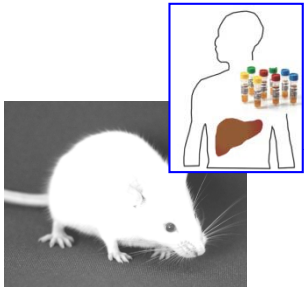
2012 Spring Symposium Sponsored by the
North Jersey ACS Drug Metabolism Discussion Group
April 26, 2012

# Drug Metabolism in Chimeric Mice with Humanized Liver




**Prof. Hiroshi Yamazaki, PhD**

**hyamazak@ac.shoyaku.ac.jp**

**Lab. Drug Metabolism and Pharmacokinetics,**

**Showa Pharmaceutical University**

**Machida, Tokyo 194-8543, JAPAN**

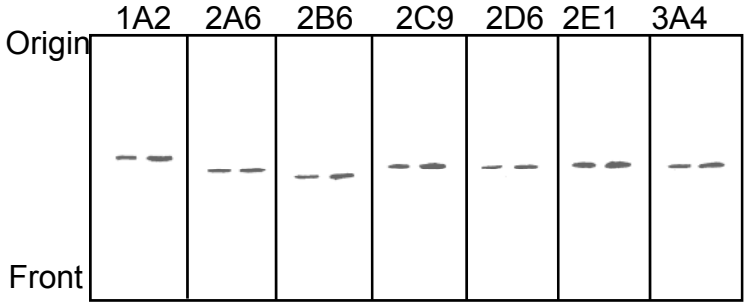
T Shimada, H Yamazaki, M Mimura, Y Inui, and FP Guengerich

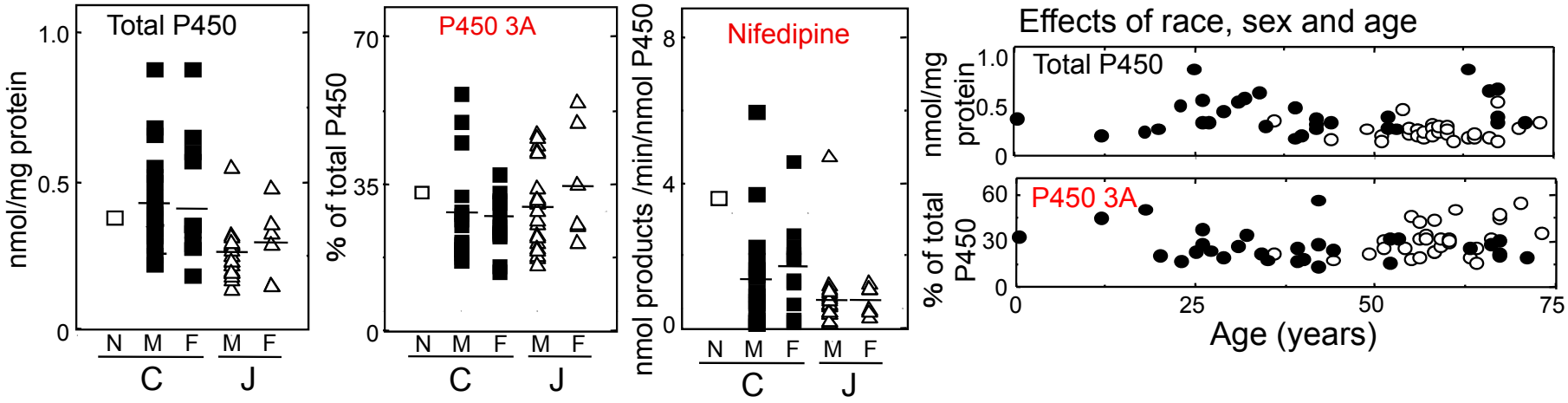
J Pharmacol Exp Ther,1994 270:414-423.

This article has been cited **1811** times in Scopus (04/21/2012).

# Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians.

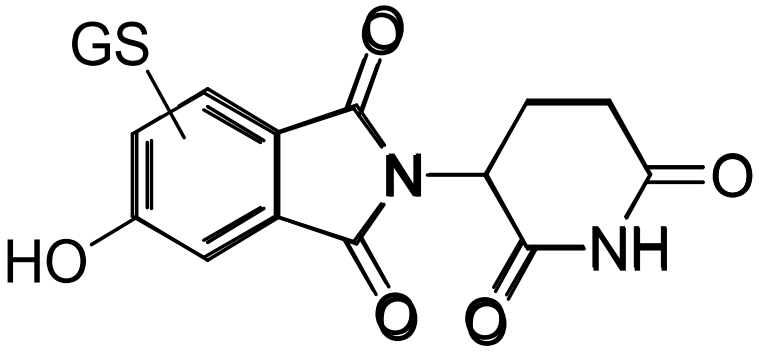
Contents of total P-450 determined spectrally and individual P-450 forms determined immunochemically in liver microsomes from 60 human samples were reported.



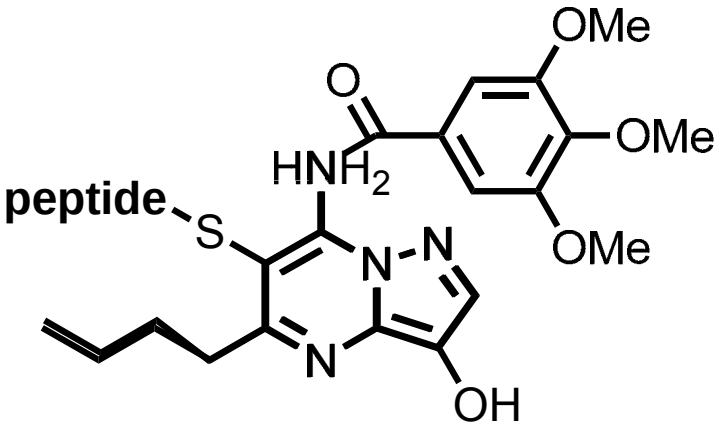
# Outline: Human Metabolites in This Presentation

- Reactive metabolite from thalidomide by **P450 3A4/5**




Glutathione adduct

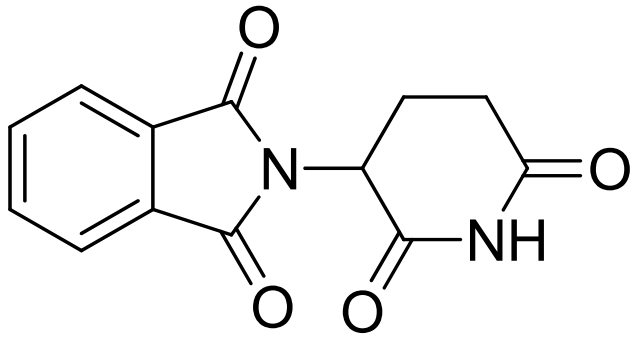
- Troublesome metabolite from analgesic drug candidate (OT-7100) by **P450 1A2**




C&EN, August 31, 2009, Volume 87, Number 35 pp. 27–28

# Thalidomide

- Thalidomide was previously withdrawn because of its teratogenic effects in humans.

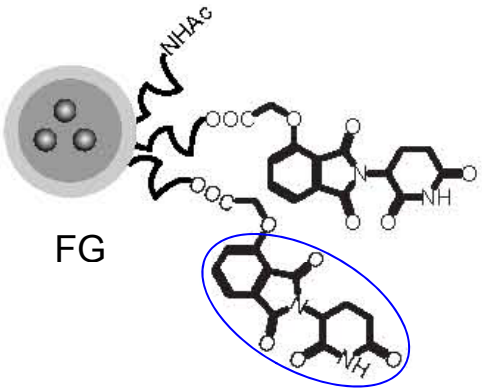



- Thalidomide has been approved for the treatment of refractory multiple myeloma.
- It has been shown that rabbits are thalidomide-sensitive and rodents are thalidomide-resistant.

# Cereblon as Reported Thalidomide-binding Protein

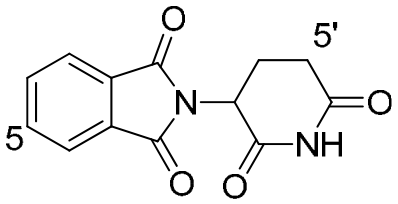
Cereblon has been reported as a thalidomide-binding protein.

Thalidomide would initiate its teratogenic effects by binding to cereblon and inhibiting the associated ubiquitin ligase activity.

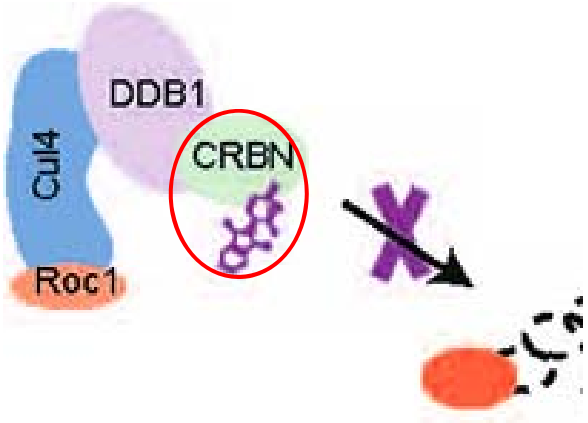



Thal-immobilized beads

FG, Ferriteglycidyl methacrylate beads ("Handa" beads)




Thalidomide



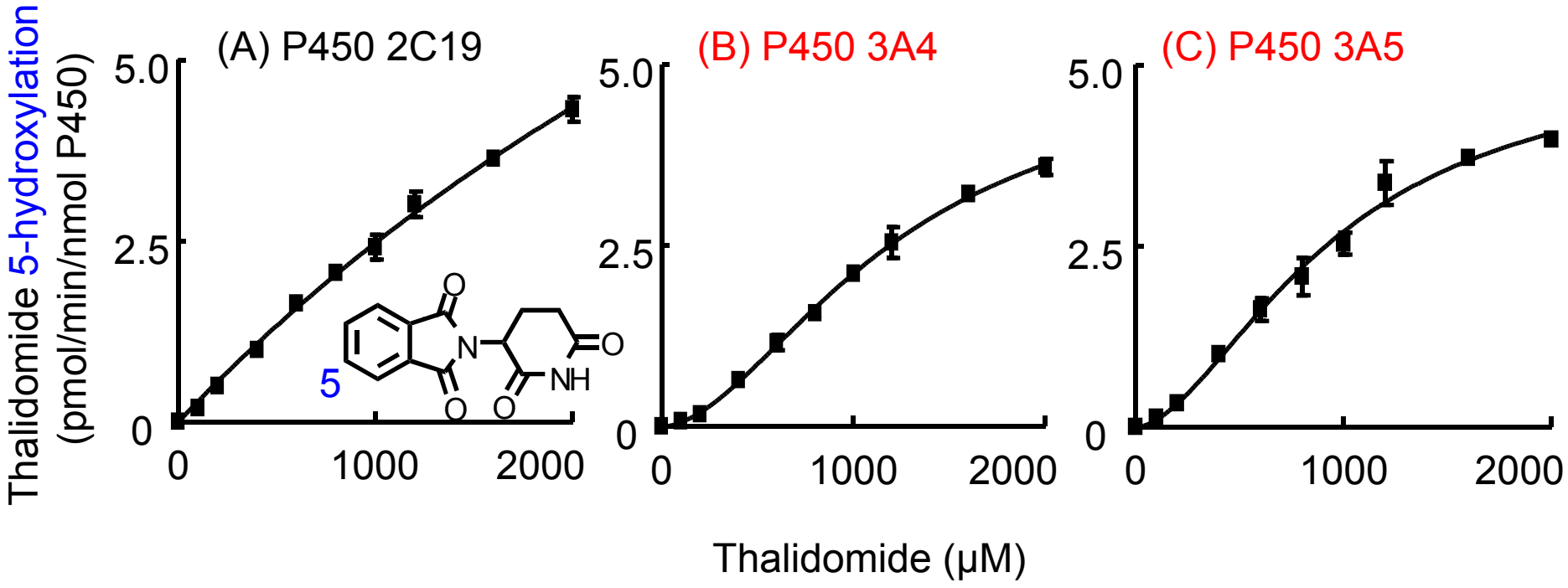

Cereblon (CRBN) forms an E3 ubiquitin ligase complex with damaged DNA binding protein 1 (DDB1) and Cul4A that is important for limb outgrowth.

Ito et al., *Science* 327, 1345-1350 (2010).

# Purpose

- The purpose of this first part was to investigate the oxidative metabolism of thalidomide by human liver *in vitro* and *in vivo* and to better understand the metabolic activation of thalidomide.
- These oxidations of thalidomide were studied *in vitro* liver microsomes and recombinant P450 enzymes, and *in vivo* in chimeric mice with humanized liver.

