ผลของ N-(2-propylpentanoyl) urea ต่อเอนไซม์ไซโตโครมพี 450 และกลูตาไธโอน เอส-ทรานสเฟอเรส ในตับหนูขาว

นางสาว ธีรภรณ์ เกียรติโกศลกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา ภาควิชาเภสัชวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2542

ISBN 974-333-827-6

EFFECT OF N-(2-PROPYLPENTANOYL) UREA ON RAT HEPATIC CYTOCHROME P450 AND GLUTATHIONE S-TRANSFERASE

Miss Teeraporn Kiatkosolkul

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy

Department of Pharmacology

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic Year 1999

ISBN 974-333-827-6

Thesis Title	Effect of N-(2-propylpentanoyl) urea on rat hepatic cytochrome P450 and glutathione S-transferase
By	Miss Teeraporn Kiatkosolkul
Department	Pharmacology
Thesis Advisor	Pol. Col. Lt. Somsong Lawanprasert, Ph.D.
Thesis Co-advisor	Assistant Professor Pornpimol Kijsanayotin

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University

In Partial Fulfillment of the Requirements for Master's Degree

..... Dean of The Faculty of Pharmaceutical Sciences

(Associate Professor Sunibhond Pummangura, Ph.D)

THESIS COMMITTEE

..... Chairman

(Associate Professor Siripon Foongwithaya)

..... Thesis Advisor

(Pol. Col. Lt. Somsong Lawanprasert, Ph.D.)

..... Thesis Co-advisor

(Assistant Professor Pornpimol Kijsanayotin)

..... Member

(Assistant Professor Chamnan Patarapanich, Ph.D.)

นางสาว ธีรภรณ์ เกียรติโกศลกุล : ผลของ N-(2-PROPYLPENTANOYL) UREA ต่อเอนไซม์ไซโตโครมพี 450 และกลูตาไธโอน เอส-ทรานสเฟอเรส ในตับหนูขาว. (EFFECT OF N-(2-PROPYLPENTANOYL) UREA ON RAT HEPATIC CYTOCHROME P 450 AND GLUTATHIONE S-TRANSFERASE) อ. ที่ปรึกษา : อ. พ.ต.ท. หญิง ดร. สมทรง ลาวัณย์ประเสริฐ, อ. ที่ปรึกษาร่วม : ผศ. พรพิมล กิจสนาโยธิน, 76 หน้า. ISBN 974-333-827-6.

การวิจัยนี้มุ่งหมายที่จะศึกษาผลของ เอ็น-(2-โพรพิลเพนทาโนอิล) ยูเรีย (วีพียู) ต่อเอนไซม์ไซโตโครมพี 450 และกลูตาไธโอน เอส-ทรานสเฟอเรส รวมทั้งปริมาณกลูตาไธโอนรวมในตับหนูขาว โดยการฉีดวีพียู และวัลโปรอิค แอซิด (วีพีเอ) ในขนาด 80 และ 250 มิลลิกรัม/กิโลกรัม วันละครั้ง แก่หนูขาวเพศผู้ทางหน้าท้อง เป็นเวลา 7 วัน หลังจากนั้นฆ่าหนูโดยวิธีดึงคอ เตรียมไมโครโซม และไซโตซอลจากตับ ทำการวิเคราะห์หาปริมาณไซโตโครมพี 450 รวม และแอคติวิตีของไซโตโครมพี 450 จากไมโครโซม แอคติวิตีของกลูตาไธโอน เอส-ทรานสเฟอเรส จากไซโตซอล และปริมาณกลูตาไธโอนรวมจากตับ ผลการวิจัยพบว่า ทั้งวีพียูและวีพีเอไม่มีผลเปลี่ยนแปลงค่าต่าง ๆ เหล่านี้ : ปริมาณไซโตโครมพี 450 รวม แอคติวิตีของ ethoxy- และ methoxyresorufin o-dealkylases (แสดงถึงแอคติวิตีของ CYP1A1 และ CYP1A2) แอคติวิตีของ aniline 4-hydroxylase (แสดงถึงแอคติวิตีของ CYP2E1) แอคติวิตีของกลูตาไธโอน เอส-ทรานสเฟอเรส และ ปริมาณกลูตาไธโอนรวม อย่างไรก็ตามพบว่า วีพียูมีผลเพิ่มแอคติวิตีของ bezyloxy- และ pentoxyresorufin o-dealkylases (แสดงถึงแอคติวิตีของ CYP2B1 และ CYP2B2) อย่างมีนัยสำคัญทางสถิติ และเมื่อทำการศึกษาหาปริมาณโปรตีนของเอนไซม์ทั้งสองตัวนี้ โดยวิธี Western blot พบว่าปริมาณโปรตีนของ CYP2B1 และ CYP2B2 ในกลุ่มที่ได้รับวีพียูจะมีมากกว่ากลุ่มที่ได้รับวีพีเอ และกลุ่มควบคุม ผลการศึกษานี้แสดงให้เห็นว่าวีพียูมีฤทธิ์เหนี่ยวนำเอนไซม์ CYP2B1 และ CYP2B2 ซึ่งควรที่จะมีการศึกษาถึงกลไกการเกิดการเหนี่ยวนำ ศึกษาผลการเหนี่ยวนำเอนไซม์เมื่อให้ขนาดยาและระยะเวลาที่ให้ยาต่าง ๆ กัน รวมทั้งศึกษาผลของวีพียูต่อ CYP isoforms อื่น ๆ ที่สำคัญในขบวนการเปลี่ยนแปลงของยาในคนต่อไป

ภาควิชา เภสัชวิทยา
สาขาวิชา เภสัชวิทยา
ปีการศึกษา 2542

ลายมือชื่อนิสิต Teeraporn Kiatkosolkul
ลายมือชื่ออาจารย์ที่ปรึกษา Pol. Col. Lt Sumrong Lawpoolsri
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม P. Kyssamayotin

TEERAPORN KIATKOSOLKUL : EFFECT OF N-(2-PROPYLPENTANOLY) UREA ON RAT HEPATIC CYTOCHROME P450 AND GLUTATHIONE S-TRANSFERASE. THESIS ADVISOR : POL. COL. LT. DR. SOMSONG LAWANPRASERT, THESIS COADVISOR : ASSIST. PROF. PORNPIMOL KIJSANAYOTIN, 76 pp. ISBN 974-333-827-6.

Effects of N-(2-propylpentanoyl) urea (VPU) on rat hepatic cytochrome P450, glutathione S-transferase and total glutathione were investigated. VPU and valproic acid (VPA) were given intraperitoneally to male Wistar rats at a dosage of 80 and 250 mg/kg, respectively, once daily for 7 days. On the day after, the animals were sacrificed by cervical dislocation and the livers were prepared for microsomal and cytosolic subfractions. Microsomal total cytochrome P450 contents and the enzyme activities, cytosolic glutathione S-transferase activities as well as hepatic total glutathione contents were determined. No effects of VPU and VPA were observed on total cytochrome P450 contents, ethoxy- and methoxyresorufin o-dealkylase activities (representing CYP1A1 & CYP1A2 activities), aniline 4-hydroxylase activities (representing CYP2E1 activities), glutathione S-transferase activities and hepatic total glutathione contents. However, benzyloxy- and pentoxyresorufin o-dealkylase activities (representing CYP2B1 & CYP2B2 activities) were significantly increased by VPU, correspondingly to a slight increase of CYP2B1 & CYP2B2 proteins detected by Western blotting. Further study to clarify the mechanism of this enzyme induction should be proceeded. Moreover, various doses and extended duration of VPU administrations should be additionally studies. Effect of VPU on other isoforms of CYP, involving human drug metabolism, was also suggested exploring.

ภาควิชา เภสัชวิทยา
สาขาวิชา เภสัชวิทยา
ปีการศึกษา 2542

ลายมือชื่อนิสิต Teeraporn Kiatkosolkul
ลายมือชื่ออาจารย์ที่ปรึกษา Pol. Col. Lt Somsong Lawanprasert
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม P. Kijsanayotin

Acknowledgments

I wish to express my sincere gratitude to my advisor, Pol. Col. Lt. Dr. Somsong Lawanprasert and my co-advisor, Assistant Professor Pompimol Kijsanayotin, as well as Associate Professor Nuansri Niwattisaiwong for their valuable advise and guidance, kindness, and encouragement during the course of experimental work and presentation of the thesis.

A grant support from the Faculty of Pharmaceutical Sciences, Chulalongkorn University and a partial support from the Graduate School, Chulalongkorn University are gratefully acknowledged.

I would like to thank Assistant Professor Dr. Chamnan Patarapanich, Department of Pharmaceutical Chemistry, Chulalongkorn University, for kindly supplying the N-(2-propylpentanoyl) urea and thanks are also extened to Associate Professor Dr. Supatra Srichairat for the laboratorial facilities.

I also wish to thank Professor Dr. Kan Chiba and all staff members of Laboratory of Biochemical Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Chiba University, Japan, for the assistance on Western blot analysis.

I really thank to all staff members of Deparment of Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for their valuable helps and kindness.

Finally, I would like to thank my family and my friends for their love and encouragement.

Contents

Page

Page

List of Tables

Table Page

List of Figures

Figure Page

List of Abbreviations

CYP	=	cytochrome P450
β	=	beta
ω	=	omega
°C	=	degree celcius
µl	=	microlitre
2,4-diene VPA	=	2-n-propyl-2,4-pentadienoate
2-ene VPA	=	2-propyl-2-pentanoic acid
3-keto VPA	=	2-propyl-3-keto-pentanoic acid
4-ene VPA	=	2-n-propyl-4-pentenoate
4-PA	=	4-pentenoic acid
ATP	=	adenine triphosphate
BROD	=	benzyloxyresorufin o-dealkylation
BSA	=	bovine serum albumin
CDNB	=	1-chloro-2,4-dinitrobenzene
cm	=	centrimetre
DMSO	=	dimethyl sulfoxide
DTNB	=	5,5'-dithiobis-(2-nitrobenzoic acid)
ED_{50}	=	median effective dose
EDTA	=	ethylenediamine tetraacetic acid
EROD	=	ethoxyresorufin o-dealkylation
et al.	=	et alii (and other)
g	=	gram
G6P	=	glucose 6-phosphate
G6PD	=	glucose 6-phosphate dehydrogenase
GABA	=	gamma-aminobutyric acid
GABA-T	=	gamma-aminobutyric acid transaminase
GAD	=	L-glutamate decarboxylase
GSH	=	reduced glutathione
GSSG	=	disulfide of GSH

GST	=	glutathione S-transferase
HPLC	=	high performance liquid chromatography
i.p.	=	intraperitonium
kg	=	kilogram
L	=	litre
LD_{50}	=	median lethal dose
M	=	molar
mA	=	milliampere
MES	=	maximal electroshock
mg	=	milligram
mg/kg	=	milligram per kilogram body weight
mM	=	millimolar
MROD	=	methoxyresorufin o-dealkylation
NADP	=	nicotinamide adenine dinucleotide phosphate (reduced form)
NADPH	=	nicotinamide adenine dinucleotide phosphate
nmol	=	nanomol
PEG400	=	polyethylene glycol 400
PGA	=	2-propyl glutaric acid
pmol	=	picomol
PROD	=	pentoxyresorufin o-dealkylation
PTZ	=	pentylenetetrazole
SSADH	=	succinic semialdehyde dehydrogenase
TCA	=	trichloroacetic acid
total GSH	=	total glutathione (GSH+GSSG)
Tris	=	Tris (hydroxymethyl) aminomethane
UDPGT	=	uridine-5'-diphospho-glucuronyltransferase
VPA	=	valproic acid
VPU	=	N-(2-propylpentanoyl) urea
v/v	=	volume by volume
w/v	=	weight by volume

Chapter I

Introduction

Valproic acid (VPA), a widely used anticonvulsant drug, has been known of its effectiveness for the treatment of absence, myoclonic, partial and generalized tonic-clonic seizure (Alber and Peroutka, 1992). However, it become increasingly evident that VPA therapy may be associated with serious hepatotoxicity (Zimmerman and Ishak, 1982 ; Zarfrani and Berthelot, 1982) as well as embryotoxicity (Gomez, 1981). Therefore, there are many scientists trying to develop improved derivatives of VPA in order to increase therapeutic efficacy and reduce side effects.

N-(2-propylpentanoyl) urea (VPU) is one of VPA derivatives which was synthesized in 1992 by staffs of the department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Saisorn et al., 1992). A study of Tantisira and collaborates in 1997 on the anticonvulsant activity and neurotoxicity of VPU showed that VPU possessed a higher anticonvulsant activity either against maximal electroshock (MES) seizure or pentylenetetrazole (PTZ)-induced convulsion, comparing with its parent compound, VPA. Moreover, VPU possessed greater therapeutic index but less neurotoxicity than those of VPA .

Meesomboon et al. (1997) reported that VPU produced less developmental toxicity than did VPA. Regarding to a hepatotoxicity study, VPU demonstrated hepatotoxic effect and depleted hepatic total glutathione at high dose of administration (700 and 1,400 mg/kg) in rats (Patchamart, 1996).

Pharmacokinetic study utilizing ^{14}C-VPU and autoradiographic technique was performed by Kijsanayotin and collaborates in 1997. VPU was found rapidly absorbed from injected area as well as gastrointestinal tract and instantaneously distributed to various tissue organs, including liver, kidneys, lungs, muscle, and brain. Moreover, VPU was rarely hydrolyzed to VPA by carboxyesterase obtained from phenobarbital-induced mice liver and human liver. This result indicated that anticonvulsant activity of VPU may be attributed from VPU and/or its metabolites, not VPA.

VPU demonstrated a prolong barbiturate sleeping time characteristic (Tantisira et al., 1997). This was probably resulted from the effect of VPU directly to CNS or the effect on cytochrome P450 (CYP), an important phase I drug-metabolizing enzyme. Concerning the effect of VPU on CYP, Kijsanayotin et al. (1997) found that VPU demonstrated an inhibition effect on CYP 2C9 and CYP 1A2 *in vitro* using human liver microsomes.

A major complication of epilepsy treatment with polytherapy are associated with a wide range of drug-drug interactions, including hepatic enzyme induction and inhibition as well as protein binding displacement. Clinical consequences may be drug toxicity, seizure worsening and/or inappropriate evaluation of the efficacy of polytherapy. Phenobarbital, phenyltoin, primidone and carbamazepine are inducers of CYP2C and CYP3A, whereas VPA is an inhibitor of CYP2C, UDP-glucuronyltransferase (UDPGT), glutathione S-transferase (GST) and epoxide hydroxylase (Riva et. al., 1996; Anderson, 1998). Therefore, preclinical testing involving effects of a new compound on hepatic drug metabolizing enzymes is recommended in the drug development process of a new antiepileptic drug (Cereghino and Kupferberg, 1993).

In this study, we followed the protocol of the Anticonvulsant Screening Project (ASP) of the Antiepileptic Drug Development (ADD) program that suggested several hepatic parameter measurements following 7 days of a compound dosing (Cereghino and Kupferberg, 1993). Dosage of both VPU and VPA used in this study were median effective doses (ED_{50}) protected rats against maximal electroshock convulsions which were 80 mg/kg and 250 mg/kg, respectively (Tantisira et al., 1997). The aim of this study was primarily to assess the effect of VPU on phase I metabolizing enzymes, CYP, including CYP1A1/2, CYP2B1/2 and CYP2E1. Effects of VPU on GST, an important phase II metabolizing enzymes, as well as its effect on hepatic total glutathione (GSH) were also evaluated.

Hypothesis

VPU, at ED_{50} protected rats against maximal electroshock convulsions, demonstrated an induction and/or an inhibition effects on rat hepatic drug metabolizing enzymes, CYP and GST. In addition, the compound at such dose, depleted rat hepatic total GSH.

Benefit gained from the study

Preliminary data whether VPU at ED_{50} protected rats against maximal electroshock convulsions, possessed an induction and/or an inhibition effects on hepatic drug metabolizing enzymes, CYP and GST. Results from the study were expected to provide some information for a further study so as to predict potential drug-drug interactions. In addition, the information assuring that the compound at such dose was safe from the reactive metabolites was gained.

Study design and process

An *ex vivo* study model was used in the study. The following process was performed :

1. Animal dosing for 7 days
2. Preparation of microsome and cytosolic subfractions
3. Microsomal and cytosolic enzyme assays
4. Hepatic total GSH assays
5. Data collecting and analysis
6. Writing a thesis

Chapter II

Literature reviews

Metabolism

Metabolism is a biological process which convert lipophilic xenobiotics to more hydrophilic metabolites in order to facilitate subsequent renal or fecal excretion. The enzyme systems responsible for the biotransformation of many drugs are located in the smooth endoplasmic reticulum and cytosol of the liver cells. These enzymes are also found in other organs such as kidney, lung and gastrointestinal tract, but in small quantities .The chemical reaction of enzymatic biotransformation are divided into two phases, both of which are phase I and phase II reactions.

