Bioactivation (Metabolic Activation) of Drugs – Principles, Protocols and Impacts
(药物的生物活化 --- 原理，常规分析方法和影响)

张洲鹏 博士
文波 博士

Outlines

1. Introduction (介绍)
2. Bioactivation of drugs and metabolism-dependent drug-induced toxicities
   (药物的生物活化和药物代谢引起的的毒性)
3. Functional group metabolism to reactive metabolites
   (由相关药物的化学结构代谢为具有反应活性的代谢物
4. Experimental strategies to detect reactive metabolites
   (具有反应活性代谢物的常用分析检测方法)
   a. Common trapping agents for reactive metabolites (常用诱捕剂)
   b. LC/MS analysis of protein and DNA adducts (蛋白质和DNA加合物的质谱分析)
   c. Analytical strategies for high-throughput screening of reactive metabolites
      (高通量筛选反应性代谢物的分析检测方法)
5. Assessment of exposure and magnitude of reactive metabolite formation
   (具有反应活性代谢物的半定量分析与方法)

a. In vitro covalent protein binding assays (体外共价蛋白结合实验与步骤)
b. In vivo covalent protein binding assays (体内共价蛋白结合实验与步骤)

6. Structural alerts and rational drug design (药物分子结构预警与合理药物设计)
7. Data interpretation and risk mitigation strategies (数据与风险评估方法)
8. Panel discussion and Q&A (小组讨论与问答)

1. Introduction (介绍)

Discovery and development of new drugs involve:

chemistry, biology, pharmacology, toxicology,

safety, process chemistry, formulation,

regulatory affairs, clinical research, manufacturing,

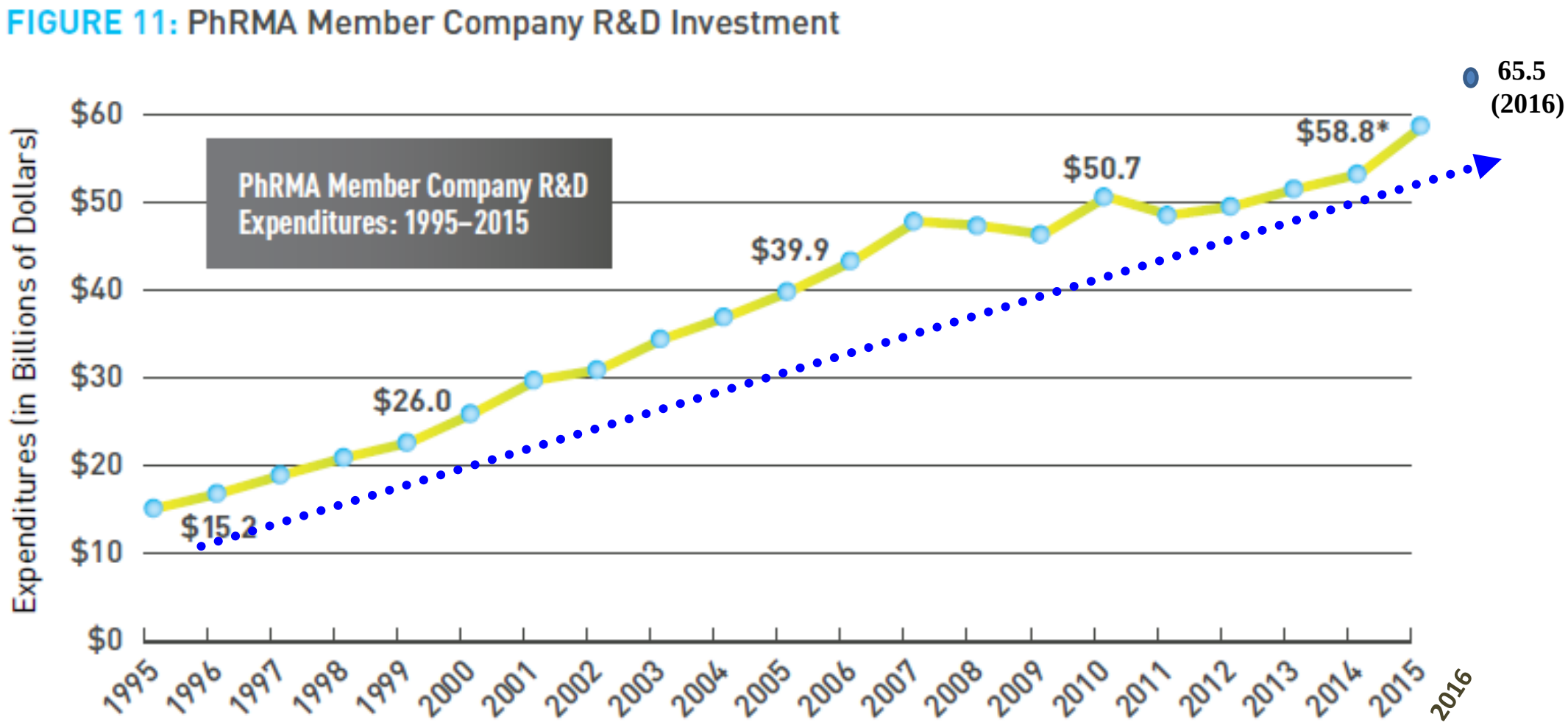
marketing, etc.

The 10 biggest R&D spenders worldwide in 2013

	Volkswagen	Samsung	Intel	Microsoft	Roche	Novartis	Toyota	J&J	Google	Merck
$ (Billions)	13.5	13.4	10.6	10.4	10.0	9.9	9.1	8.2	8.0	7.5
% of Revenue	5.2%	6.4%	20.1%	13.4%	19.0%	16.8%	3.5%	11.5%	13.2%	17.0%

(http://fortune.com/2014/11/17/top-10-research-development/)

R&D spending trend of PhRMA member companies*



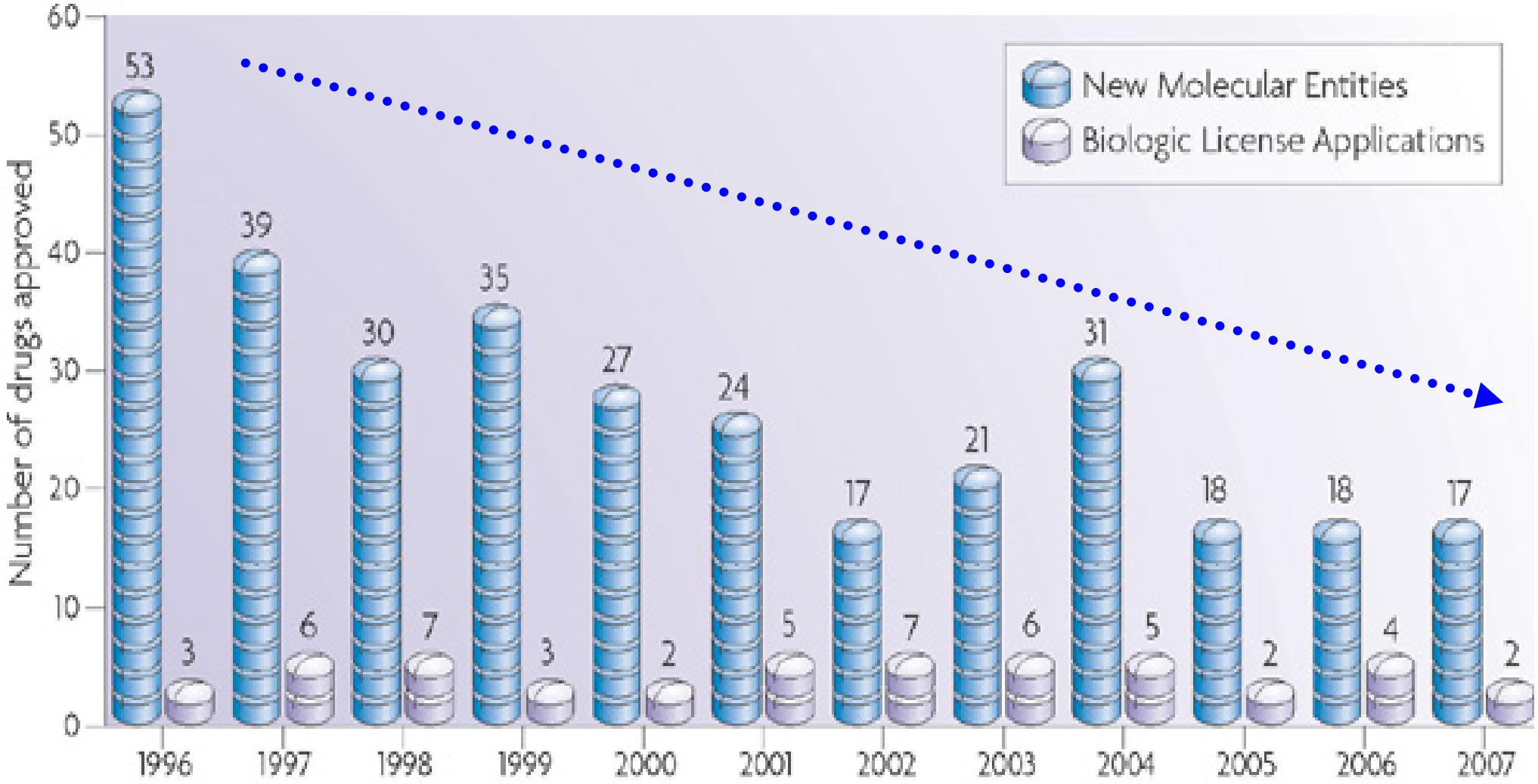
*Estimated fiscal year 2015

Sources: Congressional Budget Office (CBO). A CBO study: research and development in the pharmaceutical industry. www.cbo.gov/sites/default/files/cbofiles/ftpdocs/76xx/doc7615/10-02-drugr-d.pdf. Published October 2006. Accessed April 2016; Pharmaceutical Research and Manufacturers of America (PhRMA). PhRMA Annual Membership Survey, 1995-2015. Washington, DC: PhRMA; 2016.

http://www.phrma.org/sites/default/files/pdf/2016_phrma_profile.pdf

(PhRMA : PHARMACEUTICAL RESEARCH AND MANUFACTURERS OF AMERICA)

Numbers of drugs approved by FDA (1996 – 2007)



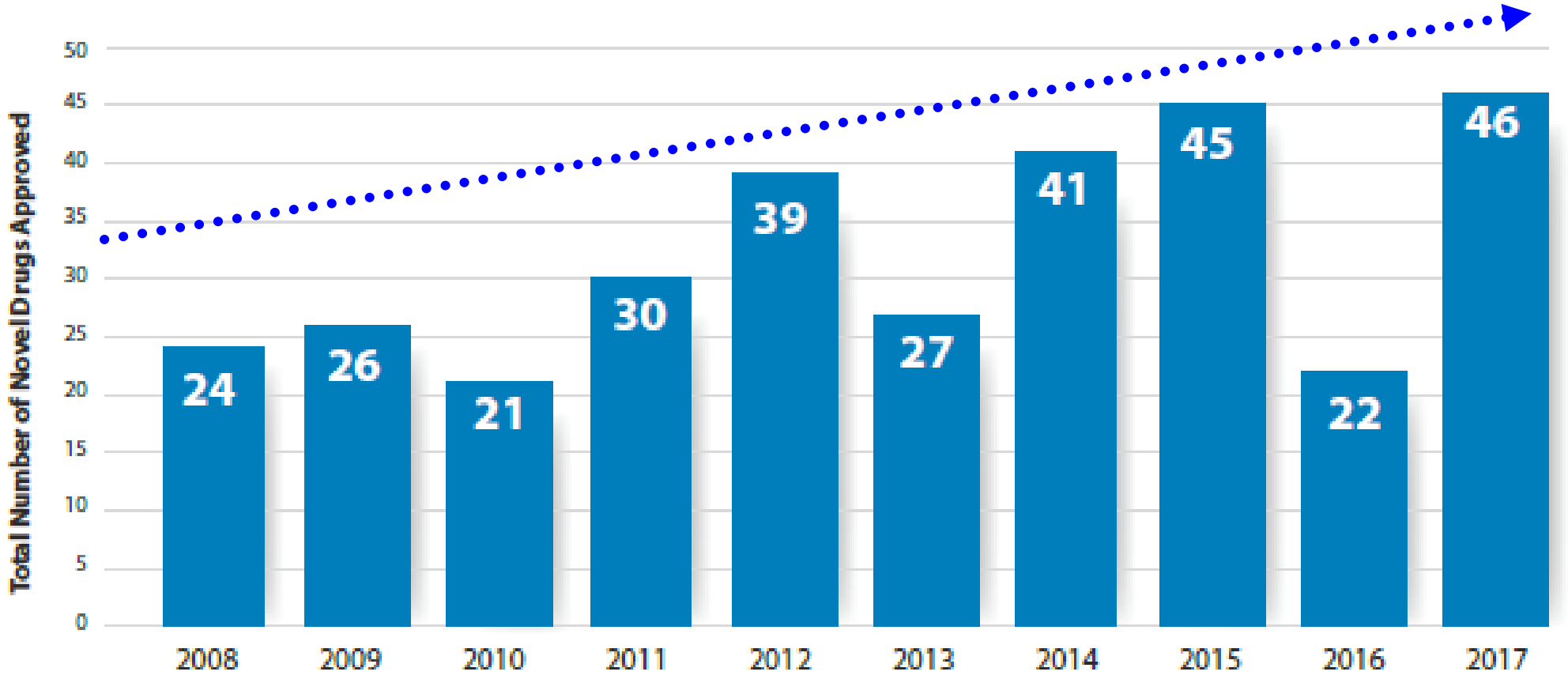
(Hughes B, Nature Reviews Drug Discovery 7, 107, 2008)

Nature Reviews | Drug Discovery

Numbers of drugs approved by FDA (2008 – 2017)

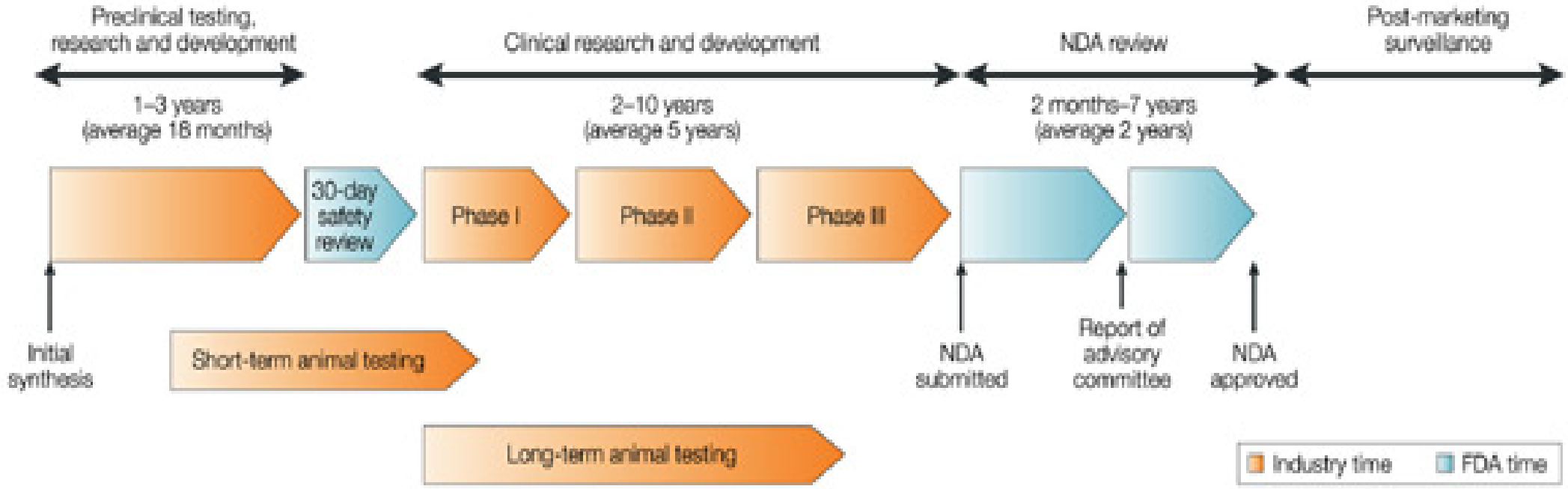
CDER's Annual Novel Drug Approvals: 2008 - 2017

In 2017, CDER approved 46 novel drugs. The ten-year graph below shows that from 2008 through 2016, CDER has averaged about 31 novel drug approvals per year.



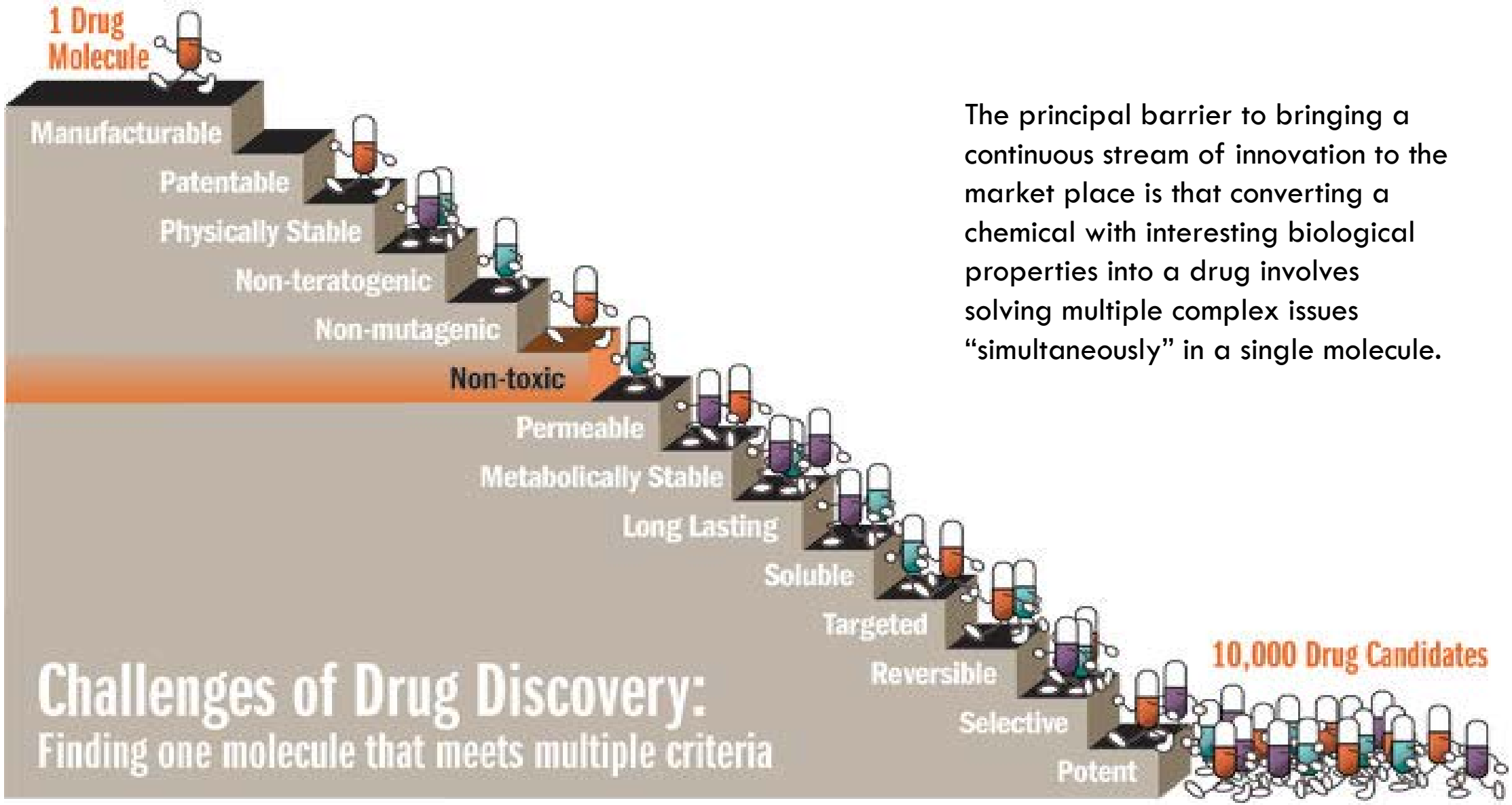
(https://www.fda.gov/downloads/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/ReportsBudgets/UCM591976.pdf)

Drug discovery and development processes



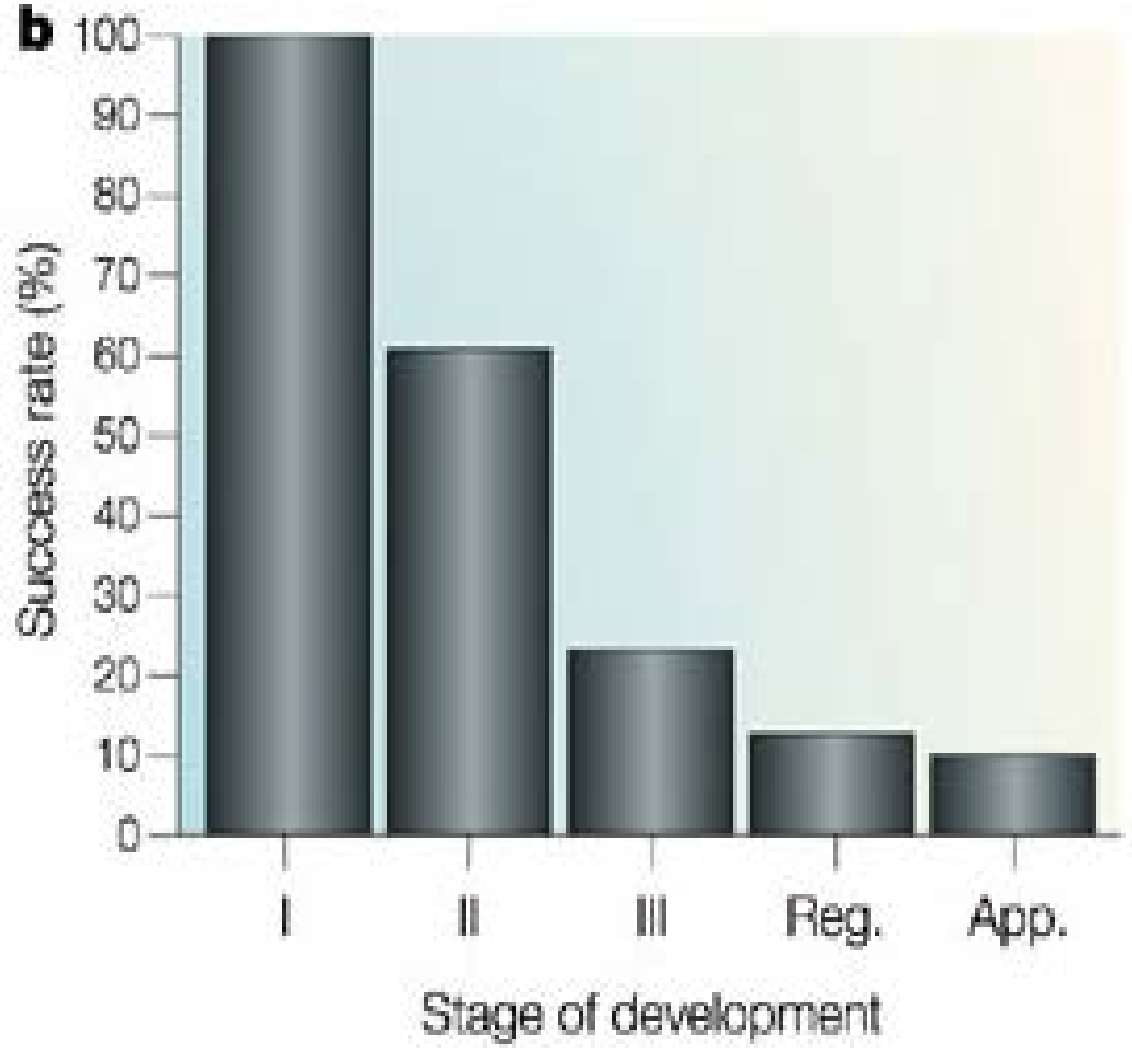
(Dickson M and Gagnon J P, Nature Reviews Drug Discovery 3, 417, 2004)

The Challenge for Pharmaceutical Industry: Solving the Multiple Issues Simultaneously



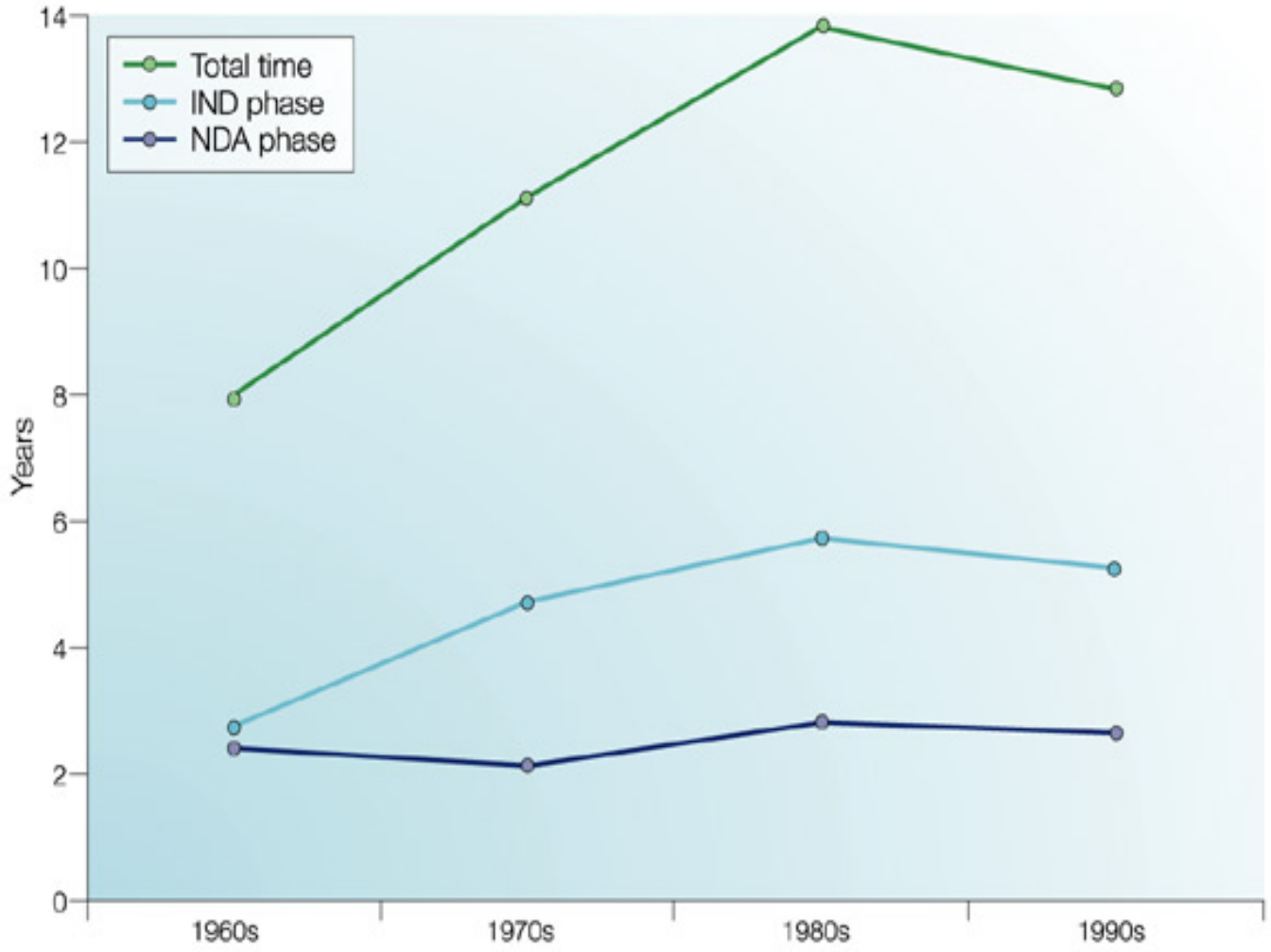
The principal barrier to bringing a continuous stream of innovation to the market place is that converting a chemical with interesting biological properties into a drug involves solving multiple complex issues "simultaneously" in a single molecule.

Success rate by phase of clinical development



Kola I and Landis J, Nature Reviews Drug Discovery **3, 711, 2004**

Trends in times for development of a NCE



Dickson M and Gagnon J P, Nature Reviews Drug Discovery 3, 417, 2004

Estimated cost and time for a NCE

1990s - early 2000s:

- > $ 0.8 billions!
- ~ 8 – 12 years !

(Dickson M and Gagnon J P, Nature Reviews Drug Discovery 3, 417, 2004)

Early 2000s- early 2010s:

- $ 2.6 billions!

(http://www.phrma.org/sites/default/files/pdf/2015_phrma_profile.pdf)

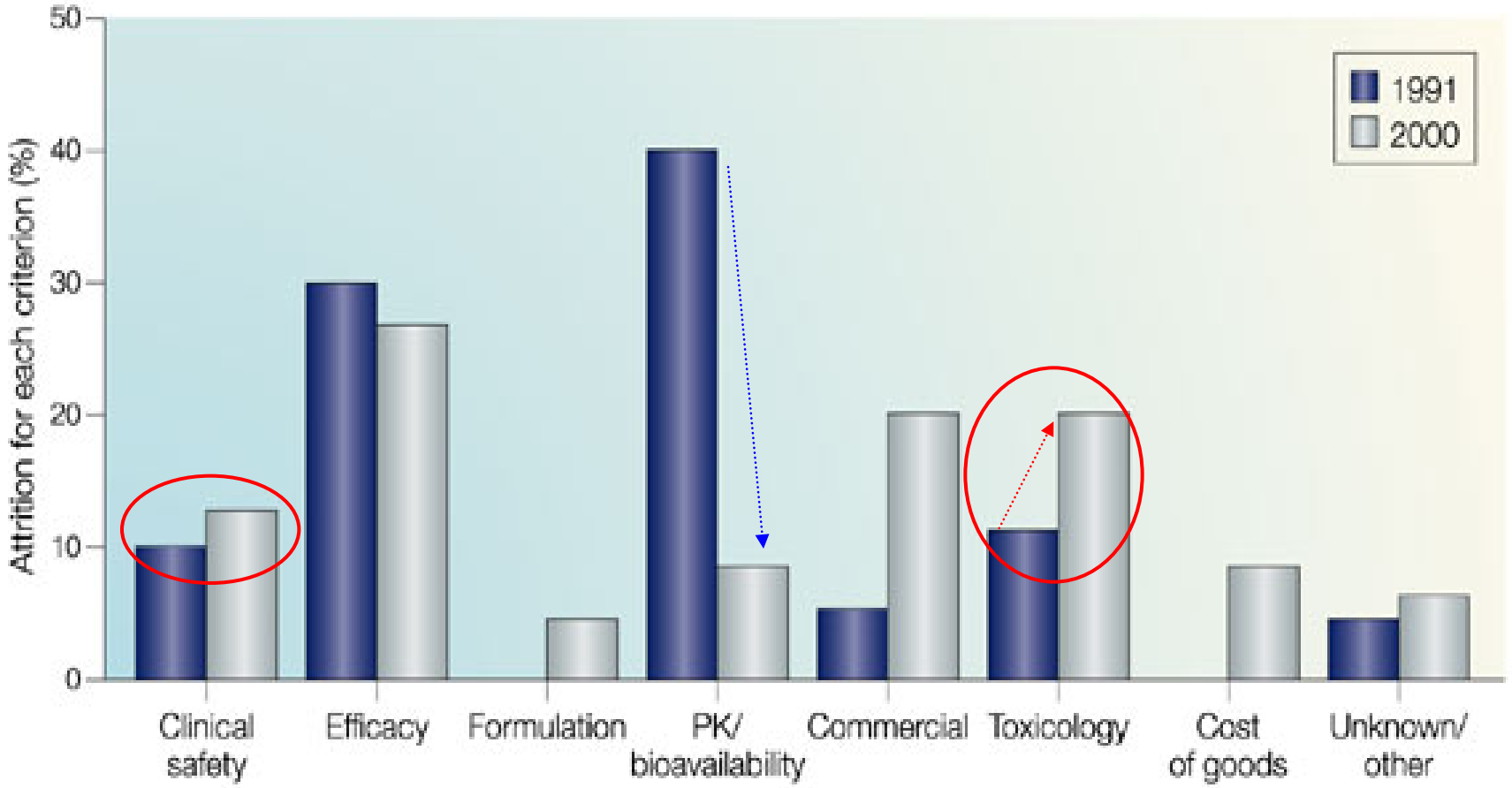
(http://www.phrma.org/sites/default/files/pdf/2015_phrma_profile.pdf)

Reasons for termination of drug candidates in development

Years	Human PK	Human AEs	Animal Toxicity	Lack of efficacy	Financial	Others
1964-1985	39%	10%	11%	29%	6%	5%

(Prentis, R.A., 1988)

Reasons for termination of drug candidates in development



Nature Reviews | Drug Discovery

Kola I and Landis J, Nature Reviews Drug Discovery 3, 711, 2004

Requirements for a successful new drug

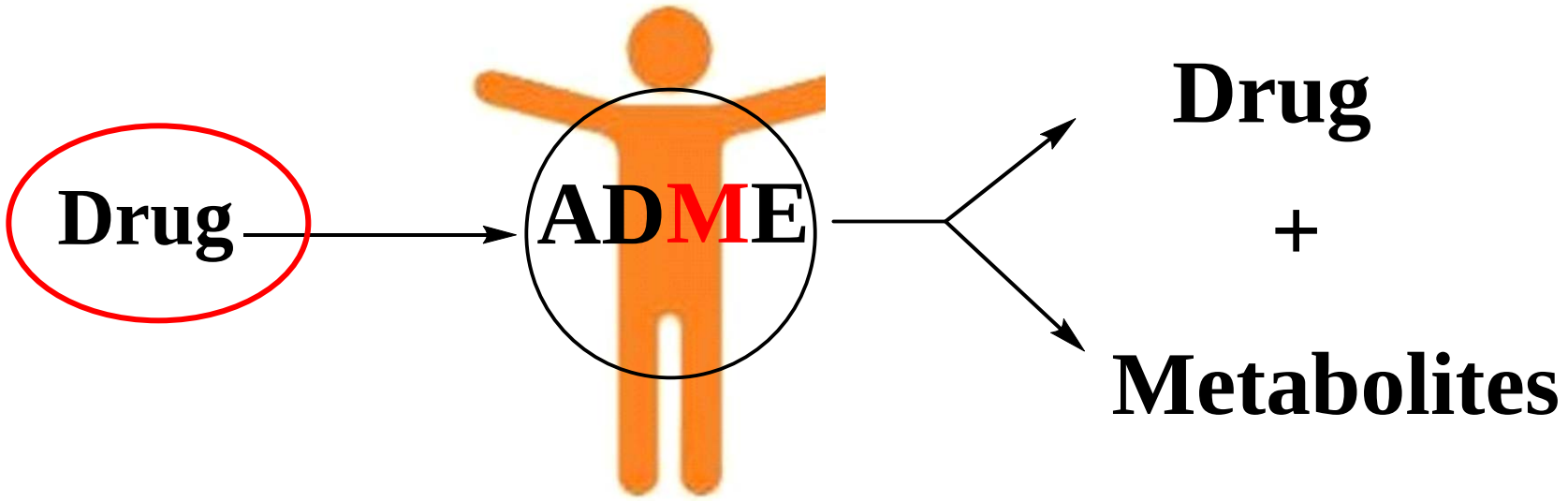
- **Satisfies an unmet medical need**
- **Exhibits superiority over existing treatments**
  - **new mechanism of action**
  - **improved potency or selectivity**
  - **improved safety profile**
  - **superior pharmacokinetics**
  - **improved metabolic characteristics**

The “Ideal” Drug Candidate – DMPK Point of View

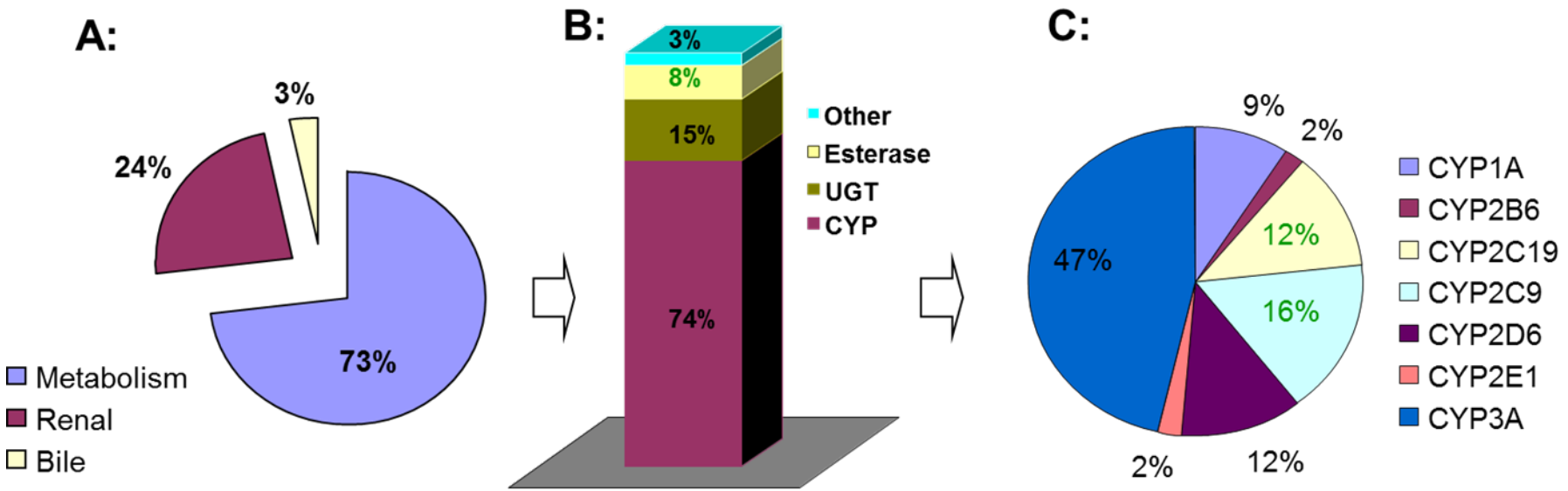
- **Good aqueous solubility for IV formulation and oral absorption**
- **Good permeability coefficient in Caco-2 cells**
- **High bioavailability and acceptable PK profiles for intended route**
- **Small “first-pass” effect (liver/gut) – minimize inter-subject variability**
- **‘Balanced’ clearance mechanisms:**
  - **renal excretion of intact drug**
  - **biliary elimination of intact drug**
  - **metabolism to limited number of products**
- **No pharmacologically active metabolite (unless prodrug)**
- **Minimal Pgp activity (eg. CNS programs)**
- **Minimal P450 induction and inhibition (especially mechanism-based)**
- **Metabolism catalyzed by multiple human P450 enzymes, eg. CYP3A4,**
  not largely dependent on a polymorphically-expressed P450, eg. CYP2D6, 2C9, 2C19
- **Desirable animal and human PK/PD (eg. maximal efficacy at low plasma conc.)**
- **Comparable in vitro and plasma metabolite profiles in humans and toxicological species**
- **Low or no level of chemically reactive metabolites (metabolic activation)**

The principal challenge is to solve multiple issues ‘simultaneously’ in a single molecule.

2. Bioactivation of drugs and metabolism-dependent drug-induced toxicities (药物的生物活化和药物代谢引起的的毒性)



Metabolism by P450 Enzymes – A Key Determinant of Drug Clearance



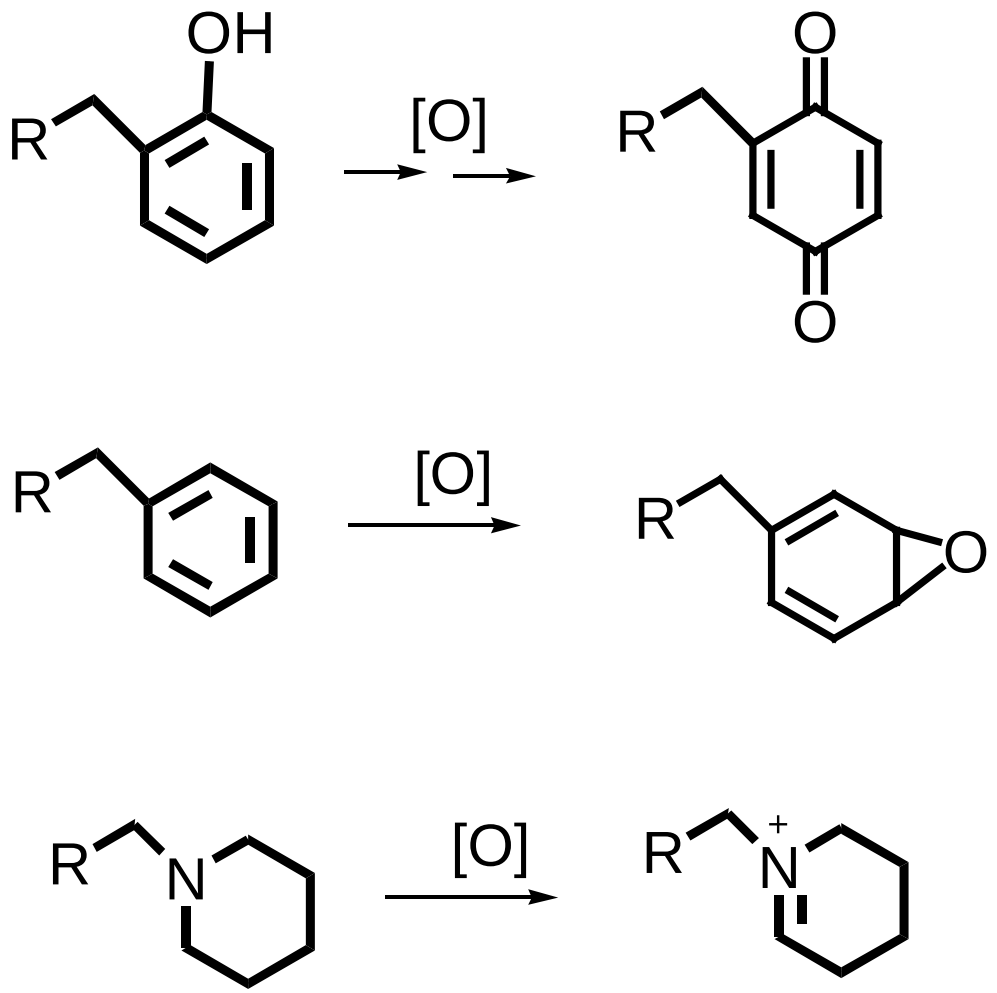
A: Elimination pathways of the top 200 prescribed drugs in 2002.

B: Enzymes contribute to clearance for metabolized drugs.

C: Proportion of individual CYP substrates in CYP mediated drug metabolism.

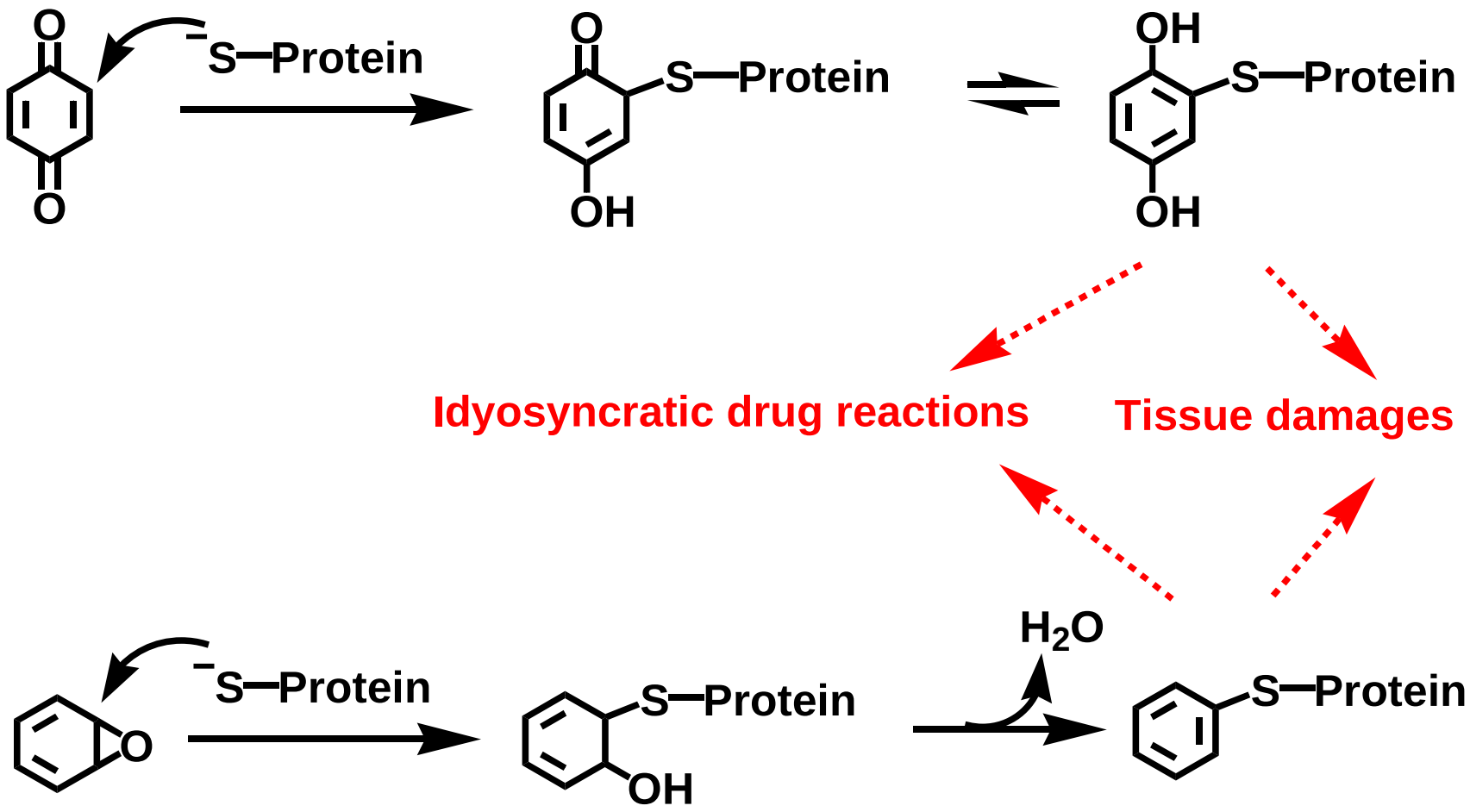
Ref. JA Williams et al, DMD 32:1201, 2004

Metabolism of drugs to reactive metabolites



19

Formation of drug-protein adducts

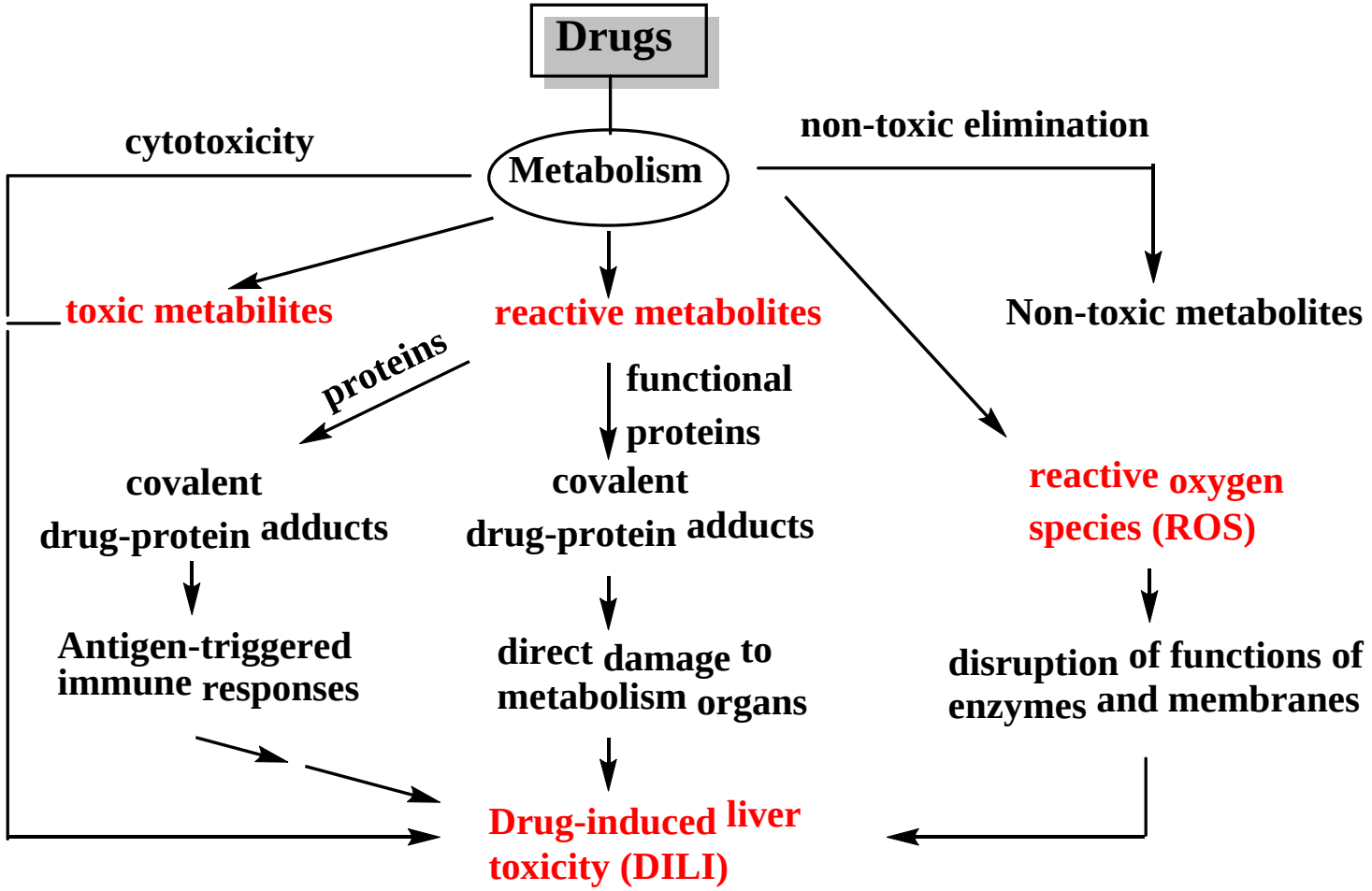


- Direct correlation between bioactivation (protein adduct formation) and toxicity is not clear.
- Some toxicity findings were thought to be related to bioactivation of drug molecules.

