2.1. Herb-Drug Interaction:

2.1.1. Introduction:

Herbal medicines are becoming popular worldwide, despite their mechanisms of action being generally unknown, the lack of evidence of efficacy, and inadequate toxicological data. An estimated one third of adults in developed nations and more than 80% of the population in many developing countries use herbal medicines. To date, there are more than 11 000 species of herbal plants that are in use medicinally and, of these, about 500 species are commonly used in Asian and other countries. These herbs are often co-administered with therapeutic drugs raising the potential of drug-herb interactions, which may have important clinical significance based on an increasing number of clinical reports of such interactions. The interaction of drugs with herbal medicines is a significant safety concern, especially for drugs with narrow therapeutic indices (e.g. warfarin and digoxin). Because the pharmacokinetics and/or pharmacodynamics of the drug may be altered by combination with herbal remedies, potentially severe and perhaps even life-threatening adverse reactions may occur. Because of the clinical significance of drug interactions with herbs, it is important to identify herb drug interaction.

2.1.2. Mechanisms for drug interactions with herbal medicines:

The underlying mechanisms for most reported drug interactions with herbal medicines have not been fully elucidated. As with drug-drug interactions, both pharmacokinetic and pharmacodynamic mechanisms are implicated in these interactions (Figure 1.1). Alterations in absorption, metabolism, distribution or excretion of drugs are the cause of pharmacokinetic interactions. Altered drug metabolism by herbal medicines is often a result of cytochrome P450 (CYP) induction and/or inhibition [1]. The most well studied and understood example of this is the induction of CYP3A4 and CYP2B6 by St John's wort in humans [2-5]. Of the components of St. John's wort, hyperforin is purported to be the active constituent and it is the most potent agonist for pregnane X receptor (PXR) with a Ki of 27 nM [2]. Because of the important role of P-glycoprotein (P-gp) in drug transport and excretion, modulation of P-gp by herbal medicines may have significant pharmacokinetic consequences [6]. St John's wort induces intestinal P-gp in vitro and in vivo [7-9]. Oral administration of St John's wort for 14 days in healthy volunteers resulted in a 1.4-fold increase in P-gp expression [7]. The
substrates of P-gp, fexofenadine and digoxin, which are often used as probes for examining P-gp activity in vivo, were found to have increased clearance in healthy subjects treated with St John’s wort [10]. However, there is rare clinical evidence for altered protein binding of drugs by herbal medicines. Given that many herbal components are highly bound by plasma proteins, they may displace the drugs from the binding sites [11]. Herbal medicines are often administered orally and they can attain moderate to high concentrations in the gut lumen (the primary site of absorption for most orally-administered drugs) and liver, and may exert a significant effect on enterocytes and hepatocytes. Both P-gp and CYP3A4 are abundantly expressed in the villus tip of enterocytes and hepatocytes [12]. The interplay of both intestinal P-gp and CYP3A4 has a strong effect on the bioavailability of most orally administered drugs including cyclosporine, midazolam, talinolol, statins, HIV protease inhibitors and verapamil [12]. Thus, the modulation of intestinal and hepatic P-gp and CYP3A4 by herbal medicines represents a potentially important mechanism by which the bioavailability of co-administered drugs can be modulated. Altered pharmacokinetics almost inevitably leads to a significant change in response to drugs that have narrow therapeutic indices; however, given that a single herbal preparation may contain more than 100 components, all of which may have unknown biological activities, a herb can potentially mimic, increase, or reduce the effects of co-administered drugs through simultaneous effects on the same drug targets (Figure 1.1) [13]. If the effect of the drug in combination with the herbal medicine is enhanced (e.g. synergistic or additive effect), unfavourable on-target toxicity may occur. By contrast, some herbal remedies may contain compounds with antagonistic properties, which are likely to reduce drug efficacy and produce therapeutic failure. The synergistic or antagonistic effects between herbs and drugs often result from the competitive or complementary effect of the drug and the co-administered herbal constituents at the same drug targets [13].

2.1.3. Herbal products contain several chemicals:

There is a general belief among the general public that herbal preparations are “good for humans” as they are “all natural” [14]. There is little public understanding or appreciation of the fact that these “all natural” extracts are actually a combination of potentially biologically active compounds that exist in these marketed products in unknown quantities. Marketed herbal products contain structurally diverse chemicals and several of them possess inherent
pharmacological activity and some may even be toxic. Since components of herbal products consumed must also be eliminated from the body by the same mechanism that removes drugs, there is a potential for interaction between herbal components and drugs. Among the more popular herbal products used worldwide and in the U.S. are St. John's wort, used for its reported antidepressant activity, and milk thistle, used clinically because of its hepatoprotective properties [15, 16]. St. John's wort accounted for 15 million U.S. dollars in sales in 2003, making it the seventh highest grossing botanical supplement [17]. Several clinical studies have demonstrated the potential benefits of St. John's wort compared with conventional therapy in the treatment of mild to moderate depression [18, 19]. Studies conducted in vitro and in animals have shown that St. John's wort constituents inhibit the reuptake of neurotransmitters linked to depression in humans [20]. Marketed St. John's wort, an extract of the flowering portion of the plant Hypericum perforatum L., is a mixture of a number of biologically active, complex compounds (Figure 2.1.1). At 0.3 mg per capsule, the napthodianthrone, hypericin is used as a means of standardization of the marketed product. The phloroglucinol, hyperforin, the most plentiful lipophilic compound in the extract, is a potent inhibitor of serotonin, norepinephrine and dopamine reuptake. In addition other components such as pseudohypericin, adhyperforin, biapigenin, quercetin, quercitrin, isoquercitrin, hyperoside and rutin are also present in the marketed extracts. Milk thistle [Silybum marianum (L.) Gaertn. (Fam. Asteraceae)] extract is one of the most commonly used nontraditional therapies, particularly in Germany. In accordance with the Dietary Supplement Health and Education Act (DSHEA) legislation, it is marketed in the U.S. as a dietary supplement that "promotes liver health." The annual sale of this product is about $180 million in Germany alone [21]. In the U.S., milk thistle is the 11th most popular herbal product in retail sales with an annual increase of almost 10% [17]. Clinically, milk thistle is being studied as a therapy in the treatment of prostate cancer and has been used in the treatment of a variety of liver disorders. Milk thistle is known to contain a number of flavonolignans, compounds that are produced in plants by radical coupling of a flavonoid and a phenylpropanoid [22]. A mixture of these flavonolignans, termed silymarin, is known to be composed of mainly silybin (about 50–70%), isosilybin, silychristin, silydianin, toxifolin and other closely related flavonolignans [23]. A standardized extract of milk thistle contains at least 70% silymarin [24, 25]. Currently, valerian is among the ten most widely used herbal
supplements. Historically, valerian extract has been used as a sedative, antispasmodic, carminative and mild analgesic. It has been used to treat insomnia and nervous disorders. The commercially used preparation is an extract of the roots and rhizomes from Valeriana officinalis. Valerian extracts contain valerenic acid and its derivatives (acetoxyvalerenic acid, hydroxyvalerenic acid), valepotriates, valtrate, valerenal and others and are often standardized to the content of valerenic acid. It is clear that structurally very diverse chemicals are present in herbal extracts. These chemicals must be eliminated from the body either through the kidney or through the liver.
Chapter 2: Review of Literature

Figure 2.1.1. Chemical structure of major components of St. John’s wort

Quercetin
R = H

Quercitrin
R = L-rhamnosyl

Isoquercitrin
R = D-glucosyl

Hyperoside
R = D-galactosyl

Rutin
R = rutinosyl

Hypericin
R = CH₃

Pseudohypericin
R = CH₃OH

Hyperforin
R = H

Adhyperforin
R = CH₃
2.1.4. Herbal constituents are substrates for drug metabolizing enzymes:

