ผลของวัลโปรอิล มอร์โฟลีน ต่อเอนไซม์ไซโตโครม พี 450 ในตับหนูขาว

นางสาวกัลยาณี ยานะ



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# EFFECT OF VALPROYL MORPHOLINE ON RAT HEPATIC CYTOCHROME P450

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For the Degree of Master of Science in Pharmacy

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ศึกษาผลของวัลโปรอิล มอร์โฟลีน (วีพีเอ็ม) ต่อเอนไซม์ไซโตโครม พี 450 (CYP) ในตับหนูขาว โดยการฉีดวีพีเอ็ม และวัลโปรอิค แอซิด (วีพีเอ) ซึ่งเป็นสารต้นแบบของวีพีเอ็ม ในขนาดที่ต้านชักได้ในหนูถีบจักรจำนวนครึ่งหนึ่ง (100 มิลลิกรัม/กิโลกรัม/วัน สำหรับวีพีเอ็ม และ 250 มิลลิกรัม/กิโลกรัม/วัน สำหรับวีพีเอ) และวีพีเอ็ม ขนาด 200 มิลลิกรัม/กิโลกรัม/วัน แก่หนูขาวเพศผู้ ทางหน้าท้อง เป็นเวลา 7 วัน หลังจากนั้นฆ่าหนูแล้วเตรียมไมโครโซมจากตับ ทำการวิเคราะห์หาปริมาณไซโตโครม พี 450 รวม และแอคติวิตีของไซโตโครม พี 450 จากไมโครโซม นอกเหนือจากนี้ยังศึกษาผลของวีพีเอ็มในการยับยั้งเอนไซม์ไซโตโครม พี 450 ด้วย ผลการวิจัยพบว่า ทั้งวีพีเอ็ม และวีพีเอ ไม่มีผลเปลี่ยนแปลงค่าต่างๆเหล่านี้ : ปริมาณไซโตโครม พี 450 รวม แอคติวิตีของ CYP1A1 CYP1A2 และ CYP2E1 ยกเว้นเฉพาะวีพีเอ็มขนาด 200 มิลลิกรัม/กิโลกรัม/วัน ที่มีผลเพิ่มแอคติวิตีของ CYP1A1 วีพีเอ็มทั้งสองขนาดมีผลเพิ่มแอคติวิตีของ CYP2B1 และ CYP2B2 อย่างมีนัยสำคัญทางสถิติ และวีพีเอ็มขนาด 200 มิลลิกรัม/กิโลกรัม/วัน มีผลเพิ่มแอคติวิตีของ CYP2B1 และ CYP2B2 มากกว่าเมื่อให้ในขนาด 100 มิลลิกรัม/กิโลกรัม/วัน จากการศึกษาในหลอดทดลองพบว่า วีพีเอ็มไม่มีผลในการยับยั้ง CYP2E1 ที่ทุกความเข้มข้นของวีพีเอ็มที่ใช้ในการศึกษานี้ (0, 0.1, 1, 10, 100 และ 1000 ไมโครโมลาร์) วีพีเอ็มไม่มีผลในการยับยั้ง CYP1A1 CYP1A2 CYP2B1 และ CYP2B2 ที่ความเข้มข้นต่ำ แต่มีผลยับยั้งเอนไซม์เหล่านี้ที่ความเข้มข้นสูง กล่าวคือมีผลยับยั้ง CYP1A1 และ CYP1A2 ที่ความเข้มข้น 1000 ไมโครโมลาร์ มีผลยับยั้ง CYP2B1 และ CYP2B2 ตามความเข้มข้นที่มากขึ้น ที่ความเข้มข้น 100 และ 1000 ไมโครโมลาร์ ควรทำการศึกษาต่อไปถึงผลของวีพีเอ็มต่อ CYP isoforms อื่นๆที่สำคัญเกี่ยวข้องในการเปลี่ยนแปลงยาหรือสารแปลกปลอมต่างๆในคน รวมถึงการศึกษากลไกที่ใช้อธิบายการที่วีพีเอ็มมีผลเหนี่ยวนำและยับยั้ง CYP isoforms ต่างๆที่พบในการศึกษาครั้งนี้

ภาควิชาเภสัชวิทยา ลายมือชื่อนิสิต.....

สาขาวิชาเภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEY WORD: VALPROYL MORPHOLINE, CYTOCHROME P450

KANLAYANEE YANA : EFFECT OF VALPROYL MORPHOLINE ON RAT HEPATIC CYTOCHROME P450. THESIS ADVISOR : POL. COL. LT. DR. SOMSONG LAWANPRASERT, THESIS CO-ADVISOR : ASSIST. PROF. PORNPIMOL KIJSANAYOTIN, 62 pp. ISBN 974-347-018-2

Effect of valproyl morpholine (VPM) on rat hepatic cytochrome P450 (CYP) was investigated. Median effective anticonvulsant dose of VPM in mice (100 mg/kg/day) as well as of valproic acid (VPA, the prototype of VPM)(250 mg/kg/day) were given intraperitoneally to male Wistar rats for 7 days. VPM at a dose of 200 mg/kg/day was also given to another group of animals in the same manner. On the day after, the animals were sacrificed and hepatic microsomal subfractions were prepared. Microsomal total CYP contents and CYP activities were determined. In addition, an inhibition effect of VPM on CYP was also studied. The results showed that there were neither induction effects of VPM nor VPA on total CYP contents as well as on the activities of CYP1A1 CYP1A2 and CYP2E1. The exception was observed with VPM at a dosage of 200 mg/kg/day that showed significant induction effect on CYP1A1 activity. Both dosage of VPM administered to the animals in this study induced CYP2B1 and CYP2B2 of which the activities were more enhanced by 200 mg/kg/day of VPM than by the dosage regimen of 100 mg/kg/day. No inhibition effect of VPM at any concentrations used in the study (0, 0.1, 1, 10, 100, and 1000 μM) on CYP 2E1 in an *in vitro* study was noted. VPM demonstrated no inhibition effect on CYP1A1 CYP1A2 CYP2B1 and CYP2B2 at the lower concentrations but exhibited significant inhibition effect on these isoforms of CYP at the higher concentrations as following: CYP1A1 and CYP1A2 were inhibited by VPM at 1000 μM whereas CYP2B1 and CYP2B2 were inhibited at 100 and 1000 μM in the dose-related manner. Effect of VPM on other isoforms of CYP involving human drug metabolism, should be further explored. Further study to clarify the mechanism of induction or inhibition of VPM on the corresponding affected isoforms of CYP should also be proceeded.

Department…….Pharmacology…….Student's signature…….

Field of study… Pharmacology…….Advisor's signature…….

Academic year ..2000……..Co-advisor's signature……...

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# List of Abbreviations

| | |
|---|---|
| α | = alpha |
| β | = beta |
| °C | = degree celcius |
| ω | = omega |
| μl | = microlitre |
| 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) |
| FAD | = flavin adenine dinucleotide |
| FMN | = flavin mononucleotide |
| g | = gram |
| G6P | = glucose 6-phosphate |
| G6PD | = glucose 6-phosphate dehydrogenase |
| i.p. | = intraperitonial |
| kg | = kilogram |
| L | = litre |
| $LD_{50}$ | = median lethal dose |
| M | = molar |
| MES | = maximal electroshock |
| mg | = milligram |
| mg/kg | = milligram per kilogram body weight |

mM = millimolar

MROD = methoxyresorufin O-dealkylation

NADP = nicotinamide adenine dinucleotide phosphate

NADPH = nicotinamide adenine dinucleotide phosphate (reduced form)

nmol = nanomol

PEG400 = polyethylene glycol 400

PGA = 2-propyl-glutaric acid

PI = protective index

pmol = picromol

PROD = pentoxyresorufin O-dealkylation

PTZ = pentylenetetrazole

TCA = trichloroacetic acid

$TD_{50}$ = median neurotoxic dose

Tris = Tris (hydroxymethyl) aminomethane

UDPGT = uridine-5'-diphospho-glucuronyltransferase

VPA = valproic acid

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-pentanoate

3-OH-VPA = 2-propyl-3-hydroxypentanoic acid

4-OH-VPA = 2-propyl-4-hydroxypentanoic acid

5-OH-VPA = 2-propyl-5-hydroxypentanoic acid

VPM = valproyl morpholine

v/v = volume by volume

w/v = weight by volume

# Chapter I

# Introduction

Valproyl morpholine (VPM), one of derivatives of valproic acid (VPA), was synthesized by Assistant Professor Chamnan Patarapanich and collaborators at the Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. This compound was synthesized and developed in an attemp to overcome a number of drawbacks of VPA. VPA is a commonly used anticonvulsant being effective in the treatment of many types of seizures, including generalized and partial seizures (Alber et al., 1992). However, VPA possesses moderate anticonvulsant activity and produces many unwanted effects, such as hepatotoxicity (Gerber et al., 1979; Zimmerman and Ishak, 1982), teratogenicity (Dalen et al, 1980; Gomez, 1981; Robert and Guibaud, 1982), etc. A study of Namthongsakun (2000) on the anticonvulsant activity and neurotoxicity of VPM showed that VPM possessed a higher anticonvulsant activity against maximal electroshock seizure as well as a greater relative safety margin, as compared to VPA. Despite demonstrating no change with locomotor activity test, VPM prolonged barbiturate sleeping time. This probably resulted from the effect of VPM directly on CNS or the inhibition of cytochrome P450 (CYP) responsible for the degradation of barbiturate.

CYP, an important phase I enzyme, is responsible for many drug/xenobiotic oxidation reactions. CYP in families 1-3, such as CYPs1A1, 1A2, 2B1, 2B2, 2E1, 3A4 etc., have been reported to bioactivate certain classes of chemicals to mutagenic and carcinogenic intermediates (Robertson et al., 1983; Aoyama et al., 1990; Levin et al., 1986; Yoo et al., 1988). Effect of a new compound on CYP, either induction and inhibition, not only is a beneficial information for the process of drug development regarding the possibility of drug-drug interactions but also give an information of the substance possibility to increase the risk of chemical carcinogenesis. Therefore, the aim of this study was primarily to investigate the effect of VPM on CYPs, including CYPs1A1, 1A2, 2B1, 2B2 and 2E1 both in *ex vivo* and *in vitro*.

**Hypothesis**

Valproyl morpholine demonstrated an induction and/or inhibition effects on rat hepatic cytochrome P450.

**Benefit gained from the study**

To obtain preliminary data on whether VPM possessed an induction and/or inhibition effects on hepatic CYP, especially CYP isoforms involving in a bioactivation certain classes of drugs, chemicals and environmental pollutants resulting in reactive metabolites. Results from this study, are not only a beneficial information for the process of drug development regarding the possibility of drug-drug interactions but also providing an information of the substance possibility to increase the risk of chemical carcinogenesis.