20

Drug-induced liver injury (DILI)

- A rare but potentially serious idiosyncratic adverse drug reaction associated with treatment of certain drugs

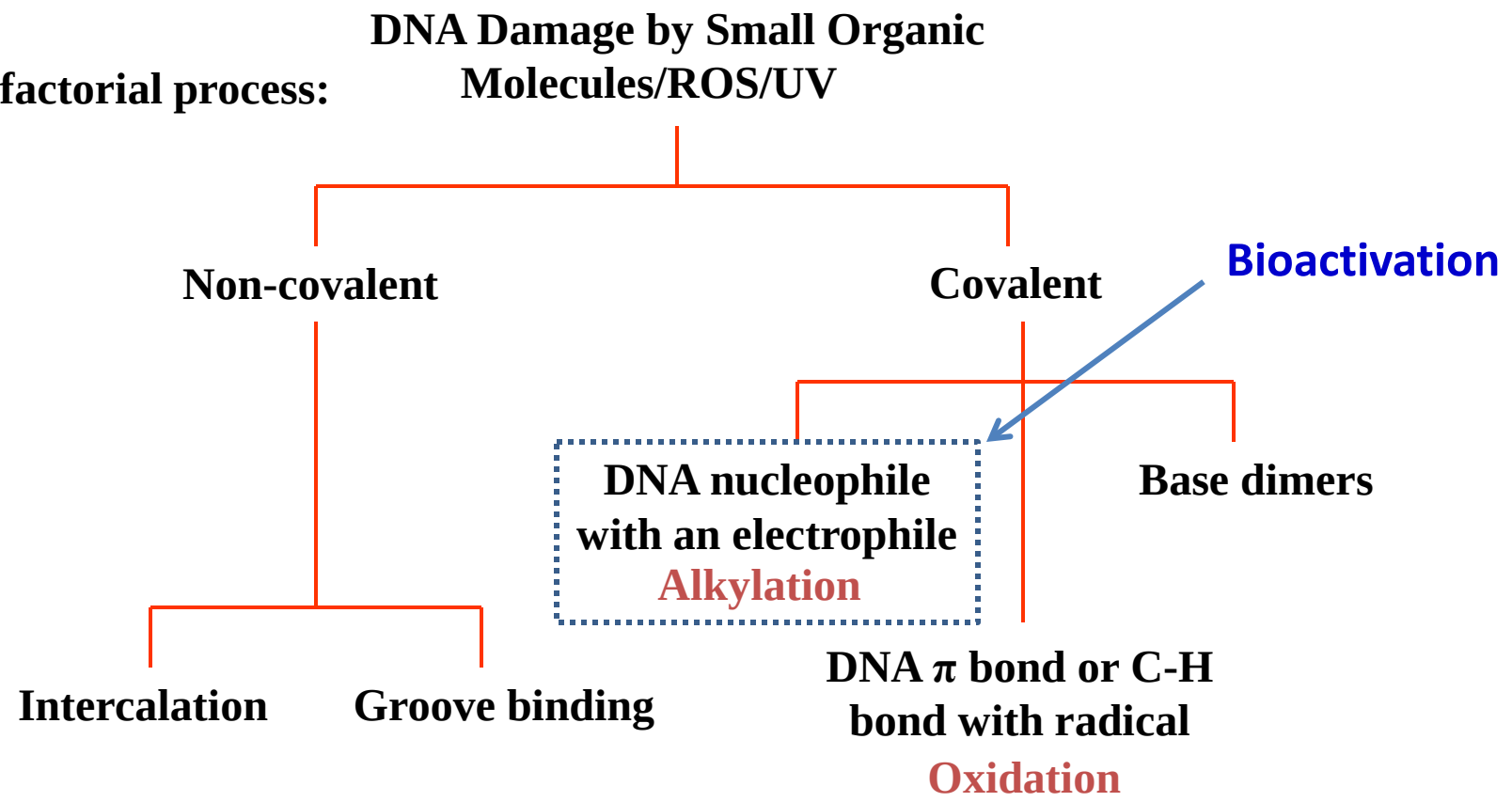


- Bioactivation is only ONE of the several possible causes for DILI

Genotoxicity

- **The property of chemical agents that damages the genetic information within a cell causing mutations, which may lead to cancer.**

A multifactorial process:



- Metabolism is only ONE of the causes for genotoxicity
- Drug-induced liver injury and genotoxcity may or may not share the same mechanism

22

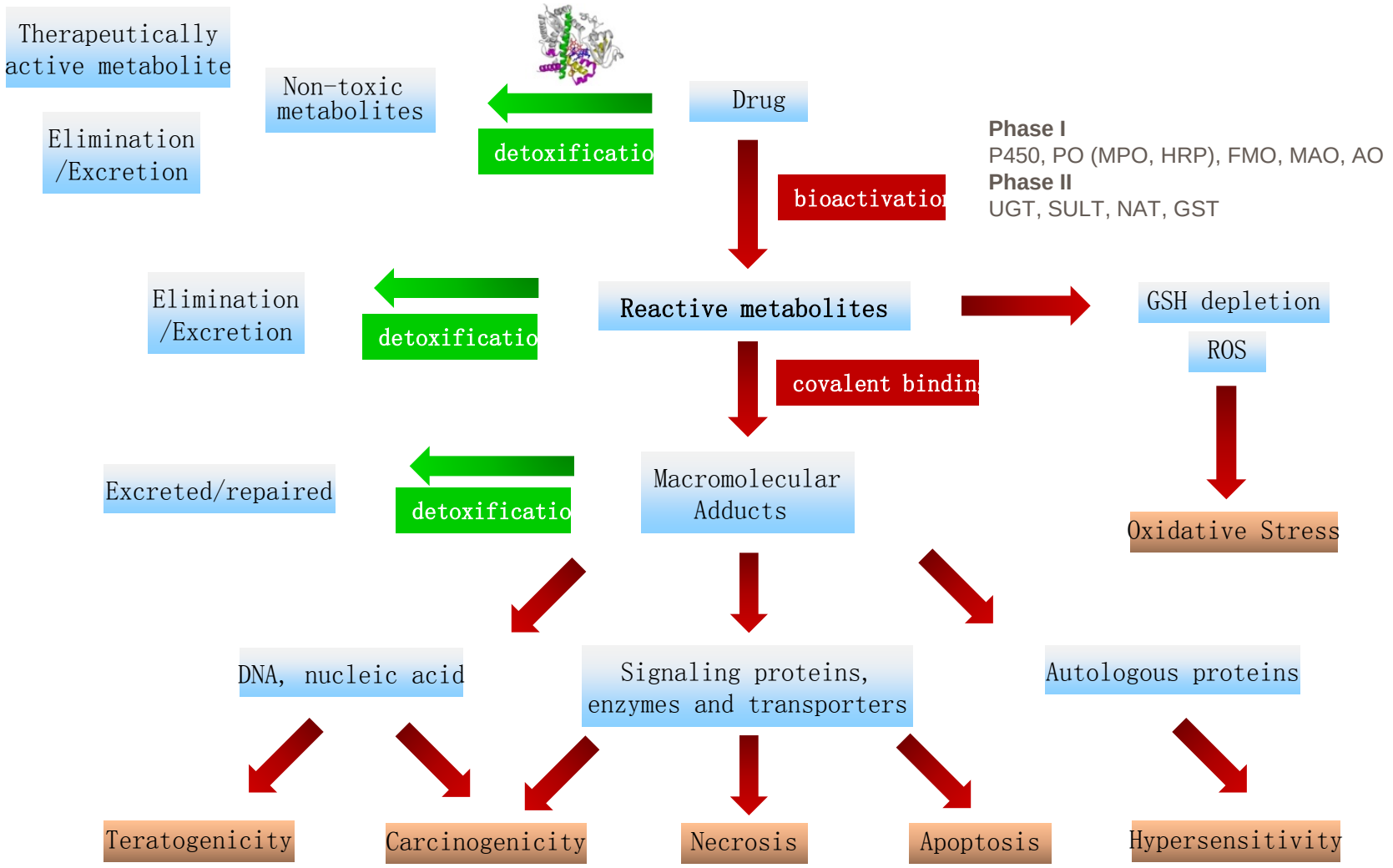
Reactive metabolites *vs.* idiosyncratic drug reactions (IDRs)

- **Most drugs with IDRs have generated significant amount of reactive metabolites in metabolism.**
- **Drugs which form less reactive metabolites are safer than their analogs (isoflurane vs. halothane).**
- **Some drugs that form a lot of reactive metabolite do not cause a significant incidence of IDRs.**
- **Some drugs that cause IDRs do not appear to form reactive metabolites.**
- **The evidence for involvement of reactive metabolite is circumstantial, and it is difficult to prove a specific reactive metabolite responsible for a specific IDR**.

(Jack Uetrecht)

23

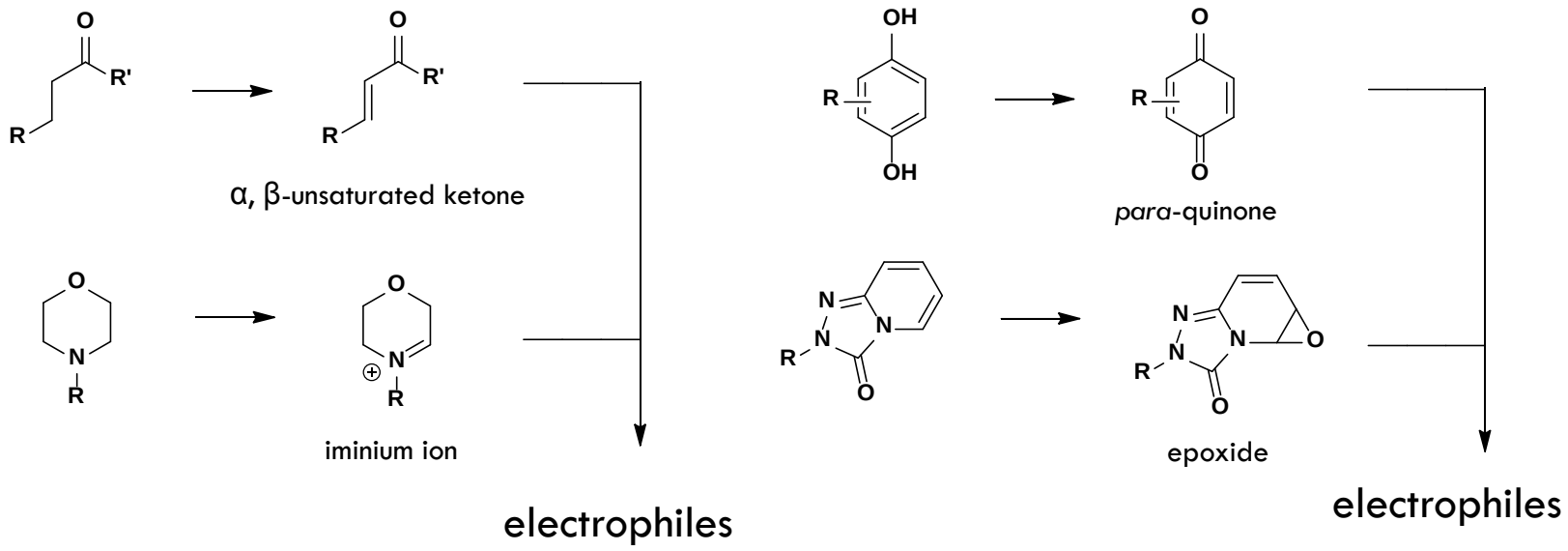
Circumstantial Evidence Links Reactive Metabolites to Adverse Drug Reactions (ADRs)



3. Functional group metabolism to reactive metabolites
(由相关药物的化学结构代谢为具有反应活性的代谢物)

Principles of reactivity – Electrophiles

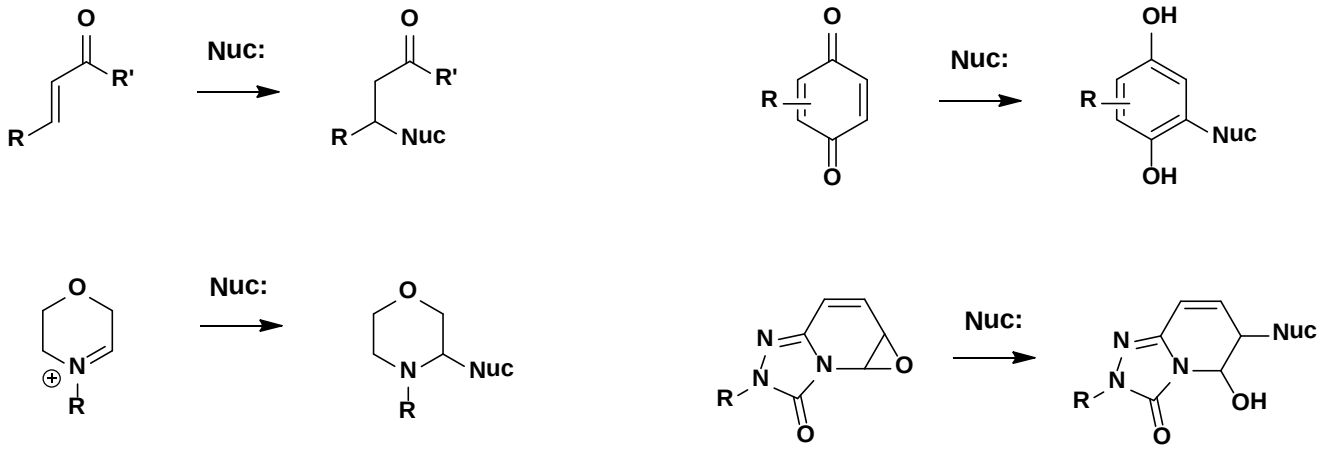
- An “electrophile” is a “lover of electrons”
- Outer orbitals in electrophilic molecules are below capacity, rendering them reactive with electron-rich molecules



“Oxidative metabolism may generate strong electrophiles”

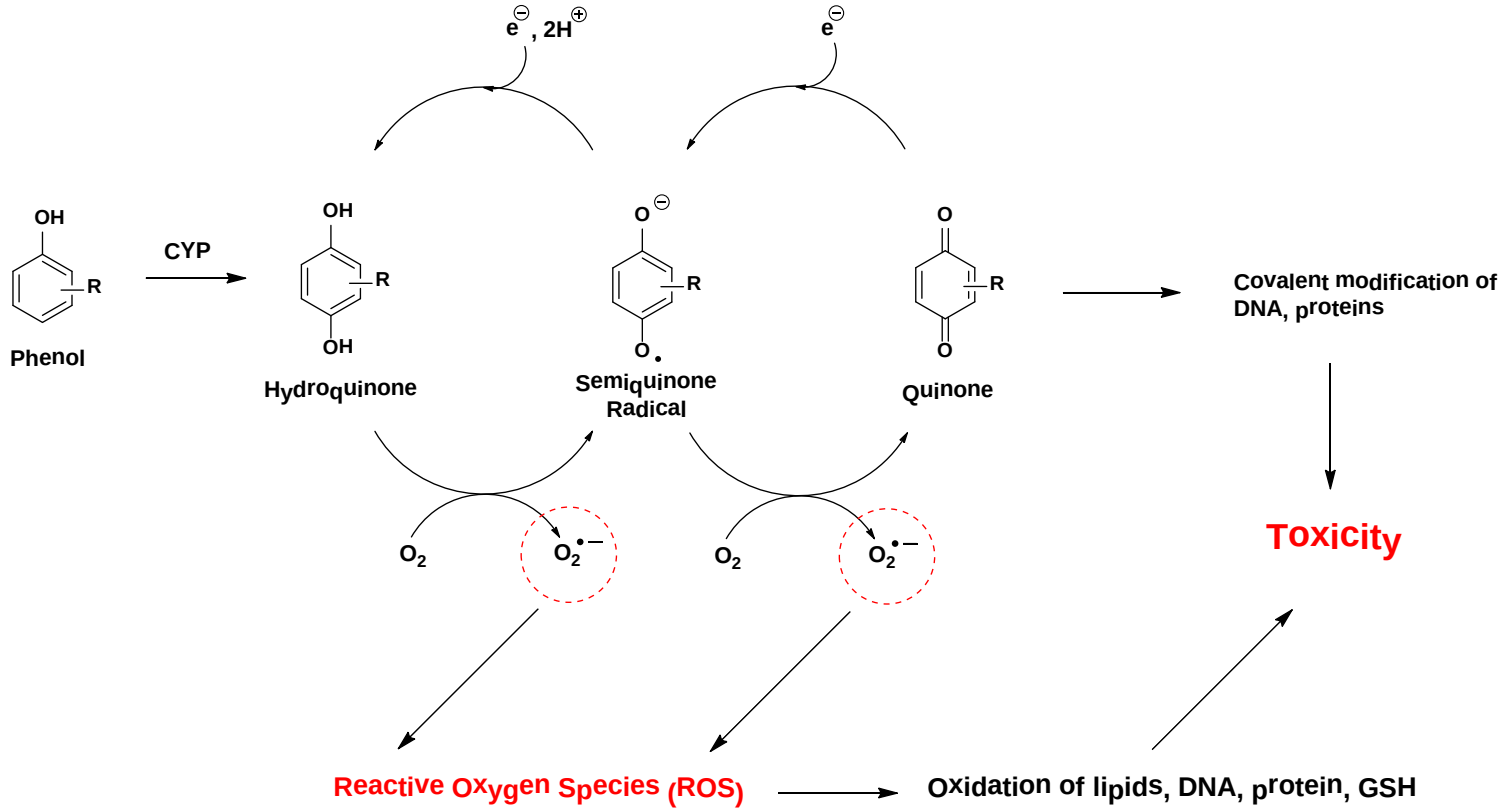
Principles of reactivity – Nucleophiles

- An “nucleophile” is a “lover of nuclei”
- Outer orbitals in nucleophilic molecules are near capacity, rendering them reactive with electron-poor molecules
- A nucleophile can be a protein, DNA, or small molecules (i.e. GSH)



“Electrophilic metabolites react with small and large nucleophiles”

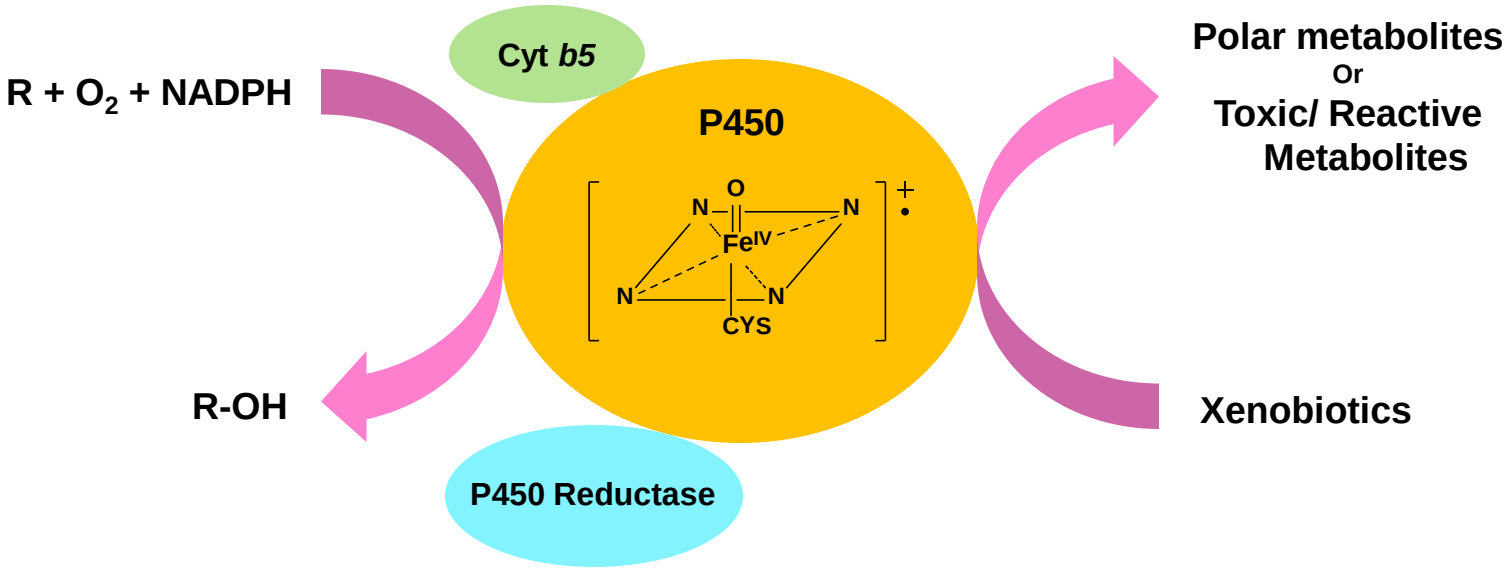
Principles of reactivity – Red/Ox cycling



- Reactive electrophiles may be generated via red/ox cycling
- ROS generated during this process may contribute to toxicity

(Attention: Not all reactive metabolites can be trapped!!)

CYP-mediated Bioactivation

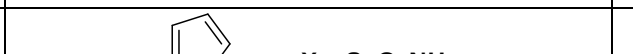
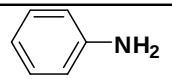
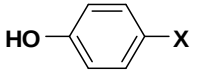
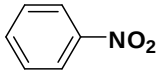
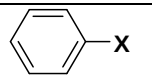
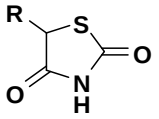
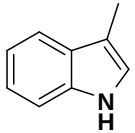
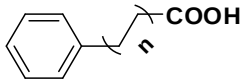


❖ CYPs are the most likely drug metabolizing enzymes to generate reactive metabolites

Examples of functional groups potentially susceptible to bioactivation

- Anilines (or masked anilines)
- *p*-Aminophenols
- Nitrobenzenes
- Hydrazines (phenylhydrazines)
- Benzylamines
- Cyclopropylamines
- 1,2,3,6-Tetrahydropyridines
- 2-Halopyridines and pyrimidines
- Haloalkanes
- Unsubstituted alkenes
- Acetylenes
- Imides
- Formamides
- Sulfonylureas
- Thioureas
- Methylenedioxy groups
- 3-Methylindoles
- 5-Hydroxy (or methoxy) indoles
- Unsubstituted furans
- Unsubstituted thiophenes
- Unsubstituted thiazoles
- Unsubstituted oxazoles
- Thiazolidinediones
- Reduced aromatic thiols
- Arylacetic acids and arylpropionic acids
- Hydroxylamines
- Hydroxamic acids
- Michael acceptors
- Fatty acids (medium to long chain)
- Hydroquinones
- Bromobenzene
- **Benzene !!!**

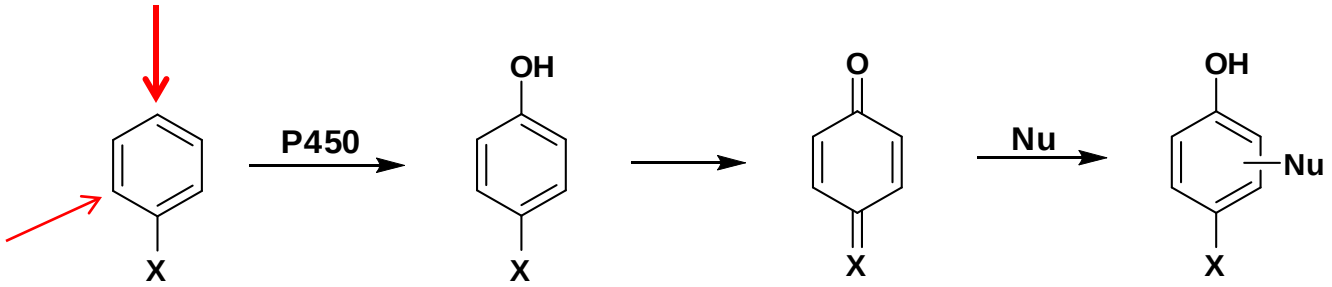
Structural alerts: a starting point for testing and beyond

Name	Substructure	Reactive species
Hydrazines, hydrazides	 R_1, R_2 = alkyl or aromatic groups	diazonium ions, free radicals
Thiophenes, furans, pyrroles	X = S, O, NH	epoxides, *S*-oxides (thiophenes),α, β-unsaturated dicarbonyl (furans)
Anilines, anilides and precursors		nitrosos, quinoneimines, free radicals
Quinones and precursors	 X = NH_2, OH, etc	quinones, quinoneimines, free radicals
Nitroaromatics		nitroso intermediates, free radicals
Halogenated aromatics (Br > Cl > F)	 X = Br, Cl, F	arene oxides
Thiazolidinediones	 R = alkyl or aromatic groups	isocyanates, isothiocyanates
3-Alkylindoles		3-methyleneindolenines
Alkynes, acetylenes	$R_1-C\equiv C-R_2$ R_1, R_2 = H or alkyl groups	oxirenes, ketenes
Arylacetic acids, arylpropionic acids	 n = 1, 2	acyl glucuronides

Wen B & Fitch W, *Expert Opin Drug Metab Toxicol.* 5:39-55 (2009)

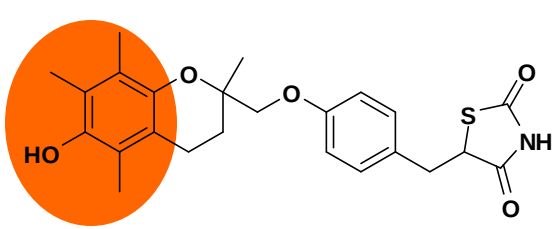
Mechanism of bioactivation – Michael acceptors

A. Quinones or quinoid containing structures

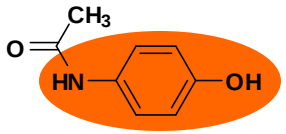


X = O, N, C

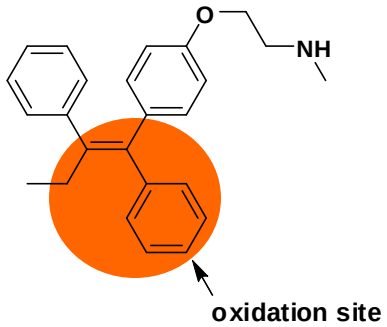
Examples



Troglitazone



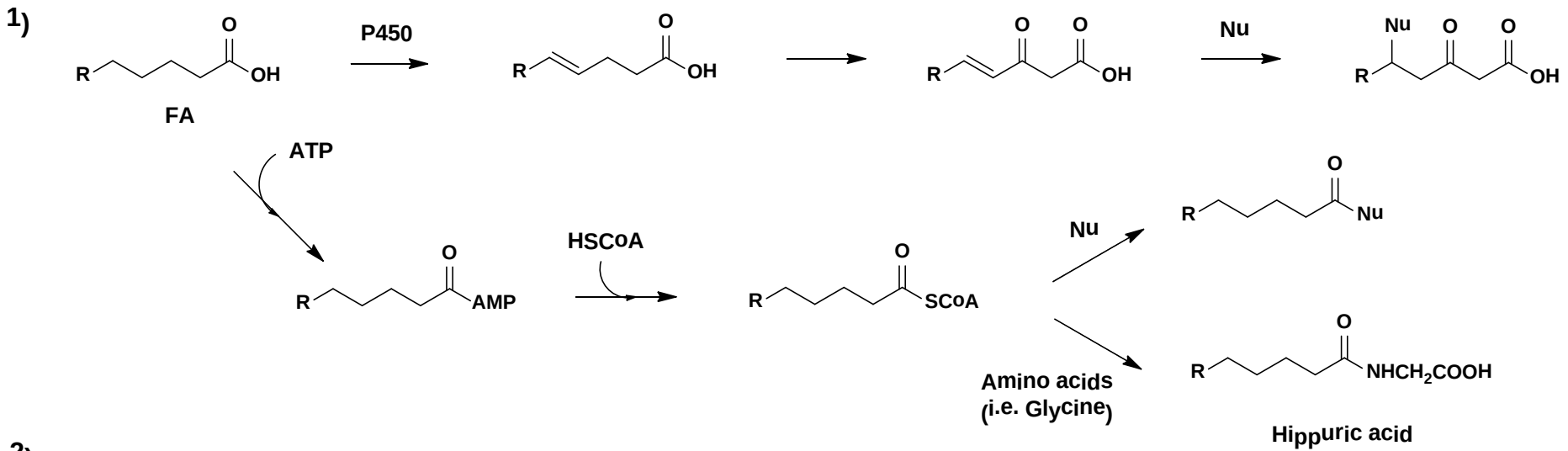
Acetaminophen



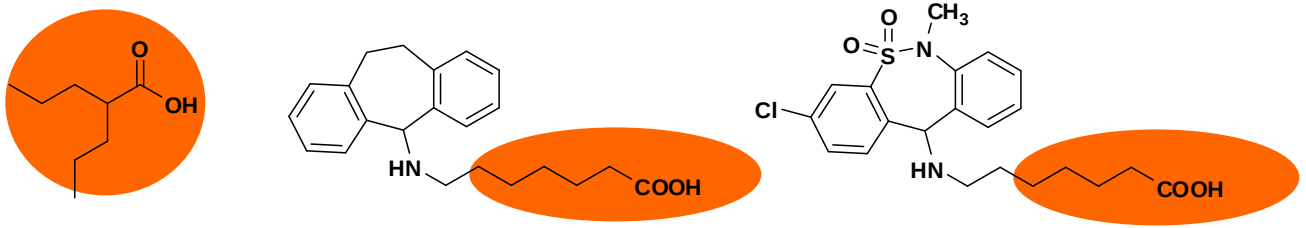
Tamoxifen

Mechanism of bioactivation – Michael acceptors

B. Fatty acids/Allylic alcohols



Examples

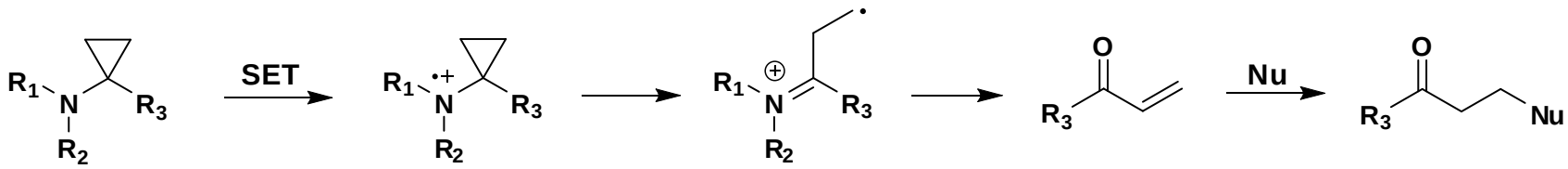


Valproic acid | Amineptine | Tianeptine

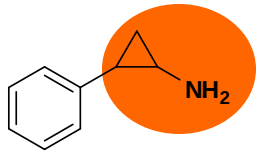
Kalgutkar AS, et al. *Curr Drug Metab.* 6:161-225 (2005)
Darnell M and Weidolf L, *Chem Res Toxicol.* 26:1139-55 (2013)

Mechanism of bioactivation – Michael acceptors

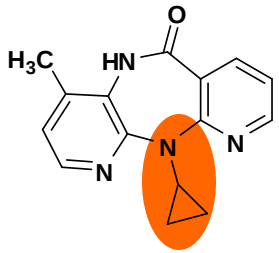
C. Cyclopropylamines



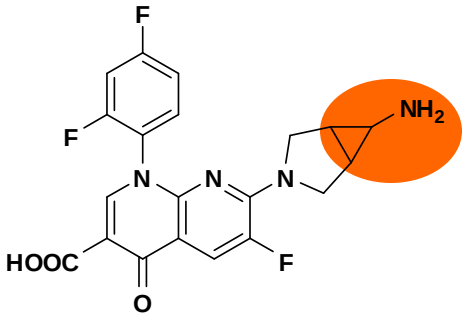
Examples



Tranylcypromine



Nevirapine

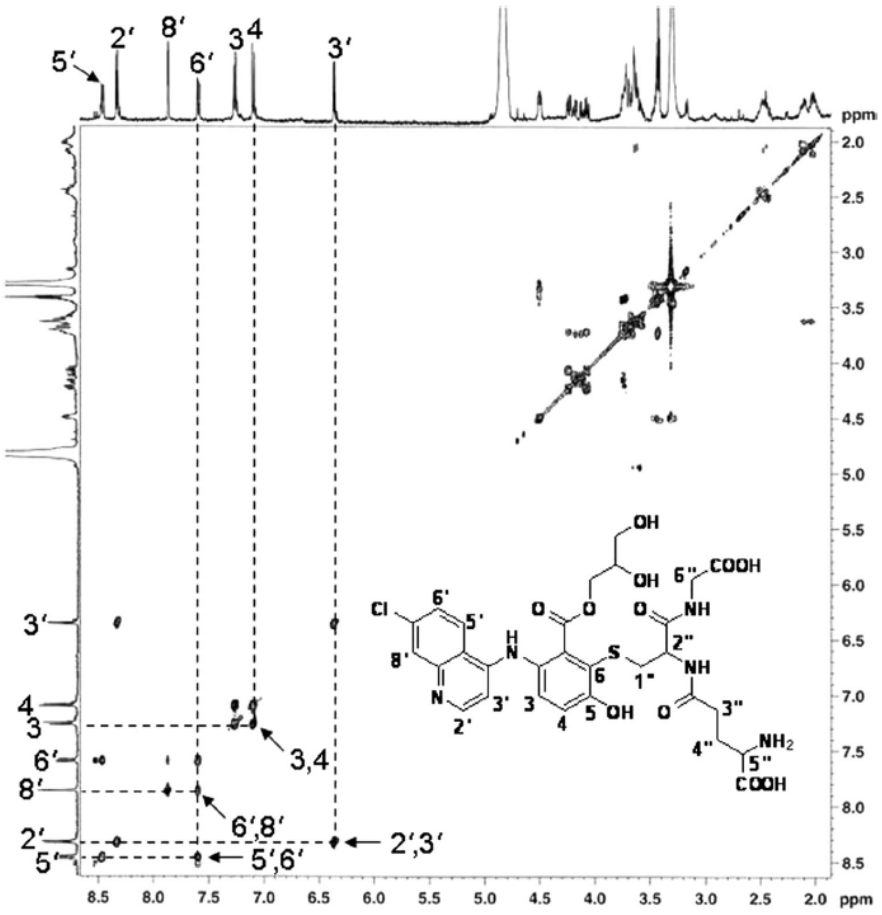


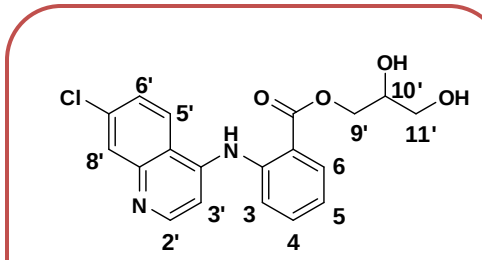
Trovafloxacin

Kalgutkar AS, et al. *Curr Drug Metab.* 6:161-225 (2005)

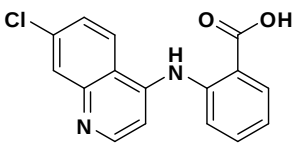
Example of Michael acceptors - Glafenine

A. Quinones or quinoid containing structures

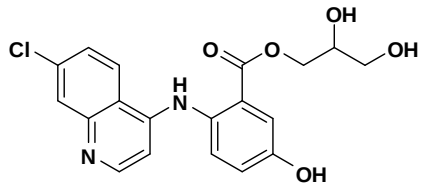




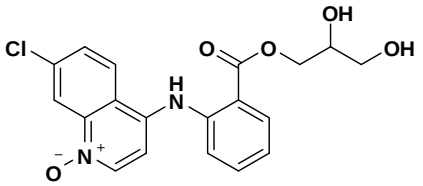
Glafenine



Glafenic acid



5-Hydroxyglafenine



Glafenine N-oxide

FIG. 6. ^{1}H-^{1}H COSY spectrum of M1 showing the coupling between aromatic protons at C3 and C4.

TABLE 1

^{1}H NMR data of glafenine, 5-hydroxyglafenine (M3), and its GSH conjugate (M1) (δ in CD_3OD)

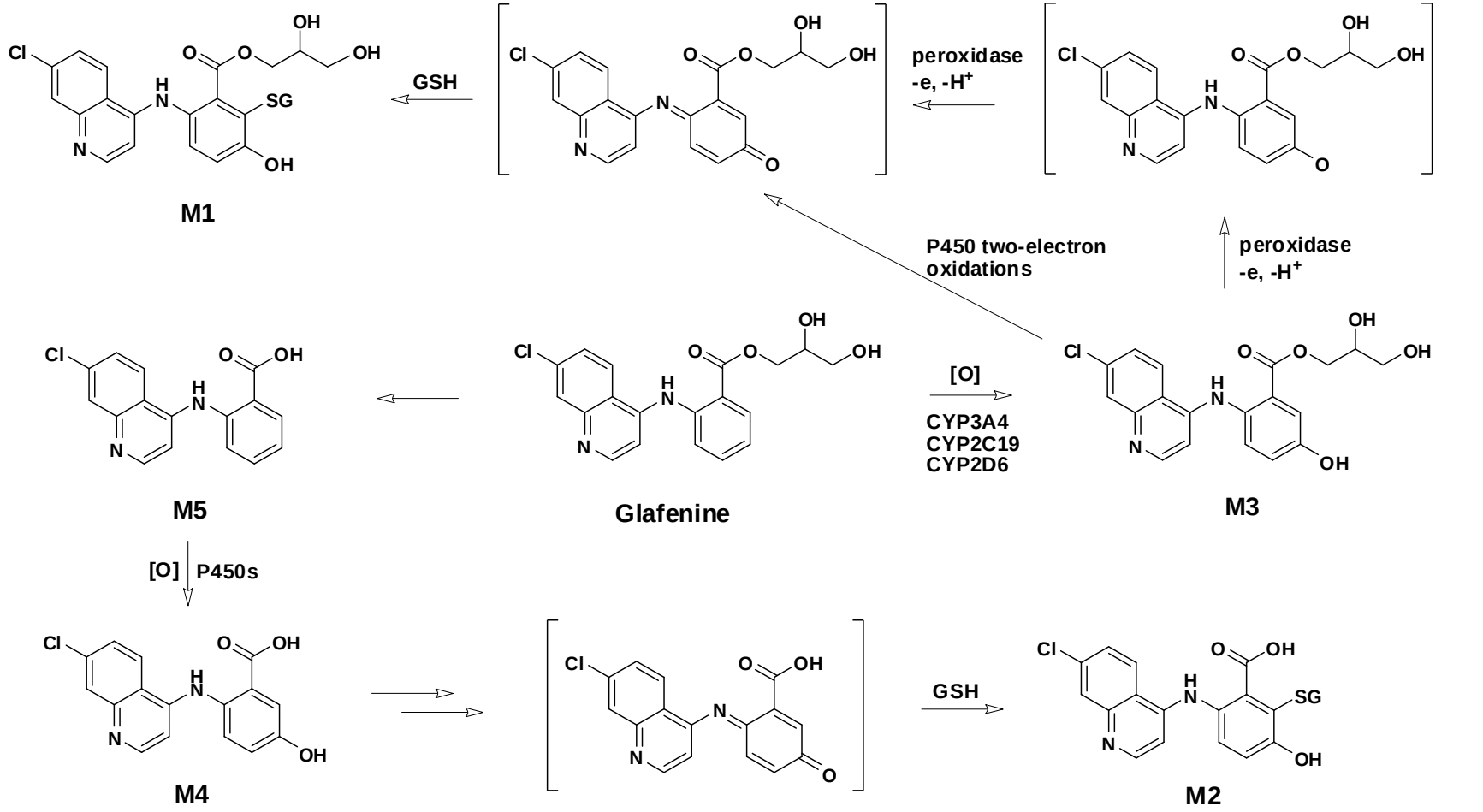
Refer to Fig. 6 for signal assignments of the thioether moiety; cysteine 1″-H: δ 3.3/3.4, cysteine 2″-H: δ 4.4, glutamate 3″-H: δ 2.5, glutamate 4″-H: δ 2.1/2.3, glutamate 5″-H: δ 3.6, glycine 6″-H: δ 3.7.

