ผลของ (เอน-ไฮดรอกซีเมทธิล)-2-โพรพิลเพนทามายด์ ต่อเอนไซม์ไซโตโครมพี 450 ในตับหนูขาว

นางสาว นารีรัตน์ รักสุนทร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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EFFECT OF (N-HYDROXYMETHYL)-2-PROPYLPENTAMIDE ON RAT HEPATIC CYTOCHROME P450

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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

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(เอน-ไฮดรอกซีเมทธิล)-2-โพรพิลเพนทามายด์ (เอชพีพี) เป็นอนุพันธ์ตัวหนึ่งของวัลโปรอิค แอซิด (วีพีเอ) ซึ่งมีฤทธิ์ต้านการชัก ได้ถูกนำมาศึกษาผลต่อเอนไซม์ไซโตโครมพี 450 ในตับหนูขาวแบบ *ex vivo* และ *in vitro* การศึกษาใน *ex vivo* เป็นการศึกษาผลของการให้ HPP ในระยะสั้นต่อเอนไซม์ ทำโดยการฉีด เอชพีพีขนาด 100, 200 มิลลิกรัม/กิโลกรัม/วัน หรือ วีพีเอขนาด 250 มิลลิกรัม/กิโลกรัม/วัน แก่หนูขาวเพศผู้พันธุ์ Wistar ทางหน้าท้องเป็นเวลา 7 วัน วันถัดมาจึงเตรียมไมโครโซมจากตับหนู เปรียบเทียบปริมาณไซโตโครมพี 450 รวมและสมรรถนะของไซโตโครมพี 450 ซึ่งเกี่ยวข้องกับการกระตุ้นฤทธิ์ของสารก่อมะเร็งได้แก่ CYP1A1, CYP1A2, CYP2B1/2B2 และ CYP2E1 โดยใช้ปฏิกิริยาของ เอทอกซีรีโซรูฟิน-, เมทอกซีรีโซรูฟิน-, เบนซิลอกซีรีโซรูฟิน- & เพนทอกซีรีโซรูฟิน โอ-ดีอัลคิเลส และอนิลิน-4-ไฮดรอกซีเลส ตามลำดับ ส่วนใน *in vitro* เป็นการศึกษาฤทธิ์ยับยั้งเอนไซม์ โดยศึกษาผลของเอชพีพีที่ความเข้มข้นสุดท้าย 0.1, 1, 10, 100 และ 1000 ไมโครโมลาร์ ต่อเอนไซม์ CYP1A1/1A2, CYP2B1/2B2 และ CYP2E1 ซึ่งถูกเหนี่ยวนำให้เพิ่มขึ้นก่อนโดยใช้ เบต้าแนพโทฟลาโวน ฟีโนบาบิทัล และ เอธานอล ตามลำดับ ผลการศึกษาพบว่า วีพีเอในขนาดที่ใช้ศึกษาไม่มีผลต่อปริมาณไซโตโครมพี 450 รวมและสมรรถนะของ CYP ที่ศึกษา แต่เอชพีพีในขนาด 100 และ 200 มิลลิกรัม/กิโลกรัม/วัน มีผลเหนี่ยวนำสมรรถนะของ CYP1A1 และ CYP2B1/2B2 อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม โดยพบผลเหนี่ยวนำต่อ CYP2B1/2B2 แรงกว่าต่อ CYP1A1 นอกจากนี้ยังพบว่าเอชพีพีในความเข้มข้น 100 และ 1000 ไมโครโมลาร์ มีผลยับยั้งสมรรถนะของ CYP2B1/2B2 ใน *in vitro* อย่างมีนัยสำคัญทางสถิติโดยมีค่า IC_{50} ประมาณ 752 ไมโครโมลาร์ ผลการศึกษานี้ชี้ให้เห็นว่าการที่ เอชพีพีมีฤทธิ์เพิ่ม barbiturate sleeping time ในหนูถีบจักรเมื่อให้เอชพีพีเพียงครั้งเดียวตามที่มีรายงานมาก่อนอาจเป็นผลบางส่วนจากฤทธิ์ยับยั้ง CYP2B1/2B2 ซึ่งทำหน้าที่เปลี่ยนแปลง barbiturate ในหนูถีบจักร ส่วนการที่เอชพีพีในขนาด 100 และ 200 มิลลิกรัม/กิโลกรัม/วัน เมื่อให้ติดต่อกัน 7 วัน กลับมีฤทธิ์เหนี่ยวนำสมรรถนะของ CYP1A1 และ CYP2B1/2B2 ซึ่งไม่พบจากการให้วีพีเอในขนาด 250 มิลลิกรัม/กิโลกรัม/วัน (ประมาณ ED_{50}) อาจเป็นผลจากการเหนี่ยวนำของเอชพีพีเองหรือเมตาบอไลท์ของเอชพีพี อย่างไรก็ตามเนื่องจาก CYP2B1/2B2 ซึ่งพบว่าถูกเหนี่ยวนำมากเป็น isoform ที่ไม่พบในคนจึงควรมีการศึกษาเพิ่มเติมเพื่อหาวิถีการเปลี่ยนแปลงของเอชพีพีโดยเฉพาะ CYP ที่เกี่ยวข้องและผลของเอชพีพีต่อ CYP isoforms ต่าง ๆ ในคนตลอดจนการศึกษาเพิ่มเติมเพื่อประเมินศักยภาพในการก่อให้เกิดปฏิกิริยาระหว่างยาและการเพิ่มความเสี่ยงต่อสารก่อมะเร็งในคนได้ชัดเจนยิ่งขึ้น

ภาควิชา	เภสัชวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา	เภสัชวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....
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The effect of (N-hydroxymethyl)-2-propylpentamide (HPP), one of valproic acid (VPA) derivative possessing anticonvulsant activity, on rat hepatic cytochrome P450 (CYP) was studied in *ex vivo* and *in vitro* systems. In *ex vivo* study, to study the short-term effect of HPP, HPP at a dosage of 100, 200 mg/kg/day or VPA 250 mg/kg/day were given intraperitoneally to male Wistar rats once daily for 7 days. On the day after, rat liver microsomes were prepared and total CYP contents as well as CYP activities were determined using ethoxyresorufin o-dealkylase for CYP1A1, methoxyresorufin o-dealkylase for CYP1A2, benzyloxy- & pentoxyresorufin o-dealkylase for CYP2B1/2B2 and aniline 4-hydroxylase for CYP2E1. In *in vitro* study, inhibitory effects of HPP at final concentrations of 0.1, 1, 10, 100 and 1000 μM on β–napthoflavone-induced CYP1A1/1A2, phenobarbital-induced CYP2B1/2B2 and ethanol-induced CYP2E1 activities were studied. The results showed that VPA at the dose studied did not have any effect on total CYP contents and all CYP activities. However, HPP 100 and 200 mg/kg/day significantly induced CYP1A1 and CYP2B1/2B2 activities. Stronger induction effect was seen on CYP2B1/2B2 than on CYP1A1. In addition, HPP at 100 and 1000 μM significantly inhibited CYP2B1/2B2 activities *in vitro* with IC_{50} of about 752 μM. These results suggested that the inhibitory effect of HPP on CYP2B1/2B2 activities may be, in part, responsible for prolongation of barbiturate sleeping time after single dose administration of HPP. The induction effect of HPP, but not VPA, on CYP1A1 and CYP2B1/2B2 activities after being administered for 7 days may be resulted from direct effect of HPP or its metabolites. Since CYP2B1/2B2 which were found to be strongly induced in rats are not expressed in human, further studies are needed to clarify the metabolic pathways of HPP and the CYPs involved as well as the effect of HPP on human CYPs. Furthermore, *in vivo* studies to verify the potential of drug interaction and carcinogenic risk are also needed.

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Co-advisor's signature……

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I wish to thank Assistant Professor Dr. Chamnan Patarapanich for providing (N-hydroxymethyl)-2-propylpentamide for this study. Thanks are also extended to Associate Professor Nuansri Niwattisaiwong, Associate Professor Dr. Supatra Srichairat, Associate Professor Dr. Nikhom Chaisiri, Associate Professor Dr. Prasan Dhumma-Upakorn and Dr. Chanchai Hosanguan for their kind assistance.

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I also would like to thank all staff members of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for their helps.

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

Contents

List of Tables

Table Page

Table Page

List of Figures

Figure Page

List of Abbreviations

BROD	= benzyloxyresorufin o-dealkylation
BSA	= bovine serum albumin
cm	= centrimetre
CYP	= cytochrome P450
DMSO	= dimethyl sulfoxide
ED_{50}	= median effective dose
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
HPP	= (N-hydroxymethyl)-2-propylpentamide
IC_{50}	= 50% inhibition concentration
i.p.	= intraperitonium
kg	= kilogram
L	= litre
LD_{50}	= median lethal dose
M	= molar
MES	= maximal electroshock
mg	= milligram
mg/kg	= milligram per kilogram body weight
MI	= metabolic intermediate complex
mM	= millimolar
MFO	= mixed-function oxidase
MROD	= methoxyresorufin o-dealkylation

NADP	= nicotinamide adenine dinucleotide phosphate (reduced form)
NADPH	= nicotinamide adenine dinucleotide phosphate
β-NF	= beta-napthoflavone
nmol	= nanomole
PEG400	= polyethylene glycol 400
pmol	= picromole
PROD	= pentoxyresorufin o-dealkylation
PTZ	= pentylenetetrazole
S-N-K	= Student-Newman-Keuls
TCA	= trichloroacetic acid
TD_{50}	= median neurotoxic dose
Tris	= Tris (hydroxymethyl) aminomethane
UGT	= uridine diphosphate glucuronosyltransferase
VPA	= valproic acid
VPM	= valproyl morpholine
VPU	= N-(2-propylpentanoyl) urea
β	= beta
ω	= omega
^{o}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
3-OH-VPA	= 2-propyl-3-hydroxypentanoic acid
4-OH-VPA	= 2-propyl-4-hydroxypentanoic acid
5-OH-VPA	= 2-propyl-5-hydroxypentanoic acid
v/v	= volume by volume
w/v	= weight by volume

Chapter I

Introduction

(N-hydroxymethyl)-2-propylpentamide (HPP), a hydroxymethylamide derivative of valproic acid (VPA), was synthesized by Assistant Professor Dr.Chamnan Patarapanich and colleagues (Lomlim, 1998). Study in mice has shown that HPP possessed a higher anticonvulsant activity and relative safety margin, comparing to its parent compound, VPA (Patarapanich, Tantisira and Tantisira, 1998; Supatchaipisit, 1995).

It has been known that while VPA has a broad spectrum of antiepileptic activity, two serious fatal adverse effects, teratogenicity and hepatotoxicity, have been associated with VPA therapy (Davis, Peter and McTavish, 1994). Furthermore, drug interaction is a common problem of combination antiepileptic drug therapy, including VPA. Regarding the mechanism of drug interaction, the inhibition or induction effects on hepatic enzymes are the common causes. VPA is an inhibitor of human hepatic enzymes, including cytochrome P450 (CYP) 2C9, uridine diphosphate glucuronosyltransferase (UGT) and epoxide hydrolase (Anderson, 1998). Additionally, a study of Rogiers et al. (1995) has shown that VPA is a potent inducer of rat CYP2B1/2B2. In line with these findings, n-(2-propylpentanoyl) urea (VPU), one of VPA derivatives has demonstrated an inhibitory effect on human CYP2C9 and CYP1A1/1A2 *in vitro* (Kijsanayotin et al., 1997), and an induction effect on rat CYP2B1/2B2 (Kaitkosolkul, 1999).

VPA is potentially teratogenic and hepatotoxic to both human and experimental animals (Cotariu and Zaidman, 1991; Dreifuss and Langer, 1984; Rall and Schleifer, 1996). The mechanisms of VPA-induced teratogenicity and hepatotoxicity are still unclear. However, it is postulated that these toxicities might be resulted from VPA itself or its cytochrome P450 metabolite, 2-n-propyl-4-pentanoic acid (4-ene-VPA) (Di Carlo, Bickart and Auer, 1986; Powell-Jackson, Tredger and Williams, 1984). It has been demonstrated that 4-ene-VPA was formed by CYP2B subfamily in rat (Rettie et al., 1987) and CYP2C9, CYP2A6 in human (Sadeque et al., 1997). Coadministered of VPA with phenobarbital (PB), a strong inducer of CYP2B, was associated with increasing

hepatotoxicity in rats (Heinemayer et al., 1985). Study with HPP (75 mg/kg) has shown that HPP significantly prolonged barbiturate sleeping time (Patarapanich et al., 1998; Supatchaipisit, 1995). This may probably result from the direct depressant effect of HPP on central nervous system (CNS) or indirect inhibitory effect on CYP2B which is responsible for barbiturate clearance. Since many adverse effects of VPA, the parent compound of HPP, resulted from inhibitory or induction effects on CYPs involving in its own metabolism and this could be the case for HPP which its metabolic pathways are still unknown. It is worthwhile to explore effect of HPP on rat CYP2B which involve in VPA metabolism. The effect of xenobiotics on CYP activity may indicate not only the tendency of drug interaction but also the toxicity from bioactivated metabolites, particularly mutagenic or carcinogenic metabolites. The known CYP isoforms that are toxicologically important due to their ability to bioactivate promutagens or procarcinogens are CYPs 1A1, 2B1, 2B2, 2E1 in rats and CYPs 1A1, 1A2, 2B7, 2E1, 3A4 in human (Soucek and Gut, 1992).

The aim of this study was primarily to assess the effect of HPP on five isoforms of rat hepatic CYP , including CYPs 1A1, 1A2, 2B1, 2B2 and 2E1 which are responsible for the formation of mutagenic or carcinogenic metabolites. The study was designed to investigate the short-term effect of HPP on rat liver microsomal CYP by using *ex vivo* model, and its inhibition effect by using *in vitro* model.

Hypothesis

HPP demonstrated an induction and/ or an inhibition effect on rat hepatic CYP.

Benefit gained from the study

Results from this study, whether HPP possesses an induction and/ or inhibition effects on rat liver microsomal CYP, particularly CYP isoforms involving in bioactivation of promutagens or procarcinogens, would provide not only preliminary information about the potential risk of carcinogenic toxicity or drug interaction associated with HPP but also beneficial information for the development of other new derivatives of VPA with lower toxicities.

Chapter II

Literature reviews

Drug metabolism

Drugs and other xenobiotics usually have low solubilities in aqueous system and require enzyme biotransformation to metabolites that are more hydrophilic and hence more readily excreted from the body (Murray, 1999). The metabolism of drugs and other xenobiotics normally is classified into two phases, phase I functionalization reactions and phase II conjugative reactions. They occur predominantly in the liver, mainly by CYP system (Gibson and Skett, 1994). The reactions in phase I and Phase II are shown in table 1.

Phase I functionalization reactions proceed a functional group such as –OH, -COOH, $-NH_2$, on the parent compound which is prepared for the phase II reactions. However, products from the phase I reactions can be excreted from the body if they are sufficiently polar. There are many reactions in phase I reactions such as oxidation, reduction, hydrolysis and hydration. The most common reaction is oxidation, especially oxidation involving the microsomal mixed-function oxidase (CYP-dependent).

Phase II conjugative reactions involve coupling the parent compound to an endogenous substitute group such as glucuronic acid, sulfate, glutathione, which further increase the polarity of the parent compound. There are many reactions in phase II reactions. The most common reaction is glucuronidation.

The microsomal mixed-function oxidase (MFO) system is capable of catalyzing a wide variety of oxidation reactions. The MFO system comprises of CYP, NADPH-cytochrome P450 reductase and phospholipid. CYP is the substrate- and oxygen-binding site for the mixed function oxidase reactions, whereas the NADPH-cytochrome P450 reductase transfer electron to CYP. Phospholipid may be required for substrate

binding, electron transferring or facilitating the interaction between CYP and NADPH-cytochrome P450 reductase system (Gibson and Skett, 1994).

Table 1. Reactions classed as phase I or phase II reactions (from Gibson and Skett, 1994).

Phase I reactions	Phase II reactions
Oxidation	Glucuronidation/ glucosidation
-involving cytochrome P450	Sulfation
-other	Methylation
Reduction	Acetylation
Hydrolysis	Amino acid conjugation
Hydration	Glutathione conjugation
Isomerisation	Fatty acid conjugation
Miscellaneous	Condensation

In order to be metabolized by the MFO system, substrate binds to a lipophilic substrate pocket of CYP protein (type I substrates) or directly to the ferric CYP (type II substrates) resulting in a complex of enzyme-substrate. The complex is subsequently reduced by an electron which is derived from NADPH. The electron is transferred via NADPH-CYP reductase to a ferrous state. Molecular oxygen binds to the reduced CYP-substrate complex. An electronic rearrangement is thought to occur, the precise oxidation states of iron and oxygen in these intermediates are not precisely known. A second electron transfer occurs. It is usually transferred from NADPH-CYP reductase or perhaps transferred from cytochrome b_5. The oxygen-oxygen bond is split with the uptake of two protons resulting in the release of water and the generation of a ferric oxene $(Fe\text{-}O)^{3+}$ drug complex. Hydrogen abstraction from the substrate is thought to occur resulting in the production of transient hydroxyl and substrate carbon radical species. Recombination of the hydroxyl and carbon radicals occurs to give the product (ROH). Dissociation of ROH restores the CYP to its ferric state (Gibson and Skett, 1994; Riddick, 1998). The catalytic cycle of CYP is shown in figure 1.

