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THÈSE

Evaluation de possibles Interactions Médicamenteuses: Effets d'extraits de *Andrographis paniculata* sur les enzymes du métabolisme hépatique chez le rat et chez l'Homme

Présentée pour l'obtention du grade de Docteur de l'université de Franche-Comté

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LIST OF ABBREVIATIONS

5-HT	Serotonin
ABC	ATP-binding cassette
ACTH	Andrenocorticotrophic hormone
ADP	Adenosine diphosphate
AhR	Aryl hydrocarbon receptor
AND	Andrographolide
AP	*Andrographis paniculata*
AUC	Area under the curve
BCRP	Breast cancer resistance protein
cAMP	Cyclic adenosine monophosphate
CAR	Constitutive androstane receptor
CAM	Complementary and alternative medicine
CDK	Cyclin-dependent kinase
CK	Creatine kinase
CYP	Cytochrome P450
DDM	Disc Diffusion Method
DMEs	Drug metabolizing enzymes
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EMs	Extensive metabolizers
EPH	epoxide hydrolases
ERK	Extracellular signal-regulated kinase
ESWL	Extracorporeal shock wave lithotripsy
FDA	Food and drug administration
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamic pyruvic transaminase
GST	Glutathioe S-transferase
IC_{50}	Half maximum (50%) inhibitory concentration

MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
MRSA	Methicillin Resistant *Staphylococcus aureus*
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAT	*N*-acetyltransferases
OATP	Organic anion transporting polypeptide
PAPS	3’-phosphoadenosine-5’-phosphosulfate
PCH	primary cultures of hepatocytes
PG	Prostacyclin
P-gp	P-glycoprotein
PKC	Protein kinase C
PLC	Phospholipase C
PMs	Poor metabolizers
PPAR	Peroxisome proliferator activated receptor
PXR	Pregnane X receptor
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SAM	S-adenosylmethionine
SLC	Solute carrier
SULT	Sulfotransferases
TCM	Traditional Chinese Medicine
TX	Thromboxane
UGT	UDP-glucuronosyltransferases

LITERATURE SURVEY

CHAPTER 1: ANDROGRAPHIS PANICULATA

1. Description of the plant

Andrographis paniculata (AP), also known commonly as "King of Bitters," is a member of the plant family *Acanthaceae*, and has been used for centuries in Asia to treat gastrointestinal tract and upper respiratory infections, fever, herpes, sore throat, and a variety of other chronic and infectious diseases. It is found in the *Indian Pharmacopoeia* and is the prominent component of at least 26 Ayurvedic formulas; whereas in Traditional Chinese Medicine (TCM), *Andrographis* is an herb used for its important "cold properties": it is used to rid the body of heat, as in fevers, and to dispel toxins from the body. In Scandinavian countries, it is commonly used to prevent and treat colds. Research conducted in the '80's and '90's has confirmed that *Andrographis*, when properly administered, has a surprisingly broad range of pharmacological effects, some of them extremely beneficial: Analgesic, Anti-inflammatory, Antibacterial, Antipyretic, Antithrombotic, Antiviral, Cytotoxic effect, Cardioprotective effect, Digestive, Expectorant, Hepatoprotective effect, Hypoglycemic, Immune Enhancement, Laxative, Sedative, Thrombolytic, Vermicidal (detailed in paragraphs below).

Andrographis paniculata Nees is an annual herb about 30-100 cm high, having stem erect, 4-angled, much branches. Leaves are simple, opposite, sessile or short-petioled, elliptic or lanceolate, 2.5-8 cm long, 1-3 cm wide, glabrous on both surfaces. Flower are in racemes, 2.5-10 cm long, consisting of flowers distant; frequently 1-sided; bract small, linear; pedicel 0-6 mm; calyx 1, green, about 3 mm long, connate at the base, divided into 5 linear segments, hairy; corolla white, tubular, divided into 2 lips upper lip 3-lobed, rose-purple spotted, hairy; lower lip small, 2-lobed; stamen 2, filaments hairy, upwards, anther dark-purple; ovary 1, style slender, tip minutely bifid. They distribute in evergreen, pine and deciduous forests, and along road sides and some are cultivated. They are grown in all types of soil. Moreover, it grows in soil types where almost no other plant can be cultivated, particularly "serpentine soil," which is relatively high in aluminium, copper and zinc.

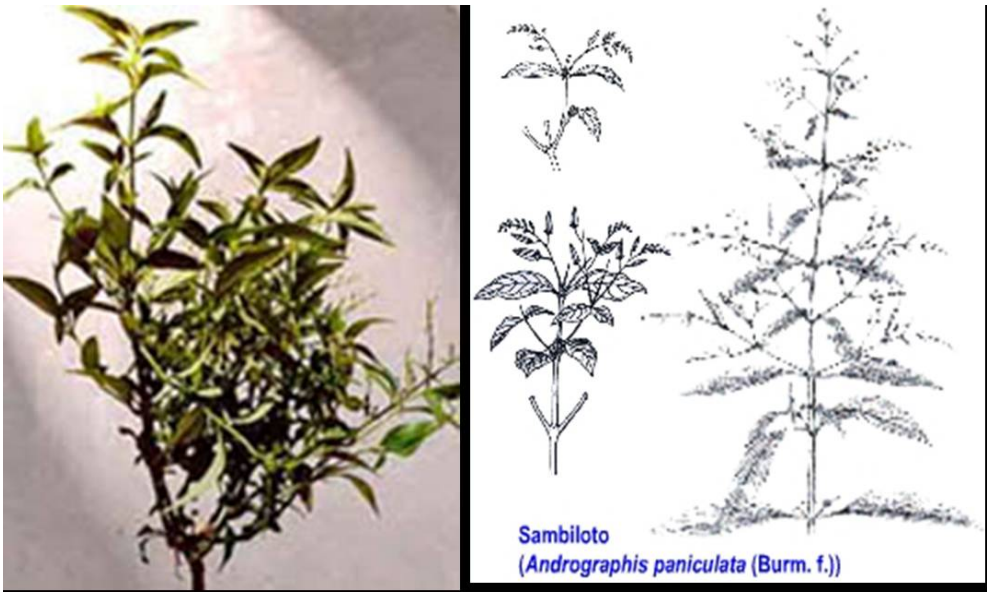


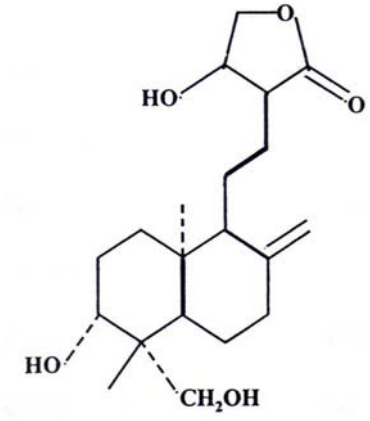
Figure 1: *Andrographis paniculata* Wall.ex Nees. It is an annual herb whose leaves are the major sources of andrographolide and many other lactones (Fah Talai Jone, 1995).

2. Chemical constituents

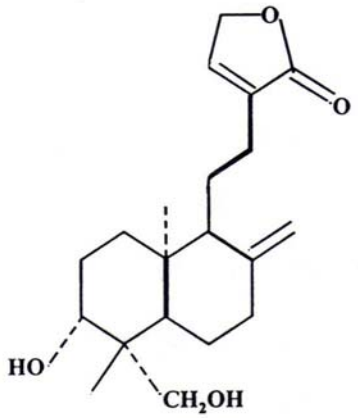
The leaves from *Andrographis paniculata* contain the highest amount of andrographolide (2.39%), which has been reported to be the most medicinally active phytochemical in the plant (Sharma et al., 1992), while the seeds contain the lowest. It has a very bitter taste, is a colourless crystalline in appearance, and is called a "diterpene lactone" - a chemical name that describes its ring-like structure.

The other medicinal chemicals isolated from *Andrographis paniculata* are also bitter principles: diterpenoids and deoxyandrographolide, -19ß-D-glucoside, and neo-andrographolide, all of which have been isolated from the leaves (Chem W. and Liang X., 1982).

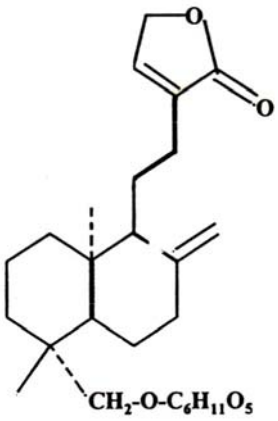
Besides the bitters cited above, other active components of *Andrographis paniculata* include 14-deoxy-11, 12- didehydroandrographolide (andrographolide D), homoandrographolide, andrographan, andrographon, andrographosterin, and stigmasterol, the last of which was isolated from an Astrographis preparation (Siripong et al., 1992). The structures of lactones are shown in Figure 2.



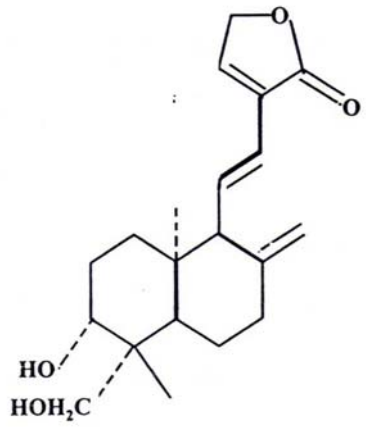
andrographolide



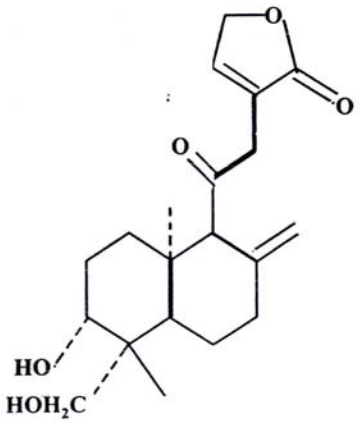
deoxyandrographolide



neoandrographolide



dehydroandrographolide



14-deoxy-11-oxoandrographolide

Figure 2: Chemical structure of the main constituents of AP.

3. Pharmacological activities

3.1 Hepatoprotective effect

Efficacy of an aqueous extract of *Andrographis paniculata* in the treatment of liver damage against galactosamine induced hepatotoxicity in experimental male albino rats has been studied. Histopathology of liver and biochemical parameters suggested a significant protective effect of the extract. Animals pretreated with drug suffered less stress as compared with the untreated animals (Shahid, 1987).

The hepatoprotective effects of andrographolide, the major active diterpenoid lactone of the plant *Andrographis paniculata,* has also been studied in rats submitted to single high doses of hepatotoxins inducing acute hepatotoxicity. It was found that rats pre- or post-treated at different time intervals with various doses of andrographolide in addition to treatment with acute hepatotoxins, significantly improved their liver status. For example, rats administered with andrographolide, 400 mg/kg ip or 800 mg/kg po, 48, 24 and 2h before galactosamine administration were protected against the hepatotoxic effect of acute galactosamine administration, as evaluated by the levels of serum biochemical parameters and histopathology. Similarly, administration of andrographolide, 200 mg/kg ip, 1, 4 and 7 h after paracetamol challenge leaded to complete normalisation of toxin-induced increase in the levels of the liver biochemical parameters and significantly ameliorated toxin-induced histopathological changes. Taken together, these results confirm the *in vivo* hepatoprotective effect of andrographolide against both galactosamine and paracetamol-induced hepatotoxicity in rats (Handa and Sharma, 1990).

The anti-hepatotoxic activity of pure andrographolide and various extracts from the plant *Andrographis paniculata* was compared in CCl_4-intoxicated rats. Animals were treated either with Andrographolide (100 mg/kg, ip), the methanolic extract of the plant (861.33 mg/kg, ip, equivalent to 100 mg/kg of

andrographolide) and the andrographolide-free methanolic extract (761.33 mg/kg, ip, equivalent to 861.33 mg/kg of the methanolic extract). Overall, an inhibition of the CCl_4 - induced increases in biochemical parameters of acute liver failure was found to be 48.6 % for andrographolide, 32.0 % for the methanolic extract and 15.0 % for the Andrographolide–free methanolic extract. Furthermore, andrographolide (100 mg/kg, ip) was found to normalize completely the CCl_4-induced increase in the pentobarbitone induced sleep time of mice. Another study reported that alcoholic extract of the leaves of *Andrographis paniculata* at 300 mg/kg prevented against CCl_4-induced liver damage, as evidenced by morphological, biochemical and functional parameters (Rana and Avadhoot, 1991).

Prajjal et al (2007) found that pretreatment of mice with andrographolide protected mice against ethanol-induced liver and kidney toxicity. Supplementation of ethanol-treated mice with andrographolide showed a protective action at 500 mg/kg of body weight, equivalent to the protection observed with a known hepatoprotective drug, silymarin.

Taken together, these results suggest that andrographolide is the major active antihepatotoxic principle present in *Andrographis paniculata* (Handa and Sharma, 1990b).

Andrographolide has also been shown to exhibit a strong choleretic action when administered intraperitoneally to albino rats. This substance induced an increase in bile flow together with a change in the physical properties of the bile secretion (Tripathi and Tripathi, 1991). Andrographolide produced a dose (1.5 to 12 mg/kg po x 7) dependent choleretic activity in conscious rat as well as anaesthetized guinea pig.

Andrographolide has also been shown to display an anticholestatic effect (40-100%) against galactosamine-induced liver cholestasis.

Finally, effects of Andrographolide on hepatocyte viability *in vitro* in culture, by increasing the viability of hepatocytes as tested by trypan blue

exclusion and oxygen uptake tests, GOT, GPT and alkaline phosphatase in rat hepatocytes (Visen et al., 1993).

3.2 Effect on cardiac and vascular disorders

As mentioned above, the presence of many flavones in *Andrographis paniculata* has been reported. Flavone extracted from the root of *Andrographis paniculata* was found to inhibit platelet aggregation and the production of thromboxane B2 (TXB2) as reported by Zhao and Fang (1991). They found that when the aqueous extract of *Andrographis paniculata* was injected intravenously to dogs that had developed the myocardial infarction 1 hr before by formation of thrombus, prostacycline (PGI_2) was increased remarkably. Synthesis of thromboxane (TXA_2) was inhibited whereas the level of cyclic adenosine monophosphate (cAMP) in platelets was elevated. Creatine kinase (CK) peak was lowered and appeared earlier. Platelet maximum aggregation was inhibited and the size of ischemic area recorded by epicardial ECG was reduced. Pathologically, the myocardial structure surrounding the initially appearing ischemic area, i.e., the reversibly damaged area, became relatively normal, while the degree of myocardial degeneration and necrosis in the central part of the ischemic area was mild. *Andrographis paniculata* might limit the expansion of ischemic focus, exert marked protective effect on reversibly ischemic myocardium and demonstrate a weak fibrinolytic action.

In a clinical study, Zhang et al. (1994) had observed 63 patients of cardiac and cerebral vascular disease, 3 h and 1 week after having taken *Andrographis paniculata* extracts and reported that platelet aggregation induced by ADP was significantly inhibited ($P<0.001$) and serotonin (5-HT) released from the platelet decreased ($P<0.01$), but plasma 5-HT levels remained unchanged. They also suggested that a rise in the platelet cAMP level might be another mechanism of the antiplatelet effect of the *Andrographis paniculata* extract.

Both *Andrographis paniculata* and fish oil have been shown to significantly alleviate atherosclerotic iliac artery stenosis induced by both deendothelialization and high cholesterol diet and restenosis following angioplasty in rabbits, while the former had a more marked effect. They both significantly inhibited blood monocytes to secrete growth factors *in vivo*. Ca^{2+}-ATPase activity of cell membrane of atherosclerotic rabbits was significantly decreased. *Andrographis paniculata* might play an important role in preventing restenosis after coronary angioplasty, but fish oil may be useful in reducing the extent of restenosis after coronary angioplasty (Wang and Zhao, 1994).

Thisoda et al. (2006) also reported that andrographolide (AP1) and 14-deoxy-11, 12-didehydroandrographolide (AP3) significantly inhibited thrombin-induced platelet aggregation in a concentration-(1–100 μM) and time-dependent manner while neoandrographolide (AP4) had little or no activity. AP3 exhibited higher antiplatelet activity than AP1 with IC_{50} values ranging from 10 to 50 μM. The inhibition of extracellular signal-regulated kinase1/2 (ERK1/2) pathway may contribute to antiplatelet activity of these two compounds. It appears that *Andrographis paniculata* extract and its active diterpenoids, AP1 and AP3, can inhibit platelet aggregation in vitro; therefore, they may be used to treat or prevent some cardiovascular diseases.

3.3 Effect on gastrointestinal tract

Extracts of *Andrographis paniculata* have been shown to have significant effects against the diarrhea associated with *E. coli* bacterial infections (Gupta et al., 1990). The *Andrographis paniculata* components, andrographolide and neoandrographolide, showed similar activity to loperamide (Imodium), the most common antidiarrheal drug. Acute bacterial diarrhea in patients was treated with a total dose of 500 mg andrographolide divided over three dosing periods *per* day for six days (2.5 to 3.0 mg/kg of body weight). This regimen was combined with rehydration. There were 66 cures of 80 patients treated - an 82.5% cure rate.

Seven additional patients responded favourably to the treatment and only seven patients (8.8%) did not respond. It had been believed that *Andrographis paniculata* was effective against bacterial dysentry and diarrhea because it was antibacterial, but studies could not confirm this effect. However, the andrographolides were very effective in stopping the diarrhea. How this is accomplished is not completely understood at present.

Chronic inflammation of the colon was treated with a decoction of a combination of *Andrographis paniculata* (60 g) and *Rehmannia glutinosa* (30 g). The latter is a Chinese herb used to treat anemia, fatigue, and to promote the healing of injured bones. The liquid part of the mixture was taken at doses of 100 to 150 ml each night for 14 days. Of a total of 85 patients, 61 (72%) were considered clinically cured and 22 (26%) had symptomatic relief (Yin and Guo; 1993).

3.4 Antibacterial / Antimalarial activity

Leelarasamee et al. (1990) designed an experiment to detect serum bactericidal activity after an oral intake of dry herb powder (stem and leaf of AP). The volunteers received a single oral dose of *Andrographis paniculata* (1, 2, 3 and 6 g) in a randomized, cross-over manner. Blood samples were taken at different time intervals after ingestion for the assay of the bactericidal activity by agar diffusion technique. Serum bactericidal activity was not detected in any of the sera tested. Accordingly they had studied in 96 rats which were daily fed with high doses of *Andrographis paniculata* ranging 0.12-24 g/kg, for 6 months. Antibacterial activity was still undetectable when lung parenchyma and liver tissue were placed on culture media containing bacteria tested.

In a clinical study, 100 patients having renal stones less than 3 cm in size and normal renal function underwent extracorporeal shock wave lithotripsy (ESWL). It appeared that that the incidence of post ESWL pyuria and hematuria

in patients receiving *Andrographis paniculata* (1 g, t.i.d., for 5 days) was reduced to 0.69 and 0.55 time of pre ESWL value. The results obtained with *Andrographis paniculata* treatment were equivalent to those obtained when comparing these results with routine treatments using western drugs such as cotrimoxazole and norfloxacin. Besides the efficacy in treatment of urinary tract infection, its side effects were negligible. Patients having history of allergy to sulfa drug and receiving *Andrographis paniculata* instead showed no adverse reactions. Thus this herbal medicine is a promising drug, beneficial for the treatment of post ESWL urinary tract infection, especially in the group of patients allergic to modern medicines (Muangman et al., 1995).

Zaidan et al.,(2005) determined the presence of antibacterial activity in the crude extracts of some of the commonly used medicinal plants in Malaysia including *Andrographis paniculata*, In this preliminary investigation, the leaves were used and the crude extracts were subjected to screening against five strains of bacteria species, Methicillin Resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*, using standard protocol of Disc Diffusion Method (DDM). A water extract of *Andrographis paniculata* presented potential antibacterial activities to both gram positive *S. aureus* and Methicillin Resistant *S. aureus* (MRSA). *Andrographis paniculata* also showed antibacterial activities to gram negative *P. aeruginosa* with MIC of 2 μg/disc.

Extracts of *Andrographis paniculata* containing the four active components previously mentioned were evaluated for antimalarial activity against *Plasmodium berghei,* one of the parasites that transmit malaria. The extract was found to produce considerably inhibition of the multiplication of the parasites (Misra et al., 1992). Two of the *Andrographis paniculata* components, neoandrographolide and deoxandrographolide, were found to be the most effective of the four. Pre-treating animals with neoandrographolide for 15 - 21 days prior to exposure was found to

be more effective than when treatment was started only after infection occurred. However, noteworthy is the fact that when treatment was started after infection, the protection by *Andrographis paniculata* was better than by chloroquine, a commonly used antimalarial drug.

3.5 Antipyretic activity

Andrographis paniculata is also used as a folk medicine remedy for fever, pain reduction, and disorders of the intestinal tract. The ability of *Andrographis paniculata* to lower fever has been demonstrated in several laboratories. Rat studies done in China have shown that andrographolide, neoandrographolide, and dehydroandrographolide can lower the fever produced by different fever-inducing agents, such as bacterial endotoxins (toxic chemicals released from bacteria), pneumococcus, hemolytic streptococcus, typhoid, paratyphoid, and the chemical 2,4-dinitrophenol (Deng, 1978). In another study, *Andrographis paniculata* extracts were found to produce results comparable to 200 mg of aspirin/kg body weight (Vedavathy and Rao, 1991). The researchers also established that there was a wide margin of safety in using *Andrographis paniculata* extract, an indication of lack of toxicity.

The prevention of the common cold with an extract of *Andrographis paniculata* was shown in a pilot double-blind study. Students were given Kan Jang, a formulation of *Andrographis paniculata* produced by the Swedish Herbal Institute, and were diagnosed for the presence or absence of colds during a three-month period. A dose of 200 mg/day was given to the study group. After one month there was no significant difference in the number of colds. However, after the third month of intake of Kan Jang there was a significant decrease in the incidence of colds as compared to the placebo group. The students that got the Kan Jang had a rate of incidence of colds of 30% compared to 62% for students that received the placebo. The relative risk of catching a code indicated that the

preventive effect could be due to the presence of andrographolide, which has known immunostimulant effects (Caceres et al., 1997).

3.6 Anti-inflammatory activity

The anti-inflammatory effects of various compounds extracted from *Andrographis paniculata* have been shown in many studies in which the inflammation was produced by chemicals. Inflammation caused by histamine, dimethyl benzene, croton oil (hemolytic necrosis), and acute pneumocystis produced by adrenaline was significantly reduced or relieved (Deng, 1978). This effect was observed for all major andrographolides: deoxyandrographolide, andrographolide, neoandrographolide, and dehydroandrographolide. Dehydro-andrographolide had the most pronounced effect, followed by neoandrographolide and andrographolide. This anti-inflammatory effect seemed to work by a mechanism that involved the adrenal glands. The effect disappeared when adrenal glands were removed from experimental animals (Yin and Guo, 1993). Further study confirmed that the anti-inflammatory action of dehydroandrographolide was due to its effect on increasing the synthesis and release of andrenocorticotrophic hormone (ACTH) of the pituitary gland of the brain. ACTH signals the adrenal gland to make cortisol, a natural anti-inflammatory (Deng, 1978b).

The 85% ethanolic extract of *Andrographis paniculata* at the dose of 2 g/kg could decrease the swelling of the carrageenan-induced hind paw edema in albino rat. This inflammatory effect was not found in this animal model when aqueous extract and 50% ethanolic extract were used (Sawasdimongkol et al., 1990). In a subsequent study, researchers have reported that the anti-inflammatory effect of andrographolide could be explained by its ability to inhibit neutrophil adhesion/transmigration through suppression of Mac-1 up-regulation. The inhibitory effect of andrographolide on Mac-1 expression could be mediated by down regulation of ROS production via a PKC-dependent but calcium independent mechanism. As effective anti-adhesive and anti-transmigration drug

at pharmacological concentrations (0.1-10 mM), andrographolide may be useful for the improvement of inflammatory disorders by limiting the early phases of neutrophil infiltration (Shen et al., 2002).

3.7 Hypoglycemic effect

Experiments in rabbits showed that water extracts of *Andrographis paniculata* at the dose of 10 mg/kg could prevent induction of hyperglycemia by oral administration of glucose 2 mg/kg but not in adrenalin-induced hyperglycemia. It also failed to demonstrate any "fasting blood sugar lowering effect" upon chronic administration (6 weeks). It has been suggested that *Andrographis paniculata* prevents glucose absorption from gut (Borhanuddin et al., 1994). In C2C12 cells, andrographolide increased radioactive glucose uptake in a concentration-dependent manner that was abolished by pre-treatment with prazosin. Activation of alpha1-adrenoceptors by andrographolide may increase the glucose uptake via the phospholipase C (PLC)-protein kinase C (PKC) pathway (Hsu et al., 2004). Rafidah et al. (2004) reported a significant 50% reduction in blood glucose levels when hyperglycaemic rats were treated with 50 mg/kg body weight (BW) aqueous extract of *Andrographis paniculata*. This effect was enhanced when freeze–dried material was used, where 6.25 mg/kg BW gave a 60% reduction in blood glucose level.

3.8 Antifertility effect

Dry leaf powder of *Andrographis paniculata* , when fed orally to rats, at a dose of 20 mg per day for 60 days, resulted in cessation of spermatogenesis, degenerative changes in the seminiferous tubules, regression of Leydig cells and regressive and/or degenerative changes in the epididymis, seminal vesicle, ventral prostate and coagulating gland. The results suggest antispermatogenic and/or antiandrogenic effect of this plant (Akbarsha et al., 1990). On the other hand, Burgos et al., (1997) reported no testicular toxicity with the treatment of 20, 200

and 1000 mg/kg during 60 days as evaluated by reproductive organ weight, testicular histology, ultrastructural analysis of Leydig cells and testosterone levels after 60 days of treatment. It was concluded that *Andrographis paniculata* dried extract induced its antifertility effect without any subchronic testicular toxicity effect in male rats.

3.9 Immunological benefits: cancer, HIV & other viruses

Mice studies have shown that *Andrographis paniculata* is a potent stimulator of the immune system, and this by two mechanisms, i.e. by modulating the **antigen-specific response,** the immune reaction by which antibodies are made to counteract invading microbes, and by modulating the **non-specific immune response**, the immune mechanism by which macrophage cells scavenge and destroy invaders. *Andrographis paniculata* has been shown to activate both responses and thus making it effective against a variety of infectious and oncogenic agents (Puri et al., 1993).

In a study with mice, researchers searched for naturally occurring substances that would cause differentiation *in vitro* of leukemia cells. *Andrographis paniculata* was chosen because it contained substances (terpenes) that were similar to substances found in other plants and were known to cause differentiation of cancer cells. The results of the study demonstrated that *Andrographis paniculata* had potent cell differentiation-inducing activity on leukemia cells (Matsuda et al., 1994).

In addition, *Andrographis paniculata* extracts from the leaves of the plant have also been found cytotoxic *in vitro* against cancer cells. This cancer cell-killing ability was demonstrated against human epidermoid carcinoma skin cell lines of the nasopharynx and against lymphocytic leukemia cells (Talukdar and Banerjee, 1968). It was the andrographolide component that was found to have the cancer cell-killing ability. This ability for killing cancer cells was superior to the

levels of the effectiveness recommended by the National Cancer Institute for a cytotoxic substance.

Japanese researchers have reported that *Andrographis paniculata* stopped stomach cancer cells from multiplying *in vitro*. After three days, there were less than 8 cancer cells growing in the presence of *Andrographis paniculata* while the untreated cancer cells numbered 120. Another group of Japanese researchers tested *Andrographis paniculata* on sarcoma cells. These usually very malignant cancers affect muscle, connective tissue, and bones. When tumor samples were examined under the microscope, *Andrographis paniculata* was found to inhibit the growth of the tumors.

Laboratory tests conducted in Buffalo, New York, demonstrated that *Andrographis paniculata* inhibited the growth of human breast cancer cells at levels similar to the drug tamoxifen. Extracts of *Andrographis paniculata* were much less toxic than most chemotherapeutic agents used to fight cancer. Although more studies need to be done to determine just which types of cancer respond to *Andrographis paniculata*, the results so far have been promising.

In 1977, a human study was conducted using *Andrographis paniculata* in sixty skin cancer patients, including forty-one with confirmed metastases. As reported in the *Journal of Chinese Medicine*, twelve patients given *Andrographis paniculata* and its compounds (andrographolide) alone, recovered. All other patients were given *Andrographis paniculata* along with standard drugs; there was no tumor regrowth in forty-seven of these patients. Based on this report, American investigators obtained investigational new drug status from the FDA to test *Andrographis paniculata* extract. In 1996, early trials showed that the extract safely and effectively blocked growth of prostate and breast cancers, as well as non-Hodgkin's lymphomas. Based on the results of using *Andrographis paniculata* on breast cancer cells grown in the laboratory, researchers believe that *Andrographis paniculata* probably inhibits synthesis of cancer cell DNA. Additional details of cancer trials are given in the book, *Miracle Herbs* by Stephen

Holt, M.D., wherein cancer studies done at Roswell Park Cancer Institute in Buffalo, New York, showed that *Andrographis paniculata* extract has an antiprostate cancer action comparable to that of the widely used and highly toxic agent cisplatin without the toxicity.

Cheung et al., (2005) reported that andrographolide, in comparison to other two diterpenoids: deoxyandrographolide and neoandrographolide, had more potent anti-cancer activity against human leukemia HL-60 cells and other cancer cells. As demonstrated by chromosomal DNA fragmentation, accumulation of HL-60 cells in the sub-G1 cell cycle stage, disappearance of mitochondrial cytochrome c and an increased expression of Bax and downregulation of Bcl-2 in the inhibited cells, it induced apoptosis of HL-60 cells. In a subsequent study, they indicated that andrographolide induced cell cycle arrest at the G2/M phase and late cell death in HepG2 via an intrinsic pathway to decrease the level of GSH and by means of excessive accumulation of intracellular hydrogen peroxide (Li et al., 2007).

Exciting recent research has indicated that extracts of *Andrographis paniculata* may have great promise for interfering with the viability of the HIV virus. Scientists now believe that *Andrographis paniculata* can join with modern technology in the fight against AIDS. Cells, when they grow and reproduce, go through a series of steps collectively termed the cell cycle. During this process, chemical messages are carried to various parts of the cell in order to turn on functions. This process is called signal transduction. The HIV virus actually subverts the cells messengers, tricking them into producing more viral particles. Using signal transduction technology, scientists found that *Andrographis paniculata* contained substances that destroyed the communications mechanism of virus. One component of the herb “andrographolide” prevented transmission of the virus to other cells and stopped the progress of the disease by modifying cellular signal transduction. Andrographolide probably does this by inhibiting enzymes that facilitate the transfer of phosphates. Phosphates are molecules that

are the energy storehouses of the cell. During the cell cycle, phosphates are created or chemically changed and energy is produced. This energy is used in the regulation of the cell cycle and for the many cellular functions that go on during reproduction of the cell. *Andrographis paniculata* can thus interfere with key enzymes that result in viral reproduction (Holt and Comac, 1998).

HIV alters regulation of the cell cycle by causing the process to stop at a particular phase. What the virus specifically does is to alter the action of a central information-processing enzyme that coordinates all events relating to cell division. This regulatory enzyme is called cyclin-dependent kinase (CDK). A particular CDK (CDK-1) is the prime target of HIV. When the cell moves through its cycle, all information about cellular activities is sent to CDK-1. Several diseases in addition to the AIDS infection *per se*, such as cancer, heart disease, and viral infections, are associated with aberrant functioning of CDK-1. The virus causes CDK-1 to misfunction by attaching molecules to it is a process called phosphorylation. Agents that can prevent this phosphorylation can less the severity of AIDS. The new class of antiviral compounds with this ability is called tyrosine kinase inhibitors. This class includes the andrographolides. Work done by researchers at the National Institutes of Health (NIH - USA) in 1995 showed that T-cells infected with HIV accumulate high levels of over phosphorylated CDK-1. An extract of *Andrographis paniculata* can, in fact, inhibit CDK-1 that has been altered by HIV. In 1992, NIH researchers reported that these inhibitors could halt the disease-causing components of HIV. These compounds are amino acids that can inhibit the viral enzymes involved in the production of high-energy phosphates.

Cooperative research at the National Cancer Institute has shown that andrographolide can also inhibit HIV's toxic effect on cells. It does this by inhibiting c-mos, a genetic component involved in HIV propagation and T-cell death. C-mos is integrated into the DNA of the cell and usually is inactive. Normally found only in reproductive system cells, c-mos is not expressed in CD4

cells or other body cells. When CD4 cells are infected by the HIV virus, c-mos expression is activated. For this to happen, an enzyme (c-mos kinase) is needed. *Andrographis paniculata* extracts can inhibit this enzyme and so can support normal immune function. A hypothesis for the mechanism of action of *Andrographis paniculata* in AIDS is that the herbal extract appears to induce apoptosis or programmed cell death. In this process, cells break up into particles which are then scavenged by immune system cells. The HIV virus may generate apoptotic signals to uninfected immune cells. This would explain the extensive T-cell destruction caused by HIV infection, which is far more than the amount of virus present.

Testing of *Andrographis paniculata* done at the Frederick Research Centre demonstrated that extracts of AP increased AZT's ability to inhibit replication of HIV. The effect of the combination was greater than that of either compound alone. An added benefit is that lower doses of AZT could be used.

Some researchers believe that *Andrographis paniculata* extracts may also be useful in combating other viruses, including the Ebola virus and the viruses associated with herpes, hepatitis, and influenza. In a study examining 27 types of "heat clearing" and detoxifying medicinal herbs, researchers at the China Academy of Traditional Chinese Medicine in Beijing reported that *Andrographis paniculata* was one of the herbs that had an inhibitory effect on HIV replication (Weibo, 1995).

3.10 Nervous system effect

Many herbal compounds do not penetrate the blood-brain barrier. However, andrographolide does so and concentrates in the brain and particularly in the spinal cord (Weibo, 1995). Several studies have shown that *Andrographis paniculata* products have a sedative effect. In mice given barbital as anesthesia, the animals became sedated more quickly and the anesthesia lasted longer when co-administered with extracts of *Andrographis paniculata*. Also, it was possible to

give less of the anesthesia if it was given along with *Andrographis paniculata* (Deng, 1978). The studies indicate that products from *Andrographis paniculata* may act at the barbital receptors in the brain.

3.11 Respiratory system effect

Andrographolide has been used to treat tonsilitis, respiratory infections, and tuberculosis. In one study, *Andrographis paniculata* was used to treat 129 cases of acute tonsilitis. Sixty-five percent of patients responded to the therapy. The same authors used andrographolide to treat 49 pneumonia patients. Thirty five cases were found to show positive changes and nine patients completely recovered. In another study, andrographolide was used to treat 111 patients with pneumonia and twenty with chronic bronchitis and lung infection. The overall effectiveness of *Andrographis paniculata* treatment was 91%. Fever subsided within three days in 72% of the patients and 40% of these patients had smaller areas of infection within one week.

Tuberculosis is usually treated within the antibiotic rifampin. When used alone, rifampin therapy still results in 22.5% of patients dying. In a study using an injectable solution of 2.5% andrographolide given so as to provide 50 to 80 mg/kg body weight per day for two months, results were improved. Of seventy cases of tubercular meningitis, 30% of patients were considered cured with a fatality rate of 8.6%. The combination of andrographolide plus rifampin resulted in a 2.6-fold decrease in fatality rates.

A three-arm clinical study comparing the efficacy of Kan Jang, a fixed herbal combination containing standardized *Andrographis paniculata* (N.) SHA-10 extract, with Immunal, a preparation containing *Echinacea purpurea* (L.) extract, in uncomplicated common colds was carried out in 130 children aged between 4 and 11 years over a period of 10 days. It was found that the adjuvant treatment with Kan Jang, was significantly more effective than Immunal, when started at an early stage of uncomplicated common colds. The effect of Kan Jang

was particularly pronounced on nasal secretion (g/day) and nasal congestion. Kan Jang also accelerated the recovery time, whereas Immunal did not show the same efficacy (Spasov et al., 2004).

4. Pharmacokinetics

Wang et al., (1995) reported the pharmacokinetic parameters of andrographolide in the blood plasma of rabbits after oral administration of a water extract of *Andrographis paniculata*. It was found that the maximal concentration (22.4 mg/ml) of Andrographolide (AND) in the blood occurred two hours after drug administration.

Andrographolide is quickly and almost completely absorbed into the blood following the oral administration of the extract of *Andrographis paniculata* at a dose of 20 mg/kg body wt in rats, and is 55% bound to plasma proteins.

Following the oral administration of four Kan Jang tablets (a single therapeutic dose, equal to 20 mg of AND) to humans, maximum plasma levels of 393 ng/ml (approx. 1.12 μM) were reached after 1.5-2 hours, half-life time was 6.6 hours, respectively. The calculated steady state plasma concentration of AND for multiple doses after the normal therapeutic dose regimen, about 1 mg/kg/day) was 660 ng/ml, and the concentration in blood was 1342 ng/ml (approx. 3.8 μM). It is intensely metabolized and renal excretion is not the main route for elimination (Panossian et al., 2000).

5. Toxicity

Acute toxicity testing of 50 % alcoholic extract of *Andrographis paniculata* revealed no evidence of toxicity in mice when given at the dose of 15 g/kg, with LD50 of the extract given orally or subcutaneously was >>> 15 g/kg. When given intraperitoneally *Andrographis paniculata* was more toxic, with an LD50 it was 14.98 g/kg (Sithisomwongse et al., 1989).

Oral administration of a suspension of leaf powder at 2 g/kg, a suspension of alcohol extract at 2.4 g/kg, or andrographolide at 3 mg/kg, showed no acute toxic effects in both male and female mice (Dhammaupakorn and Chaichantipyuth, 1989).

Subchronic toxicity testing of *Andrographis paniculata* powder was evaluated for 6 months in 96 Wistar rats divided into four groups consisting of control, 0.12, 1.2 and 2.4 g/kg/day equivalent to 1, 10 and 20 times of human therapeutic doses (6 g/day/man 50 kg). Potential toxic effects were assessed by observing and measuring growth rate (body weight), food consumption, clinical signs, hematological, serum biochemical values and histopathological changes. No evidence of abnormalities has been found at any of the tested doses (Sithisomwongse et al., 1989).