	Proton Signals		
	Glafenine	M3	M1
C3	7.74 (1H, bd, J = 8.2 Hz)	7.50 (1H, d, J = 8.8 Hz)	7.27 (1H, d, J = 8.6 Hz)
C4	7.62 (1H, ddd, J = 8.2, 7.9 and 1.8 Hz)	7.13 (1H. dd, J = 8.8 and 2.9 Hz)	7.10 (1H, d, J = 8.6 Hz)
C2′	8.57 (1H, d, J = 5.4 Hz)	8.41 (1H, d, J = 5.8 Hz)	8.33 (1H, d, J = 6.2 Hz)
C3′	7.41 (1H, d, J = 5.4 Hz)	6.93 (1H, d, J = 5.8 Hz)	6.36 (1H, d, J = 6.2 Hz)
C5′	8.20 (1H, d, J = 9.0 Hz)	8.28 (1H, d, J = 9.0 Hz)	8.46 (1H, d, J = 9.0 Hz)
C6′	7.60 (1H, dd, J = 9.0 and 2.1 Hz)	7.60 (1H, dd, J = 9.0 and 1.9 Hz)	7.60 (1H, dd, J = 9.0 and 2.0 Hz)
C8′	7.94 (1H, d, J = 2.1 Hz)	7.89 (1H, d, J = 1.9 Hz)	7.87 (1H, d, J = 2.0 Hz)
C6	8.19 (1H, dd, J = 8.1 and 1.8 Hz)	7.57 (1H, d, J = 2.9 Hz)	
C5	7.16 (1H, bdd, J = 8.1 and 7.9 Hz)		

Wen B, et al. *Drug Metab. Dispos.* 39:1511-1521, 2011

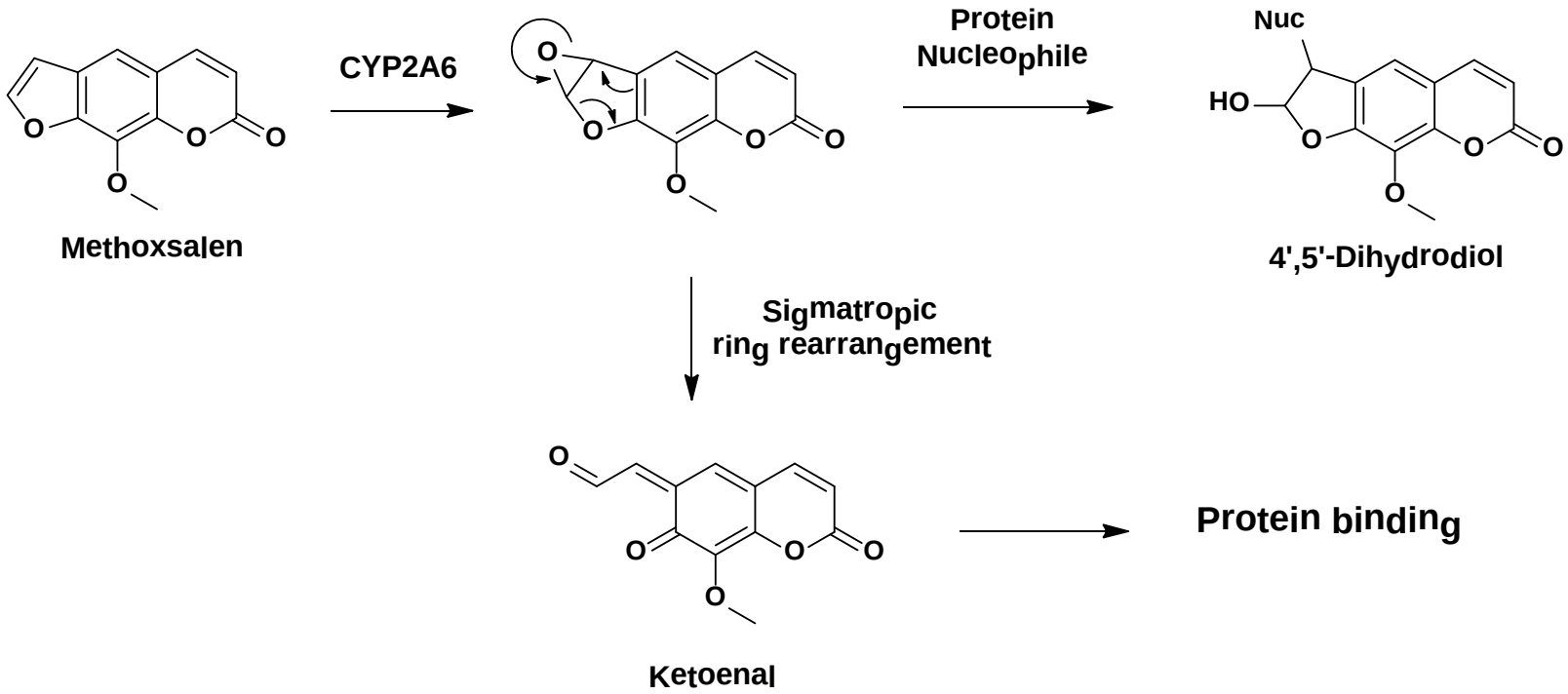
Example of Michael acceptors - Glafenine

A. Quinones or quinoid containing structures



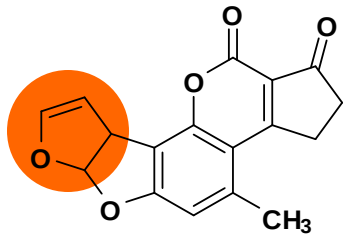
Wen B, et al. *Drug Metab. Dispos.* 39:1511-1521, 2011

Mechanism of bioactivation - Furans

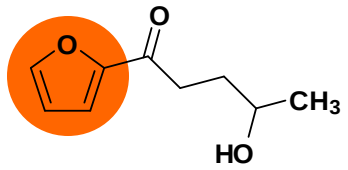


Mays DC, et al, *J Pharmacol Exp Ther.* 254:720-31 (1990)
Kalgutkar AS, et al. *Curr Drug Metab.* 6:161-225 (2005)

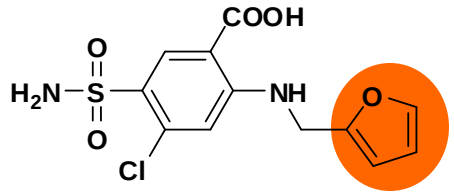
Examples of bioactivation - Furans



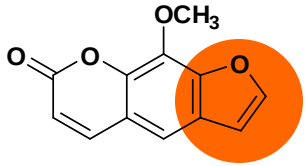
Aflatoxin B1



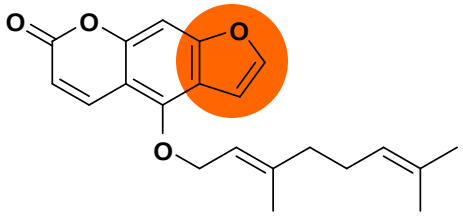
Ipomeanol



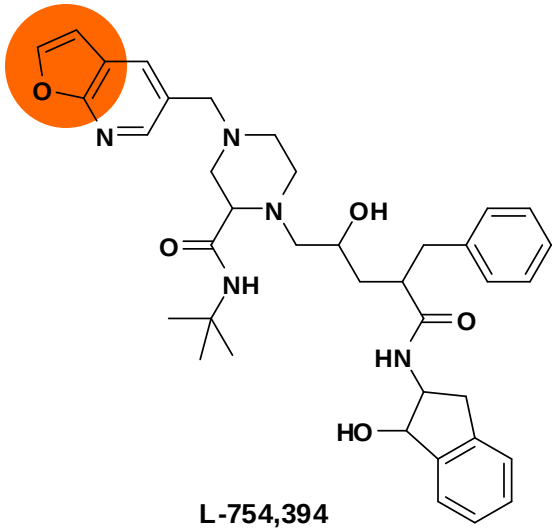
Furosemide



8-Methoxypsoralen



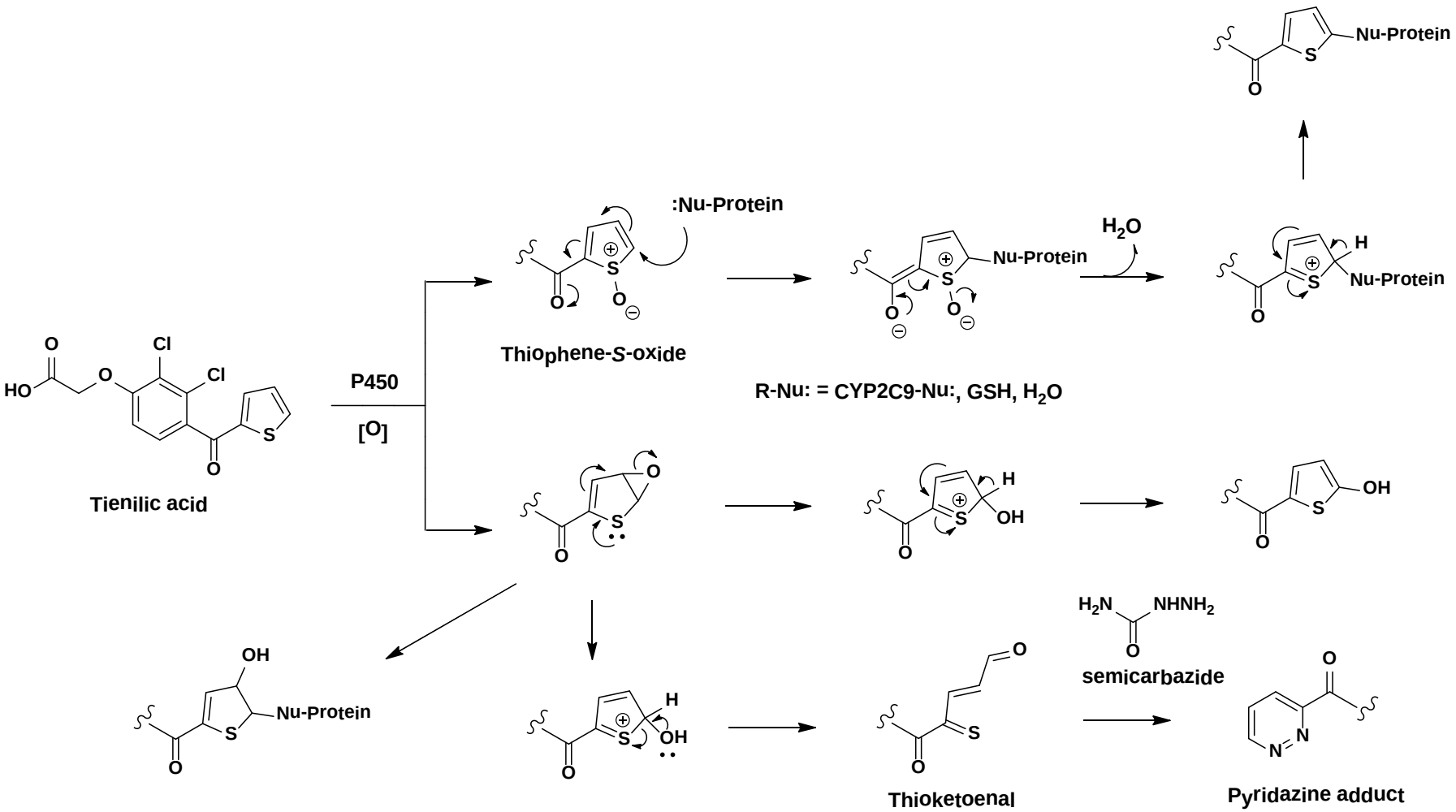
Bergamottin



L-754,394

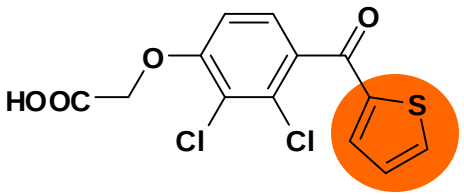
Kalgutkar AS, et al. *Curr Drug Metab.* 6:161-225 (2005)

Mechanism of bioactivation - Thiophenes

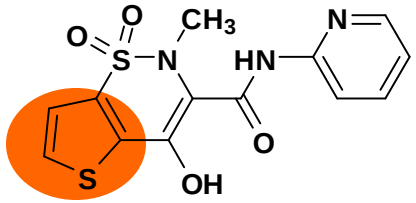


Rademacher PM, et al. Chem Res Toxicol. 25:895-903 (2012)

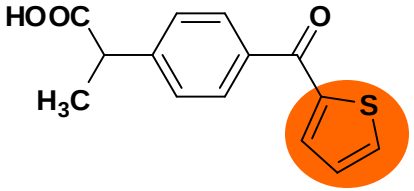
Examples of bioactivation - Thiophenes



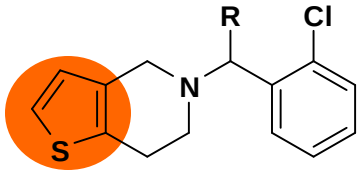
Tienilic acid



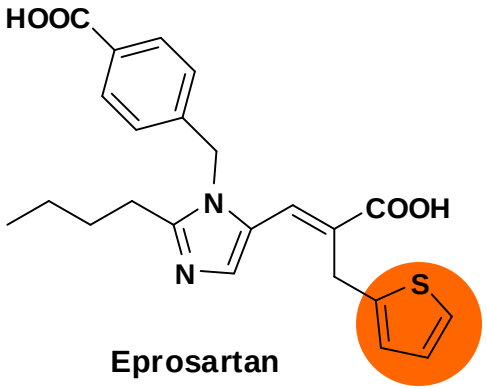
Tenoxicam



Suprofen



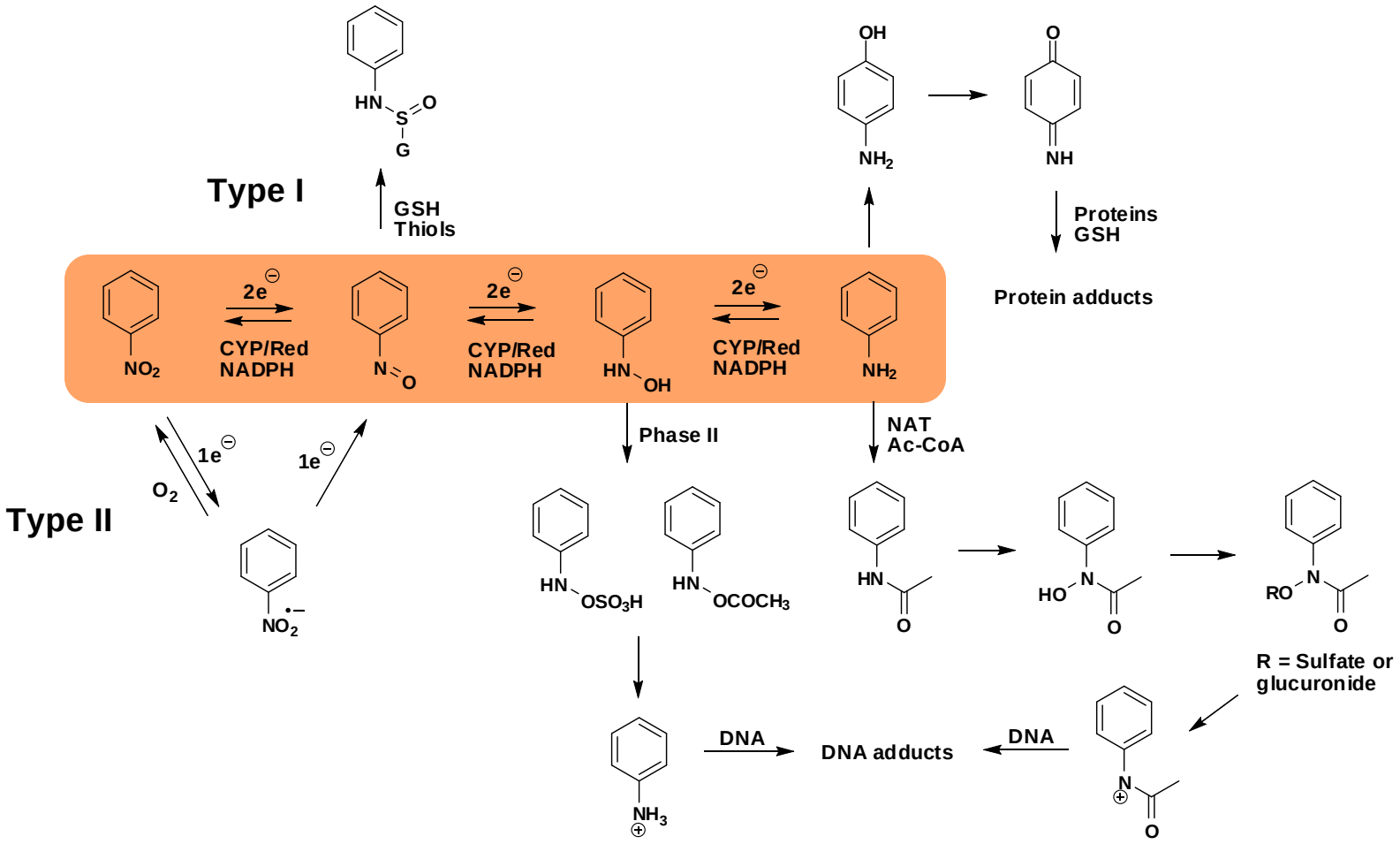
R = H, Ticlopidine
R = $COOCH_3$, Clopidigrel



Eprosartan

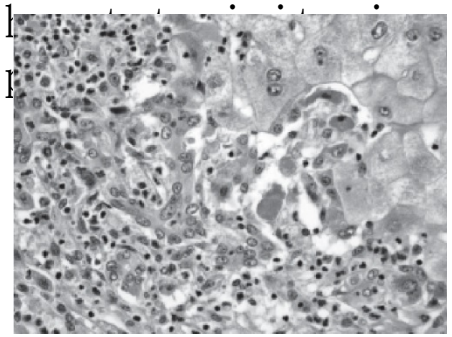
Kalgutkar AS and Soglia JR, *Expert Opin Drug Metab Toxicol*. 1:91-142 (2005)
Kalgutkar AS, et al. *Curr Drug Metab.* 6:161-225 (2005)

Mechanism of bioactivation – Anilines or masked anilines

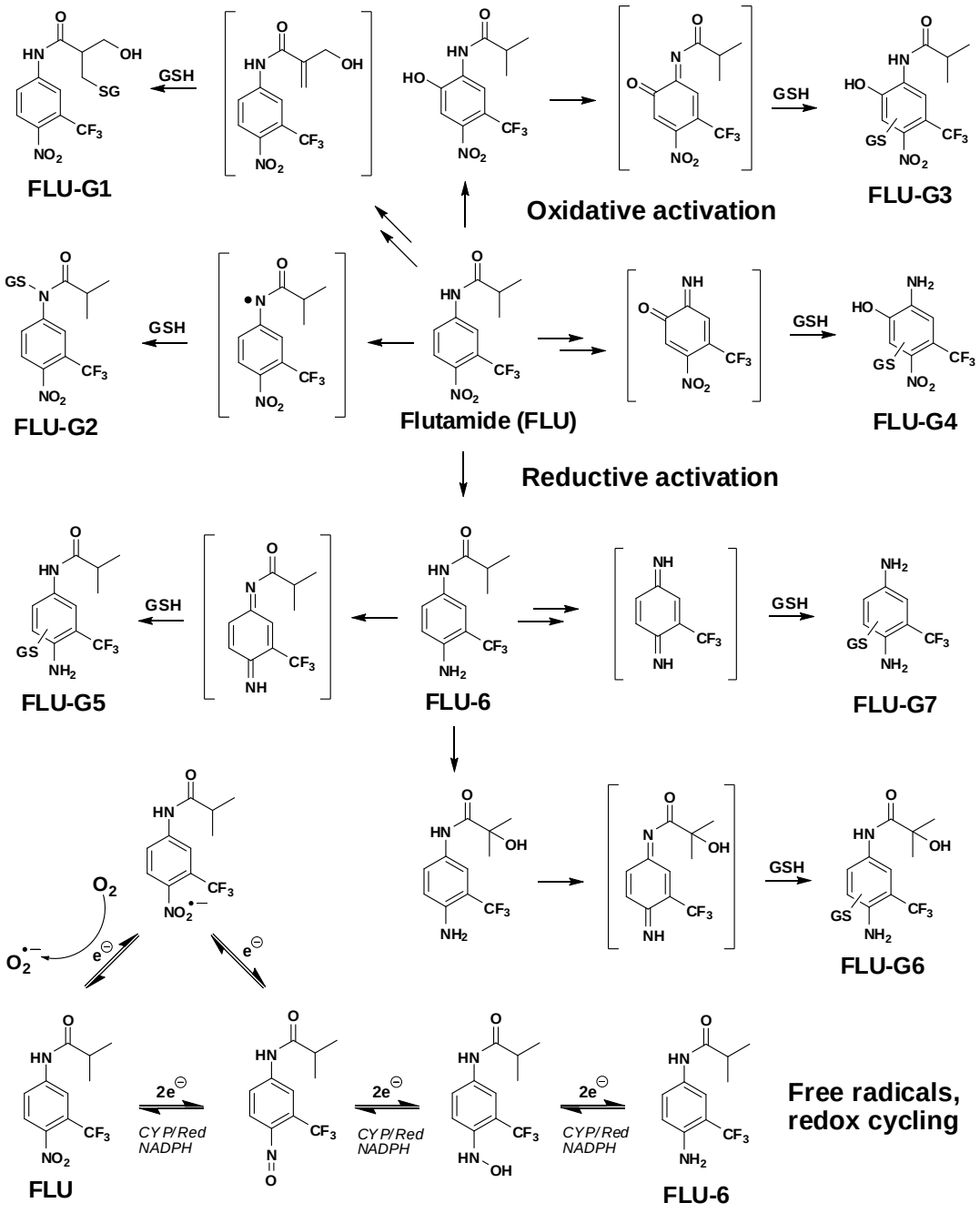


Examples of Anilines or masked anilines: Flutamide

- a nonsteroidal antiandrogen drug that is widely used for the treatment of prostate cancer
- Nitroaromatics associated with idiosyncratic h
  p

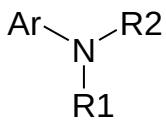


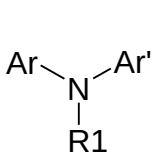
* Rare but severe liver injury caused by flutamide in patients

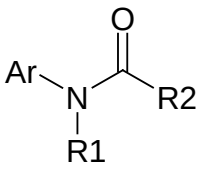


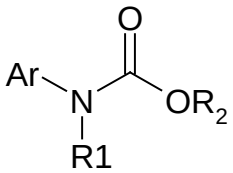
Wen B, et al. *Chem. Res. Toxicol.* 21:2393-2406, 2008

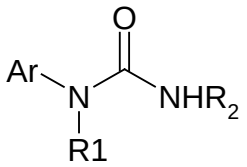
Masked arylamine linkages and relative risk assessment for enzyme-mediated arylamine release







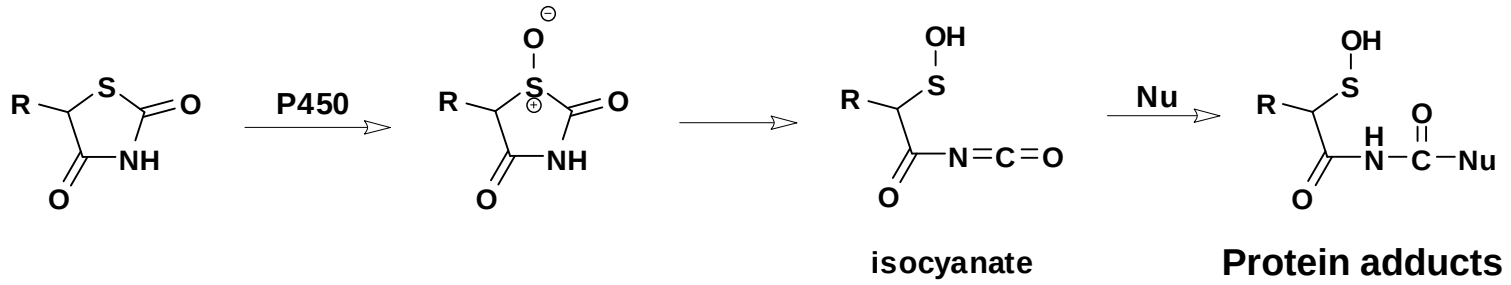




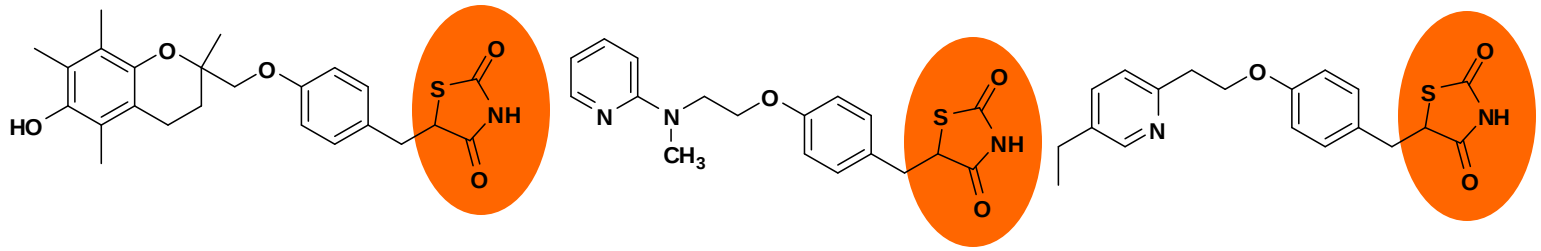
- Amide linkage: **high** risk for releasing arylamine
- Alkyl or carbamate linkage: **moderate** risk for releasing arylamine
- Aryl or urea linkage: **low** risk for releasing arylamine

Kalgutkar AS and Soglia JR. *Expert Opin Drug Metab Toxicol.* 1:91-142 (2005)
Wen B and Fitch WL, *Expert Opin Drug Metab Toxicol.* 5:39-55 (2009)

Mechanism of bioactivation – Thiazolidinediones



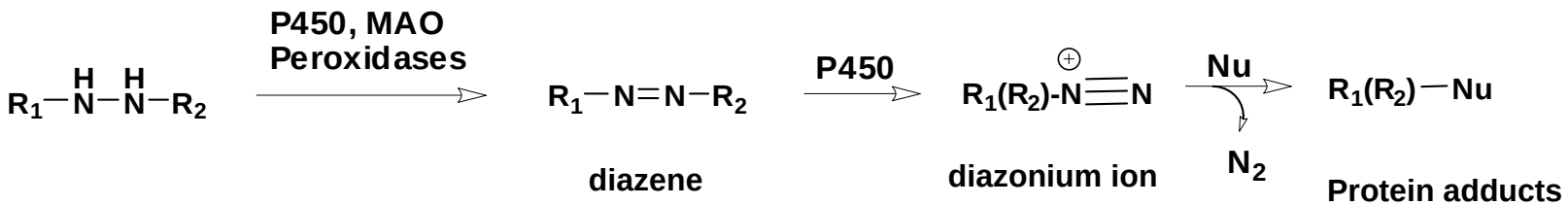
Examples



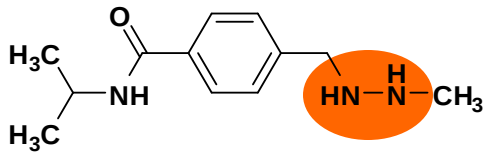
Troglitazone **Rosiglitazone** **Pioglitazone**

Kassahun K, et al, *Chem. Res. Toxicol.* 14:62-70 (2001)

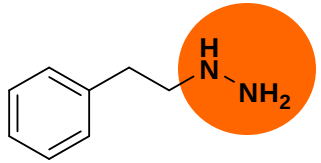
Mechanism of bioactivation – Hydrazines, Hydrazides



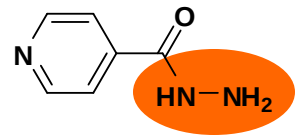
Examples



Procarbazine

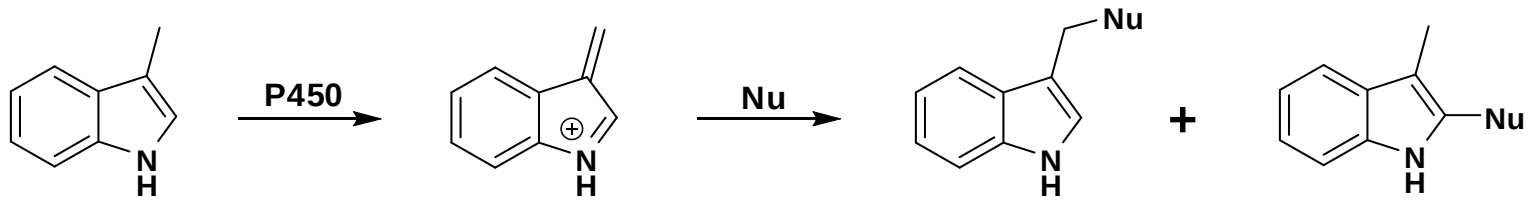


Phenelzine



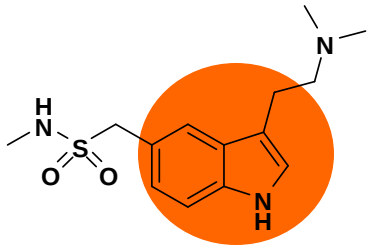
Isoniazid

Mechanism of bioactivation – 3-Alkylindoles and analogs

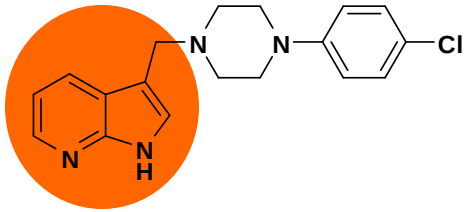


Protein adducts

Examples



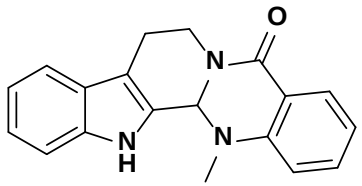
Sumatriptan



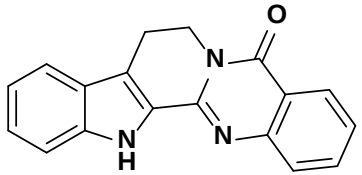
L-745,870

Kalgutkar AS, et al. Curr Drug Metab. 6:161-225 (2005)

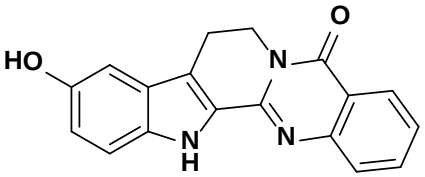
Examples of 3-Alkylindoles and analogs: Evodiamine and Rutaecarpine



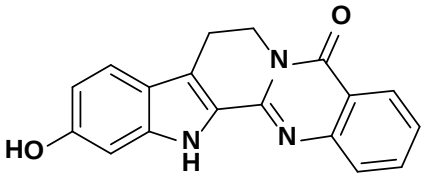
Evodiamine



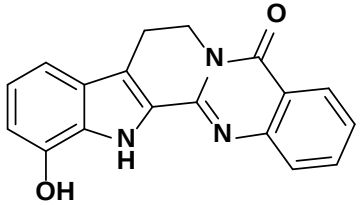
Rutaecarpine



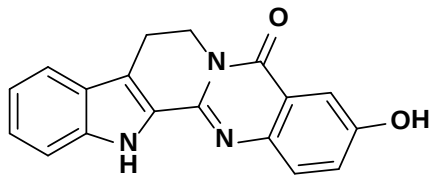
10-Hydroxyrutaecarpine



11-Hydroxyrutaecarpine



12-Hydroxyrutaecarpine



3-Hydroxyrutaecarpine

- Main active alkaloids of the herbal medicine Evodia rutaecarpa (吴茱萸); 具有镇痛、降血压及体温上升等药理作用。用作利尿剂和发汗剂。
- However, immune-mediated toxicity and acute toxicity were reported in mice.
- Mechanism-base inactivation of CYP3A4 and CYP1A2 by Rutaecarpine.

Wen B, et al, *Drug Metab. Dispos.* 42:1044-1054 (2014)
Zhang FL, et al, *Xenobiotica*, 45:978-89 (2015)

Examples of 3-Alkylindoles and analogs: Evodiamine and Rutaecarpine

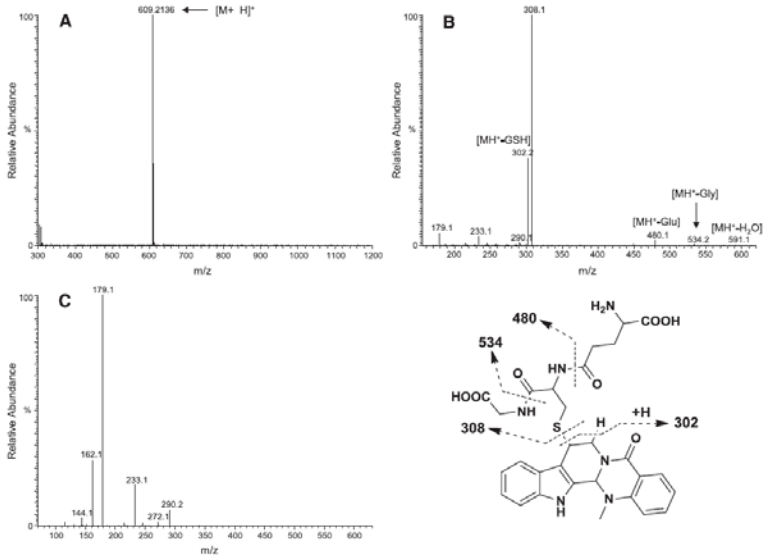


Fig. 3. Mass spectra of evodiamine GSH conjugate (EM1) obtained in the positive ion mode. (A) Full-scan mass spectrum of EM1. (B) MS/MS spectrum of EM1 at *m/z* 609 ([M + H]+). (C) MS^3 mass spectrum of the fragment ion of EM1 at *m/z* 308.

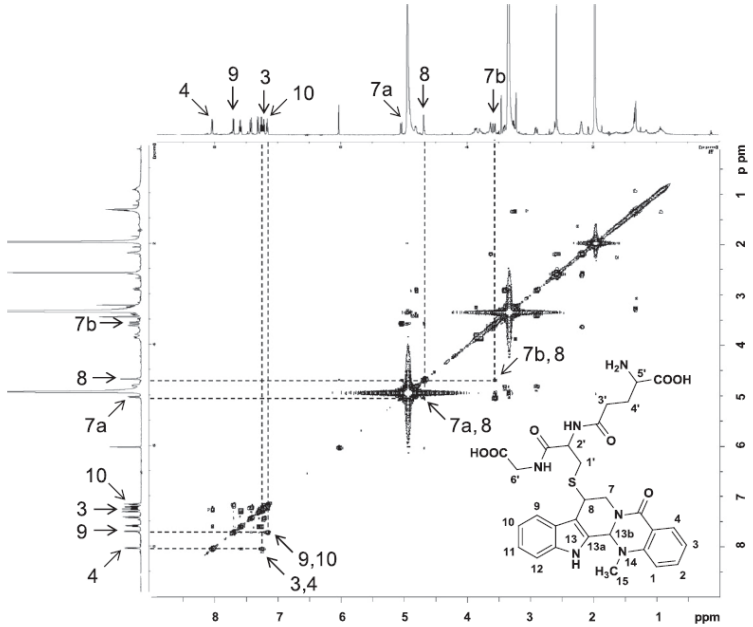


Fig. 7. ^1H-^1H COSY spectrum of EM1 showing the coupling between aliphatic protons at C-7 and C-8.

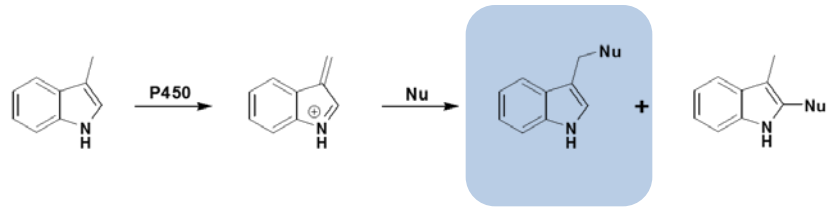
TABLE 1

^1H NMR data of evodiamine and its GSH conjugate (EM1) (δ in CD_3OD)

Refer to Fig. 7 for signal assignments of the thioether moiety.

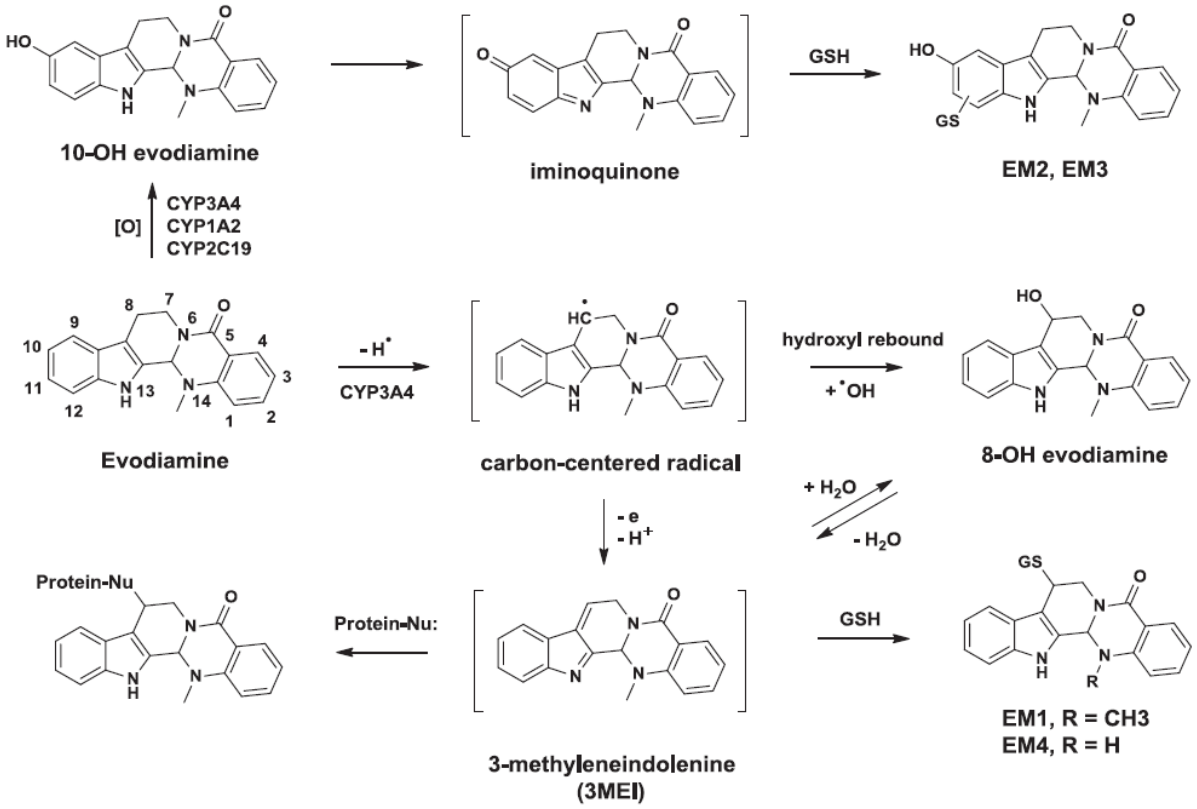
	Proton Signals	
	Evodiamine	EM1
C-1	7.24 (1H, bd, *J* = 8.1 Hz)	7.30 (1H, bd, *J* = 8.2 Hz)
C-2	7.56 (1H, ddd, *J* = 8.1, 7.9 and 1.8 Hz)	7.59 (1H. bdd, *J* = 8.2, 7.9 and 1.8 Hz)
C-3	7.16 (1H, bdd, *J* = 8.2 and 7.9 Hz)	7.26 (1H, bdd, *J* = 8.2 and 7.9 Hz)
C-4	7.98 (1H, dd, *J* = 8.2 and 1.8 Hz)	8.03 (1H, dd, *J* = 8.2 and 1.8 Hz)
C-9	7.57 (1H, bd, *J* = 8.5 Hz)	7.70 (1H, bd, *J* = 8.5 Hz)
C-10	7.09 (1H, ddd, *J* = 8.5, 8.2 and 2.1 Hz)	7.16 (1H, ddd, *J* = 8.5, 8.2 and 2.1 Hz)
C-11	7.19 (1H, bdd, *J* = 8.4 and 8.2 Hz)	7.22 (1H, bdd, *J* = 8.3 and 8.2 Hz)
C-12	7.42 (1H, dd, *J* = 8.4 and 2.1 Hz)	7.42 (1H, dd, *J* = 8.3 and 2.1 Hz)
C-13b	6.03 (1H, s)	6.03 (1H, s)
C-15	2.68 (3H, s)	2.58 (3H, s)
C-7a	4.81 (1H, dd, *J* = 5.8 and 3.1 Hz)	5.03 (1H, dd, *J* = 5.3 and 1.4 Hz)
C-7b	3.31 (1H, dd, *J* = 5.8 and 3.2 Hz)	3.55 (1H, dd, *J* = 5.3 and 1.6 Hz)
C-8	2.99 (2H, dd, *J* = 3.1 and 3.2 Hz)	4.68 (1H, dd, *J* = 1.4 and 1.6 Hz)

cysteine 1′ –H, δ 2.9/3.4; cysteine 2′ –H, δ 4.8; glutamate 3′ –H, δ 2.6; glutamate 4′ –H, δ 2.1/2.3; glutamate 5′ –H, δ 3.6; glycine 6′ –H, δ 3.8.



Wen B, et al, *Drug Metab. Dispos.* 42:1044-1054 (2014)

Examples of 3-Alkylindoles and analogs: Evodiamine and Rutaecarpine



Scheme 1. Proposed mechanisms for the bioactivation and formation of GSH conjugates of evodiamine in human liver microsomes.

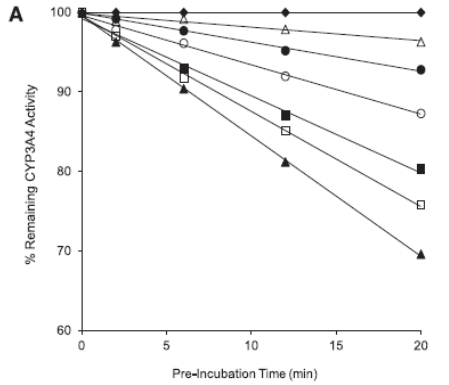


Fig. 9. (A) Time- and concentration-dependent inactivation of CYP3A4 by evodiamine. ♦, 0 μM;Δ, 1 μM;●, 3 μM;○, 10 μM; ■, 30 μM; □, 50 μM;▲, 100 μM. (B) The hyperbolic plot of k_{obs} versus evodiamine concentrations in HLM.

- The 3-alkylindole moiety in evodiamine and rutaecarpine undergoes CYP-catalyzed dehydrogenation to form an electrophile 3-methyleneindolenine.
- Evodiamine was a mechanism-based inactivator of CYP3A4, with K_I = 29 μM and k_{inact} = 0.029 minute^{-1}, respectively.

Wen B, et al, *Drug Metab. Dispos.* 42:1044-1054 (2014)

Mechanism of bioactivation – Alicyclic Amines

Drug Metabolism Reviews

http://informahealthcare.com/dmr
ISSN: 0360-2532 (print), 1097-9883 (electronic)

Drug Metab Rev, 2014; 46(3): 379–419
 DOI: 10.3109/03602532.2014.924962

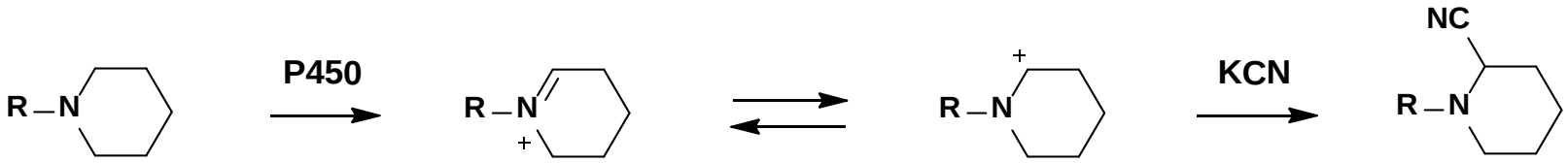
informa healthcare

REVIEW ARTICLE

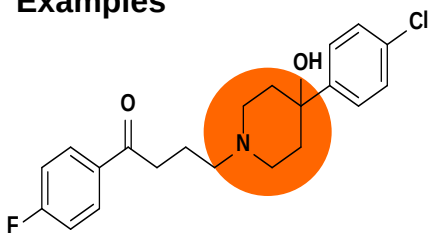
Biotransformation and bioactivation reactions of alicyclic amines in drug molecules

Jayaprakasam Bolleddula, Kevin DeMent, James P. Driscoll*, Philip Worboys, Patrick J. Brassil, and David L. Bourdet

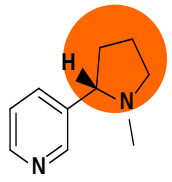
Department of Drug Metabolism and Pharmacokinetics, Theravance, Inc., 901 Gateway Blvd, South San Francisco, CA, USA



Examples



Haloperidol

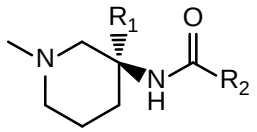


(*S*)-Nicotine

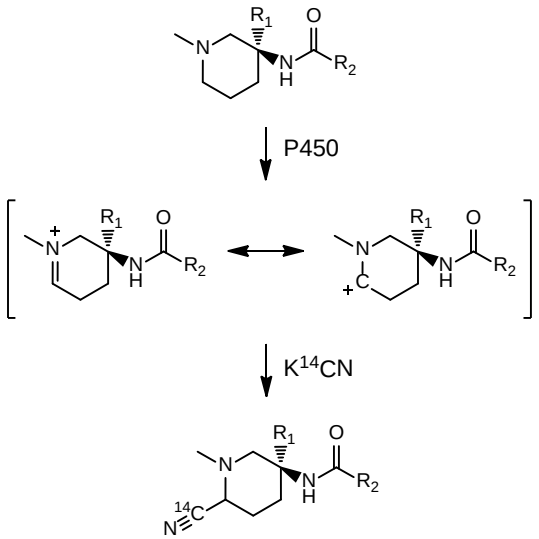
Bolleddula J, et al, *Drug Metab Rev. 46:379-419 (2014)*

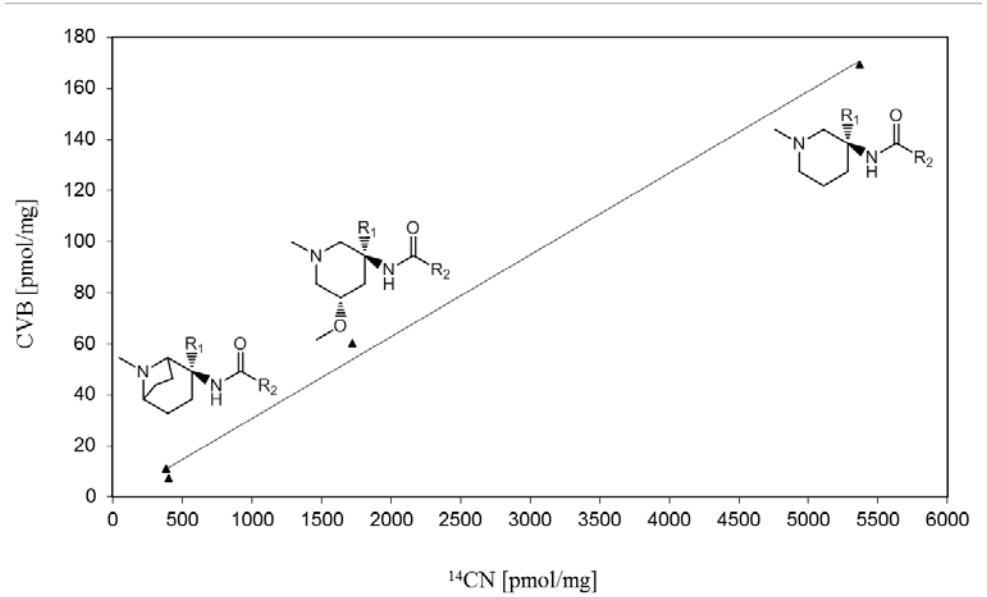
Examples of Cyclic Amines

QSAR and Association of ^{14}CN Trapping and CVB



- No GSH adduct formation was observed within the entire chemical series
- CVB values clearly exceeded 100 pmol/mg protein (HLM:170)
- GSH assay was not predictive for high CVB





- The results suggested that CVB was mediated by iminium ion formation, which could not be trapped by the ‘soft’ nucleophile GSH.
- Quantitative ^{14}CN-based cyanide trapping should be considered for GSH negative compounds upon ‘structural alert’.
- SAR: Substitutions on the piperidine ring substantially decrease ^{14}CN trapping and CVB.