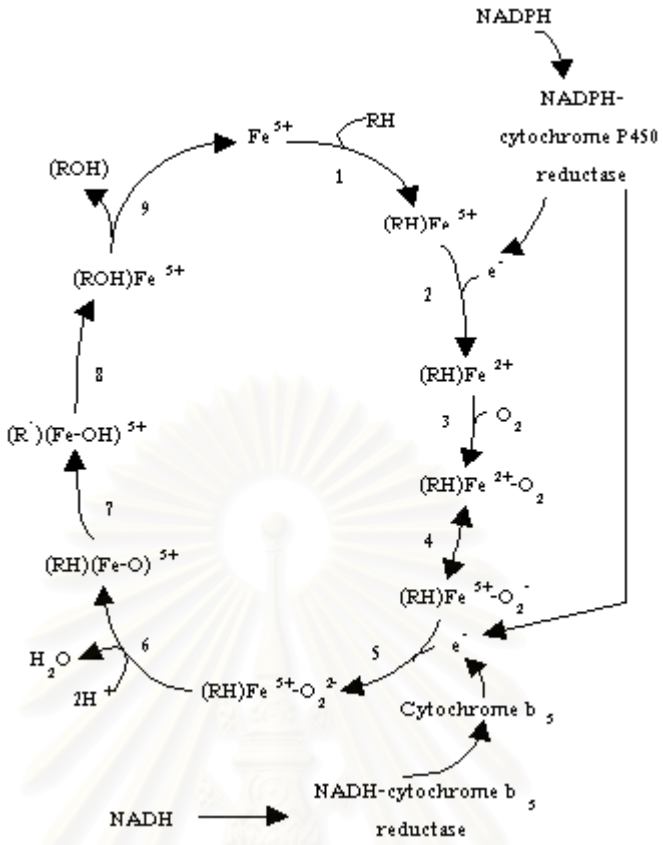


Figure 1. The catalytic cycle of cytochrome P450 (from Riddick, 1998).

CYPs are a superfamily of heme-containing enzymes. CYP consists of two components, an iron protoporphyrin IX heme moiety and a single polypeptide chain or apoprotein (figure 2). The name CYP is derived from the ability of CYP to form a biologically inactive ferrous carbonyl complex with carbon monoxide that has a unique absorption spectrum with a maximum at 450 nm (Gibson and Skett, 1994). Mammalian CYP can be divided into two major classes, mitochondrial CYP and microsomal CYP. The mitochondrial CYPs are found in the mitochondria of the adrenal cortex and are involved in steroid synthesis. They receive electron from the iron sulfur protein adrenodoxin via NADPH-adrenodoxin oxidoreductase. The second class is found primarily in endoplasmic reticulum membrane called microsomal CYP. Electrons are donated to microsomal CYP via the flavoprotein NADPH-CYP reductase or cytochrome b5 (Gonzalez, 1990). CYPs playing important roles in drugs and xenobiotic metabolism are microsomal CYP (Murray, 1999). The superfamily of CYP has been classified as different families in according to the degree of homology of amino acid sequence in

their protein structure. The CYP superfamily is subdivided into families, subfamilies and individual CYP. Members in the same family generally have more than 40% amino acid sequence identity. Mammalian members of the same subfamily have a greater than 55% amino acid sequence identity (Schenkman, 2000). Families are denoted by the first Arabic numbers, e.g. CYP1, CYP2 and subfamilies are denoted by capital letters, e.g. CYP1A, CYP2B. Individual CYPs are denoted by the last Arabic numbers, e.g. CYP1A1, CYP1A2 (Gibson and Skett, 1994). Orthologous gene are those that have counterparts in different species, that is, that have been derived from a common ancestral gene before the species diverged and often exhibit the same catalytic activities. These are given the same designation such as a human ortholog of rat CYP1A1 also called CYP1A1. Sequence homology between rat and human CYPs is shown in table 2. However, orthologous CYP from different animal species may differ in catalytic activities (Soucek and Gut, 1992).

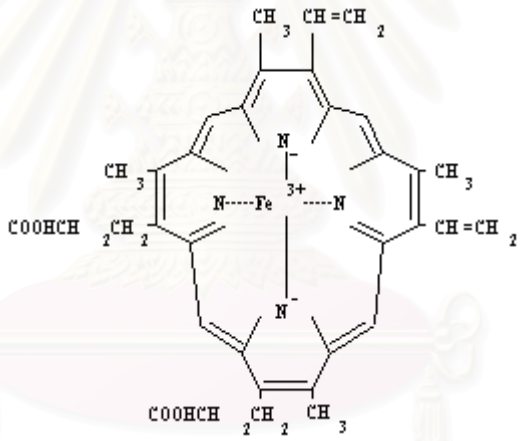


Figure 2. Structure of ferric protoporphyrin IX, the prosthetic group of cytochrome P450 (Gibson and Skett, 1994).

Table 2. Sequence homology between rat and human CYPs (from Soucek and Gut, 1992).

P-450 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]
CYP2C6	CYP2C10	(75)
CYP2C11	CYP2C9	80(77)
CYP2C12	———	—[b]
CYP2C13	CYP2C8	74(68)
CYP2D1	CYP2D6	(71)
CYP2E1	CYP2E1	75(78)
CYP3A1	CYP3A3	(78)
———	CYP3A4	(73)
CYP3A2	CYP3A5	(71)
	CYP3A7	(65)
CYP3A9	———	—[b]
CYP4A1	CYP4A9	—[b]
CYP4A3	———	—[b]
CYP11A1	CYP11A1	79(76)

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

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

CYPs families 1, 2 and 3 play a major role in drug and xenobiotic metabolism. These three families account for about 70% of total CYPs in the human liver. While CYP4 family involves in fatty acid and prostaglandins metabolism (Gonzalez, 1990). Furthermore, some of CYP isoforms play a role in the bioactivation of xenobiotics to mutagens or carcinogens (Denision and Whiflock, 1995; Gonzalez and Lee, 1996; Watkins, 1990). These isoforms including rat CYPs 1A1, 2B1, 2B2, 2E1 as well as human CYPs 1A1, 1A2, 2B7, 2E1 and 3A4 (Soucek and Gut, 1992). An example of rat and human CYPs which can activate some of potential carcinogens/ mutagens was shown in table 3.

CYP1A subfamily includes CYP isoforms that have the capacity to activate a range of environmental procarcinogens. In human and other mammalian species, the CYP1A subfamily appears to comprise of just two members, CYP1A1 and CYP1A2 (Miners and McKinnon, 2000). CYP1A1 and CYP1A2 have been isolated from rat, hamster, rabbit, dog, monkey and human (Nelson et al., 1993). CYP1A2 is constitutively expressed in the liver, whereas CYP1A1 is expressed at very low level in both liver and extrahepatic tissue but highly inducible (Gonzalez, 1990). CYP1A1 is active toward carcinogenic polycyclic aromatic hydrocarbon (PAH), while CYP1A2 is primary responsible for metabolic activation of aflatoxin B1 and a number of arylamines (Gonzalez and Gelboin, 1994).

CYP 2B family has been extensively studied in rat because it can be induced by phenobarbital (Gonzalez, 1990). CYP2B1/2B2 are highly similar in nucleotide sequence (97% amino sequence similarity). CYP2B2 is constitutively expressed in rat liver, while CYP2B1 is constitutively expressed in lung and testis (Soucket and Gut, 1992). They have similar substrate specificities. However, CYP2B1 has two-to-ten fold higher level of activity than CYP2B2 when the enzymes are purified and reconstituted, depending on the substrate (Gonzalez, 1990). For certain analogues of resorufin, CYP2B1 displayed up to a 100-fold higher activity relative to CYP2B2 (Gonzalez, 1989). CYP2B1/2B2 have an important role in the metabolism of drugs and compounds

(Waxman and Azoroff, 1992). They are also responsible for metabolic activation of carcinogens or mutagens (Soucket and Gut, 1992).

CYP 2E1 is constitutively expressed primarily in the liver. It is also expressed in extrahepatic tissue such as lung and kidney (Gonzalez and Lee, 1996). Because of its responsibility for the metabolic activation of some drugs, organic solvents, small molecular-weight compounds and environmental procarcinogens to cytotoxic and/or carcinogenic metabolites, CYP2E1 is clinically and toxicologically important (Tanaka, Terada and Misawa, 2000). Furthermore, CYP2E1 possibly plays a role in the formation of reactive oxygen intermediates such as hydrogen peroxide, superoxide, and hydroxyl radicals during substrate oxidation. These reactive oxygen intermediates lead to lipid peroxidation and cytotoxicity (Lieber, 1997).

Each CYP isoform differs from others in amino acid sequence to give a unique enzyme active site for substrates, inducers, inhibitors and specificity of the reactions that they catalyze. It also possesses a broad spectrum of substrates catalytic activities (table 4). Furthermore, a single CYP can catalyze several different types of reactions at different locations on the same drug (Watkin, 1990). Different forms of CYP may have overlapping substrate specificities (table 4), but in the same substrate they act at different rates (Rang, Dale and Ritter, 1999). The activities of CYPs are induced or inhibited by a wide variety of compounds called an inducer or an inhibitor, respectively (table 5). A change of enzyme activity either increase or decrease can result in a change of pharmacological response or toxicological response. Specific substrates and inhibitors of CYPs are used as experimental tools to examine metabolism through these pathways and thereby add to the ability to predict potential drug interactions (Ring and Wrighton, 2000).

Drug interaction exists when two or more drugs are co-administered. Mechanism of drug interactions is divided into two types, pharmacodynamic and pharmacokinetic interactions. Pharmacodynamic interactions occur when a drug either enhances or diminishes the efficacy or toxicity of another drug without affecting drug

concentration. Pharmacokinetic interactions occur when a drug alters the concentration of a second drug by affecting absorption, distribution, metabolism and/or excretion. The most frequent and important interactions involve alterations in metabolism, particularly hepatic enzyme induction and inhibition (Cloyd and Remmel, 2000). The clinical significance of drug interaction will depend on the magnitude of the change in the concentration of parent drugs or metabolites at the site of pharmacological action and the therapeutic index of the drug (Lin and Lu, 1998).

Enzyme induction is a protective mechanism by increasing enzyme activity when receiving enzyme inducer. Although, enzyme induction generally reduces the pharmacological response, it can enhance production of toxic metabolites (Lin and lu, 1998). Induction response is dose-dependent and reversible (Mayer, 1996). Inducers can take several days or longer to exert their effects (US. FDA, 1998). Mechanism of CYP induction may arise as a consequence of an increase in gene transcription, decrease rate of protein degradation or mRNA stabilization, or combination of these process (Lin and Lu, 1998).

Enzyme inhibition is reduced enzyme activity. This process generally exerts its effects more rapidly than induction effects (US. FDA, 1998) and usually begins with the first dose of inhibitor (Tanaka, 1998). Mechanism of CYP inhibition can be divided into three categories: reversible inhibition, quasi-irreversible inhibition and irreversible inhibition (Lin and Lu, 1998). The reversible inhibition is resulted from competitive inhibition between inhibitor and substrate at the same binding site of CYP. The effect of this inhibition will be dissipated after discontinuing the inhibitor. The quasi-irreversible inhibition occurs when a reactive metabolite forms stable complex with prosthetic heme of CYP. The stable complex is called metabolic intermediate (MI) complex. The MI complex can be reversed and the catalytic activity of CYP can be restored by incubating *in vitro* with lipophilic compounds that can displace the inhibitor from the active site. However, synthesis of *de novo* enzyme is required to restore CYP activity in *in vivo*. The irreversible inhibition occurs when a reactive metabolite binds irreversibly to protein or the prosthetic heme of CYP or a combination of both resulting in irreversible

inactivation of CYP prior its release from the active site. This process called mechanism-based inhibition or suicide inhibition (Lin and Lu, 1998).

Table 3. Role of rat and human CYPs in the activation of potential carcinogen/mutagens (modified from Gonzalez and Gelboin, 1994; Guengerich, 1993; Soucek and Gut, 1992).

CYP	Potential carcinogens/mutagens	
	Rat	Human
1A1	Aflatoxin B1	Benzo(a)pyrene
	Benzo(a)pyrene	Dimethylbenz(a)anthrancene
	7,12-Dimethylbenzo(a)pyrene	6-Nitrochrysene
	2-Naphthylamine	
1A2		2-Acetylfluorene
		Aflatoxin B1
		4-Aminobiphenyl
		2-Naphthylamine
2B1	2-Acetylfluorene	
	Aflatoxin B1	
	Benzo(a)pyrene	
	3-Methylcholanthrene	
2B2	4,4'-(bis) Methylene chloroaniline	
2E1	N-N'-Nitrosodimethylamine	Acrylonitrile
	N-Nitroso-N-diethylamine	Benzene
		Carbon tetrachloride
		Chloroform
		N-Nitrosodiethylamine
		N-Nitrosodimethylamine
3A4		Aflatoxin B1
		Aflatoxin G1
		Benzo(a)pyrene
		Sterigmatocystin

Table 4. Participation of CYP in drug metabolism (modified from Guengerich, 1995; Rendic and Di Carlo, 1997; Tanaka, 1998).

CYP	Participation in drug metabolism (%)	Substrate
1A1	2.5	(R)-Warfarin
1A2	8.2	Acetaminophen
		Antipyrine
		Caffeine
		Clomipramine
		Clozapine
		Haloperidol
		Ondansetron
		Phenacetin
		Theophylline
		(R)-Warfarin
2B6	3.4	Cyclophosphamide
		Testosterone
2C8,9	15.8	Diclofenac
		Hexobarbital
		Phenytoin
		Tolbutamide
		(S)-Warfarin
2E1	4.7	Acetaminophen
		Chlorzoxazone
		Dapsone
		Enflurane
		Ethanol
		Halothane

Table 5. Common inducers and inhibitors of CYPs (modifies from Di Carlo and Rendic, 1997; Lieber, 1997; Murray and Reidy, 1990).

CYP	Inducer		Inhibitor	
	Rat	Human	Rat	Human
1A1	ACLR	ACLR	α-NF	Isosafrole
	3-MC	Benzo(a)pyrene		Propofol
	β-NF	Cigarette smoke		Quercetin
	TCDD	β-NF		
		TCDD		
1A2	Isosafrole	Benzo(a)pyrene		Cimetidine
	3-MC	Cigarette smoke		Ciprofoxacin
	β-NF	3-MC		Furafylline
	TCDD	β-NF		Norfloxacin
		TCDD		
2B1	ACLR		Orphenadrine	
	PB		Secobarbital	
			Diphenhydramine	
2B2	PB			
2E1	Acetone	Acetone	Diallyl sulfide	Disulfiram
	DMSO	Benzene	Disulfiram	4-Methylpyrazole
	Ethanol	Clofibrate		
	Ether	DMSO		
		Ethanol		
		Isoniazid		
		Pyrazole		
		Toluene		

Abbreviation: ACLR, aroclor 1254; DMSO, dimethyl sulfoxide; 3-MC, 3-methylcholanthrene; β-NF, β-napthoflavone; α-NF, α-napthoflavone; PB, phenobarbital; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Valproic acid

Valproic acid (2-propylpentanoic acid; VPA) was synthesized by Burton in 1882 and it was approved for use as antiepileptic drugs in the United State in 1978 (Rowan, 1997). Chemical structure of VPA differs from other antiepileptic drugs as it is a branched-chain fatty acid as shown in figure 3 (Davis et al., 1994).

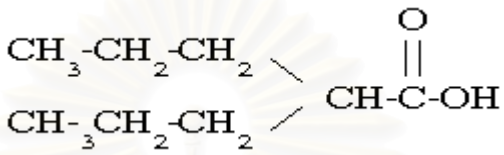


Figure 6. Chemical structure of VPA

Pharmacological effects

VPA has a broad spectrum of antiepileptic activity in generalized seizure as well as partial seizure. It is more effective for the treatment of generalized seizure than partial seizure (Davis et al., 1994). In generalized seizure, the efficiency of VPA was not significantly different from carbamazepine or phenytoin (Pellock, 2000).

Mechanism of action

The mechanism of action of VPA is not clarified yet. It has been suggested that VPA had multiple mechanism (Davis et al., 1994). It has been shown to increase GABA level in brain by enhancement of glutamic acid decarboxylase, a major enzyme in GABA synthesis, or inhibition of GABA transminase/succinic semialdehyde dehydrogenase, an enzyme used in GABA degradation (Davis et al., 1994; Johnston and slater, 1982). There are also evidences that VPA appears to reduce excitatory amino acids such as aspartate, glutamate or gamma-hydroxybutyrate. In addition, VPA appears to reduce sustained high-frequency repetitive firing of sodium and potassium conductance (Davis et al., 1994; Rowan, 1997).

Pharmacokinetics

VPA is rapidly and nearly completely absorbed from the gastrointestinal tract. It is distributed mainly in circulation and extracellular fluid with an apparent volume of distribution of 0.1-0.4 L/kg, due to high plasma protein binding. The drug is about 90% plasma protein bound, mainly albumin. The elimination half-life of VPA range from 9 to 16 hours (Davis et al., 1994). VPA is primarily metabolized in the liver and excreted as the glucuronide conjugation (Bruni, 1996). There are many several metabolic pathways of VPA, including glucuronidation, β-oxidation, ω-, ω1-, and ω2– oxidation and dehydrogenation (Davis et al., 1994; Siemes et al., 1993). The major pathways appear to be glucuronidation and mitochondrial β-oxidation with minor CYP-dependent metabolism, including CYPs 2C9, 2C19 and 2A6 in human (Anderson, 1998). β-Oxidation results in 2-propyl-2-pentanoic acid (2-ene-VPA) and 2-propyl-3-keto-pentanoic acid (3-keto-VPA) which are both major metabolites in human, dog, rat and mouse (Nau and Loscher, 1984). 2-Ene-VPA may contribute significantly to anticonvulsant effect in long-term VPA therapy (Nau and Loscher, 1984). Furthermore, Siemes et al.(1993) suggested that VPA-induced hepatotoxicity might be associated with impairment of VPA β-oxidation and increase of metabolites of ω- and ω1-hydroxylation and dehydrogenation reaction. The ω-, ω1- and ω2– oxidation are CYP-dependent resulting in 5-OH VPA, 4-OH VPA and 3-OH VPA, respectively (Prickelt and Baillie, 1984). 4-Ene-VPA and 2,4-diene-VPA are formed by dehydrogenation reaction. It has been evidenced that VPA was metabolized to 4-ene-VPA by CYP2B subfamily in rat (Rettie et al., 1987) and CYP2C9, CYP2A6 in human (Sadeque et al., 1997). Metabolic pathway of VPA was shown in figure 4.

