The role of metabolic inhibition-interactions on toxicity

Muhammad Umer Naeem

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Supervisors:
Professor Markku Pasanen
Dr. Risto Juvonen
Humans are exposed to foreign compounds like drugs, pesticides and environmental pollutants, collectively known as xenobiotics. Being exposed in household or occupationally, xenobiotics exert their harmful effects on human health. Metabolism an important parameter in pharmacokinetics plays an important role in the breakdown of toxicologically active compounds into less toxic, water soluble metabolites or bioactivation of inactive compound to toxic metabolites. A change in the metabolism of xenobiotic being co-exposed to another chemical substance is an important cause of xenobiotic interactions and brings considerable changes in the metabolic fate of xenobiotics. Inhibition of CYP enzymes is the most common mechanism of xenobiotic interactions resulting in toxicity. This inhibition can be either reversible or irreversible (suicidal inhibition) depending on the nature of enzyme inhibitor. Reversible inhibition being the most common type of inhibition is further classified into competitive, uncompetitive and non-competitive (mixed-type) type of inhibition. Chemical inhibitors are of much importance in determination of in-vivo toxicities of exposed xenobiotics and in the treatment of many poisoning cases.

The focus of experiments of this thesis work was to study inhibition of pig CYP2A19 enzymes. In this study 96-well plate assay format was used to determine inhibition of coumarin 7-hydroxylation by five benzothiophene derivatives and to study the effect of coumarin concentration on IC$_{50}$ value of inhibitory compounds. Benzothiophene derivatives were proved to be efficient inhibitors for CYP2A19 in an animal (pig) model experiments. The IC$_{50}$ values of 1-benzothiophen-3-carbaldehyde, 1-benzothiophene-3-ylmethylamine, 1-benzothiophen-5-carbaldeyde, 1-benzothiophene-5-ylmethylamine and 5-choro-1-benzothio-3-carbonitrile were 2.4 (± 0.5) µM, 2.6 (± 1.0) µM, 5.3 (± 2.0) µM, 42 (± 12 µM) and 50 (± 8.0) µM respectively at 25 µM coumarin concentration. Three most potent compounds were also checked to find out the type of inhibition. 1-benzothiophene-3-carbaldehyde, 1-benzothiophen-3-ylmethylamine and 1-benzothiophen-5-carbaldeyde showed competitive inhibition for CYP2A19. Ki values for these three inhibitors were 2.21 µM, 1.73 µM and 0.91 µM respectively. The results provide initial structure-activity information about the interaction of benzothiophene derivatives with pig CYP2A19 enzyme. The data obtained in this study can be used for QSAR modelling for CYP 2A19 in future as the position and type of substitution affects potency of inhibition.
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Abbreviations

ACh    Acetylcholine
AChE   Acetylcholine esterase
C<sub>max</sub>  Maximum plasma concentration
CNS    Central nervous system
CYP    Cytochrome P450
DDI    Drug-drug interaction
DMSO   Dimethyl sulfoxide
EIS complex Enzyme inhibitor substrate complex
(E)    Enzyme
GSTs   Glutathione S-transferase
HIV    Human immunodeficiency virus
(I)    Inhibitor concentration (unbound)
IC<sub>50</sub>  Concentration required for 50% inhibition
Ki     Inhibition constant
Km     Michaelis-Menten constant for a substrate
NADPH  Nicotinamide adenine dinucleotide phosphate
OPs    Organophosphates
PAH    Polycyclic aromatic hydrocarbons
QSAR   Quantitative structure activity relationship
(S)    Substrate
Sd. dev. Standard deviation
TCA    Tricarboxylic acid
UDP    Uridine Diphosphate
UDGPA  Uridine Diphosphate Glucuronic Acid
µM     Micro-molar
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1. INTRODUCTION

Foreign compounds also called xenobiotics include therapeutic drugs, plant derived or fungal derived metabolites used in food, environmental pollutants-e.g. polycyclic aromatic hydrocarbons, halogenated hydrocarbons, industrial mixtures, pesticides and herbicides (Nebert & Russell, 2002). After exposure to living organisms, majority of foreign xenobiotics undergo chemical reactions known as metabolism or biotransformation. These reactions normally intend to convert active xenobiotics into inactive and water soluble metabolites. But sometimes the metabolites formed get more toxic properties than parent compound and have potency to react with tissue macromolecules and exert unwanted effects. Hepatic and extra-hepatic enzymes are involved in such biotransformation reactions which produce metabolites to get excreted via urine or bile (Nebbia, 2001).

Many chemicals found in food are mutagenic and carcinogenic (Lall et al. 1999). Drug-drug and drug-food interactions are very common in systemic drug therapy. Normally these interactions are not so intense but still can lead to mild or moderate toxicity and even hospitalization in poisoning cases. Inhibition of drug metabolizing enzymes by other drugs is mostly competitive and can lead to increased exposure of drug or chemical whose metabolism is inhibited. Induction of enzymes can lead to increased metabolism and less internal exposure of drug can lead to non-response situation (Dresser et al. 2000; Parkinson & Ogilvie, 2008). Metabolism of many xenobiotics occurs mostly in liver but also other places like kidney, skin, lungs and intestine are also important for metabolism of many compounds (Miksys & Tyndale, 2011). Xenobiotic metabolism is mainly divided into two types of reactions, namely phase I (hydrolysis, oxidation and reduction) and phase II conjugation reactions. Xenobiotics are metabolized in one or both types of reactions to get polar, water soluble and inactive metabolites (Martikainen, 2012).

Cytochrome P450 are a major family of enzymes involved in metabolism of drugs, chemical compounds and carcinogens. CYP enzymes are heme containing proteins which metabolizes toxic and pharmacologically active xenobiotics into non-toxic and inactive metabolites (Liu et al. 2011; Miksys & Tyndale, 2011). CYP enzymes play their vital role in the breakdown of lipophilic substances into polar and water soluble low molecular weight metabolites, which are easily excreted through bile or urine. CYP1, CYP2 and CYP3 are major families involved in the metabolism of many xenobiotics exposed to humans (Nebert & Russell, 2002). Metabolic enzymes are of much importance in the breakdown of toxic substance into inactive
metabolites. Metabolic activity of these enzymes is largely affected by interaction with xenobiotics. Chemical compounds having closer structural similarity to substrate molecules compete for the active site of enzymes and block the binding of substrate to enzyme. This bonding can be strong (covalent bond) and enzyme can then completely lose its function. In other case the bonding is not strong and as chemical inhibitor leaves the enzyme, normal function and binding with substrate restores the enzyme (Sharma, 2012). Chemical inhibitor technique is used when it is assessed, if CYPs metabolize different xenobiotics including drugs (Martikainen, 2012). A change in P450 activities affects activation or inactivation of xenobiotics (Monostory & Dvorak, 2011). The main aim in conducting in-vitro metabolic inhibition reaction is the qualitative and quantitative prediction of drug-drug interaction in-vivo. Quantitative in-vitro metabolic data can be extrapolated to in-vivo situations by taking into account suitable pharmacokinetic principles. This could be wisely used in understanding of competitive inhibitions (Zhou & Zhou, 2009).

The aim of the present study was to find out the IC\textsubscript{50} values of benzothiophene derivatives against pig liver coumarin 7-hydroxylation. These results give information about effect of substrate on inhibition and structural activity relationship with inhibition potency of chemical compounds.

2. REVIEW OF THE LITERATURE

2.1 General mechanism of toxicity of xenobiotics

Xenobiotic interactions occur when effects of chemical substances are altered by the coadministration of another drug or chemical compound. Alteration in xenobiotic metabolism is a common cause behind the pharmacokinetics interactions (Dresser et al. 2000). The chemical compounds are metabolized to yield numerous metabolites; some of these produced metabolites are active and react with body tissues to cause toxicity (Nebbia, 2001; Bozina et al. 2009; Johnson et al. 2012). Depending upon the dose and route of exposure, all chemical compounds can adversely affect the structure and function of tissue or organ. Concentration and persistence of ultimate toxicant at the target site determines the intensity of the toxicity. The ultimate toxicants can be either a parent compound to which living organisms are exposed directly, the metabolite of the parent compound or can be reactive oxygen or nitrogen species (ROS or RNS) generated during metabolism process in the body of living organisms. A number of xenobiotic compounds like (strong acids, strong bases, heavy metals, nicotine, aminoglycosides, ethylene oxide, CO) are directly toxic while many chemicals exert
their toxicity by their metabolites. Many factors like life style, chemical nature of xenobiotic, genetics and environment affect upcoming toxicity level (Pelkonen et al. 2008).

Chemical exposure can result in different gene expression, which could bring different pharmacodynamics effects. A link between chemicals with different doses, tissue pathology, site of action and level of toxicity has been an important and traditional tool in toxicity assessment. Many cytochrome P450 enzymes have ability to metabolize various exogenous compounds including therapeutic drugs, natural plant products and environmental toxins and pollutants (Nebert & Russell, 2002). CYP’s are mostly involved in metabolism of chemicals and but could also activate the pre-carcinogens metabolically (Bozina et al. 2009). As food having different xenobiotics is digested in gastrointestinal tract, the intestinal micro-flora are also involved in xenobiotic metabolism and could modulate the xenobiotic –induced toxicity by production of active or inactive metabolites (Jeong et al. 2013). Reactive and unstable metabolites formed during xenobiotic metabolism attack DNA of cell and cause cell toxicity and transformation (Bozina et al. 2009). Active metabolites formed could have ability to exert their own effects or block the action of enzyme or receptors involved in endogenous metabolism (Johnson et al. 2012). Codeine a pharmaceutical agent is extensively metabolized by CYP2D6 to get clinical analgesic effect, which is mainly attributed to its conversation to morphine (active metabolite) inside the body (Salminen et al. 2010; Miksys & Tyndale, 2011). Metabolism of xenobiotics is stimulated or inhibited by other co-administered therapy and inhibition of metabolism is quicker as compared to enzyme induction, which depends upon the tissue concentration of inhibitor (Dresser et al. 2000; Horn, 2012).