Non-CYP mediated bioactivation: a review

DRUG METABOLISM REVIEWS, 2016
VOL. 48, NO. 4, 473–501
http://dx.doi.org/10.1080/03602532.2016.1225756

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

Non-cytochrome P450-mediated bioactivation and its toxicological relevance

Jinping Gan[a], Shuguang Ma[b] and Donglu Zhang[b]*

[a]Pharmaceutical Candidate Optimization, Bristol-Myers Squibb, Princeton, NJ, USA; [b]Department of Drug Metabolism & Pharmacokinetics, Genentech, South San Francisco, CA, USA

ABSTRACT

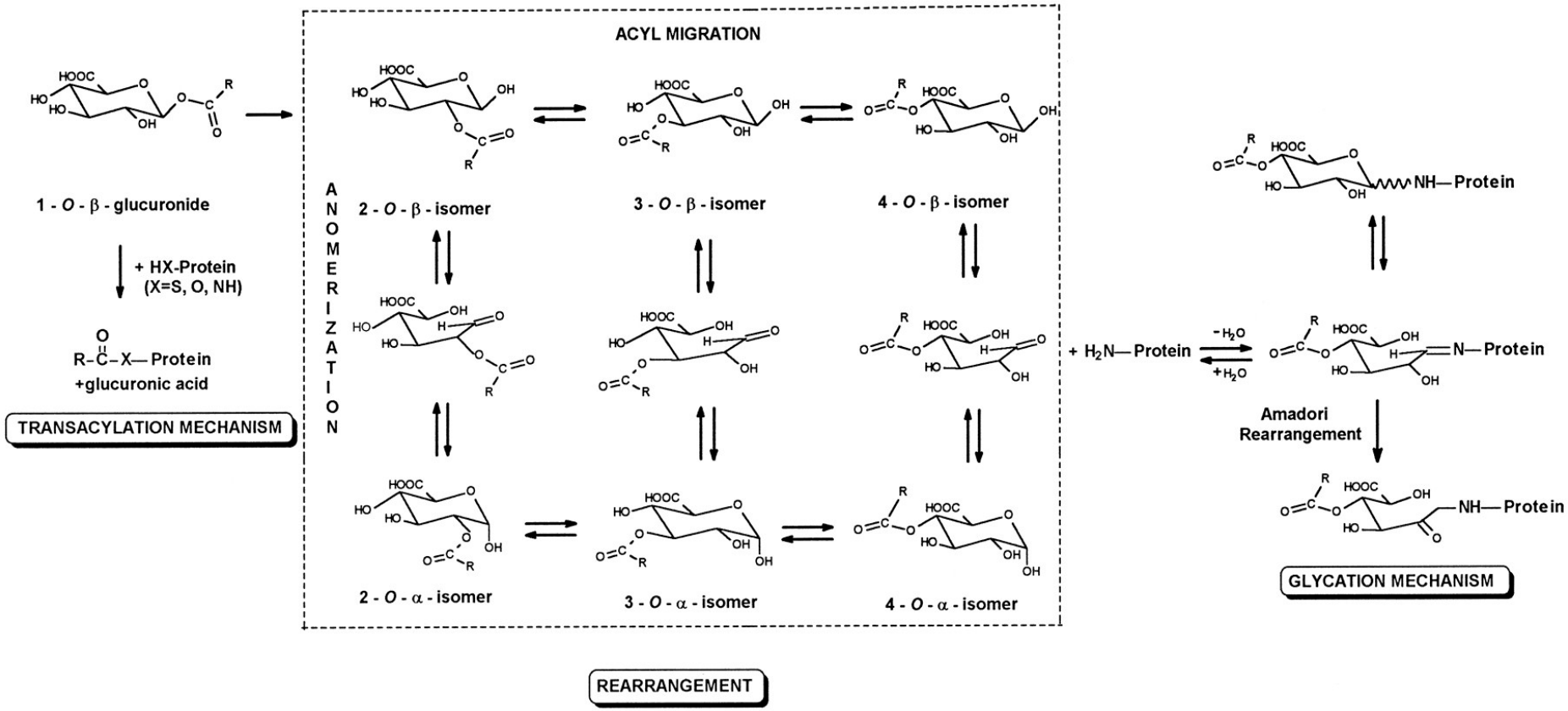
The bioactivation of drugs is often associated with toxicological outcomes; however, for most cases, the causal relationship between bioactivation and toxicity is not well established despite extensive research that attempts to elucidate the mechanisms leading to the formation of chemically reactive species, presumably the initial step towards adverse reactions. Due to rapid advancement in the research of cytochrome P450s (CYPs) and the prevalence of CYP involvement in the metabolic clearance of pharmaceuticals, CYP-mediated bioactivation is widely investigated and reviewed, while non-CYP-mediated bioactivation has not been emphasized. The widespread use of metabolic stability screening in drug discovery, however, has led to the identification of new chemical entities that rely on non-CYP enzymes for clearance, and the number of drugs that undergo metabolism via these enzymes has increased. Non-CYP enzymes can be divided into four general categories according to their enzymatic function, namely, oxidative, reductive, conjugative and hydrolytic. The aim of this review is to complement the existing literature on CYP-mediated metabolism by focusing on bioactivation mediated non-CYP enzymes and provide representative examples in each category.

Abbreviations: ABCB1: p-glycoprotein efflux transporter; ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase; CES: carboxylesterase; CYP: cytochrome P450 enzymes; DA: dopamine; DOPAL: 3,4-dihydroxyphenyl acetaldehyde; EH: epoxide hydrolase; FAD: flavin adenine dinucleotide; FMO: flavin-containing monooxygenase; GGT: γ-glutamyl transpeptidase; GSH: glutathione; GST: glutathione-S-transferase; IDR: idiosyncratic reactions; KT: ketoconazole; MAO: monoamine oxidase; MPO: myeloperoxidase; MPP+: N-methyl-4-phenylpyridinium; MPTP: N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NADH: reduced form nicotinamide adenine dinucleotide; NADPH: nicotinamide adenine dinucleotide phosphate; NAT: N-acetyltransferase; NCE: new chemical entity; SNP: single-nucleotide polymorphism; SULT: sulfotransferase; UGT: uridine 5′-diphosphoglucuronosyltransferase.

ARTICLE HISTORY
Received 1 May 2016
Revised 12 August 2016
Accepted 12 August 2016
Published online 12 September 2016

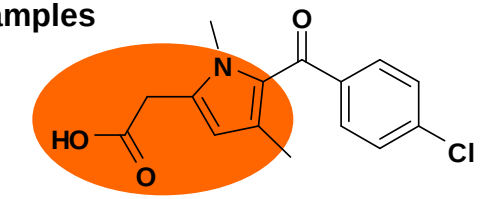
KEYWORDS
Drug bioactivation; non-CYP enzymes; reductive reactions; conjugation reactions; hydrolytic reactions

Mechanism of bioactivation – Acyl glucuronides

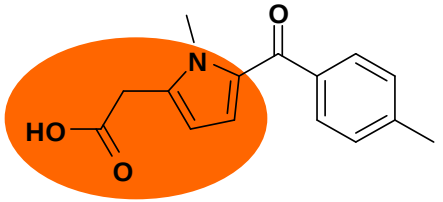


- The half-life and the rearrangement rate of the primary 1β AG isomer reflect its reactivity.

Examples



Zomepirac



Tolmetin

Assessing Stability and Toxicity of Acyl Glucuronides

1) The stability of acyl glucuronides is assessed in 100 mM phosphate buffer, pH 7.4, at 37C
2) The half-life of the parent acyl glucuronide is determined
3) If the half-life is less than 0.5 hour, the acyl glucuronide is likely to cause liver toxicity (or possibly other adverse effects) if drug is given at high dose and acyl glucuronide formation is major metabolic pathway

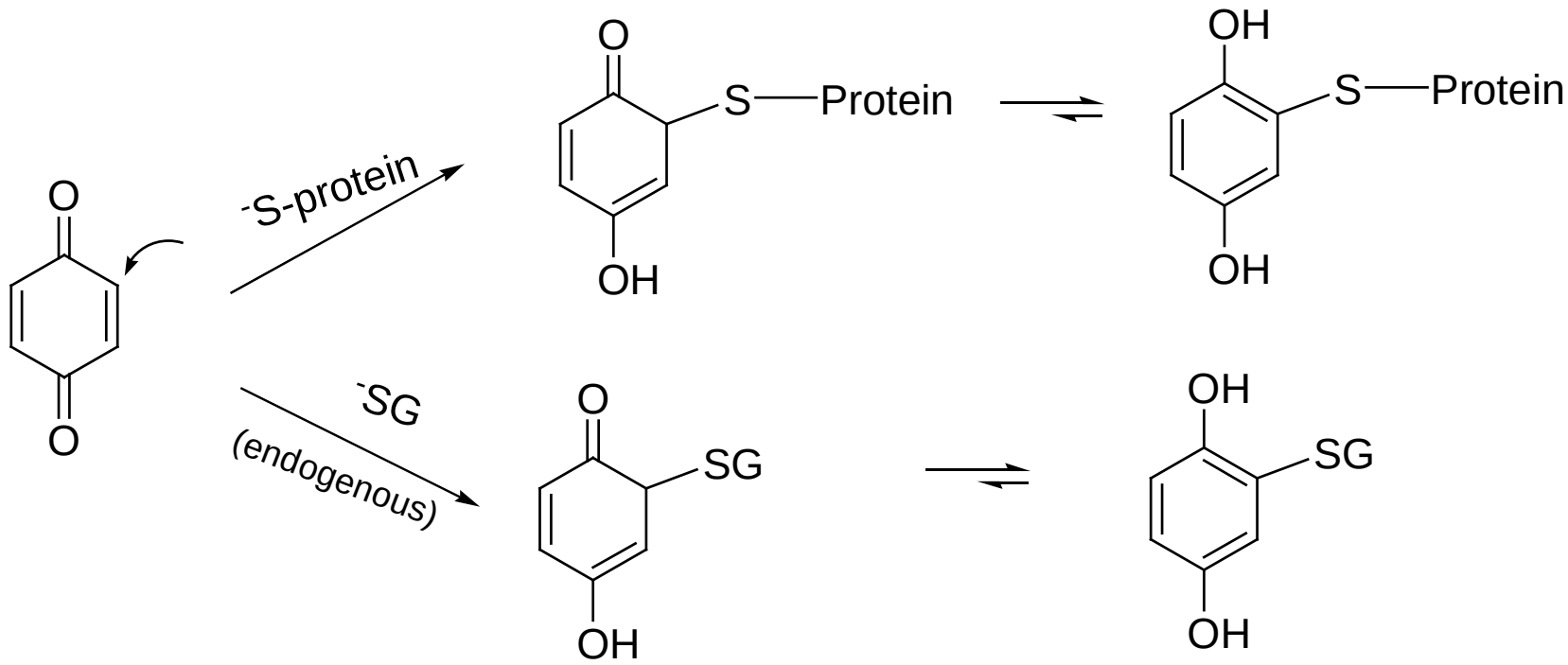
Acid drug	Half-life (h)
Tolmetin*	0.26
Isoxepac*	0.29
Probenecid	0.40
Zenarestat*	0.42
Zomepirac*	0.45
Diclofenac	0.51
Diflunisal	0.67
(R)-Naproxen	0.92
Salicylic acid	1.3
Indomethacin	1.4
(S)-Naproxen	1.8
Ibuprofen	3.3
Bilirubin	4.4
Furosemide	5.3
Flufenamic acid	7.0
Clofibric acid	7.3
Mefenamic acid	16.5
Telmisartan	26
Gemfibrozil	44
Valproic acid	79
*withdrawn	

Boelsterli UA, *Curr. Drug Metab.* 3:439-450 (2002).
Skonberg C, et al. *Expert Opin Drug Metab Toxicol.* 4:425-438 (2008)

4. Experimental strategies to detect reactive metabolites
(具有反应活性代谢物的常用分析检测方法)

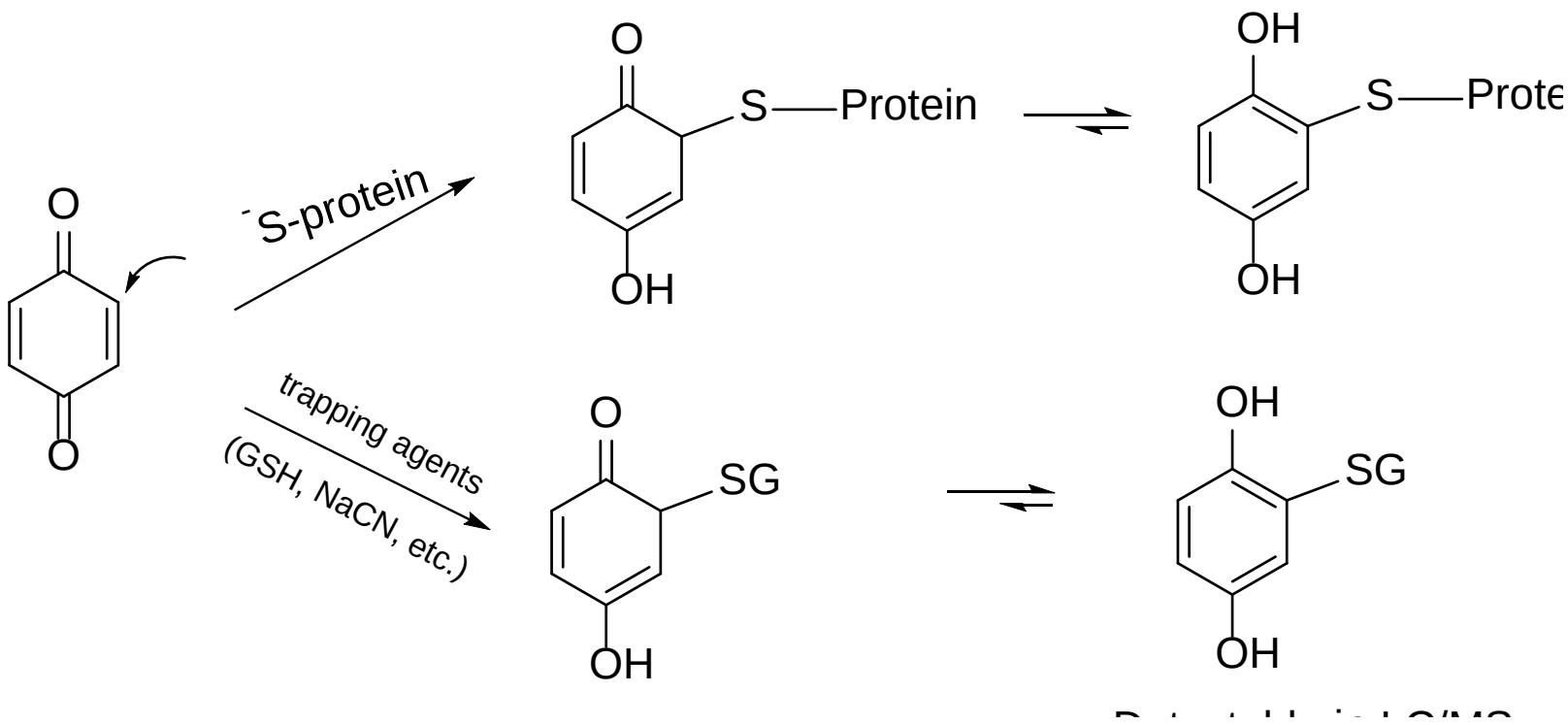
a. Common trapping agents for reactive metabolites (常用诱捕剂)

Formation of drug-protein and drug-GSH adducts



Detectable in LC/MS

In vitro trapping studies

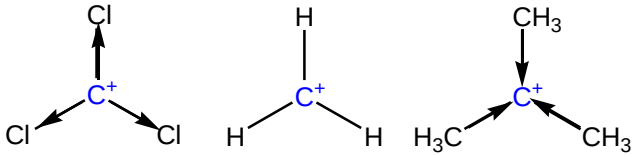


57

“Hard” vs. “soft” electrophiles/nucleophiles

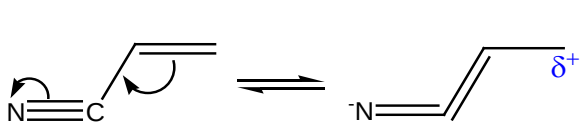
- Chemical “hardness” and “softness” is a function of polarization.

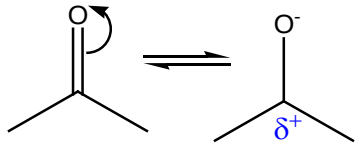
- Hard electrophiles have high positive charge density at the electrophilic center (the charge is localized, ex. carbocations)



Harder----------Softer
(EWG) (EDG)

- Soft electrophiles have low positive charge density at the electrophilic center (the charge is delocalized) – often as a result of diffuse electron density of π bond





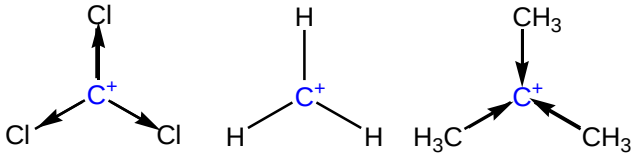
- Hard nucleophiles have high negative charge density (not diffuse and localized charge)

- Soft nucleophiles have less negative charge density (more diffuse and delocalized charge)

“Hard” vs. “soft” electrophiles/nucleophiles

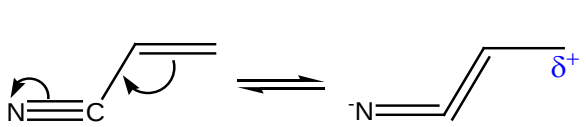
- Chemical “hardness” and “softness” is a function of polarization.

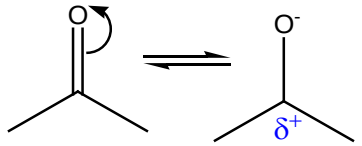
- Hard electrophiles have high positive charge density at the electrophilic center (the charge is localized, ex. carbocations)



Harder----------Softer
(EWG) (EDG)

- Soft electrophiles have low positive charge density at the electrophilic center (the charge is delocalized) – often as a result of diffuse electron density of π bond

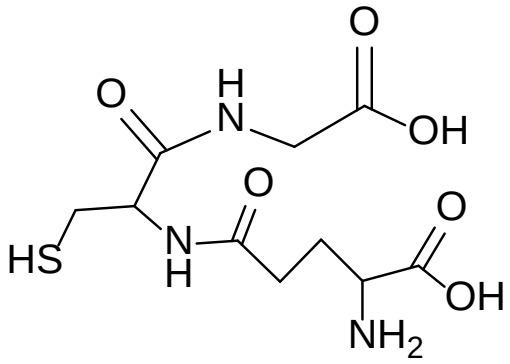




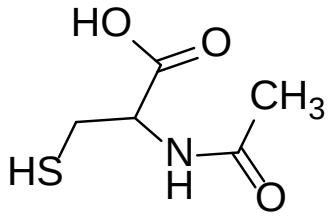
- Hard nucleophiles have high negative charge density (not diffuse and localized charge)

- Soft nucleophiles have less negative charge density (more diffuse and delocalized charge)

Common *in vitro* trapping agents



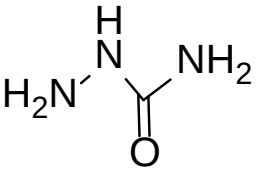
Glutathinone (GSH)



N-acetylcysteine (NAc)

CN^-

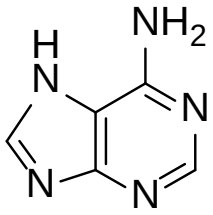
Cyanide



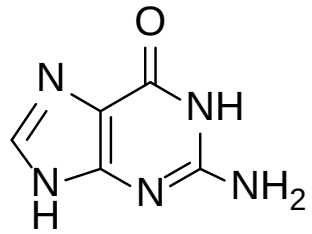
Semicarbazide

NH_2-OCH_3

Hydroxyamine

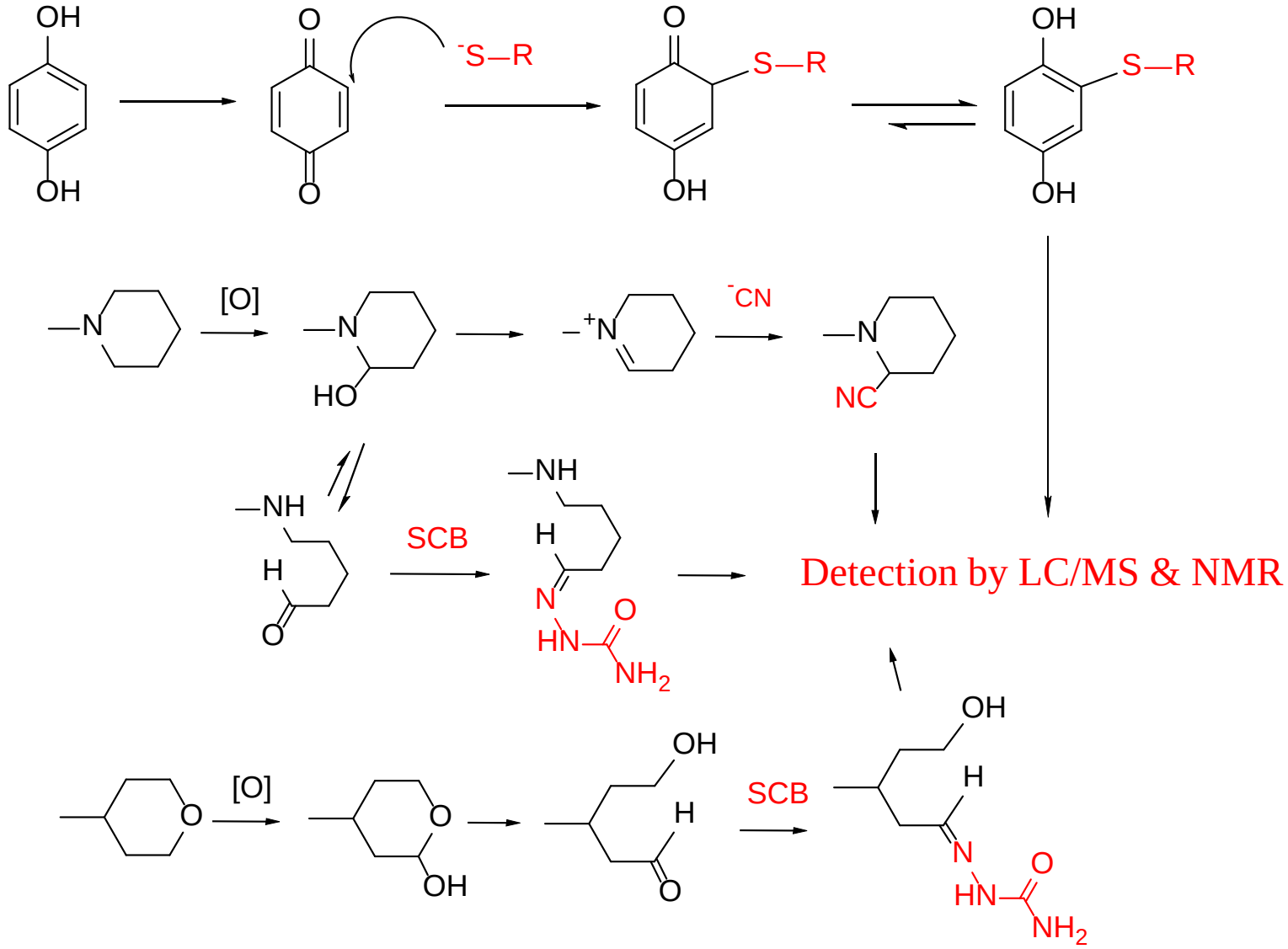


Adenine



Guanine

In vitro trapping



- **Select appropriate trapping agents based on structures of compounds and SAR**

Incubation for trapping reactive metabolites using trapping agents

Human or rat liver microsomes (1 mg protein/mL) are suspended in phosphate buffer (100 mM, pH 7.4) containing EDTA (1 mM), MgCl2 (0.1 mM) and GSH (5 mM or NAc (5 mM) or KCN/K^{13}C^{15}N (1 mM) in a total volume of 1 mL. A test compound in methanol is added to a final concentration of 50 μM, such that the concentration of methanol in the incubation mixture does not exceed 0.2% (v/v). Incubations are performed in the presence of NADPH (1.2 mM) at 37 ºC for 60 min. Control experiments contain liver microsomes and a test compound in the absence of either NADPH or trapping agents. The reaction is quenched by adding 2 mL of acetonitrile. The suspension then is sonicated for 5 min and centrifuged at 20,800 × *g* for 10 min. The supernatants are removed and the pellets are extracted twice with 1 mL of methanol-water (3:1, v/v). The extracts are combined with the above supernatants and are evaporated to dryness under nitrogen at room temperature. The residues are dissolved in 300 μL of methanol-water (3:1, v/v), and an aliquot (75 μL) is loaded onto an HPLC column for LC/MS/MS analysis.

(Zhang Z. et al. In: *Drug Metabolism in Drug Design and Development* (2008) p447-476)

Incubation for trapping reactive metabolites using trapping agents

(simplified)

1 mL (1 mg protein/mL) of human or rat liver microsomes + GSH (5 mM) or

NaCN (1 mM) + 10 - 50 μM test compound + NADPH (1.2 mM) --- >

at 37 ºC for 0 and 60 min --- > quenched with acetonitrile (2 mL) --- >

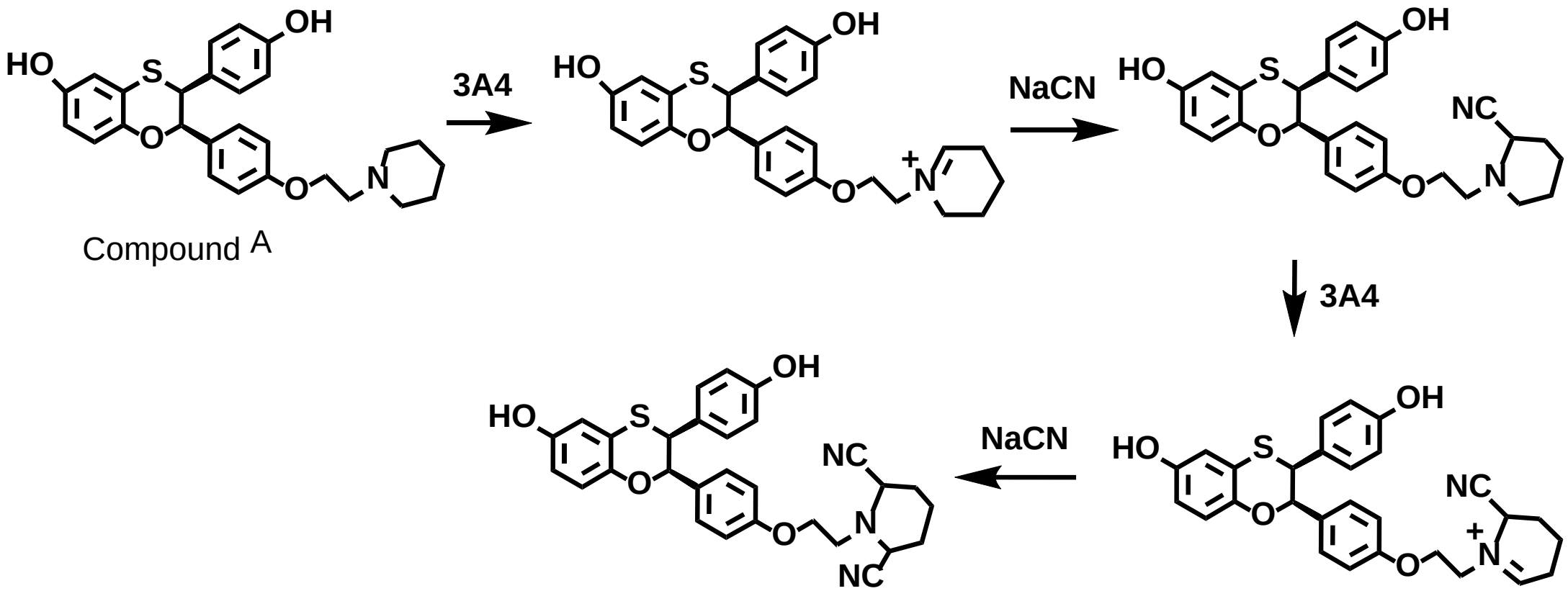
Sonicated for 5 min and centrifuged at 20,800 × *g* for 10 min -- >

The supernatants evaporated to dryness under N2 at room temperature -- >

The residues reconstituted in MeOH-water (3:1, v/v) for LC/MS.

(Zhang Z. et al. In: *Drug Metabolism in Drug Design and Development* (2008) p447-476)

Formation of a bis-cyano adduct of Compound A in CYP3A4

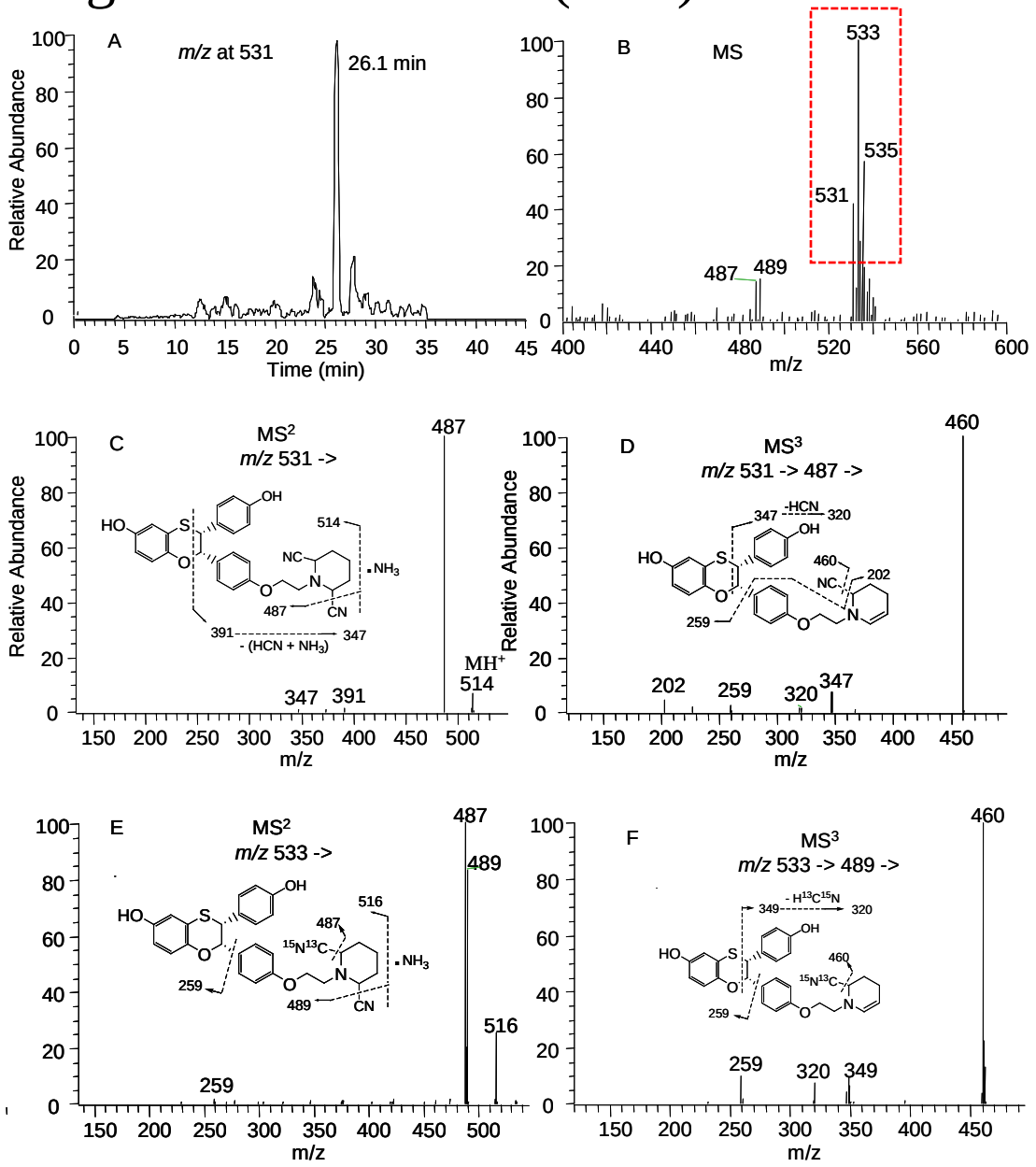


Compound A

(Zhang, et al., Chem. Res. Toxicol. 2005, 18, 675)

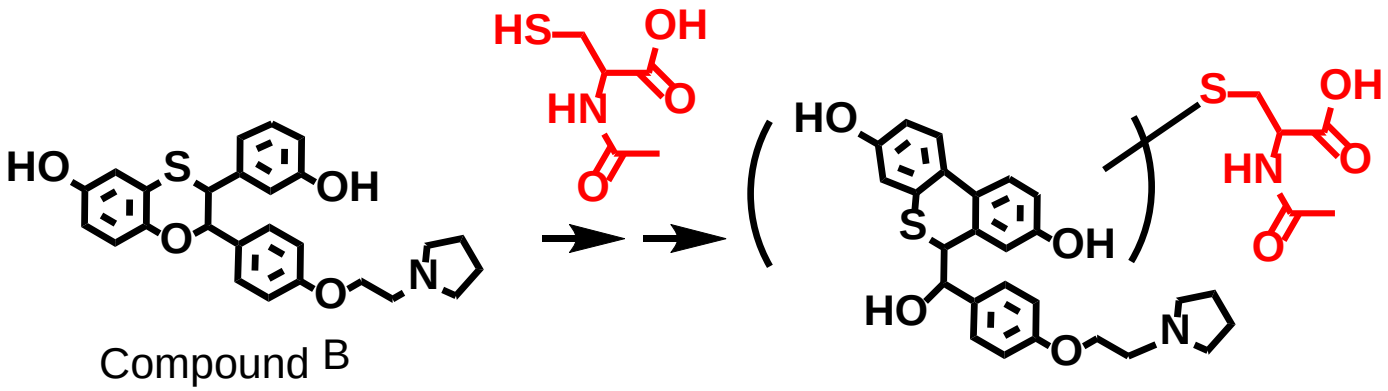
64

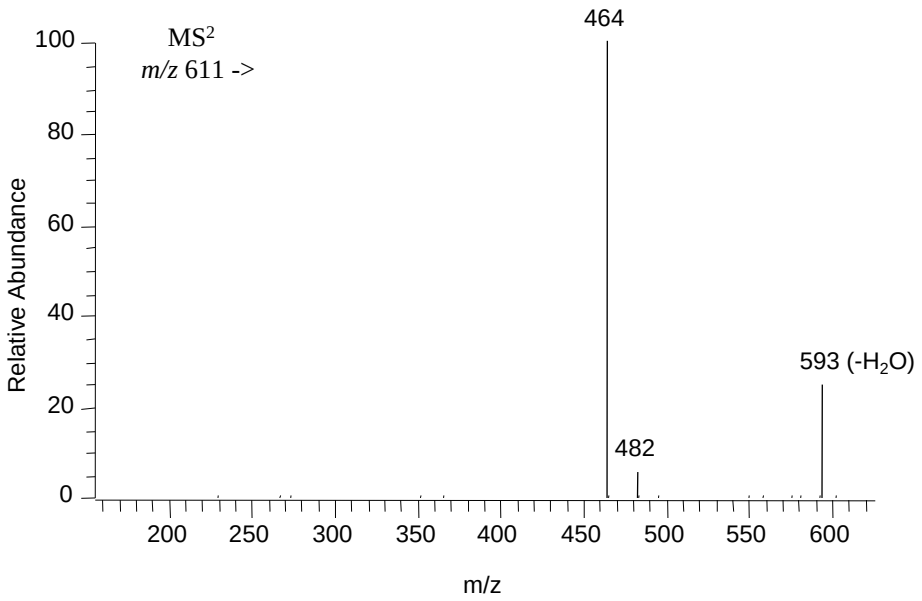
Formation of a Bis-cyano Adduct of Compound A in HLM Using NaCN-Na^{13}C^{15}N (1 : 1) as an In Vitro Trapping Agent

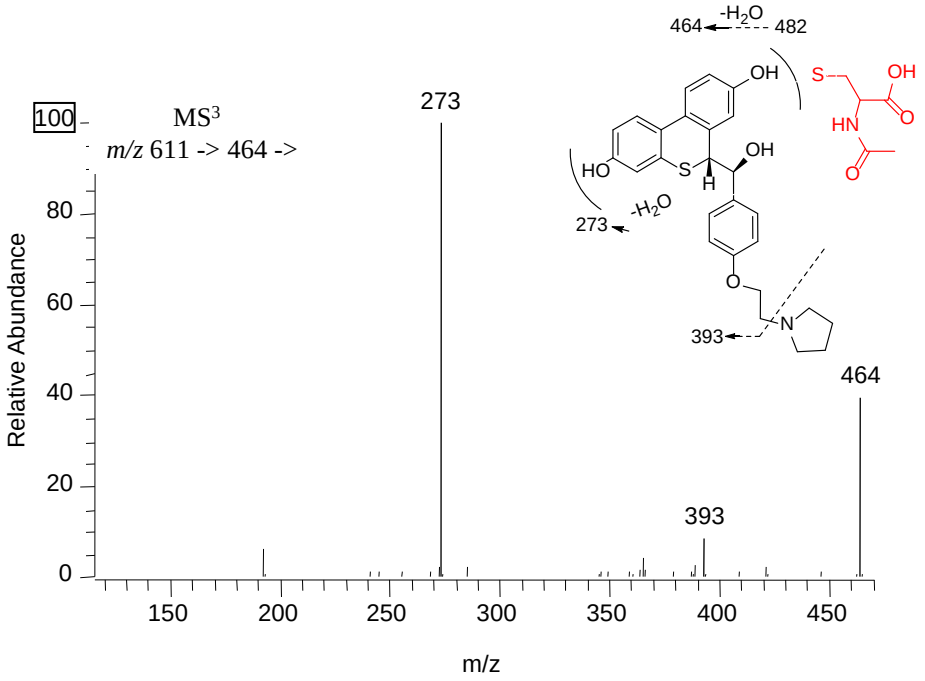


(Zhang, et al., Chem. Res. Toxicol. 2005, 18, 675)

LC/MS/MS analysis of the major NAc adduct of II in HLM and CYP3A4

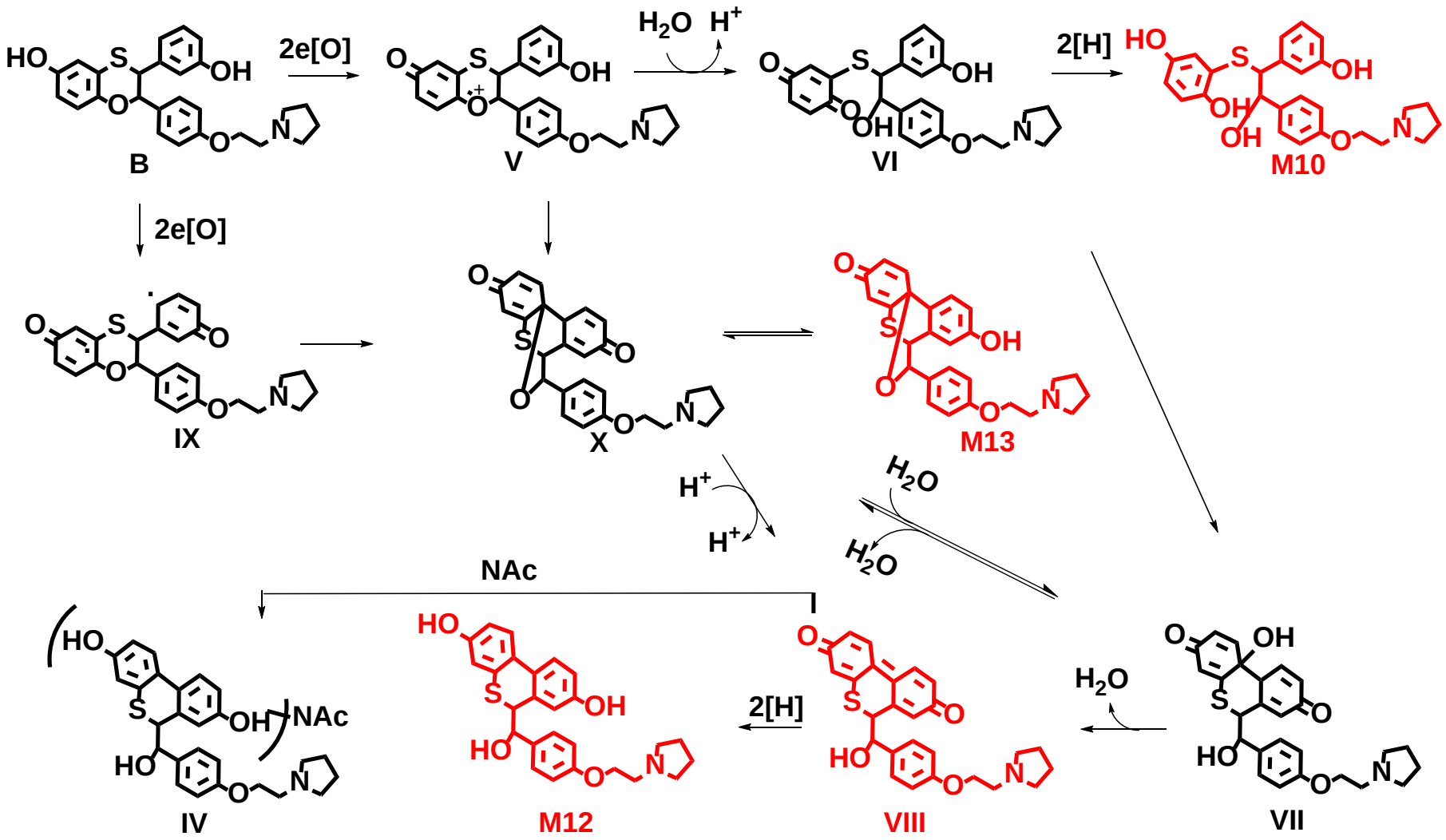






(Zhang, et al., Chem. Res. Toxicol. 2005, 18, 675)

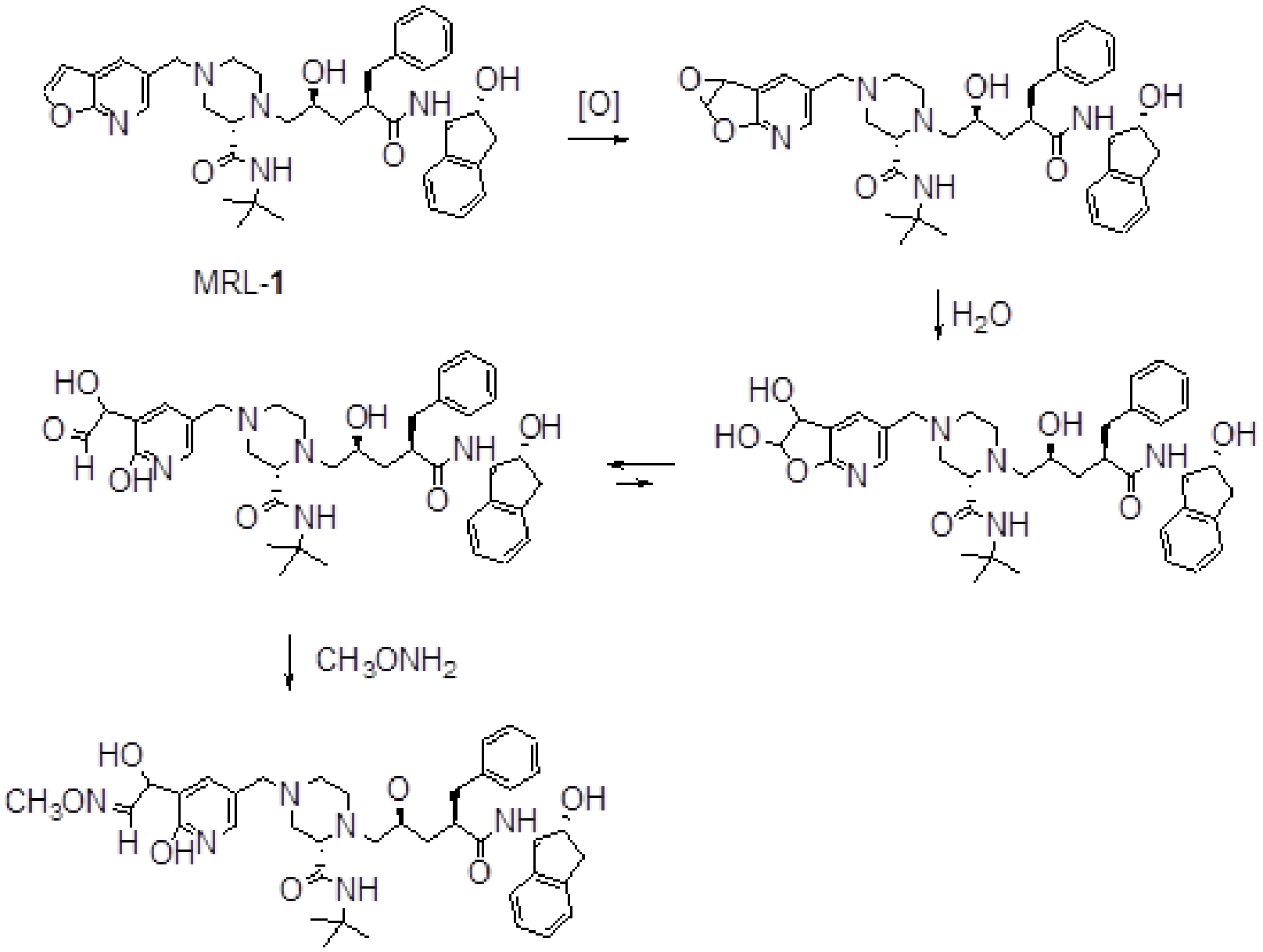
CYP3A4-mediated biotransformation of compound B



(Zhang, et al., Chem. Res. Toxicol. 2005, 18, 675)

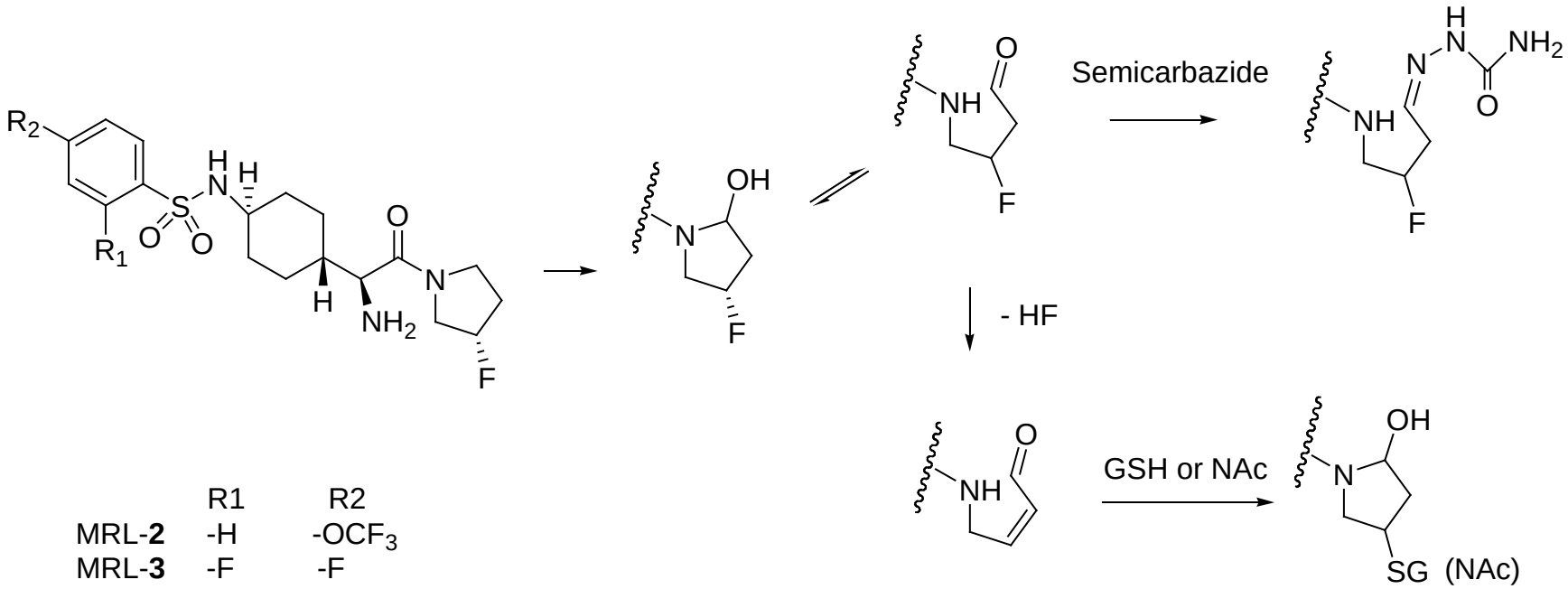
67

Formation of methyloxime adducts of MRL-1 with methoxyamine



(Sahali-Sahly Y, et al. Chem. Res. Toxicol 1996, 9:1007)

Formation of adducts of MRL-2 and MRL-3 with semicarbazide or GSH/NAc



	R1	R2
MRL-**2**	-H	-OCF_3
MRL-**3**	-F	-F

(Xu S, et al. Drug Metab. Dispos. 2005, 33:121)

LC/MS methods for detection of GSH/cyano adducts

1. **HRMS instruments**

2. **Q-trap:**
   EMS-EPI
   NL/Prec -EPI

3. **QQQ:**
   Neutral loss of m/z 129 (GSH adducts)
   Neutral loss of m/z 27 (Cyano adducts)