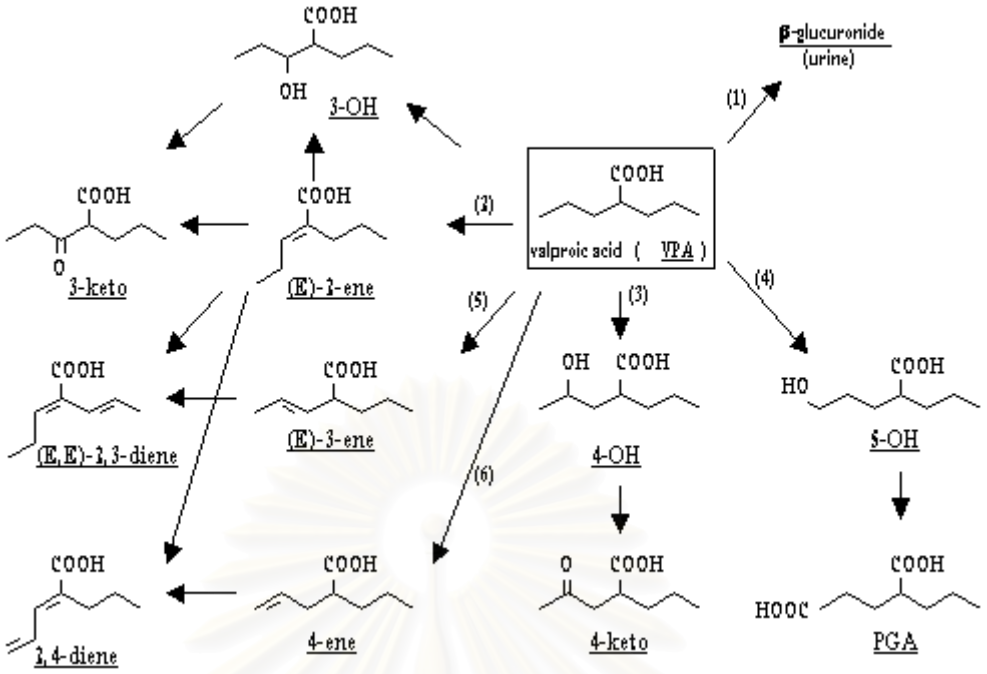


Figure 4. Metabolic pathways of VPA. (1) Conjugation of VPA with glucuronic acid. (2) Mitochondrial β-oxidation (3) ω1-Hydroxylation (4) ω-Hydroxylation (5) Two important "non-classical" products of mitochondrial β-oxidation, 3-ene-VPA and the major diene-derivatives (E)-2,3'-diene-VPA (6) The main toxic metabolites 4-ene-VPA and 2,4'-diene-VPA are formed by dehydrogenation reaction (Siemes et al., 1993).

Adverse effects

The most common adverse effects of VPA are gastrointestinal disturbance, neurological side effects and weight gain. The serious adverse effects of VPA are hepatotoxicity and teratogenicity (Davis et al., 1994).

The characteristic tissue lesion in VPA-induced hepatotoxicity showed microvesicular steatosis without immunologically mediated hepatotoxicity reactions (Rall and Schleifer, 1996). Fatal hepatotoxicity occurs more frequently in children under the age of 2 years with polytherapy (Bryant and Dreifuss, 1996; Dreifuss et al., 1989). The overall incidence of hepatic facility was approximately 1/ 37,000 in monotherapy use (Bruni, 1996). It has been suggested that valproate may directly interfere with mitochondrial function and fatty β-oxidation (Seimes et al., 1993) or result from its

metabolite, 4-ene-VPA (Powell-Jackson et al., 1984). 4-Ene-VPA is most likely associated with hepatotoxicity because its structure is similar to 4-pentenoic acid as well as a toxic metabolite of hypoglycine A (Nau and Loscher, 1984). A study of Kingsley et al.(1983) reported that 4-ene-VPA was shown to be the most toxic metabolite of VPA in rat hepatocytes culture. It has been suggested that 4-ene-VPA was a potent inhibitor of medium-chain fatty acid β-oxidation *in vitro* of endogenous fatty acid, a property which may be of key toxicological importance of VPA-induced hepatotoxicity (Bjorge and Baillie, 1985).

The use of valproate in the first trimester of pregnancy has been reported to increase the risk of neural-tube defects in the offspring, particularly spina bifida aperta (Bruni, 1996). In a prospective cohort study of Omtzigt et al.(1992) reported that the prevalence of spina bifida aperta in fetuses of maternal use of VPA was 5.4%. It has been suggested that VPA or 4-ene-VPA (Di Carlo et al., 1986) may interfere with embryonic folate metabolism (Wegner and Nau, 1992).

Drug interactions

In human, VPA is an inhibitor of liver enzymes, including CYP2C9, UGT and epoxide hydrolase (Anderson, 1998). The effect of VPA on hepatic enzymes is a cause of drug interactions. VPA primarily inhibits drug metabolized by CYP2C9 subfamily such as phenytoin and phenobarbital resulting in a decrease in the rate of metabolism of these drugs (Tanaka, 1999). On the other hand, metabolism of VPA is affected from other drugs. A study of Wagner et al.(1994) reported that felbamate significantly decreased VPA clearance. Felbamate might inhibit CYP2C19 and β–oxidation which were responsible for VPA metabolism (Anderson, 1998). In addition, co-administration of VPA and two or more enzyme-inducing antiepileptic drugs (carbamazepine, phenytoin, phenobarbital or primidone) resulted in a 10% increase in VPA clearance (Yukawa et al., 1997). This could be resulted from induction effect on CYP2C subfamily which was responsible for VPA metabolism (Anderson, 1998). In rats, prolonged exposure of valproate results in the drug inducing its own metabolism. A study of Rogiers et al.(1995) demonstrated that VPA was a potent inducer of CYP2B1/2B2 *in vitro* rat

hepatocyte cultures as well as *in vivo* when it is administrated to rats by continuous for two weeks.

(N-hydroxymethyl)-2-propylpentamide (HPP)

HPP is one of VPA derivatives which was synthesizes by Assistant Professor Dr.Chamnan Patarapanich and colleagues (Lomlim, 1998). The HPP is hydroxymethylamide of VPA. Chemical structure of HPP is shown in figure 5.

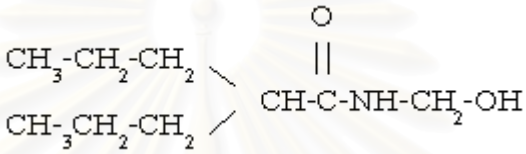


Figure 5. Chemical structure of HPP

A study of anticonvulsant activity of HPP in mice revealed that HPP possessed a higher anticonvulsant activity either against maximal electroshock (MES) or pentylenetetrazol (PTZ) test, comparing with its parent compound, VPA. In MES, the median effective dose (ED_{50}) of intraperitoneally administered HPP and VPA were 77 and 214 mg/kg of body weight, respectively, meanwhile in PTZ, they were 35 and 108 mg/kg of body weight. In addition, the relative safety margin of HPP is about three times higher than VPA (Patarapanich et al., 1998; Supatchaipisit, 1995). However, HPP exhibited higher neurotoxicity than VPA (TD_{50} of HPP:VPA = 89:274). Furthermore, a single dose of HPP (75 mg/kg of body weight) significantly prolonged barbiturate sleeping time (Patarapanich et al., 1998; Supatchaipisit, 1995). This probable resulted from the direct depressant effect on CNS or indirect inhibition effect on CYP2B which is responsible for barbiturate clearance. Since HPP is a derivative of VPA whose many clinical significant adverse effects resulted from induction or inhibition effects on CYPs involving in its own metabolism, it is interesting to investigate the effect of HPP on rat CYPs, especially CYP2B which involves in VPA metabolism as well as other CYPs involving in bioactivatation of procarcinogens and promutagens, including CYPs 1A1, 1A2, 2B1, 2B2 and 2E1.

Chapter III

Materials and Methods

Experimental animals

Adult male Wistar rats of initial weight between 250-300 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. The animals were maintained in animal care facility at the Faculty of Pharmaceutical Science, Chulalongkorn University and acclimatized for about 1 week before the experiment. All animals were given free access to food (S.W.T. Ltd., Thailand) and water.

Experimental instruments

1. Autopipets 20, 100, 200, 1000 and 5000 μl (Gilson, France)
2. Centrifuge (Kokusan, Japan)
3. Fluorescence spectrophotometer (Jasco, Japan)
4. Metabolic shaker bath (Heto, Denmark)
5. Potter-Elvehjem homogenizer with teflon pestle and glass homogenizer (Heidolph, Germany)
6. pH meter (Beckman Intruments, USA)
7. Refrigerated superspeed centrifuge (Hitachi, Japan)
8. Refrigerated ultracentrifuge (Beckman, USA)
9. Sonicator (Elma, Germany)
10. Spectrophotometer (Jasco, Japan)
11. Surgical equipments
12. Ultra-low temperature freezer (Forma Scientific Inc., USA)
13. Vortex mixer (Clay adams, USA)
14. Tank of carbon monoxide gas (T.I.G., Thailand)

Experimental chemicals

(N-hydroxymethyl)-2-propylpentamide (HPP) was synthesized by Assistant Professor Dr.Chamnan Patarapanich and collaborates (Lomlim, 1998) at the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

All other chemicals were purchased from the following suppliers:

Acetonitrile was purchased from J.T.Baker, USA.

4-Aminophenol, aniline hydrochloride, benzyloxyresorufin (BR), bovine serum albumin (BSA), cupric sulfate, dimethylsulfoxide (DMSO), ethoxyresorufin (ER), Folin&Ciocalteu's phenol reagent, glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD), methoxyresorufin (MR), β-napthoflavone (β-NF), nicotinamide adenine dinucleotide phosphate (NADP), pentoxyresorufin (PR), potassium phosphate, resorufin, sodium carbonate, sodium citrate, sodium phosphate dibasic anhydrous, Trisma® base and valproic acid were purchased from Sigma, USA.

Ethanol absolute and glycerol were purchased from Carlo Erba, USA.

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

Methanol HPLC grade was purchased from BDH Laboratory Supplies, England.

Phenobarbital sodium (PB) was purchased from May&Baker, England.

Polyethyleneglycol 400 (PEG400) was purchased from T.Chemical Ltd. Partnership, Thailand.

Sodium dithionite was purchased from Fluka Chemic, Japan.

Experimental methods

1. Animal treatment

1.1 For *ex vivo* study

An *ex vivo* study model was used to investigate the effect of HPP in repeated dosage on rat liver cytochrome P450. Rats were randomly assigned into 4 treatment groups. Each treatment group comprised of 6 rats.

1. Control group: rats were given PEG400 (the diluent of VPA and HPP) intraperitoneally, once daily for 7 days.
2. VPA treatment group: rats were given VPA in PEG400 (250 mg/kg/day) intraperitoneally, once daily for 7 days.
3. HPP treatment group1: rats were given HPP suspended in PEG400 (100 mg/kg/day) intraperitoneally, once daily for 7 days.

4. HPP treatment group 2: rats were given HPP suspended in PEG400 (200 mg/kg/day) intraperitoneally, once daily for 7 days.

One animal from each treatment group was treated simultaneously during each experimental period. On the day after the seven days of compound administrations, rats were sacrificed for preparation of liver microsomes. The microsomes prepared from these 4 treatment groups were used for studying the effects of HPP and VPA on total CYP contents and CYP isoforms catalytic activities (CYP1A1, CYP1A2, CYP2B1/2B2 and CYP2E1).

1.2 For *in vitro* study

An *in vitro* study model was used to investigate the inhibitory effect of HPP on rat liver cytochrome P450. Rats were ramdomly assigned into 3 treatment groups. Each treatment groups comprised of four rats and were pretreated with different inducers to induce individaul CYP isoforms of interest.

1. β-NF pretreatment group: For an induction of CYP1A1 and CYP1A2, rats were given β-NF suspended in corn oil (80 mg/kg/day) intraperitoneally, once daily for 2 days. Four rats were given corn oil in the same manner.

The β-NF-induced microsomes prepared from these animals were used for *in vitro* inhibition studies of HPP on CYP1A1 and CYP1A2.

2. PB pretreatment group: For an induction of CYP2B1 and CYP2B2, rats were given phenobarbital sodium (80 mg/kg/day) intraperitoneally, once daily for 3 days. Four rats were given sterile water in the same manner.

The PB-induced microsomes prepared from these animals were used for *in vitro* inhibition studies of HPP on CYP2B1 and CYP2B2.

3. Ethanol pretreatment group: For an induction of CYP2E1, short-term heavy ethanol treatment was used according to the method of Hu, Ingelman-Sundberg and Lindros (1995) with some modification. At first, tolerance to ethanol was initiated by addition of ethanol to the drinking water to rats for 6 days by stepwise increase from 3 to 7% (3, 4, 5, 6, 7 and 7% respectively). On day seventh to ninth, rats were given orally a 20% (v/v) ethanol solution three times daily at 7 a.m., 1 p.m. and 7 p.m. The total daily dose of ethanol

was increased from 8, 10 and 12 g/kg/day. During these 3 days, all rats given ethanol were maintained on the 7% ethanol drinking water. On day tenth, they were given one third of the last dose at 7 a.m., 2 hours before the rat liver microsomes were prepared. Four rats were given water in the same manner.

The ethanol-induced microsomes prepared from these animals were used for *in vitro* inhibition studies of HPP on CYP2E1.

2. Preparation of liver microsomes

2.1 Reagents

1. 0.9% w/v NaCl
2. Phosphate buffer, pH 7.4

One liter of phosphate buffer, pH 7.4 comprised of KH_2PO_4 1.78 g, Na_2HPO_4 9.55 g and KCl 11.50 g. The solution was adjusted to pH 7.4 with NaOH or HCl.

3. Phosphate buffer, pH 7.4 containing 20% v/v glycerol

2.2 Procedure

1. Under light ether anesthesia, the rat's abdominal cavity was opened. The liver was perfused *in situ* via the portal vein with ice-cold 0.9% w/v NaCl until the entire organ became pale.
2. The liver was taken out, washed out the blood with ice-cold 0.9% w/v NaCl and blotted dry with gauze.
3. The whole liver were weighted, cut into pieces and homogenized with 3 volume of phosphate buffer, pH 7.4.
4. 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.
5. The supernatant was transferred into ultracentrifuge tubes and centrifuged at 100,000 g for 60 minutes at 4 °C, using refrigerated ultracentrifuge.
6. The pellets (microsomal fractions) were resuspended with phosphate buffer, pH 7.4 containing 20% glycerol about 3-5 ml. The microsomal suspensions

were kept in microtubes and stored at -80 °C until the time of enzyme activity assays.

3. Determination of protein concentrations

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

3.1 Reagents

1. 1 mg/ml Bovine serum albumin (BSA) in 0.5 M sodium hydroxide
2. 1% w/v Cupric sulfate
3. Folin & Ciocalteu's phenol reagent
4. 2% w/v Sodium carbonate
5. 2% w/v Sodium citrate
6. 0.5 M Sodium hydroxide
7. Working protein reagent. This reagent comprised of sodium carbonate, sodium hydroxide, sodium citrate and cupric sulfate solution in a 100/10/1/1 ratio, respectively. The solution was freshly prepared before using.

3.2 Procedure

All each unknown and standard samples were determined in duplicate.

1. For each standard protein tubes, the following reagents were added.

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

Each tube was mixed thoroughly after addition of the reagents.

2. For each unknown sample tubes, 490 μl of 0.5 M NaOH and 10 μl of microsomal sample were added and mixed thoroughly.
3. After 6.5 ml of working protein reagent was added to each tube in steps 1. and 2. and mixed thoroughly, the tubes were allowed to stand at room temperature for 10 minutes.
4. Folin & Ciocalteu's phenol reagent 200 μl was then added to each tube, mixed immediately for a minimum of 30 seconds and stood at room temperature for a minimum of 30 minutes.
5. The absorbances of the solution were measured by spectrophotometer at 500 nm using the 0 μg standard as blank.

3.3 Calculations

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 absorbance against the standard curve. The protein concentration (mg/ml or μg/μl) in each unknown sample was obtained by dividing its amount of protein with the volume of microsomal sample used in the reaction.

4. Spectral determination of total CYP contents

Total CYP contents in microsomal samples were determined spectrophotometrically according to the method of Omura and Sato, 1964.

4.1 Reagents

1. Carbon monoxide
2. Solid sodium dithionite
3. 0.1 M Tris buffer, pH 7.4 containing 20% v/v glycerol

4.2 Procedure

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

2. Of the total 5 ml diluted samples, 2.5 ml each was placed in the sample and reference cuvettes. A few grains of solid sodium dithionite was added to both cuvettes with gentle stirring, and put into a spectrophotometer, adjusted to zero and corrected to a baseline between 400 nm to 500 nm.
3. 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

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 total CYP contents were given by:

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

5. Microsomal incubation for the determination of cytochrome P450 enzyme-substrate activities

5.1 Alkoxyresorufin o-dealkylation assays

The catalytic activities of CYP1A1, CYP1A2, CYP2B1/2B2 were estimated by measuring the rate of o-dealkylation of ethoxyresorufin, methoxyresorufin and benzyloxyresorufin & pentoxyresorufin, respectively, using the method of Burke and Mayer (1974) and Lubet et al. (1985) with some modification.

