ผลการยับยั้งของโนมิลินต่อเอนไซม์ไซโตโครม พี450 3เอ4

นางสาวอลิสา สุขเอม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา   ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

INHIBITORY EFFECTS OF NOMILIN ON THE CYTOCHROME P450 3A4

Miss Alisa Suk-aim

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Pharmacology

Department of Pharmacology and Physiology

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Acedemic Year 2011

Thesis Title	INHIBITORY EFFECTS OF NOMILIN ON THE CYTOCHROME P450 3A4
By	Miss Alisa Suk-aim
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Suree Jianmongkol, Ph.D.
Thesis Co-advisor	Associate Professor Nusara Piyapolrungroj, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……………………………………. Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

……………………………………. Chairman

(Associate Professor Police Lieutenant Colonel Somsong Lawanprasert, Ph.D.)

……………………………………. Thesis Advisor

(Assistant Professor Suree Jianmongkol, Ph.D.)

……………………………………. Thesis Co-advisor

(Associate Professor Nusara Piyapolrungroj, Ph.D.)

……………………………………. Examiner

(Assistant Professor Rataya Luechapudiporn, Ph.D.)

……………………………………. External Examiner

(Associate Professor Pornpen Pramyothin, Ph.D.)

อลิสา สุขเอม : ผลการยับยั้งของโนมิลินต่อเอนไซม์ไซโตโครม พี450 3เอ4. (INHIBITORY EFFECTS OF NOMILIN ON THE CYTOCHROME P450 3A4) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.สุรีย์ เจียรณ์มงคล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.นุศรา ปิยะพลรุ่งโรจน์, 87 หน้า.

อันตรกิริยาระหว่างยาที่เกิดจากการยับยั้งเอนไซม์ไซโตโครม พี450 โดยเฉพาะเอนไซม์ไซโตโครม พี3เอ4 อาจทำให้เกิดอาการไม่พึงประสงค์ที่มีความสำคัญทางคลินิก งานวิจัยนี้ได้ศึกษาผลของ nomilin ซึ่งเป็นสารในกลุ่มลิโมนอยด์ที่พบได้มากในพืชตระกูลส้ม ต่อการยับยั้งสมรรถนะเอนไซม์ไซโตโครม พี450 3เอ4 ของมนุษย์ โดยวัดปริมาณ 6β-hydroxytestosterone ที่เกิดจากปฏิกิริยาการเปลี่ยนแปลงของ testosterone ด้วยเทคนิค high-performance liquid chromatography (HPLC) ผลการทดลองพบว่า nomilin สามารถยับยั้งสมรรถนะของเอนไซม์ไซโตโครม พี3เอ4 ได้ โดยมีค่า IC_{50} 14.54 $\pm$ 2.77 μM และ มีรูปแบบการยับยั้งเป็นชนิดผันกลับแบบผสม (mixed type reversible inhibition) โดยมีค่าคงที่ K_i 4.83 μM ผลการทดลองยังแสดงให้เห็นว่า nomilin ไม่ใช่ mechanism-based inhibitor ของเอนไซม์ไซโตโครม พี450 3เอ4 และจากการที่ค่า IC_{50} และ K_i ของ nomilin อยู่ในระดับไมโครโมลาร์นั้น ทำให้เป็นไปได้ว่า nomilin และผลิตภัณฑ์ธรรมชาติที่มี nomilin เป็นส่วนประกอบสามารถรบกวนกระบวนการเมแทบอลิซึมที่เกี่ยวข้องกับการทำงานของเอนไซม์ไซโตโครม พี3เอ4 ซึ่งอาจนำไปสู่ปัญหาอันตรกิริยาระหว่างอาหารและยาได้ และควรมีการศึกษาแบบ *in vivo* เพิ่มเติมในเรื่องดังกล่าวต่อไป

ภาควิชา : เภสัชวิทยาและสรีรวิทยา　　　　ลายมือชื่อนิสิต

สาขาวิชา : เภสัชวิทยา　　　　ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

ปีการศึกษา :2554　　　　ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

5176607033   : MAJOR PHARMACOLOGY

KEYWORDS : NOMILIN / CYTOCHROME P450 / CYP3A4 / INHIBITION

ALISA SUK-AIM: INHIBITORY EFFECTS OF NOMILIN ON CYTOCHROME P450 3A4. ADVISOR: ASST. PROF. SUREE JIANMONGKOL, Ph.D., CO-ADVISOR: ASSOC. PROF. NUSARA PIYAPOLRUNGROJ, Ph.D., 87 pp.

Drug interaction caused by inhibition of the cytochrome P450 especially CYP3A4, may result in clinically important adverse reactions due to a change in metabolism of the drugs. Nomilin, one of the limonoid compounds found abundantly in citrus species, was examined for its inhibition against recombinant human CYP3A4 activity. The enzyme activity was determined using the high-performance liquid chromatography (HPLC) technique to quantify the production of 6β-hydroxytestosterone from testosterone. The results demonstrated that nomilin inhibited CYP3A4 activity with the IC_{50} value of 14.54 ± 2.77 μM. The inhibitory profile suggested the mixed type reversible inhibition with the apparent K_i value of 4.83 μM. Furthermore, nomilin was not a mechanism-based inhibitor of CYP3A4 activity. However, its apparent IC_{50} and K_i values were in the micromolar range, it was possible that nomilin and natural product containing nomilin could interfere CYP3A4-mediated metabolisms, and potentially lead to food-drug interaction problems. The in vivo study may be needed to assess this further.

Department : Pharmacology and Physiology   Student's Signature

Field of Study : Pharmacology   Advisor's Signature   .

Academic Year : 2011   Co-advisor's Signature

ACKNOWLEDGEMENTS

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

β	=	beta
μg	=	microgram
μl	=	microlitre
μM	=	micromolar
^{0}C	=	degree celcius
CYP	=	cytochrome P450
e.g.	=	example gratia
et al.	=	et alii
g	=	gram
G6P	=	Glucose 6 phosphate
G6PD	=	Glucose 6 phosphate dehydrogenase
i.e.	=	id est (that is)
IC_{50}	=	median inhibition concentration
K_i	=	inhibition constant
K_m	=	the substrate concentration at which the reaction velocity is half maximal
L	=	litre
M	=	Molar
mg	=	milligram
min	=	minute
ml	=	millitre
mM	=	millimolar
MW	=	molecular weight
NADP	=	nicotinamide adenine dinucleotide phosphate
NADPH	=	nicotinamide adenine dinucleotide phosphate (reduced form)
nmole	=	nanomole

pH	= potential of hydrogen
R^2	= coefficient of determination
SEM	= standard error of the mean
U	= unit
V	= the velocity of the reaction
V_{max}	= the maximum velocity of the reaction
vs	= versus

CHAPTER I

INTRODUCTION

Cytochrome P450 (CYPs) are drug metabolizing enzymes that play an important role in the metabolism of various endogenous and exogenous substances including steroids, biogenic amines, alcohol, drugs and chemicals. The basic principle of drug metabolism in the body is to increase water solubility which enables the compound to be excreted more readily in the urine or bile (Hasler et al., 1999). CYPs can be found mainly in the liver tissues and to a lesser extent in the kidney, skin, gut and lung. A number of CYP450 isoforms including, CYP3A4, CYP2C9, CYP2C19, CYP1A2, CYP2E1, and CYP2D6 are responsible for xenobiotics and drug metabolism. Among those CYPs, CYP3A4 is the major isoform found approximately 30% in human liver total CYP content. This isoform involves with drug metabolism up to 55% of all prescribed drugs (Mukherjee, 2011; Ogu and Maxa, 2000). As known, a number of drugs from a broad range of therapeutic categories are CYP3A4 substrates. Thus, alteration in CYP3A4 activity either through induction or inhibition often causes uncertain outcomes and toxicities due to a shift in metabolic clearance and pharmacokinetic profiles of the compounds being CYPs substrates (Martin, 2001).

The effect on drug metabolism through alteration of CYPs activity can result in clinical drug interaction issues. Clinical adverse events have been reported, ranging from relatively mild and transient to life threatening outcomes (Paine et al., 2004). For example, torsades de points, a life-threatening ventricular arrhythmia associated with QT prolongation, has been reported in the cases that CYP3A4 inhibitors (ketoconazole, clarithromycin, erythromycin, ritonavir) were coadministered with terfenadine, astemizole, cisapride or pimozide (Dresser et al., 2000). In addition to drug, a number of phytochemicals found in fruits, vegetables, herbs, spices, and beverages have great potential for inhibiting the activity of CYP isozymes which may lead to clinical food-drug interaction event. For example, Bergamottin and 6, 7-dihydroxybergamottin, the two chemical constituents in grapefruit juice, were able to increase serum level of calcium channel antagonists due to their inhibitory action against CYP3A4 activity (Pal and Mitra,

2006). Piperine, an alkaloid found abundantly in *Piper nigrum* Linn. and *Piper longum* Linn. elicited its inhibitory effect on CYP3A4 activity in the *in vitro* model of human liver microsomes and increased the bioavailability of propranolol and theophylline in healthy volunteers (Chen and Raymond, 2006).

Limonoids are a group of highly oxygenated terpenoids found abundantly in the plant families of *Meliaceae* and *Rutaceae*. The terpenoids have been reported their broad range of biological activities including insecticidal, antiviral, antioxidant, antifeedant, and anticancer (Roy and Saraf, 2005). In addition, these compounds have elicited their inhibitory effect on CYP3A4 activity (Han et al., 2011). Nomilin is a bitter taste limonoid found in citrus species such as *Citrus grandis, Citrus limon, Citrus paradisi, Citrus sinensis* (Hasegawa and Herman, 1985; Rouseff, 1982; Girard and Mazza, 1998). Several studies demonstrated that nomilin has a range of significant biological activities including anti-feedant (Ruberto et al., 2002; Jayaprakasha et al., 1997), antitumor (Tian et al., 2001), immune enhancer (Raphael and Kuttan, 2003), anti-obesity (Ono et al., 2011), antioxidant (Mandadi et al., 2007) and anti-inflammation (Kim et al., 2011). It was reported that nomilin given to the mice was able to shorten the sleeping time induced by alpha-chloralose and urethane (Wada et al., 1992). This compound was able to affect activity and expression of various enzymes such as glutathione S- transferase (Perez et al., 2010), reverse transcriptase (Balestrieri et al., 2011). In addition, nomilin which was extracted from pomelo juice was shown to inhibit CYP3A4 activity (นุศรา ปิยะพลรุ่งโรจน์, 2551).

The inhibition of CYP can be related to the drug interaction issues. There were a few studies of the inhibitory effect of nomilin on drug metabolizing enzymes. In this study, the kinetic inhibition of nomilin on the catalytic activity of CYP3A4 was further investigated and characterized using the *in vitro* model of recombinant human c-DNA baculovirus expressed CYP3A4. The information from this study might be used for risk evaluation of the potential metabolic-based interaction related to consumption of nomilin and natural products containing nomilin.

Objectives

The objectives of this study were to determine the type and mechanism of nomilin-mediated inhibition on CYP3A4 activity. In addition, the kinetic parameters of nomilin-mediated inhibition were determined.

Experimental design

```
┌─────────────────────────────────────────────┐
│   Determination of inhibitory effects of     │
│   nomilin on CYP3A4 activity                 │
└─────────────────────────────────────────────┘
        │                          │
        ▼                          ▼
┌──────────────────┐      ┌────────────────────────────┐
│ Determination of │      │ Assessment of mechanism-    │
│ the type of      │      │ based inhibition            │
│ reversible       │      └────────────────────────────┘
│ inhibition and   │              │           │
│ kinetic          │              ▼           ▼
│ parameters       │      ┌──────────┐  ┌──────────┐
└──────────────────┘      │ Time     │  │ NADPH    │
                          │ dependent│  │ dependent│
                          │ effect   │  │ effect   │
                          └──────────┘  └──────────┘
```
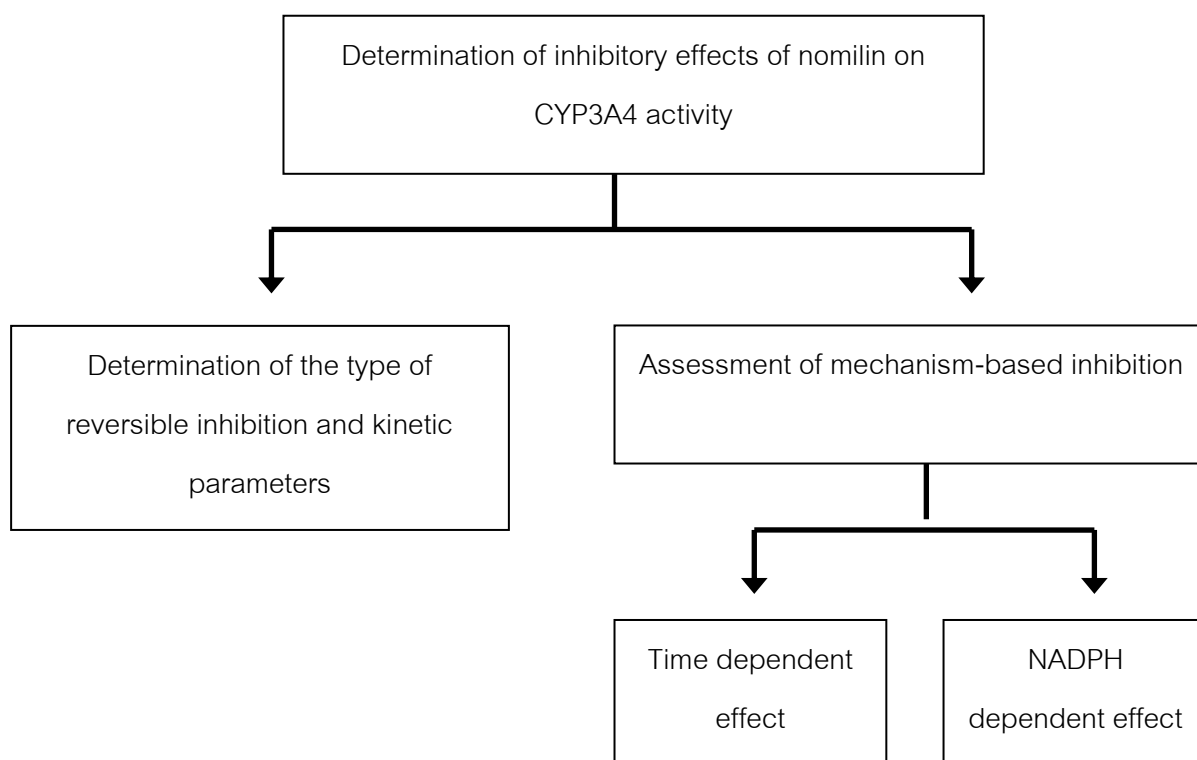
CHAPTER II

LITERATURE REVIEWS

Drug metabolism

Drug metabolism is one major process to rapidly eliminate various drugs and other xenobiotics including food additives, cosmetic products, and agrochemicals. Generally, the metabolism serves to enzymatically convert the hydrophobic compound into its hydrophilic derivative, which can be readily eliminated through the urine or the bile. The metabolism can be divided into phase 1 and phase 2 reactions. The reactions in phase 1 lead to addition of a functional group to the parent compound. Those reactions include oxidation, reduction, hydrolysis, and hydration which are carried out by cytochrome P (CYP)s, flavin-containing monooxygenases (FMO), and epoxide hydrolases (EH) (Gonzalez and Tukey, 2005). In the phase 2 reactions, drugs and phase 1 metabolites are conjugated with endogenous substance to produce a more water-soluble metabolite. Hence, the phase 2 reactions facilitate the inactivation of potentially toxic phase 1 metabolites as well as their elimination from the tissue. The phase 2 enzymes include several superfamilies of conjugating enzymes such as glutathione-S-transferases (GST), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), *N*-acetyltransferases (NAT), and methyltransferases (MT) (Gonzalez and Tukey, 2005).

Cytochrome P450

Cytochrome P450 (CYP) enzymes are the most prominent phase 1 enzyme. They are a superfamily of hemoproteins that play an important role in the metabolism of various endogenous compounds such as steroids, bile acids, fat-soluble vitamins, and fatty acid as well as exogenous compounds such as drugs, food, food additives, cosmetic products and agrochemicals (Hasler et al., 1999). CYP enzymes are mainly located in the liver which is the major organ for drug metabolism. In addition, CYPs are also found in other tissues such as gut, skin, kidney, and lung (Ogu and Maxa, 2000). As an oxidoreductase, CYP contains a heme-catalytic center in the catalytic domain that

binds to the substrate and oxygen molecules. The catalytic reaction requires electron transfer from NADPH in the reductase domain to the heme-containing oxidase domain. Then, the oxygen molecules split. One atom of oxygen is incorporated into substrate and the other forms water (Glue and Clement, 1999; Gonzalez and Tukey, 2005). The catalytic cycle of P-450 was illustrated in figure 1.

The abbreviation for cytochrome P450 (CYP) is followed by a number referring to the gene family, a letter denoting the subfamily and another number indicating the enzyme. For example, CYP3A4 is the CYP in gene family 3 and subfamily A while the number 4 refers to its individuality. Enzymes with at least 40% sequence homology belong to the same family. Within the family, enzymes with sequence homology of greater than 55% are in the same subfamily (Glue and Clement., 1999). Currently, there are more than 40 different isoforms of human P450 proteins, as shown in figure 2 (Hasler et al., 1999).
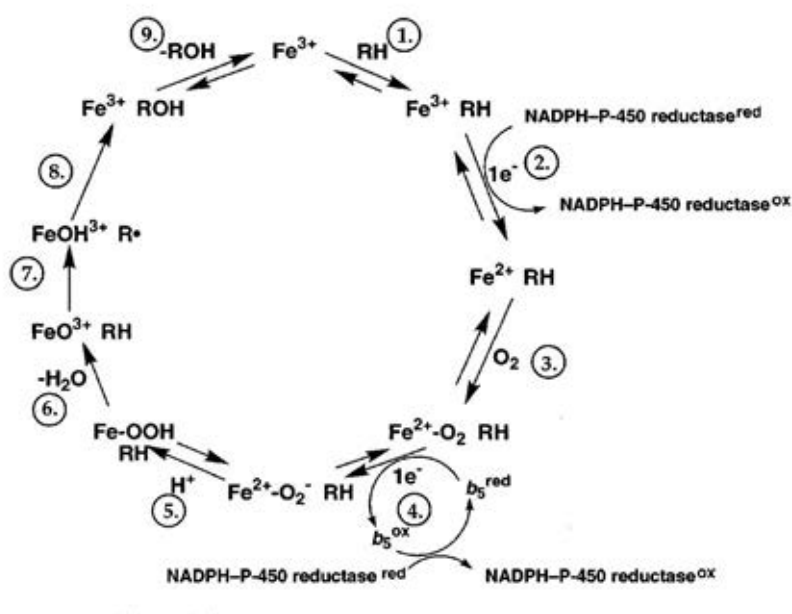
Figure 1 Catalytic cycle of CYP450 (Guengerich, 1999)
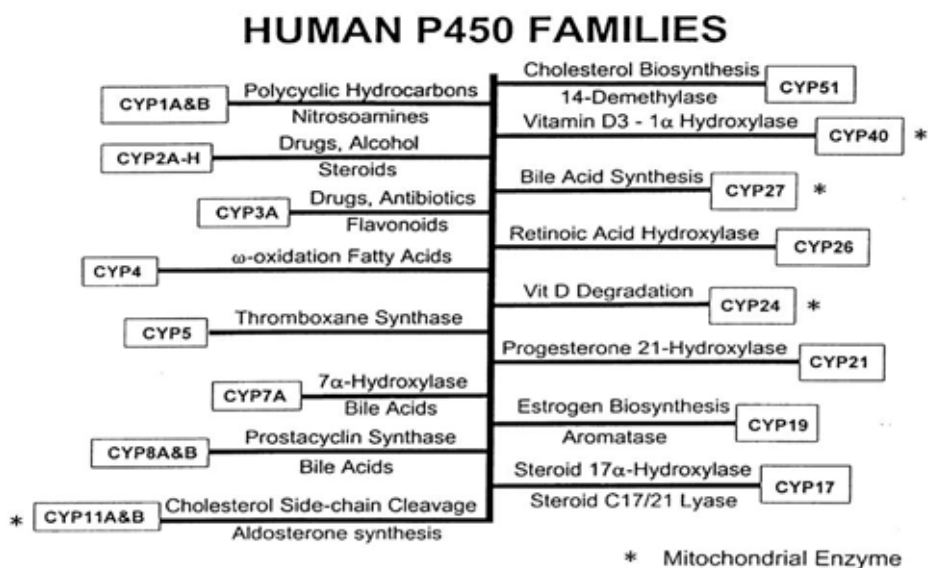
Figure 2 Human CYP450 families (Hasler, 1999)

The CYP3A subfamily

CYP3A is the most abundant enzyme found in human. The human P-450 3A subfamily contains three members including 3A4, 3A5, and 3A7. These isoforms share at least 85% amino acid sequence homology. CYP3A4 and CYP3A5 are found in liver and gut, and CYP3A7 is in human embryonic, fetal and newborn liver (De Wildt et al., 1999; Yuan et al., 2002). It has been reported that CYP3A4 mRNA was detected at approximately 10% of adult levels in fetal liver microsomes (between 11 and 30 weeks gestational age). The mRNA levels increased immediately after birth and reached 50% of the adult levels at the age between 6 and 12 months (De Wildt et al., 1999). CYP3A4 is the most abundantly expressed CYP in human liver and in small intestine, which is accounted for approximately 30 to 40% of the total CYP content in both organs (De Wildt et al., 1999).

