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Human hepatocytes as a key *in vitro* model to improve preclinical drug development

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SUMMARY

Over past decades, numerous in vitro and/or ex vivo models have been developped to investigate drug metabolism. In the order of complexity we found the isolated perfused liver, hepatocytes in co-culture with epithelial cells, hepatocytes in suspension and in primary culture and subcellular hepatic microsomal fractions. Because they can be easily prepared from both animals (pharmacological and toxicological species) and humans (whole livers as well as biopsies obtained during surgery) hepatocytes in primary culture provide the most powerfull model to better elucidate drug behavior at an early stage of preclinical develompent such as: the characterization of main biotransformation reactions, the identification of phase I and phase II isozymes involved in such reactions, the evaluation of inter-species differences allowing the selection of a second toxicological animal species more closely related to man on the basis of metabolic profiles, the detection of the inducing and/or inhibitory effects of a drug on metabolic enzymes, the prediction of drug interactions, the estimation of inter-individual variability in biotransformation reactions. The use of hepatocytes, and in particular those obtained from humans, at an early stage of drug development allows the obtention of more predictive preclinical data and a better knowledge of drug behavior in humans before the first administration of the drug in healthy volunteers.

INTRODUCTION

Human *in vitro* models, and in particular hepatocytes in suspension or in primary culture have been increasingly used over the past decade for pharmacological and toxicological studies. Indeed, it is now well recognized that hepatocytes express most of the functional activities of the intact liver and they are therefore suitable for investigating xenobiotic metabolism and toxicity.

In recent reports (1-5) many authors have clearly demonstrated that *in vitro* cultured hepatocytes retained their *in vivo* specific drug-metabolizing activities, including inter-species polymorphism. Various authors (6,7) have demonstrated that cytochrome P450 dependent reactions could be selectively increased following exposure of human hepatocytes to various inducers. This suggests that they may provide a useful system for investigating the regulation of drug metabolism and toxicity in humans.

With the availability of good preparations of human adult hepatocytes (with a viability greater than 80%) which have been shown to conserve both cellular integrity, biochemical function and phase I and phase II processes, these *in vitro* tools can be, at a preclinical stage of drug development, very powerful in determining the qualitative and quantitative importance of the biotransformation reactions, the inter-species variability in these metabolic pathways, the potential drug interactions which can be observed following multi-drug therapy in humans and the effect of the drug on the expression and the regulation (induction and/or inhibition) of phase I and phase II isoenzymes.

The results presented in this paper demonstrate the practical feasibility of the use of *in vitro* human models, both hepatocytes in primary culture and microsomal fractions, for the improvement of preclinical drug development.

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RESULTS AND DISCUSSION

Human *in vitro* models are increasingly used during preclinical drug development in order to better predict the behavior of a drug in humans. They can bring valuable information to the *in vivo* human situation and allow more accurate studies :

In metabolism - by the early kwowledge of main biotransformation processes of a drug in humans

Metabolism of tetrazepam (figure 1) has been studied following exposure to human hepatocytes in primary culture.

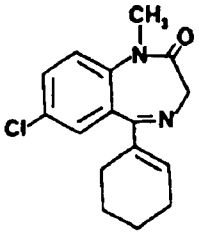

Fig.1: Chemical structure of tetrazepam

As illustrated in figure 2, tetrazepam is rapidly and intensively metabolized by human hepatocytes firstly to its 3'-hydroxylated derivative followed by its rapid conjugation with glucuronic acid.

After a 24-hour exposure to 20 µM 14C - tetrazepam, unchanged drug represented only 20-25% of extracellular drug concentration.

The predictability from the human hepatocyte culture model to the *in vivo* human situation has been confirmed following tetrazepam administration in humans. Hence, after a 4-hour per os administration of tetrazepam in healthy volunteers, the main circulating tetrazepam derivative was the 3'-hydroxylated metabolite while the main urinary excreted derivative was the glucuronidated conjugate of the 3'-hydroxy-tetrazepam.

In experimental toxicology - by the choice of the animal species most closely related to man on the basis of metabolic pattern

Experimental toxicology is usually performed in two different animal species : firstly in Sprague Dawley rats and secondly in a non-rodent species, such as Beagle dogs or Macaca Fascicularis and Baboon Papio Papio monkeys.