# 5-Hydroxylation of Thalidomide

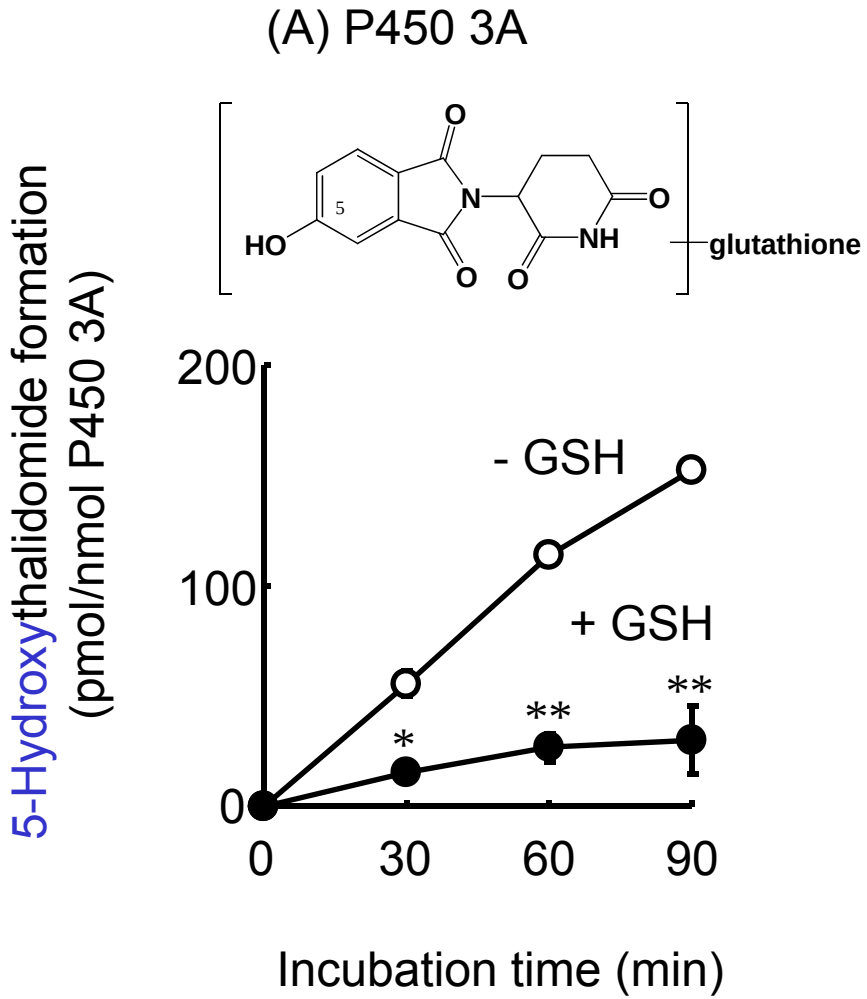


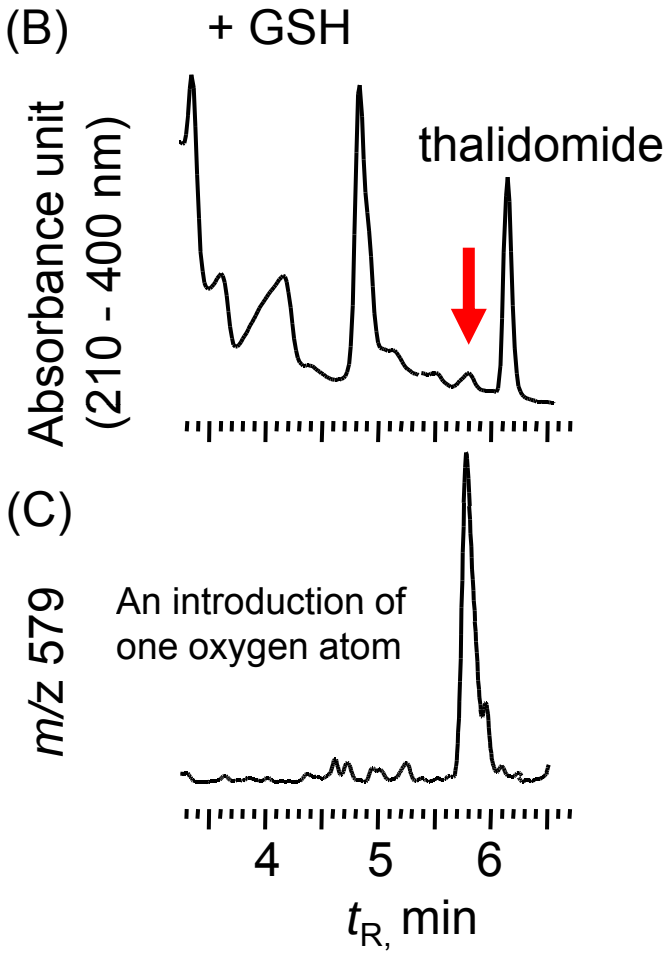

(*R*)-Thalidomide 5-hydroxylation, $min^{-1}$

| | | | |
|---|---|---|---|
| Human | | Monkey | |
| Liver Ms | 0.24 | Liver Ms | 0.63 |
| 3A4 | 2.0 | 3A4 | 12 |
| 3A5 | 2.3 | 3A5 | 9.2 |
| 3A7 | 2.8 | | |

Chowdhury, Yamazaki et al., *Chem Res Toxicol,* 23, 1018-1024 (2010).

# Reactive Metabolite(s) of Thalidomide

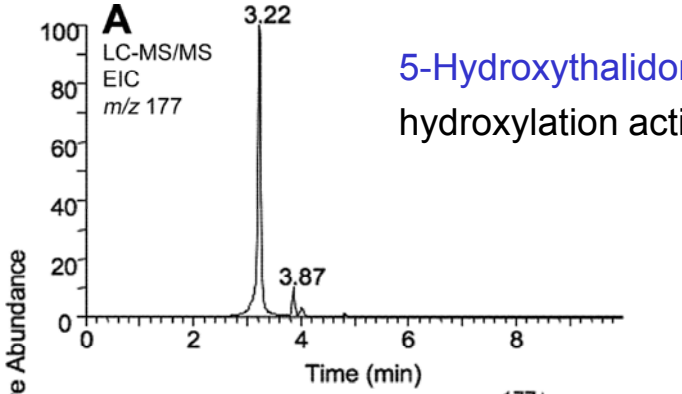


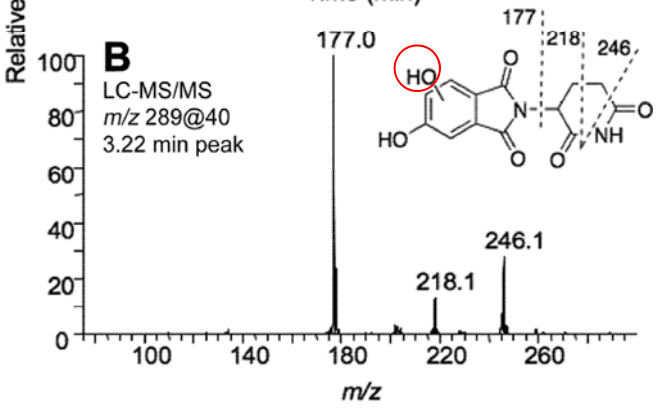

Glutathione (*m/z* 306) and thalidomide (*m/z* 257) were eluted at retention times of 3.7 min 6.3 min, respectively.

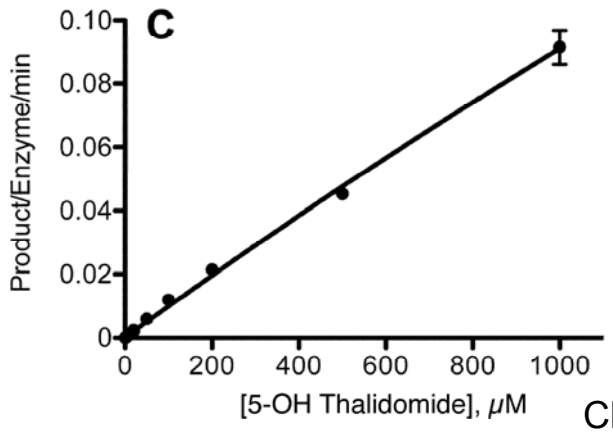
Chowdhury, Yamazaki et al., *Chem Res Toxicol,* 23, 1018-1024 (2010).

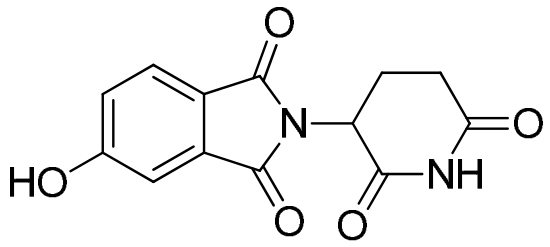
# Secondary Hydroxylation of 5-Hydroxythalidomide




5-Hydroxythalidomide
hydroxylation activity

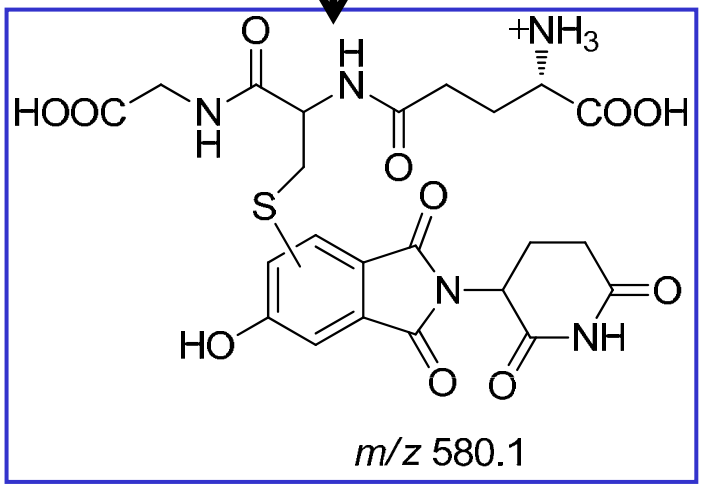


5-Hydroxythalidomide

P450 3A4/GSH

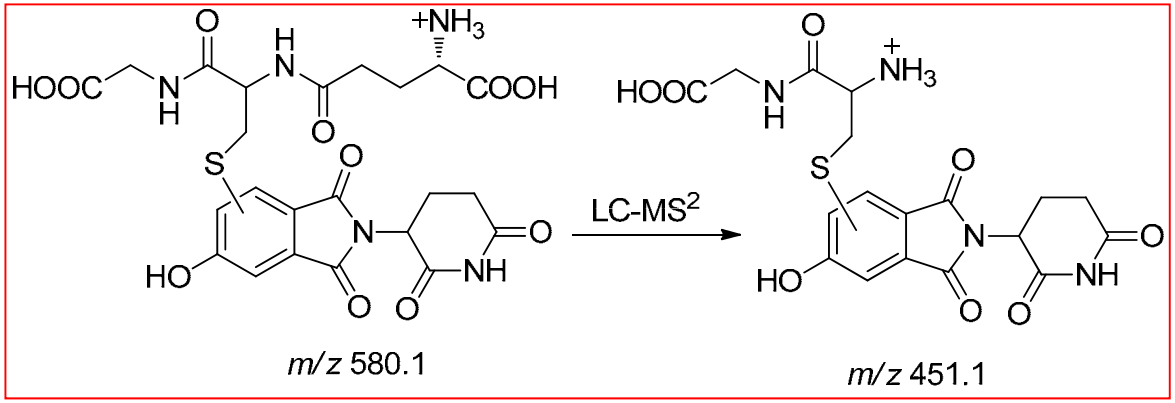


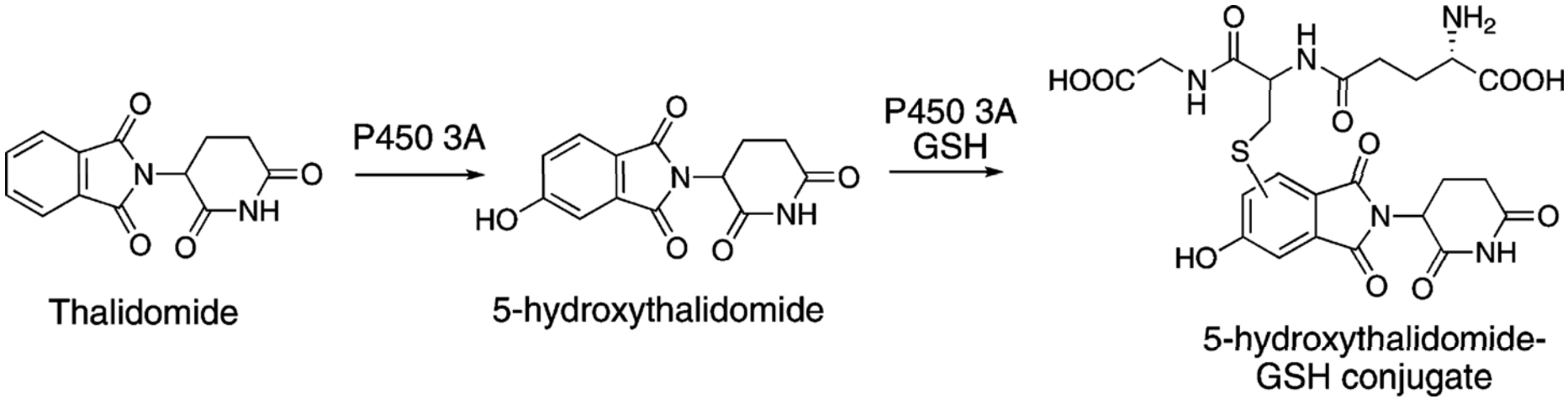

Assigned as a conjugate of 5-hydroxythalidomide formed on the phenyl ring.
5-Hydroxythalidomide-GSH conjugate

Chowdhury, Yamazaki et al., *Chem Res Toxicol,* 23, 1018-1024 (2010).

