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DRUG METABOLISM: REGULATION AND IMPORTANCE

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Abstracts - MONDAY, September 17, 2001

The biology and clinical importance of the CYP-gene superfamily
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Cytochrome P450 (CYP) heme proteins are one of the largest superfamilies of enzymes found in virtually all organisms. Based on information of the human genome sequence, there exist more than 50 human CYP genes encoding enzymes that can be grouped into 17 families. These enzymes are involved in the synthesis of steroid hormones, cholesterol and bile acids, catalyze the biotransformation of eicosanoids, fatty acids and retinoic acid, and metabolize innumerable drugs, procarcinogens and carcinogens as well as other xenobiotics. The reactions CYP enzymes carry out can be extremely diverse or even surprising.

The clinical importance of CYP enzymes relates to their numerous physiological functions and their role in drug metabolism. Of particular clinical significance are their overlapping substrate specificities, their induction and repression by endogenous and exogenous factors (food components, drugs, other xenobiotics) and their genetic diversity. Genetic polymorphisms are important contributors to interindividual variation in drug response as are competitive inhibition and induction. Phenotyping and genotyping tests can assess this variation in patients and volunteers. There has been considerable recent progress in understanding the regulation by xenobiotics of the expression of CYP genes, particularly those of families 1-4. Interaction of drugs with nuclear receptor signaling can explain and predict the clinical induction response.

Metabolism of CYP-generated reactive intermediates by epoxide hydrolases
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CYP-generated reactive epoxides are primarily inactivated by epoxide hydrolases and glutathione S-transferases, further systems such as epoxide reduction not significantly contributing to the overall control in all cases sufficiently investigated so far. Where the structural substrate specificities are not discriminating, in all cases known to date epoxide hydrolases represent by far higher affinity systems than glutathione S-transferases and, hence, play the decisive role at the usually low concentration of either environmental or metabolically formed epoxides.

Of the several mammalian epoxide hydrolases only 2 significantly contribute to foreign compound metabolism: Microsomal and soluble epoxide hydrolase. Recent studies have shown that – in contrast to previous assumption – they work by forming an intermediary enzyme-bound ester which is subsequently cleaved by enzymically activated water. The recognition that the first enzyme-catalyzed step is very fast and the enzyme present in high amounts compared with low concentrations of environmental or metabolically formed epoxides defines a threshold level below which epoxide-dependent toxicities are not visible.

Moreover, the cellular topographical relationship of epoxide producing to epoxide inactivating to precursor sequestering systems and the consequences for the control of toxic metabolites have been investigated.

Analyzing the function of genes encoding human drug metabolizing enzymes using humanized transgenic mice
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To address the role of P450s in drug metabolism, chemical toxicities and whole animal chemical carcinogenesis, gene knockout and transgenic mice are being produced and analysed. Mice were made that lack the P450s known to catalyse carcinogen and toxin activation. Mouse lines lacking expression of genes encoding P450s CYP1A2, CYP2E1 and CYP1B1 were developed. These mice exhibit no grossly abnormal phenotypes, suggesting that the xenobiotic-metabolizing enzymes have no critical roles in mammalian development and physiological homeostasis thus offering an explanation for the existence of P450 polymorphisms in mammals. However, P450-null mice do show marked differences in sensitivities to acute chemical toxicities, and chemical carcinogenesis. These studies establish the importance of xenobiotic metabolism in activation pathways that lead to gene mutations and cell death and validate ongoing molecular epidemiology studies to investigate the role of P450 polymorphisms in cancer susceptibility. Since

marked species differences occur in the catalytic activities and regulation of P450s enzymes, especially between rodents and humans, P450 “humanized” mice are being produced using bacterial artificial chromosomes. Human P450 regulation and catalytic activities reflect the native human P450s. These studies will provide a framework for the production of transgenic mice that may be of value in drug research.

Poster NO. I-5

Investigation of diazinon activation by P450 enzymes in human liver

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Phosphorothioate compounds are used worldwide as agricultural pesticides. We investigated the activation of the phosphorothioate diazinon to diazoxon in human liver. In a study using three human liver microsomal samples, K_m for diazoxon formation varied markedly (31, 208 and 660 mM, V_{max} 1125, 685, and 1028 pmol/min/mg protein respectively), suggesting the involvement of more than one P450 enzyme. A wide variation in activity was found using 50 mM diazinon as substrate (11–648 pmol/min/mg protein, n=15), whereas with 500 mM variation was less (164–978 pmol/min/mg protein). Amongst eight P450-catalysed reactions, the putative high affinity component (50 mM diazinon) correlated with *S*-mephenytoin 4'-hydroxylase activity (r=0.686, p<0.01), suggesting the involvement of CYP2C19. The putative low affinity component (500 mM diazinon) correlated with both *S*-mephenytoin 4'-hydroxylase (r=0.714; p<0.005) and high-affinity phenacetin O-deethylase activity (r=0.625; p<0.05). This activity was partially inhibited by furafylline, troleandomycin and ketoconazole indicating contributions from CYP2C19, CYP1A2 and CYP3A4. Of several heterologously expressed human P450 enzymes, CYP2C19 activated diazinon (500 mM) at the fastest rate, followed by CYP3A4, CYP1A2 and CYP2C9. Both microsomal *S*-mephenytoin 4’-hydroxylase and high-affinity phenacetin O-deethylase activities were strongly inhibited by diazinon (IC_{50}<2.5 mM), while no effect was seen on midazolam 1'-hydroxylase activity. These data indicate that CYP2C19 is the major enzyme involved in diazinon activation in human liver, while other enzymes including CYP1A2 may play a more minor role.

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Poster NO. II-4

CYP 2D6 and 2E1 expression in human individuals: stability, effect of genotype, disulfiram and alcohol pretreatment

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In individuals phenotyped for CYP2D6 polymorphism by sparteine, a subsequent dextromethorphan test after four years revealed a good correlation except one supposed PM individual, whose real EM status was confirmed by analysis of three principal inactivating mutations. This revealed the phenotype stability within four years and a good phenotype/genotype correlation. The analysis of CYP2E1 genotype polymorphisms in a group of 110 volunteers enabled to select individuals for phenotype study using chlorzoxazone (CLZ) metabolism to 6-hydroxyCLZ (6OHCLZ) excreted in urine. Pretreatment with disulfiram inhibiting CYP2E1 decreased 6OHCLZ excretion in the first hours 5.5-fold, i.e. in the same sensitivity as plasma elimination. Mild alcohol doses (0.8g/kg or 1.5g/kg b.w.) increased CLZ metabolism in young men and women 2-fold and indicated significant CYP2E1 induction, which was unrelated to CYP2E1 polymorphisms, but inversely related to their habitual alcohol intake. Our further in vitro and in vivo study revealed that the rates of styrene oxidation and DNA adduct formation in rats, mice and humans were comparable, catalyzed primarily by CYP2E1 and CYP3A, and in human individuals exposed to styrene inhalation, were related to CYP2E1 expression. The in vitro study devoted to benzene, chlorobenzene, and dichlorobenzenes revealed similar in vitro rates of metabolism in rats, mice and humans and indicated dominant role of CYP2E1 and CYP3A in their oxidation.

Poster NO. VI-3

In vitro Identification of the Cytochrome P450 Enzymes Responsible for the Metabolism of Dietary Flavonoids

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Human and mouse liver microsomes and membranes isolated from Escherichia coli which expressed cytochrome P450 (P450) 1A2, 3A4, 2C9 or 2D6 together with NADPH-P450 reductase were used to identify the human and murine P450 enzymes involved in the metabolism of dietary flavonoids. In human liver microsomes, kaempferol, apigenin and naringenin were all hydroxylated at the 3’-position to yield their corresponding analogs quercetin, luteolin and eriodictyol, whereas hesperetin and tamarixetin were demethylated at the 4’-position to yield eriodictyol and quercetin, respectively. The same pattern of metabolism was evident in mouse liver microsomes. Recombinant P4501A2 was capable of metabolizing all five investigated flavonoids, whereas recombinant P4503A4 protein did not catalyze hesperetin demethylation, but showed similar metabolic profiles for the remaining compounds as did human

microsomes and recombinant P4501A2, although the reaction rates in general were lower as compared to P4501A2. P4502C9 catalyzed the 4'-demethylation of tamarixetin, whereas P4502D6 did not seem to play any role in the metabolism of the selected flavonoids. Potent inhibition of flavonoid metabolism by the P4501A inhibitor a-naphthoflavone, and the CYP1A2 inhibitor fluvoxamine in mouse and human microsomes, respectively, verified major involvement of the P4501A isozymes in hepatic flavonoid biotransformation. The present data suggest that human P450s, although with varying capacities/affinities, are capable of metabolizing a wide range of flavonoids giving rise to structurally diverse metabolites, which in other studies have been found to possess biochemical properties different from the parent compound. The major involvement in flavonoid metabolism of human P4501A, which is known to be polymorphic, raises the important question of whether individual differences in the P450 enzyme activity, might affect the beneficial outcome of dietary flavonoids rendering some individuals more or less refractory to the health-promoting potential of dietary flavonoids.

Poster NO. I-11

Directly coupled HPLC-NMR-MS approaches for the rapid characterisation of drug metabolites: application to the human metabolism of naproxen

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High resolution NMR spectroscopy is a very powerful tool for the structural identification of xenobiotic metabolites in complex biological matrices such as plasma, urine and bile. However, these fluids are dominated by thousands of signals resulting from endogenous metabolites and it is advantageous when investigating drug metabolites in such matrices to simplify the spectra by including a separation step in the experiment by directly-coupling HPLC and NMR. Traditionally peak picking in directly coupled HPLC-NMR experiments has been performed by means of UV-detection however adding an additional detection step namely MS allows the detection of compounds with no chromophoric groups. In addition the combination of NMR and MS is complementary in information content since the NMR spectra provide evidence to distinguish isomers such as the type of glucuronides formed, and the HPLC-MS data allow identification of molecules containing NMR-silent fragments such as occur in the sulfate ester.

HPLC-NMR-MS applications will be described and the application to the investigation of the metabolism of naproxen in urine samples with a very simple sample preparation. Naproxen (6-methoxy-a-methyl-2-naphthyl acetic acid) is metabolised *in vivo* to form its demethylated metabolite that is subsequently conjugated with b-D-glucuronic acid as well as with sulfate. Naproxen is also metabolised by phase II metabolism directly to form a glycine conjugate as well as a glucuronic acid conjugate at the carboxyl group.

Genetic Polymorphisms in the human CYP2C subfamily

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The human CYP2C subfamily consists of four members, all of which are polymorphic. CYP2C19 metabolizes mephenytoin, the proton pump inhibitor omeprazole, diazepam, and the antimalarial proguanil. 11 alleles of CYP2C19 have been previously reported including 9 defective alleles. Omeprazole is more effective in extensive metabolizers (EMs) than poor metabolizers (PMs). PMs require a lower dose of diazepam. New (8) CYP2C19 coding alleles will be reported and their effects on mephenytoin activity assessed *in vitro*. CYP2C8 is found in liver, heart, coronary vessels and aorta. CYP2C8 is proposed to be the endothelial derived hyperpolarizing factor synthetase (EDHF), which mediates vasodilatation. 20 SNPs were discovered in CYP2C8 including the upstream region, coding region, and introns. The SNPs show a complex linkage pattern. Two alleles contain coding changes: CY2C8*2 contains I269F and CYP2C8*3 contains R139K and K399R. CYP2C8*3 is defective in the metabolism of the anticancer drug taxol and an endogenous substance arachidonic acid.

CYP2C9 metabolizes many clinically important drugs such as warfarin, phenytoin, tolbutamide, glipizide, losartin and nonsteroidal antiinflammatory drugs. Some of these drugs (warfarin, phenytoin) have narrow therapeutic indices, and polymorphisms in CYP2C9 affect their clinical toxicity. At least four defective alleles of CYP2C9 have been previously reported. Our studies have identified a new null mutation that dramatically affects phenytoin toxicity in humans. We have also found 6 new alleles containing coding changes, and the catalytic activity of the new recombinant human CYP2C9 alleles toward tolbutamide will be assessed in a cDNA expression system.

Polymorphism in biotransformation: CYP2D6

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Cytochrome P450 2D6 (CYP2D6) is a polymorphic enzyme accounting for about 30 % of hepatic metabolism of clinically used drugs. The enzyme is mainly distributed in the liver and accounts for 3 % of all hepatic P450, but is also present in the brain to some extent. No endogenous substrate is known, but it has a high affinity for alkaloids. Seven per cent of the Caucasian population lack the enzyme because of inactivating gene mutations and are so called *Poor Metabolisers (PMs)*. In total more than 84 different allelic forms are known. In Asia, the general capability for CYP2D6 metabolism is decreased due to the frequent distribution of a partially defect allele. Some individuals,

preferentially in Ethiopia, Arabia and around the Mediterranean Sea, carry multiple gene copies causing increased metabolism and are called *Ultrarapid Metabolisers (UMs)*. Such subjects are frequently. At ordinary drug doses, UMs might not achieve therapeutic plasma levels and PMs are at risk for developing side reactions as a consequence of too high plasma drug levels. The lecture will give a state of the art situation regarding the functional importance of this enzyme and the impact of the polymorphism on drug therapy.

Polymorphisms of Human Arylamine N-Acetyltransferases

Grant DM, Orchid BioSciences Inc., Princeton, NJ 08540, USA

One of the earliest discovered pharmacogenetic polymorphisms of drug biotransformation is that affecting the acetylation of certain homo- and heterocyclic aromatic amines and hydrazines. This so-called acetylation polymorphism was first observed in the 1950s, based on its influence on the efficacy and toxicity of the tuberculostatic drug isoniazid, and has subsequently been shown to alter the disposition and effects of a variety of therapeutic agents and environmental carcinogens. The classical isoniazid acetylation polymorphism is now known to be based upon allelic variation in one of the two known human arylamine *N*-acetyltransferase genes, namely *NAT2*, which results in the production of NAT2 proteins with variable enzyme activity or stability. The *NAT1* gene locus encodes a structurally related enzyme, NAT1, with distinct catalytic specificity for arylamine acceptor substrates from that exhibited by NAT2. NAT1 function has also been shown to be genetically variable in human populations, as a result of allelic variation that produces proteins with defective enzyme activity. Consequences of variable drug acetylation capacity can range from variable dosing requirements and dose-related toxicity to altered drug selectivity and increased metabolic activation of procarcinogenic arylamines. Epidemiological studies to test the latter effects have produced variable results, suggesting that the effects of genetic polymorphism in metabolic activation are likely significantly influenced by issues of carcinogen exposure and other environmental and lifestyle factors.

Poster NO. I-1

CYP2C8 and CYP3A4 are the principle enzymes involved in the *in vitro* biotransformation of the insulin secretagogue repaglinide

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Repaglinide is a short acting, insulin secretagogue that is structurally unrelated to the sulphonylurea drugs. Repaglinide undergoes extensive hepatic metabolism, mainly by oxidative opening of the piperidine ring, with or without subsequent oxidative N-dealkylation, by hydroxylation of the piperidine ring, or by direct conjugation with glucuronic acid (Bauer E. et al; Diabetologia 1997,40(Suppl 1), A326). Less than 2% of the administered dose is excreted unchanged in humans (Van Heiningen PNM. et al; Eur. J. Clin. Pharmacol. 1999, 55, 521–5).

Metabolism of ^{14}C-repaglinide by recombinant cytochromes P450 and Human Liver Microsomes (HLM) from individual donors in the presence of inhibitory antibodies was performed, and metabolites were analysed by High-Performance Liquid Chromatography (HPLC) with on-line radiochemical detection and by Liquid Chromatography-Mass Spectrophotometry (LC-MS). Biotransformation by individually expressed P450 enzymes and the degree of inhibition in different HLM samples was measured and metabolites were identified.

CYP3A4 and CYP2C8 were found to be responsible for the conversion of repaglinide into two primary metabolites. Metabolites were shown to be formed by both CYP´s, but each enzyme contributed predominantly to one biotransformation pathway only. The possible consequence of this balanced CYP biotransformation in relation to clinical pharmacokinetics and drug-drug interactions of repaglinide is discussed.

Poster NO. I-2

Low doses of fluvoxamine inhibit the metabolism of both caffeine (CYP1A2) and omeprazole (CYP2C19)

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Fluvoxamine is metabolised by CYP2D6 and CYP1A2. Therapeutic doses of fluvoxamine inhibit CYP1A2 and CYP2C19. We used 5 extensive metabolizers (EM) and 5 poor metabolizers (PM) of debrisoquin (CYP2D6) and two probes, caffeine (CYP1A2) and omeprazole (CYP2C19) to investigate if low doses of fluvoxamine inhibit CYP1A2, but not CYP2C19. Single oral doses of 100 mg caffeine and 20 mg omeprazole were given separately, followed by one week daily doses (25 mg x 2 to EM and 25 mg x 1 to PM) of fluvoxamine and again caffeine (day 6) and omeprazole (day 7). The same protocol, but with fluvoxamine, 10 mg x 2 to EM and 10 mg x 1 to PM was then performed. Concentrations of all drugs were analysed by HPLC. At steady-state the 25/50 mg fluvoxamine dose caused an inhibition of both CYP1A2 and CYP2C19 indices by 75-80%. The inhibition after the 10/20 mg dose was 40-50%. It does not seem possible to selectively inhibit CYP1A2. One EM subject had a very low oral clearance of fluvoxamine and she might have a deficient transporter protein in the gut leading to an increased absorption of fluvoxamine. Other factors than metabolism might be more important for the disposition of fluvoxamine.