Hepatocytes in primary culture isolated from these different animal species as well as from humans can bring valuable information for the identification of the various animal species for which metabolic patterns are most similar to those of man.

The inter-species variability in drug metabolism can be illustrated by the use of two different drugs : firstly midazolam, a 1,4-benzodiazepine (figure 3) and secondly a new anticancer drug belonging to the ellipticin family (figure 4), 2-(diethylamino-2-ethyl) -9-hydroxyellipticinium chloride (SR 95156B).

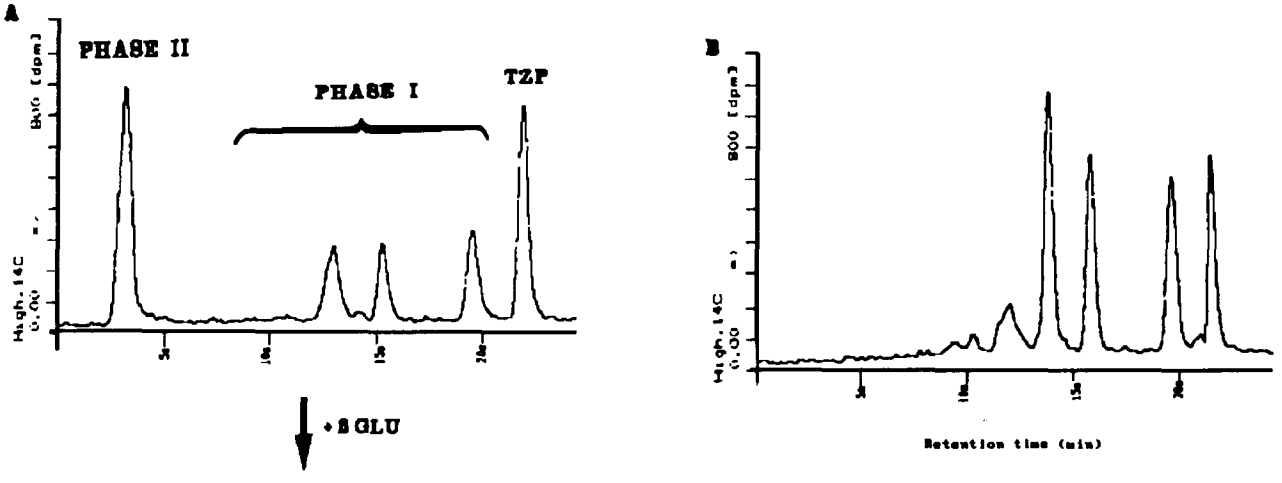

Fig. 2: HPLC Analyses of extracellular medium recovered following a 24-hour exposure to human hepatocytes in primary cultures (A). The effect of a 24-hour subsequent incubation with helix pomatia juice is illustrated (B)

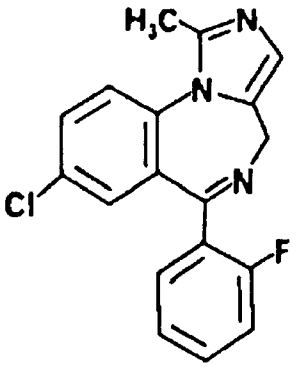

Fig.3: Chemical structure of midazolam

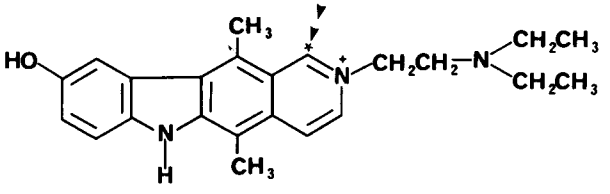

Fig.4: Chemical structure of 2-(diethylamino-2-ethyl) -9-hydroxyellipticinium chloride (SR 95156B).

Midazolam is known to be metabolized to its 1-hydroxy-, 4-hydroxy- and 1,4-dihydroxy-midazolam which are subsequently glucuronidated before renal elimination (8,9).

Metabolism of midazolam has been studied on hepatocytes in suspension isolated from various animal species including Wistar rats, New Zealand rabbits, Beagle dog, pig and Caucasian humans. As illustrated in figure 5, midazolam was rapidly cleared from the extracellular medium, accumulated within the hepatocytes and metabolized into different derivatives which were then recovered in the extracellular compartment.

