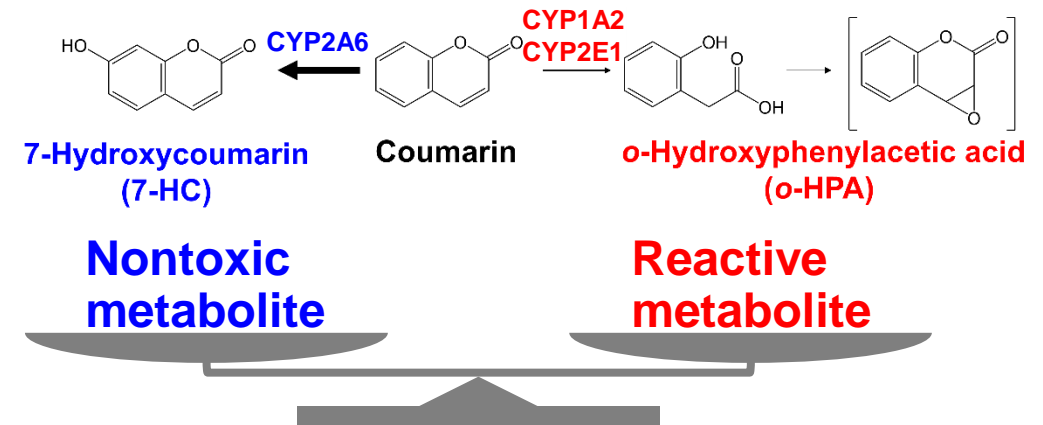


## Individual variability of CYP enzymes:

**CYP2A6**: Gene polymorphism

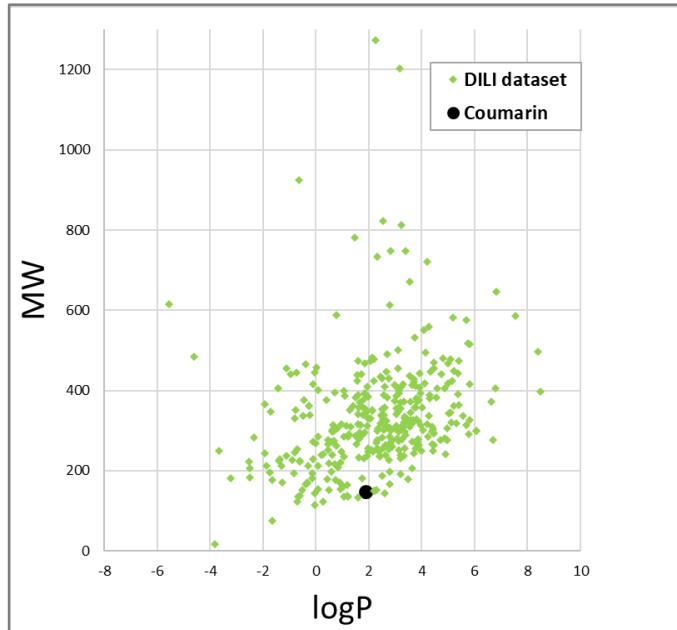
**CYP1A2**: Environmentally inducible (ex. smoking)

**CYP2E1**: Environmentally inducible (ex. drinking)



A series of analyses showed that further research on individual variability is required to refine the assessment to protect highly susceptible subpopulation.

- Since the target of coumarin is the liver, we applied DILI score model.
- DILI score model predicts incidence of drug-induced liver injury (DILI) caused by drug candidates from their information about daily intake, hydrophobicity, reactive metabolite formation (developed by FDA NCTR).
- No food-related substances have been reported to be applied to the model so far, so the applicability and predictivity is unknown. We examined its usability by trial under the cooperation of FDA NCTR.