**Study design and process**

Experimental design: *ex vivo* and *in vitro* study

The following processes were performed:

1. An *ex vivo* study was used for an induction study
   - 1.1. Animal dosing for 7 days
   - 1.2. Preparation of liver microsomes
   - 1.3. Determination of hepatic microsomal enzyme activities
2. An *in vitro* study was used for an inhibition study
   - 2.1. Induction of the animals' hepatic enzymes by various inducers such as phenobarbital, β-naphthoflavone and ethanol
   - 2.2. Preparation of liver microsomes
   - 2.3. Determination of hepatic microsomal enzyme activities
   - 2.4. *In vitro* inhibition studies
3. Data collecting and analysis
4. Writing a thesis

# Chapter II

# Literature reviews

## Metabolism

Most pharmacologically active molecules are lipid soluble and remain un-ionized or only partially ionized at physiological pH. These substances are altered chemically by the metabolizing enzymes in the liver and extrahepatic tissues, usually into less active than the parent compound or may be inactive. However, in some cases numerous chemical procarcinogens form reactive metabolites capable of binding covalently to protein or nucleic acid—a critical step to mutagenicity, cytotoxicity, and carcinogenicity. Therefore, insight into the biotransformation and bioactivation of xenobiotics becomes an undisputable prerequisite for the assessment of drug safety and risk estimation of chemicals and drugs (Mayer, 1996., Williams, 1995).

The pathways of xenobiotic metabolism have been divided into two major categories (Table 1). Phase I reactions include oxidation, reduction, hydrolysis and hydration as well as other rarer reactions. In these enzymatic reactions the xenobiotics are transformed of the functional groups resulting more water-soluble metabolites. Phase II reactions (conjugation) increase the polarity of the drugs or xenobiotics and therefore more readily excreted. Most substances undergo both phase I and phase II reactions, sequentially.

**Table 1** Reactions classed as phase I and phase II metabolism (Gibson and Skett, 1994)

| Phase I | Phase II |
|---|---|
| Oxidation | Glucuronidation/Glucosidation |
| Reduction | Sulfation |
| Hydrolysis | Methylation |
| Hydration | Acetylation |
| Dethioacetylation | Amino acid conjugation |
| Isomerization | Glutathione conjugation |
| | Fatty acid conjugation |
| | Condensation |

Oxidation is probably the most common reaction in xenobiotic metabolism catalyzed by a group of membrane-bound mixed-function oxidases in the smooth endoplasmic reticulum of the liver and other extrahepatic tissues. This group of enzymes is called cytochrome P450 monooxygenase enzyme system.

**Cytochrome P450 (CYP)**

The cytochrome P450 monoxygenase enzyme system consists of at least two protein components: a haem-containing enzyme (a haemoprotein) with iron protoporphyrin IX as the prosthetic group (Figure 1) called cytochrome P450 (CYP) and a flavoprotein called NADPH-cytochrome P450 reductase containing both flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). CYP is the substrate and oxygen-binding site of the enzyme system, whereas the reductase serves as an electron carrier, shuttling electrons from NADPH to CYP. The third component essential for electron transport from NADPH to CYP is a phosphatidylcholine. In some cases, cytochrome $b_5$ increase the rate of catalysis because of its ability to shuttle the second electron.

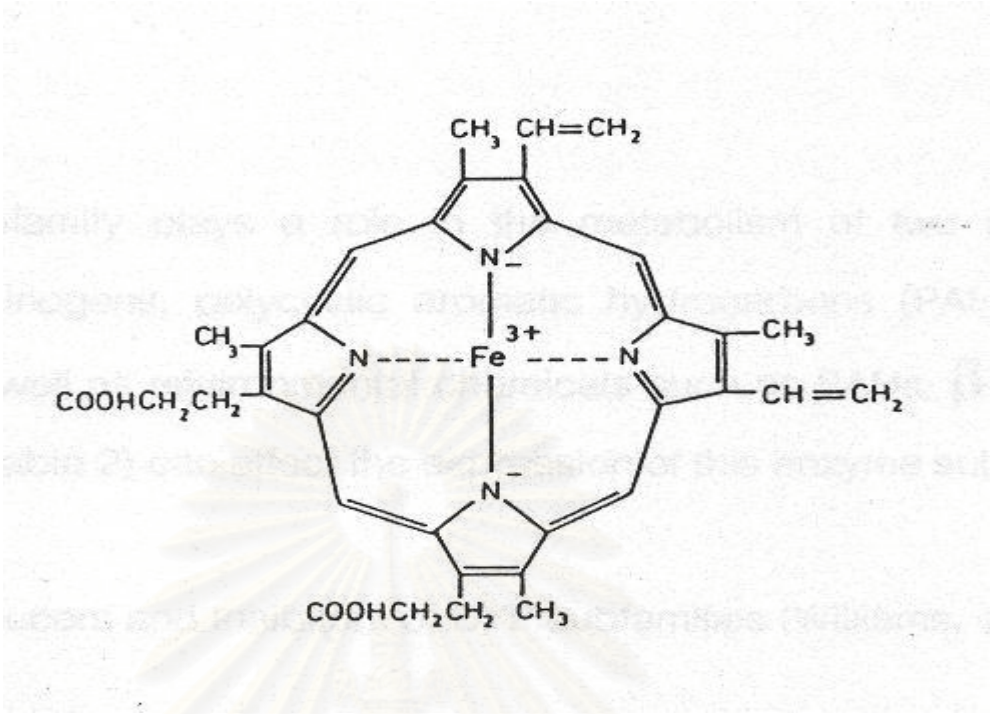


**Figure 1** Structure of ferric protoporphyrin IX, the prosthetic group of CYP (Gibson and Skett, 1994)

CYP functions as a multicomponent electron-transport system responsible for the oxidative metabolism of a variety of endogenous substrates (such as steroids, fatty acids, prostaglandins, and bile acids) and exogenous substrates (xenobiotics), including drugs, carcinogens, insecticides, plant toxins, environmental pollutants, and many other foreign chemicals. This enzyme catalyzes xenobiotic transformation in ways that usually lead to detoxification, but in many cases, they lead to products with greater cytotoxic, mutagenic, or carcinogenic properties. CYP occurs in many different forms (“isozymes” or “isoforms”) which differ from each other by their amino acid sequences. They also differ in spectral, electrophoretical, and immunological properties as well as different substrate affinities. In addition, these isozymes differ in their regulation and tissue distribution (Mayer, 1996). Due to the fact that liver contains multiple forms of CYP, CYPs involved in drug metabolism were often refered to as having broad and somewhat overlapping substrate specificities (Wrighton et al., 1996).

Mammalian CYP superfamily consists of six families (CYP7, CYP11, CYP17, CYP19, CYP21, and CYP27) involved in steroid and bile acid metabolism; and four families (CYP1, CYP2, CYP3, and CYP4) appearing to be responsible for xenobiotic metabolism (Williams, 1995).

### Family 1

CYP1A subfamily plays a role in the metabolism of two important classes of environmental carcinogens, polycyclic aromatic hydrocarbons (PAHs) and aryl amines. Genetic factors as well as environmental chemicals such as PAHs, β-naphthoflavone, and indole-3-carbinol (Table 2) can affect the expression of this enzyme subfamily.

**Table 2** Inducers and Inhibitors of CYP subfamilies (Williams, 1995)

| Inducers | Inhibitors |
|---|---|
| **CYP1A** | **CYP3A** |
| 3-Methylcholanthrene and PAH | Imidazole("conazoles") antifungals (ketoconazole, |
| Tetrachlorodioxin | fluconazole, clotrimazole, miconazole) |
| Smoking | Erythromycin and analogs |
| β-Naphthoflavone | 17α-Ethynylestradiol |
| Indole-3-carbinol (from cruciferous plants) | Triacetyloleandomycin |
| **CYP2E** | Gestodene |
| Ethanol | Quinidine |
| Dimethyl sulfoxide | **Others** |
| Acetone | Chloramphenicol (CYP2B, CYP2C) |
| Isoniazid | 21-Halosteroids (CYP2C, CYP21) |
| Pyrazoles | Quinidine (CYP2D6) |
| **CYP3A** | Cimetidine (nonselective) |
| Phenobarbital (and CYP2B, CYP2C) | Fluoroquinolones (CYP1A2) |
| Phenytoin | Sulfinpyrazone (CYP2C9/10) |
| Carbamazepine | Disulfiram (CYP2E1) |
| Dexamethasone (glucocorticoids) | Methoxypsoralen (CYP2E1) |
| Rifampicin, rifabutin, and analogs | Fluvoxamine (CYP1A2) |
| Erythromycin, its analogs, and others macrolides | SKF525A (CYP2B, CYP2C, CYP3A) |
| Phenylbutazone | Spironolactone |
| "Conazoles" antifungals | |
| **CYP4A** | |
| Clofibrate and hypolipidemics | |

CYP1A1 is expressed in the liver, small intestine, placenta, skin, and lung in response to the presence of inducers. This isozyme of CYP metabolizes a range of PAHs, including a large number of procarcinogens and promutagens. Mechanism of induction of the CYP1A1 gene begins with binding of the inducing agents to a cytosolic receptor protein, the Ah receptor, which is translocated to the nucleus and bind to the DNA of the CYP1A1 gene, thus enhancing its rate of transcription.

CYP1A2 is constitutively expressed in the liver, intestine, and stomach; and is also induced by smoking, PAHs, and isosafrole. CYP1A2 metabolizes aryl amines, nitrosamines, and aromatic hydrocarbons, including the bioactivation of procarcinogens and promutagens such as aflatoxin-B1, the aryl amines, caffeine, and many other substances as shown in table 3.

**Table 3** Some procarcinogens and other toxins activated by human CYP (Williams, 1995)

| CYP1A1 | CYP1A2 | CYP2E1 |
|---|---|---|
| Benzo[a]pyrene and other | 4-Aminobiphenyl | Benzene |
| polycyclic aromatic | 2-Naphthylamine | Styrene |
| hydrocarbons | 2-Aminofluorene | Acrylonitrile |
| CYP3A4 | 2-Acetylaminofluorene | Vinylbromide |
| Aflatoxin B1 | 2-Aminoanthracene | Trichloroethylene |
| Aflatoxin G1 | Heteropolycyclic amines | Carbon tetrachloride |
| Estradiol | (2-aminoquinolines) | Chloroform |
| 6-Aminochrysene | Aflatoxin B1 | Methylene chloride |
| Polycyclic hydrocarbon | Ipomeanol | N-nitrosodimethylamine |
| dihydrodiols | | 1,2-Dichloropropane |
| | | Ethyl carbamate |

Family 2

CYP2B subfamily can be induced by phenobarbital. Two isozymes, 2B1 and 2B2, have similar broad, overlapping specificities toward a number of substrates. They were found to possess 97% c-DNA deduced amino acid sequence similarity (Kumar et al., 1983). Mechanism of the CYP2B gene is poorly understood. Phenobarbital administration to rats causes a rapid transcriptional activation of the CYP2B1/2B2 genes (Hardwick et al., 1983). Eventhough CYP2B1 and CYP2B2 genes are very similar, marked differences exist in their expression (Gonzalez, 1990). Rat CYP2B1 and CYP2B2 genes contain different tissue-specific enhancer and inducer control elements. Rat CYP2B1 is 76% similar to human CYP2B6 (Williams, 1995). Human CYP2B6 is also inducible by phenobarbital in liver cells (Waxman, 1999). However, the role of CYP2B6 in human drug metabolism is questionable.