# Hydroxylation of Thalidomide

LC-MS/MS



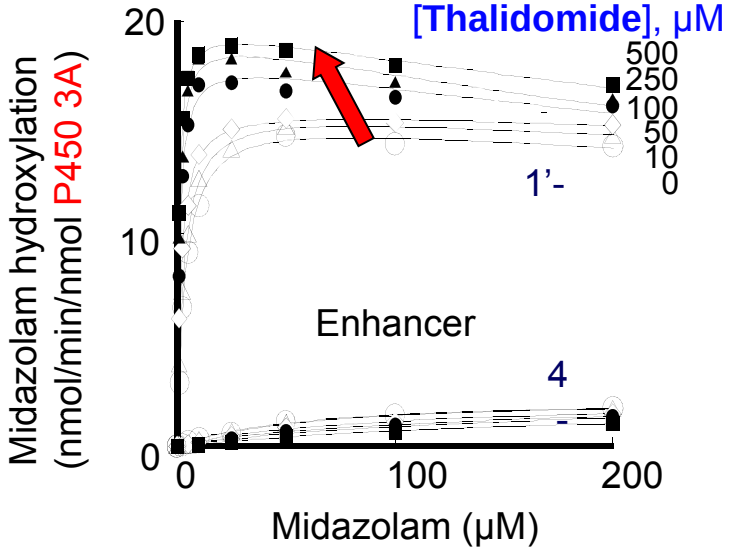



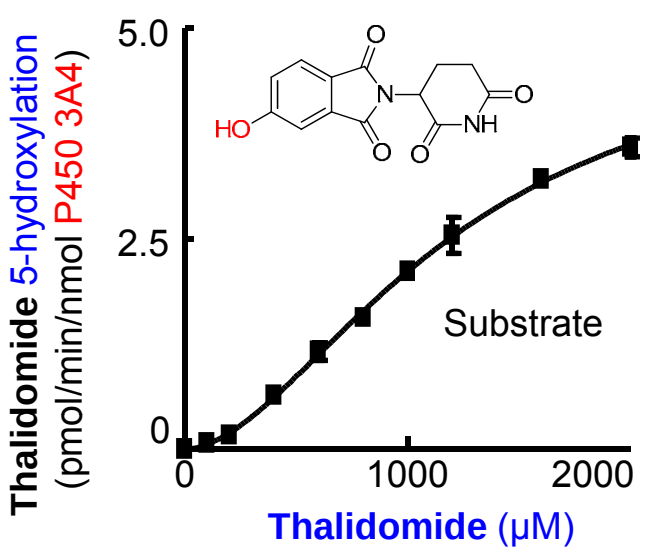

- This study reveals a basis for thalidomide teratogenicity and may contribute to the development of new thalidomide derivatives without teratogenic activity.

Chowdhury, Yamazaki et al., *Chem Res Toxicol,* 23, 1018-1024 (2010).

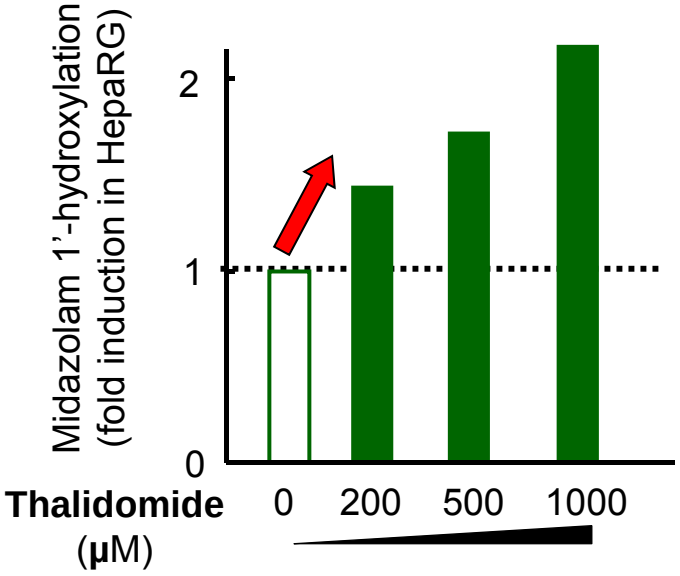
# Drug Interaction Study with **Thalidomide:** Cooperativity of P450 3A and Unique Metabolite(s)

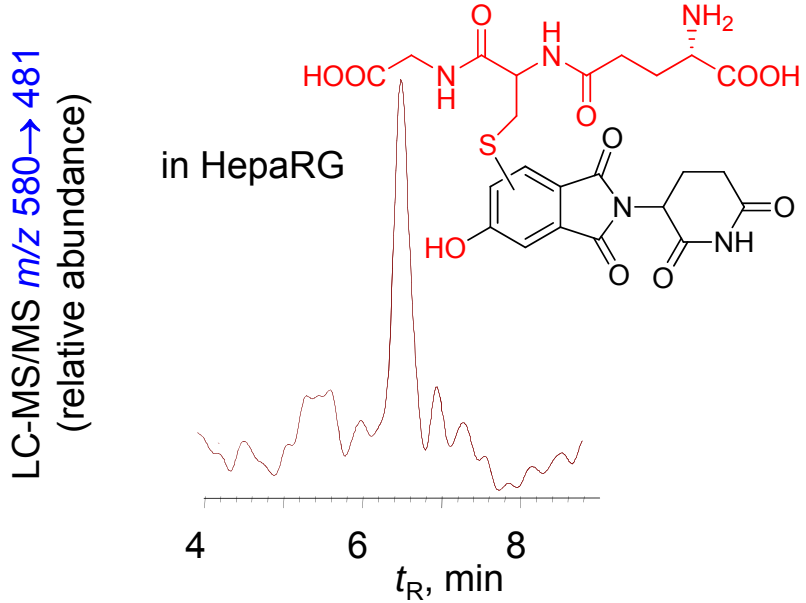
Recombinant P450




Cell system

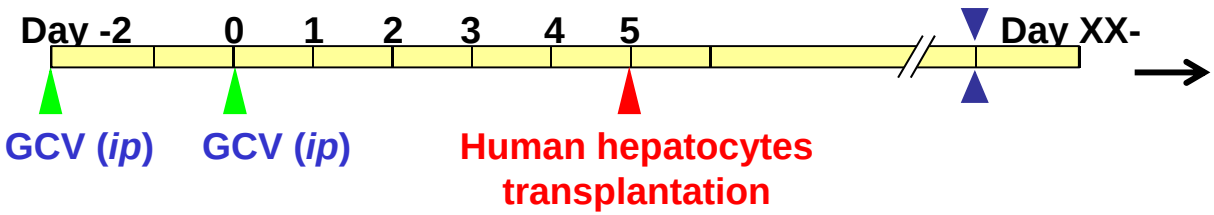


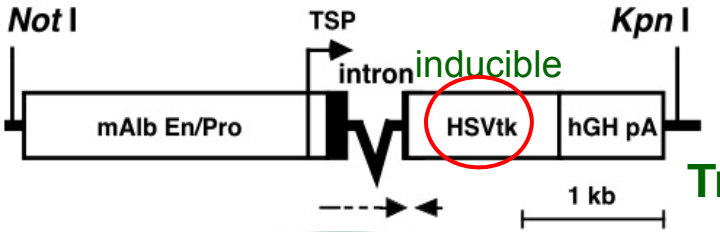




Okada et al., Drug Metab Dispos 2009; Chowdhury et al., Chem Res Toxicol 2010; Murayama et al, 26th JSSX, 1-C-O8-4, 2011

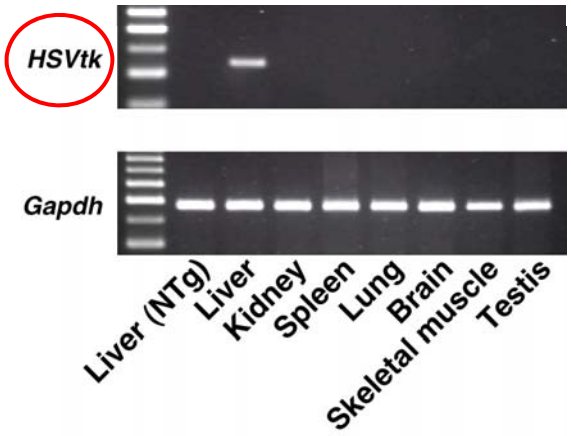
# Humanized TK-NOG mice by Central Institute for Experimental Animals (CIEA) , Kawasaki, Japan

## Protocol for transplantation in TK-NOG mice

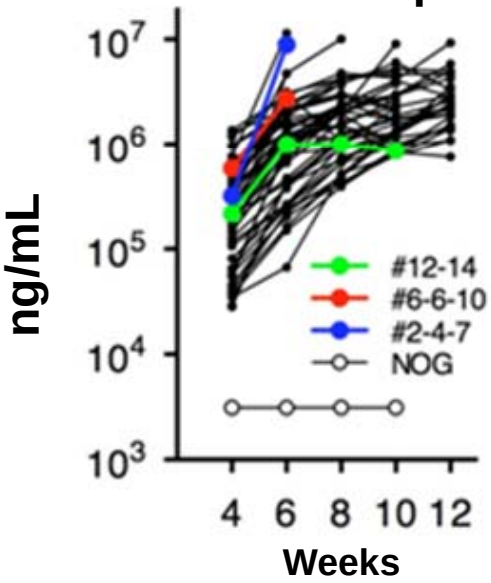


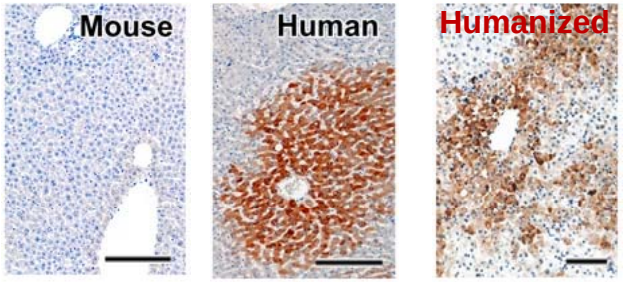
## Transgene expression




## Human albumin expression



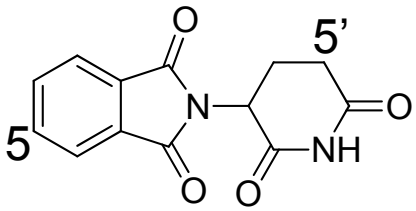

## Immunodetection of human P450 3A by anti-human P450 3A antibodies




TK-NOG mice were treated to express a herpes simplex virus type 1 thymidine kinase (HSVtk) within the livers of severely immunodeficient NOG mice and induced by a non-toxic dose of ganciclovir, and human liver cells were transplanted in the absence of ongoing drug treatment.

From Hiroshi SUEMIZU, Ph.D, CIEA

# Metabolism of Thalidomide and 5-Hydroxythalidomide




| Enzyme source | substrate: (*R*)-thalidomide: 5-OH-thalidomide | (*R*)-thalidomide: 5'-OH-thalidomide | (*R*)-thalidomide: GSH adduct | (*R*)-5-OH-thalidomide: GSH adduct |
|---|---|---|---|---|
| Liver microsomes | pmol formed $min^{-1}$ (mg protein)$^{-1}$ | | | |
| NOG mice | 1.6 | 48 | <0.1 | 2.0 |
| NOG mice with transplantation of human liver cells | 3.3 | 4.9 | <0.1 | 2.1 |
| Pooled human liver (H150) | 0.4 | 1.1 | <0.1 | 3.2 |
| Baculovirus expression system | pmol formed $min^{-1}$ (nmol P450)$^{-1}$ | | | |
| P450 2C19 | 23 | 66 | <0.1 | 12 |
| P450 3A4 | 1.4 | 26 | <0.1 | 11 |
| P450 3A5 | 1.1 | 9.8 | 0.8 | 9.1 |

# Metabolites in Humanized Mice *In Vivo* after Oral Administration of Thalidomide

Showa Pharmaceutical University

(A) Thalidomide
*m/z* 257 → 146

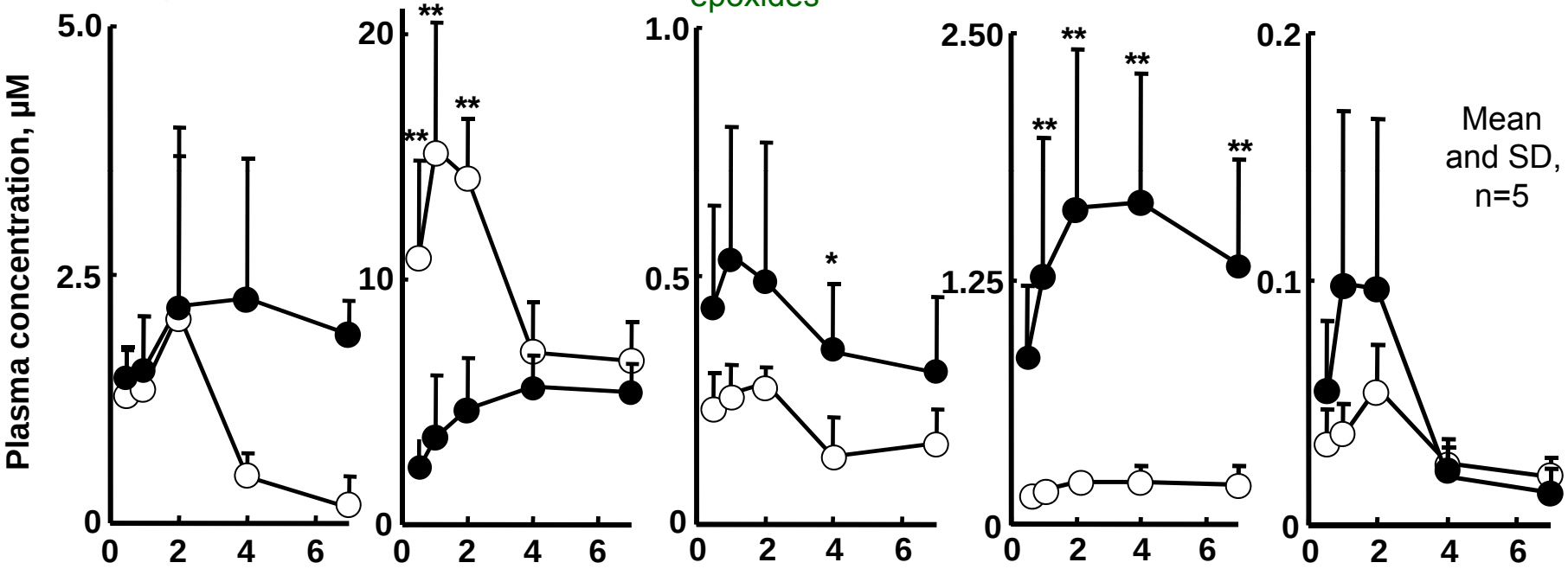
(B) 5'-Hydroxy-thalidomide
*m/z* 273 → 146

(C) 5-Hydroxy-thalidomide
*m/z* 273 → 161
activated to reactive epoxides

(D) Dihydroxy-thalidomide
*m/z* 289 → 177

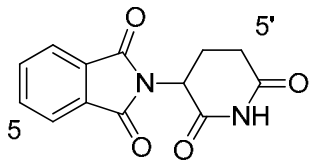
(E) 5-Hydroxy-thalidomide GSH conjugate
*m/z* 580→451

○, control
●, humanized

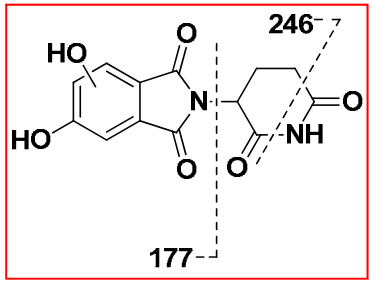



**Time after oral administration of thalidomide, h**

*$p$ <0.05 two-way ANOVA with Bonferroni post tests.



