2. Species differences in CYP enzymes

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RESUMEN

La selección de especies animales óptimas que puedan servir para estudiar el metabolismo de fármacos en humanos es todavía un gran reto en el programa de desarrollo de fármacos. Las especies utilizadas en estudios de metabolismo deberían también incluir parámetros de toxicidad relacionados con el metabolismo. El uso de proteínas CYP recombinantes humanas y de sistemas de expresión no puede resolver este problema. Las especies más usadas en estudios metabólicos son: ratón, rata, conejo, perro y mono, y en menor grado cobayas y hamsters. Todos ellos son de alguna manera defectivos en su perfil de CYP cuando se comparan al humano. Por ejemplo, la presencia del enzima CYP1A2 en mono es controvertida; las actividades marcadoras clásicas de los CYP2C y CYP2E en perro están cuestionadas; el CYP2A de hígado de rata no cataliza la reacción de la 7-hidroxilación de la cumarina; la CYP2D de cerdo no cataliza la reacción de la hidroxilación de la debrisoquina y diversas otras formas CYP tienen pesos moleculares diferentes a los CYP humanos o de rata. Excepto en el hombre y la rata, las proporciones relativas de diversas formas CYP a nivel basal no han sido investigadas y cuantificadas en otras especies. Aunque los primates no humanos se han considerado el mejor modelo para estudiar el metabolismo de fármacos en humanos, debido a rezones éticas el uso de monos puede no ser la primera opción para el estudio *in vivo* de las características metabólicas de una nueva entidad química. Debido a las diferencias dependientes de las especies en la estructura primaria de las distintas formas CYP y en la expresion génica que conduce las cascadas reguladoras, pueden encontrarse notables diferencias en el nivel basal de la expresión de CYP en su inducibilidad y en las propiedades de unión a sustratos, inhibidores y anticuerpos. Por tanto, el principal obstáculo en la interpretación de los resultados asociados a CYP de una especie a otra es la carencia de conocimientos básicos necesarios para hacer el diagnóstico en otras especies.

1. ABSTRACT

Selection of the optimal animal species to mimic drug metabolism in man is still a big challenge in a drug development program. The species used in metabolism studies should also pick up possible metabolism-linked toxicity end points. The use of human recombinant CYP proteins and expression systems could not resolve that specific question. The mostly used species in metabolism studies are mouse, rat, rabbit, dog and monkey, lesser extent guinea pig and hamster. All of them are somehow "defective" in their CYP profile compared to man. For instance the presence of CYP1A2 enzyme in cynomolgous monkeys is controversial; classical marker activities for CYP2C and CYP2E in dogs are contested; rat hepatic CYP2A does not catalyze coumarin7-hydroxylation reaction, pig CYP2D does not catalyze debrisoquine hydroxylation reaction and several other CYP forms have different molecular weight compared to rat and human counterpart. Except man and rat, relative proportions of various CYP forms at basal level have not been investigated and quantitated for other species. Although non-human primates have considered to be the "best fit" models to mimic drug metabolism in man still due to ethical reasons the use of monkeys may not be the first option to study the metabolic characteristics of a new chemical entity in vivo. Due to species-dependent differences in the primary structure of distinct CYP forms and differences in the gene expression guiding regulatory cascades, remarkable differences in the basal CYP expression level, inducibility, substrate, inhibitor and antibody binding properties can be found. Except man and rat, relative proportions of various CYP forms at basal level have not been investigated and quantitated for other species. Therefore, the main obstacle in interpretation of CYP-associated results from one species to the other is the lack of basic knowledge in "is the finding diagnostic for other species".

2. INTRODUCTION

In a drug development program, or drug research in general, species-dependent differences in the expression of drug metabolizing enzymes and species-dependent differences in substrate specificities and responses to

diagnostic chemical inhibitors is still a big problem. Although human recombinant enzymes offer a considerable straightforward approach to study and detect metabolic characteristics in human tissues and organelles still the main obstacle is: How to select the optimal species to mimic drug metabolism in man so that in following toxicological studies relevant, possibly metabolism-linked toxicity end points, will be picked up. In order to overcome with this question I'll highlight an up to-date comparative overview based on published literature on cytochrome P450 (CYP) linked species-dependent characteristics in terms of «major» xenobiotic-metabolizing CYP enzymes in rodents, rabbit, dogs, pigs, non-human primates and man. Although extrahepatic CYP-mediated metabolism can be of great significance, the hepatic drug metabolism is still the predominant factor in species-dependent differences. Therefore, the main focus is in hepatic CYP forms. All animal CYP genes and accession numbers are available at Internet at Dr Nelson's web pages (http://drnelson.utmem.edu/CytochromeP450.html; www.imm.ki.se/cypalleles/www.icgeb.org/~p450srv/P450Nom_Animals). This review does not go in depth into each CYP form, rather a more general overview is given.