5.1.1 Reagents

1. 0.1 M Tris buffer, pH 7.4
2. Resorufin and alkoxyresorufins
   a. 10.5 mM Benzyloxyresorufin (BR; MW227)

BR 1.515mg was dissolved with DMSO qs to 10 ml.

b. 20.5 mM Ethoxyresorufin (ER; MW241)

ER 1.205 mg was dissolved with DMSO qs to 10 ml.

c. 30.5 mM Methoxyresorufin (MR; MW227)

MR 1.135 mg was dissolved with DMSO to 10 ml.

d. 40.5 mM Pentoxyresorufin (PR; MW283)

PR 1.415 mg was dissolved with DMSO to 10 ml.

3. NADPH regenerating system

a. 10 μl of 0.1 M NADP, pH 7.4

NADP 0.765 g 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 mmoles of NADP).

b. 10 μl of 0.5 M G6P, pH 7.4

G6P 1.41 g 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 5 mmoles of G6P).

c. 10 μl of 0.3 M $MgCl_2$, pH 7.4

$MgCl_2$ 603.93 mg 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 3 mmoles of $MgCl_2$).

NADPH regenerating system comprised of 0.1 M NDAP, 0.5 M G6P, 0.3 M $MgCl_2$ in a 1/1/1 ratio, respectively. The solution was freshly prepared before using.

4.G6PD, pH7.4

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

5.1.2 Procedure

1. Diluted microsomal samples were prepared by using 100 μM of each microsome diluted with 0.1 M Tris buffer, pH 7.4 qs to 5000 μM.
2. Microsomal incubations were carried out in a final volume of 1.5 ml. Each preincubation mixture contained:
   a. NADPH regenerating system 45 μl
   b. 0.5 mM Alkoxyresorufin 15 μl (BR, ER, MR or PR)
   c. Varied volume of diluted microsomes to obtained 150 μg protein per one incubation mixture.
   d. 0.1 M Tris buffer, pH 7.4 qs to 1485 μl

   Tree tubes were needed for each microsomal sample (one is a sample blank and the others are samples).
3. The incubation mixtures were preincubated at 37 °C in shaking water bath for 2 minutes.
4. The reaction was started by the addition of 15 μl of G6PD (1 unit of G6PD per 1 ml of incubation mixture). For a sample blank, 15 μl of 0.1 M Tris buffer, pH 7.4 was used instead of G6PD.
5. After 5 minutes incubation, the reaction was stopped by 1.5 ml of methanol.
6. The absorbance was measured by fluorescence spectrophotometer using an excitation wavelength of 556 nm. and an emission wavelength of 588 nm.
7. A resorufin standard curve was performed using 8 concentrations of resorufin: 0.025, 0.05, 0.075, 0.1, 0.2, 0.4, 0.5 and 1.0 nmol/ml.

5.1.3 Calculations

The rate of dealkylation which was represented as amount of resorufin formed per minute per mg of protein was calculated by determining the amount of resorufin formed, dividing by 5 minutes, then extrapolating to mg of protein.

5.2 Aniline 4-hydroxylation assay

The catalytic activity of CYP2E1 was estimated by determining the rate of aniline 4-hydroxylation, using the method of Schenkman et al. (1967).

5.2.1 Reagents

1. 10 μM 4-Aminophenol (MW145.6)

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

2. 10 mM Aniline HCl (MW129.6)

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

3. G6PD, pH 7.4

4. 1 M Na_2CO_3

   Anhydrous Na_2CO_3 200 g was dissolved to 2 liters of double distilled water.

5. NADPH regenerating system (as described in 5.1.1).

6. 1% w/v Phenol

   Phenol 20 g and NaOH 40 g were dissolved to two liters of distilled water.

7. 6% w/v Trichloroacetic acid (TCA)

   TCA 60 g was dissolved with 1 liter of double distilled water.

8. 20% w/v TCA

   200 g of TCA was dissolved with 1 liter of double distilled water.

9. 0.1 M Tris buffer, pH 7.4

5.2.2 Procedure

1. Microsomal incubations were carried out in a final volume of 2 ml. Each preincubation mixture contained:

   a. NADPH regenerating system 30 μl

   b. 10 mM Aniline HCl 500 μl

   c. Varied volume of microsomes to obtained 5 mg protein in final mixture.

   d. 0.1 M Tris buffer, pH 7.4 qs to 1980 μl.

2. All tubes were preincubated at 37 °C in shaking water bath for 2 minutes.
3. The reaction was started by the addition of 20 μl of G6PD. For a sample blank, 20 μl of 0.1 M Tris buffer, pH 7.4 was used instead of G6PD.
4. After 30 minutes incubation, the reaction was stopped by 1 ml of ice-cold 20% w/v TCA, and the tubes were kept on ice for 5 minutes.
5. The solution was then centrifuged at 3000 rpm for 5 minutes and 1 ml of the supernatant was transferred to a new tube.
6. After 1 ml of 1% w/v phenol and 1 ml of 1 M $NaCO_3$ were added to each tube and mixed well, the tubes were allowed to stand at room temperature for a minimum of 30 minutes.
7. The absorbance of the solution was measured by spectrophotometer at 630 nm.
8. A standard curve was performed by using 5 concentrations of 4-aminophenol standard solutions (2, 4, 6, 8 and 10 μM), following step 6 as described above.

5.2.3 Calculations

The 4-hydroxylation of aniline was calculated by determining the amount of 4-aminophenol formed (product), dividing by 30 minutes and, then extrapolating to mg of protein.

6. *In vitro* inhibition study

The inhibition effects of HPP on CYP isoform catalytic activities were investigated by performing co-incubation of HPP with marker substrates for each CYP isoforms.

6.1 Procedure

6.1.1 Inhibition studies of HPP on CYP1A1, CYP1A2 and CYP2B1/2B2 activities

1. Four concentrations (0.1, 1, 10 and 100 mM) of HPP-substrate solutions were prepared by dissolving HPP in each of 0.5 mM substrate solutions (BR, ER or MR). The addition of 15 μl of the corresponding substrate or these HPP-substrate solutions in preincubation mixtures will give final concentrations of 0, 0.1, 1, 10, 100 and 1000 μM HPP, respectively, in 1.5 ml incubations.
2. Microsomal incubations were carried out with or without HPP (at final concentrations of 0.1, 1, 10, 100 and 1000 μM) as the method described in 5.1 by using the following selective microsomes:

   β-NF- induced microsomes were used for studying the inhibition effects on CYP1A1 (ethoxyresorufin o-dealkylation activity) and CYP1A2 (methoxyresorufin o-dealkyltion activity).

   PB-induced microsomes were used for studying the inhibition effects on CYP2B1/2B2 (benzyloxyresorufiin o-dealkylation activity).

6.1.2 Inhibition effect of HPP on CYP2E1 activity

1. Since HPP is not water soluble, four concentrations (0.1, 1, 10 and 100 mM) of HPP were prepared by dissolving HPP in acetonitrile. The addition of 20 μl of the acetonitrile or these HPP solutions to preincubation mixtures will give final concentrations of 0, 0.1, 1, 10, 100 and 1000 μM HPP, respectively, in 2.0 ml incubations. The final concentration of acetonitrile in all the incubations was 1% v/v.
2. Inhibition effect of HPP on CYP2E1 (aniline 4-hydroxylation) activity was studied by addition of 20 μl of HPP in acetonitrile (at final concentrations of 0.1, 1, 10, 100 and 1000 μM) or acetonitrile (at final concentration 1% v/v) simultaneously with substrate. Additional buffer control incubation contained the same volume of 0.1 M Tris buffer, pH 7.4. The microsomal

incubation was carried out as the method described in 5.2 by using ethanol-induced microsomes.

7.Data analysis

All numeric data were presented as mean ± SD or % of control activity. A student's *t* test was used for the comparison between two groups, whereas an one way analysis of variance (ANOVA) followed by Student-Newman-Keuls test were used for statistical comparisons among more than two groups. The statistical significant level was $p<0.05$. All the statistical analysis was performed by using SPSS program version 7.5.

For estimation of IC_{50}, the % of inhibition was transformed to probit unit by using transformation table of Fisher and Yates. The linear regression method was used to fit a curve between probit unit and log dose by using Crikcet graph program (Macintosh ® computer). The IC_{50} was calculated from the log dose probit line.

Chapter IV

Results

1. *Ex vivo* study

The effects of seven-days treatment of HPP and VPA on rat hepatic total CYP contents and five CYP isoforms catalytic activities were studied (ethoxyresorufin o-dealkylase for CYP1A1, methoxyresorufin o-dealkylase for CYP1A2, benzyloxy resorufin o-dealkylase and pentoxyresorufin o-dealkylase for CYP2B1/2B2, and aniline 4-hydroxylase for CYP2E1) by using rat liver microsomes.

The results of this study showed that neither VPA treatment (250 mg/kg/day) nor HPP treatment (100 and 200 mg/kg/day) for seven days had significant effect on total CYP contents (figure 6). Whereas VPA had no significant effect on any CYP catalytic activities, HPP showed induction effect on some CYPs activities (figure 7-11). The strongest induction effects were seen on the CYP2B1/2B2 activities with both substrates used, meanwhile the relative weaker effect was seen on CYP1A1 activity. HPP both 100 and 200 mg/kg/day significantly ($p<0.05$) increased the rate of ethoxyresorufin o-dealkyation by CYP1A1 for about two-fold of control (figure 7). Additionally, HPP also significantly increased the rate of CYP2B1/2B2 catalyzed benzyloxyresorufin o-dealkylation for about eight-fold of control at both dosages (figure 9), as well as pentoxyresorufin o-dealkylation for about eleven to fifteen-fold of control (figure 10). Slightly increased, but not significantly, in the rate of methoxyresorufin o-dealkyaltion by CYP1A2 was also seen (figure 8). In contrast, there was no significant effect on aniline 4-hydroxylation by CYP2E1 (figure 11).

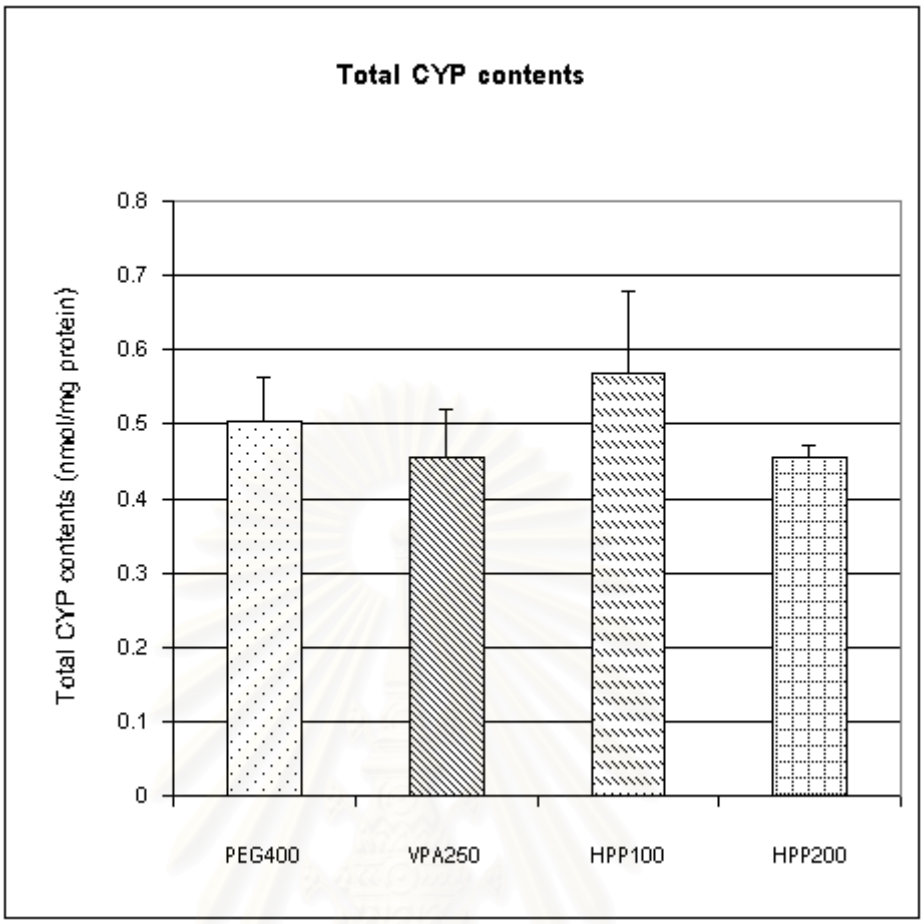


Figure 6. Effect of HPP and VPA on rat liver microsomal total CYP contents in *ex vivo* system. Rats were given PEG400 (control), VPA (250 mg/kg/day) or HPP (100 and 200 mg/kg/day) for seven days. Liver microsomes were prepared and determined for the total CYP contents. Each bar represents the mean of total CYP contents (nmol/mg protein, n=6). The error bars indicate standard deviations.

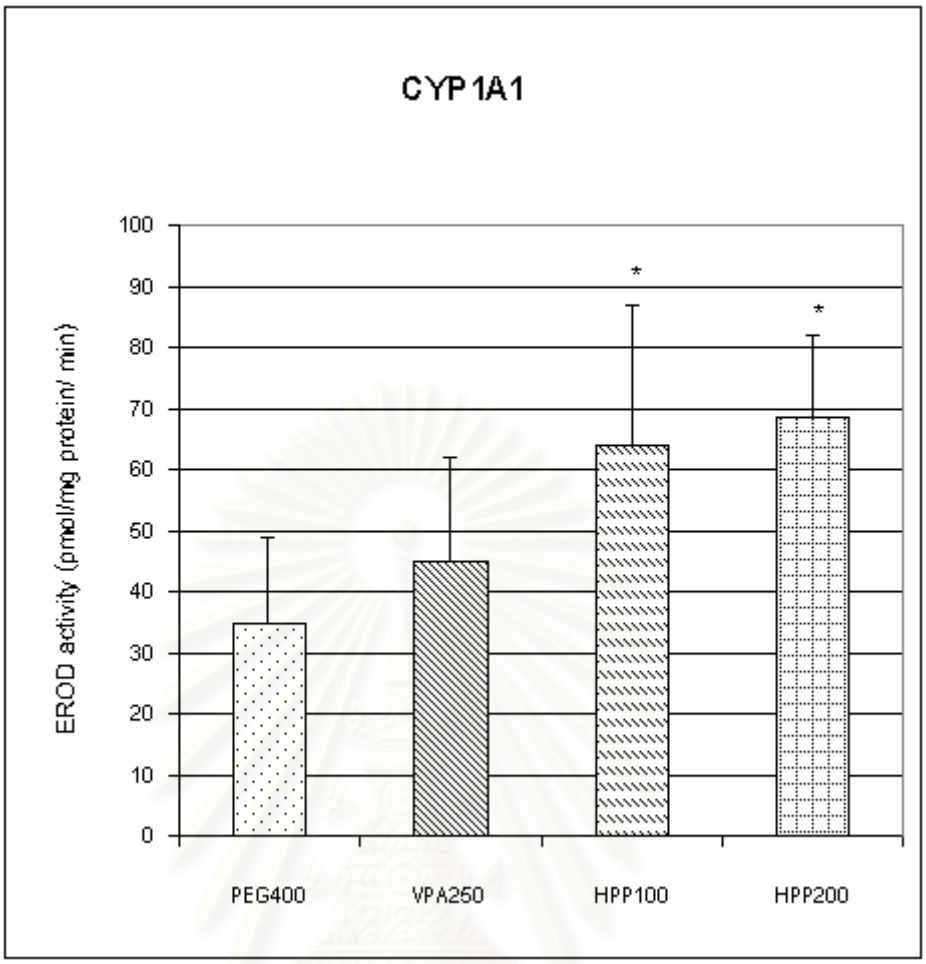


Figure 7. Effect of HPP and VPA on rat liver microsomal CYP1A1 activity in *ex vivo* system. Rats were given PEG400 (control), VPA (250 mg/kg/day) or HPP (100 and 200 mg/kg/day) for seven days. Liver microsomes were prepared and determined for ethoxyresorufin o-dealkylase (EROD) activity. Each bar represents the mean of EROD activity (pmol/mg protein/min, n=6). The error bars indicate standard deviations. *Significantly different from control was determined by One-way ANOVA followed by S-N-K at $p<0.05$.

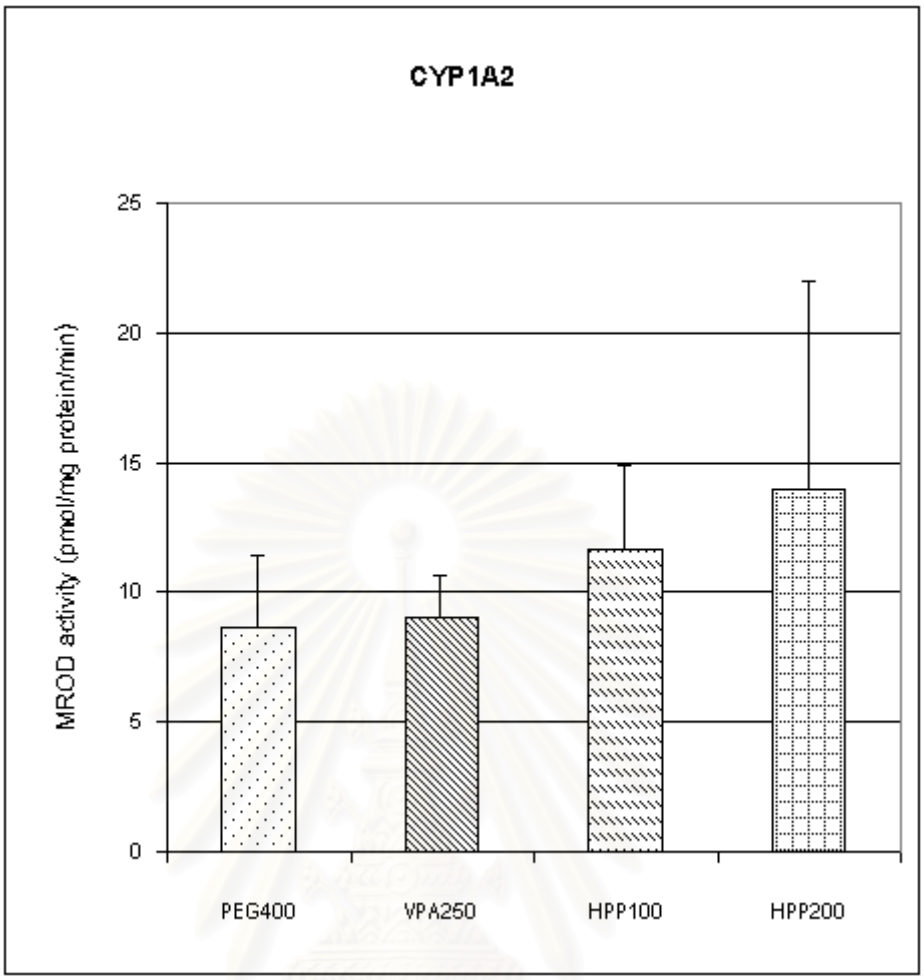


Figure 8. Effect of HPP and VPA on rat liver microsomal CYP1A2 activity in *ex vivo* system. Rats were given PEG400 (control), VPA (250 mg/kg/day) or HPP (100 and 200 mg/kg/day) for seven days. Liver microsomes were prepared and determined for methoxyresorufin o-dealkylase (MROD) activity. Each bar represents the mean of MROD activity (pmol/mg protein/min, n=6). The error bars indicate standard deviations.