Poster NO. I-3

Involvement of multiple CYP450 isoforms in the oxidative metabolism of tamoxifen

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Tamoxifen (tam) is extensively metabolised in the human liver by cytochrome P450 (CYP450) isoforms. Previous studies investigating the formation of Z-4-hydroxy-tam (Z-4-OH-tam), the potent anti-estrogen metabolite, have reported varying roles for CYP2C8/9, 2C19, 2D6, 2E1, and 3A4. Furthermore, a detailed account of involvement of CYP450 in the formation of a-hydroxy- (a-OH-), N-desmethyl- and N-didesmethyl-tam and tam-N-oxide has not been reported. This study aimed to elucidate the role of CYP450(s) in the oxidative metabolism of tam. Data was complied from; 1. western blots of human liver microsomes (n=50); 2. microsomal metabolism of 10µM tam (1% dimethyl sulfoxide, n=50); 3. chemical and monoclonal antibody inhibition (n=6-10); and 4. metabolism of tam by expressed CYP450 (1-10µM, 1% dimethyl sulfoxide). Tam metabolites were quantified by LC-MS analysis. It is concluded that the predominant CYP450 mediating the metabolism of tam to a-OH-, N-desmethyl- and N-didesmethyl-tam was CYP3A4, and CYP2C19 and 3A4 had equal roles in the formation of tam-N-oxide. In contrast, the formation of Z-4-OH-tam was mediated predominantly by CYP2C9 and CYP2D6. As these isoforms both exhibit polymorphic expression, it is plausible that a large degree of variability in the ability to form Z-4-OH-tam, and subsequent therapeutic outcome may occur clinically.

Poster NO. I-6

3-Hydroxylation of quinine is the major metabolic pathway catalyzed by CYP3A4 in human liver microsomes

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The aim of this study was to investigate the importance of 3-hydroxyquinine, 2´-quininone, 10(R),11-dihydroxydihydroquinine and 10(S),11-dihydroxydihydroquinine in the metabolism of quinine *in vitro* and to study the involvement of CYP3A4 in these metabolic pathways. Incubations with human liver microsomes (n=6) were carried out at substrate concentrations of 1-400 umol/L. Michaelis-Mentens constant K_m, V_{max} and intrinsic clearance V_{max} / K_m were determined for each metabolic pathway. Inhibition studies were performed (n=4) using different concentrations of ketoconazole and troleandomycin at quinine concentrations of 20 and 50 umol/L, which are relevant to *in vivo*. The intrinsic clearance (V_{max} / K_m) for the formation of 3-hydroxyquinine, 2´-quininone, 10(R),11-dihydroxydihydroquinine and 10(S),11-dihydroxydihydroquinine was 22±7.8, 2.4±1.5, 3.2±2.7 and 1.4±1, respectively. At 10 umol/L of ketoconazole the 3-hydroxylation of quinine and the 2´-quininone formation was completely inhibited while the formation of 10 (S),11-dihydroxydihydroquinine and 10 (R),11-dihydroxydihydroquinine was inhibited by 74 and 40 % respectively. At 80 umol/L of troleandomycin the 3-hydroxylation of quinine was completely inhibited while the formation of 2´-quininone, 10 (S)-11-dihydroxydihydroquinine and 10 (R),11-dihydroxydihydroquinine was inhibited by 84, 69 and 22%, respectively. The results from this study show that quinine is mainly metabolized to 3-hydroxyquinine by CYP3A4.

Poster NO. I-7

Chiral aspects of flobufen biotransformation in primary cultures of human hepatocytes

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Flobufen (**F**), a new anti-inflammatory drug exhibits one chiral and one prochiral centre in its structure. Reduction, the principal biotransformation pathway of **F**, leads to the formation of four diastereoisomers of 4-dihydroflobufen (**DHF**).

Chiral inversion of **F** enantiomers and stereoselectivity and stereospecificity of **F** reductases were studied in primary cultures of human hepatocytes. The (+)-R-**F,** (-)-S-**F** and *rac*-**F** were used as substrates. Samples of culture medium were collected during 24 hours. The amount and ratio of **DHF** diastereoisomers and **F** enantiomers were assessed using HPLC, with (R,R)-ULMO column and column with terguride bonded.

Human hepatocytes significantly converted (+)-R-**F** to its antipode. Weak chiral inversion of (-)-S-**F** was also observed. From both **F** enantiomers the (+)-R-**F** was also predominantly metabolised in human hepatocytes. The (+)(-)-**DHF** was the main metabolite formed through reduction of all substrates. On the other hand (-)(+)-**DHF** was hardly detected. The changes in DHF diastereoisomers ratio during 24 h indicate not only the differences in their formation but also differences in their subsequent transformation.

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Poster NO. I-8

Reduction of the potential anticancer drug oracin in the rat gut lumen and wall

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Oracin is a potential cytostatic agent for oral use. The main *in vivo* and *in vitro* metabolite in all laboratory animals studied as well as in man is 11-dihydrooracin (**DHO**). Enantiomers of **DHO** are formed through the metabolic conversion of oracin prochiral centre on carbon 11.

Metabolism in the gut lumen and wall can make a variable contribution to the overall bioavailability and hence to the pharmacological activity of a wide range of drugs. To evaluate the extension and stereospecificity of oracin reduction to **DHO** in different parts of intestine we have isolated microsomal and cytosolic fractions from the small intestine, caecum and large intestine. Also intestinal bacteria have been incubated anaerobically with oracin. The amount of **DHO** enantiomers was measured by the HPLC method with Chiralcel OD-R as chiral column.

Reductive biotransformation of oracin is stereospecific; the chiral metabolites were formed in different rate in all models used. Stereospecificity for **(-)-DHO** (60%) was observed in bacterial oracin reduction in the lumen of small intestine, caecum and large intestine. On the other hand the high stereospecificity for **(+)-DHO** was found in intestinal cytosol; 82% **(+)-DHO** in small intestine and 80% **(+)-DHO** both in caecum and large intestine. Microsomal reduction in intestine was found to be lower than in cytosolic intestinal fraction and formation of **DHO** enantiomers was comparable.

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Poster NO. I-9

Losartan metabolism *in vitro*: The role of different genetic variants of CYP2C9

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Losartan is an angiotensin 2-receptor antagonist used in the treatment of hypertension and heart failure. Losartan is converted to an active carboxylic acid metabolite, E-3174, by CYP2C9 and CYP3A4 (Stearns *et. al*., DMD, 23:207). We have studied the relative importance of these P450 enzymes for losartan oxidation *in vitro*, and compared the kinetics of losartan oxidation between different genetic variants of CYP2C9. Yeast microsomes expressing CYP2C9.1, .2, or .3, and human liver microsomes defined for *CYP2C9* genotypes (**1*1* (n=9), **1*2* (n=5), **1*3* (n=4), **2*2* (n=4), **2*3* (n=2), **3*3* (n=1)) were incubated with losartan (0.05-50 mM) and E-3174 formation was analysed by HPLC. Sulfaphenazole, a specific inhibitor of CYP2C9, completely blocked the formation of E-3174 at low concentrations of losartan (up to 1 mM, corresponding to therapeutic plasma concentrations) while a partial inhibition by troleandomycin (a specific CYP3A4-inhibitor) was only detectable at high losartan concentrations (>25 mM). The intrinsic clearance (V_{max}/K_m) was markedly lower for CYP2C9.3 compared to CYP2C9.1 in both yeast and human liver microsomes (8- and 20-fold lower, respectively) while the activity of CYP2C9.2 was at an intermediate level. These *in vitro*-results indicate that CYP2C9 is the major enzyme for losartan oxidation, and that the *CYP2C9* genotype contributes to interindividual differences in losartan metabolism.

Poster NO. I-10

Effects of refampicin on the pharmacokinetics and pharmacodynamics of glibenclamide and glipizide

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Glibenclamide and glipizide are two commonly used second generation sulphonylurea antidiabetic drugs. They are both almost completely bioavailable from the gastrointestinal tract and are eliminated by metabolism by CYP2C9. The effects of rifampicin, a potent inducer of several CYP enzymes, on glibenclamide and glipizide were studied in two separate, randomized, two-phase cross-over studies with 10 healthy volunteers in each study. The volunteers received 600 mg rifampicin or placebo once daily for 5 days. On day 6, a single dose of 1.75 mg glibenclamide (study I) or 2.5 mg glipizide (study II) was administered orally. In study I, rifampicin decreased the total AUC of glibenclamide by 39% ($P < .001$) and the C_{max} by 22% ($P = .01$). The $t_{½}$ of glibenclamide was shortened from 2.0 to 1.7 h ($P < .05$) by rifampicin. The blood glucose decremental AUC (0-7) and the maximum decrease in the blood glucose concentration were decreased by 44% ($P = .05$) and 36% ($P < .001$), respectively, by rifampicin. In study II, rifampicin decreased the total AUC of glipizide by 22% ($P < .05$) and shortened the $t_{½}$ from 3.0 to 1.9 h ($P = .01$). No statistically significant differences in the blood glucose variables were found between the phases. In conclusion, rifampicin moderately decreased the plasma concentrations and effects of glibenclamide but had only a slight effect on glipizide. The mechanism underlying the interaction between rifampicin and glibenclamide may be induction of both CYP2C9 and P-glycoprotein. Induction of CYP2C9 would explain the increased systemic elimination of glipizide. It is probable that the blood glucose lowering effect of glibenclamide, and, in some patients, that of glipizide, is reduced during concomitant treatment with rifampicin.

Poster NO. II-1

CYP2D6 and risperidone metabolism in psychiatric patients

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The implication of the cytochrome P450 CYP2D6 enzyme activity in the metabolism of the antipsychotic drug, risperidone has been reported in vitro and in healthy volunteers. Around seven percent of Caucasians has inherited impaired capacity of this enzyme (poor metabolizers). The aim of the study was to determine the relationship of debrisoquine hydroxylation (CYP2D6 enzyme activity) and risperidone plasma levels in psychiatric patients. A population of forty Spanish and Hungarian schizophrenic patients was studied. The possible inhibition of CYP2D6 enzyme was also evaluated in a group of patients comedicated with inhibitors of CYP2D6. Risperidone/9-hydroxy-risperidone ratio correlated significantly to the CYP2D6 enzyme activity ($p<0.001$). In patients comedicated with strong inhibitors of CYP2D6 the plasma levels of risperidone were significantly higher and CYP2D6 activity was lower compared to patients with monotherapy ($p<0.01$). According to the data clinicians should be aware that poor metabolizers and patients comedicated with CYP2D6 inhibitors are prone to interactions, unexpected side effects or changes in the clinical efficacy. The evaluation of risperidone/9-hydroxy-risperidone ratio may reflect the actual enzyme activity of the CYP2D6 enzyme and therefore may help to assess potential pharmacokinetic interactions and to improve the balance between clinical efficacy and side effects during risperidone treatment.

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Poster NO. II-2

Polymorphic oxidative metabolism of proguanil (CYP2C19) in a Nigerian population

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Proguanil (PG) is widely used in the tropics for antimalaria prophylaxis. Its antimalarial activity depends on its metabolism to the therapeutically active metabolite, cycloguanil (CG). This oxidative metabolic reaction co-segregates with the genetically determined metabolism of S-mephenytoin, mediated by CYP2C19. Because of adverse side effects of mephenytoin and its unavailability in most countries, PG has been proposed as a more suitable probe for phenotyping purposes for CYP2C19.

Due to the limited and conflicting data on the genetic polymorphism of PG and CYP2C19 in Africans, we investigated the proguanil oxidation capacity in 126 healthy, unrelated Nigerians using the 8 h urinary metabolic ratio of PG to CG following administration of a single oral dose of 200 mg PG

The distribution of the PG metabolic ratio, which ranged from 0.01 to 39.64 with a median of 1.38 in the 126 subjects, was bimodal. On the basis of the antimode value of 10, for the PG/CG ratio, the prevalence of poor metabolizers (PM) of proguanil in this Nigerian population was estimated to be 4.8 % (6 out of 126). This is very similar to that of S-mephenytoin (4.3 %) found in a previous study in Nigerians and also compares favourably to those reported in Caucasians (3%), Zimbabweans (4%), Ethiopians (5%) but is much lower than those reported for Kenyans (35%) and Orientals (18%).

Our data demonstrated a clear distinction between extensive and poor metabolizers and supports previous studies that PG can be used to phenotype for CYP2C19 activity.

Poster NO. II-3

Pharmacokinetic consequences of *CYP2D6* genotypes with emphasis on gene duplication/amplification

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The relationship between the major Caucasian (including ultrarapid metabolisers) and Oriental *CYP2D6* genotypes and the disposition of two CYP2D6 substrates nortriptyline and debrisoquine was investigated. We conclude that in Caucasians the number of functional *CYP2D6* genes, and especially the presence of multiple functional *CYP2D6*-genes is of quantitative importance for the pharmacokinetics of drugs, whose metabolism is to a major extent catalysed by CYP2D6. The *CYP2D6*10*, which occurs at an allele frequency of 50 % in Asians, encodes an enzymes with decreased metabolism of nortriptyline and debrisoquine. In a pilot study Caucasian ultrarapid metabolisers with duplicated/multiduplicated *CYP2D6* genes received single oral doses of 5-160 mg quinidine followed by debrisoquine phenotyping. A dose-effect relationship could be established for quinidine with regard to the inhibitory effect on CYP2D6 activity. Ultrarapid metabolisers may develop low plasma concentrations and show poor response due to inadequate drug plasma concentrations despite normal or high doses of the drug assuming the parent compound is active. Such patients may also produce large amounts of metabolite(s), which, in case they are pharmacologically active, may contribute to the clinical effects of the parent compound. Metabolites may also produce toxicological effects or undesirable side-effects. The utilisation of drugs may be improved and made more rational by using both therapeutic drug monitoring, pheno- and genotyping tests to establish individualised dosage strategies. Further

prospective clinical studies with evaluation of clinical efficacy and adverse drug reactions in relation to different genotypes/phenotypes are required to improve such an adjustment of drug doses to the individual metabolic capacity.

Poster NO. II-5

Comparison of methods to estimate CYP2E1 activity in humans

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In 19 healthy volunteers CYP2E1 activity was determined by chlorzoxazone clearance and metabolite/drug plasma concentration ratio. Multisample oral clearance (Cl) and multisample fractional clearance (Cl_{fe}) were compared with one-time-point clearance estimation at 3, 4, 5 and 6 hours. Cl and Cl_{fe} were calculated from AUC, the latter by multiplying Cl and fe (fraction of dose recovered in urine). The concordance between Cl, Cl_{fe} and a single postdose measurement of plasma chlorzoxazone clearance were highest at 3 and 6 hours, respectively. The minimal mean prediction error (MPE) of clearance estimates from one-time-point measurements of plasma chlorzoxazone (Cl_{est}) as a percentage of actual mean Cl_{fe} or Cl was –0,9% at 3 and -16,2% at t=6 hours, respectively. We also compared metabolite/drug plasma concentration ratio at t=1,2,3,4,5, and 6 hours with metaboliteAUC/drugAUC ratio and Cl_{fe}. The best concordance was found at 2 (MPE=3,2%) and 3 hours (r=74; p<0,001), respectively. No significant differences were found between Cl_{fe} and Cl_{est} at 3 hours, between Cl and Cl_{est} at 6 hours, or between AUCs ratio and metabolite/drug plasma concentration ratio at 2 hours, suggesting that all three single dose-sample estimates can serve as markers of CYP2E1 activity. Cl_{fe} and consequently Cl_{est} at 3 hours is, however, a more direct measure of hydroxylating activity. In conclusion, Cl_{est} at 3 hours represents the best surrogate marker of CYP 2E1 activity.

Poster NO. II-7

CYP2D6 and CYP2C9 genetic polymorphisms in a Cuban population

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The genetic polymorphism of the cytochrome P450 enzymes is one of the major determinants of the interindividual and interethnic variability of pharmacokinetics and drug response. Genetic polymorphism is known for both enzymes, CYP2C9 and CYP2D6. The most extensively studied genetic polymorphism is that of CYP2D6. Two phenotypes have been described "poor metabolizers" (PM), and "extensive metabolizers" (EM). *CYP2D6* mutated alleles causing enzyme deficiency has been described. Of the various *CYP2C9* mutants, the variants *CYP2C9*2* and *CYP2*3* have been reported with altered catalytic activities compared to the wild type *CYP2C9*1*. Interethnic differences in cytochrome P450 polymorphism might be responsible, at least in part, for the variations in drug disposition between ethnic groups. The present study was aimed to analyze the CYP-enzymes polymorphism in a Cuban population. Among the 260 Cubans studied (196 females and 64 males) 4.8% were PMs of debrisoquine hydroxilation. Debrisoquine metabolic ratio among EMs seems to be similar in Cubans and Spaniards (0.8±1.2 vs 0.9±1.3, average±SD). The urinary metabolic ratios of diclofenac/3-OH, 4-OH and 5-OH diclofenac in 236 Cuban healthy volunteers (181 females and 55 males) were 7.9±4.5, 0.8±0.4, 2.7±5.9 respectively. Blood was obtained from 256 subjects. The analysis of *CYP2C9* and *CYP2D6* genotypes will be shown during the meeting

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Poster NO. II-8

The impact of CYP2C9 genotype on celecoxib metabolism in vitro

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Celecoxib, a selective COX-2 inhibitor, is converted by hepatic P450-enzymes to an inactive hydroxy-metabolite. We studied celecoxib hydroxylation in human liver microsomes in order to assess the relative contribution of CYP2C9 and CYP3A4, and evaluated the importance of different genetic variants of CYP2C9. Human liver microsomes with different CYP2C9 genotypes (**1*1* (n=12), **1*2* (n=6), **1*3* (n=7), **2*2* (n=4), **2*3* (n=2), **3*3* (n=1)) as well as *CYP2C19*1*2* (n=4) were incubated with celecoxib (1 or 20 mM) in the absence or presence of sulfaphenazole (a CYP2C9-inhibitor) or troleandomycin (a CYP3A4-inhibitor). Sulfaphenazole caused major inhibition of celecoxib metabolism in all liver samples (a mean inhibition by 70%), in contrast to troleandomycin. The rate of hydroxy-celecoxib formation was approximately 5-, 2- and 1.5- lower in liver microsomes with *CYP2C9*3*3*-, **1/*3*- ($p<0.01$) and **2*2*-genotypes ($p=0.15$), respectively, compared to *CYP2C9*1*1*. Celecoxib hydroxylation was also studied in yeast microsomes expressing CYP2C9.1, .2, or .3. The intrinsic clearance of celecoxib hydroxylation by CYP2C9.3 was approximately 3 times lower than CYP2C9.1 or CYP2C9.2. Further experiments with yeast microsomes expressing CYP2C19.1, revealed that this related P450 had an almost 10-fold lower affinity for celecoxib, compared to CYP2C9.1. In summary, the results obtained *in vitro* indicate that CYP2C9 is the major enzyme for celecoxib hydroxylation and that the CYP2C9.3-variant is associated with markedly slower metabolism.