Use of prescribed and over-the-counter pharmacological agents at the same time increases the risk of overloading the detoxification process. This detoxification system becomes more inefficient in poor nutritional status. Cytochrome P450 and other conjugation enzymes present in liver, which metabolizes lipophilic xenobiotics to more water soluble metabolites to enhance their excretion from body. Balance of these reactions determines the rate of metabolism and elimination of xenobiotic compound and extent of intracellular damage and toxicity from parent compound or activated metabolites (Bidlack et al. 1986). Metabolism of foreign compounds normally leads toward successful detoxification process resulting in inactive metabolites production, which is easy to excrete from the body. But formation of active metabolites in metabolism, which react with body tissues and contribute increase risk of toxicity, cancer and birth defects in newborns (Nebert & Russell, 2002).
Figure 1. Potential stages in the development of toxicity after chemical exposure, and the effect of metabolic inhibitor on toxicity.

Metabolic inhibitors can inhibit metabolism pathways of other chemicals and can alter the toxicity level. Metabolism rate affects the intensity of up-coming toxicity.
2.2 Metabolism of Xenobiotics

Biotransformation enzymes catalyze either detoxification or bio-activation reactions. Many of xenobiotics are metabolized into active electrophilic metabolites, which interact with nucleophilic sites on cellular macromolecules. Generally metabolism increases the xenobiotic hydrophobicity and favors the excretion of lipophilic compounds. Metabolism prevents the bioaccumulation of the most xenobiotics inside the body up to toxic level (Testai, 2001). Cytochrome P450 acts on many endogenous and exogenous substrates and introduces oxidative, reductive and peroxidative changes into chemical structure. Rate of detoxification and sometime of metabolic activation of xenobiotics can be totally different between individuals depending on different CYP genetics (Nebert & Russell 2002).

**Figure 2.** Xenobiotic metabolism pathways. More common route is functionalization followed by conjugation reactions and then excretion. Besides some xenobiotics undergoes conjugation directly and get excreted (modified from EOLSS).

Figure 2 shows the major (bold) and minor (blank) pathway for xenobiotic metabolism. Majority of xenobiotics undergo functionalization reactions to form metabolites, which are further conjugated in phase-II reaction to get excreted from the body. Mostly xenobiotics
follows the same route but rarely some xenobiotics go directly to conjugation reactions and are excreted with or without Phase-I reactions (EOLSS).

Drug metabolism is divided into two main phases: phase I and phase II. In phase I so-called functionalization reactions, enzymes introduce polar groups into the parent xenobiotic compound structure. These polar groups are inherently variable reactive. This phase reactions include oxidation, reduction and hydrolysis. After oxidation these compounds are then conjugated to polar compounds in conjugation reactions. Conjugation or phase II metabolism involves the introduction of hydrophilic endogenous species like glucuronic acid or sulphate to the xenobiotic molecule (Parkinson & Ogilvie, 2008; Puccinelli et al. 2011; Martikainen 2012).

Liver is the main place for the metabolism of xenobiotics by CYPs but these metabolizing enzymes are also found in other parts of body, e.g. brain, kidney and intestine (Miksys & Tyndale, 2013). Action of CYP enzymes on xenobiotics leads to biotransformation of many xenobiotics to inactive water soluble metabolites but sometime it also produces aggressive metabolites, which are responsible for toxicity in cell or in tissue (Daniel, 2013). Usually xenobiotic molecules undergo functionalization reactions followed by conjugation reaction, but sometimes the parent molecule can be conjugated without prior functionalization. This way parent xenobiotic molecules are converted into less lipophilic metabolites by having polar substituents. Metabolism plays an important role in the inactivation and breakdown of drug/xenobiotic lipophilic molecule into easily excreted metabolites. But sometimes this way brings a big concern by metabolizing inactive parent compound into active and toxic metabolites (Nebbia, 2001; Pelkonen et al. 2008). Metabolism of xenobiotic normally results in successful detoxification but sometime cytochrome P450 enzymes can also generate toxic, reactive and unstable metabolites formed during some xenobiotics metabolism which can attack DNA of cell and cause cell toxicity and transformation ultimately leading to cancer and even defects in tissue structure and function (Daniel & David, 2002; Bozina et al. 2009).

### 2.2.1 Phase I – Functionalization

Several enzyme systems participate in phase I metabolism of xenobiotics. The major pathway for the metabolism in phase I reactions is the monoxygenation function by cytochrome P450s (Parkinson & Ogilvie, 2008). Liver is the main place for the metabolism of xenobiotics while CYP enzymes are also found in other parts of body like brain (Sharon et al. 2012). CYP-
mediated interactions are of much importance as about 70% of prescribed drugs are metabolized in phase-I reaction by CYP enzymes (Ramasamy et al. 2014).

2.2.1 Oxidation

Majority of CYP reactions are oxidation (Guengerich, 2001). Cytochrome P450 mediated oxidation biotransformation reactions are identified in bioactivation of many xenobiotics. CYP P450 could activate or detoxify a toxicant at the same time depending upon the type of reaction. In some oxidation reactions the metabolites retains toxic actions of parent compounds (Nebbia 2001). CYP enzymes catalyze the oxidative and reductive halogenation of many xenobiotics. Alcohol dehydrogenase, aldehyde oxidase and cytochrome P450 are important enzymes which can catalyze the reactions and are influenced by nature of substrate and condition of reaction (Parkinson & Ogilvie, 2008). Organophosphorus insecticides like malathion, parathion, diazinon and fenithrothion undergo metabolic activation to develop fully anticholinesterase activity. These derivatives are result of CYP mediated oxidative desulphuration (Nebbia, 2001).

2.2.1.2 Reduction

Reductive reactions are carried out by either NADPH cytochrome reductase or even cytochrome P450 under anaerobic conditions (Nebbia, 2001). However CYP do not participate generally in biological reduction of endogenous substrates as do flavoproteins. Nitrogen oxides and quinones are reduced by CYP and other enzymes too. Substrates which undergo reduction reactions include N-oxides, aldehyde, ketone, alkene, sulfoxide, quinone and nitroxides containing xenobiotics. CYP are also involved in the reduction of inorganic molecules like Cr and SO₂ (Guengerich, 2001). Many endogenous and exogenous chemicals are also reduced inside body either enzymatically or nonenzymatically by other reducing agents (Parkinson & Ogilvie, 2008).

2.2.1.3 Hydrolysis

Microsomal and cytosolic enzymes; esterases and amidases play vital role in the hydrolysis of esters and amides. Many toxic compounds like OPs, pyrethroids and mycotoxins like T-2 toxins are bio-transformed into inactive metabolites in hydrolysis reactions (Nebbia, 2001). Carboxylesterases, cholinesterases and paraoxonases are important hydrolytic enzymes involved in the xenobiotic metabolism. Hydrolytic enzymes like carboxylesterases could
produce toxic and tumorigenic metabolites and therefore hydrolysis is not always a detoxification process (Parkinson & Ogilvie, 2008).

2.2.2 Phase-II reactions

Phase II or conjugation reactions include glucuronidation, sulfonation, acetylation, methylation or conjugation with glutathione and with amino acids moieties. In these reactions the cofactors reacts with functional group present on the xenobiotic or the added during phase I reactions. Majority of conjugation reactions increase the hydrophilicity of xenobiotics and promote their excretion from the body. Conjugation enzymes are mainly found in the cytosol, only UDP-glucuronosyltransferases are microsomal enzymes. Glucuronidation, sulfonation, acetylation and methylation are reactions with activated cofactors, while phase II reaction involving activated xenobiotic occurs with amino acids or glutathione moiety (Parkinson & Ogilvie, 2008).

2.2.2.1 Glucuronidation

Glucuronidation; a major pathway of xenobiotic metabolism in mammals requires mainly cofactor UDPGA but can also use UDP-glucose, UDP-xylose and UDP-galactose. UDP-glucuronosyltransferases located in the endoplasmic reticulum of liver and other parts of body catalyzes the reaction. As the electron rich nucleophilic like O, N or S heteroatom are site of glucuronidation in the atom; examples of substrate for glucuronidation are alcohols, phenols, carboxylic acid, aliphatic amines, primary and secondary amines. Carboxylic acid structures containing xenobiotics are glucuronidated to form acylglucoronides which can be reactive and cause toxicity. After Glucuronidation, the xenobiotic conjugates became polar and water soluble and are excreted from body through urine or in bile. Inhibition of UGT’s has been reported to involve in drug-drug interactions. Glucuronide conjugate could also go under further metabolism stages by oxidation or even by further conjugation (Parkinson & Ogilvie 2008).