6. In summary

Andrographis paniculata is an annual plant with characteristic white-purple or spotted purple flowers that flourishes in South-East Asia, China and India. It has been valued for centuries by herbalists as a treatment for upper respiratory infections, fever, sore throat and herpes. Other reported applications include its use in cases of malaria, dysentery and even snakebites. Now, important new research has confirmed a host of pharmacological benefits for this herb, including potent anti-inflammatory, anti-bacterial, hepatoprotective effects and anti-viral effects as summarize in table 1. In addition, scientists have discovered that *Andrographis paniculata* helps boost the immune system, protects against cancer, prevents blood clots and maintains efficient digestive functioning. Andrographolide, the major constituent of the extract is implicated towards its pharmacological activity (table 1). When consumed, andrographolides appear to accumulate in organs throughout the viscera. Absorption and excretion is rapid: 80% is removed within eight hours via the kidney (urine) and gastrointestinal tract. Ninety percent is eliminated within forty-eight hours. In Traditional Chinese Medicine (TCM) and in systems of healing in Thailand and India, *Andrographis*

paniculata has long been perceived as safe. Formal toxicological studies in animal models and in animal and human clinical trials confirmed that andrographolide and other members of this *Andrographis paniculata* family of compounds have very low toxicity. To date, the herb is best known in Western society as a popular over-the-counter alternative remedy for the common cold. Several double-blind clinical trials have shown that andrographis can reduce the severity of symptoms.

Table1. Summary of the Pharmacological activities of *Andrographis paniculata* and andrographolide.

Pharmacological properties	Effects	Dose/Route of administration	System
Hepatoprotective effect	- against galactosamine-induced hepatotoxic	- AND 400 mg/kg ip or 800 mg/kg po	- in vivo, rat
	- against paracetamol-induced hepatotoxic	- AND 200 mg/kg ip	- in vivo, rat
	- against paracetamol-induced hepatotoxic	- AND 100 mg/kg, ip or APE 861.33 mg/kg, ip	- in vivo, rat
	- against ethanol-induced liver and kidney toxicity	- AND 500 mg/kg	- in vivo, mice
	- against paracetamol-induced hepatotoxic	- AND 0.75-12 mg/kg po	- in vitro, rat hepatocytes
Cardiovascular effects	- inhibit platelet aggregation	- APE, iv	- in vivo, dog
	- inhibit platelet aggregation	- APE, po	- clinical study
	- alleviate atherosclerotic iliac artery stenosis	- APE	- in vivo, rabbit
	- inhibit platelet aggregation	- APE 10-100 μg/ml	- in vitro
Gastrointestinal effects	- against the diarrhea	- 500 mg AND, po	- clinical study
	- chronic colon inflammation	- 100-150 ml liquid mixture, po	- clinical study
Antimicrobial activity	- treatment UTI	- 1 g tid of dry powder	- clinical study
	- antibacterial activity	- MIC < 2 μg/disc	- in vitro, disc diffusion method
	- activity against *Plasmodium berghei,*	- APE	- in vivo, animal

Table1. Summary of the Pharmacological activities of *Andrographis paniculata* and andrographolide. (Continue)

Pharmacological properties	Effects	Dose/Route of administration	System
Antipyretics	- lower the fever produced by endotoxins - treat common cold	- AND and other compound - 200 mg/day	- in vivo, rat - clinical study
Anti-inflammatory activity	- increase release ACTH - decrease carrageenan-induced hind paw edema	- dehydroandrographolide - APE 2 g/kg	- in vivo, albino rat
Hypoglycemic effects	- prevent induction of hyperglycemia - reduce blood glucose level	- APE 10 mg/kg, po - APE 50 mg/kg, po	- in vivo, rabbit - hyperglycaemic rats
Antifertility effects	- antispermatogenic and/or antiandrogenic effect - antifertility effect without any testicular toxicity	-20 mg dry powder per day, po - 20-1000 mg/kg APE	-in vivo, rat -in vivo, rat
Immunostimulant, Anticancer,	- inhibit formation of oxygen derived free radicals -induce cell cycle arrest in HL-60 cells - cytotoxic effect, induces cell cycle arrest at G2/M pase - inhibits the proliferation of HT-29 (colon cancer) cells and augments the proliferation human peripheral blood lymphocytes (HPBLs)	- APE - APE, AND - AND (IC_{50} 40.2μM) -APE	- in vitro - in vitro, HL-60 cells - HepG2 cells - human cancer and immune cells
Nervous system effect	- produce sedative effect	- APE	- in vivo, mice
Respiratory system effects	- treat uncomplicated common colds - treat tuberculosis	- APE - AND 50 to 80 mg/kg	- clinical study - clinical study

CHAPTER 2: XENOBIOTICS BIOTRANSFORMATION

1 Introduction

Xenobiotics are chemical compounds that do not belong to the normal composition of the human body. These compounds enter the body via the diet, air and medication. The principal route of elimination of xenobiotics from the body is biotransformation. Drug metabolizing enzymes (DMEs) play central roles in the metabolism, elimination and/or detoxification of xenobiotics or exogenous compounds introduced into the body (Meyer, 1996). In general, DMEs protect the body against the potentially harmful exposure to xenobiotics from the environment. In order to minimize the potential injury caused by these compounds, most of the tissues and organs are well equipped with diverse and various DMEs including phase I, phase II metabolizing enzymes (Figure 1) as well as phase III transporters, which are present in abundance either at a basal expression level, and/or are induced and present at an increased level after xenobiotic exposure (Meyer, 1996; Wang and LeCluyse, 2003).

Phase I DMEs consist primarily of the cytochrome P450 (CYP) superfamily of microsomal enzymes, which are found abundantly in the liver, gastrointestinal tract, lung and kidney, consisting of families and subfamilies of enzymes that are classified based on their amino acid sequence identities or similarities (Gonzalez and Nebert, 1990; Guengerich, 2003; Meyer, 1996). In human, five CYP gene families, i.e. CYP1, CYP2, CYP3, CYP4 and CYP7 are believed to play crucial roles in hepatic as well as extra-hepatic metabolism and elimination of xenobiotics and drugs (Gonzalez and Nebert, 1990; Lewis, 2003).

The phase II metabolizing or conjugating enzymes, consists of a superfamily of enzymes including sulfotransferases (SULT), UDP-glucuronosyltransferases (UGT), epoxide hydrolases (EPH), glutathione *S*-transferases (GST) and *N*-acetyltransferases (NAT). Each superfamily of phase II

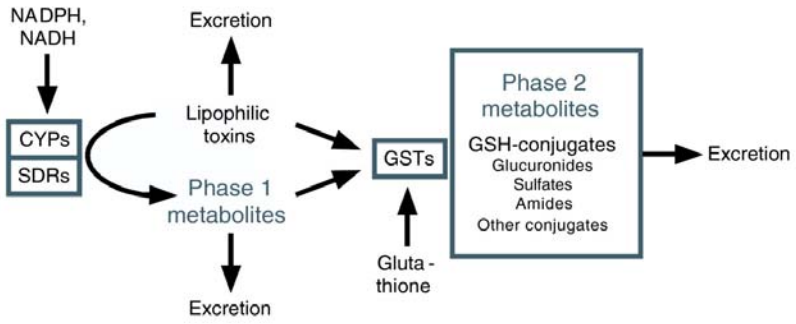


Figure 1: Cellular detoxification (drug metabolism). This process entails two phases: phase 1 (functionalization), and phase 2 (conjugation); CYPs=cytochromeP450; SDRs=short-chain dehydrogenase reductases; GSTs=Glucosyltransferase

DMEs consists of families and subfamilies of genes encoding the various isoforms with different substrate specificity, tissue and developmental expression, as well as inducibility and inhibitory by xenobiotics (Hinson and Forkert, 1995). Conjugation with phase II DMEs generally increases hydrophilicity, and thereby enhances excretion in the bile and/or the urine.

Drug transporters, including P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and organic anion transporting polypeptide 2 (OATP2) are expressed in many tissues such as the liver, intestine, kidney, and brain, where they provide a formidable barrier against drug penetration, and play crucial roles in drug absorption, distribution, and excretion (Staudinger et al., 2003).

Variations in the expression of phase I, phase II DMEs and drug transporters has potential impact on the metabolism, elimination, pharmacokinetics/dynamics, toxicokinetics/dynamics, drug-drug interactions of many therapeutic agents, as well as their ability in the protection of the human body against exposure of environmental xenobiotics (Guengerich, 2003;Wang and LeCluyse, 2003).

2 Phase I metabolism

Phase I metabolism includes oxidation, reduction, hydrolysis and hydration reactions. Oxidations performed by the micosomal mixed-function oxidase system (cytochrome P450-dependent) is considered separately because of its importance

and the diversity of reactions performed by this enzyme system. Oxidations involving cytochrome P450 system found in microsomes (endoplasmic reticulum) of many cells (notably of liver, kidney, lung and intestine) perform many different functionalisation reactions e.g. aromatic hydroxylation, aliphatic hydroxylation, epoxidation, *N*-dealkylation, *O*-dealkylation, *S*-dealkylation, oxidative deamination, *N*-oxidation, *S*-oxidation, dehalogenation and alcohol oxidation. All of these reactions require the presence of molecular oxygen and NADPH as well as the complete mixed-function oxidase system (cytochrome P450, NADPH-cytochrome P450 reductase and lipid) All reactions involve the initial insertion of a single oxygen atom into the drug molecule. A subsequent rearrangement and/or decomposition of this product may occur, leading to the final product seen (Figure 2). Reductive reactions usually require NADPH but are generally inhibited by oxygen, unlike the mixed-function oxidase reactions that require oxygen. Examples of compounds undergoing reduction are azo-compound, nitro-compound, epoxide, heterocyclic ring compound and halogenated hydrocarbon. In most cases the final product after phase I contains a functional group, such as –OH, $–NH_2$, –SH, –COOH, etc in the adequate chemical state to be by substrates of the phase II or conjugative enzyme.

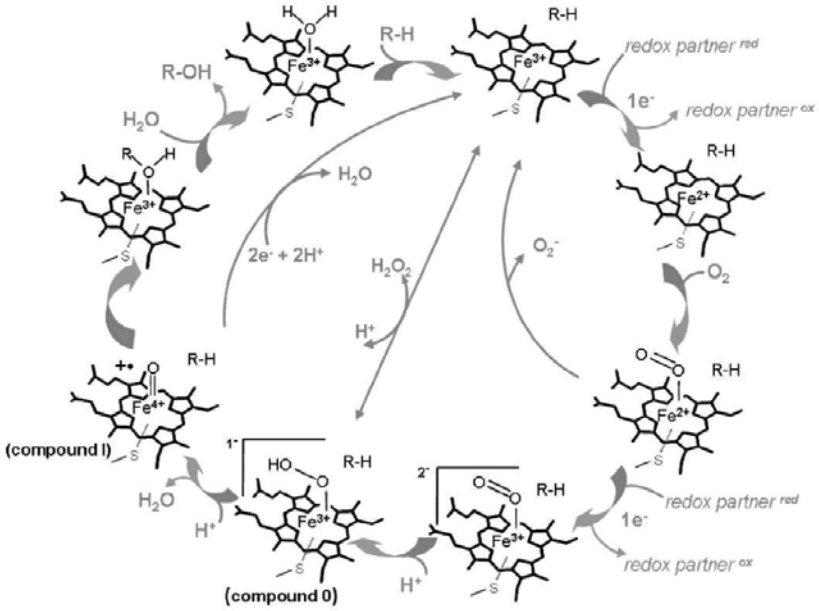


Figure 2: The catalytic cycle of cytochrome P450.

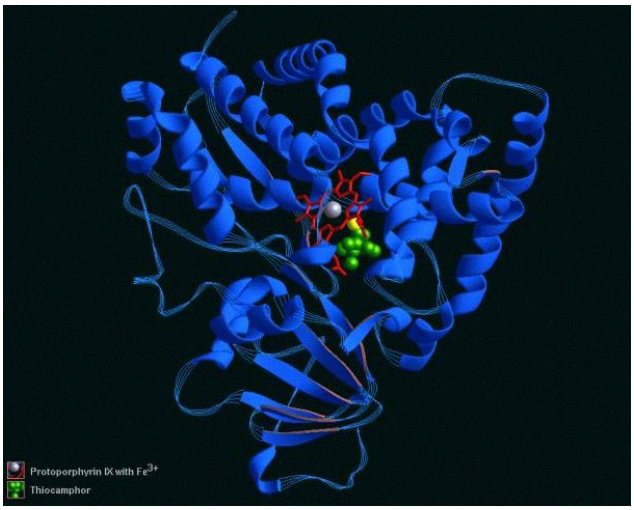


Figure 3: Cytochrome P450 oxidases are an important set of enzymes involved in xenobiotic metabolism.

2.1 Cytochrome P450 enzymes - interspecies differences

Animal models are commonly used in the preclinical development of new drugs to predict the metabolic behavior of new compounds in humans including potential drug-drug interactions. It is generally believed that pharmacokinetic data can be extrapolated to human reasonably well, using the appropriate pharmacokinetic principles. Generally, rodents are used because of their short lifespan, allowing the growth of a large number of animals in a short period of time and, consequently, the feasibility of many studies. In contrast, large animals live longer, allowing for longitudinal studies and they are more close in size to humans providing an opportunity to address issues related to scaling up to human therapy. It is however important to realize that humans differ from animals with regarding to isoform composition, expression and catalytic activities of drug metabolizing enzymes and that the validity of animal testing to predict drug metabolism and pharmacokinetic in humans needs to be questioned as it is for the assessment of efficacy and safety in human for a given drug.

Cytochrome P450 (CYP) is a group of hemoproteins that play a central role in the oxidative metabolism (phase I) of clinically-used drugs and other xenobiotics (Figure 3). The P450 superfamily is divided into families (e.g. CYP1, CYP2, CYP3, etc.) where the primary structure is more than 40% identical, and in subfamilies (labeled with letters A, B, C, etc.) where the primary structure is more

than 55% identical (Nelson et al., 1993), and finally by an Arabic number, representing the individual enzyme.

In human, more than 50 isoforms have been isolated and ~35 CYP isoenzymes are clinically relevant, although P450s 1, 2, and 3 families appear to be mainly responsible for the metabolism of drugs and other xenobiotics (Table 1), and are also involved in metabolic conversion of a variety of endogenous compounds such as vitamins, bile acids and hormones. The CYP isoenzymes from the other families are generally involved in endogenous processes, particularly hormone biosynthesis.

Table 1. Summary of xenobiotic-metabolising human hepatic CYPs.

CYP	Relative amount In liver (%)	Substrates (reaction in parenthesis)	Selective inhibitors	Other characteristics
1A2	~10	Ethoxyresorufin (*O*-deethylation) Phenacetin (*O*-deethylation)	Furafylline	Inducible
2A6	~10	Coumarin (7-hydroxylation)		Polymorphic
2B6	~1	*S*-Mephenytoin (*N*-demethylation) Bupropion (hydroxylation)	Orphenadrine	
2C8	<1	Paclitaxel (6α-hydroxylation)	Quercetin	
2C9	~20	Tolbutamide (hydroxylation) Diclofenac (hydroxylation) *S*-Warfarin (7-hydroxylation)	Sulfaphenazole	Polymorphic
2C19	~5	*S*-mephenytoin (4'-hydroxylation) Omeprazole (oxidation)		Polymorphic
2D6	~5	Dextromethorphan (*O*-demethylation) Debrisoquine (4-hydroxylation) Bufuralol (1'-hydroxylation)	Quinidine	Polymorphic
2E1	~10	Chlorzoxazone (6-hydroxylation) Aniline (4-hydroxylation)	Pyridine	Inducible
3A4	~30	Midazolam (1'- and 4-hydroxylation) Testosterone (6β-hydroxylation) Nifedipine (dehydrogenation)	Azole antimycotics	Inducible

Data adapted from Pelkonen and Breimer (1994) and Pelkonen et al. (1998).

In animals and in humans, CYPs can be found in virtually all organs notably the liver, intestine, skin, nasal epithelia, lung and kidney, but also in testis, brain etc. However, the liver (300 pmol of total CYPs/mg microsomal protein) and the intestinal epithelium (~20 pmol of total CYPs/mg microsomal protein) are the predominant sites for P450-mediated drug elimination, while the other tissues contribute to a much smaller extent to drug elimination (Peters and Kremers, 1989). Although all members of this superfamily possess highly conserved regions of amino acid residues, there are small differences in the primary amino acid sequences of the cytochrome P450s across species. However, even small changes in the amino acid sequences can give rise to profound differences in substrate specificity and catalytic activity. Thus differences in CYP isoforms between species are a major cause of species differences in drug metabolism.

2.1.1 CYP1A

This P450 subfamily consists of two members, CYP1A1 and CYP1A2 (Table 2) in mouse, rat, dog, monkey and human. CYP1A shows a quite strong conservation among species with an identity to human higher than 80%. Both isoforms have been studied extensively because of their roles in the metabolism of two important classes of environmental carcinogens, polycyclic aromatic hydrocarbons (PAHs) and arylamines (Sugimura and Sato, 1983). CYP1A1 is expressed at very low levels in mouse, rat, dog, monkey and human liver but it is present predominantly in the intestine, lung, placenta and kidney (Pelkonen et al., 1998). The expression levels of CYP1A1 in human intestine are reported to be variable. According to McDonnell et al., the CYP1A1 catalytic activity varied considerably in human intestine and microsomal preparations. In fact, higher levels of CYP1A1 are often associated with increased smoking, physical exercise and ingestion of char grilled meats. CYP1A1 preferentially catalyzes the oxygenation of PAHs, such as benzo[a]pyrene, into reactive epoxide intermediates, whereas CYP1A2 transforms aryl and heterocyclic amines to N-

Table 2. CYP enzymes of the major drug metabolizing CYPs family in human, mouse, rat, dog and monkey. Bold type indicates the predominant forms.

Family	Subfamily	Human	Mouse	Rat	Dog	Monkey
CYP1	A	1A1, **1A2**	1a1, **1a2**	1A1, **1A2**	1A1, **1A2**	1A1, 1A2
	B	1B1	1b1	1B1	1B1	1B1
CYP2	A	**2A6**, 2A7, 2A13	2a4, 2a5, 2a12, 2a22	**2A1**, 2A2, 2A3	2A13, 2A25	2A23, 2A24
	B	**2B6**, 2B7	2b9, 2b10	**2B1**, 2B2, 2B3	2B11	2B17
	C	2C8, **2C9**, 2C18, 2C19	2c29, 2c37, 2c38, 2c39, 2c40, 2c44, 2c50, 2c54, 2c55	2C6, 2C7*, **2C11***, 2C12*, 2C13*, 2C22, 2C23	2C21, 2C41, 2C42,	2C20, 2C43
	D	**2D6**, 2D7, 2D8	2d9, 2d10, 2d11, 2d12, 2d13, 2d22, 2d26, 2d34, 2d40	2D1, 2D2, 2D3, 2D4, 2D5, 2D18	2D15	2D17**, 2D19**, 2D29**, 2D30** 2D42**
	E	2E1	2e1	2E1	2E1	2E1
CYP3	A	**3A4**, 3A5, 3A7, 3A43	3a11, 3a13, 3a16, 3a25, 3a41, 3a44	**3A1**/3A23, 3A2*, 3A9*, 3A18*, 3A62	3A12, 3A26	3A8

* gender difference; ** strain specific

Main references for CYPs in difference species: Nelson et al. (1993); Hewitt et al. (2007); Zuber et al. (2002); http://drnelson.utmem.edu/cytochromeP450.html

hydroxylated metabolites, which are further modified by phase II enzymes to carcinogens (Ma and Lu, 2007; Shimada and Fujii-Kuriyama, 2004). CYP1A2 is expressed mainly in the liver and is not or less expressed in extrahepatic tissues in human, rat and mouse. In human liver CYP1A2 accounts for 13% of the total CYP content and is involved in the metabolism of ~4% of total drugs (Nashimura et al., 2003). In contrast, in monkey and dog, CYP1A2 is expressed at low levels in the liver of untreated animals, even though a quite strong similarity in amino acid sequence to human (95% in monkey). In human, CYP1A2 metabolizes several drugs, including phenacetin, tacrine, ropinirole, acetaminophen, riluzole, theophillyne and caffeine (Cupp and Tracy, 1998). The expression of CYP1A family members is highly inducible in response to some substrates, including PAHs (e.g. 3-methyl-cholantrene and benzo[a]pyrene), polyhalogenated aromatic hydrocarbons (e.g. tetrachlorodibenzo-p-dioxin, TCDD), aromatic amines and flavonoids (Hashimoto and Degawa, 1995; Moon et al., 2006)

2.1.2 CYP1B

In human, CYP1B was discovered when it was found to be transcriptionally induced by 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin within a human keratinocyte cell line. CYP1B1 is constitutively expressed in normal tissues, such as heart, brain, placenta, lung, liver, kidney, prostate, but it is express at much higher levels in tumor cells compared with the surrounding normal tissue. Thus, CYP1B1 induction is an important factor in determining risk associated with hormone mediated cancers. In addition, CYP1B1 is involved in the metabolism of some clinically relevant anticancer agents used in the treatment of hormone-mediated cancer. Human CYP1B1 also catalyzes estrogens to active 4-hydroxylated derivatives that may cause breast cancer. In rat, CYP1B1 is expressed in liver and lung. In mouse, CYP1B1 has been detected in several tissues such as testis, kidney, skeletal muscle, lung, spleen, brain, liver and heart. In human and rodents species, CYP1B1 can bioactivate carcinogenic polycyclic aromatic hydrocarbons, such as benzo[a]pyrene to DNA-reactive species associated with toxicity,

mutagenesis and carcinogenesis. Furthermore benzo[a]pyrene can induce expression of CYP1B1 via the aryl hydrocarbon receptor (Harrigan et. al., 2006).

2.1.3 CYP2A

In human, CYP2A consists of 3 subfamilies CYP2A6, CYP2A7 and CYP2A13 (Table 2). CYP2A6 is expressed in human liver and accounts for about 4% of total hepatic P450, whereas other subfamily isoforms (2A7 and 2A13) expressed at lower levels. Human CYP2A6 shows different substrate specificity, in comparison to CYP2A enzymes in animal species. CYP2A6 is involved in the metabolism of xenobiotics such as *O*-deethylation of 7-ethoxycoumarin, 7-hydroxylation of coumarin (a marker reaction), oxidation of nicotine, cyclophosphamide, iphosphamide, fadrozole and aflatoxin B1 (Guengerich, 1997). In rat, the CYP2A family consists of CYP2A1, CYP2A2 and CYP2A3 (Cytochrome P450 Homepage, 2008). Rat CYP2A1 (female dominant) and CYP2A2 (male dominant) are expressed in the liver (2%) (Haduch, 2005). CYP2A3 is constitutively expressed in the esophagus, lung and nasal epithelium, but not in intestine, liver and kidney. The rat CYP2A1/2 has about 60% homology in amino acid sequence to human CYP2A6. CYP2A1 was catalyses 7α-hydroxylation of testosterone, while CYP2A2 catalyses 15α- and 7α-hydroxylation of testosterone.

In mouse, CYP2A4, CYP2A5, CYP2A12 and CYP2A22 belong to CYP2A family. CYP2A5 is expressed mainly in the liver, olfactory mucosa, kidney, lung, brain and small intestine. CYP2A5 resembles the human orthologue in catalyzing 7-hydroxylation of coumarin. CYP2A4 is a female-predominant form in liver, and the transcription is repressed by growth hormone in males (Guengerich, 1997).

In dog, CYP2A13 and CYP2A25 and in monkey, CYP2A23 and CYP2A24, are members of CYP2A family. As reported by Bogaards *et al.* dog and monkey microsomes catalyse coumarin 7-hydroxylation. The human CYP2A6 antibody showed moderate to strong inhibition of coumarin 7-hydroxylase activities towards monkey, dog, human and mouse. In human,

CYP2A6 is induced by phenobarbital, rifampicin, dexamethasone and nicotine (Honkakoski and Negishi, 1997). In rats, CYP2A3 mRNA is increased by treatment with 3-methylcholanthrene and pyrazole in the esophagus, in the kidney and in the distal part of the intestine. The mechanism of CYP2A gene induction is not well understood, but recent studies concerning the murine CYP2A5 indicate the role of constitutive androstane receptor (CAR), pregnane X receptor (PXR) and peroxisome proliferators-activated receptor (PPAR) in transcriptional activation of CYP2A5. Moreover, human CYP2A6 may be regulated post-transcriptional via interaction of the nuclear ribonucleoprotein A1 with CYP2A6 mRNA.

2.1.4 CYP2B

Several CYP2B isoforms have been identified in several mammalian species (Table 1). These isoforms were among the first microsomal cytochrome P450s purified and show the most dramatic induction by barbiturates. In human, the CYP2B family is CYP2B6 and CYP2B7. CYP2B6 is expressed in liver and in some extrahepatic tissues, whereas CYP2B7 mRNA expression was detected in lung tissue (Czerwinski, 1994). CYP2B6 is involved in the metabolism of nearly 25% of drugs, such as the anticancer drugs cyclophosphamide and tamoxifen (Sridar et al., 2002), the anesthetics ketamine and propofol, drug of abuse (methylenedioxymethamphetamine; MDMA, Ectacy, Adam) (Kreth et al., 2000) and procarcinogens such as the environmental pollutant (pesticides and industrial chemicals) (Hodson and Rose, 2007) and environmental contaminants (e.g. aflatoxin B1, dibenzanthracene). The average relative abundance of CYP2B6 in human liver is ranges from 2 to 10% of the total CYP content. In addition, significant interindividual differences in hepatic CYP2B6 expression, which varies in some studies from 25- to 250- fold, have been reported (Code, 1997). These large differences may be due to both polymorphism and induction. This finding of CYP2B6 variability suggests that there are significant interindividual differences in the systemic exposure to a variety of drugs that are metabolized by

CYP2B6, with the consequent variation in therapeutic and toxic responses. In mouse, among several CYP2B isoenzymes, CYP2B9 and CYP2B10 are the major CYP2B isoenzymes expressed constitutively. CY2B10 mRNA is detected in the liver and in the intestine and its expression seems to be higher in the duodenum than in the liver (Martignoni et al., 2005). Similar to rats, the CYP2B family is sexual dimorphic, but female mice express more CYP2B9 isoenzymes than males. In contrast, CYP2B10 is equally expressed in both sexes. Rats express three CYP2B isoenzymes, CYP2B1, CYP2B2 and CYP2B3. CYP2B1 and CYP2B2 are structurally related isoenzymes (97% identical) with very similar substrate specificities. However, CYP2B1 is generally much more catalytically active than CYP2B2. Both are expressed constitutively in liver and extrahepatic tissues such as intestine and lung (Lindell et al., 2003). Their constitutive expression in liver is sexually dimorphic, with male expressing higher CYP2B levels than female rats. This sexual dimorphism may be explained by a sex-dependent secretion of pituitary growth hormone which suppresses CYP2B expression more in female than in male rats. In dog, the main 2B isoform is CYP2B11 which has a 75% identity with respect to amino acid sequence to that of rat CYP2B1. Remarkably, CYP2B11 catalyses the *N*-demethylation of dextromethorphan (mediated in human by CYP3A) and the 4'-hydroxylation of mephenytoin (mediated in human by CYP2C19), and, together to CYP3A12, dog CYP2B11 also contributes to *S*-warfarin hydroxylation (mediated in human by CYP2C9) (Graham et al., 2003). Interestingly, the dog is the only mammalian species able to metabolize polycyclic aromatic hydrocarbons through its CYP2B isoenzyme (Guengerich, 1997).

2.1.5 CYP2C

The CYP2C subfamily is the most complex subfamily of the P450s found in human and animal species with several different isoforms. In human, the CYP2C family (Table 1) is involved in the metabolism of about 16% of drugs currently on the market. CYP2C8 and CYP2C9 are the major forms, accounting for 35% and 60%, respectively, of total human CYP2C, while CYP2C18 (4%) and CYP2C19

(1%) are the minor expressed CYP2C isoforms (Romkes et al., 1991). CYP2C8, CYP2C9 and CYP2C19 proteins are primarily located in the liver where they account for approximately 20% of total cytochrome P450. However, also other expression levels were reported and the expression appears to show race-related differences and genetic polymorphism, CYP2C8 is expressed mainly in the liver, but its mRNA was also detected in kidney, adrenal glands, brain, uterus, mammary glands, ovary and duodenum (Lapple et al., 2003). CYP2C8 is involved in the metabolism of retinol and retinoic acid, arachidonic acid, benzo[a]pyrene and in the oxidation of the anticancer drug paclitaxel. CYP2C9 metabolizes many clinically important drugs including tolbutamide, phenytoin, *S*-warfarin, losartan, glipizide, torasemide and numerous anti-inflammatory drugs such as ibuprofen, diclofenac, piroxicam, tenoxicam, mefenamic acid (Miners and Birkett, 1998) CYP2C19 has been detected in liver and duodenum. CYP2C19 has been shown to metabolise several drugs such as *S*-mephenytoin, omeprazole, imipramine, diazepam, proguanil. CYP2C19 is highly polymorphic. Poor metabolizers (PMs) of CYP2C19 represent approximately 3-5% of Caucasians and of African-Americans and 12-23% in oriental population (Goldstein, 2001). Toxic effects can occur in PMs exposed to diazepam, and the efficacy of some proton pomp inhibitors may be greater in PMs than in EMs at low doses of these drugs.

The mouse CYP2C family is larger and more complex than its human counterpart, with more than 10 members published to date, including CYP2C29, CYP2C37, CYP2C38, CYP2C39, CYP2C40, CYP2C44, CYP2C50, CYP2C54 and CYP2C55 (Table 2). Similar to human and rat, the mouse CYP2C has an important physiological role through the oxidation of arachidonic acid into regioand stereospecific epoxyeicosatrienoic acids and hydroxyeicosatetraenoic acids. The expression of different CYP2Cs is organ selective (Tsao et al., 2001). In rats, the CYP2C family includes several isoforms, such as CYP2C6, CYP2C7, CYP2C11, CYP2C12, CYP2C13, CYP2C22 and CYP2C23. The CYP2C family is the most abundant CYP2C isoform in the liver of the rat and is involved in the

oxidation of dihydropyridines and aflatoxin B1 and in the hydroxylation of steroids. There are sex-dependent differences in the expression of CYP2C family in rats, which are developmentally regulated and manifest in adult animals. CYP2C11 is the predominant isoform in male rat liver comprising up to 50% of the total CYP content (Morgan et al., 1985) and is also expressed in extrahepatic tissues such as kidney and intestine at lower levels. Therefore the suppression of this isoform in the liver helps to explain the decline in drug-metabolizing capacity.

Despite the common use of the dog in safety evaluation as the most used non-rodent species, obliged for any new drug prior to use in man, knowledge concerning the canine cytochrome P450 system and in particular the CYP2C family is limited. Two canine CYP2C isoenzymes have been isolated so far, CYP2C21 and CYP2C41. These two canine CYP2C isoforms exhibit 70% nucleotide and amino acid identity. Moreover, they exhibit 74-83% nucleotide and 67-76% amino acid identity with the human CYP2Cs. Both isoenzymes were found in dog liver, but the expression is highly variable: In addition, the metabolism of specific human CYP2C substrates, such as tolbutamide, warfarin and S-mephenytoin, is impaired in dog compared to human liver, illustrating an important species difference between dog and human drug metabolism (Graham et al., 2003).

In monkey, the CYP2C family accounts for two isoforms, CYP2C20 and CYP2C43. These isoforms are both expressed in the liver and show an identity of 83% and 77% for the nucleotide and for the amino acid sequences, respectively. CYP2C43, but not CYP2C20, was able to metabolize S-mephenytoin, a probe substrate of CYP2C19 in human. In contrast, CYP2C43 was not able to metabolize tolbutamide, a probe substrate of CYP2C9 in human. Therefore monkey CYP2C43 appears to be functionally related to human CYPC19 but not to human CYP2C9, although the N-terminal sequence (first 18 residues) was identical for CYP2C43 and CYP2C9 (Matsunaga et al., 2002).

2.1.6 CYP2D

CYP2D isoforms have been identified in several mammalian species (Table 2) and are involved in the mono-oxygenation of various chemicals including antidepressants (e.g.desipramine), β-blockers (e.g. propanolol), anti-arrhythmics (e.g. sparteine) and others such as dextromethorphan and methadone (Zanger et al., 2004). CYP2D was the first isoform shown to be polymorphic. Induction of CYP2D has not been reported to date.

Although CYP2D6 is expressed at a low level in human liver accounting for about 4% of total P450 (12.8 pmol/mg microsomal protein), this enzyme is involved in the biotransformation of 30% of drug. In human, only one isoform, CYP2D6, is expressed in various tissues including the liver, kidney, placenta, brain, breast, lung and intestine (Huang et al., 1997). CYP2D7 and CYP2D8 are inactive pseudogenes. Problems related to polymorphism in drug metabolism became evident when sparteine and debrisoquine were found to be metabolized at different rates among individuals. Approximately 7 to 10% of the Caucasian population inherits mutant *CYP2D6* alleles as an autosomal recessive trait, leading to individual variation in response to many drugs that are cleared by CYP2D. Another polymorphism stratifies the population depending on the copy number of wild-type alleles between poor metabolizers (PM, zero copies), intermediate metabolizers (one copy), extensive metabolizers (EM, two copies), and ultrarapid metabolizers (multiple copies). In addition, this genetic variation in CYP2D6 is associated with risk for diseases and cancer, for example Parkinson's disease, lung cancer, liver cancer and melanoma (Agundez, 2004).

Only a few studies have been performed to characterize the CYP2D family in mouse. There are at least nine mouse CYP2D genes (CYP2D9, CYP2D10, CYP2D11, CYP2D12, CYP2D13, CYP2D22, CYP2D26, CYP2D34 and CYP2D40), but some of the isoenzymes have not been characterized for expression and function (Nelson et al., 2004). In rats, six isoforms (CYP2D1, CYP2D2, CYP2D3, CYP2D4, CYP2D5 and CYP2D18) have been identified by

genomic analysis. The rat and human CYP2D isoforms share a high sequence identity (>70%) (Venhorst et al., 2003). Similar to human CYP2D6, the six isoforms are expressed in various tissues such as liver, kidney, and brain. Among the six isoforms, CYP2D1 is the rat orthologue of human CYP2D6.

CYP2D15 is the major CYP2D in dog with enzymatic activities similar to human CYP2D6. CYP2D15 is expressed in the liver, with lower but detectable levels in several other tissues. Bogaards et al. reported that dog and human liver microsomes showed similar enzyme kinetics with respect to the 1'- hydroxylation of bufuralol. In cynomolgus monkey, a full length cDNA (called CYP2D17) encodes a 497-amino acid protein that is 93% identical to human CYP2D6. The recombinant CYP2D17 catalyzed the oxidation of bufuralol to 1'-hydroxybufuralol and dextromethorphan to dextrophan, reactions shown to be mediated by CYP2D6 in human, and strongly inhibited by quinidine.

2.1.7 CYP2E1

CYP2E1 shows a quite strong conservation among species (Table 2) with an identity to human CYP2E1 of 80 % for rat, mouse and dog and of 96% for monkey. In human, CYP2E1 accounts for approximately 6% of total P450 in the liver and is involved in the metabolism of 2% of the drugs (Zuber et al., 2002). The CYP2E1 appears to have a dual physiological role, namely a role in detoxification and in nutritional support. CYP2E1 is expressed in many tissues, such as the nose, the oropharynx (exposed to airborne xenobiotics), the lung and the liver. Also the inducible and adaptive responsiveness to xenobiotics is suggestive of a protective role. Regarding xenobiotics such as ethanol, CYP2E1 plays a detoxification role preventing ethanol to reach excessive levels. In terms of its nutritional role, the up-regulation of CYP2E1 plays a useful physiologic role when starvation and/or low carbohydrate diet prevail because of its contribution to the metabolism of fatty acids and its capacity to convert ketones to glucose. However, like many other useful adaptive systems, when the adaptation ceases to be homeostatic and becomes excessive, adverse consequences prevail. CYP2E1-

mediated metabolism generates oxygen radicals and, when this exceeds the cellular detoxification systems, it results in oxidative stress with its various pathologic consequences. This is true not only when excess alcohol has to be metabolized, but also when CYP2E1 is confronted with an excess of ketones and fatty acids associated with diabetes and/or obesity. A few drugs are metabolized by CYP2E1, such as acetaminophen, caffeine and chlorzoxazone, the latter being considered a marker of CYP2E1 activity (Lofgren et al., 2004). Although relatively few drugs are oxidized by CYP2E1, the list of carcinogens that can be activated by CYP2E1 is quite extensive and includes benzene, styrene, acrylonitrile, and nitrosoamines. CYP2E1 may generate reactive oxygen intermediates, such as superoxide radicals (Dai et al., 1993), which play a key role in liver injury because of the interaction with cellular proteins or DNA (Wu and Cederbaum, 2003). CYP2E1 activity is inducible by ethanol and by acetone in both rodents and non-rodents. Similar to human, many substrates such as organic solvents, nitrosamines and drugs such as paracetamol are metabolized by rodent CYP2E1. Therefore rodents, such as rats may be an appropriate model to study CYP2E1 dependent metabolism in man. However, in dogs and monkeys some discrepancies with human have been found. In dog microsomes, the antibody against human CYP1A was shown to influence the 6-hydroxylation of chlorzoxazone, a typical activity of CYP2E1 in man. In monkeys, CYP2E1 activities in liver microsomes seem to be similar to human CYP2E1 (Bogaards et al., 2000). Disulfiram and diethyldithiocarbamate are mechanism-based inhibitors of CYP2E1 in man. In addition, diethyldithiocarbamate is a potent mechanism based inhibitor of 6 OH-chlorzoxazone formation in microsomes of rodents and non-rodents species, indicating a species-conserved mechanism for the oxidative biotransformation of chlorzoxazone among species.

2.1.8 CYP3A

The P450 3A subfamily (Tables 1 and 2) plays very important roles in the metabolism of xenobiotics and has very broad substrate specificity. It is highly

inducible and can be inhibited by numerous drugs. Therefore large interindividual differences in CYP3A-mediated metabolism have been reported.