Conventionally, drug metabolism is broadly divided into phase I and phase II processes [26]. Phase I processes include oxidation, reduction, hydrolysis and hydration resulting in the formation of functional groups (OH, SH, NH2 or COOH) that impart the metabolite with increased polarity compared to the parent compound. Of the phase I processes, the CYP super family is responsible for the metabolism of a variety of xenobiotics and endobiotics [26]. Human CYP isoforms that are involved in the biotransformation of xenobiotics include CYP1A1/2, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1, CYP3A4/5 and CYP4A [26, 27]. Phase II processes include sulfation, methylation, acetylation, glutathione conjugation, fatty acid conjugation and glucuronidation [26]. The latter is catalyzed by uridine diphosphoglucuronosyltransferases (UGTs) and involves the transfer of the glucuronic acid residue from uridine diphosphoglucuronic acid to a hydroxy, either phenolic or alcoholic, or a carboxylic acid group on the compound [28]. In humans, 16 different UGT isoforms have been classified into either 1A or 2B subfamilies [29]. They metabolize a broad range of endogenous and exogenous substances with significant overlap in substrate specificity between isozymes [30]. Among the UGT1A family, UGT1A1 is most notably involved in the glucuronidation of bilirubin but also metabolizes estradiol, acetaminophen and the active metabolite of irinotecan, SN-38 [31-33]. UGT1A6 and UGT1A9 metabolize short planar phenols including catechols, acetaminophen, and 4-methylumbelliferone [32-33]. Hydroxylation mediated by CYP3A and CYP2B enzymes is the primary pathway of metabolism of hyperforin, the major component in St. John's wort extract. Silybin is primarily glucuronidated in the liver at the 7 and 20 position with the latter preferred over the former [34]. The precise UGT isoform responsible for the metabolism of silybin is currently unknown. The primary route of elimination is hepatic with both the parent and conjugate excreted into the bile accounting for 2–12% of the ingested dose [35, 36]. CYP enzymes play a minor role in the metabolism of silybin. Valerenic acid (valerian extract) is also metabolized by glucuronidation. The exact UGT isoform that is responsible for the glucuronidation of valerian is not known at this time.
2.1.5. Herbal components are also substrates for certain hepatic and extra hepatic transporters:

Hepatobiliary transport processes and subsequent bile flow serve vital roles in the maintenance of cholesterol and lipid homeostasis, the removal of endogenous and exogenous substances from the body, and adequate bile salt flow and recycling [37, 38]. Transport proteins located in the sinusoidal (basolateral) membrane of the hepatocyte enable compounds to gain access to intracellular drug metabolizing enzymes (Figure 2.1.2).

Following translocation across the cell or biotransformation, bile acids, drugs and/or their metabolites are actively secreted into canalicular spaces that exist between adjoining hepatocytes. Two classes of hepatic transporters are largely involved in the uptake and efflux of drugs, drug conjugates and endogenous substrates. They are broadly broken down into the solute carrier family (SLC) and the ATP binding cassette family (ABC). The organic anion transporters (OATP, SLC21 subfamily) are uptake carrier proteins that are involved in the Na-independent transport of a variety of structurally diverse compounds such as bromosulphophthalein, glycocholate, prostaglandin E2 and estradiol-17β-glucuronide, with new substrates still being discovered [39]. OATP-8 (SLC21A6) and OATP-C (SLC21A8) are the predominant members of this family located in the liver [40]. The hepatic uptake of bile salts is mediated by the liver specific sodium-dependent taurocholate co-transporting protein (NTCP) (SLC10A1) [41, 42]. While the substrate specificity of NTCP is narrow, including mainly bile salts, its activity is integral in ensuring the homeostatic vectoral movement of bile salts. Drugs and/or their metabolites often exit the liver through secretion into the bile. Because compounds must traverse a steep concentration gradient, efflux is mediated through the ATP utilizing ABC transporters. The transporters located in the canalicular membrane that have been described to date are multidrug resistance associated protein 2 (MRP2, ABCC2), bile salt export pump (BSEP, ABCB11), multidrug resistant protein 3 (MDR3, ABCB4), multidrug resistance protein 1 (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) (reviewed in [43]. BSEP, as with NTCP, is integral in the cellular handling of conjugated and unconjugated bile salts, such as taurocholate, via active secretion into the canalicular space [44]. MDR1 is the most extensively studied ABC transporter and is responsible for the transport of a wide variety of compounds usually containing planar aromatic motifs and a molecular weight greater than 400 [45]. However, in contrast to
rodents, human hepatic expression of MDR1 is low compared to the intestine [46]. MRP2 is responsible for the biliary secretion of organic anions such as acetaminophen glucuronide, camptothecin, SN-38, bile salts, glutathione, glucuronide and sulfate conjugates [47, 48].

Chemical constituents in herbal products, similar to prescription drugs, are eliminated by various metabolic enzymes in the body and may be substrates for various transporters. The derivatives of the flavanolignan silybin display high in vitro affinities for direct binding to P-glycoprotein. The potential for involvement of drug metabolizing enzymes and transporters in the handling of herbal components leads to a predisposition of herb drug interactions. Such herb drug interactions may involve inhibition of drug metabolizing enzymes and/or drug transporters resulting in increased levels of one or both drugs leading to adverse drug reactions. Conversely, the induction of these same enzyme systems leads to a decrease in the overall body exposure to the drug creating a situation where the patient will be underdosed. It is of importance that potential drug–herb interactions be identified in order to prevent adverse outcomes in patients taking combinations of drugs and herbal supplements. Also, the identification of the mechanism involved in any such interaction will offer insight into the approaches to be taken to minimize their impact and to design appropriate studies in humans.

Figure 2.1.2. Uptake and efflux transporters in human liver
2.1.6. Approaches (in vitro and in vivo) to evaluate herb drug interactions:

Most research on drug-herb interactions has focused on the in vitro evaluation of herbal constituents in microsomal systems, supersomes, cytosols, expressed enzymes or cell culture systems such as transfected cell lines, primary cultures of human hepatocytes and tumor derived cells. In addition, studies have also been carried out in vivo in animals (normal, transgenic, humanized) and in humans (primarily healthy individuals). Most of the studies have used the commercially available products or a crude extracts of the herbal product or isolated purified individual components. These studies so far have paid particular attention to the effect of herbal components on CYP enzymes.

2.1.6.1. In vitro studies using microsomes:

 Constituents of St. John's wort, biapigenin, hypericin, hyperforin and quercetin have been documented to be competitive or non competitive inhibitors of several CYP enzymes [41]. However, hyperforin has been shown to be a potent inducer of h-PXR in a cells transfected with expression plasmid for h-PXR and CYP3A1-tk-CAT [49]. PXR regulates the expression of several enzymes and transporters such as CYP3A, CYP2C and p-glycoprotein. This observation would indicate that hyperforin will be an inducer of certain CYP enzymes. This has been actually documented in humans and in human hepatocyte cultures.

 The interaction of silybin with CYPs in microsomal system and the possibility of drug-herb interactions have only recently been shown [2]. Silybin noncompetitively inhibited CYP3A4 activity (IC50=29 μM; Ki=9 μM) and CYP2C9 activity (IC50=44 μM; Ki=19 μM) in liver microsomes [50, 51]. Interestingly, it has recently been shown that this inhibition may result from irreversible binding of a reactive intermediate to the heme moiety of both CYP3A4 and CYP2C9 in human liver microsomes [52]. Silymarin also inhibits certain hepatic enzymes such as aminopyrine demethylase, benzopyrene hydroxylase, hexobarbital hydroxylase, and ethoxy coumarin O-deethylase in rats [53]. Using expressed liver enzymes, it was shown that silybin inhibited UGT1A1 (IC50=1.4 μM), UGT1A6 (IC50=28 μM), UGT1A9 (IC50=20 μM), UGT2B7 (IC50=92 μM) and UGT2B15 (IC50=75 μM) [52]. This suggests that the effect of silybin on glucuronidation will be isoenzyme specific and is most likely to occur with UGT1A substrates. Studies in microsomes while providing information on the potential
Chapter 2: Review of Literature

of a chemical to alter enzyme activity are limited in that they are useful only to evaluate acute inhibition of metabolism and not induction of metabolism as they are not intact cell systems. Furthermore, it is not possible to evaluate the effect of herbal components on transporters using microsomes. Since excess co-substrate is added in the system it is not possible to evaluate co-substrate depletion as a potential mechanism of any interactions. Microsomal studies also do not provide complete mechanistic information of any interactions (effects on m-RNA or protein and the potential role of any metabolite formed).

2.1.6.2. Primary cultures are valuable in vitro tools in evaluating herb drug interactions:

In selecting in vitro systems one must pay particular attention to the test system used. The use of more physiologically relevant in vitro models, such as primary cultures of human hepatocytes (PCHH), are necessary if better predictions of drug-herb interactions are to be made in humans. These systems will also facilitate determination of whether there is a need to conduct more demanding clinical studies. Primary cultures of human hepatocytes are viable for up to 2 weeks (or one month if placed in a three-dimensional culture) and retain all cofactors and co-substrates necessary for phase I and phase II metabolic pathways and transporter function, making them a versatile in vitro system to study induction and inhibition of drug metabolizing enzymes and certain transporters (Table 2.1) [54].