CYP2E1 is constitutively expressed in the liver, kidney, intestine, and lung; and is induced by ethanol, isoniazid, and other chemicals as shown in table 2. This isoform is toxicologically important in that it converts many low- molecular weight organic compound, including carbon tetrachloride, N-nitrosodimethylamine, acetaminophen, and ethanol to relative reactive intermediates (Wrighton et al., 1996). Most of the same compounds that induce CYP2E1 are also substrates for this enzyme. The induction of this enzyme in human can cause enhanced susceptibility to toxicity and carcinogenesis of CYP2E1 substrates. The mechanism of induction appears to be a combination of an increase in CYP2E1 transcription, m-RNA translation efficiency, and stabilization of CYP2E1 against proteolytic degradation.

Family 3

At least four isoforms of CYP constitute the human CYP3A subfamily (Table 4). Enzymes in CYP3A subfamily is the most abundant CYPs in human liver, especially CYP3A4 comprises approximately 25% of total CYP. This subfamily are responsible for the metabolism of a large number of structurally diverse drugs, xenobiotics, and

endobiotics. They also appear to be induced or inhibited by a number of xenobiotics (Table 2&4)

**Table 4** The human CYP3A subfamily (Wrighton et al., 1996)

| CYP | Marker catalytic activities | Inhibitors | Inducer |
|---|---|---|---|
| 3A3/4 | Erythromycin N-demethylase | Troleandomycin | Troleandomycin |
| | Cyclosporin metabolism | Gestodene | Rifampicin |
| | Nifedipine oxidase | Quercetin | Barbiturates |
| | Midazolam hydroxylase | | Phenytoin |
| | Testosterone 6$\beta$-hydroxylase | | Dexamethasone |
| | Cortisol 6$\beta$-hydroxylase | | |
| 3A5 | Testosterone 6$\beta$-hydroxylase | Troleandomycin | |
| | Nifedipine oxidase | Gestodene | |
| | Midazolam hydroxylase | | |
| 3A7 | Dehydroepiandrosterone-3-sulfate 16$\alpha$-hydroxylase | | |

CYP3A3 and CYP3A4 are so highly related that the majority of studies performed to date have not been able to distinguish their contribution. CYP3A5 appears to metabolize fewer substrates than CYP3A3/3A4. CYP3A7 is expressed only in human fetal livers (approximately 50% of total fetal CYP) and little is known about its substrate specificity, except of the 16$\alpha$-hydroxylation of dehydroepiandrosterone-3-sulfate. In view of the toxicological implications, CYP3A4 has been reported to bioactivate many procarcinogens and xenobiotics (Table 3).

Family 4

A CYP induced by hypolipidemic drugs such as clofibrate was purified from rats and found to metabolite lauric acid at its ω-position (Tamburini et al., 1984). Clofibrate administration results in a rapid transcriptional activation of CYP4A1 gene (Hardwick et al., 1987). This enzyme is also active in the metabolism of arachidonic acid (Bains et al., 1985). CYP4A1 gene is induced in both liver and kidney (Hardwick et al., 1987) whereas CYP4A2 is constitutively expressed in kidney (Kimura et al., 1989). The high level of activity of the CYP4 family toward fatty acids suggests that they may carry out physiologically important hydroxylation reactions.

**Mechanism of induction of CYP**

Induction is an adaptive response that protects cells from toxic xenobiotics by increasing the detoxification activity. Drugs, environmental chemicals, and many other xenobiotics enhance the metabolism of themselves and/or of other co-ingested/inhaled compounds, resulting in a reduction of pharmacological effects or an increase of toxicity as a result of an increase formation of reactive metabolites. A number of chemicals which induce the individual CYP were shown in table 2. The time course of induction varies with different inducing agents and different isoforms. Also, the induction response is dose-dependent and reversible. Enzyme induction can also enhance the activation of procarcinogens or promutagens. Therefore, enzyme induction is important in interpreting the results of chronic toxicity, mutagenicity, or carcinogenicity and explaining certain unexpected drug interactions in patients. The precise mechanisms of CYP induction are not fully understood, except for the induction of certain CYP1A subfamily by PAHs via the Ah receptor (Whitlock, 1995). PAHs interact with a specific cytoplasmic receptor (Ah receptor) and the PAH-receptor complex is then translocated to the nucleus of hepatocyte, sequencing interact with a regulatory site on the CYP1A gene, resulting in an increased transcription of mRNA. Other biochemical effects have been noted on the inductive response of an individual CYP isozyme to the enzyme inducers (Table 5).

**Table 5** Differences in induction mechanisms for CYP (Gibson and Skett, 1994)

| P450 isozyme | Representative inducer | Main induction mechanism |
|---|---|---|
| 1A1 | Dioxin | Transcriptional activation by ligand activated Ah receptor |
| 1A2 | 3-Methylcholanthrene | mRNA stabilisation |
| 2B1/2B1 | Phenobarbital | Transcriptional gene activation |
| 2E1 | Ethanol, acetone isoniazid | Protein stabilization (in part) |
| 3A1 | Dexamethasone | Transcriptional gene activation |
| 4A6 | Clofibrate | Transcriptional activation, mediated by peroxisome proliferator activated receptor |

It is clear that enzyme inducers have a variety of effects on the functional components of the mixed function oxidase system, particularly on the terminal haemoprotein, CYP. The proposed mechanism of induction involves binding of the particular RNA polymerase to the promoter segment of gene causing expression of the respective CYP structural gene with increased transcription of mRNA, resulting in increased CYP isoform synthesis. Moreover, induction of CYP may arise as a consequence of decreased degradation of the protein enzyme or the corresponding mRNA, activation of preexisting components, or a combination of these two processes.

**Mechanism of inhibition of CYP**

Many therapeutic drugs and environmental xenobiotics have been reported to inhibit CYP in the liver via different mechanisms. The inhibition effects can take place in several ways including the destruction of pre-existing enzymes, an inhibition of enzyme synthesis, an inactivating of the drug-metabolizing enzymes and a competition for the enzyme catalytic sites. The inhibition of drug metabolism may result in undesirable

elevations in plasma drug concentrations. Thus, the inhibition of CYP is of clinical importance for both therapeutic and toxicological reasons.

Mechanisms of CYP inhibition can be divided into three categories (Lin and Lu, 1998; Williams, 1995):

**1. Reversible inhibition**

Reversible inhibition is probably the most common mechanism responsible for the documented drug interactions. Generally, this interactions and as a result of competition at the CYP active site and probably involve only the first step of the CYP catalytic cycle. Many of the potent reversible CYP inhibitors are nitrogen-containing drugs, including imidazoles, pyridines, and quinolines. These compounds can interact at the prosthetic haem iron and/or bind to the lipophilic region of the protein. Inhibitors that bind to both regions of CYP are more potent inhibitors. The potency of an inhibitor is determined both by its lipophilicity and by the strength of the bond between its nitrogen lone pair electron and the prosthetic haem iron. Drugs interacting reversibly with CYP include the fluoroquinolone antimicrobials, cimetidine, the "conazole" antifungals, quinidine, and diltiazem.

**2. Quasi-irreversible inhibition via metabolic intermediate complexation**

A large number of compounds, including methylenedioxybenzenes, alkylamines, macrolide antibiotics, and hydrazines; undergo metabolic activation by CYP enzyme to form inhibitory metabolites called an metabolic intermediates (MI). These metabolites have a high affinity for forming stable complexes with the prosthetic haem of CYP, sequestering in a functionally inactive state. MI complexation can be reversed and the catalytic function of ferric CYP can be restored by *in vitro* incubation with highly lipophilic compounds that displace the metabolic intermediate from the active site. Dissociation or displacement of the MI complex results in the reactivation of CYP functional activity. However, in an *in vivo* situations, the MI complex is so stable that the

CYP involved in the complex is unavailable for drug metabolism, and synthesis of new enzymes is the only means by which activity can be restored.

### 3. Irreversible inactivation of CYP

Drugs containing certain functional groups can be oxidized by CYP to reactive intermediates that cause irreversible inactivation of the enzyme prior to its release from the active site. Because metabolic activation is required for enzyme inactivation, these drugs are classified as mechanism-based inactivators or suicide substrates. The mechanism-based inactivation of CYP may result from irreversible alteration of haem or protein, or a combination of both. Drugs that are mechanism-based inactivators of CYP, including 17$\alpha$-ethynylestradiol, norethisterone, chloramphenicol, spironolactone, etc.

### Valproic acid (VPA)

VPA is a branched-chain fatty acid (Figure 2). This drug is commonly used and effective in the treatment of many types of seizures, including generalized and partial seizures (Alber et al., 1992).

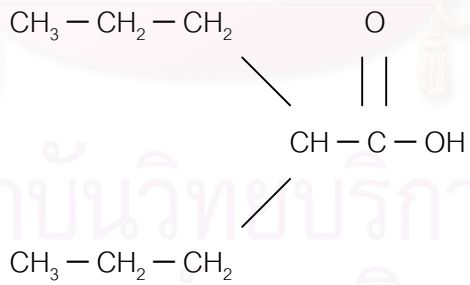

**Figure 2** Structure of valproic acid (VPA)

VPA predominately undergoes hepatic metabolism, with less than 5 % of the dose excreated unchanged in urine. Major metabolism occurs by UDPGT-catalyzed glucuronide conjugation and $\beta$-oxidation with minor CYP-dependent metabolism (Anderson, 1998).

β-oxidation of VPA results in a formation of 2-propyl-2-pentanoic acid (2-ene-VPA) and 2-propyl-3-keto-pentanoic acid (3-keto-VPA), which are both major plasma metabolites. Oxidation, probably by the microsomal CYP (Heinemeyer et al., 1984) results in the formation of three minor metabolites, including 2-propyl-3-hydroxypentanoic acid (3-OH-VPA), 2-propyl-4-hydroxypentanoic acid (4-OH-VPA) and 2-propyl-5-hydroxypentanoic acid (5-OH-VPA)(Figure 3)(Nau et al., 1984). The 2-n-propyl-4-pentanoic acid (4-ene-VPA), an unsaturated metabolite, is proposed occurred during the formation of 4-OH-VPA and 5-OH-VPA (Figure 4) (Rettie et al., 1987). Other studies support that both 4-ene-VPA and its principal metabolite, 2-n-propyl-2,4-pentadienoic acid (2,4-diene-VPA) are potent inducers of microvesicular steatosis via an inhibition of fatty acid β-oxidation (Gerber et al., 1979; Zimmerman and Ishak, 1982).