4. **Ion trap: Data-dependent MS^n**

(Zhang, Z, et al. Current Pharmaceutical Design 2009, 15:2220)

Common LC/MS Platforms Used for Metabolite Identification

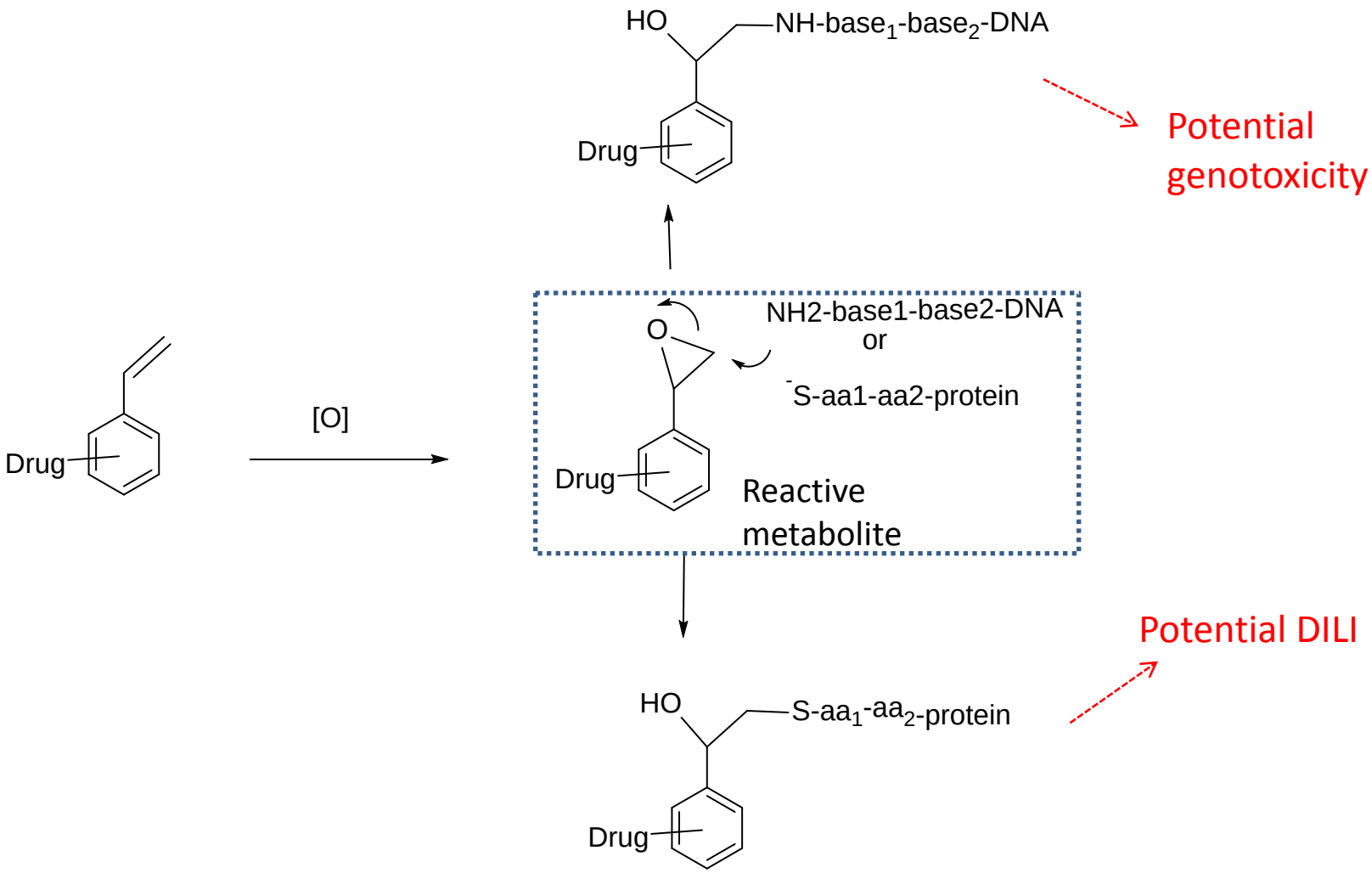
LC/MS instrument	Scan functions for metabolite ID	Preferred method for GSH adduct screening	Preferred method for metabolic soft spot determination	Additional comments
Triple quadrupole	PI, NL and MRM	• Negative PI scan followed by positive product ion scan[90] • NL scan using stable labeled GSH as a trapping agent	Not available	• Able to search for uncommon metabolites • New models provide NL-, PI- or MRM-dependent MS/MS scanning function
Ion trap	Full MS scan and Data-dependent MS/MS scan	Isotope-dependent MS/MS scan followed by post-acquisition data processing using NLF and PIF	Intensity dependent MS/MS scan followed by data mining	• Not suitable for low levels of uncommon oxidative metabolites • MS^n capability • Sensitive full scan MS-dependent MS/MS acquisition
Triple quadrupole-linear ion trap	Full MS scan-, PI-, NL-, MRM- and MIM-EPI.	• PI-EPI combined with polarity switching	EMS-EPI or MIM-EPI-EMS followed by data mining	• Well suited for high throughput screening of reactive metabolites. • Useful in both metabolite profiling and drug and metabolite quantification • Sensitive and selective MRM-EPI
LTQ-Orbitrap	Full MS scan and data-dependent MS/MS scan	• Intensity-dependent MS/MS scan followed by MDF or background subtraction[100] • Isotope-dependent MS/MS scan followed by NLF	Intensity-dependent MS/MS scan followed by data mining	• Suitable for high throughput profiling of various metabolites
Q-tof	Full MS scan and MS^E	MS^E scan followed by data mining	MS^E scan followed by data mining	• Suitable for high throughput profiling of various metabolites

(Zhang, Z, et al. Current Pharmaceutical Design 2009, 15:2220)

4. Experimental strategies to detect reactive metabolites
(具有反应活性代谢物的常用分析检测方法)

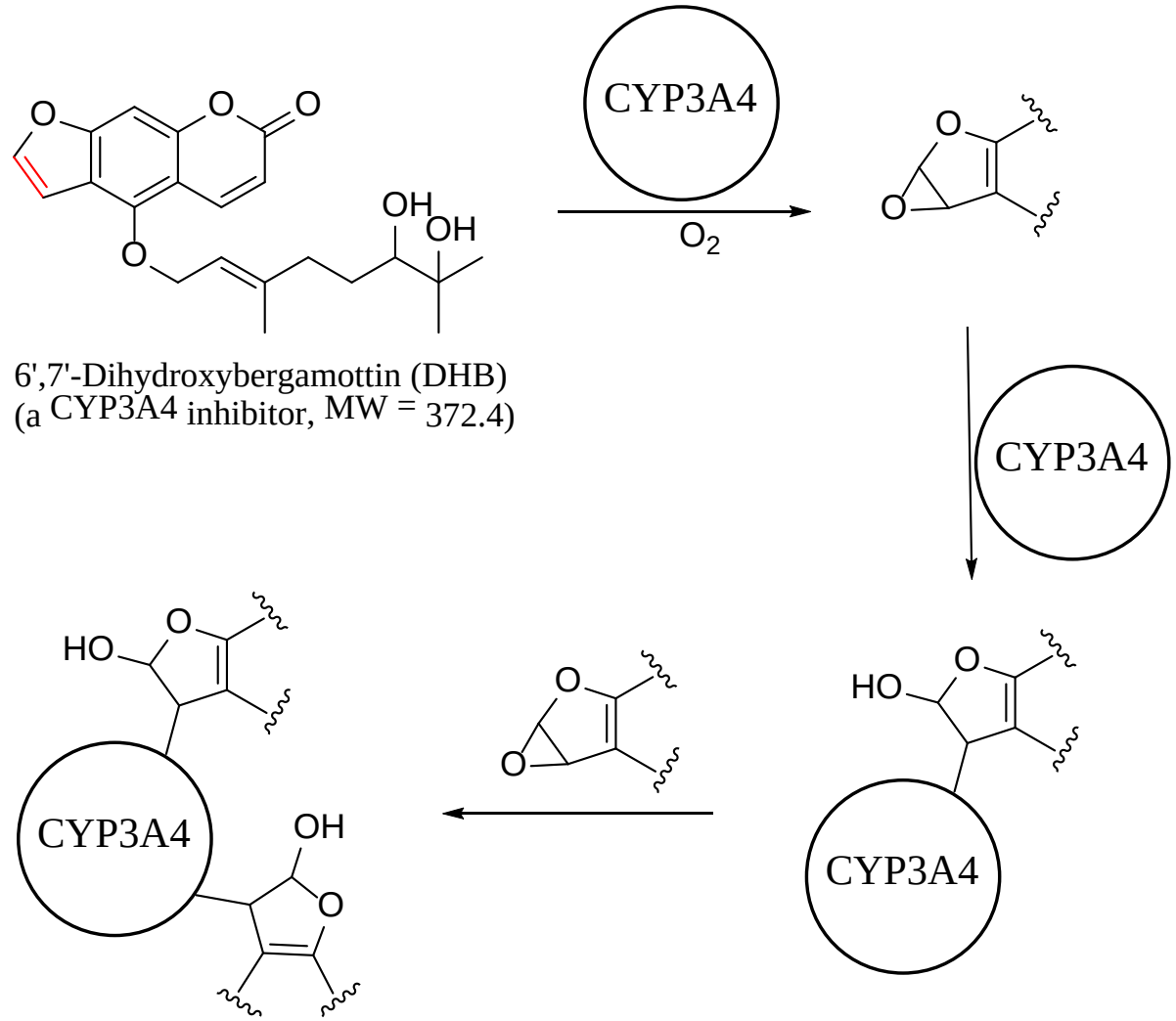
a. Common trapping agents for reactive metabolites (常用诱捕剂)
b. **LC/MS analysis of protein and DNA adducts**
   (蛋白质和DNA加合物的质谱分析)

Formation of drug-protein or drug-DNA adducts



73
73

Detection of covalent adducts to CYP 3A4 using LCMS



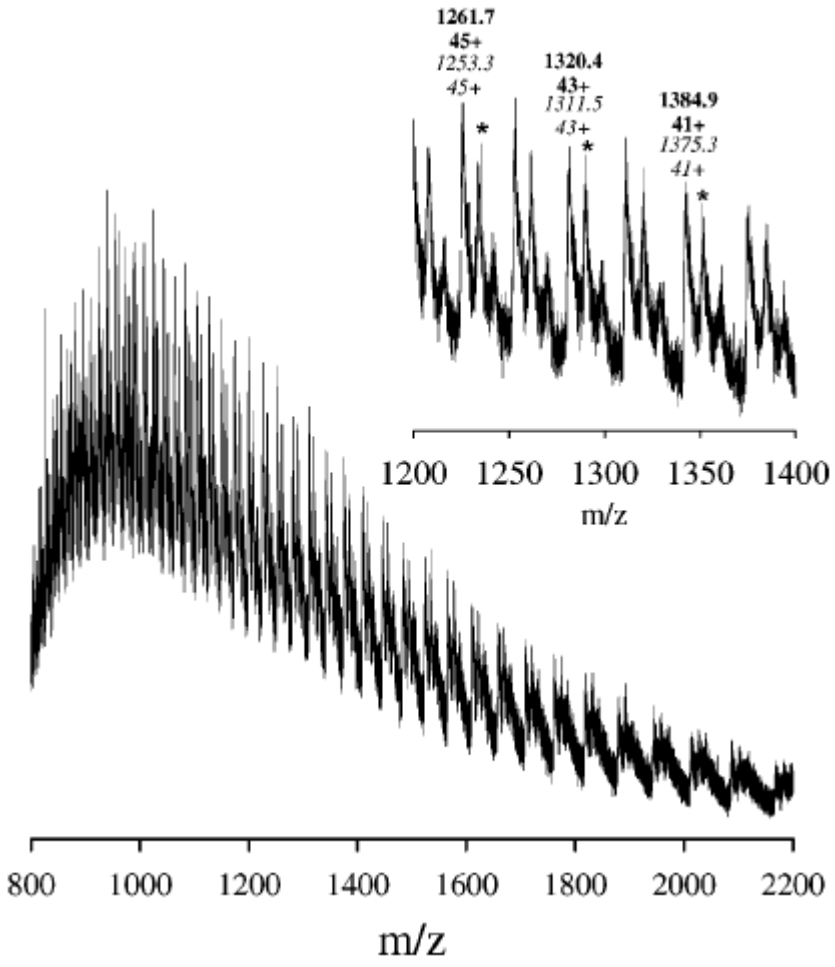
6',7'-Dihydroxybergamottin (DHB)
(a CYP3A4 inhibitor, MW = 372.4)

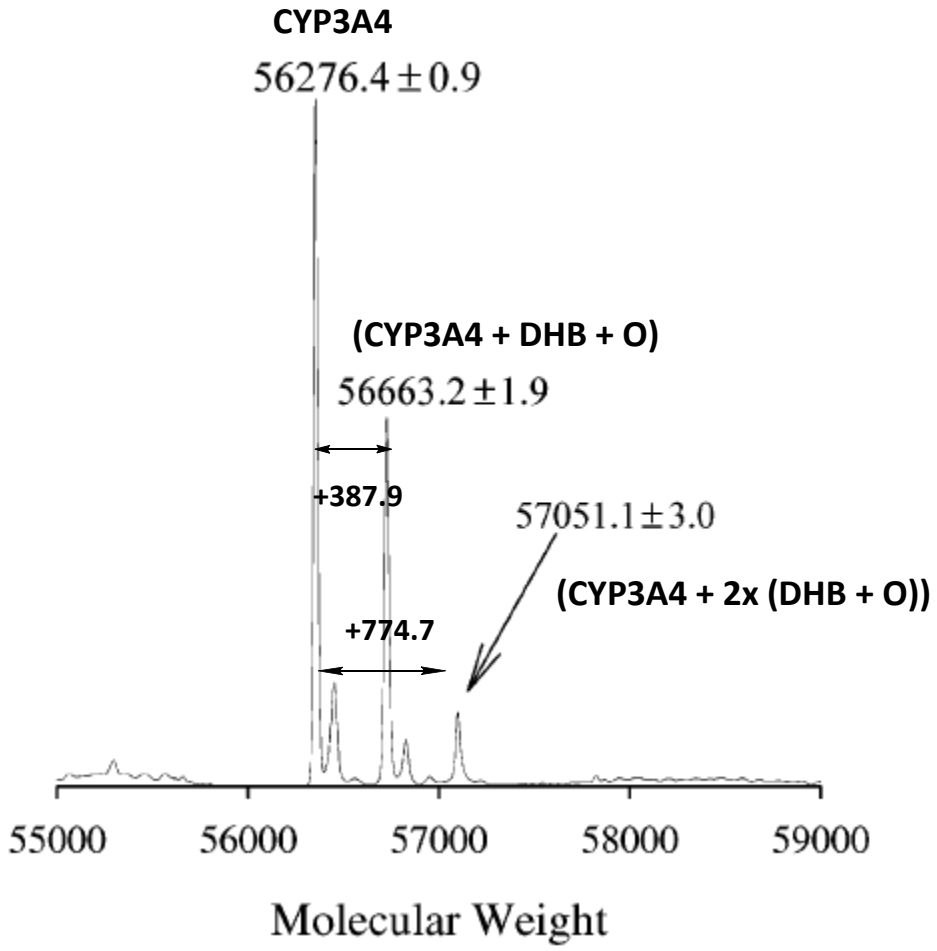
CYP3A4 + 386.8 ± 2.1
(CYP3A4 + DHB + O)

CYP3A4 +774.7 ± 3.0
(CYP3A4 + 2x (DHB + O))

(Bateman KP et al. Chem Res Toxicol 2004, 17:1356-61)

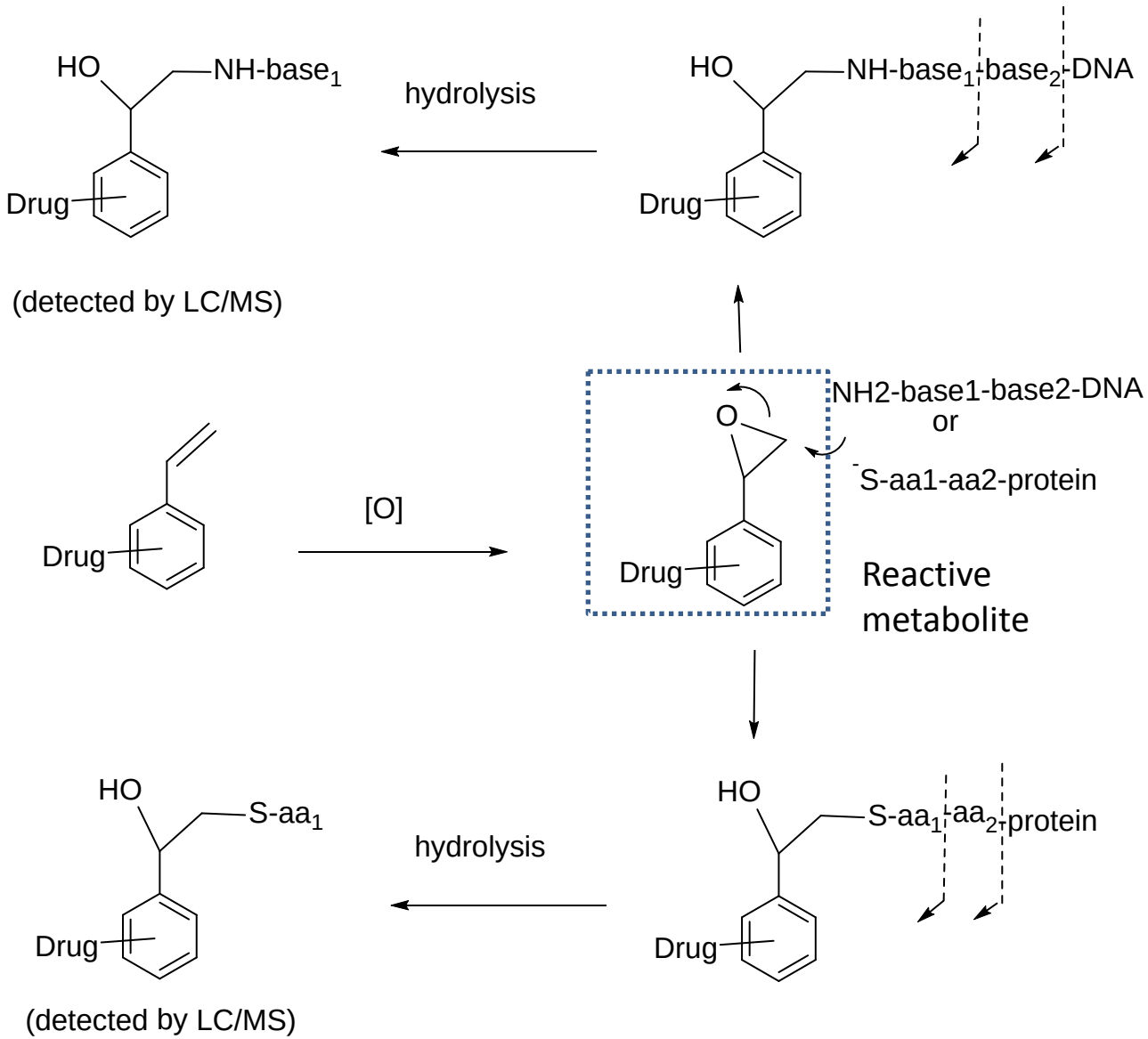
LC/MS detection of (CYP3A4+DHB+O) and (CYP3A4+2x(DHB+O)) adducts





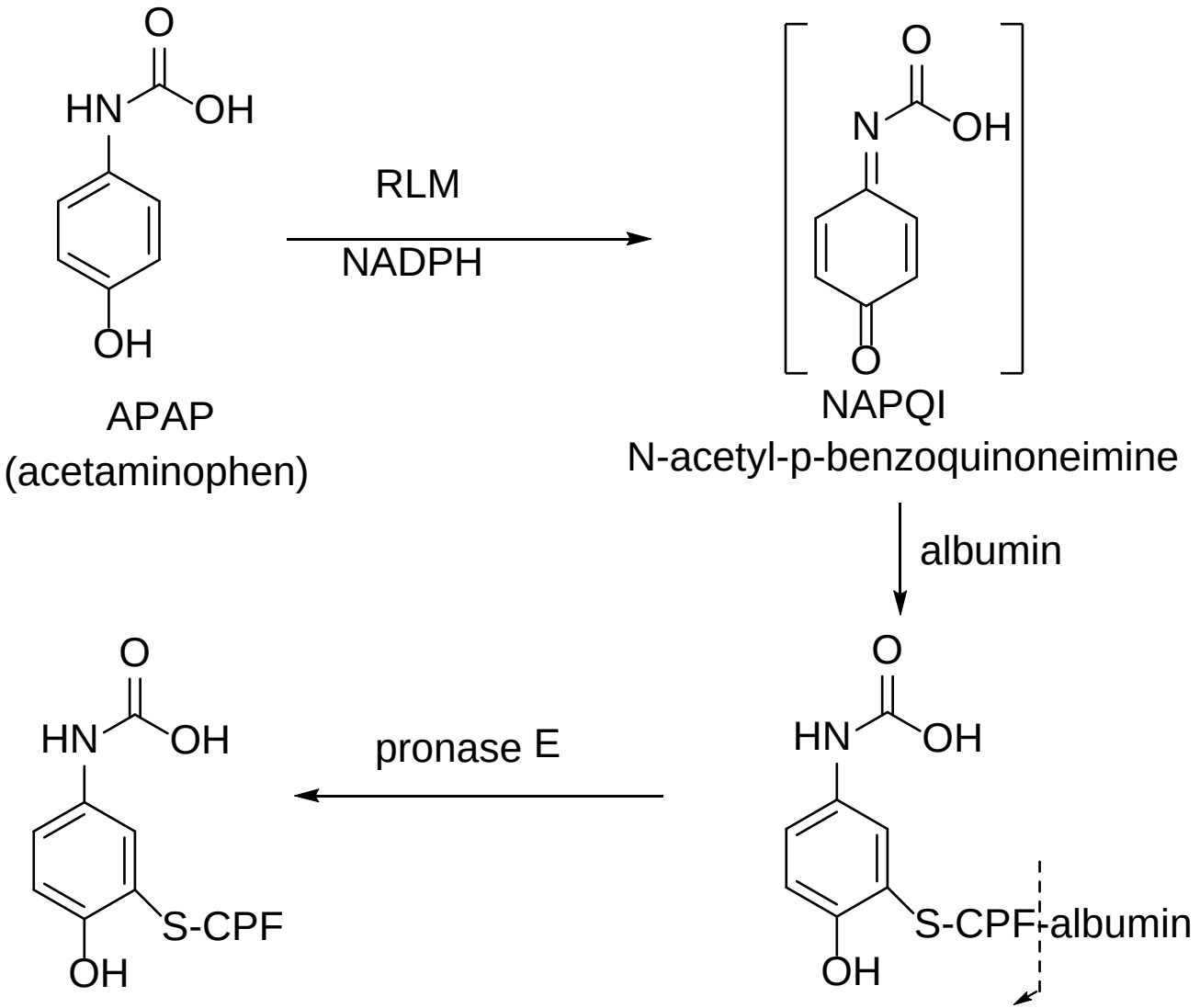
(Bateman KP et al. Chem Res Toxicol 2004, 17:1356-61)

LC/MS analysis of drug-amino acid or drug-DNA base adducts



76
76

LCMS detection of covalent binding of acetaminophen to human serum albumin

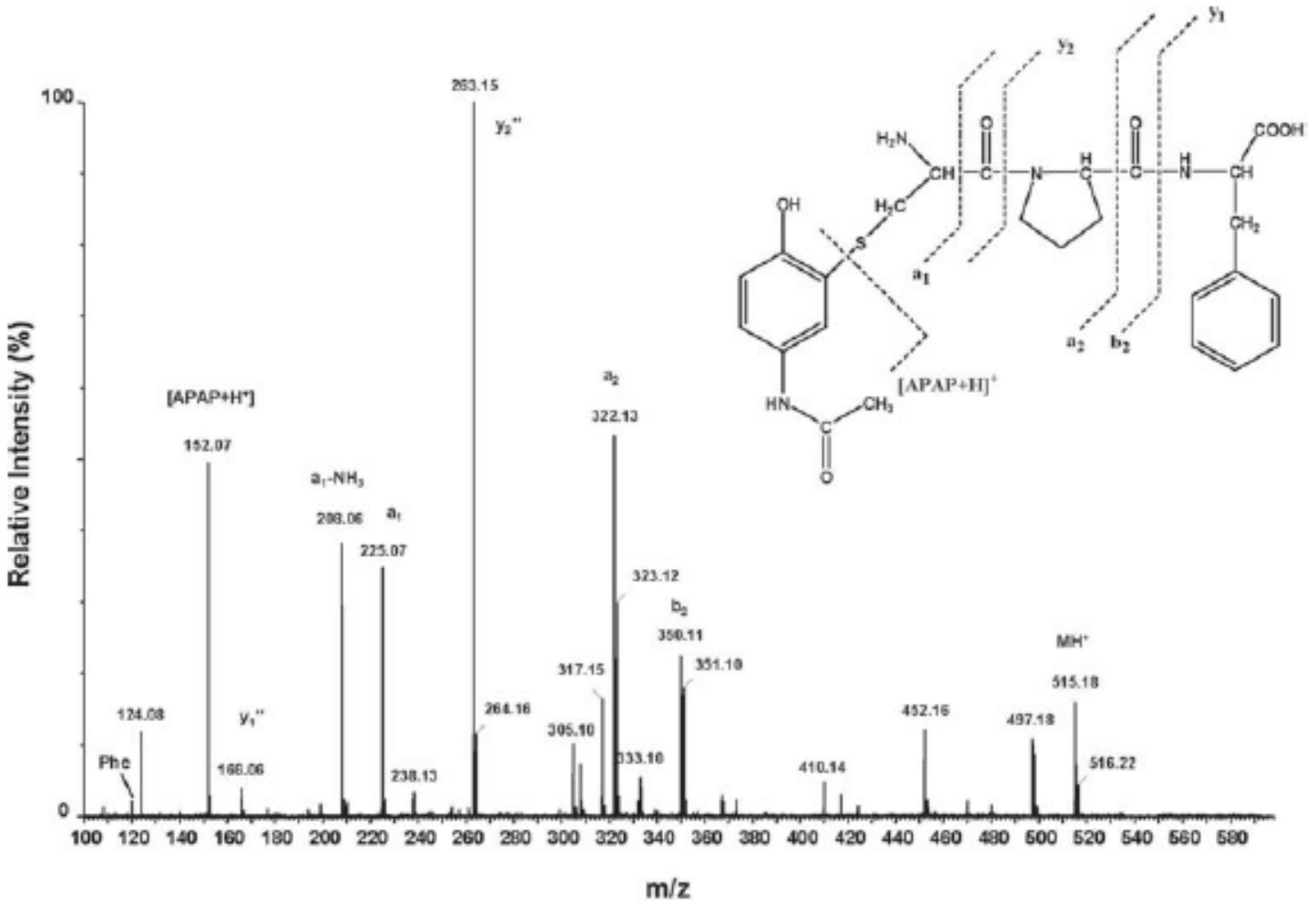


APAP
(acetaminophen)

NAPQI
N-acetyl-p-benzoquinoneimine

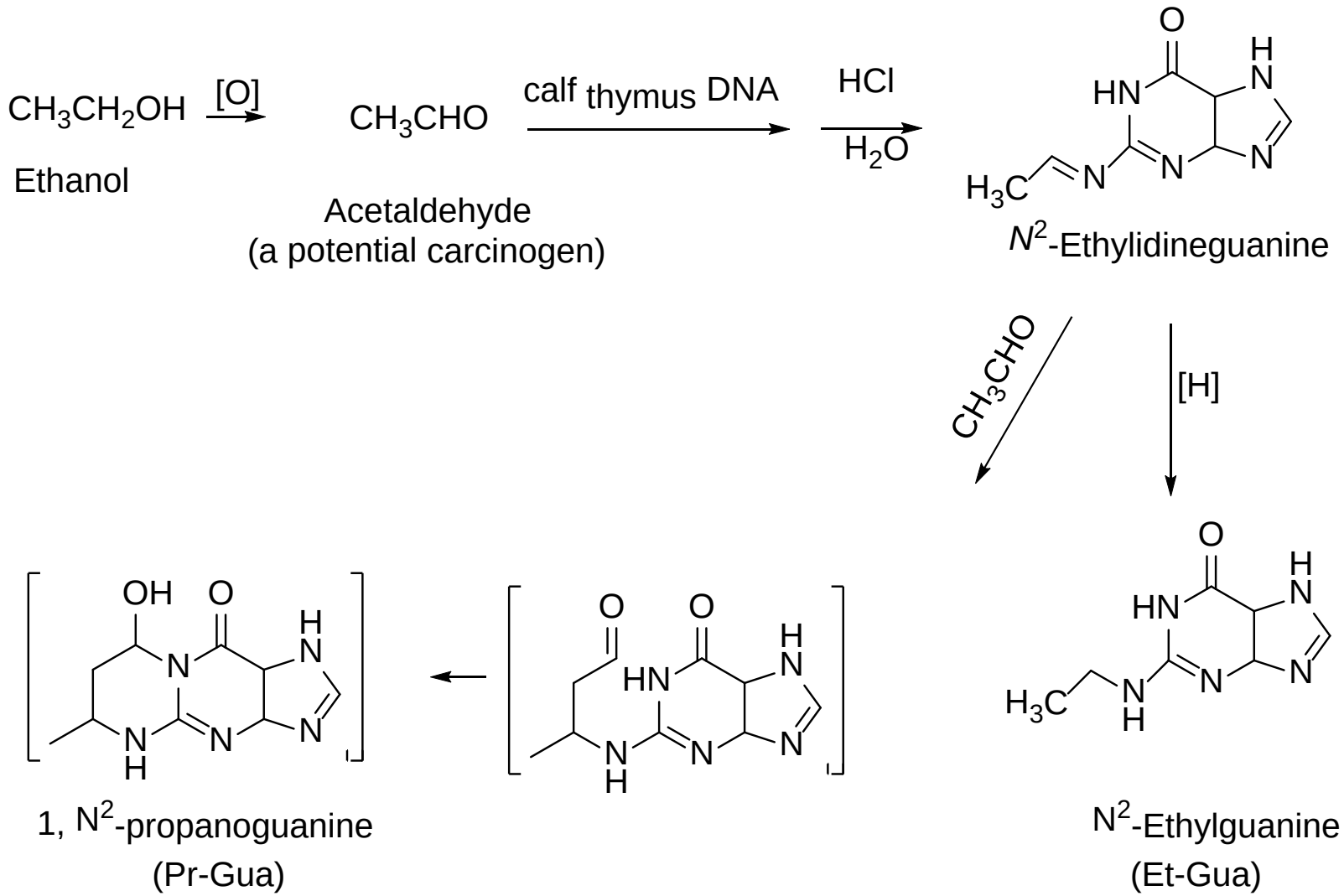
(Micaela D et al. Drug Metab Dispos 2007, 35: 1408)

LC/MS of the NAPQI-CPF adduct



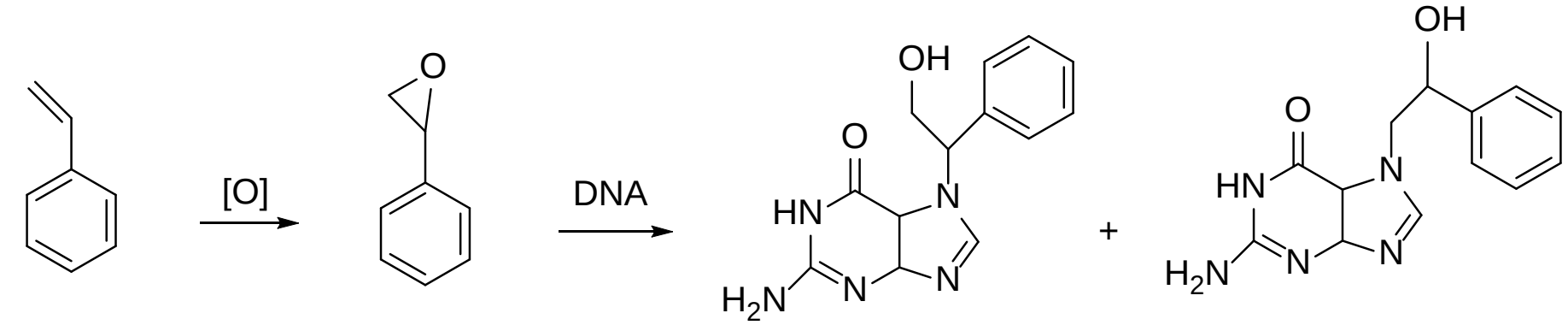
(Micaela D et al. Drug Metab Dispos 2007, 35: 1408)

In vitro reactions of acetaldehyde with calf thymus DNA



(Inagaki S et al., J Chromatogr A. 2003, 987:341-7)

In vitro reactions of acetaldehyde styrene 7,8-oxide with salmon testis DNA



Styrene

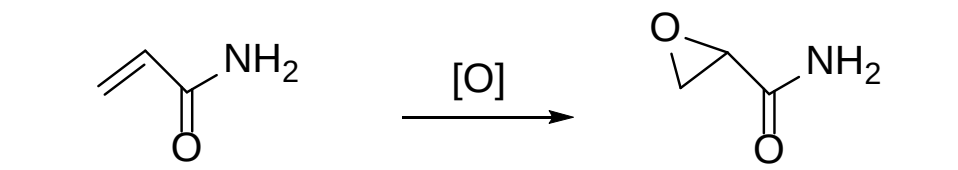
Styrene-7,8-oxide
(a mutagen and carcinogen)

αN-7 Gua adduct

βN-7 Gua adduct

(Koskinen M. Chem Biol Interact 2000, 124:13-27)

Formation of DNA adducts in mice dosed with acrylamide

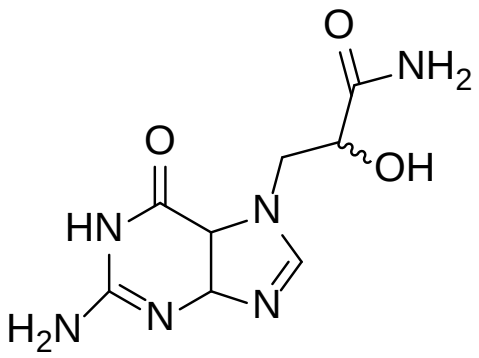


Acrylamide (AA)
(in cigarette smoking)

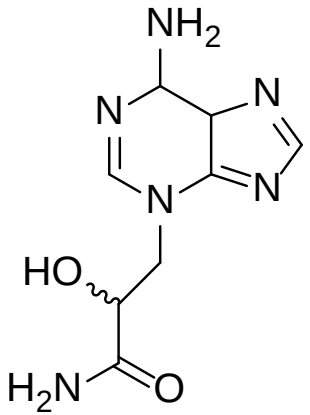
Glycidamide (GA)

DNA

hydrolysis



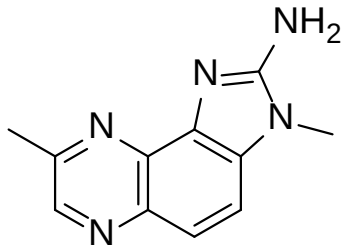
N^7-GA-Gua



N^3-GA-Ade

(Solomon JJ. Cancer Res 1985, 45:3465-3470)

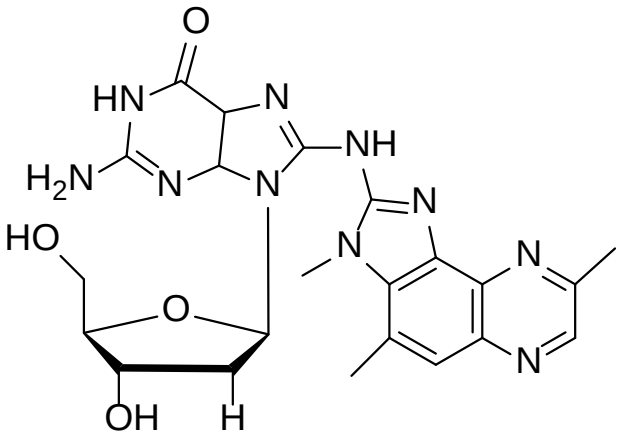
Formation of DNA adducts in rats dosed with MeIQx



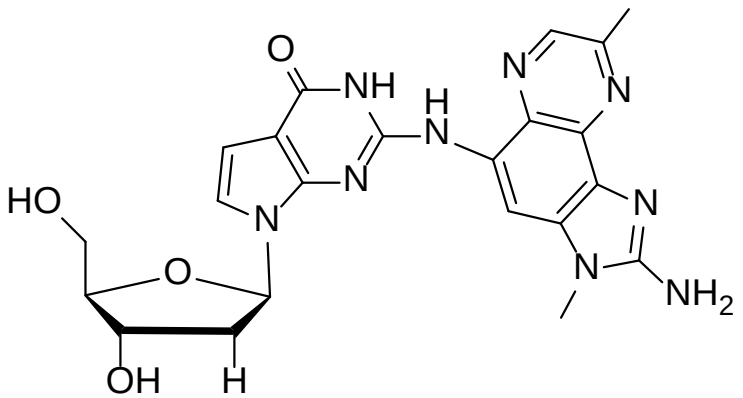
MeIQx
(2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline)
(a carcinogen)

DNA

hydrolysis



dG-C^8-MeIQx



dG-N^2-MeIQx

(Paehler A. Chem Res Toxicol. 2002, 15:551-61)

4. Experimental strategies to detect reactive metabolites (具有反应活性代谢物的常用分析检测方法)

a. Common trapping agents for reactive metabolites (常用诱捕剂)
b. LC/MS analysis of protein and DNA adducts (蛋白质和DNA加合物的质谱分析)
c. **Analytical strategies for high-throughput screening of reactive metabolites (高通量筛选反应性代谢物的分析检测方法)**

83

Analytical strategies for screening and characterization of GSH conjugates

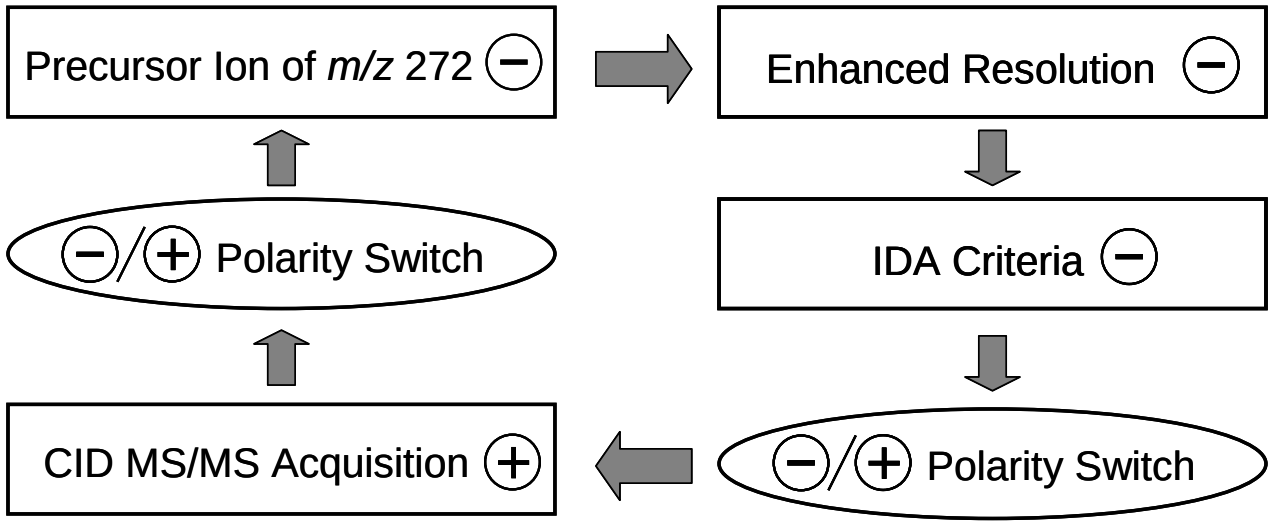
Analytical type	*m/z*	Polarity	Instruments	Comments
Neutral loss (NL)	129 Da	+	• Triple quadrupole • Q-trap • LTQ	• Poor selectivity • Not all structural classes afford a NL of 129 Da • Generic approach
Precursor ion (PI)	272 Da, 254 Da	–	• Triple quadrupole • Q-trap • LTQ	• Good selectivity and sensitivity • Broader coverage of different structural classes • Generic approach • Suitable for high-throughput screening
Multiple reaction monitoring (MRM)	$P^{\dagger} \rightarrow (P - 129)$, $P \rightarrow (P - 307)$; $P^{\ddagger} \rightarrow 272$, $P \rightarrow 254$	+, –	• Triple quadrupole • Q-trap	• Superior selectivity and sensitivity • Requires construction of MRM transitions; not suitable for unknown metabolites • Not generic approach
Accurate mass	129.0426 Da (NL); 272.0888 Da (PI), 254.0782 Da (PI)	+, –	• Q-TOF • LTQ-Orbitrap • LTQ-FTICR MS	• Superior selectivity and sensitivity • Good for structural elucidation • Mass defect filtering and background subtraction; suitable for In Vivo samples • Generic approach

† Protonated molecular weight $[M + H]^{+}$;
‡ Deprotonated molecular weight $[M - H]^{-}$.