Previously some outstanding articles under the similar title have been published [1-4] which should be read together with the present overview. Already at this point I have to emphasize that in the literature there are not very many papers in which this topic has been comparatively studied with relevant species and using validated methodology. This bias have to be kept in mind when reading the text and comparing catalytic activity data in the Tables included.

3. XENOBIOTIC METABOLIZING CYP FAMILIES - GENERAL BACKGROUND AND PRESENT REGULATORY PLATFORM

3.1. Cytochrome P450 content and conditions affecting metabolic activities

According to Shimada and coworkers [5] and Stevens and coworkers [6] the species-dependent differences can be identified already from the total microsomal P450 content (Table 1) which is 3-4 fold higher in monkeys, two fold higher in rat compared to human value, and within

TABLA 1

Hepatic microsomal CYP450 content in different species

Species	*P450 Content pmol/mg protein*
Human (Our Lab)	400 up to 1000
Human	307 ± 160
Monkey	1030 ± 106
Dog	386 ± 36
Pig	488 ± 83 (f)
Minipig	821 ± 183 (f)
Guinea pig	1235 ± 95
Rabbit	681 + 36
Rat	673 ± 50
Mouse	719 + 41

data from [5, 35, 36, 77]; f, female;

human value in beagle dog microsomes [7]. In addition, in man diseases and clinical drug therapies may significantly affect or increase total P450 content and metabolizing activities and drug cleareance, thus complicating species selection. Consequently, total P450 content in human microsomes can rise up to 1 nmol/mg protein as was the clinical case after a long term phenobarbital therapy (Table 1). As a general remark from Table 1 and the others presented in the article, remarkable laboratory-dependent differences in the total P450 content and catalytic activities can be found. Obvious reasons for that are interlaboratory differences in the quality and processing methodology of the starting material. The best indicator for such differences is the human value, which —if low— reflects the differences of the quality of starting material (delay in starting preparation of subcellular organelles, methodologies used, *etc*).

Based on *in vitro* and *in vivo* data immunomodulators (see [8]) and inflammatory diseases [9-11] downregulate CYP expression. On the other hand type 1 diabetes in children has been demonstrated to enhance antipyrine clearance and drug metabolism in children [12]. Moreover, in experimental and clinical cases of hepatic injuries individual total CYP profile may change [13-15]. Interestingly, except man and rat relative proportions of various CYP forms at basal level have not been investigated and quantitated for other species. Therefore, no actual relative CYP distribution profile for other species cannot be demonstrated. Surprisingly, mouse and rabbits are perhaps the less studied species in this respect (see [16]).

3.2. Effect of diagnostic inhibitors

Major qualitative species-dependent differences have been described in general pharmacological and toxicological text books demonstrating that some basic metabolic reactions can be even species restricted ones. Moreover, although sequence homology of CYP enzymes between species is considerably high, amino acid mutations in their active center can result in remarkable substrate specificity and/or reaction velocity differences and wide variation in their response to chemical inhibitors. As an example of that ketoconazole has its main inhibitory potential against human microsomal CYP3A4 and also in a lesser potency for CYP1A1/2, 2C9 and 2D6 [17]. However, no such inhibition can be found in beagle dog microsomes against CYP1/2, 2C12 and 2D15 [18]. In rat at 10 μM concentrations considerable inhibition of CYP1A2 and 2C6 in addition of "diagnostic" CYP3A1 and 3A2 inhibition was found [19]. Moreover, in rat cDNA-expressed P450s system out of seven "diagnostic for human CYPs" inhibitors only sulfaphenazole can be considered as a selective inhibitor for rat CYP2C6 [19]. Therefore, what is "diagnostic" for one species may not be adopted across the species.