2.2.2.2 Sulfonation

Xenobiotic and other endogenous substrate undergoing O-glucuronidation also go under sulfonation reactions. The reaction is catalyzed by sulfotransferases enzymes family which produces highly water soluble sulfuric acid ester. Xenobiotic conjugates are excreted mainly in the urine. Sulfonation is supposed to be a benign metabolic pathway as compared to other xenobiotic metabolism as it can prevent or reduce the activation of promutagens and
procarcinogens. Sulfonation decreases the pharmacological and toxicological activity of xenobiotics but in some cases sulfonate conjugates are chemically unstable and form potent electrophilic species which cause toxicity inside the body. However some pharmacological drug molecules are sulfonated and get active to exert their desired effects (Parkinson & Ogilvie, 2008).

### 2.2.2.3 Methylation

Methylation a minor pathway for xenobiotic metabolism differ from other conjugation reactions as it decreases the water solubility and covers the functional group which could go under further metabolism by other enzymes. But N-methylation of pyridine containing xenobiotics like nicotine makes positively charged ion that are water soluble and are easily excreted from the body. Some inorganic compounds like arsenic undergo methylation and get more cytotoxic and genotoxic as compared to arsenate or arsenite (Parkinson & Ogilvie, 2008).

### 2.2.2.4 Acetylation

Many xenobiotics are N-acetylated after their biotransformation by hydrolysis, reduction and oxidation. N-acetyltransferases enzymes are basically cytosolic enzymes found in the liver and play an important role in the biotransformation of xenobiotics containing an aromatic amine or a hydrazine group. Same like methylated conjugates, N-acetylated metabolites are less water soluble than parent compound. N-acetyltransferases have ability to detoxify or activate the aromatic amines which depends upon the early phase of biotransformation (Parkinson & Ogilvie, 2008).

### 2.2.2.5 Amino acid conjugation

Steric hindrance around the carboxylic group and the substitution on aromatic ring or on aliphatic ring are two factors which play an important role in ability of xenobiotic undergoing amino acid conjugation. Endogenous amino acid substitution to xenobiotic favors the interaction with tubular organic anion transport system of kidney and facilitates the elimination from the body. Amino acid conjugation of carboxylic acid containing xenobiotics leads to inactive metabolites and is a detoxification process, whereas xenobiotic containing carboxylic acid group with glucuronidation produces potentially toxic acylglucuronides (Parkinson & Ogilvie, 2008).
2.2.6 Glutathione conjugation

Glutathione conjugation is the conjugation of xenobiotic with tripeptide glutathione (GSH) and substrate for glutathione conjugation include electrophilic xenobiotics or xenobiotics that could be biotransformed to electrophiles. The reaction is catalyzed by glutathione transferases found in the cytosolic, microsomal and mitochondrial cell fractions (Parkinson & Ogilvie, 2008). Xenobiotic having hydrophobic properties, having electrophilic atom and tendency to react with non-enzymatically with glutathione are main substrate of glutathione transferase. Other substrate for glutathione conjugation includes reactive intermediates produced during other biotransformation reactions, free radicals and xenobiotic with electrophilic heteroatom. Many enzymes involved in xenobiotic metabolism have ability to produce reactive metabolites, which are then detoxified by conjugation with glutathione. In some cases the conjugation with glutathione produces reactive form of xenobiotic which enhances toxicity (Parkinson & Ogilvie, 2008).

2.2.3 Human cytochrome P450 (CYP) enzyme system

The cytochromes P450 are responsible for the metabolism of many endogenous and exogenous compounds and rank first in term of catalytic versatility to detoxify or activate a wide range of xenobiotics to reactive metabolites (Benedetti, 2001; Ingelman-Sundberg, 2002). Cytochrome P450 is a wide heme-containing complex superfamily of enzymes and is the major catalytic component of liver mixed function oxidase system, which catalyse the oxidative metabolism of many xenobiotics including drugs, plant or fungal derived secondary metabolites consumed with food, environmental pollutants, pesticides, herbicides and other industrial compounds (Salminen et al. 2012; Emanuela et al. 2011; Van-lersel et al, 1999).

Some CYPs are highly polymorphic enzymes regulated at multiple molecular levels and they act as bridge between our body and environment, and their functions are linked in many ways to initiation/prevention of carcinogenesis (Tamási et al. 2011). Humans have 57 CYP genes and 33 pseudogenes, which are arranged into 18 families and 42 subfamilies (Nebert & Russell, 2002). Out of 57 functional human CYP about a dozen of enzymes belonging to 1, 2 and 3 cytochrome P450 families are involved in the metabolism of many xenobiotics and about 80% of drugs in use (Zanger & Schwab, 2013). Cytochromes P450 are divided into families and sub families on the basis of aminoacid percentage sequence and gene nucleotide sequence identity. Cytochrome P450 metabolizes many substrates by introducing oxidative, peroxidative and reductive alterations (Daniel & David, 2002). CYPs are supposed to be
present in all living species of Earth including archaea bacteria, plants and in animal species (Anzenbacher & Anzenbacherová, 2001). Cytochrome P450 being involved in the metabolism of therapeutic drugs, are major determinants of drug half-life (Singh et al. 2011). Major CYP involved in hepatic biotransformation of xenobiotics includes CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Figure 3) (Ramasamy et al. 2014). Metabolic activation is of concern with CYP1A1, CYP1A2, CYP1B1, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 metabolism. In some cases these enzymes are associated with increased risk of certain types of cancer and toxic effects (Nebert & Russell, 2002).

Many members of CYP family exhibit selectivity towards specific substrate and inhibitors (Pelkonen et al. 2008). Human liver microsomes contain more complete hepatic metabolizing enzymes as compared to recombinant enzymes, so it is taken as more physiological to assess CYP inhibition (Martikainen, 2012). Extra-hepatic organs like lungs, kidney and brain also have ability to metabolize through cytochrome P450 (Ravindranath & Strobel, 2013). Many substrates for CYP enzymes effect the metabolism of other compounds, so called drug-drug interactions and is basis for toxicity (Dresser et al. 2000; Nebert & Russel, 2002). Cytochrome P450 can activate or detoxify a toxicant at the same time depending upon the reaction performed (Nebbia, 2001)

**Figure 3.** Cytochrome P450 abundance in human liver (modified from Galetin et al. 2008)
2.2.3.1 CYP1-family

The CYP1- family includes three members: CYP1A1, CYP1A2 and CYP1B1. CYP1A1 is expressed e.g. in lung, skin, larynx and placenta and induced in liver by polycyclic aromatic hydrocarbons. CYP1A1 has capacity to activate the compounds with carcinogenic properties and is detected after induction of polycyclic aromatic hydrocarbons (PAH). CYP1A2 enzyme is expressed in human liver and is about 12% of total content of CYP enzymes in human liver. CYP1A2 metabolizes many important drugs e.g. caffeine, theophylline, clozapine and olanzapine. CYP1A2 is also involved in the metabolism of several environmental toxins and endogenous substances like bilirubin, melatonin and estradiol and as well as for the activation of dietary heterocyclic amines, mycotoxins, aryl amines and tobacco specific nitrosamines. Potent inhibitors for CYP1A2 are rofecoxib, ciprofloxacin, fluvoxamine and alpha napthoflavone (Pelkonen et al. 2008; Martikainen, 2012). CYP1A1 and CYP1B1 are expressed in many tissues in various concentrations and are efficient metabolizers of compounds like polycyclic aromatic hydrocarbons.

CYP1B1 is the only member of CYP1B subfamily. CYP1B1 is involved in metabolism of many compounds including N-heterocyclic amines, azo dyes, aryl amine, many PAHs and other carcinogens. It has been also proposed as marker of tumorigenic potential (Pelkonen et al. 2008). All three CYP1 enzymes are involved in detoxification or activation of many environmental carcinogens (Nebert & Russell, 2002). Ciprofloxacin, rofecoxib used as therapeutic drugs oral contraceptives have shown significant inhibition of CYP1A2 mediated metabolism in humans (Pelkonen et al. 2008). Inhibitors for porcine CYP1A subfamily include ellipticine and alpha-napthoflavone (Puccinelli et al. 2011).

2.2.3.2 CYP2-family

CYP2-family involved in mammalian drug metabolism is one of largest and most diverse family in CYP enzymes (Martikainen, 2012). Many therapeutic drugs (more than 100) are metabolized into active and inactive metabolites by CYP2 family. Different CYP2 families are involved greatly in the metabolism of anti-depressants, antipsychotics, drug of abuse like ethanol and amphetamine, endogenous neurochemicals like dopamine and serotonin (Sharon et al. 2012). CYP2A6 mediates the oxidative metabolism of many xenobiotic with procarcinogenic activity targeting human liver (Raunio et al. 1998). Human CYP2A6 is the key enzyme in the elimination of nicotine and it is known to be involved in bio-activation of some toxicologically important substances such as aflatoxin and nitrosamines and CYP2B6 is
of considerable importance due to bio-activation of many procarcinogens (Pelkonen et al. 2008). Some natural and herbal products are metabolized by CYP2C9, which could lead to formation of toxic metabolites. However it is also involved the metabolism of anticoagulant warfarin and in many endogenous metabolisms of steroids, melatonin and retinoids. Mostly weak acids are substrate of CYP2C9, but it could also metabolize many neutral or highly lipophilic compounds. Irreversible inhibitors for CYP2C9 include tienilic acid, suprofen and silybin which potentially give rises to adverse drug interactions (Zhou et al. 2009). CYP2C9 and CYP2C19 also metabolize centrally acting antidepressants, anxiolytics and anticonvulsants. Inhibitors for these enzymes will inhibit the metabolism process of ingested drugs and will lead to increased plasma concentration of these active xenobiotics. This increased plasma concentration will result in adverse reaction and different toxicities (Ramasamy et al. 2014). Many drugs acting on central nervous system are metabolized by members of CYP2 family into active or inactive metabolites (Miksys & Tyndale, 2013).