Phase I reactions consist of many reactions, for example, oxidation, reduction and hydrolysis (Table 1). The reactions of phase I change many xenobiotics to more polar metabolites which are more active, less active or inactive than the parent compounds. Generally, phase I reactions create reactive functional groups on the molecule of the parent compounds, such as -OH, -COOH, $-NH_2$, and –SH. Therefore, phase I reactions can be called as "functionalisation" or "nonsynthetic reaction" (Gibson and Skett, 1994). The most important reaction in this phase is oxidation, especially using cytochrome P450.

Table 1 Phase I reactions (Gibson and Skett, 1994)

1. Oxidation involving cytochrome P450
Oxidation, not involving cytochrome P450
2. Reduction
3. Hydrolysis
4. Hydration
5. Isomerisation
6. Miscellaneous

Phase II reactions, which are also called "conjugation" or "synthetic reaction", involve coupling the drug or its polar metabolite with an endogenous substrate; for example, UDP-glucuronic acid, glutathione, acetyl group or an amino acid, etc. The phase II enzymes and the functional groups on the parent compound/phase I metabolite, which are likely to be attacked by the corresponding enzymes, were shown in table 2. This phase is generally thought to be a real detoxification pathway because after conjugation reaction, the xenobiotics will be more readily excreted from the body.

Table 2 Conjugation reactions (Gibson and Skett, 1994)

Reaction	Enzyme	Functional group
Glucuronidation	UDP-Glucuronyl transferase	-OH
		-COOH
		$-NH_2$
		-SH
Glycosidation	UDP-Glycosyltransferase	-OH
		-COOH
		-SH
Sulfation	Sulfotransferase	$-NH_2$
		$-SO_2NH_2$
		-OH
Methylation	Methyltransferase	-OH
		$-NH_2$
Acetylation	Acetyltransferase	$-NH_2$
		$-SO_2NH_2$
		-OH
Amino acid conjugation		-COOH
Glutathione conjugation	Glutathione-S-transferase	Epoxide
		Orgnic halide
Fatty acid conjugation		-OH
Condensation		Various

Cytochrome P450 (CYP)

It is now well established that *CYP* exists as a gene superfamily where each gene encodes a separate isoform of CYP. CYP is responsible for not only the metabolism of many xenobiotics (e.g. drugs, industrial chemicals, environmental pollutants, plant products and toxins) but also the biosynthesis and catabolism of a broad range of endogenous compounds, including bile acids, biogenic amines, eicosanoids, fatty acids and steroid hormones.

Although metabolism of xenobiotics by CYP is normally associated with inactivation of the parent compound, it is now recognized that certain compounds may be converted to highly reactive intermediates which are capable of interacting with cellular macromolecules. Moreover, many studies show that CYP – catalysed metabolic activation results in the toxicity, mutagenicity and carcinogenicity of many foreign compounds.

CYP, a haem-containing enzyme, is composed of apoprotein and ferroprotoporphyrin IX prosthetic group (Figure 1) at which molecular oxygen is coordinated and activated during the oxidative reaction.

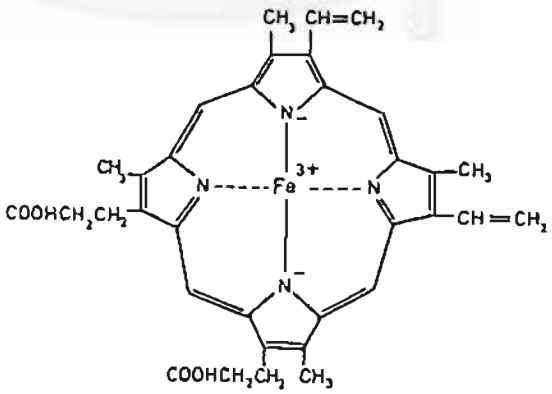

Figure 1 A ferroprotoporphyrin IX prosthetic group of CYP (Gibson and Skett, 1994)

CYP enzymes are membrane-bound haem proteins. They are embeded either in the endoplasmic reticulum membrane or in the mitochondrial inner membrane. Mitochrondrial CYPs , which are involved in steroid-biosynthetic reactions, are found mainly in steroidogenic organs and generally do not metabolize foreign compounds. Mitochrondrial CYPs receive electron from the ion sulfur protein adrenodoxin via NADPH-adrenodoxin oxidoreductase, whereas microsomal CYPs receive electron from the flavoprotein NADPH-CYP oxidoreductase and, in some case, cytochrome b_5.

CYP superfamily is subdivided into gene families and gene subfamilies that are defined on the basis of amino acid sequence homology. Within a given CYP gene family, there are greater than 40% identical in their amino acid sequence to each other. If two CYP proteins are at least 70% similar in their amino acid sequence, they are classified in the same subfamily (Gibson and Skett, 1994). Proteins in different families are indicated by an arabic numeral, e.g. CYP1, and those in different subfamilies are indicated by a capital letter, e.g. CYP1A, CYP1B, etc. Individual CYPs are then termed, for example, CYP1A1, CYP1A2, etc.

CYP superfamily comprises at least 79 gene families, 14 of which are found in mammals. CYP families 1-4 are the functionally most important ones in the metabolism of xenobiotics (Nelson et al., 1996). CYP1, CYP2 and CYP3 families are responsible for the metabolism of xenobiotics and steroid synthesis, while CYP4 family is mainly responsible for catalysis of fatty acid. Multiple forms of CYP have overlapping substrate specificities. Each CYP isoform possesses a characteristic broad spectrum of catalytic activities of substrate (Table 3).

The regulation of these enzymes has been most extensively studied using rat, mouse and rabbit as model systems. Species differences have been noted in the expression of CYP, particularly those in the CYP2 family because of gene duplications and gene conversions in each species (Gonzalez, 1990). Table 4 presents sequential homology between rat and human CYP forms known at the end of 1990. Sequential homology of cDNA and amino acid sequence between rat human CYP isoforms is high in similarity, approximately 70%. There are generally conserved regions (for P-450 reductase, haem,

signal peptide) which increase this similarity. On the other hand, change in a single amino acid may markedly or even completely alter CYP function, such as increase or decrease its activity, or even completely change its substrate specificity (Soucek and Gut, 1992).

Table 3 Human CYP isoforms and the corresponding selective substrates (modified from Redic and Di Carlo, 1997)

CYP isoform	Substrates
1A2	Caffeine (3-demthylation)
	Phenacetin
	7-Ethoxyresorufin
2A6	Coumarin
2C9/10	Phenytoin
	Tolbutamide
	Warfarin
2C19	S-mephenytoin
	Omeprazole
	Proquanil
2D6	Bufuralol
	Debrisoquine
	Dextromethorphan
	Metoprolol
	Sparteine
2E1	Chlorzoxazone
	Ethanol
	4-Nitrophenol
	Aniline
3A4 and 3A5	Erythromycin
	Dapsone
	Lignocaine
	Nifedipine
	Midazolam

Important members of CYP families 1-4

CYP1

The CYP1 family, of which CYP1A1 and CYP1A2 are the most important members, is responsible for the metabolic activation of some known carcinogenic environmental chemicals, toxins and drugs (Gonzalez and Gelboin, 1994). CYP1A1 and CYP1A2 have been isolated and sequenced from rat, mouse and man. Both CYP1A1 and CYP1A2 have overlapping substrate specificities. CYP1A1 is not expressed in the absence of inducers, such as 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), β-naphthoflavone (β-NF) and benzo[a]pyrene (Goldstein et al., 1982; Guengerich et al.,1982, Gonzalez and Gelboin, 1994). In contrast to CYP1A1, CYP1A2 is constitutively expressed in livers and may also be induced by foreign chemicals, such as aromatic hydrocarbons. It activates arylamines and dietary promutagens, including nitrosamines and aflatoxins (Gonzalez and Gelboin, 1994). Moreover, it also participates in metaboic activation pathways of several drugs; for example, paracetamol, tacrine and clozapine (Rendic and Di carlo, 1997). Therefore, these enzymes may play a very important role in carcinogen activation (Gonzalez, 1990).

CYP2

CYP2A1 and CYP2A2 were purified from rats and found to specially hydroxylate testosterone (Waxman et al.,1983 ; Matsunaga et al., 1988). CYP2A6 is a minor form of CYP in human liver, accounting for about 4% of total CYP contents (Rendic and Di Carlo,1997). It metabolically activates many xenobiotics, such as aflatoxin B_1, nitrosamines and coumarin, but it is only weakly active on steroid (Yun et al., 1991). CYP2A6 is the human ortholoque of rat CYP2A3, but the enzymes are functionally different (Gonzalez, 1990; Soucek and Gut, 1992; Murray,1999).

Two isoforms, CYP2B1 and CYP2B2, have been extensively studied in rats because they are induced by phenobarbital. In experimental animals the hepatic CYP2B proteins are expressed very poorly under normal conditions (usually <5% of the total CYP contents) but can be highly induced by a number of chemicals, including the prototypic inducer phenobarbital (Guengerich et al., 1982 ; Ryan et al.,1982 ; Waxman and Azaroff, 1992). Human CYP2B6 is orthologous to rat CYP2B1 (Yamano et al, 1989 ; Soucek and Gut, 1992),

but its expression is quite low, approximately 0.2% of total CYP contents (Rendic and Di Carlo, 1997). It is unknown whether CYP2B6 is inducible by the phenobarbital class of compounds similar to its rodent counterparts.

CYP2C subfamily is generally thought to represent a class of constitutively expressed genes. These enzymes are noted for their sex-specific and developmentally regulated expression in rats. This subfamily in rats includes an adult female-specific CYP (2C12), two adult male-specific CYP (2C11 and 2C13) and two enzymes expressed in both males and females (2C6 and2C7) (Gozalez, 1990). Four enzymes of CYP2C subfamily have been identified in humans, including CYP2C8, 2C9, 2C18 and 2C19 (Rendic and Di Carlo, 1997). Human CYP2C9 is the most abundant enzyme in this family. CYP2C enzymes catalyze the oxidations of a number of drugs, the examples of which are shown in Table 3. There is interindividual variation in drug metabolism due to genetic polymorphism of an enzyme in this subfamily, CYP2C19 which is resposible to (S)-mephenytoin 4-hydroxylation (Mayer et al., 1994). No carcinogens are known to be metabolically activated by CYP2C (Gonzalez and Gelboin, 1994).

CYPs of the 2D subfamily were purified from rat (Larray et al., 1984) and human (Distlerath et al., 1985 ; Gut et al., 1986). Furthermore, cloning studies showed that at least five genes, designated *2D1, 2D2, 2D3, 2D4* and *2D5* are present in rat (Matsunaga et al., 1989). CYP2D6 is an important member of the CYP2D subfamily that expressed in humans. Table 3 lists some examples of drugs which are substrates of CYP2D6. In addition, genetic polymorphism was demonstrated with CYP2D6 known as sparteine/debrisoquine polymorphism (Gut et al., 1986)

CYP2E1 gene is constitutively expressed primarily in humans and in rodents. It has been shown to be induced by ethanol and some substrates ; for example, acetone, isopropanol, toluene and benzene. Furthermore, it is also induced by the pathophysiologic state of diabetes. CYP2E1 is of major toxicological importance because it is responsible for the formation of reactive metabolites/intermediates from a number of laboratory and environmental chemicals (Koop, 1992 ; Rendic and Di Carlo, 1997).

CYP3

CYP3A1 and CYP3A2 are found in rats. Expression of CYP3A1 is very low in normal rat liver, whereas CYP3A2 is the adult male-specific CYP which is absent from the livers of adult females (Gonzalez, 1990). In humans, three important CYP3A isoforms, including CYP3A4, 3A5 and 3A7, have been identified in liver (Redic and Di Carlo, 1997). CYP3A4/5 are involved in the metabolism of more than 150 drugs belonging to about 38 classes ; for instance, opoid analgesics, corticosteroids and antiarrhythmics. CYP3A7, responsible for catalyzing steroid hormones, is highly expressed only in human fetal, but not adult, liver. (Redic and Di Carlo, 1997).

CYP4

In rats, CYP4A1, 4A2 and 4A3 are responsible to the metabolism of fatty acid at its ω position and arachidonic acid. CYP4A1 is induced by clofibrate both in liver and kidney, but rat CYP4A2 is induced only in liver and constitutively expressed in kidney. CYP4A11, found in human kidney, metabolizes fatty acids through ω- and ω_1 hydroxylation. Human CYP4B1 is constitutively expressed in the lung, not in liver (Gonzalez,1990 and Gonzalez and Gelboin, 1994).

Table 4 Sequential homology between rat and human CYP forms (Soucek and Gut, 1992)

CYP form		Sequential homology[a]
Rat	Human orthologue	(%)
CYP1A1	CYP1A1	80 (78)
CYP1A2	CYP1A2	75 (70)
CYP2A1	———	—[b]
CYP2A2	———	—[b]
CYP2A3	CYP2A6	(85)
———	CYP2A7	—[b]
CYP2B1	CYP2B6	78 (74)
	CYP2B7	(76)
CYP2B2	———	—[b]
CYP2B3	———	—[b]
CYP2B18	———	—[b]
CYP2C6	CYP2C10	(75)
CYP2C7	———	—[b]
CYP2C11	CYP2C9	80 (77)
CYP2C12	———	—[b]
CYP2C13	CYP2C8	74 (68)
CYP2D1	CYP2D6	(71)
CYP2D2	———	—[b]
CYP2D3	———	—[b]
CYP2D4	———	—[b]
CYP2D5	———	—[b]
———	CYP2D7	—[b]
———	CYP2D8	—[b]
CYP2E1	CYP2E1	75 (78)
———	CYP2F1	—[b]
CYP3A1	CYP3A3	(78)
———	CYP3A4	(73)
CYP3A2	CYP3A5	(71)
	CYP3A7	(65)
CYP3A9	———	—[b]
CYP4A1	CYP4A9	—[b]
CYP4A2	———	—[b]
CYP4A3	———	—[b]
CYP4A8	———	—[b]
CYP4B1	CYP4B1	(>80)

[a] Similarity of cDNA and amino acid (in parentheses) sequence stated.

[b] No data available regarding existence of orthologous form.

Catalytic cycle of CYP

At inactive state, CYP is substrate-free with ferric form of haem. During the oxidation reaction, substrates bind reversibly to ferric CYP and the resultant bimolecular complex accepts an eletron from NADPH that is delivered by the coenzyme NADPH-CYP-reductase. A molecular oxygen is coordinated to the ferrous CYP-substrate complex which under goes reduction by a second electron from NADPH/NADPH-CYP-reductase or NADH/NADH-cytochrome b_5 reductase / cytochrome b_5. The O-O bond which is clevaged releases one of the atoms of oxygen as water and the other remains binded to the CYP heme, possibly as a highly reactive intermediate, ready for oxidation of substrate (Figure 2).

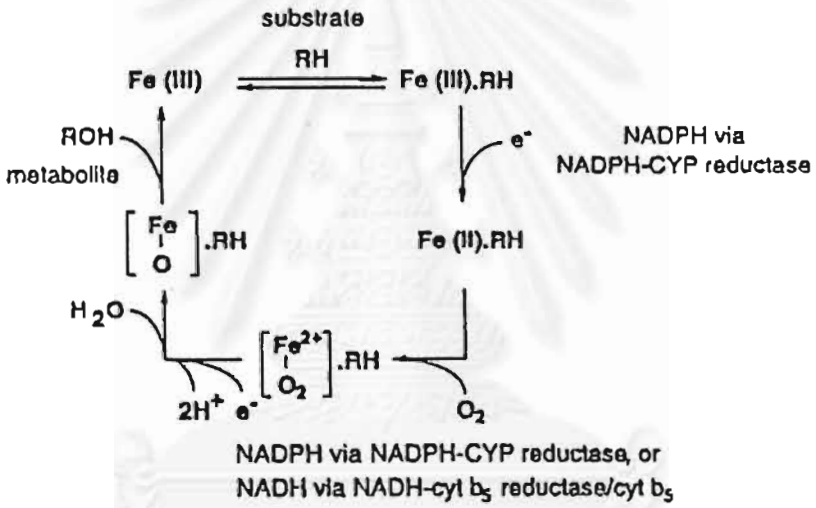

Figure 2 CYP reaction cycle (Murray, 1999)

Glutathione S-transferase (GST)

One of the most important reactions in phase II is glutathione conjugation. Many drugs and endogeneous compounds are changed by this reaction, for example, paracetamol, parathion, 1-chloro-2,4-dinitrobenzene, and vitamin K_3. The responsible enzyme in glutathione conjugation is glutathione S-transferase (GST).

GSTs are soluble enzymes located in cytosol of liver, kidney, gut and other tissues. They play a physiological role in initiating the detoxification of potentially toxic electrophilic

compounds/intermediates, including pharmacologically active compounds/their reactive intermediates. These enzymes catalyze the conjugated reaction of considerable compounds with the endogenous tripeptide glutathione (glutamyl-cysteinylglycine : GSH), thereby neutralizing their electrophilic sites and contributing to the products more water-soluble and non-toxic. GSTs are induced by some inducers, such as 2-acetylaminofluorene, 3-methylcholanthrene, phenobarbital, 2,3,7,8-tetrachlorodibenzo-p-dioxin and trans-stilbene oxide (Gibson and Skett, 1994). Glutathione conjugates are thought to be metabolized further by clevage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of cysteinyl residue, to produce the final product, a mercapturic acid which is then excreted (Figure 3). Today, at least 6 isoforms are known with differing substrate specificities as shown in Table 5.