3.3. Regulatory platform

Overall pharmacokinetic characteristics of a new chemical entity have been for a long time the most crucial factor for its coming success in a

drug development program. In this respect, predictability of drug metabolism in non-clinical studies is of major value. At present several recombinant-based alternatives are available to study metabolism of a drug molecule *in vitro*. However, still the basic pharmacokinetic and toxicokinetic evaluations have to be carried out in different animal models *in vivo*. According to the up-to-date regulatory guidelines these animal models should be based on the "best fit model" to mimic physiology, disease pathology and the metabolic characteristics of man.

It is well known that species differences, both qualitatively and quantitatively, in drug metabolizing pathways are genetically determined. Therefore, to find the "best fit" model may not be an easy task to resolve. It has even been suggested that a priori knowledge of metabolizing enzymes gives no guide in identifying the failure or success of allometry for prediction of clearance [20]. I wonder if this claim can be widened to pharmacological and toxicological responses, too.

4. EXAMPLES OF SPECIES-DEPENDENT INDUCIBILITY OF CYP ENZYMES

Remarkable species-dependent differences in the inducibility of drug metabolism and mechanisms behind the phenomena can be found. A few examples will be highlighted at a very general level only; induction of CYP1A and CYP3A *in vitro* and *in vivo*.

Polycyclic aromatic hydrocarbons (PAH), 3-methylcholantrene and benzoflavone are effective inducers of CYP1A enzyme throughout the species when assayed by 7-ethoxyresorufin O-deethylase [21, 22], i.e. CYP1A1 induction seems to be a universal phenomenon. Interestingly, oral PAH are more effective inducers of minipig duodenal CYP1A than the hepatic form which follows just an opposite pattern reported in rats [22]. On the other hand, omeprazole produces a "human like" CYP1A induction in the dog, rabbit and minipig hepatocytes while rat, mouse and monkey hepatocytes are reasonably resistant to the effect of omeprazole (23, 24; Table 2).

CYP3A inducibility follows also both inducer and species-dependent pattern. In *in vitro* test system human and rat CYP3A res-

TABLE 2

The effect of omeprazole and 3-methylcholanthrene on CYP1A mRNA or enzyme activity (EROD) in vitro

Species	*Omeprazole*	*3-Methylcholanthrene*
Human	++	++
Rat	(+)	+++
Mouse	?? (high conc)	++
Rabbit	++	+++
Monkey	?? (high conc)	++
Dog	++	++
Mini pig	++	++

(+), marginal induction; ++, < 10 fold dose and time dependent increase; +++, > 10 fold increase in the parameter studies, ?? induced by high concentrations only. Data from [23, 24]

ponses to rifampin and dexamethasone are opposite. Comparison of dexamethasone and rifampin in the induction of CYP3A activity in rat, dog, pig and human hepatocytes demonstrated that rifampin is the most potent inducer of CYP3A in human hepatocytes *in vitro* followed by dog > pig but in rat hepatocytes it is not an inducer at all (Table 3). Instead, dexamethasone seems to be a more potent inducer of rat CYP3A. Moreover, in dogs the induction was more potent in males than females. In pigs no such sex-linked differences was observed [24].

Autoinduction of metabolism caused by the drug under investigation may also complicate predictability from animal metabolism data to humans. Voriconazole is an example with its nonlinear pharmacokinetic characteristics. Autoinduction is not observed in humans but multiple dosing of voriconazole results in substantially decreased exposure levels in mouse and rat, less decreased in dog and not at all in quinea pig or rabbit [25].

TABLE 3

The effect of rifampin, dexamethasone and phenobarbital on human, minipig, beagle dog and rat hepatocyte CYP3A activity in vitro

Species	*Rifampin*	*Dexamethasone*	*Phenobarbital*
Man	++++	+	+++
Minipig	++	–	??
Beagle dog	++	–	??
Rat	(+)	+++	++

(–) no induction; (+) marginal induction; (++) medium induction; (+++) strong induction; (++++) very strong induction; (??) not studied.