The CYP3A subfamily is the most important of all human drug-metabolizing enzymes because this subfamily is involved in the biotransformation of approximately 50% of therapeutic drugs currently on the market, although its content in the liver is only 30% of total P450. Some examples of drugs metabolized by CYP3A are terfenadine, the benzodiazepines (e.g. midazolam and triazolam), quinidine, lidocaine, carbamazepine, nifedipine, tacrolimus, dapsone, erythromycin, dextromethorphan (Nebert and Russell, 2002), lipid-lowerling drugs (e.g. statins) (Neuvonen et al., 2006) etc. In addition to drugs, CYP3A is involved in the oxidation of a variety of endogenous substrates, such as steroids, bile acids and retinoic acid (Marill et al., 2000). CYP3A4 and its related CYP3A5 are the most abundant CYP isoforms in human liver. It is also located at the apex of the enterocytes and plays a major role in the first-pass metabolism of xenobiotics. CYP3A protein and catalytic activity decrease longitudinally along the small intestine. Although the levels of CYP3A in the intestine expressed per mg microsomal protein are generally 10 to 50% lower than those found in the liver, in some individuals CYP3A concentration is equal to or even higher than those in the liver. This, together with its strategic localisation at the tip of the villus makes the intestinal CYP3A play a major role in drug metabolism. In addition, P-glycoprotein can influence the metabolism process by recycling drugs between enterocytes and lumen, thereby increasing drug exposure to intestinal metabolic enzymes. Thus, the amount of an orally administered drug that reaches the systemic circulation can be reduced by both intestinal and hepatic metabolism. Initial data suggested that CYP3A5 accounted for only a small proportion of the total hepatic CYP3A content in only about 20% of samples and when expressed it accounts for one-third of CYP3A4. However, recent evidence indicates that CYP3A5 may represent more than 50% of the total CYP3A in some individuals (Kuehl et al., 2001). In addition, within mucosa of the colon and the stomach,

CYP3A5 protein and mRNA appear to be more prominent than the corresponding CYP3A4 protein and mRNA. Furthermore, CYP3A5 is expressed in one-third of Caucasian livers and over one-half of African-American livers examined.

In mouse, there are six CYP3A isoforms identified so far. CYP3A11 and CYP3A13 show a maximal level of expression at 4-8 weeks of age (Yanagimoto et al., 1997). CYP3A11 isoforms is the most similar to human CYP3A4, with 76% amino acid homology. In addition, CYP3A11 is also expressed in the small intestine like CYP3A4 in man. The catalytic activity of the mouse CYP3A form towards clinically active drugs has not been extensively tested, but it has been shown that compounds such as aflatoxin B1 and ethylmorphine are metabolized by mouse CYP3A isoforms similar to human (Anakk et.al.,2003).

In rats, CYP3A1/3A23, CYP3A2, CYP3A9, CYP3A18, and CYP3A62 have been reported as CYP3A forms. CYP3A1 and CYP3A2 were detected only in the liver. These CYP3A forms appear to be expressed in a sex-specific manner in rats. For example, CYP3A2 and CYP3A18 are male specific forms, whereas CYP3A9 is a female dominant form (Guengerich , 1997).

In dog, the CYP3A family accounts for two isoforms, including the CYP3A12 and CYP3A26. Both have been detected in the liver. Several distinctions in catalytic activity have been identified between these two enzymes. The major differences in steroid hydroxylases identified clearly demonstrate that CYP3A26 is less active than CYP3A12 (Fraser et al., 1997).

In cynomolgus monkey CYP3A8 represents ~ 20% of the P450 in monkey liver, and it is 93% similar to the human CYP3A4 protein. In addition, the human 3A marker midazolam 1'-hydroxylase and the erythromycin *N*-demethylation had 4-fold and 19-fold higher activity, respectively, in cynomolgus monkeys than human, suggesting that there are some differences in affinity and enzymatic rates for CYP3A substrates (Sharer et al., 1995).

3 Phase II metabolism

The phase II or conjugation reactions are listed in table 3. It is seen that they involve a diverse group of enzymes often involving an “activated” (or high energy) co-factor or substrate derivative, generally leading to a water-soluble product which can be excreted in bile or urine (Stine and Brown, 2006).

3.1 Glucuronidation

Glucuronidation is the most widespread of the conjugation reaction probably due to the relative abundance of the co-factor for the reaction, UDP-glucuronic acid and the ubiquitous nature of the enzyme, UDP-Glucuronosyltransferase. UDP-glucuronic acid, being part of the intermeadiary metabolism and closely to glycogen synthesis, is found in all tissue of the body. The enzymes are located in the cytosol. Glucuronide formation is quantitatively the most important form of conjugation for drug and endogenouscompound and can occur with alcohols, phenols, hydroxylamines, carboxylic acids, amines, sulfonamides and thiols.

3.2 Sulfate conjugation

Sulfation is the major conjugation for phenol but can also occur for alcohols, amines and, to a lesser extent, thiols. As with sugar conjugation, an energy-rich donor is required – in this case 3’-phosphoadenosine-5’-phosphosulfate (PAPS). PAPS is produced by two-stage reaction from ATP and sulfate. These reactions occur in the cytosol. Sulfation occurs by interaction of the drug and PAPS in the presence of the cytosolic enzyme sulfotransferase.

3.3 Methylation

Methylation mainly involves endogenous compounds but some drugs may be methylated by non-specific methyltransferases. The co-factor, S-adenosylmethionine (SAM), is required to form methyl conjugates.

Table3. Conjugation reactions

Reaction	Enzyme	Functional group
Glucuronidation	UDP-Glucuronosyltransferase	-OH -COOH $-NH_2$ -SH
Glycosidation	UDP-Glycosyltransferase	-OH -COOH -SH
Sulfation	Sulfotransferase	$-NH_2$ $-SO_2NH_2$ -OH
Methylation	Methyltransferase	-OH $-NH_2$
Acetylation	Acetyltransferase	-NH2 $-SO_2NH_2$ -OH
Amino acid conjugation		-COOH
Glutathione conjugation	Glutathione-S-transferase	Epoxide Organic halide
Fatty acid conjugation		-OH
Condensation		various

3.4 Acetylation

Acetylation reactions are common for aromatic amines and sulfonamides and required the co-factor, acetyl-CoA, which may be obtained from the glycolysis pathway or via direct interaction of acetate and coenzyme A. Acetylation takes place mainly in the liver and, interestingly, is found in the Kupffer cells and not in the more common location of other enzymes, i.e. the hepatocytes.

3.5 Amino acid conjugation

Exogenous carboxylic acids and acetate can form CoA derivatives in the body under the action of the ATP-dependent acid-CoA ligase, and can then react with endogenous amines, such as amino acids, to form conjugates. Amino acid conjugation is, thus, a special form of N-acylation, where the drug and not the endogenous co-factor can be activated. The most common amino acids involved are glycine, glutamine, ornithine, arginine and taurine.

3.6 Glutathion conjugation

Glutathione is recognised as protective within the body for the removal of potentially toxic electrophilic compounds. The enzymes catalyzing this reaction are the glutathione-S-transferases which are located in the cell cytosol of liver, kidney, gut and other tissues. The glutathione conjugates may be excreted directly in urine or more usually bile.

4 An extended phase concept; Phase 0, Phase III and Phase IV metabolism

Drug transporter / drug metabolism interplay represents a new challenge in cellular pharmaco- and toxicokinetics (Liu and Pang, 2005; Kim, 2006). Reflecting the meaning of drug transport in cellular pharmacokinetics, the historical two-phase concept, which has only considered the relevance of biotransformation of drugs for drug evasion, has needed extension.

Figure 4 shows an extended phase concept encompassing interactions between transporter phases for uptake and excretion, and the metabolic steps. The metabolic phases 1 and 2 are flanked by drug transporter phases 0 and 4, while intracellular cytoplasmic drug traffic is regarded as phase 3.

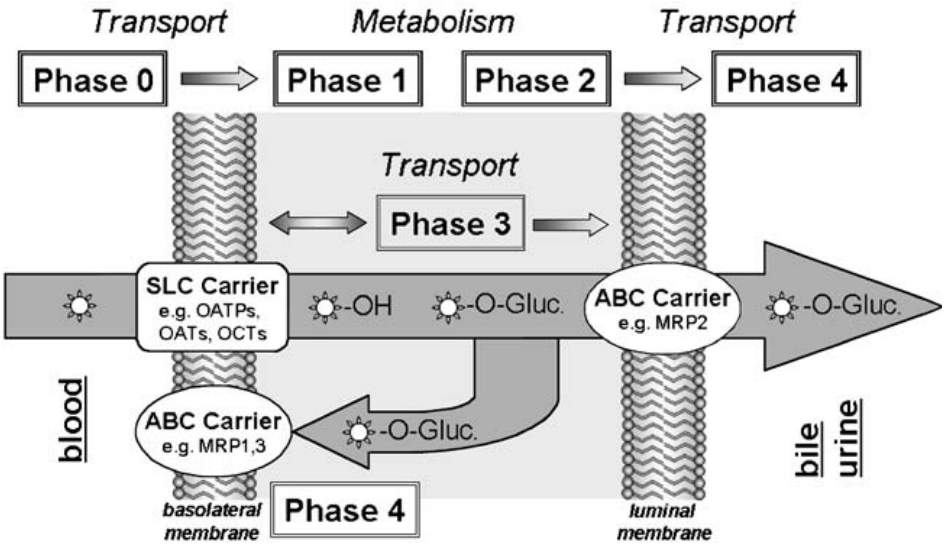


Figure 4: Schematic principle of vectorial drug evasion in liver and kidney. Phase 0 = drug uptake out of blood, Phases 1 and 2 = biotransformation exemplified by hydroxylation and glucuronidation, Phase 3 = transport of xenobiotics/metabolites towards excretion, Phase 4 = efflux into excreted fluids and/or backward into blood.

Phase 0 solute carrier (SLC) transporters mediate the earliest phase in drug kinetics on cells, which is here termed phase zero (phase 0). Phase 0 is the first step of drug elimination from blood via uptake across the basolateral membrane into the cells, i.e. of the proximal tubule or into hepatocytes respectively, or the first step of absorption from the gut, i.e. the uptake across the luminal membrane into enterocytes. Thus, SLC transporters are located in both the basolateral and the luminal cell membrane. This type of carrier imposes a selection for certain classes of xenobiotics. SLC-drug carriers are multispecific, which means they allow permeation of a spectrum of compounds with variable chemical structures. An example of this type of multispecificity are members of the organic anion transporting polypeptide carriers OATPs (SLC21 / SLCO) which transport weak organic acids, neutral compounds and even a few cationic compounds (Hagenbuch and Meier, 2003). Similarly, another family of organic anion transporters, the OAT-family, transports negatively and positively charged compounds, and is very closely related to the OCT-family, specialised for organic cation transport (Koepsell and Endou, 2004; Biermann et al., 2006). Phase 0 drug transporters jointly influence compound allocation for drug metabolism.

Phase 4 pathways are well-defined. They comprise final steps of excretion, e.g. in the bile canaliculus of the liver, or secretory steps in the luminal membrane, e.g. in the gut, counteracting absorption. Phase 4 is predominantly maintained by directly driven "primary" uphill transport of xenobiotics across cell membranes, which is achieved by ATP-consuming transport ATPases belonging to the ABC-carrier proteins (Chan et al., 2004). A prominent member of these transport proteins, first detected in drug-resistant tumor cells, is P-glycoprotein. This drug resistance was conferred by a gene named multidrug resistance (*MDR*) gene, which was characterized in 1986. P-glycoprotein substrates are lipophilic, and are generally non-conjugated compounds, whereas water-soluble drug conjugates (sulfated, glucuronidated and glutathione-conjugated drugs) are transported in the liver by the multidrug resistance associated protein MRP2 (Fardel et al., 2005). ABC-carriers for xenobiotics, i.e. P-glycoprotein, MRP2 and BCRP (breast cancer resistance protein), are mainly located at the luminal membrane of a cell facing, for example, the bile canaliculus, the lumen of the gut or the tubule lumen of a nephron. The general function which results from this location is drug excretion, providing evidence that these types of ABC-carriers convey protection against xenobiotics (Leslie et al., 2005). In the gut, these ABC-carriers are gatekeepers of drug absorption, limiting drug bioavailability (Dietrich et al., 2003) (Figure 5). The other members of the MRP (MRP1,3,4,6) family are located on the basolateral membrane and are responsible for the removal of conjugates formed in the liver via the urine. They mediate the excretion of organic anions into the sinusoidal bloodstream as well as the basolateral excretion of glutathione and leukotriene C4. MRP1 and 3 are expressed in low levels under normal conditions, but are upregulated under conditions in which MRP2 is downregulated (for instance in cholestatic conditions). They have similar substrate specificities with the exception of glutathione conjugates, which are poor substrates for MRP3, and bile salts, which are poor substrates for MRP1. BCRP (or ABCG2) is a half transporter and it is suggested to function as homodimer. It is involved in the

biliary excretion of a variety of anticancer drugs and may also transport endogenous compounds such as porphyrins (Doyle et al., 2003). As transporters may influence significantly the pharmacokinetics of a drug, it is important to study the involvement of drug transporters when the pharmacokinetic behavior of a new drug is studied. Transporters mediate nonmetabolic clearance of compounds into bile. Moreover, transporters determine the intracellular concentration of drugs and their metabolites and thereby the exposure of the organ to the drug itself and its metabolites. As metabolites may be toxic, the toxicity of drugs can also be related to transporter function. Comprehensive knowledge of transporters may help to understand species and interindividual differences, as well as predict drug-drug and food-drug interactions (Marchetti et al., 2007).

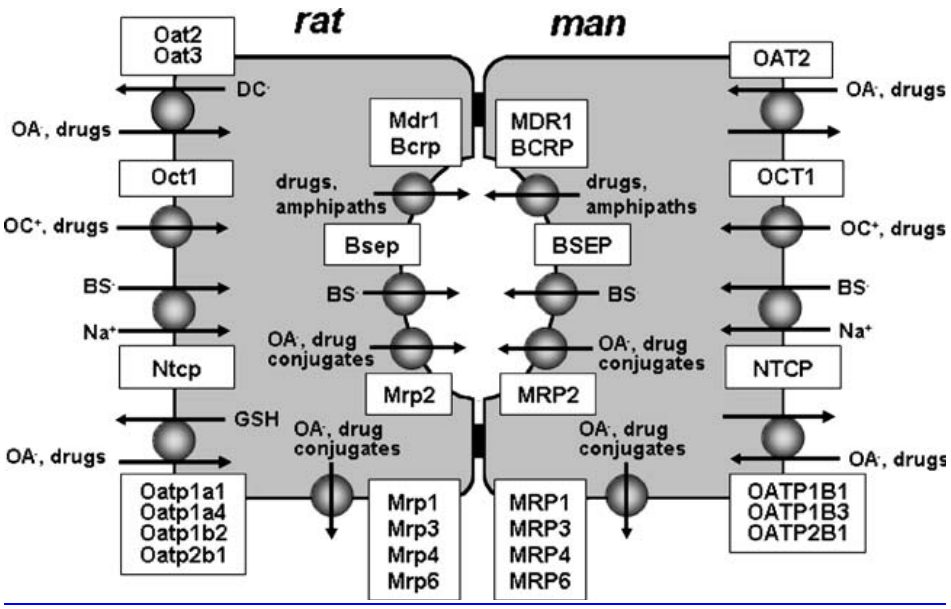


Figure 5: Individual carriers in the hepatocyte which are involved in drug uptake and secretion. OAT/Oat = organic anion transporter belonging to the SLC22 family; OCT/Oct = organic cation transporter belonging to the SLC22 family; NTCP/Ntcp = Na+/ taurocholate cotransporting polypeptide belonging to the SLC10 family; OATP/Oatp = organic anion transporting polypeptide belonging to the SLCO family (previously called SLC21). MDR1/ Mdr1 = multidrug resistance protein (ABCB1), BCRP/Bcrp = breast cancer resistance protein (ABCG2), MRP2/Mrp2 = multidrug resistance-associated protein (ABCC2), BSEP/Bsep = bile salt export pump (ABCB11) all belonging to ABC-carrier proteins; OA− = organic anion; BS− = bile salt; OC+ = organic cation; DC− = dicarboxylate; GSH = glutathion

Furthermore, various disease states may influence the expression of the transporters and thereby influence the pharmacokinetics of a drug. It is, thus, important to increase our knowledge of organ specificity and cellular localization of transporters, their substrates and inhibitor specificity, species differences, and changes in expression due to disease and compounds, and so on (Hewitt et al., 2007).

5 Factors involved in drug biotransformation

5.1 Genetic polymorphism (Meyer and Rodvold, 1996)

Genetic differences are important contributors to the inter-individual differences in drug biotransformation seen within a population of patients. These differences are called genetic polymorphisms and are linked to inherited autosomal recessive traits. The definition of a polymorphism is the presence within a population of at least 2 groups with distinctly different abilities to metabolize drugs (Poulsen and Loft, 1992). Individuals can be characterized as extensive (rapid) or poor (slow) metabolizers. Poor metabolizers often have an increased incidence of adverse effects. Among P450 polymorphisms, those affecting CYP2C9, CYP2C19, and CYP2D6 have the highest impact on drug metabolism (Table 4). In particular, CYP2D6 polymorphism requires special mention as it has been estimated that this isoform accounts for the metabolism of 25–30% of drugs used in clinical practice (Smith et al., 1998, Ingelman-Sundberg, 2005). In contrast, CYP1A1, CYP2E1, and CYP3A4 genes are relatively well conserved and only a few, if any, rare variants yielding changes in catalytic enzyme activity have been found (Ingelman-Sundberg, 2005).

5.2 Disease (Meyer and Rodvold, 1996)

Impaired liver functions can lead to decreased drug biotransformation and is a function of the severity of the disease. Disease states that can impair liver

Table 4 Polymorphic P450 Drug-Metabolising Enzymes.

Enzyme	Functional allelic variants	Major variant	Allele frequency	Phenotype
CYP1A2	13	CYP1A2*1B	12 % Japanese	Decreased activity
CYP2A6	16	CYP2A6* 2	1–3% Caucasians	Inactive enzyme
CYP2C9	12	CYP2C9* 3	7–9% Caucasians	Altered substrate specificity
CYP2C19	16	CYP2C19* 2	13% Caucasians	Inactive enzyme
CYP2D6	46	CYP2D6* 4	12–21% Caucasians	Inactive enzyme
CYP2E1	2	CYP2E1* 3	<1% Caucasians	No effects

From: Ingelman-Sundberg (2005).

function include hepatitis, alcoholic liver disease, biliary cirrhosis and hepatocarcinoma. Infection can also alter drug biotransformation. There have been reports of impaired drug elimination during viral infections such as influenza, rhino virus, adenovirus, herpes simplex virus and infectious mononucleosis.

5.3 Age (Meyer and Rodvold, 1996)

Infants do not develop a mature enzyme system until more than two weeks after birth. Elderly have age-related decreases in liver mass, hepatic enzyme activity and hepatic blood flow. In addition the overall metabolic capacity of the liver is decreased, although the considerable inter individual variability in age and disease related changes in organ function makes it difficult to form generalizations.

5.4 Concomitant drugs

Two of the most common causes of altered drug biotransformation reactions are induction and inhibition of cytochrome P450 enzymes. (Meyer and Rodvold, 1996).

5.4.1 Induction of CYPs

Upon repeated administration, certain drugs can alter their own metabolism, or that of other simultaneously or subsequently administered therapeutic agents, by changing the expression of drug-metabolising enzymes. Exposure to certain chemicals (pollutants, cigarette smoke, alcohol and dietary constituents) can also induce drug metabolizing enzymes. Induction may result in rapid metabolism of the drug and lower plasma concentrations to levels that are no longer efficacious (Finch et al., 2002). Enzyme induction also accounts for the onset of tolerance to some therapeutic agents. On the other hand, a possible consequence of enzyme induction is the increased formation of pharmacologically or toxicologically active metabolites. Thus, enzyme induction significantly contributes to interindividual differences in drug metabolism and toxicity (Ronis et al., 1999). The phenomenon of CYP induction was first discovered and studied in experimental animals, but it was soon recognised to occur in humans. All human CYPs can be influenced to a certain extent, some of them being clearly induced by xenobiotics (Ronis et al., 1999). Notably, there appears to be more variation in response to enzyme inducers among humans than in animals, probably due to varying genetics, lifestyles and dietary habits (Hollenberg, 2002). Enzyme inducers are usually classified on the basis of their action on individual CYP isoenzymes. Table 5 shows representative inducers for human CYPs. Some compounds show CYP inductive potential across species (particularly for the CYP1A family). For other substances, significant differences exist in their inducing abilities in animals. Rifampicin (rifampin) for example is a potent inducer in humans and rabbits but it is a poor inducer in rat (Strolin-Benedetti and Dostert, 1994). In contrast, pregnenolone 16α-carbonitrile, a potent inducer of CYP3A in the rat, is not an inducer in either rabbits or humans (Kocarek, 1995). Different mechanisms are known to operate in CYP enzyme induction, but in general it involves transcriptional activation of CYP genes by a receptor-

dependent mechanism (Table 6), resulting in increased levels of specific CYP mRNAs.

Table 5. Inducers of human cytochrome P450 isoenzymes

CYP	Model inducer	Others
1A2	3-Methylcholanthrene	Polycyclic aromatic hydrocarbons, 3-methyl-indole (cruciferous vegetables), omeprazole
2B6	Phenobarbital	Pesticides?
2E1	Ethanol	Isoniazid, organic solvents (acetone, benzene, pyridine)
3A4	Dexamethasone, rifampicin (rifampin)	Corticosteroids, macrolide antibacterials, phenobarbital, lovastatin, simvastatin, omeprazole?, ethanol?
4A	Clofibrate	Peroxisome proliferators (fibrates)

CYP = cytochrome P450.

The first discovered inducing receptor, the aryl hydrocarbon (Ah) receptor, belongs to the PAS family of transcription factors. It stimulates transcription of CYP1A genes via direct interaction with the promoter region of the gene (Figure 6). Upon binding of the inducer to cytosolic Ah receptor, the complex is translocated to the nucleus, where it heterodimerises with the nuclear factor Arnt (Ah receptor nuclear translocator protein), and binds to an enhancer/promoter DNA region of CYP1A genes. This is a well conserved mechanism across species and accounts for the consistent induction of CYP1A by polycyclic aromatic hydrocarbons in many cell types.

Three nuclear receptors belonging to the nuclear receptor/steroid superfamily are recognized players in the inductive mechanisms of the CYP 2, 3 and 4 families. The constitutive androstane receptor (CAR) mediates CYP2B induction by phenobarbital and ‘phenobarbital-like’ chemicals; the pregnane X receptor (PXR) is involved in CYP3A induction by steroids and other chemicals; and the peroxisome proliferator activated receptor (PPAR) activates CYP4A genes in response to peroxisome proliferators (Figure 6).

Table 6. Mechanisms of induction of cytochrome P450 (Ronin, 1999; Waxman, 1999)

Inducer	Nuclear receptor	Regulatory mechanism	CYP
Polycyclic aromatic hydrocarbons	AhR	Transcriptional activation	1A1, 1A2, 1B1
Phenobarbital	CAR	Transcriptional activation	2B6 (1A2, 2C8, 2C9, 3A4)
Corticosteroids, rifampicin (rifampin)	PXR	Transcriptional activation	3A
Peroxisome proliferators	PPAR	Transcriptional activation	4A
Ethanol	None	Enzyme stabilisation[a]	2E1

[a] Some evidence that increased transcription, mRNA stabilisation and increases in translational efficiency are also involved.
AhR = aryl hydrocarbon receptor; **CAR** = constitutive androstane receptor; **CYP** = cytochrome P450; **PPAR** = peroxisome proliferator
activated receptor; **PXR** = pregnane X receptor.

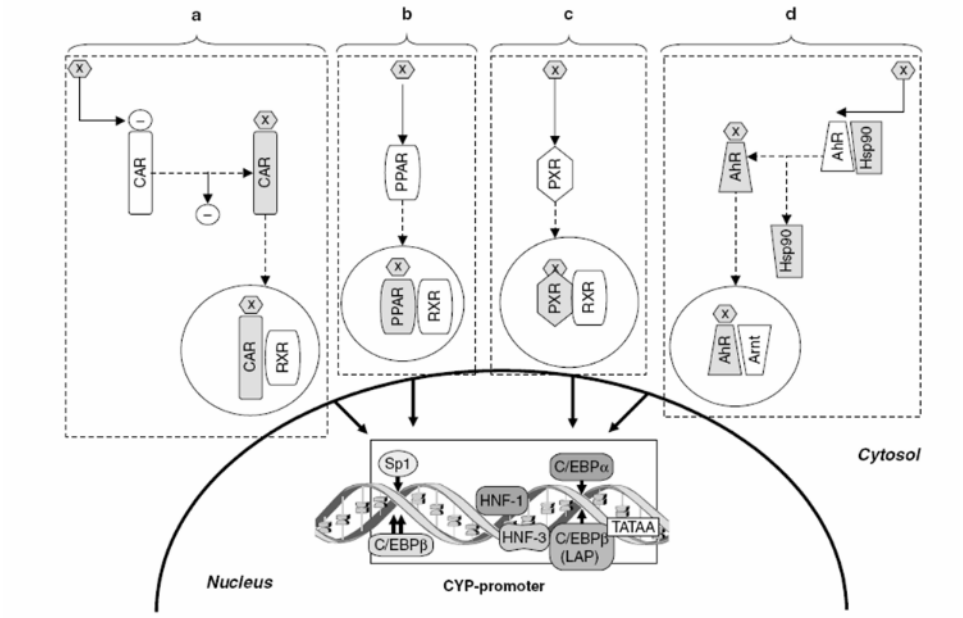


Figure 6: Role of nuclear receptors in cytochrome P450 (CYP) gene induction. Three nuclear receptors belonging to the nuclear receptor/steroid superfamily are recognised players in the inductive mechanisms of the CYP 2, 3 and 4 families: the constitutive androstane receptor (CAR) mediates CYP2B induction by phenobarbital and 'phenobarbital-like' chemicals (**a**), the peroxisome proliferator activated receptor (PPAR) activates CYP4A genes in response to peroxisome proliferators (**b**) and the pregnane X receptor (PXR) is involved in CYP3A induction by steroids and other chemicals (**c**). The aryl hydrocarbon receptor (AhR) activates transcription of CYP1A genes upon binding to polycyclic aromatic hydroxarbons (**d**) (Ronin1 1999; Waxman 1999). **Arnt** = Ah receptor nuclear translocator protein; **C/EBP** = CCAAT/enhancer binding protein; **HNF** = hepatocyte nuclear factor; **Hsp** = heat shock protein; **LAP**=liver-enriched activating protein;**RXR** = retinoid X receptor;**Sp1** = stimulatory protein 1.

All these nuclear receptors share a common heterodimerisation partner, the retinoid X receptor (RXR), and are subject to cross-talk interactions with other receptors. A number of endogenous ligands for these nuclear receptors have been identified: androstanes (CAR ligands), pregnenolone derivatives and other steroids (PXR ligands) (Lim and Huang, 2008; Gibson et al., 2006) and certain polyunsaturated long-chain fatty acids and their metabolites (PPAR ligands). Endogenous ligands stimulate receptor activity, with the exception of CAR ligands, which are inhibitory. CAR is a constitutively active receptor and endogenous ligands may bind directly to the DNA-bound CAR-RXR heterodimer in a manner that alters the conformation of nuclear receptor complex and prevents its interaction with SRC-1 (a general transducer between DNA-bound nuclear receptors and the basal transcriptional machinery). In the presence of phenobarbital, however, the binding of androstanes to CAR is abolished and receptor activity is thereby de-repressed. The mechanisms of CYP induction mediated by PXR and PPAR are different from that of CAR. Upon binding to an exogenous/endogenous ligand, the nuclear receptor displays enhanced binding to DNA as a PXR-RXR or PPAR-RXR heterodimer, activating the transcription of the gene. Ethanol-type induction of CYP2E1 is a non receptor-mediated mechanism. Regulation of CYP-2E1 expression by xenobiotics and/or pathophysiological factors occurs at transcriptional, translational and post-translational levels. At low ethanol concentrations, protein levels are increased without changes in mRNA by a mechanism mainly involving protein stabilisation after binding of the inducer to the active site of the enzyme. One proposed mechanism for CYP2E1 stabilisation is protection of the protein from proteolytic degradation by the enzyme-bound substrate. No changes in mRNA are generally observed. At higher concentrations, ethanol produces additional induction by increases in transcription.

5.4.2 Inhibition of CYPs

Competitive inhibition is the most common mechanism of inhibition and occurs when 2 or more drugs compete for the same enzyme. The clinical significance of an inhibition interaction depends primarily on the relative concentrations of the drugs, as well as a variety of other patient specific factors. Some drugs are capable of binding to, and acting as competitive inhibitors of different P450 enzymes from the ones that are responsible for the biotransformation of that particular drug (Meyer and Rodvold, 1996).

Drugs can also bind irreversibly (mechanism-based inhibition) or reversibly with the haem-binding site of the enzyme and inhibit other drugs from binding. Mechanism-based inhibition occurs when certain drugs are metabolized by the cytochrome P450 system to active metabolites that bind to the enzyme and cause irreversible loss of function. Activity can only be restored by synthesis of new enzymes, which may take several days (Meyer and Rodvold, 1996).

More complex mechanisms of inhibition can also occur. Some drugs undergo metabolic activation by the cytochrome P450 system to inhibitory products. The metabolites may generate relatively stable complexes with cytochrome P450 so that the cytochrome is held in an inactive state. There can be great clinical significance to this interaction, since it is relatively long in duration. Additionally, when the interaction involves drugs with narrow therapeutic ranges, there is the potential for toxicity (Meyer and Rodvold, 1996).

Unlike induction, enzyme inhibition usually begins with the first dose of the inhibitor (Dossing et al., 1983). Inhibition is maximal when the inhibitor reaches steady state (four to seven half-lives), and the maximum concentration of the inhibitory drug occurs when it reaches steady state at its new, longer half-life. The time required for the interaction to resolve also depends on the half-lives of the drugs involved (Dossing et al., 1983).

6. In summary

The reactions catalyzed by drug (xenobiotic)-biotransforming enzymes are generally divided into two groups, namely, phase I and phase II reactions. Phase I reactions involve hydrolysis, reduction, and oxidation, whereas phase II biotransformation reactions including glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione and amino acids. Phase I biotransformation of drug often precedes and is slower than phase II biotransformation. For this reason, phase I biotransformation tends to be the rate-limiting step in the overall metabolism and, at time, the elimination of drugs. Therefore, a decrease or increase in the content/activity of phase I drug-metabolizing enzymes often results in alternation of the pharmacokinetics of drugs. Decreased content/activity of an enzyme may result from the following mechanism: expression of mutant enzyme, inhibition of the activity of a pre-existing enzyme, inactivation of a pre-existing enzyme or suppression of the expression of an enzyme. On the other hand, an increase in the content/activity of an enzyme may result from the following mechanism: expression of several copies of the gene, stimulation of the activity of a pre-existing enzyme or increase expression or induction of an enzyme. The involvement of drug transporters in drug interaction has recently been recognized. It is becoming increasingly evident that drug transporters (uptake transporters, efflux transporters, bile duct transporters, etc.) are primarily responsible for determining the intracellular concentration of a large number of drugs. Consequently, inhibition or induction of these transporters may alter the absorption (e.g., intestine), distribution (e.g., blood-brain barrier), or elimination (e.g., liver and kidney) of drugs, thereby altering the pharmacokinetics of drugs.

CHAPTER 3 HERB-DRUG INTERACTION

1. Introduction

Herbal supplements and vitamins have long been self administered primarily to manage side effects of drugs and/or to improve overall physical and mental health. According to recent epidemiological reports, almost 40% of American population use complementary and alternative medicine (CAM) during their lifetime (Kessler et al., 2001). In Thailand, herbal products are increasingly consumed and accounted for 48,000 million Baht (900 million Euros) in sales in 2005 (National Drugs Committee, 2005). Due to the high therapeutics rates cited in pharmacopoeia and the recent renaissance of the interest in traditional medicine, the availability of medicinal herb has been widespread throughout Thailand. Some of herbal medicine including *Andrographis paniculata* has been chosen by the Ministry of Public Health of Thailand to be promoted by village health workers, traditional practitioners as well as doctors throughout the country.

Safety issues associated with these herbal medicines are mostly unknown and remain under-researched. Although herbal plants have been used from ancient times in Thailand, data concerning the interaction between Thai herbs and prescription drugs are still very limited. Herbs are very often self-administered along with therapeutic drugs. The reasons for limited informations on herbal-drug interactions may be due to various reasons, including the following:

(1) most of herbal users do not inform their doctors about complementary and alternative medicine (CAM) intake;

(2) herbal supplements are generally considered as traditional health aid which do not require stringent preclinical and clinical assessments by appropriate regulatory agencies;

(3) currently, no proper surveillance procedure exists for quality control and for monitoring adverse effects of herbs or herb–drug combinations;

(4) a general misconception persists among most CAM users that herbal medicines are safe and free from side effects and drug interactions, because these products are of natural origin. Such widely held view is unfortunately not true and

often misleading. A single herb generally contains a number of putative biochemicals, each of which may exert some degree of pharmacological effects. Phytochemicals, similar to therapeutic drugs, may be biologically active and capable of modulating physiological actions through synergistic and antagonistic effects. Natural products, as taken by the general population, are usually complex mixtures of many molecular entities. Both the putative active ingredient(s) and other constituents present in that mixture have the potential to interact with various classes of drugs.

2. Herbal products and their potential interactions with therapeutic drugs

Conventional pharmacokinetic literature generally deals with drug–drug interactions, but recently such interactions between herbal agents and prescription drugs have drawn attention, because of increasing physician awareness of the widespread adverse effects of undisclosed herbal use by the patients. Useful data about actual and potential drug–herbal interactions are accumulating from various sources, including *in vitro* and *in vivo* laboratory studies, clinical and preclinical trials.

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 potential for involvement of drug metabolizing enzymes and transporters in the handling of herbal components leads to a predisposition of herb–drug interactions in several ways:

(1) an herbal component can be a substrate of one or several isoforms of CYP enzymes and/or efflux systems (P-gp, MRP and BCRP). Therefore, one substrate can compete for another substrate for either metabolism by the same CYP isozyme and/or efflux system resulting in higher plasma concentrations due to competitive inhibition,

(2) an herbal constituent can also be an inducer of one or several CYP isoforms and/or efflux systems, thereby lowering plasma concentrations due to either higher metabolism and/or higher efflux. Such interactions may produce sub-therapeutic plasma drug concentrations,

(3) an herbal compound can also be an inhibitor of CYP450 enzymes resulting in reduced activity of one or several isoforms of CYPs. Inhibition is rapid and can produce results within a very short period of time, particularly if the inhibition is competitive in nature. If a compound is an inhibitor of efflux system, it will reduce drug efflux resulting in improved absorption. In contrast, induction is a slow process, dependent on the rate of protein synthesis. Expression of specific mRNA may be possible within a few hours, but functional expression and maturation of such proteins may require longer duration.

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. Pharmacokinetic interactions of the popular herbal products with other medicinal agents are summarized in Table 1.

St. John's wort (*Hypericum perforatum*) is the most commonly used herbal product causing severe drug–herbal interaction. St. John's wort (SJW) has been implicated in a number of clinically relevant drug interactions reducing the therapeutic efficacy of many therapies, i.e., transplantation, AIDS, cancer, etc.). SJW is commonly consumed for the relief of depression, anxiety, inflammation of the skin and blunt injuries, these being not an exhaustive list. The crude extract is a complex mixture of several active compounds such as hypericin, quercetin, isoquercitin, biflavonoids, hyperforin, naphthodanthrones, procyanidines, catechin tannins, chlorogenic acid, etc. Among these, hyperforin is the main constituent responsible for its antidepressant action, which is primarily mediated through

Table 1 Examples of Herb-drug interactions

Drug	Interaction	Comment
Garlic (Allium sativum)		
Warfarin (anticoagulant)	Additive blood thinning effects	Possible of spontaneous bleeding
Saquinavir (protease inhibitor)	Herb reduces blood level drug, lowers drug effect	May cause failure of therapy in AIDS
Ginko (Ginkgo biloba)		
Aspirin	Increased anticoagulation	Spontaneous bleeding
Warfarin	Increased anticoagulation	Brain hemorrhage (intracerebral)
Acetaminophen, caffeine and ergotamine	Unknown	Brain hemorrhage (subdural)
Ginseng (Panax ginseng)		
Warfarin	Potentiation	Possible of spontaneous bleeding
Alcohol	Increased alcohol clearance	Stimulate alcohol metabolism
Influenza vaccine	Synergy, enhances resistance	Reduces flu symptoms frequency and severity
Insulin	Synergy, antidiabetic actions of herb	Improves blood sugar and diabetic Sx
St. John's wort (Hypericum perforatum)		
SSRIs	Synergy-additive serotonin like effects	Risk of serotonin syndrome; avoid concurrent use
Amitriptyline	↓ AUC of amitriptyline and its metabolite nortriptyline	Notify prescribing physician
Warfarin	↓ INR	
Midazolan	↓ Oral bioavailability by 39.3%	May cause treatment failure due to decrease of drug concentration probably by enhancing CYP3A4
Indinavir, ritonavir, saquinavir, lopinavi, amprenavir	Herb reduces blood level drug and lowers drug effect	May cause failure of therapy in AIDS
Theophylline	Herb reduces blood drug level	enhances the metabolism of theophylline
Cyclosporine	Herb reduces blood level drug and lowers drug effect	Risk of transplant rejection; notify prescribing physician
Digoxin (cardiac glycoside)	Herb reduces blood level drug and lowers drug effect	May cause arrhythmias; notify prescribing physician
Oral contraceptives	Herb possibly reduces blood level drug and lowers drug effect	Intermenstrual bleeding and unplanned pregnancies

inhibition of synaptic reuptake of neurotransmitters (serotonin, norepinephrine and dopamine) (Moore et al., 2000). A number of clinical studies have indicated that SJW lowered steady state plasma concentrations of amitriptyline, cyclosporin, digoxin, fexofenadine, indanavir, methadone, midazolam, nevirapine,

phenprocoumon, saquinavir, simvastatin, tacrolimus, theophyline and warfarin (Zhou et al., 2004). Conversely, simultaneous administration of SJW extract with ritonavir caused 2-fold elevation of ritonavir uptake by MDCK-MDR1 cells.