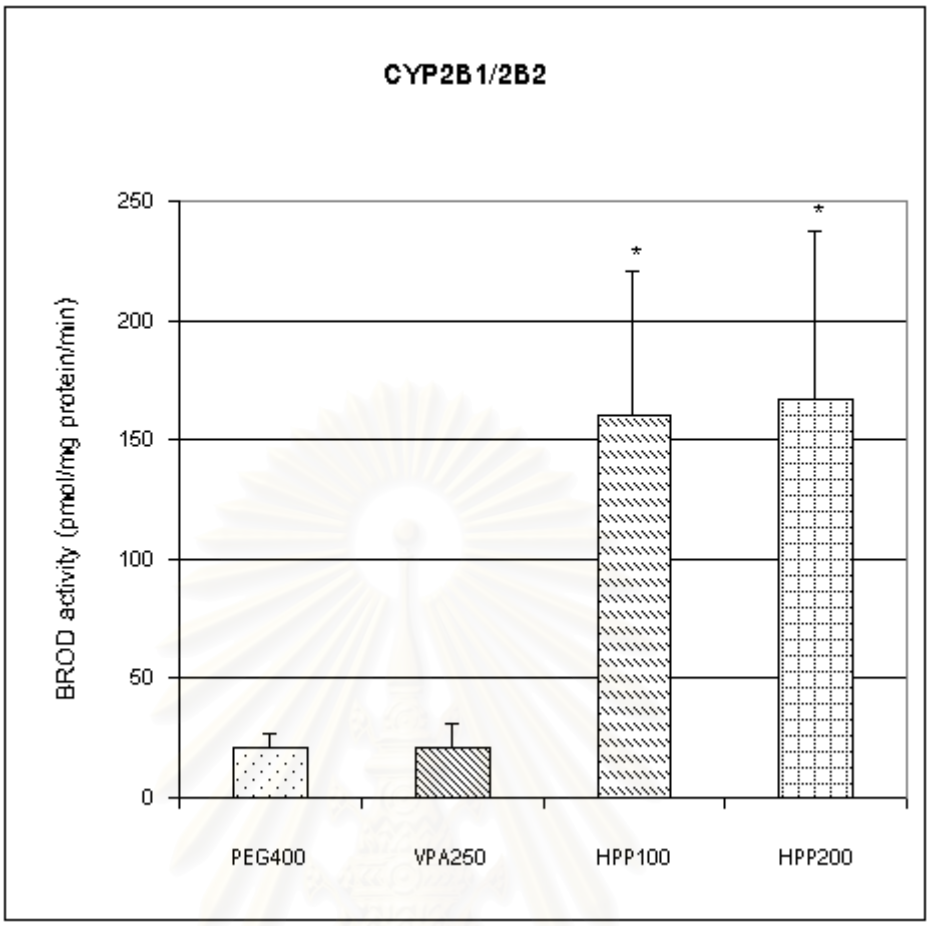


Figure 9. Effect of HPP and VPA on rat liver microsomal CYP2B1/2B2 activities in *ex vivo* system. Rats were given PEG400 (control), VPA (250 mg/kg/day) or HPP (100 and 200 mg/kg/day) for seven days. Liver microsomes were prepared and determined for benzyloxyresorufin o-dealkylase (BROD) activity. Each bar represents the mean of BROD activity (pmol/mg protein/min, n=6). The error bars indicate standard deviations. *Significantly different from control was determined by One-way ANOVA followed by S-N-K at $p<0.05$.

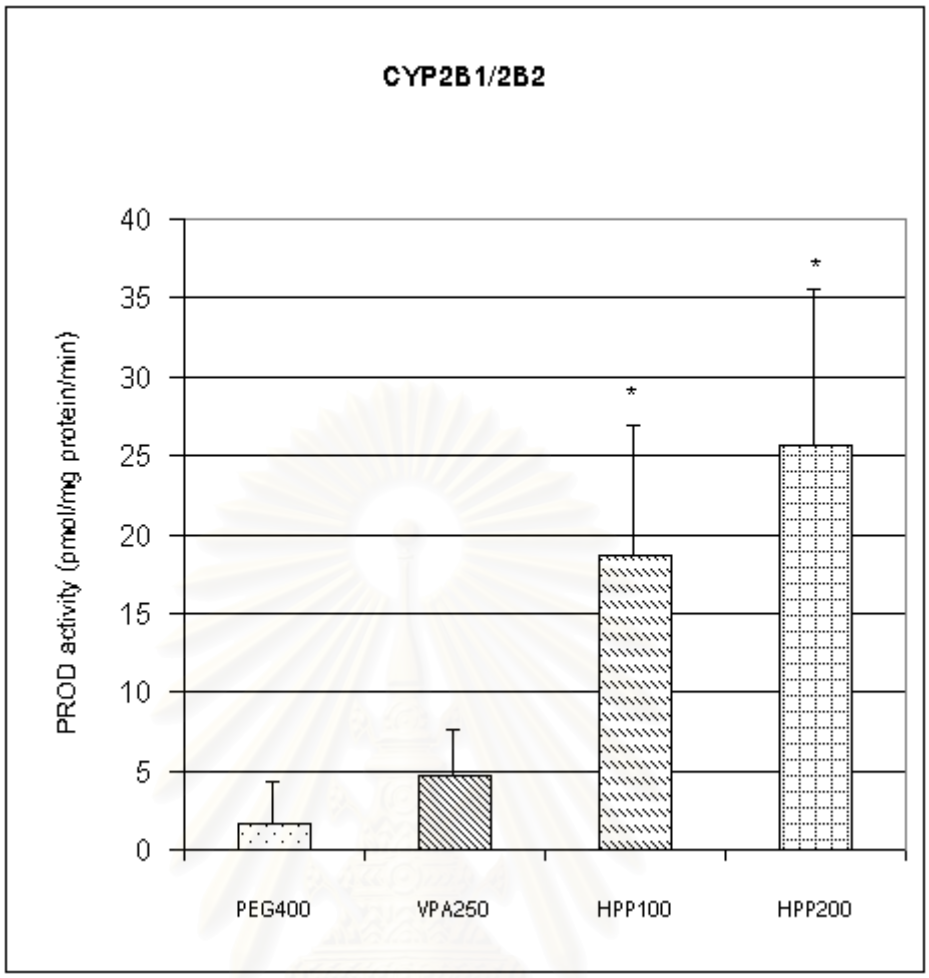


Figure 10. Effect of HPP and VPA on rat liver microsomal CYP2B1/2B2 activities in *ex vivo* system. Rats were given PEG400 (control), VPA (250 mg/kg/day) or HPP (100 and 200 mg/kg/day) for seven days. Liver microsomes were prepared and determined for pentoxyresorufin o-dealkylase (PROD) activity. Each bar represents the mean of PROD activity (pmol/mg protein/min, n=6). The error bars indicate standard deviations. *Significantly different from control was determined by One-way ANOVA followed by S-N-K at $p<0.05$.

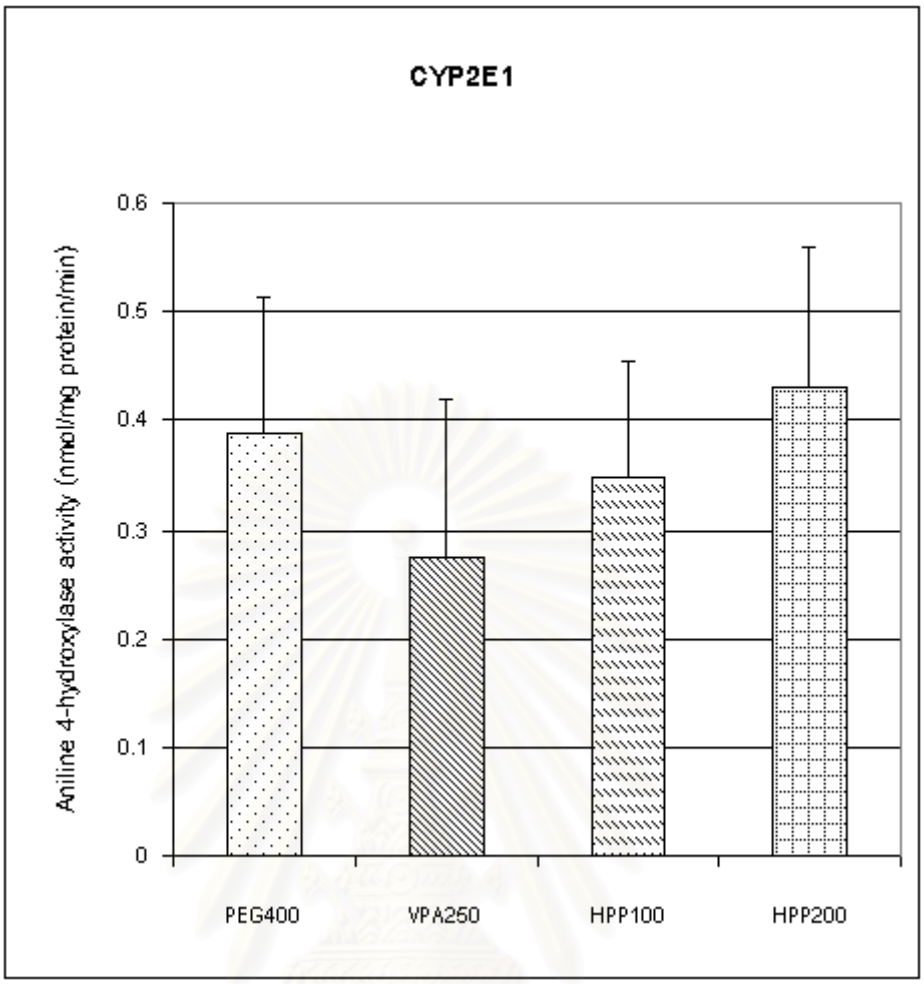


Figure 11. Effect of HPP and VPA on rat liver microsomal CYP2E1activity in *ex vivo* system. Rats were given PEG400 (control), VPA (250 mg/kg/day) or HPP (100 and 200 mg/kg/day) for seven days. Liver microsomes were prepared and determined for aniline 4-hydroxylase activity. Each bar represents the mean of aniline 4-hydroxylase activity (nmol/mg protein/min, n=6). The error bars indicate standard deviations.

2. *In vitro* study

The inhibitory effect of HPP (0.1-1000 μM final concentration) on the same CYP isoforms catalytic activities was studied by *in vitro* co-incubation of HPP with rat liver microsomes. The CYP1A1/1A2, CYP2B1/2B2 and CYP2E1 activities were induced by pretreatment with β–NF, PB and ethanol, respectively *in vivo* before the *in vitro* study.

As shown in figure 12-13, both β–NF and PB pretreatment significantly ($p<0.05$) increased the total CYP contents and CYP1A1 (about fourteen-fold of control), CYP1A2 (about nine-fold of control) and CYP2B1/2B2 (about thirty-one-fold of control) activities. On the other hand, short-term ethanol pretreatment only significantly ($p<0.05$) increased CYP2E1 activity (about two-fold of control), without increasing the total CYP contents.

Regarding the effect of solvents used for dissolving HPP on CYP activity, the rate of aniline 4-hydroxylation by CYP2E1 was almost completely inhibited by DMSO at 1% (v/v) final concentration (figure 14). Whereas acetonitrile at 0.1% and 1% (v/v) final concentrations did not significantly affect the same catalytic activity of CYP2E1. Since the limitation of HPP solubility, 1% (v/v) of acetonitrile final concentration was used in the study of effect of HPP on CYP2E1.

The results of *in vitro* inhibition studies showed that HPP at high concentrations exhibited selective inhibitory effect on CYP isoforms activities. Whereas all the concentration studied exhibited no inhibitory effect on CYP1A1 (figure 15), CYP1A2 (figure 16) and CYP2E1 (figure18) activities. HPP at 100 and 1000 μM significantly ($p<0.05$) decreased the rate of benzyloxyresorufin o-dealkylation by CYP2B1/2B2 (figure 17) with IC_{50} of about 752 μM. The inhibition effect seemed to be dose-dependent.

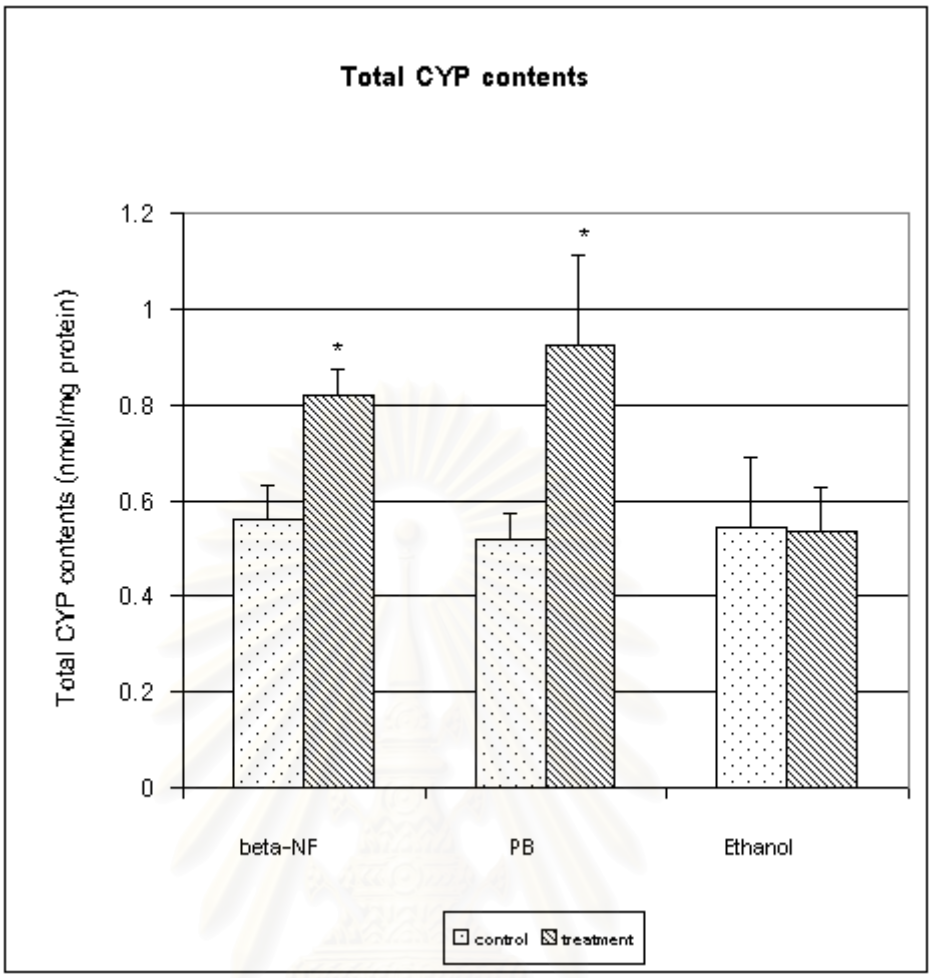


Figure 12. Effect of β-napthoflavone (β-NF), phenobarbital (PB) and ethanol pretreatment on rat liver microsomal total CYP contents. Rats were given β-NF (for an induction of CYP1A1/1A2), PB (for an induction of CYP2B1/2B2) or ethanol (for an induction of CYP2E1) as described in materials and methods. Controls of β-NF, PB and ethanol treatment groups were corn oil, sterile water and water, respectively. Rat liver microsomes were prepared and determined for total CYP contents. Each bar represents the mean of total CYP contents (nmol/mg protein, n=4). The error bars indicate standard deviations. *Significantly different from control was determined by student's *t*-test at $p<0.05$.