Human CYP2C8, CYP2C9, CYP2C18 and CYP2C19 in various amount metabolizes more than half of prescribed medicines and some endogenous steroids. Other members of this family have important role in drug metabolism and synthesis of vasodilator by vascular endothelium (Nebert & Russell, 2002). Androgens and diethyldithiocarbamate are well known porcine CYP2A inhibitor. Sulfaphenazole, ticlopidine and beta-naphthoflavone are porcine CYP2C inhibitors. Quinine, quinidine and phenobarbital have porcine CYP2D inhibition properties (Puccinelli et al. 2011).

2.2.3.3 CYP3-family

CYP3A4, CYP3A5, CYP3A7 and CYP3A43 are four isoforms grouped in CYP3A subfamily of enzymes. CYP3A4 is found in largest amount of CYP in the liver, while CYP3A5 is found in minor quantity expressed in lungs. CYP3A7 is majorly present in human fetal liver. CYP3A4 is majorly expressed in human liver where it accounts for about 35% of total liver CYP enzyme content. Being responsible for about 50% of known drugs metabolism, CYP3A4 is the most abundant human hepatic CYP isoform (Dresser et al. 2000; Salminen et al. 2011). CYP3A4 inhibition is related to many of drugs interactions inside the body. CYP3A4 with large active site favors the binding of multiple substrates and could be cause of atypical kinetics. Antiarrhythmic agents, anxiolytics, HIV protease inhibitors, lipid-lowering agents and opioids are mostly substrate for CYP3A4 (Parkinson & Ogilvie, 2008). Ligand competition with CYP3A4 originates many drug-drug interactions (Dresser et al. 2000;
CYP3A4 inhibitor such as midazolam probably binds covalently to CYP apoprotein and finally inactivates the enzyme. Other CYP3A4 inactivators like erythromycin and nelfinavir can bind with heme part of CYP and consequently inactivates the enzyme (Zhou & Zhou, 2009). Many anticancer drugs can induce and inhibit CYP3A4 in liver and extrahepatic tissues (Kivistö et al. 1995). CYP3A interactions favor the formation of drug reactive metabolite–CYP3A to initiate toxicity (Zhou & Zhou, 2009). Inhibition of CYP3A4 mediated drug metabolism is thought to be major cause of drug interactions. CYP3A4 interactions could lead to adverse clinical consequences of rhabdomyolysis, symptomatic hypotension, excessive sedation and ataxia. Many inhibitors of CYP3A4 are of clinical relevance and can be used to achieve beneficial therapeutic goals; cost saving or enhanced efficacy of other xenobiotics (Dresser et al. 2000). Porcine CYP3A inhibitors include ketoconazole, triacytlyoleandomycin and tiamulin (Puccinelli et al. 2011)

2.3 Inhibition of xenobiotic metabolism

Acute or chronic exposure to certain xenobiotics may cause adaptive changes in overall metabolism capacity of living organisms. The resulting decrease in metabolic rate of endogenous or exogenous substrate is referred as inhibition (Nebbia, 2001). Enzymes are protein molecules acting as catalyst in reactions (Sharma, 2012). Generally inhibition brings decrease in enzyme activity (Martikainen, 2012). Enzyme inhibition is phenomenon of enzyme-substrate reaction influenced by presence of any organic or inorganic chemical, any metal or biosynthetic compound which interacts with active site of enzymes by covalent or non-covalent bonds (Sharma, 2012). Inhibitions of CYP enzymes plays key role in the drug-drug interactions and have been a main cause in the removal of many drugs from market. Knowledge about CYP inhibition gives information about potential drug-drug and other exposed xenobiotics interactions (Turpeinen et al. 2006). Metabolic interactions are mainly linked to CYP enzymes and are result of inhibition or induction of CYP enzymes (Martikainen, 2012). Many mechanisms could lead towards enzyme inhibition like decreased protein synthesis or accelerated protein breakdown, reversible or irreversible interactions and competition between two xenobiotic toward same binding site (Nebbia, 2001). Inhibition of metabolism can lead to increased concentration of parent compound in the circulation and decreased elimination of parent compound (Pelkonen et al. 2008). Inhibition of CYP enzymes is the most common mechanism which leads finally to drug-drug interactions (Fontana et al. 2005; Kalgutkar et al. 2007). Broadly inhibitory drug-drug interactions are categorized into reversible and irreversible inhibition. Irreversible inhibition is also known as mechanism
based inhibition or suicide inhibition (Ring et al. 2014; Zhou & Zhou, 2009; Kalgutkar et al. 2007). Competitive inhibition is the most common type of reversible enzyme inhibition (Martikainen, 2012). Changes in intracellular metabolism can alter enzyme level and also the availability of their cofactors; NADPH, UDPGA and GSH. Diets with protein or high sugar content affect the component enzyme, cytochrome P450 and cytochrome P450 reductase towards a variety of xenobiotics (Bidlack et al. 1986).

2.3.1 Reversible inhibition

Reversible inhibition is the most common in all enzyme inhibition and is further subdivided into competitive, uncompetitive and mixed type inhibition (Ring et al. 2014; Martikainen, 2012; Pelkonen et al. 2008). Reversible inhibition develops as a result of non-covalent interaction between inhibitory compound and amino acid residue in the active site of enzyme involved. Bonds formed are weak as compared to covalent bonds of irreversible inhibition. As bonds are weak so they don’t inactivate enzyme for longer time or completely (Ring et al. 2014).

![Figure 4](http://www.wiley.com/college/boyer/0470003790/animations/enzyme_inhibition/enzyme_inhibition.htm)

**Figure 4.** Schematic representation of “Enzyme-substrate complex (a) and three types (b,c,d) of reversible inhibition”.
Reversible inhibitors have three mechanisms by which perpetrator interact with enzyme including competitive, uncompetitive and mixed type (noncompetitive) inhibition. Potential for a xenobiotic to be a clinically significant reversible inhibitor is the equilibrium dissociation constant ($K_i$) for enzyme-inhibitor complex (Ring et al. 2014; Sharma, 2012). Omeprazole and diazepam both are metabolized by CYP2C19. If both are administered together, omeprazole competitively inhibits the metabolism of diazepam and increase its plasma half-life. Inhibition of diazepam is linked with the omeprazole competition for inhibition by CYP2C19. This inhibition interaction between these two drugs is only seen in population having CYP2C19 (Parkinson & Ogilvie, 2008).

2.3.1.1 Competitive inhibition

The competitive inhibition usually occurs between the substrates for same enzyme (Miners & Birkett, 1998; Sharma, 2012). Principally in the competitive inhibition there is a mutually exclusive binding of either the substrate or the inhibitor at a single active or binding site of a drug metabolizing enzyme. Thus two different drugs/ chemical compounds compete with each other for binding to single active site (Ring et al. 2014). In the competitive inhibition the substrate and the inhibitor compete for the same active site of enzyme, the inhibitor replaces the substrate and get bind to active site of enzyme. As seen in figure 5, substrate concentration decreases the inhibition by inhibitors in competitive inhibition reactions and IC$_{50}$ values increases by increasing substrate concentration (fig. 5) (Ramasamy et al. 2014).

![Figure 5](image)

**Figure 5.** The effect of substrate concentration on the IC$_{50}$-value of competitive inhibitors.

The inhibitor binds to the active site of the free enzyme reversibly. Enzyme inhibitor complex formed prevents binding of the substrate as active site is already been taken by inhibitor.
Competitive inhibitors reduce the availability of free enzymes for substrate. Competitive inhibitors do not make strong covalent bonds with active site of enzymes. Once the inhibitor leaves the enzyme, enzymes work normally in binding and catalytic reactions with substrates (Sharma, 2012). Products of enzymatic reactions also behave as reversible inhibitors of enzymes (Sharma, 2012).

Isoniazid a therapeutic drug at clinically concentration reversibly inhibits CYP2C19 and CYP3A4 activities and inactivates CYP1A2 and CYP2A6 in human liver microsomes. Co-administration of isoniazid with other substrate of CYP2C19 and CYP3A4 could lead to inhibition of their metabolism and may result in toxicity reactions (Wen et al, 2002).

2.3.1.2 Un-competitive inhibition

Uncompetitive inhibitors have no similarity to the substrate. It’s a rare type of inhibition and inhibitor could be a reaction product. Inhibitor does not bind to the free enzyme but with enzyme-substrate complex that exposes the inhibitor binding site (Martikainen, 2012). Although it binds away from the active site, but still it causes structural distortion of the active site and allosteric sites which finally brings inactivation to catalysis function. The resultant EIS-complex formed is catalytically inactive (Sharma 2012).

![Figure 6. The effect of substrate concentration on the IC\textsubscript{50}-value of uncompetitive inhibitors.](image)

As the figure (6) shows, inhibition increases until substrate concentration increases up to saturation level (Martikainen, 2012). In uncompetitive inhibition $V_{\text{max}}$ decreases and $K_m$ of substrate increases. This type of inhibition could be seen in multi substrate enzymes, where inhibitor competes only for one substrate having structural similarity but is uncompetitive for other substrates. Dialysis could be beneficial in reversal of inhibition as by increasing
substrate concentration does not affect the inhibition process. Examples of commonly uncompetitive inhibition include; inhibitions of lactate dehydrogenase by oxalate, inhibition of heme synthetase by heavy metal ion e.g., lead. These inhibitions require treatment with reducing agents and/ or dialysis (Sharma, 2012).