Garlic (*Allium sativum*) is generally taken to treat arteriosclerosis, hypertension, high cholesterol, respiratory inflammation, hooping cough, bronchitis and joint pains. The active compounds include organosulfur, allicin, fructosans and saponins. Compared to SJW, very limited information is available on drug–garlic interactions. A substantial decrease in plasma levels resulting in 51% and 17% reduction in AUC of saquinavir and ritonavir respectively in the presence of garlic was reported in healthy volunteers (Gallicano et al., 2003). Garlic altered pharmacokinetic parameters of acetaminophen and also produced hypoglycemia with chlorpropamide (Izzo and Ernst, 2001). Concomitant administration of garlic with anticoagulants such as cumadin and antiplatelets (aspirin and dipyridamole) may increase the risk of bleeding due to garlic's effect on fibrinogen and platelet aggregation (Bordia et al., 1998) (Table 1).

Ginseng (*Panax ginseng*): Varieties of ginseng including Chinese ginseng (*Panax ginseng*) and Siberian ginseng (*Eleutheroccus senticosus*) are widely available. It is commonly used for alleviation of many aliments such as lack of stamina, fatigue and debility, lack of concentration, impotence and anxiety. Limited information is available on drug interactions with ginseng. Although Siberian ginseng did not exhibit any effect on plasma levels of alprazolan or dextromethorphan, Chinese ginseng produced adverse effects with phenelzine, warfarin and alcohol (Janetzky and Morreale, 1997).

Ginkgo (*Ginkgo biloba*): This herb is frequently taken for the enhancement of memory functions. It is also used for symptomatic relief of organic brain dysfunction, intermittent claudication, vertigo and tinnitus. The extract contains

various flavonoids (quercetin, kaempferol, isorhamnetis, p-coumaric acid), biflavonoids and proanthocyanidins. This herbal agent has been found to produce adverse effects with several drugs such as aspirin, acetaminophen and warfarin (Rosenblatt and Mindel, 1997).

Moreover, many of Chinese, Japanese, Indonesian and Thai herbs (e.g. *Alyxia reinwardtii, Curcuma heyneana, Glycyrrhiza glabra, Piper cubeba, Piper nigrum, Rheum palmatum, Syzygium aromaticum and Tinospora crispa*) have been reported as the inhibitors of several CYP isoforms *in vitro* studies (Usia et al., 2006; Tang et al., 2006).

Generally speaking, herb-drug interactions are avoided, due to the possibility of poor or unexpected outcomes. However, some of herb-drug interactions can be consider as beneficial, such as curcumin was found to be a potent inhibitor of rat liver CYP1A1/2 and a weak inhibitor of CYP2E1. These isozymes are important in the metabolic activation of certain carcinogenic polycyclic hydrocarbons and aromatic amines/amides (e.g. benzo[a]pyrene, 2-acetylaminofluorene and dibenzanthracene) and nitrosamines. From this report, curcumin could also act as an anticarcinogen because of its strong and specific inhibitory activity towards CYP1A1/2 and CYP2E1 (Oetari et al., 1995)

3. Potential of *Andrographis paniculata* on drug-metabolizing enzymes (DMEs)

Andrographis paniculata Nees. (Family Acanthaceae), traditionally employed for centuries in Asia and Europe as a folklore remedy for a wide spectrum of ailments, or an herbal supplement for health promotion, is nowadays incorporated into a number of herbal medicinal preparations. It is found in the Indian Pharmacopoeias and is a prominent component in at least 26 Ayurvedic formulas (Madav et al., 1995). In traditional Chinese medicine, it is an important “cold property” herb used to rid the body of heat, as in fevers, and to dispel toxins

from the body (Deng, 1978). In Scandinavian countries, it is commonly used to prevent and treat the common cold (Caceres et al., 1997). *Andrographis paniculata* is one of the top 10 herbal medicines, which the Thai Food and Drug Administration (Thai FDA) has promoted as an alternative medicinal therapy for fever and inflammation. Andrographolide is the most medicinally active phytochemical found in the plant *Andrographis paniculata*, including other constituents such as deoxyandrographolide, 19β-Dglucoside, neo-andrographolide, 14-deoxy-11,12-didehydro andrographolide, homoandrographolide, andrographan, andrographosterin, and stigmasterol (Shama et al., 1992; Siripong et al., 1992). Extensive research has revealed that the whole-plant extract possesses many useful bioactivities, such as anti-inflammatory (Shen et al., 2002), antiviral (Calabrese et al, 2000), anticancer (Kumar et al., 2004), and immunostimulatory (Puri et al., 1993; Iruretagoneya et al., 2005) activities. On the other hand, male reproductive toxicity (Akbarsha and Murugaian, 2000) and cytotoxicity (Nanduri et al., 2004) of this plant have been reported as well. Pharmacokinetic studies showed that andrographolide is quickly absorbed and extensively metabolized in rats and humans (Panossian et al., 2000). Andrographolide metabolites are mainly identified as sulfonic acid adducts and sulfate compounds (He et al., 2003a, b, c), as well as glucuronide conjugations (Cui et al., 2005).

The aerial parts of *Andrographis paniculata* have been traditionally used as a hepatoprotective and hepatostimulative agent in Southeast Asian folklore remedy to treat a broad range of disorders including liver disorders and jaundice (Trivedi and Rawal, 2000). The extract of *Andrographis paniculata* including andrographolide, the major diterpenoid component and its analogues have been reported to exhibit a marked effect on hepatic bio-transformation enzymes, i.e., aniline hydroxylase, *N*- and *O*-demethylase (Choudhary and Poddar, 1984), alanine aminotransferase and aspartate aminotransferase (Trivedi and Rawal, 2000), including phase II enzymes, i.e., glutathione *S*transferase and DT-diaphorase (Singh et al., 2001). Modulatory influence of *Andrographis paniculata*

extract on a responsive isoform of hepatic CYPs was recently reported in mice (Jarukamjorn et al., 2006). The effects of *Andrographis paniculata* extract were compared to prototypical CYP-inducers (3-methylcholanthrene for CYP1A induction and Phenobarbital for CYP2B induction), in terms of total CYP content and related alkoxyresorufin *O*-dealkylase activities. The results conveyed CYP1A1 and CYP2B10 as responsive CYP isoforms for *Andrographis paniculata*. How the components within the chemical pool of the crude extract of *Andrographis paniculata* affect the hepatic CYP pathway is not well understood; to date evaluation of the individual chemical components present in the plant extract on the aspect of specific CYP isoforms has not been carried out. The results of such an inquiry might provide valuable guidelines for the rational administration and precautions for the use of this herbal plant.

4. *In vitro* and *In vivo* approaches for the evaluation of drug-drug and herb-drug interaction

A key question in human drug biotransformation research is how to make reliable extrapolations from the *in vitro* or *in vivo* model to clinical practice. Thus, the objective is to establish a useful model system with a strong predictive power for human biotransformation. Several models have been developed in the past, ranging from (recombinant) isolated enzymes to the intact perfused liver (see Fig. 1). They are used to obtain early information about biotransformation pathways and to predict drug–drug and herb-drug interactions at the metabolic level (Ekins et al., 2000). The quality of the human liver used in the preparations of the different *in vitro* methods described is a dominant factor in the outcome of the *in vitro* studies, especially in precision cut liver slices and isolated hepatocytes (Fisher et al.,2001). Livers that are not suitable for transplantation or liver sections from biopsies are used and, in order to ensure a viable cell yield as high as possible or, in the case of cell fractions, the highest enzyme activity, the liver or liver section needs to be processed as soon as possible after the resection. The

optimal model system in a given situation depends on a number of factors, such as *in vivo* resemblance, expense, availability of the model, and ethical considerations. *In vitro* data from human and animal models can be used to choose the best *in vivo* model (e.g., mouse, rat dog) for further testing. In conclusion, it can be stated that an *in vitro* model is always a compromise between convenience and relevance. Current guidelines for human drug development allow *in vitro* systems to be used in supportive studies, and therefore *in vitro* data should be used mainly qualitatively. For example, when *in vitro* data show a lack of drug–drug interaction, no *in vivo* experiments have to be performed, but when a drug–drug interaction is demonstrated, then *in vivo* experiments have to follow (http://www.fda.gov).

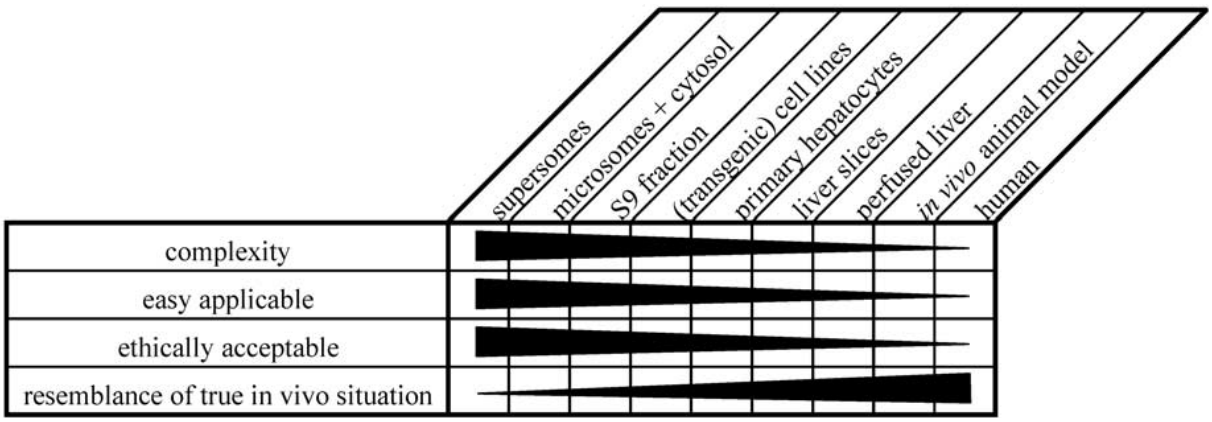


Figure 1: *In vitro* and *in vivo* models used in the development of new drugs, ranging from human to isolated enzymes, in order of *in vivo* resemblance.

In vivo studies in animals, this involved treating laboratory animals with the test compound, followed by analysis of liver CYP enzymes *ex vivo*. This raises several major issues: First, there is the requirement of large numbers of animals. Second, *in vivo* studies are not high throughput and finally, it well known that species differences in CYP induction exist, making the extrapolation from animals to humans unreliable. However, *in vivo* studies in animal are resemblance of true *in vivo* situation because these account for poor bioavailability of the active herbal components *in vivo* and account for binding of active herbal components to plasma protein (Figure 1).

In vivo studies in humans are costly, time consuming, may be unethical in certain cases, difficult and may not provide mechanistic information. However, *in vivo* human studies are valuable follow up studies to *in vitro* observations and are the only ones that are most definitive. There are advantages and limitations to each of the method used (Table 2).

In vitro studies are valuable for evaluating multiple products and multiple components, provide mechanistic information about any potential interaction and are simple to conduct. Recently there has been an increase in the use of in silico approaches for the evaluation of drug metabolism, drug transport and drug interaction studies. Such an approach is expected to be of use to predict herb-drug interactions as well in the future.

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). These studies so far have paid particular attention to the effect of herbal components on CYP enzymes. Only a small number of studies have examined the effects of herbal products on phase II metabolism or drug transport.

4.1 *In vitro* studies using microsomes

Microsomes consist of vesicles of the hepatocyte endoplasmic reticulum and are prepared by differential centrifugation and thus contain almost only CYP and UGT enzymes. Microsomes are prepared from fresh human liver, liver slices, liver cell lines, and primary hepatocytes (Skaanild and Friis, 2000). Liver microsomes account for the most popular *in vitro* model, providing an affordable way to give a good indication of the CYP and UGT metabolic profile. Also, the influence of

Table 2 Limitations and advantages of *in vivo* and *in vitro* systems for the evaluation of drug-drug and herb-drug interaction studies

Drug interaction studies	Limitations	Advantages
In vitro studies	-Single components used in test. -Typically use higher concentrations than clinically relevant -Does not account for poor bioavailability of the active component *in vivo* -Does not account for binding of active component *in vivo* to plasma proteins	-Easy to carry out -Simple systems -Provide mechanistic information
In vivo studies in animals	-Difficult to interpret-species variation -Often large non-physiologic doses are used	-To some extent addresses issues such as poor bioavailability of herbal components
In vivo studies in humans	-Selection of products: There is large variability in actual content of different ingredients-batch to batch, product to product variations in disintegration and dissolution complicates interpretation. Difficult to extrapolate from one formulation to another. There are differences in the bioavailability of selected components from different formulations. -Study design: Often carried out in healthy humans and not in patients who may have additional compromising conditions -Positive and reference controls not normally used (use of rifampin to show induction and use of ketoconazole or other inhibitors to show inhibition of pathways tested and to serve as a reference for magnitude of any changes observed). -Need an evaluation of the bioavailability of the known ingredients. -Does not distinguish gut versus liver effect of herbal products. -Generally costly. Difficult to obtain mechanistic information	-Will provide the final answer

specific isozymes can be studied in the presence of specific inhibitors (Birkett et al., 1993). Studies with microsomes, while providing information on the potential 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, they do also not allow to the evaluation of the effect of herbal components on transporters. Since excess co-substrate is added to 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).

4.2 *In vitro* studies using primary cultures

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 hepatocytes (PCH), including those from human origin , are necessary if better predictions of drug-drug and 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. PCH are viable for up to 2 weeks (or one month if placed in a three-dimensional culture) and retain all co-factors 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 (Gebhardt et al., 2003). As the use of PCH has advanced, modified culturing techniques have enabled the examination of other processes involved in drug metabolism, namely the uptake and efflux of drugs and their metabolites by hepatic drug transporters. Comparative studies using hepatic models (e.g. primary cultures of hepatocytes) are the most useful for demonstrating species differences in the metabolism of a test compound, and are of great value in the judicious and justifiable selection of animals for subsequent pharmacokinetic and toxicological

studies. Hence, the choice of experimental model needs to be rationally justified so that the data obtained in animal studies can be safely extrapolated to man.

4.3 *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. Table 3 lists a suggested ideal design for *in vivo* human studies.

Table 3 Suggested ideal *in vivo* study design in humans

1. Evaluate the composition of the herbal product used
2. Evaluate the disintegration and dissolution property of product used
3. Use chronic dosing of the herbal products (at least one week)
4. Co-administer herb and drug product on study day to maximize potential for interaction
5. Use positive controls in the study design (for example, rifampin to document induction and ketoconazole to document inhibition and give a comparative effect of the herbal product being tested)
6. Measure some herbal component in the blood or plasma to verify systemic levels of some components from the herbal product

In summary

The use of herbal products worldwide has skyrocketed in the past decade as a result of the public's pursuit of finding an ‘all natural’ alternative to the conventional western medicine. Herbal products contain several chemicals that are metabolized by phase I and phase II pathways and also serve as substrates for certain transporters. Due to their interaction with these enzymes and transporters there is a potential for alteration in the activity of drug metabolizing enzymes and transporters in presence of herbal components. Induction and inhibition of drug

metabolizing enzymes and transporters by herbal component has been documented in several *in vitro* studies.

While *in vitro* studies with the microsomal system provide limited information, hepatocyte systems offers a unique opportunity to evaluate herb drug interaction and will help in focusing and minimizing *in vivo* human studies. A lack of effect in human hepatocyte would suggest lack of *in vivo* effect. A positive response in human hepatocyte culture would indicate a need for further assessment *in vivo* in humans.

While *in vitro* and *in vivo* systems in animals will provide useful information, *in vivo* studies in humans are the only definitive way to assess the magnitude and implications of herb–drug interactions. With an increased understanding of the mechanism of herb drug interactions it should be feasible to minimize or avoid therapeutic failures or increased toxicity of conventional drug therapy.

EXPERIMENTAL WORK

General Aims of the thesis

In order to assess the potency of *Andrographis paniculata* extract (APE) and andrographolide (AND), the most medicinally active phytochemical in the extract, to induce drug-drug interactions, our strategy was as follows:

Our first aim was to assess the inhibitory effect of APE and AND on hepatic P450. Indeed, as outlined above, the liver is the major organ of xenobiotic biotransformation, where P450 play a crucial role in their metabolism and therefore, **P450 inhibition** is the most important mechanism for metabolic herb-drug interactions. A strong inhibition of P450 activities by a molecule is expected to seriously interfere with the metabolism of other simultaneously or subsequently administered drugs. Considerable progress has been made in the development of reliable *in vitro* screening methods to identify potent P450 inhibitors. Microsomes are the preferred test system as they are more readily available than hepatocytes. Because of the species-species differences in metabolic pathways and/or inhibitory effects of compounds, we examined P450 inhibition in liver microsomes from both rat, the animal species routinely used in *in vivo* toxicological studies, and human origin, in order to best predict the possible effects in Human. The data we obtained are summarized in a published manuscript (**Pekthong *et al*., Journal of Ethnopharmacology 115 (2008) 432–440).**

Our second aim was to assess the potential of APE and AND to interfere with the P450 expression. Herb-drug interactions can also occur as a consequence of **P450 induction or repression**. Metabolic interactions due to enzyme induction/repression are far less frequent than those caused by inhibition; however, their consequences can be clinically relevant. A molecule with inductive/repressive properties can accelerate/decrease its own metabolism or those of other co-administered drugs, resulting in either therapeutic inefficacy or in an exaggerated / lower response. Screening of inducers/repressors cannot be done in microsomes or recombinant models as it requires a cellular system that is

fully capable of expressing genes. Currently, primary hepatocytes are still the unique *in vitro* model for global examination of the inductive/repressive potential of drugs. The use of the human model in addition to rat model is advisable given the known interspecies differences found in P450 induction. For such assays, hepatocytes are exposed for 24–72 h to non-cytotoxic concentrations of the test compound. Upon incubation, assessment of P450 activities is performed using specific substrates. One should however be aware that enzyme catalytic activity after treatment can be the consequence of various effects at the P450 level: it can indeed be the direct consequence of an enhancement /lowering of enzyme amount, through protein synthesis modulation, it can however also be the consequence of both enzyme induction/repression and inhibition. For this reason, it is important to assess enzyme amount. The determination of enzyme amount by western blotting has the drawback of its low sensitivity, for this reason, mRNA expression is more often used to assess enzyme expression.

We participated to a study aimed to **assess the correlation between mRNA expression and drug metabolizing enzymes** in response to reference inducers in both rat and human hepatocytes. To do this, we expressed both enzyme activities and mRNA expression as “a percentage of positive control”. The use of this new calculation brings the many-fold induction of mRNA into context with enzyme activities. The results are summarized in a manuscript in revision (**Richert *et al.*, Toxicology and Applied Pharmacology 2008).**

As *in vivo* assessments are the best models to use since they give the definitive answer for a given species, we first assessed the effects of APE and AND on various hepatic CYP activities after *in vivo* administration to Wistar rats. The *in vivo – in vitro* correlation of the effects of APE and AND in rats was then evaluated after treatment of rat hepatocytes with AND and APE. As huge species differences have been widely reported, a stepwise approach, including *in vitro* human models in addition to *in vivo* and *in vitro* animal models, is required for a

proper evaluation and prediction of herb-drug interactions in Humans. These systems also facilitate determination of whether there is a need to conduct more demanding clinical studies. The data we obtained are reported in a manuscript in revision (**Pekthong *et al*., Chemico-Biological Interaction 2008).**

CHAPTER 4: PUBLICATION 1

Differential inhibition of rat and human hepatic cytochrome P450 by *Andrographis paniculata* extract and andrographolide

Publication 1: Differential inhibition of rat and human hepatic cytochrome P450 by *Andrographis paniculata* extract and andrographolide
Journal of Ethnopharmacology 115 (2008) 432–440.

P450 enzymes play a crucial role in the metabolism of drug and, therefore, **P450 inhibition** is the most important mechanism for metabolic drug-drug or herb-drug interactions. A strong inhibition of P450 activities by a molecule is expected to seriously interfere with the metabolism of other simultaneously or subsequently administered drugs. Considerable progress has been made in the development of reliable *in vitro* screening methods to identify potent P450 inhibitors. Microsomes are the preferred test system as they are more readily available than hepatocyte. In this publication, the inhibitory effect of *Andrographis paniculata* extract (APE) and andrographolide (AND), the most medicinally active phytochemical in the extract, on various hepatic P450s activities was examined using rat and human liver microsomes. The inhibitory effects are expressed as a percentage of the control activity value and IC_{50} values. The mechanism of inhibition was estimated graphically from Lineweaver–Burk plots, and from the enzyme inhibition models. *Ki* values were calculated via second plot of the slopes from Lineweaver–Burk plots *versus* inhibitor concentrations.

APE inhibited ethoxyresorufin-*O*-deethylation (EROD, CYP1A2) activity in rat and human liver microsomes, with apparent *Ki* values of 8.85 and 24.46 μM, respectively. In each case, the mode of inhibition was noncompetitive. APE also inhibited tolbutamide hydroxylation (CYP2C) both in rat and human microsomes with apparent *Ki* values of 8.21 and 7.51 μM, respectively and the mode of inhibition was mixed type. In addition, APE showed a competitive inhibition only on CYP3A4 in human microsomes with *Ki* of 25.43μM. AND was found to be a weak inhibitor of rat CYP2E1 with a *Ki* of 61.1 μM but did not affect human CYP2E1.

In conclusion, from the data presented in the paper it cannot be excluded that APE could cause drug–drug interactions in humans through CYP3A4 and 2C9 inhibition.

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Differential inhibition of rat and human hepatic cytochrome P450 by *Andrographis paniculata* extract and andrographolide

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Abstract

The inhibitory effect of *Andrographis paniculata* extract (APE) and andrographolide (AND), the most medicinally active phytochemical in the extract, on hepatic cytochrome P450s (CYPs) activities was examined using rat and human liver microsomes. For this purpose, CYP1A2-dependent ethoxyresorufin-*O*-deethylation, CYP2B1-dependent benzyloxyresorufin-*O*-dealkylation, CYP2B6-dependent bupropion hydroxylation, CYP2C-dependent tolbutamide hydroxylation, CYP2E1-dependent *p*-nitrophenol hydroxylation and CYP3A-dependent testosterone 6β-hydroxylation activities, were determined in the presence and absence of APE or AND (0–200 μM). APE inhibited ethoxyresorufin-*O*-deethylation activity in rat and human liver microsomes, with apparent K_i values of 8.85 and 24.46 μM, respectively. In each case, the mode of inhibition was noncompetitive. APE also inhibited tolbutamide hydroxylation both in rat and human microsomes with apparent K_i values of 8.21 and 7.51 μM, respectively and the mode of inhibition was mixed type. In addition, APE showed a competitive inhibition only on CYP3A4 in human microsomes with K_i of 25.43 μM. AND was found to be a weak inhibitor of rat CYP2E1 with a K_i of 61.1 μM but did not affect human CYP2E1. In conclusion, it cannot be excluded from the present study that APE could cause drug–drug interactions in humans through CYP3A and 2C9 inhibition.

Keywords: *Andrographis paniculata*; Andrographolide; Cytochrome P450; Inhibition

1. Introduction

Herbal medicines are often used with western modern drugs with the aim to decrease side effects or toxicity, or to obtain a synergistic or additive effect in terms of pharmacological effects. For this reason herbal medicine has received increasing popularity. However, it has been reported that herbal products containing a number of natural compounds can cause pharmacokinetic interaction with modern drugs when they were administrated simultaneously (Ioannides, 2002; Sorensen, 2002; Brazier and Levine, 2003).

Cytochrome P450s (CYPs) play central roles in drug metabolism (Gonzalez, 1990). The expression of individual P450s is regulated by both endogenous factors and foreign compounds including drugs and natural compounds (Nebert et al., 1991). In addition, since both endogenous and exogenous compound are CYP substrate, co-administration of such compound can lead to pharmacokinetic interactions. Thus grapefruit juice has been shown to inhibit drug metabolism (Goff-Klein et al., 2003) or change the plasma concentrations of many drugs substrates for CYP3A4 when taken simultaneously (Quo et al., 2000). St. John's wort (*Hypericum perforatum*) which is an herbal component widely used for the treatment of depression, has been found to induce cytochrome P450s (particularly CYP3A4) in the liver. It has been reported that St. John's wort co-

Abbreviations: BROD, benzyloxyresorufin-*O*-deethylation; BuOH, bupropion hydroxylation; CYP, cytochrome P450; EROD, ethoxyresorufin-*O*-deethylation; HLM, human liver microsome; PNP-OH, paranitrophenol hydroxylation; TolOH, tolbutamide hydroxylation; 6βTesto-OH, testosterone 6β-hydroxylation.

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administration with cyclosporin leaded to decrease cyclosporin plasma concentrations and kidney transplant rejection (Barone et al., 2000; Moschella and Jaber, 2001). Also, many case reports exist on irregular bleeding and unwanted pregnancies with St. John's wort taken simultaneously with ethynylestradiol (Schwarz et al., 2003). *Ginkgo biloba*, which has been shown to display beneficial effects on the vascular system, memory, cognition and gene regulation, was also found *in vitro* to strongly inhibit CYP2C9, and to a lesser extent, CYP1A2, CYP2E1 and CYP3A4 (Gaudineau et al., 2004).

Andrographis paniculata is one of the most important medicinal plants used in China, Thailand and Ayurvedic medicine to treat gastric disorder, colds, influenza and other infectious diseases. An extract of *Andrographis paniculata* (APE), standardized for its content of andrographolide (AND) and deoxyandrographolide, called 'Kan Jang', has been used extensively in Scandinavia for the last 20 years for the treatment of the common cold (Caceres et al., 1997). It has been shown in several animal studies that extracts of *Andrographis paniculata* have anti-inflammatory (Shen et al., 2002; Sheeja et al., 2006), antiallergic, anticancer (Kumar et al., 2004; Nanduri et al., 2004; Cheung et al., 2005), immuno-stimulatory (Puri et al., 1993; Iruretagoneya et al., 2005) and antiviral activities (Calabrese et al., 2000).

On the other hand, male reproductive toxicity (Akbarsha and Murugaian, 2000) of this plant has been reported. Also, an *in vivo* study indicated that a single dose of APE and AND inhibited hepatic microsomal CYP2C/2E1-dependent aniline hydroxylase, CYP2B-dependent *N*-demethylation of *N*,*N*-dimethyl aniline, and CYP2E1-dependent *O*-demethylation of *p*-nitroanisole in albino rats, while in long term treatment its administration produced induction of all these isoenzymes in this species. In *in vitro* incubation studies using rat microsomes, APE as well as AND did not produce any effect on CYP2B-dependent *N*-demethylation of *N*,*N*-dimethyl aniline, and CYP2E1-dependent *O*-demethylation of *p*-nitroanisole but only inhibit CYP2C/2E1-dependent aniline hydroxylase (Choudhury et al., 1987). *Andrographis paniculata* extracts have recently been reported to increase CYP1A1-dependent ethoxyresorufin *O*-dealkylase and CYP2B10-pentoxyresorufin *O*-dealkylase activities *in vivo* in mice (Jarukamjorn et al., 2006).

In light of its widespread use and few literature reports, more studies are needed on the impact of *Andrographis paniculata* on selective hepatic P450 enzyme activities. In particular, as there are marked differences in the expression and regulation of CYPs, assessment of the inhibitory and/or induction potential of CYPs is biologically relevant, particularly when performed in models from various species, including human, ultimately leading to better models for screening and predicting drug–drug interactions in humans (Fujii-Kuriyama, 1993; Gotoh, 1993; Negishi et al., 1993). In the present study APE and AND (Fig. 1), the most medicinally active phytochemical in the plant, were examined for their effects on microsomal hepatic P450 enzyme activities both using rat and human liver microsomes, in order to give more insight into the guideline of rational administration and precaution to be taken for using this herbal medicine.

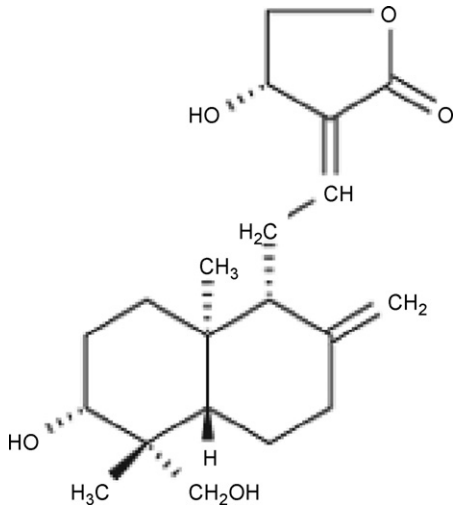

Fig. 1. Structure of andrographolide.

2. Materials and methods

2.1. Chemicals and authentic metabolite standards

AND, testosterone, 6β-hydroxytestosterone, 11β-hydroxytestosterone, bupropion, hydroxybupropion, tolbutamide, 4-hydroxytolbutamide, bovine serum albumin (BSA), *p*-nitrophenol, 4-nitrocatechol, ethoxyresorufin, benzyloxyresorufin, resorufin, ketoconazole, α-naphthoflavone, tetraethylthiuram disulfide (disulfiram), sulfaphenazole, reduced nicotinamide-adenine dinucleotide phosphate (NADPH), were purchased from Sigma–Aldrich (France). All other laboratory chemicals were used as the highest purity and from commercial suppliers.

2.2. Preparation of microsomes

Male Wistar rats (8 weeks old) were purchased from Charles River (France) and housed in metal cages with a 12 h light/dark cycle and were fed *ad lib* for 48 h prior to be sacrificed. The liver was immediately removed, weighed and washed in cold homogenization buffer (50 mM Tris–HCl, 150 mM KCl, 2 mM EDTA, pH 7.4). Routinely, the liver was then homogenized in 4 ml buffer per 1 g of liver. The homogenates were submitted to several differential centrifugations, as previously described (Richert et al., 2002). Microsomal samples were finally aliquoted and frozen at −80 °C until analysis. Human livers from three French autopsy samples (Table 1) were used with permission of Local Research Ethic Committee. Human liver microsomes were prepared as above. Protein content was determined using the bicinchoninic

Table 1
Clinical history of human donors

Donor	Age (years)	Sex	Race	Disease
HLM-D24	47	Male	Caucasian	Cholangiocarcinoma
HLM-B2	73	Male	Caucasian	Adrenocarcinoma
HLM-B12	20	Female	Caucasian	Liver cancer

acid protein determination kit (Sigma) and BSA was used as a standard.

2.3. Preparation of Andrographis paniculata extract (APE)

Medicinal capsules containing powder of *Andrographis paniculata* Nees. (Acanthaceae) dried leaves (350 mg per capsule) were from the Government Pharmaceutical Organization drug store (Bangkok, Thailand, lot number CFT 4713). The capsule was crushed to coarse powder and sieved through No. 20 mesh size. The extraction was carried out by mixing the powdered of *Andrographis paniculata* Nees. with 1:3 (w/v) in 60% ethanol (v/v) by Soxhlet apparatus for 12 h. The extract was filtered and the solvent from the filtrate was removed by rotary evaporator under reduced pressure and low temperature. The yield of extract was 10% (w/w) in terms of dried starting material. The extract was stored at 4 °C until it cooled down, filtered through a 0.45 μm nylon membrane prior to quantification of the AND content by using HPLC-UV (Jain et al., 2000) and found to contain 1.60% AND. The extract was then dissolved in distilled water in order to obtain a 10 mM AND containing stock solution. The extract stock solution was stored at −20 °C.

2.4. Cytochrome P-450 monooxygenase activities

2.4.1. CYP1A2 ethoxyresorufin-O-deethylase assay for rat and human liver microsome

Microsomal ethoxyresorufin-*O*-deethylation (EROD) was determined according to Burke et al. (1985). Briefly, hepatic microsomes (0.04 mg protein) were incubated for 2 min at 37 °C with 7-ethoxyresorufin (6.5 μM) as substrate. The reaction was initiated by adding NADPH (2 mM) and was terminated by addition of $ZnSO_4$ (87 mM) and $Ba(OH)_2$ (79 mM). Following centrifugation (800 g; 5 min) to remove precipitated protein the fluorescent metabolite resorufin was measured (excitation 530 nm and emission 580 nm). Results were expressed as pmol resorufin formed/min/mg microsomal proteins.

2.4.2. CYP2B1/2 benzyloxyresorufin-O-dealkylase assay for rat liver microsome

Microsomal benzyloxyresorufin-*O*-deethylation (BROD) was determined as described for EROD, except that incubation was carried for 3 min at 37 °C with benzyloxyresorufin (20.5 μM) as substrate.

2.4.3. CYP2B6 bupropion hydroxylase assay for human liver microsome

Bupropion hydroxylation (BuOH) was measured according to Faucette et al. (2000). Briefly, hepatic microsomes (0.1 mg protein) were incubated with bupropion (0.5 mM) and NADPH (2 mM) for 30 min at 37 °C. The reaction was stopped by adding ice-cold acetonitrile and placing samples on ice. After centrifugation, the supernatant was analyzed by HPLC with UV spectrophotometric detection at 210 nm. Quantification was performed by using the standard curve of hydroxybupropion. Results were expressed as pmol hydroxybupropion formed/min/mg microsomal proteins.

2.4.4. CYP2C tolbutamide hydroxylase assay for rat and human liver microsome

Tolbutamide hydroxylation (TolOH) was measured according to Relling et al. (1990). Briefly, hepatic microsomes (0.04 mg protein) were incubated with tolbutamide (0.25 mM) and NADPH (2 mM) for 45 min at 37 °C. The reaction was stopped by adding ice-cold acetonitrile and placing samples on ice. After centrifugation, the supernatant was analyzed by HPLC with UV spectrophotometric detection at 230 nm. Quantification was performed by using the standard curve of 4-hydroxytolbutamide. Results were expressed as pmol 4-hydroxytolbutamide formed/min/mg microsomal proteins.

2.4.5. CYP2E1 p-nitrophenol hydroxylase assay for rat and human liver microsome

Paranitrophenol hydroxylation (PNP-OH) was assessed in liver microsomes according to Allis and Robinson (1994) by evaluating the formation of 4-nitrocatechol. After 30 min of incubation of microsomes (0.25 mg protein) with *p*-nitrophenol (0.5 mM) and NADPH (1 mM) at 37 °C, the reaction was stopped with 50% trichloroacetic acid. The reaction mixture was centrifuged and NaOH (10 M, 20 μl) was added to the supernatant before measuring the absorbance at 530 nm. Results were expressed as pmol 4-nitrocatheol formed/min/mg microsomal proteins.

2.4.6. CYP3A testosterone 6β-hydroxylase assay for rat and human liver microsome

Testosterone 6β-hydroxylation (6βTesto-OH) was measured according to Pearce et al. (1996), by incubating hepatic microsomes (0.2 mg protein) with testosterone (0.25 mM) and NADPH (2 mM) for 8 min at 37 °C. The reaction was stopped by adding ice-cold acetonitrile and placing samples on ice. After centrifugation, the supernatant was analyzed by HPLC with UV spectrophotometric detection at 230 nm. Quantification was performed by internal standardization with 11β-hydroxytestosterone. Results were expressed as pmol 6β-hydroxytestosterone formed/min/mg microsomal proteins.

2.5. IC_{50} determinations

In the primary screening of the IC_{50} valves, the appropriate concentrations of ethoxyresorufin (1 μM), benzyloxyresorufin (4 μM), bupropion (100 μM), tolbutamide (90 μM), testosterone (100 μM) and *p*-nitrophenol (100 μM) were chosen in order to determine the effect of APE and AND on the high capacity/low affinity components (final concentrations of AND in the incubation mixture were 0.01, 0.1, 1, 5, 10, 50, 100, 200 μM, pure or in the extract) of metabolism for each substrate. The compounds α-naphthoflavone, proadifen, sulfaphenazole, disulfiram and ketoconazole were used as selective inhibitors of CYP1A, CYP2B, CYP2C, CYP2E and CYP3A activity, respectively. The IC_{50} values for inhibitors, APE and AND were determined graphically by nonlinear regression analysis of the

plot of the logarithm of inhibitor concentration *versus* percentage of remaining activity using GrapPadPrism3 (GrapPad Co. Ltd., USA). The enzyme activities in the presence of inhibitors were compared with the control incubation (incubation containing solvent but no inhibitor). Three determinations were carried out at each inhibitor concentration.

2.6. Determination of enzyme kinetics

For determining the apparent K_i values and mode of inhibition of APE and AND, 4–5 concentrations of specific substrate corresponding approximately to $K_m/4$, $K_m/2$, K_m, $2 \times K_m$ and $4 \times K_m$ were incubated with a range of inhibitor concentration in a presence of rat or human liver microsomes as described above. All incubations were carried out in duplicate and the average was presented as the result, the variation was usually less than 10%. The kinetic parameters for each probe reaction (i.e., V_{max} and K_m) were estimated from the best-fit least-squares linear regression of double reciprocal plot of velocity *versus* substrate concentration (Lineweaver–Burk plots). The mechanism of inhibition was estimated graphically from Lineweaver–Burk plots, and from the enzyme inhibition models. K_i values were calculated via second plot of the slopes from Lineweaver–Burk plots *versus* inhibitor concentrations.

2.7. Statistics

The significance of the difference between the groups was assessed by one-way analysis of variance and the student Newman–Keuls multiple range test (SigmaStat™, SPSS Science, Chicaco, IL, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Inhibition of rat and human CYPs by selective inhibitors

Compounds known as selective inhibitors of CYP1A2, 2C, 2E1 and 3A activities were used to confirm the selectivity of our assays (Table 2). α-Naphthoflavone caused the greatest reduction in CYP1A-dependent activity with a IC_{50} of 23.0 and 0.085 μM in rat and human microsomes, respectively, in accordance with the report of Kim et al. (2003). Disulfiram decreased CYP2E1-dependent activity with an IC_{50} of 10.1 and 1.0 μM in rat and human microsomes, respectively and sulfaphenazole decreased CYP2C-dependent activity with an IC_{50} of 56.7 and 1.01 μM in rat and human microsomes, respectively as previously reported (Eagling et al., 1998). Ketoconazole (1 μM) completely abolished CYP3A-dependent activity both in rat and human microsomes as found by Emoto et al. (2003). These results confirm previous findings on the marked species differences of the kinetic parameters generated with a given substrate using human and rat liver microsomes.