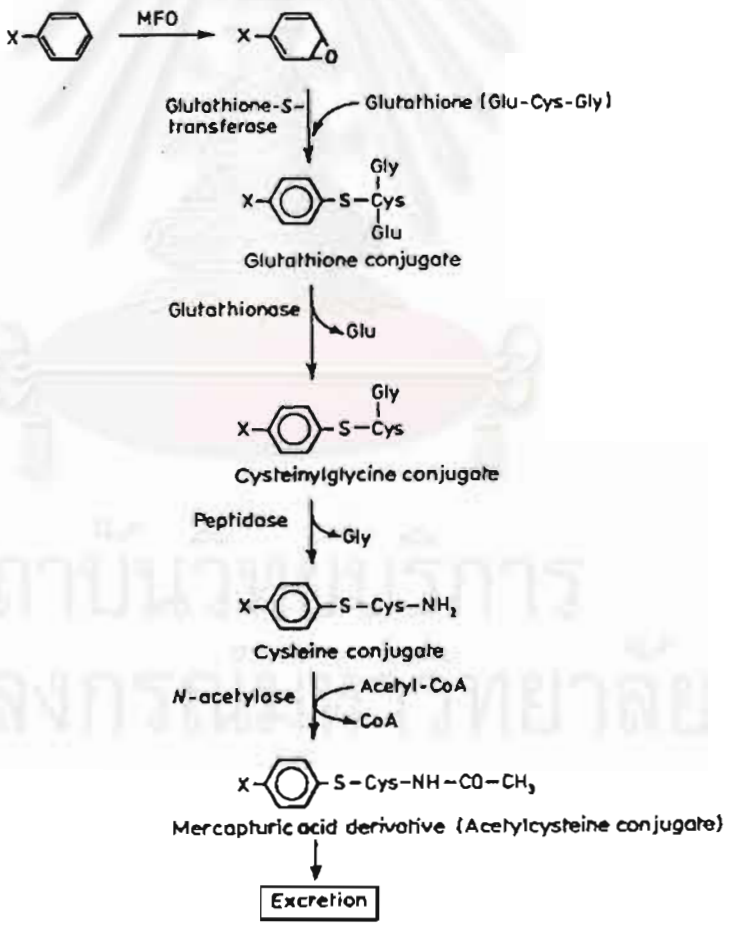

Figure 3 Glutathione conjugation and a mercapturic acid biosynthesis (Gibson and Skett, 1994)

Table 5 The isoenzymes of glutathione-S-transferases and their specific substrates (Gibson and Skett,1994)

Old name	New name	Substrate
L2	1-1	4-Nitrophenyl acetate
BL	1-2	Mixture of L2 and B2
B2	2-2	Ethacrynic acid
A2	3-3	1,2-Dichloro-4-nitrobenzene
AC	3-4	Mixture of A2 and C2
P	3-6	
C2	4-4	*trans*-4-phenyl-3-buten-2-one
S	4-6	
E	5-5	
M	6-6	
P	7-7	
K	8-8	

Glutathione (GSH)

Mammalian cells have evolved protective mechanisms to minimize injurious events that result from toxic chemicals and electrophilic oxidative products of cellular metabolism. A major endogenous protective system is the glutathione redox cycle (Figure 4).

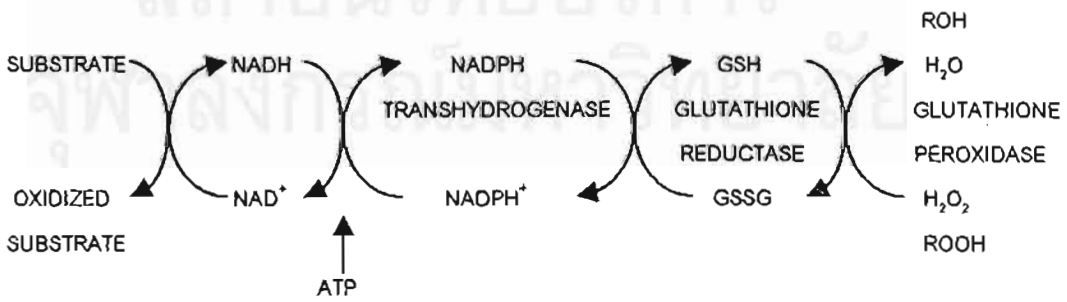

Figure 4 Glutathione redox system present in mitochondria (Reed , 1990)

Glutathione is present in high concentrations as reduced glutathione (GSH) in most mammalian cells (generally in the millimolar range), with minor fractions being the

disulfide (GSSG), mixed disulfides of GSH and other cellular thiols, and thioethers. GSH acts both as a nucleophilic "scavenger" of numerous compounds and their metabolites, via enzymatic and chemical mechanisms, converting electrophilic centers to thioether bonds, and as a substrate in the GSH peroxidase-mediated destruction of hydroperoxides. GSH depletion to about 20-30% of total glutathione levels can impair the cell's defense against the toxic actions of such compounds and may lead to cell injury and cell death (Reed, 1990).

Endogenous oxidative stress is a consequence of aerobic metabolism, which in eucaryotes, occurs mostly in the mitochondria. Reduction of oxygen in the respiratory chain involves the formation of toxic oxygen intermediates. About 2-5% of mitochodrial O_2 consumption generates hydrogen peroxide (H_2O_2) (Chance, et. al., 1979). The mitochondrial generation of H_2O_2 could require a turnover rate in the GSH redox cycle of about 10% of the GSH per minute (Sies and Moss, 1978). H_2O_2, if not reduced, can lead to the formation of a very reactive hydroxyl radical, and initiation of lipid peroxidation of phospholipids and a consequent damage of various cellular organelles, such as cell membranes, nuclear membrane, etc.

GSH can be depleted directly by conjugation with electrophiles and indirectly by the addition of inhibitors of GSH biosynthesis and regeneration, such as buthionine sulfoximide. GSH is synthesized in vivo in the liver from γ-glutamylcysteine and glycine via GSH synthetase. GSH is maintained in a redox couple with GSSG within the cell and is regenerated by GSH reductase, a cytosolic NADPH-dependent enzyme.

Hepatic enzyme induction and enzyme inhibition

According to drug metabolism, the purpose is generally to make the drug more polar/more water-soluble, thus facilitating subsequent renal excretion. The resulting metabolite may be pharmacologically inactive, less active, or occasionally more active than the parent molecule. Therefore, modifying of drug metabolism either by inhibition or induction of metabolism can alter the amount of drug available for action or alter its pharmacological effect. On the other words, drug- metabolizing enzyme induction and inhibition, especially in the liver, is one of the many factors that cause drug-drug interactions.

Hepatic enzyme induction

Hepatic enzyme induction results from taking some compounds, so called enzyme inducers, that interfere with hepatic drug metabolizing enzymes by accelerating activities of these enzymes because of an increase in the amount of enzyme protein or a change in enzyme's properties. As a result of the requirement for producing new proteins, the time course of induction is considerably longer than that of the inhibition and may take 2 to 3 weeks to reach a maximal effect in humans. Commonly used drugs that are microsomal enzyme inducers are listed in Table 6.

An inducing compound commonly affects the CYP isoform that catalyzes its own oxygenation, thereby accelerates its own metabolism, as well as that of other related chemicals (Oinonen and Lindros, 1998). Different inducers would have the potential to increase levels of the specific isoforms of CYP (Table 7).

It's obvious that enzyme inducers have several means to elevate the CYP, three of which are

a) Enhancement of gene transcription, e.g. the induction of CYP2B1/2 by phenobarbital (Waxman and Azaroff, 1992), the transcriptional activation of CYP1A1 by dioxins and of CYP4A by peroxisome proliferations. Two latters of CYP are receptor-mediated induction (Gonzalez et al.,1995; Gonzalez and Lee, 1996).

b) Stabilization of mRNA, e.g. the induction of CYP2E1 by ethanol (Gonzalez and Lee, 1996)

c) Stabilization of protein, e.g. the induction of the CYP3A4 by triacetyloleandomycin (Gibson and Skett, 1994; Lin and Lu, 1998)

Not only can CYP be induced by the inducers, but also other enzymes involving in drug metabolism are induced to various extents by a structurally diverse group of chemicals and drugs as shown in Table 8.

Table 6 Commonly used drugs that alter microsomal enzyme activities (Wright, 1992)

Inhibitors of Enzyme Activity	
Amiodarone	Metronidazole
Chloramaphenicol	Miconazole
Cimetidine	Oral contraceptives
Disulfiram	Phenothiazines
Ethanol (acute)	Phenylbutazone
Erythromycin	Sulfinpyrazone
Isoniazid	Sulfonamides
Ketoconazole	Tricyclic antidepressants
Methylphenidate	Valproate
Inducers of Enzyme Activity	
Barbiturates	Meprobamate
Carbamazepine	Phenytoin
Ethanol(chronic)	Phenobarbital
Ethchlorvynol	Primidone
Glutethimide	Rifampicin

Table 7 Commonly CYP inducers and inhibitors (data from Gonzalez, 1990; Rendic and Di Carlo, 1997)

CYP isoform	Species	Inducers	Inhibitors
1A1 and 1A2	Human, rat	Benzo[a]pyrene	Furafylline
		Dioxin	α-Naphthoflavone
		3-Methylcholanthrene	
		β-Naphthoflavone	
2B1and 2B2	Rat	Phenobarbital	
2C9	Human	Carbamazepine	Sulfaphenazole
		Phenytoin	
		Phenobarbital	
		Rifampicin	
2C19	Human		S-mephenytoin
2D6	Human		Quinidine
2E1	Human, rat	Ethanol	Diethydithiocarbamate
		Acetone	
		Isoniasid	
3A1	Rat	Dexamethasone	
3A4	Human	Barbiturate	Gestodene
		Carbamazepine	Troleandomycin
		Dexamethasone	Ketoconazole
		Rifampicin	Itraconazole
4A6	Rat	Clofibrate	

Enzyme induction can enhance metabolic rate of the affected drug, resulting in a decrease in the serum concentration of parent drug, and possibly a loss of clinical efficacy. For example, a number of unplanned pregnancies have occurred in women taking oral contraceptives with phenytoin, an enzyme inducer of CYP 3A4 (Janz et al., 1974).

In some case, an increased rate of metabolism associated with an enzyme induction can result in an increased formation of a toxic metabolite with serious consequences. For example, administration of carbamazepine may increase the risk of

valproic acid-induced hepatotoxicity by increasing the formation of a toxic metabolite of valproic acid (levy et al., 1990).

Table 8 Inducers of drug metabolising enzymes other than CYPs (Gibson and Skett, 1994)

Enzyme	Inducer
Epoxide hydrolase	2-Acetylaminofluorene, aldrin, Arochlor 1254, dieldrin, ethoxyquin, isosafrole, 3-mehylcholanthrene, phenobarbitone, *trans*-stibene oxide.
Glucuronosyl transferase	Dieldrin, isosafrole, phenobarbitone, 3-methycholanthrene, polychlorinated biphenyls, TCDD.
NADPH-cytochrome P450reductase	2-Acetylaminofluorene, dieldrin, isosafrole, phenobarbitone, polychlorinated biphenyls, *trans*-stilbene oxide.
Glutathione-S-transferase	2-Acetylaminofluorene, phenobarbitone 3-methylcholanthrene, TCDD, *trans*-stilbene oxide.
Cytochrome b_5	2-Acetylaminofluorene, griseofulvin, butylated hydroxytoluene.

Hepatic enzyme inhibition

Hepatic enzyme inhibition results in a decrease in the clearance, thereby an increase in the steady-state serum concentration of the affected drug. The magnitude of the effect is largely unpredictable because it depends on the specific enzyme which is inhibited and the quantitative importance of that pathway in the overall clearance of the affected drug. Unlike enzyme induction, it is important to note that a drug may inhibit an isoform whether or not it is a substrate of that isoform (Levy, 1995). For instance, quinidine

is a potent inhibitor of CYP2D6 although it is, in fact, metabolized by CYP3A subfamily (Guengerich et al., 1986). Commonly prescribed drugs that have been implicated in inhibitory drug interactions are listed in Table 6 and the selective inhibitors of CYP isoforms are shown in Table 7.

Enzyme inhibition of drug-metabolizing enzymes can be produced by a number of different mechanisms which are

a) Reversible inhibition by binding to the prosthetic haem iron or to the hydrophobic region of the apoprotein or a combination of both. For instance, ketoconazole and cimetidine are reversible inhibitor of CYPs by binding to both regions of the enzymes (Pelkonen and Puurunen, 1980 ; Gascon et al., 1991).

b) Quasi-irreversible inhibition by forming inhibitory metabolites which are enable to form stable complexes with the prosthetic haem of CYP, called metabolic intermediate complex. As a result, CYP is sequestered in a functionally inactive state. However, the complexation can be reversed, and the catalytic function of ferric CYP can be restored by an *in vitro* incubation with highly lipophilic compounds that displace the metabolic intermediate from the active site. In a situation that metabolic intermediate binds to ferrous CYP, the complex can be disrupted by an irradiation at 400 to 500 nm or oxidation CYP to the ferric stable by an addition of potassium ferricyanide. Examples of CYP inhibitor in this class are isosafrole and piperonyl butoxide (Murray et al., 1985; Lin and Lu, 1998).

c) Irreversible inhibition by forming reactive metabolites that cause irreversible inactivation of the enzyme. This mechanism-based inactivation of CYP may result from irreversible alteration of haem or apoprotein or a combination of both. Examples of CYP inhibitors in this class are 2-isopropyl-4-pentenamide (AIA), chloramphenical, etc. (Paul et al., 1983).

Antiepileptic drugs: Pharmacokinetic interactions

Pharmacokinetic interactions between antiepileptic drugs arise most frequently as a consequence of drug-induced changes in hepatic metabolism, and less frequently from changes in plasma protein binding (Anderson, 1998).

The metabolic reactions which are commonly affected by antiepileptic drugs are the reactions catalyzed by CYP enzymes superfamily, epoxide hydrolase and UDPGT (Perucca et al.,1995). The effects of antiepileptic drugs on these enzyme systems are summarized in Table 9.

The extent of a metabolic interaction depends on various factors; for example, pre-existing enzyme induction or inhibition by other drugs or xenobiotics, genetic predisposition, and dosage or concentration of inducer or inhibitor. (Riva et al., 1996).

Table 9 Effects of antiepileptic drugs on the most common drug metabolic processes (Riva et al., 1996).

Drug	CYP system	UDPGT system	Epoxide hydrolase
Carbamazepine	Induction (CYP2C/3A)	Induction	Induction
Ethosuximide	None	None	None
Felbamate	Inhibition(CYP2C19)	None?	Inhibition?
	Induction(CYP3A4)		
Gabapentin	None	None	None
Lamotrigine	None	NA	None?
Phenobarbital	Induction (CYP2C/3A)	Induction	Induction
Phenytoin	Induction (CYP2C/3A)	Induction	Induction
Primidone	Induction (CYP2C/3A)	Induction	Induction
Topiramate	Inhibition(CYP2C19)	Inhibition?	None
	Induction(CYP3A4)		
Valproic acid	Inhibition (CYP2C9)	Inhibition	Inhibition
Vigabatrin	None	None	None

Abbreviations and symbol : NA=information not available ; ? = results not definitive

Valproic acid

Valproic acid (VPA) is a branched-chain fatty acid (Figure 5), structurally unrelated to any other antiepileptic drugs. VPA is very effective for virtually all types of generalized seizures, including generalized tonic-clonic, absence, and myoclonic seizures. Moreover, it has also been recommended for a treatment of partial seizures (Albers and Peroutka, 1992).

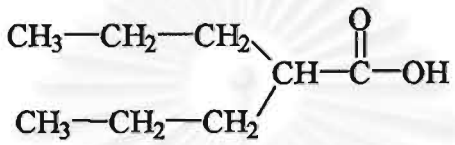

Figure 5 Structure of VPA

Mechanism of action

The mechanism of action of VPA has yet been fully elucidated, but much attention has focused on its effect on the inhibitory neurotransmitter γ-aminobutyric acid (GABA). VPA increases synaptosomal GABA concentrations via activation of a major synthetic enzyme of GABA, glutamic acid decarboxylase (GAD) (Taberner et al., 1980; Loscher ,1981; Phillips and Fowler, 1982). It also demonstrates a strong inhibition effect on the catabolic enzyme, succinic semialdehyde dehydrogenase (SSADH) (Van der Laan et al., 1979; Zeise, 1991) and GABA transaminase (GABA-T) (Godin et al.,1969).

Evidence also exists which suggests that VPA reduces the neurotransmission mediated by excitatory amino acids such as aspartic acid, glutamic acid and γ-hydroxybutyric acid (GHB) (Whittle and Turner ,1978; Chapman et al., 1982; Vayer et al., 1988).