Poster NO. II-9

Losartan and diclofenac metabolism in different genotyper of *CYP2C9*

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The aim of the present *in vivo*-study was to compare the kinetics of losartan and diclofenac in different genotypes of *CYP2C9*, and to evaluate if any of these drugs could be suitable as a CYP2C9 phenotyping agent. Losartan is converted to an active metabolite, E-3174, by CYP2C9, whereas diclofenac is subject to 4´-hydroxylation. At separate occasions, a single oral dose of losartan or diclofenac was given to healthy volunteers with *CYP2C9*1*1* (n=6), **1*2* (n=3), **1*3* (n=5), **2*3* (n=4), **2*2* (n=1) and **3*3* (n=1) genotypes. Losartan, E-3174, diclofenac and 4´-hydroxy-diclofenac were analysed by HPLC in plasma and urine samples collected up to 24 hours after drug intake. The plasma $AUC_{losartan}/AUC_{E-3174}$ ratios were higher not only in subjects with the *CYP2C9*2/*2* (4-fold), and **3/*3* (30-fold) genotypes, but also in heterozygous **1/*3* (1.7-fold), and **2/*3* (3-fold), as compared to **1/*1*. The plasma ratios correlated significantly with the 0-8 and 0-24 hour urinary losartan/E-3174 ratios (r_s=0.81; p<0.0001). No significant differences were found between the genotypes in diclofenac 4´-hydroxylation. These results suggest that especially *CYP2C9*3* but also *CYP2C9**2 are associated with decreased formation of E-3174 from losartan as compared to *CYP2C9*1*. The losartan/E-3174 ratio, but not diclofenac/4´-hydroxylation, in 0-8 hour urine might serve as a phenotyping assay for CYP2C9 activity *in vivo*. However, further studies in larger populations are required to validate this procedure.

Poster NO. II-10

Drug Metabolism: Genetic variants within the CYP3A locus and their functional significance

Hustert E, Domanski T, Eiselt R, Haberl M, Burk O, Klattig J, Zibat A, Koch I, Presecan-Siedel E, Wolbold R, Klein K, Nuessler AC, Neuhaus P, Zanger U, Brockmöller J, Klenk HP, Meyer U, Khan K K, He Y, Halpert J & Wojnowski L, Epidauros Biotechnologie AG, Bernried, Germany.

Members of the CYP3A subfamily of cytochrome P450 proteins metabolize more than 50% of currently used drugs. CYP3A activity exhibits a significant inter-individual variability which may lead to harmful drug interactions in development and application of drugs that are CYP3A substrates. The genetic component of the variability is significant but the underlying genetic factors remain largely unknown. To establish a basis for a screen for *CYP3A* genetic variants, we have recently determined the genomic organization of the *CYP3A* locus. A screen in *CYP3A4* revealed seven new protein variants of CYP3A4. Among them, two mutants, R130Q and P416L, did not result in detectable P450 holoprotein when expressed in a bacterial heterologous expression system. One mutant, T363M, expressed at significantly lower levels than wild-type CYP3A4. L373F displayed a significantly altered testosterone metabolite profile and a four-fold increase in the K_m value for 1´-OH midazolam formation. In a second screen, we investigated the expression of CYP3A5 and its genetic determinants. In Caucasians, CYP3A5 expression is elevated in 10% of livers where it accounts on average for a quarter of the combined CYP3A5 and CYP3A4 protein pool. Genotyping these liver samples led to the identification of several markers co-segregating with the increased CYP3A5 expression. Altogether, these results will aid in efforts to elucidate genetic traits responsible for the variable expression of CYP3A proteins.

BENZON SYMPOSIUM No. 48

DRUG METABOLISM: REGULATION AND IMPORTANCE

SEPTEMBER 16-20, 2001, COPENHAGEN, DENMARK

Organizing committee:
Henrik Enghusen Poulsen, Kim Brøsen, Steffen Loft, Urs Meyer, F.F. Kadlubar, P.F. Guengerich, Sven Frøkjær and Arne Svejgaard

Abstracts - TUESDAY, September 18, 2001

Molecular mechanisms of CYP induction
Gustafsson J-Å, Karolinska Institute, Huddinge Univeristy Hospital, Huddinge, Sweden.
Abstract not received.

Phase II Enzymes: Regulation, Chemistry of Inducers, Protective Functions
Talalay P, Department of Pharmacology, Johns Hopkins School of Medicine, Baltimore, MD 21210 USA.
The two sequential Phases of xenobiotic metabolism comprise a functionalization (Phase I, cytochromes P450) followed by conjugation with endogenous ligands (Phase II) such as GSH or glucuronic acid. Both classes of enzymes are highly inducible, are regulated by distinct mechanisms, and serve different cellular functions. In light of mounting evidence that induction of Phase II proteins provides powerful protection against both electrophile toxicity (including carcinogenesis) and damage by reactive oxygen species, understanding of the chemistry and molecular mechanisms of their regulation has assumed increasing importance. Monofunctional Phase II inducers belong to 9 chemical classes (e.g., isothiocyanates, Michael reaction acceptors, oxidizable diphenols), some of which are present in considerable concentrations in edible plants, and may account at least partly for the chemoprotection afforded by high vegetable consumption against the risk of malignancy. Measurement of quinone reductase inducer potency has led to the isolation from broccoli/sprouts of the isothiocyanate sulforaphane as an extremely potent inducer and inhibitor of tumor formation in animal models. The genes for many Phase II proteins contain Antioxidant Response Elements (ARE) in their upstream regions. The molecular basis for protection by Phase II enzymes was recently demonstrated in mice in which the transcription factor Nrf2 was deleted (T.W. Kensler and M. Yamamoto). Nrf2 is normally tethered in the cytosol to the chaperone Keap 1, but this combination is disrupted by Phase II inducers, resulting in the translocation of Nrf2 to the nucleus where in dimeric form with other transcription factors, it binds to the ARE element and signals enhanced transcription of Phase II proteins. In the Nrf2 knockout mice, tissue levels of Phase II enzymes are low and essentially uninducible. Moreover, these mice are particularly susceptible to benzo[a]pyrene forestomach carcinogenesis, and unlike their wild-type counterparts cannot be protected by Phase II inducers.

Induction of Glucuronidation
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Glucuronidation is arguably the most important Phase II drug metabolising mechanism in man. Therefore, an understanding of the regulation of drug glucuronidation by drugs and hormones is imperative for drug development. Glucuronidation is catalysed by a family of enzymes of similar architecture encoded by a multi-gene family. The UDP-glucuronosyltransferases (UGTs) are located in tissue endoplasmic reticulum associated with substrate and product transporters, which are essential for glucuronidation. Glucuronidation does have a profound effect on drug metabolism as observed in resistance to anticancer drugs or drug-drug interactions.

Dramatic induction of phenol UGTs by phenobarbital was observed in rat and chicken liver in the late 60's. Subsequently, Bock *et al* (1973) demonstrated differential induction of UGT activities in rat liver by 3-MC and phenobarbital. These results are reproduced by using H4IIE cells.

Studies of man have not proved to be simple because many xenobiotics seem capable of inducing UGTs. For example, alcohol, smoking, phenobarbital and cimetidine all seem to be inducers of bilirubin UGT. Similarly, several enzymes appear to be induced by the same inducer. Subsequently, the regulation of UGTs has been studied in human hepatocyte cultures where some differential regulation can be demonstrated.

Work has been done to study the regulation of the human UGT-1 gene by xenobiotics. The effects of clofibrate and cotransfection with PPAR have been evaluated. More recently, phenobarbital induction of bilirubin UGT has been suggested to be mediated through the nuclear receptor CAR by Negishi's team. We are trying to develop an *in vitro* model to study the regulation of the UGT-1 gene by xenobiotics, which will be described.

Poster NO. III-4

Characterisation of factors affecting CYP2E1 transcription

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Transcription of C*YP2E1* gene is believed to be activated mainly by the hepatocyte nuclear factor 1α (HNF1α). Otherwise little is know of the factors affecting transcription of this gene. In the current study we elucidated both the mechanism of constitutive transcriptional regulation of *CYP2E1* gene as well as the influence of factors present in patophysiological conditions.

Inflammatory process downregulates CYP2E1 expression. Furthermore several cytokines are assumed to participate in this process and are indeed know to regulate CYP2E1. We have studied the mechanism of action of these inflammatory cytokines and show that TNFα, IL-1β, IL-6 all affect the transcription of *CYP2E1* and that IL-6 acts through a mechanism distinct from the two others. Further, we have characterised the mechanism by which the anti-inflammatory cytokine IL-4 induces *CYP2E1* transcription and have identified an IL-4 response element in the *CYP2E1* promoter.

We have also characterised the factors affecting the constitutive regulation of *CYP2E1*. We show that the *CYP2E1* core promoter is an active target of transcriptional regulation and that nucleotides flanking the TATA box have critical role in this process.

Poster NO. IV-1

The Influence of St. John's Wort Extract (SJW, Esbericum® capsules) on the Pharmacokinetics of Alprazolam, Caffeine, Tolbutamide and Digoxin in Human

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Two monocentric, double-blinded, randomised, placebo-controlled, and parallel-grouped studies (a/b) were performed. 28 healthy male and female, non smoking volunteers (age 18-55) were enrolled in each study. Single doses of caffeine and alprazolam in study a, multiple doses of digoxin and single doses of tolbutamide in study b, were given on day 1. Then volunteers received SJW or placebo for 10 days. At day 11 single doses of caffeine, alprazolam, and tolbutamide and digoxin, respectively, and study medication were given. On days 1, 2, 4, 7 and 11 blood sampling for pharmacokinetic analysis was performed. Confirmative statistical analysis was done for the intra-individual differences of AUC_{0-24} on day 1 and 11, for Esbericum® capsules and for placebo treatment. Descriptive statistical analysis was done for all other parameters. No statistically significant differences were found in any pharmacokinetic parameter for alprazolam, paraxanthine, digoxin, tolbutamide and hydroxytolbutamide. A statistically trend was found in AUC ($p=0.066$) and $c_{max.}$ ($p=0.053$) for caffeine, these differences were less than 10% of the absolute value, thus not clinically relevant. Pharmacokinetics of alprazolam, caffeine, tolbutamide and digoxin are at most marginally influenced by comedication with Esbericum® capsules. Clinically relevant interactions with Cytochrome P450 enzymes 3A4, 1A2, 2C9 and p-glycoprotein activity may be excluded for the SJW extract in Esbericum® capsules.

Poster NO. IV-9

EUROCYP: *In Vitro* approaches to predict drug metabolism and interactions in man in drug development

Pelkonen O, University of Oulu, Oulu, Finland and the EUROCYP team.

The main objective of the project was to improve the design and application of *in vitro* methods for predicting the metabolic behaviour of new chemical entities (NCEs) during drug discovery and development Candidate systems included 1) homology models of P450 enzymes, 2) recombinant expressed P450 enzymes, 3) hepatic microsomal fractions, 4) cultured hepatocytes (also some permanent) and 5) precision cut liver slices. Wherever possible, human liver-derived systems were used.

The systems were characterised systematically for their substrate specificity and kinetics, level of expression of CYP enzymes, at both the RNA and protein level, and their responses to inducing compounds, as appropriate. Where necessary, specific reagents, such as enzyme-specific probes and antibodies, were developed for these purposes. A unique aspect of the study was the rigorous "blinded" evaluation of the routes, rates and specificity of metabolism and drug interactions of four "unknown" compounds supplied by industrial partners (almokalant, carbamazepine, carvedilol, selegiline). Comparison of the *in vitro* data with observations obtained *in vivo* revealed that the candidate systems performed generally satisfactorily in prediction of various aspects of *in vivo* behaviour of the four compounds. The applications for which the different systems are most suited were established. The project provided a pre-validation step for further development and testing of an optimal (integrated) approach, which will allow the effective prediction of the metabolism of drugs *in vivo* during drug discovery, during safety evaluation of NCEs and in the design and interpretation of early clinical trials.

Poster NO. VI-5

The importance of genetic polymorphisms (GSTM1 & NAT2) in urban air exposure and chromosomal aberrations

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Genetic polymorphisms in metabolism of environmental toxicants plays a significant role in exposures to traffic generated air pollution in Copenhagen, revealing statistically significant higher levels of chromosomal aberrations in non smoking bus drivers and mail carriers with Glutathion-S-transferase M1, *GSTM1* null and N-acetyltransferase 2, *NAT* 2 slow genotypes.

Our studies included blood samples from non-smoking Danish bus drivers (a total of 106 persons) and mail carriers (a total of 102 persons). Subdividing the groups according to the polymorphisms of *GSTM1* and *NAT*2 showed approximately CA levels twice the level in persons with both genes wild type. This association was statistically significant in both subgroups of bus drivers and mail carriers with *GSTM1 null/NAT2 slow* compared with groups with *GSTM1/NAT2 fast*.

In vitro techniques

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Drug metabolism is an important part of drug effect and toxicity. Therefore, its knowledge is important early in the development of drugs, in the prediction of drug efficacy and toxicity. However animal models are not very good predictors of human metabolism since metabolism is quite different between animals and humans. Therefore the understanding of drug metabolism make necessary the use of *in vitro* methods in human in order to predict the metabolites which will be produced, the enzymes responsible for this production and the potential variability in individual variability.

Many tools are necessary to decipher these metabolic pathways in human: human hepatocytes, human liver microsomes, pure enzymes, antibodies, specific chemical inhibitors… Most of them are now easily available, some of them commercially. Pure enzymes are now obtained by the use of heterologous expression of human cDNA in cell systems such as bacteria, yeast, insect cells infected by baculovirus or mammalian cells. Many questions may be addressed with such tools:

Which are the metabolites produced from a drug? by which enzymes? What is the variation of expression of an enzyme in human tissues? What are the substrates and inhibitors of an enzyme? What is the inducing capacity of a drug? What are the interactions between two drugs (induction, inhibition)? Many of these questions got clear answers in many instances. However the interpretation of the results needs caution since many pitfalls are encountered. Several approaches should be used in order to get a reliable information, the *in vitro* enzymology can be quite different from the *in vivo* situation. In general, the qualitative information are quite reliable as well as the prediction of drug-drug interactions. The quantitative extrapolations are less sure.

The cell systems (human hepatocytes, transfected cells…) can be used to understand and to predict the inducing properties of drugs and their mechanisms of action; moreover they can be used also to understand the physiologic and toxic effects of drugs at cellular level.

Finally the *in silico* methods are now appearing and begin to bring informations. In this lecture the tools their use and their limits will be described, discussed and illustrated by examples.

In Vivo Assessment of CYP3A4

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Midazolam (MDZ) and erythromycin (used in a breath test, ERMBT) have been the most widely employed in vivo CYP3A4 probes. Because ERM is a substrate for Pgp, whereas MDZ is not, it has been proposed that MDZ is a more "pure" CYP3A4 probe. The fact that the ERMBT predicts clearance of docetaxel and cyclosporine (CsA) is then attributed to the observation that like ERM, docetaxel and CsA are substrates for both Pgp and CYP3A4. However, low liver Pgp activity should result in a higher ERMBT result (analogous to the Pgp "knock out" mouse data [*Molecular Pharmacology* 2000; 58: 863-9]) whereas low Pgp should cause reduced clearance of taxotere and CsA. Thus, variation in liver Pgp activity should have opposite effects on the ERMBT result and clearance of Pgp substrates. Based on the mouse knock out studies, Pgp appears to control the flux of erythromycin across the hepatocyte, but may not influence the plasma: hepatocyte gradient (perhaps consistent with Pgp's canalicular location). Pgp may therefore influence the ERMBT result (20 minute breath $^{14}CO_2$) only to the extent that plasma level of ERM was influenced during the first 20 minutes after injection. This may explain why there was only a 60% mean increase in the $^{14}CO_2$ exhalation during the first 30 minutes after injection of radiolabeled ERM in knock out relative wild type mice. The basis for the discordance between ERMBT and MDZ tests remain unclear, but it is interesting to note that in inhibition studies performed with human liver microsomes, kinetic parameters obtained with erythromycin, testosterone and cyclosporin A track together but are different from results obtained with midazolam,

triazolam, diazepam and dextromethorphan, which also track together (Br J Clin Pharmacol 1999; 48: 716-27). The observations in vivo may therefore have an as yet unexplained in vitro correlate.