3.2. Inhibition of rat CYPs by AND and APE

To study the potential inhibitory action of APE and AND on various CYP isoforms present in rat liver microsomes, CYP1A2-dependent EROD, CYP2B1/2-dependent BROD, CYP2C11-dependent TolOH, CYP2E1-dependent PNP-OH and CYP-3A1/2-dependent 6βTesto-OH, were tested in the presence and absence of APE and AND. The results clearly demonstrated that APE is a moderate inhibitor of rat CYP1A2 and CYP2C11 with IC_{50} of 5.1 and 3.86 μM, respectively (Table 2). In con-

Table 2
IC_{50} values of *Andrographis paniculata* extract (APE), andrographolide (AND) and selective inhibitors on P450-specific model activities

P450 isoform	Reaction	Inhibitor	IC_{50} (μM)	
			Rat microsome[a]	Human microsome[b]
CYP1A2	7-Ethoxyresorufin-*O*-deethylation	APE	5.1	10.3
		AND	>100	>100
		α-Naphthoflavone	23.0	0.085
CYP2B1/2	Benzyloxyresorufin-*O*-dealkylation	APE	>100	–
		AND	>200	–
		Proadifen	20.0	–
CYP2B6	Bupropion hydroxylation	APE	–	>200
		AND	–	>200
CYP2C	Tolbutamide hydroxylation	APE	3.86	16.16
		AND	>100	>100
		Sulfaphenazole	56.7	1.01
CYP2E1	*p*-Nitrophenol hydroxylation	APE	>100	>100
		AND	35.0	>100
		Disulfiram	10.1	1.0
CYP3A	6-β-Testosterone hydroxylation	APE	>100	34.1
		AND	>100	>200
		Ketoconazole	0.10	0.11

[a] Means of three determinations in a pool of three different rat liver microsomes.
[b] Means of three determinations in a pool of three different human liver microsomes.

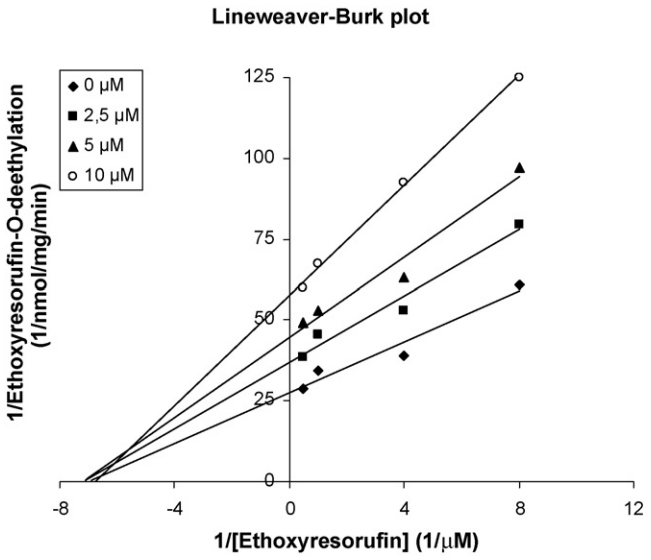

Fig. 2. Lineweaver–Burk plots for the inhibition of 7-ethoxyresorufin-*O*-deethylation by APE (♦; 0 μM, ■; 2.5 μM, ▲;5 μM, ¤ ; 10 μM) in rat liver microsomes. The final substrate concentrations were 0.125, 0.25, 1 and 2 μM. Incubations were performed with 0.04 mg microsomal protein and 2 mM NADPH for 2 min at 37 °C. Each point represents the average of duplicate determinations in a pool of three different rat liver microsomes and the variation was less than 10%.

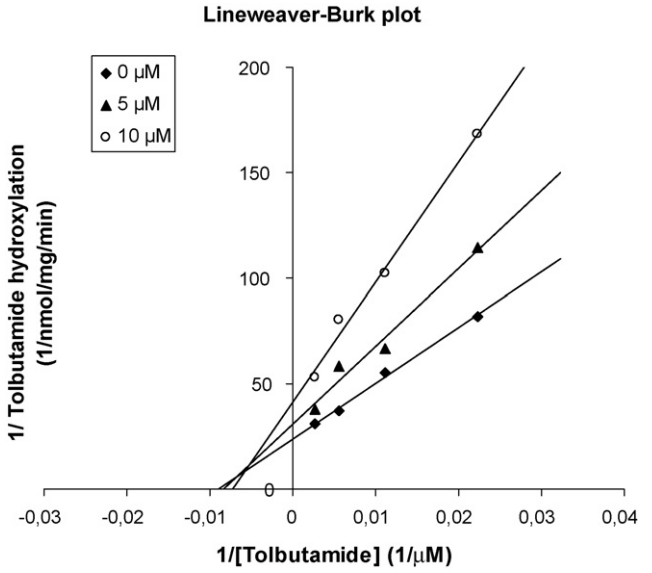

Fig. 3. Lineweaver–Burk plots for the inhibition of 4-tolbutamide hydroxylation by APE (♦; 0 μM, ▲; 5 μM, ¤; 10 μM) in rat liver microsomes. The final substrate concentrations were 45, 90, 180 and 360 μM. Incubations were performed with 0.04 mg microsomal protein and 2 mM NADPH for 45 min at 37 °C. Each point represents the average of duplicate determinations in a pool of three different rat liver microsomes and the variation was less than 10%.

trast, APE caused no significant inhibition of CYP2B1-, 2E1- and 3A1/2-dependent activities ($IC_{50} > 100$ μM). The presence of AND had minimal effect on any reaction except the hydroxylation of *p*-nitrophenol which was inhibited with an IC_{50} of 35.0 μM (Table 2).

3.2.1. Type of inhibition of CYP1A2 and CYP2C11 by APE in rat microsomes

To characterize the kinetics of CYP1A2 and 2C11 enzyme inhibition by APE, the EROD and TolOH assays were conducted with multiple concentrations of the extract and multiple concentrations of the substrates. Lineweaver–Burk plots for the inhibition of CYP1A2 and 2C11 are shown in Figs. 2 and 3. Based on nonlinear regression analysis of the enzyme kinetic data, the mode of inhibition of EROD (CYP1A2) was noncompetitive with a K_i value of 8.85 μM, and TolOH (CYP2C11) was mixed type inhibited with a K_i value of 8.21 μM.

3.2.2. Type of inhibition of CYP2E1 by AND in rat microsomes

The mechanism of inhibition of AND against PNP-OH in rat liver microsomes was determined. As shown in Fig. 4, AND exhibited a competitive inhibition of PNP-OH with K_i value being about 61.1 μM.

3.3. Inhibition of human CYPs by AND and APE

To study the potential inhibitory of APE and AND on various CYP isoforms present in human liver microsomes, CYP1A2-dependent EROD, CYP2B6-dependent BuOH, CYP2C9-dependent TolOH, CYP2E1-dependent PNP-OH and CYP-3A4/5-dependent 6βTesto-OH, were tested in the presence and absence of APE and AND, using pooled human liver microsomes. The results showed that APE is a moderate inhibitor of human CYP1A2, CYP2C9 and CYP3A4 with IC_{50} of 10.3, 16.16 and 34.1 μM, respectively (Table 2). In contrast, APE caused no significant inhibition of CYP2B6- and 2E1-dependent activities ($IC_{50} > 100$ μM). The presence of AND had minimal effect on any reaction (Table 2).

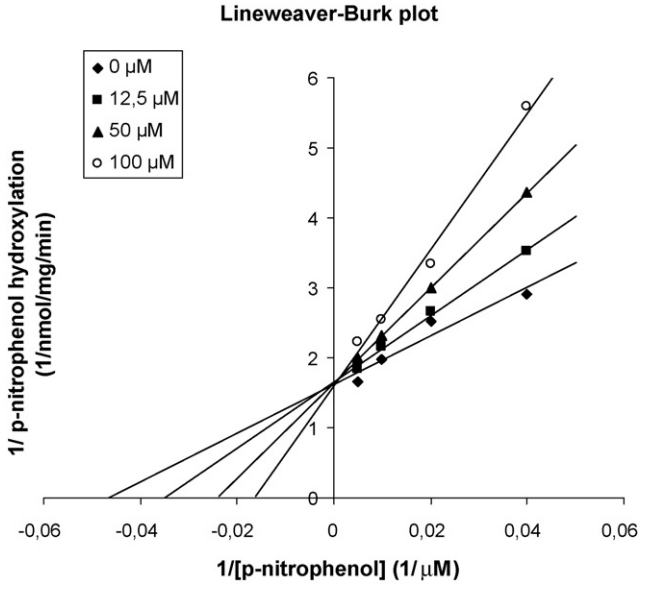

Fig. 4. Lineweaver–Burk plots for the inhibition of *p*-nitrophenol hydroxylation by AND (♦;0 μM, ■; 12.5 μM, ▲; 50 μM, ¤; 100 μM) in rat liver microsomes. The final substrate concentrations were 25, 50, 100 and 200 μM. Incubations were performed with 0.25 mg microsomal protein and 1 mM NADPH for 30 min at 37 °C. Each point represents the average of duplicate determinations in a pool of three different rat liver microsomes and the variation was less than 10%.

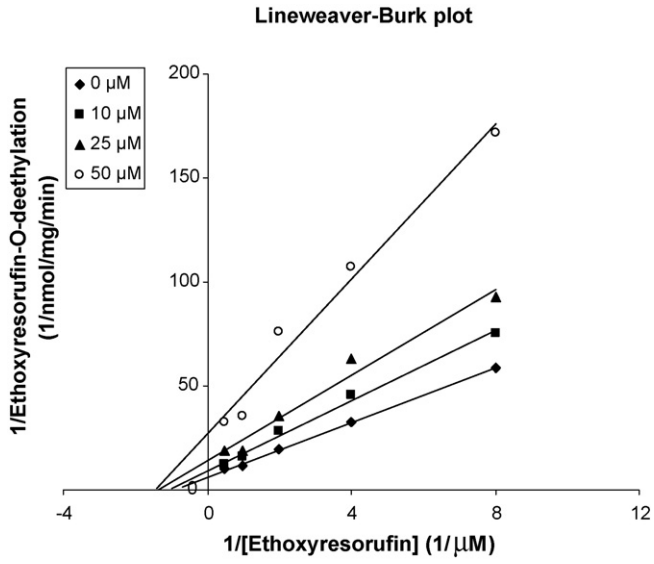

Fig. 5. Lineweaver–Burk plots for the inhibition of 7-ethoxyresorufin-*O*-deethylation by APE (♦; 0 μM, ■; 10 μM, ▲; 25 μM, ¤ ; 50 μM) in pooled human liver microsomes. The final substrate concentrations were 0.125, 0.25, 0.5, 1 and 2 μM. Incubations were performed with 0.04 mg microsomal protein and 2 mM NADPH for 2 min at 37 °C. Each point represents the average of duplicate determinations in a pool of three different human liver microsomes and the variation was less than 10%.

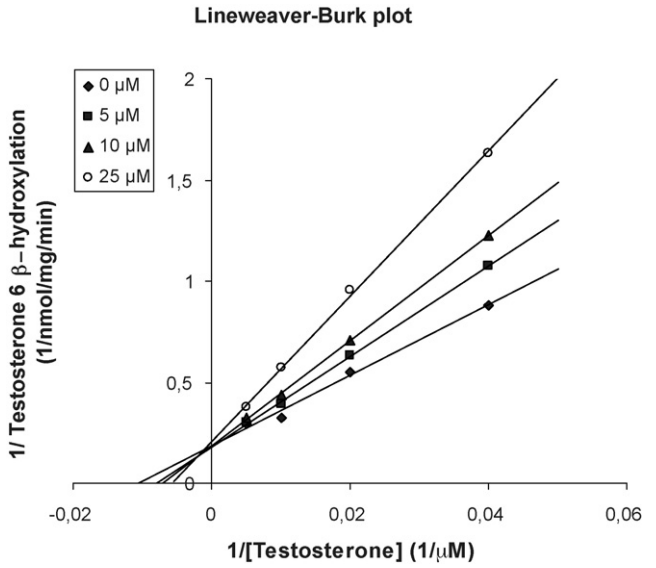

Fig. 7. Lineweaver–Burk plots for the inhibition of 6-β-testosterone hydroxylation by APE (♦; 0 μM, ■; 5 μM, ▲; 10 μM, ¤ ; 25 μM) in pooled human liver microsomes. The final substrate concentrations were 25, 50, 100 and 200 μM. Incubations were performed with 0.2 mg microsomal protein and 2 mM NADPH for 8 min at 37 °C. Each point represents the average of duplicate determinations in a pool of three different human liver microsomes and the variation was less than 10%.

3.3.1. Type of inhibition of CYP1A2, CYP2C9 and CYP3A4 by APE in human microsomes

Further kinetic studies were carried out for CYP1A2, 2C9 and 3A4 because of strong inhibition was observed. As shown in Figs. 5–7, Lineweaver–Burk plot suggested is a noncompetitive inhibition of EROD (CYP1A2) with K_i value of 24.46 μM, mixed type inhibition of TolOH (CYP2C9) with K_i value of 7.51 μM and competitive inhibition of 6βTesto-OH (CYP3A4) with K_i estimated to be 25.43 μM from second plot of the slope.

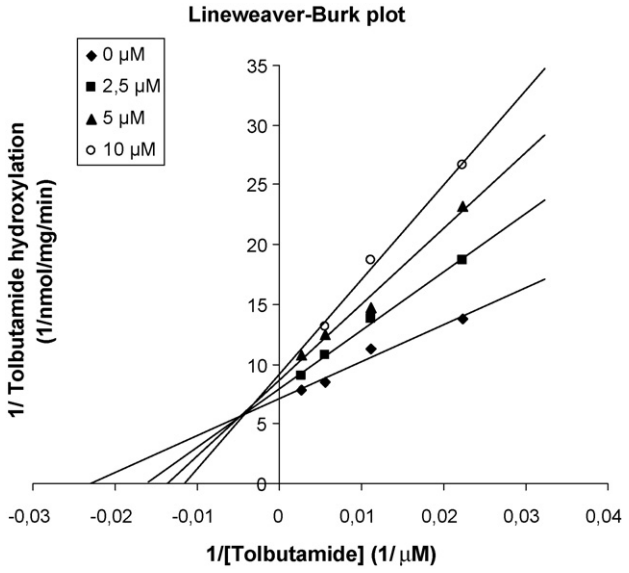

Fig. 6. Lineweaver–Burk plots for the inhibition of 4-tolbutamide hydroxylation by APE (♦; 0 μM, ■; 2.5 μM, ▲; 5 μM, ¤ ; 10 μM) in pooled human liver microsomes. The final substrate concentrations were 45, 90, 180 and 360 μM. Incubations were performed with 0.04 mg microsomal protein and 2 mM NADPH for 45 min at 37 °C. Each point represents the average of duplicate determinations in a pool of three different human liver microsomes and the variation was less than 10%.

3.3.2. Sample-to-sample variation in the inhibition of CYP1A2, CYP2C9 and CYP3A4 activities by APE

The sample-to-sample variation in the inhibition of CYP1A2, 2C9 and 3A4 were determined with three different human liver microsomes (HLMs) preparation, at substrate concentrations equal to K_m and APE concentrations was 20 μM. As expected, APE produced substantial inhibition (∼50%) of each CYP activities in all microsomal samples used (Fig. 8).

4. Discussion

We undertook to study the inhibition of CYP1A, CYP2B, CYP2C, CYP2E and CYP3A by APE and AND. For this, either pure AND (0–200 μM) or extracts containing AND at equivalent concentrations, were incubated with rat and human liver microsomes. These enzymes were selected because together they account for 80% of the total hepatic metabolism (Ortiz de Montellano, 1995).

The presence of AND had minimal effect on any rat and human liver CYP-dependent reaction measured except on the CYP2E1-dependent *p*-nitrophenol hydroxylation, which was inhibited with a K_i value of 61.1 μM in rat liver microsomes. Our observation that AND inhibited rat liver CYP2E1 is in accordance with previous results (Choudhury et al., 1987). Although *p*-nitrophenol hydroxylation is usually considered as a marker of CYP2E1 (Allis and Robinson, 1994), the selectivity is questionable in some species. Previous studies indicated

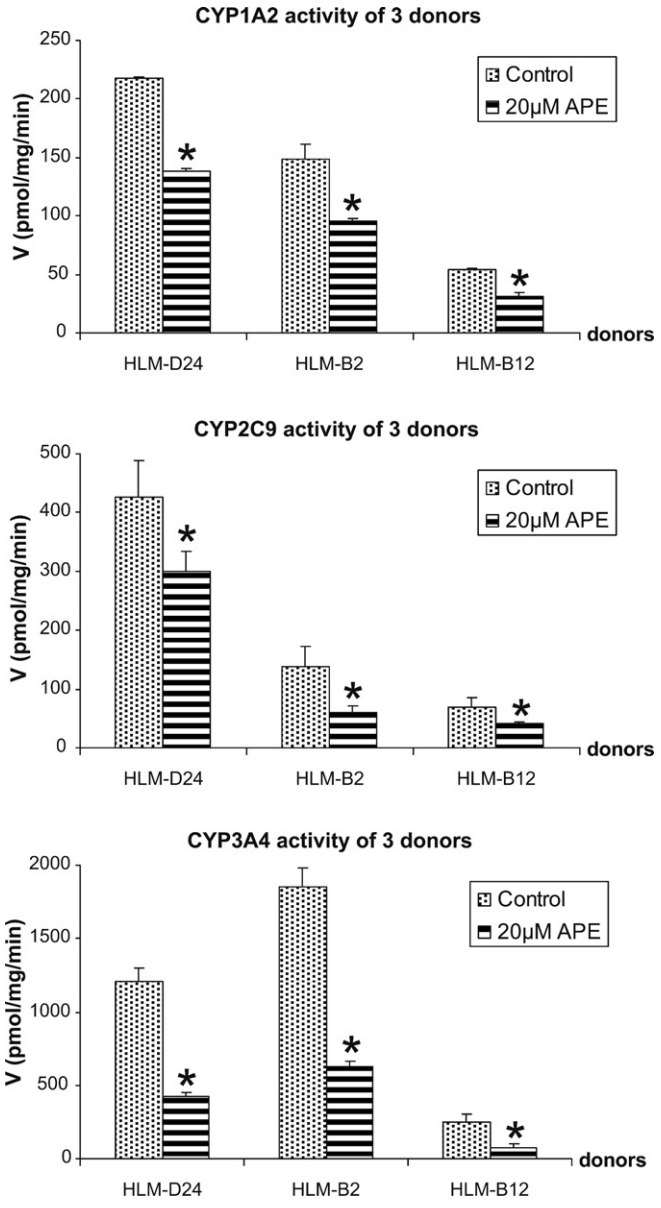

Fig. 8. Effects of 20 μM APE on the rate (expressed as pmol/mg/min) of 7-ethoxyresorufin-*O*-deethylase (CYP1A2 activity), 3-tolbutamide hydroxylase (CYP2C9 activity) and 6-β-testosterone hydroxylase (CYP3A4 activity) in human liver microsome (HLM). Data for three donors (HLM-D24, HLM-B2 and HLM-B12) are expressed from three independent determinations as mean ± S.D. **P* < 0.05, control *vs*. APE.

that CYP1A1/2, CYP2C11 and CYP3A1/2 enzymes also contribute to the formation of 4-nitrocathecol (Koop et al., 1989). When multiple isoforms of CYPs are involved in a metabolic reaction, isoform-selectivity occasionally differs depending on the substrate concentration (Kaoru et al., 2002).

The presence of APE and AND had no effect on human CYP2E1-dependent hydroxylation of *p*-nitrophenol, suggesting that no inhibitory effect on this isoenzyme occurs in humans. Our finding that tetraethylthiuram disulfide (disulfiram), used as a selective CYP2E1 inhibitor (Halpert et al., 1994) was a less potent inhibitor in rat liver microsomes (IC_{50} = 10 μM) than in human liver microsomes (IC_{50} = 1 μM) suggests that there are marked differences between rat and human CYP2E1 in their sensitivity to inhibition. This is further strengthened by previous studies showing that disulfiram was found relatively nonpotent as an inhibitor of CYP2E1-dependent aniline 4-hydroxylation (only 30% inhibition at 250 μM of disulfiram) in rat liver microsomes (Martini et al., 1997) but demonstrated profound (85–95%) inhibition of human CYP-2E1 activity, based on 6-hydroxychlorzoxazone formation (Kharasch et al., 1993).

On the other hand, in the present study, APE inhibited CYP1A2-dependent 7-ethoxyresorufin-*O*-deethylation both in rat and human microsomes, with a K_i value of 8.85 and 24.46 μM, respectively. The inhibitory effect of α-naphthoflavone, a selective CYP1A inhibitor (Halpert et al., 1994), was higher on human liver microsomal CYP1A2 than on rat liver microsomal CYP1A2. Taken together these results suggest marked species-differences in sensitivity to CYP1A2 inhibitors.

Both CYP1A and CYP2E subfamilies are specifically linked with the metabolic activation of carcinogens (Gonzalez and Gelboin, 1994). For instance, the carcinogen benzo[*a*]pyrene, requires oxidative bioactivation by CYP1A (Ioannides and Parke, 1993) to benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide, the ultimate carcinogen known to bind to DNA. In the present study, we found that APE was a moderate inhibitor of CYP1A2, measured as EROD activity in both rat and human microsomes. Since AND did not display such an inhibitory effect, our results suggest that this effect is due to other components from the crude extract APE, and suggest that APE could act as an anti-carcinogen in humans because of its specific inhibitory activity towards CYP1A. It is noteworthy that APE has also been shown to present antioxidant activity (Kapil et al., 1993).

CYP3A is one of the major CYP subfamilies involved in drug metabolism in humans accounting for the metabolism of more than 55% of current pharmaceuticals (Ortiz de Montellano, 1995). Ketoconazole is frequently used as a selective CYP3A inhibitor (Maurel, 1996; Sai et al., 2000; Zhang et al., 2002). In the present study we confirmed that ketoconazole was a CYP3A inhibitor at submicromolar concentration range both in rat and human microsomes (Ibrahim et al., 2000). It is noteworthy that the K_i of APE (K_i = 25.43 μM) on human CYP3A4 was much higher than that previously reported for ketoconazole (K_i = 0.024 μM, Walsky and Obach, 2004).

APE was also found to inhibit CYP2C both in rat and human microsomes with K_i values of 8.21 and 7.51 μM, respectively while AND alone did not. As already discussed for CYP1A2, this may be due to the presence of several other components present along with AND in the extract of *Andrographis paniculata*. Panossian et al. (2000) have reported that after multiple intake of APE at therapeutic dose regimen (3 × 4 tablets/day, i.e. about 1 mg/kg body weight/day), the steady state plasma concentration of AND in humans was approximately 1.7 μM. It is thus possible to attain concentrations of the presently unknown substance(s) capable to exhibit an inhibition of hepatic CYP activities, particularly CYP2C9. The possibility that APE could interact with CYP2C9-dependent metabolism is further strengthened by a recent study of Hovhannisyan et al. (2006) reporting that the maximum concentration and half-life of the CYP2C9 substrate

warfarin in rats was increased and its clearance was 1.8-fold decreased after APE administration.

Also, it cannot be excluded that AND or APE may inhibit several other minor CYP isoforms, such as CYP2D in both rat and human, CYP2A6, CYP2C8, CYP2C19 in human which have not been included in the present work.

5. Conclusion

The novel findings from the present *in vitro* study with rat and human liver microsomal CYPs are that APE inhibited the catalytic activities of both rat and human liver microsomal CYP1A2, CYP2C and of human liver microsomal CYP3A4. Based on K_i and IC_{50} values, these results suggest that APE could act as an anticarcinogen in humans because of its specific inhibitory effect on CYP1A2 activity. The inhibitory effect of APE on CYP3A and 2C9 activities cannot be excluded to cause drug–drug interactions, especially for CYP2C9 since its expression level in human liver is low and because it is known to metabolize several narrow therapeutic index drugs (Miners and Birkett, 1998). Further study is ongoing to fully assess the safety of AND and APE in term of CYP inhibition, after *in vivo* administration to rat and comparison of the *in vitro* effects in rat and human hepatocyte cultures.

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CHAPTER 5: PUBLICATION 2

Use of mRNA expression to detect the induction of drug metabolising enzymes in rat and human hepatocytes

Publication 2: Use of mRNA expression to detect the induction of drug metabolising enzymes in rat and human hepatocytes.
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Drug–drug or herb-drug interactions can also occur as a consequence of **P450 induction or repression**. Metabolic interactions due to enzyme induction are far less frequent than those caused by inhibition; however, their consequences can be clinically relevant. A molecule with inductive properties can accelerate its own metabolism or those of other co-administered drugs, resulting in either therapeutic inefficacy or in an exaggerated response. The use of the human model is advisable given the known interspecies differences found in P450 induction. Screening of inducers cannot be done in microsomes or recombinant models as it requires a cellular system that is fully capable of expressing genes. Currently, primary human hepatocytes are still the unique *in vitro* model for global examination of the inductive potential of drugs. P450 induction is monitored both as increases in enzyme activity or specific mRNA content. Increased catalytic activity is the direct consequence of an enhancement of enzyme amount. For such assays, hepatocytes are exposed for 24–72 h to non-cytotoxic concentrations of the test compound. Upon incubation, assessment of P450 activities (using specific substrates) or mRNA levels (by quantitative RT-PCR) is performed, and the results in induced cells are compared to those of untreated cells.

The present study was aimed to assess the correlation between mRNA expression using Taqman™ Low Density Array (TLDA) analysis and drug metablising enzymes in response to the reference inducers in both rat and human hepatocytes. There was a good correlation between the induction of CYP1A2, CYP2B6 and CYP3A4 enzyme activities and mRNA expression in human hepatocytes. In contrast, BROD activities and mRNA expression in rat hepatocytes correlated poorly. However, bupropion hydroxylation correlated well with CYP2B1 expression in rat hepatocytes. The further evaluations are requested to confirm that bupropion is a selective probe substrate for CYP2B1. This is currently under investigation. In conclusion, application of TLDA methodology to investigate the potential of compounds to induce enzymes in rat and human hepatocytes increases the throughout and information gained from one assay, without reducing the predictive capacity.

Use of mRNA expression to detect the induction of drug metabolising enzymes in rat and human hepatocytes

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Running title: Enzyme induction using TaqMan analysis in rat and human hepatocytes

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Abstract

It is important to investigate the induction of cytochrome P450 (CYP) enzymes by drugs. The most relevant end point is enzyme activity; however, this requires many cells and is low throughput. We have compared the CYP1A, CYP2B and CYP3A induction response to eight inducers in rat and human hepatocytes using enzyme activities (CYP1A2 (ethoxyresorufin), 2B (benzoxyresorufin for rat and buproprion for human) and CYP3A (testosterone)) and Taqman low density array (TDLA) analysis. There was a good correlation between the induction of CYP1A2, CYP2B6 and CYP3A4 enzyme activities and mRNA expression in human hepatocytes. In contrast, BROD activities and mRNA expression in rat hepatocytes correlated poorly. However, bupropion hydroxylation correlated well with Cyp2b1 expression in rat hepatocytes. TLDA analysis of a panel of mRNAs encoding for CYPs, phase 2 enzymes, nuclear receptors and transporters revealed that the main genes induced by the 8 compounds tested were the CYPs. AhR ligands also induced UGTs and GSTs in rat and human hepatocytes. The transporters, MDR1, MDR3 and OATPA were the only transporter genes significantly up-regulated in human hepatocytes. In rat hepatocytes bsep, Mdr2, Mrp2, Mrp3 and Oatp2 were up-regulated. In conclusion, application of TLDA methodology to investigate the potential of compounds to induce enzymes in rat and human hepatocytes increases the throughout and information gained from one assay, without reducing the predictive capacity.

Introduction

Induction of drug metabolising enzymes (DMEs) can cause alterations in the pharmacokinetics, efficacy and/or toxicity of a drug (either the inducer itself or a co-administered drug). Therefore, it is important to investigate the induction potential of compounds in development. In a survey based on in vitro induction methods carried out in the pharmaceutical industry, 17% of the researchers considered stopping the development of a compound if it was shown to cause enzyme induction in an in vitro assay (Hewitt et al., 2007). The FDA recommends the use of human hepatocytes to determine enzyme induction in vitro and, if the outcome is negative (i.e. no induction of CYP1A2 and CYP3A4 is measured), then drug interaction studies in the clinics are not necessary (Huang and Stifano, 2006). The use of the 2-fold threshold to define a 'positive' result for an enzyme inducer was dropped from the 2004 draft FDA paper (Huang, 2004) and replaced by the recommendation to compare the induction response to a test compound with that caused by the positive control (PC). This is likely to have been due to the large donor-to-donor variability in the induction response, resulting in difficulties in data interpretation. The more recent FDA guidelines recommends also incorporating the 'background' vehicle control activities into the calculation, since this may influence the induction response (Kostrubsky et al., 1998).

The most relevant end point is enzyme activity, since this ultimately affects the clearance of a metabolised drug; however, the methods for determining enzyme activity are time consuming and require large numbers of cells and are therefore low throughput. With the exception of CYP2E1, CYP induction occurs via activation of nuclear receptors (NRs) and gene transcription (Honkakoski and Negishi, 2000). Therefore, for initial screening, the use of mRNA expression profiling increases the throughput and provides information on the potential of a compound to induce DMEs. The main NRs involved in human DME regulation are: AhR, which regulates CYP1A1/2 (Safe and Krishnan, 1995) and a number of glutathione S-transferases (GSTs, Hayes and Pulford, 1995) and UDP-glucuronosyltransferases (UGTs, Munzel et al., 1998, Erichsen et al., 2008); CAR, which regulates CYP3A4, CYP2B6, CYP2C9 and UGTs (Sueyoshi and Negishi, 2001); and PXR, which regulates CYP3A and UGT1A1 (Faucette et al., 2004, Sugatani et al., 2005). The regulation of transporters is via the same NRs as CYPs; therefore, inducers that cause PXR activation can concomitantly induce CYPs and transporters (e.g. CYP3A4 and MDR1 in human hepatocytes by avasimibe (Sahi et al., 2000).

It is well known that induction responses in the rat differ from those in the human due to sequence differences in the ligand domain of the NR genes and CYP response elements (Lin, 2006). However, information on the induction response of rat hepatocytes to test compounds is still needed to interpret early rodent pharmacokinetic studies (Hewitt et al., 2007). The compounds chosen for these studies therefore included those which preferentially induce human or rat CYPs. For example, rifampin (RIF) induces human CYP3A4 (Maurel 1996; Lu & Li 2001) whereas RIF is a weak inducer of rat CYP3A1/2 but dexamethasone (DEX) and pregnenalone-16-carbonitrile (PCN) both potently induce this CYP (Maurel 1996; Lu & Li 2001). In total, the effect of eight compounds on the expression of a number of DMEs, NRs and transporter genes in rat and human hepatocytes were studied. The compounds were known inducers of human CYP1A1/2 (3-methylcholantrine (3-MC), β-naphthoflavone (BNF), omeprazole (OMP)); CYP2B6 (phenobarbitone (PB)); CYP3A4 (RIF) and CYP4A (fenofibrate) and rat CYP3A1 (DEX, PCN). Enzyme activities (CYP1A2 (ethoxyresorufin O-deethylation), 2B (benzoxyresorufin-O-dealkylation for rat and buproprion hydroxylation for human hepatocytes) and CYP3A

(testosterone 6β-hydroxylation)) were measured in hepatocyte microsomes after 72h of treatment and mRNA was analysed after 24h and 72h.

TaqMan™ Low Density Arrays (TLDAs) were used to monitor the effects of compounds on the expression of a number of phase 1 and 2 DMEs, NRs and transporters. Their quantification using RT-PCR has been carried out before, with mostly good correlations between expression levels and enzyme activities (Kostrubsky et al., 1998, Pérez et al., 2003, Burczynski et al., 2001). The use of low density microarrays allows for the simultaneous measurement of multiple genes using relatively little mRNA. Moreover, the quantitative fold changes of mRNA measured using this technique is comparable with that measured using RT-PCR (de Longueville et al., 2003). We have combined the low density array technology with the FDA recommended induction calculation in order to make a comparison between mRNA expression and CYP enzyme activities. By expressing the data as a percent of the PC, the maximal induction measured using both end points are always 100%. This is helpful because the fold induction of mRNA may be over an order of magnitude greater than that measured for enzyme activities.

Methods

Materials

All chemicals were obtained from Sigma-Aldrich (St. Quentin-Fallavier, France) and reagents for cell culture were from Invitrogen (Cergy Pontoise, France) unless stated otherwise. Cell culture plastics were purchased from Becton Dickinson (Grenoble, France).

Hepatocyte isolation and culture

Male Wistar rat hepatocytes and human hepatocytes were isolated as previously described by Richert et al. (2002) and Alexandre et al. (2002). Human liver samples were from patients undergoing liver resection for different pathologies (Table 1). All experimental procedures were performed in compliance with French law and regulations, after approval by the National Ethics Committee (France). Informed consent was obtained from all patients for the use of liver tissue for research purposes. Initial cell viabilities were determined using Trypan blue exclusion. Rat and human hepatocytes were seeded onto 60 mm^2 dishes, 12-well and 24-well plates in DMEM medium supplemented with 5% foetal calf serum, gentamycin (50mg/l), insulin (4mg/l) and DEX (10µM). Seeding densities were 3.5million cells in 3ml complete medium per 60mm^2 dish, 0.75million cells in 1ml complete medium per well in a 12-well plate and 0.3million cells in 0.5ml complete medium per well in a 24-well plate. Cells were allowed to attach by incubating under a CO_2/air (5%/95%) humidified atmosphere maintained at 37°C.

Treatment of hepatocyte cultures

After 48h of culture, the cultures medium was removed and replaced with fresh serum-free medium containing test compound. Test compounds were dissolved in medium to give final concentrations of: 5µM 3-MC, 10µM BNF), 20µM OMP, 1000µM PB, 10µM RIF, 50µM DEX, 10µM (PCN), 100µM FEN. Control cultures were treated with the solvent, DMSO (0.2% v/v final concentration). The medium containing test compound was replaced every 24h up to a maximum of 72h. At 24h and 72h, medium was discarded and hepatocyte monolayers were washed 3 times with ice cold PBS.

Hepatocytes treated for 24h (24-well plates) and 72h (12-well plates) were lysed by the addition of 125µL and 250µl Trizol/well, respectively. Samples were stored at -80°C until mRNA extraction and analysis. Microsomes were prepared from hepatocytes cultured for 72h in 60mm^2 dishes (3 dishes per sample). Homogenisation buffer (1ml per dish) was added before the hepatocytes were harvested and pooled into a single tube. Samples were kept on ice until they were placed in storage at -80°C until microsome preparation. After thawing, hepatocytes were sonicated and centrifuged at 9000g for 20min at 4°C. Supernatant fractions were collected and centrifuged at 100000g for 60min at 4°C. The final microsomal pellets were suspended in 80-120µL of 0.25M sucrose. All samples were stored at -80°C. The protein content of each sample was determined using the Pierce Protein Assay kit.

Microsomal Cytochrome-P450 dependent monooxygenase activity determination

Hepatocyte microsomal enzyme activity determinations at 72h were carried out by incubating hepatocyte microsomes with respective probe substrates for the CYPs under study: CYP 1A2: ethoxyresorufin-O-

dealkylation (Burke et al., 1985), CYP 2B1: benzoxyresorufin-O-dealkylation (Burke et al., 1985), CYP2B6: bupropion hydroxylation (Faucette et al., 2000), CYP 3A4/5: testosterone 6β-hydroxylation (McKillop et al., (1998).

Enzyme activity calculations

Enzyme activities are expressed as pmol/min/mg microsomal protein. Fold induction was calculated by dividing the activity in treated hepatocytes by the activity in cells treated with the vehicle control. A comparison of induction responses was made by comparing the enzyme activity or mRNA expression in treated cells with that in cells treated with the positive control (PC). The basal activities in vehicle control (VC) cultures were also incorporated into the calculation:

$$\% \text{ Positive control} = \frac{\text{CYP activity in treated cells} - \text{CYP activity in VC cells}}{\text{CYP activity in PC cells} - \text{CYP activity in VC cells}} \times 100$$

For mRNA expression, values were also normalised to the PC response:

$$\% \text{ Positive control} = \frac{\text{Fold induction of mRNA in treated cells}}{\text{Fold induction of mRNA in PC cells}} \times 100$$

mRNA analysis by TaqMan

At the end of the incubation periods (24 h and 72h), hepatocyte cultures were rinsed twice with ice cold PBS and 500µl of TRI Reagent™ was added to each well. Cells were harvested from three wells per treatment group and pooled. Total RNA was isolated according to the TRI Reagent™ standard protocol provided by the manufacturer. RNA pellets were dissolved in nuclease-free water and stored at -80°C until analysis.

cDNA synthesis and analysis

An aliquot of 5µg RNA was reverse transcribed to cDNA using random hexamer primers with the "Transcriptor first strand cDNA synthesis kit" (Roche, Mannheim, Germany) according to the protocol provided by the manufacturer. cDNA quality and concentration were determined using the Agilent Bioanalyzer 2100 applying the mRNA Pico Assay (Agilent Technologies, Waldbronn, Germany).

TaqMan™ analysis

TLDAs (Applied Biosystems, Darmstadt, Germany) were used to analyze rat and human mRNA. An aliquot of cDNA (50ng) was used per sample and loaded into a single sample loading port. The human and rat genes with the corresponding gene expression assays present on the respective TLDAs were the same as that reported by Richert et al. (2007). Thermal cycling and fluorescence detection was performed on Applied Biosystems ABI Prism 7900HT Sequence Detection System with ABI Prism 7900HT SDS Software 2.1. Analysis of gene expression values was performed using the efficiency-corrected comparative CT method. Gene expression ratios were calculated using the following formula:

$$R = \frac{(E_{target})^{\Delta CT_{target}(control-sample)}}{(E_{18S})^{\Delta CT_{18S}(control-sample)}}$$

mRNA analysis

Quality and concentration of total RNA were determined using the NanoDrop spectrophotometer (Kisker, Steinfurt, Germany) and the Agilent Bioanalyzer 2100 applying the Total RNA Nano Assay (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocols.