2.3.1.3 Non-competitive inhibition

Non-competitive inhibition is a form of mixed type inhibition reactions (Martikainen, 2012). These types of inhibitors have also no structural similarity to substrate but in this way they have ability to bind both to free enzyme and enzyme-substrate complex as well. The presence of substrate has no influence on the ability of non-competitive inhibitor to bind an enzyme and vice versa.

Fig 7. The effect of substrate concentration on the IC$_{50}$-value of non-competitive inhibitors.

As shown in figure 7 by increase or decrease in the substrate concentration does not affect the inhibition reaction and have no influence on reversibility of inhibition reaction and there is a reduced metabolic rate regardless of substrate concentration (Martikainen, 2012). Although the inhibitor binds away from active site but its changes the conformation of enzyme by affecting the nature of catalytic groups on active site and then reduces its catalytic performance. EI and ESI complexes formed are non-productive and increasing substrate concentration is ineffective.

Many poisons including cyanide and azide are common examples of noncompetitive inhibitors (Sharma, 2012).
2.3.2 Irreversible inhibition

The irreversible inhibitors bind covalently to enzyme and have no structural relationship to substrate (Sharma, 2012). Irreversible inhibition also known as mechanism based inhibition results from either strong covalent bond formation between heme and protein of CYP and reactive metabolites or metabolite intermediate complexes are formed (Van-lersel et al. 1999). Mechanism based CYP inactivation usually involves bioactivation of xenobiotic to reactive metabolite, which in some cases triggers auto immune response and can lead to toxicity. Irreversible inhibition have more chances to elicit drug-drug interactions as the inactivated CYP enzyme has to be replaced by newly synthesized protein (Kalgutkar et al. 2007; Kamel & Harriman, 2013). Mechanism-based inhibition of CYPs is characterized by NADPH, time and concentration dependent enzyme inactivation and by substrate protection. As CYPs have important role in metabolism, inactivation of CYPs leads toward clinical drug-drug interactions and adverse drug reactions with final toxicity outcome (Zhou & Zhou, 2009). Irreversible inhibition is usually long lasting as it could be overcome by resynthesis of enzyme. Time, concentration and NADPH dependent enzyme inactivation are important factors in irreversible inhibition. Suicide inhibition is an irreversible inhibition in which metabolic product inactivates the enzyme totally (Pelkonen et al. 2008). Kinetically irreversibly inhibitors decrease the concentration of active enzymes with ultimate reduction in ES complex formation and decrease in reaction rate of inactivated enzyme. Remaining unbound enzymes molecules are normally functional and have free active site for substrate binding (Sharma, 2012). Many drugs are irreversible inhibitor of CYP enzymes like CYP3A4. These bind to CYP3A4 and inactivate the enzyme irreversibly. In the data extrapolation from in-vitro to in-vivo more factors should be taken into account for mechanism based CYP inhibition (Zhou & Zhou 2009).

2.4 Inhibition interactions, toxicity and clinical use

Drugs, pesticides and environmental pollutants have ability of enzyme inhibition (Nebbia, 2001). Inhibition or induction of CYP enzymes give rises to metabolic interactions and toxicities (Martikainen, 2012). Inhibition of metabolism of a drug results in increase in exposure to drug in patients, probably outside the therapeutic window and increases the possibilities of adverse drug reactions in patients (Ring et al. 2014). Generally it is suggested that even small changes in pharmacokinetics of xenobiotics could bring alterations in efficacy and toxicity profile (Kivistö et al. 1995). Inhibition of CYP enzymes results in increased
plasma concentration of parent drug through decreased drug metabolism, which results in increased risk of adverse effects or toxicity from parent drug molecules (Ramasamy et al. 2014). In nature animals and plants have and are exposed to many poisons as secondary metabolites, peptides and proteins that behave as enzyme inhibitors. Reversible interaction of a xenobiotic or drug with the enzyme results in inhibition of drug metabolizing enzyme, which decreases the metabolism of another drug and is the major cause of clinically significant drug-drug interactions (Ring et al. 2014). Ethanol consumption is widespread and has effect on the absorption, plasma protein binding, blood flow and distribution of other xenobiotics as well as on phase I and phase II metabolism of xenobiotics. Inhibition of CYP2E1 function is caused by acute consumption of ethanol via competitive inhibition (Yang et al. 1992). Enzyme inhibition is of practical importance in the prediction of drug-drug interactions. Sometimes enzyme inhibition technique is used to inhibit the formation of toxic metabolites of an exposed xenobiotic in poisoning treatment (Nebbia, 2001).

Methanol also known as wood alcohol is widely present in the cleaning solutions, stains, dyes and other industrial and household chemicals. Absorption of methanol occurs through oral, dermal or inhalation routes mostly in accidental or suicidal attempts. The lethal dose of pure methanol is about 1-2 mL/kg. Methanol toxicity may lead to permanent blindness and death. Methanol is metabolized in liver by alcoholdehydrogenase phase-I reaction. The metabolites formed are more toxic then the parent molecule and exert severe toxicity in exposed population. Methanol is metabolized to formaldehyde in the presence of alcoholdehydrogenase enzyme. Then formaldehyde is oxidized to formic acid by formaldehyde dehydrogenase. Formed formic acid is the toxic metabolite of the methanol and is very persistent inside the body with half-life of about 20 hours. Another industrial solvent ethylene glycol is also used as coolant and anti-freeze exert toxicity inside the body after accidental or suicidal ingestion. After metabolism by hepatic enzymes more active metabolites; glyoxylic acid and oxalic acid are produced. These metabolites accumulation leads toward fatal acidosis and renal failure. This metabolism makes more complex toxicity rather than parent ethylene glycol. Inhibition of further metabolism of ethylene glycol and elimination of both parent and metabolites molecules are important steps in therapy. The use of antidote like high concentration of ethanol saturates the alcohol dehydrogenase and prevents the metabolism of ethylene glycol. Fomepizole with less adverse effects is alternative to ethanol is successfully used inhibitor metabolizes in a predictable manner and is safe to use in methanol and ethylene glycol poisonings and inhibits the formation and
accumulation of formate (Brent, 200; Jammalamadaka & Raissi, 2010). Fomepizole or 4-methylpyrazole acts as potent competitive inhibitor of alcohol dehydrogenase inhibits the initial steps in the conversion of methanol to toxic metabolites. 4-methylpyrazole is used extensively in methanol and ethylene glycol poisoning due to its longer duration of action and lesser adverse effects. It can be seen in data that ethanol also inhibits the metabolism of fomepizole in the body and thereby increases the therapeutic blood levels of 4-methylpyrazole. Normally self-poisoned or accidental have ingested ethanol with methanol or ethylene glycol so this mutual interaction could have good clinical result (Jacobsen et al. 1996). Methanol poisoning without treatment causes serious intoxications complicated by visual impairment, coma, metabolic acidosis, respiratory and circulatory insufficiency and finally death (De Brabander et al. 2005).

Steroid hormones are biologically active compounds play a vital role in the physiological process by endocrine signaling pathways. Xenobiotic exposure can change endocrine function either directly as hormone agonist/antagonist or indirectly by altering hormone metabolism rate. Xenobiotics by modulating P450 expressions have effects on both foreign chemicals and on endogenous steroid hormones. This disturbance in hormone metabolism leads towards imbalance in sexual and reproductive development/function, glucose, lipid and salt/water balance inside body (Monostory & Dvorak, 2011). Testosterone and its metabolically active form dihydrotestosterone are vital for male reproductive system and spermatogenesis. Testosterone is biosynthesized in the leydig cells of testis from cholesterol. Many industrial chemicals like phthalates, bisphenol A, benzophenone and pesticides/biocides acts as antiandrogens, because they inhibit one or more enzymes involved for testosterone biosynthesis and metabolic activation. Thus inhibition in testosterone biosynthesis and activation leads to reproductive and developmental toxicity (Ye et al. 2011).

Organophosphate compounds used as insecticides are extremely useful in agricultural pest control all over the world, but their extensive use targeted many other species including humans. Organophosphate pesticides inhibit the acetylcholinesterase enzyme from breaking down acetylcholine, thereby increasing level and duration of action of the acetylcholine. The inhibition could be reversible or irreversible (Sultatos, 1994). Inhibition of AChE leads to accumulation of ACh in the synaptic cleft and results in impeded neurotransmission. Irreversible inhibitors of AChE may lead to muscular paralysis, convulsions, bronchial constriction, and death by asphyxiation (Harper et al. 2009).
Cholesterol is present in high concentration in liver. With low solubility and equilibrium with bound forms, still high concentration is of potential risk factor in human beings. Cholesterol is metabolized (hydroxylated) by CYP3A4 in an in-vivo reaction. Other than as a substrate for CYP3A4, cholesterol showed non-competitive inhibition toward three oxidation reactions catalyzed by CYP3A4. Increased cholesterol level have ability to inhibit the CYP3A4 activity in hepatocytes, resulting in conclusion that basal and elevated levels of cholesterol in human liver could affect metabolism of clinical drugs and other compounds by CYP3A4 (Shinkyo & Guengerich, 2011).