CYP3A4 has been known of its ability to metabolize a broad variety of pharmaceuticals and other xenobiotics such as procarcinogens and pesticides (De

Wildt et al., 1999; Guengerich, 1999). In addition, drugs and xenobiotics can be either inducer or inhibitor of CYP3A4, leading to clinically significant drug-drug interactions. Consequently, alteration of CYP activities can cause adverse reactions or therapeutic failures. Examples of CYP3A4 substrates, inhibitors and inducers were shown in Table 1.

Table 1 CYP3A4 substrates, inhibitors and inducers (Ogu and Maxa, 2000)

Substrates	Inhibitors	Inducers
alprazolam, astemizole, buspirone, calcium channel blockers, carbamazepine, cisapride, cyclosporine, doxorubicin, erythromycin, etoposide, felodipine, fentanyl, HIV protease inhibitors, ifosphamide, lovastatin, midazolam, nifedipine, pimozide, quinidine, quinine, simvastatin, tacrolimus, terfenadine, triazolam	amiodarone, cimetidine, cyclosporine, danazol, diltiazem, fluconazole, grapefruit juice, HIV protease inhibitors, itaconazole, ketoconazole, macrolide (not azithromycin), miconazole, nefazadone, omeprazole, quinidine, ritonavir, verapamil	carbamazepine, rifabutin, rifampin, ritonavir

CYP-mediated drug interaction

Uses of multiple drugs can result in metabolism-based interactions in particular when these drugs are metabolized by the same enzyme. Consequently, the pharmacokinetic profiles of each drug may alter, leading to changes in either therapeutic efficacy or adverse effects. As known, CYP is the most predominant phase 1 drug metabolizing enzyme which has been linked to drug interaction. Clinical adverse events have been reported, ranging from relatively mild and transient to life threatening outcomes (Paine et al., 2004). For example, torsades de points, a life-threatening ventricular arrhythmia associated with QT prolongation has been reported in the cases that terfenadine, astemizole, cisapride or pimozide were coadministered with CYP3A4 inhibitors (ketoconazole, clarithromycin, erythromycin, ritonavir) (Dresser et al., 2000). Subsequently, terfenadine, astemizole and cisapride were withdrawn from the market. On the other hand, some drug interactions might have advantages in therapeutic outcomes. For example, ritonavir (a potent CYP3A4 inhibitor) was reported to increase serum lopinavir in HIV patients (Lynch and Price, 2007).

Phytochemicals and natural substances in food have been reported to interfere with CYP3A4 function and expression. Given that there is an increasing consumption of medicinal herbs worldwide, the P450-meadiated herb-drug interactions could be anticipated especially the combination with conventional therapeutic drugs. For example, hyperforin in St John's wort, which induced the expression of CYP3A4, was responsible for a decrease in oral bioavailability of midazolam in healthy subjects (Chen and Raymond, 2006). Bergamottin and 6,7-dihydroxybergamottin found in grapefruit juice increased serum level of calcium channel antagonists via their inhibition against CYP3A4 activity (Pal and Mitra, 2006). Piperine, a CYP3A4 inhibitor found abundantly in *Piper nigrum* Linn. and *Piper longum* Linn. markedly increased the bioavailability of propranolol and theophylline in human subjects The mechanism of action involved with an inhibition of CYP3A4 (Chen and Raymond, 2006).

Enzyme kinetic

      Like other enzymes, the catalytic reactions of CYP can be initiated by formation of reversible complex (enzyme-substrate complex) followed by decomposes to products and free enzyme. The velocity of the product formation can be estimated by the following Michaelis-Menten equation (1) (Voet and Voet, 2011).

$$V = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{Equation 1}$$

where V and V_{max} = the velocity and maximum velocity of the reaction; [S] = the concentration of substrate; K_m = the substrate concentration at which the reaction velocity is half maximal

      Generally, the kinetic parameters (V_{max} and K_m) can be estimated from the Lineweaver-Burk plot which is the plot between the reciprocal values of velocity (V) and substrate concentration ([S]) as shown in figure 3. The double-reciprocal derived form of equation 1 was shown in equation 2.
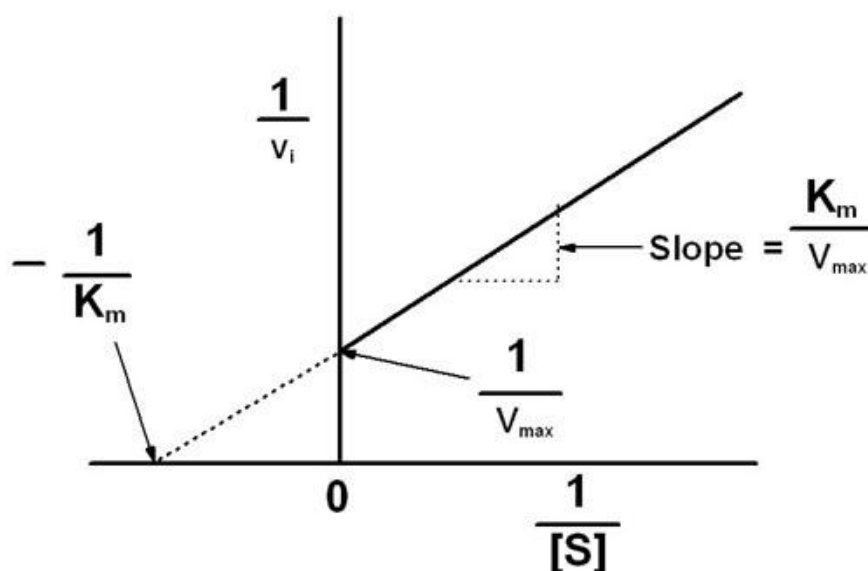
Figure 3 The Lineweaver-Burk plot (www.themedicalbiochemistrypage.org/enzyme-kinetics.php)

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \qquad\qquad \text{Equation 2}$$

Kinetic of enzyme inhibition (Honkalammi, 2011; Zhang, 2007; Zhou et al., 2007)

The enzyme catalytic reaction can be impeded in the presence of an inhibitor. A Michaelis-Menten equation for inhibition (equation 3) can be derived to estimate the type of inhibition. The inhibition constant (K_i) is introduced to describe the inhibitory ability of an inhibitor.

$$K_i = \frac{[E][I]}{[EI]} \qquad\qquad \text{Equation 3}$$

where [E] = the free enzyme concentration; [I] = the inhibitor concentration; [EI] is the enzyme – inhibitor complex

In addition, the inhibitory potency can be estimated from an IC_{50} (the concentration of the inhibitor that results in 50% inhibition of enzyme activity). Based on the Michaelis-Menten model, several classes of inhibition are defined as follows.

1. Reversible inhibition

Reversible inhibition is the type of inhibition which disappears soon after the removal of an inhibitor (Hasler et al., 1999). The inhibitor noncovalently binds to the enzyme catalytic site, which can readily dissociate from the enzyme. The reversible inhibition can be further grouped into competitive, noncompetitive and uncompetitive inhibition (Thummel and Wilkinson, 1998).

1.1 **Competitive inhibition** In the competitive reversible inhibition, the inhibitor competes with the substrate for the same binding site on the enzyme. Once the enzyme-inhibitor complex is formed, the substrate cannot bind to that enzyme (Figure 4). The Michaelis-Menten equation for competitive inhibition (Equation 4) is shown

below. The inhibitor does not affect the maximal velocity of the product turnover (V_{max}), but certainly increase the K_m of substrate (Tracy, 2008). This type of inhibition can be overcome by increasing the concentration of the substrate. A Lineweaver-Burk plot in the presence of competitive inhibitor is depicted in figure 5.
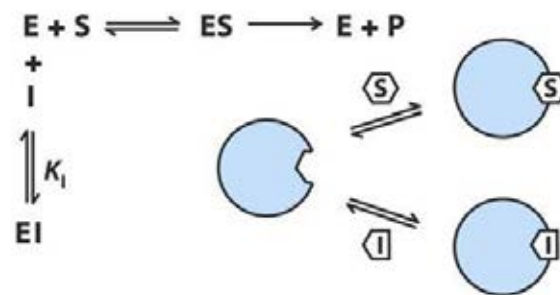
Figure 4 Interaction between enzyme, substrate and inhibitor in competitive inhibition (www.2.fiu.edu)

$$V = \frac{V_{max}[S]}{[S]+K_m\left(1+\dfrac{[I]}{K_i}\right)}$$

Equation 4

where [I] = the free inhibitor concentration, K_i = the inhibition constant
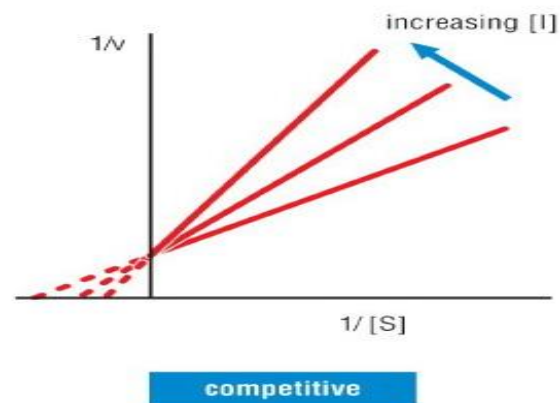
Figure 5 The Linewever-Burk plots for competitive inhibition

(http://chemistry.ewu.edu/jcorkill/biochem/com_nono_unIH.jpg)

      1.2 Noncompetitive inhibition In the noncompetitive reversible inhibition, the substrate and inhibitor independently occupy the different binding sites on the enzyme. Thus, the inhibitor has no effect on the binding of the substrate to the enzyme. Eventually, the enzyme can be inactivated through the formation of either enzyme-inhibitor or enzyme-inhibitor-substrate complexes (Figure 6). In this situation, the free enzyme and the enzyme-substrate complex would exhibit equal binding constant for the inhibitor. Hence, the inhibitor affects the catalytic properties of the enzyme but does not affect substrate binding. The Michaelis-Menten equation for noncompetitive inhibition (equation 5) is shown below. The inhibitor does not affect the K_m but impedes the catalytic velocity (Figure 7). In this circumstance, an increase of the substrate concentration does not decrease the inhibitory action of the inhibitor.
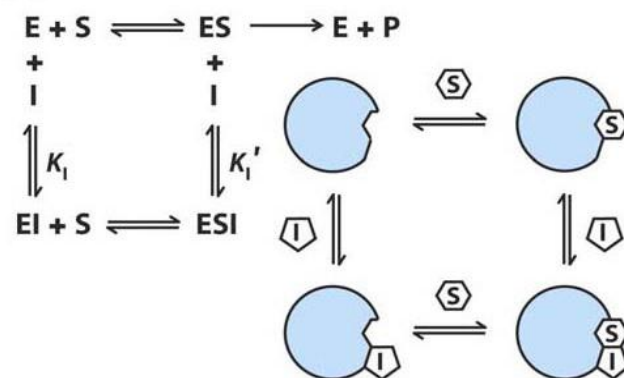
Figure 6 Interaction between enzyme, substrate and inhibitor in noncompetitive inhibition (www.2.fiu.edu)

$$V = \frac{V_{max}[S]}{\left([S] + K_m\right)\left(1 + \frac{[I]}{K_i}\right)}$$

Equation 5
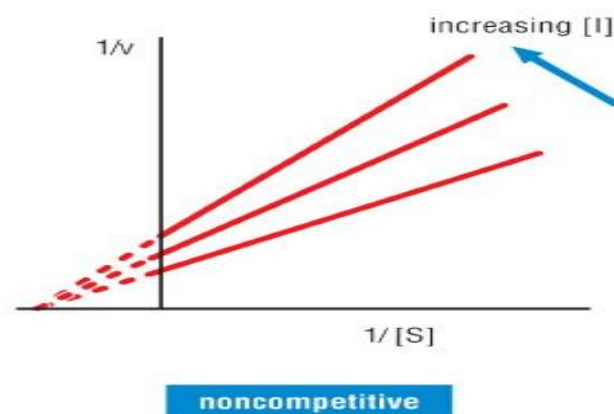
Figure 7 The Linewever-Burk plots for noncompetitive inhibition
(http://chemistry.ewu.edu/jcorkill/biochem/com_nono_unIH.jpg)

1.3 **Mixed-type inhibition** In this type of inhibition, the inhibitor can bind to either the free enzyme [E] or the enzyme substrate complex [ES] (Figure 8). The enzyme- substrate-inhibitor complex [ESI] is quite unstable. The inhibitor can dissociate from this complex. Thereafter, the substrate can be turnover into the product. Hence, the inhibitor inactivates the enzyme via both competitive and uncompetitive inhibition with the inhibition constant of K_i and K'_i, respectively. The Michaelis-Menten equation (equation 6) is shown below. In this condition, the ability of the inhibitor to bind the free enzyme does not equal to the enzyme-substrate complex. As a result, V_{max} and K_m are changed in the presence of an inhibitor (Figure 9).
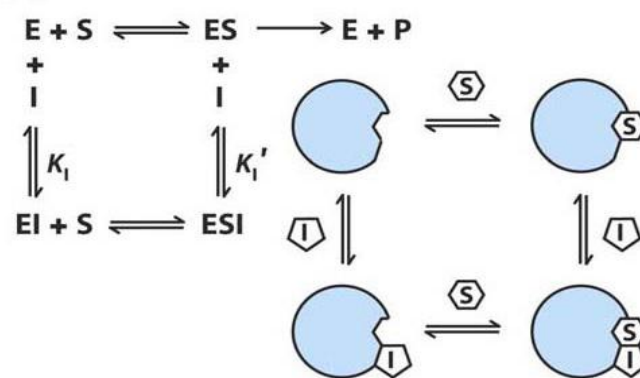
Figure 8 Interaction between enzyme, substrate and inhibitor in mixed type inhibition (www.2.fiu.edu)

$$V = \frac{V_{max}[S]}{K_m \left(1 + \frac{[I]}{K_I}\right) + [S]\left(1 + \frac{[I]}{K'_I}\right)} \qquad \text{Equation 6}$$

where K_i = the inhibition constant for competitive inhibition; K'_I = the inhibition constant for uncompetitive inhibition.
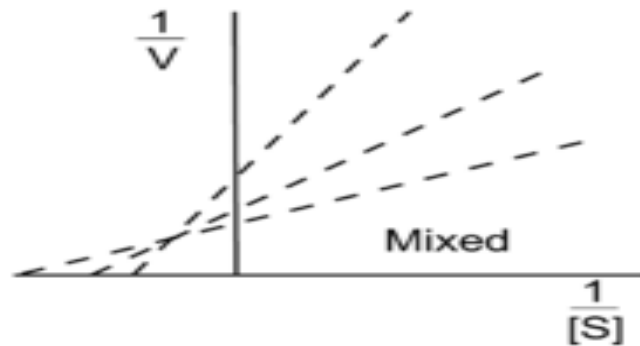
Figure 9 The Linewever-Burk plots for mixed type inhibition (www.en.wikipedia.org)

1.4 Uncompetitive inhibition In this type of inhibition, the inhibitor binds only to the enzyme-substrate complex [ES], but not the free enzyme [E] (Figure 10). The binding of uncompetitive inhibitor inactivates the catalytic activity of enzyme without any interference on the binding between enzyme and substrate. The Michaelis-Menten equation for uncompetitive inhibition is shown in equation 7. As a result, both V_{max} and K_m decrease in the presence of an inhibitor (Figure 11) (Tracy, 2008).
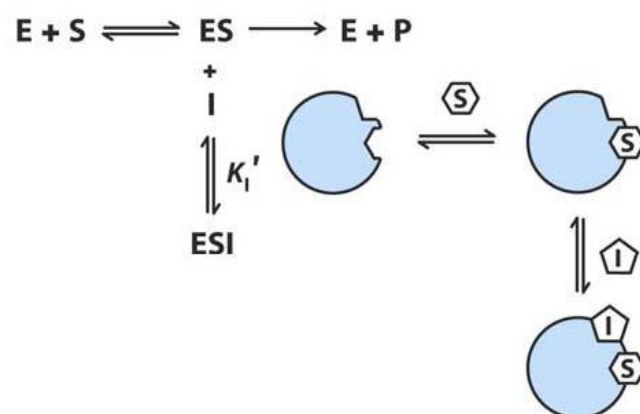
Figure 10 Interaction between enzyme, substrate and inhibitor in uncompetitive inhibition (www.2.fiu.edu)

$$V = \frac{V_{max}\,[S]}{[S]\left(1 + \dfrac{[I]}{K_i}\right) + K_m} \qquad \text{Equation 7}$$
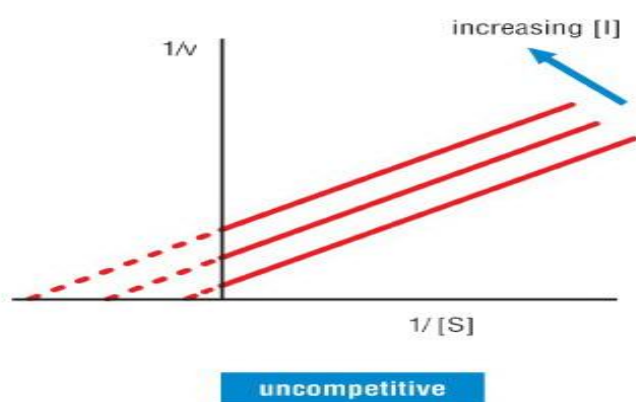
Figure 11 The Linewever-Burk plots for uncompetitive inhibition

(http://chemistry.ewu.edu/jcorkill/biochem/com_nono_unIH.jpg)

The effect of inhibition types on K_m and V_{max} was summarized in Table 2

Table 2 The changes in the kinetic constants in different subtypes of reversible inhibition (Tracy, 2008)

Type of inhibition	V^{app}_{max}	$V^{app}_{max}/K^{app}_{m}$	K^{app}_{m}
competitive	no change	increase	increase
mixed	decrease	increase or decrease	increase
pure noncompetitive	decrease	decrease	no change
uncompetitive	decrease	no change	decrease

2. Quasi-irreversible inhibition via metabolic intermediate complexation

In the quasi-irreversible inhibition, the inhibitor is catalytic turnover by CYP450 to form metabolite. This metabolite non-covalently binds to heme prosthetic group of the enzyme to form a quasi-irrevesible complex (or metabolic intermediate (MI) complex). Consequently, CYP was inactivated (Lin and Lu, 1998). In certain experimental condition, the quasi-irrevesible inhibition can be reversible. For example, it was reported that addition of lipophilic drugs could replace the metabolic intermediate at the enzyme active site and reactivate the enzyme activity (Honkalammi, 2011).