Table 2.1. Hepatocytes are valuable for metabolism and transport studies

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact system</td>
<td>Not readily available</td>
</tr>
<tr>
<td>Human Specific</td>
<td>Variable basal expression in different hepatocytes (need to run controls)</td>
</tr>
<tr>
<td>Animal sparing</td>
<td>May be costly</td>
</tr>
<tr>
<td>More physiologic (not engineered; no external co-factor needed)</td>
<td>Loss of activity in culture</td>
</tr>
<tr>
<td>Phase I and II metabolism present</td>
<td>Not all transporters can be efficiently characterized at this time</td>
</tr>
<tr>
<td>Transporters present</td>
<td></td>
</tr>
<tr>
<td>Induction and inhibition can be studied</td>
<td></td>
</tr>
<tr>
<td>Possible to get mechanistic information (mRNA, protein and activity can be simultaneously measured)</td>
<td></td>
</tr>
</tbody>
</table>
In human hepatocytes, treatment with hyperforin resulted in significant increases in mRNA, protein and activity of CYP3A4 and CYP2C9, but had no effect on CYP1A2 or CYP2D6. However, acute administration of hyperforin at 5 and 10 μM 1 h prior to, and along with probe substrate, inhibited CYP3A4 activity. Hypericin had no effect on any of the enzymes tested. These results demonstrate, while hyperforin on acute exposure at high concentrations can inhibit CYP3A4, with chronic exposure the inductive effect of St. John's wort on drug metabolizing enzymes predominates over any inhibition [55]. Furthermore, this observation points out that while human liver microsomes are a valuable tool to evaluate large numbers of herbal constituents in a cost and time efficient manner, such results must be interpreted with caution. It is also important to realize that certain chemicals can have a dual effect based on their concentrations and the effect may depend on acute or chronic exposure. Silymarin was first shown to decrease the expression of CYP3A protein and CYP3A activity in human hepatocyte cultures [56]. Since components of silymarin are known to be conjugated they were expected to alter glucuronide conjugation of other drugs and endogenous chemicals. Silymarin is known to deplete the pool of uridine diphosphoglucuronic acid (UDPGA) in hepatocytes and decrease glucuronidation of bilirubin in rats [57]. Silybin (0–300 μM), when added simultaneously with SN-38, acetaminophen or testosterone, inhibited glucuronidation of SN-38 by UGT1A1 (IC50=12.4 μM). When applied to PCHH for 3 or 6 days, silybin (0–100 μM) did not alter enzyme mRNA expression or protein content for UGT1A1 [55]. The reduction in UGT1A activity in human hepatocyte can be partially compensated by the addition of the co-substrate uridine diphosphoglucuronic acid suggesting co-substrate depletion as a potential component in this interaction. Inhibition of the UGT1A1 mediated metabolism of SN-38 by silybin indicates a potential for an interaction of silybin with irinotecan, a topoisomerase inhibitor used to treat a variety of solid tumors. Given the adverse events associated with elevated SN-38 levels, patients taking milk thistle should be monitored for increased incidence of neutropenia and diarrhea. Interestingly, certain herbal components such as chrysin can increase the activity of UGT1A1 as documented in HepG-2 cells, indicating the potential of increased clearance of certain drugs undergoing glucuronide conjugation, in presence of certain herbal products. Increased understanding of how the liver handles compounds, which involve the sinusoidal uptake and canalicular efflux along with phase I and phase II metabolic pathways, has shown that these processes do not occur
independently, but are rather interconnected by similar regulatory elements and substrates and through a complex network of feedback mechanisms.

2.1.6.3. In vivo studies:

In vivo studies in humans have been carried out with various experimental designs. Typically subjects receive a single dose of a test drug or a cocktail of drugs that are markers for various enzymes on day 1. This is followed by multiple daily dose treatment with the herbal product (typically one week) and on the last day of treatment, administration of the test drug or the cocktail of drugs. A comparison of the various pharmacokinetic parameters or phenotypic measures is used as a method to evaluate the effect of herbal products on the pharmacokinetics of test drug or activity of various drug metabolizing enzymes. Several reports have documented decreased blood/plasma levels of CYP3A4 substrates, such as digoxin, indinavir, cyclosporine A and imatinib, in patients concomitantly taking St. John's wort [58-61]. Additional in vivo evidence has demonstrated that St. John's wort increased CYP3A4 and P-gp protein levels in rats [62]. St. John's wort extract containing low content of hyperforin appear not to alter the activity of CYP1A2, CYP2C9, CYP3A and MDR1 [63].
Chapter 2: Review of Literature

2.1.7. References:


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


2.2. Phytoestrogens:

2.2.1 Introduction:

Phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian estrogens and therefore are considered to play an important role in the prevention of cancers, heart disease, menopausal symptoms and osteoporosis [1-3]. The diverse biological activity of phytoestrogens is due in part to their ability to act estrogenically as estrogen agonists and antiestrogenically as antagonists. As estrogen agonists, phytoestrogens mimic endogenous estrogens and cause estrogenic effects. As estrogen antagonists, they may block or alter estrogen receptors (ER) and prevent estrogenic activity, causing antiestrogenic effects [4]. As estrogen agonists and antagonists, phytoestrogens can also be classified as selective estrogen receptor modulators (SERMs) [4]. SERMs are non-steroidal chemicals with a similar structure to 17β-estradiol (E2) and an affinity toward estrogen receptors [5]. They are unique in that they can function as agonists or antagonists depending on the tissue, ER and concentration of circulating endogenous estrogens [6]. Tamoxifen and raloxifene are well-known SERMs. Tamoxifen has been used in clinical practice for breast cancer patients because it acts as an estrogen antagonist in breast tissue, slowing cancer cell proliferation and an estrogen agonist in bone tissue and in the cardiovascular system to prevent osteoporosis and heart disease. However, tamoxifen has shown estrogenic activity in the uterus and therefore may increase the risk of endometrial cancer [7, 8].

Mechanistically phytoestrogens have been shown to bind to two types of estrogen receptors: estrogen receptor α (ERα), which was cloned in 1986, and estrogen receptor β (ERβ) cloned in rats [9] and in humans [10]. The two receptors differ in their tissue distribution and affinity to ligands, yet there is some overlap. In rats, ERα and ERβ both are clearly expressed in ovary and uterus tissue [11]. ERβ has been shown to have ligand specificity toward phytoestrogens and is distributed in humans in ovary, spleen, testis and thymus tissue [10] and in rats in bladder, brain, lung, ovary, prostate, testis and uterus tissue [11]. Phytoestrogens show a lower binding affinity than E2 and some show a higher binding affinity for ERβ than for ERα, which may suggest different pathways for their actions and explain tissue specific variability of phytoestrogenic action [12]. The complexity of phytoestrogens and ERs appears to be further compounded because different transcriptional activities in vitro are activated...
depending on the ligands, as well as the environment of the promoter region of specific genes for translated ERα and ERβ receptors [13].

2.2.2. Classification of Phytoestrogens:

Phytoestrogenic compounds occur in many different plants [14], mainly in legumes [15]. Soy is the major dietary source of phytoestrogens but it contains a smaller number of estrogenically active substances compared to red clover. The three main categories of phytoestrogens are isoflavones, lignans and coumestans (Figure 2.2.1.). They are contained in high concentrations in red clover and in soybean and can be absorbed from nutrients in the gut. Red clover contains the isoflavones genistein, daidzein, biochanin A and formononetin, whereas soy only contains two of those compounds (genistein and daidzein) and additionally glycitein [16]. Other classes of phytoestrogens that have been reported include: anthraquinones [17], chalcones [18], flavones [19], flavonols, prenylflavonoids [20] and saponins [21]. Figure 2.2.2. shows the structures of phytoestrogens. Out of these phytoestrogens, isoflavones have received the most attention regarding their food sources, absorption, metabolism and human health effects.