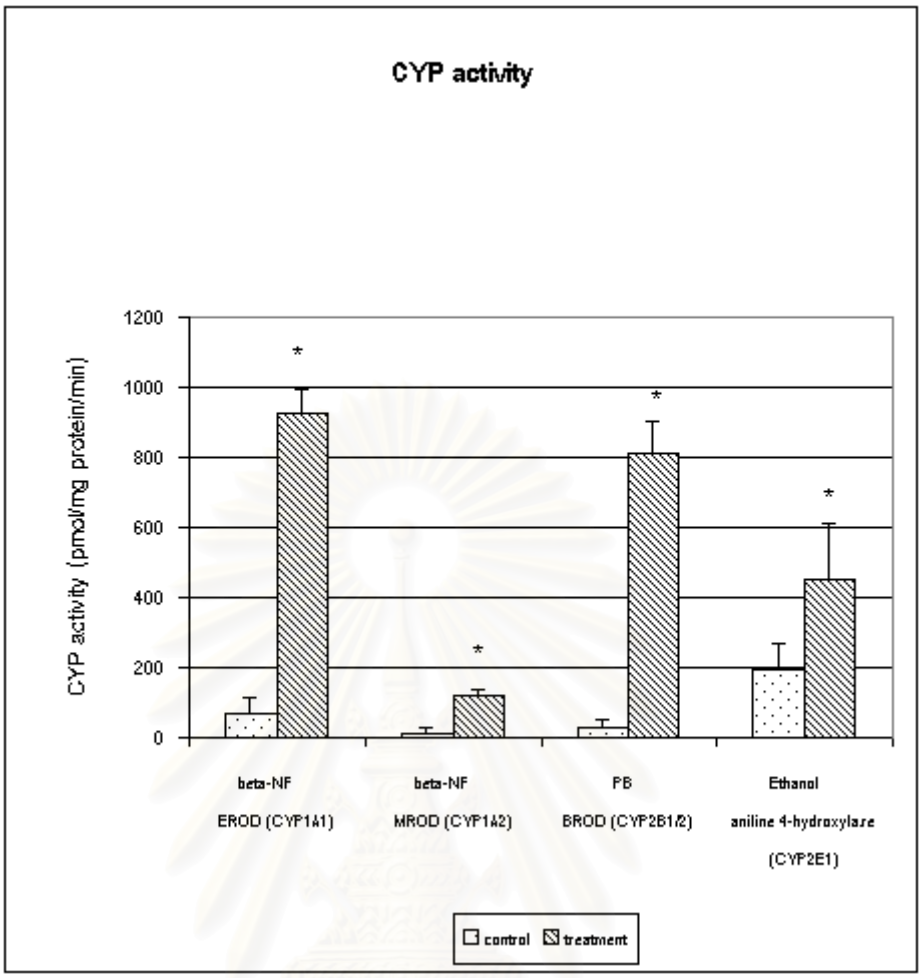


Figure 13. Effect of β-napthoflavone (β-NF), phenobarbital (PB) and ethanol pretreatment on rat liver CYP1A1, CYP1A2, CYP2B1/2B2 and CYP2E1 activiteis. Rats were given β-NF (for an induction of CYP1A1/1A2), PB (for an induction of CYP2B1/2B2) or ethanol (for an induction of CYP2E1) as described in materials and methods. Controls of β-NF, PB and ethanol treatment groups were corn oil, sterile water and water, respectively. Rat liver microsomes were prepared and determined for EROD (CYP1A1), MROD (1A2), BROD (2B1/2B2) and aniline 4-hydroxylase (2E1) activities. Each bar represents the mean of activities (pmol/mg protein, n=4). The error bars indicate standard deviations. *Significantly different from control was determined by student's *t*-test at $p<0.05$.

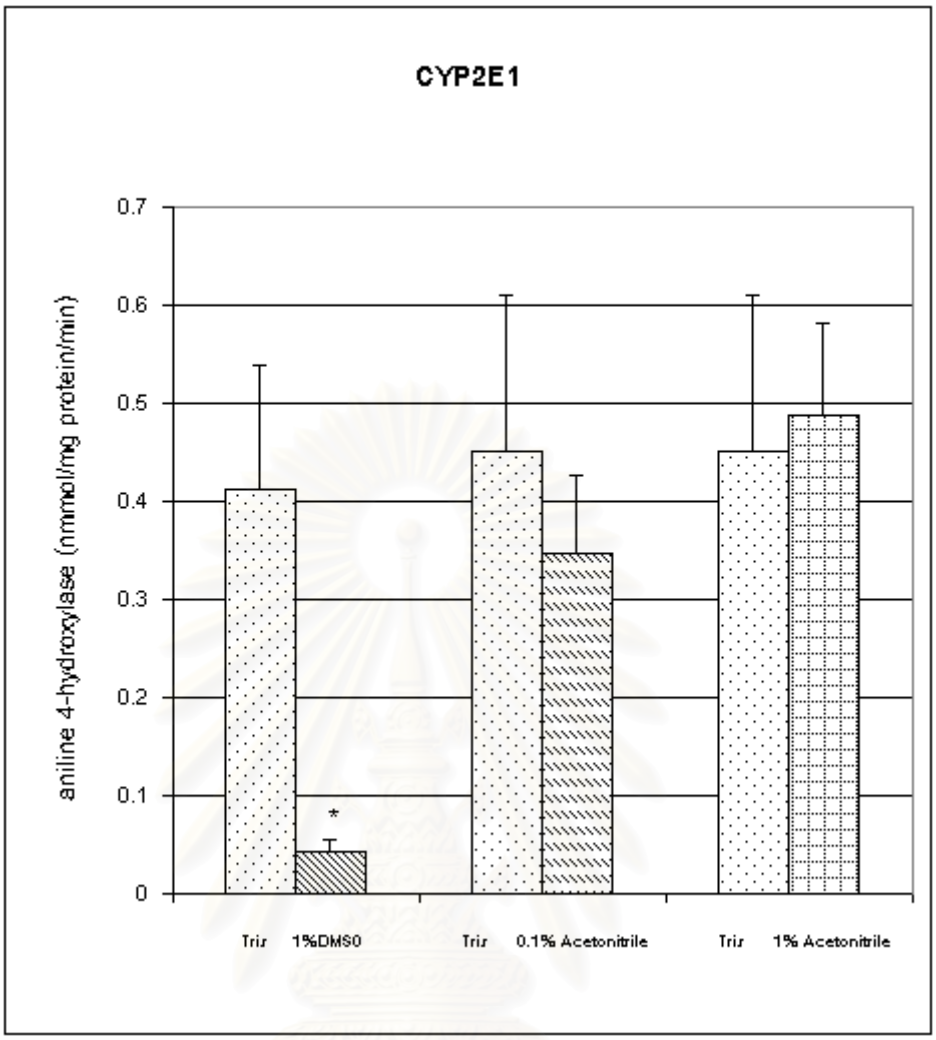


Figure 14. Effect of organic solvents on aniline 4-hydroxylation activity of CYP2E1 in *in vitro* system. The effects of 1% v/v DMSO, 0.1% v/v acetonitrile and 1% v/v acetonitrile at final concentration were determined by co-incubation of each solvent with rat liver microsomes, NADPH regenerating system, G6PD and 10 mM aniline hydrochloride (as described in materials and methods). Each bar represents the mean of aniline 4-hydroxylase activity (nmol/mg protein/min, n=4). The error bars indicate standard deviations. *Significantly different from control (Tris buffer, pH7.4) was determined by student's *t*-test at $p<0.05$.

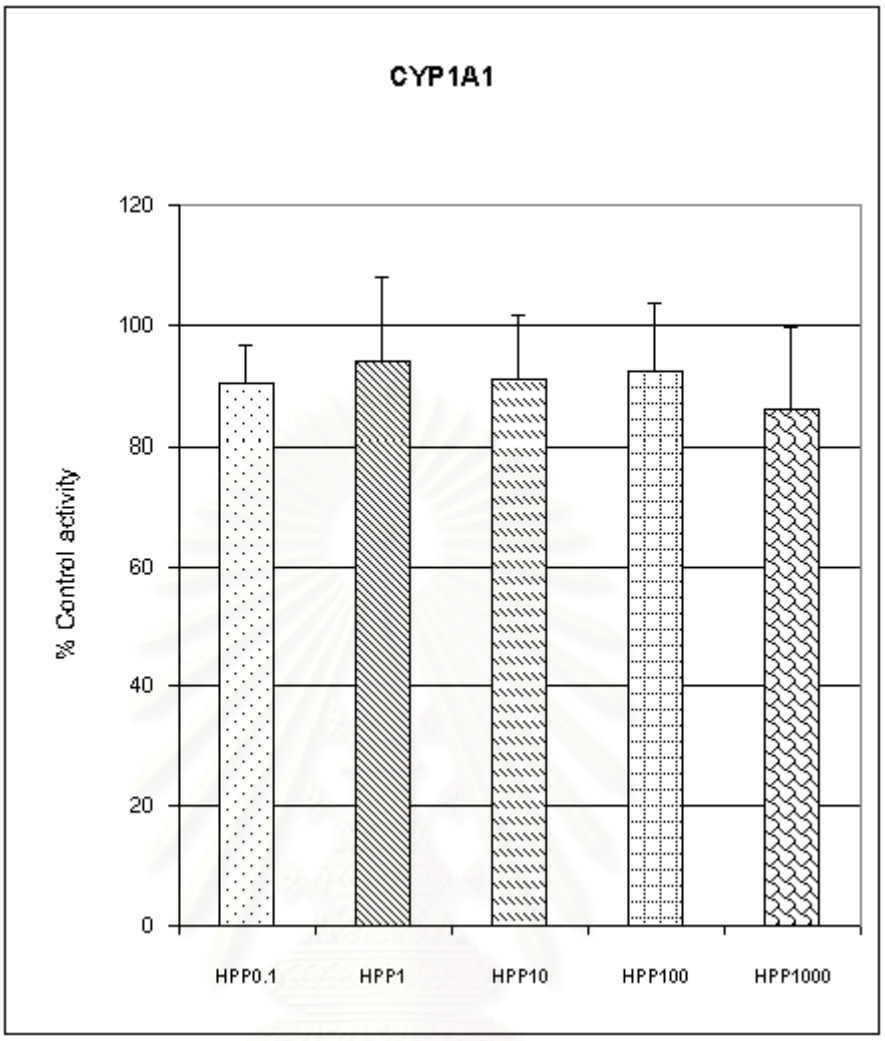


Figure 15. Effect of HPP on ethoxyresorufin o-dealkylase (EROD) activity of CYP1A1 in *in vitro* system. The effects of HPP at final concentrations of 0.1, 1, 10, 100 and 1000 μM on CYP1A1 activity were determined by co-incubation of each concentration of HPP with β-NF-induced rat liver microsomes, NADPH regenerating system, G6PD and 0.5 mM ethoxyresorufin (as described in materials and methods). Each bar represents the mean of EROD activity (pmol/mg protein/min) of four experiments that have been expressed individually as a percent of control (Tris buffer, pH7.4) activity. The error bars indicate standard deviations.

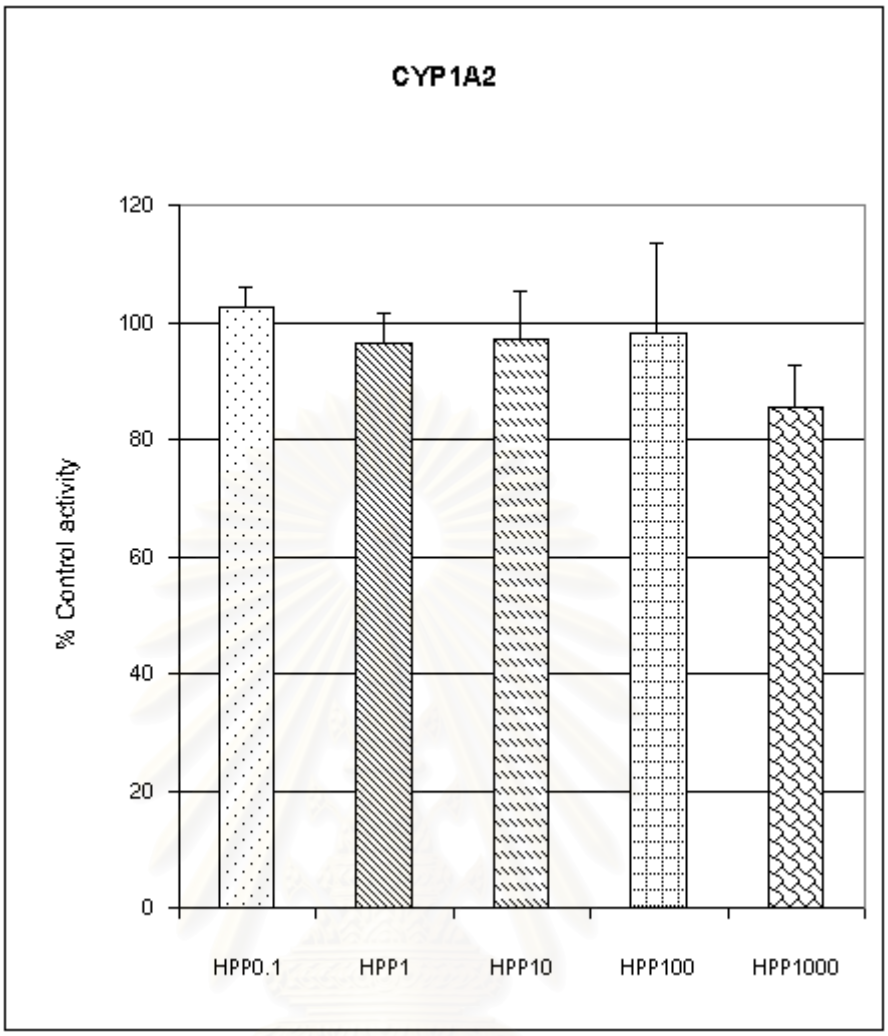


Figure 16. Effect of HPP on methoxyresorufin o-dealkylase (MROD) activity of CYP1A2 in *in vitro* system. The effects of HPP at final concentrations of 0.1, 1, 10, 100 and 1000 μM on CYP1A2 activity were determined by co-incubation of each concentration of HPP with β-NF-induced rat liver microsomes, NADPH regenerating system, G6PD and 0.5 mM methoxyresorufin (as described in materials and methods). Each bar represents the mean of MROD activity (pmol/mg protein/min) of four experiments that have been expressed individually as a percent of control (Tris buffer, pH7.4) activity. The error bars indicate standard deviations.

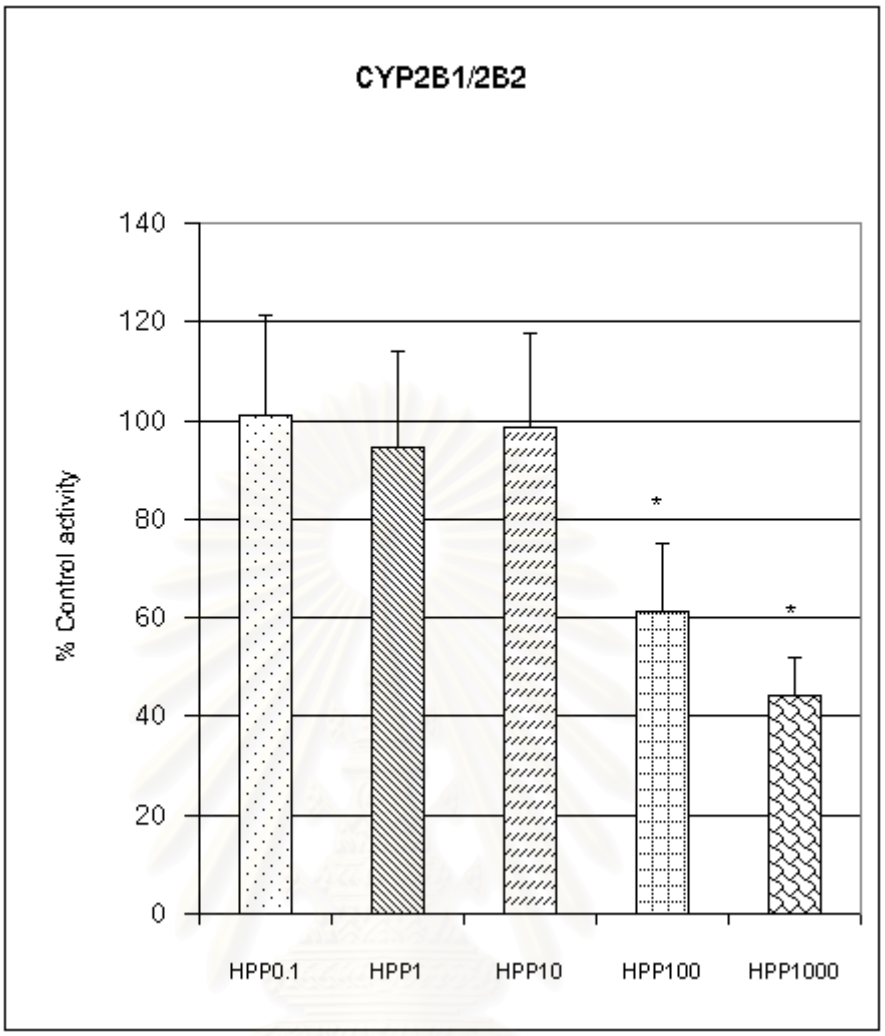


Figure 17. Effect of HPP on benzyloxyresorufin o-dealkylase (BROD) activity of CYP2B1/2B2 in *in vitro* system. The effects of HPP at final concentrations of 0.1, 1, 10, 100 and 1000 μM on CYP2B1/2B2 activities were determined by co-incubation of each concentration of HPP with PB-induced rat liver microsomes, NADPH regenerating system, G6PD and 0.5 mM benzyloxyresorufin (as described in materials and methods). Each bar represents the mean of BROD activity (pmol/mg protein/min) of four experiments that have been expressed individually as a percent of control (Tris buffer, pH7.4) activity. The error bars indicate standard deviations. *Significantly different from control was determined by One-way ANOVA followed by S-N-K at $p<0.05$.