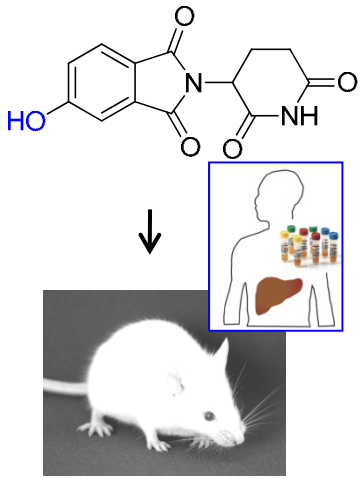

Thalidomide (100 mg/kg)




Yamazaki et al., *Chem Res Toxicol* 2012

# Dihydroxythaidomide and GSH Conjugate in Humanized Mice *In Vivo* after Oral Administration of 5-Hydroxythalidomide

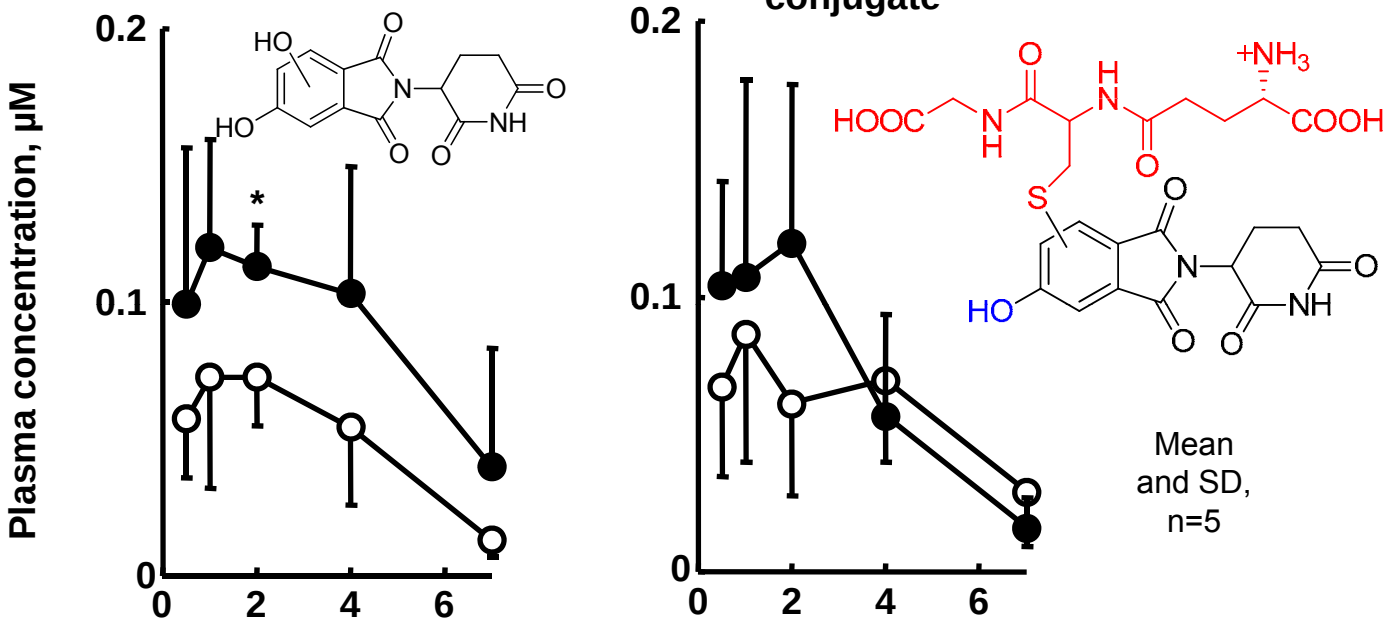
5- Hydroxythalidomide (10 mg/kg)




**Humanized NOG mouse** →

**(A) Dihydroxy-thalidomide**

**(B) 5-Hydroxy-thalidomide GSH conjugate**



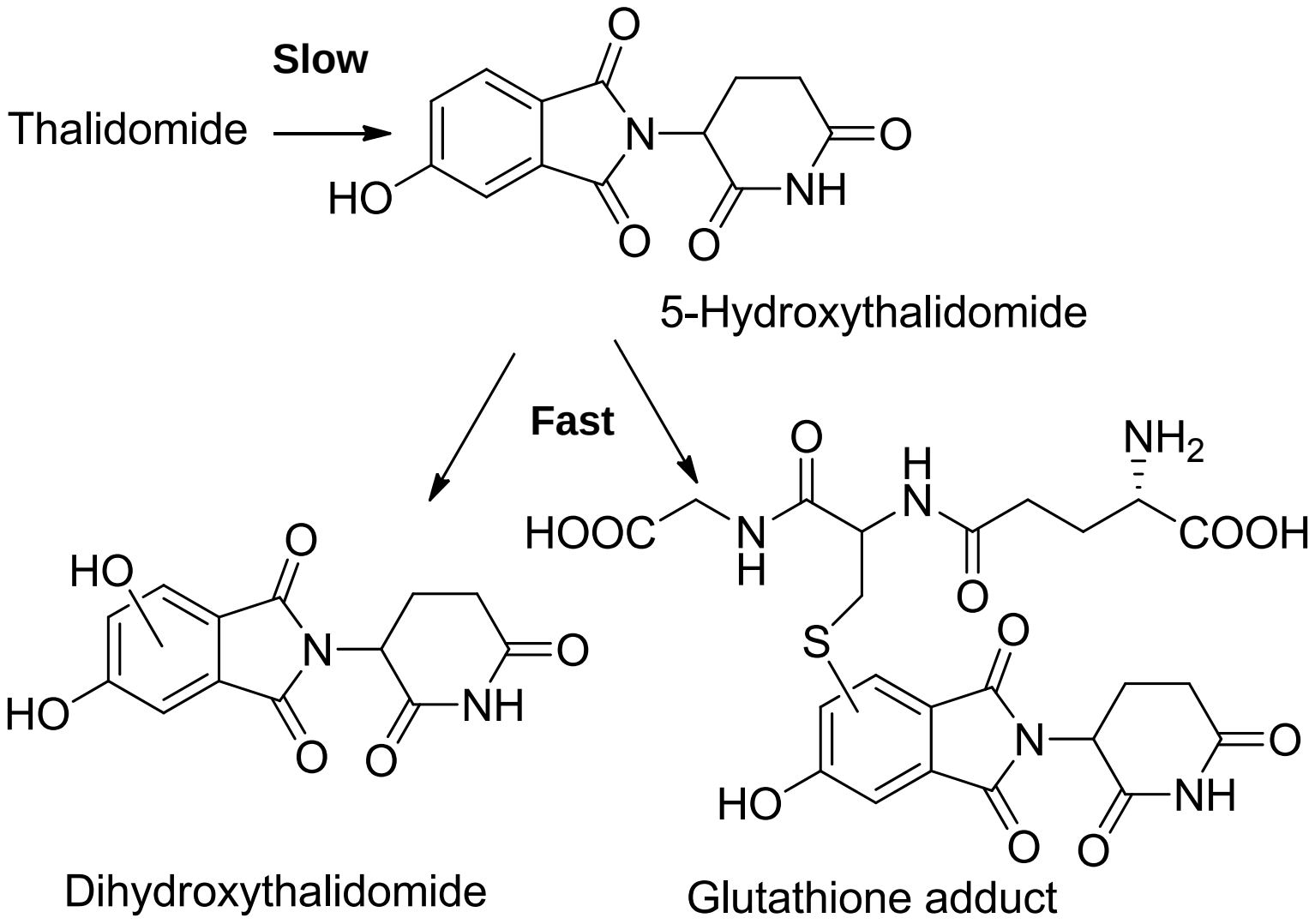

**Time after oral administration of 5-hydroxythalidomide, h**

*$p$ <0.05 two-way ANOVA with Bonferroni post tests

- The 5-hydroxythalidomide-GSH conjugate formed *in vivo* was measured using LC-MS/MS (using the *m/z* 580 → 451 transition), on the basis of a standard curve of 5-hydroxythalidomide.
- 5-Hydroxythalidomide concentrations were lower than the detection level.
- ***In Vivo* formation of a glutathione conjugate derived from thalidomide in humanized mice.**

Yamazaki et al., *Chem Res Toxicol* 2012

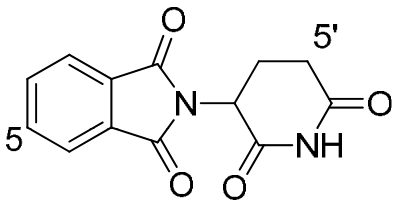
# Hydroxylation of Thalidomide



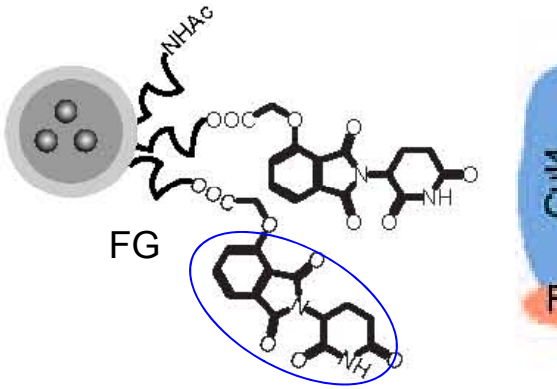

Chowdhury, Yamazaki et al., *Chem Res Toxicol,* 23, 1018-1024 (2010).
Yamazaki et al., *Chem Res Toxicol,* 25, 274-279 (2012).

# Thalidomide: Original Issue Is Birth Defects

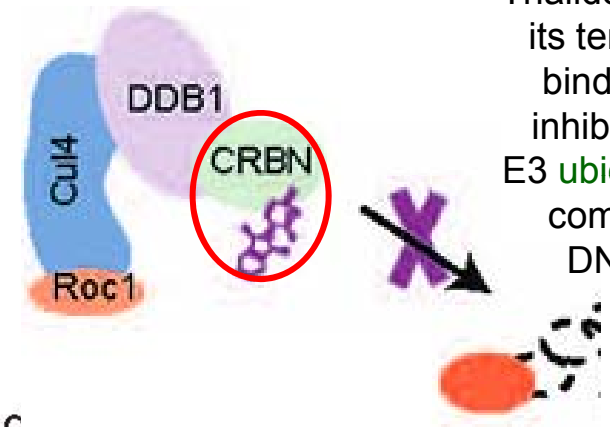
- Cereblon (CRBN) has been reported as a thalidomide-binding protein important for limb outgrowth (Ito et al., Science, 2010) using thalidomide-ferriteglycidyl methacrylate beads (FG, “Handa” beads).




Thalidomide




Thal-immobilized beads




Thalidomide would initiate its teratogenic effects by binding to cereblon and inhibiting the associated E3 ubiquitin ligase activity complex with damaged DNA binding protein 1 (DDB1) and Cul4A

- Thalidomide chemically connected to “Handa” beads is unable to be oxidized at its aromatic ring. Reactive metabolite(s) from aromatic 5-hydroxythalidomide was trapped in our study..
- In this situation, what is the target protein(s) by the activated metabolite? Are these bindings specific or non-specific? They are not known.
- The issue of human-specific metabolites is complex. Much remains to be learned.

# Unique Human Metabolites

- Metabolite
- Conjugate
- Pharmacologically active metabolite
- Major metabolite
- Reactive metabolite
- Unique human metabolite
- Disproportionate drug metabolite

In February, 2008, the US FDA issued the Guidance for Industry: Safety Testing of Drug Metabolites. Any human drug metabolite "...formed at greater than 10 percent of parent drug systemic exposure at steady state" is subject to separate safety testing, that is, by synthesis and administration to test animals.