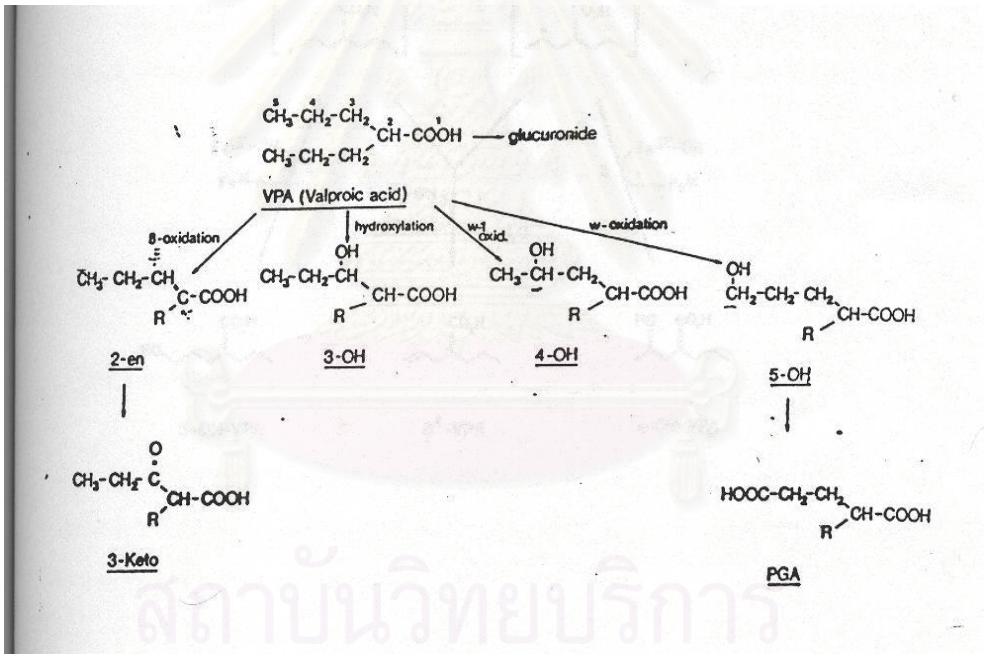


**Figure 3** Structure of valproic acid metabolites that have been measured by gas chromatography-mass spectrometry. [2-ene, 2-propyl-2-pentanoic acid; 3-keto, 2-propyl-3-keto-pentanoic acid; 3-OH, 2-propyl-3-hydroxypentanoic acid; 4-OH, 2-propyl-4-hydroxypentanoic acid; 5-OH, 2-propyl-5-hydroxypentanoic acid; PGA, 2-propyl-glutaric acid (Nau et al., 1984)]

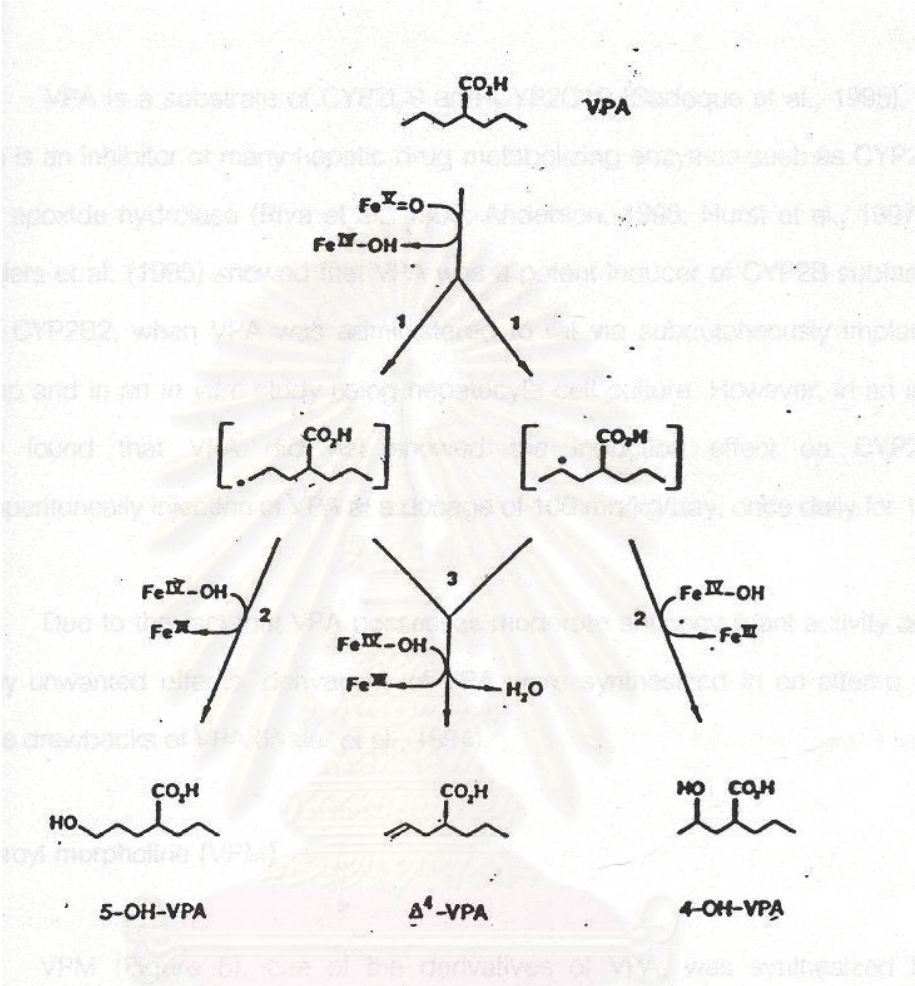


**Figure 4** Proposed scheme for the cytochrome P450-dependent metabolism of VPA to 4-OH-, 5-OH- and $\Delta^4$-VPA (Rettie et al., 1987)

One previous study reported that the metabolic flux through the 4-ene-VPA in human was mediated by CYP2C9 and CYP2A6 (Sadeque et al., 1997). Also, a study in rat showed that CYP2B1&2B2 were involved in the production of hepatotoxin 4-ene-VPA metabolite (Rogiers et al., 1995).

Beside the hepatotoxicity, VPA were reported to produce another side effects, including embryotoxicity (Dalen et al., 1980; Gomez, 1981; Robert and Guibaud, 1982), GI side effects (nausea, vomiting, diarrhea, abdominal cramp) and CNS side effects (drawsiness, ataxia, tremor), etc. (McNamara, 1996).

VPA is a substrate of CYP2C9 and CYP2C19 (Sadeque et al., 1995). Furthermore, VPA is an inhibitor of many hepatic drug metabolizing enzymes such as CYP2C9, UDPGT, and epoxide hydrolase (Riva et al., 1996; Anderson, 1998; Hurst et al., 1997). A study of Rogiers et al. (1995) showed that VPA was a potent inducer of CYP2B subfamily, CYP2B1 and CYP2B2, when VPA was administered to rat via subcutaneously implanted osmotic pump and in an *in vitro* study using hepatocyte cell culture. However, in an *in vivo* study, they found that VPA did not showed the induction effect on CYP2B following intraperitoneally injection of VPA at a dosage of 100 mg/kg/day, once daily for 10 days.

Due to the fact that VPA possesses moderate anticonvulsant activity and produces many unwanted effects, derivatives of VPA were synthesized in an attemp to overcome these drawbacks of VPA (Bialer et al., 1994).

**Valproyl morpholine (VPM)**

VPM (Figure 5), one of the derivatives of VPA, was synthesized by Assistant Professor Chamnan Patarapanich and collaborators at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

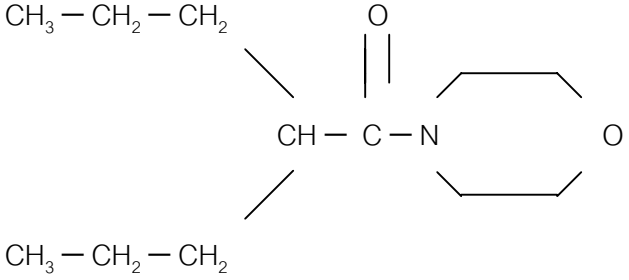


**Figure 5** Structure of valproyl morpholine (VPM)

A study of Namthongsakun (2000) on the anticonvulsant activity and neurotoxicity of VPM, showed that VPM was effective in the maximal electroshock (MES) induced seizure but not in the pentylenetetrazole (PTZ) induced seizure whereas VPA was effective in both MES and PTZ tests. VPM was more potent than VPA. The median effective dose ($ED_{50}$) of VPM was 107 mg/kg body weight whereas the corresponding value of VPA was 230 mg/kg body weight. In term of safety, based on the results that the relative safety margin ($LD_{50}/ED_{50}$) of VPM (5.89) was higher than that of VPA (2.97), VPM seemed to be safer than VPA. The median neurotoxic doses ($TD_{50}$) of VPM and VPA as determined by the rotorod test were 151 and 309 mg/kg body weight, respectively. However, both VPM and VPA possessed similar protective index ($PI=TD_{50}/ED_{50}$) of 1.41 and 1.34, respectively (Table 6).

Eventhough VPM showed no change with locomotor activity test, it significantly prolonged barbiturate sleeping time at the dosage of 100 and 200 mg/kg body weight.

**Table 6** $ED_{50}$, $TD_{50}$, $LD_{50}$, PI ($TD_{50}/ED_{50}$), and relative safety margin ($LD_{50}/ED_{50}$) of an intraperitoneal administration of VPM and VPA in MES and PTZ models (Namthongsakun, 2000)

| Parameters | Animal models | VPM | VPA |
|---|---|---|---|
| $ED_{50}$ (mg/kg BW.) | MES | 107 | 230 |
| | PTZ | - | 77 |
| $TD_{50}$ (mg/kg BW.) | Rotorod | 151 | 309 |
| PI ($TD_{50}/ED_{50}$) | MES | 1.41 | 1.34 |
| | PTZ | - | 4.01 |
| $LD_{50}$ (mg/kg BW.) | - | 631 | 685 |
| Relative safety margin ($LD_{50}/ED_{50}$) | MES | 5.89 | 2.97 |
| | PTZ | - | 8.89 |

The prolonged barbiturate sleeping time characteristic of VPM probably resulted from the effect of VPM directly on CNS or its inhibitory effect on hepatic drug metabolizing enzymes, especially CYPs. CYP, an important phase I enzymes, is responsible for many drug/xenobiotic oxidation reactions. CYPs in the families of 1-3, especially CYP1A1&1A2, CYP2B1&2B2, CYP2E1, CYP3A4 etc., have been reported to bioactivate certain classes of chemicals to mutagenic and carcinogenic intermediates (Soucek and Gut, 1992). Effect of a new compound on CYP, either induction or inhibition, not only is beneficial information for the process of drug development regarding the possibility of drug-drug interaction but also give an information of the substance possibility to increase the risk of chemical carcinogenesis.

Therefore, the aim of this study was primarily to investigate the effect of VPM on CYP, including CYP1A1&1A2, CYP2B1&2B2 and CYP2E1 both *in vivo* and *in vitro*.

# Chapter III

# Materials and Methods

## Animals

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

## Chemicals

These following chemicals were used in the experimentation:

4-Aminophenol, aniline hydrochloride, bovine serum albumin, benzyloxy resorufin, cupric sulfate, dimethylsulfoxide (DMSO), ethoxyresorufin, Folin& Ciocalteu's phenol reagent, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PD), methoxyresorufin, β-naphthoflavone, nicotinamide adenine dinucleotide phosphate (NADP), potassium phosphate, potassium phosphate monobasic anhydrous, pentoxyresorufin, resorufin, sodium carbonate, sodium citrate, sodium phosphate dibasic anhydrous, Trisma® base and valproic acid (Sigma, U.S.A.)