Data from [24, 78, 79]

5. GENERAL OVERVIEW- CYP EXPRESSIONS IN SELECTED SPECIES

5.1. Monkey

Cynomolgus monkeys express constitutively CYP1A, 2A, 2B, 2C and 3A proteins similar to man but the enzymatic profile *in vitro* using alkoxyresorufin derivatives as diagnostic probe substrates is more close to the profile obtained in rat than man [21]. Moreover, considerable differences in the total P450 content and specific immunoreactive CYP protein in hepatic microsomes from nonhuman primates has been described [26]. It has been demonstrated that a monoclonal antibody against human CYP3A4 can specifically differentiate human and Rhesus monkeys CYP3A proteins from mouse, rat and dog counterparts [27] and that in African green monkey CYP2A6 mediated nicotine metabolism mimic human situation [28]. Moreover, phenobarbital has been shown to increase CYP2B6 immunoreactive protein in African green monkey [28]. Therefore, because of their similar CYP profile, monkeys in general can be considered a species of choice in drug metabolism studies. However due to ethical considerations the use of primates may not be the first option in the ranking list when selecting the species for the first pharmacokinetic evaluations. In drug development program the selection of species has to be put in the perspective with both the chemical and indication under study.

5.2. Dogs

In beagle dogs CYP families 1A, 2A, 2B, 2C, 2E and 3A are expressed [7, 18, 29]. Interestingly, in one comparative study of CYP2C proteins using tolbutamide hydroxylation assay as a probe the activity could not be detected in dog microsomes [29] but after phenobarbital induction a protein, an N-terminal amino acid sequence of which resembles CYP2C protein, has been purified [30]. In dogs phenobarbital, rifampin, 3 methylcholanthrene and β-naphthofalvone were the most potent inducers of the total CYP450 content *in vivo*. Unfortunately, no other species was used as a reference to compare the potency of induction and profile of CYP enzymes involved. Two bands in Western blotting were detected after β-naphthofalvone induction, a well-known inducer of CYP1A enzymes. The expression of CYP2B11 was increased after phenobarbital treatment but no effect after ethanol treatment on 4-nitrophenol hydroxylase or chlorzoxazone 6-hydroxylation activites was found. Rifampicin and phenobarbital enhanced testosterone 6β– hydroxylase activity *ex vivo* and *in vitro*, both of them affecting the expression of CYP3A12 [7]. Whether, CYP2C and 2E1 proteins are expressed at basal level in dogs seems to depend on experimental conditions and methodology used.

A good example for species-dependent enantiomer specific metabolism is dog-associated stereoselective metabolism of bisoprolol. In dogs bisoprolol is extensively metabolized by both high affinity CYP2D and low-affinity CYP3A enzymes; with the overall R(+)/S(-) ratio of 1.46 favoring the R isomer while in humans stereosectivity is performed only via the low-affinity CYP2D form that represents only for less than 5% of the metabolism of bisoprolol in man [31]. Suggesting that in man stereoselectivity has no clinical value. On the other hand, in baculovirus expressed CYP proteins dog enzymes catalyzed dextromethophan O-demethylation and N-demethylation by CYP2D15 and 3A12, orthologus to human CYP2D6 and 3A4 [32], thus mimicing human metabolic characteristics. In general dog is a good alternative model for drug metabolism studies.

5.3. Pig

Pig and mini/micropig is increasingly used in pharmacokinetic and toxicity studies. Its use is relevant; it is omnivore as man and the pig

is suggested to be the species of choice for hepatocyte xenograft in humans. However, thus far characterization of CYP enzyme profile in pig/minipig has resulted in contradictory CYP expression results. Especially problematic is CYP2D expression. In one study all main CYP activities excluding 7-pentoxyresorufin O-dealkylation (CYP2B) were detectable in reasonable quantity in mirosomal fraction of minipig liver [33]. In the other studies [34-36] CYP 2C and 2D linked enzymatic activities could not be detected reliably, although the CYP2D25 gene with 75% homology with the human 2D6 has been identified in pig liver. In addition, swine homologs to CYP1A, 2B and 2E posses different molecular weight from their rodent and human counterparts [37]. In further studies a human cDNA could detect a CYP2D6-like mRNA and positive immunoreactive protein was found from pig hepatocytes [38].