In addition to its effect on amino acid neurotransmitters, VPA appears to possess a direct neuronal membrane depressant effect through its influence on sodium or potassium conductance (Davis et al., 1994).

Pharmacokinetics

VPA is rapidly and completely absorbed after oral administration. Peak concentration is observed within 1 to 4 hours. However the onset can be delayed for several hours if the drug is administered in enteric-coated tablets or with meals. The apparent volume of distribution (Vd) is about 0.2 liter per kilogram. The drug is about 90% plasma protein bound. The half-life of valproate is approximately 15 hours but is reduced in pateints taking other antiepeleptic drugs (Rall and Schleifer, 1992).

Biotransformation and metabolites of VPA

In humans and animals, VPA is metabolized by several pathways. These include conjugation with UDP-glucuronic acid, β-oxidation and microsomal hydroxylation (ω-, ω_1-, ω_2- oxidation) (Gugler et al.,1980; Zaccara et al.,1988). Glucuronidation is a major pathway resulting in several metabolites excreted in urine. β-oxidation reaction yields 2 metabolites, 2-propyl-2 pentanoic acid (2-ene- VPA) and 2-propyl-3-keto-pentanoic acid (3-keto VPA). ω-, ω_1-, ω_2- Oxidations, probably catalyzed by the microsomal CYP system (Heinemeyer et al., 1984), result in the formation of 3-OH, 4-OH and 5-OH metabolites. 5-OH VPA was further oxidized to 2- propyl glutaric acid (PGA). Moreover, in a minor amounts, two other unsaturated metabolites (4-ene-VPA and 2,4-diene-VPA) are also found (Figure 6) (Granneman et al., 1984; Nau and Loscher, 1984).

2-Ene-VPA, a β-oxidation derivative, probably contributes to the anticonvulsant activity of VPA, while another metabolite, 4-ene-VPA, may be involved in both hepatic toxicity and embryotoxicity (Nau et al., 1984).

From the data presently available it seems likely that rat CYP2B (Rettie et al., 1987), human CYP2C9 and CYP2A6 (Sadeque et al., 1997) as well as rabbit CYP4B1 mediate the formation of the hepatotoxin, 4-ene-VPA.

Toxicology of VPA

The most common adverse effects associated with valproic acid are mild to moderate in severity. These include gastrointestinal disturbances (nausea, vomiting, dyspepsia), weight gain, neurological effects (tremor, fatique, dizziness, headache) and

transient hair loss. Dematological (skin rash, hirsutism) and neurological adverse effects occur less frequently with VPA than do other antiepileptic drugs. Moreover, it has become increasingly evident that VPA therapy may be associated with serious hepatotoxicity as well as embryotoxicity in a very small portion of patients (Nau and Loscher, 1984; Davis et al., 1994).

Neurotoxicity of VPA

VPA is generally associated with fewer neurological adverse effects than other antiepileptic drugs. Neuronal side effects are tremor, fatique, somnolence, dizziness and headache (Davis et al., 1994).

Embryotoxicity of VPA

VPA is embryotoxic in rabbit, rat and mouse. The most pronounced effects observed were neural tube defects, skeletal abnormalities, embryolethality and fetal weight retardation. Use of antiepileptic drug during pregnancy is associated with an increased risk of congenital malformations. (Nau and Loscher, 1984; Davis et al., 1994). A single dose of 400 mg/kg sodium valproate during the period of gestation most sensitive to VPA resulted in a significant incidence of neural tube defects. Repeated administration of this dose, once between days 6 and 14 of gestation, increased the incidence of neural tube defects to 45 % (Nau and Loscher, 1984).

Hepatotoxicity of VPA

Apparently, valproate induced hepatic toxicity can be seen in many different forms, ranging from transient elevation of hepatic enzymes to irreversible liver damage. In the majority of cases the observed changes have been non-specific, consisting of hepatocellular necrosis, cholestatic changs and/or hepatitis. In addition, in some instances microvesicular fatty change has been observed (Zimmerman and Ishak, 1982).

Many of the syndromal, biochemical and histological features in the patients with microvesicular steatosis caused by VPA are similar to those in Reye's syndrome, poisoning by hypoglycin (Jamaican vomiting sickness) and 4-pentenoic acid (4-PA) (Gerber et al., 1979). Because of the structural similarity of the toxic metabolite of hypoglycin as well as 4-

PA to the 4-ene metabolite of VPA (Figure 7), it is fascinating to consider that ω-oxidation pathway and the resulted reactive metabolies, 4-ene-VPA and 2,4-diene-VPA, may be of particular significance with regard to hepatotoxicity of VPA (Gerber et al., 1979; Zimmerman and Ishak, 1982; Gram, 1983; Nau and Loscher, 1984).

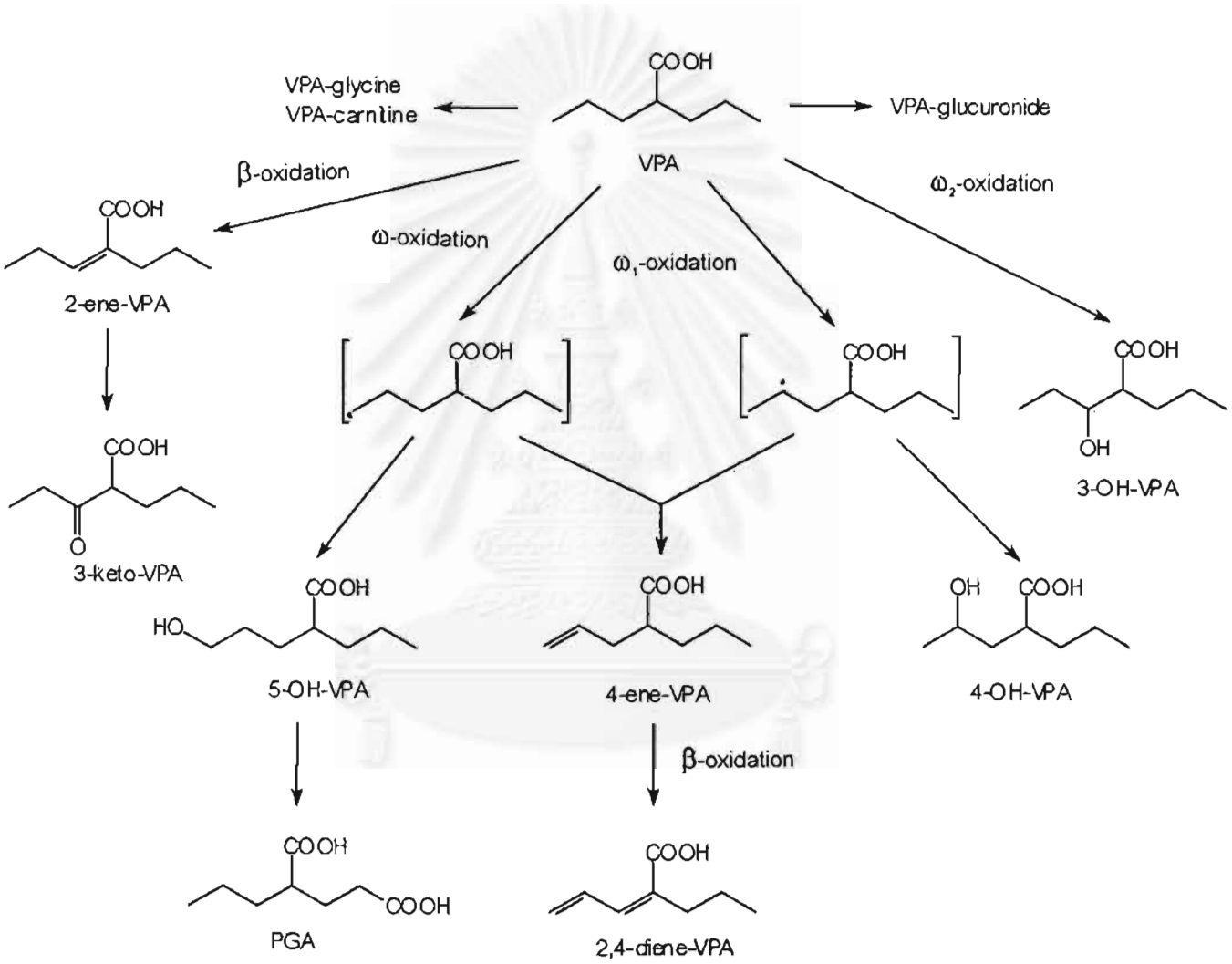

Figure 6 Metabolic pathways of VPA (modified from Nau and Loscher, 1984 ; Rettie et al., 1987)

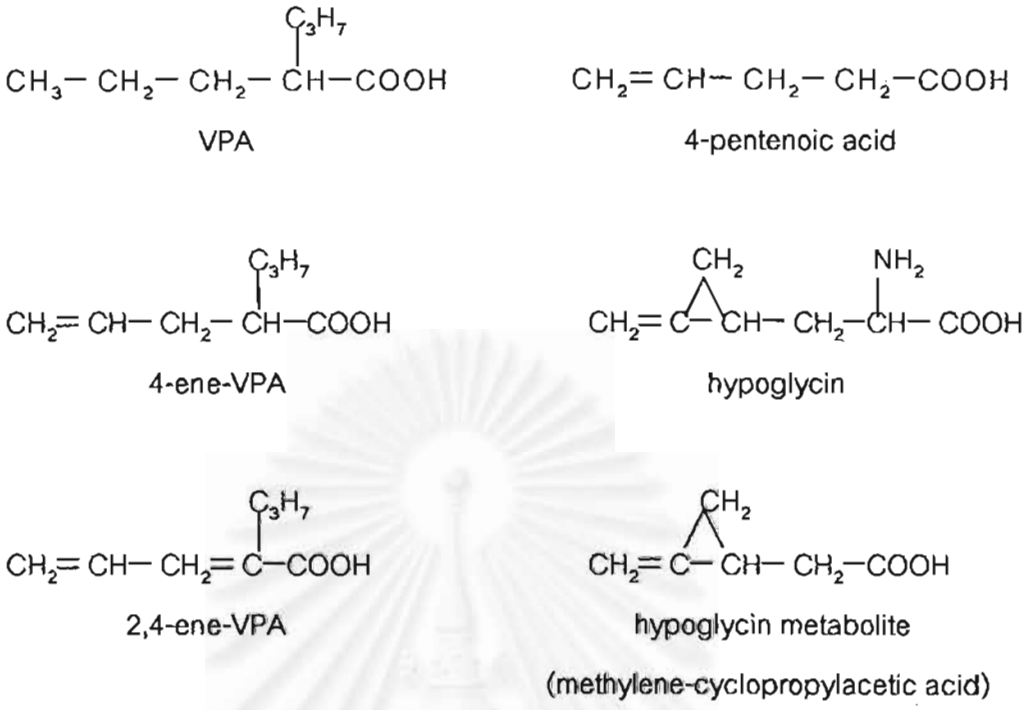

Figure 7 Structure of VPA, 4-ene-VPA, 2,4-diene-VPA , 4-pentenoic acid, hypoglycin and hypoglycin metabolite (Neu and Loscher, 1984)

According to the close structural resemblance of 4-ene VPA to 4-PA, which is a powerful β-oxidation inhibitor (Bressler et al., 1969; Billington et al., 1978), VPA-induced hepatotoxicity may share a similar biochemical ethiology with inhibition of fatty acid β-oxidation as a common link (Zimmerman and Ishak, 1982).

In the study of Kesterson et al. (1984), young rats (150 g) were treated with a single 100 mg/kg dose of various VPA- unsaturated metabolites or with a single 700 mg/kg dose of VPA. Ranking in the severity of hepatotoxicity, based on the degree of microvesicular steatosis, was 4-ene-VPA > 4-PA (possitive control) > 2,4-diene-VPA > 3-ene-VPA > 2-ene-VPA >VPA. Young rats were found to be susceptible to VPA (350 mg/kg/day) induced steatosis following enzyme induction with phenobarbital. By contrast, steatosis developed in mature rats (250 g) but only at near lethal dose (700 mg/kg/day).

Corresponding to the *in vivo* study of Kesterson et al. (1984), the *in vitro* study of Bjorge (1985) indicated that VPA and its hepatotoxic metabolite, 4-ene- VPA are potent,

broad spectrum inhibitors of fatty acid β-oxidation, a property which may be one of a key toxicological important explanation of VPA-induced liver injury.

It has been postulated that an electrophilic epoxide intermediate formed during the metabolism of 4-ene-VPA, may be the ultimate toxic chemical species (Granneman et al., 1984; Rettenmeier et al., 1986). The available evidence strongly suggests that 4-ene- VPA, a terminal olefin, acts to destroy CYP by the "suicide substrate" mechanism (Prickett and Baillie, 1986).

The unsaturated metabolites of VPA, particularly 4-ene-VPA, undergo further metabolic activation yielding electrophilic intermediates that bind covalently to liver macromolecules (Porubek et al., 1988). This phenomenon may explain the depletion of hepatic GSH after high doses of VPA administration.

Jezequel et al. (1984) demonstrated that hepatic GSH was significantly decrease by 25 % in both 200 and 600 mg/kg VPA-treated rats at 3 hours after VPA i.p. injection. At 5 hours, the level of hepatic GSH return to control values in the 200 mg/kg VPA treated rats, while reduced levels persisted in rats given 600 mg/kg of VPA.

Furthermore, the *in vivo* study of Cotariu et al. (1990) showed that GSH level decreased in a dose-dependent relationship after a single i.p. administration of VPA. At the end of 3 hours, GSH levels either return to control values following injection of 300 mg/kg VPA or tended to revert to normal following injection of 500 mg/kg. No change of GSH level was observed in animals given a non-toxic dose (100 mg/kg) of VPA.

A study of Kassahun and collaborates in 1990 regarding the identification and characterization of GSH and N-acetylcysteine conjugates of 2,4-diene-VPA in rats and humans, indicated that VPA was metabolized to chemicallly reactive intermediates, especially 2,4-diene-VPA, that may contribute to the hepatotoxicity of VPA.

Pharmacokinetic interactions of VPA

In human, VPA is an inhibitor of hepatic metabolizing enzymes, including CYP2C9, UDPGT and epoxide hydrolase. Therefore, VPA can inhibit the metabolism of some drugs metabolized by these enzymes. For example, VPA inhibits the metabolism of phenobarbital and phenytoin of which the metabolic pathways are catalyzed by CYP2C9. In addition to the enzyme inhibition characteristic, VPA can displace other drugs, such as phenytoin, carbamazepine and diazepam, from albumin binding sites because of its highly protein binding property (Riva, et al, 1996; Anderson, 1998).

On the other hand, some drugs that can inhibit or induce the metabolic pathways of VPA will cause pharmacokinetic drug interaction with VPA. For instance, VPA plasma concetrations are decreased in the presence of CYP2C and UDPGT inducing drugs, such as phenobarbital, phenytoin and carbamazepine(Riva, et al, 1996; Anderson, 1998).

In an isolated rat hepatocyte model, when VPA was given i.p a dosage of 100 mg/kg once daily for 10 days, the following enzymes were significant induced ; CYP, cytochrome b_5, aldrin epoxidase and GST. In contrast, VPA administered by osmotic pumps, only 7-ethoxycoumarin-o-deethylase activity was induced, whereas aldrin epoxidase and GST activities were significantly inhibited (Rogiers et al., 1988).

Using rat hepatocyte co-cultured with rat primitive biliary epithelial cells, VPA was shown to be a potent inducer of CYP2B1 and CYP2B2. In contrast, to phenobarbital which induced GST, VPA selectively decreased the subunit of 3 and/or 4 of GST. Moreover, VPA was also more effective as an inducer of CYP2B when it was administrated to rat via subcutaneously implanted osmotic minipumps (Rogiers et. al., 1995).

N-(2-propylpentanoyl) urea

N-(2-propylpentanoyl) urea (VPU) (Figure 8) is one of the VPA derivatives which was synthesized in 1992 by staffs of the department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Saisorn et al., 1992). The synthetic pathways of monoureide analogues of VPA, VPU is shown in Figure 9.

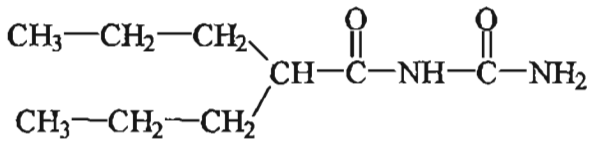

Figure 8 Structure of VPU

A study of Tantisira and collaborates in 1997 on the anticonvulsant activity and neurotoxicity of VPU showed that VPU possessed a higher broad spectrum anticonvulsant activity in maximal electroshock seizure (MES) and pentylenetetrazole (PTZ) induced convulsions in rats and mice, comparing with its parent compound, VPA (Table 10). Concerning with testing about dose-lethality relationship in mice, VPU had a higher LD_{50} than that of VPA. A higher LD_{50} but lower ED_{50} value of VPU compared with those of VPA indicated that VPU possessed a greater margin of safety. Moreover, VPU appeared to have less neurotoxicity than did VPA, using rotarod test (Tantisira et al., 1997).