Figure 2.2.1. Classification of phytoestrogens
Figure 2.2.2. Structures of phytoestrogens
2.2.3. Isoflavones:

Isoflavones are the most well-known of the phytoestrogens. Naturally occurring isoflavones that have shown estrogenic activity are: the aglycones, daidzein (4',7-dihydroxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone); the glycosides, daidzin and genistin; and biochanin A and formononetin, 4'-methyl ethers of daidzein and genistein [22, 23]. In plants, they can often be found as glycosides [24]. In processing, isolation and analysis, these compounds are readily degraded chemically or enzymically to the aglycone [22]. Glycitein is another isoflavone reported in soy that has also shown estrogenic activity. After mammals consume isoflavones, are metabolized in the gastrointestinal tract. Biochanin A and formononetin can metabolize to genistein and daidzein respectively. Daidzein may be further metabolized to dihydroidaidzein and then to O-desmethylandangolensin (O-DMA) and equol [23]. Equol is not metabolized equally in all humans and an individual’s ability to transform soy isoflavones into equol may offer an explanation for the varied results of present phytoestrogen studies. Genistein metabolizes to dihydrogenistein and then to 6'-hydroxy-O-DMA [23] and hormonally inert p-ethylphenol in sheep and humans [22, 24]. These new compounds produced from metabolism may have different biological effects than the original isoflavones digested [25]. Isoflavones are primarily found in the Fabaceae family, which has food legumes such as soy, peanut (Arachis hypogaea L.) and clover (Trifolium spp.). Soy seeds show high levels of formononetin and biochanin A (both 729 μg/g dry weight) [24]. Other food sources of isoflavones are oilseeds and nuts, such as the sunflower seed (Helianthus spp., Asteraceae) and walnut (Juglans nigra L., Juglandaceae) from different botanical families [26, 27]. They are primarily extracted from soy and red clover. Raw soybeans contain 1.2–4.2 mg/g dry weight of isoflavones, while high protein soy ingredients like soy flour contain 1.1–1.4 mg/g dry weight [23].

2.2.4. Human health and phytoestrogens:

2.2.4.1. Breast cancer

In western countries, breast cancer is the most common cancer affecting women [28]. Historically, the risk of breast cancer was much higher in American women than in Asian women prior to the influence of the western diet in Asian cultures [29]. Epidemiological
studies of breast cancer and the dietary intake of soy and lignan have recently been reviewed [30], as well as the mechanisms of phytoestrogenic action in breast tissue [31]. One task has been to find an estrogen replacement therapy for women at risk for breast cancer or who have survived breast cancer. A diet rich in phytoestrogens has been suggested as a preventative agent against breast cancer although there is conflicting evidence [32, 33]. Phytoestrogens act as weak estrogens and exhibit estrogenic activity in a low-estrogen environment; therefore it has been postulated that they show antiestrogenic activity in a high-estrogen environment [34]. This explanation suggests that prior to menopause when there is a high-estrogen environment phytoestrogens may protect against breast cancer and after menopause when there is a low estrogen environment they may stimulate breast cancer [35]. This theory is highly debated and many studies show conflicting evidence about the action of phytoestrogens and breast cancer.

2.2.4.2. Osteoporosis/bone health

After menopause bone density in women often is reduced due to ovarian hormone deficiency and many patients suffer from osteoporosis. Hormone replacement therapy (HRT) is an effective tool to reduce postmenopausal osteoporosis in women. Also phytoestrogens that can act as SERMs can mediate these beneficial effects on bone density [36]. Osteoporosis is caused by an imbalance between bone formation and bone resorption, with more resorption than formation. Rassi et al. [37] investigated the effects of daidzein and 17β-estradiol on the development and activity of cells that resorb bone (osteoclasts) in vitro. Daidzein could inhibit osteoclast differentiation and activity in the same degree as 17β-estradiol. This could be due to an increase in apoptosis of osteoclast progenitors mediated by ERs. In contrast to these findings other studies report a phytoestrogen-mediated stimulation of osteoblasts (cells responsible for bone growth) rather than an inhibition of osteoclasts and thus a mechanism of action of isoflavones different from estrogens [38]. Estrogens exert an inhibitory effect on osteoclasts and do not stimulate osteoblast activity. Some studies about the effect of dietary phytoestrogens on the prevention of osteoporosis have been performed to date. There is evidence that the intake of soy, which contains isoflavones, has modest effects on bone [39]. According to Clifton-Bligh et al. [40] the effects of a red clover-derived preparation were similar to soy preparations.
2.2.4.3. Menopausal symptoms

The symptoms associated with menopause cause many women to seek medical solutions. Hormone replacement therapy has proven effective in the reduction of hot flushes, yet it is still controversial if HRT may be associated with increased risks of breast and endometrial cancer. Initial findings from the Women's Health Initiative (WHI) randomized controlled trial in which women received a daily dose of conjugated equine estrogen (0.625 mg) and medroxyprogesterone acetate (2.5 mg) have shown increased risks to benefits ratio [41]. The investigators detected increased risks for invasive breast cancer and coronary heart disease with the consumption of the combined hormone preparation after 5.2 years of average follow-up. Due to controversial evidence on HRTs, alternative therapies have been sought, such as phytoestrogens and SERMs. The mechanisms of action are still poorly understood because of the complex biological actions observed in phytoestrogens. As discussed before, the target tissue, the kinds of ERs and the concentration of endogenous estrogens are all factors that affect phytoestrogens activity at the cellular level. Other non-receptor mechanisms may also explain the biological effects of phytoestrogens on menopausal symptoms, such as antioxidation, blocking of enzymes involved in the biosynthesis of estrogen, inhibition of protein kinase which is part of intracellular signaling, and inhibition of 5α-reductase and aromatase [42].

2.2.4.4. Cardiovascular disease

The leading cause of death in women in industrialized nations is CHD. In menopause the risk of CHD greatly increases and it is proposed that this is due to the loss of estrogen [43]. Lipid profiles, vascular reactivity, cellular proliferation and thrombosis are factors that affect CHD and on which phytoestrogens have shown beneficial effects [35]. Mechanisms suggested to explain the prevention of cardiovascular disease and the reduction of atherosclerosis are: improvement of plasma lipid concentrations, reduction of thrombus formation such as inhibition of platelet action, improvement of systemic arterial compliance and antioxidant activity [44].
Chapter 2: Review of Literature

2.2.4.5. Cognition

Cognition and memory functioning have been reported to decrease around menopause, and therefore studies have investigated the association of ERT and cognition, as well as phytoestrogens and cognition [42]. However, limited studies are available on the effects of phytoestrogens on cognitive functioning. A study with female rats proposed that soy phytoestrogens function as estrogen agonists because they increased choline acetyltransferase and nerve growth factor messenger RNA in the frontal cortex and hippocampus. Other human studies have also suggested improved memory with dietary phytoestrogens [45, 46].
Chapter 2: Review of Literature

2.2.5. References:


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


2.3. Osteoporosis

2.3.1. Introduction:

Osteoporosis is an emerging medical and socioeconomic threat characterised by a systemic impairment of bone mass, strength, and microarchitecture, which increases the propensity of fragility fractures [1]. Bone mineral density (BMD) can be assessed with dual x-ray absorptiometry (DXA), and osteoporosis is defined by a T score of less than 2.5, i.e., more than 2.5 standard deviations below the average of a young adult. About 40% of white postmenopausal women are affected by osteoporosis and, with an ageing population; this number is expected to steadily increase in the near future [2–4]. The lifetime fracture risk of a patient with osteoporosis is as high as 40%, and fractures most commonly occur in the spine, hip, or wrist, but other bones such as the trochanter, humerus, or ribs can also be affected (Figure 2.3.1.)

![Common sites of fracture](image)

**Figure 2.3.1. Common sites of fracture**

2.3.2. Recent developments in bone biology:

In the past decade, the pathogenesis of osteoporosis has been linked to tissue, cellular, and molecular processes. Master signals that integrate various endocrine, neuroendocrine, inflammatory, and mechanical stimuli have been defined. At the cellular level,
communication and coupling between the main bone-cell types, the bone-forming osteoblasts and the bone-degrading osteoclasts, constitute the smallest functional unit. Several key molecules coordinate activities of osteoblasts and osteoclasts during bone remodelling. Detailed knowledge of the molecular and cellular players has created a new concept in bone pathophysiology.