From enzyme kinetics to pharmacokinetics

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In predicting quantitative aspects of drug metabolism in man, we are confronted with the issue of using *in vitro* enzyme kinetic data obtained using, for example, human liver microsomes or hepatocytes and enzyme expression systems to extrapolate to whole body pharmacokinetics. Thus, in addition to the biochemical data, there is a need to factor in physiological parameters in defining a structural model for the kinetic phenomena. The process may require either only a model of eliminating organ clearance (*e.g.* a "well-stirred liver") if predicting steady-state conditions, or a fuller physiologically - based pharmacokinetic model to assess concentration – time profiles. In addition, since it is desirable that inter-subject variability is accommodated, a Monte Carlo approach is appropriate. To this end, we have been developing "SIMCYP", a Windows – based software that uses demographic, physiological and genetic information, along with data on *in vitro* enzyme kinetics and the relative abundance of cytochromes P450 (CYPs), to generate distributions of drug clearance in virtual populations, and to simulate the potential impact of drug – drug interactions and ethnic variation. Further linkage to pharmacodnamic models allows, for example, the assessment of genetic polymorphism with respect not just to pharmacokinetic behaviour but also to therapeutic utility. Thus, by summarizing experimental data in the context of the current state-of-the-science at the interface between pre-clinical and clinical drug development, a simulation approach has the potential to integrate understanding, to aid in the design of *in vivo* experiments, and to put variability in drug metabolism into pharmacological and toxicological perspective.

Poster NO. III-1

The effect of dexamethasone on UGT1A1 and UGT1A6 activity induced by 3-methylcholanthrene, clofibrate and rifampicin in cultured rat hepatocytes

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Induction of UDP-glucuronosyltransferases UGT1A1 and UGT1A6 toward bilirubin and p-nitrophenol, respectively by methylcholanthrene (MC), dexamethasone (DEX), clofibrate (Cl), rifampicin (RIF) and MC and Cl combined with DEX was studied in cultured rat hepatocytes. RIF, Cl and DEX increased the activity of bilirubin-UGT after 96 hours of induction. In the case of DEX the rate of induction was concentration dependent. Combination of DEX and Cl showed additive effect, enzyme activity was enhanced compared to either DEX or Cl treatment alone. Both MC and DEX enhanced the paranitrophenol- UGT activity. Combination of MC with DEX resulted in additive effect. Cl treatment did not affect the conjugation of paranitrophenol either alone or in combination with MC. Western blot analysis revealed that only the amount of UGT1A1 was elevated by RIF, Cl and DEX. In contrast concentration of UGT1A6 was increased by MC.

It is well documented that DEX potentiates the inductory effect of PAH on UGT1A6. In our study DEX increased the rate of Cl and RIF induction on bilirubin-glucuronidation as well, suggesting, that glucocorticoids play roll in the regulation of UGT1A1 too, as has been demonstrated in the case of CYP2C induction by RIF.

Poster NO. III-2

Inhibition and induction of 24-hydroxylase (CYP24) in vitamin D_3 target cells

Kissmeyer AM, Mørk Hansen C, Holm, PK, Løgsted Nielsen J & Binderup L, Leo Pharmaceutical Products, Ballerup, Denmark.

CYP24 is a mitochondrial cytochrome P450 enzyme that is present in a number of vitamin D target cells (e.g. kidney, bone, cancer and skin cells). CYP24 is the major enzyme involved in the catabolism of $1\alpha,25(OH)_2D_3$ (the physiological active metabolite of vitamin D_3). A high activity of CYP24 in target cells is suggested to suppress the biological activity of $1\alpha,25(OH)_2D_3$. It was recently demonstrated that inhibition of CYP24 increased the biological effect of $1\alpha,25(OH)_2D_3$ in a prostate cancer cell line. We have studied the effect of ketoconazole (a cytochrome P450 inhibitor) both on the level of $1\alpha,25(OH)_2D_3$ and on cell growth using 3 cell lines representing different human vitamin D target cells (HaCaT (keratinocytes), MG-63 (osteosarcoma) and MCF-7 cells (breast cancer). After 5 days' incubation, we found higher concentrations of intact $1\alpha,25(OH)_2D_3$ in the ketoconazole treated cells combined with an increased antiproliferative effect of $1\alpha,25(OH)_2D_3$. Beside being a substrate for CYP24 $1\alpha,25(OH)_2D_3$ can easily induce CYP24, and consequently the biological activities of $1\alpha,25(OH)_2D_3$ is further decreased. To study the induction of CYP24 in target cells, we have set up an assay in MG 63 cells. After 24 hours preincubation with $1\alpha,25(OH)_2D_3$, $25OHD_3$ was added, and following another 24 hours, the relative concentration of $24,25(OH)_2D_3$ (a measure for CYP 24 activity) was 3-fold higher in the $1\alpha,25(OH)_2D_3$ pretreated cells compared to vehicle treated cells. The present models are useful for studying inhibition and induction of CYP24 by either $1\alpha,25(OH)_2D_3$ or by synthetic vitamin D analogs.

Poster NO. IV-2

Isolated Hepatocytes: A valuable tool to study the metabolism of NCE and to "predict" the excretion route of their metabolites

Badolo, L, H. Lundbeck A/S, Valby, Denmark:

Lu 28-179 is a compound in development in Lundbeck for treatment of anxiety. Previous studies performed in vivo (rats, human and dogs) have shown that, Lu 28-179 was extensively metabolised. The metabolism involved several phase I oxidation steps as well as Phase II conjugation with glucuronic acid. The extensive biliary excretion of numerous metabolites suggests that the liver is the major site of metabolism.

Objective: In order to set up hepatocytes as an valuable model for metabolism study, Lu 28-179 was incubated with freshly isolated rat hepatocytes and cryopreserved rat and human hepatocytes in suspension for short-term incubation (3 hours) and rat hepatocytes in culture for long-term study (24 hours). The major metabolites were identified and semi quantified with LC-MS.

Results: The results showed that hepatocytes extensively metabolised Lu 28-179 into 9 of 10 metabolites identified from previous in vivo and ex vivo experiments. The major metabolites were similar to those obtained in vivo.

The metabolic profiles in rat hepatocytes (suspension test and cultured cells) matched very well human hepatocytes metabolism.

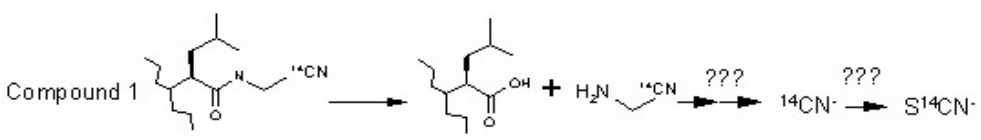
Conclusions: The in vivo versus in vitro correlation showed that hepatocytes would have been able to highly predict the metabolism (both phase 1 and phase 2) of Lu 28-179 in vivo. Furthermore, they might have allowed speculating on the routes of excretion of metabolites based on their distribution between supernatants and cell pellets.

Poster NO. IV-3

Elucidation of the cyanide/thiocyanate metabolite produced upon metabolism of a drug candidate containing a peptide-acetonitrile side-chain

Chauret N[1], Oballa R[1], Bateman K[1], Day S[1], Falgueyret JP[2], Li C[1], Percival D[2], Silva J[1] & Nicoll-Griffith DA[1], Department of [1]Medicinal Chemistry and [2]Biochemistry, Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec.

Understanding metabolic pathways of new drug candidates is an important aspect of drug discovery. This presentation will describe the various *in vivo* and *in vitro* studies that were performed to elucidate an important metabolic route of Compound 1. Pharmacokinetic studies in rat showed that, upon dosing with ^{14}C Compound 1, a long-lived radioactive metabolite was detected in the supernatant obtained from protein precipitated plasma samples. *In vitro* experiments showed that Compound 1 was extensively metabolised in rat hepatocytes, mainly to the acid indicating that the amino acetonitrile side-chain, containing the ^{14}C, was lost upon metabolism. Additional experiments were performed to elucidate if the amino-acetonitrile side-chain could undergo further metabolism to release cyanide. Based on the physical properties, as well as MS data, it was found that the metabolite responsible for the long-lived radioactivity was in fact, thiocyanate, which is probably produced from cyanide by rhodanese, a cytosolic enzyme. This presentation will focus on the work that has led to the elucidation of the thiocyanate metabolite and will also describe a fluorimetric assay that was developed to monitor cyanide level in blood samples obtained from animals that had been dosed with Compound 1.



Poster NO. IV-4

Correlation between caffeome metabolic indices in 12 hours urine samples, spot urine and plasma samples

Damkier P & Brøsen K, Clinical Pharmacology, Institute of Public Health, University of Southern Denmark, Odense, Denmark.

Background: Various metabolic indices of caffeine metabolism in plasma and urine have been used to assess P4501A2 activity. Plasma ratios of 1.7-dimethyl-xanthine to caffeine in plasma samples six hours after an oral administration of caffeine provides the best validated estimate. We wanted to assess if urine ratios in a 12 hour sample or in a spot urine sample provided a reliable estimate of CYP1A2 activity.

Methods: Fourty-two healthy young male volunteers were ad-ministered 200 mg of caffeine orally. A ten ml plasma blood sample was drawn six hours after ad-ministration of caffeine. Urine were collected un-til twelve hours after administration of caffeine, with the exeption of a 10 ml spot urine sample voided after six hours. The volunteers refrained from intake of xanthine containing foods during the study.

Results: Urine ratios of (1-methylxanthine + 1-methyluric acid + 5-acetylamino-6-formylamino-3-methyluracil) to 1.7-dimethylxanthine correlated to the ratio of 1.7-dimethylxanthine to caffeine in plasma with Spearman's rank

correlation coefficients of 0.68 [95% CI 0.58-0.78] and 0.71 [95% CI 0.62-0.78] for spot urine and 12 hour urine sample, respectively.
Conclusion: A 12 hour urine sample or a spot urine sample six hours after oral administration of caffeine produces reasonable estimates of CYP1A2 activity in young healthy males.

Poster NO. IV-5
CYP2C9 genotype and diclofenac hydroxylation in a Spanish population
Dorado P, Norberto MJ, Berecz R, Martínez M, De la Rubia A, Yasar Ü, Dahl M-L, LLerena A. Department of Pharmacology and Psychiatry, University of Extremadura. Spain. Division of Clinical Pharmacology, Karolinska Institute, Stockholm, Sweden.
One of the major determinants of the interindividual variability of pharmacokinetics and drug response is the genetic polymorphism of the drug metabolizing cytochrome P450-system enzymes. CYP2C9 seems to be implicated in the metabolism of several important drugs. Previously, we have shown the genetic polymorphism of CYP2D6 among Spaniards. The present study is aimed to analyze the CYP2C9 polymorphism in a Spanish Population. Diclofenac hydroxylation and *CYP2C9* genotype was studied in a population of 250 healthy volunteers, 129 females and 121 males, mean age 26,9 years. The urinary concentrations of diclofenac and its main metabolites 4-OH, 3-OH and 5-OH after a single oral dose of 50 mg were analyzed by HPLC with a newly developed method. The diclofenac/4-OH diclofenac metabolic ratio seems to be related to *CYP2C9* alleles, however 3-OH and 5-OH ratios were not. The diclofenac/4-OH ratio was slightly higher among subjects carrying the *CYP2C9*3* allele (0.9±0.5, mean±SD, n=16) compared to *CYP2C9*2* (0.8±0.4, n=24) and *CYP2C9*1* (0.7±0.4, n=78). The *CYP2C9* allele frequencies found among 119 Spaniards are similar to those in previously published populations: 78.1%, 13.9% and 8% for *1, *2 and *3 alleles, respectively.
Supported partly by grants from Junta de Extremadura, Consejeria de Educacion, Ciencia y Tecnología IPRI98B006.

Poster NO. IV-6
Determination of NAT2 Phenotype From Dietary Caffeine Metabolites Using LC-MS/MS
Kinzig-Schippers M[1], Illauer M[1], Jetter A[2], Hermann R[3], Erb K[3], Borlak J[4], Sörgel F[1] & Fuhr U[2], [1] Institute for Biomedical and Pharmaceutical Research, Nürnberg-Heroldsberg; [2] Institute of Pharmacology, Clinical Pharmacology, University of Köln; [3] ASTA Medica AG, Frankfurt/Main; [4]ITA Fraunhofer-Institut, Hannover; Germany.
Urinary concentration ratios of caffeine metabolites following a caffeine test dose are used for *N*-acetyltransferase type 2 (NAT2) phenotyping. Problems of this method include the need for methylxanthine abstinence and difficult metabolite quantification by HPLC. In the present study, a novel LC-MS/MS method was developed to measure urinary concentrations of 5-acetylamino-6-formylamino-3-methyluracil (AFMU), 5-acetylamino-6-amino-3-methyluracil (AAMU), 1-methylxanthine (1X) and 1-methylurate (1U). The limit of quantification (LOQ) was 10 ng/ml. As a NAT2 metric, the molar ratio (AFMU+AAMU) / (1X+1U+AFMU+AAMU) was determined in samples taken prior to a 150 mg caffeine test dose given after overnight methylxanthine abstinence and 4-6 hours thereafter in 59 healthy male and female volunteers. In postdose samples, a bimodal distribution was determined using a maximum likelihood procedure, with ratios <0.21 for poor and >0.32 for extensive metabolisers, respectively. Genotyping limited to NAT2*4, *5A, *5B, *5C *6A, *7A in 15 and to NAT2*4, *5A, *6A, *7A in further 8 individuals confirmed the phenotyping results. Predose AFMU and AAMU concentrations above LOQ originating from dietary caffeine sources enabled calculation of NAT2 ratios in 47 volunteers. Interestingly, predose ratios were closely correlated to those from postdose samples ($r = 0.891$, $p<0.001$). NAT2 ratios could even be measured in infants suckled by a mother drinking coffee. Thus, NAT2 phenotyping is feasible using urinary metabolic ratios from dietary caffeine sources.

Poster NO. IV-7
Prediction of the human clearance of an acidic drug from pre-clinical and *in vitro* data: Studies with the nsaid lornoxicam.
Kohl C, Kocian M, Steinkellner M & Palmetzhofer H, Drug Metabolism, Sandwich Laboratories, Pfizer Global R&D, Sandwich, UK.
Reliable prediction of human clearance from early *in vitro* and pre-clinical data is important for the selection of drug candidates. Earlier work has shown that the prediction of acidic compounds is particularly unreliable (Obach; *Drug Met. Dispos.* **27**, 1350, 1999). Lornoxicam is a relatively lipophilic acidic enol that is completely ionised at pH 7.4 and predominantly metabolised by CYP2C9 in humans. The clearance of lornoxicam in humans was predicted from allometric scaling of its total and unbound clearance in mouse, rat, dog and monkey. In addition, human clearance was calculated from human liver microsomal data. The *in vitro* disappearance $t_{1/2}$ of lornoxicam and the Cl_{int} of the formation of 5'-hydroxy lornoxicam, the major metabolic pathway of lornoxicam in humans, was used in these predictions. The influence of plasma protein binding and microsomal binding on the calculations was investigated. Allometric scaling of total and unbound clearance of the pre-clinical species underpredicted the human clearance by about tenfold. Scaling of in *vivo* Cl_{int} from rat to human allowing for the difference in *in vitro* metabolic rates yielded

even greater underestimation of human clearance. Direct projection of *in vivo* clearance from *in vitro* data achieved the best results when the disappearance half-life of lornoxicam in liver microsomes was used and the free fraction in plasma and in liver microsomes was taken into account. This method yielded underpredictions of about twofold, irrespectively of the well-stirred or the parallel tube model of hepatic clearance being used. This study highlights species differences in the clearance of acidic compounds metabolised by CYP2C9 in humans. It is an example of the superiority of human or humanised *in vitro* systems over animal models in the prediction of human clearance.

Poster NO. IV-8

Evaluation of the value of quinine as phenotyping probe for CYP3A4

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It has been shown that the metabolism of quinine to 3-hydroxyquinine is catalyzed by CYP3A4 both *in vitro* and *in vivo*. We evaluated the value of quinine as an *in vivo* probe drug for CYP3A4 activity. In a randomized three-way crossover study 9 healthy Swedish volunteers were given a single oral dose of quinine (500mg), quinine plus ketoconazole, (100 mg twice a day for three days) and quinine plus fluvoxamine (25 mg twice a day for two days), on three different occasions. Blood and urine samples were collected before quinine intake and up to 96 h thereafter. They were analyzed for both quinine and 3-hydroxyquinine using HPLC. The ratio of the plasma concentration of quinine to 3-hydroxyquinine was determined at 24, 32, 48, 72 and 96h after quinine intake. The quinine to 3-hydroxyquinine ratio was found to be constant for each subject at the different time points. There was a significant increase ($p= 0.006$) in mean ratio at 24h from 5.8 when quinine was given alone to 12.2 when the drug was given together with ketoconazole. When quinine was given with fluvoxamine the mean ratio at 24h was unchanged.

In conclusion the 24h plasma sample can be used for estimation of CYP3A4 activity. These results need to be confirmed in a defined population pharmacokinetic study and preferably by linking phenotype to genotype.

Poster NO. IV-10

Prediction of clearance from *in vitro* metabolism data for a set of benzamides and nicotinamides

Tuvesson H, Halvarsson L, Hallin I, Ellman M, Svanström C, Gunnarsson PO, Birgitta Eriksson B & Seidegård J, Pharmacokinetics and Metabolism, Active Biotech Research AB, Lund, Sweden, Department of Cell and Molecular Biology, Lund University, Sweden.