3. Mechanism-based inactivation

Mechanism-based inactivation produces more severe and long-lasting suppression of enzyme activity than other types of inactivation do. In this inhibition, the inhibitor is catalytic turnover by the enzyme to generate the reactive intermediates. These metabolites can covalently attach to the active site of the enzyme (amino acid residues) or the heme prosthetic group or a combination of both (Lin and Lu, 1998; Zhou, 2005). In other word, the inhibitor is a suicide substrate of the enzyme. Consequently, the enzyme is irreversible inactivated. The major characteristics of the mechanism-based inactivation include time dependency, saturable reaction, and catalytic reaction for the inhibitor. Generally, the stoichiometry between inhibitor and enzyme is 1:1 (Zhou, 2005).

Limonoids

Limonoids are a group of chemically related triterpene derivatives found abundantly in the *Rutaceae* and *Meliaceae* families (Sun, 2005). The structure of limonoids contained tetranortriterpenoid (Manners, 2007) (Figure 12). Limonoids are of moderate polarity, insoluble in water and hexane but soluble in hydrocarbons, alcohol and ketone. Limonoids have a substituted furan moiety, attached to the D-ring at C-17 as well as oxygen containing functional groups at C-3, C-4, C-7, C-16 and C-17 (Silalahi, 2002; Roy and Saraf, 2006).
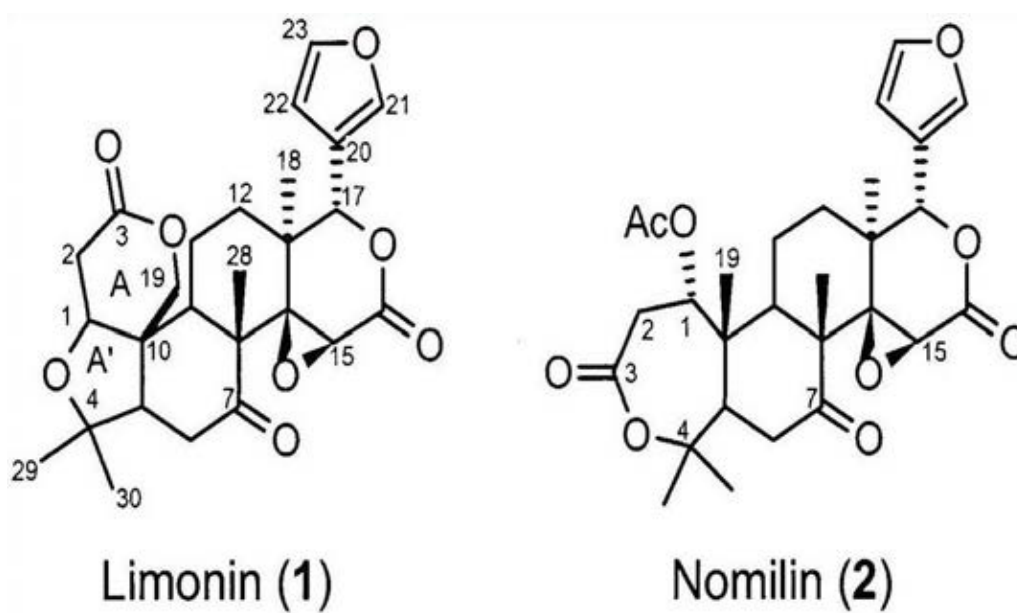
Figure 12 The structure of limonoids, 1: limonin, 2: nomiin (Ruberto et al., 2002).

Limonin and nomilin (figure 12) are the most prominent limonoids aglycone found in citrus species such as *Citrus grandis, Citrus limon, Citrus paradisi, Citrus sinensis* (Hasegawa and Herman, 1985; Rouseff, 1982; Girard and Mazza, 1998). These compounds have bitter taste (Girard and Mazza, 1998). Both limonin and nomilin have broad spectrum of biological activities such as insecticidal, antiviral, antioxidant, antifeedant, anticancer (Roy and Saraf, 2005). In addition, limonin elicited their inhibitory effect on CYP1A2, CYP1B1, CYP3A4 activity (Poulose, 2005; Han et al., 2011). It was reported that the content of nomilin in pummelo juices from *Citrus grandis* grown in Thailand was approximately 29.40 mg/L (ranging from 10.90 – 41.83 mg/L) (Pichaiyongvongdee and Haruenkit, 2009). Several studies demonstrated that nomilin has a range of significant biological activities including anti-feedant against the fourth instar larvae of mosquito *Culex quinquefasciatus* (Jayaprakasha et al., 1997), the late third or early fourth instar *Aedes albopictus* (Hafeez et al., 2011), the fifth instar larvae *Spodoptera frugiperda* (Ruberto et al., 2002), antitumor (Poulose et al., 2006; Tian et al., 2001), antioxidant (Mandadi et al., 2007), anti-inflammation (Kim et al., 2011) and anti-obesity (Ono et al., 2011). In addition, nomilin was an immune enhancer which could inhibit delayed hypersensitivity reaction (Raphael and Kuttan, 2003). It was reported that nomilin was able to shorten the sleeping time induced by alpha-chloralose and urethane in the mouse model (Wada et al., 1992). Furthermore, this compound was able to affect activity and expression of various enzymes such as glutathione S- transferase by inducing enzyme activity (Perez et al., 2010), inhibition reverse transcriptase (Balestrieri et al., 2011). In addition, the effect of nomilin on the activity of CYP enzyme was also reported. In 2005, Iwata et al. demonstrated that nomilin extracted from *Evodiae Fructus* fruit could inhibit human liver microsomal erythromycin N-demethylation activity, reflecting its effect on CYP3A4 (Iwata et al., 2005). The inhibitory effects of nomilin on various CYP450 isoforms including CYP1A2, CYP1B1, CYP3A4 and CYP19 were investigated using specific substrates such as ethoxyresorufin (ethoxyresorufin *o*-dealkylase, (EROD), methoxyresorufin (methoxyresorufin *o*-dealkylase, (MROD) and dibenzylfluorescein (DBF). It was found that nomilin exhibited inhibition action against

CYP1B1, CYP1A2, CYP19 and CYP3A4. The IC_{50} values of nomilin on CYP1B1 (EROD), CYP1B1 (MROD), CYP1A2 (MROD), CYP19 and CYP3A4 of 20.62, 24.06, 31.01, 16.43 and 10.43 μM respectively (Poulose, 2005). Moreover, nomilin from pomelo juice could inhibit CYP3A4 activity with the IC_{50} value of 17.20 μM (นุศรา ปิยะพลรุ่งโรจน์, 2551).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Instruments

Analytical balance (Mettler$^\circledR$AJ, Switzerland)

Autopipet (Pipetman Glison$^\circledR$, France)

Centrifuge (Hermle$^\circledR$Z383K, Germany)

High Performance Liquid Chromatography Apparatus: Shimadzu$^\circledR$ LC-20AD HPLC pump with a communication bus module (CBM-20A), and an autoinjector (SIL-20A). The HPLC was connected to a spectro UV-VIS detector (SPD-20A) and computerized integrator (LC solution).

Hot air oven (Memmert$^\circledR$, Germany)

pH meter (Schott$^\circledR$, England)

Ultra-low temperature freezer (Forma Scientific$^\circledR$ Inc., USA)

Ultrapurifier (ELGA MAXIMA$^\circledR$, England)

Vacuum pump (GE$^\circledR$motors, USA)

Vortex mixture (Vortex Genie 2$^\circledR$, USA)

Water bath (Memmert$^\circledR$, Germany)

2. Chemicals

Acetonitrile (HPLC grade) was purchased from Honeywell-Burdick & Jackson, Muskegon, MI, USA.

Baculovirus expressed recombinant cDNA Cytochrome P450 3A4 including cytochrome b5 (Cytochrome P450 BACULOSOMES$^\circledR$ Invitrogen, USA).

Erythromycin ethylsuccinate was obtained from Siam Pharmaceutical Co. Ltd., Thailand.

Glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Merck, Germany.

Ketoconazole was kindly provided by Government Pharmaceutical Organization, Thailand.

Magnesium Chloride, potassium phosphate, and dipotassium phosphate were purchased from Ajax Finechem, Sydney, Australia.

Methanol (HPLC grade) was purchased from Honeywell-Burdick & Jackson, Muskegon, MI, USA.

Nicotinamide adenine dinucleotide phosphate (NADP) was obtained from Sigma Chemical Co., St Louis, MO, USA.

Nomilin was purchased from Chromadex, Irvine, CA, USA.

Potassium phosphate monobasic anhydrous, potassium phosphate dibasic and magnesium chloride were purchased from Ajax Finechem, Sydney, Australia.

Testosterone (Sigma Chemical Co.,St Louis, MO, USA).

Ultrapure water was prepared by ELGASTAT MAXIMA UF$^{®}$ (ELGA Ltd., England)

6β-hydroxytestosterone (Sigma Chemical Co., St Louis, MO, USA).

Methods

1. High performance liquid chromatography (HPLC) system

In this study, the 6β-hydroxytestosterone, a metabolite of testosterone hydroxylation, was determined using the HPLC system. The sample was prepared and injected onto a BDS Hypersil column (4.2 mm x 250 mm, 5 μm). The total run time was 35 min under the condition of mobile phase methanol : water (55:45), and flow rate at 1 ml/min (Engdal and Nilsen, 2009). The UV absorbance was determined at the wavelength of 254 nm. The metabolite 6β-hydroxytestosterone was detected at the retention time 7.8 min whereas testosterone was detected at the retention time 30 min.

In this study, the stock solution of 6β-hydroxytestosterone and testosterone were prepared in methanol at the concentration of 2 mM and 20 mM, respectively. The stock solutions were kept at -20°C. For each experiment, the stock solution was diluted with 0.1 M potassium phosphate buffer (Appendix A).

1.1 Linearity of the detection (Engdal and Nilsen, 2009)

In this study, the HPLC method to detect 6β-hydroxytestosterone was tested for its linearity. In brief, the stock solution of 6β-hydroxytestosterone was diluted with 0.1 M potassium phosphate buffer to six concentrations (0.25, 1.25, 2.5, 5, 10 and 20 μM). One hundred microliters of each standard solution was, then, injected into the HPLC system. The retention time and peak area were recorded. The plot of concentration and peak area was constructed and analyzed for the linearity using coefficient of determination (R^2).

1.2 Stability of 6β-hydroxytestosterone in reaction sample

The stability of 6β-hydroxytestosterone kept in the experimental condition was monitored at various storage times.

1.2.1 The samples were kept on ice and subjected to HPLC analysis at 0, 5, 7 hour after the reaction stopped.

1.2.2 The samples were kept in a freezer at -20^{0} C for 17 days. On the day of experiment, the samples were freshly thawed and subjected to HPLC analysis.

The retention time and peak area were recorded and analyzed. Deviation of the values from those of the first time or first day analysis was calculated. The acceptance values were in between minus 15% and plus 15% of the original data.

Percentage of deviation was calculated from the following equation.

$$\text{Percentage of deviation} = \frac{100 \times (\text{Measured value at certain time} - \text{Measured value at time 0/day 0})}{\text{Measured value at time 0/day 0}}$$

2. CYP3A4 activity assay

The activity of CYP3A4 was determined via the testosterone 6β-hydroxylation with the use of the method of Takanaga et al. (2000) with some modification. The metabolite of testosterone hydroxylation, 6β-hydroxytestosterone, and the substrate, testosterone, in reaction mixture were detected under HPLC system. The reaction mixtures (112.5 μl) consisted of CYP3A4 (20 pmol/ml) and testosterone (200 μM) in 0.1 M potassium phosphate buffer (pH 7.45) were pre-incubated at 37°C for 5 min in a shaking water bath. The reaction was initiated by addition of 12.5 μl NADPH regenerating system (1.3 mM NADP, 3.3mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenese, and 3.3 mM magnesium chloride) (Appendix A) and incubated for 15 min at 37°C. At the end of 15 min-incubation period, 50 μl ice-cold acetonitrile (100%) was added into the mixture to stop the reaction, followed by centrifugation at 15,000 rpm at 4°C for 45 min. The supernatant was subjected to HPLC analysis for 6β-hydroxytestosterone and testosterone under HPLC system. The 15 min-incubation time used in this study was within the linear range (see Appendix C).

3. Effects of nomilin on CYP3A4-mediated metabolism of testosterone

3.1 Determination of the IC$_{50}$ value

The concentration of inhibitor causing a 50% reduction in CYP activity of nomilin (IC$_{50}$) was determined. The reaction mixture (112.5 μl) containing CYP3A4 (20 pmol/ml), testosterone (200 μM), and nomilin at various concentrations (ranging from 2 to 150 μM), was pre-incubated at 37°C for 5 min. The reaction started by addition of 12.5 μl NADPH regenerating system. After the incubation period of 15 min at 37°C, 50 μl ice-cold acetonitrile (100%) was added into the mixture to stop the reaction, followed by centrifugation at 15,000 rpm at 4°C for 45 min. The supernatant was subjected to HPLC analysis for 6β-hydroxytestosterone.

In this experiment, ketoconazole at the concentration of 0.5 μM was used as a positive control group. In addition, nomilin and ketoconazole were prepared as stock solutions in acetonitrile at the concentration of 15 mM and 0.05 mM, respectively.

The stock solutions were kept at -20°C. On the day of experiment, the stock solutions were freshly thawed and diluted with 0.1 M potassium phosphate buffer (pH 7.45). The final concentration of acetonitrile in reaction mixture was 1%.

3.2 Determination of the type of inhibition and inhibition constant

In this experiment, the inhibition of CYP3A4 was performed in the reaction mixtures (112.5 μl) containing testosterone (25, 50, 100, 200 μM), nomilin (0, 5, 10, 20 μM) and CYP3A4 (20 pmol/ml) in 0.1 M potassium phosphate buffer (pH 7.45). The reaction mixture was pre-incubated at 37°C for 5 min prior to addition of 12.5 μl NADPH regenerating system to start the reaction. After the 15 min incubation period, the reaction was terminated by 50 μl ice-cold acetonitrile (100%), followed by centrifugation at 15,000 rpm at 4°C for 45 min. The supernatant was subjected to HPLC analysis. The metabolite, 6β-hydroxytestosterone was quantified with the use of a standard curve of 6β-hydroxytestosterone (0.25 to 20 μM).

The enzyme activity was calculated as the formation of 6β-hydroxytestosterone in the 15 min incubation of CYP3A4. The enzyme activity was expressed as the amount of 6β-hydroxytestosterone formed (pmol) per pmol CYP3A4 and time (min).

For this assay, nomilin was prepared as stock solutions in acetonitrile at the concentration of 5 mM. The stock solutions were kept at -20°C. On the day of experiment, the stock solutions were freshly thawed and diluted with 0.1 M potassium phosphate buffer (pH 7.45). The final concentration of acetonitrile in reaction mixture was 1%.

3.3 Assessment of mechanism-based inhibition

3.3.1 NADPH-dependent inhibition assay

The effect of NADPH on CYP3A4 inactivation was determined using a method modified from that of Obach et al. (2007). In this experiment, reaction mixture (8.5 μl) containing CYP3A4 (147 pmol/ml) and an inactivator (either erythromycin 30 μM or ketoconazole 0.5 μM or nomilin 50 μM) in 0.1 M potassium phosphate buffer (pH 7.45) was incubated in the presence and absence of the NADPH regenerating

system for 30 min at 37°C. At the end of 30-min inhibition period, a 65 μl of substrate solution containing testosterone 100 μM and NADPH regernerating system was added to stop the inhibition. In order to determine residual CYP3A4 activity, the mixture was further incubated at 37°C for another 15 min. The reaction was terminated by addition of ice cold acetonitrile (29.4 μl). After centrifugation at 15,000 rpm at 4°C for 45 min, the supernatants were collected for HPLC analysis.

In this experiment, erythromycin 30 μM was used as a positive control group and ketoconazole 0.5 μM was used as a negative control group. Furthermore, erythromycin, ketoconazole and nomilin were prepared as stock solutions in acetonitrile at the concentration of 6 mM, 0.1 mM and 30 mM, respectively. The stock solutions were kept at -20°C. On the day of experiment, the stock solutions were freshly thawed and diluted with 0.1 M potassium phosphate buffer (pH 7.45). The final concentration of acetonitrile in reaction mixture was 0.5%.

3.3.2 Time-dependent inhibition assay

The time dependent inhibition of nomilin on CYP3A4 was determined using a method modified from that of Iwata et al. (2005). The inhibition mixture (50 μl) contained CYP3A4 (147 pmol/ml), nomilin (25 and 100 μM) and NADPH regenerating system in 0.1 M potassium phosphate buffer (pH 7.45), and incubated at 37°C for certain period of time (0, 10, 20 and 30 min). At the end of each inhibition period, an 8.5 μl of inhibition mixture was transferred to the secondary mixture (65 μl) containing testosterone 250 μM and NADPH regenerating system, and further incubated for another 15 min at 37°C. The reaction was terminated on ice by addition of 29.4 μl ice cold acetonitrile. After centrifugation at 15,000 rpm at 4°C for 45 min, the supernatants were collected and subjected to HPLC analysis.

For this assay, erythromycin, ketoconazole and nomilin were prepared as stock solutions in acetonitrile at the concentration of 6 mM, 0.1 mM and 30 mM, respectively. The stock solutions were kept at -20°C. On the day of experiment, the

stock solutions were freshly thawed and diluted with 0.1 M potassium phosphate buffer (pH 7.45). The final concentration of acetonitrile in reaction mixture was 0.5%.

4. Data analysis

The IC_{50} value (the concentration of inhibitor causing a 50% reduction in CYP activity) was determined using Probit analysis in SPSS 16.0 software. The type of inhibition was initially estimated graphically from the Lineweaver-Burk plots between reciprocal of CYP3A4 activity and reciprocal of testosterone concentrations. The apparent K_i value was estimated from the secondary plots of the slopes of Lineweaver-Burk plots against individual nomilin concentration.

A two-sample t-test was used to test the effect of NADPH dependent inhibition. P-value of ≤ 0.05 was set to be statistically significant.