Pig microsomes do not metabolize debrisoquine. Instead pig microsomes metabolize dextromethorphan with a biphasic kinetics, and bufuralol, but once again no correlation between these two enzymatic activity and CYP2D protein could be demonstrated [38, 39]. Instead, a positive correlation with CYP2B immunoreactive protein content and an inhibitory response to chemical inhibitors of CYP2B was observed [39]. Moreover, remarkable sex-dependent differences in CYP1A and 2E enzymes was observed [35]. The basal activities of CYP1A are reported to be 6- fold, CYP2A 6-fold and CYP2C 3 —fold higher in human liver microsomes compared to minipig enzymes levels. On the other hand "CYP2D-linked" activity was 4-fold higher in minipig liver microsomes compared to that obtained in human liver microsomes [33]. Due to these extremely contradictory results (missing activities, different molecular weights) more confirmatory studies are needed to validate pig for CYP2D panel. Probably this discrepancy is due to pig strain differences [34, 35]. On the other hand minipig has been shown to be a reasonable good model for alcohol-induced liver damage in man [40] and polycyclic aromatic hydrocarbon contaminated soil has been demonstrated to induce CYP1A expression in hepatic and extrahepatic tissues in minipigs after oral exposure [22] thus mimicking cicarette smoke induction of CYP1A in man [41].

6. SPECIES-DEPENDENT EXPRESSION OF CYP ENZYMES

6.1. Comparative studies within the CYP1A enzymes

CYP family 1A is the most conserved amongst all. Their substrates are common throughout the species; mainly planar polycyclic aromatic hydrocarbons for CYP1A1/2. According to the microsomal fraction *in vitro* enzymatic data throughout the species (mouse, rat, rabbit, dog, micropig, monkey, man) 7-ethoxyresorufin O-deethylation is characteristic for this enzyme. Other relevant marker activities are dealkylation reactions of other alkoxyresorufin derivatives, phenacetin O-deethylation, acetanilide 4-hydroxylation and N-hydroxylation reaction of several heterocyclic amines.

Remarkable species-dependent differences can be observed in the metabolic capacity towards the substrates listed above. For example, ethoxyresorufin O-deethylase activity was inhibited by 80 –100% by human anti-CYP1A antiserum and α-napthoflavone in mouse, rabbit, dog and human liver microsomes, but only moderately in rat, micropig and monkey liver microsomes [3]. Furafylline which have considered to be preferentially CYP1A2 specific inhibitor in human liver microsomes [42, 43] produced a very strong inhibition only in human samples, moderate or even less inhibition in rat, mouse, rabbit and dog microsomes and did not inhibit at all in monkey and micropig microsomes [3]. Representative examples concerning CYP1A enzymatic activities in different species from comparative studies is shown in Table 4.

Although catalytic characteristics of CYP1A subfamily are quite well conserved throughout the species, still some species-dependent differences in the substrate specificity of CYP1A can be found. For instance, rat CYP1A2 protein is more active in 7-methoxyresorufin O-deethylation than the human counterpart but the human CYP1A2 was more active in the N-hydroxylation of 2-amino-3,8-dimethylimadozo(4,5-f)quinoxaline (MeIQ) [1]. In another comparative study on CYP1A2 linked procarcinogen activation of 2-amino-3-methyl-imidazole(4,5-f)quinone was the most active in rats followed by monkey > dog=human microsomes. However, antibody sensitive responses followed the order human=rat > dog > monkey [44]. Studies with anti-human CYP1A antibody and chemical

TABLE 4

Representative examples of CYP1A associated enzymatic activities in different species and laboratories.

Species	*Phenacetin O-deethylation Ref. [5]*	*Ethoxyresorufin O-deethylation*		
		[80]	*[36]*	*[3]*
		pmol/min/mg protein		
Human	32±30	21±14	18.3±6.5	95 m; 25 f
Monkey	110±20	240±85	ND	129 m; 118 f
Dog	28±10	46±25	23.1±3.9	409 m; 411 f
Guinea pig	172±20	ND		
Rat	36±20	ND	31.1±10.9	78 m; 67 f
Pig/minipig	ND	ND	5.4±2.1	57 m; 116 f

ND, not determined; f, female; m, male

inhibitor (α-naphthoflavone) have resulted remarkable interspecies differences and picked up a group of species in which a similar inhibitory power in 7-ethoxyresorufin O-deethylase activity was obtained. Similar inhibitory characteristics were obtained in mouse, rabbit, dog and human liver samples [3]. It is worthy to emphasize that thus far no real comparison between species and enzyme sources with all suggested substrates of CYP1A subfamily have been carried out. Therefore, real interspecies differences still remain to be resolved.