Table 10 Anticonvulsant activity of intraperitoneally given VPU and VPA on MES and PTZ seizure test models of epilepsy (Tantisira et al., 1997)

Animal model	Animals	ED_{50} (mg/kg of body weight)	
	(n=8)	VPU	VPA
MES induced seizure	mice	66 (58-75)*	242 (233-247)
	rats	67 (55-81)	233 (220-247)
PTZ induced seizure	mice	57 (54-60)	95 (86-105)
	rats	80 (71-90)	140 (132-148)

* Values in parenthesis are range.

Meesomboon et al. (1997) reported that VPU seem to produce less developmental toxicity, regarding effects on axial rotation and embryonic growth, than did VPA. Concerning a hepatotoxicity study, VPU exhibited hepatotoxic effects, including an increase of transaminase activities and a depletion of hepatic total glutathione at high dose of VPU administration in an *in vitro* study using isolated hepatocyte cells. The author suggested that the depletion of GSH might indicate a production of VPU reactive intermedieates. Furthermore, VPU increased serum transaminase activities as well as induced fat vacuolar degeneration in the periportal and midzonal area following at high doses (700 and 1400 mg/kg/day) (Patchamart, 1996) of VPU in rats in an *in vivo* study.

Pharmacokinetic study utilizing ^{14}C-VPU and autoradiographic technique was performed by Kijsanayotin and collaborates in 1997. VPU was found rapidly absorbed from injected area as well as gastrointestinal tract and instantaneously distributed to various tissue organs, including liver, kidneys, lungs, muscle and brain. Moreover, VPU was rarely hydrolyzed to VPA by carboxyesterase obtained from phenobarbital-induced mice livers and human livers. This result indicated that anticonvulsant activity of VPU may be attributed from VPU and/or its metabolites, not VPA.

VPU demonstrated a prolong barbiturate sleeping time characteristic (Tantisira et al., 1997). This was probably resulted from the effect of VPU directly on CNS or the effect on hepatic drug metabolizing enzymes. Concerning the effect of VPU on CYP, Kijsanayotin et al. (1997) found that VPU demonstrated an inhibition effect on CYP 2C9 and CYP 1A2 *in vitro* using human liver microsomes.

Anticonvulsant drugs are mostly associated with a wide range of drug interactions, including hepatic enzyme induction and inhibition. In drug development process, a new compound of anticonvulsant purpose is recommended to be evaluated for the potential effects on liver drug metabolizing enzymes (Cereghino and Kupferberg, 1993). Therefore, the aim of this study was primarily to assess the effect of VPU on phase I metabolizing enzymes, CYP. Effects of VPU on GST, an important phase II metabolizing enzyme, as well as its effect on total hepatic GSH were also evaluated.

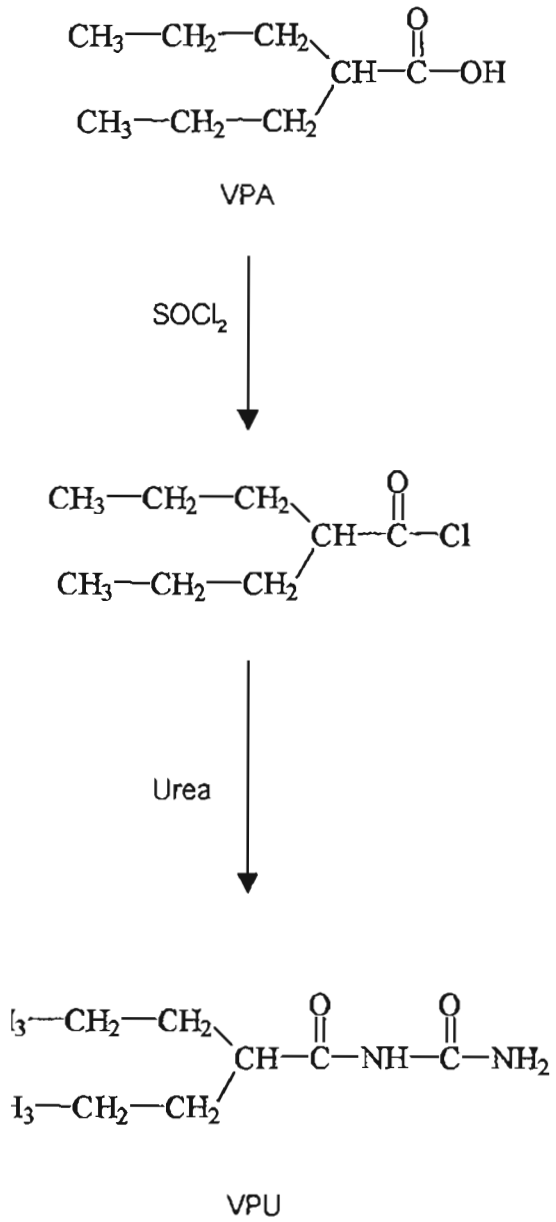

Figure 9 The synthetic pathway of VPU (Saisorn et al., 1992)

Chapter III

Materials and Methods

Experimental animals

Adult male Wistar rats of body weight between 250-300 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. The animals were housed in animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University and acclimatized for at least 1 week before the experimentation.

Chemicals

The following chemicals were used in the experimentation :

4-aminophenol, aniline hydrochloride, bovin serum albumin, 1-chloro-2,4-dinitrobenzene, cupric sulfate, dimethylsulfoxide, 5,5' dinitrobenzene , ethylenediamine tetraacetic acid, Folin & Ciocalteu's phenol reagent, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, glutathione (reduced form), nicotinamide adenine dinucleotide phosphate, potassium phosphate, potassium phosphate monobasic anhydrous, sodium carbonate, sodium citrate, sodium phosphate dibasic anhydrous, Trisma® base, valproic acid, benzyloxyresorufin, ethoxyresorufin, methoxyresorufin, pentoxyresorufin and resorufin (Sigma, U.S.A.)

Magnesium chloride, phenol, potassium chloride, sodium chloride, sodium hydroxide and trichloroacetic acid (E. Merck, Germany)

Ethanol absolute and glycerol (Carlo Erba, U.S.A.)

Methanol (HPLC grade) (BDH Laboratory Supplies, England)

Sodium dithionite (Fluka Chemic, Japan)

Polyethylene glycol 400 (PEG400) (T. Chemical Ltd. Partnership, Thailand)

N-(2-propylpentanoyl) urea (VPU) was synthesized by Associated Professor Dr. Chamnan Patarapanich and collaborates at the department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

All chemicals used in Western blot analysis were generously obtained from Professor Kan Chiba.

Experimental instruments

The following instruments were used in the experimentation :

Potter-Elvehjem homogenizer with teflon pestle and glass homogenizer (Heidolph, Germany)

Vortex mixer (Clay adams, U.S.A.)

Refrigerated superspeed centrifuge (Beckman, U.S.A.)

Refrigerated ultracentrifuge (Hitachi, Japan)

Centrifuge (Kokusan, Japan)

Ultra-low temperature freezer (Forma Scientific Inc., U.S.A.)

Metabolic shaker bath (Heto, Denmark)

Spectrophotometer (Jasco, Japan)

Fluorescence spectrophotometer (Jasco, Japan)

Tank of carbon monoxide gas (T.I.G. , Thailand)

pH meter (Backman Intruments, U.S.A.)

Autopipets 20, 100, 200, 1000 and 5000 μl (Gilson, France)

All instruments used in Western blot analysis were available in Professor Kan Chiba's laboratory.

Experimental methods

1. Rats were divided into 4 treatment groups. Each treatment group comprised 6 rats.

1.1 Control group 1 : rats were administered with an i.p. injection of sterile water, once daily for 7 days.

1.2 Control group 2 : rats were administered with an i.p. injection of PEG400 (which was used for suspending of VPU), once daily for 7 days.

1.3 VPA treatment group : rats were administered with an i.p. injection of a solution of VPA in PEG 400, at a dosage of 250 mg/kg/day, once daily for 7 days.

1.4 VPU treatment group : rats were administered with an i.p. injection of VPU suspended in PEG400 at a dosage of 80 mg/kg/day, once daily for 7 days.

Four animals were studied simultaneously during each experimental period (1animal / each treatment group).

2. Preparation of liver microsomes and cytosols

2.1 Reagents

1. Phosphate buffer pH7.4

For a preparation of 1 L of phosphate buffer pH 7.4 , these following materials were used ; 1.78 g of KH_2PO_4, 9.55 g of Na_2HPO_4 and 11.50 g of KCl. The solution was adjusted to pH 7.4 with NaOH or HCl.

2. Phosphate buffer pH 7.4 , containing 20% glycerol

3. 0.9 % w/v NaCl

2.2 Procedure

1. On the day after the seven days of compound administrations, animals were sacrificed by cervical dislocation under light ether anesthesia.

2. The livers were perfused *in situ* via the portal vein with ice-cold 0.9 % w/v NaCl until the entire organ became pale.

3. The livers were taken out, rinsed with ice-cold 0.9 % w/v NaCl, blotted dry with a gauze.

4. The whole livers were weighed. A small portion of livers were kept at -80°C for total GSH assay. The remaining portion of livers were homogenized with 3 volume of phosphate buffer pH 7.4.

5. The liver homogenates were centrifuged at 10,000 g for 30 minutes at 4°C using refrigerated superspeed centrifuge, to pellet intact cells, cell debris, nuclei and mitochondria.

6. The supernatant (S9, post mitochondrial fraction) were transferred into the ultracentrifuge tubes and centrifuged at 100,000 g for 60 minutes at 4°C, using refrigerated ultracentrifuge.

7. The supernatants were kept for cytosolic enzyme assays. The pellets (microsomal fractions) were resuspended with phosphate buffer pH 7.4 containing 20% glycerol about 3-5 ml. Transfer the obtained microsomal suspensions into eppendroff tubes. The tubes containing cytosolic and microsomal samples were stored at -80°C until the time of enzyme activity asssays.

3. Determination of protein concentrations

Liver microsomal and cytosolic protein concentrations were determined according to the method of Lowry et. al. (1951).

3.1 Reagents

1. 2% w/v Sodium carbonate
2. 0.5 M Sodium hydroxide (NaOH)
3. 2% w/v Sodium citrate
4. 1% w/v Cupric sulfate
5. 1 mg/ml Bovine serum albumin (BSA) in 0.5 M NaOH
6. Folin & Ciocalteu's phenol reagent
7. Working protein reagent : The solution was prepared freshly in a sufficient amount for all tubes in the assay (6.5 ml of the solution was required for each tube). This reagent comprised sodium carbonate, sodium hydroxide, sodium citrate and cupric sulfate solutions in a 100/10/1/1 ratio, respectively.

3.2 Procedure

1. 16 x 125 mm Tubes were labelled in duplicate for 8 standards (0, 50,100, 150, 200, 250, 300 μg) and for each unknown sample.

2. The following reagents were added in μl to each standard tube:

Standard tube	0	50	100	150	200	250	300	(μg)
BSA, 1 mg/ml	0	50	100	150	200	250	300	(μl)
NaOH, 0.5 M	500	450	400	350	300	250	200	(μl)

Each tube was mixed thoroughly after addition of the reagents.

3. To each of the unknown tube, 490 μl of 0.5 M NaOH and 10 μl of microsomal or cytosolic sample were added and mixed thoroughly.

4. After 6.5 ml of working protein reagent was added to each tube in the assay, the tubes were allowed to stand at room temperature for 10 minutes.

5. While 200 μl of Folin & Ciocalteu's phenol reagent was added to each tube in the assay, the tubes were vortexed thoroughly for a minimum of 30 seconds.

6. After the tubes were allowed to stand at room temperature for a minimum of 30 minutes, the absorbances of the solutions were measured by spectrophotometer against the 0 μg standard at 500 nm.

3.3 Calculations

1. The average absorbance of each standard was plot against its amount of protein. The best fit regression line was drawn through the points. The amount of protein in each unknown sample was obtained by comparing its abosorbance against the standard curve.

2. The protein concentration (mg/ml) in each unknown sample was obtained by dividing its amount of protein (from1) with the volume of microsomal or cytosolic sample used in the reaction.

4. Spectral determination of total CYP contents

Total CYP contents in microsomal samples were determined spectrophotometrically by the method of Omura and Sato (1964).

4.1 Reagents

1. 0.1 M Tris buffer, pH 7.4 containing 20% v/v glycerol

2. Solid sodium dithionite

3. Carbon monoxide

4.2 Procedure

1. Microsomal samples were diluted to 2 mg/ml in 0.1 M Tris buffer, pH 7.4 containing 20% glycerol.

2. Of the total 4 ml diluted samples, 2 ml each was divided and placed in sample and reference cuvettes.

3. After a few grains of solid sodium dithionite were added to the sample and reference cuvettes with gentle mixing, both cuvettes were put in a spectrophotometer, adjusted to zero and corrected to a baseline between 400 nm to 500 nm.

4. Immediately after the sample cuvette was bubbled with carbon monoxide (approximately 1 bubble/second) for approximately 1 minute, the cuvette was placed back to the spectrophotometer and scanned from 400 nm to 500 nm. The absorbance difference between 450 nm and 490 nm was recorded.

4.3 Calculations

1. Total CYP contents were calculated based on the absorbance difference between 450 nm and 490 nm as well as an extinction coefficient of 91 $mM^{-1}cm^{-1}$. Using Beer's law and an assuming cuvette path length of 1 cm, the CYP contents were given by :

$$\text{Total CYP contents (nmol/mg protein)} = \frac{\text{Absorbance difference (450-490 nm)} \times 1000}{91 \times \text{concentration(mg/ml) of the diluted sample}}$$

5. Alkoxyresorufin o-dealkylation assays

The o-dealkylation of benzyloxy-, ethoxy-, methoxy- and pentoxyresorufins by liver microsomes were determined according to the method of Burke and Mayer, 1974 and Lubet et al., 1985 with slight modifications.

5.1 Reagents

5.1.1 0.1 M Tris buffer, pH 7.4

5.1.2 Resorufin & Alkoxyresorufins

1. 0.5 mM Benzyloxyresorufin (MW 303)

   1.515 mg of benzyloxyresorufin was dissolved with dimethyl sulfoxide (DMSO) qs to 10 ml.

2. 0.5 mM Ethoxyresorufin (MW 241)

   1.205 mg of ethoxyresorufin was dissolved with DMSO qs to 10 ml.

3. 0.5 mM Methoxyresorufin (MW 227)

   1.135 mg of methoxyresorufin was dissolved with DMSO qs to 10 ml.

4. 0.5 mM Pentoxyresorufin (MW 283)

   1.415 mg of pentoxyresorufin was dissovled with DMSO qs to 10 ml.

5. 0.5 mM Resorufin (MW 235)

   1.175 mg of resorufin was dissovled with DMSO qs to 10 ml.

5.1.3 NADPH regenerating system

1. 0.1 M NADP, pH 7.4

   0.765 g of NADP (Sigma N-0505) was dissolved with 20 mM K_3PO_4 qs to 10 ml, and adjusted pH to 7.4 with HCl or NaOH (10 µl contains 1 mmole of NADP).

2. 0.5 M Glucose 6-Phosphate (G6P), pH 7.4

   1.41 g of glucose 6-phosphate (Sigma G-7879) was dissovled with 20 mM K_3PO_4 qs to 10 ml, and adjusted pH to 7.4 with HCl and NaOH (10 µl contains 5 mmoles of G6P).

3. Glucose 6-Phosphate Dehydrogenase (G6PD), pH 7.4

   G6PD (Sigma G-8289) was diluted to 100 units per ml with 20 mM K_3PO_4 and adjusted pH to 7.4 with HCl and NaOH (10 µl contains 1 unit of G6PD).

4. 0.3 M $MgCl_2$, pH 7.4

   609.93 mg of $MgCl_2$ was dissolved with 20 mM K_3PO_4 qs to 10 ml, and adjusted pH to 7.4 with HCl and NaOH (10 µl contains 3 mmoles of $MgCl_2$).

5.2 Procedure

1. Microsomes were diluted with 0.1 M Tris buffer, pH7.4 to measure out 100 µg of protein (100 µl of microsome was diluted with 0.1 M Tris buffer, pH7.4 qs to 5000 µl)

2. For each ml of the reaction mixture, the following reagents were added :

   a. 30 µl of NADPH regenerating system comprised
      10 µl of 0.1 M NADP
      10 µl of 0.5 M G6P
      10 µl of 0.3 M $MgCl_2$
   b. 10µl of 0.5 mM Alkoxyresorufin
   c. Varied volume of diluted microsomal suspension containing 100 µg of microsomal protein.
   d. 0.1 M Tris buffer, pH 7.4 qs to 990 µl

3. Three reaction tubes were prepared for each microsomal sample. One tube is a sample blank tube and the others are sample tubes. All tubes were preincubated in a 37°C shaking water bath for 2 minutes.

4.The reaction was started by adding 10 µl of G6PD (1 unit of G6PD/1 ml of reaction mixture). For a sample blank, 10 µl of 0.1 M Tris buffer was added instead of G6PD.