2.3.2.1. Osteoclasts and bone resorption

Osteoclasts originate from haemopoietic stem cells and are closely related to monocytes and macrophages. Differentiation from osteoclast precursor to fully activated multinucleated osteoclast depends essentially on receptor activator of NF-κB ligand (RANKL), a member of the tumour necrosis factor (TNF) family, and the permissive role of macrophage-colony stimulating factor (M-CSF). RANKL, abundantly expressed by bone-forming osteoblasts, bone-marrow stromal cells, and T and B lymphocytes, activates its receptor, RANK, expressed on osteoclasts. After RANKL induced RANK stimulation, several key regulatory transcription factors and enzymes are induced to promote the differentiation, proliferation, multinucleation, activation, and survival of osteoclasts. The result is profound resorption of bone. Of note, mice with deletion of RANKL or RANK lack mature osteoclasts [5]. Osteoprotegerin (OPG) is a naturally occurring antagonist of RANKL [6]. In early menopause, the acute phase of oestrogen deficiency, RANKL expression by bone marrow stromal cells and lymphocytes increases and is associated with enhanced bone loss [7]. In addition to menopause, conditions in which suppression of sex hormones is therapeutically induced (eg, in men with prostate cancer or in women with receptor-positive breast cancer), are also associated with an activated RANKL/RANK pathway and enhanced bone resorption. Various hormones [8, 9] and inflammatory cytokines modulate osteoclast biology through the RANKL pathway. Additionally, immunological and malignant bone disorders that destroy bone locally are associated with high RANKL activity, including rheumatoid arthritis, [10] periodontal disease, [11] myeloma bone disease, [12] and osteolytic bone metastasis [13]. The src kinase is highly expressed in osteoclasts and mediates multiple pathways regulating osteoclast activity. Src-deficient mice have osteopetrosis because their osteoclasts do not have an intact ruffled border [14, 15].
Interestingly, the absence of src does not alter the number of osteoclasts and is associated with an enhanced rate of osteoblastic bone formation [16]. With their jelly-fish-like shape, motile cytoskeleton, and adhesion molecules such as integrins (Figure 2.3.2.), osteoclasts attach to bone and create a sealing zone on the bone surface, which provides a highly enriched acidic microenvironment. Cathepsin K is a key cystein proteinase of the mature osteoclast that degrades collagen and breaks down bone. Cathepsin K is a crucial determinant of resorptive activity by osteoclasts; bone of poor quality in which microcracks have accumulated is removed, and hole-like lacunae appear. Thus people without functioning cathepsin K have pycnodysostosis, a rare disease characterised by osteosclerosis, a dense, but brittle bone phenotype, short stature, and lytic lesions of the distal phalanges because of poorly functioning osteoclasts [17]. A more severe phenotype, osteopetrosis (so-called marble bone disease) has been described in cathepsin K-deficient mice [18].

Figure 2.3.2. Osteoclast physiology and potential therapeutic targets
Osteoblasts and bone formation

The osteoblast is a unique bone-forming cell derived from mesenchymal stem cells (Figure 2.3.3.). The rate of bone formation is determined by the speed and effectiveness of precursor cells differentiating into mature osteoblasts which secrete matrix that can be mineralized and by their life span. These processes are enhanced by vitamin D as well as by intermittent pulses of parathyroid hormone, a treatment scheme used by daily injections of teriparatide. By contrast, bone formation can be suppressed by exogenous glucocorticoids or be impaired in some elderly patients. At sites of resorption lacunae, a team of osteoblasts produces an extracellular matrix containing type-I collagen and various non-collagenous proteins, such as osteocalcin, osteonectin, osteopontin and others. Vitamin D, calcium, and phosphate help this matrix mineralise. The calcium-sensing receptor (CaSR) on the parathyroid gland controls PTH release to maintain serum calcium concentrations within a narrow physiological range. Whereas hypocalcaemia stimulates the CaSR and release of PTH to increase serum calcium, hypercalcaemia has the opposite effects [19]. Accordingly, drugs that mimic high concentrations of calcium at the CaSR and suppress PTH secretion are termed calcimimetic drugs (eg, cinacalcet), whereas drugs that mimic low concentrations of calcium are termed calcilytic drugs (eg, MK-5442) [20]. Although the physiological role of the CaSR expressed on osteoblasts and osteoclasts is not fully understood, it might mediate some of the effects of the osteoporosis drug strontium ranelate [21]. At the molecular level, activation of the canonical Wnt/β-catenin pathway is the master switch for osteoblastic differentiation [22]. This key bone-anabolic pathway is negatively regulated by Wnt inhibitors such as dickkopf-1 (Dkk-1) and sclerostin, which bind and block the Wnt receptor LRP-5 (Figure 2.3.3.) [23].
Chapter 2: Review of Literature

Figure 2.3.3. Osteoblast physiology and potential therapeutic targets

2.3.2.3. Osteocytes:

Osteocytes account for more than 90% of all bone cells and are found scattered throughout the mineralized matrix. They are terminally differentiated osteoblasts and share morphological similarities with neural cells. Their long dendritic processes form a sensory network, whereby they can sense and communicate mechanical stress within the bone. Osteocytes also express several factors known to regulate phosphate metabolism, which suggests a role in matrix mineralisation. Additionally, osteocytes exclusively produce and secrete sclerostin, [24] an inhibitor of the Wnt-signalling pathway, which inhibits osteoblast differentiation and bone formation.
Chapter 2: Review of Literature

2.3.3. Osteoporosis therapies:

Osteoporosis therapies fall into two classes, anti-resorptive drugs, which slow down bone resorption, and anabolic drugs, which stimulate bone formation.

2.3.3.1. Anti-resorptive drugs

*Calcium and vitamin D*

Calcium and vitamin D are necessary for normal skeletal homeostasis and are considered the first step in osteoporosis treatment. Inadequate intake of calcium and vitamin D leads to reduced calcium absorption, a compensatory rise in parathyroid hormone, higher bone remodelling rates and increased bone loss. The recommended dose of calcium, usually in the form of calcium carbonate, is 1 g daily and common side effects include dyspepsia and constipation. However, an increased risk of myocardial infarction in patients on calcium supplements, without coprescribed vitamin D, was recently reported in a meta analysis by Bolland and colleagues [25], which raises concerns about its widespread use. Because of the limited evidence available of its benefit when used alone, it is advised that calcium should not be prescribed to patients with osteoporosis unless they are also receiving vitamin D and/or an effective treatment for osteoporosis.

*Bisphosphonates*

Of the anti-resorptive drugs, bisphosphonates, with their high affinity for bone and long safety record, constitute the largest class. Bisphosphonates can be given orally or intravenously, and are most widely used because they can be inexpensive and used across a broad spectrum of osteoporosis types, including postmenopausal, male, and steroid-induced osteoporosis, as well as Paget's disease. Alendronate, risedronate, ibandronate and zoledronic acid are all nitrogen containing bisphosphonates and have demonstrated efficacy in fracture risk reduction [26, 27]. Alendronate and risedronate are most commonly given in weekly oral formulations and zoledronic acid (ZA) is administered annually as an intravenous infusion. Ibandronate comes in monthly oral or 3-monthly intravenous formulations.
Numerous randomised placebo-controlled studies including the Women's Health Initiative (WHI) [28] and the Postmenopausal Estrogen/Progesterone Interventions (PEPI) [29] have established that the postmenopausal decrease in BMD is attenuated by oestrogen, resulting in a lower risk of fracture. However, data from the WHI also suggested that oestrogen/progesterone therapy increases the incidence of breast cancer, coronary heart disease, stroke and venous thromboembolism. Unopposed oestrogen therapy has been shown to increase stroke and thromboembolism only. Therefore, as the overall health risks exceed the benefits, hormone replacement is not considered first-line therapy in the management of postmenopausal osteoporosis.

Selective oestrogen receptor modulators

Raloxifene, lasofoxifene and bazedoxifene are currently the selective estrogen receptor modulators (SERMs) available for use in the treatment of osteoporosis and their effects on vertebral and non-vertebral fractures have been studied in numerous trials reporting beneficial results. The first SERM used to treat osteoporosis, raloxifene, demonstrated a reduced risk of vertebral fractures (HR 0.7), but not non-vertebral fracture in a large 3-year study of postmenopausal women [30]. Although SERMs have demonstrated benefits with regard to ER-positive breast cancer, coronary heart disease, and stroke, the main concern with treatment is the three-fold increased risk of venous thromboembolism. Tamoxifen is the main drug used for the breast cancer. Ospemifene and arzoxifene are the new SERM molecules currently under investigation [31, 32].

2.3.3.2. Anabolic therapy

Parathyroid hormone

Recombinant parathyroid hormone (PTH) 1-34 and 1-84 are anabolic agents that work by stimulating pre-osteoblasts to mature into bone-forming osteoblasts. When given at low dose by daily subcutaneous injection they enhance bone remodelling.
2.3.4. Novel targets for treatment of osteoporosis:

2.3.4.1. Anti-resorptive therapies

Denosumab

The prominent role of RANKL in osteoclastogenesis has made it a prime target in diseases characterised by excessive bone loss. Denosumab (AMG 162) is a human monoclonal antibody with a high affinity and specificity for receptor activator of NF-kB ligand (RANKL), a key mediator of osteoclastic bone resorption. By reducing RANKL binding to the osteoclast receptor RANK, there is a decrease in differentiation, activity, and survival of osteoclasts, lowering the rate of bone resorption [33].