Results

Induction of CYPs in rat hepatocytes

The positive control inducers of rat CYP1A2, CYP2B1 and CYP3A1 were 3-MC, PB and DEX, respectively. The basal CYP1A2 activities, using EROD as the marker activity, in hepatocytes from all three rats were similar, with an average of 35.5 ± 7.2pmol/min/mg protein. Treatment with 3-MC caused an increase in CYP1A2 activity of 8.3 ± 3.4-fold over control activities. BNF also caused an increase in CYP1A2 activities (4.5 ± 1.3 fold increase over control), which was 46.4 ± 13.4% of the PC response (Figure 1A). In contrast, OMP only induced CYP1A2 activities by 1.6 ± 0.9-fold, which was 6.0 ± 8.5% of the PC response. When the induction response of rat hepatocytes to all 8 compounds were compared, there was a good correlation between CYP1A2 activities (which were measured at 72h only) and mRNA at both time points (24h and 72h). Table 2 shows the correlation between the two end points, enzyme activities and mRNA expression, for the CYP1A2, CYP2B1 and CYP3A4 induction responses in rat hepatocytes to the compounds tested. The induction responses (using either EROD activities or mRNA as end point) of rat hepatocytes to PB, RIF, DEX, PCN and FEN were all less than 10% of the PC (Figure 1A). The response to OMP was less than 20% of the PC.

The average basal CYP2B1 activity, using BROD as the marker activity, in hepatocytes from all three rats was 3.2 ± 3.6pmol/min/mg protein. There was a significant increase in BROD activities (14.1 ± 5.4-fold above control) in rat hepatocytes treated with PB, which was also reflected in an increase in Cyp2b1 expression at both time points (21.2 ± 21.4-fold and 10.7 ± 7.1-fold above control at 24h and 72h, respectively). However, CYP2B1 activity in rat hepatocytes treated with all test compounds did not correlate well with the corresponding mRNA expression in these cells (Table 2). A notable difference between BROD and mRNA expression was with respect to 3-MC and BNF treatment. Both compounds induced BROD activity (91 ± 11% of the PC by 3-MC and 27 ± 13% of the PC) but suppressed Cyp2b1 expression down to -57± 60% (3-MC) and 40 ± 38% (BNF) of the PC response.

A second substrate was selected to determine if it was more suitable for reflecting CYP2B1 activities in rat hepatocytes. Buproprion was chosen since it was the substrate of choice for CYP2B6 activities in human hepatocytes. Using this substrate, the average basal CYP2B1 activity in microsomes prepared from the same rat hepatocytes used in the previous study was 13.4 ± 12.1pmol/min/mg microsomal protein. Figure 1B shows the induction response of rat hepatocytes treated with the positive control, PB and the 7 remaining compounds. The main effect of using bupropion was to decrease the induction response of rat hepatocytes to 3-MC, BNF and PB. Using BROD to determine CYP2B1 activity, the fold induction response to 3-MC, BNF and PB was 13.2 ± 5.9, 5.0 ± 3.1 and 14.1 ± 5.4, respectively. In contrast, using bupropion hydroxylation to determine CYP2B1 activity, the fold induction in response to 3-MC, BNF and PB was 0.6 ± 0.1, 1.0 ± 1.2 and 7.4 ± 2.8, respectively. The correlation between bupropion activities and mRNA expression at both time points was markedly improved (Table 2).

The average basal CYP3A1 activity, using testosterone 6β-hydroxylation as the marker activity, in rat hepatocytes was 137.1 ± 63.7pmol/min/mg protein. There was a good correlation between CYP3A1 activities and mRNA expression at both time points (Table 2, Figure 1B). PCN and PB both induced CYP3A1 activity by

at least 40% of the PC (DEX) response. By contrast, the induction response of rat hepatocytes to RIF was less than 20% of the PC (7 ± 5% of the PC activity and 20 ± 7% and 6 ± 3% of the PC Cyp3a1 expression at 24h and 72h, respectively). The expression of Cyb3a1 in rat hepatocytes treated with PB was increased over control levels by 5.6 ± 2.0-fold at 24h and 13.8 ± 4.6-fold at 72h, however, when expressed as a percentage of the PC, the relative expression was decreased from 24h (54 ± 9% of PC) to 72h (24 ± 3% of PC). This effect was also observed in the Cyp2b1 expression in hepatocytes treated with other compounds.

Induction of CYPs in human hepatocytes

The CYP1A2 activities in hepatocytes from the three donors were similar and the average activity was 36.4 ± 3.8pmol/min/mg microsomal protein. There was a clear difference in the induction response between inducers and non-inducers, whereby EROD activities in hepatocytes treated with 3-MC, BNF and OMP were increased above control activities by 7.4 ± 0.9-fold, 7.5 ± 1.1-fold and 6.6 ±0.4-fold, respectively and EROD activities in hepatocytes treated with PB, RIF, DEX, PCN and FEN were unchanged. These responses were reflected in the mRNA expression (Figure 2A), whereby CYP1A2 mRNA levels in BNF and OMP treated human hepatocytes were at least 40% and 25% of the PC (3-MC), respectively and the expression of CYP1A2 in human hepatocytes treated with PB, RIF, DEX, PCN and FEN were unchanged. There was a good correlation between CYP1A2 activities and mRNA encoding for this CYP at both time points (Table 2).

The mean CYP2B6 activity of microsomes from human hepatocytes using buproprion as the substrate was 1.4 ± 0.5pmol/min/mg microsomal protein. The three most potent inducers of CYP2B6 activities were PB (the PC, 7.4 ± 3.9-fold above control activities), RIF (3.8 ± 1.4-fold above control activities) and OMP (3.3 ± 1.2-fold above control activities). With the exception of mRNA at 24h in OMP treated cells, RIF and OMP responses were greater than 40% of the PC response (Figure 2B). BNF caused a small increase in CYP2B6 activity (20 ± 25% of the PC response) and a transient marked increase the CYP2B6 mRNA at 24h (95 ± 58% of the PC), which decreased to the corresponding CYP activity after 72h (28 ± 16% of PC). There was also a good correlation between CYP2B6 activities and mRNA levels, which was better at 72h than at 24h (Figure 2B and Table 2).

CYP3A4 activities in human hepatocytes varied between donors and were 373pmol/min/mg microsomal protein, 143pmol/min/mg microsomal protein and 74pmol/min/mg microsomal protein in donor 1, 2 and 3, respectively. The fold induction of CYP3A4 activity in response to RIF also varied but was not related to the basal activity (3.4-fold in donor 1, 14.7-fold in donor 2 and 5.5-fold in donor 3). When the responses were expressed as a percentage of the PC (RIF), the variation in responses between donors was decreased (data not shown). There was a good correlation between CYP3A4 activities and mRNA expression at both time points (Figure 2C and Table 2). The induction responses, measured using enzyme activities and mRNA expression, of human hepatocytes to PB and DEX were above 70% of PC and 20% of PC, respectively. OMP induced a similar increase in CYP3A4 expression to that induced by DEX but, in contrast to DEX, OMP did not cause an overall increase in CYP3A4 activity. However, this was due to OMP causing a decrease in CYP3A4 activity in hepatocytes from donor 1 (45% of control activities) whereas it caused an increase in activity in hepatocytes from donor 2 (320% of control activities).

TaqMan™ Low Density Array analysis

TLDA analysis of a panel of mRNAs encoding for CYPs, phase 2 enzymes, NRs and transporters revealed that the main genes induced by the 8 compounds tested were the CYPs (Figure 3). A summary of the genes up-regulated by at least 2-fold over control is shown in Table 3. Of the CYPs, those coding for CYP1A1 and CYP1A2 were the most strongly induced (CYP1A1 mRNA was induced by 3-MC by up to 104-fold and 1744-fold in rat and human hepatocytes, respectively; and CYP1A2 mRNA was maximally induced by 3-MC in rat and human hepatocytes by 216-fold and 187-fold, respectively), compared to the gene encoding for CYP3A (58-fold by DEX in rat hepatocytes and 27-fold by PB and RIF in human hepatocytes) and the remaining genes (maximal induction was 37-fold in rat and 14-fold in human hepatocytes).

The effect of the compounds on expression of the genes encoding for CYP1A2, CYP2B and CYP3A in rat and human hepatocytes has been described above. Cyp2a1 was strongly increased (between 4- and 9-fold) in rat hepatocytes by 3-MC and BNF and the human homologue, CYP2A6, was induced by PB, RIF and BNF (maximal induction was 9-fold, 3.2-fold and 2.4-fold, respectively). In rat hepatocytes Cyp2c was induced by PB, DEX and PCN by 2.5-fold, 4.8-fold and 3.4-fold, respectively. Of the human CYP2C genes analysed, CYP2C19, CYP2C8 and CYP2C9 were induced by PB, RIF and in the case of CYP2C8, also by DEX. CYP2D6 expression in human hepatocytes was not affected by any of the compounds but rat Cyp2d9 was induced at 24h (but not 72h) in rat hepatocytes treated with 3-MC (3.9-fold), BNF (3.9-fold), OMP (6.0-fold), PB (4.1-fold), DEX (2.3-fold) and PCN (2.6-fold). Cyp2e1 was only induced in rat hepatocytes and only by FEN (2.9-fold induction). Cyp4a14 and Cyp4a22 in rat hepatocytes were both strongly induced by FEN (17.7-fold and 37.2-fold, respectively) but suppressed by up to 5-fold lower than control cells by the other compounds tested. CYP 4A11/22 expression in human hepatocytes was also induced only by FEN (4.4-fold), suppressed by 3-MC (12-fold lower), PB (2.8-fold lower), RIF (2.7-fold lower), and unaffected by BNF, OMP, DEX and PCN.

AhR activators (3-MC, BNF and OMP) induced Gstp2 mRNA in rat hepatocytes up to 12-fold higher than control levels, whereas, GSTa1 mRNA was induced in human hepatocytes by PB (2.5-fold) and RIF (2.1-fold). The gene for UGT1A6 was induced by 3-MC, BNF (19.9-fold, 15.4-fold induction, respectively) in rat but not human hepatocytes. UGT1A1 expression was induced by 3-MC, BNF and OMP by up to 2.5-fold in human hepatocytes. PB, DEX and OMP also induced Ugt1a6 in rat hepatocytes but the response was lower than for 3-MC and BNF (2-3-fold). Ugt1a7 was induced only by DEX in rat hepatocytes (2-fold) and UGT2B7 mRNA was induced in human hepatocytes only by 3-MC (2.6-fold induction).

The NR genes for AHR, PXR, FXR and GR were not up-regulated by any of the compounds tested in rat and human hepatocytes. CAR expression was up-regulated by FEN (8.5-fold), OMP (2.0-fold) and RIF (3.1-fold) in rat hepatocytes and by only 3-MC in human hepatocytes. The genes encoding PPARα and PPARγ in rat hepatocytes were up-regulated approximately 2-fold by DEX but not by any of the other compounds tested. PB, PCN and FEN caused an increase in PPARα at 24h but not 72h in human hepatocytes.

The expression of the efflux transporter, MDR1, was up-regulated only in human hepatocytes treated with PB and RIF (approximately 2-fold induction). MDR3 was also up-regulated in human hepatocytes treated with FEN

only (2.3-fold). In rat hepatocytes DEX was the only compound which induced Bsep (2.3-fold) and Mdr2 (2.7-fold). Mrp2 was up-regulated in rat hepatocytes by 2.3-fold and 4.1-fold by PCN and DEX, respectively. At 24h, Mrp3 in rat hepatocytes was up-regulated by 3-MC, BNF and OMP by 2.3-fold, 3.7-fold and 2.1-fold, respectively. In rat hepatocytes, Oatp2 but not Oatp1 or Oct1 was induced by PCN, DEX and PCN and to a lesser extent, RIF. In contrast, in human hepatocytes, OATPA was induced by PCN and OMP but only at 24h of treatment.

Discussion

The induction of DMEs can have serious consequences on the efficacy and toxicity of the inducer itself or a co-administered drug; therefore, it is important to determine the induction potential of new chemical entities early on in development. A comprehensive induction assay, whereby hepatocytes are incubated for three days with the test compound and then harvested to make microsomes, which are subsequently incubated with CYP selective substrates, is time consuming and uses a large amount of cells. With the increase in the use and acceptance of cryopreserved human hepatocytes for the determination of enzyme induction (Hewitt et al., 2007, Huang and Stifano, 2006), comes a need to miniaturise the induction protocol to minimise the use of hepatocytes. Also, as our knowledge of the regulation of CYPs and transporters increases, and the recognition that some drug-drug interactions occur due to induction of proteins other than CYPs, the need to determine the effects of compounds on a broad spectrum of genes has increased. For example, St John's wort and Avisimbe are both known to alter the pharmacokinetics of co-administered drugs by inducing both CYP3A4 and MDR1 (Dresser et al., 2003, Sahi et al., 2003). We have used TaqMan™ Low Density Arrays to monitor the induction (or suppression) of a number genes in hepatocytes to confirm the selectivity of the inducers, demonstrate species differences in induction responses and to evaluate the usefulness of the higher throughput screening for induction screening.

One of the challenges of measuring mRNA as an end point for induction responses is interpreting the significance of the fold-induction. The large inter-individual variation in the responses of CYP and transporter expression to inducers complicates the interpretation further (Nishimura et al., 2006). For enzyme activities, a threshold of 2-fold above control is generally considered to be a significant induction, although this is no longer recommend by the FDA (Huang and Stifano, 2006). However, does this also hold true for mRNA induction - and for all DMEs? In the current study, BNF increased CYP1A2 activity in rat hepatocytes by 4.5-fold and Cyp1a2 gene expression by 133-fold – both of which are quite clearly above the threshold considered to be 'positive' but the mRNA induction is nearly 30 times that of enzyme activity. By nominating a PC and expressing the induction as a percentage of the PC response, the comparisons using enzyme activities and gene expression are placed on the same scale. The FDA have recommended that compounds whose induction responses are greater than 40% of the PC should be considered to have positive induction effects. Using this comparison, the BNF induction in rat hepatocytes was 46 ± 13% and 77 ± 27% of the PC according to the induction of enzyme activities and mRNA, respectively. Although the responses to CYP1A2 inducers were variable, they were distinct from non-inducers of this CYP (Figure 1A and Figure 2A). Moreover, OMP, an inducer of human CYP1A2 (Diaz et al., 1990), does not induce CYP1A2 activity in rat hepatocytes (Lu and Li, 2001), and this species difference was reflected in the % of the PC (2-16% and 27-88% of the PC (mRNA and activities) in rat and human hepatocytes, respectively) but not in the fold-induction of mRNA (which were all above the 2-fold threshold: 3.1-3.6-fold and 7-62-fold (mRNA and activities), in rat and human hepatocytes, respectively). In contrast, the induction of CYP1A2 activity and mRNA in human hepatocytes by OMP was 88.1 ± 6.8% and 27-36% of the PC, respectively. The interpretation is more difficult when the fold induction values are closer to the 2-fold threshold. For example, in the case of FEN, CYP3A1 activity in rat hepatocytes was 1.7-fold higher than control activity but the Cyp3a1 expression was induced by 4.1-fold, however, when these responses were expressed as a percentage of the PC, the values were comparable (6 ± 4% and 7 ± 2% of the PC, respectively).

Our data support the findings of others that there is a good correlation between enzyme activities and mRNA expression in hepatocytes treated with inducers (Kostrubsky et al., 1998, Pérez et al., 2003, Burczynski et al., 2001, Richert et al., 2008). In contrast to other reports in which the expression of a specific CYP was measured with and without a selective inducer of that enzyme, we have determined CYP1A2, CYP2B and CYP3 activities and gene expression of hepatocytes treated with a number of AhR, CAR, PXR and PPAR activators to determine the selectivity of the induction response in each species. This resulted in data for potent, mild and non-inducers for each CYP. The correlations between CYP1A2, CYP2B6 and CYP3A activities and their corresponding mRNA levels were similar at 24h and 72h, suggesting that either time point would be appropriate for determining the induction response of these two CYPs. The poor correlation between BROD activities and mRNA expression in rat hepatocytes treated with different inducers was attributed to the non-selective properties of the substrate. BROD activity was used because it was shown to be selectively induced in hepatic microsomes from rats treated with PB and can be inhibited by monoclonal antibodies selective for CYP2B (Nerurkar et al., 1993). In addition to induction by PB, BROD was significantly induced by 3-MC and BNF, despite these compounds causing suppression of Cyp2b1 at both 24h and 72h. This suggests that BROD was not selective for CYP2B1 activity, since neither 3-MC nor BNF would be expected to induce CYP2B1. It is likely that BR is also a substrate for CYP1A1/2 and that the effect of 3-MC and BNF was to induce this enzyme rather than CYP2B1. This theory is supported by Kobayashi et al. (2002) who reported that BROD is also catalyzed by recombinant rat CYP1A2 in addition to CYP2B1. The same authors also reported that EROD was catalyzed both by rat CYP1A2 and by CYP2C6, however, induction of CYP2C by DEX and PCN (measured by TaqMan™) did not coincide with an increase in EROD activity and therefore, in our studies we can conclude that EROD was selective for CYP1A1/2. As an alternative to BROD, bupropion was chosen as an alternative substrate for CYP2B1 since it is known to be a selective substrate for human CYP2B6 (Faucette et al., 2000). The correlation between bupropion hydroxylation and Cyp2b1 expression at both time points was markedly improved, suggesting that bupropion is a selective substrate for rat CYP2B1. Further work is needed to support this hypothesis.

Known species differences in CYP induction were also observed in the present study. For example, OMP induced CYP1A2 mRNA and activities in human but not in rat hepatocytes, which has been demonstrated by others (Nishimura et al., 2007, Lu and Li, 2001). CYP3A4 was potently induced by RIF in human hepatocytes, whereas DEX was a moderate inducer (20-40% of the PC) and PCN did not induce CYP3A4 (less than 20% of the PC). In contrast, DEX and PCN significantly induced CYP3A1 in rat hepatocytes (60-100% of the PC) whereas the response of RIF in rat hepatocytes was less than 20% of the PC.

OMP is recommended by the FDA as a preferred human CYP1A2 inducer (Huang and Stifano, 2006), but it is not a specific inducer of this enzyme and has been reported to also induce CYP2B6 and CYP3A4 (Hewitt et al., 2007). Likewise, OMP induced CYP2B6 and CYP3A4 mRNA in the present study. The TLDAs allowed for an analysis of the different genes which were either up or down-regulated by each compound. In these comparisons, only the fold induction over control was used since the significance of individual gene induction is unknown. CYP1A1 is not constitutively expressed in the liver (Wrighton and Stevens, 1992) but it can be induced by OMP (Nishimura et al., 2007). Our studies also showed that mRNA encoding for rat and human CYP1A1 was potently

induced (up to 1700-fold) by AhR activators (OMP, BNF and 3-MC) but also by CAR and PXR activators (PB, RIF, DEX and, in rat hepatocytes, PCN) but to a much lower extent (up to 6-fold). In contrast, Nishimura et al. (2007) found that OMP but not DEX and RIF (at similar concentrations used here) increased CYP1A1 expression in human hepatocytes and in rat hepatocytes, DEX induced cyp1a1 which was not dose-dependent and was 3-fold higher than control expression. This difference is most likely to be due to human donor variation, rather than to method of detection because Taqman™ was used in both studies.

The mechanism of enzyme induction is a receptor-mediated process whereby a ligand binds to a NR causing it to translocate to the nucleus if it is located in the cytosol (AhR and CAR) and to bind to a transcription factor (AhR nuclear translocator (Arnt for AhR) and retinoid X receptor (RXR for both PXR and CAR)) response element and the resulting heterodomer binds to response elements leading to the transcription of the respective CYP isoform (Lin, 2006). PXR and CAR in turn are regulated by the glucocorticoid receptor (GR) which is up-regulated by sub-micromolar concentrations of glucocorticoids (Pascussi et al., 2000). Without GR activation by glucocorticoids such as DEX, rat hepatocytes do not respond to PB induction of CYP2B1 (Waxman et al., 1990, Burczynski et a., 2001), therefore, in our studies all hepatocyte culture media contained 10μM DEX. At this concentration, GR was likely to be maximally induced since the expression of GR was not increased further by any of the compounds. Small heterodimer protein (SHP), an atypical orphan NR, was induced by 3-MC in human hepatocytes. SHP expression has been shown to inhibit PXR transcription activity (Ourlin et al., 2000), which may explain some of the broad suppression of genes caused by this compound. CAR was up-regulated by 3-MC (2.5-fold) in human hepatocytes and relatively potently (8.5-fold) by FEN in rat hepatocytes. The increase in CAR expression by 3-MC did not lead to induction of CAR-mediated CYP2B6 induction, in fact this enzyme was suppressed by 3-MC. The induction by FEN is likely to be due to its PPAR activation properties since other PPAR activators are also known to induce CAR in rat hepatocytes as part of an adaptation to fasting (Wieneke et al., 2007). With the exception of CAR, SHP and PPAR, few of the NRs were up-regulated by the compounds tested and, in fact, there was an inverse correlation between PXR and CYP3A4 expression in human hepatocytes (inducers of CYP3A4 down-regulated PXR). This could be a negative feedback mechanism of the cells which respond may limit over transcription of PXR-regulated genes but this theory needs to be investigated further.

AhR activators (3-MC, BNF and OMP) which induced CYP1A2 also induced Gstp2 and Cyp1a6 expression in rat hepatocytes but the magnitude of Cyp1A2 and Gstp2 induction was not directly correlated. In contrast, there was a good correlation between the fold-induction of Cyp1a2 and Ugt1a6. Both phase 2 enzymes have been linked to BNF and CYP1A induction in rat hepatocytes by others (de Longueville et al., 2003, Maheo et al., 1997, Saarikoski et al., 1998). In contrast to rat hepatocytes, GSTp1 expression in human hepatocytes was not induced by AhR activators, however, GSTa1 mRNA was induced in human hepatocytes by PXR/CAR activators (OMP, PB and RIF). The magnitude of induction of phase 2 enzymes in human hepatocytes is reported to be lower than that of CYPs (Smith et al., 2005), and this was reflected our results. Maximum fold-inductions of CYP1A2, CYP2C, CYP2B6, CYP3A4, UGTs and GSTs in human hepatocytes were 1743-fold, 4.6-fold, 13.8-fold, 27.7-fold, 2.6-fold and 2.5-fold, respectively. Therefore, the 2.6-fold induction of UGT2B7 mRNA in human hepatocytes by 3-MC may be considered to be of significance.

Gene suppression is also an important consideration since this can also lead to an alteration in the metabolism of xenobiotics and endogenous compounds. For example, PB suppressed Cyp4a1 in rat hepatocytes and since this CYP is involved in the metabolism of fatty acids (Gibson et al., 1982), its suppression can be considered deleterious. With the exception of FEN (a PPAR ligand), all other compounds caused a down-regulation of Cyp4a1 in rat hepatocytes (up to 5-fold lower than control cells). Likewise, CYP4A1 expression was down-regulated by most of the compounds (with the exception of FEN which induced this CYP and BNF which had no effect) in human hepatocytes. Most notable was the suppression of genes caused by 3-MC in both rat and human hepatocytes. The suppression was mainly of the CYP genes (apart from CYP1A which was potently induced) in human hepatocytes but in rat hepatocytes, in addition of CYPs, NRs (with the exception of SHP, which was up-regulated), phase 2 enzymes and transporters were also down-regulated by this compound. The significance of this effect is not known but was not connected to the type of compound since the other two AhR activators did not cause the same degree of widespread gene suppression.

There was a link between the expression of the efflux transporter, MDR1, and CYP3A4 induction in human hepatocytes. This is line with the findings that they are both regulated by PXR (Gupta et al 2008). The induction of rat and human MDR1 and MRP2 transporters have been investigated by Nishimura at al. (2006b). They reported that RIF up-regulated MDR1, MRP1 and MRP2 in human hepatocytes by 1.5-fold, 1.3-fold and 1.8-fold, respectively. In our studies MDR1, MRP1 and MRP2 mRNA levels were 2.3-fold, 1.2-fold and 1.7-fold higher than control expression, respectively. Given the 2-fold threshold criteria, we would only consider MDR1 to be markedly induced. We also observed a good correlation ($R^2 = 0.96$) of the magnitude of CYP3A4 induction by PXR/CAR activators (OMP, PB, RIF, DEX and PCN) and MDR1 induction. The correlation between MRP2 and CYP3A4 mRNA was relatively poor (0.68). In contrast to human hepatocytes, none of the compounds influenced Mdr1a expression in rat hepatocytes; likewise others have reported that DEX does not alter this gene (Annaert et al., 2001, Luttringer et al., 2002, Salphati and Benet, 1998). In rat hepatocytes, Mrp2 was up-regulated in rat hepatocytes by PCN and DEX, which was in accordance with the findings of others (Turncliff et al., 2004, Luttringer et al., 2002, Nishimura et al., 2006). Ligands for FXR and CAR have been shown to alter the expression of Mrp2 in rat hepatocytes (as reviewed by Kast et al., 2002) which supports our findings that Mrp2 expression correlated well with Cyp3a1 expression in these cells treated with OMP, PB, RIF, DEX and PCN ($R^2 = 0.93$). In rat hepatocytes, DEX was the only compound which induced Bsep (2.3-fold) and is in agreement with others who found DEX to be a potent inducer of this biliary transporter, possibly via GR (Warskulat et al., 1999, Fardel et al., 2001). Of the up-take transporters, Oatp2 but not Oatp1 or Oct1 was induced in rat hepatocytes treated with PCN, DEX, PCN and RIF and the magnitude of induction of Oatp2 correlated relatively well with Cyp3a1 expression (R2 = 0.73), suggesting a link between this transporter and PXR. In contrast, in human hepatocytes, OATPA was induced by PCN and OMP but only at 24h of treatment. Some drug-metabolizing enzyme inducers, especially the CYP3A inducers, regulate oatp2 protein levels, however, there is no correlation between increases in levels of oatp2 protein and mRNA, suggesting that regulation of oatp2 occurs at both the transcriptional and post-translational levels (Guo et al., 2002).

In conclusion, the effects of selective CYP inducers on the gene expression of rat and human hepatocytes correlated well with measured CYP1A2, CYP2B and CYP3A activities. The use of TaqMan™ Low Density

Arrays allowed for an analysis of different genes and the effects of the inducers, which were in accordance with the known gene expression effects of inducers reported by others. The arrays increase the throughout and information gained from one assay, without reducing the predictive capacity. We therefore support the use of this technology to investigate the potential of compounds to induce DMEs, NRs and transporters in rat and human hepatocytes.

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List of figures

Table 1. Human donor demographics.

Donor	Age (years)	Sex	Disease	Viability (%)
1	79	Male	Metastasis (rectal)	84
2	67	Male	Colic adenocarcinoma	90
3	75	Male	Metastasis (hepatic)	87

Table 2. Comparison of CYP activities and mRNA expression in rat and human hepatocytes treated with prototypical inducers and non-inducers.

	R^2 value			
	CYP1A2 (EROD activity)	**CYP2B (BROD activity)**	**CYP2B (Bupropion hydroxylase activity)**	**CYP3A (Testosterone 6β-hydroxylase activity)**
Rat				
mRNA at 24h	0.94	0.27	0.91	0.91
mRNA at 72h	0.93	0.07	0.72	0.91
Human				
mRNA at 24h	0.83	n.d.	0.67	0.86
mRNA at 72h	0.82	n.d.	0.81	0.87

Correlations between CYP activities and mRNA expression at 24h and 72h were made using a linear regression function in Excel.

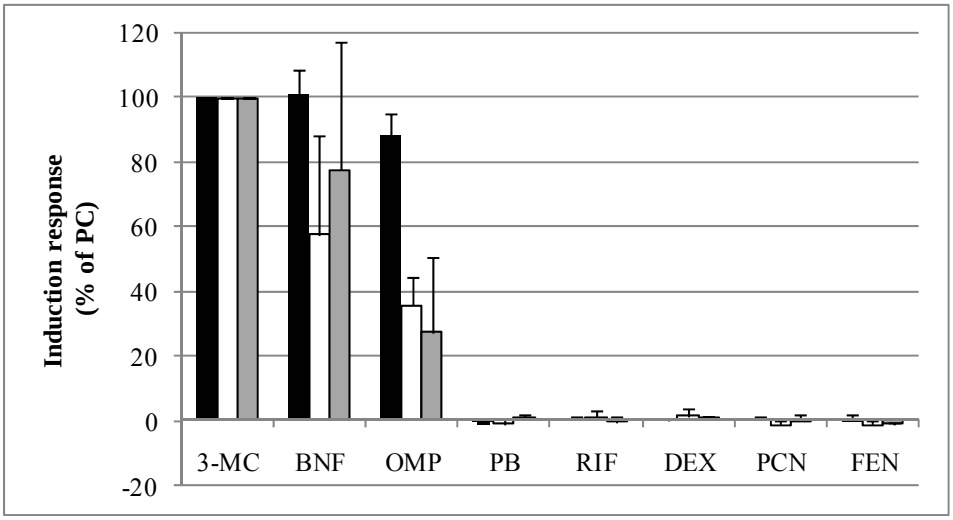
Table 3. Genes up-regulated in rat and human hepatocytes treated with different inducers.

Rat gene	Rat	Human gene	Human
Cyp1a1	**3-MC, BNF** >> OMP, PB, RIF, PCN, FEN	CYP1A1	**3-MC, BNF** >> OMP, PB, RIF
Cyp1a2	**3-MC, BNF** >> OMP	CYP1A2	3-MC, BNF, OMP
Cyp2a1	**3-MC, BNF** > RIF	CYP2A6	PB > RIF, BNF
Cyp2b1	PB, FEN OMP,	CYP2B6	PB, RIF > OMP, BNF, DEX
Cyp2c	DEX, PCN, PB	CYP2C19	PB, RIF
		CYP2C8	PB, RIF, DEX
		CYP2C9	PB, RIF
Cyp2d9	3-MC, BNF, OMP, PB, DEX, PCN		
Cyp2e1	FEN		
Cyp3a11	DEX, PCN, PB	CYP3A4	PB, RIF, DEX, OMP
Cyp3a3/1	**DEX, PCN** > PB > OMP	CYP3A5	PB, RIF, DEX, OMP
		CYP3A7	PB, RIF, DEX, OMP, FEN
Cyp4a14	FEN	CYP4A11/22	FEN
Cyp4a22	FEN		
		SHP	3-MC
CAR	**FEN** > RIF, OMP	CAR	3-MC
PPARα	DEX	PPARα	PB, PCN, FEN
PPARγ	DEX		
		GSTa1	PB, RIF
GSTp2	BNF, 3-MC > OMP		
UGT1A6	**3-MC, BNF**,OMP, PB, DEX		
UGT1A7	DEX	UGT1A1-10	BNF, 3-MC, OMP
		UGT2B7	3-MC
		MDR1	PB, RIF
		MDR3	FEN
BSEP	DEX		
MDR2	DEX		
MRP2	DEX, PCN		
MRP3	3-MC, BNF, OMP		
OATP2	DEX, PCN, PB > RIF	OATPA	PCN > OMP

The genes listed are those that were up-regulated by at least 2-fold over control levels at 24h or 72h. Compounds in bold represent potent inducers.

Figure 1.

(A)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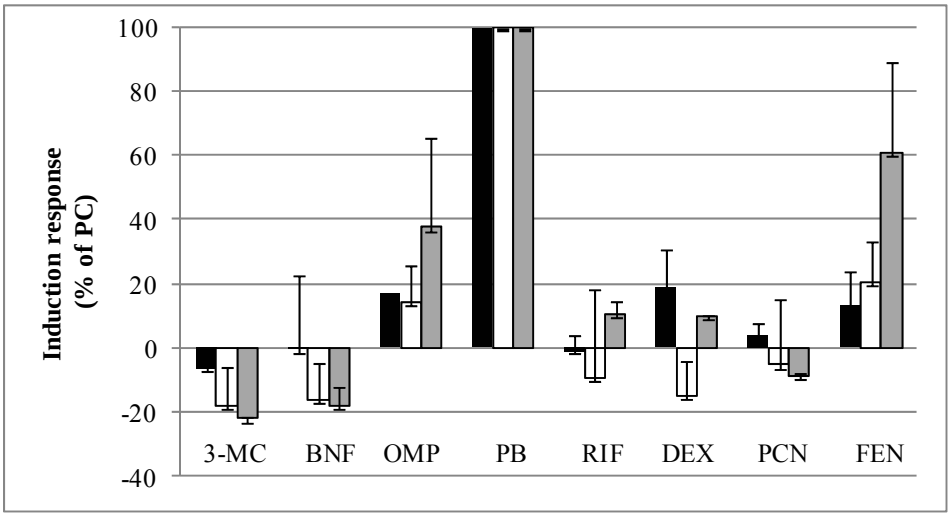
(B)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(C)

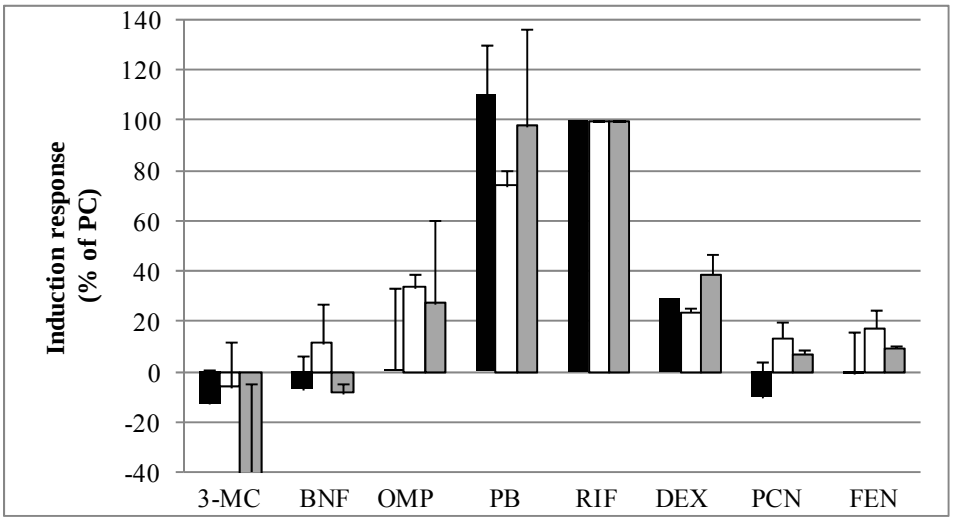
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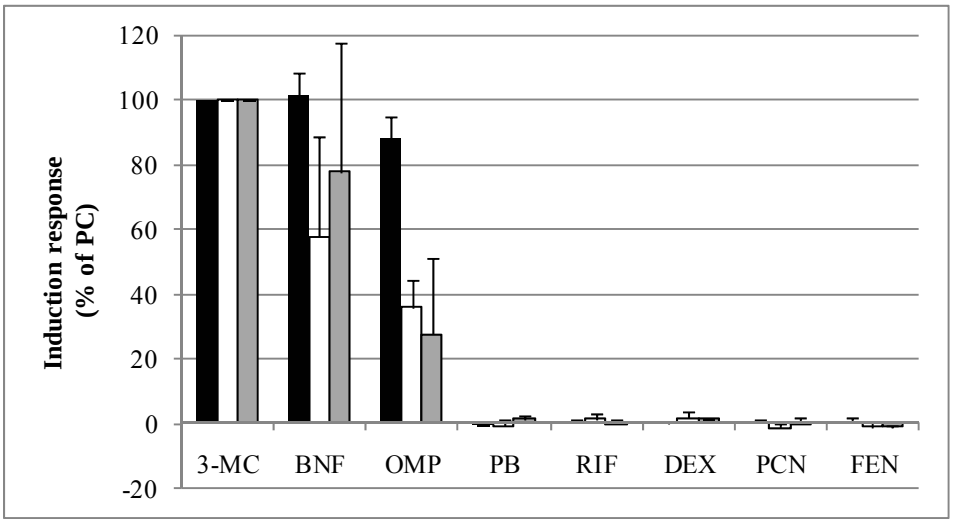
Figure 2.