In vitro drug metabolism screening has become an important tool in the selection of new drug candidates. Metabolism data from such studies can be used to predict the pharmacokinetics and facilitate the selection of drugs with acceptable pharmacokinetic profiles for further development.

The aim of this study was to use metabolic half-life data, obtained from microsomal incubations, to predict clearance (CL_{Int}) for a number of benzamides and nicotinamides, which are chemical entities under evaluation for possible therapeutic use in inflammatory diseases.

The metabolic stability of the compounds investigated varied greatly. A comparison of the CL_{Int} values with the clearance values obtained *in vivo* demonstrated a good correlation for many of the tested compounds. However, for some compounds, the CL_{Int} data failed to predict clearance *in vivo*, and additional studies were performed to investigate other possible elimination pathways. Studies using plasma and whole blood demonstrated that deacetylation was the major route of elimination for these compounds.

The elimination of many drugs is considered to be controlled by hepatic clearance, and liver microsomal systems are widely used for metabolism screening. However, this study demonstrates the limitations of such systems for compounds eliminated primarily by extrahepatic metabolism.

Poster NO. IV-11

A combination of *in vitro* and *in vivo* drug metabolism studies to guide selection of species for toxicological evaluation of new drug candidates before first human dose

Vanggaard J, Department of Drug Metabolism, Novo Nordisk A/S, Måløv, Denmark.

According to the ICH M3 guideline for evaluation of new pharmaceuticals in humans, toxicological evaluation has to be made in a rodent and a non-rodent species before first administration to man. The selection of species should be based on among others metabolic considerations, which mean that metabolites expected to be formed in humans, should also be present in species selected for toxicological evaluation. Since the metabolism in humans is unknown at this stage, the evaluation may be based on *in vitro* metabolism in human tissue.

This work has focused on an approach, which combine *in vitro* and *in vivo* metabolism studies for a rapid selection of appropriate species. The *in vitro* metabolism profile in primary human hepatocytes is compared to the metabolism profile in hepatocytes from a range of animal species for selection of rodent and non-rodent species. Furthermore, the metabolism is investigated *in vivo* in one species, to evaluate the validity of the *in vitro* metabolism results.

The *in vitro* metabolism of ^{14}C-NNC 55-0118 was studied in mouse, rat, rabbit, dog, pig, monkey and human hepatocytes as well as the *in vivo* metabolism was studies in the rat. A high degree of *in vitro* to *in vivo* correlation was observed, indication good validity of the *in vitro* metabolism results. The *in vitro* metabolism data divided the species in two different groups metabolizing ^{14}C-NNC 55-0118 by either N-dealkylation or glucuronidation, making it easy to guide the selection of species based on the *in vitro* metabolism profiles.

The applicability of the used approach compared to other methods will be discussed, as well as the use of metabolism data for selection of species for toxicological evaluation in general.

Poster NO. IV-12

Rapid assessment of cytochrome P450 activities

Zbaida S, Ghosal A, Hapangama N, Yuan Y, Lu X, Horne D & Patrick JE, Department of Drug Metabolism and Pharmacokinetics, Schering-Plough Research Institute, Kenilworth, New Jersey, USA.

Cytochrome P450s (CYP) substrates that yield fluorescent metabolites can be used for the rapid screening of drug metabolism activities of intact cells (hepatocytes) and subcellular fractions (microsomes and recombinant cytochrome P450s). The selection of substrate probes for hepatocytes should take into account further possible biotransformation of the metabolites by cytosolic diaphorase and sulfotransferase, or microsomal UDP-glucuronosyltransferase which may lead to a decrease in the fluorescence. A CytoFluor 4000/TC-fluorescence plate reader, equipped with a temperature-controlled chamber in which plates were scanned, provided the ability to collect data from kinetic experiments. This system was used to monitor the biotransformation of 3-cyano-7-ethoxycoumarin (CEC) by CYP1A1, CYP1A2, CYP2C9, and CYP2C19; 7-methoxy-4-trifluoromethylcoumarin (7-MFC) by CYP2B6, CYP2C18, and CYP2E1; dibenzylfluorescein (DBF) by CYP2C8, CYP3A4, and CYP3A5; 7-ethoxyresorufin (7-ER) by CYP1A1, CYP1A2, and CYP1B1; coumarin by CYP2A6 and 3-[2-(N, N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC) by CYP2D6. The reproducible results, obtained more quickly than those generated using conventional HPLC methods, were found to be useful for rapid assessment of CYP activities (positive control) in both intact cells and subcellular fractions.

BENZON SYMPOSIUM No. 48

DRUG METABOLISM: REGULATION AND IMPORTANCE

SEPTEMBER 16-20, 2001, COPENHAGEN, DENMARK

Organizing committee:
Henrik Enghusen Poulsen, Kim Brøsen, Steffen Loft, Urs Meyer, F.F. Kadlubar, P.F. Guengerich, Sven Frøkjær and Arne Svejgaard

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Abstracts - WEDNESDAY, September 19, 2001

Enzymatic Activation of Carcinogens
Guengerich FP, Dept. of Biochemistry and Center in Molecular Toxicology, Vanderbilt University, Nashville, TN 37232, USA.
Halogenated chemicals can be activated by a variety of enzymatic mechanisms. Oxidation of trichloroethylene (TCE) by P450s yields TCE oxide, which rearranges to reactive acyl halides before reacting with proteins and DNA. An interesting feature with TCE is the large fraction of unstable protein and DNA adducts formed, which can be demonstrated by mass spectrometry and reactivation kinetics. Dihaloalkanes are activated by conjugation reactions. Earlier work with ethylene dibromide (EDB) has been extended to methylene dihalides, where glutathione conjugation yields DNA adducts. Another phenomenon of interest is the analogous activation of EDB by the active site cysteine residue of the DNA repair protein guanine O^6-alkyl transferase. Expression of the enzyme in bacteria is accompanied by increased EDB mutagenicity, and the enzyme is crosslinked to DNA. (Supported in part by USPHS R35 CA44353, P01 ES00267)

Drug Metabolizing Enzymes and Carcinogen-DNA Adduct Formation in Man
Kadlubar K, Gorlewska K, Green B, DiPaolo O, Thompson P & Coles B, Division of Molecular Epidemiology, National Center for Toxicological Research, Jefferson, AR 72079 USA.
Our laboratory has focused on carcinogenic aromatic and heterocyclic amines that are found in environmental and dietary sources. The drug metabolizing enzymes that activate and/or detoxify these compounds involve primarily cytochrome P4501A2 (CYP1A2), several UDP-glucuronosyltransferases (UGTs), *N*-acetyltransferases -1 and -2 (NAT1 and NAT2), the major phenol sulfotransferase, SULT1A1, an ATP-dependent kinase, and glutathione *S*-transferases (GSTs) A1 and P1. CYP1A2, which catalyzes the *N*-oxidation of these amines, exhibits several genetic variants, but its phenotype may be largely controlled by HNFs in the 5'-regulatory region. UGTs appear to be critically important their detoxification by conjugating and excreting the N-hydroxy metabolite, although the major isoform(s) involved is as yet unknown. The well described, genetically polymorphic NATs can both activate by *O*-acetylating the *N*-hydroxy metabolite or detoxify by *N*-acetylating the parent amine; but this is substrate-specific. The same is true for the sulfonating activity of SULT1A1, except that its genetic variants account for only a portion of its phenotypic variation. The kinase appears to be an activation mechanism, by *O*-phosphorylating *N*-hydroxy derivatives. The GSTs, whose genetic polymorphisms reasonably well describe the phenotype, serve as final detoxification step and act directing on the *O*-ester, blocking its formation of DNA adducts. We are currently examining the effect of genotype on DNA adduct levels in human breast ductal epithelial cells and in prostate tissue using ^{32}P-postlabelling/HPLC. Initial data are consistent with the presence of the C8-deoxyguanosine adducts of 4-aminobiphenyl in human breast DNA and of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in human prostate; and genotype-adduct comparisons are in progress.

On the origin of p53 mutations in lung cancer
Rodin S & Rodin A, Beckman Research Institute of the City of Hope, Duarte, CA, USA; Human Genetics Center, University of Texas, Houston, TX, USA.
The high frequency of G→T transversions in the p53 gene is a distinctive feature of lung cancer patients with a smoking history and is commonly believed to reflect the direct mutagenic signature of polycyclic aromatic hydrocarbon (PAH) adducts along the gene. Our study of p53 mutations from the IARC p53 database and the primary literature points to the other sources of the G→T excess, in addition to PAH adducts. We found that, in contrast to the 3-fold increased frequency of p53 G→T transversions in lung cancer of smokers (as compared to nonsmokers and to most non-lung cancers), C→A transversions, the DNA- strand mirror G→T counterparts, appeared to be equally

frequent in virtually all human cancers. Along with the results of our comparison of strand-specific p53 mutational patterns and certain other data, this strand bias led us to suggest that smoking may inhibit repair of G→T primary lesions on the non-transcribed strand rather than activate their transcription-coupled repair. We also found that cell lines derived from lung cancers, but not from other cancers, demonstrate significant additional excess of G→T transversions when compared to p53 mutations in parent tumors. A detailed codon-by-codon comparison provides evidence in favor of an in vitro origin of this culture-associated G→T augmentation. Since in culture lung cancer cell lines are not exposed to carcinogens from smoke, one would rather ascribe these new G→T transversions to some other primary lesions, such as 7,8-dihydro-8-oxoguanine, which is the most frequent mutagenic base lesion generated by reactive oxygen species.

Mutations in the tumor suppressor gene TP53 in human cancers associated with exposure to tobacco: facts, interpretations and controversies.

Hainaut P, International Agency for Research on Cancer, World Health Organization, Lyon, France,

TP53 is a tumor suppressor, which plays a multiple, coordinated role in the control of cell cycle, apoptosis, differentiation and DNA repair. The p53 protein is constitutively repressed in normal cells, but this repression is alleviated in response to both genotoxic (mutagenic) and non-genotoxic signals. In human cancers, TP53 is often altered by mutations, with a high prevalence of single base substitutions. Over 15,000 mutations have been reported to date and are compiled in a public database maintained at the International Agency for Research on Cancer.

Tobacco smoke is a complex mixture of chemicals containing multiple agents capable of taking part in cancer initiation and progression. Tobacco smoking is the causative agent in the pathogenesis of several types of cancers, including common forms of bronchiopulmonar cancers, and cancers of the oral cavity, larynx, pharynx, esophagus and bladder. In lung cancers of smokers, the pattern of TP53 mutations shows a high prevalence of G to T transversions, notably at bases that *in vitro* are known as sites of adduction of metabolites of benzo(a)pyrene (BP), one of the most potent tobacco carcinogens. This pattern is not observed in lung cancers of non-smokers.

Mutations in TP53 are the consequence of complex biological processes in which DNA damage, DNA repair and biological selection of meaningful mutants play important contributions. Mutant p53 may display different functional properties according to the location of the mutation and its suspected impact on protein structure. Weighing these different contributions requires a detailed understanding of the natural history of cancers. In the case of lung cancers, the weight of evidence indicates that tobacco carcinogens can leave their mark in the genome of exposed cell populations in the respiratory tract of heavy smokers.

Is there a relationship between drug metabolism and disease?

Eichelbaum M, Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany.

The topic can be viewed from different perspectives.

1. Exposure and metabolism of environmental and dietary chemicals as risk factors in the development of idiopathic or spontaneous diseases and tumors.
2. The role of metabolising enzymes in the development of drug induced diseases.
3. Modification of drug metabolising enzymes by diseases.

All living organisms including humans are continuously exposed to approximately 10,000 chemicals present in food, drinks and the environment. Many naturally occurring compounds are toxic, such as mold aflatoxins, which are among the most potent carcinogens. Various defense mechanisms have evolved to protect organisms from their effects. Of particular importance are cytochrome P450 enzymes, heme-containing mono-oxygenases which catalyze the final step in the incorporation of oxygen into organic molecules. They convert xenobiotics including manmade chemicals and drugs into less toxic, more watersoluble products, thereby facilitating their elimination from the body. These enzymes can also transform nontoxic chemicals drugs into reactive intermediates that are toxic or carcinogenic.

Thus differences in the activity of these enzymes can influence individual susceptibility to adverse drug reactions, drug induced diseases and certain types of cancers. Genetic polymorphisms of drug metabolizing enzymes have proven as useful tools enzymes by demonstrating that genotype related differences in enzyme activities are associated with the risk to develop drug induce diseases and cancer.

In addition to drug metabolizing enzymes proteins involved in the transport of both endogenous and exogenous substances constitute another defense mechanism of the organism against potential harmful chemicals including drugs. In the case of MDR1 genetically determined differences in the expression of its gene product Pgp constitute a factor predisposing to the development of certain types of cancer and inflammatory diseases.

Susceptibility factors in lung cancer

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Historically, the effort to identify genetic susceptibility factors for lung cancer was motivated by critical role of specific Phase 1 and Phase 2 enzymes in determining the disposition of carcinogenic products of tobacco smoke. In the past, our group and many others have conducted population studies to evaluate specific plausible candidate genes.

The sum total of these and many other studies, however, has not to date produced a clear risk profile for genetic factors in lung cancer. For example, it is difficult to improve an individual's risk assessment for lung cancer based on traditional assessment of tobacco expsoure, using the existing biomarkers and genetic susceptibility factors studied to date.

A review of the literature and methodological studies over the past few years considered in the context of current technological advances suggest some lessons that will be applied to the current generation of studies. Adequete study size and attention to design features are central concerns. Study sizes in the thousands are required to achieve adequete power to identify plausible main effects of genes, as well as postulated gene-environment and gene-gene effects. In order to unravel the effects of genes in lung cancer, collection of detailed exposure data is required. Both family studies and population–based designs have been suggested as approaches to identify critical genes in lung cancer. Although studying lung cancer families is worthwhile, both the expected relative risk of the genes (modest) and their prevalence in the population (likely high) warrent a focus on studies in the general population. Our work suggests that population stratification is not likley to be a significant source of bias in these studies when conducted appropriately, although ethnic variation in metabolic genes must be considered. New categories of genes including those that influence smoking itself, or act distally in the carcinogenic pathway (i.e. influence on angiogenesis or DNA repair) need to be taken into account. Finally, new technologies to accomplish high throughput genotyping, identify novel candidate genes, and apply SNP (and haplotype) based-analyses will be discussed.

Lung cancer is the paridigm of a cancer deriving from a gene-environment interaction, and therefore considering genes that act proximally in the carcinogenic pathway to influence the disposition of carcinogens will remain a key focus of future work.

Ethnic and genetic differences in susceptibility genes that affect individual risk of toxicity and cancer

Nebert DW, University of Cincinnati Medical Center, Cincinnati, OH, USA.

Pharmacogenomics, "the field of new drug discovery based on our rapidly expanding knowledge of the human genome," is the obvious extension of the past seven decades of pharmacogenetic studies. In human genetics it is becoming increasingly appreciated that no human disease is caused exclusively by a single gene. Some diseases are predominantly monogenic (*e.g.* phenylketonuria, Gaucher disease), but the same DNA sequence variant can result in strikingly different phenotypes (*e.g.* the rate at which the disease might progress). Other diseases are polygenic (encoded by two, and usually many more than two, genes) and multifactorial (affected by environmental and epistatic factors). The same can be said for toxicity and cancer caused by drugs or other environmental agents**:** some are predominantly monogenic, whereas others represent multiplex phenotypes, *i.e.* interaction between the drug/pollutant (or its metabolites) and the products (enzymes, receptors, transporters, transcription factors, other targets) of many genes.

The usual clinical pharmacology study generally includes all patients on a given medication, and often "control patients" are "matched for age, gender, social status, etc.," or selected by arbitrary telephone-dialing or some other random statistical means. Often there is little regard for the dosage or time the drug has been administered, the threshold at which toxicity is observed in the most sensitive patient group, the varying degrees of toxicity, or the relative dose needed for efficacy versus the dose that causes toxicity in some patients. Because drug efficacy or toxicity always represents a *gradient*, one approach to quantitate the clinical phenotype would be to examine the extreme ends of a distribution, in much the same way as genetic studies have been done in the dissection and identification of genes responsible for blood pressure homeostasis, a truly multiplex phenotype [Halushka et al., 1999; *Nature Genet* **22:** 239-247 *& refs therein*]. In studying pharmacogenetic diseases, it is extremely important to select patients having the most unequivocal phenotype possible—if one wishes to find the gene(s) responsible for the trait. The method of "extreme discordant phenotype" (EDP) is therefore proposed. Using EDP methodology, DNA sequence variants (genotype) can be unequivocally correlated with drug efficacy or toxicity (phenotype). EDP methodology is mathematically intuitive and, in essence, has been used in a number of previous clinical pharmacogenetic studies. This EDP approach should be applicable to virtually any pharmaceutical agent (and, perhaps, certain environmental pollutants as well) in patient populations.

Supported, in part, by NIH grants P30 ES06096, R01 ES06321, R01 ES08147 and R01 ES10416.

Poster NO. V-1

Oxidative stress markers in zinc protected diabetic rats

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Numerous hypotheses have been proposed to understand the multifactorial pathogenesis of diabetic vascular disease. One of them is the enhancing of "oxidative stress" by ROS (reactive oxygen species) or their oxidative products (lipid peroxides, oxidized LDL) that causes dysfunction of the endothelium and activates blood platelets and other blood cells that consequently initiate a cascade of pathophysiological reactions leading to diabetic angiopathy. In our study, we have studied the effect of Zn salts in hyperglycemic and hyperlipidemic diabetic rats. Diabetes was induced by injecting a unique dose of alloxan, i.v. and the zinc salts was administered by gavage (daily). After 6 weeks, blood was

drawn from the retroocular sinus in order to assess the antioxidants parameters. So, we studied the antioxidant metalloenzymes glutathione peroxidase (GPx) and catalase (CAT) activity, the synthesis of glutathione (G-SH), plasmatic and hepatic malondialdehyde (MDA). Our results showed a decrease of CAT and GPx activity in the diabetic rats. The therapy with zinc remakes and even amplifies the activity of CAT as compared with the group of normal animals. GPx is less sensitive to zinc treatment, its value increasing only slightly. More relevant for the lipid peroxidation is the G-SH that decreases significantly in the diabetic rats. The treatment with zinc causes the restoration of the thyol groups, almost to normalization, which shows the protecting effect of zinc in oxidative stress. We noticed a major vascular damage only in diabetic rats unprotected by zinc.