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

Acetonitrile, ethanol and glycerol (Carlo Erba, U.S.A.)

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

Sodium dithionite (Fluka Chemic, Japan)

Phenobarbital (Gardinal®)(Zuelling)

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

Valproyl morpholine (VPM) was synthesized by Assistant Professor Dr. Chamnan Patarapanich and collaborators at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

**Instruments**

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

Centrifuge (Kokusan, Japan)

Fluorescence spectrophotometer (Jasco, Japan)

Metabolic shaker bath (Heto, Denmark)

Potter-Elvehjem homogenizer with Teflon pestle and glass homogenizing vessel (Heidoph, Germany)

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

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

Refrigerated ultracentrifuge (Hitachi, Japan)

Spectrophotometer (Jasco, Japan)

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

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

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

**Methods**

1. **Animal treatment**

**1.1. An *ex vivo* study**

Rats were randomly assigned into 4 treatment groups. Each treatment group comprised 6 rats.

1. Control group: PEG 400, which was used as vehicle for VPA and VPM, was administered intraperitoneally to rats, once daily for 7 days.
2. VPA treatment group: VPA, at a dosage of 250 mg/kg/day, was administered intraperitoneally to rats, once daily for 7 days.
3. VPM treatment group 1: VPM, at a dosage of 100 mg/kg/day, was administered intraperitoneally to rats, once daily for 7 days.
4. VPM treatment group2: VPM, at a dosage of 200 mg/kg/day, was administered intraperitoneally to rats, once daily for 7 days.

Four animals were used simultaneously in each experimental period (1 rat / each treatment group). On the day after the seven days of compound administrations, rats were sacrificed for the preparation of liver microsomes. Liver microsomes prepared from the animals in these 4 treatment groups were used for studying the induction and inhibition effects of VPM and VPA on total CYP contents and activities of CYP1A1&1A2, CYP2B1&2B2 and CYP2E1.

1.2. An *in vitro* study

Rats were randomly assigned into 3 treatment groups. Each treatment group comprised 4 rats.

1. Phenobarbital treatment group: Phenobarbital, at a dosage of 80 mg/kg/day, was administered intraperitoneally to rats, once daily for 3 days. Liver microsomes prepared from the animals in this treatment group were used for studying the inhibition effect of VPM on CYP2B1&2B2.

   Additional four rats were also given sterile water for injection, which was used as a solvent of phenobarbital, in the same manner.

2. β-Naphthoflavone treatment group: β-Naphthoflavone, at a dosage of 80 mg/kg/day, was administered intraperitoneally to rats, once daily for 2 days. Liver microsomes prepared from the animals in this treatment group were used for studying the inhibition effect of VPM on CYP1A1&1A2.

   Additional four rats were also given corn oil, which was used as a solvent of β-naphthoflavone, in the same manner.

3. Ethanol treatment group: For an induction of CYP 2E1, the method of Hu et al. (1995) was used with slight modification. Briefly, ethanol was added to the drinking water by stepwise increase from 3 to 7 %, for 6 days (3, 4, 5, 6, 7, and 7 %, respectively). After 6 days, the rats were given orally a 20 %v/v ethanol solution three time daily (at 7 a.m., 1 p.m., and 7 p.m.) for three day. The total daily dose of ethanol was increased from 8 to 12 g/kg body weight/day (8, 10, 12 g/kg body weight/day, respectively). During three days of the oral administration of ethanol, all rats were maintained on the 7 % ethanol drinking

water. Liver microsomes prepared from the animals in this treatment group were used for studying the inhibition effect of VPM on CYP2E1.

Additional four rats were also given double distilled water, which was used to dilute ethanol, in the same manner.

**2. Preparation of liver microsomes**

**2.1. Reagents**

1. Phosphate buffer, pH 7.4
   One litre of phosphate buffer, pH 7.4 consisted of 1.78 g of $KH_2PO_4$, 9.55 g of $Na_2HPO_4$, and 11.50 g of KCl. The solution was adjusted to pH 7.4 with NaOH or HCl.
2. Phosphate buffer, pH 7.4, containing 20 % glycerol.
3. 0.9 % w/v NaCl.

**2.2. Procedure**

1. On the subsequent day following the treatment period, animals were anesthesia using light ether vapor.
2. After openning the abdominal cavity, livers were perfused *in situ* via portal veins with ice-cold 0.9 % w/v NaCl through the inferior vena cava until the entire organ became pale.
3. The livers were taken out, rinsed with ice-cold 0.9 % w/v NaCl, and blotted dry with gauzes.
4. The whole livers were weighed, cut into pieces and homogenized with 3 volume of phosphate buffer, pH 7.4.
5. The liver homogenates were centrifuged at 10,000 g for 30 minutes at 4°C, using refrigerated superspeed centrifuge, to pellet intact cells, cell debris, nuclei and mitochondria.
6. The supernatant were transferred into ultracentrifuge tubes and centrifuged at

100,000 g for 60 minutes at 4°C, using refrigerated ultracentrifuge.

7. The pellets (microsomal subfractions) were resuspended with 5 ml of phosphate buffer, pH 7.4, containing 20 % glycerol. The microsomal suspensions were aliquoted kept in microtubes and stored at -80°C until the time of enzyme activity assays.

**3. Determination of protein concentrations.**

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

**3.1. Reagents**

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

**3.2. Procedure**

1. 16x125 mm Tubes were labeled in duplicate for 9 standards (0, 50, 100, 150, 200, 250, 300, 350, 400 μg) and for each unknown sample.
2. The following reagents were added in μl to each standard solution tube:

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Standard tube | 0 | 50 | 100 | 150 | 200 | 250 | 300 | 350 | 400 | μg |
| BSA, 1mg/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.

3. To each of the unknown tube, 490 μl of 0.5 M NaOH and 10 μl of microsomal sample was added and mixed thoroughly.
4. After 6.5 ml of working protein reagent was added to each tube in the assay, the tubes were allowed to stand at room temperature for 10 minutes.
5. While 200 μl of Folin & Ciocalteu's phenol reagent was added to each tube in the assay, the tubes were vortexed thoroughly for a minimum of 30 seconds.
6. After the tubes were allowed to stand at room temperature for a minimum of 30 minutes, the absorbances of the solutions were measured by spectrophotometer against the 0 μg standard at 500 nm.

### 3.3. Calculations

1. The average absorbance of each standard was plot against its amount of protein. The best fit regression line was drawn through the points. The amount of protein in each unknown sample was obtained by comparing its absorbance against the standard curve.
2. The protein concentration (mg/ml or μg/μl) in each unknown sample was obtained by dividing its amount of protein (from step 1) with the volume of microsomal sample used (i.e.,10 μl) in the reaction.

**4. Spectral determination of total CYP contents**

Microsomal total CYP contents were determined according to the method of Omura and Sato (1964).

**4.1. Reagents**

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

**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. After a few grains of solid sodium dithionite were added to the 5 ml diluted sample with gentle mixing, the solution was then added to the sample and reference cuvettes (2.5 ml for each cuvette). Both cuvettes were put in 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 one 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 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:

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

**5. Analysis of alkoxyresorufin O-dealkylation**

The rate of hepatic microsomal alkoxyresorufin O-dealkylation was determined according to the method of Burke and Mayer (1974; 1985) and Lubet et al. (1985) with slight modifications. Benzyloxyresorufin and pentoxyresorufin were used as specific substrates of CYP2B1&2B2. Ethoxyresorufin and methoxyresorufin were used as specific substrates of CYP1A1 and CYP1A2, respectively.

**5.1.Reagents**

1. 0.1 M Tris buffer, pH 7.4
2. Resorufin & Alkoxyresorufins

   0.5 mM Resorufin (MW 235)

   1.175 mg of resorufin was dissolved with dimethylsulfoxide (DMSO) qs to 10 ml.

   0.5 mM Benzyloxyresorufin (MW 303)

   1.515 mg of benzyloxyresorufin was dissolved with DMSO qs to 10 ml.

   0.5 mM Ethoxyresorufin (MW 241)

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

   0.5 mM Methoxyresorufin (MW 227)

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

   0.5 mM Pentoxyresorufin (MW 283)

   1.415 mg of pentoxyresorufin was dissolved with DMSO qs to 10 ml.
3. NADPH regenerating system

   Glucose 6-phosphate dehydrogenase (G6PD), pH 7.4

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

   0.5 M Glucose 6-phosphate (G6P), pH 7.4

   1.41 g of glucose 6-phosphate was dissolved with 20 mM $K_3PO_4$ qs to 10 ml, adjusting pH to 7.4 with HCl or NaOH (10 μl contains 5 mmoles of G6P).

0.1 M NADP, pH 7.4

0.765 g of NADP was dissolved with 20 mM $K_3PO_4$ qs to 10 ml, adjusting pH to 7.4 with HCl or NaOH (10 μl contains 1 mmole of NADP).

0.3 M $MgCl_2$, pH 7.4

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

**5.2. Procedure**

1. Microsomes were diluted with 0.1 M Tris buffer, pH 7.4 to measure out 100 μg of protein for the 1 ml of the reaction mixture (100 μl of microsome was diluted with 0.1 M Tris buffer, pH 7.4 qs to 5000 μl).
2. For each ml of the reaction mixture, the following reagents were added:
   a. 30 μl of NADPH regenerating system comprised
      10 μl of 0.1 M NADP
      10 μl of 0.5 M G6P
      10 μl of 0.3 M $MgCl_2$
   b. 10 μl of 0.5 mM Alkoxyresorufin
   c. Varied volume of diluted microsomal suspension containing 100 μg of microsomal protein
   d. 0.1 M Tris buffer, pH 7.4 qs to 990 μl.
3. Three tubes were prepared for each microsomal sample. One tube was a sample blank tube and the others were sample tubes. All tubes were preincubated in a 37°C shaking water bath for 2 minutes.
4. The reaction was started by the addition of 10 μl of G6PD (1 unit of G6PD / 1 ml of reaction mixture volume). For a sample blank, 10 μl of 0.1 M Tris buffer, pH 7.4 was added instead of G6PD.
5. After a 5 minutes incubution, the reaction was stopped by adding 1 ml of

methanol (HPLC grade).

6. The absorbance was measured by fluorescence spectrophotometer using an excitation wavelength of 556 and an emission wavelength of 588.
7. A resorufin standard curve was carried out using 8 concentrations of resorufin: 0.025, 0.05, 0.075, 0.1, 0.2, 0.4, 0.5, 1.0 nmol/ml.

### 5.3. Calculations

Rate of alkoxyresorufin O-dealkylation was calculated by dividing the amount of resorufin formed by the time of incubation (5 minutes) and an amount of microsomal protein (100 μg) used in the reaction. The units were expressed as pmol/mg protein/min.

## 6. Analysis of aniline 4-hydroxylation

The rate of hepatic microsomal aniline 4-hydroxylation was determined according to the method of Schenkman et al. (1967). Aniline hydrochloride was used as a substrate of CYP2E1.