5. After a 5 minute incubation, the reaction was stopped by adding 1 ml of methanol (HPLC grade) (1 ml/ml of reaction mixture).

6. The absorbance was measured by fluorescence spectrophotometer using an excitation wavelength of 556 and an emission wavelength of 588.

7. A resorufin standard curve was constructed using 8 concentrations of resorufin : 0.025, 0.05, 0.075 0.1, 0.2, 0.4, 0.5, 1.0 nmol/ml.

5.3 Calculations

The o-dealkylations of benzyloxy-, ethoxy-, methoxy-, and pentoxyresorufins were calculated by dividing the amount of resorufin formed, by the time of incubation (5 minutes) and an amount of microsomal protein (100 µg) used in the reaction.

6. Aniline 4-hydroxylation assay

The 4-hydroxylation of aniline by liver microsomes was determined according to the method of Schenkman et. al. (1967).

6.1 Reagents

1. 10 mM Aniline HCl

   93 mg of aniline HCl was dissolved with 100 ml of double distilled water. The solution was stored in dark brown bottle.

2. 6% w/v Trichloroacetic acid

   60 g of trichloroacetic acid was dissolved with 1 L of double distilled water.

3. 20% w/v Trichloroacetic acid

200 g of trichloroacetic acid was dissolved with 1 L of double distilled water.

4. 1% w/v Phenol

20 g of phenol and 40 g of NaOH were dissolved with 2 L of double distilled water.

5. 1 M Na_2CO_3

200 g of anhydrous Na_2CO_3 was dissolved with 2 L of double distilled water.

6. 10 μM 4-Aminophenol

36.5 mg of 4-aminophenol was made up to 10 ml with double distilled water and added 0.1 ml of this aminophenol solution to 15 g trichloroacetic acid and made up to 250 ml with double distilled water.

7. 0.1 M Tris buffer, pH 7.4

8. 0.1 M NADP

9. 0.5 M G6P

10. 0.3 $MgCl_2$

6.2 Procedure

1. Microsomes were diluted with 0.1 M Tris buffer, pH 7.4 to measure out 5 mg of protein.

2. For each ml of the reaction mixture, the following reagents were added :

a. 30 μl NADPH regenerating system comprised

10 μl of 0.1 M NADP

10 μl of 0.5 M G6P

10 μl of 0.3 M $MgCl_2$

b. 500 μl of 10 mM Aniline HCl

c. Vaired volume of microsomal suspension containing 5 mg of microsomal protein

d. 0.1 M Tris buffer, pH 7.4 qs to 2 ml

3.Three reaction tubes were prepared for each microsomal sample.

One tube is a sample blank tube and the other are sample tubes.

4. All tubes were preincubated in a 37°C shaking water bath for 2 minutes. The reaction was initiated by adding 20 µl of G6PD. For a sample blank, 20 µl of 0.1 M Tris buffer was added instead of G6PD.

5. After a 30 minute incubation time, the reaction was stopped with 1 ml, ice-cold 20% w/v trichloroacetic acid and the tubes were kept on ice for 5 minutes.

6. The solution was then centrifuged at 3000 rpm for 5 minutes.

7. After 1 ml of the supernatant was transferred to a new test tube, 1 ml of 1% w/v phenol and 1 ml of 1 M $NaCO_3$ were added. The solution was mixed well by vortex mixer.

8. The solution was kept at room temperature for 30 minutes and its absorbance was measured by spectrophotometer at a wavelength of 630 nm.

9. A standard curve was constructed using 5 known concentrations of 4-aminophenol standard solutions (2, 4, 6, 8, 10 µM), following the procedure from step 7 in the same manner as sample.

6.3 Calculations

The 4-hydroxylation of aniline was calculated by dividing the amount of product formed (4-aminophenol) by the time of incubation (30 minutes) and an amount of microsomal protein (5 mg) used in the reaction.

7. Determination of glutathione S-transferase activity

Liver cytosolic GST activity was determined according to the method of Warholm (1985).

7.1 Reagents

1. 0.1 M Sodium phosphate buffer , pH 6.5 containing 1 mM sodium ethylenediamine tetracetic acid (EDTA)

2. 20 mM 1-Chloro-2,4-dinitrobenzene (CDNB) in 95% ethanol

4. 20 mM GSH

7.2 Procedure

1. Spectrophotometer was operated ready for use, at 340 nm and using "time scan" mode.

2. The reaction was done in cuvettes. Total volume of the reaction mixture was 0.5 ml.

3. For each 1 ml of the reaction mixture, the following reagents were added in both blank and sample cuvettes:

a. 890 µl of the sodium phosphate buffer

b. 50 µl of 20 mM GSH

c. 50 µl of 20 mM CDNB

4. Both cuvettes were covered with parafilm and mixed well. The blank cuvette was placed at the referent cuvette position and the sample cuvette was placed at the sample cuvette position in the spectrophotometer.

5. 10 µl of Cytosol was pipetted into the sample cuvette to initiate the reaction, whereas the sodium phosphate buffer was prior added into the blank cuvette with the same volume.

6. Immediately after put the sample cuvette into the spectrophotometer, the absorbance was recorded until the reaction reach plateau.

7.3 Calculations

Cytosolic GST activity was calculated based on the rate of an product formed (2,4-dinitrophenylglutathione) and expressed as a function of time and amount of cytosolic protein used in the reaction. The extrinction coefficient of 9.6 $mM^{-1}cm^{-1}$ was used in the calculation as following :

$$\text{Cytosolic GST activity (nmol/min/mg protein)} = \frac{\Delta A \times 60 \times 1000 \times 1000}{\Delta t \times 9.6 \times V \times Y}$$

ΔA : Absorbance difference

Δt : Time difference (second)

V : Volume of cytosol used in the reaction (µl)

Y : Protein concentration of cytosol (mg/ml or µg/µl)

8. Determination of hepatic total glutathione

Hepatic total GSH was determined according to the method of Sedlak and Lindsay (1968).

8.1 Reagents

1. 0.1 M Tris buffer, pH 7.4
2. 0.4 M Tris buffer, pH 8.9
3. 5 mM 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) in absolute methanol
4. 10% w/v Trichloroacetic acid (TCA)

8.2 Procedure

1. A portion of liver was homogenized with 0.1 M Tris buffer, pH 7.4 to make 20 % w/v of liver homogenate.

2. 1 ml of liver homogenate was added with 1 ml of 10% w/v TCA, mixed vigorously by vortex mixer, and centrifuged at about 3,000 rpm for 10 minutes. The obtained supernatant was used for the reaction in next step.

3. The reaction was done in a cuvette and the absorbance was measured at 412 nm by spectrophotometer. The following reagents were added in seperated cuvette :

	Blank	Standard	Sample
a) 0.4 M Tris buffer, pH 8.9	3000 µl	3000 µl	3000 µl
b) pipette	0.4 M Tris buffer, pH 8.9	Std. (GSH) Varied conc.	Supernatant
	200 µl	200 µl	200 µl
c) 5 mM DTNB	100 µl	100 µl	100 µl

4. The cuvettes were covered with parafilm and mixed well. The absorbance was measured spectrophotometrically at 412 nm using the blank to set spectrophotometer to zero before measuring the absorbance of the standards and samples.

8.3 Calculations

A standard curve was constructed between the 5 concentrations of GSH (0.0625, 0.125, 0.25, 0.5, 1 mM) and their absorbance at 412 nm. Total hepatic GSH concentrations were calculated as a function of liver protein or weight of the liver .

9. Western blotting analysis

Microsomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) and proteins were transferred onto a nitrocellulose membrane in the Towbin buffer system (Towbin et al., 1979).

9.1 Reagents

1. Goat anti-rabbit IgG
2. Rabbit peroxidase-antiperoxidase complex (PAP) solution
3. 3,3'-Diaminobenzidine hydrochloride-H_2O_2 solution comprised 30 ml of 50 mM Tris-HCl, pH 7.6, 7.5 mg of 3,3'-diaminobezidine and 7.5 µl of 30 % H_2O_2
4. 3 % Bovine serum albumin (BSA)
5. Phosphate buffer saline (PBS) solution comprised 80 g of NaCl, 11 g of Na_2HPO_4, 2 g of KCl and 2 g of KH_2PO_4 in 1 L of double distilled water. Before using, PBS was diluted with double distilled water (PBS: H_2O=1:9).
6. Solubilization buffer comprised 20 ml of 2 M Tris-HCl buffer, pH 6.8, 120 ml of 50 % glycerol, 6 mg of pyronin Y and 52 ml of double distilled water.

7. Transblotting buffer comprised 14.4 g of glycin, 3.04 g of Tris-base, 200 ml of methanol, adjusted volume to 1 L with double distilled water.

8. 10 % Separation gel comprised 6.67 ml of acrylamide solution, 5 ml of Tris-HCl buffer, pH 8.8, 1 ml of 2 % SDS, 7.13 ml of double distilled water, 0.2 ml of ammonium persalfate and 10 µl of N, N, N',N' tetramethylethylenediamine (TEMD).

9. Stacking gel comprised 1ml of acrylamide, 2.5 ml of Tris-HCl buffer, pH 6.8, 0.5 ml of 2% SDS, 5.83 ml of double distilled water, 0.17 ml of ammonium persulfate and 7 µl of TEMD.

10. CYP2B1/2B2 antibody

9.2 Procedure

1. Separation gel and stacking gel were freshly prepared.
2. Microsomal samples were diluted to 1 mg/ml in distilled water.
3. 10 µl of Solubilization buffer was added to 10 µl of samples.
4. After boiling for 2 minutes, the mixtures were stood at room temparature and 15 µl of the mixture was applied to the stacking gel.
5. A current setting of 10 mA was used for a stacking gel and 20 mA was used for a separating gel. After the electrophoresis was finished, the proteins were transferred to a nitrocellulose sheet using 110 mA of current for 1 hour.
6. Nitrocellulose sheets were washed gently for 5 minutes, twice at room temperature with PBS. The sheets were then shaken gently with a minimal amount of PBS containing 3% BSA for 15 minutes at room temperature.
7. The sheets were washed 6 times with PBS solution (1 minute for each wash). Subsequently, the sheets were shaken with an appropriate antibody to CYP2B1/2, over night at 4^{o}C.
8. The sheets were washed 6 times with PBS as before and shaken with PBS containing 1% goat anti-rabbit IgG solution for 15 minutes at room temperature.

9. After another 6 washes with PBS, the sheets were shaken with a 1/2000 dilution of rabbit PAP in PBS for 15 minutes at room temperature and then washed 6 times with PBS.

10. To each tray containing a nitrocellulose sheet, a fresh solution of 3,3'-diaminobenzidine-H_2O_2 solution was added to develop the stain.

11. After 5-15 minutes of shaking at room temperature, the sheets were washed 5 to 7 times with water. The visualized sheets were dried under filter paper and stored.

9.2 Calculations

The intensities of the immunoblots were measured with an Epson GT-9600 Scanner equipped with NIH Image/Gel Analysis Program adapted for Macintosh computers.

10. Statistics

All quantitative data were presented as mean ± SEM. An one way analysis of variance (ANOVA) and Student-Newman-Keuls test were used for statistical comparisons at significant level of $p < 0.05$.

Chapter IV

Results

Effect of VPU on hepatic microsomal total CYP contents

The dosage of both VPU and VPA used in this study were median effective doses (ED_{50}) protected rat against maximal electroshock convulsions which were 80 mg/kg and 250 mg/kg, respectively (Tantisira, et al.,1997) and the duration of treatment was 7 days.

PEG400, which was used for suspending of VPU, exhibited no effects on total CYP contents, comparing with sterile water. No significant effects of both VPU and VPA on total CYP contents as compared to those in control group 1 & 2 (Table 11).

Table 11 Effect of VPU on hepatic microsomal total CYP contents

Treatment group	Total CYP contents [a,b] (n = 6)
1. Control group 1	0.620 ± 0.031
2. Control group 2	0.639 ± 0.031
3. VPA treatment group	0.534 ± 0.026
4. VPU treatment group	0.621 ± 0.031

[a] Unit expressed as nmol/mg protein

[b] Data were presented as mean ± SEM

Effect of VPU on hepatic microsomal alkoxyresorufin o-dealkylase activities

No effects of PEG400 were observed on the o-dealkylation of all alkoxyresorufins used in this study. Both ethoxy- and methoxyresorufin o-dealkylase (EROD and MROD, respectively) activities, which represented CYP 1A1 and CYP 1A2 activities, demonstrated no change following multiple dose of VPU and VPA as compared to those in control group 1 & 2. Benzyloxy- and pentoxyresorufin o-dealkylase (BROD and PROD, respectively)

activities, which represented CYP 2B1 and CYP 2B2 activities, showed no change after VPA treatment. Significant increases of BROD and PROD activities were observed following VPU treatment as compared to control group 1&2 (Table 12). Corresponding to the activities data, CYP 2B1& 2B2 proteins detected by Western blotting were slightly increased following VPU treatment (Figure 10).

Table 12 Effect of VPU on hepatic microsomal alkoxyresorufin o-dealkylase activities

Treatment group	Alkoxyresorufin o-dealkylase activities [a,b] (n=6)			
	EROD	MROD	BROD	PROD
1. Control group 1	60.67 ± 8.27	22.00 ± 3.76	28.67 ± 4.81	5.00 ± 0.86
2. Control group 2	58.33 ± 8.07	14.33 ± 2.80	27.00 ± 4.73	2.33 ± 1.31
3. VPA treatment group	57.33 ± 10.46	19.33 ± 3.68	28.67 ± 8.89	3.00 ± 1.44
4. VPU treatment group	70.33 ± 7.14	20.00 ± 3.58	68.33 ± 10.20*	9.67 ± 0.95*

[a] Unit expressed as pmol/mg protein/min.

[b] Data were presented as mean ± SEM

* $p < 0.05$; VPU vs control group 1&2

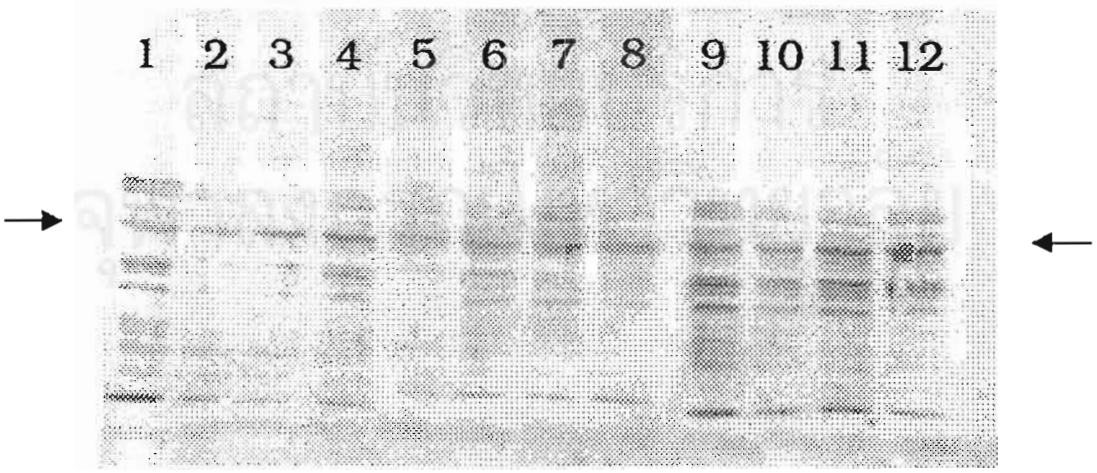

Figure 10 Western blot analysis of liver microsomes isolated from PEG400 treated rats (lane 1-4), VPA treated rats (lane 5-8) and VPU treated rats (lane 9-12); n=4.

Effect of VPU on hepatic microsomal aniline 4-hydroxylase activity

No effect of PEG400 was observed on hepatic microsomal aniline 4-hydroxylase activity, which represented CYP2E1 activity. Likewise, both VPU and VPA exhibited no effect on this enzyme activity as compared to those in control group 1&2 (Table 13).

Table 13 Effect of VPU on hepatic microsomal aniline 4-hydroxylase activity

Treatment group	Aniline 4-hydroxylase activity [a,b] (n=6)
1. Control group 1	0.259 ± 0.017
2. Control group 2	0.295 ± 0.029
3. VPA treatment group	0.210 ± 0.015
4. VPU treatment group	0.272 ± 0.028

[a] Unit expressed as nmol/mg protein/min

[b] Data were presented as mean ± SEM

Effect of VPU on hepatic cytosolic GST activities

No effect of PEG400 was observed on cytosolic GST activities, Likewise, both VPU and VPA treatment exhibited no effect on cytosolic GST activities as compared to those in control group 1&2 (Table 14).

Effect of VPU on hepatic total GSH concentration

PEG400 exhibited no effect on total GSH concentration. Similarly, both VPU and VPA treatment demonstrated no effect on total GSH concentration as compared to those in control group 1&2 (Table 15).