Poster NO. V-2

Degenerative effect of alcohol and tobacco abuse on digestive tract

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In many areas of the world, tobacco and alcohol have been identified as major risk factors in the aetiopathogenesis of digestive cancer. We investigated the effect of a high tobacco and ethanol intake on the production of malondialdehyde (MDA) in blood and hepatic tissue. We also studied the antioxidant metalloenzymes glutathione peroxidase (GPx) and catalase (CAT) activity, the synthesis of glutathione (G-SH) and the histological images of all digestive tract. We used young male Wistar rats. Tobacco-alcohol solution was administered by intubation, 30 minute before lunch. The animals were divided into 2 groups, each comprising 20 subjects. Rats from first group received a regular diet; those in the second group received a diet with tobacco extract solved in alcohol (0,5 mg tobacco/ml alcohol/100 mg body weight) daily, for 8 weeks. Our results showed a decrease of CAT and GPx activity in the tobacco and alcohol diet rats. GPx is less sensitive to both drugs administration. More relevant for the lipid peroxidation is the reduced glutathione that decrease significantly in the second group. Histological fragments of esophagus, stomach, jejunum, ileum and colon noticed a variety of damage between linear ulceration of the mucosal surface, inflammation and mucosal epithelial dysplasia. Apoptosis might be a mechanism which eliminates ROS (reactive oxygen species) damaged cells. Inefficiency of the process can lead to a vicious cycle: ROS →damage of DNA → respiratory chain abnormalities → ROS → protooncogene mutation → loss of additional cancer suppressor genes.

Poster NO. V-3

Antioxidant system of the liver as a 'retention' factor in the development of ethanol hepatotoxicity*

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In order to evaluate the antioxidant system, partial hepatectomy was performed to 112 male rats, and 2 months later ethanol intoxication was started (through a probe, intragastrically, 5 g/kg, as a 30% water solution, once per day, daily, for 57 days). Correlation analysis demonstrated that hepatite vacuolization is associated with low retinol levels, whereas fat infiltration is depends on low glutathion levels. Increased activity of aspartate aminotransferase (AA) in plasma correlates with enzimopathy of CDNB glutathione S-transferase (CDNB-GT), and activation of alcaline phosphatase (AP) in the plasma if affected by enzimopathy of superoxidedismutasa. The mathematical model of interrelation between cytosol and microsomal (CDNB-GT) and AA in plasma in informationally significant ($p<0,003$), as confirmed by the ANOVA results. Cannonical analysis revealed a close relationship between antioxidant system, especially glutathionreductase, and liver damage, especially alcaline phosphatase and γ-glutamyl transferase. These findings demonstrate that antioxidant system of the liver serves as a "retaining" factor in the development of ethanol hepatotoxicity.

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Poster NO. V-4

Identification of human hepatic P450 isoforms responsible of *'in vitro'* interindividual variability in chloroform bioactivation

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Chloroform toxicity and carcinogenicity are dependent on its P450-mediated metabolic bioactivation to oxidative and reductive metabolites. Their relative amount depends on $CHCl_3$ concentration and pO2. To study human interindividual variability in hepatic bioactivation of $CHCl_3$ and the contribute of specific CYP to $CHCl_3$ metabolism we measured phosfolipid adducts as markers of phosgene (PL-PHOS) and dichlorometil radical (PL-RAD) production in hepatic microsomes from 12 human samples (Hepatoscreen™, HBI). Liver microsomes showed a high variability in the bioactivation of 14C-$CHCl_3$ to $COCl_2$: levels of PL-PHOS production varied 40 and 58 times among different samples at 0.1 and 5 mM $CHCl_3$, respectively. PL-RAD levels showed only a 10-fold variation between the tested biopsies. The correlation of PL-PHOS and PL-RAD levels with each single CYP activity showed that at low $CHCl_3$ concentration PL-PHOS strongly correlated only with CYP2E1 activity ($p<0.001$); these data were confirmed by the use of a panel of monoclonal antibodies (90% of inhibition with anti-CYP2E1). At 5 mM the

correlation was significant with CYP2A6 ($p<0.001$). The formation of PL-RAD correlated with CYP2E1 ($p<0.01$) and CYP2C9 ($p<0.05$); anti-CYP2E1 gave 79% of reductive products inhibition. These data, confirming our previous results with cDNA expressed CYPs, show that oxidation is a CYP2E1-catalyzed high affinity reaction and human liver microsomes are able to activate CHCl3 with a high interindividual variability. The highest differences in $CHCl_3$ metabolism in humans seem to be characteristic of $COCl_2$ formation and may be expecially relevant at low chloroform concentrations, typical of human exposure due to chlorination of drinking water and swimming pools.

Poster NO. V-5

Different Metabolic Endpoints of *In Vitro* Cytochrome P450 metabolism and *In Vivo* Metabolism of Flavonoids

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The metabolism of structurally related flavonoids was investigated *in vitro* by rat liver mirosomal preparations and *in vivo* in female rats. Sixteen different flavonoids were investigated in control and Aroclor induced rat liver microsomes. Rats were p.o. administered the flavanone naringenin, the flavones chrysin, apigenin, and tangeretin, the flavonol quercetin and the isoflavone genistein. Metabolites with intact flavane nucleus were separated and identified in microsomal incubations and urine by HPLC and the structures elucidated by LC/MS and ^{1}H NMR.

Overall, the B-ring in the flavonoid molecule was the primary site of biotransformation both *in vitro* and *in vivo*, and the extent of metabolism was highly dependent on the number and position of the hydroxyl and methoxyl groups in the B-ring. *In vitro*, the majority of the identified metabolites were consistent with metabolic pathways leading to the corresponding catechol (3',4'-dihydroxylated) structure. E.g. flavonoids lacking hydroxy groups in the B-ring were hydroxylated in the 3'-position and subsequently in the 4'position. Similarly, flavonoids having a CH_3O-group in the 4'-position (but not in the 3'-position) were effectively demethylated to the corresponding hydroxylated compound. Flavonoids already having the catechol structure were not further metabolized. Thus regardless of the parent compound, the end-point of the *in vitro* metabolism was the 3', 4'-dihydroxylated derivative.

In contrast to the *in vitro* metabolism, the major metabolic end-product *in vivo* was the B-ring *mono*hydroxylated flavonoid structure with the hydroxyl group in the 4'-position. Compounds already exhibiting a catechol moiety were methylated to give a structure with only one free hydroxyl group. Furthermore, compounds already possessing a single hydroxyl group in the B-ring were excreted unchanged in the urine, and in much higher amounts than the other compounds investigated rather than being metabolised.

Poster NO. V-6

The effect of *CYP* and *GST* genotypes on biomarkers of polycyclic aromatic hydrocarbon exposure in an occupationally exposed population

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Biomonitoring of human exposures to environmental genotoxic agents reveal large interindividual variations of the levels of biomarkers of exposure. Those differences may mostly originate from the multiple interactions of individual Phase I and Phase II xenobiotic-metabolising enzymes. Here we investigated the single and combined effects of *CYP1A1* Ile462Val, *CYP1A1 Msp*I, *CYP1B1* Leu432Val, *CYP2C9* Ile359Leu, *GSTM1* and *GSTP1* genotypes on urinary 1-hydroxypyrene (1-OHPY) and peripheral blood lymphocyte DNA adduct levels in a total of 161 aluminium plant workers occupationally exposed to polycyclic aromatic hydrocarbons (PAHs). The *CYP* genotypes did not have recognisable influence on the biomarker levels. We observed a statistically significant positive linear correlation ($p=0.011$) between 1-OHPY and aromatic DNA adduct levels, as determined by ^{32}P-postlabelling, in *GSTM1* null individuals. However, no such correlation was found for PAH-DNA adduct levels determined by immunoassay. Significantly elevated DNA adduct levels, as determined by immunoassay, were associated with *GSTM1* homozygous deletion as compared to the presence of *GSTM1*. However, no such association existed for the DNA adduct levels measured by ^{32}P-postlabelling. The results clearly indicate that the apparent presence or lack of the effect of a genotype on a biomarker of exposure very much depends on the applied methodology for the particular biomarker.

Poster NO. V-7

Metabolic genotypes and aromatic DNA adducts in bronchial tissue from lung patients

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Genetic susceptibility to lung cancer and interindividual variability in the activation or inactivation of carcinogenic components of cigarette smoke in the target tissue are likely influenced by polymorphisms of the genes encoding the metabolising enzymes. Multiple interactions among *CYP1A1* Ile462Val, *CYP1A1 Msp*I, *CYP1B1* Leu432Val, *CYP2C9* Arg144Cys, *CYP2C9* Ile359Leu, *NQO1* Pro189Ser, *GSTM1* gene deletion and *GSTP1* Ile105Val genotypes were investigated on the levels of aromatic DNA adducts in macroscopically normal bronchial tissue from lung patients. The basic study population comprised of 150 patients undergoing pulmonary surgery for lung cancer or other

lung conditions. Metabolic genotypes were determined by PCR-based methods. Aromatic DNA adduct levels were determined by the ^{32}P-postlabelling technique with nuclease P1 adduct enrichment. There was no statistically significant correlation between variants of single polymorphisms and DNA adduct levels after adjustment to smoking status. Among current smokers, no significant correlation was observed between daily cigarette dose and DNA adduct levels in subgroups with selected genotypes. Among smokers, after adjustment to multiple *CYP* genotypes, interactions of *GSTM1* and *GSTP1* were recognised in the formation of bronchial DNA adduct levels. The results underline the importance of the size of the study population and the adjustment of the comparable groups for potential modulating factors when the impact of metabolic genotypes on DNA adduct formation is investigated.

Poster NO. V-8

Air pollution and metabolising enzymes

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Air pollution in the urban environment is suspected to have important consequences on human health. Long term exposure to high pollution levels increase the risk of cancer and respiratory diseases, whereas short term exposure peaks are thought to cause exacerbation of bronchitis, asthma and other respiratory tract diseases as well as precipitate ischaemic coronary attacks. One factor believed to influence the individual susceptibility to air pollution is a genetic polymorphism in metabolising enzymes. One study investigated Copenhagen bus drivers from the City centre and found an increased in DNA damage in subjects with gluthatione S-transferase (GST) M1 and N-acetyl transferase genotypes.

We are studying air pollution-induced damages and genetic polymorphisms in metabolising enzymes in population studies. In a study where personal exposure to benzene was monitored on 40 volunteers living and working in Copenhagen, we found that men with genotype GST M1 or GST T1 wild types had significantly higher excretion of trans,trans mucunic acid (ttMA), which is a biomarker of metabolic toxification of benzene, than men with the null genotypes. A significant correlation between the ttMA excretion and DNA damage, estimated by the comet assay, in men with the GSTM1 wild type was found. In another study we measure diesel exposure ($PM_{2.5}$) in 50 students living and studying in Copenhagen, four times to account for seasonal variation. From these students we have collected blood samples, which are currently being analysed for biomarkers for DNA damage, protein damage, inflammation status and others. In addition to this, we determine the genotype of GST, quinone reductase, cytochrome P450 A1, myeloperoxidase and N-acetyltransferase.

Poster NO. V-9

A novel aldo-keto reductase in mouse

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Exposure to chemicals in the environment is thought to be a factor initiating many forms of cancer. Aldehydes and ketones are a group of chemicals, which form the reactive group in many toxic, mutagenic and carcinogenic compounds. The ability of mammalian cells to survive exposure to these chemicals is dependent on the presence of detoxification enzymes. A major family of enzymes carrying out the reduction of aldehydes and ketones to their corresponding alcohols are known as aldo-keto reductases.

We have identified and cloned a novel mouse aldo-keto reductase that has extensive amino acid similarity to the AKR7 family, the founder member of which is rat aflatoxin-B_1 dialdehyde reductase (AFAR; AKR7A1). The rat enzyme has been shown to be inducible in the liver by a number of naturally occurring dietary components. We have shown that, like other AKRs, the mouse enzyme has broad substrate specificity and is able to reduce both aldehydes and ketones. It displays highest affinity towards 9,10 phenanthrenequinone and 2-carboxybenzaldehyde. It can also reduce AFB_1-dialdehyde arising from AFB_1-dihydrodiol (pH 8.5), a metabolite of a hepatocarcinogen which is activated by cytochrome P450.

The effect of environmental and dietary factors on the level of the mouse enzyme in liver cells is being investigated in order to unravel the mechanism by which diet can protect against cancer.

Poster NO. V-10

The effect of tamoxifen on hepatoblastoma cells (HepG2subline) stably transfected with human CYP3A4 or CYP2E1

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Tamoxifen (Tam) is a site-specific antiestrogen used mostly in the prevention and treatment of breast cancers and in the adjuvant therapy for node-positive breast cancers. The drug is metabolized via 4-hydroxylation (to 4-OHT) and/or demethylation of its side chain (to N-desmethyltamoxifen and then to didesmethyltamoxifen). In humans, N-desmethyl-tamoxifen (weak antiestrogen) is a major product, while 4-OHT is a minor drug metabolite (but strong antiestrogen). CYP3A4 seems to be the most important human CYP metabolizing Tam, but other CYPs like CYP2E1

may also mediate metabolic activation of the drug. The aim of this study was to compare effects of Tam in actively proliferating hepatoblastoma cells (HepG2 subline), which do no express estrogen receptors but are stably transfected with human CYP3A4 (cells metabolizing 1.5 pmol fentanyl min/mg protein), with CYP2E1 (cells hydroxylating 320 pm PNP/min/mg protein) or cells transfected with empty vector (C34 cells). All cells were treated with 1-20 microM Tam for 24-72 hours. The drug induced cell cycle changes and decreased proliferation index in all cell lines in a time and dose dependent manner. Tam cytotoxicity correlated with cell membrane changes measured as phosphatidylserine externalization leading to apoptotic cell death. Dihydrodichlorofluorescein diacetate fluorescence was increased and pretreatment with buthionine sulfoximine (decreasing cellular glutathione) significantly increased this fluorescence. The effects of the drug were diverse in different cells, generally the strongest effects were observed in 3A4 cells. Ketoconazole or 4-methylpyrazole (inhibitors of CYP3A4 and CYP2E1 respectively) blocked some of the drug-induced effect pointing to the role of metabolic activation of Tam.

Poster NO. VI-1

Effect of grapefruit juice on the pharmacokinetics of oral midazolamin cirrhosis

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Grapefruit juice increases the bioavailability of many drugs by down-regulating CYP3A4 in the intestinal wall, leading to a reduced intestinal first pass metabolism of the drug. Thus, after grapefruit juice the area under the plasma concentration-versus-time curve (AUC) of oral midazolam increased by 52 % in healthy subjects (Kupferschmidt HH et all, Clin Pharmacol Ther 1995; 58, 20-8). However, knowledge on the effects of grape fruit juice in patients are scarce. Particularly patients with impaired liver function may be more susceptible to the consequences of this interaction. The effect of grapefruit juice on the AUC of oral midazolam in liver cirrhosis was investigated. A randomised cross-over study was performed in ten patients (3 female, 7 male) with liver cirrhosis based on biopsi or clinical criteria. Following either tap water or grapefruit juice the patients were given midazolam (7.5 or 15 mg) orally. Blood samples were analysed for midazolam and alpha-hydroxy-midazolam by HPLC. The AUC of midazolam increased 2.3 times, corresponding to 128% (124-132) (mean (95%CI)) after grapefruit juice. Simultaneously the AUC of the CYP3A4 mediated metabolite, alpha-hydroxymidazolam, decreased by 71%. Thus, grapefruit juice more than doubled the systemic availability of oral midazolam in patients with liver cirrhosis. This results emphasize the importance of investigating drug interactions in patients.

Poster NO. VI-2

Effect of Grape fruit juice on Carbamazepine bioavailability in patients with epilepsy

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Grapefruit juice has been shown to enhance the bioavailability of number of drugs. Carbamazepine an anticonvulsant drug with narrow therapeutic index is the drug of first choice for partial and clonic seizures. It is metabolised by CYP3A4 microsomal enzymes. The present study was conducted to investigate the effect of grape fruit juice on carbamazepine pharmacokinetic.

A randomized crossover study in 2 phases was conducted. Ten epileptic patients receiving carbamazepine 200mg three times a day for the last three to four weeks were selected. They were given either grapefruit juice or 300ml water at 8 AM along with 200mg carbamazepine. Each treatment was separated by two days; however subjects continued to receive carbamazepine during two days. On both occasion blood samples were collected at different time intervals between 0 to 8 hours. Carbamazepine levels were estimated by reversed-phase HPLC technique..Compaired with water, the steady-state Cmax, Cmin and AUC(0-8) of carbamazepine were significantly increased with grape fruit juice which is likely due to inhibition of intestinal metabolism resulting in increase oral bioavailability. Kinwoo fruit juice, a hybrid variety of grape fruit commonly used in India also produced similar effect on carbamazepine pharmacokinetics in healthy volunteers.