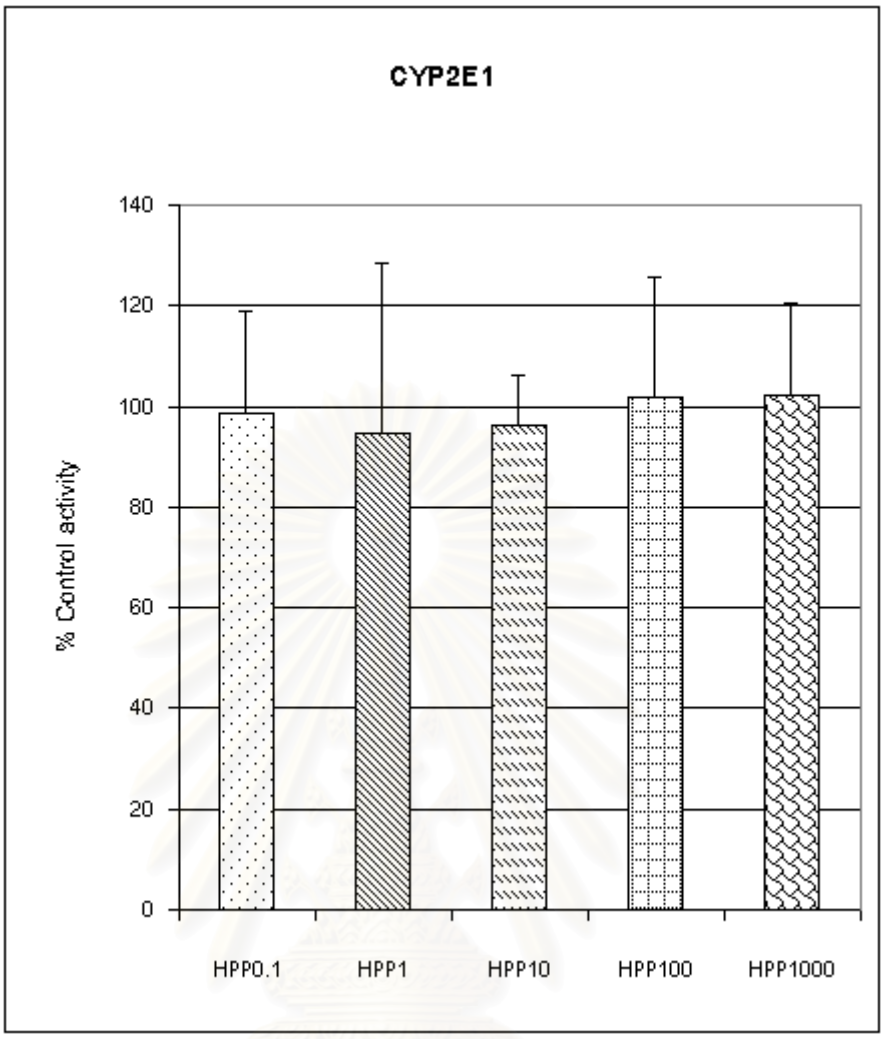


Figure 18. Effect of HPP on aniline 4-hydroxylase activity of CYP2E1 in *in vitro* system. The effects of HPP at final concentrations of 0.1, 1, 10, 100 and 1000 μM on CYP2E1 activity were determined by co-incubation of each concentration of HPP with ethanol-induced rat liver microsomes, NADPH regenerating system, G6PD and 10 mM aniline hydrochloride (as described in materials and methods). Each bar represents the mean of aniline 4-hydroxylase activity (nmol/mg protein/min) of four experiments that have been expressed individually as a percent of control (1% v/v acetonitrile) activity. The error bars indicate standard deviations.

Chapter V

Discussion and Conclusion

Short-term (seven days) treatment of HPP at 100 (approximate ED_{50} in mice) and 200 mg/kg/day exhibited induction effect on rat hepatic microsomal enzymes, not only CYP2B1/2B2 but also CYP1A1 activities. The CYP2B is more highly induced than CYP1A1. Moreover, slightly increased in CYP1A2 activity was also seen. Only CYP2E1 was not found to be affected. Previously it has been shown that intraperitoneal injection of PEG400 had no effect on rat liver microsomal total CYP contents as well as all CYP activities as compared to sterile water (Kiatkosolkul, 1999) therefore effects of HPP on rat liver microsomal activities in this study are not contributed by PEG400. The induction effects of HPP found in this study were weaker than those of β–NF and PB. β–NF was found to strongly induced CYP1A1 (about fourteen-fold) and CYP1A2 (about nine-fold), while PB was found to strongly induced CYP2B1/2B2 (about thirty-one- fold), following 2-3 days administration in this study.

VPA at 250 mg/kg/day (approximate ED_{50} in rat) given intraperitoneally showed no effect on all CYP isoforms studied. This result is consistent with the finding of Kiatkosolkul (1999). Furthermore, an earlier study by Rogier et al. (1995) also shown that an IP injection of 100 mg/kg/day of VPA in rat for 10 days demonstrated no induction effect on CYP2B1/2B2 activities. However, VPA demonstrated a potent induction effect on CYP2B1/2B2 in *in vitro* hepatic cell culture system as well as in *in vivo*, when administered by continuous infusion for two weeks. It has been proposed that the absence of induction effect on CYP2B following IP administration of VPA may be a consequence of the short half-life of VPA in rats (10-20 minutes) (Dickinson et al., 1979). In human, VPA has been documented as an inhibitor of CYP2C9, UGT and epoxide hydrolase.

In line with this finding, it has also been found that other derivatives of VPA exhibited induction and inhibition effects on CYPs, namely VPU has been demonstrated to be an inducer of rat liver CYP2B1/2B2 *in vivo* (Kiatkosolkul, 1999) as well as an

inhibitor of human liver CYP2C9 and CYP1A1/1A2 (Kijsanayotin et al., 1997). Valproyl morpholine (VPM), another derivative of VPA, was recently shown to be an inducer of rat liver CYP2B1/2B2 and CYP1A1 *in vivo* as well as an inhibitor of CYP2B1/2B2 and CYP1A1 and CYP1A2 *in vitro* (Yana, 2000).

Although CYP2B1/2B2 are not expressed in human, they play an important role in anticovulsant metabolism including VPA and PB in rat. Regarding VPA metabolism, CYP2B subfamily is responsible for the formation of 4-ene-VPA, a potential hepatotoxic and teratogenic metabolite of VPA (Nau and Loscher, 1984; Powell-Jackson et al., 1984). Prolong exposure of valproate resulted in inducing its own metabolism. Lewis et al. (1982) has reported that the incidence of VPA-induced liver injury was increased in rats treated with PB, a strong inducer of CYP2B. Induction effect of VPA on CYP2B1 has also been suggested to contribute substantially to the hepatotoxic effect of VPA (Rogiers et al., 1995). In parallel with VPA, all derivatives of VPA including VPU, VPM and HPP have shown an induction effect as well as inhibition effect on CYP2B. It is interesting to explore whether CYP2B involved in their metabolism or the formation of toxic metabolites, as VPA. Thus the induction effect of HPP as well as other VPA derivatives on CYP2B, would be of pharmacological or toxicological importance, as they could increase their own clearance or increase toxicity. Although human CYP2B6 is analogous to rat CYP2B1 (Soucket and Gut, 1992), it has been noted that there are species differences in CYP catalytic activity and regulation (Lin and Lu, 1998). Thus, the induction effect of HPP on rat CYP2B may not be directly extrapolated to human CYP2B6 which has no known important function.

CYP1A has been of particular interest due to their ability to activate procarcinogens and promutagens both in rat and human, namely PAH, aromatic amine and aflatoxin B1. Meanwhile CYP2B is active toward aflatoxin B1, benzo(a)pyrene and 4,4'-(bis)methylcholanthrene. Since about one tenth of human population exhibited a high CYP1A1 inducibility (Miner and McKinnon, 2000), increasing these CYP activities by HPP may increase susceptibility to the adverse effect of CYP mediated activation of toxins and carcinogens. Further study to verify its carcinogenicity should be conducted.

Furthermore, since CYP1A1 and CYP1A2 are also inducible in extrahepatic tissues (Gonzalez, 1990), study of the effect of HPP on extrahepatic CYP1A should also be considered. Moreover, HPP had neither induction nor inhibition effects on rat liver microsomal total CYP contents and CYP2E1 activity. These findings should be an advantage of HPP in terms of drug interaction and a potential risk of toxicity from reactive metabolites mediated by CYP2E1.

A study of Levi, Yagen and Bialer (1997) has demonstrated that valpromide (VPD), a primary amide of VPA, serves as a prodrug of VPA. Whereas the substitution of a hydrogen attached to the nitrogen in the valpromide molecule by a hydroxyl (valproyl hydroxamic acid) or a β–hydroxyethyl (N-(1-hydroxyethyl)-VPD) moiety prevented the biotransformation of the amide to VPA. In this regard, HPP, a hydroxymethyl substitute of valpromide could be considered as a metabolically stable derivative of VPD and its induction effects on rat liver CYPs may be responsible by HPP itself or its any other metabolites than VPA. On the other hand, if this hypothesis was true, it should be of advantage that HPP would not be metabolited to VPA and subsequently its toxic metabolite, 4-ene-VPA. However, the study of Levi et al. (1997) has also shown that the hydroxyl substitution of VPD reduced neurotoxicity of the compounds as compared to VPD, but the toxicity is still higher than VPA. This was confirmed by the acute toxicity study with HPP, the TD_{50} (neurotoxicity) of HPP in mice was lower than VPA (Patarapanich et al., 1998; Supatchaipisit, 1995). In this study, it was found that rats treated with 200 mg/kg/day of HPP exhibited ataxia which was not seen in VPA treated rats.

In order to observe the apparent inhibitory effect of HPP *in vitro*, rats were pretreated *in vivo* with different inducers capable of inducing certain types of CYPs activities. In contrast to β–NF and PB, ethanol pretreatment slightly increased total CYP contents but significantly ($p<0.05$) increased CYP2E1 activity. This finding may be resulted from the mechanism of induction. The induction of CYP2E1 may arise through multiple mechanisms. The possible mechanism of ethanol induction appeared to occur via stabilization of the CYP2E1 mRNA. Transcriptional activation of CYP2E1 gene has

also been reported (Lieber, 1997). Induction effects of PB and β–NF are involved transcriptional activation. The later effect was associated with a specific cytosolic receptor, termed the Ah receptor (Gibson and Skett, 1994).

In *in vitro* inhibition study, HPP at final concentration of 100 and 1000 μM inhibited rat hepatic CYP2B1/2B2 activities with IC_{50} of about 752 μM. The loss of CYP2B1/2B2 catalyzed BROD activity occurred without preincubation, indicating that HPP should be a competitive reversible inhibitor. This finding suggested that HPP may be a substrate for CYP2B1/2B2 similar to VPA (Retties et al., 1987) and may be, in part, responsible for prolongation of barbiturate sleeping time after single dose administration of HPP (Patarapanich et al., 1998; Supatchaipisit, 1995). This effect may be resulted from the competition between HPP and barbiturate for the same binding site on CYP2B which are responsible for barbiturate clearance in rat (Lewis and Lake, 1997). However, further *in vitro* study to investigate whether HPP could be a mechanism-based inhibitor, should be conducted.

In vitro model is a useful tool to screen for the effect of new drug on common CYP pathways (US. FDA, 1998). Although *in vitro* model can assess inhibitory effect with rapid, simple and inexpensive, it has a limitation in the solubility of the test compounds. HPP requires water miscible organic solvents for effective solubilization. It is known that some organic solvents can affect the activities of several CYPs (Busby, Ackermann and Crespi, 1999; Chauret, Gauthier and Nicoll-Griffith, 1998; Hickman, Wang and Unakat, 1998). The presence of an organic solvent can strongly affect the reliability and interpretation of *in vitro* data. A study of Busby et al., (1999) suggested that induction or inhibition effects of solvent were substrate-dependent for a given cytochrome P450. Thus, it should be considered to examine the solvent effect on each substrate used for individual CYP activity assay. In this study, the organic solvents used to solubilize HPP were kept minimal and constant. Regarding CYP1A1, CYP1A2 and CYP2B1/2B2 assays, all the substrates used were dissolved in DMSO at 1% (v/v) final concentration which has been shown to have no effect on these CYP activities (Busby et al., 1999; Chauret et al., 1998; Hickman et al., 1998). In order to keep organic solvent minimal and constant,

HPP were solubilized in each substrate solutions. Whereas DMSO showed an inhibition effect on CYP2E1 activity, acetonitrile did not show noticeable change on this enzyme activity at concentration $\leq$ 1%. Due to the limit solubility of HPP in acetonitrile, 1% acetonitrile was used as the solvent of HPP in the inhibition study on CYP2E1.

In conclusion, the present study indicated that short-term (seven days) administration of HPP exhibited selective induction effect on rat hepatic microsomal cytochrome P450. HPP 100 and 200 mg/kg/day demonstrated no effects on rat hepatic microsomal total CYP contents, CYP1A2 and CYP2E1 activities. In contrast, HPP 100 and 200 mg/kg/day induced CYP1A1 and CYP2B1/2B2 activities. The induction effect on CYP2B was stronger than on CYP1A1. Furthermore, HPP at final concentrations of 100 and 1000 μM inhibited CYP2B1/2B2 activities with IC_{50} of about 752 μM. This finding suggested that HPP may be, in part, responsible for prolongation of barbiturate sleeping time after single dose administration. Further studies are needed to clarify the metabolic pathways of HPP and the CYPs involved as well as the effect of HPP on human common CYPs. *In vivo* studies to verify the potential of drug interaction and carcinogenic risk are also needed.

References

Anderson, G. D. 1998. A mechanistic Approach to antiepileptic drug interactions. Annals of Pharmacotherapy 32: 554-563.

Bjorge, S. M., and Baillie, T. A. 1985. Inhibition of medium-chain fatty acid β–oxidation *in vitro* by valproic acid and its unsaturated metabolite, 2-n-propyl-4-pentenoic acid. Biochem. Biochem. Biophys. Res. Commun. 132 (1): 245-252.

Bruni, J. 1996. Valproate. In Shorvon, S. (ed.), The treatment of epilepsy, 482-490. Massachusetts: Blackwell Science.

Bryant, A. E., and Dreifuss, F. E. 1996. Valproic acid hepatic fatalities. III. U. S. expression since 1986. Neurology 46: 465-469.

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

Busby, W. F., Ackermann, J. J., and Crespi, C. L. 1999. Effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on *in vitro* activities of cDNA-expressed human cytochromes P-450. Drug. Metab. Dispos. 27 (2): 246-249.

Chauret, N., Gauthier, A., and Nicoll-Griffith, D. A. 1998. Effect of common organic solvents on *in vitro* cytochrome P450-mediated metabolic activities in human liver microsomes. Drug. Metab. Dispos. 26 (1): 1-4.

Cloyd, J. C., and Remmel, R. P. 2000. Antiepileptic drug pharmacokinetics and interactions: Impact on treatment of Epilepsy. Pharmacotherapy 20 (8 Pt 2): 139s-151s.

Cotariu, D., and Zaidman, J. L. 1991. Developmental toxicity of valproic acid. Life Sciences 48: 1341-1350.

Davis, R,. Peter, D. H., and McTavish, D. 1994. Valproic acid: A reappraisal of its pharmacological properties and clinical efficacy in epilepsy. Drug 47 (2): 332-372.

Denision, M. S., and Whiflock, J. P. 1995. Xenobiotic-inducible transcription of cytochrome P450 genes. J. Biol. Chem. 270 (31): 18175-18178.

Di Carlo, F. J., Bickart, P., and Auer, C. M. 1986. Structure-metabolism relationship (SMR) for the prediction of health hazards by the environmental protection agency. II. Application to teratogenicity and other toxic effects caused by aliphatic acids. Drug Metab. Rev. 17 (3&4): 187-220.

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

Dreifuss, F. E., Langer, D. H., Moline, K. A., and Maxwell, J. E. 1989. Valproic acid hepatic fatalities. II. U. S. experience since 1984. Neurology 39: 201-207.

Gibson, G. G., and Skett, P. 1994. Introduction to drug methabolism. 2 nd ed., London: Chapmansh.

Gonzalez, F. J. 1989. The molecular bilology of cytochrome P450s. Pharm. Rev. 40 (4): 243-288.

Gonzalez, F. J. 1990. Molecular genetics of the P-450 superfamily. Pharmaco. Ther. 45: 1-38.

Gonzalez, F, J., and Gelboin, H. V. 1994. Role of human cytochrome P450 in the metabolic activation of chemical carcinogens and toxins. Drug. Metab. Rev. 26 (142): 165-183.

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

Guengerich, F. P. 1993. Bioactivation and detoxication of toxic and carcinogen chemical. Drug. Metab. Dispos. 21 (1): 1-6.

Guengerich, F.P. 1995. Human cytochrome P450 In P. R Ortiz de Metellano, (ed.), Cytochrome P450: Structure, mechanism, and biochemistry, pp. 473-535, New York: Plenum Press.

Heinemayer, G., Nau, H., Hildebrandt, G. A., and Roots, I. 1985. Oxidation and glucuronidation of valproic acid in male rats-influence of phenobarbital, 3-methylcholanthrene, beta-napthoflavone and clofibrate. Biochem. Pharmocol. 34: 133-139.

Hickman, D., Wang, Ji-Ping., and Unakat, J. D. 1998. Evaluation of the selectivity of *in vitro* probes and suitability of organic solvents for the measurement of human cytochrome P450 monooxygenase activities. Drug. Metab. Dispos. 26 (3): 207-215.

Hu, Y., Ingelman-Sundberg, M., and Lindros, K. O. 1995. Induction mechanism of cytochrome P450 2E1 in liver: Interplay between ethanol treatment and starvation. Biochem. Pharmocol. 50 (2): 155-161.

Johnston, D., and Slater, G. E. 1982. Valproate: Mechanism of action. In M.D.Woodbury, K. J., Penry, E. C., and Pippenger, C. E (eds.), Antiepileptic Drugs, pp. 611-616. New York: Raven Press.

Kiatkosolkul, T. 1999. Effect of N-(2-propylpentanoyl) urea on rat hepatic cytochrome P450 and glutathione s-transferase. Master's Thesis, Department of Pharmacology, Graduate School, Chulalongkorn University.

Kijsanayotin, P., Hayama, E., Nanbo, T., Patarapanich, C., Satoh, T., and Shigematsu, A. 1997. 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.