Wen B, et al. *Expert Opin. Drug Metab. Toxicol.* 5:39-55, 2009.
Ma S, et al, *Chem. Biol. Interact.* 179:25-37, 2009

Polarity switching between MS detection and CID MS/MS acquisition:

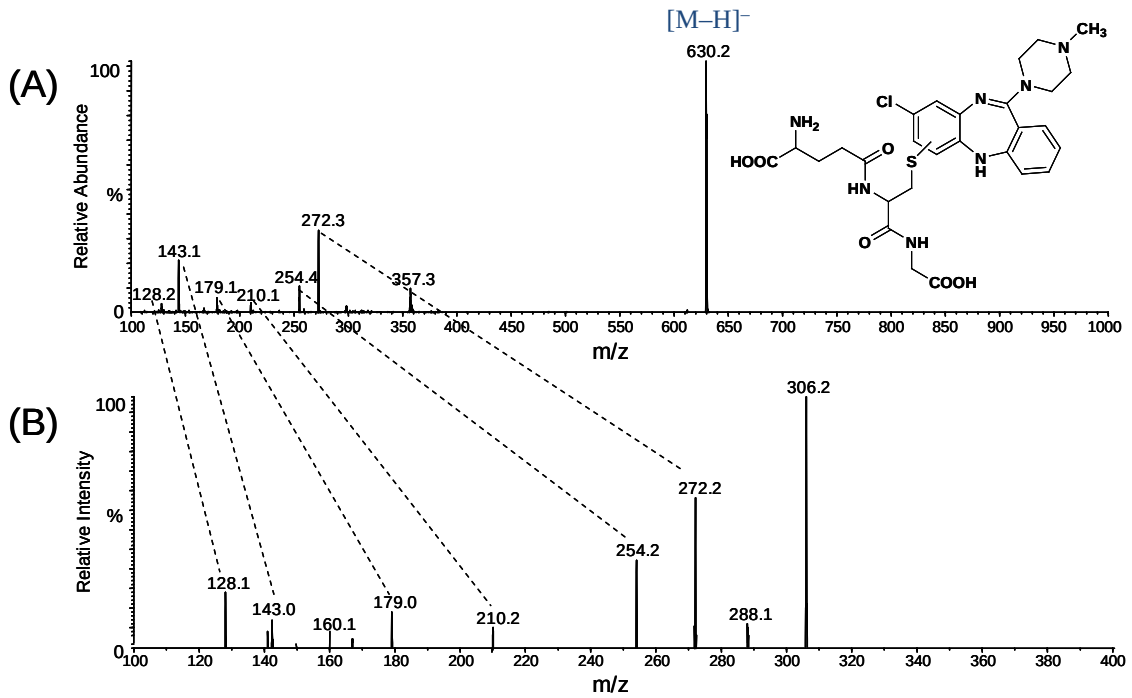
The PI-EPI approach with QTRAP LC-MS/MS systems



PI: precursor ion
EPI: enhanced product ion

Wen B, et al. *Anal. Chem.* 80:1788-1799, 2008
Wen B, et al. *J. Mass. Spectrom.* 44:90-100, 2009

LC/MS/MS analysis of GSH adducts in the negative ion mode



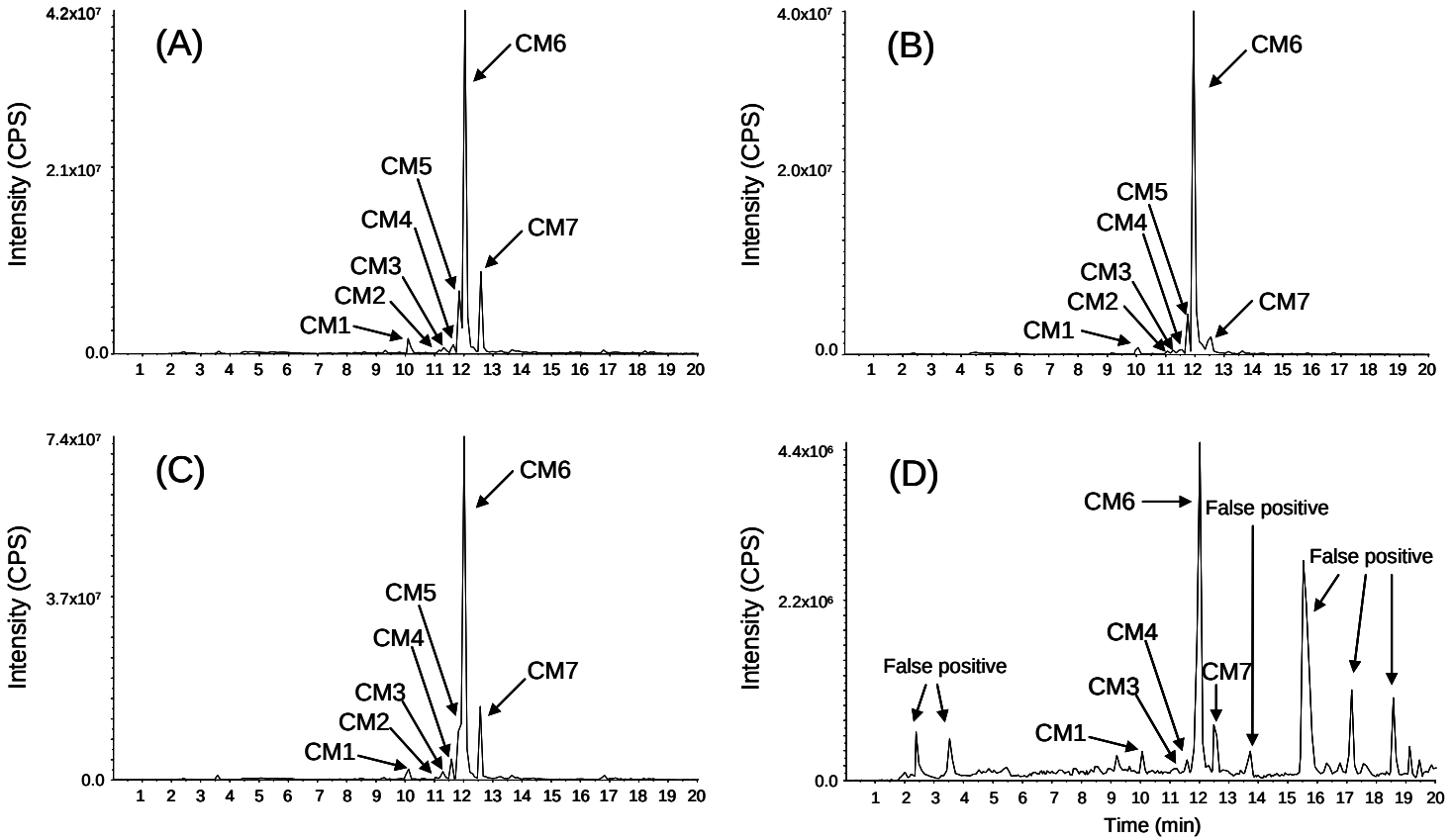
(A) MS/MS spectrum of the $[M–H]^-$ ion of a clozapine GSH adduct at *m/z* 630

(B) MS/MS spectrum of the $[M–H]^-$ ion of glutathione at *m/z* 306

- Fragmentation of GSH adducts in the negative ion mode often does not provide structurally informative product ions

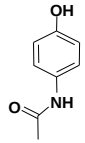
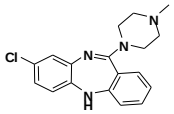
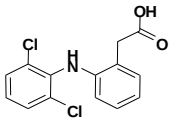
Wen B, et al. *Expert Opin. Drug Metab. Toxicol.* 5:39-55, 2009.
Ma S, et al, *Chem. Biol. Interact.* 179:25-37, 2009

LC-MS/MS analysis of GSH adducts in HLM incubations of clozapine



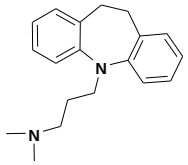
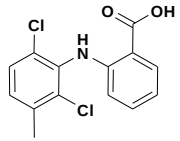
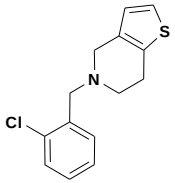
(A) TIC of negative PI scan of *m/z* 272; (B) TIC of negative PI scan of *m/z* 254;
(C) TIC of EPI triggered by PI scan of *m/z* 272; (D) TIC of positive NL scan of *m/z* 129

Table 1. Summary of Glutathione Conjugates Identified by the PI-EPI Approach

Compound (MH+)	Structure	GSH Conjugate	MH+ and (major fragment) of GSH Conjugate [a]	Postulated Conjugate Composition	GSH Conjugate Detected by NL [b]
Acetaminophen (152)		AM1	473 (**344**, 274, 256, 227, 199, 181, 145)	P + GSH + O – 2H	–
		AM2	473 (398, **344**, 327, 285, 224, 164, 156)	P + GSH + O – 2H	–
		AM3	457 (411, **382**, 336, **328**, 311, 208, 166, 140)	P + GSH – 2H	+
		AM4	457 (411, **382**, 336, **328**, 311, 208, 166, 140)	P + GSH – 2H	+
		AM5	489 (414, **360**, 184, 172, 138)	P + GSH + 2O – 2H	–
Clozapine (327)		CM1	648 (**519**, 375, 318, 300)	P + GSH + O – 2H	–
		CM2	598 (**469**, 412, 325, 293, 268, 209)	P + GSH – HCl	–
		CM3	632 (**503**, 359, 327, 302, 270, 243)	P + GSH – 2H	+
		CM4	618 (**489**, 345, 328, 302, 276)	P + GSH – 2H – CH2	–
		CM5	664 (**535**, 325, 270, 243)	P + GSH + 2O – 2H	–
		CM6	632 (**503**, 359, 327, 302, 270, 243)	P + GSH – 2H	+
		CM7	648 (630, **519**, 357, 327, 302, 275)	P + GSH + O – 2H	+
Diclofenac (296)		DM1	599 (551, **470**, 452, 307, 290, 262, 199, 181, 145)	P + GSH + 2O – HCl	–
		DM2	583 (**508**, 490, **454**, 436, 419, 334, 315, 290, 230, 199)	P + GSH + O – HCl	–
		DM3	617 (**542**, **488**, 470, 452, 367, 350, 342, 331, 296)	P + GSH + O – 2H	+
		DM4	567 (**492**, **438**, 420, 403, 318, 299, 246, 214)	P + GSH – HCl	–
		DM5	583 (**508**, **454**, 316, 308, 262)	P + GSH + O – HCl	+

Wen B, et al. *Anal. Chem.* 80:1788-1799, 2008

Table 1. ***Continued***

Compound (MH+)	Structure	GSH Conjugate	MH+ and (major fragment) of GSH Conjugate [a]	Postulated Conjugate Composition	GSH Conjugate Detected by NL [b]
Imipramine (281)		IM1	574 (**499** , **445** , 301, 159, 117)	P + GSH + O – 2 H – 2CH2	–
		IM2	602 (584, **473** , 329, 256, 155)	P + GSH + O – 2H	–
		IM3	517 (499, **442** , **388** , 242, 211)	P + GSH + O – 2H after N-dealkylation	–
		IM4	586 (568, **457** , 279, 234, 206)	P + GSH – 2H	+
Meclofenamic acid (296)		MM1	583 (**508** , **454** , 334, 282, 260, 256, 177)	P + GSH + O – HCl	–
		MM2	617 (573, **542** , **488** , 470, 444, 331, 298)	P + GSH + O – 2H	–
		MM3	617 (**542** , **488** , 342, 296, 175, 125)	P + GSH + O – 2H	–
		MM4	617 (573, **542** , **488** , 470, 444, 331, 298)	P + GSH + O – 2H	–
Ticlopidine (264)		TM1	569 (525, **494** , **440** , 296, 211, 154, 125)	P + GSH – 2H	+
		TM2	587 (**512** , 494 , **458** , 440, 355, 308, 280, 253, 154)	P + GSH + O	–
		TM3	587 (494, **458** , 440, 416, 341, 287, 280, 184, 125)	P + GSH + O	–
		TM4	603 (571, **528** , **474** , 330, 296, 265, 232, 199, 154, 125)	P + GSH + 2O	–
		TM5	585 (**456** , 438, 312, 278, 250, 200, 154, 125)	P + GSH + O – 2H	–

[a] The boldface type denotes characteristic product ions resulting from neutral losses of 75 and 129 Da, respectively. [b] The + denotes GSH conjugates identified in the NL scanning previously reported in the literature; the – denotes those not detected.

Several major advantages (vs. conventional NL method):

1) Significantly improved sensitivity (~ 10 X) for detecting minor GSH conjugates at low levels due to less matrix background noise in the negative mode.
2) Excellent selectivity for different classes of GSH conjugates with essentially no false positive signals.
3) Highly efficient in the detection and structural characterization of GSH conjugates in a single LC/MS/MS run.
4) Suitable for high-throughput screening of reactive metabolites in a drug discovery setting.

5. Assessment of exposure and magnitude of reactive metabolite formation (具有反应活性代谢物的半定量分析与方法)

a. **In vitro covalent protein binding assays**
(体外共价蛋白结合实验与步骤)

Chem. Res. Toxicol. **2004,** *17,* 3−16

Perspective

Drug−Protein Adducts: An Industry Perspective on Minimizing the Potential for Drug Bioactivation in Drug Discovery and Development

David C. Evans,*,† Alan P. Watt,‡ Deborah A. Nicoll-Griffith,§ and Thomas A. Baillie∥

Drug Metabolism, Merck & Co., Inc., Rahway, New Jersey, Medicinal Chemistry, Drug Metabolism Section, Merck Sharp & Dohme, Terlings Park, United Kingdom, Medicinal Chemistry, Merck Frosst Center for Therapeutic Research, Kirkland, Quebec, Canada, and Drug Metabolism, Merck & Co., Inc., West Point, Pennsylvania

Table 2. Summary of Covalent Binding Data for Prototypical Hepatotoxins in Vivo and in Vitro

compound	species	dose	covalent binding to liver protein homogenate in vivo	evaluation of covalent binding in vitro[a]	ref
APAP	mouse	250 mg/kg ip phenobarbital-induced animals	1.3−1.6 nmol equiv/mg at 3 h	not determined	*23*
amodiaquine (AQ)	rat	18 mg/kg portal vein	only negligible levels of binding determined in vivo	following incubation of 10 μM AQ with RLMs or HLMs; levels of covalent binding were ~600 (rat) and ~180 (human) pmol equiv/mg; incubation of AQ−quinoneimine with RLMs or HLMs produced levels of binding >3 nmol equiv/mg protein	*51, 62*
bromobenzene	rat	~300 mg/kg ip	5.6 nmol equiv/mg at 4 h	following incubation of 1 mM bromobenzene with rat liver microsomes (45 min, 2 mg of protein), the level of covalent binding was ~20 nmol equiv (~13 nmol equiv/mg)	*17−19*
EE	rat	5, 50, and 100 mg/kg iv	<5 pmol equiv/mg at 3 h	250 μM EE incubated with RLMs (20 min) = 1.2 ± 0.06 nmol equiv/mg (<10% EE metabolized)	*63−65*
furosemide	mouse	400 mg/kg ip	1.2 nmol equiv/mg at 3 h	50−250 μM furosemide incubated with mouse liver microsomes; 4−6 nmol equiv/mg	*14, 31*
iproniazid	rat	200 mg/kg ip	0.26 nmol equiv/mg at 6 h	isopropyl[2-^{3}H]hydrazine (1 mM) covalently labeled RLM and HLM protein; 0.58 and 0.37 nmol equiv/mg (15 min incubation time)	*66, 67*
4-ipomeanol	rat	45 mg/kg ip	liver, 1.9 nmol equiv/mg at 24 h lung, 3.8 nmol equiv/mg at 24 h	in liver and lung microsomes in vitro, the level of covalent binding observed following a 6 min incubation with 1 μmol of [3,5-^{14}C] ipomeanol approximated to 3−6 nmol equiv/mg protein	*6−8*
isoniazid	rat	200 mg/kg acetylisoniazid	0.5 nmol equiv/mg at 24 h	acetyl[^{14}C]hydrazine (1 mM) covalently labeled RLM and HLM protein; 0.55 and 0.16 nmol equiv/mg (15 min incubation time)	*67*
phenacetin	mouse	500 mg/kg ip	liver, 0.60 nmol equiv/mg at 4 h kidney, 0.53 nmol equiv/mg at 4 h lung, 0.64 nmol equiv/mg at 4 h	incubation of *p*-[^{3}H]nitrosophenetole with RLMs (4.5 mg) resulted in levels of covalent binding, which approximated to 40 nmol equiv following a 1 h incubation.	*68*

[a] Covalent binding data have been normalized to an incubation period of 1 h to allow for comparison of data unless otherwise stated.

Having determined the level of covalent binding of a drug candidate, the question then becomes, "How much apparent covalent binding is acceptable in deciding whether to advance a drug candidate into development?" Merck's approach to this question has been to take the levels of covalent adducts typically found in the livers of animals given a dose of a prototypic hepatotoxin (e.g., APAP, bromobenzene, furosemide, or 4-ipomeanol) (Table 2) associated with the expression of hepatic necrosis (approximately 1 nmol drug equiv/mg protein) and to reduce this figure by 20-fold to give a conservative target "threshold" value for in vivo covalent binding of 50 pmol drug equiv/mg total liver protein. This value also corresponds to a level of radioactivity that is approximately 10 times background under normal conditions and thus provides a suitable dynamic range for measurement of covalently bound drug−protein adducts. It should be

Evans et al. Chem. Res. Toxicol., Vol. 17, No. 1, 2004

A protocol for *in vitro* covalent protein binding in human or rat liver microsomes

Rat or human liver microsomes (1 mg protein/mL) are suspended in phosphate buffer (100 mM, pH 7.4) containing EDTA (1 mM) and $MgCl_2$ (0.1 mM) in a total volume of 1 mL. The stock solution of 5 mM of a ^3H-labeled compound in methanol is prepared by mixing unlabeled material with radiolabeled material with a final specific activity of 100 Ci/mol, and is added to the above suspension with a final concentration of 10 μM, such that the concentration of methanol in the incubation mixture does not exceed 0.2% (v/v). Incubations are performed in duplicate in the presence of NADPH (1.2 mM) at 37 ºC, and quenched with 5 mL of acetonitrile at 0, 30, and 60 min. The duration of the incubations conducted in the presence of sodium cyanide (1 mM) and glutathione (5 mM) is 60 min. Samples are centrifuged at 2,500 × *g* to afford Protein pellets, which then are suspended in 1 mL of water and sonicated for 10 min. Four mL of ethanol is added to the above suspension and the mixture is vortexed and sonicated for 10 min. Samples are placed at –20 ºC for 30 min, and are centrifuged at 4 ºC for 10 min. Supernatants are aspirated and the residues are resuspended in 1 mL of water. The above washing procedures are repeated until radioactivity in the supernatant is less than 2-fold background. The protein pellets then are dissolved in 0.1 M sodium hydroxide (1 mL), 50% of which is neutralized with 0.1 M hydrochloric acid (0.5 mL) and analyzed by a Beckman Counter liquid scintillation counter (LS6500, Fullerton, CA). The protein concentration in the remaining aliquot is determined using a Pierce bicinchoninic (BCA) protein assay kit (Rockford, IL). Covalent protein binding values in pmol-equiv./mg protein are estimated based on the residual radioactivity in the protein pellets. Control experiments are performed in the absence of NADPH for 60 min. It was reported that the covalent protein binding values of reference model compounds in human and rat liver microsomes were ~ 136 and 574 pmol-equiv./mg protein for [^3H]imipramine, 68 and 413 pmol-equiv./mg protein for [^{14}C]diclofenac, 1405 and 1578 pmol-equiv./mg protein for [^{14}C]naphthalene, respectively.

(Zhang Z. et al. In: *Drug Metabolism in Drug Design and Development* (2008) p447-476)

I*n vitro* covalent protein binding in human/rat liver microsomes

(simplified)

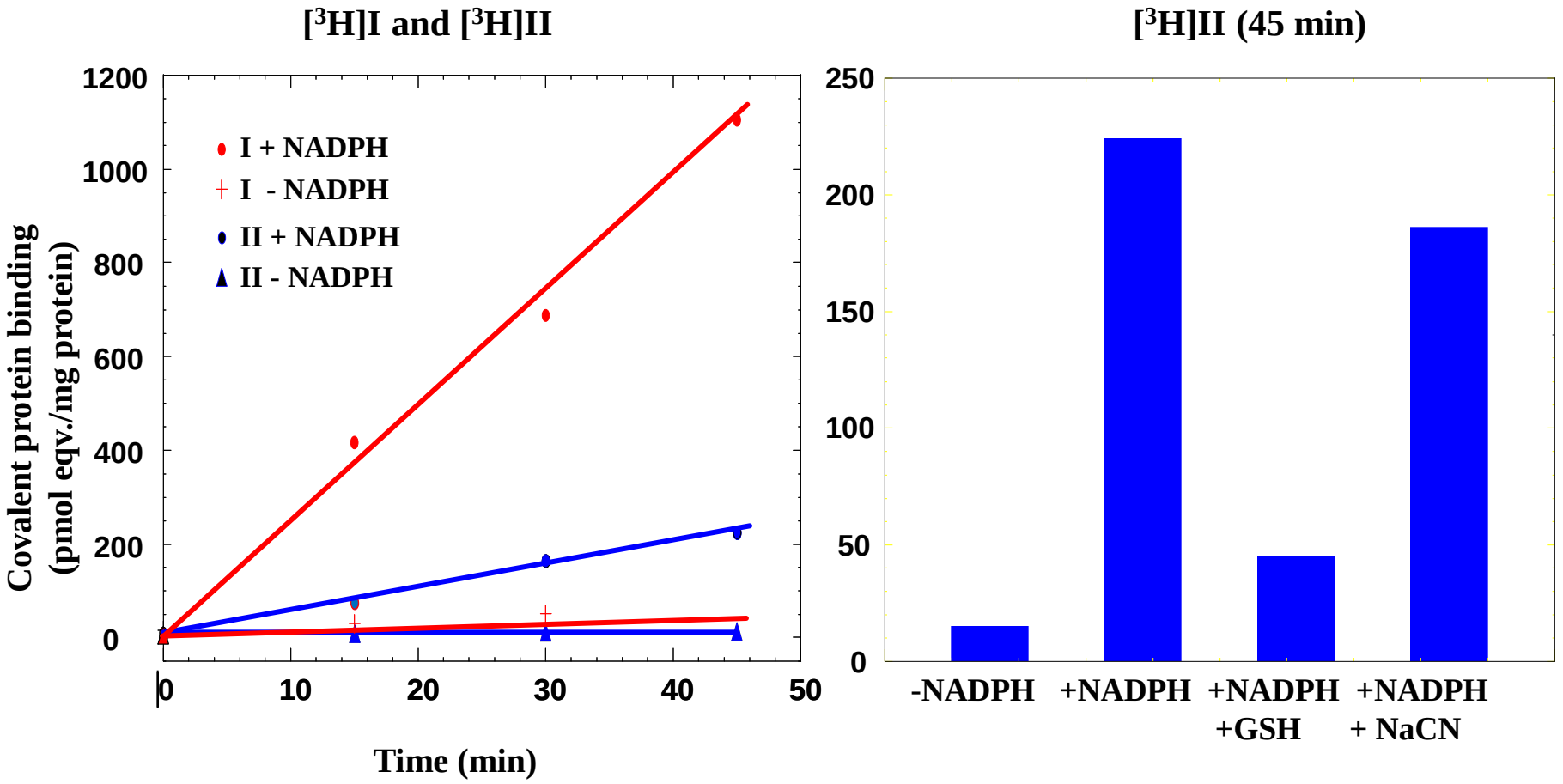
1 mL (1mg protein/mL) of human/rat liver microsomes + 10 μM of $^{3}H/^{14}C$-labeled test (~100 Ci/mol) + NADPH (1.2 mM) at 37 ºC for 0, 30, and 60 min. --- > quenched with ACN (5 mL) --- > Sonicated and centrifuged --- > protein pellets --- > suspended in 1 mL of H_2O and sonicated for 10 min --- > 4 mL of EtOH added --- > vortexed and sonicated for 10 min --- >placed at –20 ºC for 30 min --- > centrifuged at 4 ºC for 10 min --- > Supernatants removed and the residues resuspended in 1 mL of H_2O --- > Repeat the above washing procedures until radioactivity in the supernatant is < 2x background --- > protein pellets dissolved in 0.1 M NaOH (1 mL) --- > 50% of which is neutralized with 0.1 M HCl (0.5 mL) and radioactivity is counted using liquid scintillation counter --- > The protein concentration in the remaining aliquot is determined using a Pierce bicinchoninic (BCA) protein assay kit --- > Covalent protein binding = x pmol-equiv./mg protein.

Control experiments are performed in the absence of NADPH for 60 min.

Positive control experiements using reference model compounds

(Zhang Z. et al. In: *Drug Metabolism in Drug Design and Development* (2008) p447-476)

Covalent binding of [^{3}H]I and [^{3}H]II to proteins of human liver microsomes*



*: Substrate, 10 μM; 1 mg protein/mL; GSH, 5 mM; NaCN, 1 mM; NADPH, 1.2 mM.

(Zhang, Z. et al. Chem. Res. Toxicol. 2005, 18, 675)

A protocol for *in vitro* covalent protein binding in human hepatocytes

Cryopreserved human hepatocytes from three male and two female donors or freshly isolated male rat hepatocytes are analyzed for viabilities (75-85%) using the trypan blue exclusion methods. Incubations are performed by suspending the hepatocytes in Krebs-biocarbonate buffer followed by addition of a ^{3}H-labeled compound in methanol. The specific activity of the compounds is 100 Ci/mol. The final concentration of test compound in the suspension is 10 μM in a final volume of 1 mL ($1x10^{6}$ cells/mL), and the final concentration of methanol does not exceed 0.2% (v/v). Incubations are allowed to proceed at 37 ºC for 1 h, and are quenched with acetonitrile (5 mL). The remaining procedures are the same as described in the previous slide (the protocol for in vitro covalent protein binding in human or rat liver microsomes). Covalent protein binding values in pmol-equiv./mg protein are estimated based on the residual radioactivity in the protein pellets.

(Zhang Z. et al. In: *Drug Metabolism in Drug Design and Development* (2008) p447-476)

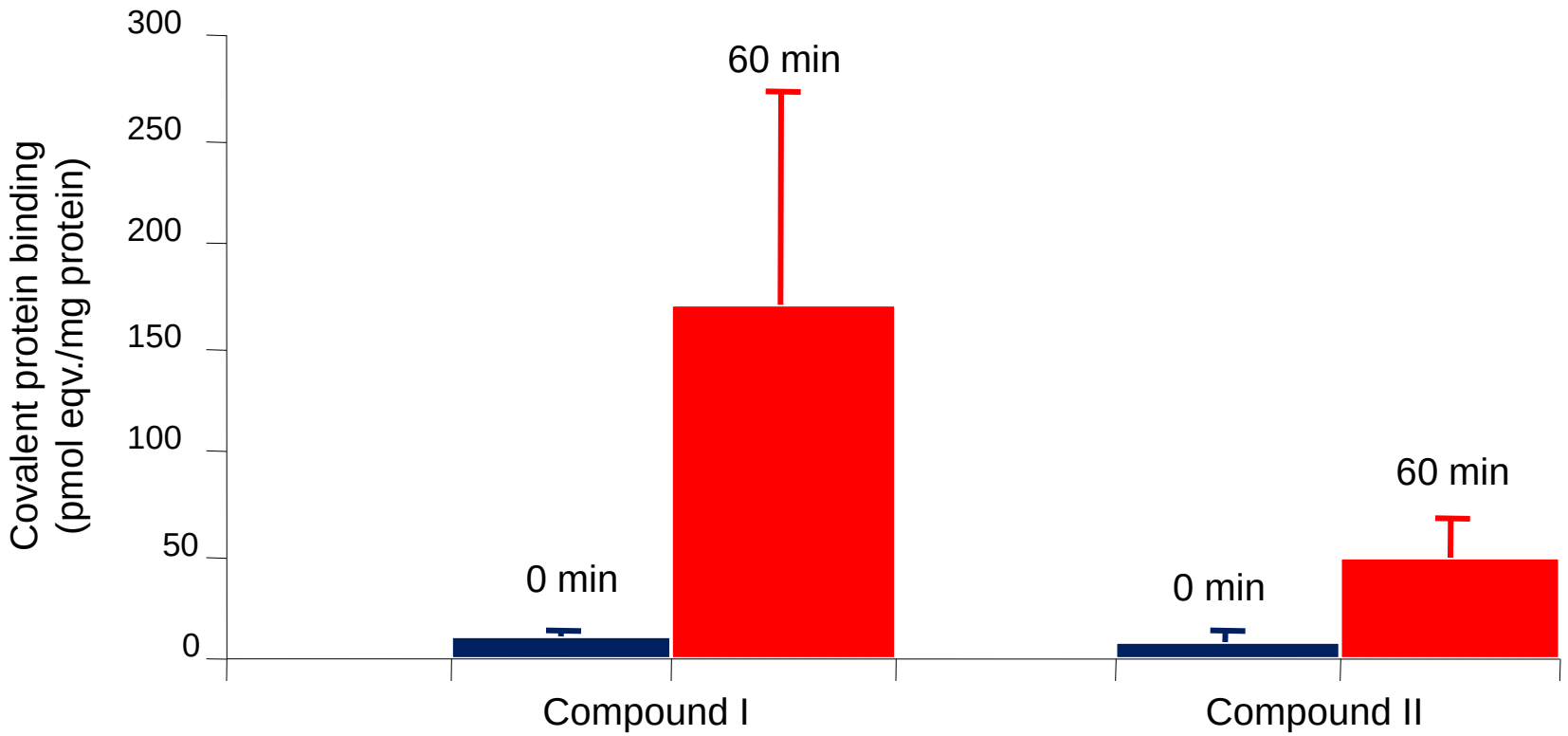
I*n vitro* covalent protein binding in human hepatocytes

(simplified)

1 mL (1x10^6 cells/mL) of human hepatocytes + 10 μM of ^{3}H/^{14}C-labeled test compound (~100 Ci/mol) 37 ºC for 1 h --- > quenched with acetonitrile (5 mL) --- > --- > --- > the remaining procedures are similar to that shown in previous slides --- > Covalent protein binding values in pmol-equiv./mg protein are estimated based on the residual radioactivity in the protein pellets.

(Zhang Z. et al. In: *Drug Metabolism in Drug Design and Development* (2008) p447-476)

Covalent binding of [^{3}H]I and [^{3}H]II to proteins of human hepatocytes*



*: Substrate, 10 μM; 1 x 10^6 cells/mL; 37 °C; 60 min; N = 5.

(Zhang, Z. et al. Chem. Res. Toxicol. 2005, 18, 675)

5. Assessment of exposure and magnitude of reactive metabolite formation (具有反应活性代谢物的半定量分析与方法)

a. In vitro covalent protein binding assays (体外共价蛋白结合实验与步骤)

b. In vivo covalent protein binding assays (体内共价蛋白结合实验与步骤)

A protocol for *in vivo* covalent protein binding in plasma and liver of rats

A dose solution of 4 mg/mL is prepared by dissolving/suspending a test compound in ethanol/PEG400/water (1:4:5, v/v/v) with a final specific activity of 3-10 Ci/mol (^{3}H-tracer) or 1.5-3 Ci/mol (^{14}C-tracer). Nine male Sprague-Dawley rats are orally dosed with a test compound at 20 mg/kg. Blood and liver samples of rats are taken at 2, 6, and 24 h post dosing and urine samples are collected at 24 h (3 rats/time point). Plasma samples are prepared by centrifugation of blood at 4 ºC for 30 min. Liver is suspended in PBS buffer (3 mL/g tissue) and the mixture is homogenized to give liver homogenate. The washing procedures are as follows:

1. **Aliquots of samples (0.5 mL of liver homogenates or 0.15 mL of plasma) in duplicate are placed in test tubes, and 1.5 mL of acetonitrile is added to each tube.**
2. **The mixtures are sonicated, vortexed for 10 min, and then are placed at –20 ºC for 30 min.**
3. **Samples are centrifuged at 2,500 x *g* at 4 ºC for 10 min, and 50 mL of supernatant in each tube is taken for measurement of radioactivity.**
4. **The remaining supernatant is carefully aspirated under vacuum, and the remaining pellets are resuspended in 1 mL of water, sonicated, and vortexed.**
5. **Four mL of ethanol is added to each tube, and the resulting suspensions are sonicated, vortexed, and placed at –20 ºC for 30 min.**
6. **Steps 3-5 are repeated for 4-6 times until the radioactivity of the supernatant (0.5 mL) in the last washing is below 2 times of background readout.**

The protein pellets are then dissolved in 4 mL of 0.1 M sodium hydroxide. The remaining procedures for measurement of radioactivity and protein concentrations of the resulting samples are the same as described in the previous slide (the protocol for in vitro covalent protein binding in human or rat liver microsomes). Covalent protein binding in pmol-equiv./mg protein is estimated based on the residual radioactivity in the proteins. An example of covalent protein binding studies in liver and plasma of male rats orally dosed with the tritium-labeled dihydrobenzoxazhiin analog E at 20 mg/kg is shown in Table 2. The covalent protein binding of A was below 20 pmol-equiv./mg protein in rat liver and plasma samples.

(Zhang Z. et al. In: *Drug Metabolism in Drug Design and Development* (2008) p447-476)

In vivo covalent protein binding of [^{3}H]II in rats*

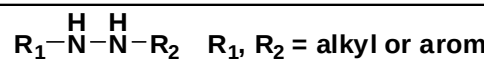
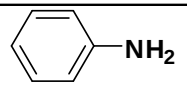
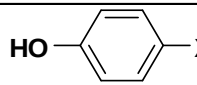
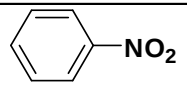
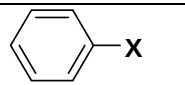
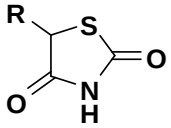
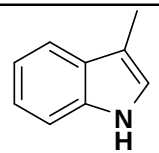
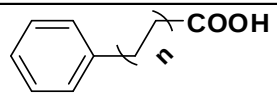
Tissues	Covalent binding (pmol/mg protein)			Drug concentration (μM)		
	2 h	6 h	24 h	2 h	6 h	24 h
Plasma	0.4 ± 0.6	0	0.6 ± 2.4	1.8	1.1	0.1
Liver	2.8 ± 1.7	6.7 ± 2.8	8.2 ± 0.7	7.0	3.4	0.1

*: Male Sprague-Dawley rats were dosed orally with [^{3}H]II at 10 mg/kg (N = 3 each time point).

(Zhang, Z. et al. Chem. Res. Toxicol. 2005, 18, 675)

6. Structural alerts and rational drug design
(药物分子结构预警与合理药物设计)

Structural alerts: a starting point for testing and beyond

Name	Substructure	Reactive species
Hydrazines, hydrazides	 R_1, R_2 = alkyl or aromatic groups	diazonium ions, free radicals
Thiophenes, furans, pyrroles	 X = S, O, NH	epoxides, *S*-oxides (thiophenes),α, β-unsaturated dicarbonyl (furans)
Anilines, anilides and precursors		nitrosos, quinoneimines, free radicals
Quinones and precursors	 X = NH_2, OH, etc	quinones, quinoneimines, free radicals
Nitroaromatics		nitroso intermediates, free radicals
Halogenated aromatics (Br > Cl > F)	 X = Br, Cl, F	arene oxides
Thiazolidinediones	 R = alkyl or aromatic groups	isocyanates, isothiocyanates
3-Alkylindoles		3-methyleneindolenines
Alkynes, acetylenes	$R_1-C\equiv C-R_2$ R_1, R_2 = H or alkyl groups	oxirenes, ketenes
Arylacetic acids, arylpropionic acids	 n = 1, 2	acyl glucuronides

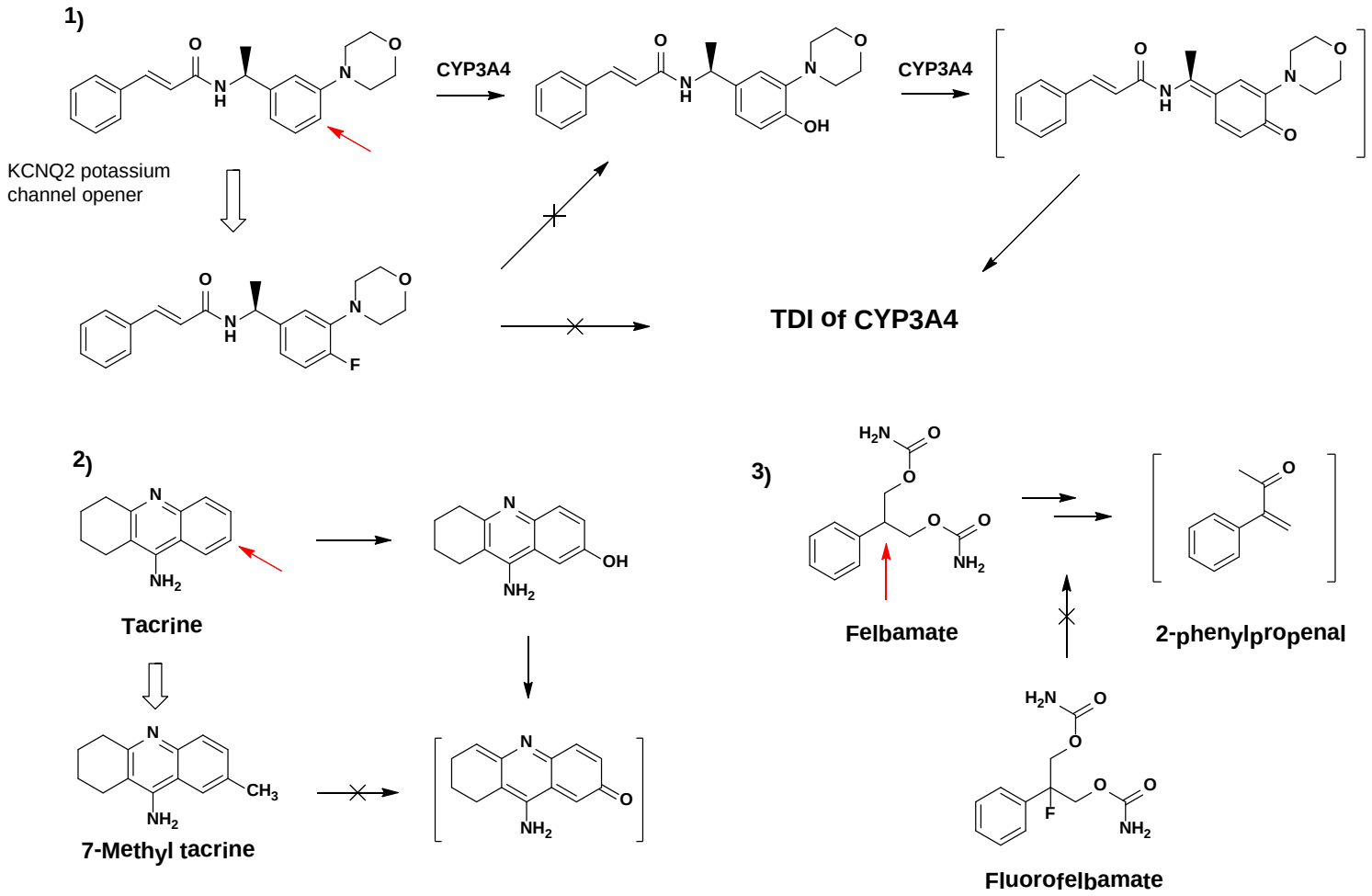
Wen B & Fitch W, *Expert Opin Drug Metab Toxicol.* 5:39-55 (2009)

Widely accepted that Attrition Must Occur Earlier!

- ✓ “Dialing-out” the risk of reactive metabolites the earlier the better
- ✓ **Can the offending substructure be designed out of the series?**

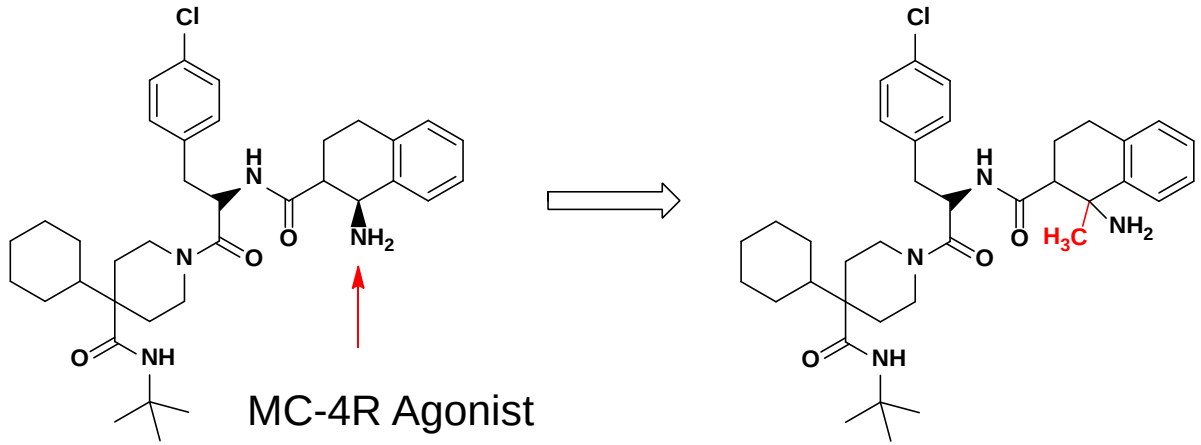
  -- A prerequisite is no loss of pharmacological activity

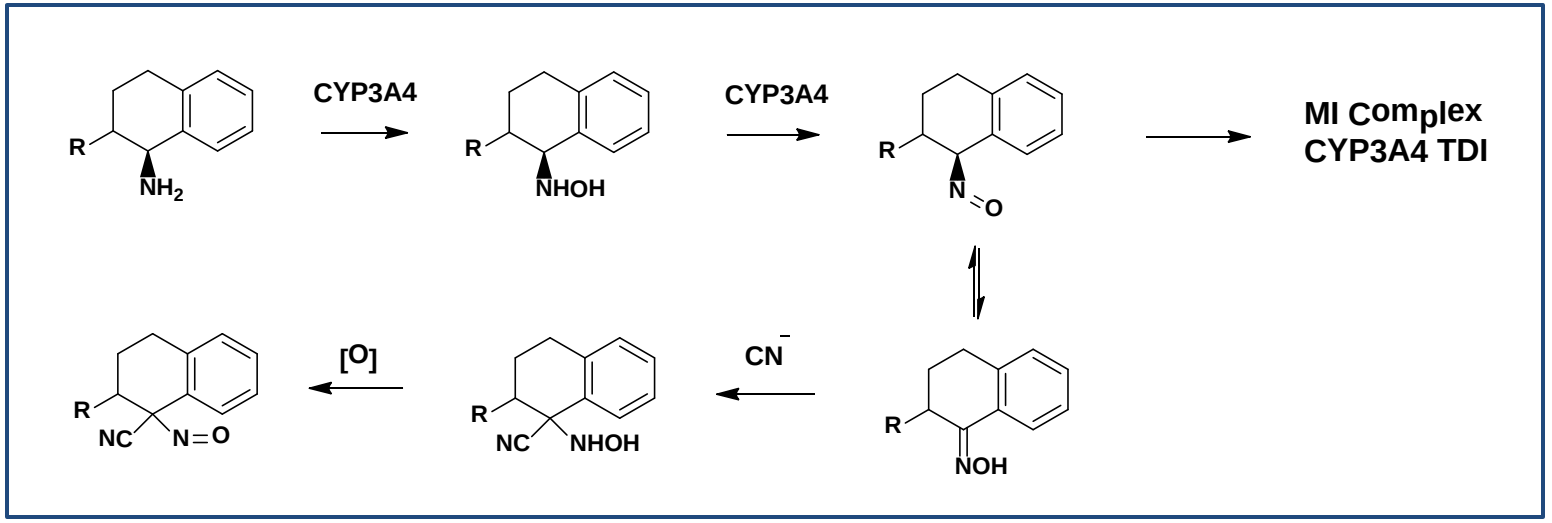
Minimizing Metabolic Activation:
(1) Block Site of Metabolism



Wu YJ, et al, *J. Med. Chem.* 46, 3778-3781 (2003)
Diekhaus CM. *Chem. Biol Interact*, 142, 99-117 (2002)

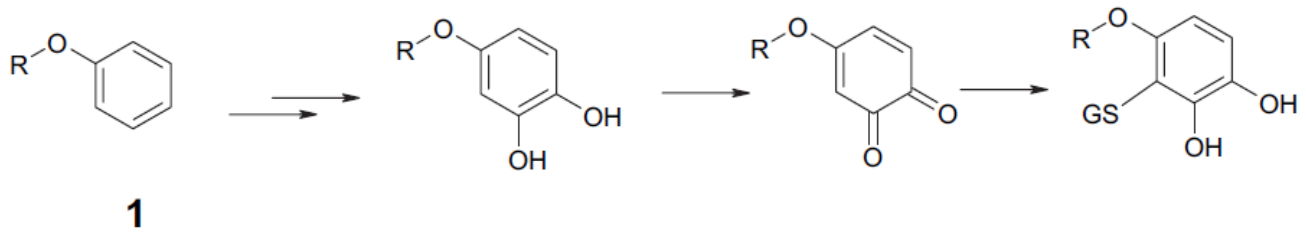
Minimizing Metabolic Activation:
(2) Introduce Steric Hindrance





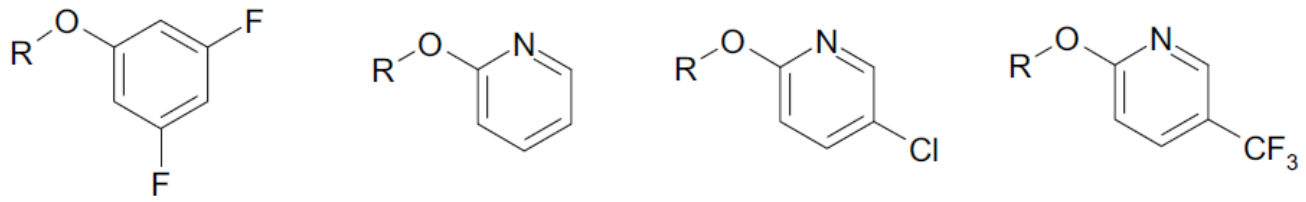
Tang W et al., Xenobiotica, 38, 1437-1451 (2008)

Minimizing Metabolic Activation:
(3) Introduce Electronic Changes



1

Figure 1 Metabolic activation of an aryloxy-substituted drug candidate (**1**).

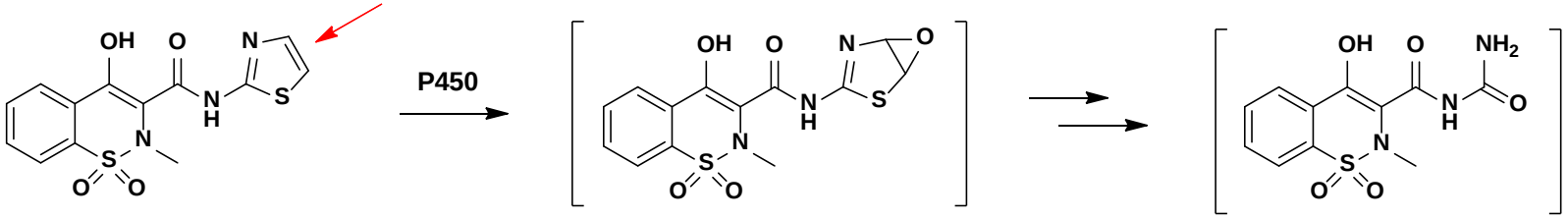


(1690) (911) (303) (88)

Figure 2 Improved analogs of the lead compound **1** showing reduced levels of covalent binding (pmol-equiv/mg protein, shown in parentheses).

Doss GA and Baillie TA., *Drug Metab Rev,* 38, 641-649 (2006)

Minimizing Metabolic Activation:
(4) Introduce Metabolic "Soft Spots"

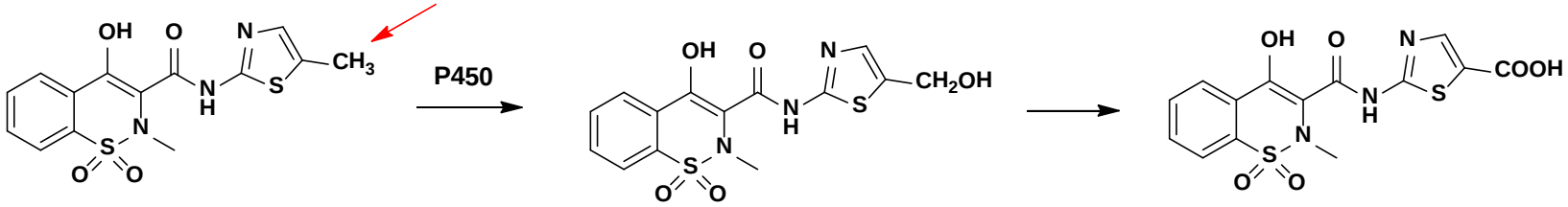


Sudoxicam
(Withdrawn during Phase III due to hepatotoxicity)

Epoxide

Acylthiourea

Toxic !!

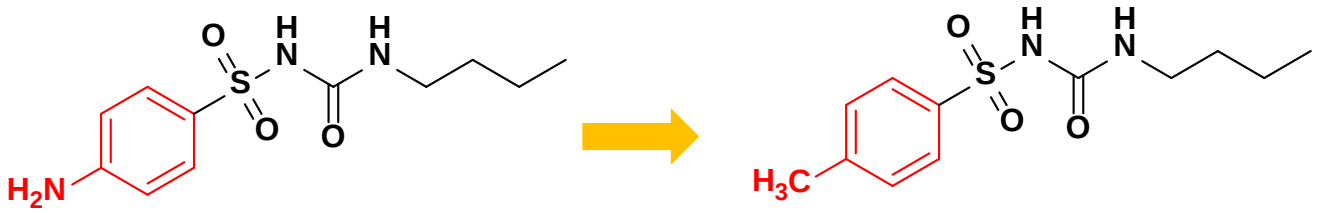


Meloxicam
(Non-hepatotoxicity)

✓ Introduction of methyl group dramatically alters the metabolic profile

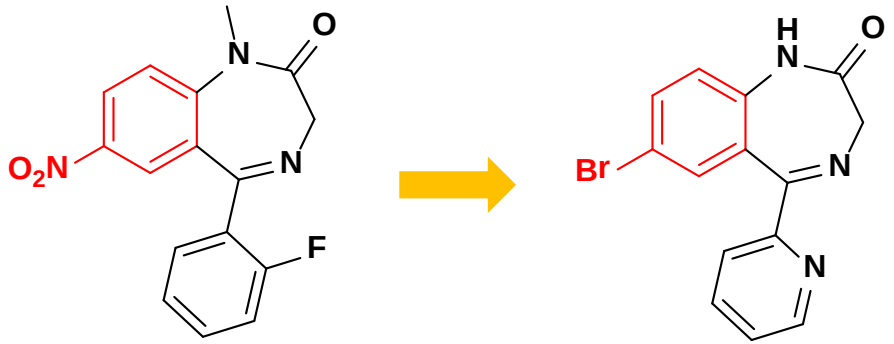
Obach RS et al., *Chem. Res. Toxicol,* 21, 1890-1899 (2008)

Minimizing Metabolic Activation:
(5) Replacement of structural alerts or elements



Carbutamide

Tobutamide



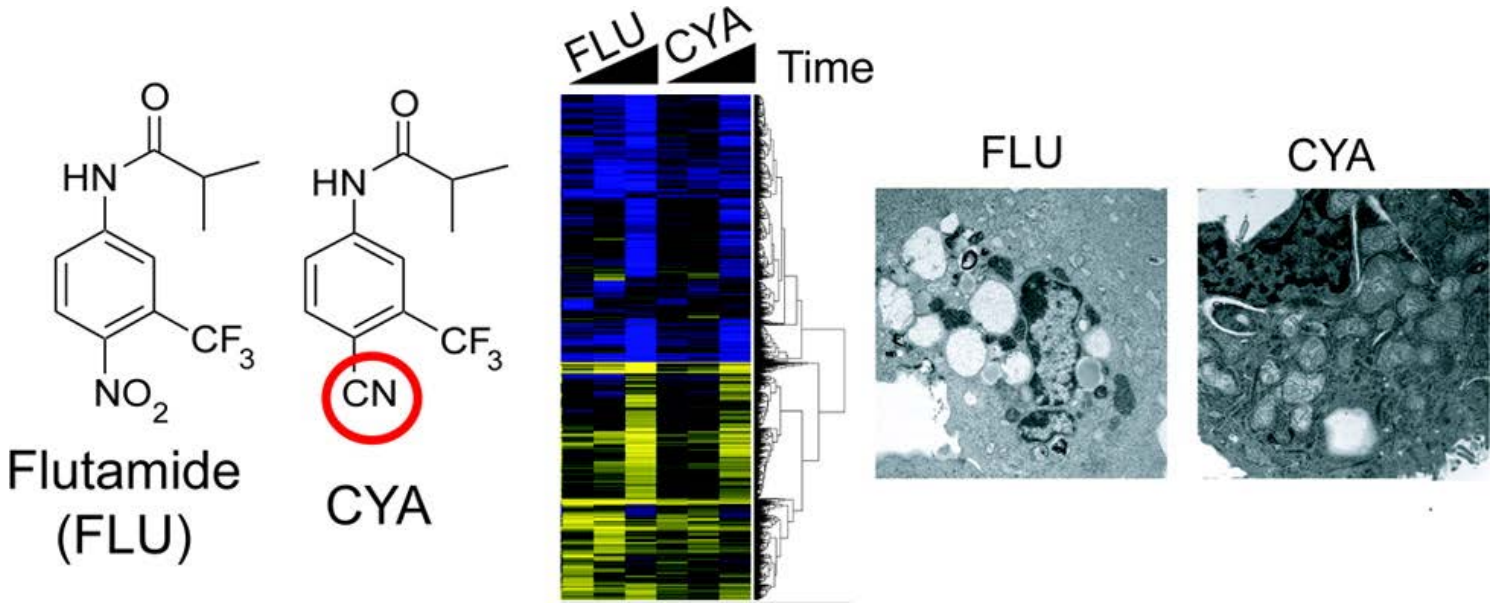
Flunitrazepam

Bromazepam

- The principle here is to minimize the electronic changes (replace EDW with EDG, and EWG with EWG), and its impact on pharmacological activity.