Poster NO. VI-4

QTc interval lengthening is related to CYP2D6 activity and thioridazine plasma concentrations in psychiatric patients

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Thioridazine cardiotoxicity has been associated with prolonged QTc intervals. The present study was aimed at evaluating the influence of the dose and plasma concentration of thioridazine and CYP2D6 enzyme status on the QTc interval in psychiatric patients treated at therapeutic doses. Sixty-five Caucasian patients receiving thioridazine antipsychotic monotherapy were studied. The plasma levels of thioridazine and its metabolites were determined. All patients were phenotyped for CYP2D6 activity with debrisoquine during treatment. Thirty-three patients had a QTc interval over the physiological normal level (420 msec). Thioridazine daily dose was correlated with the QTc interval ($p<0.01$). Patients with high risk of cardiac side effects (QTc interval > 456 msec) received a daily dose of 150 mg or

higher. The plasma concentration of thioridazine was also correlated with the QTc ($p<0.01$), as well as with debrisoquine metabolic ratio (MR) ($p<0.001$) and thioridazine/mesoridazine ratio ($p<0.01$). Thioridazine dose and also plasma concentration were related to the lengthening of QTc interval among psychiatric patients. Since debrisoquine MR was correlated with the QTc intervals, it may be concluded that CYP2D6 enzyme activity is important in determining the risk for QTc interval lengthening. Patients with impaired CYP2D6 enzyme activity due to genetic factors or drug interactions might be more prone to increased risk of sudden death due to torsade de pointes type cardiac arrhythmia.

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Poster NO. VI-6

Metabolic activation of imidazoacridinone antitumor drug, C-1311, with myeloperoxidase and rat liver microsomes

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5-Diethylaminoethylamino-8-hydroxyimidazoacridinone, C-1311, is a highly active antitumour compound developed in our laboratory, which was selected to the phase I clinical studies [1].

We showed earlier that metabolic activation is a necessary step to biological action of this compound. Therefore, the studies on the transformation pathway of C-1311 have been undertaken. The model activation system with plant peroxidase was applied in the first step of these studies. The simplicity of this model enabled us to isolate several products of this reaction with the amount sufficient for NMR structural studies. The identified metabolites turned out to be the derivatives of C-1311, which was modified in the aminoalkyl side chain as well as in the imidazoacridinone core [2].

The presented studies are aimed at analysis of the products of metabolic transformation observed for C-1311 in the animal activation systems. We investigated the transformation of this agent in the presence of myeloperoxidase and rat liver microsomes. The reaction mixtures were analysed by means of HPLC with multidiode array detection and with MS analysis of the fractions. The studies revealed that meyloperoxidase mediated activation gave rise to at least three products identical to those observed with the model system. Two of them, C1 and C3, were the result of dealkylation occured at position 5 of imidazoacridinone ring and one, C2, was the reactive N-hydroxy derivative. Transformation performed in the presence of microsome enzymes led to the similar metabolites. These and our previous results allowed us to propose the chemical pathway of C-1311 metabolic transformation occurred under in vitro conditions.

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2. Z.Mazerska, J Dziegielewski, J.Konopa, *Biochem.Pharmacol.* 61, 685-694, (2001)

Poster NO. VI-7

A new principle to study the metabolism of selective muscarinic cholinoreceptor antagonists

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The search of the heterogeneityof muscarinic cholinergic receptors (M-ChR) and the selectivity of muscarinic antagonists have allowed to make correlation in our knowledge of pathogenesis and treatment of organophosphorus compounds poisonings and some deseases. It is well known that some selective M-ChR antagonists are widely used for treatment of Parkinson's desease. At present, the results of *in vitro* experiments with the radioligand analysis of cholinolytics interaction with M-ChR usually serve as a basis for the conclusion on selective action, but these data do not always allow to predict their pharmacological activity *in vivo.* In the experiments with rats we have developed a new methodological approach the evaluation of the selectivity for M-ChR antagonists action. According to our results, the protective effect of M-cholinolytics during acute organophosphate (DDVP, DFP etc.) poisoning depends on M_1 subtype M-ChR occupation. The efficiency of cholinolytics in inhibition of tremor reaction caused by agonist arecoline depends on M_2 subtype M-ChR occupation. A high degree of correlation was observed for different M cholinolytics between the ratios of average effective doses (ED_{50}) of M antagonists (in the tests with organophosphates) and arecoline, and the ratio of dissociation constants of antagonists complexes with M-ChR from the homogenates of rat's cerebral cortex and heart containing M_1 and M_2- ChR subtypes, respectively. Using this methodological approach we found that some muscarinic antagonists change their selectivity, which depends on the duration of their presence in the rat's organism. The biotransformation of the different cholinolytics with usage of inducer (phenobarbital) and inhibitor (SKF-525a) of cytochrome P-450 was also investigated.

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Abstracts - THURSDAY, September 20, 2001

The discovery of the MDR-pump. A rationale for selecting extracellular targets for cancer chemotherapy
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Reduced cellular uptake is a widespread mechanism of resistance to anticancer drugs. In a study[1] of the mechanism of a decreased accumulation of daunomycin in resistant compared to wild-type Ehrlich ascites tumor cells, several findings indicated an active extrusion of daunomycin from resistant cells: (1) Over a certain range of daunomycin concentrations, the steady state accumulation was considerably higher in isolated nuclei from resistant cells than in the corresponding whole cells. (2) The distribution ratio at steady state of daunomycin in resistant whole cells to that in the medium increased with concentration, in contrast to a decrease for isolated resistant nuclei. (3) Accumulation of daunomycin in resistant cells was enhanced by structural analogs (N-acetyldaunomycin and daunorubicinol) and by metabolic inhibitors (2-deoxyglucose and iodiacetate) suggesting that there also is an active extrusion of daunomycin from wild-type cells. The decreased accumulation in resistant cells may thus be due either to a higher rate of active efflux, a lower rate of influx (in the presence of active efflux), or both. Vincristine and vinblastine increased the accumulation of daunomycin in resistant cells. Together with previous findings of reciprocal cross-resistance between daunomycin and the vinca alkaloids, and a decreased accumulation of daunomycin in cells selected for resistance to vincristine and vinblastin, this effect suggested that these drugs are transported by the same extrusion mechanism as daunomycin. These findings have since been confirmed and extended by many other studies, demonstrating that a variety of drugs are transported by this outward pump, now known as the multidrug resistance (MDR) pump.
The pronounced tendency to development of resistance to anticancer drugs may be seen in relation to the fact that until recently, practically all anticancer drugs were selected on an empirical basis. There are reasons to expect that drugs targeted to mechanisms involved in cancer progression will be less prone to development of resistance. Particularly interesting are extracellular targets. As an example of this approach will be discussed therapy directed against receptor binding of the proteolytic matrix degrading enzyme uPA, that plays a central role in cancer invasion. Gene inactivation studies do however indicate that also against such types of drugs, resistance is likely to occur due to a widespread redundancy in matrix degrading protease systems.
[1]Danø, K.: Active outward transport of Daunomycin in resistant Ehrlich ascites tumor cells. Biochim. Biophys. Acta 1973, 323, 466-481

Multidrug transporters in bacteria
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Bacteria when exposed to cytotoxic agents develop rapidly resistance to these compounds. This resistance can be at the level of drug inactivation, drug target alteration, inhibition of drug influx and active drug efflux from the cells. The latter process can be mediated by transporters which extrude only one drug. An increasing number of transporters are found which can extrude a wide variety of drugs. These transporters belong to the class of multidrug transporters (MDRs). MDRs have been extensively studied in the lactic acid bacterium *Lactococcus lactis.* This organism possesses two multidrug resistance systems. One of the systems, LmrP, belongs to the secondary transporters and mediates the extrusion of drugs in exchange for protons. The second transporter is the ABC transporter LmrA. It mediates multidrug resistance by extrusion of drugs at the expense of ATP. Both systems have been overexpressed in *L.lactis,* solubilised and purified to homogeneity and functionally reconstituted in liposomes. Both transport systems were found to function as vacuum-cleaner systems by which the lipophilic substrates are picked up in the inner leaflet of the cytoplasmic membrane and removed directly to the external water phase. Kinetic transport and binding studies indicate that LmrP and LmrA have at least two allosterically interacting binding sites. LmrA functions as a

homodimer and is both structurally and functionally homologous to the human MDR P-glycoprotein. Equilibrium binding experiments, photoaffinity labelling and drug transport assays revealed that LmrA mediates drug transport by an alternating two-site transport mechanism.

The Functional Coupling of P-Glycoprotein and Drug Metabolism

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P-Glycoprotein (P-gp) has been first described as an efflux pump contributing to resistance of cancer cells to chemotherapy. More recently, P-gp and other proteins of the ABC-transporter family have been identified as functional barriers against uptake of xenobiotics and drugs (eg. in the intestine or the blood / brain barrier) or as proteins with excretory function for various compounds via liver or kidneys. A wide interindividual variability in basal expression of P-gp has resulted in efforts to identify factors involved in regulation of P-gp. Aside from genetic factors expression of P-gp can be altered by classical enzyme inducers (eg. rifampin).

Substrates for P-gp originate from a broad range of therapeutic indications covering diverse chemical structures. Interestingly, many substrates, inhibitors or inducers of P-gp demonstrate a broad overlap in specificity with Cytochrome P450 enzymes, in particular CYP3A4, suggesting a functional coupling of both proteins. Moreover, both Pgp and CYP3A4 are often expressed in the same cellular systems (eg villus tip of enterocytes of the small intestine). Recent data from animal experiments suggest that the lack of P-gp in knockout mice altered the level of expression of various Cytochrome P450 enzymes (Schuetz EG et al, Molecular Pharmacology 57, 188-197, 2000). In such a system of concerted action of both detoxification systems, the level of P-gp may modify the amount of drug reaching CYP3A4. Again, experiments in knock out mice indicated erythromycin-N-demethylase activity to be reduced in absence of P-gp (Lan L et al, Molecular Pharmacology 58, 863-869, 2000). In turn, metabolites produced by P-450 enzymes could have a high affinity to the efflux pumps leading to an effective chain of different clearance mechanisms.

Moreover, (patho)-physiological processes (eg age, various diseases) may alter the functional coupling of P-gp and drug metabolism.

Poster NO. VIII-1

CYP1A2 phenotyping using orally given melatonin

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Melatonin has been recently reported to be almost exclusively metabolized *in vitro* by the hepatic cytochrome P450 (CYP) 1A2. The Km for the formation of 6-hydroxymelatonin was in the low micromolar range (6.3±3.6 μM). Since the Km of the caffeine N3-demethylation, the established marker reaction for the CYP1A2 phenotyping, is about a magnitude higher it was suggested that orally given melatonin might serve as a more specific and sensitive probe drug for CYP1A2. 12 healthy Swedish subjects (6 male) were phenotyped for CYP1A2 with caffeine. About 12 months later melatonin (25 mg) was given at 9:30 and blood samples were taken hourly between 0.5 and 6.5 hours after intake. Serum concentrations of melatonin and conjugated 6-hydroxymelatonin were analyzed by LC/MS. Concentrations of melatonin and conjugated 6-hydroxymelatonin, or their ratio at different time points, were tested for correlation to either the apparent oral caffeine clearance (CL) or the melatonin AUC. We found a significant correlation between the caffeine CL and the melatonin AUC with a Spearman rank correlation coefficient (Rs) of –0.685; $p < 0.01$. The melatonin concentration 1.5 h after administration also closely correlated to the caffeine CL as well as the melatonin AUC ($Rs = -0.62$; $p < 0.05$ and $Rs = -0.57$, $p < 0.05$, respectively). Inclusion of conjugated 6-hydroxymelatonin gave no closer correlations.

Melatonin might be used as an alternative to caffeine as a probe drug for CYP1A2 phenotyping.

Poster NO. VIII-10

The impact of paroxetine on tramadol analgesia

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The analgesic drug tramadol probably acts by a monoaminergic effect of tramadol itself and an opioid effect of its metabolite (+)-M1. (+)-M1 is formed by O-demethylation of tramadol via CYP2D6. The aim of this study was to evaluate the impact on tramadol analgesia of the SSRI paroxetine, which is a potent inhibitior of CYP2D6. The study, which included 16 healthy volunteers that were extensive metabolisers of sparteine, was a randomised, doubleblind and crossover study. Paroxetine 20 mg daily or placebo was given for 3 days. On the fourth day, 150 mg tramadol or placebo was dosed orally and pain tolerance threshold to transcutaneous electrical sural nerve stimulation and pain during a cold pressor test was determined before and 2, 4, 6 and 8 hours after dosing of tramadol or its placebo. Pharmacodynamically, tramadol with placebo pretreatment caused a more pronounced increase in pain tolerance thresholds (sum of differences) to sural nerve stimulation (median difference from placebo 13.9 mA 95% c.i.

=[+3.8;+22.3] vs 8.0 mA c.i. =[+2.3;+15.2]) and a more pronounced decrease of mean pain ratings during the cold pressor test (median difference -5.0 cm c.i. =[-8.4;-1.5] vs -2.1 cm c.i. =[-5.3;+0.1]) than tramadol with paroxetine pretreatment. Two hours after tramadol administration, the paroxetine pretreatment caused an increase in the median serum concentrations of (+)-tramadol from 0.856 μM (95% c.i. = [0.725;0.915]) to 1.212 μM (95% c.i. = [1.016;1.268]), and a decrease in the median (+)-M1 concentration from 0.257 μM (95% c.i. = [0.170;0.341]) to 0.069 μM (95% c.i. = [0;0.123]). The concentrations of (-)-tramadol and (-)-M1 are changed similarly to, but less extensively than, the (+)-enantiomers. The mean metabolic ratio of (+)-M1/(+)-tramadol was decreased by paroxetine pretreatment from 0.30 to 0.07 ($p < 0.0004$). It is concluded, that paroxetine, via inhibition of CYP2D6, inhibits the metabolism of tramadol, thus increasing the serum concentrations of (+)- and (-)-tramadol and lowering the serum concentrations of the metabolites (+)- and (-)-M1. Further, via inhibited formation of (+)-M1, paroxetine reduces but does not abolish the analgesic effect of tramadol.

Poster NO. VIII-9

Alcohol in relation to other risk factors in paracetamol-induced hepatotoxicity

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Background: The aim was to determine how alcohol and other factors affected the clinical course and outcome in patients with paracetamol poisoning.

Methods: 645 consecutive patients admitted from 1994 to 2000 with single-dose paracetamol poisoning were studied giving special attention to alcohol history, time between overdose and intravenous N-acetylcysteine treatment ('time to NAC'), and other data available at the time of admittance.

Results: Up until 72 hours post ingestion, 'time to NAC' was the single most important independent risk factor. With a 'time to NAC' below 12 hours, the mortality rate was 0.42% (95% confidence interval 0.05-2.7). When 'time to NAC' exceeded 12 hours, 24 hours, and 48 hours, the mortality rate increased to 6.1%, 13%, and 19%, respectively. Chronic alcohol abuse was an independent risk factor of mortality (odds ratio 3.52; 95% confidence interval 1.78-6.97). Acute alcohol was an independent protective factor regarding mortality in alcoholics (odds ratio 0.08; 95% confidence interval 0.01-0.66), but not in non-alcoholics (odds ratio 0.21; 95% confidence interval 0.03-1.67). Age and quantity of paracetamol were independent risk factors.

Conclusions: 'Time to NAC' was confirmed as the major risk factor in paracetamol-induced hepatotoxicity and mortality. Chronic alcohol consumption was an independent risk factor that was counteracted by concomitant acute alcohol ingestion. Age and quantity of paracetamol were independent risk factors. We suggest that chronic alcoholics with suspected paracetamol poisoning should be treated with NAC regardless of risk estimation, in particular older alcoholics and those with recent alcohol withdrawal.

Poster No. VIII-6

CYP2D6 and CYP2C9 genetic polymorphisms and psychoactive-drug treatment outcome

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Cytochrome P450s (CYP) genetic polymorphisms cause interindividual variability in the sensitivity to many clinically used psychoactive drugs. *CYP2D6* and *CYP2C9* genotypes have been evaluated in 132 unrelated Italian healthy volunteers (75 males and 57 females, aged 21-64 years). Genotyping has been performed on peripheral leukocytes DNA by molecular biology techniques (PCR, RFLP, long-PCR). Volunteers could be divided into four *CYP2D6* genotype groups: 72 case with no mutated alleles (54.5%), 51 cases with one mutated allele (38.6%), 3 cases with two mutated alleles (2.3%) and 6 cases with extracopies of a functional gene (4.6%). Forty-one subjects (31.1%) carried one and 9 subjects (6.8%) carried two *CYP2C9* mutated alleles. In a study performed on 119 schizophrenic patients treated with antipsychotics mainly metabolised by CYP2D6, all the *CYP2D6* PMs (n=4) had developed neuroleptic-induced extrapyramidal syndromes, suggesting that the PM condition might represent a predisposing factor for such adverse effects. The *CYP2D6* deficiency might have contributed to a clinically important interaction in a PM schizophrenic patient in whom addition of carbamazepine to preexisting risperidone therapy resulted in a marked decrease in the plasma levels of risperidone and 9-hydroxyrisperidone with consequent acute exacerbation of his psychosis. Similarly, in a 31-old woman, the *CYP2C9*3* homozygous condition was associated with a severe phenytoin intoxication, with an unusual pharmacokinetic behaviour. In conclusion, our findings clearly suggest that the genetically based interindividual variability in the CYP2D6 and CYP2C9 activity may lead to differences in clinical response to psychoactive drugs.