Kingsley, E. D., Gray, P., Tolman, K. G., and Tweedale, R. 1983. The toxicity of metabolites of sodium valproate in cultured hepatocytes. J. Clin. Pharmacol. 23: 178-185.

Levi, M., Yagen, B., and Bialer. 1997. Pharmacokinetics and antiepileptic activity of valproyl hydroxamic acid derivatives. Pharm. Res. 14 (2): 213-217.

Lewis, D. F. V., and Lake, B. G. 1997. Molecular modeling of mammalian CYP2B isoforms and their interaction with substrates, inhibitors and redox partners. Xenobiotica 27 (5): 443-478.

Lewis, J. H., Zimmerman, H. J., Garrett, C. T., and Rosenberg, E. 1982. Valproate-induced hepatic steatogenesis in rats. Hepatology 2: 870-3.

Lieber, C. S. 1997. Cytochrome P450 2E1: Its physiological and pathological role. Physiological Reviews 77 (2): 517-544.

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

Lomlim, L. 1998. Synthesis of amide derivatives of valproic acid. Master's Thesis, Department of Pharmaceutical Chemistry, Graduate School, Chulalongkorn University.

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

Lubet, A. R., Mayer, T. R., Cameron, W. J., Burke, M. D., Wolft, T., and Guengerich, F. P. 1985. 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 (1): 43-48.

Mayer, A. 1996. Overview of enzymes of drug metabolism. J. Pharmacokinet, Biopharm. 24 (5): 449-459.

Miners, J. O., and McKinnon, R. A. 2000. CYP1A in R. H. Levy, K. E. Thummel, W. F. Trager, P. D. Hansten, and M. Eichelbaum (eds.), Metabolic drug interaction, pp. 61-73. Philadelphia: Lippincott Williams&Wilkins.

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

Murray, M., and Reidy, G. 1990. Selectivity in the inhibition of mammalian cytochromes P-450 by chemical agents. Pharm. Rev. 42 (2): 85-98.

Nau, H., and Loscher, W. 1984. Valproic acid and metabolites: Pharmocological and toxicological studies. Epilepsia 25 (suppl. 1): s14-s22.

Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. S., Coon., M. J., Gunsalus, I. C., Gotoh, O., Okuda, K., and Nebert, D. W. 1993. The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA and Cell Biology 12 (1): 1-51.

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

Omtzigt, J. G. C., Los, F. J., Grobbee, D. E., Pijers, J., Johoda, M. G. J., Brandenburg, H., Stewart, P. A., Gaillard, H. L. J., Sachs, E. S., and Wladimiroff, J. W. 1992. The risk of spina bifida aperta after first-trimester exposure to valproate in a prenatal cohort. Neurology 42 (suppl 5): 119-125.

Patarapanich, C., Tantisira, M. H., and Tantisira, B. 1998. Pharmocological evaluation of anticonvulsant activity of (N-hydroxymethyl)-2-propylpentamide. Final report to the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Pellock, M. J. 2000. Treatment of epilepsy in new millennium. Pharmacotherapy 20 (8); 129s-138s.

Powell-Jackson, P. R., Tredger, J. M., and Williams, R. 1984. Hepatotoxicity to sodium valproate: A review. Gut 25: 673-681.

Prickett, K. S., and Baillie, T. A. 1984. Metabolism of valproic acid by hepatic microsomal cytochrome P-450. Biochem. Biophys. Res. Commun. 122 (3): 1166-1173.

Rall, T. W., and Schleifer, L. S. 1996. Drug effective in the therapy of the epilepsies. In J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Ruddon, A. G. Gilman, (eds.), Goodman & Gilman's the pharmacological basis of therapeutics, pp. 436-462. Singapore: McGraw-Hill.

Rang, H. P., Dale, M. M., and Ritter, J. M. 1999. Pharmacology. 4th ed. Edinburgh: Churchill Livingstone.

Rendic, S., and Di Chalo, F. J. 1997. Human cytochreme P450 enzymes: A status report summerizing their reactions, substrates, inducers, and inhibitors. Drug. Metab. Rev. 29(1&2): 413-580.

Rettie, A. E., Rettenmeier, A. W., Howald, W. N., and Baillie, T. A. 1987. Cytochrome P-450-catalyzed formation of Δ^4-VPA, a toxic metabolite of valproic acid. Science 235: 890-893.

Riddick, D. S. 1998. Drug biotransformation. In H. Kalant, W. H. E. Roschlau (eds.), Principles of medicinal pharmacology, pp. 38-54. New york: Oxford University press.

Ring B.J., and Wrightorn, S.A. 2000. Industrial viewpoint: Application of *In vitro* drug metabolism in various phases of drug development In R. H. Levy, K. E. Thummel, W. F. Trager, P. D. Hansten, and M. Eichelbaum (eds.), Metabolic drug interaction, pp. 29-47. Philadelphia: Lippincott Williams&Wilkins.

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

Rowan, A. J. 1997. Valproate. In J. Engel, and T. A. Pedley, (eds.), Epilepsy: A comprehensive textbook, pp. 1599-1607. Philadelphia: Lippincott-Raven Publishers.

US. Food and drug Administration. 1998. Guidance for industry *in vivo* drug metabolism/drug interaction studies – study design, data analysis, and recommendations for dosing and labeling. Rockville, MD.

Sadeque, A. J. M., Fisher, M. B., Korzekwa, K. R., Gonzalez, F. J., and Rettie, A. E. 1997. Human CYP2C9 and CYP2A6 mediate formation of the hepatotoxin 4-ene-valproic acid. J. Pharmacol. Exp. Ther. 283: 698-730.

Schenkman, J. B. 2000. Cytochrome P450: Historical overview. In R. H. Levy, K. E. Thummel, W. F. Trager, P. D. Hansten, and M. Eichelbaum (eds.), Metabolic drug interaction, pp. 51-60. Philadelphia: Lippincott Williams&Wilkins.

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

Siemes, H., Nau, H., Schultze, K., Wittfoht, W., Drews, E., Penzien, J., and Seidel, U. 1993. Valproate (VPA) metabolites in various clinical conditions of probable VPA-associated hepatotoxicity. Epilepsia 34 (2): 332-346.

Supatchaipisit, P. 1995. Anticonvulsant activity and effects of (N-hydroxymethyl) -2-propylpentamide on the central nervous system. Master's Thesis, Inter-Department of Pharmacology, Graduate School, Chulalongkorn University.

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

Tanaka, E. 1998. Clinically important pharmacokinetic drug-drug interaction: Role of cytochrome P450. enzymes. J. Clin. Pharm. Ther. 23: 403-416.

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

Tanaka, E., Terada, M., and Misawa, S. 2000. Cytochrome P450 2E1: its clinical and toxicological role. J. Clin. Pharm. Ther. 25: 165-175.

Wagner, M. L., Graves, N. M., Leppik, I. E., Remmel, R. P., Shumaker, R. C., Ward, D. L., and Perhach, J. L. 1994. The effect of felbamate on valproic acid disposition. Clin. Pharmacol. Ther. 56: 494-502.

Watkins, P. B. 1990. Role of cytochrome P 450 in drug metabolism and hepatotoxicity. Seminars in Liver Disease 10 (4): 235-250.

Waxman, D.J., and Azaroff, L. 1992. Phenobarbital induction of cytochrome P-450 gene expression. Biochem. J. 281: 577-592.

Wegner, C., and Nau, H. 1992. Alteration of embryonic folate metabolism by valproic acid during organogenesis: Implications for mechanism of teratogenesis. Neurology 42 (suppl 5): 17-24.

Yana, K. 2000. Effect of valproyl morpholine on rat hepatic cytochrome P450. Master's Thesis, Department of Pharmacology, Graduate School, Chulalongkorn University.

Yukawa, E., Honda, T., Ohdo, S., Higuchi, S., and Aoyama, T. 1997. Detection of carbamazepine-induced changes in valproic acid relative clearance in man by simple pharmacokinetic screening. J. Pharm. Pharmacol. 49: 751-756.

APPENDIX

1. Enzyme induction study

Table 6. The concentration of protein in individual rat microsomal subfraction in *ex vivo* *

Rat number	Treatment group			
	PEG 400	VPA	HPP 1	HPP 2
1	33.18	26.12	37.78	28.01
2	25.78	30.76	29.12	24.43
3	31.53	20.22	26.41	29.12
4	25.65	23.29	23.71	30.60
5	25.15	27.09	26.57	25.86
6	29.19	25.99	30.78	23.54

* unit expressed as mg/ml

Table 7. The hepatic microsomal total CYP contents in individual rat in *ex vivo* *

Rat number	Treatment group			
	PEG 400	VPA	HPP 1	HPP 2
1	0.484	0.489	0.648	0.505
2	0.566	0.361	0.604	0.467
3	0.544	0.547	0.659	0.667
4	0.500	0.478	0.615	0.472
5	0.533	0.406	0.516	0.308
6	0.398	0.450	0.366	0.305

* unit expressed as nmol/mg protein

Table 8. The hepatic microsomal benzyloxyresorufin o-dealkylase activity in individual rat in *ex vivo* *

Rat Number	Treatment group			
	PEG 400	VPA	HPP 1	HPP 2
1	16	18	74	86
2	16	6	176	174
3	14	36	228	272
4	26	24	222	180
5	28	24	112	92
6	24	20	148	200

* Unit expressed as pmol/mg protein/min

Table 9. The hepatic microsomal ethoxyresorufin o-dealkylase activity in individual rat in *ex vivo* *

Rat Number	Treatment group			
	PEG 400	VPA	HPP 1	HPP 2
1	12	54	62	54
2	46	14	54	64
3	22	48	80	82
4	40	64	100	70
5	46	50	52	56
6	42	40	36	86

* Unit expressed as pmol/mg protein/min

Table 10. The hepatic microsomal methoxyresorufin o-dealkylase activity in individual rat in *ex vivo* *

Rat Number	Treatment group			
	PEG 400	VPA	HPP 1	HPP 2
1	4	8	14	12
2	8	8	12	14
3	8	10	8	26
4	10	8	16	20
5	10	12	8	4
6	12	8	12	8

* Unit expressed as pmol/mg protein/min

Table 11. The hepatic microsomal pentoxyresorufin o-dealkylase activity in individual rat in *ex vivo* *

Rat Number	Treatment group			
	PEG 400	VPA	HPP 1	HPP 2
1	0	6	14	14
2	6	2	20	30
3	0	8	30	40
4	4	2	24	18
5	0	8	18	20
6	0	2	6	32

* Unit expressed as pmole/mg protein/min

Table 12. The hepatic microsomal aniline 4-hydroxylase activity in individual rat in *ex vivo* *

Rat Number	Treatment group			
	PEG 400	VPA	HPP 1	HPP 2
1	0.303	0.340	0.310	0.412
2	0.602	0.061	0.423	0.581
3	0.248	0.488	0.484	0.576
4	0.326	0.316	0.392	0.239
5	0.445	0.243	0.294	0.402
6	0.402	0.202	0.187	0.372

* Unit expressed as nmol/mg protein/min

2. Enzyme inhibition study

Table 13. The concentration of protein in individual rat microsomal subfraction in *in vitro* *

Rat number	Treatment group					
	Corn oil	β-NF	Sterile water	PB	Water	Ethanol
1	27.26	29.44	30.43	43.10	27.35	39.25
2	26.47	30.58	27.97	42.60	37.72	39.25
3	32.20	30.56	29.75	45.05	28.44	44.53
4	26.37	30.76	25.72	42.14	46.95	43.56

* Unit expressed as mg/ml

Table 14. The hepatic microsomal total CYP contents in individual rat in *in vitro**

Rat	Treatment group					
number	Corn oil	β-NF	Sterile water	PB	Water	Ethanol
1	0.668	0.882	0.445	1.190	0.527	0.426
2	0.656	0.761	0.516	0.885	0.358	0.514
3	0.352	0.796	0.577	0.742	0.662	0.568
4	0.566	0.844	0.533	0.876	0.668	0.640

* Unit expressed as nmol/mg protein

Table 15. The hepatic microsomal alkoxyresorufin o-dealkylase activity in individual rat individual in *in vitro* *

Rat number	EROD		MROD		BROD	
	Corn oil	β-NF	Water	PB	Water	Ethanol
1	68	1004	32	112	26	910
2	132	840	4	108	62	829.2
3	20	952	8	140	8	822.4
4	48	908	12	124	8	695.2

* Unit expressed as pmol/mg protein/min

Table 16. The hepatic microsomal aniline 4-hydroxylase activity in individual rat in *in vitro* *

Rat number	Aniline 4-hydroxylase activity	
	Water	Ethanol
1	0.155	0.520
2	0.160	0.361
3	0.303	0.289
4	0.154	0.639

* Unit expressed as nmol/mg protein/min

Table 17. Effect of 1%DMSO on aniline 4-hydroxylase activity in individual rat in *in vitro*

Rat number	Protein concentration*	Aniline 4-hydroxylase**	
		control	1% DMSO
1.	28.40	0.403	0.035
2.	26.10	0.534	0.051
3.	29.14	0.472	0.031
4.	30.56	0.243	0.055

* Unit expressed as mg/ml

** Unit expressed as nmol/mg protein/min

Table 18. Effect of acetonitrile on aniline 4-hydroxylase activity in individual rat in *in vitro*

Rat	Protein	Aniline 4-hydroxylase**			
number	Concentration*	Control	0.1% Acetonitrile	Control	1% Acetonitrile
1.	39.25	0.520	0.403	0.520	0.497
2.	39.25	0.361	0.340	0.361	0.379
3.	44.53	0.289	0.236	0.289	0.466
4.	43.56	0.639	0.409	0.639	0.607

* Unit expressed as mg/ml

** Unit expressed as nmol/mg protein/min

Table 19. Effect of HPP on ethoxyresorufin o-dealkylase activity in individual rat in *in vitro* *

Rat number	Concentration of HPP (μM)					
	0	0.1	1	10	100	1000
1	1004	812	736	828	760	672
2	840	776	816	896	832	740
3	952	900	952	816	916	840
4	908	844	956	804	896	908

* Unit expressed as pmol/mg protein/min

Table 20. Effect of HPP on methoxyresorufin o-dealkylase activity in individual rat in *in vitro* *

Rat number	Concentration of HPP (μM)					
	0	0.1	1	10	100	1000
1	112	112	108	116	100	92
2	108	116	96	112	128	100
3	140	144	140	132	128	108
4	124	124	124	108	124	112

* Unit expressed as pmol/mg protein/min

Table 21. Effect of HPP on benzyloxyresorufin o-dealkylase activity in individual rat in *in vitro* *

Rat number	Concentration of HPP (μM)					
	0	0.1	1	10	100	1000
1	910	708	768	678	396	318
2	829.2	888	718	766	484	376
3	822.4	766	682	916	550	356
4	695.2	870	858	802	528	372

* Unit expressed as pmol/mg protein/min

Table 22. Effect of HPP on aniline 4-hydroxylase activity in individual rats in *in vitro**

Rat	Concentration of HPP (μM)					
Number	0	0.1	1	10	100	1000
1	0.497	0.518	0.616	0.531	0.581	0.543
2	0.379	0.406	0.446	0.347	0.443	0.465
3	0.466	0.323	0.232	0.398	0.310	0.453
4	0.607	0.696	0.530	0.617	0.647	0.487

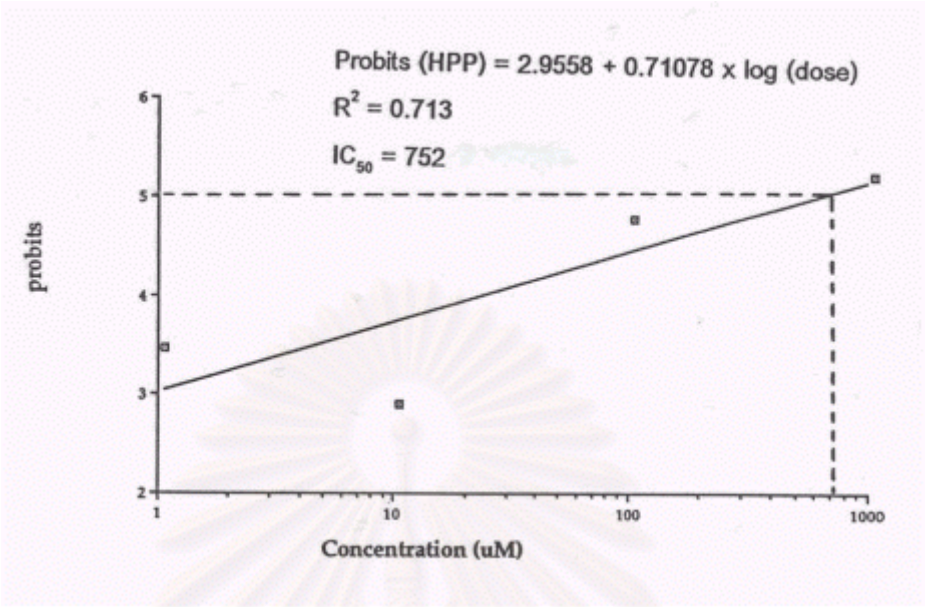
*Unit expressed as nmol/mg protein/min

Table 23. Transformation of % inhibition to probit unit

Concentration of HPP (μM)	% Inhibition*	Probits
1	5.53	3.4018
10	1.47	2.8299
100	38.74	4.7129
1000	55.67	5.1434

*Data were presented as mean ± SD (N = 4)

Figure 19. Estimation of IC_{50} by Probit Analysis



The IC_{50} was calculated from the log dose probits line as:

$Y = 2.9558 + 0.71078 \times \log(dose)$

Curriculum Vitae

Miss Nareerat Ruksuntorn was born in January 19, 1972 in Roi-Et, Thailand. She graduated with a Bachelor of Science in Pharmacy in 1996 from the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. After graduation, she worked as a pharmacist in Roi-Et Hospital for three years