From a quantitative point of view, no obvious difference was found in the metabolism of midazolam by the different animal species. Following analyses of the extracellular medium by high performance liquid chromatography, we could demonstrate interspecies variabilities, mainly between pigs and other animal species including humans. Hence in the latter, 1-hydroxy-midazolam represented the main phase I derivative (2,10,11) while in pigs 4-hydroxy-midazolam concentration was much higher than that of the 1-hydroxylated derivative. The pharmacokinetics of midazolam and its main hydroxylated derivatives have been studied in both pigs (12) and humans (8,9) and the relationship between metabolism *in vivo* with metabolism in the hepatocyte model was clearly established.

Metabolism of SR 95156B has been studied following incubation of hepatocytes in a primary culture treated by 20 µM ^{14}C - SR 95156. Hepatocytes from

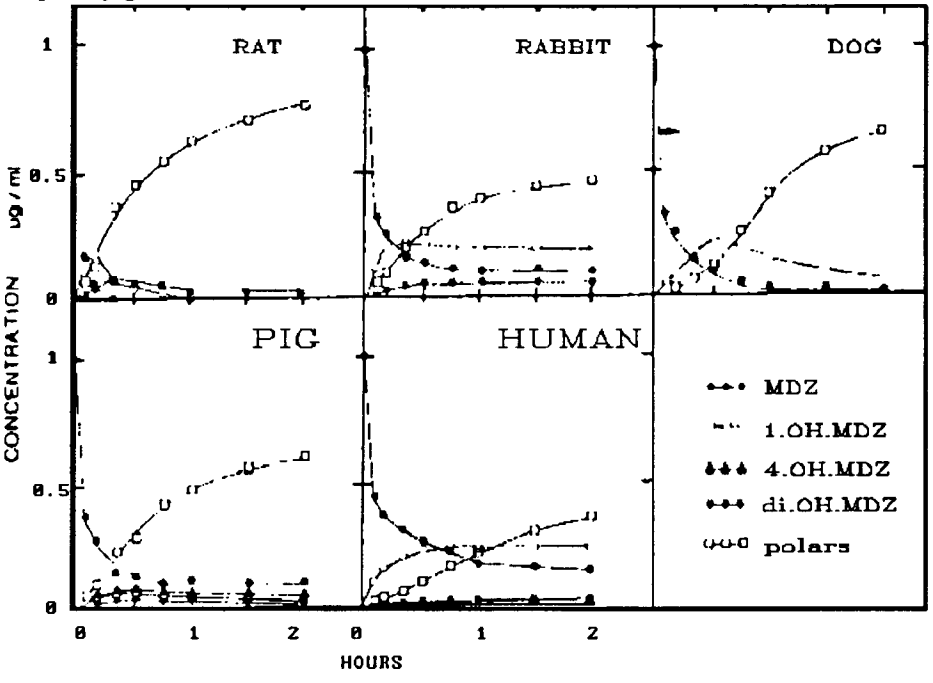

Fig. 5: Extracellular kinetic of midazolam following incubation of 1 µM^{14}C-midazolam with hepatocytes in suspension isolated from rat, rabbit, dog, pig and humans

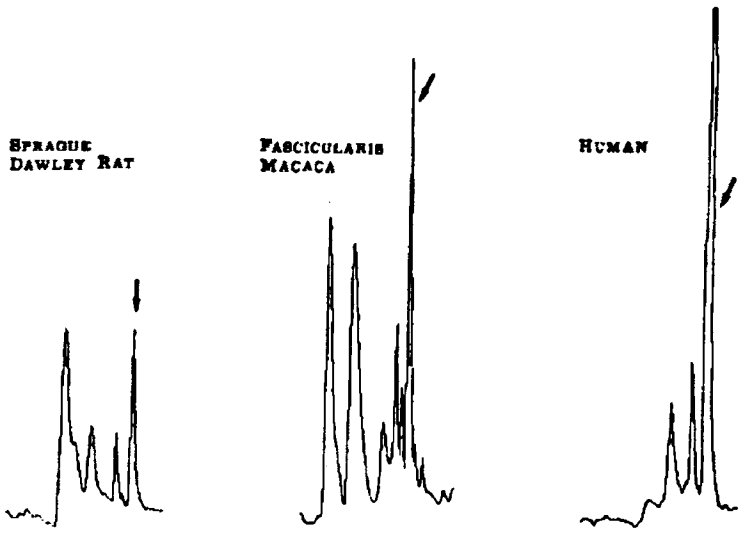

Fig. 6: High performance liquid chromatograms of extra- cellular medium obtained following a 12-hour incubation of hepatocytes in primary culture with 20 $\mu M^{14}C$ - SR 95156B. (The arrows refer to unchanged drug).