Odanacatib (MK-822)

On the basis of the concept that the protease cathepsin K has an important role in enzymatic bone degradation, the use of cathepsin-K inhibitors has emerged as a novel therapeutic approach. A high specificity and affinity for cathepsin K over other cathepsins (B, L, and S) that are widely expressed, particularly in the skin, was crucial for this class of compound [34]. Odanacatib is currently the most advanced inhibitor of cathepsin K under clinical investigation. Cathepsin K is a lysosomal cysteine protease expressed in abundance by osteoclasts that degrades Type I collagen and is responsible for degradation of the organic bone matrix during bone remodelling. Odanacatib (MK-822) is a potent and selective inhibitor of Cathepsin K [35] and has been shown in studies to reduce osteoclast-mediated bone resorption.

2.3.4.2. Anabolic therapies

Calcilytic drugs (MK-5442)

Calcilytic drugs represent a new class of bone-forming drugs. They act as antagonists of the CaSR and mimic hypocalcaemia, thus evoking a short pulse of PTH secretion (Figure 2.3.3.) [36]. Calcilytics are given orally and obviate the need for injections, as opposed to PTH treatment. A major practical obstacle for these drugs has been their narrow therapeutic index.
Chapter 2: Review of Literature

Inhibitors of Wnt antagonists

Wnt-dependent nuclear accumulation of β-catenin (Figure 2.3.3.) is a major trigger of osteoblastic differentiation and bone formation [37]. The endogenous inhibitors of Wnt signalling, sclerostin and Dkk-1, present potential therapeutic targets to enhance osteoblastic bone formation and are under clinical investigation [38].
Chapter 2: Review of Literature

2.3.5. References:


Chapter 2: Review of Literature


Chapter 2: Review of Literature


2.4. Drug metabolism and CYP enzymes:

2.4.1. Drug metabolism:

Most drugs are lipophilic compounds, which need to be enzymatically transformed into more polar, water-soluble, and excretable metabolites that can be easily eliminated from organisms [1, 2]. The biotransformation of drugs can be classified into phase I and phase II reactions. Phase I metabolism includes oxidation, reduction and hydrolysis reactions, while phase II reactions include glucuronidation, acetylation, sulfation, methylation and glutathione and amino acids conjugation. Usually phase II reactions generate inactive and more water-soluble compounds that can be easily eliminated from an organism via urine or bile [3, 4]. The major site of biotransformation of drugs is the liver, which contains a large number of metabolising enzymes. In addition, enzymes in the intestinal mucosa also contribute significantly to the metabolism of drugs [5]. Other extrahepatic sites of drug metabolism include the kidneys, lungs, skin, brain and nasal epithelium. However, these sites contribute to a minor extent to the systemic elimination of drugs compared with the liver and intestines [6].

2.4.2. CYP enzymes in man:

Cytochrome P450 (CYP) enzymes, are the most important phase I enzymes that are involved in the metabolism of many endogenous compounds and a majority of clinically used drugs [3]. CYP proteins, named according to the absorption band at 450 nm of their reduced carbon-monoxide-bound forms, consist of large superfamilies of enzyme proteins [7]. The root symbol, CYP, is followed by a number for the family (a general group of protein with more than 40% amino-acid sequence identity), a letter for the subfamily (greater than 55% identity), and a number for the gene denoting a specific CYP form. Distinct CYP forms differ from each other with respect to their chemical, immunological properties, and they have different substrate affinities [2]. In mammals, at least 17 CYP gene families have been identified. Among these, the CYP1, CYP2, and CYP3 subfamilies are mainly involved in the biotransformation of pharmaceuticals and xenobiotics. The other CYP families mainly take part in the biosynthesis of steroids, metabolism of bile acids and arachidonic acid, and metabolism of other endogenous compounds [8]. The major drug metabolizing CYP subfamilies in humans are CYP3A (3A4 and 3A5) (<30% of total P450 content in the liver),
CYP2C (2C8, 2C9, 2C18 and 2C19) (<20%), CYP1A2 (<13%), CYP2E1 (<7%), CYP2A6 (<4%), and CYP2D6 (<2%) [9]. Among these, several CYP forms, e.g. CYP2A6, 2C8, 2C9, 2C19, 2D6, and 2E1 are polymorphic expressed in human subjects [9-13].

2.4.2.1. CYP1A2 enzyme

The human CYP1A subfamily comprises two members, CYP1A1 and CYP1A2. CYP1A1 is expressed at a very low level in the liver, while it is primarily an extrahepatic enzyme found in the lungs and placenta [14]. CYP1A2 is the predominant enzyme of the CYP1A subfamily constituting approximately 13% of the total CYP protein in human livers [9]. CYP1A2 activity can be induced by cigarette smoking, resulting in an almost two-fold increase of the metabolic clearances of substrates of this enzyme compared with nonsmokers [15]. In addition, omeprazole has been shown to be a dose-dependent inducer of CYP1A2 in man [16]. CYP1A2 is responsible for the metabolism of several drugs, including phenacetin, caffeine, theophylline, paracetamol, olanzapine, lidocaine, and some procarcinogens [14, 17, 18]. The major route of caffeine metabolism in man (N-3-demethylation of caffeine to paraxanthine) is mediated by CYP1A2 [19, 20]. Therefore, caffeine has been used in vivo as a CYP1A2 probe substrate. In in vitro studies, ethoxyresorufin and phenacetin have been used as preferential probe substrates [21, 22]. Furfurylline (a mechanism-based inhibitor of CYP1A2, KI = 3 < 23 μM and Kinact = 0.07 < 0.87 min-1) and α-naphthoflavone are used as relatively specific and potent inhibitors of CYP1A2 in vitro [23, 24].

2.4.2.2. CYP2A6 enzyme

CYP2A6 is a quantitatively minor component (1-5%) of the human hepatic CYP forms. A number of compounds such as phenobarbital and pyrazole increased CYP2A6 activity at the mRNA level in human hepatocytes in primary culture [25, 26]. CYP2A6 is active in the metabolism of a few drugs, such as coumarin, methoxyflurane, halothane, valproic acid, disulfiram, losigamone, letrozole and a number of procarcinogens [27-29]. In humans, CYP2A6 is polymorphically expressed. To date, several defective alleles of CYP2A6 have been reported with the most prevalence of them being a Leu 160 His substitution (CYP2A6*2) that yields an inactive enzyme [30, 31]. By contrast, a deletion mutation (CYP2A6*4) is the most common variant (15-20%) in Asian populations [32].
Coumarin 7-hydroxylation is selectively catalyzed by CYP2A6, and coumarin has therefore been used as a CYP2A6 probe drug both in vitro and in vivo [33]. Compounds including 8-methoxypsoralen, menthofuran, pilocarpine and tranylcypromine have been found to be relatively potent inhibitors of CYP2A6 [34-37]. Among these, 8-methoxypsoralen is a probe and potent mechanism-based inhibitor of CYP2A6 (KI = 1.9 μM and K\text{inact} = 2 \text{ min}^{-1}) which has been used as a useful in vitro tool for evaluation of the contribution of CYP2A6 metabolic reactions [34]. In addition, tranylcypromine might be an adequately selective CYP2A6 inhibitor for in vitro use [37, 38].

2.4.2.3. CYP2B6 enzyme

CYP2B6 comprises on average only about 0.2% of the total CYP in human livers, but its expression has large inter-individual variability [13]. In primary cultured human hepatocytes and in different human cell lines, CYP2B6 can be induced at protein and mRNA levels by phenobarbital, and cyclophosphamide which is an anticancer drug known to be metabolised by CYP2B6 [39]. CYP2B6 can be involved in the metabolism of a number of substrates such as, nicotine, aminochrysene, tamoxifen, testosterone, diazepam, S-mephenytoin (N-demethylation), S-mephobarbital, cyclophosphamide and propofol [40-42]. The activity of CYP2B6 can be inhibited by fluvoxamine, sertraline and paroxetine [43].

