ฤทธิ์ยับยั้งการทำงานของเอนไซม์ไลเปสจากตับอ่อนของยากลุ่มเตตร้าไซคลิน

นางสาวทิพวรรณ   มณีท่าโพธิ์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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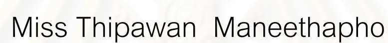
ปีการศึกษา 2551

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

INHIBITORY EFFECT OF TETRACYCLINES ON PANCREATIC LIPASE ACTIVITY



Miss Thipawan Maneethapho

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Pharmacology
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2008

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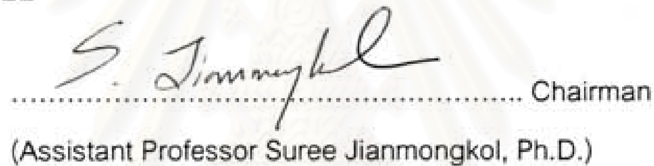
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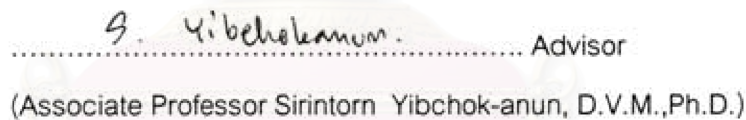
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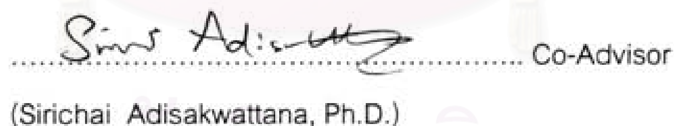
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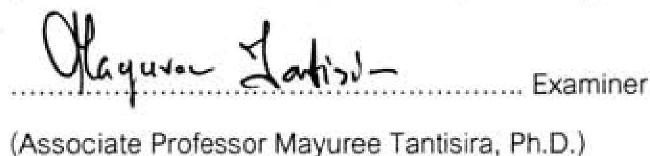
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ทิพวรรณ มณีท่าโพธิ์ : ฤทธิ์ยับยั้งการทำงานของเอนไซม์ไลเปสจากตับอ่อนของยากลุ่มเตตร้าไซคลิน. (INHIBITORY EFFECT OF TETRACYCLINES ON PANCREATIC LIPASE ACTIVITY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.สพ.ญ.ดร.ศิรินทร หยิบโชคอนันต์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ดร.สิริชัย อดิศักดิ์วัฒนา, 81 หน้า.

วัตถุประสงค์ของการวิจัยครั้งนี้ เพื่อศึกษาฤทธิ์ในการยับยั้งการทำงานเอนไซม์ไลเปสของยา 4 ชนิด ซึ่งเป็นอนุพันธ์ในกลุ่มเตตร้าไซคลินส์ ประกอบด้วย เตตร้าไซคลิน คลอเตตร้าไซคลิน ด็อกซีไซคลิน และอ็อกซีเตตร้าไซคลิน ซึ่งเป็นยาต้านจุลชีพที่ออกฤทธิ์ยับยั้งการเจริญเติบโตของแบคทีเรียแกรมบวกและแกรมลบได้ดี นิยมใช้ในการรักษาอาการติดเชื้อกันอย่างแพร่หลาย จากผลการทดลอง พบว่า ความแรงในการยับยั้งเอนไซม์ไลเปสเรียงตามลำดับ คือ ด็อกซีไซคลิน (IC_{50} = 55.42 ± 1.67 μM) > คลอเตตร้าไซคลิน (IC_{50} = 88.71 ± 11.39 μM) > เตตร้าไซคลิน (IC_{50} > 500 μM) และอ็อกซีไซคลินไม่พบฤทธิ์ยับยั้งเอนไซม์ไลเปส อย่างไรก็ตามเมื่อเปรียบเทียบฤทธิ์ยับยั้งเอนไซม์ไลเปสของดอกซี่ไซคลินกับออริสเตท ซึ่งเป็นยาที่ใช้ในทางคลินิกสำหรับลดน้ำหนัก มีกลไกการออกฤทธิ์โดยยับยั้งเอนไซม์ไลเปสโดยตรง พบว่าด็อกซีไซคลินมีฤทธิ์ในการยับยั้งเอนไซม์ไลเปสจากตับอ่อนได้น้อยกว่าออริสเตท (IC_{50} = 1.31 ± 0.13 μM) 80 เท่า และเมื่อศึกษากลไกและชนิดในการยับยั้งโดยการพล็อตกราฟแบบไลน์วีฟเวอร์เบิร์ก พบว่า ด็อกซีไซคลินมีกลไกการยับยั้งเป็นแบบย้อนกลับได้ชนิดไม่แข่งขัน ซึ่งแตกต่างจากออริสเตทซึ่งมีรูปแบบการยับยั้งเป็นแบบไม่ย้อนกลับ และเมื่อนำไปพล็อตกราฟทุติยภูมิเพื่อหาค่า K_i และ K_i' พบว่า ด็อกซีไซคลินมีค่า K_i เท่ากับ 66.41 ± 3.27 μM และ K_i' เท่ากับ 70.49 ± 7.11 μM นอกจากนี้ด็อกซีไซคลินยังสามารถเสริมฤทธิ์แบบซินเนอร์จิสซึมในการยับยั้งเอนไซม์ไลเปสเมื่อให้ร่วมกับออริสเตท และการให้ด็อกซีไซคลินโดยการกินในขนาด 20 ถึง 100 มิลลิกรัมต่อกิโลกรัม สามารถลดระดับไตรกลีเซอไรด์ในซีรัมของหนูขาวที่เหนี่ยวนำให้มีระดับไขมันสูง

จากการทดลองดังกล่าวสรุปได้ว่า ด็อกซีไซคลินลดระดับซีรัมไตรกลีเซอไรด์โดยการออกฤทธิ์ยับยั้งการทำงานของเอนไซม์ไลเปสจากตับอ่อน ซึ่งนับว่าเป็นประโยชน์ต่อการวิจัยในทางเภสัชวิทยาและพิษวิทยาคลินิกต่อไป

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

สาขาวิชา สหสาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต ทิพวรรณ มณีท่าโพธิ์

ปีการศึกษา 2551 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม

4889079220 : MAJOR PHARMACOLOGY

KEYWORDS : TETRACYCLINES / LIPASE INHIBITOR / TRIGLYCERIDE

THIPAWAN MANEETHAPHO: INHIBITORY EFFECT OF TETRACYCLINES ON PANCREATIC LIPASE ACTIVITY. THESIS ADVISOR : ASSOC. PROF. SIRINTORN YIBCHOK-ANUN D.V.M.,Ph.D., THESIS CO-ADVISOR: SIRICHAI ADISAKWATTANA, Ph.D., 81 pp.

The objective of this research was to demonstrate the inhibitory potencies of 4 chemical derivatives of tetracyclines consist of tetracycline, doxycycline, chlortetracycline and oxytetracycline. Tetracyclines are a group of broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria. The results showed inhibitory potency of doxycycline (IC_{50} = 55.42 ± 1.67 μM) > chlortetracycline (IC_{50} = 88.71 ± 11.39 μM) > tetracycline (IC_{50} > 500 μM) but it had no inhibitory activity of oxytetracycline. However, the study which compared the potency between doxycycline and orlistat, a drug clinically used for weight loss by directly inhibit pancreatic lipase indicated that doxycycline was about 80 times less potent than orlistat (IC_{50} = 1.31 ± 0.13 μM). The study inhibitions mechanism using Lineweaver-Burk plot showed that doxycycline was a reversible non-competitive type inhibition against pancreatic lipase which is differs from orlistat in which it was irreversible inhibition. The affinity inhibitor constant of doxycycline showed K_i = 66.41 ± 3.27 μM and K_i' = 70.49 ± 7.11 μM. Moreover, doxycycline exhibited the synergistic inhibition on pancreatic lipase activity when administration with a low dose of orlistat. The oral administration of doxycycline (20 – 100 mg/kg) significantly decreased serum triglyceride concentration in lipid emulsion-induced hyperlipidemia rats.

In conclusion, doxycycline markedly decreased serum triglyceride concentration in rats by inhibiting pancreatic lipase. This research may be beneficial for further studies in clinical pharmacology and toxicology.

Field of Study : Pharmacology .... Student's Signature ....

Academic Year : 2008 .... Advisor's Signature ....

Co-Advisor's Signature ....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and sincere appreciation to my advisor, Associate Professor Dr. Sirintorn Yibchok-anun for her advice, guidance, excellent, encouragement, kindness and support throughout my study. My great appreciation is also expressed to my co-advisor, Dr. Sirichai Adisakwattana for guidance, help, support, comments and suggestions on my research and laboratory work. My great appreciation is also extended to Assistant Professor Dr. Suree Jianmongkol, Associate Professor Dr. Muyaree Tantisira and Associate Professor Sumana Chompootaweep, who are the committee members, for their useful comments and suggestions on this thesis.

I wish to thank all staff members at Department of Pharmacology, Faculty of Pharmaceutical Sciences, and Department of Pharmacology Faculty of Veterinary Science, Chulalongkorn University.

My special thanks also extend to my classmates, all friends and laboratory members for their help, sincerity kindness and friendship.

This study was partly supported by the Graduate Research Funds from the Ministry of University Affairs and Graduate School, Chulalongkorn University.

Finally, I wish to express my deepest appreciation and infinite gratitude to my family for their love, encouragement, understand and support that they are deserved to be mentioned as a part of my success.

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ABBREVIATIONS

α	Alpha
β	Beta
^{o}C	Degree celcius
p	Para-
µg	Microgram
µL	Microlitre
µmol	Micromolar
µM	Micromolar
BW	Body weight
DMSO	Dimethylsulfoxide
et al.	et alii (and others)
g	Gram
GL	Gastric lipase
h	Hour
HCl	Hydrochloride
HDL	High density lipoprotein
HGL	Human gastric lipase
HPL	Human pancreatic lipase
IC_{50}	Half maximal inhibitory concentration
L	Liters
LCT	Long chain triacylglycerol
M	Molar
MAG	Monoacylglycerol
MCT	Medium chain triacylglycerol
mg	Milligram
mg/kg	Milligram per kilogram body weight
LDL	Low density lipoprotein
TG	Triglyceride
Rpm	Revolution per minute

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SEM	Standard error of mean
SFAs	Saturated fatty acids
Tris	Tris (hydroxymethyl) aminomethane
v/v	Volume by volume

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CHAPTER I

INTRODUCTION

Tetracyclines are a group of broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria as well as atypical organisms such as *Chlamydia*e, *Mycoplasmas*, and *Rickkettsiae* and protozoa. Tetracyclines were discovered in the 1940s. The favorable antimicrobial properties of these agents and the absence of major adverse side effect has led to their extensive use for the therapy of human and animal infections and widely used around the world for a long time (Chopra and Robert, 2001). The tetracyclines have function as antibiotics by inhibiting bacterial protein synthesis and preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor A site (Islam et al, 2003). Tetracyclines are used to treat pathological conditions including acne, lympogranuloma vernerum, trachoma and respiratory tract infections. Tetracyclines belong to a group of antibiotics including tetracycline, doxycycline, oxytetracycline and chlortetracycline etc. Chlortetracycline and oxytetracycline are elaborated by *Streptomyces aureofaciens* and *Streptomyces rimosus*, respectively. Tetracycline and doxycycline are semi-synthetically produced from chlortetracycline (Chopra and Robert, 2001). In general, tetracyclines are daily used for mild to moderate infections at dose of 1 to 2 grams or higher dose of 500 mg for severe infections. Moreover, tetracyclines are also used for the long-term treatment of brucellosis and acne at dose of 1 g/day daily. Tetracyclines have been reported to their common side effects in human especially nausea, fever, vomiting, skin reaction or photosensitivity, dysphagia and diarrhea.

In recent years, tetracyclines have been established, various biological effects of independent of their antimicrobial activity. For examples, tetracyclines inhibit the matrix metalloprotease (MMPs) including collagenase (MMP-1), gelatinase (MMP-2), and stromelysin (MMP-3) activities, resulting in prevention of pathogenic tissue destruction (Golub et al., 1983). Moreover, tetracyclines inhibit tumor progression (DeClerck et al., 1994), bone reabsorption (Rifkin et al., 1994), angiogenesis (Maragoudakis et al., 1994), and connective tissue breakdown (Golub et al., 1991). They also prevents glycation of various proteins that reduce the progression of diabetic neurophathy, nephropathy, and

cataract (Ryan., 1998). In addition, Truswel (1974) reported that chlortetracycline and tetracycline are well known as a pancreatic lipase inhibitor that delays fat absorption into blood circulation (Krondl et al, 1974; Shilata et al, 1970).

Moreover, the effect of tetracycline on the lypolysis in human hair used for treatment for acne vulgaris was investigated by Shaw and Grosshans (1980). The results indicate that tetracycline hydrochloride significantly reduced the total of free fatty acid and triglyceride ratio in hair lipid after treatment for 4 weeks.

The effect of tetracyclines on the inhibition of pancreatic lipase was investigated by Shilata (1970). The study was undertaken to determine the inhibitory effect of tetracycline HCl, demethyl-chlortetracycline and doxytetracycline hyclate on pancreatic lipase by using olive oil emulsion as a substrate in vitro. The results indicated that the order of the inhibitory potency was tetracycline HCl > demethyl-chlortetracycline > doxycycline hyclate.

Orlistat is a clinically used for antiobesity which acts as a pancreatic lipase inhibitor which has been available in market since 1999. Orlistat strongly inhibits the activities of both gastric and pancreatic lipase by irreversible type. In addition, orlistat also inhibited absorption of dietary ingested fats and reduced plasma triglyceride levels, leading to the reduction of body weight and lowering cholesterol (Hongan et al, 1987). However, orlistat also show the most common side effects such as oily spotting on underwear, flatulence, urgent bowel movements, fatty or oily stools, increased number of bowel movements, abdominal pain or discomfort and inability to control stool (incontinence).

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This has stimulated a growing interest that tetracyclines may have the same mechanism and side effect as orlistat. Especially, the combined use of these agents may produce serious side effects. Therefore, the study was to investigate the effect of tetracyclines including tetracycline, chlortetracycline, doxycycline and oxytetracycline on the pancreatic lipase inhibition. In addition, the type of their inhibition and the synergistic effects were also investigated. Finally, the study was also investigated the antihyperilipidemia effect of tetracyclines on serum triglyceride concentrations in normal

rats by olive oil emulsion loading test. The scientific evidence of this study would be the novel pharmacological and toxicological information of tetracyclines for further research.

1.1 Research Hypothesis

Tetracycline and its derivative inhibited pancreatic lipase and decreased serum triglyceride concentration in normal rats.

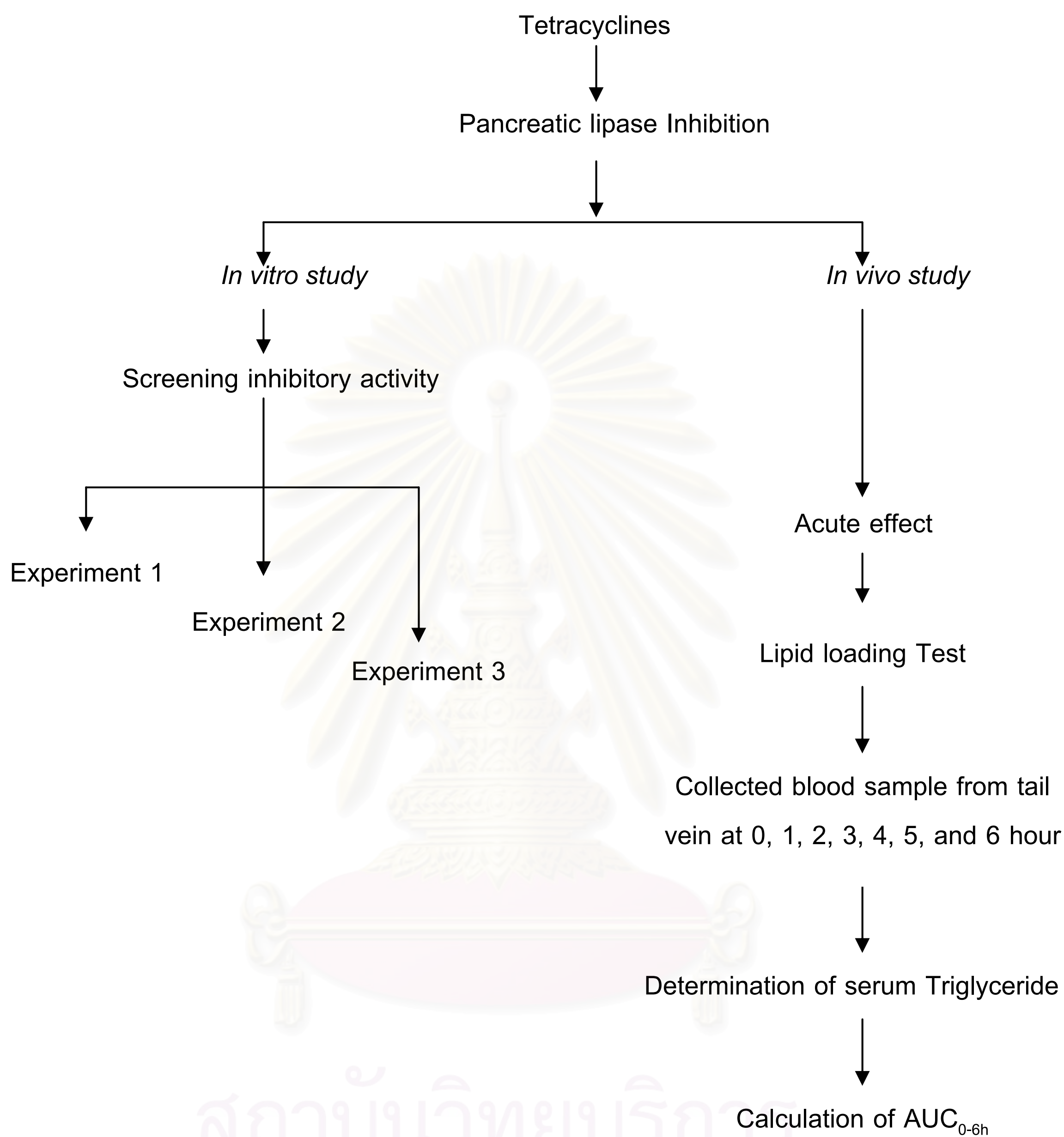
1.2 The objective of the study

1. To investigate the inhibitory effect of tetracyclines and type of their inhibition on pancreatic lipase.
2. To investigate the antihyperlipidemia effect of tetracyclines in normal rats.