# Unique Human Metabolite of Dextromethorphan by CYP2D6.49

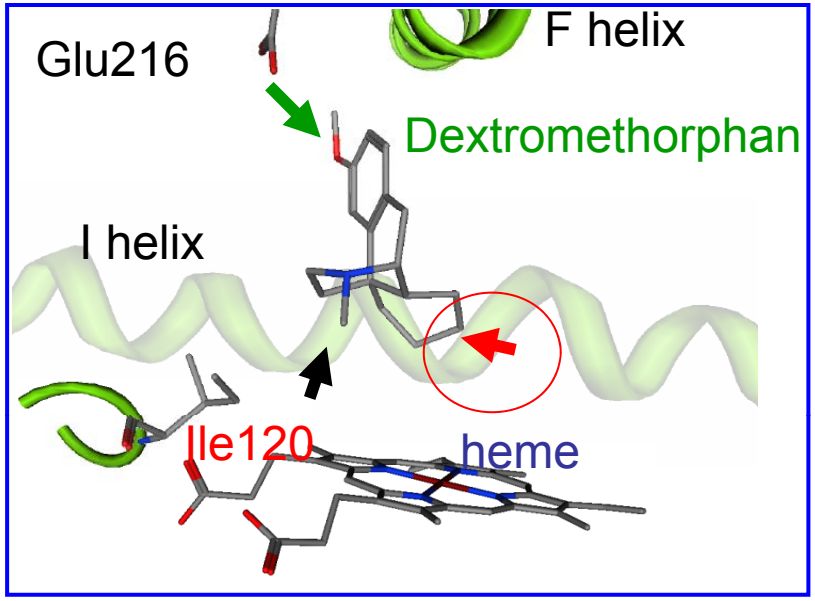
Showa Pharmaceutical University

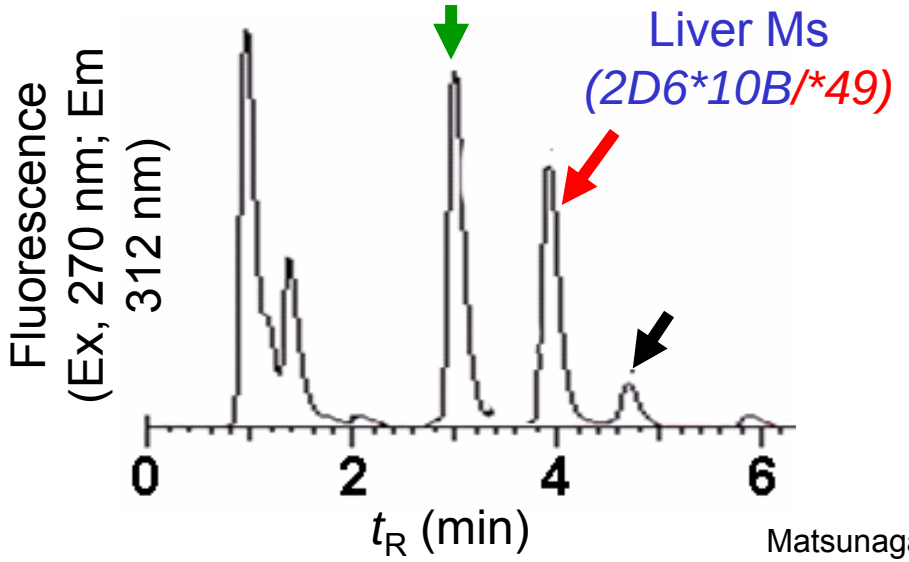
Dextromethorphan *O*-demethylation

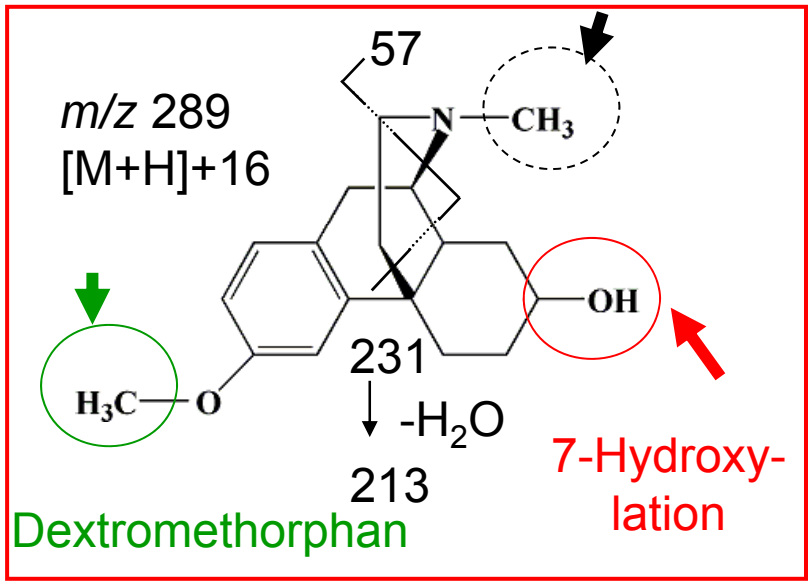
| 2D6 in *E. coli* membranes | $V_{max}/K_m$ $min^{-1}/ \mu M$ |
|---|---|
| 2D6.1 | 0.63 |
| 2D6.10 | 0.019 |
| 2D6.49 ¶ | 0.003 |

¶ P34S, F120I, S486T.

(*2D6*49* allele freq; 3/698 = 0.4%)



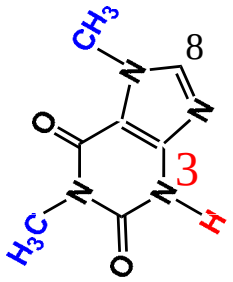



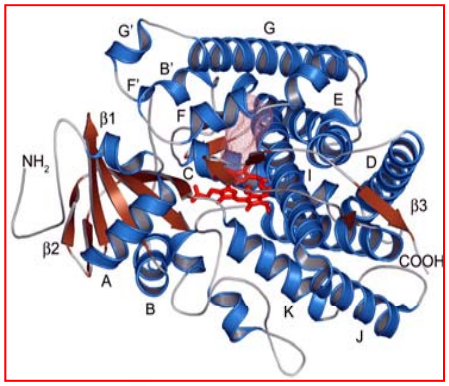

Matsunaga, Yamazaki et al. Drug Metab.Dispos. 37, 699-701 (2009)

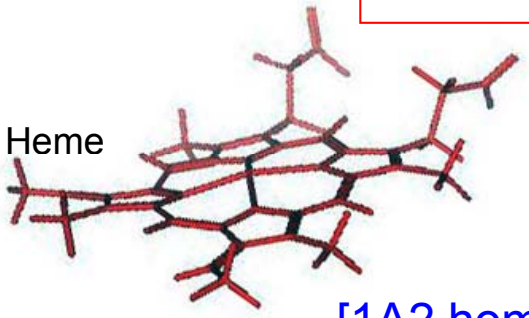
# Docking of Caffeine into Rat and Human P450 1A2 to Produce Different Main Metabolites

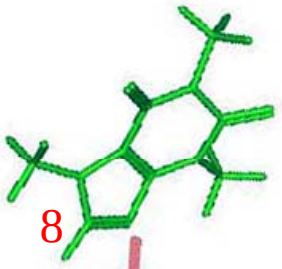
Main human metabolites from caffeine is 3-demethylated paraxanthine (Butler et al., 1989).

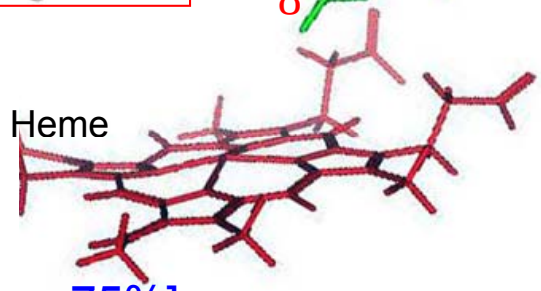


Sansen et al. *JBC* 2007; 282:14348



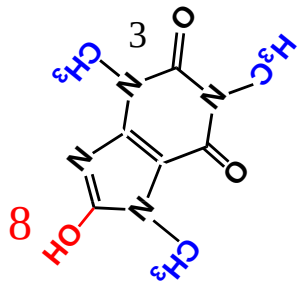




[1A2 homology, 75%]

Human P450 1A2 (PDB 2HI4) (*U* = -23.9)

Rat P450 1A2

Rat liver microsomes produce mainly 8-hydroxylated trimethyluric acid (Agúndez et al., 1992).

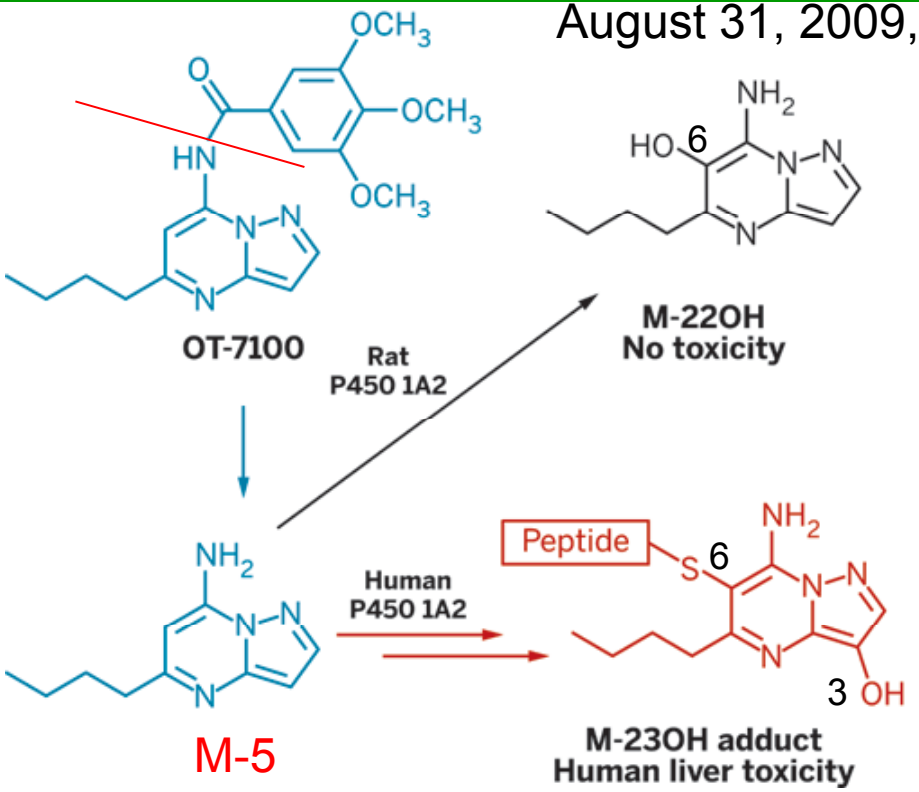



- Rat 1A2 were aligned with a crystal structure of human 1A2 using MOE software (Computing Group, Montreal) for modeling of the3-D structure. Docking simulation was carried out A typical species difference is seen in caffeine metabolism by the same enzyme forms with possibly different substrate pockets.

# C&EN "Troublesome Drug Metabolites: Diverging Paths"

## Toxicologists pool their knowledge to advance safety testing of drug candidates

August 31, 2009, Volume 87, Number 35 pp. 27 – 28.




- Treatment of rats or dogs with 1000 mg/kg of OT-7100, an experimental analgesic drug, or the primary metabolite M-5 produced no apparent hepatotoxicity.
- Limited and reversible elevations in serum levels of aspartate and alanine aminotransferase were were occasionally observed. Most of these cases (7 of 11) occurred with >1400 mg/day of OT-7100 for >14 days after 28-day treatments in a dose-escalation study.

- M-5 is metabolized by human P450 1A2 enzyme to form M-23OH, which conjugates with a peptide to form an adduct. In rats, the same enzyme forms some M-23OH but leads predominantly to M-22OH, which shows no toxicity.
- **These metabolic differences highlight some of the perils of testing drug candidates for metabolite toxicity.** (http://pubs.acs.org/cen/email/html/8735sci1.html)

Kuribayashi, Yamazaki et al., *Chem Res Toxicol,* 22, 323-331 (2009); Yamazaki et al., *Chem Res Toxicol,* 23, 152-158 (2010)

# Troublesome Drug Metabolites

- Bioactivated small organic molecules in *vivo* can adduct to biological macromolecules (~1 nmol drug equiv/mg protein) and subsequently elicit organ toxicity.
- To reduce this figure by 20-fold gave a conservative target threshold value for *in vivo* covalent binding of 50 pmol drug equiv/mg protein .

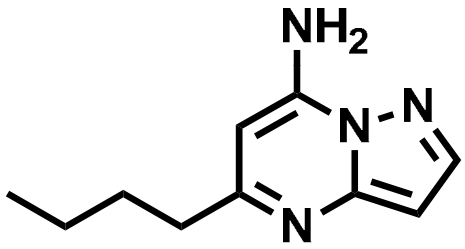
To be solved:

- What is activated metabolite(s)?
- What and how abundant is target protein(s)?
- Are they specific or non-specific?