CHAPTER IV

RESULTS

1. HPLC chromatograms of 6β-hydroxytestosterone and testosterone

The metabolite of testosterone hydroxylation, 6β-hydroxytestosterone was identified by HPLC system on the BDS C18 reversed column using methanol:water (55:45) as isocratic mobile phase at flow rate of 1 ml/min. The peaks were detected at 254 nm. This HPLC system could detect peak area of the standard 6β-hydroxytestosterone and testosterone at the retention time of 7.84 min and 28.34 min, respectively (Figure 13, 14).

The HPLC chromatograms of the sample collected from the CYP-mediated hydroxylation of testosterone were shown in figure 15. Peak of 6β-hydroxytestosterone and testosterone were detected at the retention time 7.74 and 28.61 min, respectively. Furthermore, any interferences in the sample were not observed in the usable range of data collection, indicating the selectivity of the method for the determination of 6β-hydroxytestosterone in the reaction mixture.
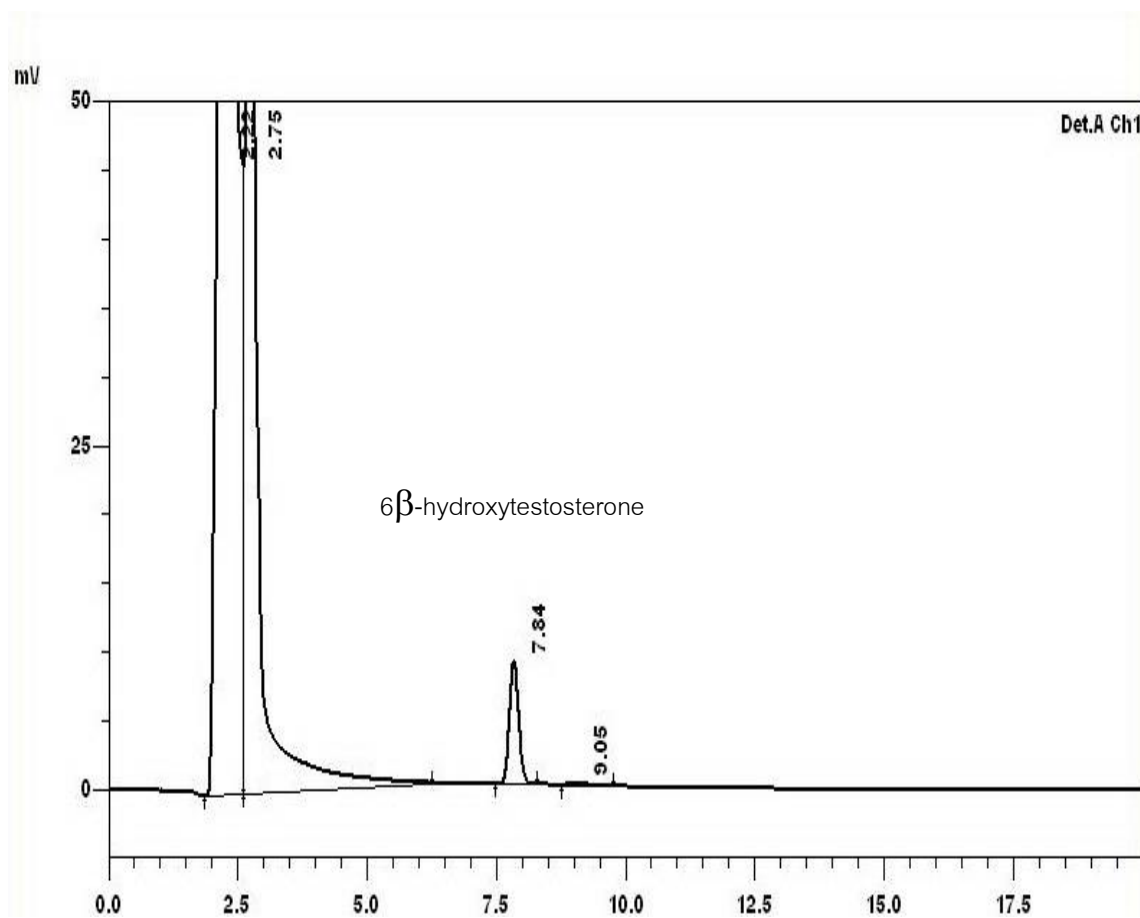
Figure 13 Representative HPLC chromatogram of the standard 6β-hydroxytestosterone (2.5 μM).
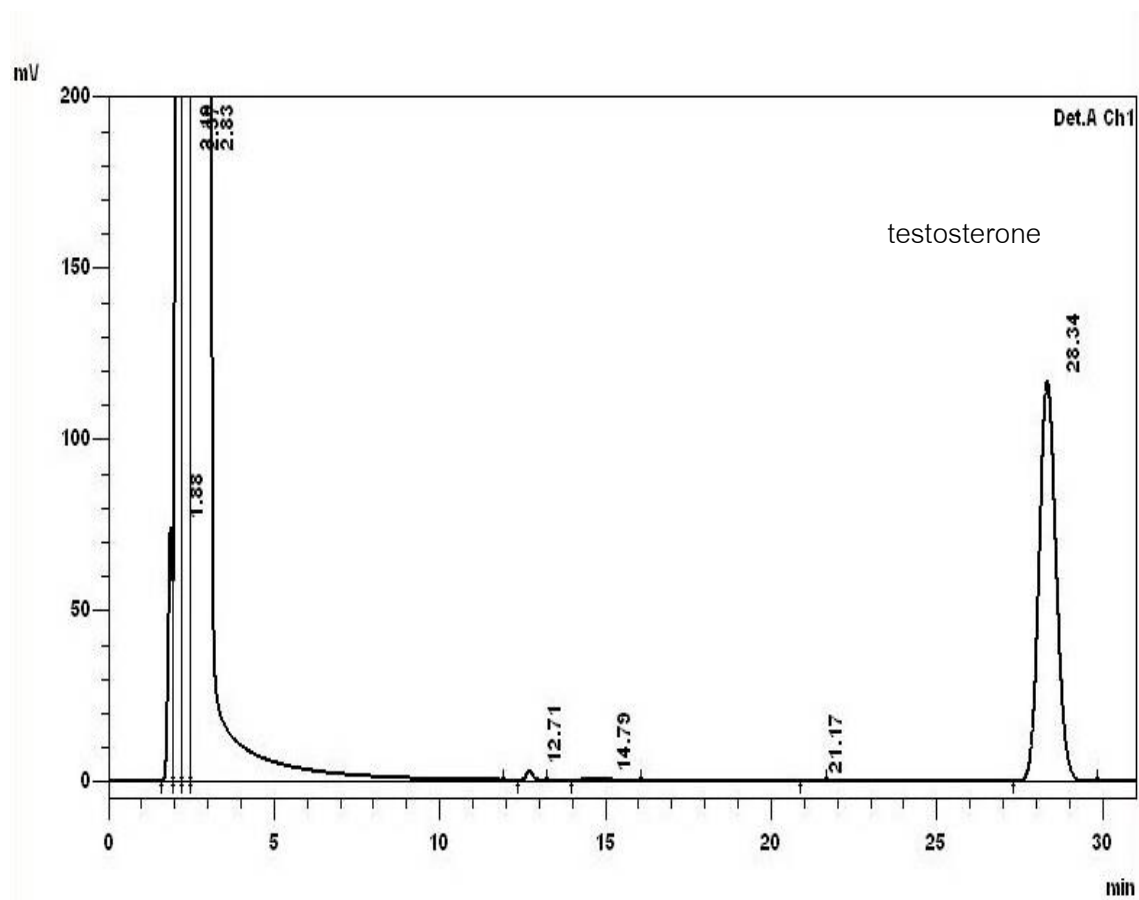
Figure 14 Representative HPLC chromatogram of the standard testosterone (200 μM).
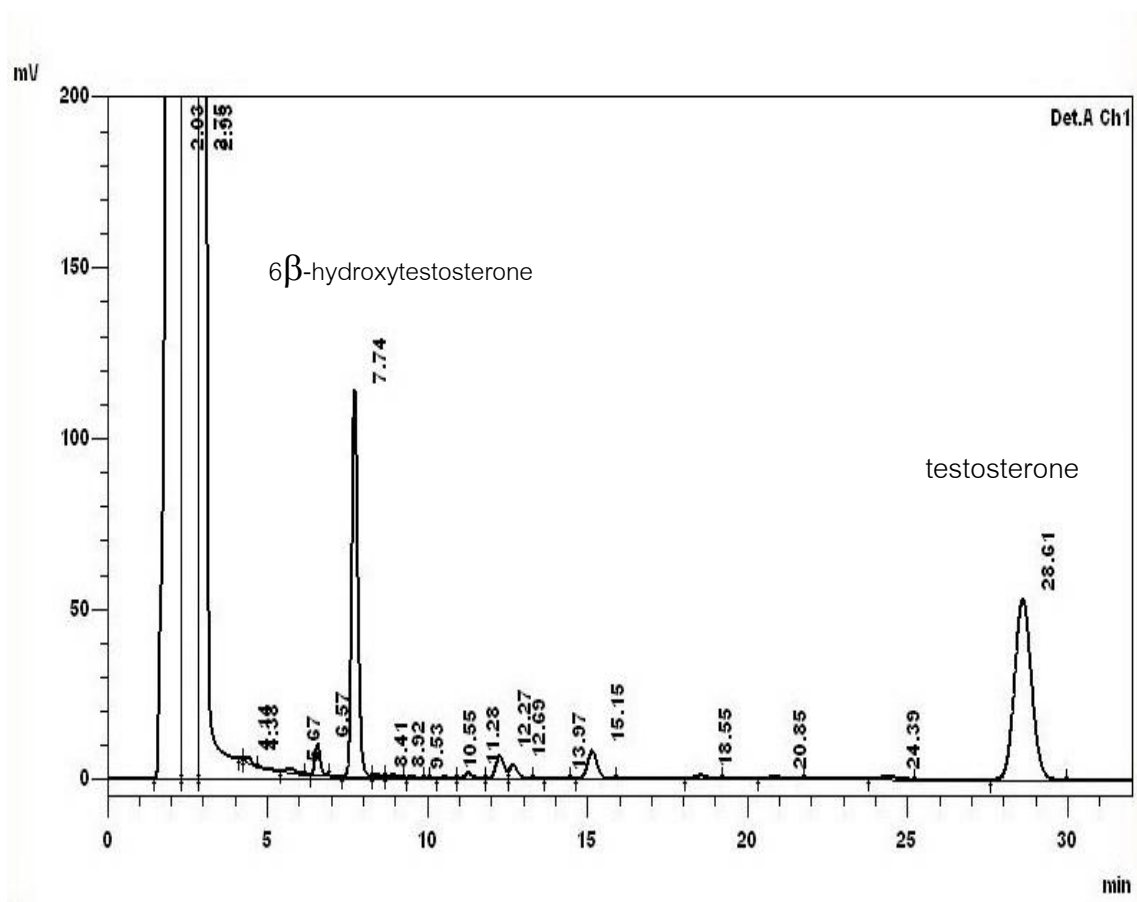
Figure 15 Representative HPLC chromatogram of the CYP-mediated hydroxylation of testosterone sample.

2. Relationship between peak areas and concentrations of 6β-hydroxytestosterone

The standard curve of the 6β-hydroxytestosterone in the range of 0.25-20 nmole/ml was illustrated in figure 16. This curve demonstrated a linear relationship between peak area of chromatograms and concentrations with the coefficient of determination (R^2) = 0.9995.

Standard curve of 6β- hydroxytestosterone

y = 76250x - 23558

R² = 0.9995
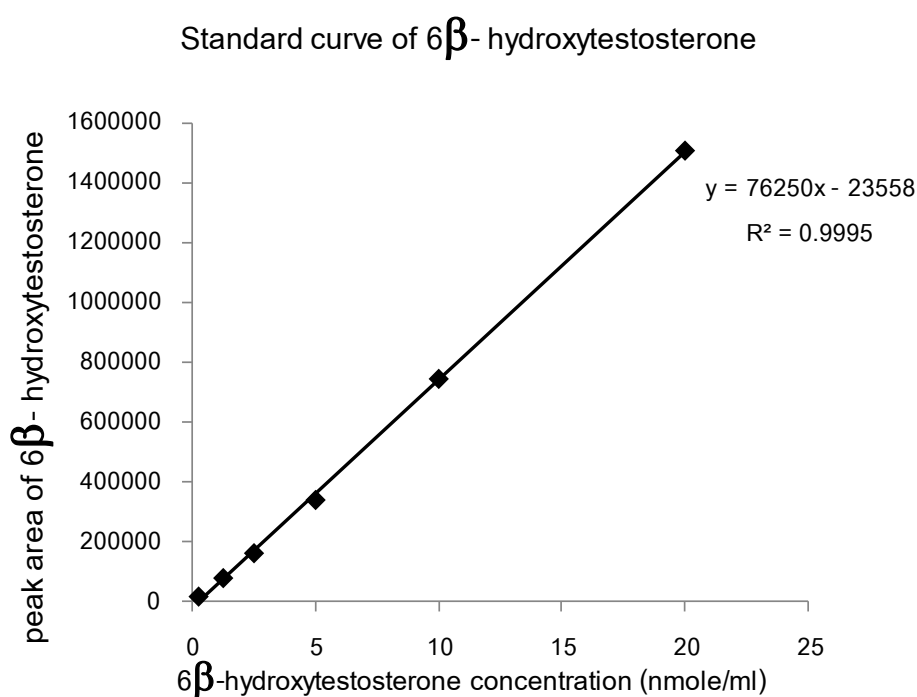
Figure 16 Standard curve of 6β-hydroxytestosterone (the concentration range of 0.25-20 nmole/ml). Various concentrations of 6β-hydroxytestosterone (100 μl) were injected into the HPLC system as mentioned in *materials and methods*. The injection was repeated 2 times for each concentration. The standard curve was plotted between peak areas calculated from the chromatogram and the corresponding concentrations of 6β-hydroxytestosterone.

3.   Stability of 6β-hydroxytestosterone in the reaction sample

　　　3.1 The supernatant of reaction mixtures were collected and subjected to HPLC analysis for 6β-hydroxytestosterone at 0, 5, 7 hour after the reaction was terminated. All the samples were kept on ice. Data were calculated as the percentage deviation from the values obtained at time 0 and presented in table 3. The results showed that percentage deviation from time 0 was less than 2%, suggesting the good stability in the experimental storage condition prior to analysis.

Table 3 The percentage deviation of the peak area of 6β-hydroxytestosterone obtained from the samples kept on ice for 5 and 7 hours.

Storage time (hour)	%deviation from time 0 hr			Mean of %deviation from time 0 hr	SEM
	1	2	3		
5	-0.06	0.89	1.20	0.67	0.38
7	-1.00	0.65	1.60	0.42	0.76

The supernatant of reaction mixtures were collected and subjected to HPLC analysis at 0 and 17 days after kept in a freezer at -20^0 C. Data were calculated as the percentage deviation from the values obtained at day 0 and presented in table 4. These result showed that peak area of 6β-hydroxytestosterone decreased very slightly with the less than 3% deviation from day 0.

Table 4 The percentage deviation of the peak area of 6β-hydroxytestosterone obtained from the sample kept at $-20^{\circ}C$ for 17 days.

Storage time (day)	%deviation from day 0			Mean of %deviation from day 0	SEM
	1	2	3		
17	-1.20	-0.93	-2.55	-1.56	0.50

4. The inhibitory effect of nomilin on CYP3A4-mediated metabolism of testosterone

Addition of nomilin to the incubation mixtures resulted in concentration-dependent inhibition of CYP3A4-mediated testosterone 6β-hydroxylation (Figure 17). The data were transformed into the Probit unit in order to estimate the IC_{50} values (Figure 18). The apparent IC_{50} value of nomilin obtained from 15 min-inhibition at 37°C, pH 7.45 was 14.54 $\pm$ 2.77 μM (n=3).
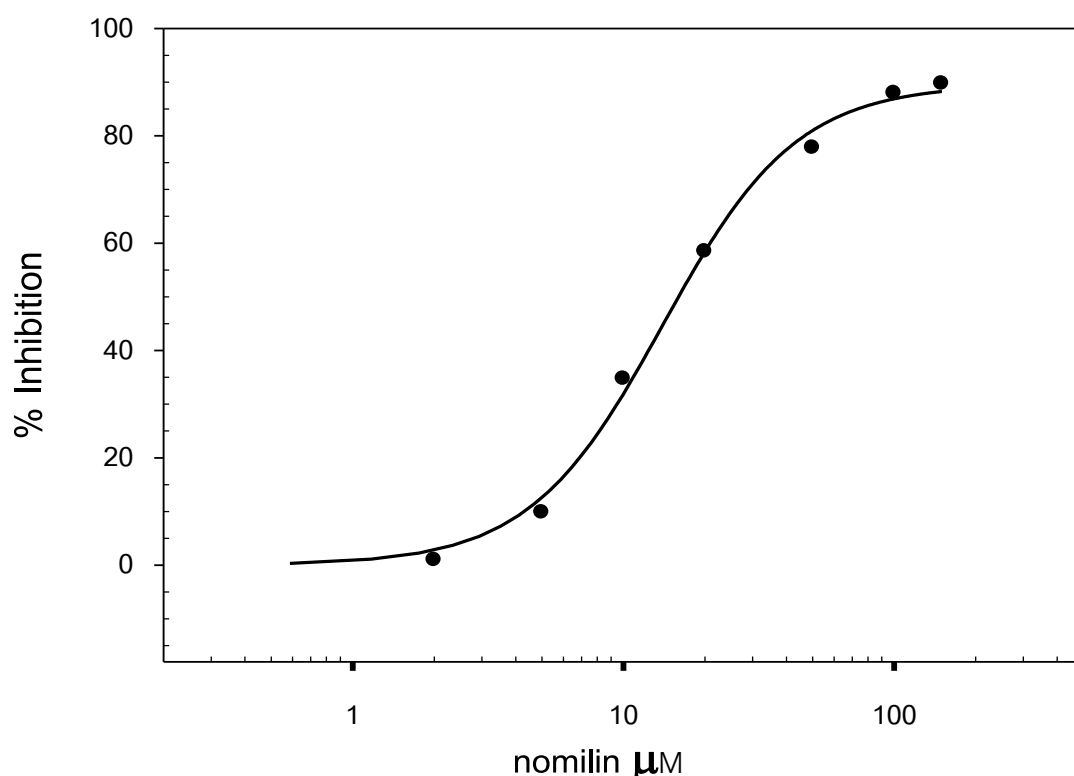
Figure 17 Representative plot for the inhibitory effect of nomilin on the CYP3A4-mediated 6β-hydroxylation of testosterone. As described in materials and methods, 200 μM of testosterone was incubated with CYP3A4 in the presence of nomilin at various concentrations (2-150 μM) for 15 minutes, and metabolite formation was measured by HPLC.
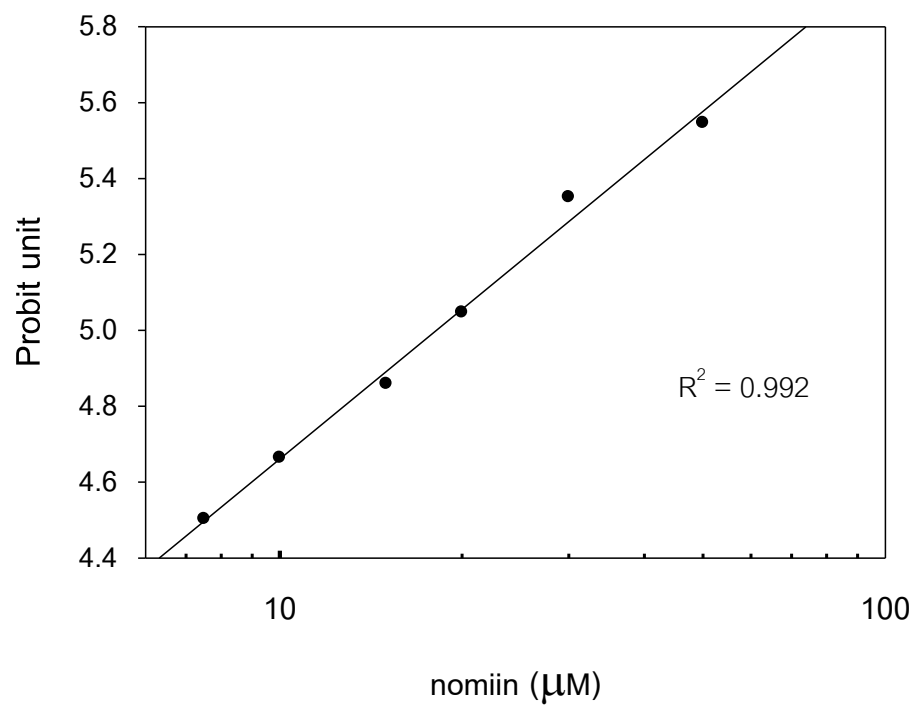
Figure 18 Representative Probit plot for inhibitory effect of nomilin on CYP3A4-mediated 6β-hydroxylation of testosterone.