Chemotype contained in coumarin	No. of duplications in the set molecules
bond:C(=O)O_carboxylicEster_alkenyl	9
bond:C=O_carbonyl_generic	228
chain:alkeneCyclic_ethylene_C_(connect_noZ)	37
chain:alkeneCyclic_ethylene_generic	67
chain:aromaticAlkane_Ph-C1_cyclic	83
chain:aromaticAlkene_Ph-C2_cyclic	11
ring:aromatic_benzene	264
ring:hetero_[6]_O_pyran_generic	15
ring:hetero_[6]_Z_1-	100
ring:hetero_[6]_Z_generic	152
ring:hetero_[6_6]_O_benzopyran	3
ring:hetero_[6_6]_O_benzopyrone_(1_2-)	1
ring:hetero_[6_6]_Z_generic	52

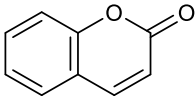
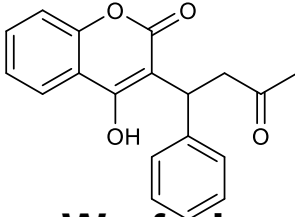
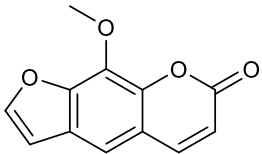
logP and MW of coumarin are within the range of the training set of drugs.

All the chemotypes in coumarin are included in the training set.



Judged that coumarin is applicable to DILI score model

# DILI scores of coumarin and related substances

Chemicals	Daily dose (mg/day)	logP	RM formation	DILI score	DILI risk (estimated)	DILI (observed)	Remarks
 <b>Coumarin</b>	2.5	1.39	Yes	3.71	Moderate	(+)  (+)	≈ ADI
	25			5.11	Moderate		Administered as a drug.
 <b>Warfarin</b>	2	2.44	No	0.98	Low	-  -	Long-term administration as anticoagulant. Hepatotoxicity is rarely reported.
	10			1.95	Low		
 <b>Methoxsalen</b>	3	1.93	Yes	3.92	Moderate	+  +	Occurrence of elevated hepatic enzymes = 2-12%*
	40			5.51	Moderate		

\* LiverTox, <https://www.ncbi.nlm.nih.gov/books/NBK547852/>

Yamada et al., Food Safety. 2022

## Conclusion 2:

- Risk assessment of food-related substances is mostly based on *in vivo* animal data. In silico models based on human data may be one of the useful methods for primary check to consider species differences or extrapolation to human.

# NAM in risk assessment of food-related substances

## -Lessons from our case studies-



- Hepatotoxicity detection considering PCP, ADME, structural features and MoA showed improved sensitivity and equivalent specificity compared to existing QSAR.
- These findings support the refinement of hazard assessment and provide suggestions on **how to select NAMs that are associated with *in vivo* toxicity**.
- Simplified PBK model, *in vitro* metabolism test with human hepatocytes and DILI score model offers useful supporting information for experts' judge in filling the data gap and extrapolating animal test results to human.
- ***In silico* approaches based on human data should be encouraged to be developed** since they will be of help in extrapolating animal test results to human. They may be also applicable in future as a part of NAM directly assessing toxicity in human without interspecies extrapolation.

# Acknowledgement

## **Toxicity database curation and analyses:**

- Taeko Maruyama-Komoda (National Institute of Health Sciences)

## **Data analyses and interpretation:**

- Yasushi Yamazoe (Food Safety Commission of Japan)

## **PBK modeling:**

- Hiroshi Yamazaki (Showa Pharmaceutical University)

## ***In vitro* metabolism study:**

- Norie Murayama (Showa Pharmaceutical University)

## **DILI score model:**

- Weida Tong (US Food and Drug Administration)

## **Discussion:**

- Akihiko Hirose (National Institute of Health Sciences)
- Toru Kawanishi (Food Safety Commission of Japan)

This study was supported by a grant from the Food Safety Commission, Cabinet Office, Government of Japan (Research program for Risk Assessment Study on Food Safety, No. JPCAFSC20202006).