1.3 The Advantage of the study

1. The study would be an important fundamental in basic science for further research.
2. The study would indicate a new pharmacological action of tetracycline on digestive system.

1.4 Conceptual Framework



Experiment 1; Dose-dependent study

Experiment 2; the enzyme kinetic inhibition

Experiment 3; the synergistic effect of orlistat and tetracyclines

CHAPTER II

LITERATURE REVIEW

The effect of dietary fat on hyperlipidaemia is well known as it is directly or indirectly associated with various diseases like obesity, diabetes, hypertension and cardiovascular problems (Sharma et al, 2005). Western diets are high in fat and tend to promote obesity. Increased intake of high caloric (energy and fat) food promotes body fat storage and greater body weight and adiposity in humans and animals (Moreno et al, 2006) The assimilation of dietary fats into the body requires that on overage, adults Western diets contain approximately 100 g of lipids per day, of which 92% to 96% are Long-chain triacylglycerols. Triacylglycerols (also referred to as triglycerides or TGs) are fatty acid trimesters of glycerol (Figure 1). TGs differ according to the identity and position of their tree fatty acid residues (termed *sn*-1; *sn*-2; *sn*-3). Most TGs nature contains long-chain free fatty acids, although for example, TGs in human milk are mixtures containing both medium- and long-chain fatty acids. The presence of difference fatty acid at different positions on the glycerol molecule will also influence its characteristics (i.e. melting point and digestibility).

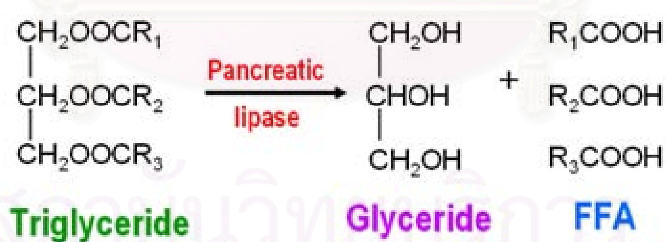


Figure 1 Structure of triacylglycerol (triglyceride) and hydrolysis of triacylglycerol.

An adequate intake of dietary lipid is essential for life and well-being. Lipids serve several important functions in the human body. Firstly, they represent the major source of energy (9 kcal/g), double that of sugars and protein. In the average Western diet, lipids provide approximately 40% of the caloric energy which can be stored in the human body for more than several days in contrast to carbohydrates and proteins. The lipid content of normal humans (21% for men, 26% for women) enables them to survive energy

starvation for 2 to 3 months. Secondly, lipids are the major constituent of cell membranes in the form of phospholipids, sphingolipids and cholesterol. Furthermore, lipids are the only source of essential fatty acids, the precursors of eicosanoids such as prostaglandins, thromboxanes, and leucotrienes. Finally, lipids are necessary for the solubilization and uptake of the fat-soluble vitamins A, D, E, and K.

2.1 Structure of lipid in human nutrition

Triacylglycerol (TG) constitutes the major energy contribution of dietary lipids. Many different fats and oils have been used in the human diet including oils that originate from fruits such as palm oil and olive oil or from seed such as corn-, rapeseed-, and soybean oil. Also fats from adipose tissues and intramuscular fat droplets from pigs, cattle, poultry and lamb as well as marine sources such as fish oils, seal and whale oils are used in the human diet. These fats possess in general complicated fatty acid profiles involving a range of fatty acids with various chain length and degree of unsaturation and different fatty acid isomers (Mu and Porsgaard, 2005).

Lipid emulsions based on long-chain TG (LCT) derived from soybean or safflower oils were the first choice in total parenteral nutrition for many years. Unfortunately, the use of pure LCT emulsions is connected with slow clearance from the bloodstream and impairment of the reticuloendothelial system (RES). Medium-chain TG (MCT) derived from palm kernel or coconut oils represents an alternative lipid source. MCT have been used for clinical nutrition because they undergo nearly complete hydrolysis, are absorbed predominantly as free fatty acids (FFAs) and transported to the liver for oxidation and thereby provide fast energy. However, long-term use of MCT may result in essential fatty acids deficiency and may have side effects such as metabolic acidosis and therefore the use of mixed LCT/MCT emulsions has attracted increased attention in patient.

Fatty acids vary in length from 2 to 80 carbons, but are typically present in food as 14, 16, 18, 20 and 22 carbon atom chains. The predominant fatty acid residues in nature are those of the C16 and C18 species palmitic, oleic, linoleic, and stearic acids. Fatty acids can be divided into three main classes according to their chain length: 1. short-chain fatty acids, less than 6 carbon atoms; 2. medium-chain fatty acids, from 6 to 12

carbon atoms; 3. long-chain fatty acids, 14 or more carbon atoms. The properties of fatty acids are markedly dependent on their chain length and degree of saturation. Unsaturated fatty acids are more fluid than saturated fatty acids of the same length. By virtue of their smaller molecular size, medium-chain fatty acids are relatively soluble in water.

2.2 Digestion of triacylglycerols by pancreatic lipase

Fat enhances the flavour and palatability of foods, and has been shown to slow the rate at which the stomach contents are emptied into the small intestine. As a result, fat leads to general feelings of satiety and satisfaction after a meal that is often difficult to mimic in reduced fat products. In order to be efficiently digested and absorbed, dietary fat must first be emulsified by bile secreted into the small intestine from the gallbladder. The intestinal absorption of dietary TGs requires firstly their enzymatic conversion into the more polar fatty acid monoacylglycerols by digestive lipolytic enzymes. Lipolysis is catalyzed by lingual lipase and gastric lipase, which are specific for the *sn*-3-position of TG. Major digestion under normal digestion and absorption conditions results from hydrolysis with pancreatic lipase. The hydrolysis of alimentary triacylglycerols begins in the stomach and is catalysed and hydrolyzed by gastric lipase (GL) which is able to hydrolyze short-chain triacylglycerols and long-chain triacylglycerols at comparable rates. Under acidic pH conditions, GL has been shown to be remarkably stable and active, whereas pancreatic lipase irreversibly loses its lipolytic capacity. The optimum pH for GL activity is around 5.4 which is close to the pH of the gastric content during a test meal, as against 8 to 9 in the case of pancreatic lipase. The partial hydrolysis of alimentary TGs which occurs at the pH levels prevailing in the stomach rapidly triggers pancreatic lipase activity in small intestine (Gargouri et al, 1997; Ros, 2000).

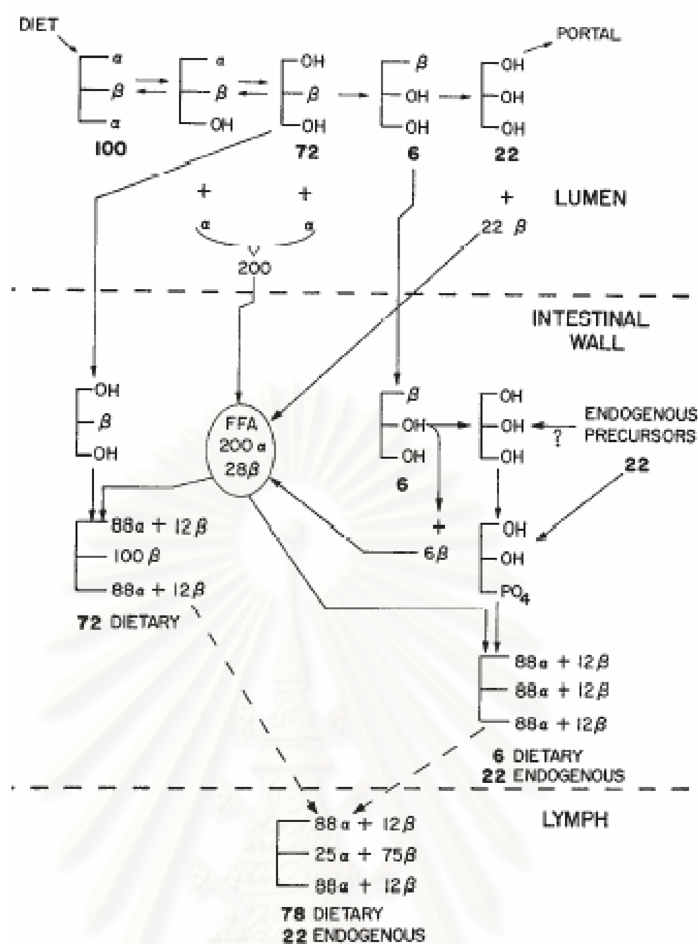


Figure 2 The digestion, absorption, and resynthesis of glycerides. The proportions of the various products of digestion and their resynthesis into triglycerides, which appear in the lymph, are shown. The bold-faced numbers beneath the formulas are the values for the glycerol moiety and the light-faced numbers are the values for the fatty acid moiety. Fatty acids are occupied the α, β or α' position in the original triglyceride.

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The digestion and absorption of TG was reported by Mattson and Volpenhein (1963). The investigated studies both *in vitro* and in *vivo* have established that the hydrolysis of TG in the intestinal tract can yield diacylglycerol, monoacylglycerol, free fatty acids and free glycerol. From these data and the results obtained by other, the following Figure 2 is proposed for the digestion and absorption of oleoyl glycerides. In the lumen of the intestine, TG are hydrolysis results in the cleavage of all of the fatty acids esterified at the α, β-Diacylglycerol to 72 parts β-Monoacylglycerol, 6 parts α-Monoacylglycerol and 22 parts free glycerol. This hydrolysis results in the cleavage of all of the fatty acids

esterified at the α and α' positions and 22% of those esterified at the β position of the dietary TG. Thus, approximately 75% of the glycerol of dietary TG is absorbed as MAG and 75% of fatty acids of dietary TG are absorbed as free acids. The free glycerol is absorbed independently of the lipids and little of it is used in glyceride synthesis.

The rate of hydrolysis affects the absorption process. An extensive review of the pancreatic lipase has been presented by Lowe (1997). Following the gastric pre-digestion, the food bolus enters the duodenum. The fat droplets are emulsified by bile acids and the available surface is thereby increased. The pancreatic lipase which is released from the pancreas into the duodenum upon the hormonal response of cholecystokinin, binds to the surface of the fat droplets with the co-lipase as a mediator to overcome the expulsion of the lipase into the water-phase caused by the bile acids and digests the TG. The degradation process is regiospecific and ideally results in the formation of *sn*-2 monoacylglycerol (MAG) and free fatty acids (FFAs). A rearrangement of *sn*-2-MAG into *sn-1*-MAG may result in complete degradation into glycerol and FFAs.

Akesson et al, 1978 using structured TG 12:0/12:0/18:1 found that approximately 25% of 12:0 initially located in the *sn-2*-position had migrated to the *sn1, 3*-positions during hydrolysis and absorption. This implies a general conservation of approximately 75% of the fatty acids located in the *sn-2*-position, which is important when considering the possible advantages in tailor-making fats with particular TG structural analysis of lymph lipids. Jensen et al (1994) estimated that less than 40% of Medium-chain fatty acids were conserved during absorption when they were located in the *sn-2*-position indicating a higher tendency for acyl migration during hydrolysis and absorption of Medium-chain fatty acids compare with Long-chain fatty acids.

Even though the pancreatic lipase is active toward fatty acids located in the *sn1, -3-* position, the activity toward *n-3* fatty acids, in particular 20:5*n-3* and 22:6*n-3*, is lower compared with the activity toward other fatty acids when located in the sn-3-positions.

The activity of the pancreatic lipase has been examined in further details using *in vitro* condition. Jandecck et al (1987) prepared 2-oleyl-1,3-dioctanoyl-*sn*-glycerol (8:0/18:1: 8:0) in pure form and compared the degradation in vitro by pancreatic lipase including bile fluid from rats with the gradation of medium chain triacylglycerol (containing

67% caprylic acid, 23% capric acid and 10% other fatty acid acids), tricaprin acid indicating that in vitro the specific structured TG was well degraded by intestinal lipase. Medium-chain fatty acids were hydrolyzed faster than long-chain fatty acids and Short-chain fatty acids especially when Medium-chain fatty acids occupied the primary position of TG. In a parallel experiment they examined the hydrolysis by a commercial porcine pancreatic lipase and found the following initial rates: MCT > 1, 3-dioctanoyl-2-linoleyl-sn-glycerol (8:0/ 18:2/ 8:0) > sunflower oil. The total digestion at the end point was similar for MCT and 1, 3-dioctanoyl-2-linoleyl-sn-glycerol. They also examined the hydrolysis of a series of randomized TG ranging in Medium chain fatty acid (MCFA) content from 32% to 80% and found that the hydrolysis rate was highest for the TG with the highest contents of medium-chain fatty acids.

The initial droplet size in the stomach may in certain cases affect the rate of hydrolysis. A small initial droplet size resulted in a finer emulsion in the duodenum also followed by higher degree of lipolysis. In normal digestion the capacity for hydrolysis is so high that is of no importance, whereas in pancreatic deficiency and gastric tube feeding it may be important.

Overall, the presence of medium-chain fatty acids in the outer positions of dietary TGs will lead to faster hydrolysis of the TG in comparison with the presence of long-chain fatty acids; however, the effect may be masked in the presence of other TG species.

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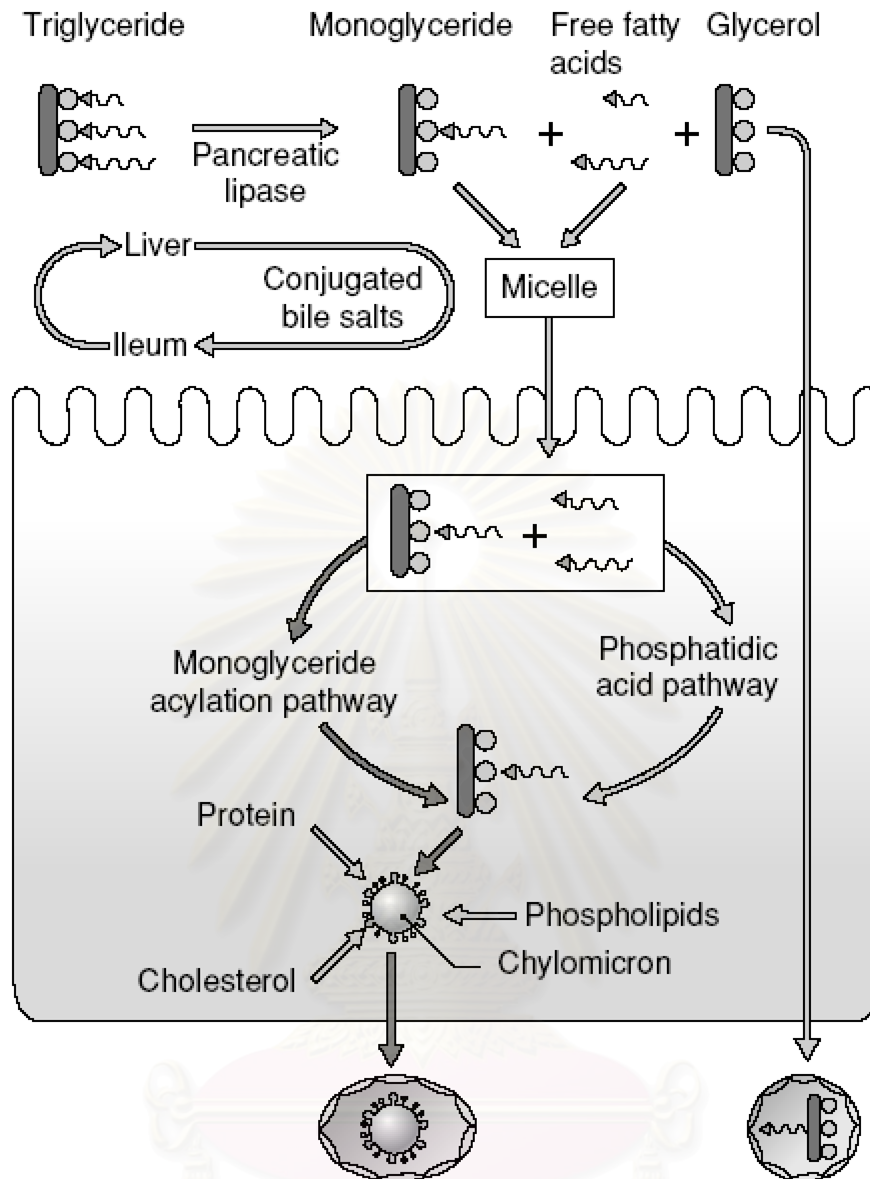

Figure 3 Overview of digestion and absorption of triglyceride (Johnson, 1997)

The fat droplets form small micelles that are dispersible in water and can be acted on by pancreatic lipases cleaving the fatty acids at the *sn-1* and *sn-3* positions of the TG. The end-products from the digestion of fat: free fatty acids, a 2-monoacylglycerol, and very limited amounts of glycerol, are absorbed by diffusion across the gut wall into the cells of the intestine. Recently, work by Stremmel et al (2001) has introduced the idea that fatty acids may be absorbed by carrier-mediated processes, involving transport proteins in the membranes of cells lining the small intestine. However, the importance of this route has been questioned, as the candidate transporter proteins are also found in other cells in the body where they have little to do with fatty acid transport (Tso et al, 2004). Regardless of

this, the digestion of fat is very efficient, with typically more than 95% of dietary fat being broken down and absorbed (Sanders and Emery, 2003).

The mode of transport away from the gut is dependent on chain length; the short to medium chain fatty acids (2–12 carbon atoms) are transported bound to the carrier protein albumin *via* the hepatic portal vein to the liver. However, dietary fat mainly consists of the longer chain fatty acids (>12 carbon atoms) which are reassembled in the intestinal cell into TG. These are then packaged into lipoprotein particles known as chylomicrons and are transported *via* the lymph system into the peripheral circulation. The rate of lipolysis, absorption of fatty acids and transport might be influenced by the degree of un-saturation (BNF, 1992). However, the nutritional significance of this difference is unclear. The fatty acid at *sn*-2 may influence subsequent metabolism of chylomicrons and ultimately low density lipoprotein (LDL) cholesterol response. It has been suggested that dietary fats containing a predominance of *sn*-2 unsaturated TG might be absorbed more slowly, and cleared more rapidly from the circulation compared with saturated fatty acids, resulting in reduced concentrations of TG in the blood after a meal (Yi-Jokipii et al, 2001) Conversely, Saturated Fatty acids in the *sn*-2 position are preferentially absorbed into the cells lining the intestines over Saturated Fatty Acids at positions *sn*-1 and *sn*-2, which also has implications for the balance of TG in the blood after a meal (Berry and Sanders, 2005). It remains controversial as to the relevance of these findings to adults, as most of the work has been conducted in animals, and any work that has been carried out in humans has been conducted in infants.