Dietary influences on xenobiotic metabolism could alter the therapeutic effects of drugs and toxicity or carcinogenicity of environmental chemicals (Yang et al. 1992). Flavonoids naturally present in fruits and vegetables with enzyme modulating activities results in decreased carcinogenicity of xenobiotics. The flavonoids have effects on CYP, which are involved in activation of procarcinogens. Flavonoids inhibit competitively or irreversibly CYP enzymes including CYP1A1, 1A2, 2E1 and 3A4. Flavonoids also activate phase II detoxifying enzyme; such as UDP-glucuronyl transferase, glutathione S-transferase and quinone reductase which play important role in detoxification of carcinogens. These flavonoids could have interactions with chemotherapeutic drugs through induction or inhibition of their metabolism (Moon et al. 2006).

Chemical inhibitors are of importance because of their widespread availability, stability and cost effectiveness (Liu et al. 2011). Inhibitors are used to find out the in-vivo toxicity outcomes of exposed xenobiotics by inhibition of their metabolism. Chemical inhibitors have been used as important tool to determine the enzyme involvement in the in-vitro P450 mediated biotransformation of xenobiotics as well as of endogenous compounds (Nebbia, 2001). Co-administration of CYP3A4 irreversible inhibitors are used to get beneficial drug interaction in some clinical treatments. Mechanism based CYP3A4 inhibitors are used with cyclosporine. This administration may allow reduction of the dosage and cost of immunosuppressant. In HIV/AIDS patents co-administration of inhibitors allows reduction of dosage and cost of immunosuppressant. Ritonavir an antiviral is used as booster in protease inhibitors therapy to inhibit CYP3A4 that normally metabolizes protease inhibitors (Zhou & Zhou, 2009).

Methotrexate an anticancer drug is the competitive inhibitor of dihydrofolate reductase. It is used as antimetabolite chemotherapy in pediatric leukemia. Methotrexate resembles
structurally to dihydrofolate and behaves as competitive inhibitor of dihydrofolate reductase. Methotrexate hinders the availability of tetrahydrofolate being important for anabolic process. This finally leads to the decreased synthesis of purine nucleotides for DNA replication. Neurotoxins are natural inhibitors, which are toxic in nature but are of therapeutic importance at low doses. Glycoalkaloids present in many plants (potato, tomato, and eggplant) inhibits acetylcholinesterase results in increase acetylcholine concentration, muscular paralysis and then death in severe conditions. Many invertebrates and vertebrates venoms contain protein and peptide enzyme inhibitors for plasmin, renin and angiotensin converting enzymes. Reversible competitive inhibitors of acetylcholinesterase; physostigmine and neostigmine are used in the treatment of myasthenia gravis and in anesthesia. Among pesticides, carbamates are commonly exposed reversible acetylcholinesterase inhibitors (Sharma, 2012).

Although xenobiotic-metabolism inhibition interactions are generally having negative adverse effects on health and are mostly to be avoided but they are also been in use to achieve beneficial therapeutic effects against diseases and poisoning treatment (Dresser et al. 2000, Sharma, 2012).
3. EXPERIMENTAL PART

Pigs are getting widely used as test animals in pharmacological and toxicological assessment of new xenobiotic substances and compounds. Many studies suggest to use pig or mini pig as a new animal model for humans with many advantages. Because of anatomical, physiological and biochemical similarity to humans, pigs are animals of choice for testing the different compounds to which humans are exposed. Similarity towards humans in heart, nasal cavity, liver, kidney, brain, reproductive and gastrointestinal system favors pigs more for in vitro and in vivo experiments rather than other animals (Puccinelli et al. 2011).

As the pigs are largely used in medical studies and research so they can also be a useful model to study the biotransformation of xenobiotics in humans (Matal et al. 2009). Pig CYP enzymes get importance to study as pig liver and hepatocytes to use as against the shortage of human organs in transplantation techniques and treatments. Pigs and minipigs could be good model species for pharmacology and toxicology studies with no need to induce biotransformation enzymes (Liu et al. 2011).

Pigs have been recognized as a model for human diseases like cardiovascular diseases (atherosclerosis), metabolic diseases (hypercholesterolemia) and neural diseases (Parkinson’s and Alzheimer’s). In the non-rodent species the generation of transgenic animals and xenotransplantation is well known in pigs (Puccinelli et al. 2011).

The in vitro metabolic inhibition studies gives information to predict the drug-drug interactions qualitatively and quantitatively inside the body. This quantitative in vitro metabolic data could be extrapolated to in vivo situations for competitive inhibition by help of appropriate pharmacokinetic principles (Zhou Z & Zhou S 2009).

Coumarin a plant derived natural product belongs to polyphenolic compounds and is well known for its pharmacological properties like anti-inflammatory, antibacterial, anti-viral, antifungal and antioxidant properties (Venuqopala et al 2013). Coumarin being a plant alkaloid being well known for its immunomodulatory and antitumour activity is metabolized mainly to 7-hydroxylated form by CYP2A6 in humans (Pelkonen et al. 2008), CYP2A5 in mice and in pigs by CYP2A19 (Kinonen et al. 1995; Skaanild & Friis, 2005; Puccinelli et al. 2011). Coumarin 7-hydroxylation is the most utilized activity marker for CYP2A6 in humans (Liu et al. 2011) and in humans liver coumarin 7-hydroxylation activity level is higher as
compared to pigs (Puccinelli et al. 2011). CYP2A6 is an important isoform of CYP involved in precarcinogen activation and oxidation of many important drugs inside human body. Five different benzo thiophene derivatives were tested as an inhibitor against pig liver microsomal coumarin 7-hydroxylation under the presence of NADPH and oxygen. Coumarin 7-hydroxylase activity has been identified in pigs as well as in conventional and minipigs (Liu et al. 2010). The method is based on the detection and measurement of fluorescence emitted by 7-hydroxy coumarin in alkaline condition (Fig. 8) (Aitio, 1978; Rahnasto et al. 2011).

3.1 Aims of the study

In the experimental part, I studied the inhibition of pig liver coumarin 7-hydroxylation with following benzothiophene derivatives:

- 1-Benzothiophene-3-carbaldehyde
- 1-Benzothiophene-3-ylmethylamine
- 1-Benzothiophene-5-ylmethylamine
- 1-Benzothiophene-5-carbaldehyde
- 5-chloro-1-benzothiophene-3-ylmethylamine

**Figure 8.** Oxidation of coumarin to 7-hydroxycoumarin.

Reaction occurs in the presence of CYP2A19 of Pig liver Microsomes and NADPH in Tris HCL buffer pH 7.4. CYP2A19 inhibiting compound decreases the catalytic efficiency and therefore fluorescence is reduced.
The aim was to

1. determine the IC$_{50}$ values of above listed benzothiophene derivative against pig liver coumarin 7-hydroxylation

2. study the effect of coumarin concentration on the IC$_{50}$ value of these compounds.

These experiments give us information about inhibition structure-activity relationship against pig coumarin 7-hydroxylation via CYP2A19.

**Table 1.** Chemical structures of inhibitory compounds

<table>
<thead>
<tr>
<th>Inhibitory compounds</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-benzothiophen-3-carbaldehyde</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>1-benzothiophene-3-ylmethylamine</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>1-benzothiophen-5-carbaldehyde</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>1-benzothiophen-5-ylmethylamine</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>5-chloro-1-benzothio-3-carbonitrile</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
</tbody>
</table>
3.2 General Experimental Procedure

3.2.1 Materials

3.2.1.1 Chemicals

- 1 M Tris-HCL buffer pH 7.4, Ultra-pure by MP Biomedicals France.
- 50 mM Tris-HCL by MP Biomedicals France
- 30 % Trichloroacetic acid (TCA) by Sigma Aldrich Germany
- 10 mM Coumarin/ 1mM coumarin/ 25 µM coumarin by Sigma Aldrich Germany
- 10 mM 7-hydroxycoumarin standards by Sigma Aldrich Germany
- Pig liver microsomes
- 100% DMSO from J.T.Baker, Holland.
- 10 mM inhibitor stock solution of compound in 100 % DMSO
- NADPH regenerating system (Prepared by department laboratory technician)
- Water (H$_2$O) was purified using MAXIMA USF ELGA instrument at 18.2 MΩ.
- 100 % ethanol was obtained from Altia Oy Finland.

3.2.1.2 Biological material

Microsomal fractions were prepared from three different pigs’ livers by differential centrifugation as described in Lang and Nebert (1981), by the department’s laboratory technician on the 4.06.2007. They were collected from the fridge stored at -80°C for the assays. The total protein concentration (mg/ml) of microsomal preparations was determined with the bicinchoninic acid (BCA) protein assay kit.

3.2.2 Inhibition assay of Coumarin 7-hydroxylation

3.2.2.1 IC$_{50}$ value determination

Assay of 7-hydroxylation activity is based on fluorescence activity emitted by 7-hydroxycoumarin in alkaline condition observed in spectrofluorometer. 96-well plate format
was used for inhibition analysis. Different concentrations of inhibitors and standard (7-hydroxycoumarin) were run in duplicate manner in 96 well plates.

96 well plate with parafilm on the base was kept on the ice in the start of the experiments. Chemicals listed table 2 were pipetted in the wells of plate. Final concentration volume in (table 2) reaction well of 100 µl of incubation volume contained 5 µl of 20 % DMSO, 5µl of 1 M Tris-HCL, 1 µl of 25 µM coumarin, 5 µl of diluted pig liver microsomes, 59 µl of water and 25 µl of NADPH regeneration system was added in order to start the reaction.