Argikar UA, et al, *Curr Top Med Chem*.11:419-449 (2011).

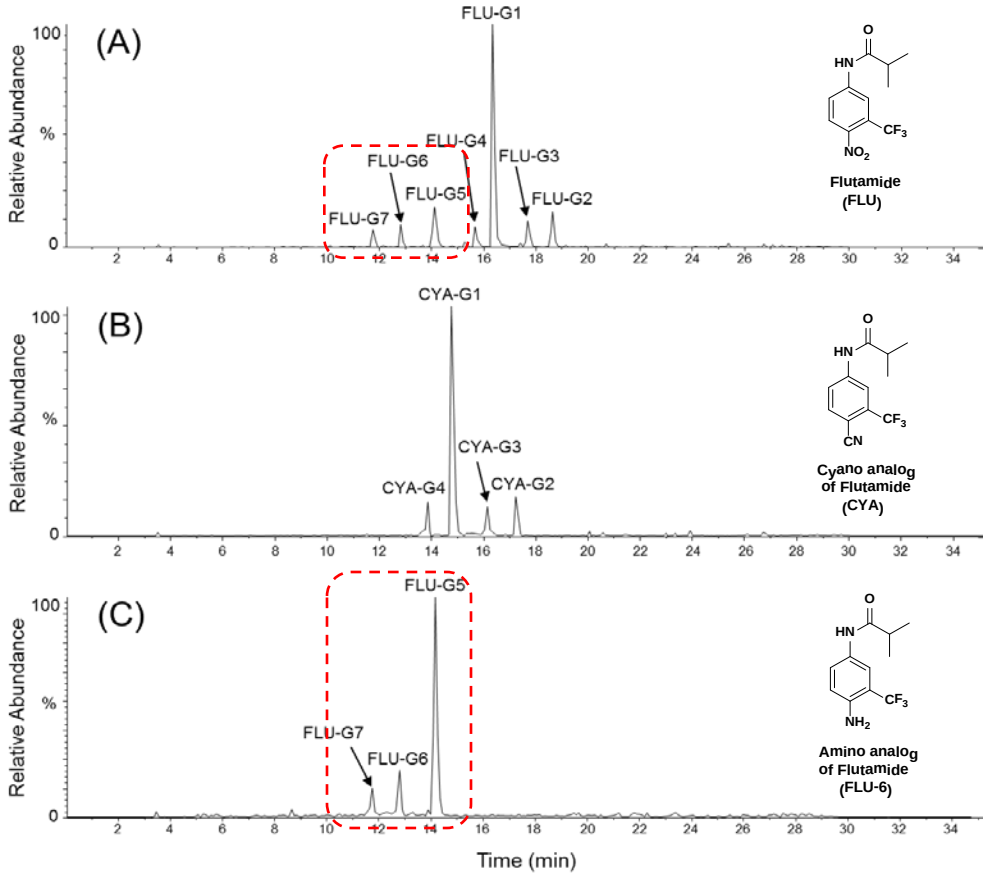
Comparison of the Cytotoxicity of Flutamide (FLU) to Its Cyano Analogue (CYA)



- Replacement of the nitro by the cyano group in CYA significantly attenuated the FLU cytotoxicity to hepatocytes via mechanisms involving mitochondrial dysfunction and ATP depletion.
- Microarray analysis comparing FLU to CYA revealed some pattern similarities, however, FLU resulted in more substantial changes than CYA in gene expressions associated with oxidative phosphorylation, antioxidant defense, and cell death pathways.

Coe KJ, et al. *Chem. Res. Toxicol.* 20:1277-1290, 2007

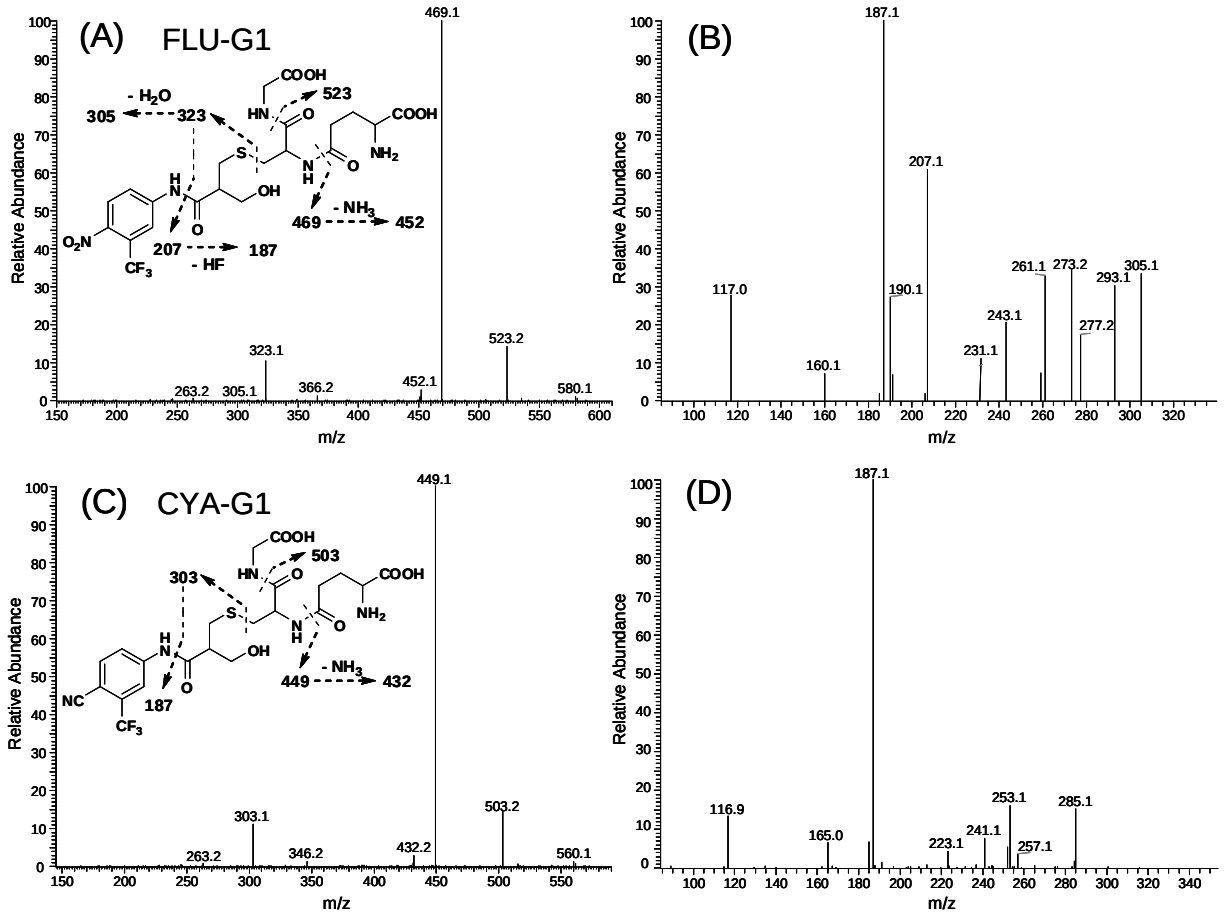
Negative PI scanning at *m/z* 272 in HLM incubations of FLU (A), CYA (B) and FLU-6 (C) using the PI-EPI approach



- FLU and CYA shared similar oxidative bioactivation pathways to generate GSH adducts FLU-G1-4 and CYA-G1-4, respectively.
- Of significance was the detection and characterization of several GSH adducts FLU-G5-7 resulting from nitroreductive metabolism of FLU.

Wen B, et al. *Chem. Res. Toxicol.* 21:2393-2406, 2008

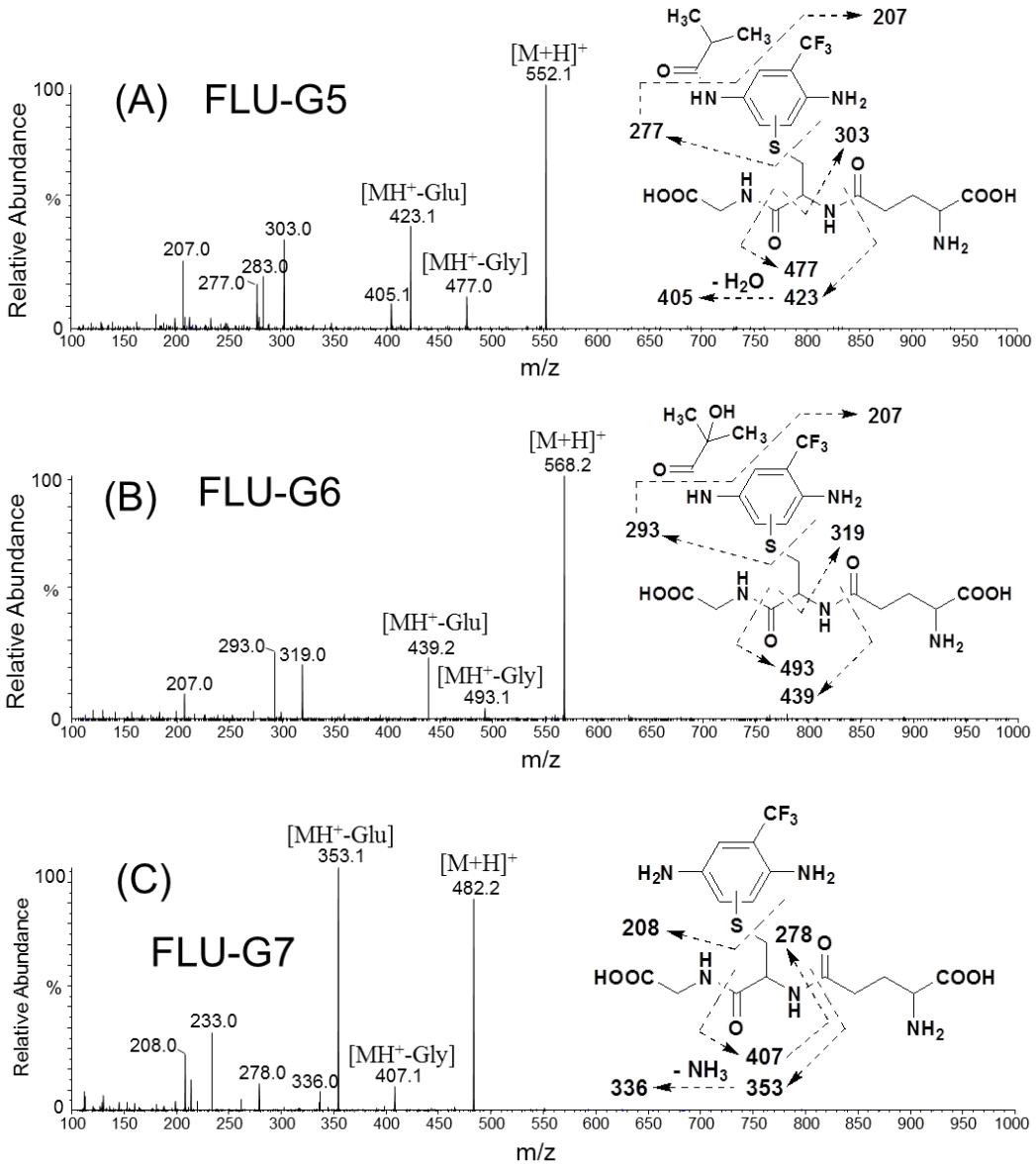
LC/MS/MS analysis of FLU-G1 (A, B) and CYA-G1 (C, D)



(A) MS/MS spectrum of FLU-G1 at m/z 598 ($[M+H]^+$); (B) MS^3 spectrum of the fragment ion of FLU-G1 at m/z 323;
(C) MS/MS spectrum of CYA-G1 at m/z 578 ($[M+H]^+$); (D) MS^3 spectrum of the fragment ion of CYA-G1 at m/z 303

- FLU and CYA shared similar bioactivation mechanisms to form the major GSH adducts FLU-G1 and CYA-G1 respectively.

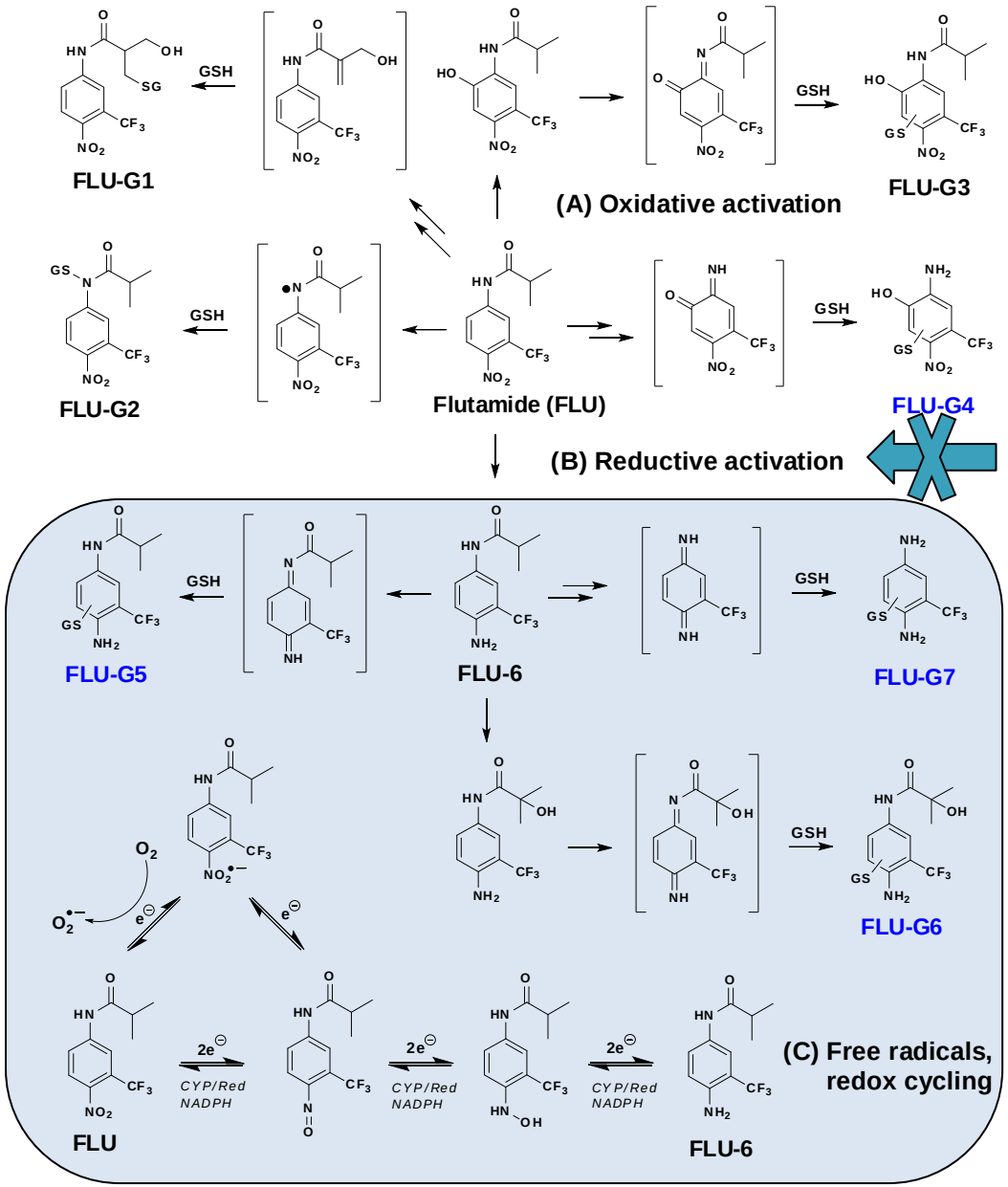
LC/MS/MS analysis of FLU-G5-7, the GSH adducts of the nitro reduction metabolite FLU-6

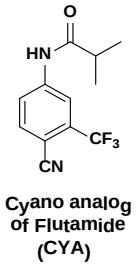


- FLU undergoes nitroreductive metabolism to form FLU-6 which is subsequently bioactivated to form GSH adducts FLU-G5-7 in HLM.
- The identities of FLU-G5-7 formed in HLM incubations of FLU were confirmed by comparing its HPLC retention time and MS spectra with those formed in HLM incubations of FLU-6.

Wen B, et al. *Chem. Res. Toxicol.* 21:2393-2406, 2008

Bioactivation pathways of flutamide



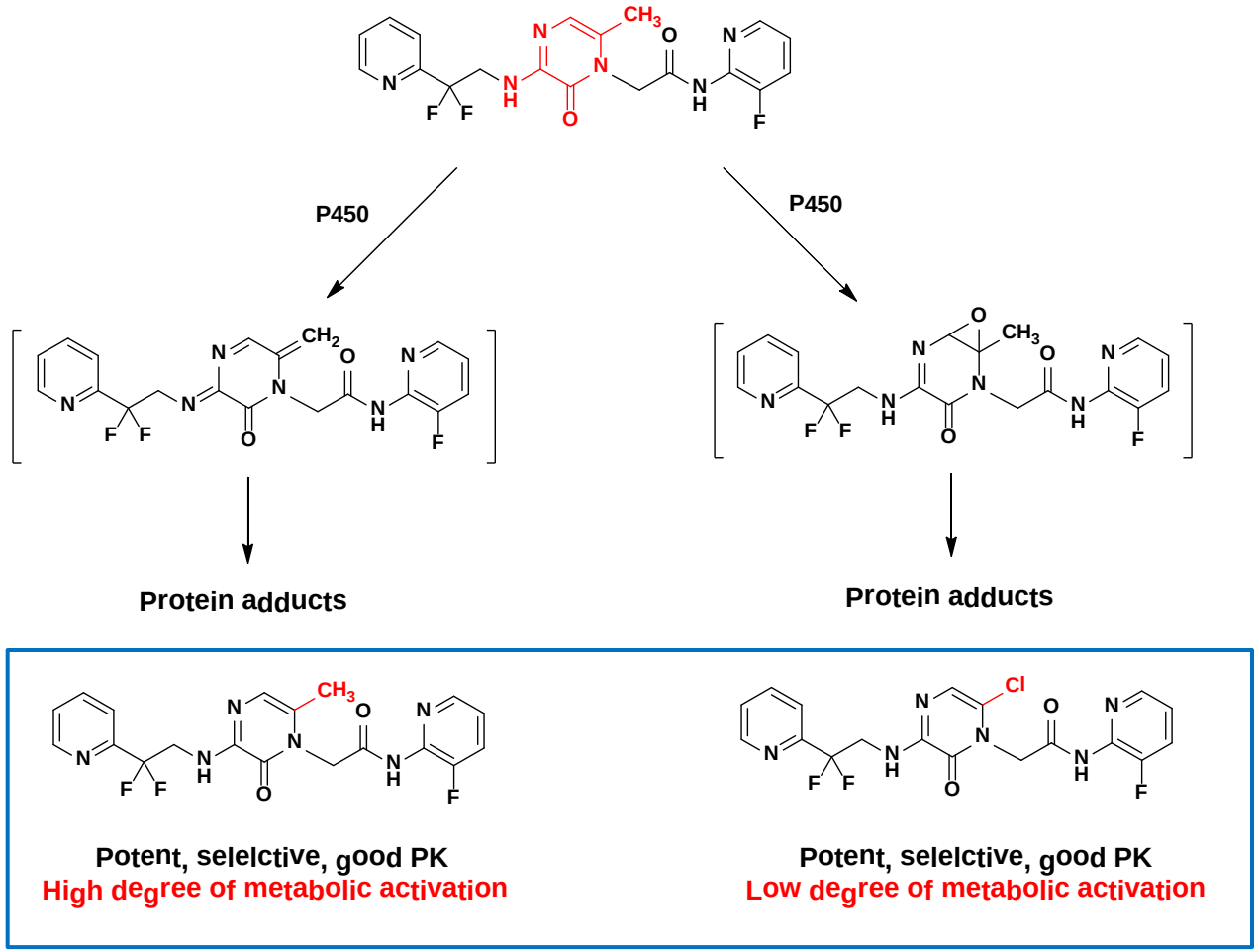


(A) Oxidative activation
(B) Reductive activation
(C) Free radical formation

- These data clearly demonstrate that the nitroaromatic group of FLU contributes to FLU bioactivation via different mechanisms
- These results provide a possible explanation for the difference in cytotoxicity between FLU and CYA

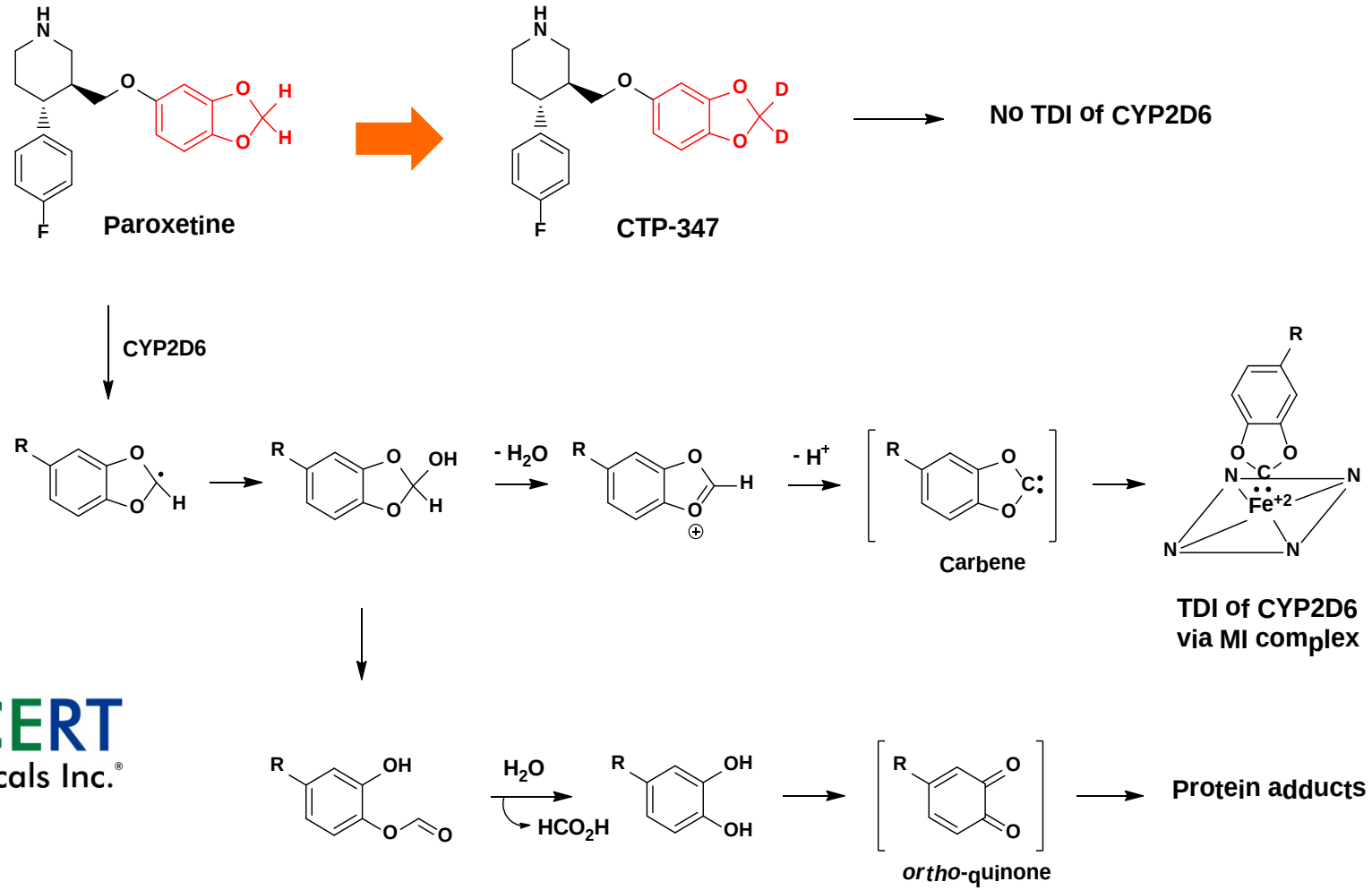
Wen B, et al. *Chem. Res. Toxicol.* 21:2393-2406, 2008

Minimizing Metabolic Activation:
(6) Combination approaches (i.e. steric and electronic changes)



Singh E et al, et al., *Chem. Res. Toxicol,* 16, 198-207 (2003)

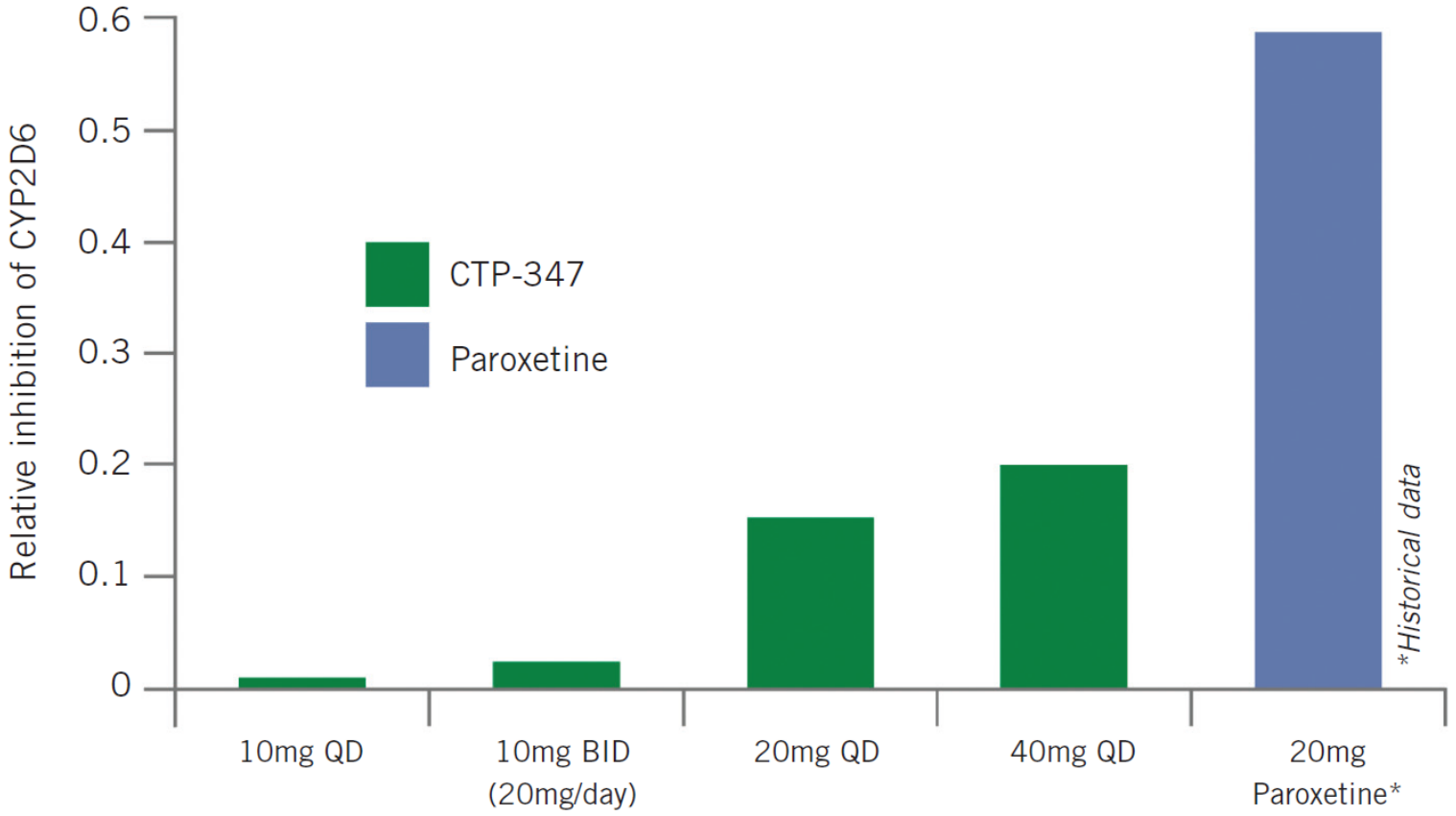
Minimizing Metabolic Activation:
(7) Other approaches (i.e. selective deuteration)



CoNCERT Pharmaceuticals Inc.®

Yarnell At, *Chem. Eng. News,* 87, 36-39 (2009)

Drug-drug interaction between CTP347 and dextromethorphan from Phase 1b study



- Y-axis shows the ratio of intact excreted dextromethorphan versus dextrorphan metabolite (9)

http://www.concertpharma.com/news/documents/IPT32ConcertPharma.pdf

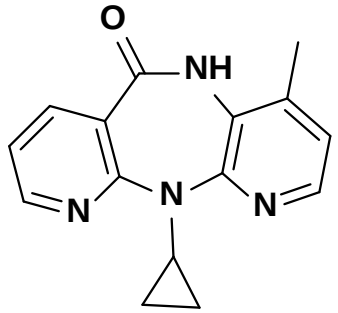
Nevirapine: Another Case of Selective Deuteration

- Nevirapine (NVP) is the first non-nucleoside reverse transcriptase inhibitor approved by FDA for use in combination therapy of HIV-1 infection

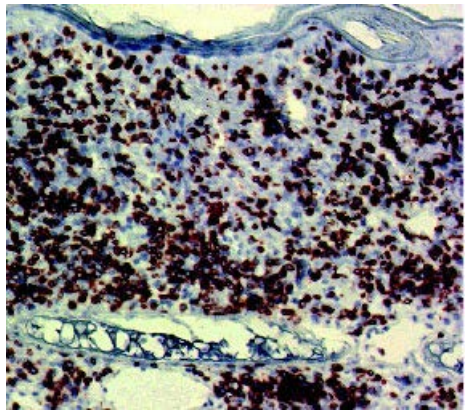
- Nevirapine causes a **skin rash** in 8-16% of patients and can also cause severe **liver toxicity**

- Nevirapine also causes a skin rash in rats

- The mechanism of skin rash and idiosyncratic hepatotoxicity remains unknown

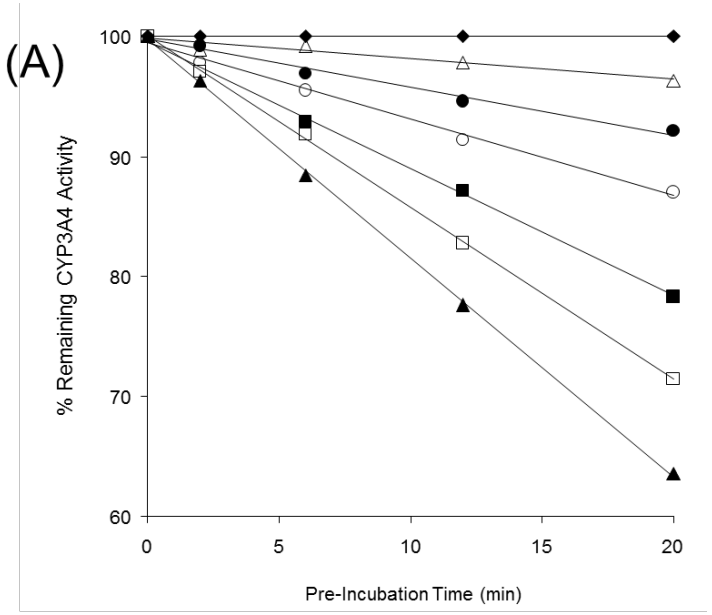


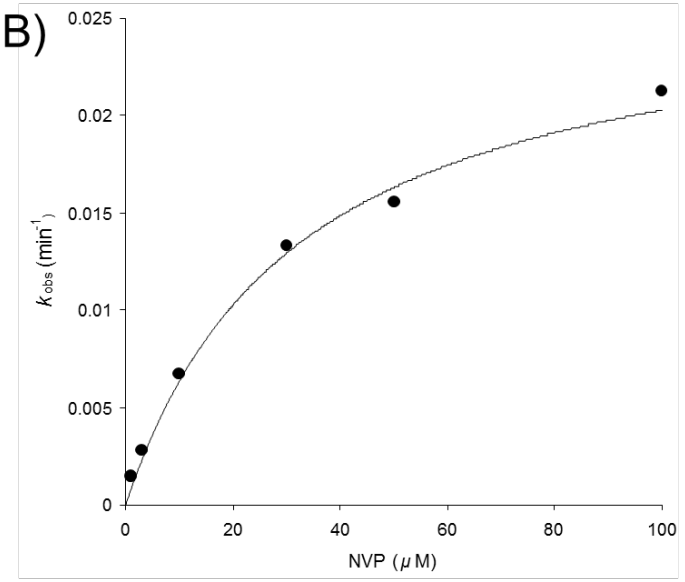
Nevirapine

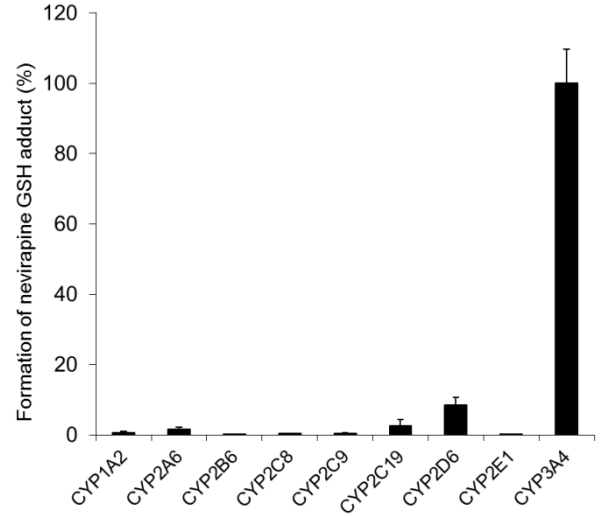


Sharma AM, et al. *Chem Res Toxicol.* 26:817-827 (2013)
Caixas U, et al, *Toxicology,* 301:33-39 (2012)

Time- and concentration-dependent inactivation of CYP3A4 by NVP



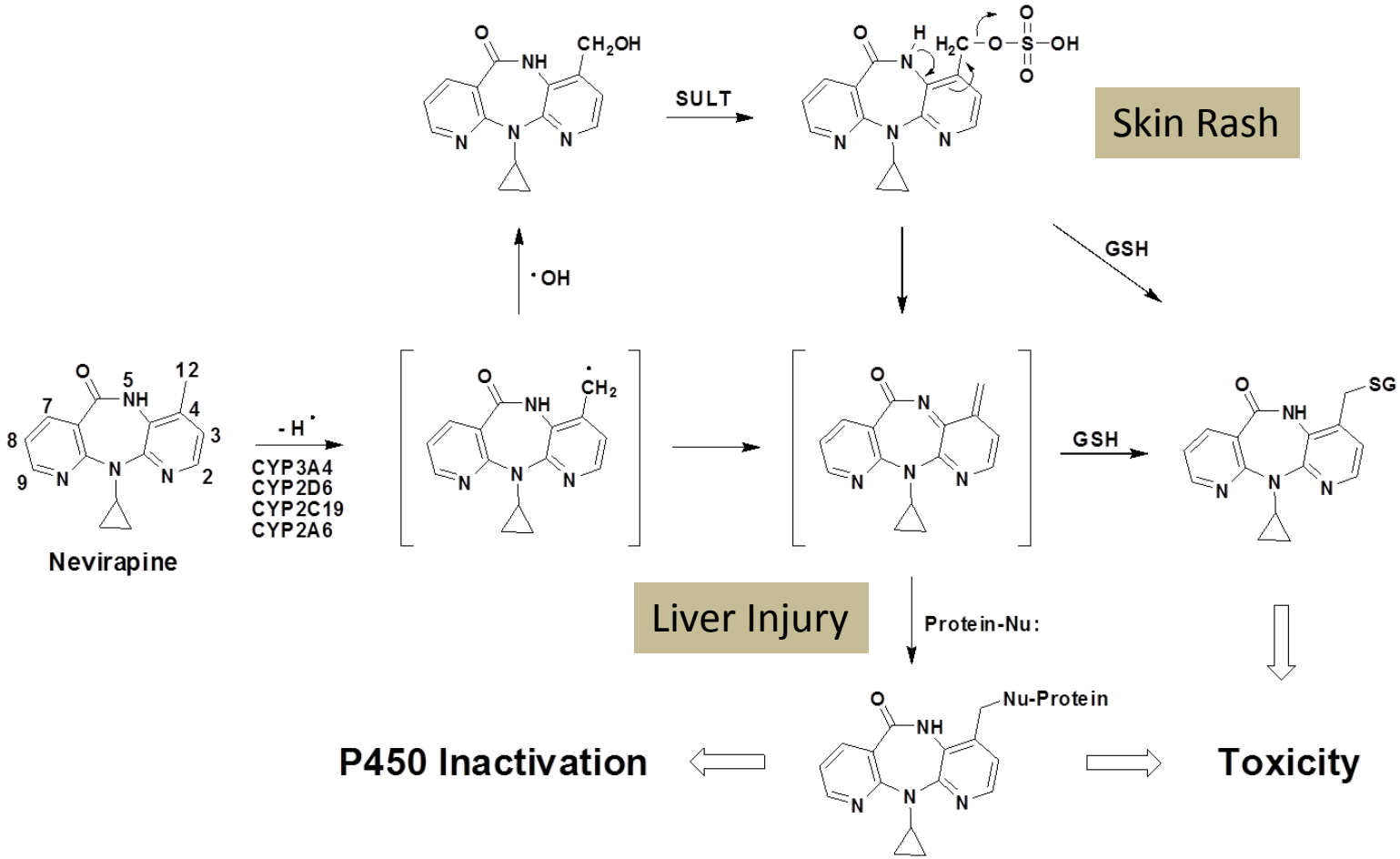




- Formation of the NVP GSH adduct was primarily catalyzed by CYP3A4.
- The NVP quinone methide intermediate was also a mechanism-based inactivator of CYP3A4, with K_I = 31 μM and k_{inact} = 0.029 min^{-1} .

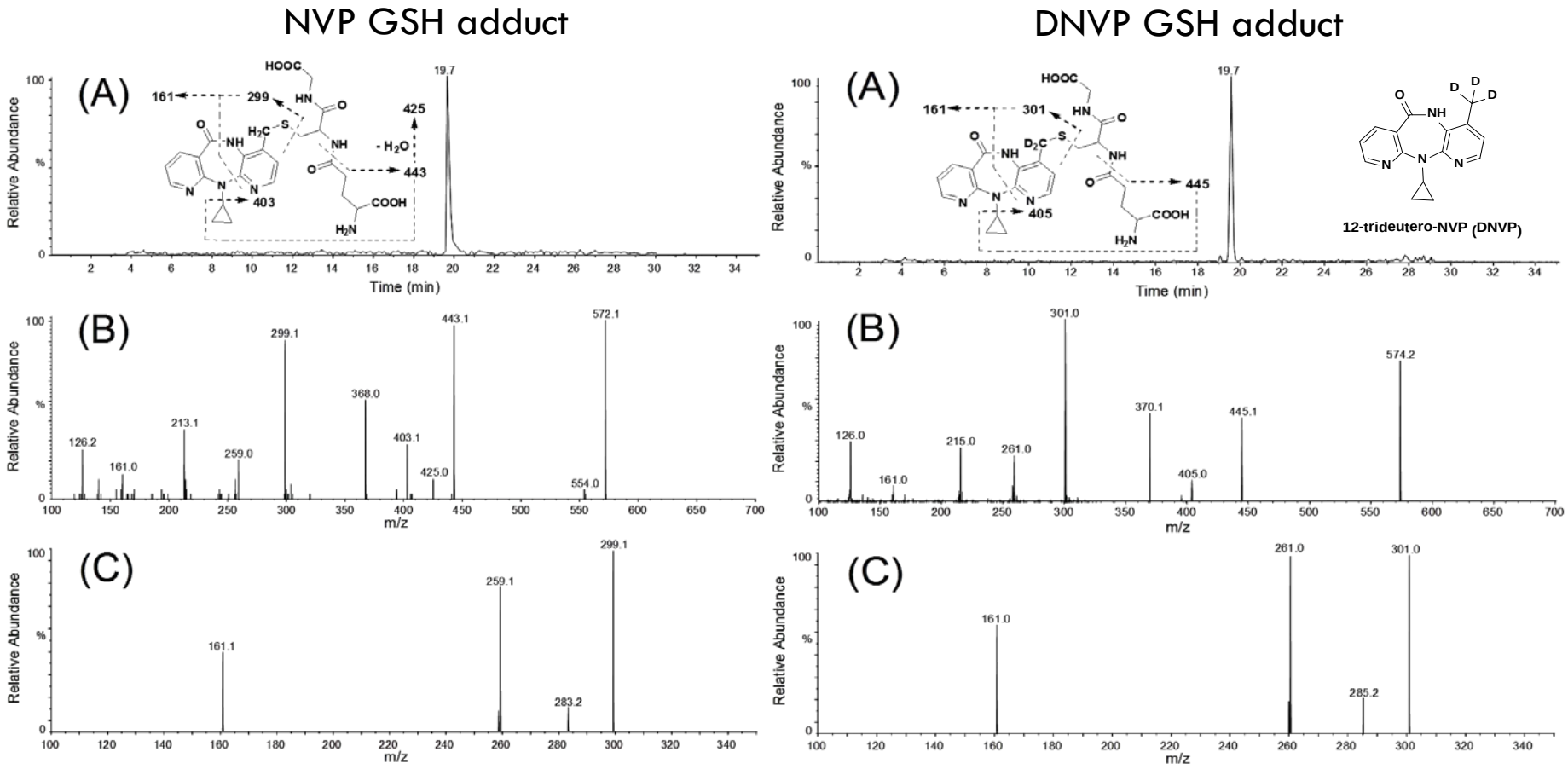
Wen B, et al. *Drug Metab. Dispos.* 37:1557-1562, 2009

Proposed bioactivation pathways of nevirapine.



Wen B, et al. *Drug Metab. Dispos.* 37:1557-1562, 2009

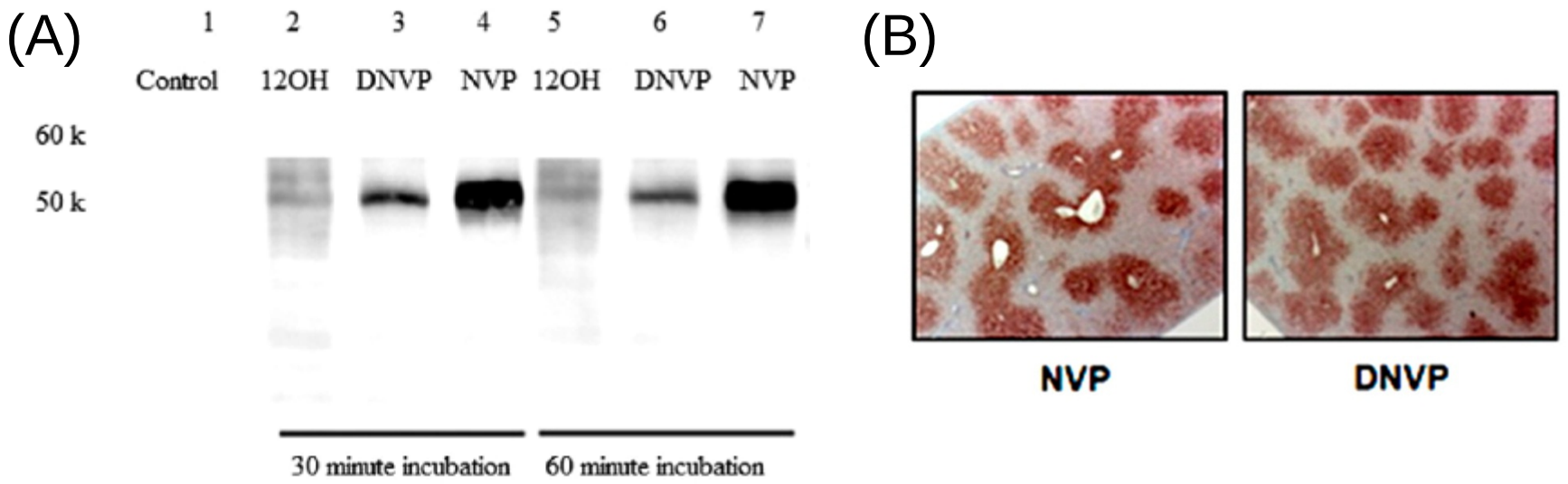
LC-MS/MS analysis of GSH adducts of NVP and DNVP



- Deuteration of 12-methyl NVP aids in structural elucidation of NVP GSH adduct and minimizes the formation of NVP quinone methide.
- Magnitude of GSH adduct formation by DNVP was substantially decreased compared to that of NVP, presumably due to **isotope effects**.

Wen B, et al. *Drug Metab. Dispos.* 37:1557-1562, 2009

Comparison of covalent binding (A) and immunohistochemistry (B) of NVP and DNVP



- Substitution of the 4-methyl hydrogens with deuterium (DNVP) led to a substantial decrease in covalent binding.
- Immunohistochemistry of liver sections from NVP- and DNVP-treated rats showed a marked decrease in covalent binding of DNVP compared to that of NVP in the centrilobular area.
- These results are in parallel with our findings in GSH adduct formation by NVP and DNVP.