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2.3 Clinical applications of a pancreatic lipase inhibitor

Pancreatic lipase plays a role in the emergence of diseases linked to the presence of an excess of lipids, such as cardiovascular diseases, hyperlipdemias and obesity (Tsujita et al, 2005). Lipase and pancreatic other enzyme supplements have been widely used for a long time to reduce fat malabsorption (Miled et al, 2000).

Obesity is a significant health problem in worldwide. It is a risk factor for clinical disorder such as hypertension, hyperlipidemia, diabetes mellitus and cardiovascular diseases (Mukherjee, 2003). Inhibition of lipase activity has been focused largely on strategies for controlling the energy intake and management of obesity. The inhibition of dietary fat absorption has been reported to be one of the effective therapeutic for treatment of obesity. For instance, orlistat ((S)-1-[[(S, 2S, 3S)-3-hexyl-4-oxo-2-oxetanyl] methyl]dodecyl-(S)-2-formamido-4-ethylvalerate) or Xenical® (Figure 4), a hydrogenated derivative of lipstatin, isolated from *Streptomyces toxitricini* is a effective lipase inhibitor. For mechanism of its binding, the β-lactone ring of tetrahydrolipstatin binds to serine 152 of pancreatic lipase to form esters (Tsujita et al, 2005) which the IC_{50} value was 0.14 μM (Mukherjee, 2003). In addition, orlistat also inhibited absorption of dietary ingested fats and reduced plasma triglyceride levels, leading to a reduction in body weight and lowering cholesterol (Hogan et al., 1987). In clinical trial, orlistat reduced the absorption of dietary fats by 30% (Kokotos, 2003) and reduced postprandial plasma triglyceride levels. Moreover, orlistat reduces the progression of atherosclerotic lesions as a result of the triglyceride-lowering effect *via* inhibition of fat absorption (Ueshima et al, 2004).

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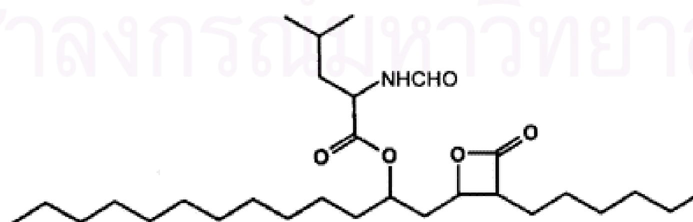

Figure 4 the structure of orlistat

2.4 Rationale of pancreatic lipase inhibitors

There were several studies reported to natural products which showed the inhibitory activity on pancreatic lipase such as teasaponin (Han et al., 2001), citrus pectin (Kawakuchi et al., 1997), carnosic acid (Ninomiya et al., 2004) and crocin (Sheng, 2006). Amphiphilic protein bovine serum albumin and β-lactoglobulin (Gargouri et al., 1984) have been shown to inhibit lipase activity towards its triacylglycerol substances. Basic materials also influence the lipase reaction rate such as chitin-chitosan which inhibited the hydrolysis of trioleoyglycerol (Mukherjee, 2003).

There have been many reports to chemical compound which showed the inhibitory effects on pancreatic lipase. Minard (1952) reported that polyoxyethylene sorbitan (Tweens) inhibited pancreatic lipase when combination with corn oil emulsions and bile salts.

Wills (1959) investigated the inhibitory effect of several divalent metal ions in presence of sulhydryl group in structure on the rate of triglyceride hydrolysis. The results indicated that divalent metal containing sulhydryl group markedly inhibited pancreatic lipase, affecting to delay in digestion and absorption of triglyceride. Moreover, Klein et al (1967) reported the effect of lecithin on the inhibitory activity of pancreatic lipase. The results indicated that 0.4 μmol/ml of lecithin inhibited pancreatic lipase and also decreased plasma TG concentration.

One of the potent pancreatic lipase inhibitors is ε-polylysine which has been used as a food additive in Japan. This compound significantly decreased plasma triglyceride in normal rats (Tsujita et al, 2006). It also inhibited a human pancreatic lipase and gastric lipase with the IC_{50} of 28.63 μmol/l and of 2,986.17 μmol/l, respectively. In addition, crocin, a natural product, markedly inhibited pancreatic lipase and gastric lipase (Kido et al, 2003)

2.5 Pancreatic lipase

Pancreatic lipase (EC 3.1.1.3), one of the exocrine enzymes of pancreatic juice, is synthesized from the pancreatic acinar cells that is essential for digestion of dietary fats in the intestinal lumen. Hydrolysis of dietary triacylglycerols by both gastric and pancreatic lipase is essential for their absorption by enterocytes, to facilitate assimilation of dietary fat in the body. Pancreatic lipase requires colipase as a cofactor for its enzymatic activity (Mukherjee, 2003). Pancreatic lipase catalyzes the hydrolysis of emulsified esters of glycerol and long-chain fatty acids. Short-chain fatty acids can be directly absorbed into the blood, while long-chain fatty acids and monoglycerides combine with bile salts to form soluble micelles. The micelles are absorbed into the mucosal cells of the intestine and the fatty acids and monoglycerides are re-synthesized into triglycerides. These triglycerides are formed into small particles known as chylomicrons which consist of triglycerides, the sterol lipid cholesterol, and apoproteins. These chylomicrons are transported to the muscle and adipose tissue.

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2.6 Tetracycline and its derivatives

Tetracyclines, discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (Figure 5). Tetracyclines are broad-spectrum agents and exhibits activity against a wide range of gram-positive and gram-negative bacteria. Tetracyclines have been classified in terms duration of action in the body.

1. Short-acting derivatives: chlortetracycline, oxytetracycline and tetracycline
2. Intermediate-acting derivatives: demeclocycline and methacycline
3. Long-acting derivatives: doxycycline and minocycline

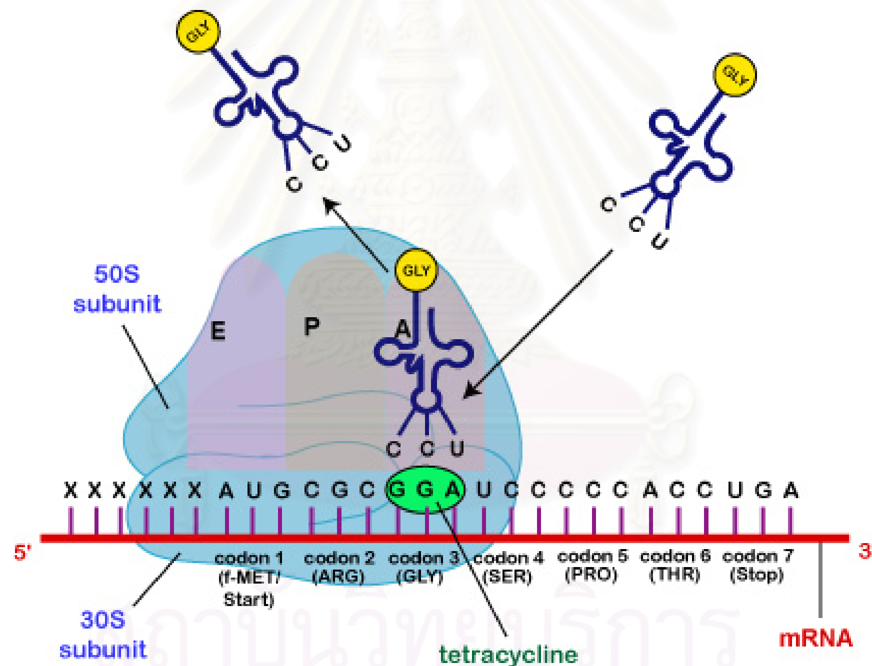

Figure 5 Mechanism of action of tetracycline

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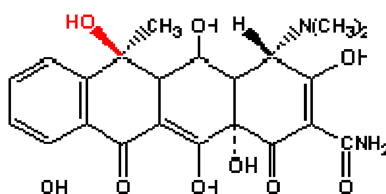
2.7 Pharmacokinetic profiles of tetracyclines

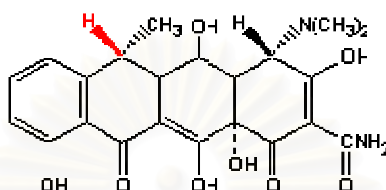
Tetracyclines are orally administered, although some are also available as parenteral products. Rolitetracycline (Tetracycline condensation with pyrolidine and formaldehyde structure) is available only as a parenteral product. The ability to use either oral or parenteral formulations of doxycycline has been used advantageously to permit switching programs from intravenous to oral administration.

Dosing regimens and pharmacokinetic properties of the tetracyclines have been extensively reviewed in various publications. Absorption of tetracyclines occurs largely in the stomach and proximal small intestine and is influenced by the presence of food, milk, or divalent cations, particularly, calcium, with which tetracyclines from nonabsorbale chelates. Levels achieved in serum after normal oral dosing are in the range 2 to 5 μg/ml, and most tetracyclines have to be given four times daily to maintain therapeutic concentrations in the serum. However, the long elimination half-lives of doxycycline and minocycline permit once- or twice-daily dosing. Tetracyclines penetrate moderately into body fluids and tissue and are excreted in the urine.

Name (date of discovery)	Structure
7-chlortetracycline (1948)	Cl, H_3C, OH, $N(CH_3)_2$, OH, $CONH_2$, OH, O, OH, OH, O; ring labels D, C, B, A; positions 1, 4, 5, 6, 7, 10, 11, 12
Tetracycline (1953)	H_3C, OH, $N(CH_3)_2$, OH, $CONH_2$, OH, O, OH, OH, O
7-dimethylamino-6-demethyl-6-deoxy-tetracycline (minocycline) (1973)	$N(CH_3)_2$, $N(CH_3)_2$, OH, $CONH_2$, OH, O, OH, OH, O
9-(*N*,*N*-dimethylglycylamido)-6-demethyl-6-deoxytetracycline (1993)	$(CH_3)_2N$, O, N, H, $N(CH_3)_2$, OH, $CONH_2$, OH, O, OH, OH, O
9-(*N*,*N*-dimethylglycylamido)-minocycline (1993)	$(CH_3)_2N$, O, N, H, $N(CH_3)_2$, $N(CH_3)_2$, OH, $CONH_2$, OH, O, OH, OH, O
9-(*t*-butylglycylamido)-minocycline (GAR-936) (1993)	CH_3, CH_3, CH_3, H, N, O, N, H, $N(CH_3)_2$, $N(CH_3)_2$, OH, $CONH_2$, OH, O, OH, OH, O



Oxytetracycline (Terramycin®)



Doxycycline (Vibramycin®)

Figure 6 Chemical structures of tetracyclines

2.8 Pharmacological effects of tetracycline and its derivatives

Tetracyclines display various biological effects independent of their antimicrobial activity. Tetracyclines have been shown to specifically decreased levels of inducible NO synthase in activated macrophages by regulating the stability of its mRNA. They also inhibits phospholipase A2, cyclooxygenase-2 (COX-2)-mediated prostaglandin synthesis. Moreover, tetracyclines specifically inhibit the expression, activation from pro-enzyme precursor, as well as the enzymatic activity of metrix metalloproteinases (MMP) (Kuzin et al., 2001) activity including collagenase (MMP-1), gelatinase (MMP-2) and stromelysin (MMP-3) and prevention of pathogenic tissue destruction. Tetracyclines also block tumor progression, bone reabsorption and angiogenesis (Patel et al., 1999). Yu et al (1992) has shown that prophylactic administration of doxycycline markedly reduced the severity of osteoarthritis (OA) in dog models. Tetracyclines exhibited the inhibitory activity on protein glycation, causing to prevent the progession of diabetic complications (Ryan et al., 1998).

The effect of tetracyclines on bone metabolism was investigated and reported by Vernillo (1998). The anti-resorptive properties of tetracyclines have enormous therapeutic potential in medicine and dentistry. Tetracyclines directly affect to several

parameters of osteoclast function and consequently inhibit bone reabsorption by altering intracellular calcium concentration and interact with the putative calcium receptor, this lead to decrease raffled border area and diminish acid production and reduce the secretion of lysosomal cysteine proteinases (cathepsins). Consequently, they also induce cell retraction by affecting podosomes and inhibit osteoclast gelatinase activity, resulting in inducing apoptosis or programmed cell death of osteoclasts. As the results mentioned, tetracyclines may act similarly to bisphosphonates and primarily affect osteoclast function (Vernillo et al, 1998). Weaber (1970) investigated the effect of tetracycline on the lipase inhibition from *corynebacterium acnes*. This study used triglycerides (tricaprylin and trilaurin) and naphthl laurate, obtained from the broth of *Corynebacterium acne* cultures by ammonium sulfate fractionation as a substrate. The results showed that tetracycline (10^{-4} M) slightly inhibited the lipase activity with 5×10^{-5} M.

The effect of tetracyclines on pancreatic lipase was investigated and reported by Shilata (1970). The study was undertaken to determine the inhibitory effect of tetracycline HCl, demethyl- chlortetracycline and doxycycline hyclate on pancreatic lipase by using olive oil emulsion as a substrate. The results indicated that the order of the inhibitory potency was tetracycline HCl > demethylchlortetracycline > doxycycline hyclate.

2.9 Analytical techniques for determination of lipase activity (Gilham, 2004)

Most lipases are water-soluble enzymes that hydrolyze ester bonds of water-insoluble substrates. A number of protocols have been developed to assay these enzymes. The majority of triglycerol lipases act on primary ester bonds such as those in the *sn*-1 and *sn*-3 positions of triacylglycerol. The activity of triacylglycerol lipases can be assayed by monitoring the release of either fatty acid esters. Determination of free glycerol is not commonly performed to measure lipolysis with purified lipases since all three acyl chain of a triacylglycerol molecule are rarely released by a single lipase. Quantitation of released glycerol by a combination of lipases can be carried out by phosphorylation of glycerol to glycerol-3-phosphate. There are many analytical techniques for determination of lipase activity reported in available publications.

2.9.1 Chromogenic assays

Chromogenic assay is a commonly used procedure for investigating esterase and lipase activity by using *p*-nitrophenyl esters with aliphatic acyl chain of various lengths. The release of *p*-nitrophenol is measured spectrophotometrically at 410 nm. A variety of *p*-nitrophenyl esters with various acyl chain length are commercially available. Short chain esters, like acetate or butyrate, are used to measure esterase activity, while longer chains such as laurate, palmitate or oleate are used investigate lipase activity.

This method is convenient because it only requires simple ultraviolet-visible spectrophotometer that is normally found in the research laboratory. Further, many of these substrates are commercially available and are relatively inexpensive. The reactions are routinely scaled to a 96-well plate and measurements can be taken in a kinetic fashion.

However, the method has limited condition because these substrate, particularly with short acyl chains, can be hydrolyzed by non-specific esterases, non-enzymatic proteins or proteases which are often found in biological samples. For example, serum albumin as well as insulin has been shown to be able to hydrolyze *p*-nitrophenyl acetate. Therefore, these assays are suitable for only use of purified lipases which exclude these interfering components. Moreover, measurement with these substrates cannot be performed at neutral or alkaline pH, which may not be suitable for some lipases. This drawback can be worked around by raising the pH of the reaction mixture upon culmination of the reaction. Because *p*-nitrophenol has different absorption coefficients at different pH values, use of standards in different pH environment is required. In addition, preparation of the sample containing the lipase is a confounded by turbidity introduced into the reaction mixture, such as assaying cell lysates. Another colorimetric lipase assay is based on hydrolysis of *p*-nitrophenyl ester of various chain length are available, the assay requires common laboratory equipment and the reaction can be monitored in a kinetic fashion.

2.9.2 Quantitation of released fatty acid

2.9.2.1 Spectroscopic assay

This technique has been employed to measure the increase in turbidity generated when fatty acids liberated by lipase activity are precipitated using calcium. The increase in turbidity is measured at 500 nm. The turbidometric method is described as being 36 times more sensitive titrimetric assays and at least four times more sensitive than a spectrophotometric method using *p*-nitrophenyl palmitate. Clearly, this method is not useful when activities in turbid solutions, such as cell extracts, are to be determined.

2.9.2.2 Chromatography

It is a common method for direct determination of the released fatty acids following lipolysis of a lipid substrate. Chromatography allows use of the most physiologically relevant substrates, which is critical when characterizing an enzyme. Although these methods of analysis allow end point analyses and cannot be followed on a kinetic basis.

2.9.2.2.1 Thin-layer chromatography

A quantitative analysis of released free fatty acids from triacylglycerols can be carried out using densitometric, autoradiographic or scintillation counting using radiolabeled triacylglycerol as a substrate. The methods are sensitive, capable of detecting picomole amount of fatty acid. Disadvantages of this method include the usual dangers and precautions required when handling radioactivity, and the procedures are time consuming.

2.9.3 Gas chromatography (GC)

GC can be used to simultaneously and quantitatively determine mono-, di-, and triacylglycerol as well as free glycerol and the methyl ester derivatives of free fatty acids. Releases of fatty acids (lipase-catalyzed cleavage of triacylglycerols) were the generation of the reaction products to trimethylsilyl ester (fatty acids) and ethers (partial acylglycerols) before performing GC. This method is sensitive (less than a nanomole of products can be detected) and is suitable with

purified lipases or when incubation mixtures do not contain other glycerolipids (that is the milieu is free of cell membranes). Inclusion of a known amount of internal standard (such as tridecanoylglycerol) after termination of the lipases reaction with an organic solvent is necessary for sensitive, it requires specialized expensive equipment very useful when fatty acyl chain length specificity of a given lipase is to be determined since the various chain lengths and saturation of the released fatty acids are easily detected.

2.9.4 High performance liquid chromatography (HPLC)

This method of detection allows identification of the products of lipolysis. A detailed HPLC method for determining lipase activity with *p*-nitrophenyl palmitate as a substrate has been developed. HPLC can also be used to separate mixtures of different triacylglycerols, and mixtures of all fat classes (monoacylglycerols, diacylglycerols, Triacylglycerols and free fatty acids).