**Table 2. Experimental protocol to determine IC50-value for 1-benzothiophene-3-carboxyaldehyde.**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Reaction</th>
<th>Blank</th>
<th>Blank compound</th>
<th>Inhibition by a compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-HCl pH 7.4</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>25 µM coumarin</td>
<td>1 µl</td>
<td>1 µl</td>
<td>----</td>
<td>1 µl</td>
</tr>
<tr>
<td>1/20 pig liver microsomes</td>
<td>5 µl</td>
<td>----</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Water</td>
<td>59 µl</td>
<td>59 µl + 5 µl</td>
<td>59 µl + 1 µl</td>
<td>59 µl</td>
</tr>
<tr>
<td>Inhibitory compound of different conc.</td>
<td>----</td>
<td>----</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>20% DMSO</td>
<td>5 µl</td>
<td>5 µl</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>NADPH regenerating system</td>
<td>25 µl</td>
<td>25 µl</td>
<td>25 µl</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

To check the inhibition potency of 5 µl inhibitory compounds of different concentrations (0-100 µM) was pipetted to above listed incubation mixture. The plate was pre warmed for 5 min at 37°C in the incubator. Then the reaction was started by pipetting 25 µl of NADPH
regenerating system. After 20 minutes incubation at 37 °C the reaction was stopped by adding 10 µl of 30 % TCA to all wells of reaction, blank and inhibition. Two different pairs of blank wells were prepared by omitting coumarin, DMSO or inhibitory compound. 100 µl of 7-hydroxycoumarin standards (0, 0.010 µM, 0.025 µM, 0.050 µM, 0.100 µM, 0.250 µM, 1.0 µM) and 10 µl of 30 % TCA were pipetted into duplicate pattern in different wells.

Just before the measurement 140 µl of 1.6 M glycine pH 10.4 buffer was added to all wells. Fluorescence was measured with VICTOR2™ plate counter fluorescence spectrophotometer (Perkin Elmer Life Sciences, Wallac, Turku, Finland). Coumarin program with excitation 355 nm and emission 450 nm wavelengths was used.

3.2.2.2 Effect of coumarin concentration (substrate) on IC$_{50}$ value

To check the effect of coumarin concentration on the IC$_{50}$ value of inhibitory compounds, the above described procedure was done at (3.125 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM and 100 µM) coumarin concentrations. Fluorescence activity was measured for each sample and results were plotted to see the effect of increasing substrate concentration on the IC$_{50}$ values of chemical inhibitors.

3.2.3 Data analysis.

IC$_{50}$ of all five benzothiophene derivatives were calculated for three different pig liver microsomes catalyzed coumarin 7-hydroxylation using equation (1) and Microsoft excel 2007 (figure 4).

\[
(1) \quad \frac{V_i}{V_0} = \frac{1}{1+\frac{[I]}{IC_{50}}} 
\]

Where

\( V_i \) represents the reaction rate with inhibitor. \( V_0 \) represents the reaction rate without inhibitor, \([I]\) is the concentration of inhibitor and IC$_{50}$ is the concentration of the inhibitor producing 50% inhibition.

Mean IC$_{50}$ and standard deviation of three different pig coumarin 7-hydroxylation inhibition experiments were calculated.
Ki values of three most potent inhibitory compounds were calculated by equation (Cheng and Prusoff 1973).

\[
Ki = \frac{IC_{50}}{1 + \frac{[S]}{Km}} + \frac{E}{2}
\]

Where IC\textsubscript{50} is the concentration of the inhibitor producing a 50% inhibition, Ki is the equilibrium dissociation constant of the inhibitor. (S) is the substrate concentration used and Km is the Michaelis constant of the coumarin (24 µM) for pig liver coumarin 7-hydroxylation (Pig as a test animal in toxicity studies) Faculty of Health Sciences, School of Pharmacy, Master’s thesis 2010.

When S = 0 and E is in nM,

then

\[Ki \approx IC_{50}\]

**Figure.9** A dose-response curve for model and experimental values of inhibition of pig liver coumarin 7-hydroxylation by 1-benzothiophen-3-carbaldehyde. Blue and red series represent model and experimental values, respectively.
3.3 Results

3.3.1 IC\textsubscript{50}-values of inhibitors.

The inhibitory potency of benzothiophene derivatives were tested towards pig coumarin 7-hydroxylase activity in a concentration-response relationship experiment (Table 3, fig. 10-14). All five compounds with different concentrations were tested in concentration-response manner with three different pig liver microsomes. Among all tested compounds the 5-chloro-1-benzothio-3-carbonitrile was the least potent with IC\textsubscript{50} value 50 µM. While the most potent compound found to be thianaphthene-3-carboxaldehyde IC\textsubscript{50} value 2.4 µM.

Table 3. IC\textsubscript{50} values of Benzothiophene derivatives towards pig liver microsomal coumarin 7-hydroxylation.

<table>
<thead>
<tr>
<th>Inhibitory Compounds</th>
<th>Pig # 4 (IC\textsubscript{50} µM)</th>
<th>Pig # 10 (IC\textsubscript{50} µM)</th>
<th>Pig # 11 (IC\textsubscript{50} µM)</th>
<th>Mean (IC\textsubscript{50} µM)</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-benzothiophen-3-carbaldeyde</td>
<td>1.85</td>
<td>2.57</td>
<td>2.74</td>
<td><strong>2.4</strong></td>
<td>± 0.5</td>
</tr>
<tr>
<td>1-benzothiophene-3-ylmethylamine</td>
<td>3.25</td>
<td>1.49</td>
<td>3.14</td>
<td><strong>2.6</strong></td>
<td>± 1.0</td>
</tr>
<tr>
<td>1-benzothiophen-5-carbaldeyde</td>
<td>5.05</td>
<td>7.30</td>
<td>3.38</td>
<td><strong>5.3</strong></td>
<td>± 2.0</td>
</tr>
<tr>
<td>1-benzothiophene-5-ylmethylamine</td>
<td>28.83</td>
<td>51.90</td>
<td>44.43</td>
<td><strong>42</strong></td>
<td>± 12</td>
</tr>
<tr>
<td>5-choro-1-benzothio-3-carbonitrile</td>
<td>44.07</td>
<td>59.01</td>
<td>45.85</td>
<td><strong>50</strong></td>
<td>± 8.0</td>
</tr>
</tbody>
</table>
Figure 10. Inhibition of pig liver coumarin 7-hydroxylation by 1-benzothiophene-3-carboxaldehyde. Blue and red series represent model and experimental values, respectively.

Figure 11. Inhibition of pig liver coumarin 7-hydroxylation by 1-benzothiophene-3-ylmethylamine. Blue and red series represent model and experimental values, respectively.
**Figure 12.** Inhibition of pig liver coumarin 7-hydroxylation by 1-benzo thiophen-5-carbaldehyde. Blue and red series represent model and experimental values, respectively.

**Figure 13.** Inhibition of pig liver coumarin 7-hydroxylation by 1-Benzo thiophen-5-ylmethylamine. Blue and red series represent model and experimental values, respectively.
Figure 14. Inhibition of pig liver coumarin 7-hydroxylation by 5-chloro-1-benzothio-3-carbonitrile. Blue and red series represent model and experimental values, respectively.

3.3.2 Effect of coumarin concentration on IC$_{50}$

To check the effect of substrate on the inhibitory potency of benzothiophene derivatives, three most potent benzothiophene derivatives were tested at different concentrations of coumarin. All samples were run in duplicate manner. Two different pig liver microsomes were used to get results. IC$_{50}$ values were identified and calculated. Increase in IC$_{50}$ values with increasing substrate concentration (Table 4–6, fig. 15-17) showed competitive inhibition manner of tested benzothiophene compounds, as the IC$_{50}$ value increased linearly with coumarin concentration. Thus tested inhibitors proved to be competitive inhibitor for CYP2A19 catalyzed coumarin 7-hydroxylation of pig liver microsomes.
Table 4. IC50 value of 1-benzothiophene-3-carbaldehyde at different coumarin concentrations.

<table>
<thead>
<tr>
<th>Pig # 4</th>
<th>Pig # 11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coumarin conc. [µM]</strong></td>
<td><strong>IC50 [µM]</strong></td>
</tr>
<tr>
<td>6.25</td>
<td>2.56</td>
</tr>
<tr>
<td>12.5</td>
<td>3.01</td>
</tr>
<tr>
<td>25</td>
<td>3.65</td>
</tr>
<tr>
<td>50</td>
<td>4.30</td>
</tr>
<tr>
<td>100</td>
<td>7.60</td>
</tr>
</tbody>
</table>

\[y = 0.0519x + 2.2142\]

\[R^2 = 0.9788\]
Figure 15. The effect of coumarin on IC50 value of 1-benzothiophene-3-carbaldehyde against pig liver coumarin 7-hydroxylatation.

Table 5. IC50 value of 1-benzothiophen-3-ylmethylamine at different coumarin concentration.