Sprague Dawley rats, Fascicularis Macaca monkey and Caucasian humans were prepared. After a 12-hour exposure to the drug, the extracellular medium was removed and analyzed by high performance liquid chromatography. Results are illustrated in Figure 6. SR 95156B was metabolized to a greater extend in Sprague Dawley rats than in macaca monkeys and humans, respectively. These data clearly demonstrate however, that metabolism was qualitatively different in the various species. In both rat and humans, three to four metabolites were detected while in Macaca, five derivatives were found. Moreover, at least two of these metabolites are not detected in rat and/or humans.

The applicability of isolated hepatocytes to the study of interspecies difference in hepatic xenobiotic metabolism has also been demonstrated by Green et al. for amphetamine (4), by Chenery et al. for diazepam (5) by Richard et al. for mitoxantrone (2,3) and by Le Bigot et al. for ketotifen (1).

In toxicology - by the detection of enzyme induction

It is now well recognized that many xenobiotics, including drugs, can be involved in the expression and/or the regulation of cytochrome P450 monooxygenases. This can have clinical implications and in particular, in polymedicated patients. Hence, for example, the decreased level of unchanged Cyclosporin A, an immunosuppressive agent, in the serum of patients treated with rifampicin is an obvious consequence of the selective and important induction of the P450(CsA) (P450IIIA3) in response to the antibiotic

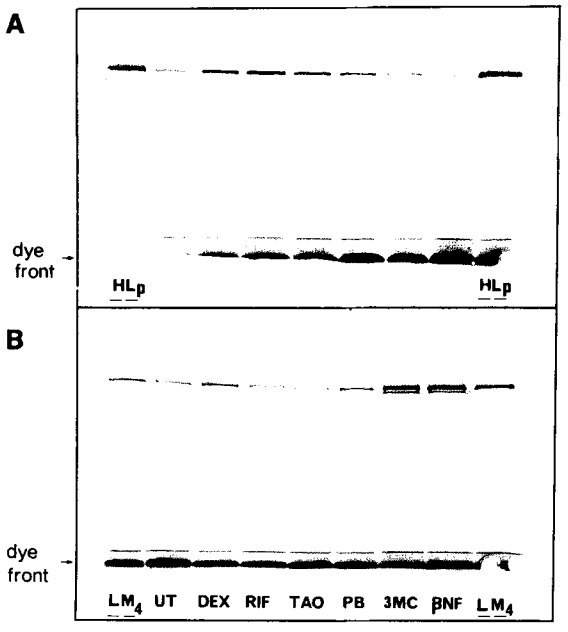

Fig. 7: Immunoblot analyses of microsomes prepared from primary cultures of human hepatocytes pretreated for 5 days with various inducers. Blots were developped with anti-LM3c (P450IIIA) IgG (A) or with anti-LM4 (P450IA) IgG (B).

(13-15). Except for special occasions (16-20), and for obvious ethical reasons, it has not yet been possible to directly study the effect of xenobiotics on the regula-

tion of metabolic enzymes, i.e. cytochrome P450, in humans. Humans hepatocytes in primary culture provide a unique and invaluable model for the investigation of these phenomena in man (6,7). A key property of P450 cytochromes is their specific inducibilities in both animals and humans by xenobiotics including polycyclic hydrocarbons (3-methylcholanthrene and β-naphthoflavone), macrolide antibiotics and glucocorticoids. In order to validate the human hepatocyte model with regard to specific cytochrome P450 isoenzyme induction, human hepatocytes in primary culture were treated over 3-4 days in the absence or the presence of various drugs known to specifically induce orthologous cytochrome P450 isozymes in animal and humans. Results are illustrated in figure 7. Microsomes prepared from cultures treated for 4 days with various inducers were analyzed by Western Blot. Immunoblots were developed with either a polyclonal antibody raised against an isozyme belonging to the P450IIIA gene subfamily (21) (figure 7, upper panel) or a monoclonal antibody raised against an isoenzyme belonging to the P450IA gene subfamily (figure 7, lower panel).