2.9.5 Fluorescent substrate

This method involves in measurement of reaction products that become fluorescent upon hydrolysis. The assays are usually very sensitive and can be continuously monitored. The overall sensitivity of a fluorescent assay using synthetic triacylglycerols or esters depends on the sensitivity of detection and on the specific activity of the lipase for that substrate. Fluorescence based assays are also much less confounded by turbidity in samples that may arise when analyzing cell lysates.

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2.10 Enzyme kinetic

Enzyme kinetic is the study of the rates of chemical reaction that are catalyzed by enzymes and focus on their reaction rates. The study of an enzyme's kinetic provides insights into the catalytic mechanism of this enzyme. Enzyme shows the role in metabolism and how drugs and poisons can inhibit its activity.

2.11 Enzyme inhibition

Enzyme inhibitors are molecules that reduce or abolish enzyme activity. These are either reversible i.e. removal of the inhibitor restores enzyme activity or irreversible i.e. the inhibitor permanently inactivates the enzyme.

2.11.1 Reversible inhibition

Reversible enzyme inhibitors can be classified as competitive, uncompetitive, non-competitive or mixed according to their effects on K_m and υ_{max}. These different effects result from the inhibitor binding to the enzyme (E), to the enzyme-substrate complex (ES) or to both. The particular type of an inhibitor can be discerned by studying the enzyme kinetics as a function of the inhibitor concentration. The four types of inhibition produce Lineweaver-Burk plots that vary in distinctive ways with inhibitor concentration.

2.11.2 Irreversible inhibition

Irreversible inhibitors covalently modify an enzyme, and inhibition cannot be reversed. Irreversible inhibition is different from irreversible enzyme inactivation. Irreversible inhibitors are generally specific for one class of enzyme and do not activate all proteins; they are unfunctioned by destroying protein structure but by specifically altering the active site of their target.

2.12 Mechanism of enzyme inhibition

Enzymes catalyze reactions in physiological systems. In equilibrium, an enzyme (E) binds a substrate (S) to form an enzyme-substrate complex (E-S). The E-S complex dissociates or irreversibly converts the substrate to a product (P) (Figure 8). The Michaelis-Menten equation describes the relationship between the rates of substrate conversion by an enzyme to the concentration of the substrate (Equation 1). In this equation, υ is the rate of conversion, υ_{max} is the maximum rate of conversion, [S] is the substrate concentration, and K_m is the Michaelis constant, the substrate concentration at which the rate of conversion is half of υ_{max}. A more illustrative version of the Michaelis-Menten equation is the Lineweaver-Burk equation (Equation 3).

2.13 Lineweaver-Burk plot

The Lineweaver-Burk plot is one way of visualizing the effect of inhibitors and determines the Michaelis Constant K_m and the Limiting Velocity υ_{max} from a set of measurements of velocity at different substrate concentrations. If $1/\upsilon_o$ is plotted against 1/[S], a straight line is obtained where the slope is equal to K_m/υ_{max} and the y-intercept is equal to $-1/\upsilon_{max}$. This method is usually used for distinguishing the type of inhibition. The information is used to select the appropriate type of plot needed to calculate the inhibition constant K_i. If the Lineweaver-Burk plots of several inhibitor concentrations intersect on the vertical axis, then the inhibitor is a competitive inhibition. Competitive inhibitors have the affect of increasing the K_m of the reaction and therefore reduce the affinity of the enzyme for its substrate. If the Lineweaver-Burk plots of several inhibitor concentrations intersect on the base line, then the inhibitor is a non-competitive inhibition. Non-competitive inhibitors do not affect to the combination of the substrate with the enzyme, but it affects to the velocity. If the Lineweaver-Burk plots of several inhibitor concentrations are parallel, then they have an uncompetitive inhibitor and should select the $1/K_m$ type of plot. Uncompetitive inhibitors have the affect of decreasing the K_m and the velocity of the reaction to the same extent. If the Lineweaver-Burk plots of several inhibitor concentrations intersect above or below the 1/[S] axis, then inhibitor is a mixed inhibition. Mixed inhibitors have the affect of decreasing the velocity of the reaction and either increasing or decreasing the K_m.

$$1/\upsilon \quad = \quad K_m + [S]/ \upsilon_{max}[S] \qquad (1)$$

Rearranging this equation

$$1/\upsilon \quad = \quad K_m/\upsilon_{max}[S] + [S]/ \upsilon_{max}[S] \qquad (2)$$

This is further simplified to

$$1/\upsilon \quad = \quad K_m/\upsilon_{max} \times 1/\upsilon_{max}/[S] + 1/\upsilon_{max} \qquad (3)$$

A plot of $1/\upsilon_0$ versus 1/[S] (a double reciprocal plot) yields a straight line with the slope of K_m/υ_{max} and ordinate intercept of $1/\upsilon_{max}$. Since the slope and intercept are readily measured from the graph, the υ_{max} and K_m can be accurately determined.

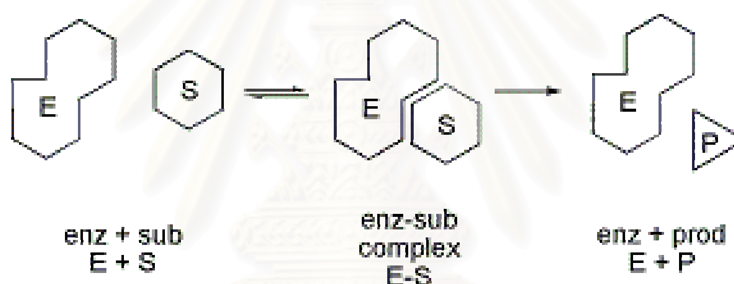

Figure 7 Enzyme-substrate complex and product formation model

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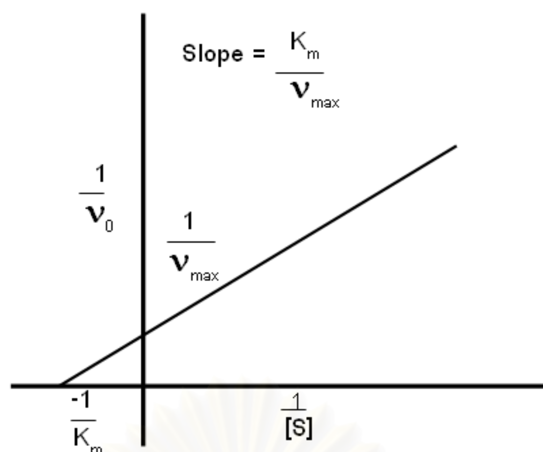

Figure 8 Lineweaver-Burk plot

Enzyme inhibition is a common goal for the pharmaceutical industry. The rates of enzyme catalysed reactions are decreased by specific inhibitors. Inhibitors are compounds that combine with enzymes and prevent enzyme and substrate from forming ES complex. All inhibitors cause the substrate to react at a lower rate than without the inhibitor. Reversible enzyme inhibitors fall into three categories; competitive; non-competitive, and uncompetitive. Furthermore, non-competitive inhibitors can be divided into two additional categories; pure and mixed types. The Lineweaver-Burk equation can be used to categorize different inhibitors. Understanding the type of inhibitor will give clues on how its structure might be modified to increase its potency.

Competitive inhibitors bind at the active site of the enzymes to form an E-I complex (Figure 9). The inhibitor blocks the active site, and the substrate cannot bind until the inhibitor dissociates. Since the inhibitor and substrate compete for the same site, raising the substrate concentration can eventually overcome the inhibitor, and υ_{max} can be achieved. Although υ_{max} can be reached, a competitive inhibitor raises K_m, indicating that the affinity of the enzyme for the substrate is lower in the presence of the inhibitor. The effect of a competitive inhibitor in a Lineweaver-Burk plot is both to move the x-intercept and increase the slope. Plots made with varying amounts of a competitive inhibitor will all cross at the same y-intercept.

Competitive inhibitors can bind to E but not to ES. Competitive inhibition increase K_m (i.e., the inhibitor interferes with substrate binding) but not affect υ_{max} (the inhibitor dose not hamper catalysis in ES because it cannot bind to ES). K_i is equal to the dissociation constant for the enzyme-inhibitor complex EI.

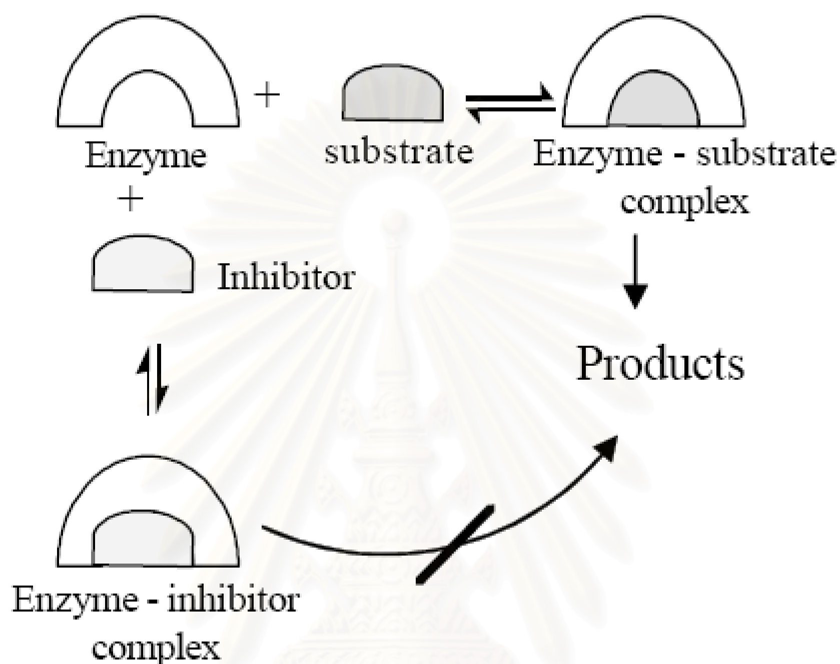

Figure 9 Model of competitive inhibition

Non-competitive inhibition occurs between the substrate and the inhibitor and the inhibitor which has no structural resemblance with the substrate and it binds with the enzyme at a place other than the active site. Non-competitive inhibitors bind at an allosteric site on the enzyme and leave the active site unblocked. In a *pure* non-competitive system, the substrate has an identical affinity for both the E-I complex and enzyme. Unlike the E-S complex, the E-I-S complex cannot convert the substrate to product (Figure 10). With a pure non-competitive inhibitor, the K_m value is unchanged while υ_{max} is lowered. So, the x-intercept will be constant, and the slope will increase with more inhibitor. With a mixed non-competitive inhibitor, the affinity of the E-I complex for the substrate is not the same as the unbound enzyme. In this case, not only is υ_{max} lowered, but K_m is also raised. The Lineweaver-Burk plot will show changes in the x-intercept and increasing slope.

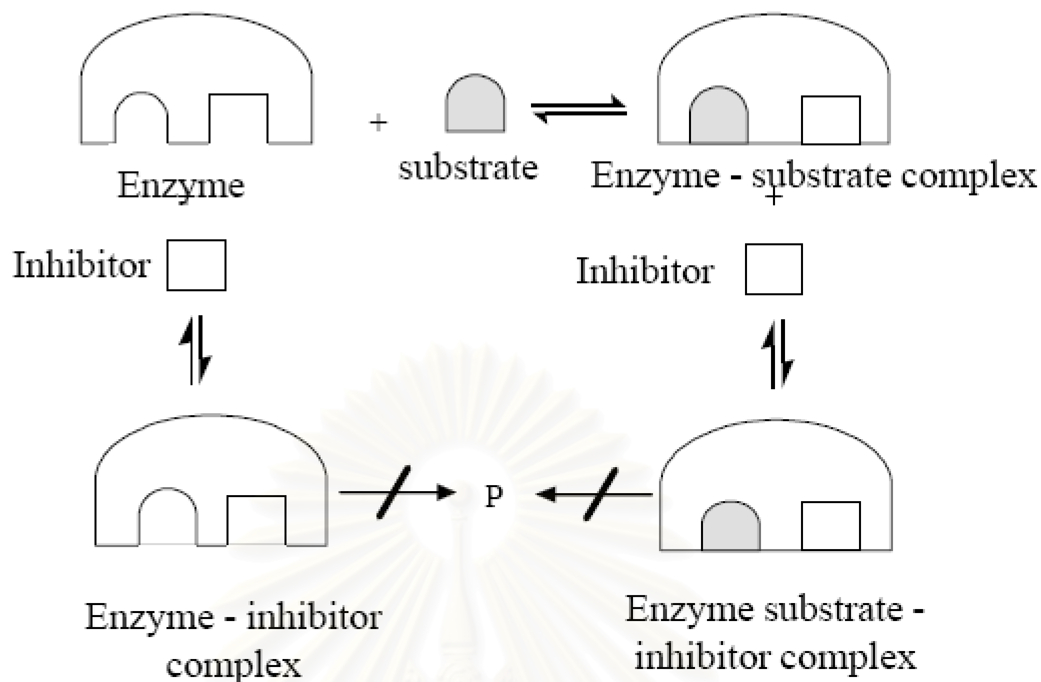

Figure 10 Model of Non-competitive inhibition

Uncompetitive inhibition occurs when an inhibitor combines reversibly only with ES to form ESI which cannot yield the products. An un-competitive inhibitor also binds at an allosteric site and the binding takes place only in enzyme substrate complex and not with the free molecule. The effect of an uncompetitive inhibitor is to decrease both υ_{max} and K_m. The drop in K_m deserves some comment. K_m is a measure of substrate affinity for the enzyme. A lower K_m corresponds to a higher affinity. The presence of an uncompetitive inhibitor actually increases the affinity of the enzyme for the substrate. This surprising fact can be understood through the binding equilibrium. Since the inhibitor binds the E-S complex, the inhibitor decreases the concentration of the E-S complex. K_i is dissociation constant of ESI complex. In a Lineweaver-Burk plot, uncompetitive inhibitors shift the line higher with a raised y-intercept.

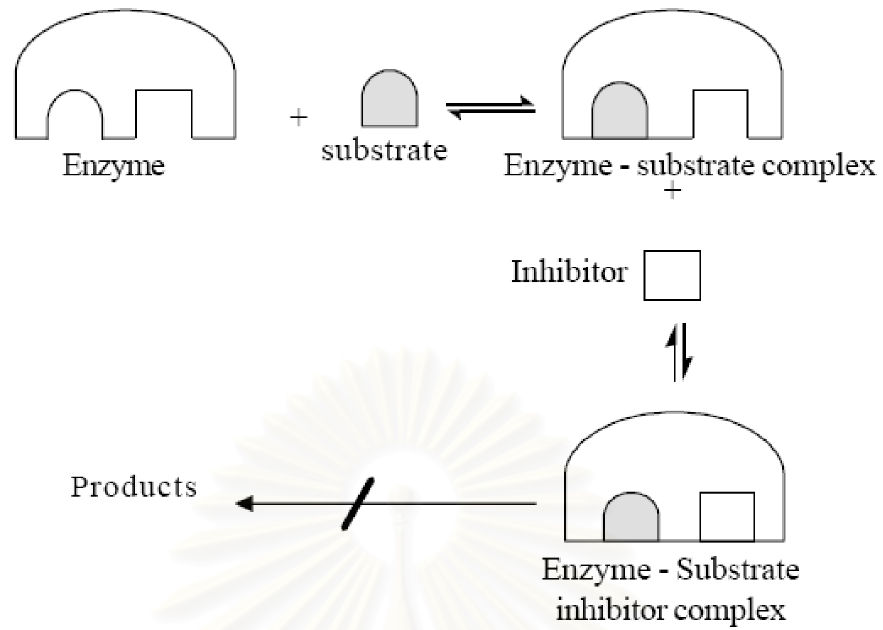

Figure 11 Model of uncompetitive inhibition

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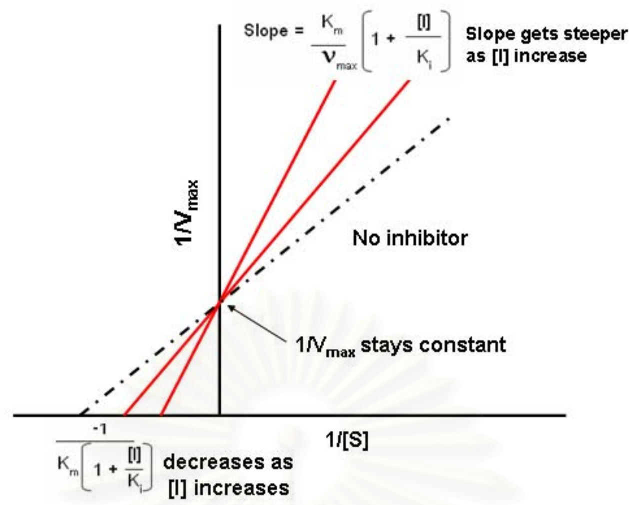
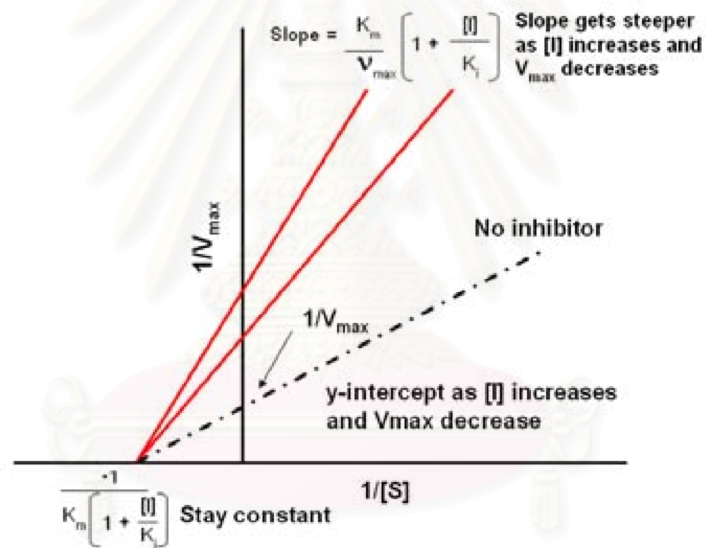
(A)



(B)



(C)