2.4.2.4. CYP2C enzymes

In human livers, the CYP2C subfamily is one of the most abundantly expressed CYP subfamilies. It includes four known members: CYP2C8, 2C9, 2C18, and 2C19. CYP2C8 and CYP2C9 are the major CYP2C forms, accounting for 35% and 60%, respectively, of the total hepatic content of human CYP2C, while CYP2C18 (4%) and CYP2C19 (1%) are the minor forms of the human CYP2C subfamily [44, 45]. It has been estimated that CYP2C8, CYP2C9, and CYP2C19 are involved in the metabolism of approximately 20% of clinically used drugs [12, 46, 47]. Although the amino-acid sequences of CYP2C9 and CYP2C19 are 91% homologous, they exhibit relatively little overlap in their substrate specificities [48].

2.4.2.5. CYP2C8 enzyme

The importance of CYP2C8 in drug metabolism has only recently been recognized [49].
CYP2C8 mRNA and protein can be induced by rifampicin and phenobarbital in primary cultures of human hepatocytes [50]. CYP2C8 is primarily responsible for the metabolism of the anti-cancer drug taxol, cerivastatin, rosiglitazone, troglitazone, and is also involved in the metabolism of zopiclone, carbamazepine, verapamil, and amiodarone [49, 51]. CYP2C8 is polymorphically expressed in human livers [11]. Quercetin has been used in vitro as an inhibitor of CYP2C8, but it also significantly inhibits CYP1A2 activity [52].

2.4.2.6. CYP2C9 enzyme

CYP2C9 is the principal CYP2C form in human liver. It metabolises many clinically important drugs including tolbutamide, phenytoin, S-warfarin, losartan, ibuprofen, diclofenac, piroxicam, tenoxicam, and mefenamic acid [11]. In addition, the antidiabetic drug glipizide and the diuretic torsemide have been reported to be metabolized by CYP2C9 [53, 54]. CYP2C9 has been found to be genetically polymorphic. S-warfarin, tolbutamide and diclofenac have been used as in vitro probe substrates of CYP2C9 [55]. Inhibitors of CYP2C9 include sulfaphenazole, sulfamethoxazole, sulfapyrazine, miconazole, and fluconazole. Sulphaphenazole (Ki < 0.3 μM) has been used as a selective inhibitor of CYP2C9 in in vitro studies [23, 24]. Inducers of CYP2C9 include barbiturates, carbamazepine, and rifampin [50, 56].

2.4.2.7. CYP2C18 enzyme

CYP2C18 expressed at a very low level in human livers, but CYP2C18 can participate, to a small extent, in the metabolism of substrates of other CYP2C forms, such as diazepam, imipramine, and tolbutamide [57, 58].

2.4.2.8. CYP2C19 enzyme

CYP2C19, is another important member of the CYP2C subfamily, it can be induced by barbiturates, carbamazepine, and rifampin, phenytoin in primary cultures of human hepatocytes [50]. CYP2C19 is involved in the metabolism of drugs including S-mephenytoin, omeprazole, diazepam, propranolol, proguanil and tricyclic antidepressants, such as imipramine, clomipramine and amitriptyline [13]. CYP2C19 exhibits genetic polymorphism.
Chapter 2: Review of Literature

S-mephenytoin and omeprazole have been used as in vitro probe substrates of CYP2C19 [59-61]. The inhibitors of CYP2C19 include fluvoxamine, omeprazole, fluconazole (Ki < 2µM), and ticlopidine [58, 62].

2.4.2.9. CYP2D6 enzyme

CYP2D6 constitutes on average about 2% to 5% of the total hepatic CYP content, and it is also found in several extrahepatic tissues, including the gastrointestinal tract, brain, and lungs [13]. CYP2D6 accounts for the hepatic metabolism of about 30% of clinically used drugs, including antiarrhythmic agents, antihypertensives, monoamine oxidase inhibitors, morphine derivatives, antipsychotics, and tricyclic antidepressants [3]. In humans, CYP2D6 is polymorphic, and at least 70 CYP2D6 alleles have been identified. Most of the known variant alleles are inactive, and produce the poor metabolizer (PM) phenotype [63, 64]. Bufuralol and dextromethorphan have been used as in vitro probe substrates of CYP2D6 [65, 66]. Inhibitors of CYP2D6 include quinidine, fluoxetine, paroxetine, perphenazine, terbinafine and ticlopidine [57]. Among these inhibitors, only quinidine has been widely used as a potent and selective probe inhibitor in in vitro studies (Ki< 0.06 µM) [24]. In contrast to most other hepatic CYP enzymes involving in human drug metabolism, CYP2D6 seems not to be inducible [13].

2.4.2.10. CYP2E1 enzyme

CYP2E1 constitutes approximately 6% of the total hepatic CYP enzymes and is present in several extrahepatic tissues, including the lungs, kidneys, nasal mucosa, bone marrow, and the white cell fraction of peripheral blood [67, 68]. Several factors including obesity and fasting can modulate human CYP2E1 activity [69-71]. In addition, ethanol is an inhibitor of CYP2E1 after transient use and an inducer after chronic use [72]. CYP2E1 is involved in the metabolic activation of many low molecular weight toxins and carcinogens, including N-nitrosamines in tobacco smoke, benzene, ethanol, and a number of drugs such as chlorzoxazone, acetaminophen, dapsone, aniline, and fluorinated general anesthetics [73]. CYP2E1 is also polymorphically expressed with up to 20-fold inter-individual variation among individuals [9]. Chlorzoxazone has been used both in vivo and in vitro as a probe substrate of CYP2E1 [74]. Disulfiram inhibits CYP2E1 activity, but it is also an almost equally potent inhibitor of
CYP2A6 [75]. Diethyldithiocarbamate (DDC), which is a mechanism-based inhibitor of CYP2E1, and pyridine have been used as useful in vitro tools for evaluation of the CYP2E1-mediated reactions [23, 76].

2.4.2.11. CYP3A enzymes

CYP3A enzymes are the most abundant CYP enzymes, comprising approximately 30-40% of the total hepatic CYP content [9]. CYP3A enzymes are involved in the metabolism of most clinically used drugs (40%-50%) in humans. The CYP3A subfamily contains 3 functional proteins: CYP3A4, CYP3A5 and CYP3A7 [8].

2.4.2.11.1. CYP3A4 enzyme

CYP3A4 is the most abundantly CYP expressed in the human liver and intestine [77], and its activity can be induced by rifampin, barbiturates, carbamazepine, nevirapine, and dexamethasone in vivo and in vitro [78, 79]. CYP3A4 plays a significant role in the metabolism of almost half of the commonly used drugs, including nifedipine, felodipine, cyclosporine, erythromycin, midazolam, alprazolam, triazolam, lovastatin, simvastatin, terfenadine, verapamil, tacrolimus, diltiazem, cicaipride, testosterone, and HIV-protease inhibitors [12, 80]. CYP3A4 activity shows large interindividual variability (up to 40-fold in hepatic microsomes), it is affected by genetic and environmental factors [9]. In some studies, several genetic polymorphisms in the CYP3A gene were found using restriction fragment length polymorphisms detected by Southern analysis, but none of these was associated with the level of nifedipine oxidation activity among various liver samples [81, 82]. There is considerable evidence for CYP3A4 behaviour allosterically, possibly due to the simultaneous binding of two or more substrate molecules to its active site [83-86]. Such binding can lead to atypical enzyme kinetics and inconsistent drug-drug interactions [86, 87]. Therefore, it was recently recommended that at least two chemically unrelated CYP3A4 substrates, such as midazolam and testosterone, should be used as probe substrates in in vitro studies [61]. A number of drugs and foreign chemicals are clinically significant inhibitors of CYP3A4 such as ketoconazole, itraconazole (Ki<270 nM), clarithromycin, erythromycin [13, 77, 80]. Troleandomycin has been characterized as a selectively mechanism-based inhibitor of CYP3A4 in vitro [23, 88]. Ketoconazole is also a potent and selective inhibitor of CYP3A4 in
vitro and in vivo with low concentrations (preferably 1 \mu M or lower) [23, 24].

2.4.2.11.2. CYP3A5 enzyme

CYP3A5 is 83\% homologous to CYP3A4, but it is expressed at a much lower level than CYP3A4 in the liver (10-30\% of CYP3A4) [13, 80]. CYP3A5 is polymorphically expressed in 30\% of individuals, and it is predominantly expressed in the kidney in most individuals [77, 89]. CYP3A5 can be induced by rifampicin and phenobarbital in hepatocyte cultures [90, 91]. CYP3A5 has been shown to be capable of metabolising most substrates of CYP3A4 [92, 93]. However, because of its lower expression level, the role of CYP3A5 in hepatic drug clearance has generally been regarded to be significantly smaller than that of CYP3A4.