Table 14 Effect of VPU on hepatic cytosolic GST activities

Treatment group	GST activities [a,b] (n=6)
1. Control group 1	919.08 ± 111.84
2. Control group 2	996.00 ± 97.06
3. VPA treatment group	969.20 ± 95.27
4. VPU treatment group	889.24 ± 74.89

[a] Unit expressed as nmol/mg protein/min

[b] Data were presented as mean ± SEM

Table 15 Effect of VPU on hepatic total GSH concentrations

Treatment group	Total GSH concentrations [a] (n=6)	
	nmol/mg protein	μmol/g of liver
1. Control group 1	30.25 ± 2.02	6.62 ± 0.43
2. Control group 2	30.17 ± 0.69	6.52 ± 0.31
3. VPA treatment group	34.77 ± 1.45	7.01 ± 0.20
4. VPU treatment group	34.19 ± 1.78	7.14 ± 0.24

[a] Data were presented as mean ± SEM

Chapter V

Discussion and conclusion

As far as the concern about drug-drug interactions that are commonly associated with antiepileptic drug therapy, an effect of a new candidate compound on hepatic drug-metabolizing enzymes is recommended (Cereghino and Kupferberg, 1993). In this study, we followed the protocol of the Anticonvulsant Screening Project (ASP) of the Antiepileptic Drug Development (ADD) program that suggested several hepatic parameter measurements following 7 days of a compound dosing. Dosage of both VPU and VPA used in this study were median effective doses (ED_{50}) protected rats against maximal electroshock convulsions which were 80 mg/kg and 250 mg/kg, respectively (Tantisira et al., 1997). Due to the simplicity of the method and the specificity of the substrates, we evaluated the effect of VPU on the activities of CYP 1A1 and CYP 1A2 by using ethoxy- and methoxyresorufins as specific substrates (Rodrigues and Prough, 1991) as well as the activities of CYP 2B1 and CYP 2B2 by using benzyloxy- and pentoxyresorufins as specific substrates (Burke et al.,1985). Besides these two major conventional inducible isoforms of CYP, we examined the effect of VPU on the other important inducible isoform, CYP2E1, by using aniline HCl as a specific substrate (Yang et al.,1991).

As an insolubility property of VPU, the compound was suspended in PEG400. The results showed that PEG400 had no effects on any parameters measured in this study similar to those of the sterile water. Therefore, the effects of VPA and VPU on the measured parameters are not contributed from PEG400 which was used as a solvent of both compounds. Our results indicated that VPU and VPA at their ED_{50} for seven consecutive days of treatments did not affect rat hepatic microsomal total CYP contents, the activities of CYP1A1, CYP1A2 and CYP2E1. However, VPU treatment appeared to slightly induce rat CYP2B1 and CYP2B2 activities. Because the induction of CYP 2B1 and CYP 2B2 activities by VPU was much less than that by phenobarbital (data was not shown), the subtle increase did not affect total CYP contents which was detected by a moderate sensitive method. Corresponding to the activity data, CYP 2B proteins detected by Western blotting were also slightly increased. Further study to elucidate the mechanism of induction of these enzymes should be proceeded. Contrast to VPU, VPA did not show an induction effect on

CYP 2B1 and CYP 2B2 in our *ex vivo* study. This was consistent to the *in vivo* study of Rogiers et. al. (1995). In that study, VPA exhibited no effects on CYP2B following i.p. injection of 100 mg/kg VPA, once daily for 10 days. In contrast, their *in vitro* study utilizing rat hepatocyte cell culture, VPA demonstrated an induction effect on CYP 2B1 and CYP 2B2 (Rogiers et al., 1995). The authors proposed that this inconsistent results between the *in vivo* and the *in vitro* study, possibly due to the short-half life of this drug in rats (10-20 min) (Dickinson et al., 1979).

Rat CYP 2B1 is analogous to human CYP 2B6 (Yamano et al.,1989; Soucek and Gut, 1992) which is expressed at very low level in human liver, approximately 0.2% of total CYP (Redic and Dicarlo, 1997). There is an evidence suggesting that CYP 2B6 may be of minimal functional significance in human liver and that variabilities exist across species in the inducibility of CYP 2B (Murray, 1999). Therefore, the induction effect of VPU on rat CYP 2B1 & 2B2 found in this study does not necessarily contribute the significant toxicological implications in humans.

Due to the fact that the extents of induction and inhibition on hepatic enzymes are dose-dependent, various doses of VPU are suggested to be administered to the animals in the further study. Unlike the inhibition of which onset of the interaction is frequently rapid usually occurs within 24 hours, the induction process is time required. The time required for induction depends on both the time to reach steady-state of the inducing agents and the rate of synthesis of new enzymes (Anderson, 1998). In this study, we administered VPU to animals for 7 days, the minimal duration recommended in the protocol of the ASP (Cereghino and Kupferberg, 1993). Therefore, a longer duration of VPU administrations should also be further proceeded. On the basis of the mechanisms of induction and inhibition of CYPs, an *ex vivo* study model utilized in this study is generally a more appropriate study design for an induction than an inhibition study since the compound can be given with repeated doses under the same conditions as truly occurring in an intact animal. In contrast, *in vitro* system is a convenient model to detect an inhibition effect and normally utilized to classify types of inhibitions (Lin and Lu, 1998).

CYP1A1 and CYP1A2, presenting both in humans and rats at low levels, are toxicologically important because they generally convert environmental chemicals (e.g. aromatic and heterocyclic hydrocarbons) and natural compounds (e.g. aflatoxin B_1) to toxic metabolites. Such metabolic activation is thus the most frequent mechanism of transformation of procarcinogen to ultimate carcinogen or carcinogenic intermediates. Therefore, these enzymes normally play a key role in carcinogen activation (Gonzalez,1990; Gonzalez and Gelboin, 1994; Redic and Di Carlo, 1997). Likewise, CYP2E1 also bioactivates a number of chemicals, to yield cytotoxic or carcinogenic intermediates (Gonzalez and Gelboin, 1994; Redic and Di Carlo, 1997). This feature, combined with the ability of CYP2E1 to convert dioxygen into reactive oxygen radicals promoting lipid peroxidation, suggests that CYP2E1 is of major toxicological importance (Oinonen and Lindnos, 1998). Thus, the induction of CYP1A1, CYP1A2 and CYP2E1 isoforms generally increase the risk of toxicological consequences following exposure to environmental chemicals or other xenobiotics which are bioactivated by these enzymes. The present results demonstrated that both VPU and VPA, at a median effective dose for 7 day treatment, did not have any effects on CYP1A1, CYP1A2 and CYP2E1 activities. This should be an advantage feature of both VPU and VPA regarding a potential increase risk of toxicity of other xenobiotics.

There are limitations of extrapolating rat data to predict the potential drug interactions in humans because of the species difference of CYP isoforms existing between rats and humans. This hindrance can be eliminated by using *in vitro* systems utilizing human liver microsomes or recombinant human CYP enzymes. Besides studying the effects of VPU on CYP1A1/2 and CYP2E1 in this study, additional study of the effect of this compound on other isoforms of human CYP which have not been determined is necessary. These can be persued using the *in vitro* systems mentioned above. Screening of inhibition effects of VPU on several human CYP isoforms, such as CYP1A2, CYP2C8/2C9, CYP2C19, CYP3A4 and CYP4A9/4A11 utilizing human liver microsomes, was performed by Kijsanayotin and collaborates in 1997. The results showed that VPU exhibited an inhibition effect on CYP1A2 and CYP2C9.

GST. These inconsistent and inconclusive effects of VPA on GST probably due to the system model used, the dosage and the duration of the compound administered.

In summary, a median effective dose of VPU administered to male Wistar rats for 7 days demonstrated no effects on rat hepatic microsomal total CYP contents, CYP1A1, CYP1A2, CYP2E1, as well as hepatic cytosolic GST and hepatic total GSH. On the other hand, CYP2B1 and CYP2B2 appeared to be slightly induced by VPU treatment. The results from this study provide basic information regarding an induction and an inhibition effects of VPU on rat hepatic drug metabolizing enzymes. Whether or not this information implies potential drug-drug interactions in humans can not be concluded unless additional studies are performed because a species difference of CYP isoforms and their inducibility exists between animals and humans (Redic and Di Carlo, 1997; Lin and Lu, 1998). Therefore, effect of VPU on other isoforms of human CYP, possibly persued by utilizing an *in vitro* model using human liver microsomes was suggested exploring. Moreover, further study to clarify the mechanism of CYP2B1/2B2 induction was suggested. Various doses and extended duration of VPU administrations should be additionally studied.

References

Alber, G.W., Peroutka, S.J. Neurologic disorders : Antiepileptic drugs. In Melmon, K.L., Morrelli, H.F., Hoffman, B.B, and Nierenberg, D.W. (eds.), Melmon and Morrel's Clinical Pharmacology, 3rd ed., pp. 318-323. USA : Arcata Graphics/Halliday, 1992.

Anderson, G. A mechanistic approach to antiepileptic drug interactions. Annuals of Pharmacotherapy 32 (1998) : 554-563.

Billington, D., Osmundsen, H., and Sherratt, H.A. Mechanisms of the metabolic disturbances caused by hypoglycin and pent-4-enoic acid *in vivo* studies. Biochem. Pharmacol. 27 (1978) : 2891-2900.

Bjorge, S.M., and Baillie, T.A. Inhition of medium chain fatty acid β-oxidation *in vitro* by valproic acid and its unsaturated metabolites, 2-n-propyl-4-pentenoic acid. Biochem. Biophys. Res. Commun. 132 (1985) : 245-252.

Bressler, R., Corredor, C., and Brendel, K. Hypoglycin and hypoglycin-like compounds. Pharmacol. Rev. 21 (1969) : 105-130.

Burke, M.D., and Mayer, R.T. Ethoxyresorufin : direct fluorimetric assay of microsomal o-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dipos. 2 (1974) : 583-588.

Burke, M.D., Thompson, S., Elcomebe, C.R., Halpert, J., Haaparanta, T., and Mayer, R.T. Ethoxy-, pentoxy- and bezyloxyphenoxazones and homologues : A series of substrates to distinguish between different induced cytochrome P-450 Biochem. Pharmacol. 34 (1985) : 3337-3345.

Cereghino, J.J., and Kupferberg, H.J. Preclinical testing. In French, J.A., Dichter, M.A., and Leppik, I.E. (eds.). New Antiepileptic Drug Development : Preclinical and Clinical Aspect. pp. 19-30. Elsevier Science Publishers, 1993.

Chance, B., Sies, H., and Boveris, A. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59 (1979) : 527-605.

Chapman, A., keane, P.E., Meldrum, B.S., Simiand, I., and Vernieres, C.I. Mechanism of anticonvulsant action of valproate. Neurobiology 13 (1982) : 315-359.

Cotariu, D. Evans, S., Zaidman, J.L., and Marcus, O. Early changes in hepatic redox homeostasis following treatment with a single dose of valproic acid. Biochem. Pharmacol. 40 (1990) : 589-593.

Davis, R., Peters, D.H. and Mctavish, D. Valproic acid : A reappraisal of its pharmacological properties and clinical efficacy in epilepsy. Drug 47 (1994) : 332-372.

Dickinson, R.G., Harland, R.C., and Ilias, A.M. Disposition of valproic acid in the rat, dose-dependent metabolism, distribution, enterohepatic recirculation and choleretic effect. J Pharmacol. Exp. Ther. 211 (1979) : 583-595.

Distlerath, L.M, Reilly, P.E., Martin M.V., Davis, G.G., Wilkinson, G.R., and Guengerich, F.P. Purification and characterization of human liver cytochrome P450 involved in desbrisoquine 4 hydroxylation and phenacetin O-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism. J. Biol. Chem. 260 (1985) : 9057-9067.

Gascon, M.P., Oestreicher-Kondo, M., and Dayer, P. Comparative effects of imidazole antifungals on liver monooxygenases (abstract). Clin. Pharmacol. Ther. 49 (1991) : 158.

Gerber, N., Dickinson, R.G., Harland, R.C., Lynn, R.K., Houghton, D., Antonias, J.I., and Schimschock, J.C. Reye-like syndrome associated with valproic acid therapy. The Journal of Pediatrics 95 (1979) : 142-144.

Gibson, G.G., and Skett, P. Introduction to drug metabolism. 2nd edition. UK : Page Bros, 1994.

Godin, Y., Heiner, L., Mark, J., and Mandel, P. Effects of di-n-propylacetate, an anticonvulsive compound, on GABA metabolism. Journal of Neurochemistry 16 (1969) : 869-873.

Goldstein, J.A., Linko, P., Luster, M.I., and Sundheimer, D.W. Purification and characterization of a second form of hepatic cytochrome P-448 from rats treated with a pure polychlorinated biphenyl isomer. J. Biol. Chem. 257 : 2702-2707.

Gomez, M.R. Possible teratogenicity of valproic acid. Prediatr. Res. 98 (1981) : 508-509.

Gonzalez, F.J. Molecular gentic of the P-450 superfamily. Pharmc. Ther. 45 (1990) : 1-38.

Gonzalez, F.J. and Gelboin, H.V. Role of human cytochrome P450 in the metabolic acivation of chemical carcinogenesis and toxin. Drug Metab. Rev. 26 (1994) : 165-183.

Gonzalez, F.J. Frenandez-Salquero, P., Lee, S.S.T, Pineau, T., and Ward, J.M. Xenobiotic receptor knockout mice. Toxicology Letters 82/83 (1995) : 117-121.

Gonzalez, F.J., and Lee, Y.H. Constitutive expression of hepatic cytochrome P450 genes. FASEB J. 10 (1996) : 1112-1117.

Gram, L. Hepatic Toxicity of Valproate : Reflections on the Pathogenesis and Proposal for and International Collaborative Registration. In Oxley, J. et. al. (eds.). Antiepilepti Therapy : Chronic Toxicity of Antiepileptic Drug, pp.69-77. New York : Raven Press, 1983.

Granneman, G.R., Wang, S., Kesterson, J.W., and Machinist, J.M. The heapatotoxiciy of Valproic acid and its metabolites in rats. II. Intermediary and valproic acid

metabolism. Hepatology 4 (1984) : 1153-1158.

Guengerich, F.P., Dannan, G.A., Wright, S.T., Martin, M.V., and Kaminsky, L.S. Purification and characterization of liver microsomal cytochrome P-450 : electrophoretic, spectral, catalytic and immunochemical properties and inducibility of eight isozymes isolated from rat treated with phenobarbital or β-naphthoflavone. Biochemsitry 21 (1982) : 6019-6030.

Guengerich, F.P., Muller-Enoch, D., and Blair, I.A. Oxidation of quinidine by human liver cytochrome P-450 . Mol. Pharmacol. 30 (1986) : 287-295.

Guengerich, F.P. Oxidation of 17α-ethylestradiol by human liver cytochrome P450. Mol. Pharmacol. 33 (1988) : 500-508.

Gugler, R., and Unruh, G.E. Clinical pharmacokinetics of valproic acid. Clin. Pharmacokinet. 5 (1980) : 67-83.

Gut, J. Catin, T., Dayer, P., Kronbach, T., Zanger, U., and Mayer, U.A. Debrisoquine / spartein-type polymorphism of drug oxidation : purification and characterization of two functionally different human liver cytochrome P-450 isozymes involved in impaired hydroxylation of the prototype substrate bufuralol. J. Biol. Chem. 261 (1986) : 11734-11743.

Janz, D., and Schmidt, D. Antiepileptic drugs and failure of oral contraceptives. Lancet 1 (1974) : 1113.

Jezequel, A.M., Bonazzi, P., Giuseppe, N., Venturini, C., and Orlandi, F. Early structural and functional changes in liver of rats treated with a single dose of valproic acid. Hepatology 4 (1984) : 1159-1166.

Kassahun, K., Farrell, K., and Abbott, F. Identification and characterization of the glutathione and N-acetylcysteine conjugates of (E) - 2 - propyl -2,4 - pentadienoic acid, a toxic

metabolite of valproic acid, in rats and humans. Drug Metab. Dispos. 19 (1990) : 525-535.

Kerr, B.M., Thummel, K.E., Wurden, C.J. et.al. Human liver carbamazepine metabolism : Role of CYP3A4 and CYP2C8 in 10, 11 – epoxide formation. Biochem. Pharmacol. 47 (1994) : 1969-1979.

Kesterson, J.W. Granneman, G.R., and Machinist, J.M. The hepatotoxicity of valproic acid and its metabolites in rats. I. Toxicologic, biochemical and histopathologic studies. Hepatology 4 (1984) : 1143-1152.

Kijsanayotin, P., Hayama, E., Nanbo, T., Patarapanich, C., Satoh, T., and Shigematsu, A. Preclinical pharmacokinetics evaluation of N-(2-propylpentanoyl) urea : a new anticonvulsant analogue of valproic acid. Presentation in Annual Meeting of American Society of Whole body Autogadiography, Ann Arbor, Michigan, USA, 21-23 September 1997.

Koop, D.R. Oxidative and reductive metabolism by cytochrome P450 2E1. FASEB J. 6 (1992) : 724-730.

Laemmli U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) : 680-685.