These data clearly demonstrated the specificity of the enzyme induction processes. Thus, macrolide antibiotics (rifampicin and troleandomycin) and glucocorticoïds (dexamethasone) strongly and specifically increased P450IIIA protein level while polycyclic hydrocarbons increased P450IA protein level.

These data are in complete agreement with those reported for *in vivo* studies of these cytochrome P450 gene subfamilies (19-22) in both animals and humans. These examples suggest that a breakthrough may be made in the development of new drugs by the predictability from human hepatocyte culture model to the *in vivo* human situation.

In pharmacogenetics : by the prediction of inter and/ or intra-individual variabilities

In clinical practice, drugs are often administered to patients who have already received numerous other drugs. It is becoming increasingly evident from different reports that cytochrome P450 isozymes but also Phase II enzymes such as UDP-glucuronosyl transferases and epoxide hydrolases are directly involved in a number of drug-induced hepatoxic effects and drug interactions. Some of these deleterious effects are explained in terms of genetic polymorphism, i.e. some subjects appear to be poor metabolizers for a given drug so that they exhibit high plasma concentrations, prolonged elimination half life value and in consequence prolonged and/or exaggerated pharmacological and adverse effects (23- 25). However, since the "poor metabolizing" genotype only represents 5 to 10% of a phenotyped population, human hepatocytes in primary culture cannot be utilized to investigate the deficiency in an oxidation step.

On the other hand, *in vitro* phenotyped human liver microsomal fractions which present low and high abilities to metabolize standard drugs such as debrisoquine, mephenytoin and biliburine can be used to identify the genetic polymorphism of a specific metabolic pathway (26).

Studies with human liver microsomal fractions suggest that poor metabolizers of debrisoquine lack the particular cytochrome P450 form i.e. P450IID gene subfamily, that hydroxylates the drug in normal individuals. Drugs in the early process of development can be screened for the possibility of metabolic pathway associated with a human genetic polymorphism using these microsomal fractions. At least four experimental approaches can be used to compare the metabolism of a drug to that of debrisoquine:

- studying the correlation between debrisoquine 4-hydroxylation rate and that of the drug on a large number of human microsomal fractions;
- evaluating the inhibitory effect of the drug on debrisoquine metabolism;
- measuring the competitive inhibition of quinidine (a specific inhibitor of cytochrome P450IID gene subfamily) on drug biotransformation;
- investigating the potential inhibition of anti-cytochrome P450IID antibodies on drug metabolism.

A retrospective study has been made on minaprine, an antidepressant drug which was found to exhibit the same genetic polymorphism as debrisoquine during clinical trials (P. Bechtel et al., unpublished data). Indeed using *in vitro* tools, mostly human phenotyped microsomal fractions, we demonstrated "a posteriori" that minaprine was only poorly hydroxylated by microsomal fractions exhibiting a slow metabolic rate for debrisoquine and was a competitive inhibitor of debrisoquine 4-hydroxylation. Moreover, quinidine specifically inhibited the 4-hydroxylation of minaprine (B. Lacarelle et al. unpublished data).

This *in vitro* investigation indicated that both minaprine and debrisoquine were metabolized by the same cytochrome P450 gene subfamily.

Other adverse effects come from interactions that are likely to occur between two drugs if both are specifically metabolized by the same isoenzyme

catalyzing either Phase I or Phase II reactions. Hepatocytes in primary culture allow for the detection of many potential drug interactions. Different targets can be involved such as :

- a transmembrane transport carrier which mediates the influx and/or the efflux of a drug through the hepatocyte membrane (27)
- a phase I enzyme and/or isoenzyme and in particular the monooxygenase cytochrome P450 complex (28,29). This kind of interaction has been particularly documented for drugs which exhibit high biotransformation pathways.
- a phase II enzyme, and in particular UDP - glucuronosyl transferase isozymes which are involved in the detoxification of drugs and which also constitute a multigenic family (30).

However to better understand the mechanism of the interaction between associated drugs, less complex *in vitro* models such as isolated membranes for transport processes or microsomal fractions or purified enzymes for metabolic processes, are required.

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