(A)



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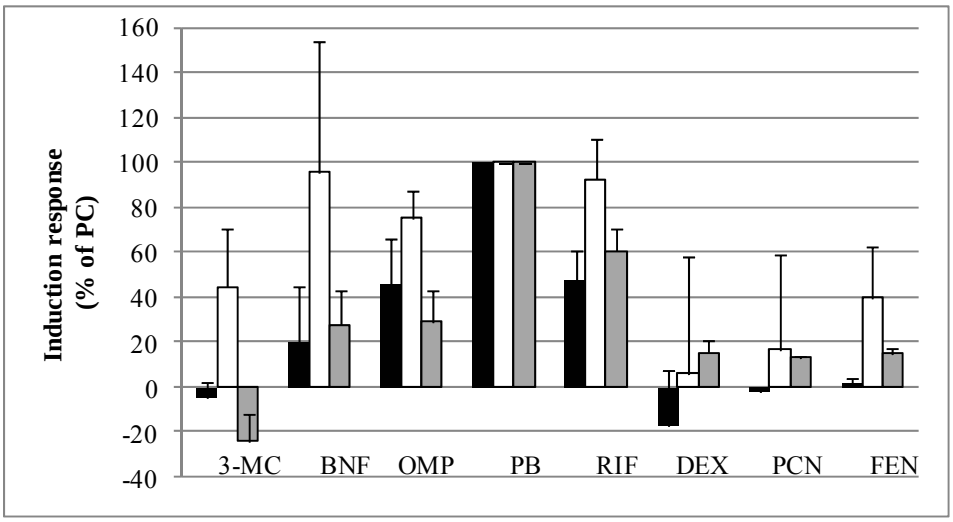

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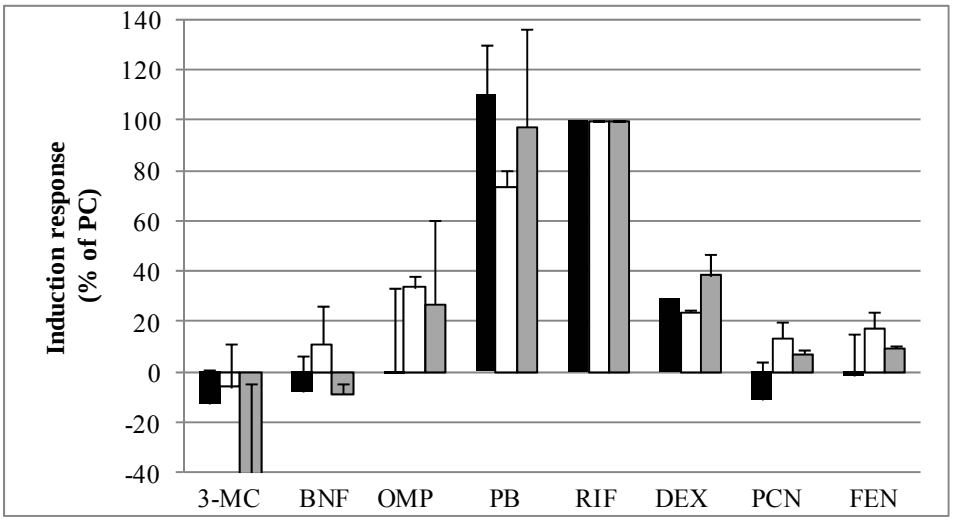
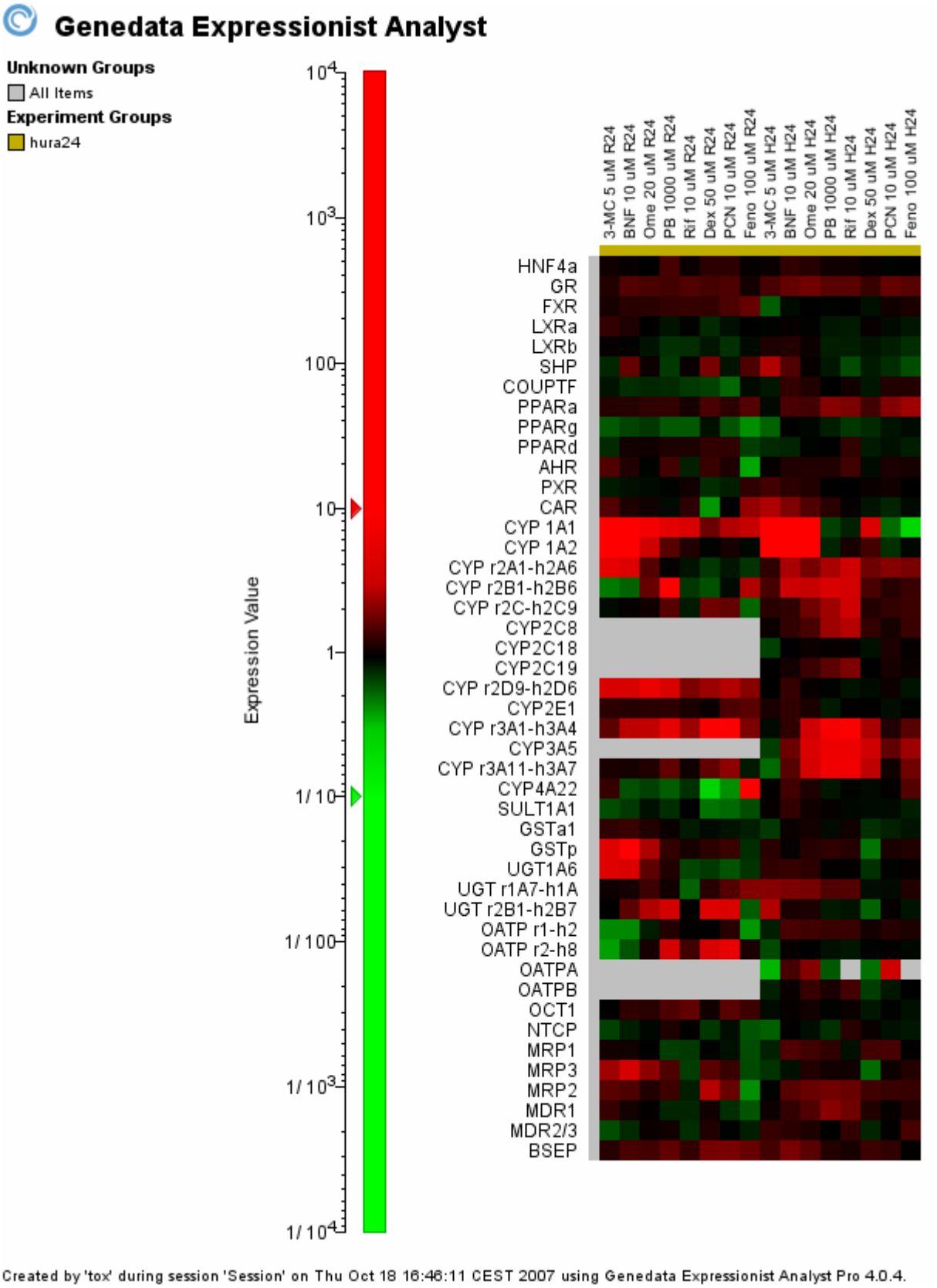


Figure 3.

(A)



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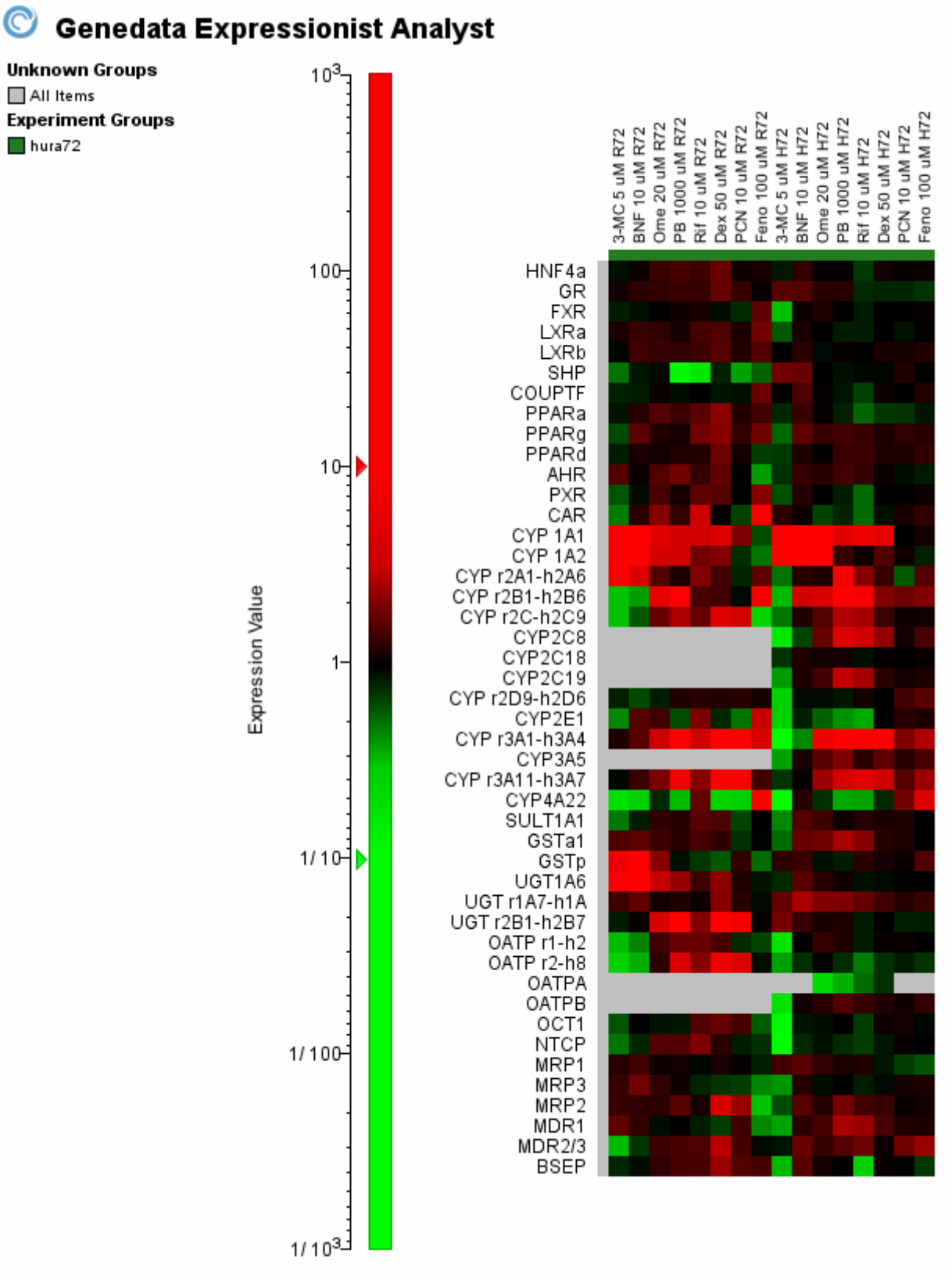
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BNF 10 uM R72
Ome 20 uM R72
PB 1000 uM R72
Rif 10 uM R72
Dex 50 uM R72
PCN 10 uM R72
Feno 100 uM R72
3-MC 5 uM H72
BNF 10 uM H72
Ome 20 uM H72
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Rif 10 uM H72
Dex 50 uM H72
PCN 10 uM H72
Feno 100 uM H72
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GR
FXR
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LXRb
SHP
COUPTF
PPARa
PPARg
PPARd
AHR
PXR
CAR
CYP 1A1
CYP 1A2
CYP r2A1-h2A6
CYP r2B1-h2B6
CYP r2C-h2C9
CYP2C8
CYP2C18
CYP2C19
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CYP2E1
CYP r3A1-h3A4
CYP3A5
CYP r3A11-h3A7
CYP4A22
SULT1A1
GSTa1
GSTp
UGT1A6
UGT r1A7-h1A
UGT r2B1-h2B7
OATP r1-h2
OATP r2-h8
OATPA
OATPB
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NTCP
MRP1
MRP3
MRP2
MDR1
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CHAPTER 6: PUBLICATION 3

Effects of *Andrographis paniculata* extract and Andrographolide on hepatic cytochrome P450 mRNA expression and mono-oxygenase activities after *in vivo* administration to rats and *in vitro* in rat and human hepatocyte cultures

Publication 3: Effects of *Andrographis paniculata* extract and Andrographolide on hepatic cytochrome P450 mRNA expression and monooxygenase activities after *in vivo* administration to rats and *in vitro* in rat and human hepatocyte cultures

Chemico-Biological Interaction 2008 (revision)

The assessment of the inhibitory and/or induction potential of CYPs, is widely accepted as ultimately leading to better predicting herb-drug interactions in humans. *In vivo* assessments are the best models to use since they give the definitive answer for a given species. However, as huge species differences have been widely reported, a stepwise approach, including in vitro human models in addition to in vivo and in vitro animal models, is required for a proper evaluation and prediction of herb-drug interactions. Studies with microsomes, while providing information on the potential of an herbal component 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 system. The use of more physiologically relevant *in vitro* models, such as primary culture of hepatocytes, are necessary if better predictions of herb-drug 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 culture of hepatocytes retain all co-factors 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. For this reason, the first aim of the present study was to compare the effects of APE and AND on various hepatic CYP activities after *in vivo* administration to Wistar rats and after *in vitro* treatment of rat hepatocytes; the second aim of the present study was to assess the effects of APE and AND *in vitro* in rat hepatocytes and human hepatocytes cultures on CYP activity, protein content and mRNA expression. After *in vivo* administration, APE at dose levels of 0.5 g/kg/d (i.e. 5 mg/kg/d AND equivalents) and at 2.5 g/kg/d (i.e. 25 mg/kg/d AND equivalents) and AND at dose levels of 5 and 25 mg/kg/d significantly decreased CYP2C11 activity. In primary cultures of rat hepatocytes, treatment with AND 50 µM and APE-containing 50 µM AND also resulted in significant decreases in CYP2C11 expression and activity, proving a good *in vivo – in vitro* correlation of the effects of APE and AND in rats. In addition, in human hepatocytes, treatment with APE and AND 50 µM resulted in a decrease in CYPEC9 and CYP3A expression and activity. In conclusion, the present study suggests that AND and APE could cause herb-drug interactions in humans through modulation of CYP2C9 and CYP3A4 expression and activities. Clinical studies in human healthy volunteers are needed to further confirm this potential herb-drug interaction with CYP2C9 and CYP3A4 metabolised drugs.

Effects of *Andrographis paniculata* extract and Andrographolide on hepatic cytochrome P450 mRNA expression and monooxygenase activities after *in vivo* administration to rats and *in vitro* in rat and human hepatocyte cultures

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Running title: *Andrographis paniculata* and cytochrome P450s

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Abstract

The expression of cytochrome P450 (CYP) is regulated by both endogenous factors and foreign compounds including drugs and natural compounds such as herbs. When herbs are co-administrated with a given drug in modern medicine it can lead to drug-herb interaction that can be clinically significant. The ability of *Andrographis paniculata* extract (APE) and Andrographolide (AND), the most medicinally active phytochemical in the extract, to modulate hepatic CYP expression was examined *in vivo* in rats and *in vitro* in rat and human hepatocyte cultures. After *in vivo* administration, APE at dose levels of 0.5 g/kg/d (i.e. 5 mg/kg/d AND equivalents) and at 2.5 g/kg/d (i.e. 25 mg/kg/d AND equivalents) and AND at dose levels of 5 and 25 mg/kg/d significantly decreased CYP2C11 activity. In primary cultures of rat and human hepatocytes, treatment with AND 50 μM and APE-containing 50μM AND also resulted in significant decreases in CYP2C expression and activity. In addition, in human hepatocytes, treatment with APE and AND 50μM resulted in a decrease in CYP3A expression and activity. In conclusion, the present study suggests that AND and APE could cause herb-drug interactions in humans through modulation of CYP2C9 and CYP3A4 expression and activities.

Key words: *Andrographis paniculata*; Andrographolide; Cytochrome P450; hepatocyte, inhibition

Abbreviations: EROD**,** ethoxyresorufin-O-deethylation; TolOH, Tolbutamide hydroxylation; 6βTesto-OH, Testosterone 6β-hydroxylation

1. Introduction

The use of herbs as alternative and/or complementary therapy in the western world is on the rise and gaining increasing popularity. As people often take different herbs in combination with prescribed modern medication, there is a potential for both pharmacokinetic and pharmacodynamic herb-drug interaction. In addition to doctor's recommendation, patients also perform self-medication with several different herbs and herbal preparations, believing it is safe, and often without informing their primary physician [1, 2]. Yet interactions have been discovered, for example between cyclosporin and St. John's wort (*Hypericum perforatum*) [3], ethynyl estradiol and St. John's wort [4], and *Ginkgo biloba* and warfarin [5].

Cytochrome P450s (CYPs) constitute a superfamily of heme-proteins that play an important role in the metabolism of xenobiotics, including drugs, endogenous compounds and herbal component as effective substrates [6]. The expression of individual CYPs is regulated by both endogenous factors and foreign compounds including drugs and natural compounds [7]. Herb-drug interactions can appear when herb and a given chemical drug are co-administered and the herbal preparation (one or more components) modulates the metabolism of the chemical drug by induction or inhibition of specific CYP enzymes. As examples, hyperforin, a component of St. John's wort (*Hypericum perforatum*) induces CYP3A4 and CYP2C9 [8], *Ginkgo biloba* strongly inhibits CYP2C9, and to a lesser extent, CYP1A2, CYP2E1, and CYP3A4 [5].

Andrographis paniculata is one of the most important medicinal plants used in China, Thailand and Ayurvedic medicine to treat gastric disorder, colds, influenza and other infectious diseases. An extract of *A. paniculata* (APE), standardized for its content of Andrographolide (AND) and Deoxyandrographolide, called 'Kan Jang', has been used extensively in Scandinavia for the last 20 years for the treatment of the common cold [9]. It has been shown in several

animal studies that extracts of *A. paniculata* have anti-inflammatory [10], anti-allergic, anticancer [11], immuno-stimulatory [12] and antiviral activities [13].

We recently reported that a crude extract of APE inhibited CYP1A2-dependent ethoxyresorufin-O-deethylation and CYP2C11-dependent tolbutamide hydroxylation in rat liver microsomes and inhibited CYP 1A2-dependent ethoxyresorufin-O-deethylation, CYP2C9-dependent tolbutamide hydroxylation and CYP3A4-dependent testosterone hydroxylation in human liver microsomes [14]. Our results of inhibition of CYP2C11 in rat liver microsomes are in accordance with the inhibitory effects on CYP2C/2E1-dependent aniline hydroxylase activity observed in albino rat microsomes and after a single administration of APE or AND *in vivo* to albino rats reported earlier [15]. However these authors found that after a long term treatment, the compounds induced hepatic microsomal CYP2C/2E1-dependent aniline hydroxylase, CYP2B-dependent N-demethylation of N,N-dimethyl aniline, and CYP2E1-dependent O-demethylation of *p*-nitroanisole in this species. APE has been recently reported to increase CYP1A1-dependent ethoxyresorufin O-dealkylase and CYP2B10-pentoxyresorufin O-dealkylase activities *in vivo* in mice [16] and one report showed that AND significantly induced the expression of CYP1A1 and CYP1A2 mRNAs in a concentration-dependent manner in primary cultures of mouse hepatocytes [17]. Clearly, more studies are needed in order to assess the potential impact of *Andrographis paniculata* on selective hepatic P450 enzyme activities and to give more insight into the guideline of rational administration and precaution to be taken for using this herbal medicine.

The assessment of the inhibitory and/or induction potential of CYPs, particularly when performed in models from various species, including human, is widely accepted as ultimately leading to better models for screening and predicting herb-drug interactions in humans [18]. For this reason, the first aim of the present study was to further assess the effects of APE and AND on various hepatic CYP activities after *in vivo* administration to Wistar rats; the second aim of

the present study was to assess the effects of APE and AND *in vitro* in rat hepatocyte and human hepatocyte cultures on CYP activity, protein content and mRNA expression.

2. Materials and methods

2.1 Chemicals

William's E culture medium, Dulbecco's Modified Eagle Medium (DMEM), dexamethasone, insulin, penicillin G/Streptomycin and fetal calf serum (FCS) were obtained from Invitrogen corporation (France). Rifampicin, dexamethasone, β-naphthoflavone, AND, testosterone, 6-β hydroxytestosterone, 11-β hydroxytestosterone, tolbutamide, 4-hydroxytolbutamide, bovine serum albumin (BSA), *p*-nitrophenol, 4-nitrocatechol, ethoxyresorufin, resorufin, reduced nicotinamide-adenine dinucleotide phosphate (NADPH), collagenase type IV were purchased from Sigma-Aldrich (France). Antibodies used to detect rat CYP1A2, 2C11, 2E1 and 3A1 and human CYP1A2, 2C9, 2E1 and 3A4 were purchased from BD Biosciences (Belgium). All other laboratory chemicals were used as the highest purity and from commercial suppliers.

2.2 Preparation of *Andrographis paniculata* extract (APE)

The extraction was carried out by mixing *Andrographis paniculata* powder with 1:3 w/v in 60% ethanol v/v using a Soxhlet apparatus for 12 h. The extract was filtered and the solvent from the filtrate was removed using a rotary evaporator under reduced pressure and low temperature [14]. The extract was stored at 4^{o}C until it cooled down, filtered through a 0.45 μm nylon membrane prior to quantification of the AND content by using HPLC-UV [19], found to contain 1.6% AND and diluted with water to reach 1.0% as a stock solution. The extract was suspended in distilled water using Tween 80 (1%) for p.o. administration at the dose of 0.5 g/kg/day and 2.5g/kg/day (corresponding respectively to AND 5 mg/kg/day and 25 mg/kg/day) and AND was suspended in distilled water using Tween 80 (1%), and administered p.o. at the dose of 5 and 25 mg/kg/day. All drug solutions were prepared extemporaneously.

2.3 *In vivo* study with Wistar rats

In this study 30 male albino rats of Wistar strain (3 weeks old) weighing 120-150 g were used. They were housed (3 rats per standard cages) for 7 days prior to the start of the study and maintained under standard laboratory conditions (temperature 24–28°C, relative humidity 60–70%, 12 h light-dark cycle) with free access to solid pellet diet and water *ad libitum* throughout the study. No animal was replaced following the beginning of the study. The study design was approved by the ethical committee of the Department for animal care and use. Rats were treated p.o. with APE (0.5 and 2.5 g/kg/day, corresponding respectively to 5 and 25 mg/kg/day AND, respectively) or AND (5 and 25 mg/kg/day) for 28 consecutive days. Rats from the control group were treated with the respective vehicle by the same route under similar condition. Rats were sacrificed 6 hr after the last treatment and the livers was immediately removed, weighed and washed in cold homogenization buffer (50 mM Tris-HCl, 150 mM KCl, 2 mM EDTA, pH 7.4). The liver was then homogenized in 4 ml buffer per g of liver. The homogenates were submitted to several differential centrifugations, as previously described [20]. Microsomal samples were finally aliquoted and frozen at -80^{o}C until analysis. Protein content was determined using the bicinchoninic acid protein determination kit (Sigma) and BSA was used as a standard.

2.4 *In vitro* studies with Wistar rat and human hepatocytes

2.4.1 Rat hepatocyte isolation and culture The liver of male albino rats of Wistar strain (8 weeks of age) was perfused with two-step collagenase digestion method as previously described [20]. Freshly isolated rat hepatocytes were seeded onto different culture supports (3.5×10^6 cells onto 60-mm collagen-coated culture dishes, 1.5×10^6 cells onto 6-well collagen-coated plates) in Williams' E culture medium supplemented with 10% FCS, 4 μg/mL insulin, 0.10 μM dexamethasone and 100 U/mL Penicillin G/100 μg/mL Streptomycin allowed to attach for 4 h at 37°C in humidified atmosphere of 95% air/5% CO_2. The medium was then changed to a

serum-free Williams' E culture medium containing 4 μg/mL insulin, 10 nM dexamethasone and 100 U/mL Penicillin G/100 μg/mL Streptomycin.

2.4.2 Human hepatocyte isolation and culture. Hepatocytes were isolated from human liver tissue obtained as surgical waste by a two-step collagenase digestion method [21]. Human livers from three French samples (Table 1) were used with permission of Local Research Ethic Committee. Freshly isolated human hepatocytes were seeded onto different culture supports (3.5×10^6 cells onto 60-mm collagen-coated culture dishes, 1.5×10^6 cells onto 6-well collagen-coated plates) in DMEM culture medium containing 5% FCS, 4 μg/mL insulin, 0.10 μM dexamethasone and 100 U/mL Penicillin G/100 μg/mL Streptomycin and allowed to attach overnight at 37°C in humidified atmosphere of 95% air/5% CO_2. The medium was then changed to a serum-free DMEM medium containing 4 μg/mL insulin, 10 nM dexamethasone and 100 U/mL Penicillin G/100 μg/mL Streptomycin.

2.4.3 Cytotoxicity Assay Primary cultures of rat hepatocytes (60 000 cells/well) were plated in 96-multiwell culture plates in Williams' E culture medium supplemented with 10% FCS, 4 μg/mL insulin, 0.10 μM dexamethasone and 100 U/mL Penicillin G/100 μg/mL Streptomycin. Four hours after plating, the medium was discarded and fresh Williams' E culture medium containing 0-100 μM of AND or APE at concentrations containing 0-100 μM AND was added to the cultures and was renewed everyday. After 3 days of treatment, cellular viability was determined by measuring the reduction of MTT to a formazan product [22]. APE-containing AND and pure AND were proven non cytotoxic up to 50 μM; at 75 and 100 μM they both exhibited a decrease of approximately 20% and 50% of mitochondrial activity, respectively, as assessed by MTT reduction (data not shown).

2.4.4 Hepatocyte treatment Hepatocytes were treated either with an APE extract containing 50 μM AND, with AND (50 μM) or with positive controls such as β-naphthoflavone (50 μM), rifampicin (10 μM) and dexamethasone (50 μM). The final concentration of the solvent

(DMSO) in the medium was 0.1%. Treatment medium was renewed everyday for up to 72 h of culture.

2.5 Microsomal Cytochrome P-450 monooxygenase activities

After 72 h of treatment, microsomes were prepared from dishes-plated hepatocytes by differential centrifugations, as previously described [20], suspended in 0.25 M sucrose at a protein concentration of 2-5 mg/ml, and stored at -80°C until analysis.

2.5.1 CYP1A2- dependent ethoxyresorufin-O-deethylase assay Microsomal ethoxyresorufin-O-deethylation (EROD) was determined according to Burke et al. [23]. Briefly, hepatic or cell microsomes (0.04 mg protein) were incubated for 2 min at 37^{o}C with 7-ethoxyresorufin (6.5 μM) as substrate in a final volume of 100 μL. The reaction was initiated by adding NADPH (2 mM) and was terminated by addition of $ZnSO_4$ (87 mM) and $Ba(OH)_2$ (79 mM). Following centrifugation (800 g; 5 min) to remove precipitated protein the fluorescent metabolite resorufin was measured (excitation 530 nm and emission 580 nm). Results were expressed as pmol resorufin formed/min/mg microsomal proteins.

2.5.2 CYP2C-dependent Tolbutamide hydroxylase assay Tolbutamide hydroxylation (TolOH) was measured according to Relling et al. [24]. Briefly, hepatic or cell microsomes (0.04 mg protein) were incubated with tolbutamide (0.25 mM) and NADPH (2 mM) for 45 min at 37^{o}C in a final volume of 200 μL. The reaction was stopped by adding perchloric acid. After centrifugation, the supernatant was analyzed by HPLC with UV spectrophotometric detection at 230 nm. Quantification was performed by using the standard curve of 4-hydroxytolbutamide. Results were expressed as pmol hydroxytolbutamide formed/min/mg microsomal proteins.

2.5.3 CYP2E1-dependent *p*-nitrophenol hydroxylase assay Paranitrophenol hydroxylation (PNP-OH) was assessed according to Allis and Robinson [25] by evaluating the formation of 4-nitrocatechol. After 30 min of incubation of microsomes (0.1 mg protein) with *p*-nitrophenol

(0.5 mM) and NADPH (1 mM) at 37°C in a final volume of 500 µL, the reaction was stopped with 50% trichloroacetic acid. The reaction mixture was centrifuged and NaOH (10 M, 20 µl) was added to the supernatant before measuring the absorbance at 530 nm. Results were expressed as pmol 4-nitrocatechol formed/min/mg microsomal proteins.

2.5.4 CYP3A-dependent Testosterone 6β-hydroxylase assay Testosterone 6β-hydroxylation (6βTesto-OH) was measured according to Pearce et al. [26], by incubating hepatic or cell microsomes (0.1 mg protein) with testosterone (0.25 mM) and NADPH (2 mM) for 8 min at 37°C in a final volume of 250 µL. The reaction was stopped by adding ice-cold acetonitrile and storing samples on ice. After centrifugation, the supernatant was analyzed by HPLC with UV spectrophotometric detection at 230 nm. Quantification was performed by internal standardization with 11β-hydroxytestosterone. Results were expressed as pmol 6β-hydroxytestosterone formed/min/mg microsomal proteins.

2.6 Microsomal CYP immunoreactive protein measurements

Microsomal liver or cell proteins (10 µg per well) were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes, according to the manufacturer's instructions (Invitrogen, France). CYP1A2 and CYP2E1 were detected with a goat anti-human and anti-rat (dilution 1:1000) antibodies. CYP2C11 and 3A1 were detected with a goat and a rabbit anti-rat antibody respectively (dilution 1:1000), CYP2C9 with a rabbit anti-human antibody (dilution 1:333) and CYP3A4 with a goat anti-human antibody (dilution 1:1000). The immunoreactive bands were revealed using a colorimetric reagent (Invitrogen, France).

2.7 CYP mRNA measurements

After 72 h of treatment, total RNA were extracted from 6-well-plated hepatocytes by using 500 µl of Trizol reagent according to the manufacturer's instruction (Invitrogen, France). RNA was quantified by spectrophotometry. cDNA were synthesized from 1 µg of total RNA using the iScript kit from Biorad (France) at 42°C for 30 min. Five microliters of diluted RT

reaction (1:10) was used for real-time PCR amplification by using SYBR Green kit from Biorad (France). The following program was used: a denaturation step at 95°C for 3 min, and 40 cycles of PCR (denaturation, 95°C, 10 sec; annealing, 58°C, 1 min) then one cycle at 55°C for 1 min. In all cases, the quality of the PCR product was assessed by monitoring of a fusion step at the end of the run. Rat and human sense and reverse primers were as follows: 5'-GACCAGGAACTATGGGGTGATCC-3' and 5'-CTCCAG TCGGCCAATGGTCT-3' for rat CYP1A2, 5'-AATCATTGGAAACACCCTTCAGA-3' and 5'-AAGGGCTTCATGCCCAAATAC-3' for rat CYP2C11, 5'-GGGACTACCTCCATTTGTTTCTGG-3' and 5'-CCCCTGGGGATTTTTAAAGAAGTG-3' for rat CYP3A1, 5'-TATCGGCAATGAGCGGTTCC-3' and 5'-GCCTGGGTACATGGTGGTG-3' for rat actin, 5'-GGGCACTTCGACCCTTACAA-3' and 5'-GCACATGGCACCAATGACG-3' for human CYP1A2, 5'-CCTATCATTGATTACTTCCCG-3' and 5'-AACTGCAGTGTTTTCCAAGC-3' for human CYP2C9, 5'-CACAAACCGGAGGCCTTTTG-3' and 5'-ATCCATGCTGTAGGCCCCAA-3'for human CYP3A4, 5'-GGGCACGAAGGCTCATCATT-3' and 5'-AGTCGGTTGGAGCGAGCATC-3' for human actin.

2.8 Statistics

The significance of the difference between the groups was assessed by one-way analysis of variance and the student Newman-Keuls multiple range test (SigmaStat^{TM}, SPSS Science, Chicaco, IL, USA). The level of significance was set at $p < 0.05$.

3. Results

3.1 Effect of the treatment of APE and AND *in vivo* in Wistar rats

Adult male Wistar rats were treated for 28 days with APE at dose levels of 0.5 g/kg/d (i.e. 5 mg/kg/d AND equivalents) and at 2.5 g/kg/d (i.e. 25 mg/kg/d AND equivalents) and AND at dose levels of 5 and 25 mg/kg/d by gavage. Body weight gain was not significantly affected by the treatment (data not shown). Table 2 shows that at necropsy body weight was not affected by APE nor AND treatment. The liver weights and the ratios of liver to body weight of all APE and AND treated rats tend to be decreased as compared with control values (Table 2) and this decrease was statistically significant in rats treated with AND 25 mg/kg/day, as compared with controls.

Microsomal CYP-dependent activities were measured in hepatic microsomes prepared from individual control, APE and AND treated rats (Table 3). AND and APE at the two dose levels tested, caused no significant changes in CYP1A2-dependent EROD, CYP2E1-dependent PNP-OH and CYP3A1/2- dependent 6β Testo-OH activities. In contrast, APE and AND at the two doses used in our study significantly inhibited Tol-OH activity, AND having a more pronounced effect than APE, but without statistically significant difference. Western blot analyses of CYP2C11 on microsomes of control, AND and APE treated rats (Fig. 1) revealed a visible decrease in microsomal CYP2C11 isoform in livers from rat treated with 2.5 g/kg/day APE. No other clear effect was observed in this immunoblot.

3.2 Effect of the treatment of APE and AND *in vitro* in rat and human hepatocyte cultures

We examined the effects of AND and APE on CYP1A2, CYP2C, CYP3A and CYP2E expression and activities in rat and human hepatocyte cultures. AND 50 µM and APE-containing 50 µM AND were tested, since this was the highest non-cytotoxic concentration (see Materials and Methods, “Cytotoxicity Assay”).

The effects of AND and APE on enzyme activities were assessed in 5 different rat hepatocyte cultures (Fig.2) and in 3 different human hepatocyte cultures (Fig. 3). In order both to assess the selectivity and the magnitude of response of AND and APE, reference inducers of CYPs were also tested. As expected, the treatment for three consecutive days with β-naphthoflavone (50 μM) caused a strong increase in CYP1A-dependent activity both in rat and human hepatocyte microsomes, with a mean 71-fold and a mean 9-fold increase, respectively. Also as expected, dexamethasone (50 μM) increased CYP3A activity by a mean of 6-fold and CYP2C activity by a mean of 1.4 fold in rat hepatocytes and rifampicin (10 μM) caused a mean 3-fold increase in CYP3A activity and a mean 1.2-fold in CYP2C activity in human hepatocytes.

Exposure of cultured rat and human hepatocytes to AND (50 μM) and to APE (containing 50 μM AND) for 3 days resulted in decreases of activity levels of all CYP450-dependent isoenzymes examined. In rat hepatocytes (Fig. 2), the effects of AND and APE are qualitatively and quantitatively similar. The enzymatic activities associated with CYP1A2 were significantly decreased up to 60% for AND and up to 55 % for APE treated cells. Tol-OH, a marker for CYP2C, was also significantly decreased about 60% by both AND and APE. Similarly, CYP3A-dependent 6βTesto-OH was significantly decreased, about 50% with both AND and APE. The enzymatic activities associated with CYP2E1 were not significantly decreased (30%) following exposure to APE and AND. In human hepatocytes (Fig. 3), the effects of APE was more important than that obtained with AND. The enzymatic activities associated with CYP1A2 and CYP2E1 were only slightly decreased (for up to 30% and 20% following APE treatment, for CYP1A2 and 2E1, respectively). In contrast, CYP2C9- and CYP3A4-dependent monooxygenase activities were significantly decreased with APE (about 40% and 60%, respectively). AND also caused a decrease in CYP2C9- and CYP3A4-dependent

monooxygenase activities (about 30%), however the latter effect was not statistically significant.

The effects of APE and AND treatment on the expression on CYP apoprotein was also assessed. Western blots depicted in Fig. 4A show a very subtle decrease in immunoreactive microsomal CYP1A2, 2C11 and 3A1 in rat hepatocytes treated with APE (containing 50 μM AND) when compared with control cells. In AND- and APE- treated human hepatocytes (Fig.4B), no visible changes in CYPs apoprotein could be revealed.

To determine the effects of AND and APE treatment on CYP1A2, CYP2C and CYP3A mRNA expression, real-time PCR analysis was performed (Fig.5). The treatment with reference compound β-naphthoflavone (50 μM) caused a strong increase in CYP1A2 mRNA expression both in rat and human hepatocytes, with a mean 253-fold and a mean 25-fold increase, respectively. Dexamethasone (50 μM) and rifampicin (10 μM) increased CYP3A mRNA expression by a mean of 167-fold and 14-fold in rat and human hepatocytes, respectively. After APE treatment of rat hepatocyte cultures (Fig. 5A), very small, not significant changes (<2-fold change) in CYP mRNA expression occurred, except an increase of CYP1A2 mRNA (4.1-fold change). AND treatment decreased CYP2C11 and CYP3A1 mRNA expression with a mean of 4.7 and 5.8-fold decrease, respectively, while it increased CYP1A2 mRNA (3.2-fold change). In treated human hepatocytes (Fig. 5B), AND and APE resulted in a significant decrease (>2-fold) in mRNA expression of CYP1A2, CYP2C9 and CYP3A4 mRNAs.

4. Discussion

Drug-drug interaction via cytochrome P450 system is widely studied since scientists agree that such interactions can often be clinically significant, but we must be aware that other foreign compounds such as food or herbs may also produce chemical interaction [27]. In the present study, the ability of APE and AND to modulate CYP450 expression at the mRNA, apoprotein and activity levels was studied *in vivo* in rat livers and *in vitro* in rat and human hepatocyte cultures. We chose to evaluate not only the effects of AND, the most medicinally active phytochemical compound in the extract, but also the effects of the whole herb as the extraction (APE) for two reasons: i) the popular behavior in countries where an active ethnomedicine exists, is usually to take the whole herb and ii) there might be other compounds present along with AND in the herb able to modulate CYP expression. Such information is important for the guideline of rational administration precautions to be taken for using this herbal medicine.

The doses of AND used for *in vivo* administration to rats were selected on the basis of a previously published report, where 5 mg AND/kg/day for 30 consecutive days was given *p.o*. to adult male albino rats [15]. Moreover, if we consider the daily recommended dose for humans that is 1 mg AND/kg/day [28], we can translate an equivalent daily dose of 6.25 mg AND /kg/day for rats, considering the pharmacological activity and toxicity difference in these two species [29]. Consequently, the AND doses we used in the present study (*i.e*. 5 and 25 mg AND/kg/day), either pure or present in APE, could be considered respectively as rat-equivalent daily recommended AND and approximately 5-fold higher AND supplementations. For *in vitro* experiments, cultures hepatocytes were treated with 50 µM AND (pure or contained in APE), that corresponds to the highest non-toxic concentration as proven by MTT assay and to a roughly equivalent concentration to those achieved in individuals ingesting *A. paniculata*. It is noteworthy that AND concentrations in blood of individuals after ingestion of *A. paniculata* at

daily recommended dose were estimated to reach approximately 5 µM [28], making it most probable that liver concentrations could reach up to 50 µM.

Since marked species differences in the expression and regulation of CYPs have been reported [30], the results obtained with treated rats could not be directly extrapolated to humans. In the present study we thus chose to compare the effects of APE and AND on rat and human hepatic CYPs, in primary rat and human hepatocyte cultures, the latter *in vitro* model being recognized as the gold standard model for the evaluation of the impact of drugs and other xenobiotics on human liver.

No significant effect of APE and AND treatments was observed on the activity of CYP2E1 in rat after *in vivo* treatment and *in vitro* in rat hepatocytes nor in human hepatocytes; therefore no further investigation was performed for this CYP isoform.

In vitro in rat hepatocyte cultures, AND and APE treatments increased CYP1A2 mRNA after 72h of treatment. Other data obtained in rodent, *i.e.* mice hepatocytes also showed that CYP1A1 and CYP1A2 mRNA were induced by 50 µM AND [17], with a maximal induction response after 24-48h of treatment. This was not accompanied by any change in CYP1A2 apoprotein expression, most probably due to sensitivity limits of immunoblotting, as previously reported even with specific reference inducers [31]. However, CYP1A2-dependent monooxygenase activity was significantly decreased, in accordance with the results of CYP1A2 inhibition previously reported [14]. As *in vivo*, APE and AND did not affect CYP1A2-dependent activity suggests that CYP1A2 is both induced and inhibited by AND and APE in rats. CYP1A2 mRNA expression and monooxygenase-dependent activity were decreased by APE and AND in human hepatocyte cultures. In contrast to the rat, the effects on mRNA expression were consistent with the effects on activity in human hepatocytes. They were also consistent with our observation of inhibition in human liver microsomes [14]. In humans, we

thus cannot exclude that CYP1A2, which accounts for about 10-15% of the total CYP of human liver and metabolizes therapeutic agents including phenacetin, theophylline, propanolol [32] could lead to clinically significant interaction, when modulated by AND and APE.