5. The type inhibition and the inhibition constant (K_i) value

For the determination of type of inhibition and inhibition constant, the Lineweaver-Burk plots between reaction rate of CYP3A4 activity and testosterone concentrations were constructed as shown in figure 19. The K_m and V_{max} values of the CYP-mediated testosterone hydroxylation were shown (Table 5). These plots also showed that the presence of nomilin altered the intercepts on both X-axis (or 1/[S]) and Y-axis (or 1/V) (Figure 19). The results indicated that nomilin apparently increased the K_m value of the testosterone-CYP3A4 complexes. Furthermore, nomilin decreased the maximal velocity (V_{max}) of the enzyme catalytic reaction (Table 5). These nomilin-mediated changes in the values of K_m and V_{max} suggested that nomilin caused a mixed type inhibition of CYP3A4-mediated testosterone 6β-hydroxylation.

The secondary plot of the slopes of Lineweaver-Burk plots (K_m/V_{max}) against concentration of nomilin was created in order to determine the inhibitior constant (K_i) of nomilin. The x-intercept of secondary plot provided the K_i value of 4.83 μM (n1 = 4.52 μM; n2 = 5.15 μM) (Figure 20).

Table 5 The apparent values of K_m and V_{max} for the nomilin-mediated inhibition CYP3A4.

Group	Average values	
	K_m	V_{max}
control	59.68	79.44
Nomilin 5 µM	197.21	82.89
Nomilin 10 µM	251.51	71.89
Nomilin 20 µM	221.86	41.60

Note: The data were obtained from two separated experiments
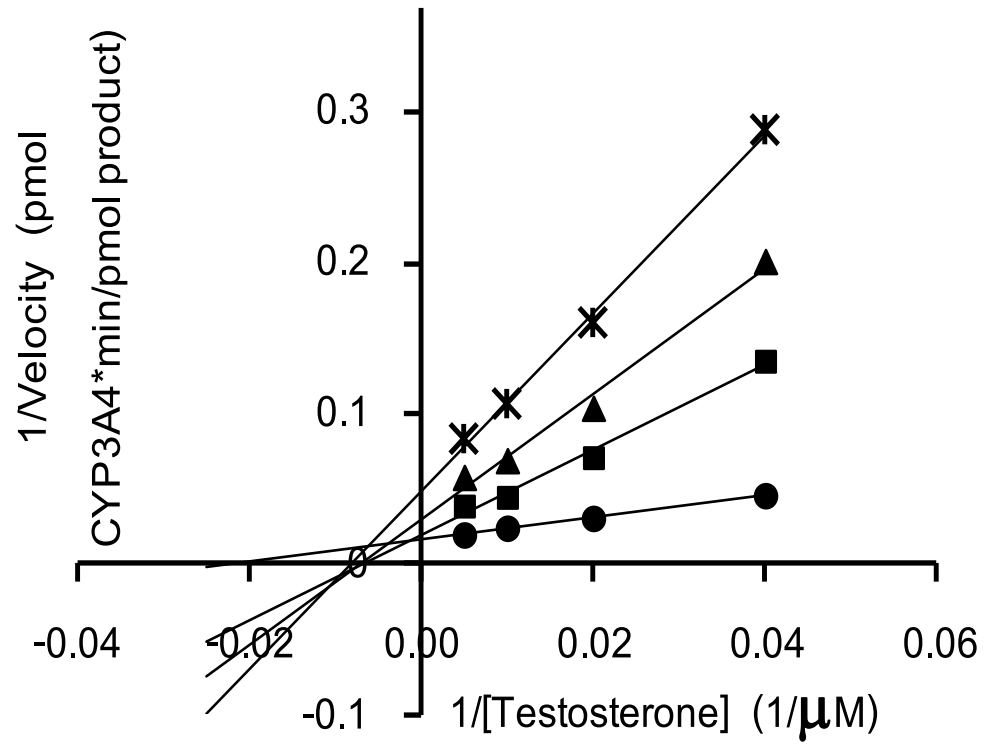
Figure 19 Lineweaver-Burk plot for the inhibition of CYP3A4 by nomilin (**A**) (●; 0 μM, ■; 5 μM, ▲; 10 μM, X; 20 μM). Each point on the Lineweaver-Burk plots was a mean of duplicate experiments.
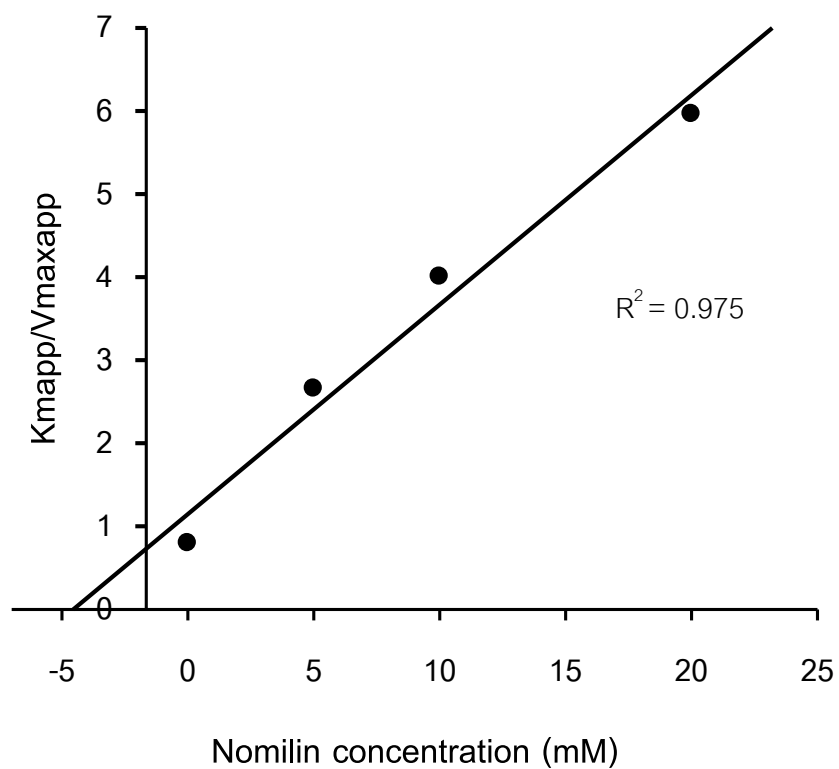
Figure 20 The secondary plot of CYP3A4 activity using the slopes of the primary Lineweaver-Burk plots *versus* the concentrations of nomilin. Each point on the Lineweaver-Burk plots was a mean of duplicate experiments.

6. Effect of NADPH on the inhibition of CYP3A4 by nomilin

The requirement of NADPH for nomilin-mediated CYP3A4 inactivation was determined in this study. As shown in figure 21, the CYP3A4 activity decreased in the presence of NADPH during the 30 min-inhibition period with various inhibitors. However, this decrease in CYP450 activity was also observed in the untreated control group. Thus, the data presentation was modified by setting the activity of untreated control as 100% as illustrated in figure 22. The results showed that erythromycin, a known mechanism-based inhibitor, inhibited the activity of CYP3A4 by 86.49% in the presence of NADPH. By contrast, the absence of NADPH in the inhibition period reduced its inhibitory effect significantly. The residual enzyme activity was 77.24%. Furthermore, the inhibitory effect of ketoconazole, a reversible inhibitor of CYP3A4, was not observed in this experimental condition which was provided for the mechanism-based inhibition. The residual enzyme activities were approximately 101.29% and 97.73% in the presence and absence of NADPH, respectively. Like ketoconazole, the presence of NADPH had no effect on nomilin-mediated inhibition of CYP3A4 in this mechanism-based experiment. The residual activity of CYP3A4 preincubated with nomilin (50 μM) were 89.47% and 82.89% in the presence and absence of NADPH, respectively.
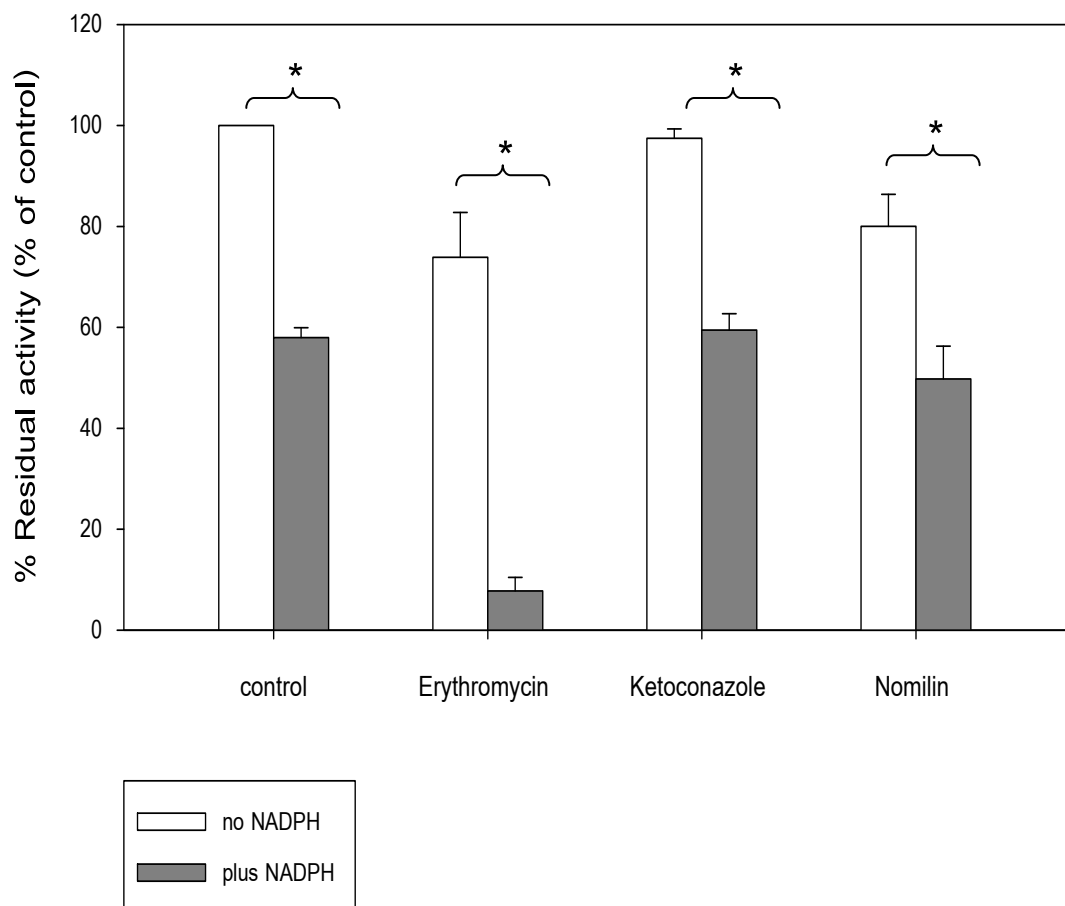
Figure 21 Requirement of NADPH in the CYP3A4 inhibition. The residual CYP3A4 activity incubated with various inhibitors (erythromycin 30 μM, ketoconazole 0.5 μM, nomilin 50 μM) in the presence and absence of NADPH regenerating system. Data were calculated as the percentage of the residual enzyme activity of the control in the absence of NADPH. Data were given as mean $\pm$ S.E.M of three or four separated experiments. * indicated statistically significance (p<0.05).
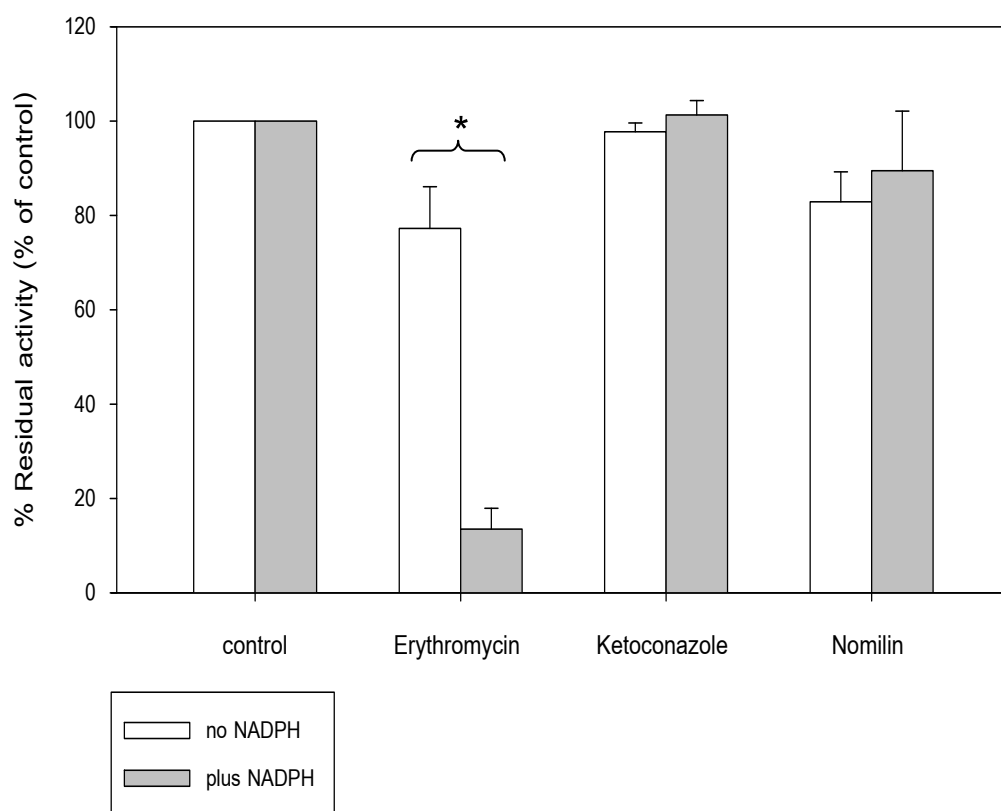
Figure 22 The modified graph of Figure 21. The effect of 30 min preincubation with erythromycin, ketoconazole and nomilin on the CYP3A4-mediated metabolism of testosterone. Data were calculated as the percentage of the residual enzyme activity of the control. Data were given as mean $\pm$ S.E.M of three or four separated experiments. * indicated statistically significance ($p < 0.05$).

7. Effect of inhibition time on the nomilin-mediated inhibition of CYP3A4

The effect of inhibition times on nomilin-mediated inactivation of CYP3A4 activity were carried out in the presence of NADPH. As shown in figure 23, erythromycin (30 μM), but not ketoconazole (0.5 μM), was able to inactivate CYP3A4 in time-dependent fashion. Nomilin neither at the concentration of 25 μM nor 100 μM could inactivate the CYP3A4 activity in this experimental condition. The results suggested that this compound was not a mechanism-based inhibitor of CYP3A4.
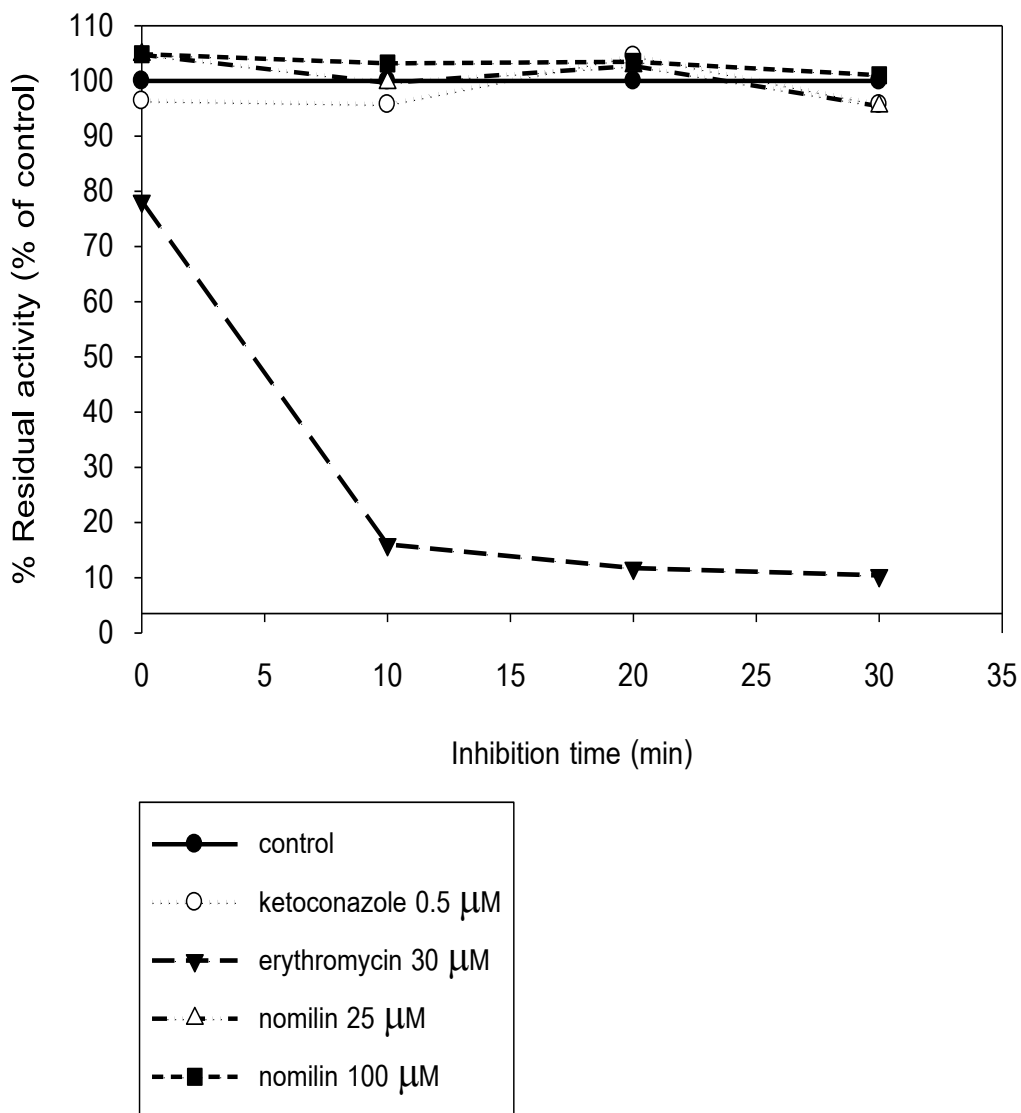
Figure 23 The effect of inhibition time on CYP3A4 activity. CYP3A4 were preincubated with erythromycin 30 μM, ketoconazole 0.5 μM, nomilin 25 μM, and nomilin 100 μM for 0, 10, 20 and 30 min at 37^0C. Then, testosterone hydroxylation was determined as described in *materials and methods*. Values were presented as percentage of the residual activity and were normalized by the activity obtained from control which was set as 100%. Each symbol represented the means of duplicate experiments.