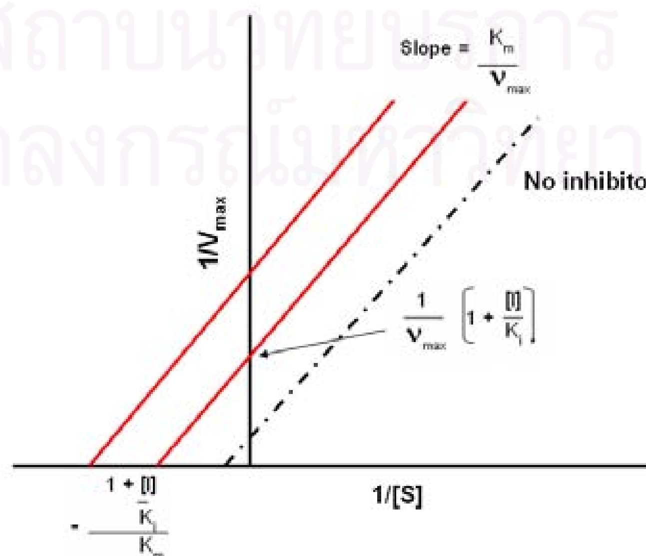


Figure 12 Lineweaver-Burk plot represent the type of enzyme inhibition. **(A)** Competitive inhibition **(B)** Non-competitive inhibition and **(C)** Uncompetitive inhibition

Table 1 Summary of kinetic parameter

Enzyme inhibition	Competitive	Non-competitive	Uncompetitive
I bind to	E only	E or ES	ES only
K_m	Increases	constant	Decreases
υ_{max}	Constant	decreases	decreases

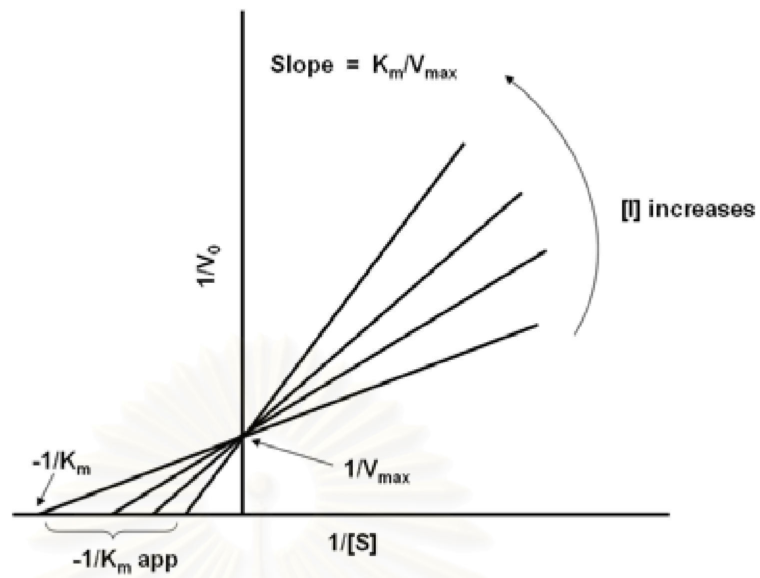
2.14 Secondary plot

Lineweaver-Burk plot represent the results of series of kinetic assays which carried out at a variety of different inhibitor concentrations. In the primary plot, each inhibitor concentration gives a different value for the maximal velocity and so called apparent maximal velocities that read from the graph as their reciprocals (1/υapp). In the secondary plot these are plotted against the inhibitor concentration. The intercept on the inhibitor axis gives us the value for $-K_i'$ and apparent maximal velocities υ app.

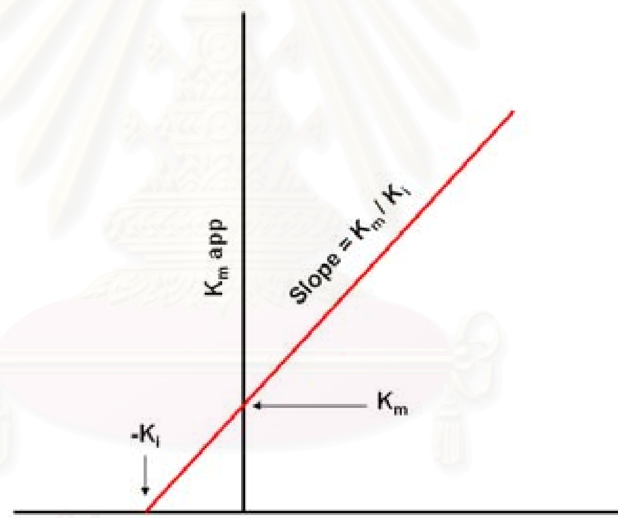
- **Competitive inhibitor**

  The secondary plots represent no change in maximal velocity. They give K'_i which is irrelevant for a competitive inhibitor.

(A)



(B)



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(C)

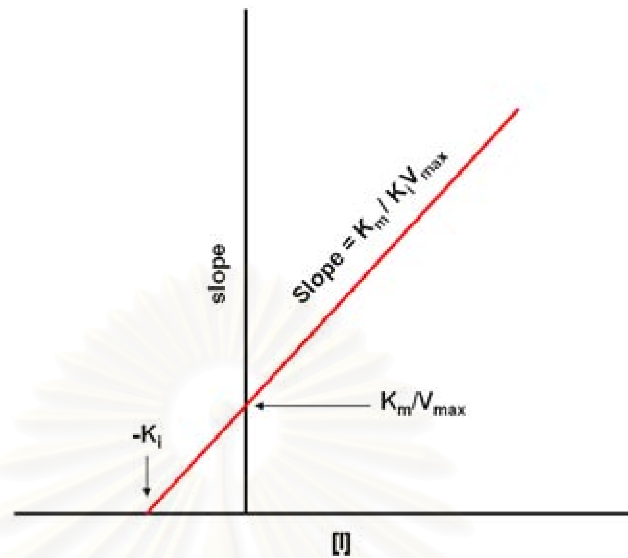

Figure 13 Competitive inhibition, Lineweaver-Burk plots **(A)** and secondary plots **(B)**; interception vs [I], **(C)** slope vs [I]. Lineweaver-Burk plots representing reciprocals of initial enzyme velocity vs. reciprocal of substrate concentration in the absence and the presence of different concentrations of inhibitor

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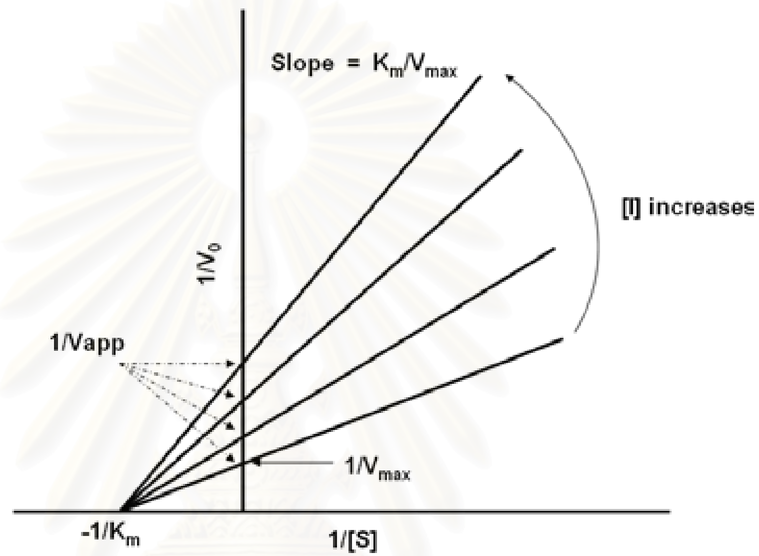
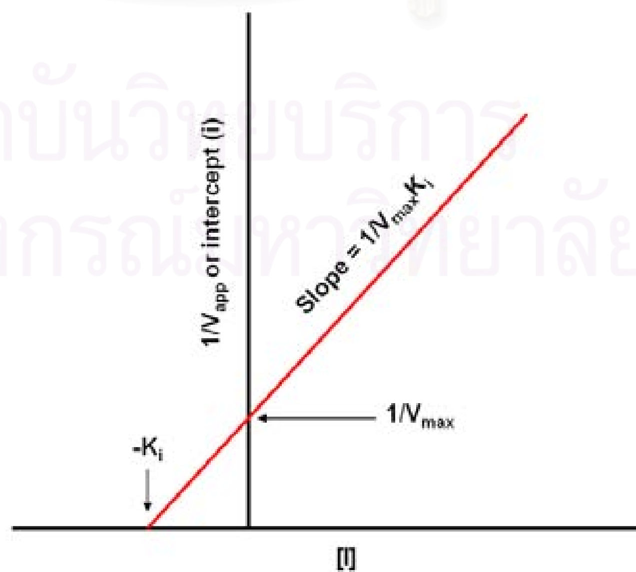
- Noncompetitive inhibitor

    The secondary plots give identical result as K_i and K'_i are equal for these inhibitors

(A)



(B)



(C)

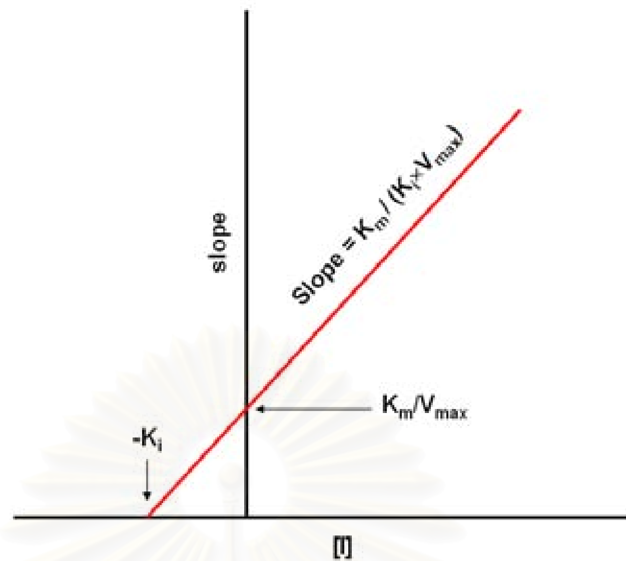
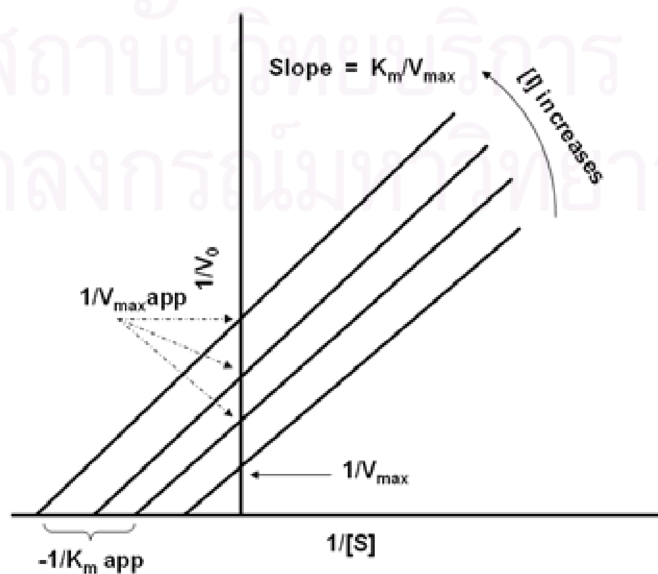


Figure 14 Non-competitive inhibition, Lineweaver-Burk plots **(A)** and secondary plots **(B)**; interception vs [I], **(C)** slope vs [I]. Lineweaver-Burk plots representing reciprocals of initial enzyme velocity vs. reciprocal of substrate concentration in the absence and the presence of different concentrations of inhibitor

- Uncompetitive inhibitor

  The secondary plots represent no change in slope. General equation for kinetic analysis in the study:

(A)



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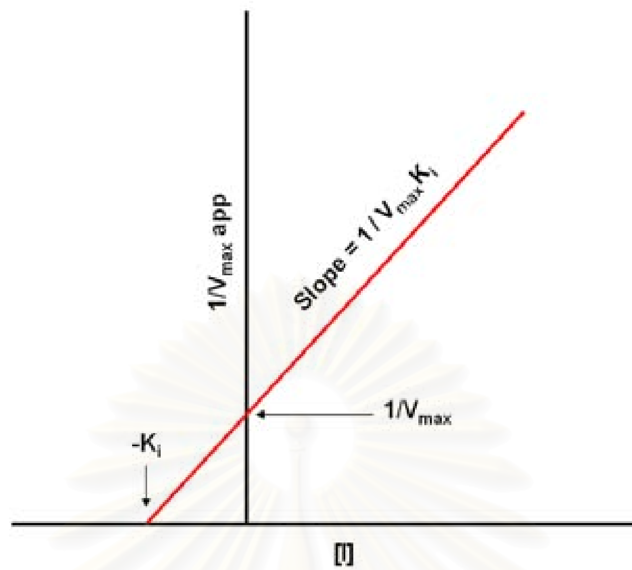

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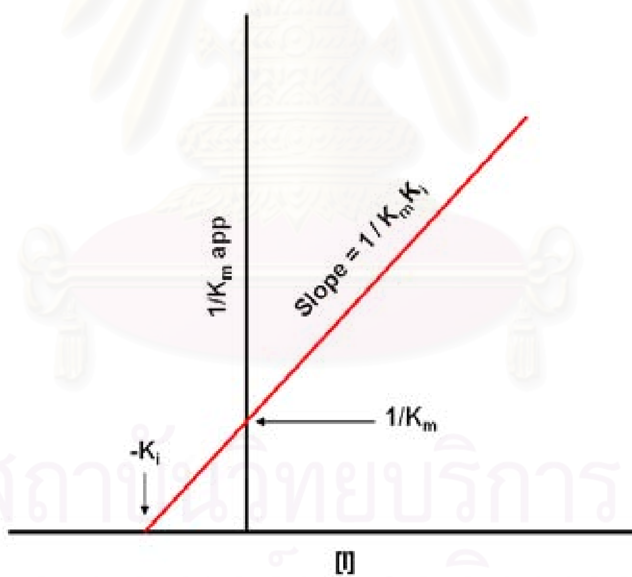

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Figure 15 Uncompetitive inhibition, Lineweaver-Burk plots **(A)** and secondary plots **(B)**; interception vs [I], **(C)** slope vs [I]. Lineweaver-Burk plots representing reciprocals of initial enzyme velocity vs. reciprocal of substrate concentration in the absence and the presence of different concentrations of inhibitor

2.15 Determination of substrate affinity

IC_{50} or the half maximal inhibitory concentration is a measure of concentration used in pharmacological research and it represents the inhibitory effect of compound on competition binding assay and functional antagonist assay. In simpler terms, it measures how much of a particular substance/molecule is needed to inhibit some biological process by 50%. This study calculated the concentration of an inhibitor that is required for 50% inhibition (IC_{50}) by using Sigma plot.

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CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Animals

Adult male Wistar rats of body weight between 230-260 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. Rats were housed in animal room of Faculty of Veterinary science, Chulalongkorn University, Bangkok and acclimatized for at least seven days prior to the experiment. They were maintained at 25°C on a 12-hour light/dark cycle and had free access to animal standard diet (CP 082 mice fed) and water throughout the study. Normal standard diet was purchased from C.P. Company.

The protocol of experimental animals was approved by Animal Ethic Committee of the Faculty of Veterinary Science Chulalongkorn University (Appendix A)

3.1.2 Instruments

The following instruments were used in the study:

Autopipettes 20, 100, 200, 1000 μL and 1 mL (Gilson, France)

Centrifuge (Heraeus, Biofuge 22R, Germany)

Feeding tube, Magnetic Stirrer (MS 101)

Microplate reader (Tecan A-5082, Austria)

Incubator (WTB binder)

pH meter (Hanna instruments, Microprocessor pH Meter model pH211),

Spectrophotometer (UV 160A Shimadzu, Japan)

Magnetic Stirrer (MS 101)

Vortex mixer (Germy industrial, Taiwan)

Water bath shaker (Grant, England)

Weighting machine (Sartorius, Germany)

Microtube

96 well microplate

Syringe

Timer

3.1.3 Chemical reagents

Tetracycline HCl (Sigma, U.S.A)

Doxycycline HCl (Sigma, U.S.A)

Orlistat (Sigma, U.S.A)

p-Nitrophenylpalmitate (Sigma, U.S.A)

Porcine pancreatic lipase (Sigma, U.S.A)

Cholic acid (Sigma, U.S.A)

Tris (hydroxymethylaminomethane) (Merck, Germany)

Chlortetracycline HCl and Oxytetracycline HCl (General drug house, Thailand)

Acetronitrile (Fisher scientific, UK)

HCl (Merck, Germany)

DMSO (Merck, Germany)

Olive oil (Bertolli classic, Unliver Best Food Italia SRL, Italy)

Triglyceride test kit (Human Gesellschaff, Germany)

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3.2 METHODS

3.2.1 Inhibitory effect of tetracyclines on porcine pancreatic lipase.

The inhibitory activity of tetracyclines against pancreatic lipase was measured according to the method of Slac (2004).

3.2.1.1 Reagents

1) Substrate

3.33 mM *p*-Nitrophenylpalmitate (PNP) was dissolved in acetronitrile to give a stock solution with a concentration of 10 mM. Ethanol was then added to final composition 1:2 (v/v) Acetronitrile: ethanol and stored at -20 °C

2) 0.061 M Tris-HCl buffer, pH 8.5

One liter of 0.061 M Tris-HCl buffer, pH 8.5 consisted of 0.122 mol/L of Tris (hydroxymethylaminomethane) 250 ml and 0.061 mol/L of HCl 130 ml. The solution was adjusted to pH 8.5 with 0.1 M HCl.

3) 5 mg/ml of enzyme

5 mg porcine pancreatic lipase (type II, crude) was dissolved in 1 ml ice-cold distilled water.

4) 10 mM tetracyclines

Tetracyclines (Tetracycline, Doxycycline, Oxytetracycline and Chlortetracycline) were dissolved in distilled water to give a stock solution with a concentration at 10 – 1,000 μM.

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3.1.3.1 Procedure

The assay mixture (200 μl) contained 10 μl of various concentration of tetracyclines, 168 μl of 3.33 mM PNP, in 0.061 M of Tris-HCl buffer, pH 8.5. Then, the reaction was initiated by adding 10 μl of porcine pancreatic lipase to the mixture. The reaction mixture was incubated for 25 min at 37°C. After the incubation, the absorbance was measured by using a microplate reader at 405 nm. For the blank test, the enzyme was replaced by the distilled water. Orlistat was used as the positive control in this study.