Sharma AM, et al. *Chem Res Toxicol.* 26:817-827 (2013)

7. Data interpretation and risk mitigation strategies
(数据与风险评估方法)

Mechanisms of Drug-Induced Toxicities

Type A - Normally reversible, involving a defined target leading to a predictable pharmacodynamic outcome (on- or off-target)

Type B - “Idiosyncratic” toxicities (not predictable, eg halothane)

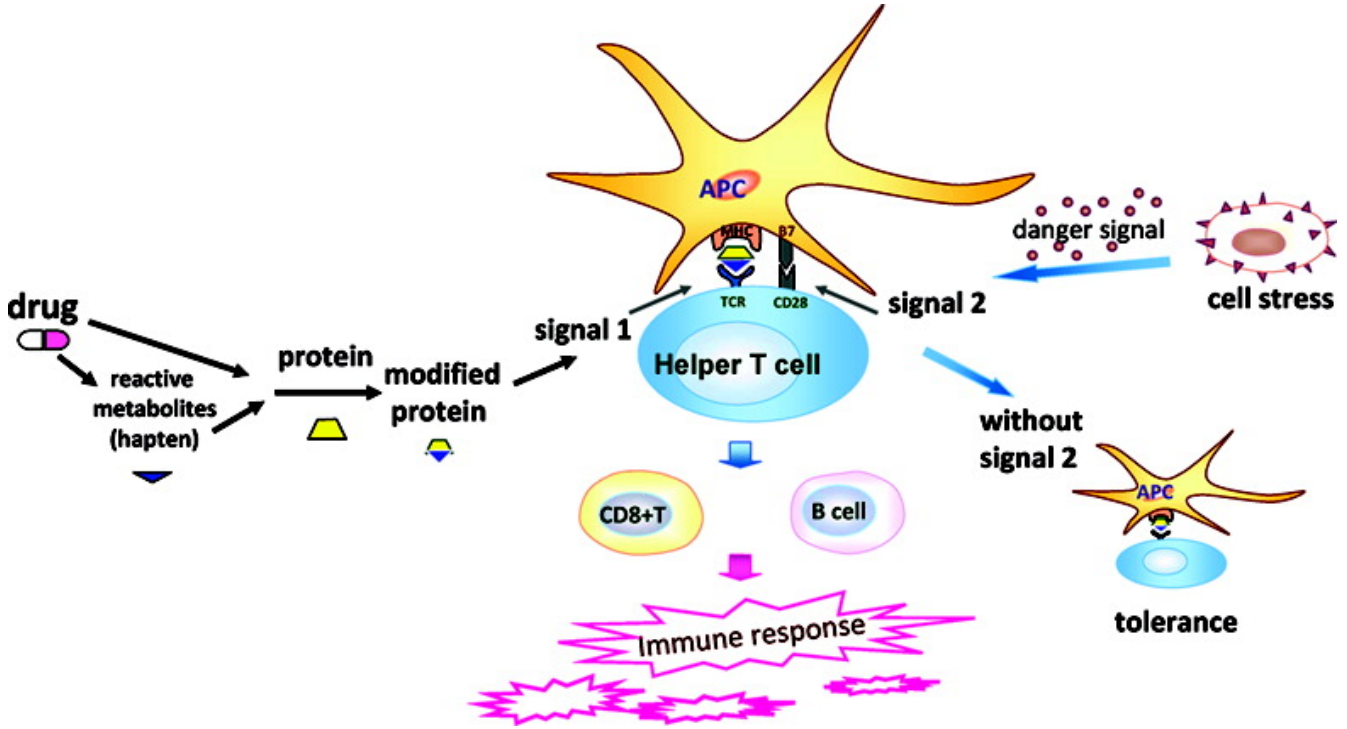
Type C - Predictable, dose-dependent toxicities (eg acetaminophen)

Type D - Occur only after prolonged dosing (carcinogenicity, teratology)

- *Evidence suggests that **reactive metabolites** may play a causative role in several forms of drug-induced toxicity (Types B, C, and D)*
- *Type B (idiosyncratic) toxicities are of greatest concern in drug development*

Park BK, et al, *Chem. Res. Toxicol.*, 11, 969-988 (1998)
Smith DA and Obach RS, *Chem. Res. Toxicol.*, 22, 267-279 (2009)
Uetrecht J, *Annu. Rev. Pharmacol. Toxicol.*, 47, 513-539 (2007)
Uetrecht J, *Chem. Res. Toxicol.*, 21, 84-92 (2008)

Idiosyncratic Drug Toxicity: *Hapten and Danger Hypothesis*

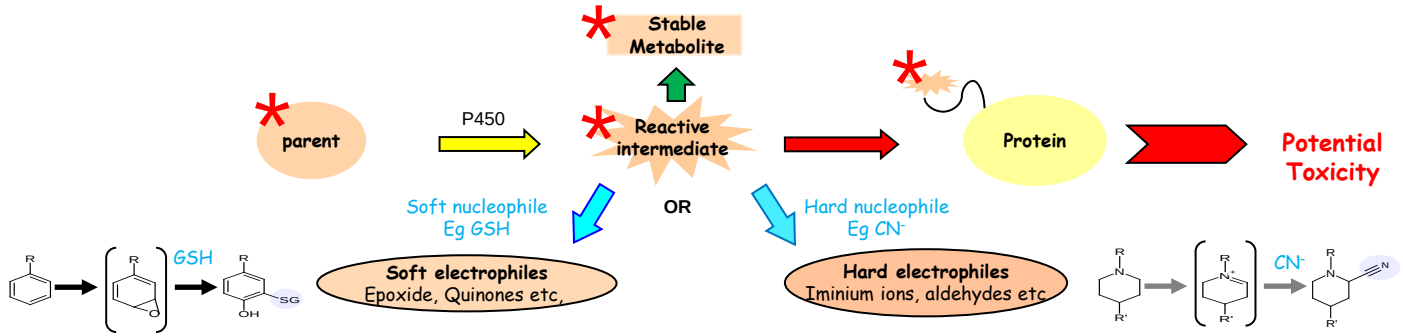


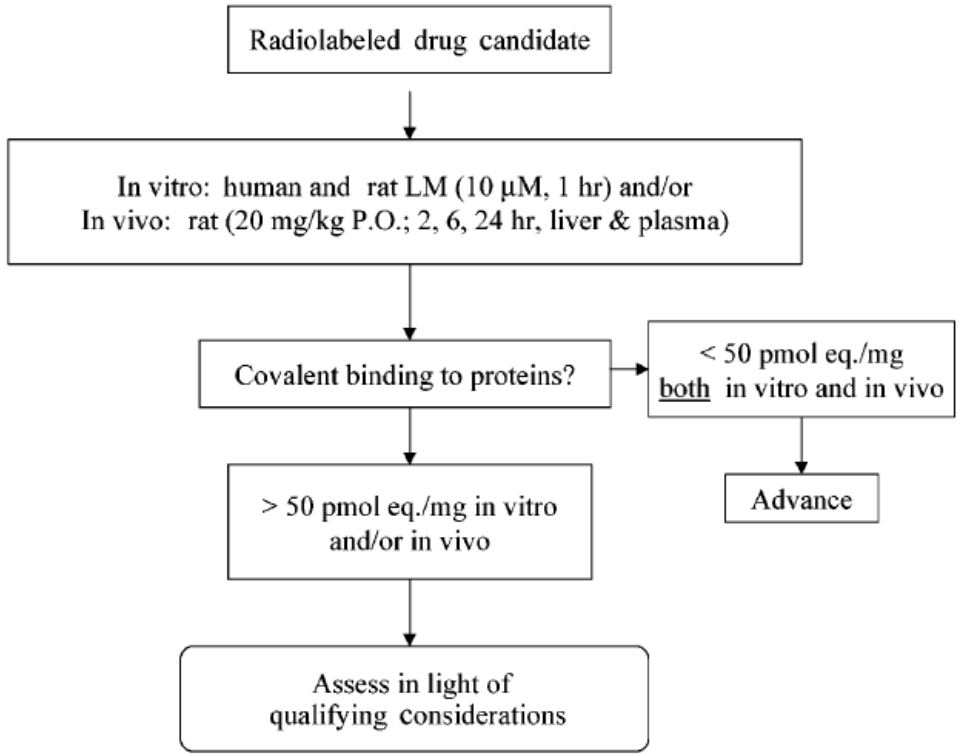
- **The Hapten hypothesis** involves a chemically reactive drug or reactive metabolite acting as a hapten by binding to protein, which is then taken up by an APC and processed. The processed antigen is presented in the context of MHC to a helper T cell; this represents signal 1.
- **The Danger hypothesis** involves cell damage or stress (possibly caused by the drug or reactive metabolite) causing the release of danger signals that lead to upregulation of costimulatory factors; this is signal 2. Without signal 2, the result is immune tolerance.

Uetrecht J, *Chem. Res. Toxicol.*, 21, 84-92 (2008)

Covalent Protein Binding: A 'Gold Standard' Assay

* = radiolabel



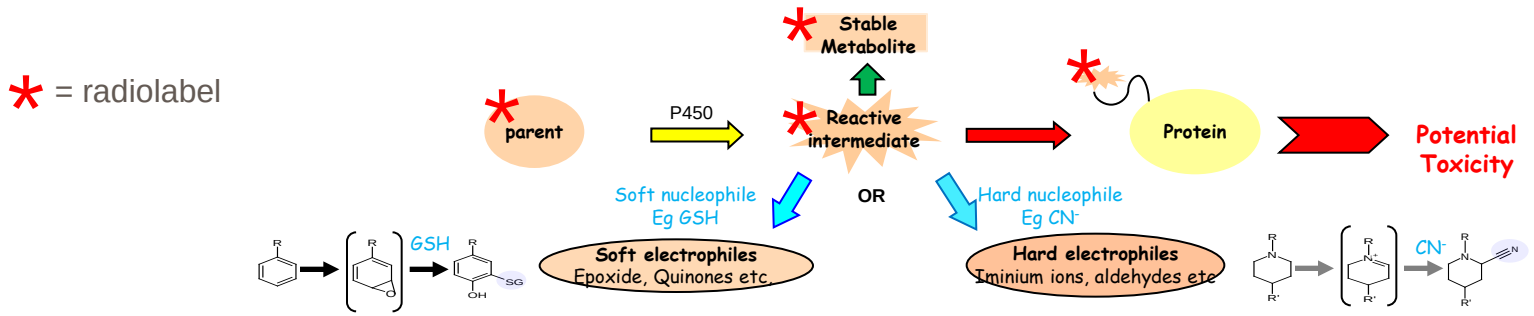


Qualifying considerations

- Chemical tractability of structural series?
  - What potential exists to modify structure?
  - Evidence of informed chemical intervention?
  - Has metabolic activation been minimized relative to the preceding compound(s)?
- Availability of existing treatments?
- Is the prognosis disabling or life-threatening?
- Is the anticipated clinical daily dose < 10 mg?
- Are the metabolic clearance routes predominantly non-Phase 1?
  - Consider studies in hepatocytes.
- Expected duration of therapy?
  - Will the drug will be used chronically/prophylactically?
- What is the intended target population?
  - Is the drug intended for a pediatric indication?

Evans DC, et al. *Chem Res Toxicol.* 17:3-16 (2004)

Screening and characterization of reactive metabolites



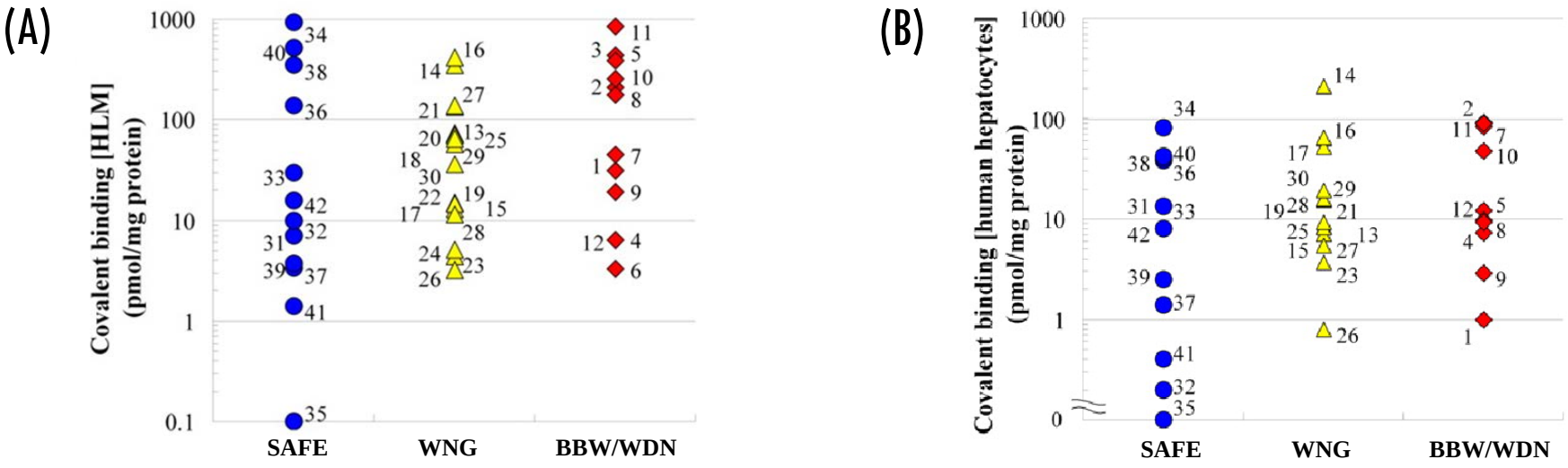
A. Covalent Binding (CVB)

- **Gold standard assay – the amount of drug-protein adduct (considered the causative agent of toxicity) is directly measured**
- **Requires radiolabeled drugs, therefore not suitable for early drug discovery**
- **Mechanistic information can be obtained through the use of trapping agents**

B. Electrophilic Trapping

- **Conducted in liver microsomes, S9 and hepatocytes**
- **LC/MS/MS and/or NMR for structure elucidation**
- Electrophiles can be classified as ‘hard’ or ‘soft’; a localized positive charge would make the electrophile ‘hard’, while a delocalized charge would make it ‘soft’
- Similarly nucleophiles can be classified as ‘hard’ or ‘soft’; a sulfur-containing nucleophile is considered ‘softer’ than a nitrogen-containing nucleophile because a sulfur atom is larger and the lone pair electrons are further away from the nucleus and there more diffuse
- ***Hard electrophiles react with hard nucleophiles and soft electrophiles react with soft nucleophiles***

Correlation of covalent binding in HLM (A) and human hepatocytes (B) with safety categories

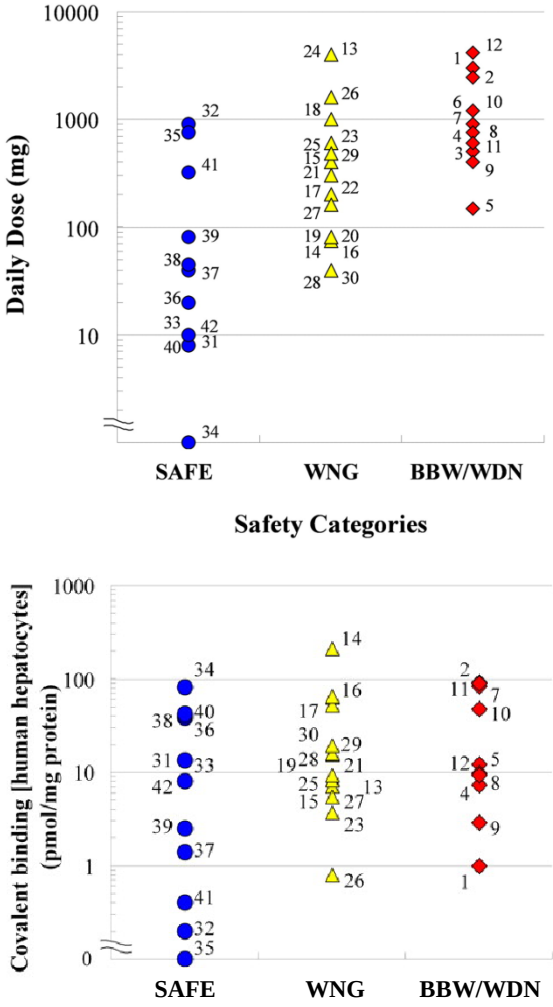


- There were 42 tested drugs in safety categories namely SAFE, WNG (no black box warning but a warning for idiosyncratic drug toxicity), BBW/WDN (black box warning/withdrawn).
- The CVB in HLM or human hepatocytes did not successfully distinguish the safety categories.

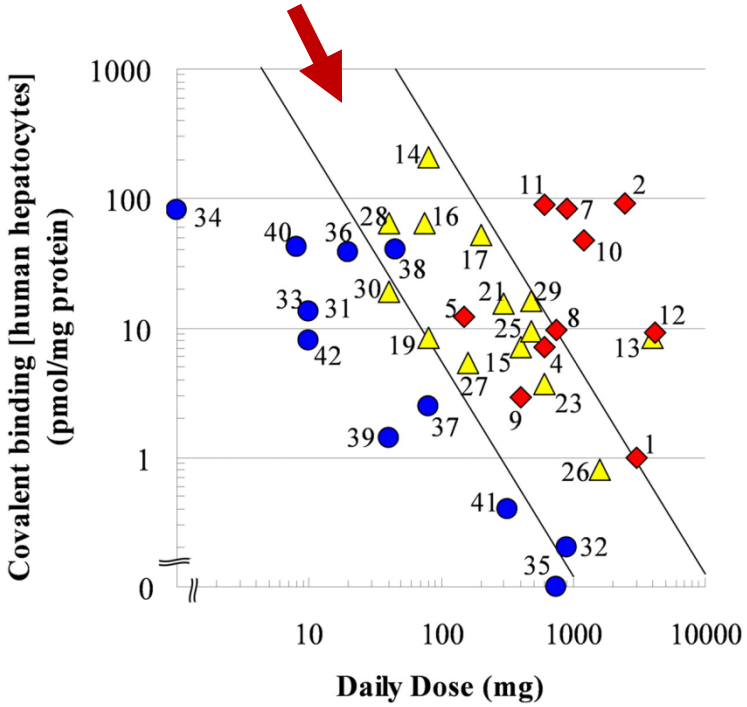
❖ The CVB values alone should not be used as a cut-off criteria or decision-making per se. For example, troglitazone (400 mg, withdrawn), pioglitazone (50 mg, safe), and rosiglitazone (8 mg, safe), all three drugs exceed the 'threshold of concern' for CVB (>50 pmol/mg protein).

Nakayama S, et al. *Drug Metab. Dispos.* 37:1970-1977, 2009

A zone classification system for risk assessment using daily dose and CVB



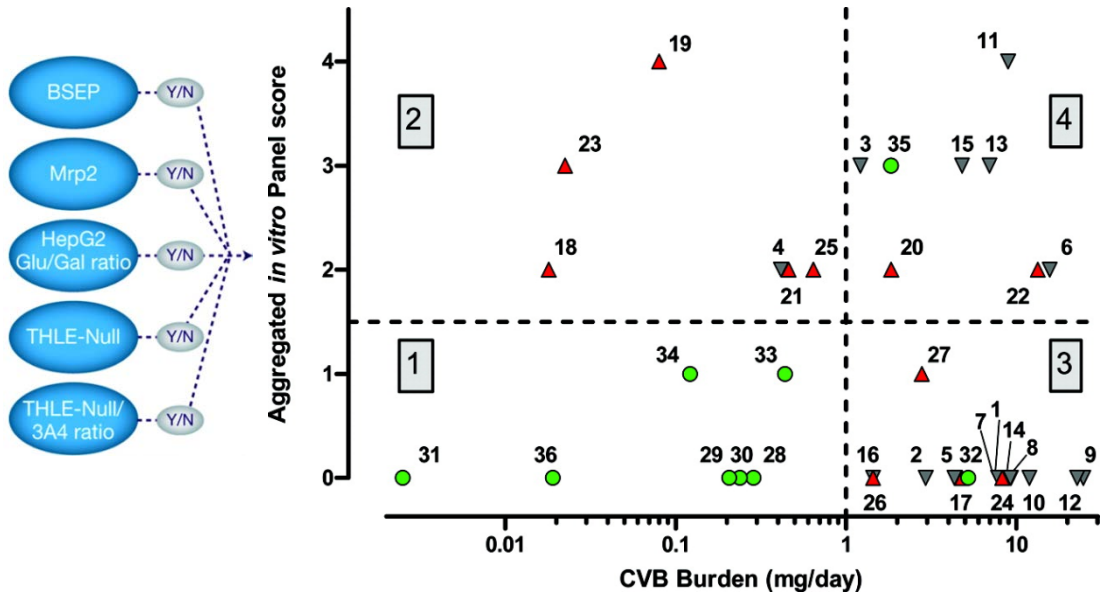
19 of 42 tested drugs are in the 'problematic' zone



- Three zones (acceptable, problematic, unacceptable) were created by a regression analysis.
- Among 37 tested drugs, 30 drugs were located in zones corresponding to their respective classified safety categories.

Nakayama S, et al. *Drug Metab. Dispos.* 37:1970-1977, 2009

Integrated *in vitro* Hepatic Hazard Matrix: 4-Zone Classification System



CVB burden = Daily Dose*f_a*f_m*f_{cvb}

- *In vitro* hepatotoxicity endpoints (eg. BSEP/Mrp2 inhibition, HepG2 mitochondrial injury, THLE cytotoxicity) have been incorporated into the Hepatic Hazard Matrix along with CVB burden.
- The 4-zone classification system demonstrated high specificity (78%) and sensitivity (100%) among 27 drugs with severe/marked risk and 9 drugs with low risk.
- *More datasets, more 'robust'?* eg. protein targets http://targetprotein.res.ku.edu/

❖ Risk assessment and mitigation strategies to date often only consider drug-related, not patient-related risk factors such as target population, age, gender, genetic variability, immune response etc.

Thompson RA et al, *Chem Res Toxicol.* 25:1616-1632 (2012)
Thompson RA et al, *Chem Res Toxicol.* 29:505-533 (2016)

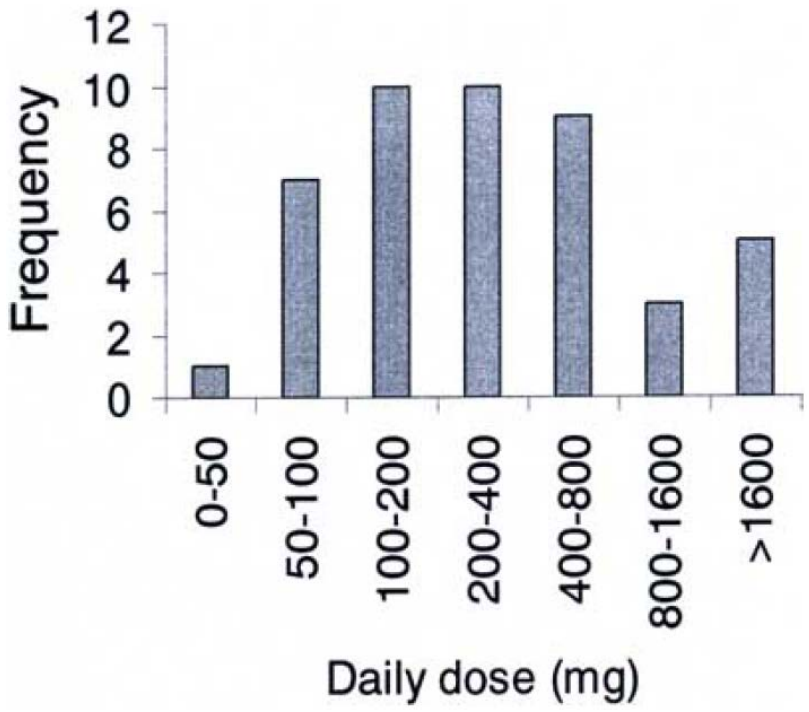
Several variables that will impact whether a drug candidate that possesses a structural alert will eventually lead to ADRs

1)Dose

2)Usage (chronic vs acute)

3)Detoxification of reactive metabolites

4)Competing metabolic pathways (f_m vs f_{rm})



Lower the Dose

↓

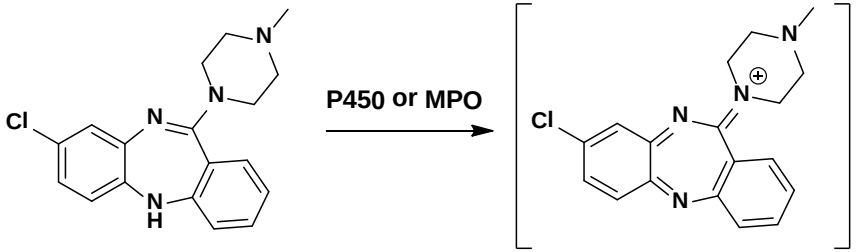
Less reactive metabolite formed

↓

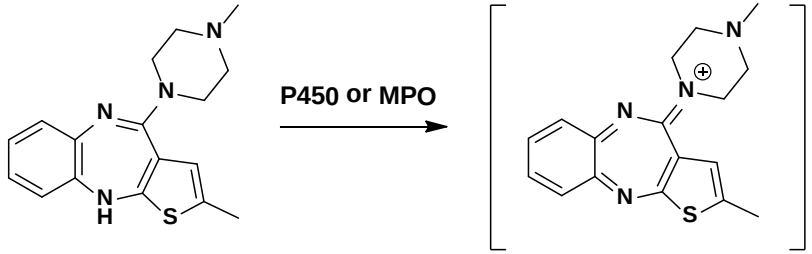
Lower the Risk

Kalgutkar AS, et al. *Curr Drug Metab.* 6:161-225 (2005)

Impact of Dose on Toxicity



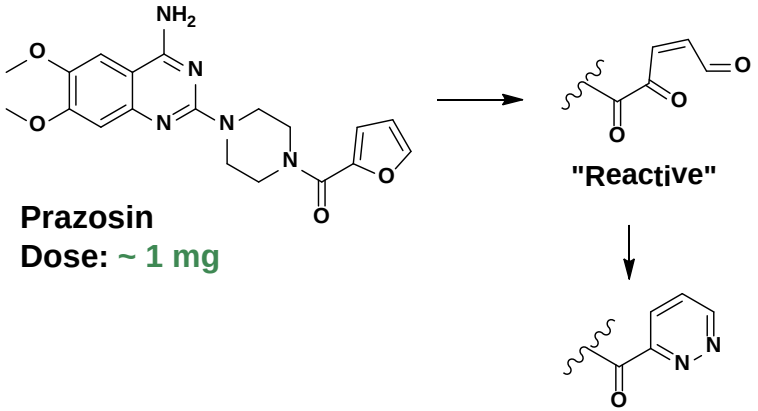
Clozapine



Olanzapine

Clozapine

- Forms reactive metabolite *in vitro*
- **Dose 300-900 mg/day**
- ~1% incidence of agranulocytosis
- 11% of the antipsychotic RX in the US (1999)
- 2-3% of the antipsychotic RX in the US (2011)

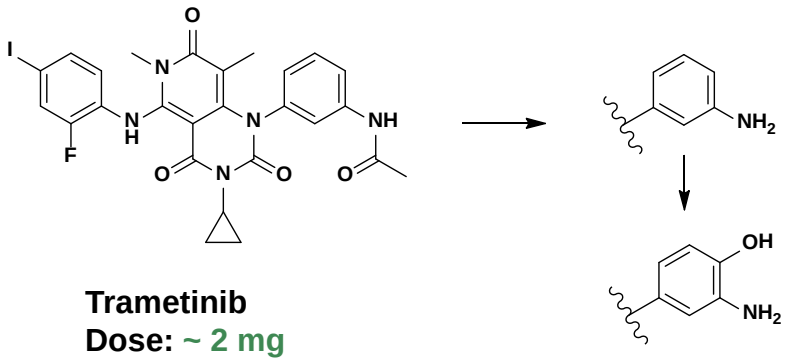


Prazosin
Dose: ~ 1 mg

Trapped

Olanzapine

- Forms reactive metabolite *in vitro*
- **Dose 10 mg/day**
- No manifestation *in vivo*
- 18% of the antipsychotic RX in the US (2011)

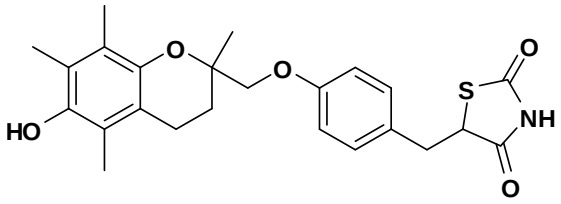


Trametinib
Dose: ~ 2 mg

Both are Structural Alerts!

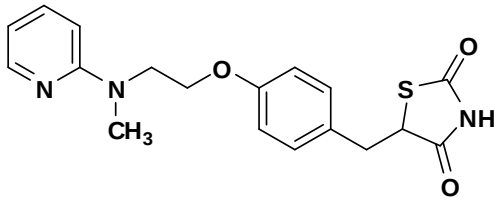
Gardner I, et al, *Mol Pharmacol.* 53:999-1008 (1998)
Erve JC, et al. *Drug Metab Dispos.* 35:908-16 (2007)

Impact of Dose on Toxicity



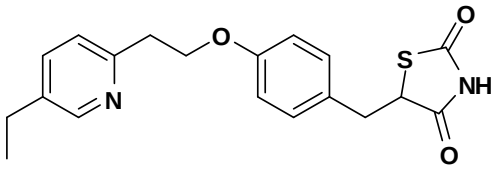
Troglitazone

200-600 mg/day
(Withdrawn from US in 2000)



Rosiglitazone

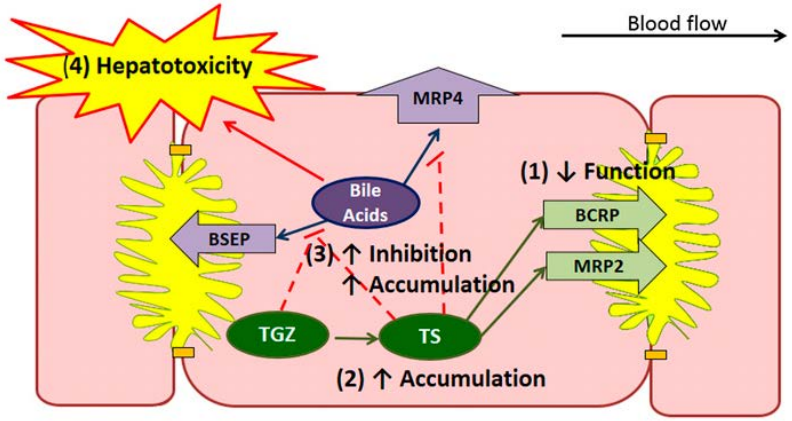
<10 mg/day



Pioglitazone

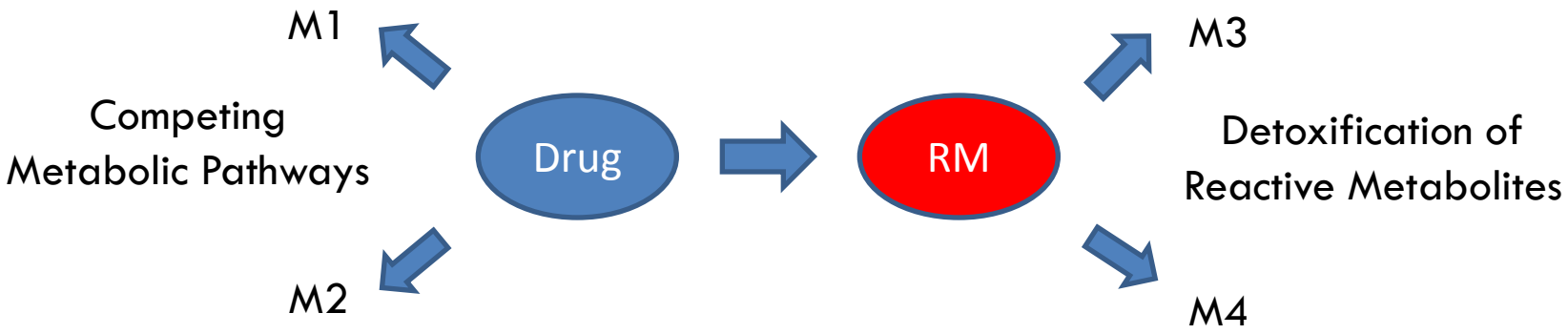
50 mg/day

- Troglitazone (Thiazolidinediones class), PPARγ agonist as oral antidiabetics to lower blood glucose levels.
- Troglitazone and, to a much greater extent, troglitazone sulfate, the main metabolite eliminated to bile, are potent BSEP transporter inhibitors, leading to cholestatic liver injury.
- High clinical doses not only increase levels of reactive metabolite formation but also lead to BSEP inhibition.



Kassahun K, et al, *Chem Res Toxicol.* 14:62-70 (2001)
Funk C, et al. *Mol Pharmacol.* 59:627-35 (2001)

Body Burden of A Reactive Metabolite Can Influence IADR



- **Amount of a reactive metabolite formed depends on:**
  - Contribution of competing metabolic routes
  - Contribution of pathways that detoxify the reactive metabolites

Daily Burden of Reactive Metabolites Calculation

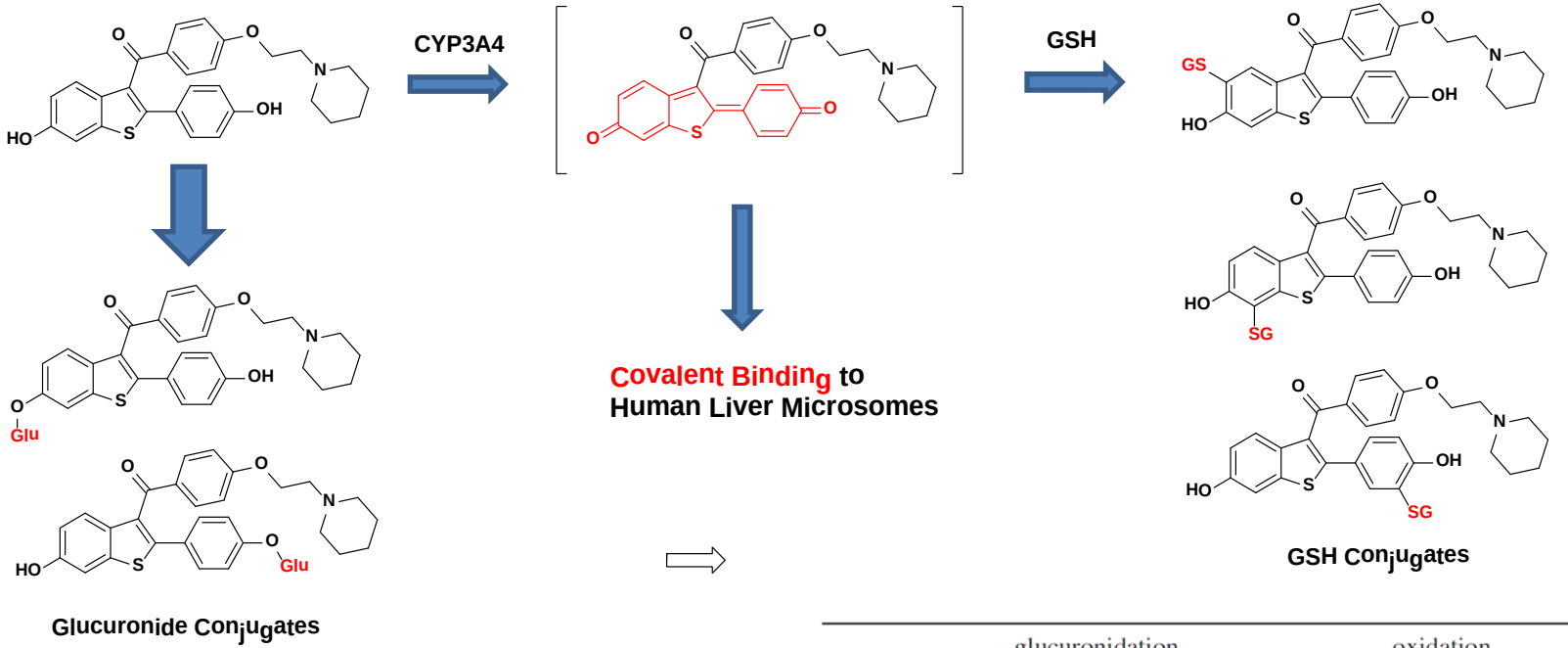
$$D_{rm} = D \times f_a \times f_m \times f_{rm}$$

D is the total daily dose (mg/day)
f_a is fraction absorbed
f_m is fractional clearance via oxidative metabolism
f_{rm} is the fraction of oxidative metabolism leading to reactive metabolite formation.

Gan J, et al., *Chem Res Toxicol.* 22:690-698 (2009)
Dalvie D, et al., *Drug Metab Rev.* 47:56-70 (2015)

The Raloxifene Case: Why Raloxifene is devoid from IADRs despite dose > 50 mg/day?

- A selective estrogen receptor modulator (SERM) approved for osteoporosis
- Bioactivated by CYP3A4 to di-quinone methide (also a mechanism-based inactivator of CYP3A4)
- Dose > 50 mg (typically 60 mg QD)
- **No IADRs or clinical DDIs reported**

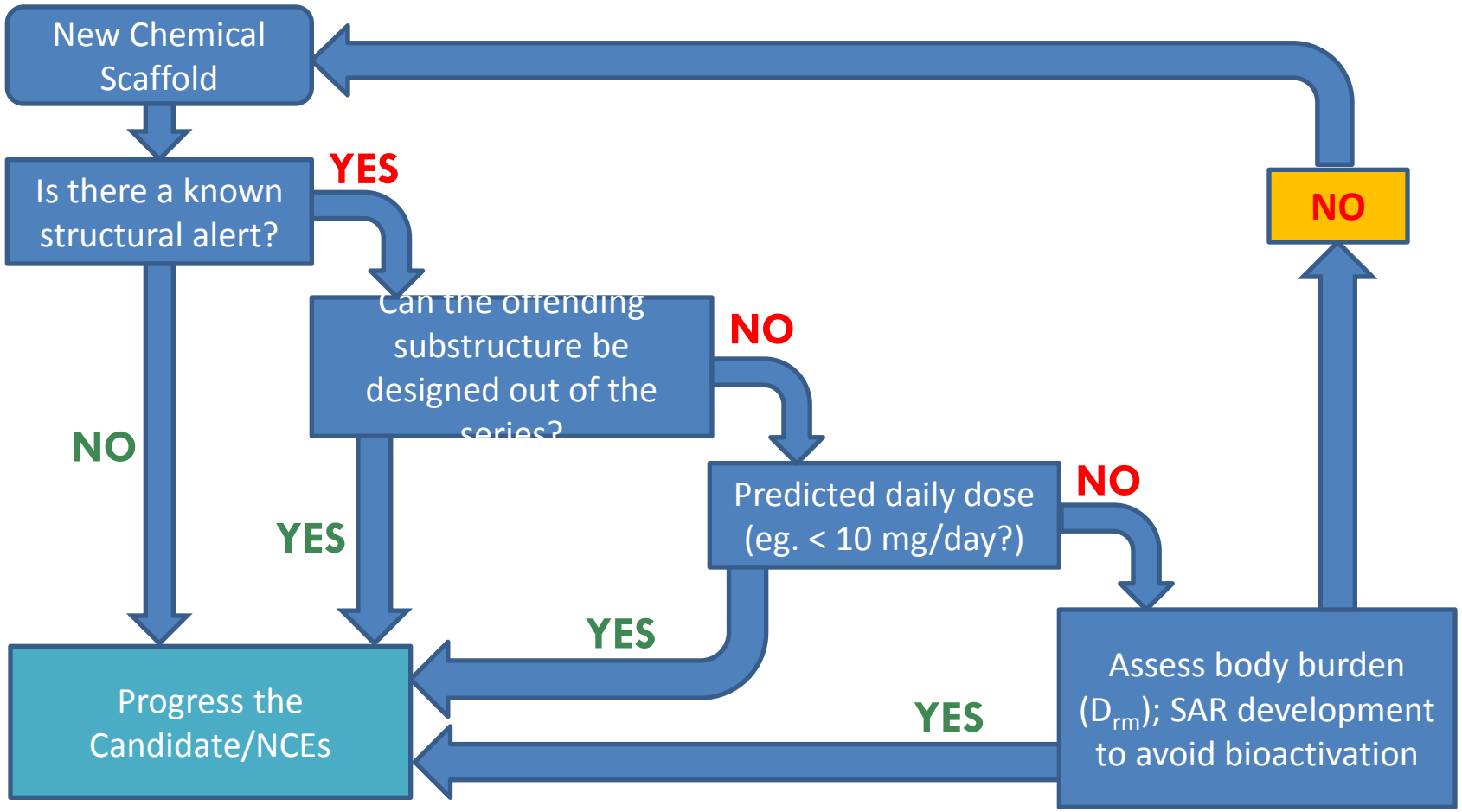


- Primary route of metabolism is glucuronidation
- Highly efficient intestinal glucuronidation limits the amount of raloxifene which undergoes bioactivation
- Take home message: importance of competing and other detoxification pathways and the overall metabolism of the drug candidate

	glucuronidation		oxidation	
	K_M (μM)	$Cl_{int\ glu}$ (μL/min/mg protein)	K_M (μM)	$Cl_{int\ oxi}$ (μL/min/mg protein)
HIM	5.5 ± 0.83	397 ± 14	3.9 + 1.5	26 + 3.1
HLM	11.2 ± 0.88	37 ± 1.6	3.8 + 0.43	142 + 5.8

Dalvie D, et al., *Chem Res Toxicol.* 21:2260-71 (2008)

The “AAA” Strategy – Aware, Assess and Avoid



Dalvie D, et al., *Drug Metab Rev.* 47:56-70 (2015)
Gan J, et al., *Chem Res Toxicol.* 22:690-698 (2009)
Wen B, et al. *Expert Opin Drug Metab Toxicol.* 5:39-55 (2009)

The “AAA” Strategy – Aware, Assess and Avoid

Aware

- Identify the structural alert(s) or substructure(s) associated with bioactivation *in silico*

Assess

- Evaluate your prediction on reactive metabolite formation, i.e. trapping agents to use, *in vitro* or *in vivo* assays etc

Avoid

- Propose chemical mitigation strategy to avoid or minimise the reactive metabolite formation

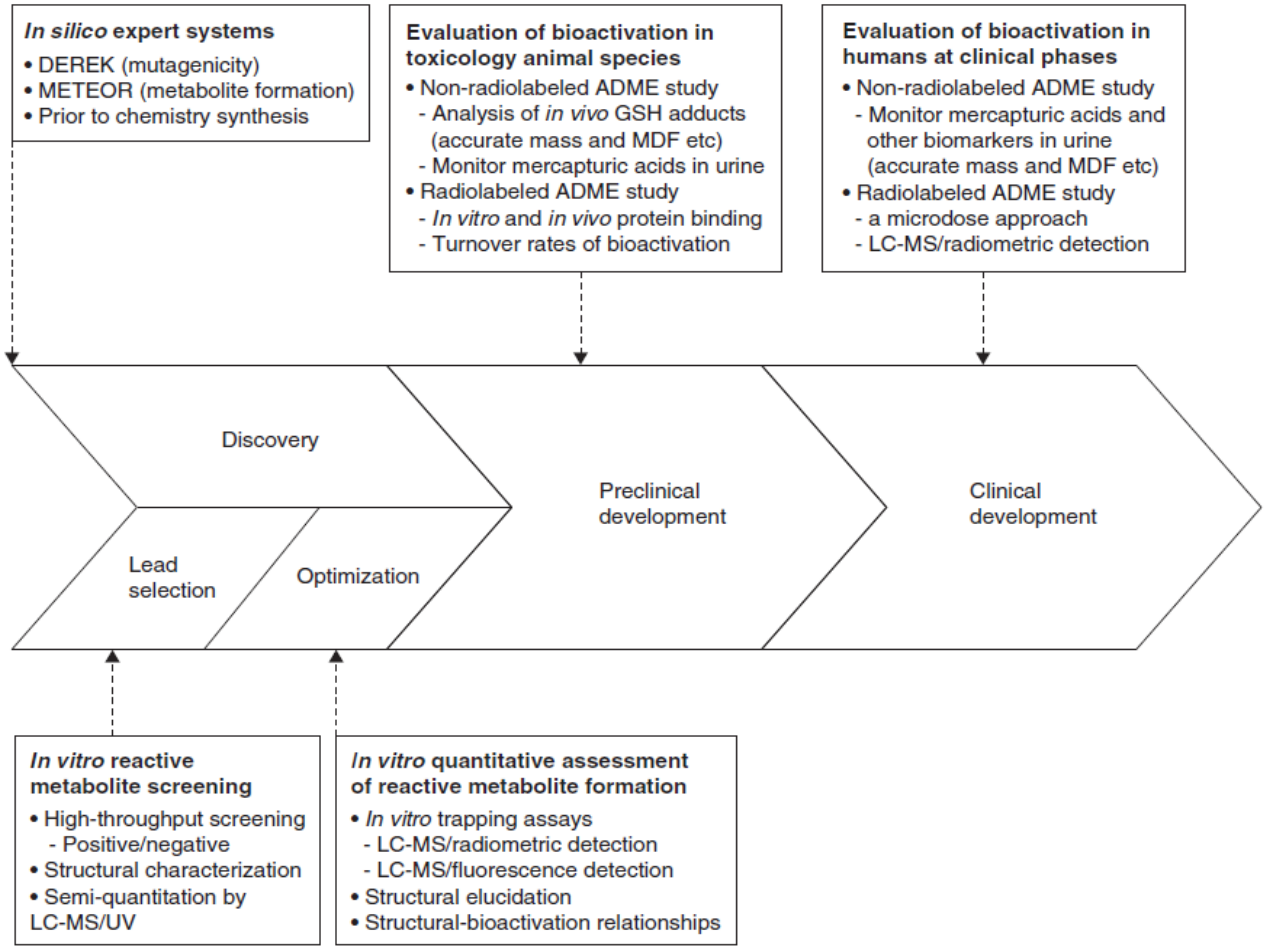
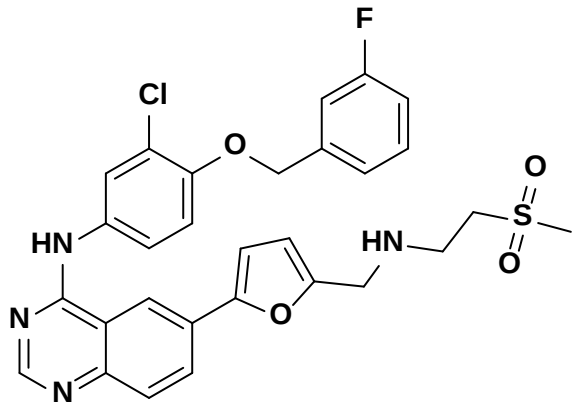


Figure 5. An integrated multitier approach for the screening, characterization and quantification of reactive metabolites in drug discovery and development.

Wen B and Fitch WL. *Expert Opin Drug Metab Toxicol.* 5:39-55 (2009)

Other Factors to Consider!

- Indication
- Unmet medical needs
- Route of administration
- Target population



Lapatinib

High Dose 1250 mg daily
Black box warning
Metabolites suggest bioactivation
Indication - ONCOLOGY

Take Home Message!

- **Predicting toxicity of reactive metabolites is challenging** (i.e. in most cases, the protein targets of reactive metabolites are not yet known)
- **Minimizing the bioactivation potential of drug candidates during the discovery/lead optimization phase has been adopted as a default strategy**
- **Good to avoid Structural Alerts**
  - -- Options for chemical mitigation if available
  - -- Prerequisite is no loss of pharmacological activity
- **Reactive metabolite assays are good "Gate Keepers"**
  - -- A 'flag' to trigger additional studies using a multitier approach
  - -- GSH adducts provide a mechanistic understanding of bioactivation
- **Keeping the dose low is important**
- **Estimation of reactive metabolite body burden is a more positive step forward towards prediction of IDR risks**

8. Panel discussion and Q&A
(小组讨论与问答)