Approach for Covalent Protein Binding *In Vivo*

# One Approach for Covalent Protein Binding:

- Chimeric mice with humanized liver by two-dimensional electrophoresis associated with accelerator mass spectrometry



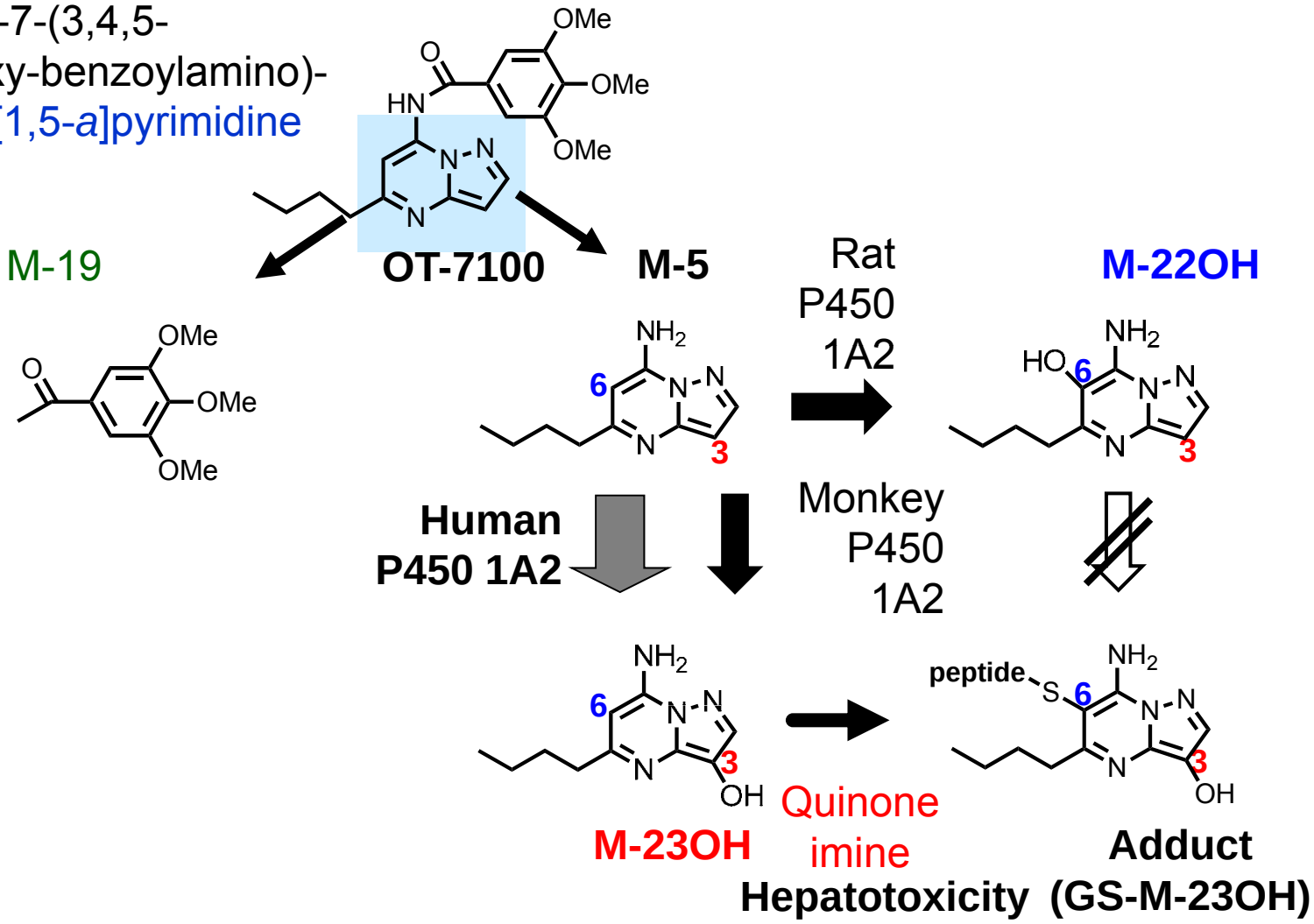

*Chem. Res. Toxicol.* **2010**, *23*, 152–158

**Approach for *in Vivo* Protein Binding of 5-*n*-Butyl-pyrazolo[1,5-*a*]pyrimidine Bioactivated in Chimeric Mice with Humanized Liver by Two-Dimensional Electrophoresis with Accelerator Mass Spectrometry**

Yamazaki et al., *Chem Res Toxicol,* 23, 152-158 (2010);

# Analgesic Drug OT-7100 Is Metabolized by Human P450 1A2

5-*n*-butyl-7-(3,4,5-trimethoxy-benzoylamino)-pyrazolo[1,5-*a*]pyrimidine



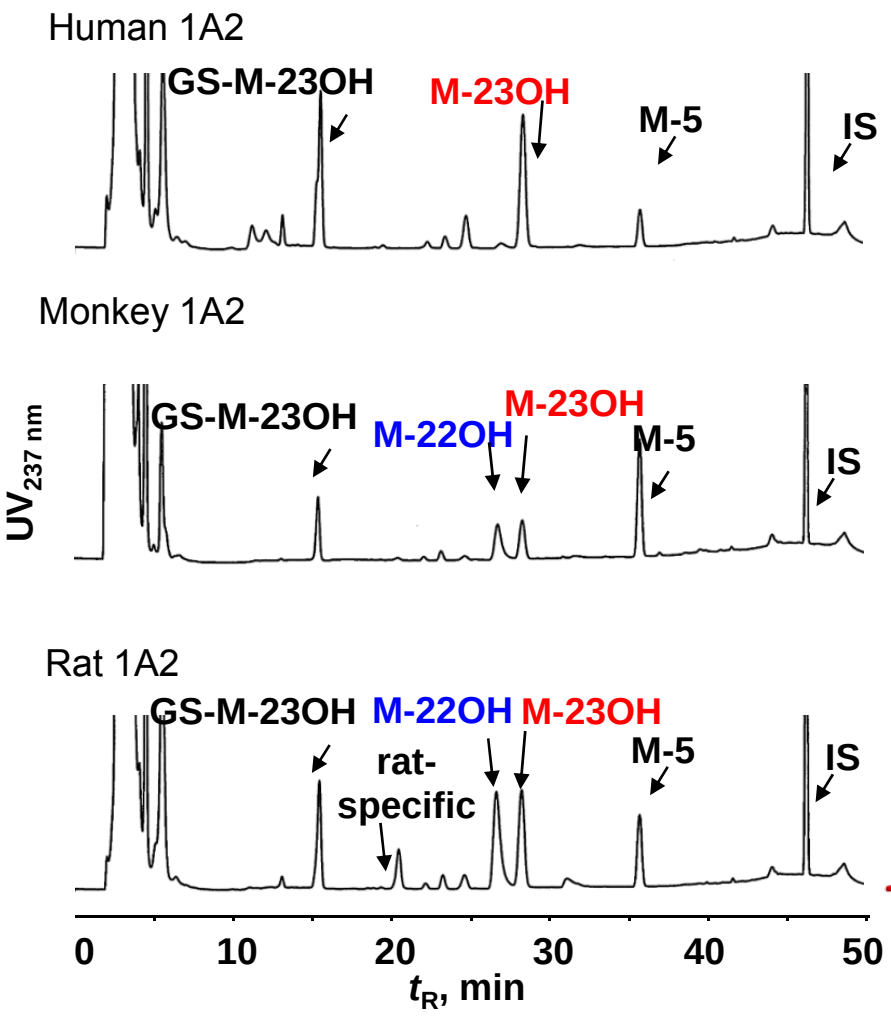


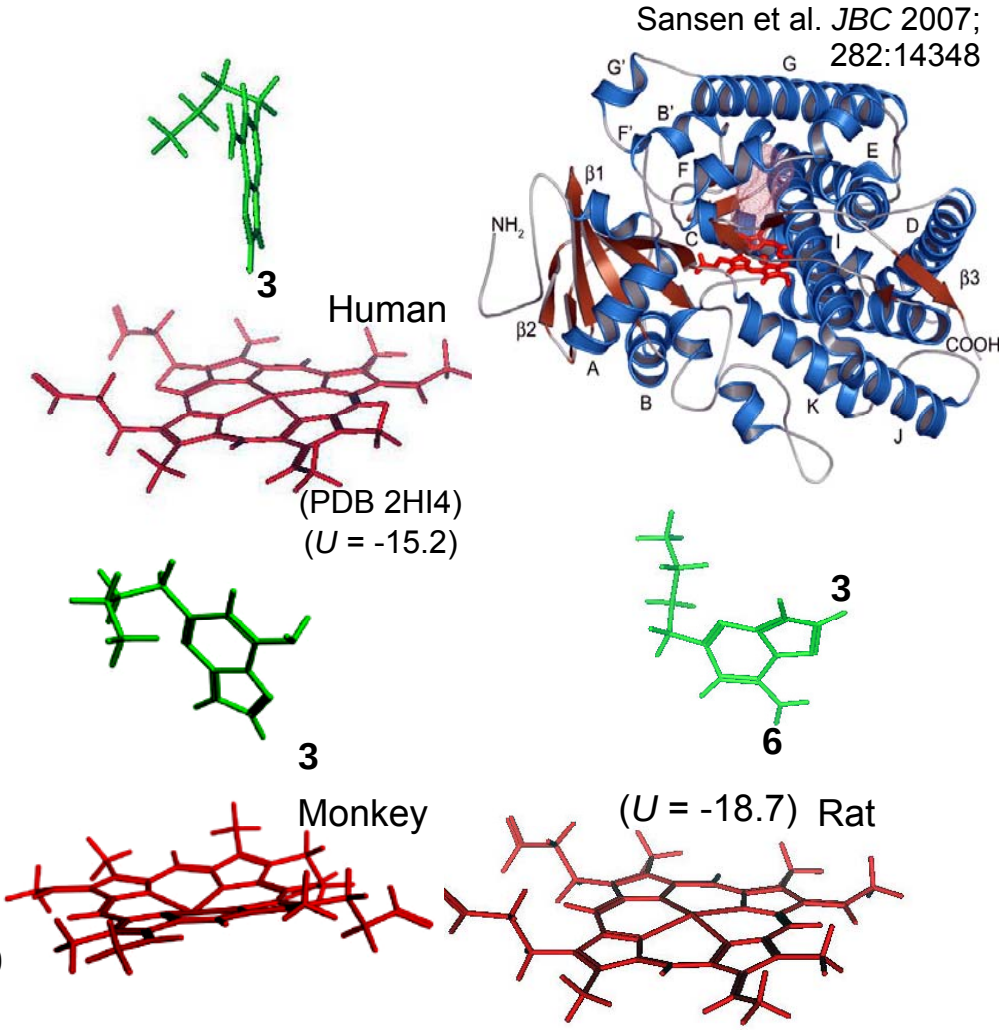
Kuribayashi, Yamazaki et al., *Chem Res Toxicol,* 22, 323-331 (2009);

Yamazaki et al., *Chem Res Toxicol,* 23, 152-158 (2010);

Kuribayashi, Yamazaki et al, *Basic Clin Pharmacol Toxicol*, 110, 405-408 (2012)

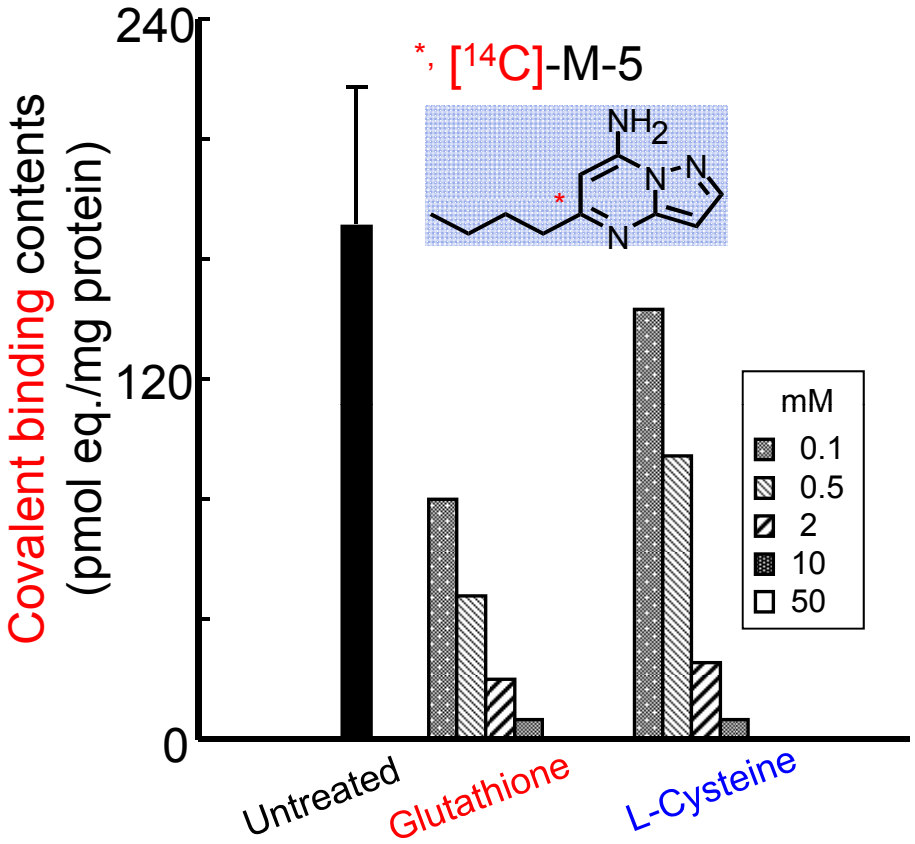
# Interaction of 5-*n*-butyl-pyrazolo[1,5-*a*]pyrimidine (M-5) with P450s 1A2

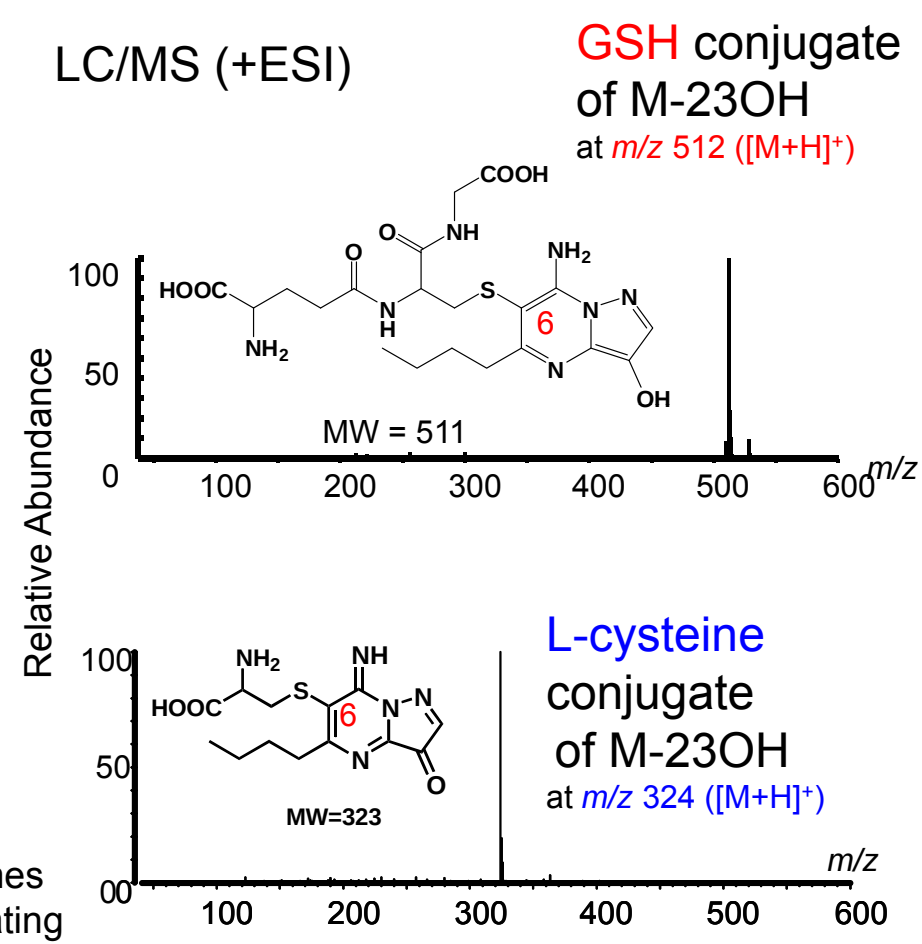



- Human P450 1A2 enzyme could form M-23OH, which conjugates with a peptide to form an adduct that has liver toxicity.