CYP3A represents the largest single portion of the CYP450 protein and activity in human and plays a substantial role in the metabolism of a vast array of pharmaceutical products [33]. Of particular concern are compounds that are predominantly or exclusively metabolized by CYP3A4 and also have a narrow therapeutic margin (e.g. cyclosporine A and ethinyl estradiol). In our study, CYP3A-dependent monooxygenase activity was significantly decreased in rat hepatocytes but not in rats after *in vivo* treatment, mRNA expression was decreased with AND but not with APE and protein expression was not affected in rat hepatocytes. Altogether it suggests that CYP3A is not affected by the treatment in rats. In human hepatocytes, CYP3A-dependent monooxygenase activity was significantly decreased, associated with decreases in mRNA and to a lesser extent protein expression. APE was a more potent inhibitor of CYP3A activity than AND, suggesting that other components present along with AND in the extract of *Andrographis paniculata* may modulate these effects. This also shows the relevance in studying the herb as a whole, as we would probably miss these effects if using only isolated compounds. Taken altogether, the results from the present study, associated with the inhibitory effect on CYP3A previously reported [14] suggest that AND and APE treatments modulate CYP3A in human and therefore can lead to herb-drug interaction.

CYP2C isoform is also an important CYP as it is implicated in the metabolism of barbiturate, non steroidal anti-inflammatory drugs and the anticoagulant warfarin [34]. In the present study, APE and AND caused a decrease in CYP2C-dependent activity *in vivo* in rat and *in vitro* in rat and human hepatocytes. Decreases in CYP2C activity were associated with decreases in corresponding mRNA and protein expression. Taken altogether, our results suggest that AND or APE interact with CYP2C-dependent drug metabolism, both by decreasing

enzyme synthesis as found in the present study and by inhibiting enzyme activity as previously reported [14]. Our conclusion is in accordance with a recent study of Hovhannisyan et al. [29] reporting that the maximum concentration and half-life of the CYP2C9 substrate warfarin in rats was increased and its clearance was 1.8-fold decreased after APE administration. This is of importance since warfarin has a narrow therapeutic index and its more active enantiomer (S-warfarin) is primarily a substrate for CYP2C9 [35].

Our studies clearly document the potential for APE and its pure constituent AND to decrease CYP2C expression *in vivo* in rat and *in vitro* in rat and human hepatocytes. Moreover, based on the decreases in CYP3A expression and activity observed *in vitro* in primary cultures of human hepatocytes observed in the present study and the inhibitory effects on human microsomal CYP3A previously reported by us [14], it cannot be excluded that APE and AND modulate CYP3A-dependent metabolism in humans. Both CYP2C and CYP3A are responsible for the metabolism of a wide variety of commonly worldwide prescribed medications. An increased blood plasma concentration of drugs that may result from decreased activity of these enzymes has the potential to seriously adverse reactions in patients concurrently taking *Andrographis paniculata*.

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Legends for figures

Fig. 1. Western blot analysis using antibody anti-CYP2C11 on rat liver microsomes (pool of six rats in each group, 10 µg microsomal protein per well) after 4 weeks of exposure. Lane 1: control rats; lane 2: APE-treated rats (0.5 g/kg/day); lane3: APE-treated rats (2.5 g/kg/day); lane 4: AND-treated rats (5 mg/kg/day); lane5: AND-treated rats (25 mg/kg/day).

Fig. 2. Effects of AND (50 µM) and APE (50 µM) on CYP1A2, CYP2C11, CYP2E1 and CYP3A1 activities in rat hepatocytes after 72 h of treatment. β-naphthoflavone (50 µM; BNF) and Dexamethasone (50 µM; Dex) were used as reference CYP inducers. P450 activities were assayed in liver microsome incubation using selective substrates. Results are expressed as percentage of activity as compared to control (DMSO-treated cells). Data are the mean ± S.E.M. of five different hepatocyte preparations. *, $p<0.05$ with respect to control.

Fig. 3. Effects of AND (50 µM) and APE (50 µM) on CYP1A2, CYP2C9, CYP2E1 and CYP3A4 activities in human hepatocytes after 72 h of treatment. β-naphthoflavone (50 µM; BNF) and Rifampicin (10 µM; Rif) were used as reference CYP inducers. P450 activities were assayed in liver microsome incubation using selective substrates. Results are expressed as percentage of activity as compared to control (DMSO-treated cells). Data are the mean ± S.E.M. of three different hepatocyte preparations. $^{*}p<0.05$ with respect to control.

Fig. 4. Effects of AND (50 µM) and APE (50 µM) on CYP1A, CYP2C and CYP3A apoprotein expression in rat (A) and human (B) hepatocytes after 72 h of treatment. β-naphthoflavone (50 µM; BNF), Dexamethasone (50 µM; Dex) and Rifampicin (10 µM; Rif) were used as reference CYP inducers. For the assay, 10 µg microsomal protein/well ware used. Immunodetectable CYP1A, CYP2C and CYP3A isoforms were analyzed.

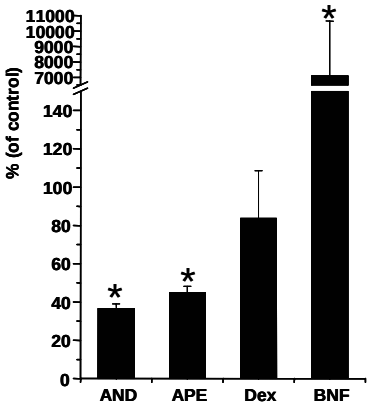
Fig. 5. Effect of AND (50 µM) and APE (50 µM) on CYP1A2, CYP2C and CYP3A mRNA expression in rat (A) and human (B) hepatocytes after 72 h of treatment. β-naphthoflavone (50 µM; BNF), Dexamethasone (50 µM; Dex) and Rifampicin (10 µM; Rif) were used as reference CYP inducers. Results are expressed as fold change as compared to control (DMSO-treated cells). Data are from one representative rat hepatocyte culture and from one human hepatocyte (HH-008) culture. Analysis were performed in duplicate with consistent results.

Figure 1

Lane 1 2 3 4 5

Figure 2

CYP1A2



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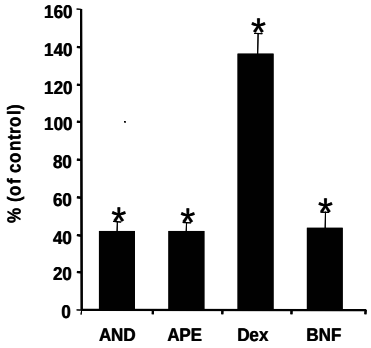

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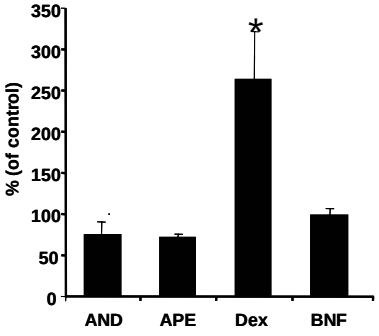

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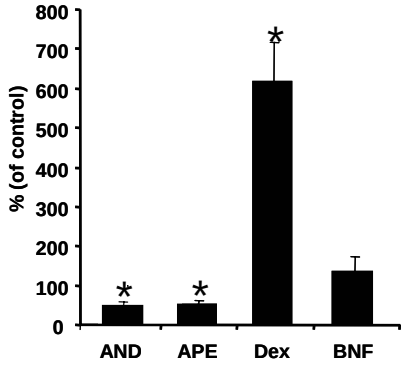
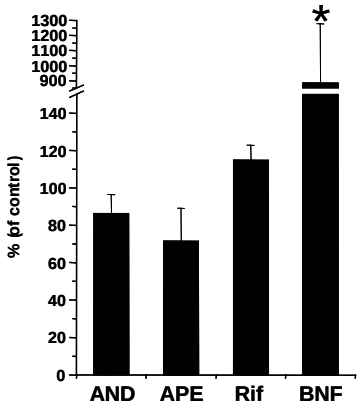
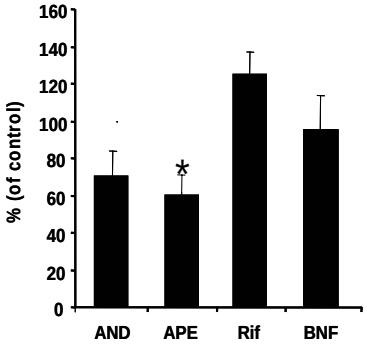


Figure 3

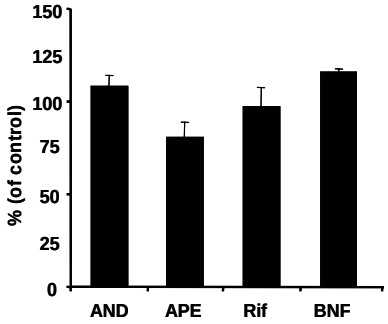
CYP1A2



CYP2C9



CYP2E1



CYP3A4

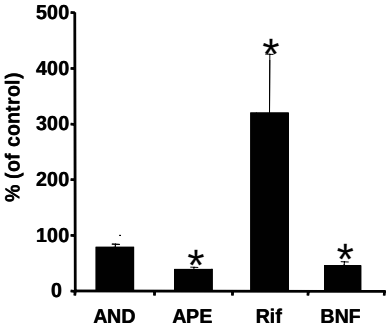


Figure 4

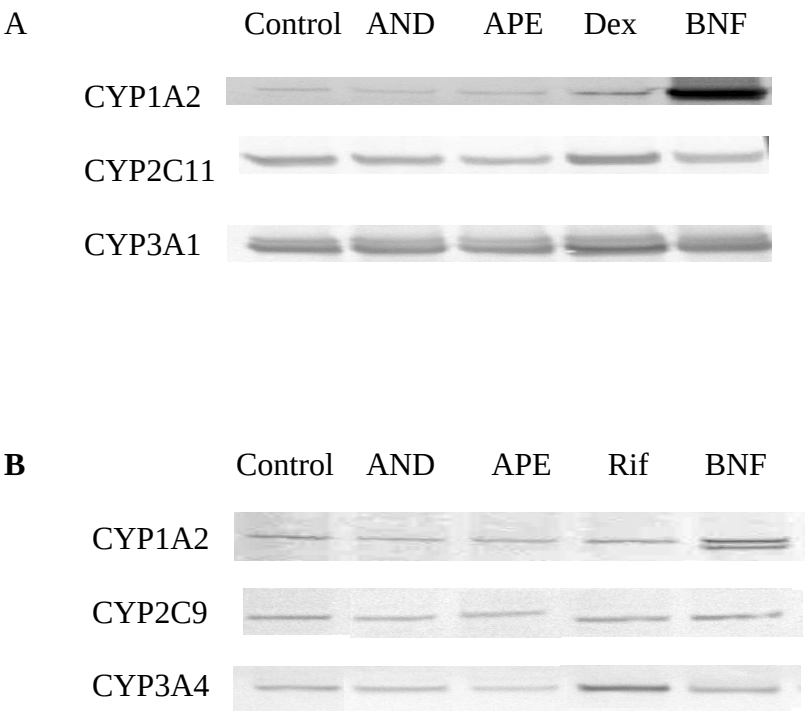
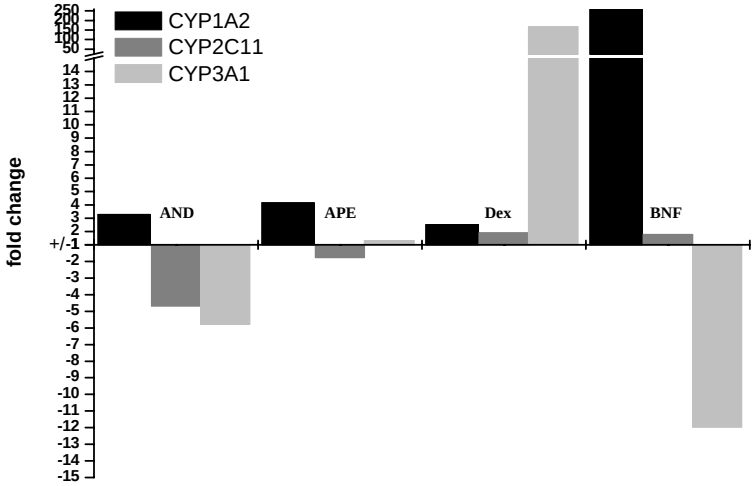

A
Control
AND
APE
Dex
BNF
CYP1A2
CYP2C11
CYP3A1
B
Control
AND
APE
Rif
BNF
CYP1A2
CYP2C9
CYP3A4

Figure 5

A


CYP1A2
CYP2C11
CYP3A1
fold change
AND
APE
Dex
BNF

B

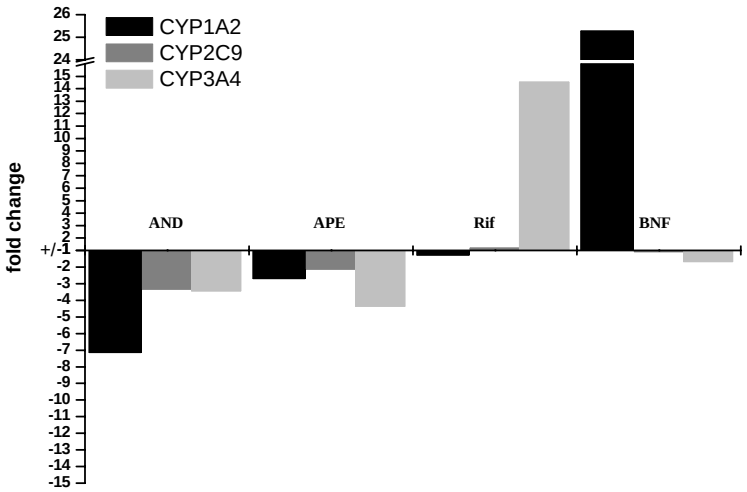

CYP1A2
CYP2C9
CYP3A4
fold change
AND
APE
Rif
BNF

Table 1. Donor information for human hepatocyte preparations

Donor	Age (years)	Sex	Race	Disease	Viability (%)
HH-005	72	Female	Caucasian	Cholangiocarcinoma	70
HH-006	67	Male	Caucasian	Metastatic hepatic	87
HH-008	21	Male	Caucasian	Wilson’s disease	89

Table 2. The effects of APE and AND on rat body and liver weight after 28 days of treatment[a]

Treatment	Body weight (g)	Liver weight (g)	Mean liver weight/body weight$x10^2$
Control	343±10	15.09±0.46	4.41±0.07
APE 0.5 g/kg/day[b]	335± 8	14.25±0.54	4.25±0. 09
APE 2.5 g/kg/day[c]	328± 5	13.74±0.43	4.18±0. 07
AND 5 mg/kg/day	340± 6	13.95±0.38	4.10±0. 07
AND 25 mg/kg/day	340±10	13.44+0.67	3.94+0. 13*

[a] Results are expressed as mean ± S.E.M., n=6 for each group.

* Significantly different from control by the student Newman-Keuls multiple range test (p<0.05).

[b] APE dose of 0.5 g/kg/day containing AND at equivalent to 5 mg/kg/day

[c] APE dose of 2.5 g/kg/day containing AND at equivalent to 25 mg/kg/day

Table 3. Effects of APE and AND treatment on CYP dependent activities in liver microsomes prepared from rat *in vivo* study [a]

Activity (pmol/mg/min)	CYP isoform	control	APE		AND	
			0.5 g/kg/d	2.5 g/kg/d	5 mg/kg/d	25 mg/kg/d
EROD	CYP1A2	33.3±2.5	30.2±3.2	45.1±4.8	44.4±4.2	33.7±3.5
Tol-OH	CYP2C11	127.6±7.4	94.5±14.4*	82.9±8.6*	63.5±11.5**	68.8±6.5**
PNP-OH	CYP2E1	696.0±22.8	569.6±57.8	571.4±34.2	570.5±21.1	531.8±32.1
6βTesto-OH	CYP3A1	149.6±12.6	135.5±13.3	153.8±18.4	132.2±16.0	113.1±14.8

[a] Results are expressed as mean ± S.E.M.; n=6. Microsomes from each rat were assayed in duplicate and the means of the duplicates were used to calculate overall means for each condition. The variances in the duplicates were less than 10%.

* Significantly different from control by the student Newman-Keuls multiple range test ($p<0.05$).

** Significantly different from control by the student Newman-Keuls multiple range test ($p<0.01$).

DISCUSSION AND PERSPECTIVES

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1. Importance of studying herb-drug interaction

The use of herbs as alternative and/or complementary therapy in the western world is on the rise and gaining increasing popularity. As people often take different herbs in combination with prescribed modern medication, there is a potential for both pharmacokinetic and pharmacodynamic herb-drug interaction. In addition to doctor's recommendation, patients also perform self-medication with several different herbs and herbal preparations, believing it is safe, and often without informing their primary physician (Kelly et al., 2005; Brazier, Levine, 2003). Herbal products contain several chemicals that are metabolized by phase I and phase II pathways and also serve as substrates for certain transporters. Phase I biotransformation of drugs often precedes and is slower than phase II biotransformation. For this reason, phase I biotransformation (such as oxidation of drugs by cytochrome P450) tends to be the rate-limiting step in the overall metabolism and, at times, the elimination of drugs. Due to their interaction with these enzymes and transporters there is a potential for alteration in the activity of drug metabolizing enzymes and transporters in presence of herbal components. Induction and inhibition of drug metabolizing enzymes and transporters by herbal component has been documented in several *in vitro* studies. The use of *Andrographis paniculata* (AP) in combination with modern drugs may also affect the metabolism of these drugs, as firstly shown in 1987 by Choudhury B.R., who report that a single dose of APE and AND in *in vivo* study inhibited hepatic microsomal CYP2C/2E1-dependent aniline hydroxylase, CYP2B-dependent *N*-demethylation of *N*,*N*-dimethyl aniline, and CYP2E1-dependent *O*-demethylation of *p*-nitroanisole in albino rats, while in long term treatment its administration produced induction of all these isoenzymes in this species. In *in vitro* incubation studies using rat microsomes, APE as well as AND did not produce any effect on CYP2B-dependent *N*-demethylation of *N*,*N*-dimethyl aniline, and CYP2E1-

dependent*O*-demethylation of *p*-nitroanisole but only inhibit CYP2C/2E1-dependent aniline hydroxylase. *Andrographis paniculata* extracts have recently been reported to increase CYP1A1-dependent ethoxyresorufin *O*-dealkylase and CYP2B10-pentoxyresorufin *O*-dealkylase activities *in vivo* in mice (Jarukamjorn et al., 2006) and one report showed that AND significantly induced the expression of CYP1A1 and CYP1A2 mRNAs in a concentration-dependent manner in primary cultures of mouse hepatocytes (Jaruchotikamol et al., 2007).

In light of its widespread use and few literature reports, more studies are needed on the impact of *Andrographis paniculata* on selective hepatic P450 enzyme activities. In particular, as there are marked differences in the expression and regulation of CYPs, assessment of the inhibitory and/or induction potential of CYPs is biologically relevant, particularly when performed in models from various species, including human, ultimately leading to better models for screening and predicting drug–drug or herb-drug interactions in humans (Fujii-Kuriyama, 1993; Gotoh, 1993; Negishi, 1993). For this reason, the first aim of the present study was to evaluate **the inhibition potential** of *Andrographis paniculata* extract (APE) and andrographolide (AND), the most medicinally active phytochemical in the extract, on microsomal hepatic P450 enzyme activities both using rat and human liver microsomes. The second aim of the present study was to assess the effects of APE and AND on various **hepatic CYP expression** after *in vivo* administration to Wistar rats and further to assess the effects *in vitro* study in rat and human hepatocytes cultures on CYP activity, protein content and mRNA expression. We chose to evaluate not only the effects of AND but also the effects of the whole herb as the extraction (APE) for two reasons: i) the popular behavior in countries where an active ethnomedicine exists, is usually to take the whole herb and ii) there might be other compounds present along with AND in the herb able to modulate CYP expression.

2. Extraction and dose selection for herb-drug interaction

There are a number of research problems associated with a study on the effects of herbal medicine. These include batch to batch variability in the amount of active constituents and a gradual decrease of each of them due to the instability of the preparation. The standard quality of AP regarding the amount of active constituent in Thai Pharmacopoeia states that the quantity of active constituent, the total lactone, must not be less than 6% w/w or the quantity of AND must not be less than 1% w/w. The preparation used in this study met this requirement since AND content was 1.6% w/w. The drug was used within 6 months after it had been produced, a period of time shown to avoid degradation.

The doses of AND used for *in vivo* administration to rats were selected on the basis of a previously published report, where 5 mg AND/kg/day for 30 consecutive days was given *p.o*. to adult male albino rats (Choudhury et al., 1987). Moreover, if we consider the daily recommended dose for humans that is 1 mg AND/kg/day (Panossian et al., 2000), we can translate an equivalent daily dose of 6.25 mg AND /kg/day for rats, considering the pharmacological activity and toxicity difference in these two species (Hovhannisyan et al., 2006). Consequently, the AND doses we used in the present study (*i.e*. 5 and 25 mg AND/kg/day), either pure or present in APE, could be considered respectively as rat-equivalent daily recommended AND and approximately 5-fold higher AND supplementations. For *in vitro* experiments, cultures hepatocytes were treated with 50 μM AND (pure or contained in APE), that corresponds to the highest non-toxic concentration as proven by MTT assay and to a roughly equivalent concentration to those achieved in individuals ingesting *A. paniculata*. It is noteworthy that AND concentrations in blood of individuals after ingestion of *A. paniculata* at daily recommended dose were estimated to reach approximately 5 μM (Panossian et al., 2000), making it most probable that liver concentrations could reach up to 50 μM.

3. Risk of herb-drug interaction in human for *Andrographis paniculata*; summary of the results and literature reviews

The following table is a summary of the effects of APE and AND *in vitro* and *in vivo* in the present study

Study	**parameters**	**rat**		**human**	
		AND	**APE**	**AND**	**APE**
In vitro (microsomes)	*Ki* (μM)	61.1 (2E1)	8.85 (1A2) 8.21 (2C11)		24.46 (1A2) 7.51 (2C9) 25.43 (3A4)
In vivo (rat liver)	activity compare to control	↓ 50% (2C11)	↓ 30% (2C11)		
In vitro (hepatocytes)	activity compare to control	↓ 60% (1A2) ↓ 60% (2C11) ↓ 50% (3A1)	↓ 55% (1A2) ↓ 60% (2C11) ↓ 50% (3A1)		↓ 40% (2C9) ↓ 60% (3A4)
	mRNA expression (fold change)	↑ 3.2 (1A2) ↓ 4.7 (2C11) ↓ 5.8 (3A1)	↑ 4.1 (1A2) ↓ < 2 (2C11)	↓ 7.2 (1A2) ↓ 3.5 (2C9) ↓ 3.6 (3A4)	↓ 2.8 (1A2) ↓ 2.0 (2C9) ↓ 4.5 (3A4)

In *in vitro* studies using liver microsomes, we undertook to study the inhibition of CYP1A, CYP2B, CYP2C, CYP2E and CYP3A by APE and AND. These enzymes were selected because together they account for more than 80% of the total hepatic metabolism (Ortiz de Montellano, 1995).

There are no inhibition effects observed in CYP2B activity both in rat and human microsome. The presence of AND had minimal effect on any rat and human liver CYP-dependent reaction measured except on the CYP2E1-dependent *p*-nitrophenol hydroxylation, which was inhibited with a *Ki* value of 61.1 μM in rat liver microsomes. Our observation that AND inhibited rat liver CYP2E1 is in accordance with previous results (Choudhury et al., 1987). APE and AND had no effect on human CYP2E1-dependent hydroxylation of *p*-nitrophenol, suggesting

that no inhibitory effect on this isoenzyme occurs in humans. No significant effect of APE and AND treatments was observed on the activity of CYP2E1 in rat after *in vivo* treatment and *in vitro* in rat hepatocytes nor in human hepatocytes; therefore no further investigation was performed for this CYP isoform.

APE inhibited CYP1A2-dependent 7-ethoxyresorufin-*O*-deethylation both in rat and human microsomes, with a *Ki* value of 8.85 and 24.46 μM, respectively. The inhibitory effect was higher on rat liver microsomal CYP1A2 than on human liver microsomal CYP1A2. These results suggest marked species-differences in sensitivity to CYP1A2 inhibitors. As *in vivo*, APE and AND did not affect CYP1A2-dependent activity suggests that CYP1A2 is both induced and inhibited by AND and APE in rats. *In vitro* in rat hepatocyte cultures, AND and APE treatments increased CYP1A2 mRNA after 72h of treatment. Other data obtained in rodent, *i.e.* mice hepatocytes also showed that CYP1A1 and CYP1A2 mRNA were induced by 50 μM AND (Jaruchotikamol et al., 2007), with a maximal induction response after 24-48h of treatment. Increase content but decrease in activity of CYP1A2 may result from the following mechanism: expression of an inactive enzyme and/or direct inhibition of the activity of CYP1A2 by AND and APE. In human hepatocyte cultures, CYP1A2 mRNA expression and monooxygenase-dependent activity were decreased by APE and AND. In contrast to the rat, the effects on mRNA expression were consistent with the effects on activity in human hepatocytes. They were also consistent with our observation of inhibition in human liver microsomes (Pekthong et al., 2008). In humans, we thus cannot exclude that CYP1A2, which accounts for about 10-15% of the total CYP of human liver and metabolizes therapeutic agents including phenacetin, theophylline, propanolol (Cupp and Tracy, 1998) could lead to clinically significant interaction, when modulated by AND and APE.

APE was also found to inhibit CYP2C both in rat and human microsomes with *Ki* values of 8.21 and 7.51 μM, respectively while AND alone did not. This may be due to the presence of several other components present along with AND

in the extract of *Andrographis paniculata*. APE and AND caused a decrease in CYP2C-dependent activity *in vivo* in rat (30% for APE and 50% for AND) and *in vitro* in rat and human hepatocytes. Decreases in CYP2C activity were associated with decreases in corresponding mRNA and protein expression. Taken altogether, our results suggest that AND or APE interact with CYP2C-dependent drug metabolism, both by decreasing enzyme synthesis as found in the present study and by inhibiting enzyme activity as previously reported (Pekthong et al., 2008). Our conclusion is in accordance with a recent study of Hovhannisyan et al. (2006) reporting that the maximum concentration and half-life of the CYP2C9 substrate warfarin in rats was increased and its clearance was 1.8-fold decreased after APE administration. This is of importance since warfarin has a narrow therapeutic index and its more active enantiomer (S-warfarin) is primarily a substrate for CYP2C9 (Rettie et al., 1992).

Finally, APE inhibited CYP3A4 with the *Ki* of 25.43 μM and much higher than that previously reported for ketoconazole (*Ki* = 0.024 μM, Walsky and Obach, 2004). CYP3A-dependent monooxygenase activity was significantly decreased in rat hepatocytes but not in rats after *in vivo* treatment, mRNA expression was decreased with AND but not with APE and protein expression was not affected in rat hepatocytes. Altogether it suggests that CYP3A is not affected by the treatment in rats. In human hepatocytes, CYP3A-dependent monooxygenase activity was significantly decreased, associated with decreases in mRNA and to a lesser extent protein expression. APE was a more potent inhibitor of CYP3A activity than AND, suggesting that other components present along with AND in the extract of *Andrographis paniculata* may modulate these effects.

Our studies clearly document the potential for APE and its pure constituent AND to decrease CYP2C expression *in vivo* in rat and *in vitro* in rat and human hepatocytes. Moreover, based on the decreases in CYP3A4 expression and activity observed *in vitro* in primary cultures of human hepatocytes observed in the present study and the inhibitory effects on human microsomal CYP3A4, it

cannot be excluded that APE modulate CYP3A-dependent metabolism in humans. Both CYP2C and CYP3A are responsible for the metabolism of a wide variety of commonly worldwide prescribed medications. An increased blood plasma concentration of drugs that may result from decreased activity of these enzymes has the potential to seriously adverse reactions in patients concurrently taking *Andrographis paniculata*. A positive response in human hepatocyte culture indicates that it **need for further assessment *in vivo* studies in humans**. It is the only definitive way to assess the magnitude and implications of herb–drug interactions. With an increased understanding of the mechanism of herb drug interactions it should be feasible to minimize or avoid therapeutic failures or increased toxicity of conventional drug therapy.

It is well known that the results of the experiments on drug metabolism in rats cannot be directly extrapolated to humans. However, many drug interactions are first described in animal studies, often when the doses of the drugs used are much greater than those used clinically. The disposition of many drugs in human and animal differs, and thus drug interactions observed in an animal *in vivo* study may not be encountered in human. And vice versa, rifampicin, a well-known inducer of liver microsomal enzymes in human, is not a good inducer in many animal species, notably the rat and rabbit, which are often used in screening studies (Timbrell, 1991). Hepatic microsomal drug-metabolizing enzymes system in rats and human though contain many similar isozymes (e.g. CYP1A2, CYP2E1), these enzymes may exert different substrate specificity. The isozymes responsible for metabolism of the same substrate are sometimes different between these two species (Eagling et al., 1998). However, an experiment in animals would give us some clues to the effect of xenobiotics on hepatic drug-metabolizing enzymes and possibility of drug interactions at this level.

4. Proposed Herb-drug interaction strategy

Nowadays, evaluation of potential drug-drug interactions has become mandatory for the registration of new drugs by most regulatory agencies (USFDA, 1999). There are two basic questions to be answered, both questions, although related, require specific experimental approaches: (i) does the drug influence the metabolism of other compounds; and (ii) is the metabolism of the drug candidate altered by other compounds that could be administered concomitantly in clinical practice?

Although legislation does not request for herb–drug interactions studies, we propose a similar strategy of experimental approaches for evaluating potential herb-drug interactions:

The inhibitory effect of the drug on a CYP isoenzyme can be readily investigated by co-incubating several concentrations of the drug and CYP-selective substrates. Kinetic measurements, for instance, will help to determine whether the tested drug acts as an inhibitor on a given CYP isoform, as well the type of inhibition caused. Liver microsomes, reconstituted enzymes and/or microsomes from CYP-engineered cells are suitable tools for this purpose, and allow an easy comparison of the inhibitory potency of related drugs. However, the question might be somewhat more difficult to answer if metabolites, rather than the parent molecule, are the chemical entities involved. For this purpose the use of metabolically competent cells is more appropriate. Another limitation of microsome assays is that inhibition parameters obtained may not accurately reflect the situation *in vivo*, since the contribution of drug transport is not considered. Inhibition assays can be performed in genetically engineered cell lines expressing individual drug-metabolising enzymes. These cells, because of the presence of membrane barriers are, in some aspects, more predictive than isolated enzymes or microsomes from hepatocytes (USFDA, 1997).

The second question is addressed by examining the rate of metabolism of the herb in the presence of other compounds (herbs or drugs) that are likely to be

administered concomitantly in clinical practice. It is highly desirable that the pharmacokinetics of a drug candidate not be influenced by the coadministration of other drugs. Hence, using liver microsomes or microsomes from CYP-expressing cells, experiments are designed to monitor the rate of catabolism of the drug in the presence of variable amount of the other compounds. Again, the use of cells, instead of subcellular fractions, although adding more complexity to the assays, better reflects the drug transport processes that are taking place in the liver. Since in the metabolism of a compound several isoenzymes may be involved, the use of cells expressing one single CYP isoenzyme may overestimate the inhibitory effect of a given drug. Hence, the best picture of the potential drug-drug interaction is obtained in metabolically competent human hepatocytes.

While in vitro studies with the microsomal system provide limited information, hepatocyte systems offers a unique opportunity to evaluate herb drug interaction and will help in focusing and minimizing *in vivo* human studies. A lack of effect in human hepatocytes, neither induction nor suppression, would suggest lack of an *in vivo* effect, and mean that no clinical study is required, since drug can be considered safe. In contrast, a positive response in human hepatocyte cultures would indicate a need for further assessment *in vivo* in humans.

While *in vitro* and *in vivo* systems in animals will provide useful information, *in vivo* studies in humans are the only definitive way to assess the magnitude and implications of herb–drug interactions. Although legislation does not request for herb–drug interactions study, we suggest that similar *in vivo* animal studies and *in vitro* animal and human studies should be performed. With an increasing understanding of the mechanism of herb-drug interactions it should be feasible to minimize or avoid therapeutic failures or increased toxicity of conventional drug therapy.

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RESUME

Evaluation de possibles interactions médicamenteuses: effets d'extraits de *Andrographis paniculata* sur les enzymes du métabolisme hépatique chez le rat et chez l'Homme.

Recently, the use of herbs as alternative and/or complementary therapy in the world is on the rise and gaining increasing popularity. As people often take different herbs in combination with prescribed modern medication, there is a potential for both pharmacokinetic and pharmacodynamic herb-drug interaction. In addition to doctor's recommendation, patients also perform self-medication with several different herbs and herbal preparations, believing it is safe, and often without informing their primary physician. It is of importance that potential herb-drug interactions be identified in order to give more insight into the guideline of rational administration and precaution to be taken for using this herbal medicine. ***Andrographis paniculata*** - plant family *Acanthaceae* - is the example of a medicinal herb that has been used for centuries in Asia to treat chronic and infectious diseases.

The first part of the present work consisted of an extensive literature review on ***Andrographis paniculata***, on **xenobiotic biotransformation,** especially on cytochrome P450, the most importance hepatic enzyme for xenobiotic metabolism and finally on examples of **herb-drug interactions**. The second part consisted of the experimental results which can be summarized as follows: the ability of *Andrographis paniculata* extract (APE) and Andrographolide (AND), the most medicinally active phytochemical in the extract, to affect hepatic cytochrome P450 (CYP) activities was examined *in vivo* rat model and *in vitro* using rat and human liver microsomes and hepatocytes. The potential for inhibition or induction of CYP1A, CYP2C, CYP2E and CYP3A were evaluated by APE and AND. In inhibition study using rat and human liver microsomes, APE inhibited CYP2C-dependent tolbutamide hydroxylation with apparent *Ki* values of 8.21 and 7.51 µM, respectively. *In vivo* study, APE and AND exhibited minimal capacity to inhibit any CYP enzyme, except CYP2C11. At a dosage of 0.5 and 2.5 g APE/kg/d (corresponding respectively to AND 5 and 25 mg/kg/d) inhibited the hydroxylation of tolbutamide with 30% decrease compare to control group. AND at a dosage of 5 and 25 mg/kg/d also inhibited CYP2C11 by 50% compare to control group. *In vitro* study, rat and human hepatocytes treated with APE (50µM) or AND (50µM) were exposed to probe substrates to determine enzyme activity, protein and RNA harvested. In rat hepatocytes, APE and AND treatment resulted in significant decrease in activity of CYP1A2, CYP2C11 and CYP3A1, but had no effect on CYP2E1. In human hepatocytes, APE significantly decrease in mRNA, protein and activity of CYP2C9 and CYP3A4 whereas not effect CYP1A2 and CYP2E1. AND exhibited minimal capacity to inhibit any CYP enzyme. In conclusion, it cannot be excluded from the present study that APE could cause herb-drug interactions in humans through CYP2C9 and CYP3A4 inhibition.

Keyword: Andrographis paniculata; Andrographolide; Cytochrome P450 inhibition; microsome; hepatocyte

L'utilisation des plantes comme alternative et/ou complément à un traitement médicamenteux classique devient actuellement populaire. Du fait de la prise conjointe de médicament et de plantes, l'interaction plantes/médicaments est envisageable en pharmacocinétique et en pharmacodynamique. Les patients pratiquent également une automédication avec plusieurs plantes ou préparations à bases de plantes, conjointement au traitement médical. Croyant cette pratique sure, l'automédication est le plus souvent faite sans même en avertir le médecin traitant. Il est donc important d'identifier les interactions plantes/médicament potentielles afin de prendre toutes les précautions qui s'imposent pour l'administration de plantes.

Andrographis paniculata - plante de la famille des *Acanthaceae* - est un exemple de plante médicinale utilisée depuis plusieurs siècle en Asie pour traiter les maladies chroniques et infectieuses.

Dans une première partie de ce travail sont présentées successivement une revue sur d'*Andrographis paniculata*, une sur la biotransformation des xénobiotiques, particulièrement celle des cytochromes P450, l'enzyme la plus importante du métabolise des xénobiotiques, et enfin une sur des exemples d'interactions plante/médicament.

Dans une seconde partie, les résultats expérimentaux sont présentés. Ils peuvent être résumés de la manière suivante : les effets de l'extrait hydroalcoolique d'*Andrographis paniculata* (EAP), plante largement utilisée en médecine Thaïlandaise, et de son diterpène majoritaire, l'Andrographolide (AND), sur l'activité des CYPs hépatiques ont été examinés *in vivo* et *in vitro* chez le rat et *in vitro* chez l'Homme. *In vitro*, dans les microsomes hépatiques, l'EAP inhibe l'hydroxylation CYP2C-dependante du tolbutamide avec un *Ki* respectivement de 8,21 pour le rat et 7,51 µM pour l'Homme. Le mode d'inhibition est de type mixte mais

probablement non métabolisme-dépendant. Après administration d'EAP et d'AND *in vivo* chez le rat, l'expression et l'activité du CYP2C11 ont significativement varié: une diminution de 30% de l'activité CYP2C11 a été observée à 0,5 et à 2,5 g d'EAP/kg/jour (correspondant respectivement à 5 et 25 mg d'AND/kg/jour), par rapport au groupe contrôle, ainsi qu'une diminution d'expression de l'isoforme CYP2C11. À 5 et 25 mg d'AND pur /kg/jour, l'activité CYP2C11-dependante est diminuée de 50% par rapport au contrôle. *In vitro* dans les cultures primaires d'hépatocytes de rat et humains, incubées avec 50 µM d'AND ou d'EAP, d'importantes diminutions des niveaux d'activité et de l'expression des ARNms des CYP2C et CYP3A ont également été mises en évidence. En conclusion, la présente étude montre qu'il n'est pas exclu que l'extrait d'*Andrographis paniculata* (EAP) ainsi que son diterpène majoritaire (AND), puissent provoquer chez l'Homme des interactions médicamenteuses en cas de co-administration avec des médicaments classiques, ceci par inhibition de l'expression et de l'activité des CYP2C9 et CYP3A4.

Keyword: Andrographis paniculata; Andrographolide; Cytochrome P450 inhibition; microsome; hépatocytes

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Présentée par ~~Madame~~, Monsieur PEKTHONG Dumrongsak

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et ayant pour titre : Evaluation de possibles interactions médicamenteuses : effets d'extraits de Andrographis paniculata sur les enzymes du métabolisme hépatique chez le rat et chez l'homme.

Vu et permis d'imprimer,

Besançon, le 24 octobre 08

Le Directeur de Thèse,

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Le Président de l'Université
et par délégation,
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