2.4.2.11.3. CYP3A7 enzyme

CYP3A7 is the major CYP form detected in human embryonic, fetal and newborn liver [94, 95]. It may also be selectively expressed in adult livers at lower levels than CYP3A4 and CYP3A5 [96]. The role of CYP3A7 in drug metabolism is unclear.

2.4.3. Enzyme kinetics in drug metabolism:

In vitro characterization of drug biotransformation generally begins with an enzyme kinetics analysis of metabolite formation rate using human liver microsomes. A typical enzyme kinetic analysis involving a mathematical description of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent biotransformation rate as a function of substrate concentration is based on the core assumptions that substrate consumption is minimal (typically less than 10-20\%), and that product formation rate is linearly related to microsomal protein concentration and duration of incubation [97]. Normally, if the conversion of substrate to product is catalyzed by a single enzyme, the enzyme kinetics can be well described by a Michaelis-Menten (MM) equation (one-enzyme model) as follows [97, 98]:

\[
V_0 = \frac{V_{\text{max}} \cdot S}{K_m + S}
\]

where \(V_0\) is the rate of product formation (or substrate disappearance), \(S\) is the substrate concentration, \(V_{\text{max}}\) is the maximal velocity of the reaction, and \(K_m\) is the MM constant representing the concentration of substrate that results in half-maximal velocity.
Two or more CYP forms with distinct affinities may catalyze a given drug biotransformation. In such cases, the relationship between $V_0$ and $S$ is biphasic, and may be described by a high-affinity and a low-affinity component using a two enzyme MM model [98]:

$$V_0 = V_{\text{max}1} \cdot S / (K_{m1} + S) + V_{\text{max}2} \cdot S / (K_{m2} + S) \quad (2)$$

Some CYP enzymes (CYP2B6 and CYP3A4) have been shown to exhibit kinetics consistent with allosteric interaction of the substrate with the enzyme, which is also known as substrate activation [85, 99, 100]. These result in an $S$-shaped substrate versus rate curve and a "hook"-shaped Eadie-Hofstee plot [12]. When allosteric interactions are observed, the Hill equation can be used to calculate kinetic constants [101]:

$$V_0 = V_{\text{max}} \cdot S^n / (S_{50}^n + S^n) \quad (3)$$

where $n$ is the Hill coefficient for cooperative substrate binding, $S_{50}$ is the substrate concentration at which half the maximal rate ($V_{\text{max}}$) is attained.

Another kinetic profile, substrate inhibition, occurs when an increase in substrate concentration beyond a certain value (usually greater than $K_m$) results in a decrease in the rate of metabolism [86, 102, 103]. Although the mechanism of substrate inhibition has yet to be fully determined, it has been described by a two-site model in which one binding site is productive, whereas the other site is inhibitory and operable at high substrate concentrations, resulting in decreased velocity with increasing concentrations [104, 105]. In general, the magnitude of the inhibition of substrate inhibition is dependent upon the structure and concentration of the substrate, the reaction type, and CYP form examined. In most cases, substrate inhibition behaves as a partial inhibition because the inhibition of CYP does not approach zero even at very high substrate concentrations [4].

2.4.4. Mechanism of drug-drug interactions involving CYP enzymes:

Inhibition of CYP enzymes is the most common cause of metabolism based drug-drug interactions. The inhibition of CYP enzymes is of clinical importance for both therapeutic and toxicological reasons. The mechanisms of CYP inhibition can be categorized into reversible inhibition and mechanism-based inhibition [4, 13, 106].
Chapter 2: Review of Literature

2.4.4.1. Reversible inhibition

Reversible inhibition is the most common type of enzyme inhibition. Reversible inhibition is transient and reversible, and the normal functions of CYPs continue after the inhibitor has been eliminated from the body [4]. Reversible inhibition can be further classified into competitive, uncompetitive, mixed-type and non-competitive inhibition [12]. Competitive inhibition is when the binding of an inhibitor to an enzyme prevents a further binding of a substrate to the active sites of the enzyme. In uncompetitive inhibition, an inhibitor does not bind to the free enzyme, but binds to the enzyme-substrate complex, resulting in a nonproductive enzyme-substrate-inhibitor complex. Mixed-type inhibition is when an inhibitor binds either to the free enzyme or to the enzyme-substrate complex [4, 12]. In the case of noncompetitive inhibition, an inhibitor binds to a nonactive binding site of the enzyme, and the binding has no effect on the binding of substrate, but the enzyme-substrate-inhibitor complex is nonproductive. Noncompetitive inhibition is a specific case of mixed-type inhibition. Mathematically, the velocity of an enzymatic reaction in the presence of an inhibitor ($V_i$), can be described by the following equations (4), (5), (6) and (7) for competitive, uncompetitive, mixed-type and non-competitive inhibition, respectively [97] (Figure 2.4.1.).

\[
V_i = V_{\text{max}} \cdot \frac{S}{K_m (1 + I / K_i) + S} \quad (4)
\]

\[
V_i = V_{\text{max}} \cdot \frac{S}{K_m + S (1 + I / K_i)} \quad (5)
\]

\[
V_i = V_{\text{max}} \cdot \frac{S}{K_m (1 + I / K_i) + S (1 + I / \alpha K_i)} \quad (6)
\]

\[
V_i = V_{\text{max}} \cdot \frac{S}{K_m (1 + I / K_i) + S (1 + I / K_i)} \quad (7)
\]

where $K_i$ is the inhibition constant, $(I)$ is the inhibitor concentration, and $\alpha$ is the factor by which $K_m$ changes when an inhibitor occupies the enzyme.
Chapter 2: Review of Literature

Figure 2.4.1. Representative double reciprocal plots of competitive (A), noncompetitive (B), mixed-type (C) and uncompetitive (D) inhibition

2.4.4.2. Mechanism-based inhibition

Mechanism-based inhibition can be mediated by covalent modification of a pyrrole nitrogen in the prosthetic heme group of CYP or by direct modification of the heme moiety or the apoprotein [107]. The mode of inhibition is highly specific because the inhibitor must both bind to and be metabolised by the enzyme [108]. The inhibitory effect of mechanism-based inhibition is terminated by enzyme resynthesis rather than inhibitor washout [109]. One mode of mechanism-based inhibition is the formation of metabolite intermediate (MI) complexes. Compounds forming MI complexes can be catalytically oxidized to intermediate or product metabolites that uncovalently bind to the prosthetic heme of the CYP. In the case of MI
complexation, the CYP activity can be restored under nonphysiological experimental conditions e.g. using potassium ferricyanide or by the in vitro dialysis method [110, 111]. However, in real in vivo situations, the MI complexes are so stable that resynthesis of new enzyme is the only means by which the enzyme activity can be restored [109]. A classic example of the MI complexation is the inhibition of CYP3A4 by troleandomycin [23].

Another mode of mechanism-based inhibition is the so-called enzyme inactivation (or suicide inhibition). Suicide inhibition results from covalent binding of reactive intermediates to the heme and/or protein of CYP [13, 112]. Typical examples of suicide inhibition are inactivation of CYP1A2 by furafylline, and inactivation of CYP3A4 by delavirdine [62, 113]. The most important phenomena of mechanism-based inhibition are time-, concentration-, and NADPH-dependent loss of the enzyme activity [112].

In vivo, the inhibitory effect of a mechanistic inactivator is thought to be more prominent after repeated dosing and last longer than that of a reversible inhibitor [106]. Many drugs have been identified as mechanism-based inactivators in vivo, and have considerable form specificity. These inhibitors include furafylline (CYP1A2 inhibitor; $K_I = 23 \mu M, K_{inact} = 0.87 \text{ min}^{-1}$) [62], menthofuran (CYP2A6 inhibitor; $K_I = 2.5 \mu M, K_{inact} = 0.22 \text{ min}^{-1}$ in human liver microsomes, and $K_I = 0.84 \mu M, K_{inact} = 0.25 \text{ min}^{-1}$ in purified expressed CYP2A6) [35], tienilic acid (CYP2C9 inhibitor; $K_I = 4.3 \mu M, K_{inact} = 0.21 \text{ min}^{-1}$) [114, 115], halothane (CYP2E1 inhibitor; data for $K_I$ and $K_{inact}$ not available), gestodene (CYP3A4 inhibitor; $K_I = 46 \mu M, K_{inact} = 0.39 \text{ min}^{-1}$) and delavirdine (CYP3A4 inhibitor; $K_I = 22 \mu M, K_{inact} = 0.59 \text{ min}^{-1}$) [113, 116].
Chapter 2: Review of Literature

2.4.5. References:


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


Chapter 2: Review of Literature