# Covalent Protein Binding of Activated [$^{14}$C]-M-5 *In Vitro*



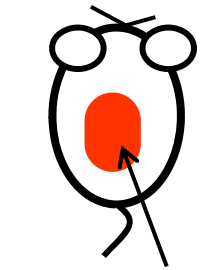




[$^{14}$C]-M-5 (5 μM) was incubated with human liver microsomes (0.5 mg protein/mL) in the presence of an NADPH-generating system and SH reagents at 37 ° for 60 min.
Data (and bars) are mean ± SD of sextuplicate determinations and average of duplicate incubations.

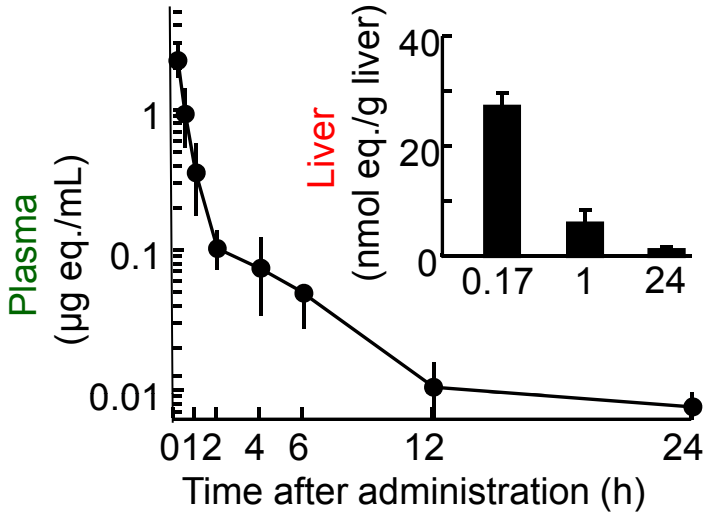
● M-23OH-GS and M-23OH-cysteine conjugates were detected *in vitro*

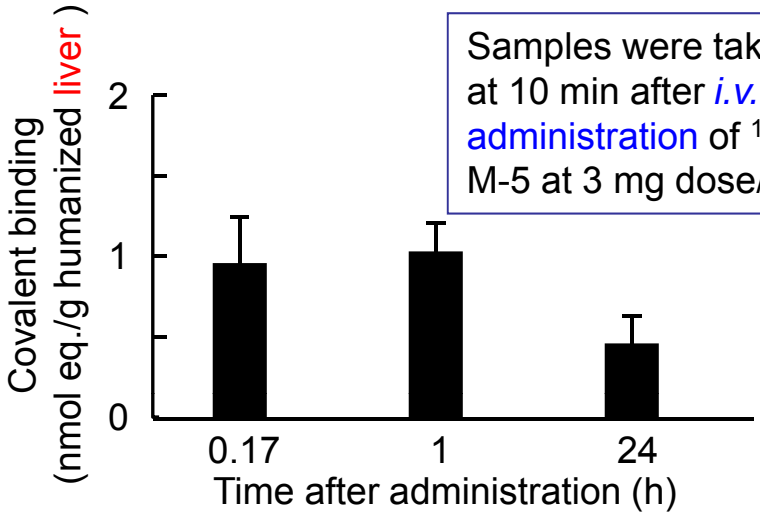
Kuribayashi, Yamazaki et al. Chem Res Toxicol (2009)

# Concentrations and LC Chromatograms after Intravenous Administration of [$^{14}$C]-M-5

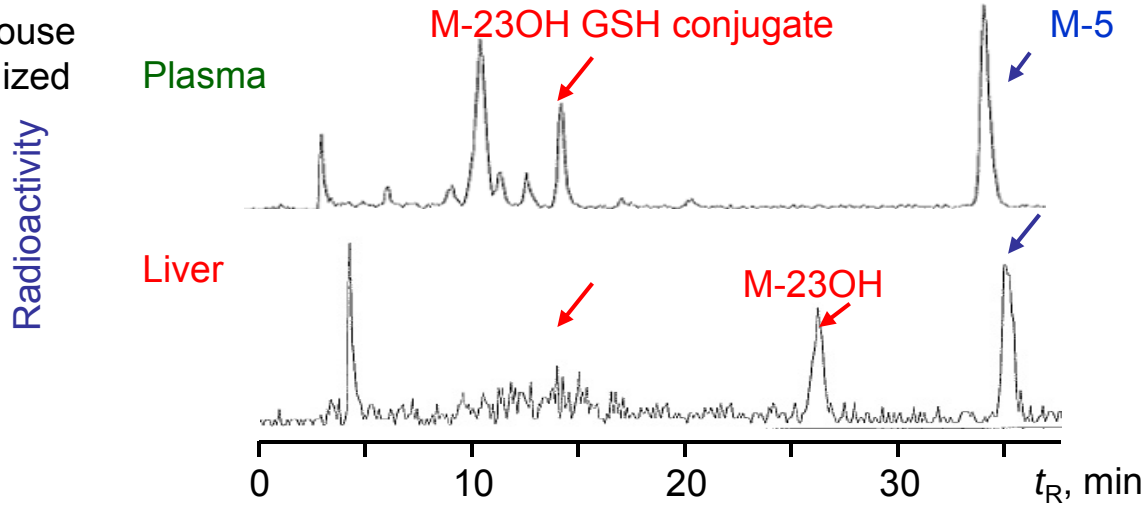


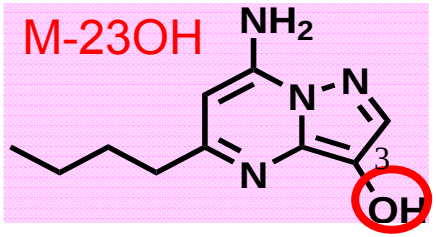
Chimeric mouse with humanized liver




Samples were taken at 10 min after *i.v.* administration of $^{14}$C-M-5 at 3 mg dose/kg.

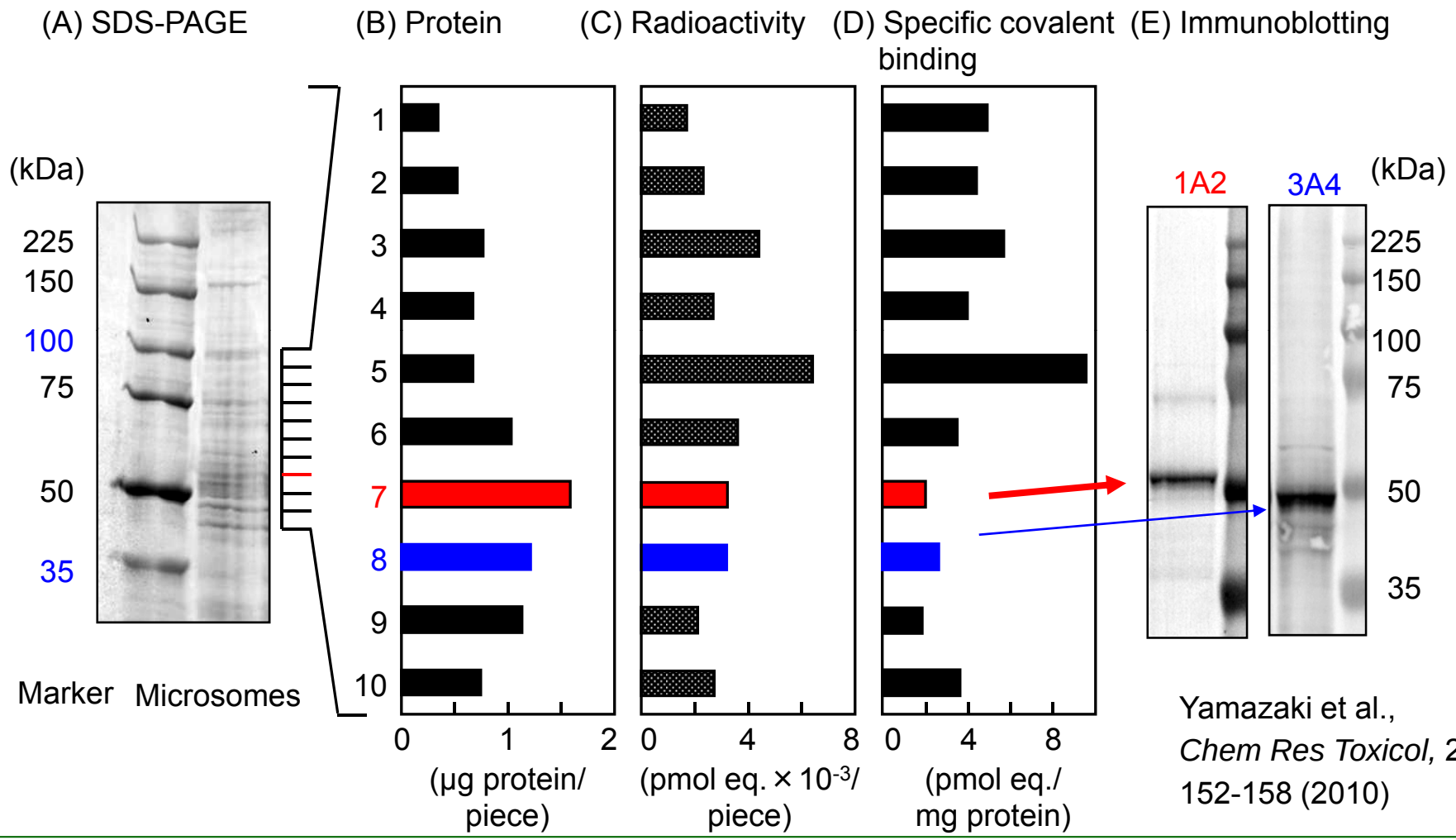



- M-23OH GSH conjugate at C-6-position was confirmed *in vivo.*

Yamazaki et al., *Chem Res Toxicol,* 23, 152-158 (2010)

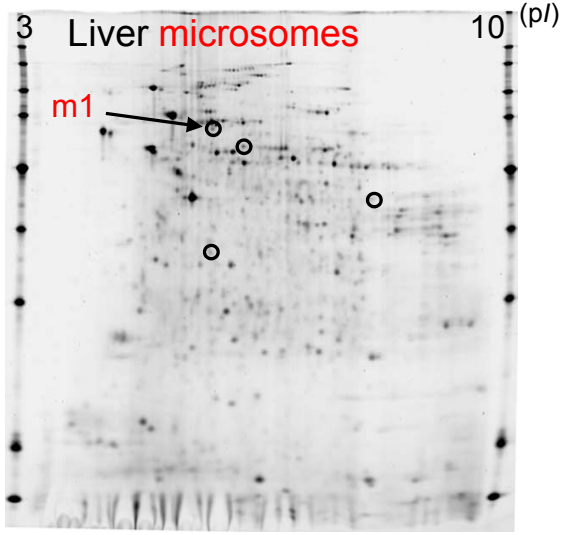
# Microsomal Covalent Binding of M-5 Bioactivated *In Vivo* by Electrophoresis

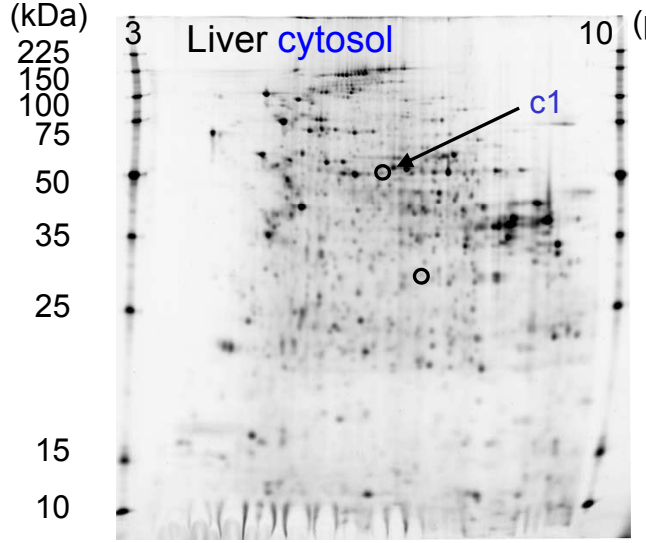



Yamazaki et al., *Chem Res Toxicol,* 23, 152-158 (2010)

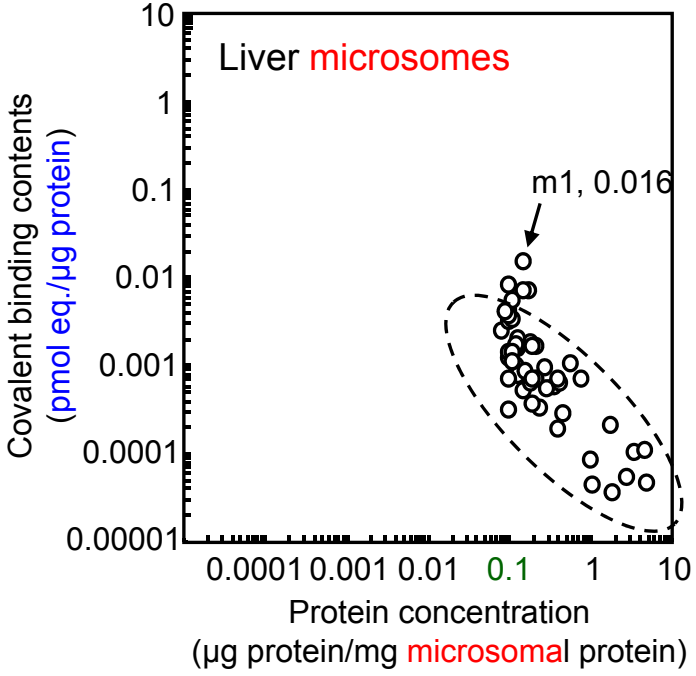
- M-5 was activated by liver microsomal P450 1A2 to reactive intermediate(s) *in vivo* in humanized mice and could relatively non-specifically bind to biomolecules such as human P450s 1A2 and 3A4 and other proteins.