### 6.1. Reagents

1. 10 mM Aniline hydrochloride
   93 mg of aniline hydrochloride was dissolved with 100 ml of double distilled water. The solution was stored in dark brown bottle.
2. 6% w/v Trichloroacetic acid
   60 g of trichloroacetic acid was dissolved with 1 L of double distilled water.
3. 20% w/v Trichloroacetic acid
   200 g of trichloroacetic acid was dissolved with 1 L of double distilled water.
4. 1% w/v Phenol
   20 g of phenol and 40 g of NaOH were dissolved with 2 L of double distilled water.

5. 1 M $Na_2CO_3$

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

6. 10 μM 4-Aminophenol

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

7. 0.1 M Tris buffer pH 7.4
8. 0.1 M NADP
9. 0.5 M G6P
10. G6PD
11. 0.3 M $MgCl_2$

### 6.2. Procedure

1. Microsomes were diluted with 0.1 M Tris buffer, pH 7.4 so as to be able to measure out 5 mg of protein for the 2 ml of the reaction mixture.
2. For each 2 ml of the reaction mixture, the following reagents were added:
   a. 30 μl NADPH regenerating system comprised
      10 μl of 0.1 M NADP
      10 μl of 0.5 M G6P
      10 μl of 0.3 M $MgCl_2$
   b. 500 μl of 10 mM Aniline hydrochloride
   c. Varied volume of microsomal suspension containing 5 mg of microsomal protein
   d. 0.1 M Tris buffer, pH 7.4 qs to 2 ml
3. Three reaction tubes were prepared for each microsomal sample. One tube was a sample blank tube and the others were sample tubes.
4. All tubes were preincubated in a 37°C shaking water bath for 2 minutes. The reaction was initiated by the addition of 20 μl of G6PD. For a sample blank, 20μl of 0.1 M Tris buffer, pH 7.4 was added instead of G6PD.

5. After a 30 minutes incubation time, the reaction was stopped by adding 1 ml of ice-cold 20% w/v trichloroacetic acid and the tubes were kept on ice for 5 minutes.
6. The solution was then centrifuged at 3,000 rpm for 10 minutes.
7. After 1 ml of the supernatant was transferred to a new tube, 1 ml of 1% w/v phenol and 1 ml of 1 M $Na_2CO_3$ were added. The solution was mixed well by vortex mixer and kept at room temperature for 30 minutes.
8. The absorbance was measured by spectrophotometer at a wavelength of 630 nm.
9. A standard curve was carried out using 5 concentrations of 4-aminophenol standard solutions (2, 4, 6, 8, 10 μM), following the procedure from step 7 in the same manner as sample.

### 6.3. Calculations

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

## 7. An *in vitro* enzyme inhibition study

7.1 Inhibition effect of VPM on CYPs1A1, 1A2, 2B1 and 2B2

7.1.1. Various concentrations of VPM (0.1, 1, 10, 100, 1000 μM) were prepared by dissolving VPM in the corresponding solution of substrate (0.5 mM benzyloxyresorufin, 0.5 mM ethoxyresorufin or 0.5 mM methoxyresorufin) used in the reaction.

7.1.2 The reactions were performed in the same manner as described in 5.2 (page 27).

a) To study the inhibition effect of VPM on CYP1A1, liver microsomes prepared from animals in the β-naphthoflavone

treatment group and 0.5 mM ethoxyresorufin with various concentrations of VPM, were used in the reactions.

b) To study the inhibition effect of VPM on CYP1A2, liver microsomes prepared from animals in the β-naphthoflavone treatment group and 0.5 mM methoxyresorufin with various concentrations of VPM, were used in the reactions.

c) To study the inhibition effect of VPM on CYP2B1&2B2, liver microsomes prepared form animals in the phenobarbital treatment group and 0.5 mM benzyloxyresorufin with various concentrations of VPM, were used in the reactions.

7.2. Inhibition effect of VPM on CYP2E1

7.2.1. Various concentrations of VPM (0.1, 1, 10, 100, 1000 μM) were prepared by dissolving VPM with acetonitrile.

7.2.2 The reactions were performed in the same manner as described in 6.2 (page 29). Liver microsomes prepared from animals in the ethanol treatment group were used in the reactions. VPM dissolving in acetonitrile was added in the reaction mixture simultaneously with an addition of aniline hydrochloride. Total amount of acetonitrile in all reaction tubes was adjusted to be an equal amount of 0.1% acetonitrile in the reaction mixture.

**8. Statistics**

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

# Chapter IV
# Results

## 1. An *ex vivo* study

### 1.1. Effect of VPM on hepatic microsomal total CYP contents

VPA at the dosage of 250 mg/kg/day as well as VPM at the dosage of 100 mg/kg/day and 200 mg/kg/day exhibited no significant effects on total CYP contents as compared to the control group (Table 7).

**Table 7** Effect of VPM on hepatic microsomal total CYP contents

| Treatment group | Total CYP contents [a,b] (n=6) |
|---|---|
| 1. Control group | 0.504 ± 0.060 |
| 2. VPA treatment group | 0.445 ± 0.065 |
| 3. VPM treatment group 1 | 0.489 ± 0.085 |
| 4. VPM treatment group 2 | 0.556 ± 0.054 |

[a] Unit expressed as nmol/mg protein

[b] Data were presented as mean ± SD

### 1.2 Effect of VPM on hepatic microsomal alkoxyresorufin O-dealkylation

Both ethoxy- and methoxyresorufin O-dealkylation (EROD and MROD, respectively), which represented CYP1A1 and CYP1A2 activities, respectively, demonstrated no changes following VPM administration. The exception was observed with VPM at a dosage of 200 mg/kg/day that showed significant increase of EROD activity as compared to the control group.

Both benzyloxy- and pentoxyresorufin O-dealkylation (BROD and PROD, respectively), which represented CYP2B1&2B2 activities, were significantly increased following VPM administration of both dosages (100 mg/kg/day and 200 mg/kg/day) as compared to the control group and VPA treatment group. The induction effect of VPM at the dosage of 200 mg/kg/day was significantly higher than that of VPM at the dosage of 100 mg/kg/day.

VPA showed no significant effects on any alkoxyresorufin O-dealkylations as compared to the control group (Table 8).

**Table 8** Effect of VPM on hepatic microsomal alkoxyresorufin O-dealkylation

| Treatment group | Alkoxyresorufin O-dealkylation [a,b] (n=6) | | | |
|---|---|---|---|---|
| | CYP 1A1 activity | CYP 1A2 activity | CYP 2B1 & 2B2 activities | |
| | EROD | MROD | BROD | PROD |
| 1.Control group | 34.67 ± 14.24 | 8.67 ± 2.73 | 20.67 ± 6.02 | 1.67 ± 2.66 |
| 2.VPA treatment group | 45.00 ± 17.10 | 9.00 ± 1.67 | 21.33 ± 9.77 | 4.67 ± 3.01 |
| 3.VPM treatment group 1 | 55.67 ± 16.85 | 11.67 ± 2.94 | 225.00 ± 82.93 *Δ | 34.33 ± 15.92 *Δ |
| 4.VPM treatment group 2 | 62.33 ± 11.62 * | 8.00 ± 4.90 | 426.00 ± 138.16 *Δt | 81.67 ± 30.71 *Δt |

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

[b] Data were presented as mean ± SD

* $P < 0.05$; VPM treatment group vs control group

Δ $P < 0.05$; VPM treatment group vs VPA treatment group

t $P < 0.05$; VPM treatment group 2 vs VPM treatment group 1

### 1.3. Effect of VPM on hepatic microsomal aniline 4-hydroxylation

No significant effects of both VPA and VPM on hepatic microsomal aniline 4-hydroxylation, which represented CYP2E1 activity, were observed as compared to the control group (Table 9).

**Table 9** Effect of VPM on hepatic microsomal aniline 4-hydroxylation

| Treatment group | Aniline 4-hydroxylation [a,b] (n=6) |
|---|---|
| 1. Control group | 0.388 ± 0.126 |
| 2. VPA treatment group | 0.275 ± 0.144 |
| 3. VPM treatment group 1 | 0.351 ± 0.099 |
| 4. VPM treatment group 2 | 0.438 ± 0.137 |

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

[b] Data were presented as mean ± SD

## 2. An *in vitro* study

### 2.1. Inhibition effect of VPM on CYP 1A1 activity

Animals treated with β-naphthoflavone, an inducer of CYP1A subfamily, exhibited an increase of hepatic microsomal total CYP contents (Table 10) as well as the activities of both CYP1A1 (ethoxyresorufin O-dealkylation) and CYP1A2 (methoxyresorufin O-dealkylation) (Table 11).

**Table 10** Effect of β-naphthoflavone on total CYP contents

| Treatment | Total CYP contents [a,b] (n=4) |
|---|---|
| Control group | 0.560 ± 0.150 |
| β-naphthoflavone treatment group | 0.820 ± 0.050 |

[a] Unit expressed as nmol/mg protein

[b] Data were presented as mean ± SD

**Table 11** Effect of β-naphthoflavone on ethoxy- and methoxyresorufin O-dealkylation.

| Treatment | Ethoxyresorufin O-dealkylation [a,b] (CYP1A1 activity) (n=4) | Methoxyresorufin O-dealkylation [a,b] (CYP1A2 activity) (n=4) |
|---|---|---|
| Control group | 67.00 ± 47.60 | 14.00 ± 12.44 |
| β-Naphthoflavone treatment group | 926.00 ± 69.47 | 121.00 ± 14.38 |

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

[b] Data were presented as mean ± SD

VPM demonstrated no inhibition effect on CYP1A1 activity of the concentrations of 0.1, 1, 10 and 100 μM in the reaction mixture. Significant inhibition effect on this enzyme activity was observed at 1000 μM of VPM as compared to the group without VPM (0 μM)(Table 12).

**Table 12** Inhibition effect of VPM on CYP 1A1 activity

| Concentration of VPM In the reaction mixture (μM) | Ethoxyresorufin O-dealkylation[a,b] (CYP1A1 activity) (n=4) |
|---|---|
| 0 | 926.00 ± 69.74 |
| 0.1 | 855.00 ± 25.17 |
| 1 | 863.00 ± 35.98 |
| 10 | 816.00 ± 120.18 |
| 100 | 852.00 ± 120.59 |
| 1000 | 642.00 ± 91.71* |

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

[b] Data were presented as mean ± SD

* $p < 0.05$; 1000 μM of VPM vs 0 μM of VPM

**2.2. Inhibition effect of VPM on CYP 1A2 activity**

VPM demonstrated no inhibition effect on CYP1A2 activity of the concentrations of 0.1, 1, 10 and 100 μM in the reaction mixture. Significant inhibition effect on this enzyme activity was observed at 1000 μM of VPM as compared to the group without VPM (0 μM)(Table 13).