3.2.1.3 Calculations

1) The absorbance of each samples were compared with control for the calculation of the percentage inhibition.

Calculation of the percentage inhibition was followed as the below equation.

$$\%\ \text{Inhibitory activity} = \frac{[(A_c - A_b) - (A_t - A_{bl})]}{(A_c - A_b)} \times 100$$

A_c = Absorbance of control

A_t = Absorbance of test samples

A_b = Absorbance of control blank

A_{bl} = Absorbance of samples blank

Table 2 Summary of analytical technique for measurement of pancreatic lipase activity

Absorbance	Enzyme	Substrate (PNP)	Tetracyclines	Tris-HCl Buffer	Distilled water
Control	12 µl	10 µl	-	168 µl	10 µl
Control blank	-	10 µl	-	180 µl	10 µl
Sample	12 µl	10 µl	10 µl	168 µl	-
Sample Blank	-	10 µl	10 µl	168 µl	12 µl

The IC_{50} values were determined by plots of concentration versus percent inhibition curves using Sigma Plot 10.0. The IC_{50} values were expressed as mean ± S.E.M; n = 3

3.2.2 The enzyme kinetic study.

The type of inhibition was determined by the Lineweaver-Burk plot. The enzyme was incubated with the various concentrations of *p*-nitrophenylpalmitate (0.83 – 6.67 mM) with or without various concentrations of inhibitor (10 – 100 µM).

In order to measure the apparent Michaelis constant (K_m), and the inhibition constant (K_i), the enzyme kinetic analysis was performed according to the above reaction.

The kinetic analysis in the study was followed as this general equation.

$$\nu_0 = \frac{\nu_{max}[S]}{K_m(1 + \frac{[I]}{K_i}) + [S](1 + \frac{[I]}{K'_i})} \qquad (1)$$

For calculation of K_i and K_i', slope and interception from Lineweaver-Burk were replotted vs. [Inhibitor] which gave the secondary plots.

The secondary plots of Lineweaver-Burk linearization are expressed as follow:

$$\text{Slope} = \frac{K_m}{v_{max}} + \frac{K_m}{v_{max}\,K_i} \quad (2)$$

$$\text{Interception} = \frac{1}{v_{max}} + \frac{1}{v_{max}\,K_i} \quad (3)$$

The symbols of the equations are as follows: v_0 represents the initial velocity. [S] and [I] are the concentrations of substrate and inhibitor, K_m represents the Michaelis-Menten constant, K_i and K_i' are the dissociation constants of the inhibitor containing complexes EI and ESI, respectively.

3.2.2.1 Data Analysis

The type of inhibition and kinetic parameter were calculated on the base of Lineweaver-Burk and its replot of slopes and vertical axis intercepts versus inhibitor concentrations as the non-linear regression using the Sigma Plot 10.0 software (IL, USA). Calculation of K_i and K_i', slope and interception from Lineweaver-Burk were replotted vs. [Inhibitor] from the secondary plots.

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3.2.3 Synergistic inhibitory effect of tetracyclines and orlistat

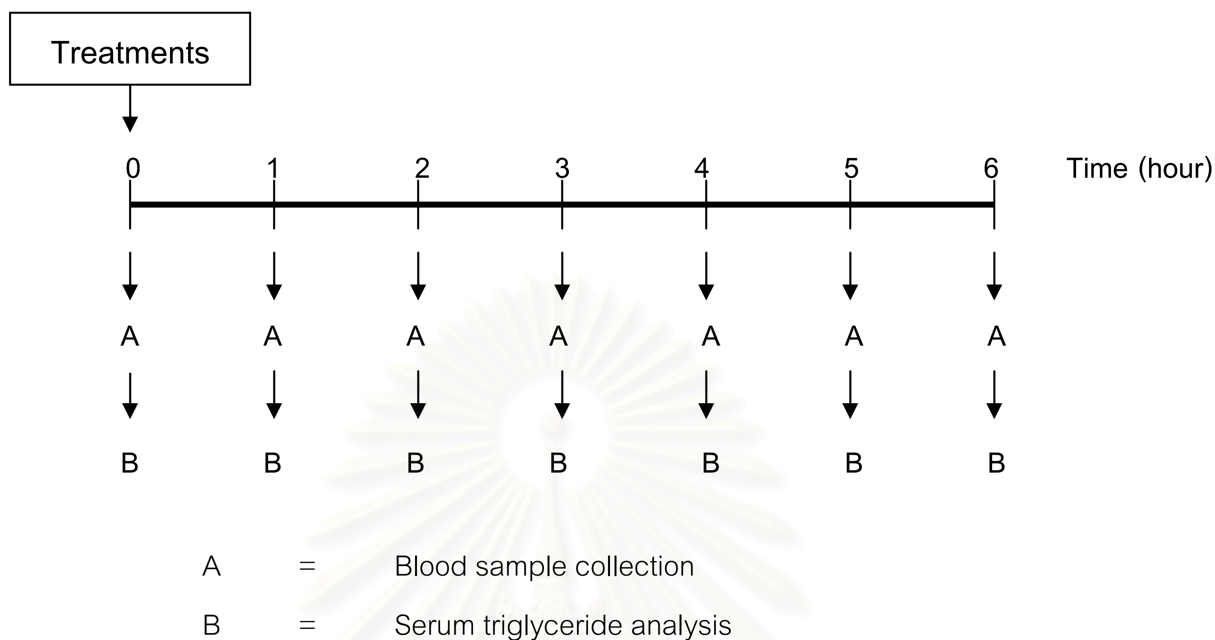
In this assay, various concentrations of orlistat (0.39-12.5 μM) were combined with or without the lowest concentration of inhibitor (1 μM doxycycline) which had no inhibitory activity on pancreatic lipase.

The reaction was performed according to the above assay. The absorbance of released *p*-nitrophenol was measured at 405 nm using a microplate reader and calculated percentage inhibition. The IC_{50} values were expressed as mean ± SE; n = 3

3.2.4 Acute effect of doxycycline on serum lipid levels in normal rats by oral administration of olive oil emulsion

The effect of doxycycline on the fat absorption was performed by the oral olive oil emulsion-loading method (Ono et al., 2006) with slightly modification. Rats were randomly divided into 6 groups which comprised 6 animals each to match average body weight. Briefly, normal rats were fasted for 12 h. Hyperlipidemia model was induced by oral administration of lipid emulsion (5 ml/kg) containing 7 ml of olive oil, 93 mg of cholic acid and 3.5 ml of distilled water. Group I received olive oil emulsion and distilled water. Group 2-5 received various single doses of doxycycline (5, 20, 40 and 100 mg/kg, po).

Group I	Control (distilled water 1 ml/kg body weight.)
Group II	Doxycycline 5 mg/ml/kg body weight.
Group III	Doxycycline 20 mg/ml/kg body weight.
Group IV	Doxycycline 40 mg/ml/kg body weight.
Group V	Doxycycline 100 mg/ml/kg body weight.
Group VI	Orlistat 5 mg/ml/kg body weight.



Blood samples were collected from tail vein before and after 1, 2, 3, 4, 5 and 6 h of lipid emulsion loading. Serum was separated by centrifugation at 4,000 rpm. Serum triglyceride concentration was measured by using triglyceride assay kits. The absorbance was measured at 500 nm using spectrophotometer (UV 160A Shimadzu). Increment in serum triglyceride levels after oral lipid emulsion-loading and the area under the curve ($AUC_{0\text{-}6h}$) were calculated.

3.3 Statistical analysis

Data are expressed as mean ± S.E.M. Area under the curve (AUC) values are reported as total areas and are using a modification of the trapzodial rule. In animal models, statistical analysis was performed by one-way analysis of variance (ANOVA), with post-hoc range tests. The Least Significant Difference (LSD) test was used for mean comparisons; $p<0.05$ was considered to be statistically significant

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CHAPTER IV

RESULTS

4.1 Inhibitory effect of tetracyclines on porcine pancreatic lipase

The effect of tetracycline and its derivatives on pancreatic lipase inhibition was shown in Figure 16 Doxycycline and chorletracycline markedly inhibited pancreatic lipase in a concentration-dependent manner. Doxycycline showed the most effective on pancreatic lipase inhibition (IC_{50} = 55.42 ± 1.67 μM) among those of tetracycline derivatives. However, it was less potent activity than that of orlistat (IC_{50} = 1.31 ± 0.13 μM) which was used as a pancreatic lipase inhibitor (Figure 17). Chlortetracycline gave a moderate inhibition, whereas tetracycline gave a weak inhibition. In contrast, oxytetracycline had no inhibitory activity (Table 3).

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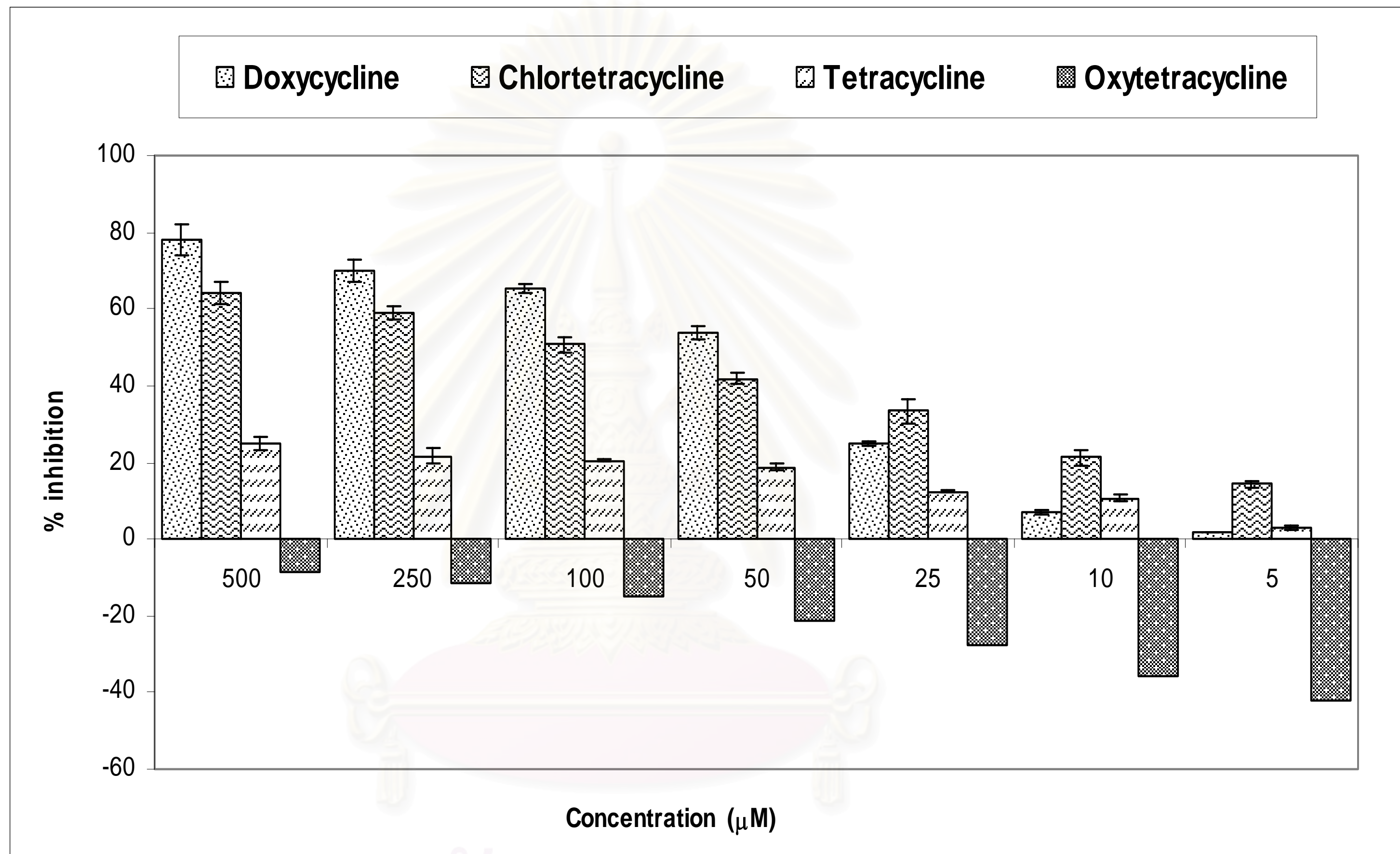

Figure 16 Dose-dependent effect of tetracycline and its derivatives on pancreatic lipase inhibition. Results were expressed as mean ± S.E.M., n = 3.

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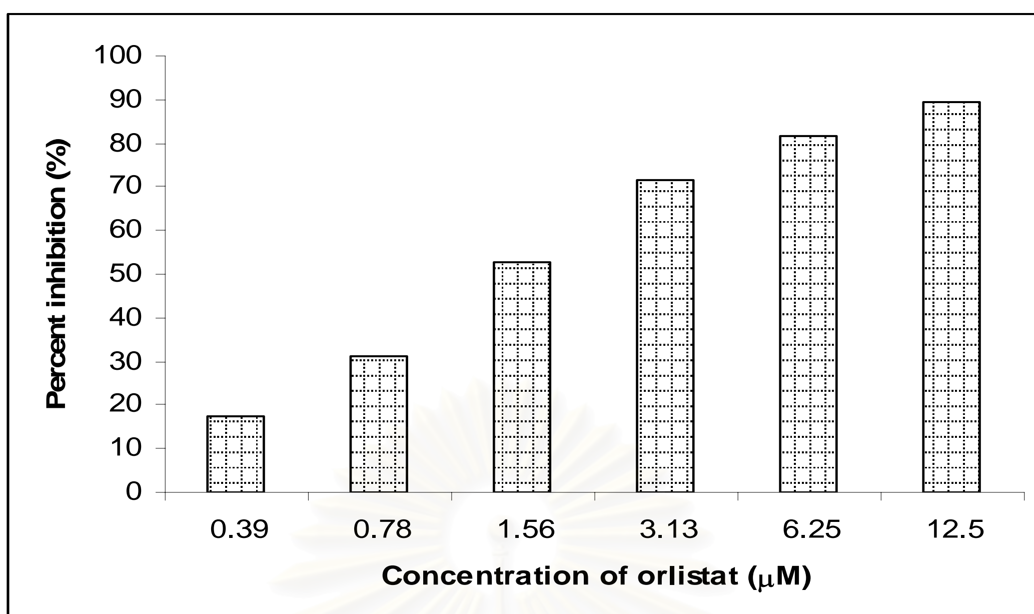

Figure 17 Dose-dependent effect of orlistat on pancreatic lipase inhibition. Results were expressed as mean ± S.E.M., n = 3.

Table 3 The IC_{50} values of tetracycline, doxycycline, chlortetracycline, oxytetracycline and orlistat.

	Doxycycline	Chlortetracycline	Tetracycline	Oxytetracycline	Orlistat
IC_{50} (μM)	55.42 ± 1.67	88.71 ± 11.39	$>$ 500	N.I.	1.31 ± 0.13

Results were expressed as mean ± S.E.M., n=3   N.I. = no inhibition

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4.2 The enzyme kinetic study

From the results above, doxycycline was the most effective compound against pancreatic lipase. Hence, doxycycline was selected for investigation the enzyme kinetic study including its type and constants of inhibition. Possible interference by doxycycline at each substrate-binding site was examined by holding the concentration of doxycycline at a constant value, and measuring the effect of increasing one substrate concentration on the initial reaction rate.

Lineweaver-Burk plots for pancreatic lipase inhibited by doxycycline and replots of slope (s) and vertical axis intercept (i) versus the various concentrations of inhibitor were shown in Fig 18. Lineweaver–Burk plot of doxycycline generated 4 straight lines (Fig 18A), clearly indicated that the inhibition was a non-competitive type with inhibition.

In the secondary plot, the slope and y-intercept obtained from Lineweaver-Burk processing of data was plotted versus the various concentration of doxycycline, a straight line was produced and the values for K_i and K'_i dissociation constant were calculated from those linear lines, respectively. The results demonstrated that an increase concentration of doxycycline resulted in the increasing of slope and the vertical axis intercept (Figure 18A and 18B). The dissociation constants for inhibitor binding, the K_i and K'_I values are 66.41 ± 3.27 μM and 70.44 ± 7.11 μM, respectively.

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Table 4 Kinetic parameter of pancreatic lipase-inhibited by doxycycline

Inhibitor	Dissociation constant	
	K_i (µM)	$K_i^{'}$ (µM)
Doxycycline	66.41 ± 3.27	70.49 ± 7.11

Results were expressed as mean ± S.E.M., n = 3.

Table 5 The linear parameters from the Lineweaver-Burk plot of doxycycline

Inhibition concentration (µM)	Slope (s)	Interception (i)	r^2
0	28.21	0.38	0.9930
10	37.07	0.50	0.9967
50	58.09	0.81	0.9860
100	76.92	1.03	0.9925

Results were expressed as mean ± S.E.M., n = 3.

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(A)

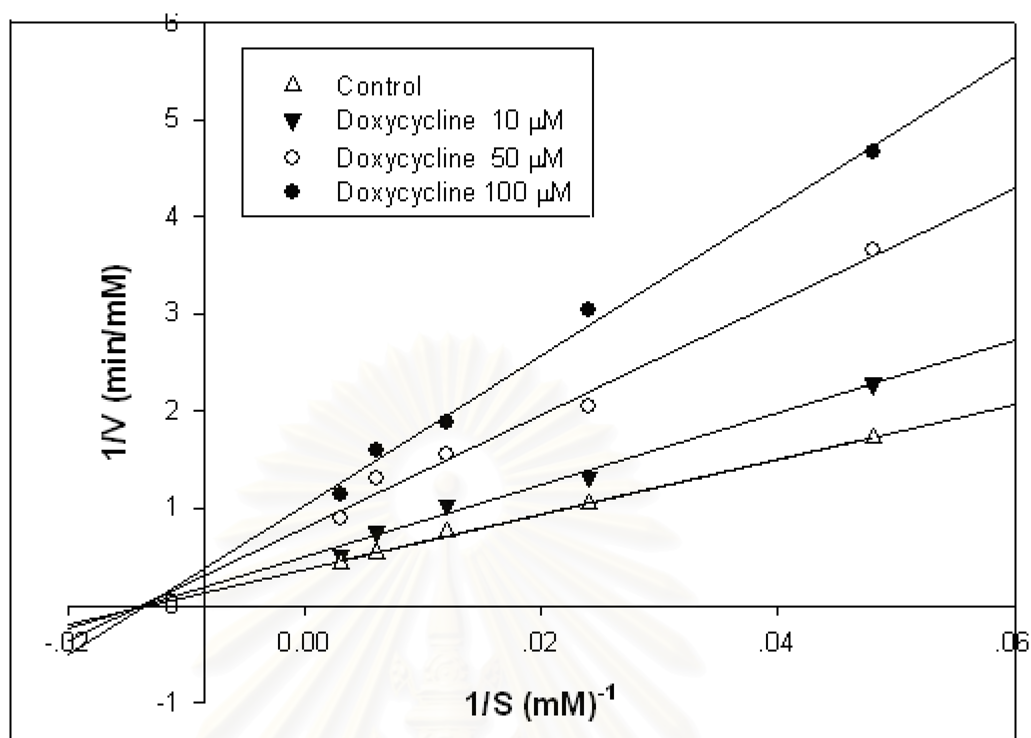

Figure 18 Lineweaver-burk plots **(A)** and secondary plots **(B)**; slope vs. [I]. **(C)** Interception vs [I]. Lineweaver-Burk plots representing reciprocals of initial enzyme (pancreatic lipase) velocity vs. reciprocal of PNP concentration in the absence and the presence of different concentrations of doxycycline. (■) 0 μM; (△) 10 μM; (▼) 25 μM; (○) 50 μM; (●) 100 μM.