CHAPTER V

DISCUSSION AND CONCLUSION

This study investigated the inhibitory action of nomilin, a bitter compound found abundantly in citrus fruits, against the recombinant human CYP3A4 metabolic activity. The activity of CYP3A4 was determined through the hydroxylation of a selective substrate testosterone. The metabolite 6β-hydroxytestosterone was determined with the use of HPLC system under the condition reported in the literature (Engdal and Nilsen, 2009). In this regard, the analytical method and instrument in the study were verified for the linearity and stability of the metabolite in the samples. The linearity of the analytical method demonstrated that the obtained HPLC data were directly, proportional to the concentration of metabolite within the linear range. The result showed that peak area and concentrations of standard 6β-hydroxytestosterone were in linearity with good correlation (R^2=0.9995). In addition, determination of stability of 6β-hydroxytestosterone in the samples kept in the experimental condition was also performed in order to validate the storage duration. The data showed that 6β-hydroxytestosterone in the reaction mixture could be stored at -20°C until the analysis for at least 17 days. In addition, the sample could be kept on ice up to 7 hours. The percentage deviation from first time or first day analysis was lower than the acceptance criteria (within 15% range) as bioanalytical method validation defined by US.FDA (US.FDA, 2001). In this study, the time course of the production of metabolite 6β-hydroxytestosterone was in linearity up to 30 min. Furthermore, nomilin did not interfere with the measurement of 6β-hydroxytestosterone (Appendix C). It was noteworthy to mention that nomilin had no effect on the production of NADPH from regenerating system (Appendix C).

The results showed that nomilin had inhibitory effect on CYP3A4 with the apparent IC_{50} value of 14.54 μM. The IC_{50} values obtained from different laboratory could have high variation due to the experimental conditions such as inhibition time, pH, substrate, source of enzyme, the method of analysis (Tracy, 2008). The IC_{50} value obtained in this study was agreeable with another reported value of 17.32 μM which

was obtained from the experiment under similar condition (นุศรา ปิยะพลรุ่งโรจน์, 2551). In another study of nomilin-mediated CYP3A4 inhibition, nomilin inhibited the erythromycin *N*-demethylation activity of human liver microsome with the reported IC_{50} value of 5.0 μM (Iwata et al., 2005). Nomilin exhibited inhibition action against dibenzylfluorescein hydroxylation of human liver expressed CYP3A4 with the IC_{50} values of 10.43 μM (Poulose, 2005). It was reported that ketoconazole, a known reversible inhibitor of CYP3A4 inhibited the testosterone hydroxylation activity of recombinant CYP3A4 with the IC_{50} value of 0.16 μM (Engdal and Nilsen, 2009). These observations suggested that nomilin was less potent than ketoconazole.

The type of nomilin-mediated inhibition and the kinetic parameters including constant of inhibition (K_i), maximal velocity (V_{max}) and the concentration at which half-maximal velocity (K_m) were further determined. Based on the Michaelis-Menten equation, the Lineweaver-Burk plots were constructed between reciprocal of substrate concentrations and reciprocal of velocity (Tracy, 2008). In this study, the velocity of the reaction was calculated from the formation of 6β-hydroxytestosterone in the 15 min reaction. The apparent K_m value was 60 μM. The value was quite agreeable with those reported previously. For example, the apparent K_m value of the CYP3A4-mediated testosterone hydroxylation in human liver microsomes was approximately 50 μM (Obach et al., 2007). The Lineweaver-Burk plots showed that the presence of nomilin in the CYP-mediated testosterone hydroxylation altered the intercepts in both X-axis (or 1/[S]) and Y-axis (or 1/V). The results indicated that nomilin apparently increased the K_m value of the testosterone-CYP3A4 complexes. Furthermore, nomilin decreased the maximal velocity (V_{max}) of the enzyme catalytic reaction. It was likely that nomilin caused a decrease in the apparent CYP3A4 affinity for testosterone and a decrease in its catalytic turnover of testosterone. These nomilin-mediated changes in the values of K_m and V_{max} suggested that nomilin caused a mixed type inhibition of CYP3A4-mediated testosterone 6β-hydroxylation. It was likely that nomilin could bind to either the free enzyme in competitive manner or the substrate-bound enzyme in uncompetitive fashion. In the competitive inhibition, nomilin could compete with testosterone for binding to the

free catalytic site of the enzyme. In the uncompetitive inhibition, nomilin could bind directly to the enzyme-bound substrate. In addition, the binding of testosterone induced a change in enzyme conformation, leading to an open access of another binding site for an inhibitor (Pan et al., 2011).

From the secondary plot of the Lineweaver-Burk plot, however, a deviation from linearity was evident (Appendix D). This deviation might suggest the possibility of the partial mixed typed inhibition (The Regents of the University of California, 2010). In the partial mixed type inhibition, inhibitor can bind with both free enzyme and enzyme-substrate complex in the similar manner to the mixed type inhibition. However, the catalytic reaction can still proceed although the enzyme-substrate-inhibitor complex is formed (The Regents of the University of California, 2010; Yoshino, 1987).

In order to determine whether nomilin was a mechanism-based inhibitor of CYP3A4, inhibition time and NADPH requirements were further investigated. In these studies, the enzyme and nomilin were co-incubated in the presence and absence of NADPH for up to 30 min. As known, the presence of NADPH was required to allow the CYP catalysis. The results showed that nomilin could not elicit its inhibitory action in the mechanism-based catalysis. Increases in inhibition time and the presence of NADPH had no effects on nomilin interaction with CYP3A4. Hence, it could be concluded that nomilin was not a mechanism-based inhibitor of CYP3A4. It should be noted that the activity of the recombinant human CYP3A4 enzyme in the presence of NADPH decreased significantly, comparing to that observed in the absence of NADPH. These result indicated that the autoinactivation of the enzyme could be triggered by addition of NADPH.

Understanding of the inhibition of CYP450 could be helpful for evaluation of the severity of drug interactions (Shen, 2011; Tracy, 2008). Uses of the *in vitro* data to predict the *in vivo* drug-drug interactions can be performed using the inhibition index ($[I]/K_i$), where $[I]$ is the plasma concentration of the CYP inhibitor and K_i is the inhibition constant (Tracy, 2008). It has been suggested that if the $[I]/K_i$ ratio is greater than 1, an *in vivo* studies are needed and that inhibitor is very likely to cause drug interaction. If

the ratio in between 0.1 to 1 (0.1< [I]/K_i <1), that compound is likely to cause drug interaction. Moreover, the drug interaction is unlikely to occur if the ratio is less than 0.1 ([I]/K_i<0.1). For example, the K_i of ketoconazole was 0.16 μM (Engdal and Nilsen, 2009) whereas its serum concentration was about 94 μM (Brass et al., 1982). The [I]/K_i ratio of ketoconazole was approximately 587 (94/0.16) (Winitthana et al., 2011) which was linked to its highly likely to cause drug-drug interaction *in vivo* (Martin, 2001). As known, ketoconazole has been related to several clinically significant drug-drug interaction (US.FDA, 1999). It was estimated that the daily intake of citrus limonoids including nomilin was approximately 1 to 100 mg/day (United States Patent 5041425). Furthermore, the concentration of nomilin in pomelo juice from seven cultivars which were grown in Thailand, was 10.90-41.83 mg/L (about 21.18 – 81.29 μM) (Pichaiyongvongdee and Haruenkit, 2009). Thus, considering the relatively low K_i value of nomilin obtained from this study, it might be possible that nomilin could cause drug interaction problems. Further pharmacokinetic study of nomilin in human might be useful for predicting possible drug-drug interaction *in vivo*.

In conclusion, the present study demonstrated that nomilin showed significant inhibitory effect on recombinant CYP3A4 through the reversible mechanism. In addition, this compound was a relatively potent inhibitor of CYP3A4 activity since its IC_{50} and K_i values were in the micromolar range. It was possible that nomilin might be able to cause drug interaction problems related to a decrease in CYP3A4 activity. Therefore, caution should be exercised with respect to possible food-drug interactions when taken nomilin or natural products containing nomilin with CYP3A4 drug substrates. Further investigation may be needed to verify the clinical significance of nomilin-mediated drug interaction.

REFERENCES

ภาษาไทย

นุศรา ปิยะพลรุ่งโรจน์. 2551. ผลของสารสกัดจากน้ำคั้นจากผลพืชสกุลส้ม ต่อการทำงานของ
cytochrome P450 3A4 และ P-glycoprotein. จุลสารสถาบันวิจัยและพัฒนา มหาวิทยาลัย
ศิลปากร 17(6): 14-22.

ภาษาอังกฤษ

Balestrieri, E., et al. 2011. Antiviral activity of seed extract from Citrus bergamia
towards human retroviruses. Bioorganic & Medicinal Chemistry 19: 2084-2089.

Brass, C., Galgiani, J.N., Blaschke, T. F., Defelice, R., O′reilly, R.A., and Stevens, D.A.
1982. Disposition of ketoconazole, an oral antifungal, in humans. Antimicrobial
Agents and Chemotherapy 21: 151-158.

Chen, J., and Raymond, K. 2006. The role of CYP3A4 and p-glycoprotein in
food-drug and herb-drug interactions. Australian Pharmacist 25: 732-738.

De Wildt, S. N., Kearns, G. L., Leeder, J. S., and Van den Anker, J. N. 1999.
Cytochrome P450 3A: Ontogeny and Drug Disposition. Clinical Pharmacokinetics
37(6): 485-505.

Dresser, G. K., Spence, J. D., and Bailey, D. G. 2000. Pharmacokinetic-
Pharmacodynamic Consequences and Clinical Relevance of Cytochrome P450 3A4
Inhibition. Clinical Pharmacokinetic. 38 (1): 41-57.

Engdal, S. and Nilsen, O. G. 2009. In vitro inhibition of CYP3A4 by herbal remedies
frequently used by cancer patients. Phytotherapy research 23: 906-912.

Florida International University. 2000. Enzymes [Online]. Available from:
http://www2.fiu.edu/~bch3033/Handouts/Lh5Ch06Enz.pdf [2012, March 23]

Girard, B., and Mazza, G. 1998. Function grape and citrus products. . In G. Mazza
(ed.), Functional Foods, pp. 139-191. USA: Technomic.

Glue, P., and Clement, R. P. 1999. Cytochrome P450 Enzymes and Drug Metabolism—Basic Concepts and Methods of Assessment. <u>Cellular and Molecular Neurobiology</u>. 19: 309-323.

Gonzalez, F. J., and Tukey, R.H. 2005. Drug metabolism. In L. L. Brunton, J. S. Lazo, and K. L. Parker (eds.), <u>Goodman & Gilman's The pharmacological Basis of Therapeutics</u>, pp. 71-9. New York: The McGraw-Hill.

Guengerich, F, P. 1999. CYTOCHROME P-450 3A4: Regulation and Role in Drug Metabolism. <u>Annual Review of Pharmacology and Toxicology</u> 39: 1-17.

Hafeez, F., Akram, W., Shaalan, E. A. 2011. Mosquito larvicidal activity of citrus limonoids against Aedes albopictus. <u>Parasitology research</u> online first 6 Jan 2011

Han, Y-L., et al. 2011. Inhibitory effects of limonin on six human cytochrome P450 enzymes and P-glycoprotein *in vitro*. <u>Toxicology in vitro</u> article in press

Hasegawa, S., and Herman, Z. 1985. Biosynthesis of obacunone from nomilin in *Citrus limon*. <u>Phytochemistry</u> 24(2): 1973-1974.

Hasler, J. A. et al. 1999. Human cytochromes P450. <u>Molecular aspects of medicine</u> 20:1-137.

Honkalammi, J. 2011. <u>Mechanism-based inhibition of CYP2C8 by gemfibrozil in humans: characterization of time and dose relationships</u> [Online]. Available from: https://helda.helsinki.fi/bitstream/handle/10138/28414/mechanis.pdf?sequence=1 [2011, December 6]

Iwata, H., Tezuka, Y., Kadota, S., Hiratsuka, A., and Watabe, T. 2005. Mechanism-based inactivation of human liver microsomal CYP3A4 by *Rutaecarpine* and limonin from Evodia fruit extract. <u>Drug Metabolism and Pharmacokinetics</u> 20(1): 34-45.

Jayaprakasha, G. K., Singh, R. P., Pereira, J., and Sakariah, K. K. 1997. Limonoids from *Citrys recticulata* and their moult inhibiting activity in mosquito *Culex quinquefasciatus* larvae. <u>Phytochemistry</u> 44(5): 843-846.

Kim, J., Jayaprakasha, G. K., Muthuchamy, N., and Patil, B. S. 2011. Structure-function relationships of citrus limonoids on p38 MAP kinase activity in human aortic smooth muscle cells.[On line]. European Journal of Pharmacology 670(1): 44-49. Abstract from: http://www.ncbi.com [2011, April 5]

King, M. W. 2012. Enzyme kinetics [Online]. Available from: http://themedicalbiochemistrypage.org/enzyme-kinetics.php [2012, April 10]

Lin, J. H., and Lu, A. Y.H. 1998. Inhibition and induction of cytochrome P450 and the clinical implications. Clinical Phramacokinetics 35(5): 361-390.

Lynch, T., and Price, A. 2007. The effect of cytochrome P450 metabolism on drug response, interaction, and adverse effects. American family physician 76(3): 391-396.

Mandadi, K. K., Jayaprakasha, G. K., Bhat, N. G., and Patil, B. S. 2007. Red Mexican grapefruit: a novel source for bioactive limonoids and their antioxidant activity [Online]. Zeitschrift fur Naturforschung C 62(3-4): 179-188. Abstract from: http://www.ncbi.com [2011, April 5]

Manners, G. D. 2007. Citrus Limonoids: Analysis, Bioactivity, and Biomedical Prospects. Journal of Agricultural and Food Chemistry 55: 8285–8294.

Martin, J. 2001. Cytochrome P450 drug interactions: are they clinically relevant. Australian prescribe 24(1): 10-12.

Mukherjee, P. K., Ponnusankar, S., Pandit, S., Hazam, P. K., Ahmmed, M., and Mukherjee, K. 2011. Botanicals as medicinal food and their effects on drug metabolizing enzymes. Food and Chemical Toxicology article in Press

Obach, R. S., Walsky, R. L., and Venkatakrishnan, K. 2007. Mechanism-based inactivation of human cytochrome P450 enzymes and the prediction of drug-drug interactions. Drug metabolism and disposition 35(2): 246-255.

Ogu, C. C., and Maxa, J. L. 2000. Drug interactions due to cytochrome P450. BUMC PROCEEDINGS 13: 421–423.

Ono, E., Inoue, J., Hashidume, T., Shimizu, M., and Sato, R. 2011. Anti-obesity and anti-hyperglycemic effects of the dietary citrus limonoid nomilin in mice fed a high-fat diet. [Online]. Biochemical and Biophysical Research Commun. 410(3): 677-681. Abstract from: http://www.ncbi.com [2011, April 5]

Paine, M. F., Criss, A. B., and Watkins, P. B. 2004. Two major grapefruit juice components differ in intestinal CY3A4 inhibition kinetic and binding properties Drug metabolism and disposition 32: 1146-1153.

Pal, D., and Mitra, A. K. 2006. MDR and CYP3A4 mediated drug herb interactions. Life sciences 78: 2131-2145.

Pan, Y., et al. 2011. *In vitro* effects of active constituents and extracts of *Orthosiphon stamineus* on the activities of three major human cDNA-expressed cytochrome P450 enzymes. Chemico Biological interactions 190: 1-8.

Perez, J.L., Jayaprakasha, G.K., Cadena, A., Martinez, E., Ahmad, H., and Patil, B. 2010. In vivo induction of phase II detoxifying enzymes,glutathione transferase and quinone reductase by citrus triterpenoids. BMC Complementary and alternative Medicine 10(51): 1-8.

Petsko, G. A., and Ringe, D. 2006. Protein structure and function 2005-2006 online update [On line]. Available from: http://chemistry.ewu.edu/jcorkill/biochem/com_nono_unIH.jpg [2012, May 10]

Pichaiyongvongdee, S., and Haruenkit, S. 2009. Investigation of limonoids, flavonones, and total polyphenol content and antioxidant activity in seven Thai pummel cultivars. Kasetsart Journal (Natural Sciences) 43(3): 458-466.

Poulose, S.M. 2005. Isolation and effects of citrus limonoids on cytochrome P450 inhibition, apoptotic induction and cytotoxicity on human cancer cells. [Online]. Available from: http://www.google.co.th/books [2011, December 6]

Poulose, S. M., Harris, E. D., and Patil, B. S. 2006. Antiproliferative effects of citrus limonoids against hunan neuroblastoma and colonic adenocarcinoma cells [Online]. Nutrition and cancer 56(1): 103-112. Abstract from: http://www.ncbi.com [2011, April 5]

Raphael, T. J., and Kuttan, G. 2003. Effect of naturally occurring triterpenoids glycyrrhizic acid, ursolic acid, oleanolic acid and nomilin on the immune system[On-line]. Phytomedicine 10(6-7): 483-489. Abstract from: http://www.ncbi.com [2011, April 5]

Roy, A., and Saraf, S. 2006. Overview of Significant Bioactive Triterpenes Distributed in Plants Kingdom. Biological and Pharmaceutical Buletin 29(2): 191—201.

Ruberto, G., Renda, A., Tringall, C., Napoll, E. M., and Simmonds, M. S. J. 2002. Citrus Limonoids and Their Semisynthetic Derivatives as Antifeedant Agents Against Spodoptera frugiperda Larvae. A Structure-Activity Relationship Study. Journal of Agricultural and Food Chemistry 50: 6766-6774.

Rouseff, R.L. 1982. Nomilin, a new bitter component in grapefruit juice. Journal of Agricultural and Food Chemistry 30(3): 504-507.

Shen, H. 2011. Cytochrome P450 3A4 [Online]. Available from: http://www.uga.edu [2011, November 11]

Silalahi, J. 2002. Anticancer and health protective properties of citrus fruit components. Asia Pacific Journal of Clinical nutrition 11(1): 79-84.

Sun, C., Chen, K., Chen, Y., and Chen, Q. 2005. Contents and antioxidant capacity of limonin and nomilin in different tissues of citrus fruit of four cultivars during fruit growth and maturation. Food Chemistry 93: 599–605.

Takanaga, H., et al. 2000. Polymethoxylated flavones in orange juice are inhibitors of P-glycoprotein but not cytochrome P450 3A4. The journal of pharmacology and experimental therapeutics 293: 230-236.