**Table 13** Inhibition effect of VPM on CYP 1A2 activity

| Concentration of VPM In the reaction mixture (μM) | Methoxyresorufin O-dealkylation [a,b] (CYP1A2 activity) (n=4) |
|---|---|
| 0 | 121.00 ± 14.38 |
| 0.1 | 119.00 ± 16.45 |
| 1 | 110.00 ± 12.44 |
| 10 | 118.00 ± 21.04 |
| 100 | 113.00 ± 17.70 |
| 1000 | 83.00 ± 8.87* |

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

[b] Data were presented as mean ± SD

* $p < 0.05$; 1000 μM of VPM vs 0 μM of VPM

### 2.3 Inhibition effect of VPM on CYP 2B1 & 2B2 activities

Animals treated with phenobarbital, an inducer of CYP2B1&2B2, exhibited an increase of hepatic microsomal total CYP contents (Table 14) as well as the activities of CYP2B1&2B2 (benzyloxyresorufin O-dealkylation) (Table 15).

**Table 14** Effect of phenobarbital on total CYP contents

| Treatment | Total CYP contents [a,b] (n=4) |
|---|---|
| Control group | 0.520 ± 0.050 |
| Phenobarbital treatment group | 0.920 ± 0.190 |

[a] Unit expressed as nmol/mg protein

[b] Data were presented as mean ± SD

**Table 15** Effect of phenobarbital on benzyloxyresorufin O-dealkylation

| Treatment | Benzyloxyresorufin O-dealkylation [(a,b)] (CYP2B1&2B2 activities) (n=4) |
|---|---|
| Control group | 26.00 ± 25.46 |
| Phenobarbital treatment group | 814.20 ± 88.75 |

[(a)] Unit expressed as pmol/mg protein/min

[(b)] Data were presented as mean ± SD

Both concentrations of VPM, 100 μM and 1000 μM, showed significantly dose-related inhibition effect on CYP 2B1 & 2B2 activities as compared to the group without VPM (0 μM). All other lower concentrations (0.1, 1, 10 μM) of VPM did not affect the activities of CYP2B1&2B2 (Table 16).

**Table 16** Inhibition effect of VPM on CYP 2B1 & 2B2 activities

| Concentration of VPM In the reaction mixture (μM) | Benzyloxyresorufin O-dealkylation [a,b] (CYP2B1&2B2 activities) (n=4) |
|---|---|
| 0 | 814.20 ± 88.75 |
| 0.1 | 775.50 ± 85.98 |
| 1 | 820.00 ± 64.42 |
| 10 | 746.50 ± 175.05 |
| 100 | 439.00 ± 49.87* |
| 1000 | 80.00 ± 14.24* [t] |

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

[b] Data were presented as mean ± SD

* $p < 0.05$; 1000 μM of VPM vs 0 μM of VPM

[t] $p < 0.05$; 1000 μM of VPM vs 100 μM of VPM

### 2.4. Inhibition effect of VPM on CYP 2E1 activity

Eventhough total CYP contents were not increased (Table 17), rate of aniline 4-hydroxylation which represented CYP2E1 activity was increased following ethanol administration (Table 18).

**Table 17** Effect of ethanol on total CYP contents

| Treatment | Total CYP contents [a,b] (n=4) |
|---|---|
| Control group | 0.550 ± 0.150 |
| Ethanol treatment group | 0.540 ± 0.090 |

[a] Unit expressed as nmol/mg protein

[b] Data were presented as mean ± SD

**Table 18** Effect of ethanol on aniline 4-hydroxylation

| Treatment | Aniline 4-hydroxylation [a,b] (CYP2E1 activity) (n=4) |
|---|---|
| Control group | 0.19 ± 0.07 |
| Ethanol treatment group | 0.45 ± 0.16 |

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

[b] Data were presented as mean ± SD

No inhibition effects of VPM at all concentrations (0.1, 1, 10, 100, 1000 μM) used in this study were observed on the activity of CYP2E1 as compared to the group without VPM (0 μM)( Table 19).

**Table 19** Inhibition effect of VPM on CYP 2E1 activity

| Concentration of VPM In the reaction mixture (μM) | Aniline 4-hydroxylation [a,b] (CYP2E1 activity) (n = 4) |
|---|---|
| 0 | 0.347 ± 0.080 |
| 0.1 | 0.375 ± 0.117 |
| 1 | 0.346 ± 0.101 |
| 10 | 0.349 ± 0.084 |
| 100 | 0.368 ± 0.091 |
| 1000 | 0.324 ± 0.120 |

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

[b] Data were presented as mean ± SD

# Chapter V
# Discussion and conclusion

Results from the *ex vivo* study indicated that VPM possessed no induction effects on CYP 1A1 (at 100 mg/kg/day dosage of VPM), CYP1A2 and CYP2E1. Induction effect on CYP2B1&2B2 was observed following VPM administration and the induction effect was dose-related. Due to the fact that CYP1A1, CYP1A2 and CYP2E1 play a key role in carcinogenic and/or mutagenic activation of many environmental chemicals (Robertson et al., 1983; Aoyama et al., 1990; Levin et al., 1986 and Yoo et al., 1988), lack of induction effects of VPM on these isozymes of CYP should be an advantageous feature of this compound regarding a potential increase risk of toxicity from many xenobiotics via metabolic bioactivation. Higher dose of VPM (200 mg/kg/day) exhibited an induction effect on CYP1A1 activity providing a preliminary information of which additional study should be proceeded to confirm the effect of this compound on this isozyme of CYP. In addition, effect of VPM on other isoforms of CYP involving human drug metabolism, especially CYP3A4 which is a major CYP isozyme in human and plays a key role in carcinogenic and/or mutagenic activation of many xenobiotics (Shimada et al., 1989), should also be further studied.

Regarding CYP2B1&2B2, an induction effect of VPM on these isozymes of CYP is not toxicologically significant in human because human CYP2B6, which is orthologous to rat CYP2B1 (Soucek and Gut, 1992), is expressed very poorly and accounted for approximately 0.2% of total CYP contents (Rendic and Di Carlo, 1997). Further study regarding the mechanism of CYP2B1&2B2 induction by VPM should be proceeded. VPA, the prototype of VPM, did not demonstrate an induction effect on CYP2B1&2B2 in this study. This result confirmed the *in vivo* study of Rogiers et al. (1995). In that study, they found that VPA did not show an induction effect on CYP2B following intraperitoneal injection of 100 mg/kg/day of VPA, once daily for 10 days. However, the induction effect of VPA on CYP2B1&2B2 were shown when they performed an *in vitro* study using rat hepatocyte cell culture as well as in an *in vivo* study that VPA was administered to the animals via subcutaneously implanted osmotic pump to maintain VPA level in blood

(Rogiers et al., 1995). The authors suggested that these inconsistent results might be a consequence of the short half life of VPA in rats (10-20 minutes)(Dickinson et al., 1979).

No inhibition effect of VPM was observed on CYP2E1 at every concentration of VPM used in the *in vitro* study. This suggested that VPM was likely not a substrate of CYP2E1. Likewise, an inhibition effect of VPM on CYP1A1&1A2 was not observed at lower concentrations (0.1, 1, 10, and 100 μM) of VPM. The inhibition effect was shown only at a very high concentration (1000 μM) of VPM of which the concentration was much higher than that of the substrates (ethoxy- and methoxyresorufin) used in the reaction which was only 5 μM. This finding possibly indicated that VPM might not be a good substrate of CYP1A1&1A2 and/or the inhibition effect shown at the highest concentration of VPM probably due to a cytotoxic effect of this compound. Further study to clarify the inhibition effect of VPM on these two isozymes of CYP should be proceeded. VPM exhibited an inhibition effect on CYP2B1&2B2 in a dose-related manner at concentrations of 100 and 1000 μM of VPM. Due to the fact that in rat, phenobarbital is metabolized to a less pharmacologically active metabolite product by CYP2B subfamily, this result might explain for the prolonged barbiturate sleeping time characteristic of VPM reported by Namthongsakun (2000). In that study, VPM was shown to prolong barbiturate sleeping time at a dosage of 100 mg/kg and 200 mg/kg and the effect increased in a dose-related manner. Moreover, an *in vitro* inhibition effect of VPM on CYP2B1&2B2 suggested a possibility that VPM might be one of the substrates of these isozymes or the metabolic pathway catalyzing by these isozymes might be one of the possible biotransformation reaction of VPM in rat. This result was similar to that of its prototype, VPA which was found to be bioactivated by CYP2B1&2B2 in rats yielding a hepatotoxic metabolite, 4-ene-VPA (Rogiers et al., 1995). However, additional studies should be proceeded to clarify the mechanism and the kinetics of the inhibition effect of VPM on these isozymes.

Opposite to the effects of VPM on activities of individual CYP, VPM demonstrated no effect on total CYP contents. This might be due to the moderate sensitive of the

classical method used in this assay and/or the mixed induction and inhibition effects of VPM on CYP were existing.

Regarding the *ex vivo* study, PEG400 was administered to animals in the control group because PEG400 was used as a solvent of VPM and VPA in the corresponding treatment groups. Previous study indicated that PEG400 had no effects on any isozymes of CYP (Kiatkosolkul, 1999). Dosage of VPM (100 mg/kg/day) and VPA (250 mg/kg/day) used in this study were median effective doses against maximal electroshock seizure in mice (Namthongsakun, 2000 and Tantisira et al., 1997). The higher dosage of VPM (200 mg/kg/day) given to animals in VPM treatment group 2 was the other dosage of VPM also given to the animals for the barbiturate sleeping time test previously studied by Namthongsakun (2000). In this study, VPM prolonged barbiturate sleeping time at the dosage of 100 and 200 mg/kg body weight with a dose-dependent manner (Namthongsakun, 2000). Two different dosages of VPM, assigned to be given to the animals in this study were also to provide a preliminary tendency whether the effect of VPM on CYP2B1&2B2 was dose-related. The seven-day duration of compound administration was a minimal duration suggested for studying an effect of any new developing anticonvulsants on various hepatic parameters (Cereghino and Kupferberg, 1993). However, various dosage regimens of VPM and various longer durations of VPM administration were suggested for a further study.

Regarding the *in vitro* study, specific inducing agents were used to induce specific isozymes of CYP on which the inhibition effect of VPM would be determined. Phenobarbital and β-naphthoflavone were utilized to induce CYP2B1&2B2 and CYP1A1&1A2, respectively according to the standard regimens generally used (Gibson and Skett, 1994; Rodrigues and Prough, 1991). Ethanol was used to induce CYP2E1 according to the method of Hu et al. (1995). Before hepatic induced microsomes were used in the *in vitro* study, an induction of interested specific CYP was checked by determining the specific CYP activity comparing to that of the control group given a solvent of the inducing agent. Because VPM was water-insoluble, appropriate organic solvent was used to dissolve the compound for an *in vitro* inhibition study. Regarding an

inhibition effect of VPM on CYP1A1, CYP1A2 and CYP2B1&2B2, the corresponding alkoxyresorufin O-dealkylations were performed. Because VPM was soluble in DMSO, various concentration of VPM were dissolved in 0.5 mM alkoxyresorufin (dissolving in DMSO) in the manner of keeping total amount of DMSO equally in all of the reaction tubes. Also, total amount of DMSO in the reaction mixture was not different from the usual reaction situation. Because DMSO inhibited CYP2E1 (Hickman et al., 1998), acetonitrile was used to dissolve VPM in the aniline 4-hydroxylation assay. Concentration of acetonitrile in the reaction mixture was the same (0.1%) in all the reaction tubes so as to avoid the variable effect of acetonitrile on the rate of aniline 4-hydroxylation reaction.