<table>
<thead>
<tr>
<th>Pig # 4</th>
<th>Pig # 11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coumarin conc. [µM]</strong></td>
<td><strong>IC50 [µM]</strong></td>
</tr>
<tr>
<td>6.25</td>
<td>2.26</td>
</tr>
<tr>
<td>12.5</td>
<td>2.94</td>
</tr>
<tr>
<td>25</td>
<td>3.46</td>
</tr>
<tr>
<td>50</td>
<td>5.17</td>
</tr>
<tr>
<td>100</td>
<td>9.47</td>
</tr>
</tbody>
</table>

\[ y = 0.0441x + 1.3209 \]

\[ R^2 = 0.9583 \]
Fig 16. The effect of coumarin on IC₅₀ value of 1-benzothiophene-3-ylmethylamine against pig liver coumarin 7-hydroxylation.
Table 6. IC\textsubscript{50} value of 1-benzothiophen-5-carbaldehyde at different coumarin concentration.

<table>
<thead>
<tr>
<th>Pig # 4</th>
<th>Pig # 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin conc. [µM]</td>
<td>IC\textsubscript{50} [µM]</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>6.09</td>
</tr>
<tr>
<td>50</td>
<td>9.46</td>
</tr>
<tr>
<td>100</td>
<td>20.18</td>
</tr>
</tbody>
</table>

\[y = 0.1917x + 0.73\]
\[R^2 = 0.9895\]
3.3.3 Ki values

Ki values were calculated by the equation of Cheng and Prusoff (1973) and are listed in table 7.

\[
Ki = \frac{IC_{50}}{(1 + [S/Km]) + E/2}
\]

Table 7. Ki values of tested chemical inhibitors (Benzothiophene derivatives)

<table>
<thead>
<tr>
<th>Chemical inhibitor</th>
<th>Ki (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-benzothiophene-3-carbaldehyde</td>
<td>2.21</td>
</tr>
<tr>
<td>1-benzothiophene-3-ylmethylamine</td>
<td>1.73</td>
</tr>
<tr>
<td>1-benzothiophene-5-carbaldehyde</td>
<td>0.91</td>
</tr>
</tbody>
</table>
3.4 Discussion.

Coumarin a commonly used experimental prodrug is used widely to investigate the enzyme activity in humans, mouse and pigs. In humans the 7-hydroxylation of coumarin is catalyzed by human CYP2A6 (Rahnasto et al. 2011). Coumarin 7-hydroxylation activity has been detected also in conventional pigs and mini pigs. In pigs coumarin 7-hydroxylation is catalyzed by CYP2A19 (Skaanild & Friis 2005; Liu et al. 2011). Therefore coumarin 7-hydroxylation activity and its inhibition reflects the catalytic capability of CYP2A19 enzyme in pigs. Inhibition of individual pig CYP enzymes by selective chemical inhibitors is very limited in pig liver microsomes. In the experimental part I examined the inhibition potency of five chemical inhibitors (benzothiophe derivatives) to inhibit pig liver microsomes catalyzed coumarin 7-hydroxylation. The main aim of the study was to find out the inhibition interaction, mechanism and effect of substrate on inhibition of pig liver coumarin 7-hydroxylation by five benzothiophene derivatives and to calculate their IC$_{50}$ values. In humans and pigs larger interindividual differences in coumarin hydroxylase activity have been noted. For this reason the Vmax values for coumarin 7-hydroxylation could be different in different samples of microsomes (Liu et al. 2011). In my experiment pig liver microsomes were collected by three pig livers to ignore the inter-individual differences.

The IC$_{50}$ values of tested inhibitory compounds (1-benzothiophen-3-carbaldehye, 1-benzothiophene-3-ylmethylamine, 1-benzothiophen-5-carbaldehye, 1-benzothiophene-5-ylmethylamine and 5-choro-1-benzothio-3-carbonitrile) for pig CYP2A19 catalyzed coumarin 7-hydroxylation were calculated in my experiments and were as 2.4 µM, 2.6 µM, 5.3 µM, 42 µM and 50 µM, respectively. In another study (Rahnasto et al. 2011) all these compounds were tested against human CYP2A6 coumarin 7-hydroxylation and IC$_{50}$ values recorded were 1.6 µM, 1.5 µM, 0.9 µM, 1.7 µM and 41 µM respectively. The pig to human IC$_{50}$ ratio from both studies result were calculated and are 1.5, 1.7, 5, 21 and 1.2 and result showed that these chemical inhibitors are more potent for human CYP2A6. 1-benzothiophene-3-carbaldehyde, 1-benzothiophene-3-ylmethylamine and 5-chloro-1-benzothio-3-carbonitrile showed quite similar inhibition potency against pig and human coumarin 7-hydroxylation in mine and Rahnasto et al. 2011 experiments. The most potent inhibitory compound found in my experiments was 1-benzothiophene-3-carbaldehyde with IC$_{50}$ value of 2.4 µM and 1-benzothiophene-3-ylmethylamine with IC$_{50}$ value of 2.6 against pig liver microsomes, these
compound showed less inhibition potency toward pig CYP2A19 than human CYP2A6 and are proved to be more potent inhibitor for human CYP2A6 and being proposed as lead compound in the development of drugs in smoking reduction therapy (Rahnasto et al. 2011). The inhibitory compound 1-benzothiophen-5-ylmethylamine (IC$_{50}$ value in pigs 42 µM) showed greater inhibition potency toward human CYP2A6 coumarin 7-hydroxylation. Larger differences in inhibition potency of this compound was seen between human and pigs coumarin 7-hydroxylation.

The compound which showed least inhibitory potency was 5-choloro-1-benzothio-3-carbonitrile against pig and human coumarin 7-hydroxylation. 1-benzothiophen with methyl amine at position 3 is much more potent than 1-benzothiophen with methyl amine at position 5. So as the methyl amine position shifted from position 3 to position 5, huge decrease in inhibitory potency was seen in pig coumarin 7-hydroxylation reactions, but there is a little difference in inhibition potency of these two compounds against human CYP2A6 (Rahnasto et al. 2011). Carbaldehyde group at position 5 of 1-benzothiophene increases the inhibition potency as compared to methyl amine at position 5 of same 1-benzothiophene. Benzothiohene ring with carbaldehyde group on position 3 or on position 5 does not make a big difference in inhibition potency of compound in both pigs and humans. Tested benzothiophene derivatives showed competitive inhibition for the CYP2A19 of pig microsomes. The Ki values were calculate for three most potent inhibitory compound’s (1-benzothiophene-3-carbaldehyde, 1-benzothiophene-3-ylmethylamine and 1-benzothiophene-5-carbaldehyde) and were 2.21 µM, 1.73 µM and 0.91 µM. The Ki values calculated in these experiments could be used in elucidating the extent to which pig CYP2A contributes to the metabolism of xenobiotics and new drugs by pig liver microsomes. The use of Kᵢ in the prediction of drug interactions for CYP inhibitors has been most frequent approach (Liu et al. 2011).

IC$_{50}$ values of 1-benzothiophene-3-carbaldehyde, 1-benzothiophene-5-carbaldehyde and 1-benzothiophene-3-ylmethylamine were below 10 µM. These derivatives were competitive inhibitors for CYP2A19 mediated pig coumarin 7-hydroxylation. In same study (Rahnasto et al. 2011) 1-benzothiophen-3-carbaldehyde, 1-benzothiophene-3-methylamine and 1-benzothiophene-5-ylmethylamine were found to be most potent inhibitor of CYP2A6 coumarin 7-hydroxylation in humans. This show the binding affinity of inhibitors is affected by electronic, hydrophobic and spatial properties of molecules. 1-benzothiophen-3-carbaldehyde an inhibitor has thiophene and aldehyde functional group, each of this group
has potential to inhibit CYP enzymes either irreversibly or become a more stable metabolite by slow isomerization process. Formation of epoxide intermediate, which can attack the heme or nitrogen atoms in the side chains of CYP, appears to be general mechanism behind the inhibitory properties of thiophene derivatives. On the other hand inactivation of CYP by aldehydes functional group favours the formation of free radicals that can form heme adducts and finally exert inhibition/ inactivation (Rahnasto et al 2011).

Knowledge about xenobiotic metabolizing enzyme provides ability to predict and understand drug-drug interactions. Xenobiotic causing reversible or irreversible inactivation of drug metabolizing enzymes causes increased exposure and bioavailability of other drugs. However in-vitro inhibitory potencies of irreversible CYP inhibitors do not necessarily relative extents of inhibition for in-vivo. Irreversible inhibition is of much importance in drug development and discovery (Zhou & Zhou, 2009). Effect of CYP inhibition on xenobiotic pharmacokinetics depends upon many factors like coadministered drug, regimen, dose, other inactivators with personal hepatic function and CYP genetics (Zhou & Zhou, 2009). Enzyme inhibition a biological process is of much importance to know and describe the nature of enzyme reaction, to check the effect of different parameters on the reaction rate and new developments in medicinal and bioengineering industry. Main objective behind in-vitro metabolic inhibition reactions is to prediction of in-vivo drug-drug interactions. In conclusion, the above experiments show and describe the inhibition potency and mechanism of inhibition of benzothiophene derivatives towards pig liver microsomal coumarin 7-hydroxylase catalysed by CYP2A19. The result obtained in these experiments could be used for QSAR modelling of CYP2A19. These benzothiophene derivatives showed similar or greater inhibition potency towards human CYP2A6 mediated coumarin 7-hydroxylation reported by Rahnasto et al. (2011).

This data could be base for further QSAR modelling and to predict the inhibition pattern of xenobiotics in humans.
4. References


33. Martikainen L (2012) In vitro and in silico methods to predict cytochrome P450 enzyme inhibition. School of pharmacy, Faculty of health sciences, University of Eastern Finland, Kuopio.