The Regents of the University of California,Davis. 2010. Enzyme inhibition [Online]
Available from: http://chemwiki.ucdavis.edu/@api/deki/pages/318/pdf [2012, May, 30]

Thummel, K.E., and Wilkinson, G. R. 1998. IN VITRO AND IN VIVO DRUG INTERACTIONS INVOLVING HUMAN CYP3A. Annual Review of Pharmacology and Toxicology 38:389-430.

Tian, Q., Miller, E. G., Ahmad, H., Tang, L., Patil, B. S. 2001. Differential inhibition of human cancer cell proliferation by citrus limonoids. [Online]. Nutrition and cancer 40(2): 180-184. Abstract from: http://www.ncbi.com [2011, April 5]

Tracy, T. S. 2008. Enzyme kinetics In D. Zhang, M. Zhu, and W. G. Humphreys (ed.), Drug metabolism in drug design and development, pp. 89-112. New York: Wiley interscience A John wiley&Sons.

U.S.FDA. 1999. Guidance for industry: drug interaction studies-study design, data analysis, and implications for dosing and labeling [Online]. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072104.pdf [2012, April 16]

U.S.FDA. 2001 Guidance for industry: Bioanalytical method validation [Online].
Available from:
http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf [2012, May 10]

Voet, D., and Voet, J. G. 1995. Biochemistry 2ed. New Jersey: John Wiley & Sons.

Wada, K., Yagi, M., Kurihara, T., and Haga, M. 1992. Studies on the constituents of edible and medicinal plants. III. Effects of seven limonoids on the sleeping time induced in mice by anesthetics [Online]. Chemical and Pharmaceutical bulletin 40(11): 3079-3080. Abstract from: http://www.ncbi.com [2011, April 5]

Wikimedia Foundation. 2012. Mixed inhibition [Online]. Available from: http://en.wikipedia.org/wiki/Mixed_inhibition [2012, May 10]

Winitthana, T., Niwattisaiwong, N., Patarapanich, C., Tantisira, M. H., and Lawanprasert, S. 2011. *In vitro* inhibitory effects of asiaticoside and medecassoside on human cytochrome P450. Toxicology in vitro 25: 890-896.

Yoshino, M. 1987. A graphical method for determining inhibition parameters for partial and complete inhibitors. Biochemical Journal 248: 815-820.

Yuan, R., Madani, S., Wei, X-X., Reynolds, K., and Huang, S-M. 2002. Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. Drug metabolism and disposition 30(2): 1311-1319.

Zhang, X. 2007. Prediction of mechanism-based inhibition of CYP3A by single and multiple inhibitors [Online]. Available from: http://www.google.co.th/books [2011, December 6]

Zhou, S., Gao, Y., Jiang, W., Huang, M., Xu, A., and Paxton, J. W. 2003. Interactions of Herbs with Cytochrome P450. Drug metabolism reviews 35(1): 35-98.

Zhou et al. 2005. Mechanism-based inhibition of cytochrome P450 3A4 by therapeutics drugs. Clinical pharmacokinetics 44(3): 279-304.

Zhou, S-F., Xue, C. C., Yu, X-Q.,Li, C., and Wang, G. 2007. Clinically Important Drug Interactions Potentially Involving Mechanism-based Inhibition of Cytochrome P450 3A4 and the Role of Therapeutic Drug Monitoring. Therapeutic drug monitoring 29 (6): 687-710.

APPENDICES

Appendix A

Preparation of stock and working standard solution

Reagents

    1. 0.1 M Potassium phosphate buffer, pH 7.45

0.2 M potassium phosphate, mono-potassium salt prepared by dissolve 1.3609 g KH_2PO_4 (MW= 136.09) in 50 ml volumetric flask and adjust to 50 ml with ultrapure water.

0.2 M potassium phosphate, di-potassium salt prepared by dissolve 3.4878 g K_2HPO_4 (MW= 174.18) in 50 ml volumetric flask and adjust to 50 ml with ultrapure water.

Nineteen militers of 0.2 M potassium phosphate, mono-potassium salt and eighty-one militers of 0.2 M potassium phosphate, di-potassium salt mixed together with 100 ml of ultrapure water to obtain a 200 ml solution with a specific pH 7.45.

    2. NADPH regenerationg system

NADPH regernerating system comprised the solution as following:

a) Glucose -6-Phosphate (G6P) 110 mM (M.W= 282.1)

    G6P 310.3 mg was dissolved with ultrapure water 10 ml

b) NADP 43.3 mM (M.W= 765.4)

    NADP 331.6 mg was dissolved with ultrapure water 10 ml

c) $MgCl_2$ 110 mM (M.W= 203.3)

    $MgCl_2 \cdot 6H_2O$ 224 mg was dissolved with ultrapure water 10 ml

d) Glucose-6-Phosphate dehydrogenase (G6PD)

    G6PD was diluted to 40 units per ml with 0.1 M Potassium phosphate buffer

For each assay, the mixture solution of NADPH regenerating system was freshly prepared in a 3:3:3:1 ratio of 110 mM G6P, 43.3 mM NADP, 110 mM $MgCl_2 \cdot 6H_2O$, and 40 unit/ml G6PD, respectively. This mixture was pre-warmed at 37^0C for 3 minutes before to use. For the reaction volume of 125 μl, 12.5 μl of this mixture was used for CYP3A4 incubation to initiate the reaction.

Stock solution

    1. 6β-hydroxytestosterone stock solution 2 mM (M.W.= 304.42)

6β-hydroxytestosterone 0.6088 mg was dissolved with 1000 μl of methanol to prepare stock solution of 2 mM, and kept at ultra-low temperature freezer -20°C until used.

    2. Testosterone 20 mM (M.W. = 288.42)

Testosterone 2.8842 mg was dissolved with 500 μl of methanol and diluted to various final concentrations with 0.1 M potassium phosphate buffer pH 7.45.

    3. Erythromycin 6 mM (MW = 862.06)

Erythromycin 5.1723 mg was dissolved with 10 ml of acetonitrole and diluted to various final concentrations with 0.1 M potassium phosphate buffer pH 7.45.

    4. Ketoconazole 0.1 mM (MW=531.431)

Ketoconazole 0.5314 mg was dissolved with 10 ml of acetonitrole and diluted to various final concentrations with 0.1 M potassium phosphate buffer pH 7.45.

    5. Ketoconazole 0.05 mM (MW=531.431)

Ketoconazole 0.2657 mg was dissolved with 10 ml of acetonitrole and diluted to various final concentrations with 0.1 M potassium phosphate buffer pH 7.45.

    6. Nomilin 30,000 μM (M.W. = 514.57)

Nomilin 3.8592 mg was dissolved with 250 μl of acetonitrile and diluted to various final concentrations with 0.1 M potassium phosphate buffer pH 7.45.

    7. Nomilin 15,000 μM (M.W. = 514.57)

Nomilin 3.8592 mg was dissolved with 500 μl of acetonitrile and diluted to various final concentrations with 0.1 M potassium phosphate buffer pH 7.45.

    8. Nomilin 10,000 μM (M.W. = 514.57)

Nomilin 2.5728 mg was dissolved with 500 μl of acetonitrile and diluted to various final concentrations with 0.1 M potassium phosphate buffer pH 7.45.

    9. Nomilin 5,000 μM (M.W. = 514.57)

Nomilin 1.2864 mg was dissolved with 500 μl of acetonitrile and diluted to various final concentrations with 0.1 M potassium phosphate buffer pH 7.45.

Appendix B

Linearity, stability of 6β-hydroxytestosterone

Table B1 Peak area of 6β-hydroxytestosterone concentrations.

6 betahydroxytestosterone (nmol/ml)	peak area of 6β-hydroxytestosterone		average
	N1	N2	
0.25	15575	11670	13623
1.25	83512	67477	75495
2.5	176477	141016	158747
5	374372	299226	336799
10	788121	695982	742052

Note: Volume of injection 100 μl

Table B2 Peak area of 6 β—hydroxytestosterone after storage at time 0, 5 and 7 hour.

Storage time (hour)	peak area of 6β—hydroxytestosterone		
	1	2	3
0	199953	286993	162698
5	199829	289535	164644
7	197956	288857	165297

Note: Sample 1, 2, 3 were from separated experiments. Volume of injection was 25 μl.

Table B3 Peak area of 6β—hydroxytestosterone after storage at time 0 and 17 days.

Storage time (day)	peak area of 6β—hydroxytestosterone		
	1	2	3
0	1007041	418829	224209
17	994966	414927	218495

Note: Sample 1, 2, 3 were from separated experiments. Volume of injection was 100 μl.

Appendix C

Verification of testosterone hydroxylation

1. **Verification of incubation time and peak area of 6β-hydroxytestosterone**

The correlation between incubation time and peak area of 6β-hydroxytestosterone were performed. The incubation time within linear range was selected for determination of testosterone hydroxylation in the subsequent experiments.

Reagents

    1. 0.1 M Potassium phosphate buffer, pH 7.45

    2. NADPH regenerating system

    3. Testosterone 20,000 μM (M.W. = 288.42)

Procedures

Reaction mixtures consisted of CYP3A4 (20 pmol/ml) and testosterone (200 μM) were pre-incubated at 37^0C for 5 min. The reaction was started by addition of NADPH regenerating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenese, and 3.3 mM magnesium chloride) and incubated for 5, 10, 15, 30, and 50 min at 37^oC. After that 100 % ice-cold acetonitrile was added into the mixture to stop the reaction, followed by centrifugation at 15,000 rpm for 45 min. The supernatant was subjected to HPLC analysis for 6β-hydroxytestosterone. Correlation between incubation time and peak area of 6β-hydroxytestosterone were calculated.

Table C1 Correlation between incubation time and peak area of 6β-hydroxytestosterone

Incubation time (min)	peak area of 6β-hydroxtestosterone
5	100939
10	391268
15	627026
30	1483458
50	1765315

Figure C1 Correlation between incubation time (5-50 min) and peak area of 6β-hydroxytestosterone were verified.

The chart shows a plot with y-axis labeled "Peak area of 6β- hydroxytestosterone" (values 0 to 1600000) and x-axis labeled "Time (min)" (values 0 to 35). The equation displayed is $y = 55103x - 175876$ and $R^2 = 0.9992$.
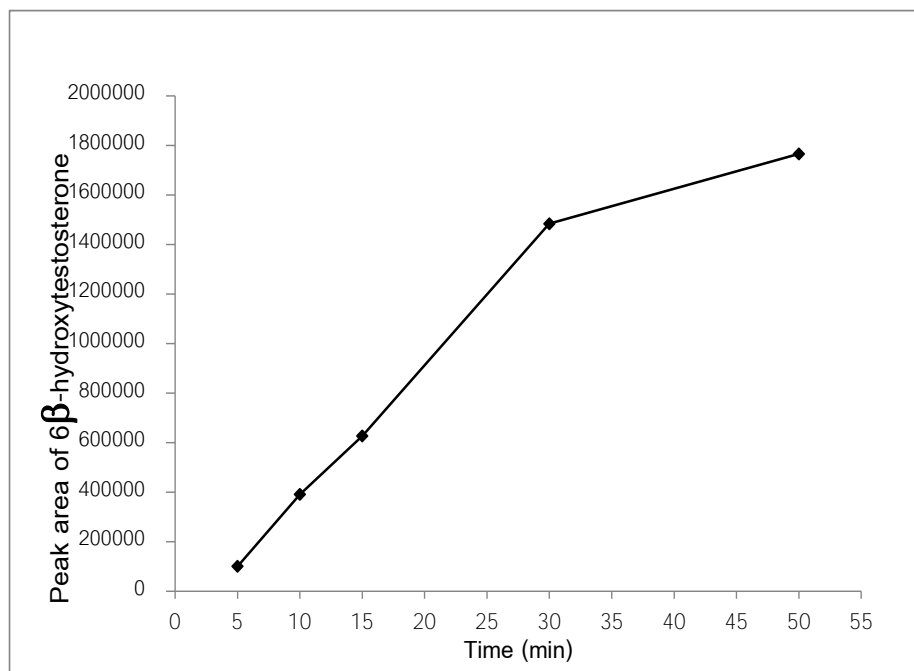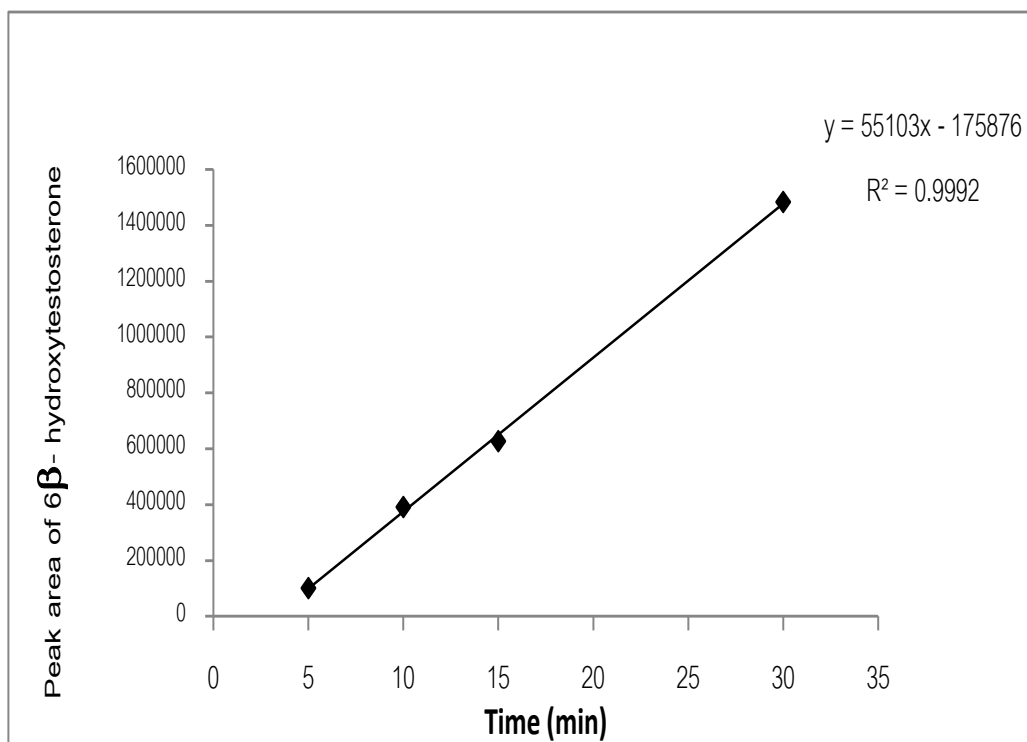
Figure C2 Correlation between incubation time (5-30 min) and peak area of 6β-hydroxytestosterone were verified. The coefficient of determination of 5-30 min was 0.9992

2. **The effect of nomilin on the peak areas of 6β-hydroxytestosterone and testosterone**

To determine whether nomilin had effect on 6β-hydroxytestosterone and testosterone peak areas. Nomilin (at final concentration 50 or 100 μM) was mixed with 6β-hydroxytestosterone and testosterone and injected into the HPLC system. The peak area of 6β-hydroxytestosterone and testosterone were recorded. Percentage of deviation of peak area was calculated in comparison between the samples with nomilin and those without nomilin.

Table C2 The percentage deviation of the peak areas of 6β-hydroxytestosterone and testosterone in the presence of nomilin from those in the absence of nomilin.

sample	% deviation of 6β hydroxytestosterone	% deviation of testosterone
6β-hydroxytestosterone 10 μM + Testosterone 100 μM +nomilin 50 μM	6.22	10.04
6β-hydroxytestosterone 10 μM + Testosterone 100 μM +nomilin 100 μM	8.15	11.61
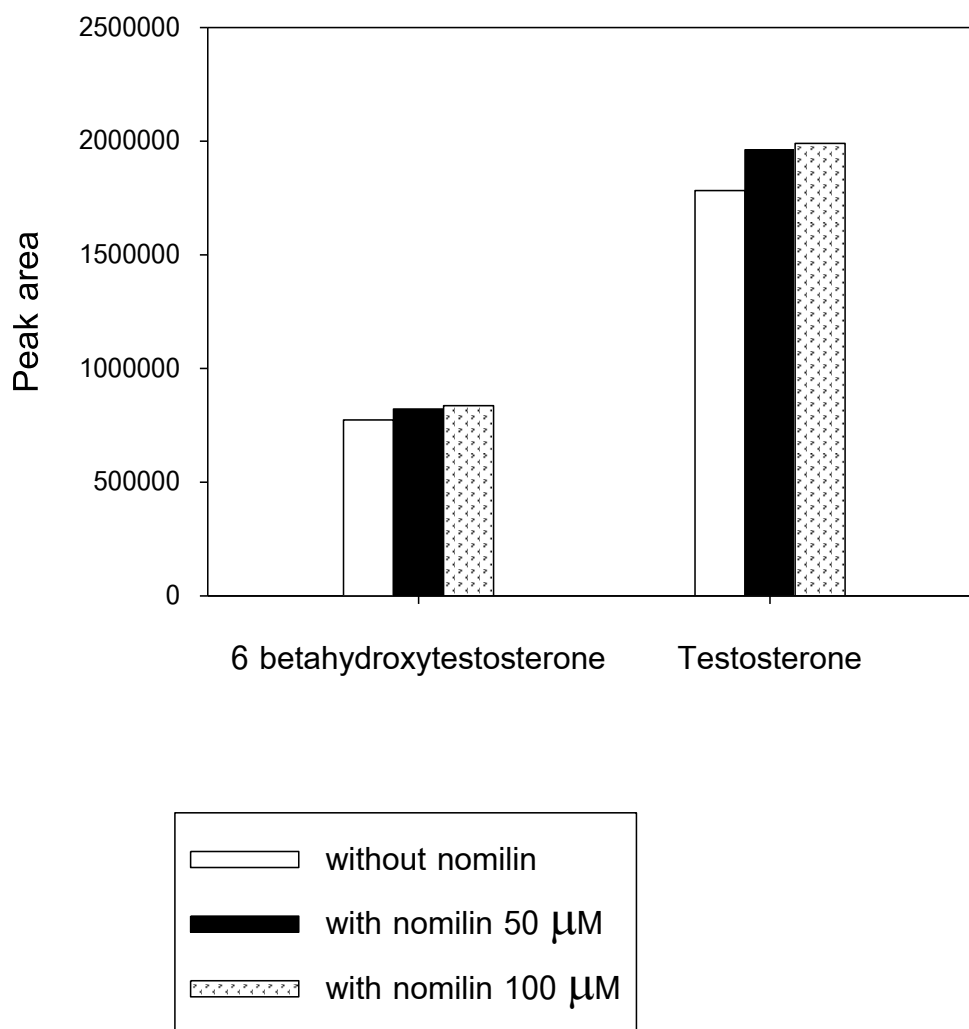
Figure C3 The effect of nomilin on the peak areas of 6β-hydroxytestosterone and testosterone.