In summary, VPM administered to male Wistar rats, at either dosage regimen of 100 mg/kg/day or 200 mg/kg/day, once daily for 7 days, exhibited no effects on total CYP contents as well as the activities of CYP1A1, CYP1A2 and CYP2E1. The only exception was observed with VPM at a dosage of 200 mg/kg/day that showed significant inductive effect on CYP 1A1 activity. Both dosages of VPM exhibited significantly inductive effect on CYP2B1&2B2 in a dose-related manner. In an *in vitro* study, VPM in all concentrations tested demonstrated no inhibition effect on CYP2E1. Inhibition effects of VPM on CYP1A1 and CYP1A2 were observed only at the highest concentration (1000 μM) of VPM. VPM exhibited an inhibition effect on CYP2B1&2B2 in a dose-related manner at concentrations of 100 and 1000 μM of VPM. Effect of VPM on other isoforms of CYPs, involving in human drug/xenobiotic metabolism remained to be investigated. Further study to clarify the mechanism of induction and inhibition of VPM on the corresponding affected isoforms of CYP should also be proceeded.

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# APPENDIX

**Table 20** The hepatic microsomal total CYP contents in individual rat*

| Rat number | Treatment group | | | |
|---|---|---|---|---|
| | PEG400 | VPA | VPM group 1 | VPM group 2 |
| 1 | 0.484 | 0.489 | 0.533 | 0.494 |
| 2 | 0.566 | 0.361 | 0.462 | 0.505 |
| 3 | 0.544 | 0.547 | 0.549 | 0.645 |
| 4 | 0.500 | 0.487 | 0.527 | 0.571 |
| 5 | 0.533 | 0.406 | 0.484 | 0.549 |
| 6 | 0.398 | 0.450 | 0.382 | 0.571 |

* Unit expressed as nmol/mg protein.

**Table 21** The hepatic microsomal ethoxyresorufin O-dealkylation in individual rat*

| Rat number | Treatment group | | | |
|---|---|---|---|---|
| | PEG400 | VPA | VPM group 1 | VPM group 2 |
| 1 | 12 | 54 | 60 | 62 |
| 2 | 46 | 14 | 44 | 42 |
| 3 | 22 | 48 | 50 | 66 |
| 4 | 40 | 64 | 46 | 58 |
| 5 | 46 | 50 | 88 | 72 |
| 6 | 42 | 40 | 46 | 74 |

* Unit expressed as pmol/mg protein/min.

**Table 22** The hepatic microsomal methoxyresorufin O-dealkylation in individual rat*

| Rat number | Treatment group | | | |
|---|---|---|---|---|
| | PEG400 | VPA | VPM group 1 | VPM group 2 |
| 1 | 4 | 8 | 10 | 8 |
| 2 | 8 | 8 | 8 | 4 |
| 3 | 8 | 10 | 16 | 8 |
| 4 | 10 | 8 | 12 | 10 |
| 5 | 10 | 12 | 14 | 2 |
| 6 | 12 | 8 | 10 | 16 |

* Unit expressed as pmol/mg protein/min.

**Table 23** The hepatic microsomal benzyloxyresorufin O-dealkylation in individual rat*

| Rat number | Treatment group | | | |
|---|---|---|---|---|
| | PEG400 | VPA | VPM group 1 | VPM group 2 |
| 1 | 16 | 18 | 246 | 310 |
| 2 | 16 | 6 | 208 | 448 |
| 3 | 14 | 36 | 260 | 526 |
| 4 | 26 | 24 | 354 | 232 |
| 5 | 28 | 24 | 170 | 430 |
| 6 | 24 | 20 | 112 | 610 |

* Unit expressed as pmol/mg protein/min.

**Table 24** The hepatic microsomal pentoxyresorufin O-dealkylation in individual rat*

| Rat number | Treatment group | | | |
|---|---|---|---|---|
| | PEG400 | VPA | VPM group 1 | VPM group 2 |
| 1 | 0 | 6 | 16 | 60 |
| 2 | 6 | 2 | 38 | 84 |
| 3 | 0 | 8 | 54 | 60 |
| 4 | 4 | 2 | 50 | 50 |
| 5 | 0 | 8 | 30 | 110 |
| 6 | 0 | 2 | 18 | 126 |

* Unit expressed as pmol/mg protein/min.

**Table 25** The hepatic microsomal aniline 4-hydroxylation in individual rat*

| Rat number | Treatment group | | | |
|---|---|---|---|---|
| | PEG400 | VPA | VPM group 1 | VPM group 2 |
| 1 | 0.303 | 0.340 | 0.467 | 0.188 |
| 2 | 0.602 | 0.061 | 0.376 | 0.426 |
| 3 | 0.248 | 0.488 | 0.340 | 0.526 |
| 4 | 0.326 | 0.316 | 0.373 | 0.410 |
| 5 | 0.445 | 0.243 | 0.382 | 0.500 |
| 6 | 0.402 | 0.202 | 0.168 | 0.576 |

* Unit expressed as nmol/mg protein/min.

**Table 26** Effect of β-naphthoflavone on total CYP contents in individual rat*

| Rat number | Treatment group | |
|---|---|---|
| | β-naphthoflavone | Corn oil |
| 1 | 0.882 | 0.668 |
| 2 | 0.761 | 0.656 |
| 3 | 0.796 | 0.352 |
| 4 | 0.844 | 0.566 |

* Unit expressed as nmol/mg protein.

**Table 27** Effect of β-naphthoflavone on methoxy- and ethoxyresorufin O-dealkylation in individual rat*

| Rat number | Treatment group | | | |
|---|---|---|---|---|
| | β-naphthoflavone | | Corn oil | |
| | MROD | EROD | MROD | EROD |
| 1 | 112 | 1004 | 32 | 68 |
| 2 | 108 | 840 | 4 | 132 |
| 3 | 140 | 952 | 8 | 20 |
| 4 | 124 | 908 | 12 | 48 |

* Unit expressed as pmol/mg protein/min.

**Table 28** Inhibition effect of VPM on CYP1A1 activity in individual rat*

| Concentration of VPM In the reaction mixture (μM) | Ethoxyresorufin O-dealkylation | | | |
|---|---|---|---|---|
| | Rat number | | | |
| | 1 | 2 | 3 | 4 |
| 0 | 1004 | 840 | 952 | 908 |
| 0.1 | 836 | 884 | 832 | 868 |
| 1 | 812 | 892 | 864 | 884 |
| 10 | 644 | 844 | 924 | 852 |
| 100 | 660 | 812 | 928 | 900 |
| 1000 | 528 | 636 | 752 | 652 |

* Unit expressed as pmol/mg protein/min.

**Table 29** Inhibition effect of VPM on CYP1A2 activity in individual rat*

| Concentration of VPM In the reaction mixture (μM) | Methoxyresorufin O-dealkylation | | | |
|---|---|---|---|---|
| | Rat number | | | |
| | 1 | 2 | 3 | 4 |
| 0 | 112 | 108 | 140 | 124 |
| 0.1 | 108 | 104 | 140 | 124 |
| 1 | 104 | 100 | 128 | 108 |
| 10 | 104 | 96 | 136 | 136 |
| 100 | 96 | 100 | 132 | 124 |
| 1000 | 72 | 80 | 92 | 88 |

* Unit expressed as pmol/mg protein/min.

**Table 30** Effect of phenobarbital on total CYP contents in individual rat*

| Rat number | Treatment group | |
|---|---|---|
| | Phenobarbital | Sterile water |
| 1 | 0.876 | 0.445 |
| 2 | 0.885 | 0.516 |
| 3 | 0.742 | 0.577 |
| 4 | 1.190 | 0.533 |

* Unit expressed as nmol/mg protein.

**Table 31** Effect of phenobarbital on benzyloxyresorufin O-dealkylation in individual rat*

| Rat number | Treatment group | |
|---|---|---|
| | Phenobarbital | Sterile water |
| 1 | 695.2 | 26 |
| 2 | 829.2 | 62 |
| 3 | 822.4 | 8 |
| 4 | 910.0 | 8 |

* Unit expressed as pmol/mg protein/min.

**Table 32** Inhibition effect of VPM on CYP2B1&2B2 activities in individual rat*

| Concentration of VPM In the reaction mixture (μM) | Benzyloxyresorufin O-dealkylation | | | |
|---|---|---|---|---|
| | Rat number | | | |
| | 1 | 2 | 3 | 4 |
| 0 | 695.2 | 829.2 | 822.4 | 910.0 |
| 0.1 | 662 | 812 | 764 | 864 |
| 1 | 728 | 828 | 876 | 848 |
| 10 | 550 | 652 | 860 | 924 |
| 100 | 376 | 440 | 442 | 498 |
| 1000 | 72 | 68 | 80 | 100 |

* Unit expressed as pmol/mg protein/min.

**Table 33** Effect of ethanol on total CYP contents in individual rat*

| Rat number | Treatment group | |
|---|---|---|
| | Ethanol | Water |
| 1 | 0.426 | 0.527 |
| 2 | 0.514 | 0.358 |
| 3 | 0.568 | 0.662 |
| 4 | 0.640 | 0.668 |

* Unit expressed as nmol/mg protein.

**Table 34** Effect of ethanol on aniline 4-hydroxylation in individual rat*

| Rat number | Treatment group | |
|---|---|---|
| | Ethanol | Water |
| 1 | 0.520 | 0.155 |
| 2 | 0.361 | 0.160 |
| 3 | 0.289 | 0.303 |
| 4 | 0.639 | 0.154 |

* Unit expressed as nmol/mg protein/min.

**Table 35** Inhibition effect of VPM on CYP2E1 activity in individual rat*

| Concentration of VPM In the reaction mixture (μM) | Aniline 4-hydroxylation | | | |
|---|---|---|---|---|
| | Rat number | | | |
| | 1 | 2 | 3 | 4 |
| 0 | 0.403 | 0.340 | 0.236 | 0.409 |
| 0.1 | 0.421 | 0.351 | 0.225 | 0.502 |
| 1 | 0.456 | 0.278 | 0.244 | 0.407 |
| 10 | 0.429 | 0.291 | 0.263 | 0.414 |
| 100 | 0.435 | 0.298 | 0.282 | 0.458 |
| 1000 | 0.470 | 0.255 | 0.202 | 0.368 |

* Unit expressed as nmol/mg protein/min.

## Curriculum Vitae

Miss Kanlayanee Yana was born in August 26, 1973 in Nan, Thailand. She graduated with a Bachelor of Science in Pharmacy in 1996 from Faculty of Pharmaceutical Sciences, Chiang Mai University, Thailand. After graduation, she worked as a clinical pharmacist in Wiengsa Hospital, Nan, Thailand, for 2 years.