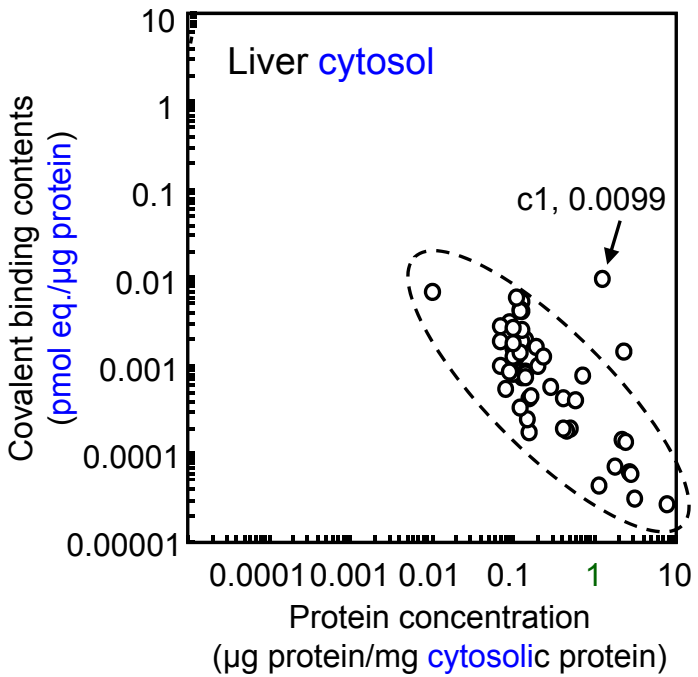
# Covalent Binding Profiles of M-5 Bioactivated in Chimeric Mice with Humanized Liver by Two-Dimensional Electrophoresis with Accelerator Mass Spectrometry




Protein samples (100 µg) were separated by isoelectric points (pH 3-10) and molecular weight (225-10 kDa). P450 forms were not evaluated because of technical reasons: the membrane bound proteins were generally difficult to resolve by the first pI-dependent separation step.

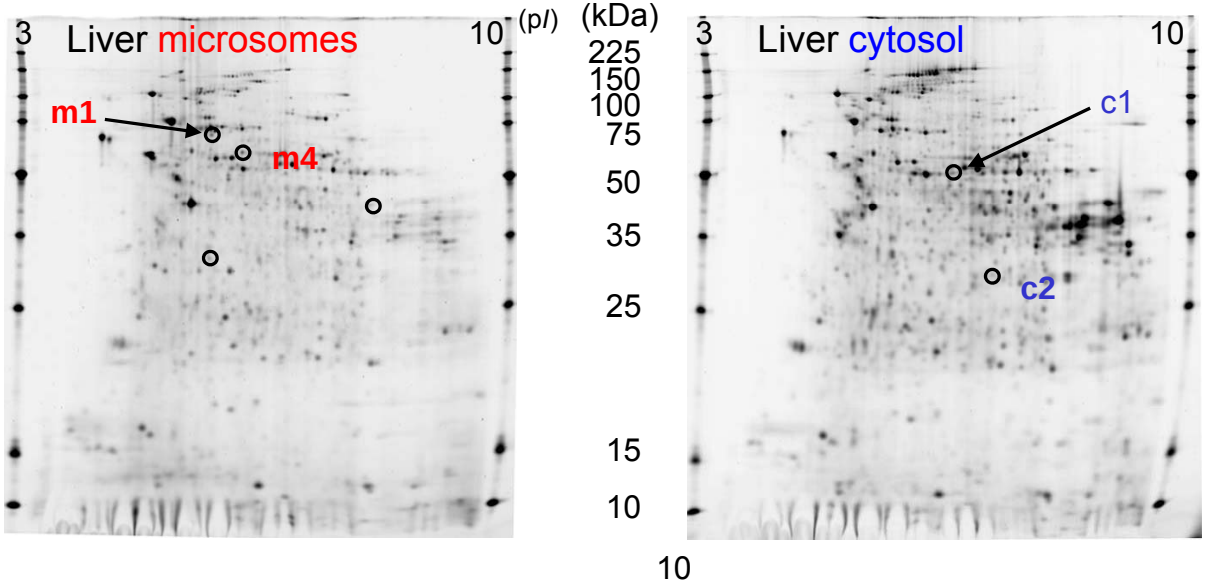



Two of the identified adducted targets were **prolyl-4-hydroxylase** (a key enzyme in the biosynthesis of collagens, m1 ) and an antioxidant enzyme **catalase** (c1) in mice with humanized livers

Yamazaki et al., *Chem Res Toxicol,* 23, 152-158 (2010)

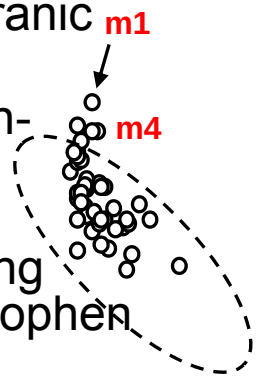
# Covalent Binding Profiles of M-5 Bioactivated in Chimeric Mice with Humanized Liver by Two-Dimensional Electrophoresis with Accelerator Mass Spectrometry




(p*I*) Protein samples (100 μg) were separated by isoelectric points (pH 3-10) and molecular weight (225-10 kDa). P450 forms were not evaluated because of technical reasons: the membrane bound proteins were generally difficult to resolve by the first pI-dependent separation step.

Liver microsomes

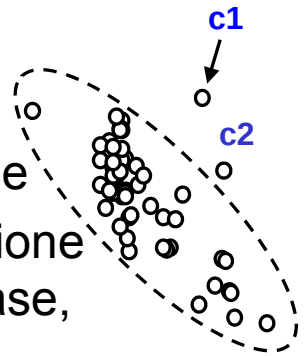
- **m1**, prolyl-4-hydroxylase
- **m2**, 3-hydroxyanthranic acid dioxygenase
- **m3**, branched-chain-amino-acid aminotransferase
- **m4**, selenium binding protein 1 (acetaminophen binding protein).




Protein concentration (μg protein/mg microsomal protein)

Liver cytosol

- **c1**, catalase
- **c2**, glutathione S-transferase, GSTM2

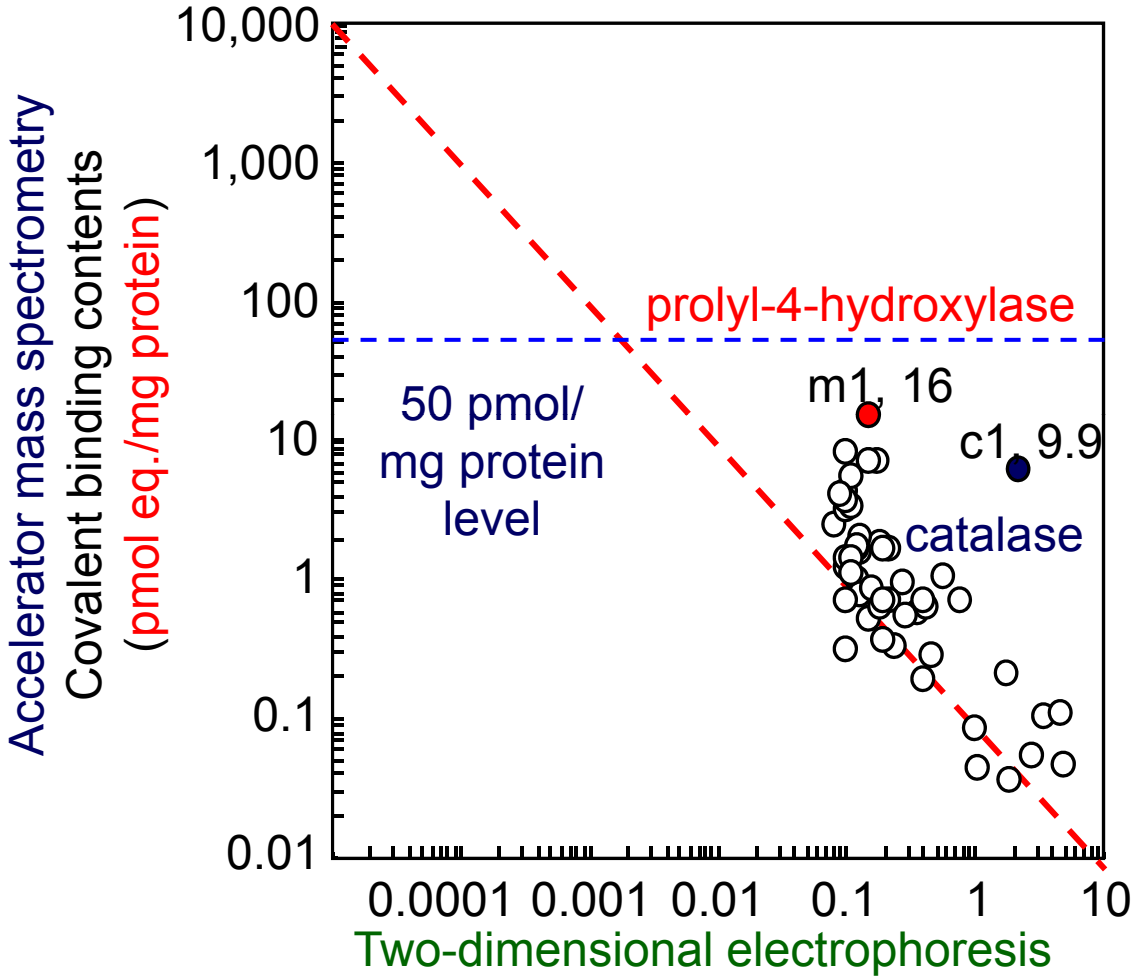



Protein concentration (μg protein/mg cytosolic protein)

Two of the identified adducted targets were **prolyl-4-hydroxylase** (a key enzyme in the biosynthesis of collagens, m1 ) and an antioxidant enzyme **catalase** (c1) in mice with humanized livers

Yamazaki et al., *Chem Res Toxicol,* 23, 152-158 (2010)

# Point of Discussion




- Estimated specific bindings (pmol drug equiv/μg protein) apparently indicated an inverse correlation with the protein contents.
- M-5 could be bioactivated by human P450 1A2 in humanized liver and would bind any proteins either in microsomes or cytosol including the catalysis of P450 1A2 itself.

- The relatively non-specific binding may be relevant to reversible effects in high doses of a hepatotoxic previous drug candidate, OT-7100.

Showa Pharmaceutical University

# Summary

- Thalidomide was metabolically activated to reactive metabolite(s) by human livers.
- The hepatotoxic proximate was activated by human livers and could non-specifically bind to biomolecules including P450 1A2.
- **The issue of human-specific metabolites is complex. Much remains to be learned.**

# 50th Anniversary Symposium on Cytochrome P450 in Fukuoka, on December 2 and 3, 2012, Japan

A grant in support of this P450 Meeting in Japan has been just decided by ISSX.

This is 50th Anniversary Symposium on Cytochrome P450 on December 2 and 3, 2012. Dr. Tuneo Omura found this important enzyme just 50 years ago so that the special meeting is planned in his present hometown, Fukuoka, Japan in this year 2012.

Committee
Chair, Yoshitsugu Shiro, RIKEN SPring-8 Center, Japan

Co-chair, Masahiko Negishi, NIEHS, USA
Co-chair, Hiroshi Yamazaki, Showa Pharmaceutical University, Japan
Co-chair, Toshiyuki Sakaki, Toyama Pref. University, Japan

**http://sites.google.com/site/50thp450/home**

# Acknowledgments


CIEA
H. Suemizu, PhD
SNBL
Y Uno, PhD
Vanderbilt Univ
F. Peter Guengerich, PhD
G. Chowdhury, PhD
APDD
T. Horie, PhD
Otsuka Pharma Factory
S. Naito, PhD
S. Kuribayashi, PhD
Towa Environ Sci
K Oofusa, PhD
Showa Pharma Univ
N. Murayama, PhD
M. Shimizu, PhD

DMPK Lab, Showa Pharma Univ, 2012 spring