Ethnic variations in drug metabolism and effects

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There are pronounced differences between populations in the metabolism and in effects of drugs. The two polymorphic cytochrome P450 (CYP) enzymes CYP2C19 and 2D6 may serve as examples to illustrate this. There are

population specific *CYP2D6* alleles: **4* in Caucasians encoding no enzyme, and **10* in Asians and **17* in Africans, which codes for enzymes with decreased activity. The *CYP2D6.17* enzyme has decreased rate of metabolism of the substrates debrisoquine and dextromethorphan, but not of codeine and metoprolol demonstrating substrate specificity (Wennerholm et al). There is a north-south gradient in the frequency of *CYP2D6* gene duplication with 1-2 % in Sweden and 7-10 % in Italy and Spain and 29 % in Ethiopia. A clear effect of the number of functional *CYP2D6* genes on the metabolism of the antidepressant nortriptyline and the neuroleptic haloperidol has been demonstrated in both healthy subjects and patients in different populations.

The *CYP2C19*2* allele is present in about the same frequency, about 20 %, in most populations investigated. In Asians there is also a frequent *CYP2C19*3* allele giving a frequency of 15-20 % poor metabolizers (PM) of the probe drugs mephenytoin and omeprazole, which is much higher than the 3 % in Caucasians. In Caucasian patients with acid related disease treated with 20 mg omeprazole for 8 days there was a more pronounced effect on both intragastric pH and gastrin release in heterozygous extensive metabolizers (EM) and PM compared to homozygous EM. A similar *CYP2C19* genotype difference in gastrin and vitamin B12 concentrations was shown in patients treated for more than a year with 20 mg daily of omeprazole. Studies in Korean patients demonstrate pronounced differences from Caucasians in both metabolism and effects (Roh et al).

Drug Interactions: mechanisms and consequenses

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Pharmacokinetic drug interactions have assumed increasing importance during the last 15-20 years, due to the introduction of many new therapeutic agents with secondary effects on drug metabolism. Interactions of greatest concern are those that involve powerful inducers or inhibitors that produce large changes in clearance of the substrate drug. Many such drugs are CYP3A substrates that ordinarily undergo extensive presystemic extraction after oral dosage, with a substantial contribution of CYP3A present in the gastrointestinal tract mucosa. Efflux transporters such as P-glycoprotein which may be modulated in parallel by agents that influence CYP3A. The 50-fold increase in plasma concentrations of saquinavir caused by co administration of ritonavir illustrates a very large pharmacokinetic interaction attributable to the effects of ritonavir on both CYP3A and P-gp. Strategic plans for drug development in the pharmaceutical industry are being significantly influenced by projected adverse consequences of drug interactions. Candidate drugs metabolized in parallel by two or more CYP isoforms are viewed positively, since induction or inhibition of a single CYP may not have a major influence on clearance. In contrast, agents that are substrates only for CYP3A – particularly if presystemic extraction is high – may well be discarded as candidates for further development. The integrated data base on drug interactions derived from basic and clinical science has had, and will continue to have, a major impact on contemporary therapeutics and drug development.

Acetaminophen Hepatotoxicity: Role of Reactive Oxygen and Nitrogen Species

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Acetaminophen (APAP)-induced hepatotoxicity correlates with metabolism to N-acetyl-*p*-benzoquinone imine. This species depletes GSH and covalently binds to protein as an APAP adduct. The mechanism of necrosis is unknown. In APAP-treated mice (300 mg/kg, 4 hrs) we recently observed nitrotyrosine adducts in the centrilobular cells, the site of the ensuing necrosis. These adducts co-localized with APAP adducts. Nitration is via peroxynitrite (PN), a reactive species that is formed from nitric oxide ($^{\cdot}NO$) and superoxide ($^{\cdot}O^{-}_{2}$). PN readily oxidizes APAP and GSH *in vitro*. At 1-hr nitration occurred in the hepatic sinusoids and by 4-hrs nitration was uniformly detected in hepatocytes. Kupffer cell inactivators decreased APAP-induced toxicity and tyrosine nitration. The role of $^{\cdot}NO$ in APAP toxicity was studied in inducible nitric oxide synthase (iNOS) knockout (KO) and wildtype (WT) mice. Histological examination indicated a similar degree of toxicity. NO synthesis (serum NO^{-}_{2} + NO^{-}_{3}) increased in WT mice only. Nitrotyrosine adducts were detected in KO mice, but greater amounts were in WT mice. Lipid peroxidation (malonaldehyde) in liver increased in KO mice only. The KO mice appeared to have more APAP adducts and APAP metabolism was more rapid. Encapsulated superoxide dismutase (SOD) (60 U/g) decreased toxicity at 4 hrs, but not at 6 hrs. However, a higher dose of SOD (120 U/g) decreased toxicity at 6 hrs. Thus, in APAP hepatotoxicity $^{\cdot}NO$ and $^{\cdot}O^{-}_{2}$ formation leading to PN are significantly increased. The major source of $^{\cdot}NO$ is from iNOS rather than endothelial NOS. NO synthesis slows APAP metabolism and plays a critical role in controlling $^{\cdot}O^{-}_{2}$ induced lipid peroxidation. In summary, the mechanism of toxicity appears to involve activation of Kupffer cells and occurs by a combination of APAP binding and oxidative and/or nitrogen stress.

Poster NO. VII-1

A potential role for P-glycoprotein in the non-proportional phamacokinetics of UK-343,664

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UK-343,664 is a potent and specific PDE5 inhibitor, which exhibits non-proportional pharmacokinetics in man. In order to investigate the molecular mechanisms for this *in vivo* observation, *in vitro* techniques, including recombinant CYP enzymes and recombinant P-glycoprotein have been employed.

UK-343,664 is a lipophilic molecule (log $D_{7.4}$ = 3.1) and as such is expected to be cleared mainly by metabolism. *In vitro* metabolism studies with human liver microsomes and recombinant CYP enzymes have demonstrated that the metabolism of UK-343,664 is predominantly mediated by CYP3A4. Kinetic studies gave a moderate Km of 76μM for this enzyme, and saturation of first-pass metabolism alone was therefore considered unlikely to account for the non-proportional pharmacokinetics.

In vitro studies with recombinant P-glycoprotein and the Gentest PGP-ATPase assay, showed that UK-343,664 had a high affinity for P-glycoprotein, with a Km of 7.3μM. Additionally, in transport studies in LLC-PK1 cell monolayers transfected with P-glycoprotein, UK-343,664 showed marked polarised transport which was concentration dependent.

The high affinity of UK-343,664 for P-glycoprotein is considered to be the primary source of the non-proportional pharmacokinetic profile observed in man.

Poster NO. VII-2

Utilization of optimized *in vitro* and *in silico* methodologies for solubility, permeability, and efflux transport studies in discovery-based compound screening.

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The use of various *in vitro* assay systems for estimating *in vivo* ADME is now commonplace in the biopharmaceutical industry. Simple issues such as solubility, and complex issues such as efflux transporter substrate affinity, have been identified as integral parameters that must be considered in any screening program. For example, P-glycoprotein (P-gp) substrate affinity should be evaluated for CNS-targeted drugs, and data regarding permeability (P_{app}), solubility, and metabolic turnover are also essential. The emergence of *in silico* methods such as polar surface area (PSA) and other calculated alerts, e.g. Rule-of-Five violations, further streamline screening strategies and QSAR-based decisions. For example, we have identified a clear relationship between calculated PSA values and experimental absorptive P_{app}, thus demonstrating the advantage of employing *in silico* methods as a predictive tool. For assessment of passive transcellular permeability, MDCK (Strain I) monolayers are nearly equivalent to Caco-2 (r^2 = 0.925) and are a superior model due to their short preparation time and inter-passage homogeneity. Caco-2 passive P_{app} studies have been optimized considerably, e.g. sodium taurocholate/pH 6.0 and BSA/pH 7.4 can be used in the AP and BL chambers, respectively, to more closely simulate *in vivo* conditions and provide more accurate P_{app} estimates for highly protein-bound or poorly soluble compounds. Also, Caco-2 monolayers remain an essential tool for identifying hPepT1 (absorptive) and P-gp (efflux) substrates; assays employed include a Taxol-based inhibition assay (to define Km) in addition to the classic BL-AP/AP-BL transport relationship. Turbidimetric solubility studies are routinely done, using nephelometry, at various physiologically relevant pH values. Thus, the careful selection of both *in vitro* and *in silico* screening tools, proper consideration of the target site of action *in vivo*, and refined data handling and ranking methods are critical for lead optimization.

Poster NO. VIII-2

Principles for treating the ultrarapid metabolizers (UM) with antidepressants by inhibition of CYP2D6 activity with paroxetine

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Normalization of the metabolic status of UMs by inhibition of CYP2D6 activity could offer a clinically acceptable method to successfully treat UMs with antidepressants. 5 UMs with a CYP2D6 gene duplication or triplication were treated with 25 mg b.i.d. nortriptyline for 3 consecutive weeks, the first week alone and during the second and third weeks concomitantly with the CYP2D6 inhibitor paroxetine 10 mg or 20 mg b.i.d., respectively, and with paroxetine 20 mg b.i.d. only during the 4th study week. At the end of each study week, steady-state pharmacokinetics of nortriptyline and/or paroxetine, as well as the debrisoquine test were assessed. All five subjects had subtherapeutic nortriptyline concentrations after 7 days' treatment with nortriptyline only. Addition of paroxetine 10 mg b.i.d. to the nortriptyline regimen turned all individual into "normal" extensive debrisoquine metabolizer phenotype and therapeutic plasma nortriptyline concentrations were achieved in 4/5 subjects after a 3-fold mean increase in nortriptyline trough concentration (P=0.0011). Doubling the paroxetine dose caused a 15-fold mean increase in paroxetine trough concentration (P<0.001), turned two subjects into poor debrisoquine metabolizer phenotype and caused a further increase in plasma nortriptyline trough concentration (P=0.0099). A strong correlation (r_s=0.89;

P<0.0001) was observed between paroxetine and nortriptyline trough concentrations. Paroxetine, with a daily dosage from 20 to 40 mg, is an effective tool in normalizing the metabolic status of UMs.

Poster NO. VIII-3

Hormone replacement therapy does not affect the pharmacokinetics of selegiline

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In our previous study, combination oral contraceptives increased serum selegiline concentrations significantly. The aim of this study was to investigate the effect of hormone replacement therapy (HRT) on the pharmacokinetics of selegiline and it's primary metabolites. In a randomized double-blind cross-over trial 12 female volunteers took for 10 days either HRT containing 250 µg levonorgestrel and 2 mg estradiol valerate or matched placebo. On day 10 the subjects took a single 10-mg oral dose of selegiline. Serum concentrations of selegiline, desmethylselegiline and methamphetamine were determined. Blood pressure and heart rate were measured to monitor the safety of selegiline. There was a statistically non-significant increase of 59% (p=0.139) in the AUC of selegiline by HRT, but only a little concomitant reduction in the AUC of desmethylselegiline (p=0.071) or metamphetamine (p=0.614) was observed. C_{max} of selegiline was not changed, but a statistically significant reduction in the C_{max} of desmethylselegiline (-17%, p=0.03) was seen in the HRT phase. The C_{max} of methamphetamine was slightly, but not significantly reduced (-5%, p=0.06). There was considerable interindividual variation in the pharmacokinetics of selegiline, as well as in the levonorgestrel concentrations. A trend was found for the correlation between the AUC of selegiline and levonorgestrel trough concentrations (r_s=0.57, p=0.06). On the contrary to oral contraceptives, hormone replacement therapy with levonorgestrel and estradiol valerate does not affect the metabolism of selegiline.

Poster NO. VIII-4

Clinical implementation of CYP2D6 and CYP2C19 genotype assays

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A new program has been initiated at The Epilepsy Hospital and the Psychiatric Hospital in Dianalund in order to implement the results that has been published during the last 10 years about CYP2D6 genotype assays in clinical practice. The aim of the program is to improve the anti-psychotic treatment of psychiatric patients and to avoid side effects.

A PCR protocol that can identify the CYP2D6 alleles *3, *4, *5, *6, *7, *8, *9, *10, *11, *12, *15, *17, *18 and the duplication of CYP2D6 will be used in the analysis. Initial experiments are in progress. An other protocol in order to identify non functional CYP2C19 alleles also will be implemented.

Initially we will determine the CYP2D6 and CYP2C19 genotype of 250 well characterized and stabile medicated psychiatric patients from the Psychiatric Hospital, Dianalund, Denmark and from district psychiatric patients. A data sheet on type and dose of drugs used, results from drug monitoring and adverse drug effects will be constructed in an anonymous form in order to study correlations between theese patient data and genotype. In addition we will get an instant picture of the frequency of CYP2D6 and CYP2C19 alleles in a population of psychiatric patients. Preliminary results will be presented.

This analysis will be followed by an CYP2D6 and CYP2C19 genotype analysis of new Psychiatric patients in order the evaluate if a rapid genotype analysis will improve the quality of the anti-psychotic medical treatment. Based on these results a decision for a future application of genotype analysis as a routine investigation before anti-psychotic medication will be taken.

The main goal of the project is to implement the genotyping a routine.

Poster NO. VIII-5

TPMT genotype distribution in IBD patients compared to healthy caucasians

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Azathioprine plays a major role in the treatment of inflammatory bowel disease (IBD). It is partly metabolized by the enzyme thiopurine methyltransferase (TPMT) to the active metabolites 6-TGN and 6-MMP. This enzyme is subject to a polymorph distribution, 90 % of a Caucasian population having a high and 10 % an intermediate (heterozygotes) activity whereas 1 in 300 inherits TPMT deficiency as an autosomal recessive trait. The most common variant allele in healthy Caucasians associated with low TPMT activity seems to be TPMT*3A, that contains 2 nucleotide transition mutations. Each TPMT*3 mutation alone (*3B, *3C) leads to a reduction in catalytic activity, while the presence of both mutations (*3A) leads to nearly complete loss of activity. The consequence of low or absent TPMT-activity is a high level of 6-TGN. TPMT-genotyping, therefore, can be used to individualize azathioprine therapy in IBD patients. TPMT-genotyping was performed in 120 randomly assigned Crohn patients using a PCR-technique. 87.5 % had a wild-type/wild-type genotype, 11.7 % had one non-functional mutant allele (*3B) and 1 in 120 had two non-functional

alleles (*3B/*3B), which compares to the distribution normally found in a Caucasian population. The *3B mutant allele has been reported in only one or two individuals today.

Poster NO. VIII-7

Influence of *CYP2C9* genotype on warfarin dose

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Bleeding complications during warfarin treatment are the most common adverse drug reactions with fatal or disabling outcome. Interindividual variation in the response to warfarin, and a low therapeutic index are two of the factors that make warfarin therapy difficult to handle.

Objective: Cytochrome P450 2C9 (CYP2C9) is the principle enzyme in warfarin inactivation. Allelic variants of *CYP2C9* generate enzymes with impaired hydroxylation of S-warfarin. We studied the association between *CYP2C9* variant alleles and low warfarin dose requirement in Swedish patients.

Method: Two hundred-and-one patients at an anticoagulation clinic in Uppsala were genotyped for *CYP2C9*1, CYP2C9*2* and *CYP2C9*3*. They had been treated with warfarin for a minimum of 2 months. Genotyping was performed by solid-phase minisequencing.

Results: The individual warfarin requirement varied between 4.5 and 77.25 mg per week. The mean maintenance doses were 35.8 mg, 29.1 mg and 11.2 mg in patients with two, one and no functional *CYP2C9* alleles, respectively.

Conclusions: The maintenance dose of warfarin was significantly related to the number of functional *CYP2C9* genes. Statistical analysis was performed by pair-wise T tests based on variance estimate from an ANOVA model. The odds ratio for the **2* or **3* alleles in patients with a low maintenance dose (<26.25 mg/week) was 1.98 (95% CI 1.07; 3.63). We are continuing the study by genotyping individuals with bleeding complications during warfarin treatment.

Poster NO. VIII-8

Early postoperative erythromycin breath test measures hepatic CYP3A and predicts nephrotoxicity and graft survival in liver transplant recipients

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Background: Interpatient differences in the pharmacokinetics of cyclosporin or tacrolimus may result from inter-individual variation of CYP3A activity. Hepatic CYP3A activity can be measured by the Erythromycin Breath Test (ERMBT). We investigated if ERMBT performed in the early postoperative phase of liver transplantation was a measure of hepatic CYP3A activity and a predictor of cyclosporin/tacrolimus toxicity, acute graft rejection, and outcome. **Methods:** In 26 liver transplant recipients, ERMBT were performed immediately post transplantation. In 18 of these patients, a liver biopsy was obtained during surgery. The biopsies were analysed for CYP3A protein content by Western blotting and for CYP3A activity using the testosterone 6-beta-hydoxylation or the erythromycin demethylation assays. The patients were observed for cyclosporin/tacrolimus toxicity, rejection, and graft survival.

Results: The ERMBT result correlated significantly to the 6-beta-hydroxylase activity (R=0.68; p=0.002), the erythromycin demethylase activity (R=0.63; p=0.005), and the CYP3A protein content (R=0.49; p=0.04) in the biopsies. A low ERMBT result was a significant predictor of cyclosporin/tacrolimus related nephrotoxicity (median 0.70 (range 0.14-0.86) vs. 1.28 (0.07-3.08) %/1 hour; p=0.02). A high ERMBT result was a significant predictor of graft survival (0.86 (0.10-3.08) vs. 0.17 (0.07-0.46) %/1 hour; p=0.003), but did not predict acute graft rejection.

Conclusions: This is the first evidence that early postoperative ERMBT reflects hepatic CYP3A activity and protein level in liver transplant recipients. ERMBT was a predictor of cyclosporin/tacrolimus toxicity and of graft survival.