3. The effect of nomilin on the production of NADPH from regenerating system-

To determine whether nomilin had effect on the production of NADPH from regenerating system. Nomilin (at final concentration 5 or 20 μM) was mixed with the NADPH regenerating system containing 1.3 mM NADP, 3.3mM glucose-6-phosphate, and 3.3 mM magnesium chloride. Then, glucose-6-phosphate dehydrogenese (0.4 U/ml) was added to the mixture in order to start the reaction. The production of NADPH was determined at the absorbance of 340 nm.
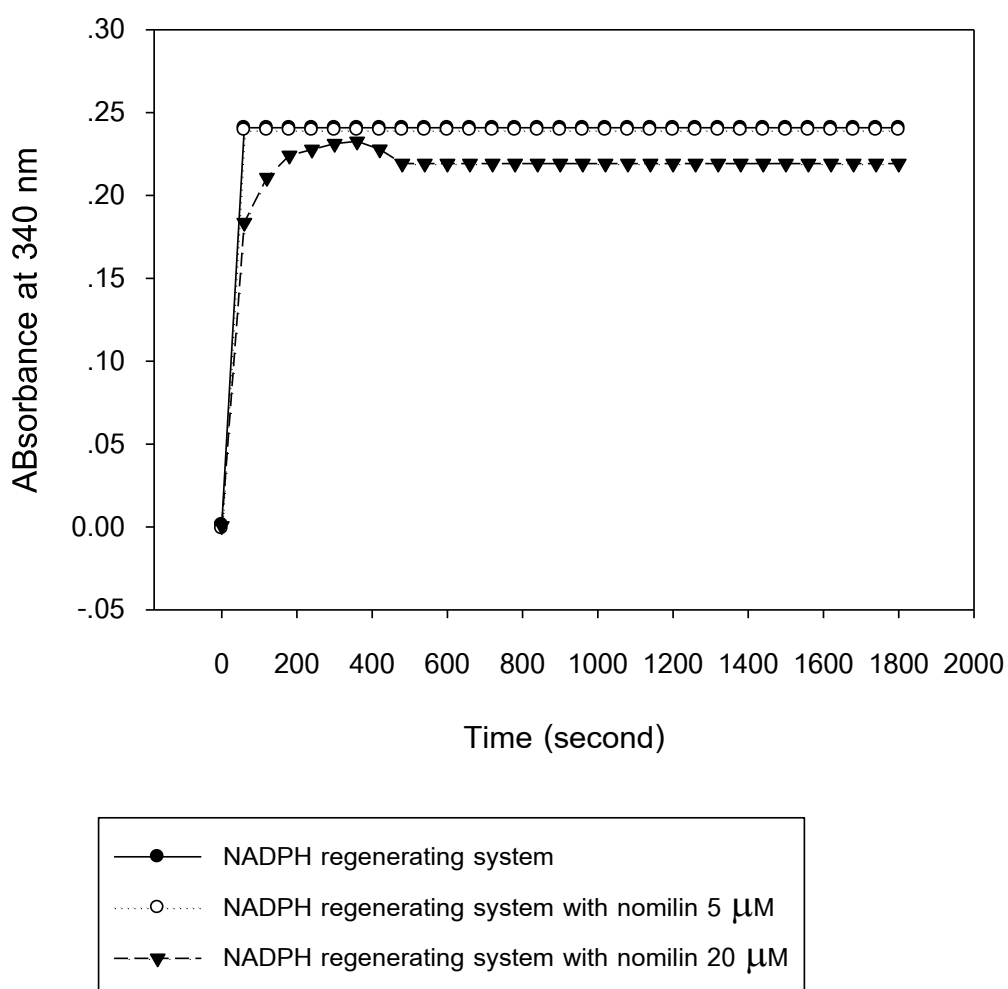
Figure C4 The effect of nomilin on the production of NADPH from regenerating system.

Appendix D

CYP3A4 inhibition assay

Table D1 Peak area of 6β-hydroxytestosteone after treatment of various concentration of nomilin and ketoconazole

Sample	peak area of 6-β OH testosterone		
	N1	N2	N3
control 15 min	1007041	1485303	1047396
Ketoconazole 0.2 µM	890007	–	–
Ketoconazole 0.5 µM	–	1197057	389002
Nomilin 2 µM	997635	–	880988
Nomilin 5 µM	908508	–	717216
Nomilin 7.5 µM	–	1025545	539758
Nomilin 10 µM	657712	938032	440262
Nomilin 15 µM	–	825874	380239
Nomilin 20 µM	418829	714865	298487
Nomilin 30 µM	–	538928	261208
Nomilin 50 µM	224209	434278	–
Nomilin 100 µM	121587	–	144044
Nomilin 150 µM	103844	–	–

Table D2 Percentage of CYP3A4 inhibition after treatment with nomilin at of various concentrations

Nomilin concentration (μM)	%CYP3A4 inhibition		
	N1	N2	N3
2	0.93	‒	15.89
5	9.78	‒	31.52
7.5	‒	30.95	48.47
10	34.69	36.85	57.97
15		44.40	63.70
20	58.41	51.87	71.50
30	‒	63.72	75.06
50	77.74	70.76	‒
100	87.93	‒	86.25
150	89.69	‒	‒
IC_{50}	16.40	18.13	9.08
average	14.54		
SD	4.81		
SEM	2.77		

Table D3 CYP3A4 activity after treatment with nomilin at the different concentrations of testosterone

Sample	CYP3A4 activity (pmol product/pmol CYP3A4*min)					
	Testosterone 25 μM		average	Testosterone 50 μM		average
	N1	N2		N1	N2	
control	25.61	18.94	22.28	43.67	25.86	34.77
Nomilin 5 μM	9.11	6.21	7.66	21.93	10.32	16.13
Nomilin 10 μM	6.25	4.11	5.18	14.24	7.25	10.74
Nomilin 20 μM	4.18	2.95	3.57	8.96	4.77	6.86

Sample	CYP3A4 activity (pmol product/pmol CYP3A4*min)					
	Testosterone 100 μM		average	Testosterone 200 μM		average
	N1	N2		N1	N2	
control	61.60	30.98	46.29	81.71	36.60	59.15
Nomilin 5 μM	37.92	15.80	26.86	49.82	17.25	33.53
Nomilin 10 μM	25.99	10.00	18.00	35.50	11.37	23.43
Nomilin 20 μM	16.16	6.60	11.38	22.77	8.14	15.45

Figure D1 Lineweaver-Burk plot for the inhibition of CYP3A4 by nomilin (A) (●; 0 μM, ■; 5 μM, ▲; 10 μM, X; 20 μM). (N1)

Figure D2 The secondary plot of CYP3A4 activity using the slopes of the primary Lineweaver-Burk plots *versus* the concentrations of nomilin. (N1; K_i = 4.52 μM)
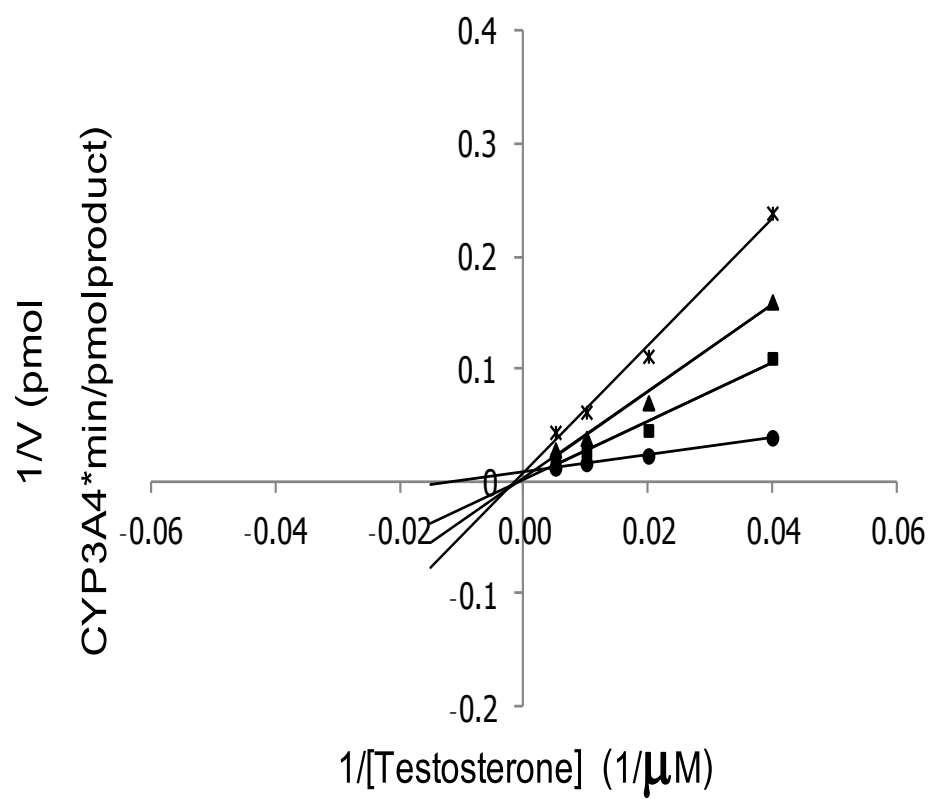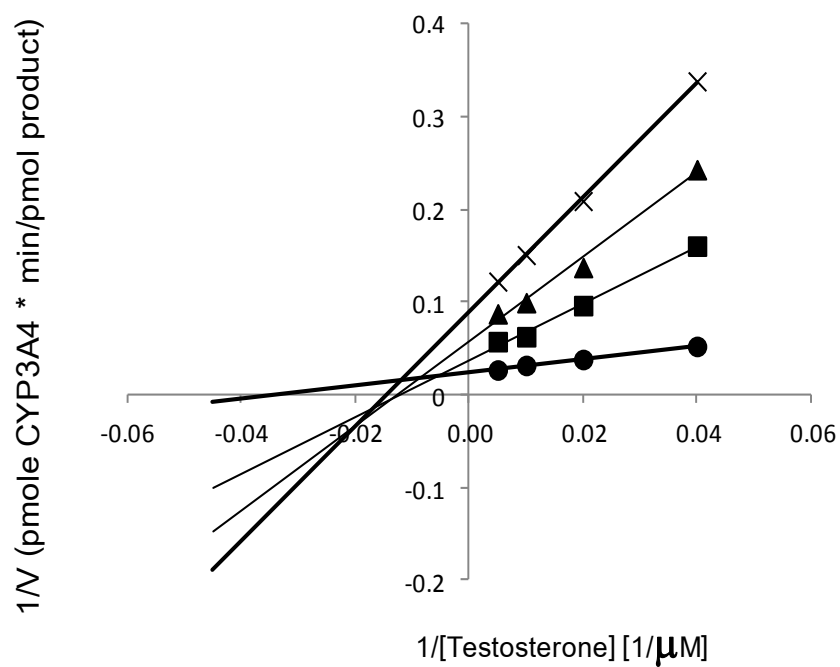
Figure D3 Lineweaver-Burk plot for the inhibition of CYP3A4 by nomilin (A) (●; 0 μM, ■; 5 μM, ▲; 10 μM, X; 20 μM). (N2)
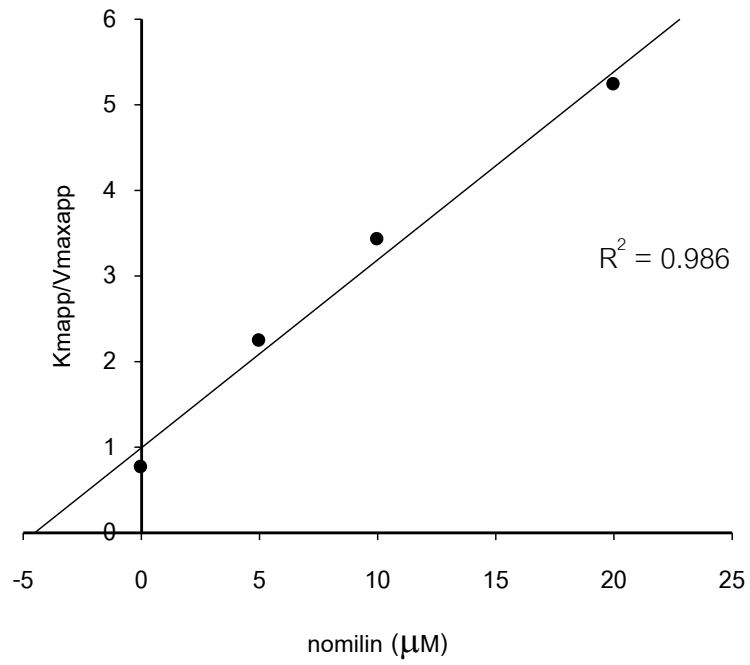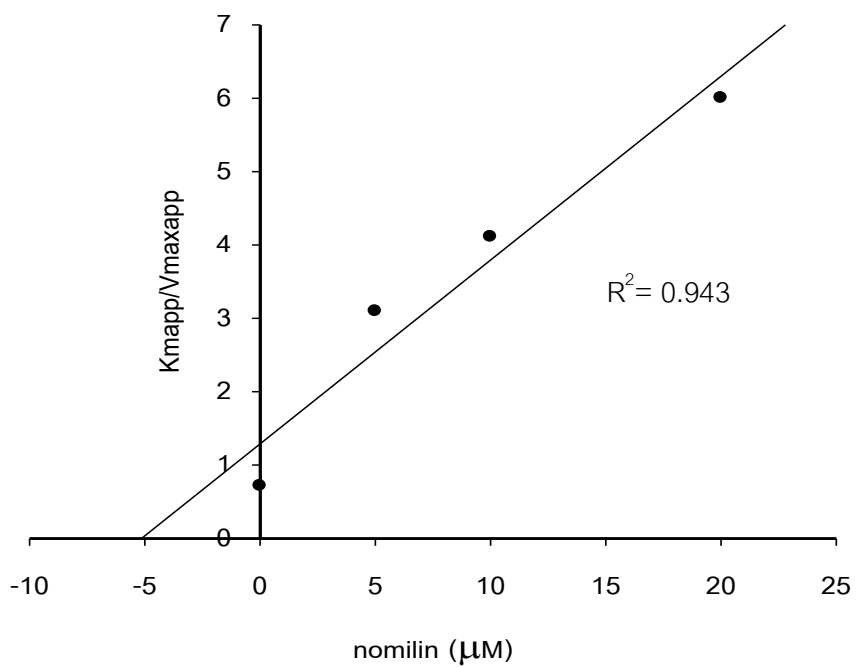
Figure D4 The secondary plot of CYP3A4 activity using the slopes of the primary Lineweaver-Burk plots *versus* the concentrations of nomilin. (N2; K_i = 5.15 μM)

Table D4 The apparent values of K_m and V_{max} for the nomilin-mediated inhibition CYP3A4.

Group	K_m		V_{max}	
	N1	N2	N1	N2
control 15 min	89.63	29.72	117.42	41.46
Nomilin 5 µM	311.26	83.15	138.93	26.86
Nomilin 10 µM	438.62	64.39	128.13	15.66
Nomilin 20 µM	378.83	64.89	72.39	10.81

Table D5 The inhibition constant for the nomilin-mediated inhibition CYP3A4.

ครั้งที่	1	2	average
K_i	4.52	5.15	4.83

Table D6 Peak area of 6β-hydroxytestosterone after pretreatment of solvent, erythromycin, ketoconazole and nomilin in the presence and absence of NADPH for 30 min.

Sample	peak area of 6β-hydroxytestosterone			
	N1	N2	N3	N4
control	344731	179161	186185	112745
control +NADPH	212541	95965	109303	69895
Erythromycin 30 µM	‒	106983	164001	94624
Erythromycin 30 µM+NADPH	‒	5634	23086	9466
Ketoconazole 0.5 µM	333418	183316	173867	111068
Ketoconazole 0.5 µM+NADPH	234833	93974	107575	68715
Nomilin 50 µM	221044	154968	166450	103205
Nomilin 50 µM +NADPH	114082	108756	103425	67268

Table D7 Percentage of the residual activity of CYP3A4 after pretreatment of solvent, erythromycin, ketoconazole and nomilin in the presence and absence of NADPH for 30 min.

Group	% residual activity (% of control)					
	N1	N2	N3	N4	average	SEM
control	100.00	100.00	100.00	100.00	100.00	0.00
control +NADPH	61.65	53.56	58.71	61.99	57.97	1.95
Erythromycin 30 µM	–	59.71	88.08	83.93	73.90	8.85
Erythromycin 30 µM+NADPH	–	3.14	12.40	8.40	7.77	2.68
Ketoconazole 0.5 µM	96.72	102.32	93.38	98.51	97.47	1.86
Ketoconazole 0.5 µM+NADPH	68.12	52.45	57.78	60.95	59.45	3.27
Nomilin 50 µM	64.12	86.50	89.40	91.54	80.01	6.34
Nomilin 50 µM +NADPH	33.09	60.70	55.55	59.66	49.78	6.48

Table D8 Percentage of the residual activity of CYP3A4 activity after pretreatment of solvent, erythromycin, ketoconazole and nomilin in the presence and absence of NADPH for 30 min.

Group	% residual activity (% of control)					
	N1	N2	N3	N4	average	SEM
control	100.00	100.00	100.00	100.00	100.00	0
control +NADPH	100.00	100.00	100.00	100.00	100.00	0
Erythromycin 30 µM	–	59.71	88.08	83.93	77.24	8.85
Erythromycin 30 µM+NADPH	–	5.87	21.12	13.54	13.51	4.40
Ketoconazole 0.5 µM	96.72	102.32	93.38	98.51	97.73	1.86
Ketoconazole 0.5 µM+NADPH	110.49	97.93	98.42	98.31	101.29	3.07
Nomilin 50 µM	64.12	86.50	89.40	91.54	82.89	6.34
Nomilin 50 µM +NADPH	53.68	113.33	94.62	96.24	89.47	12.66

Table D9 Peak area of 6β-hydroxytestosterone after pretreatment of solvent, erythromycin, ketoconazole and nomilin in the presence of NADPH for 0, 10, 20, and 30 min.

sample	peak area of 6β-hydroxytestosterone							
	Time							
	0		10		20		30	
	N1	N2	N1	N2	N1	N2	N1	N2
control	449012	286572	366179	244837	286815	200898	252783	185567
ketoconazole 0.5 μM	438250	272697	326969	250057	295974	212584	241394	178032
Erythromycin 30 μM	333746	235377	66530	34005	35946	21984	30686	16127
Nomilin 25 μM	434473	322988	344746	257454	268708	224363	237793	179512
Nomilin 100 μM	460789	306806	366720	259970	282435	217821	249137	192064

Table D10 Percentage of the residual activity after pretreatment of erythromycin, ketoconazole and nomilin in the presence of NADPH for 0, 10, 20 and 30 min.

sample	% Residual activity (%control)					
	Time (min)					
	0			10		
	N1	N2	average	N1	N2	average
control	100	100	100	100	100	100
ketoconazole 0.5 µM	97.60	95.16	96.38	89.29	102.13	95.71
Erythromycin 30 µM	74.33	82.14	78.23	18.17	13.89	16.03
Nomilin 25 µM	96.76	112.71	104.73	94.15	105.15	99.65
Nomilin 100 µM	102.62	107.06	104.84	100.15	106.18	103.16

sample	% Residual activity (%control)					
	Time (min)					
	20			30		
	N1	N2	average	N1	N2	average
control	100	100	100	100	100	100
ketoconazole 0.5 µM	103.19	105.82	104.51	95.49	95.94	95.72
Erythromycin 30 µM	12.53	10.94	11.74	12.14	8.69	10.41
Nomilin 25 µM	93.69	111.68	102.68	94.07	96.74	95.40
Nomilin 100 µM	98.47	108.42	103.45	98.56	103.50	101.03

VITAE

Name	Miss Alisa Suk-aim
Date of birth	January 26, 1978
Place of birth	Bangkok, Thailand
Institution attended	Bachelor's degree of Pharmacy (Second Class Honors), Faculty of Pharmacy, Silpakorn University, Nakorn pathom, Thailand, 2004
Position & office	2000-2003 Pharmacist at Naphalai Hospital, Samutsongkram, Thailand
	2003-present Pharmacist at Pranangklao Hospital, Nontaburi, Thailand