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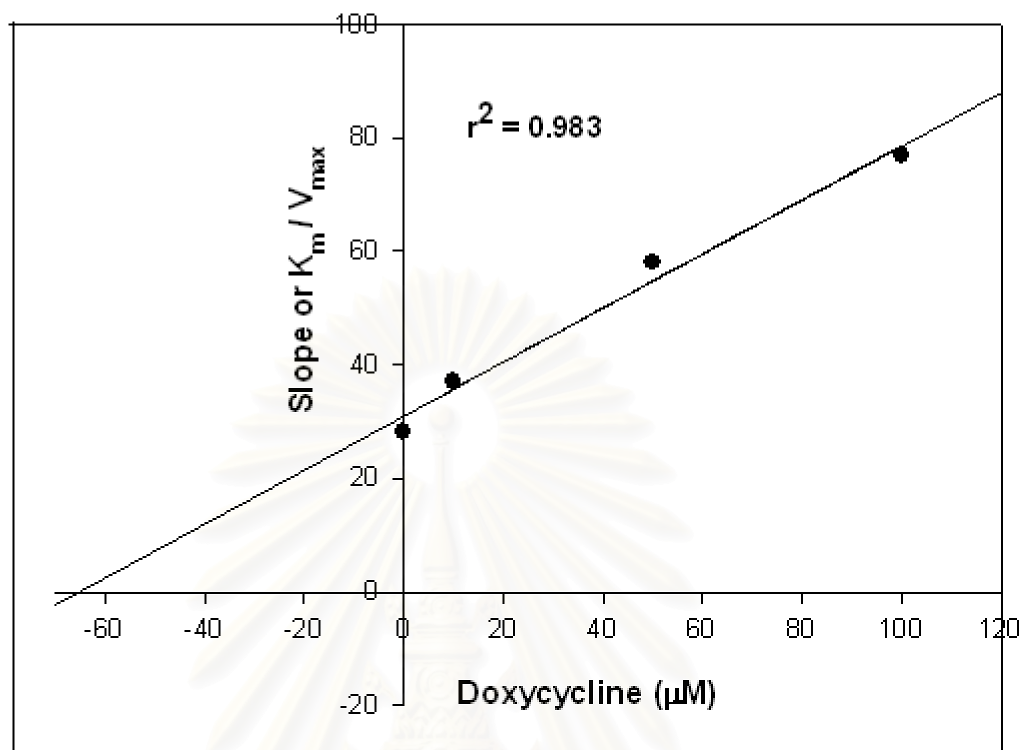
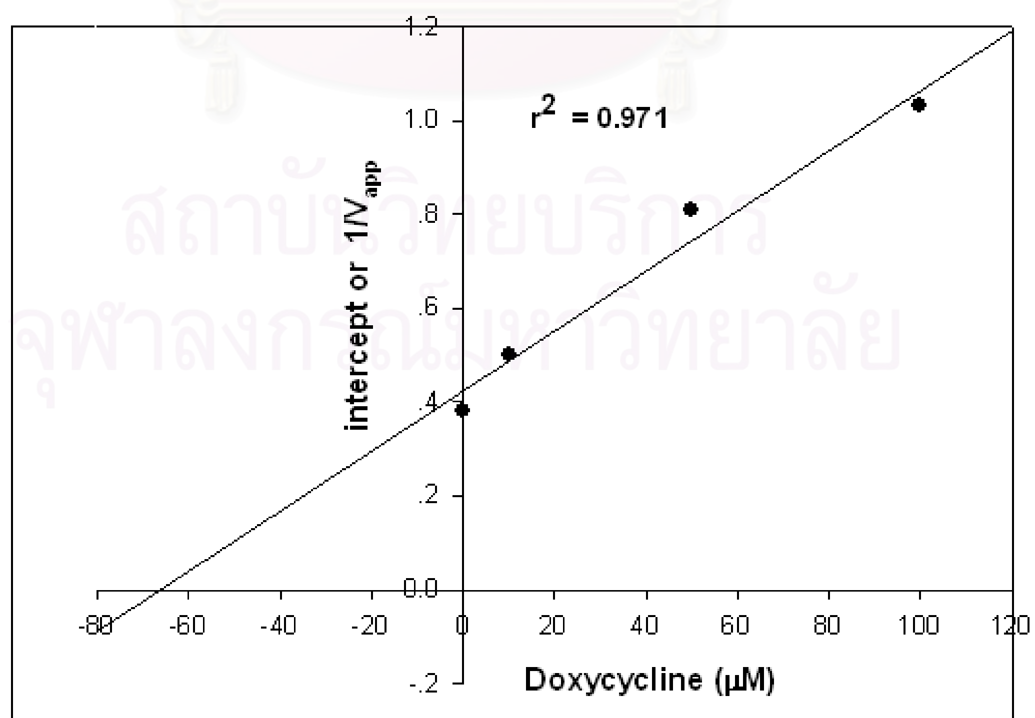
(B)



(C)



4.3 Synergistic inhibitory effect of tetracyclines and orlistat

The combined effect of doxycycline and orlistat on pancreatic lipase inhibition was shown in Table 6. The results showed that addition of 1 μM doxycycline to assay system containing various concentrations of orlistat, the percentage inhibition was significantly increased when compared with orlistat alone, indicating that doxycycline and orlistats interacts synergistically on pancreatic lipase. The IC_{50} values of combination of orlistat and doxycycline was 0.37 ± 0.01 μM which was lower than that of orlistat alone (IC_{50} = 1.31 ± 0.13 μM)

Table 6 The percentage enzyme inhibition of orlistat and combination of orlistat and doxycycline on pancreatic lipase

Concentration of orlistat (μM)	% Enzyme inhibition		
	Orlistat alone	1 μM Doxycycline	Orlistat + 1 μM doxycycline
0.39	17.27 ± 1.05		50.60 ± 0.54*
0.78	31.34 ± 0.66		66.25 ± 0.97*
1.56	52.81 ± 0.47	N.I.	74.00 ± 0.48
3.13	71.72 ± 1.52		85.67 ± 0.34
6.25	81.63 ± 1.23		87.77 ± 1.09
12.50	89.50 ± 0.59		93.77 ± 0.57

Result were expressed as mean ± S.E.M., n = 3., N.I. = no inhibition

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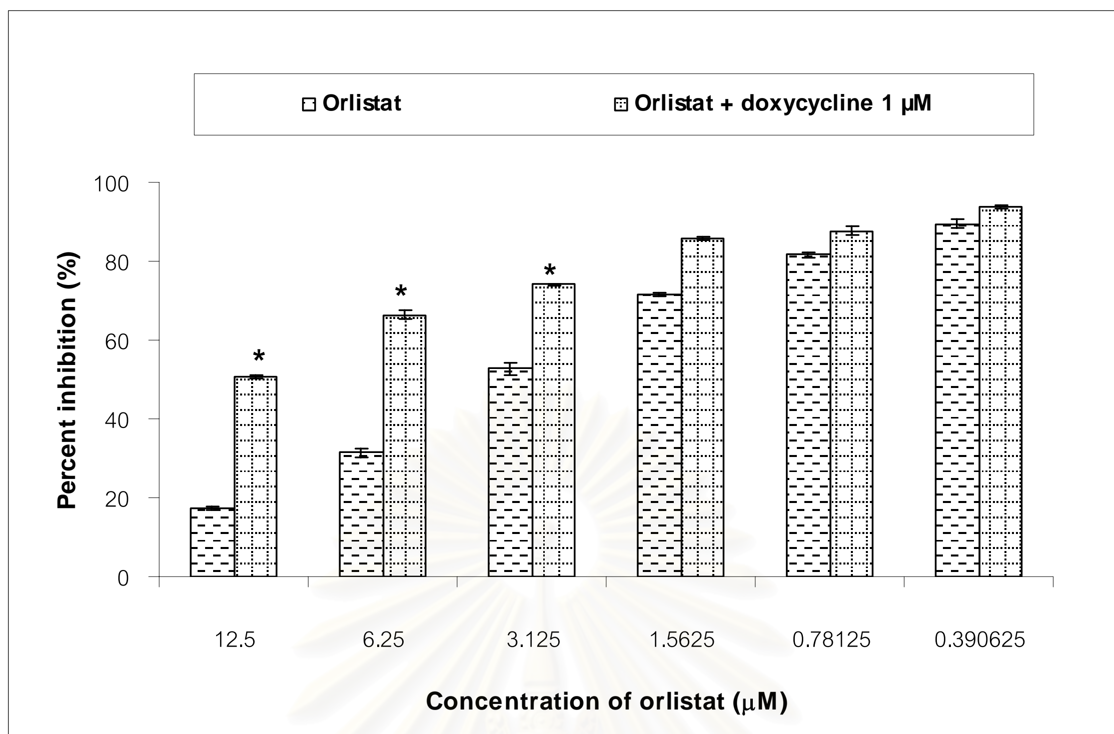

Figure 19 The percentage enzyme inhibition of orlistat and combination of orlistat and doxycycline (1 μM) on pancreatic lipase. Results were expressed as mean ± S.E.M., n = 3. *P < 0.05 compared to control group.

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4.4 Acute effect of doxycycline on serum triglyceride concentration in normal rats by oral olive oil emulsion loading test.

The serum triglyceride concentrations in normal rats are shown in Table 7 and Figure 20A. The normal rats treated with orally 20, 40, and 100 mg/kg doxycycline resulted in the significant lowering of fasting serum triglyceride concentration at 1 h after lipid administration and maintain up to 6h until the end of experiments. The maximal peak of serum triglyceride concentration in control group was 206.36 ± 9.20 mg/dL after 3h administration, whereas the group treated with doxycycline 5, 20, 40 and 100 mg/kg was 225.58 ± 5.53 mg/dL, 197.76 ± 3.02 mg/dL, 182.54 ± 5.59 mg/dL, 164.15 ± 5.20 mg/dL, respectively. The rats treated with orlistat significantly suppressed the elevation of serum triglyceride.

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(A)

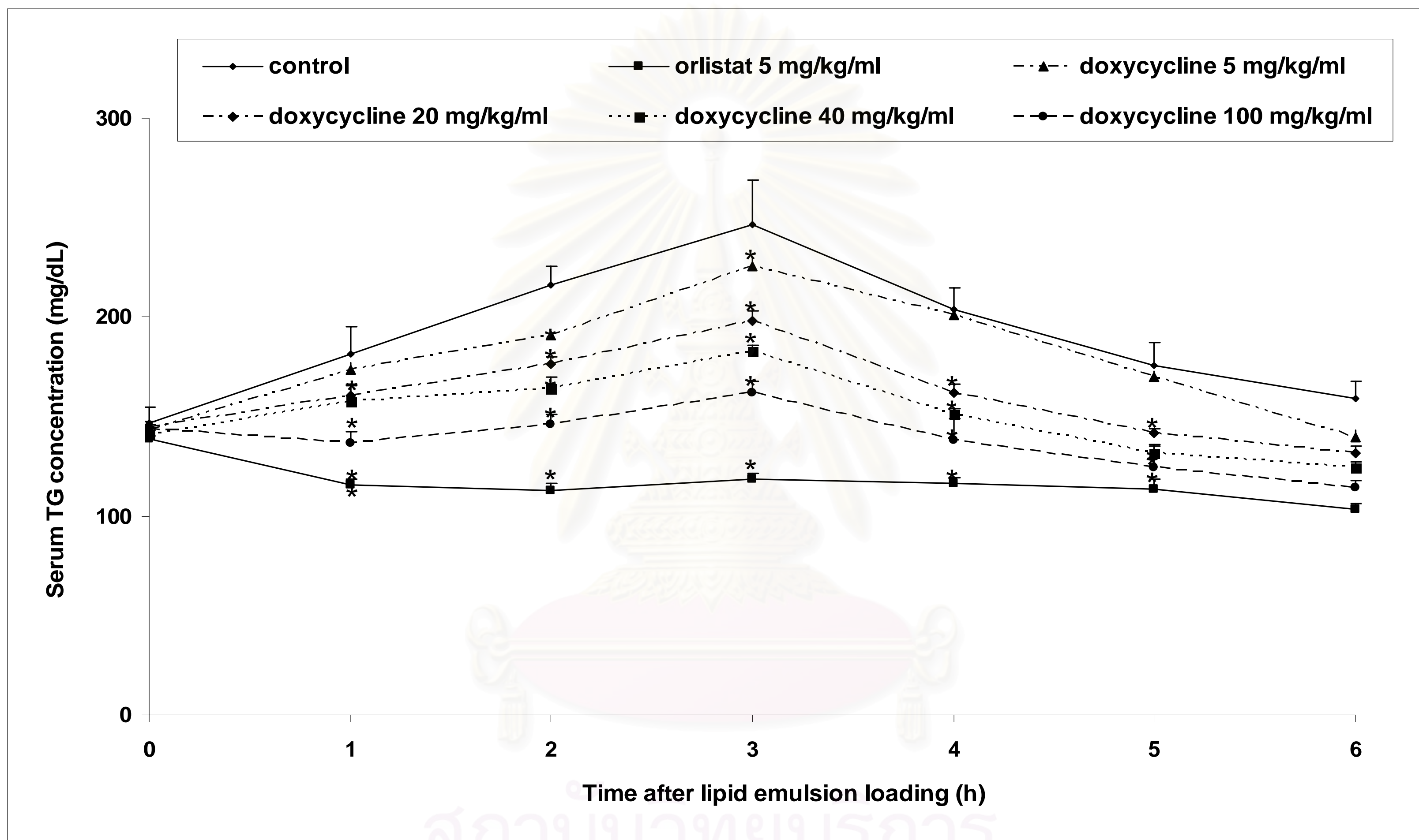

Figure 20A Effect of doxycycline on serum triglyceride concentrations in normal rats after olive oil emulsion loading. Results are expressed as mean ± S.E.M. (n=6). * P < 0.05 compared to control group.

Table 7 The serum triglyceride concentrations in normal rats after loading lipid emulsion

Time (h)	Triglyceride serum level (mg/dL)					
	Control group	Treatment group				Orlistat
		Doxycycline 5 mg/kg	Doxycycline 20 mg/kg	Doxycycline 40 mg/kg	Doxycycline 100 mg/kg	
0	146.43 ± 3.27	142.86 ± 2.42	144.91 ± 2.27	140.99 ± 2.50	143.88 ± 3.79	138.94 ± 1.30
1	181.37 ± 5.59	173.30 ± 6.15	160.22 ± 8.65*	157.33 ± 5.28*	136.60 ± 3.80*	115.31 ± 3.33*
2	216.46 ± 3.73	190.66 ± 4.66*	176.10 ± 6.05*	163.77 ± 4.19*	146.13 ± 2.52*	112.98 ± 3.51*
3	246.36 ± 9.19	225.58 ± 5.53*	197.76 ± 3.02*	182.54 ± 5.59*	162.00 ± 5.20*	118.21 ± 3.37*
4	203.85 ± 4.31	200.65 ± 11.34	161.81 ± 2.87*	150.98 ± 4.26*	137.82 ± 2.41*	116.53 ± 2.75*
5	175.35 ± 4.75	169.65 ± 11.08	141.55 ± 3.73*	131.75 ± 2.39*	124.65 ± 3.39*	113.73 ± 4.67*
6	158.75 ± 3.72	139.22 ± 3.71	131.75 ± 3.07*	124.28 ± 3.61*	114.01 ± 3.74*	103.36 ± 3.13*

Result were expressed as mean ± S.E.M., n= 6. * $P < 0.05$ compared to control group.

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The AUCs of groups treated with doxycycline (20-100 mg/kg) was significantly lower than that of control group (p < 0.05), (AUC for control group = 1,175.98 ± 1.25 mg/dL.h; doxycycline 20 mg/kg = 975.77 ± 7.31 mg/dL.h; doxycycline 40 mg/kg = 921.99 ± 3.71 mg/dL.h; doxycycline 100 mg/kg = 836.13 ± 1.10 mg/dL.h), while the AUCs of group treated with 5 mg/kg orlistat was 697.89 ± 0.05 mg/dL.h. However, there was no significant difference of AUCs between rats treated with 5 mg/kg doxycycline (AUC = 1,101.38 ± 0.98 mg/dL.h) and control group.