Larrey, D., Distlerath, L.M., Dannan, G.A., Wilkinson, G.R., and Guengerich, F.P. Purification and characterization of the rat liver microsomal cytochrome P-450 involved in the 4-hydroxylation of debrisoquine, a prototype for genetic variation in oxidative drug metabolism. Biochemistry 23 (1984) : 2787-2795.

Levy, R. H., Rettenmeier, A. W., Anderson, G., Wilensky, A. J., Friel, P. N., Baillie, T. A., Acheampong, A., Tor, J., Guyot, M., and Loiseau, P. Effects of polytherapy with phenytoin, carbamazepine, and stiripentol on formation of 4-ene-valproate, a hepatotoxic metabolite of valproic acid. Clin. Pharmacol. Ther. 48 (1990) : 225-235.

Levy, R.H. Cytochrome P450 isozymes and antiepileptic drug interaction. Epilepsia 36 (1995) : S8-S13.

Lin, J.H., and Lu, A.Y.H. Inhibition and induction of cytochrome P450 and the clinical implications. Clin. Pharmacokinet. 35 (1998) : 361-390.

Loscher, W. Valproate induced changes in GABA metabolism at the subcellular level. Biochem. Pharmacol. 30 (1981) : 1364-1366.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193 (1951) : 265-275.

Lubet, R.A., Mayer, R.T., Cameron, J.W., Nims, R.W., Burke, M.D., Wolff, T. and Guengerich, F.P. Dealkylation of pentoxyresorufin : A rapid and sensitive assay for measuring induction of cytochrome (s) P-450 by phenobarbital and other xenobiotics in the rat. Arch. Biochem. Biophys. 238 (1985) : 43-48.

Matsugana, T., Nagata, K. Holsztynska, E.J., Lapenson, D.P., Smith A.J., Kato, R., Gelboin, H.V., Waxman, D.J., and Gonzalez, F.J. Gene conversion and differential regulation in the rat P450 IIA gene subfamily : purification, catalytic activity, cDNA and deduced amino acid sequence and regulation of an adult male-specific testosterone15 α- hydroxylase. J. Biol. Chem 263 (1988) : 17995-18002.

Matsunaga, E., Zanger, U.M., Hardwick, J.P., Gelboin, H.V., Meyer, U.A., and Gonzalez, F.J. The *CYP2D* gene subfamily: analysis of the molecular basis of the debrisoquine 4-hydroxylase deficiency in DAN rats. Biochemistry 28 (1989) : 7349-7355.

Mayer, U.A. The molecular basis of genetic polymorphisms of drug metabolism. J. Pharm. Pharmacol. 46 (Suppl 1).(1994) : 409-415.

Meesomboon, R., Chongsutkaweewong, R., Tantisira, M.H., Patarapanich, C., and

Rodesitisuk, P. Investigation of embryotoxicity of N-(2-propylpentanoyl) urea in developing rat embryos in vitro. Final report to the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Mimura, M., Baba, T., Yamazaki, H., Ohmori, S., Inui, Y., Gonzalez, F.Z., Guengerich, F.P., and Shimada, T. Characterization of cytochrome P450 2B6 in human liver microsomes. Drug Metab. Dispos. 21 (1993) : 1048-1056.

Murray, M. Hetnarski, K., and Wilkinson, C. F. Selective inhibitory interactions of alkoxymethylenedioxybenzenes towards monooxygenase activity in rat hepatic microsomes. Xenobiotics 15 (1985) : 369-370.

Murray, M. Mechanisms and significance of inhibitory drug interactions involving cytochrome P450 enzymes. International Journal of Molecular Medicine 3 (1999) : 227-238.

Nau, H., and Loscher, W. Valproic acid and its metabolites : pharmacological and toxicological studies. Epilepsia 25 (Suppl. 1). (1984) : S14-S22.

Nelson, D.R., Koymans, L. Kamataki, T., Stegeman, J.J., Feyerisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., and Nelbert, D.W. P450 superfamily : update on new sequences, gene mapping, acession numbers and nomenclature. Pharmacogenetics 6 (1996) : 1-42.

Oinonen, T., and Lindros, K.O. Zonation of hepatic cytochrome P-450 expresstion and Regulation. Biochem. J. 329 (1998) : 17-35.

Omura, T., and Sato, R. The carbon monoxide-binding pigment of liver microsomes I. Evidence for its hemoprotein nature. J. Biol. Chem. 239 (1964) : 2370-2378.

Patchamart, W. Hepatotoxicity of N-(2-propylpentanoyl) urea in rats. Thesis for the Master degree of Science, Inter-Department of Pharmacology, Graduate School,

Chulalongkorn University, 1996.

Paul, R., Montellano O., and Correia, M.A. Suicidal destruction of cytochrome P-450 during oxidative drug metabolism. Ann. Rev. Pharmacol. Toxicol. 23 (1983) : 481-503.

Pelkonen, O., and Puurunen, J., The effect of cimetidine on *in vitro* and *in vivo* microsomal drug metabolism in the rat. Biochem. Pharmacol. 29 (1980) : 3075-3080.

Perucca, E., and Richens, A. General principles : biotransformation. In Levy, R.H., Mattson, R.H., and Meldrum, B.S. (eds.), Antiepileptic drugs, pp. 31-50. New York : Raven Press, 1995.

Phillips, N.I., and Fowler, L.J. The effects of sodium valproate on γ - aminobutyrate metabolism and behavior in naïve and ethanolamine-O-sulphate pretreated rats and mice. Biochem. Pharmacol 31 (1982) : 2257-2261.

Prickett, K.S., and Baillie, T.A. Metabolism of unsaturated derivatives of valproic acid in rat liver microsomes and destruction of cytochrome P450. Drug Metab. Dispos. 14 (1986) : 221-229.

Rall, T.W., and Schleifer, L.S. Drug effective in the therapy of the epilepsies. In Gilman, A.G., Rall, T.W., Nies, A.S., and Taylor, P (eds.), Goodman&Gilman's the pharmacological basis of therapeutics , pp. 436-462. Singapore : McGraw-Hill, 1992.

Rambeck, B., and Wolf, P. Lamotrigine clinical pharmacokinetics. Clin. Pharmacokinet. 25 (1993) : 433-443.

Redic, S. and Di Carlo, F.J. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. Drug Metab. Rev. 29 (1997) : 413-580.

Reed, D.J. Glutathione : Toxicological Implications. Annu. Rev. Pharmacol. Toxicol. 30

(1990) : 603-631.

Rettenmeier, A.W., Gordon, W.P., Prickett, K.S., Levy, R.H., and Baillie, T.A. Biotransformation and pharmacokinetics in the rhesus monkey of 2-n-propyl-4-pentenoic acid, a toxic metabolite of valproic acid. Drug Metab. Dispos. 14 (1986) : 454-464.

Rettie, A.E., Rettenmeier, A.W., Howald, W.N., Baillie, T.A. Cytochrome P450 catalyzed formation of 4-ene VPA, a toxic metabolite of valproic acid. Science 235 (1987) : 890-893.

Riva, R., Albani, F., Contin, M., and Baruzzi, A. Pharmacokinetic interactions between antiepileptic drugs. Clin. Pharmacokinet. 31 (1996) : 470-493.

Rodrigues, A.D., and Prough, R.A. Induction of cytochrome P450 1A1 and P450 1A2 and measurement of catalytic activities. Method in Enzymology 206 (1991) : 423-431.

Rogiers, V., Vandenberghe, Y., Callaerts, A., Sonck, W., Maes, V., and Vercruysse, A. . the inducing and inhibiting effects of sodium valproate *in vivo* on the biotransformation system of xenobiotics in isolated rat hepatocytes. Xenobiotica 18 (1988) : 665-673.

Rogiers V., Akrawi, M., Vercruysse, A., Phillips, I.R., and Shephard, E.A. Effects of the anticonvulsant, valproate, on the expression of components the cytochrome P-450-mediated monooxygenase system and glutathione S-transferases. Eur. J. Biochem. 231 (1995) : 337-343.

Ryan, D.E., Thomas, P.E., Korzeniowski D., and Levin, W. Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenoabarbital and 3-methylcholanthrene. J. Biol. Chem. 254 (1982) : 1365-1374.

Sadeque, A.J.M., Fisher, M.B., Korzekwa, K.R., Gonzalez, F.J., and Rettie, A.E. Human CYP2C9 and CYP2A6 mediated formation of the heptotoxin 4-ene-valproic acid. J. Pharmacol. Exp. Ther. 283 (1997) : 698-703.

Saisorn, B., Patarapanich, C., and janwitayanuchit, W. Synthesis of monoureide analogues Of valproic acid. Thai J. Pharm. Sci. 16 (1992) : 145-150.

Schenkman, J.B., Remmer, H., and Estabrook, R.W. Spectral studies of drug interactions With hepatic microsomal cytochrome P-450. Mol. Pharmacol. 3 (1967) : 113-123.

Sedlak, J., and Lindsay, R.H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal. Chem. 25 (1968) : 192-205.

Sies, HI, and Moss, KIM. A role of mitochondrial glutathione peroxidases in modulating Mitochondrial oxidations in liver. Eur. J. Biochem. 84 (1978) : 377-383.

Soucek, P. and Gut, I. Cytochromes P-450 in rats; structures, functions, properties and relevant human forms. Xenobiotica 22 (1992) : 83-103.

Tabemer, P.V., Charington, C.B., and Unwin, J.W. Effects of GAD and GABA-T inhibitors on GABA metabolism in vivo. Brain Research Bulletin 5 (Suppl. 2). (1980) : 621-625.

Tanaka, E. Clinical significant pharmacokinetic drug interactions between antiepileptic drugs . J. Clin. Pharm. Ther. 24 (1999) : 87-92.

Tantisira, B., Tantisira, M.H., Patarapanich, C., Sooksawate, T., and Chunngam, T. Preliminary evaluation of anticonvulsant activity of a valproic analogues : N-(2-propylpentanoyl) urea. Res. Comm. Molec. Pathol. Pharmacol. 97 (1997) : 51-62.

Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from Polyacrylamide gels to nitrocellulose sheets : Procedure and some applications.

Proc. Natl. Acad. Sci. USA. 76 (1979) : 4350-4354.

Van der Laan, J. W., De Boer, T., and Bruinvels, J. Di-n-propylacetate and GABA degradation. Preferential inhibition of succinic semialdehyde dehydrogenase and indirect inhibition of GABA-transaminase. Journal of Neurochemistry 32 (1979) : 1769-1780.

Vayer, P., Cash, C.D., and Maitre, M. Is the anticonvulsant mechanism of valproate linked to its interaction with the cerebral γ- hydroxybutyrate system. Trends in Pharmacology and Therapeutics 9 (1998) : 127-129.

Warholm, M., Guthenberg, C., Von Bahr, C., and Mannervik, B. Glutathione transferases from human liver. Method in Enzymology 113 (1985) : 499-504.

Waxman, D.J., Ko, A., and Walsh, C. Regioselectivity and stereoselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital induced rat liver. J. Biol. Chem. 258 (1983) : 11937-11947.

Waxman, D.J. and Azaroff, L. Phenobarbital induction of cytochrome P450 gene expression. Biochem. J. 281 (1992) : 577-592.

Whittle, S. R., and Turner, S. J. Effects of the anticonvulsant sodium valproate on γ-aminobutyrate and aldehyde metabolism in ox brain. Journal of Neurochemistry 31 (1978) : 1453-1459.

Wright, J.M. Drug interactions. In Melmon, K.L., Morrelli, H.F., Hoffman, B.B, and Nierenberg, D.W. (eds.), Melmon and Morrel's Clinical Pharmacology, 3rd ed., pp. 1012-1021. USA : Arcata Graphics/Halliday, 1992.

Yamano, S., Nhamburo, P.T., Aoyama, T., Meyer, U.A., Inaba, T., Kalow, W., Gelboin, H.V., McBride, O.W., and Gonzalez, F.J. cDNA cloning and sequence and cDNA-directed expression of human derived from the CYP2B locus on chromosome 19 and

differential expression of the IIB mRNAs in human liver. Biochemistry 28 (1989) : 7340-7348.

Yang, C.S., Patten, C.J., Ishizaki, H., and Yoo, J.S.H. Induction, purification, and characterization of cytochrome P450IIE. Methods in Enzymology 26 (1991) : 595-603.

Yun, C.H. Shimada, T., and Guengerich, F.P. Purification and characterization of human liver microsomal cytochrome P-450 2A6. Mol. Pharmacol. 40 (1991) : 679-685.

Zaccara, G., Messori, A., and Moroni, F. Clinical pharmacokinetics of valproic acid. Clin. Pharmacokinet. 15 (1988) : 367-389.

Zafrani, E.S., and Berthlot, P. Sodium valproate in the induction of unusual hepatotoxicity. Hepatology 2 (1982) : 648-649.

Zeise, M.L., Lasparow, S., and Zieglgansberger, W. Valproate suppresses N-methyl-D-aspartate evoked, transient depolarizations in the rat neocortex *in vitro*. Brain Research 544 (1991) : 345-348.

Zimmerman, H.J., and Ishak, K.G. Valproate-induced hepatic injury : analysis of 23 fetal cases. Hepatology 2 (1982) : 591-597.

APPENDIX

Table 16 The concentration of protein in individual rat microsomal subfraction*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	55.94	52.43	43.40	53.72
2	36.50	43.17	31.92	41.81
3	35.57	36.06	46.68	52.78
4	39.12	22.26	35.28	44.59
5	47.35	34.24	34.60	39.72
6	34.90	40.68	32.99	39.58

*Unit expressed as μg / μl.

Table 17 The concentration of protein in individual rat cytosolic subfraction*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	28.38	26.27	29.81	28.40
2	36.89	32.36	25.42	34.92
3	28.89	25.40	27.36	33.76
4	24.15	26.01	27.99	31.07
5	29.74	37.66	25.32	29.65
6	27.55	24.36	19.04	25.78

*Unit expressed as μg / μl.

Table 18 The hepatic microsomal total CYP content in individual rat*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	0.604	0.689	0.560	0.522
2	0.632	0.607	0.549	0.670
3	0.566	0.745	0.626	0.689
4	0.516	0.654	0.538	0.559
5	0.721	0.607	0.495	0.705
6	0.681	0.530	0.439	0.582

*Unit expressed as nmol/mg protein.

Table 19 The hepatic microsomal benzyloxyresorufin o-dealkylase activity in individual rat*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	28	36	32	84
2	36	28	34	48
3	38	44	68	84
4	10	20	16	64
5	20	12	12	32
6	40	22	10	98

*Unit expressed as pmol/mg protein/min.

Table 20 The hepatic microsomal ethoxyresorufin o-dealkylase activity in individual rat*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	48	30	44	46
2	68	50	68	80
3	62	84	98	70
4	38	54	32	64
5	52	54	34	64
6	96	78	70	98

*Unit expressed as pmol/mg protein/min.

Table 21 The hepatic microsomal methoxyresorufin o-dealkylase activity in individual rat*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	18	18	28	20
2	26	16	22	34
3	26	24	24	26
4	10	4	20	14
5	16	10	2	10
6	36	14	20	16

*Unit expressed as pmol/mg protein/min.

Table 22 The hepatic microsomal pentoxyresorufin o-dealkylase activity in individual rat*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	6	2	2	8
2	4	0	2	6
3	8	8	10	12
4	4	4	2	10
5	2	0	2	10
6	6	0	0	12

*Unit expressed as pmol/mg protein/min.

Table 23 The hepatic microsomal aniline 4-hydroxylase activity in individual rat*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	0.233	0.237	0.186	0.215
2	0.314	0.248	0.274	0.242
3	0.304	0.375	0.240	0.208
4	0.211	0.394	0.195	0.306
5	0.252	0.259	0.184	0.390
6	0.237	0.229	0.184	0.269

*Unit expressed as nmol/mg protein/min.

Table 24 The hepatic cytosolic glutathione s-transferase activity in individual rats*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	707.51	619.28	1072.05	692.25
2	1222.77	903.40	1139.75	1106.20
3	1158.18	1028.06	690.42	748.39
4	1103.97	1358.32	839.72	1089.88
5	578.14	1022.20	1290.64	947.10
6	743.90	1044.87	782.64	751.64

*Unit expressed as nmol/mg protein/min.

Table 25 The hepatic total glutathione concentration in individual rats*

Rat number	Treatment group			
	Sterile water	PEG400	VPA	VPU
1	24.56	28.60	39.48	24.10
2	31.62	32.22	29.88	38.47
3	23.64	32.09	37.04	31.98
4	32.14	30.44	34.50	32.97
5	35.02	28.56	36.11	37.78
6	34.52	29.12	31.60	36.86

*Unit expressed as nmol/mg protein.

Curriculum Vitae

Miss Teerapom Kiatkosolkul was born in August 22,1971 in Bangkok, Thailand. She graduated with a Bachelor of Science in Pharmacy in 1995 from Faculty of Pharmaceutical Sciences, Mahidol University, Thailand. After graduation, she worked as a medical scientist in the division of Drug Analysis, Department of Medical Sciences, Ministry of Public Health, Thailand, for 2 years.