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(B)

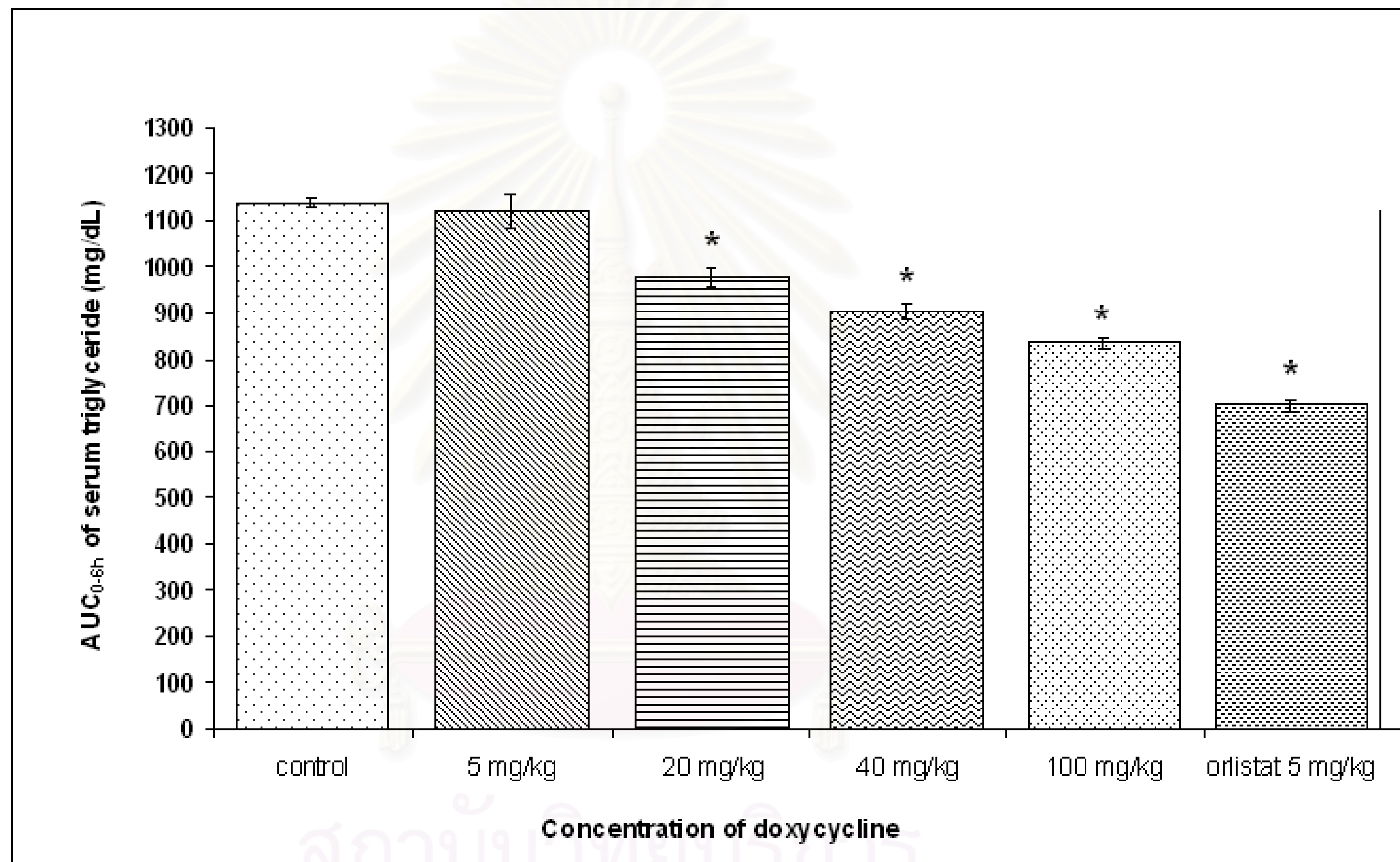

Figure 20B Total areas under triglyceride concentration response curves ($AUC_{0\text{-}6h}$) were calculated for each group of rats. Result were expressed as mean ± S.E.M., n = 6. * $P < 0.05$ compared to control group.

CHAPTER V

DISCUSSION

In recent years, many studies have reported the non-antimicrobial effect of tetracycline and its derivatives, but the inhibitory effect of tetracycline and its derivative on pancreatic lipase activity have been not studied yet. The present study to investigate the inhibitory effect of tetracycline and it derivatives and type of their inhibitions on pancreatic lipase. The study also examined the synergistic inhibition of doxycycline and orlistat. In addition, the study was evaluated the antihyperlipidemic effect of doxycycline in normal rats by lipid emulsion loading test.

Tetracycline was discovered in the late 1940s and composes of four hydrocarbon rings derivation in the chemical structure (Figure 21). From screening test, it gave a weak inhibition (IC_{50} > 500 μM) whereas the introduction of chlorine atom at R_4 position in tetracycline structure was chlortetracycline which dramatically increased the percentage inhibition (IC_{50} = 88.71 ± 11.39 μM). In the meantime, the presence of hydroxy group at R_1 in tetracycline structure was doxycycline which exhibited the most effective pancreatic lipase inhibitor (IC_{50} = 55.42 ± 1.67 μM). It concluded that the order of the inhibitory potencies was doxycycline > chlortetracycline > tetracycline > oxytetracycline. These findings suggested that tetracycline structure containing hydroxy group at R_1 position plays a key role to exhibit the most potent inhibitor against pancreatic lipase. However, adding hydroxy group at R_3 position in doxycycline, was oxytetracycline which was found to be inactive, suggesting that an increase in bulkiness at R_3 position causes to decrease the inhibitory activity.

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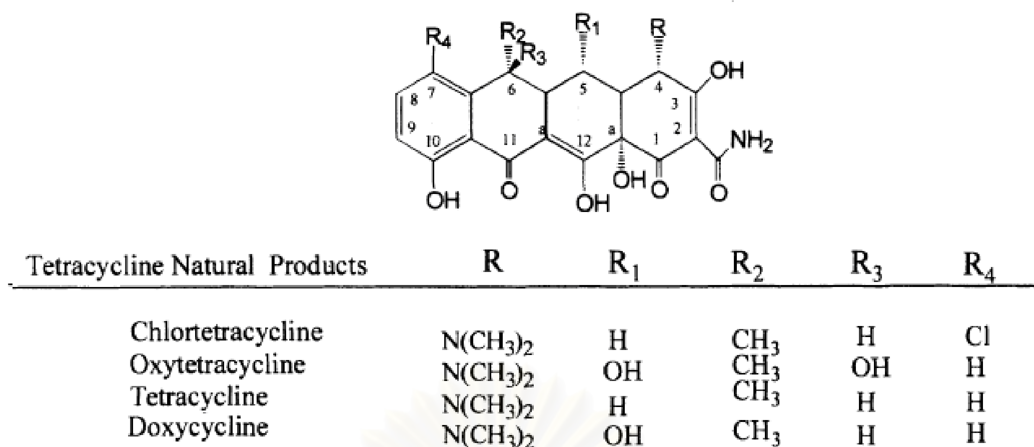

Tetracycline Natural Products	R	R_1	R_2	R_3	R_4
Chlortetracycline	$N(CH_3)_2$	H	CH_3	H	Cl
Oxytetracycline	$N(CH_3)_2$	OH	CH_3	OH	H
Tetracycline	$N(CH_3)_2$	H	CH_3	H	H
Doxycycline	$N(CH_3)_2$	OH	CH_3	H	H

Figure 21 The chemical structure of tetracycline and derivatives

To determine the type of inhibition, doxycycline was used as an inhibitor in a study which was elucidated its constants of inhibition. As findings mentioned, it clearly indicated that the doxycycline was a non-competitive inhibition. From the secondary plot, increasing the inhibitor concentrations resulted in the increasing of slope and the vertical axis intercept. The dissociation constant for doxycycline binding with free enzyme (E), K_i of 66.41 ± 3.27 μM, was obtained from the secondary plot of the slopes of these lines versus doxycycline concentrations, while the dissociation constant for doxycycline binding with enzyme-substrate complex (ES), K_i' of 70.49 ± 7.11 μM. The possible binding mode of doxycycline was assumed that it can bind either to active site of free enzyme or to the enzyme–substrate complex. When K_i and K_i' dissociation constants were compared, it was found that the K_i value of doxycycline was almost equal to K_i' value, suggesting that binding of doxycycline to the ES form equally binds to doxycycline to the enzyme alone. However, the binding site of doxycycline on pancreatic lipase remains unknown. Additional studies are needed to characterize the specific binding region of doxycycline on this enzyme.

Orlistat, which belongs to a class of anti-obesity agents, has since been developed for long-term management of obesity (Mancino et al., 2006). It exerts the therapeutic activity in the lumen of the gastrointestinal tract by strongly inhibiting pancreatic lipase, preventing the hydrolysis of dietary fat, thus reducing subsequent intestinal absorption of lipolysis products (Henness et al., 2006). As undigested fat are not

absorbed, the resulting caloric deficit may have a positive effect on weight control. The results of several clinical studies indicated that orlistat reduces fat absorption by 30% during a meal (Zhi et al., 1999). The most common adverse effect of orlistat is related to the blockade of triglyceride digestion in small intestine. Fecal fat loss and related gastrointestinal symptoms are common initially (Sidhaye et al., 2006). Moreover, absorption of fat-soluble vitamins and other fat-soluble nutrients are inhibited by long-term administration of orlistat (Chaput et al., 2007).

In general, pancreatic lipase plays a key role in lipid digestion catalyzing triglycerides to progressively release diglycerides, monoglycerides, and free fatty acid. It is well known as serine type esterase with Ser "the active site" and Ser^{152} "the substrate-binding site" (Chahinian et al., 2000). It has been reported that orlistat, a hydrogenated derivative of lipsatatin, binds to the substrate-binding site by covalently from an ester between β-lactone ring of orlistat and the side chain hydroxyl group of serine (Hadvary et al., 1991).

The explanation for interaction phenomena between tetracyclines and the pancreatic lipase is still unknown. It can be assumed that they from hydrogen bonds with the polar groups (amide, guanidine, peptide, amino and carboxyl groups) of amino acid residues in the active site of pancreatic lipase by covalent and/or non-covalent interaction. Using X-ray crystallography and computer modeling to evaluate the binding activity of these compounds on pancreatic lipase is needed to further investigation.

Doxycycline is a member of the tetracycline antibiotics group and is commonly used to treat a variety of infections such as chronic prostatitis (Madsen et al., 1983), sinusitis (Cunha, 2002), syphilis (Dayan et al., 2005), pelvic inflammatory disease (Gjønnaess et al., 1978; Sweet et al., 1988), acne (Oshendrof, 2006). In addition, doxycycline is effective in the treatment of acne, particularly during the inflammatory stage. It has previously been suggested that the beneficial effect of tetracycline is due to the inhibition of *Propionibacterium acnes* accompanied by a reduction in sebum free fatty acids and extracellular lipases. Moreover, doxycycline's effect on inflammatory acne lesions is due to its inhibition of IL-6, IL-1β, and TNF-α, inflammatory cytokines up-regulated in response to *P acnes*. In turn, this inhibits the inflammatory pathway that leads to further recruitment of cellular components, such as neutrophils and their proteolytic enzymes, for

example, MMP-8 (Eklund et al., 1999; Kirkwood et al., 1999). Furthermore, by down-regulating *P acnes* lipase, doxycycline can reduce the virulence of this organism, reducing the levels of free fatty acids that act as a potent chemokine for neutrophil recruitment. This fact is supported by a recent study that intake of doxycycline (20 mg taken twice daily) reduced the number of both inflammatory and noninflammatory lesions in patients with moderate facial acne (Skidmore et al., 2003). The cautions and side effects of doxycycline are similar to other members of tetracycline antibiotic group. The most common adverse effects of doxycycline include photosensitivity skin reactions, diarrhea, loss of appetite, nausea which are often occurred in children and adults (Dréno et al., 2004). As data mentioned above, doxycycline exhibited the most potent pancreatic lipase inhibition, causing to delay digestion of triglyceride to absorbable free fatty acids. This leads to a reduction in free fatty acid absorption and, subsequently, the rise of postprandial hypertriglycedemia is attenuated. To prove this phenomenon, an acute effect of doxycycline on serum triglyceride concentration was next performed in normal rats by oral emulsion loading test. The findings indicated that oral administration of doxycycline to normal rats significantly attenuated the rise of serum triglyceride concentration in dose dependent manner which was consistent with those of its inhibitory effect on pancreatic lipase. From the results, it indicated that the use of doxycycline may directly affect to malabsorption of fat whereas the long time use may lead to decrease the absorption of fat-soluble vitamins and other fat-soluble nutrient, resulting in the lack of sufficient nutrients to maintain healthy bodily functions. Additional studies are needed to clarify the long-term effects of doxycycline on serum lipid profiles and fat soluble nutrients.

As the above results, it suggested that doxycyline mediated *via* the same mechanism of action as orlistat by inhibiting pancreatic lipase. The question arises as to whether combination of doxycycline and orlistat might interact synergistically or the additively on pancreatic lipase. The addition of low dose doxycycline to the assay system with low dose of orlistat (0.39 μM) markedly increased the percentage pancreatic lipase inhibition by 33% when compared with orlistat alone. These findings indicated that the combination of doxycyline and orlistat produced the synergistic inhibition. It suggests that the combination use of these drugs may produce more effective inhibition of pancreatic lipase, leading to increase risk of gastrointestinal side effects including oily spotting,

flatulence and frequent loose. These adverse effects should be further investigated for the combination between doxycycline and orlistat on lowering serum triglyceride in animal experiments or clinical trials.

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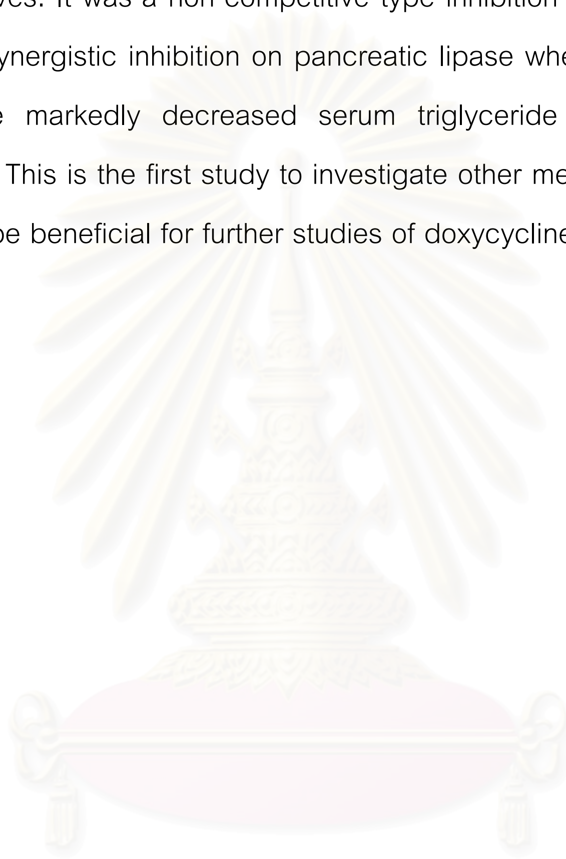
CHAPTER VI

CONCLUSION

Tetracycline and its derivative markedly inhibited pancreatic lipase. Moreover, doxycycline exhibited the most potent pancreatic lipase inhibitor among those of tetracycline derivatives. It was a non-competitive type inhibition against pancreatic lipase and exhibited the synergistic inhibition on pancreatic lipase when combined with orlistat. Finally, doxycycline markedly decreased serum triglyceride concentrations in dose dependent manner. This is the first study to investigate other mechanisms of doxycycline. This research may be beneficial for further studies of doxycycline in clinical pharmacology and toxicology.



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APPENDICES

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APPENDICES A

Ethic approval for animal experiment

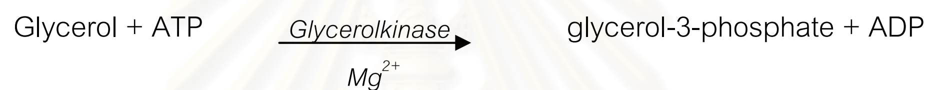
Chulalongkorn University Animal Care and Use Committee

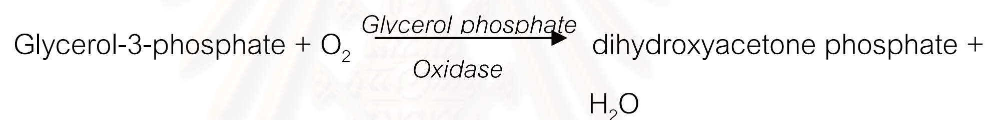
Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. **0731013**	
Protocol Title **Inhibitory effect of tetracyclines on pancreatic lipase activity**	
Principal Investigator **Assoc. Prof. Dr. Sisintorn Yibchok-A-Nun**	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval **May 2, 2007**	**Date of Expiration** **May 2, 2008**
Applicant Faculty/Institution **Faculty of Veterinary Science**	
Signature of Chairperson S. Sawangkoon	**Signature of Authorized Official** Wongtavatchai
Name and Title **Assist. Prof. Dr. Suwanakiet Sawangkoon** **Chairman**	**Name and Title** **Assoc. Prof. Dr. Janenuj Wongtavatchai** **Associate Dean (Research and Academic Service)**
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

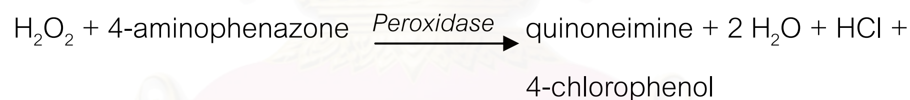
APPENDICES B

Triacylglycerol determination

Principal: Pancreatic lipase hydrolyzes triacylglycerol to glycerol followed by oxidation to dihydroxy-acetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under catalytic action of peroxidase to from a red dyestuff. The reactions are following:







Sample collections:

Collect serum, heparinized or EDTA plasma using standard sampling tubes. Plasma stable for 5-7 days at 4°C or 3 months at -20°C.

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Standard curve preparation

Standard concentration (mg/dl)	Volume (µl)	H_2O (µl)
Blank	-	10
0	-	10
50	10	
100	10	-
200	10	-

Remarks:

- It should be avoiding the carry over while pipette the triacylglycerol standard. Its may affect to the triacylglycerol concentration in the samples.
- If the triacylglycerol concentration of the sample more than linearity range, dilute sample with 0.9% NaCl or distilled/deionized water. Multiply the result by the dilution factor.

Sample preparations:

Sample	Volume (µl)
serum	10

Methods:

1. Pipette standard, samples and H_2O following the table in to the microcuvettes.
2. Add 500 µl of color reagent in each cuvette, mix wall
3. Allow the color complex developed at room temperature for 30 min. Read the OD at 500 nm. The color complex will be stable for 1 hour.

Calculation of the triacylglycerol concentration

$$C = 200 \times (\text{absorbance of sample/ absorbance of standard})$$

Remarks:

1. Determination of triacylglycerols in normal and pathogenic plasma sedentary during the sample determinations.

Interference:

1. Hemolysis: No significant interference up to the H index of 600 (hemoglobin concentration approximate: 600 mg/dl).
2. Lipemia: The L index correlates with the turbidity but not triacylglycerol level.

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