Antibodies against distinct CYP proteins, both diagnostic and inhibitory ones, and the results obtained differ remarkably between laboratories. An antibody against human CYP1A2 recognizes antigenic determinants in cynomolgus monkey and beagle dog microsomes but, interestingly, not in Sprague-Dawley rat liver microsomes [5]. However, monoclonal antibody against 3-methylchoanthrene-induced CYP1A in rat recognizes an orthologous form across the species in different test systems [45]. Interestingly, an anti-human CYP1A antibody has been shown to influence on dog microsomal CYP2E1 mediated chlorzoxazone 6-hydroxylation [3].

Although primates in general have been considered more closely mimic CYP enzymes in man, still some differences exist. Based on heterocylic ami-

ne N-oxidation studies [46] cynomolgous monkeys have been found to be defective for CYP1A2 enzyme. On the other hand, it has been shown that chronic exposure of cynomolgus monkeys to IQ induce hepatic microsomal CYP1A1 and CYP1A2, and CYP3A and CYP2C being the principal forms associated with metabolic activation of IQ [47]. Obviously this will restrict the use of this species in CYP1A2 linked metabolism and toxicity studies.

6.2. Comparative studies within the CYP2A

CYP2A protein was originally purified from pyrazole-induced mouse liver microsomes [48]. CYP2A -linked metabolic characteristics have been intensively studied in man, rabbit, rat monkey, hamster and human tissues and subcellular organelles. It became clear that these proteins differ substantially in catalytic specificity and inducibility between the species and evens strains [49]. However, still one common denominator exists; all CYP2A proteins catalyze coumarin 7-hydroxylation reaction with a variable intensity (Table 5). However, one exception exists. In the rat liver, the CYP2A3 gene is not virtually expressed and coumarin 7-hydroxylation activity is negligible [3, 50, 51]. Unlike in mouse, the gene was not inducible in rat liver by 3-methylcholanthrene or pyrazole [51]. On the other hand, dispite the immunological and catalytical similarities between mouse and hamster CYP2A enzymes in hamsters 3-methylcholantrene does not enhance the expression of CYP2A and pyrazole decrease CYP2A expression and sex-oriented expression pattern is opposite between the two species [52, 53].

Among the important toxicity study species the coumarin 7-hydrolylase activity is the lowest in dog hepatic microsomes and highest in mouse, rabbit, monkey and human liver microsomes [3, 54]. The reaction velocity is comparable between human and African green monkey microsomes but still immunological and chemical inhibitor-dependent species differences can be obtained [54] demonstrating that the proteins in these two species are not identical.

Remarkable species-dependent differences in inhibition of coumarin 7-hydroxylase activity between mouse and human have been observed. Compared to other CYP mediated reactions pilocarpine was a reasonably selective inhibitor (Ki <1 μM) for mouse and human coumarin 7-hydroxylase and of similar potency [55, 56]. However, metyrapone is two orders of mag-

TABLE 5

Representative examples of CYP2A associated coumarin 7 hydroxylase activity determinations in different species and laboratories

Species	*Ref. [5]*	*Coumarin 7-hydroxylation* *[36]*	*[3]*
		(pmol/min/mg protein)	
Human	21±12	617±256	1275 m; 357 f
Monkey	209±126	ND	2398 m; 7175 f
Dog	12±5	6.5±5.9	68 m; 180 f
Guinea pig	20±8	ND	ND
Rat	<1	1.2±0.4	<5 m/f
Pig/minipig	ND	16.6±7.9	18 m; 966 f

ND, not determined; f, female; m, male.

nitude more potent inhibitor for mouse than human CYP2A [57]. Comparative molecular field analysis and *in vitro* inhibition studies demonstrated that naphthalene is competitive inhibitor *in vitro* both for mouse and human hepatic coumarin 7-hydroxylase with the Ki of 12-26 and 1,2 –5.6 μM [58], respectively. 5- or 6-position substituted γ and δ- lactones are potent inhibitors for mouse CYP2A5 but not for human CYP2A6 [59].

Based on the above examples it can be concluded that with respect of CYP2A linked (coumarin 7-hydroxylation) metabolism the "best fit" models are mouse, dog, pig, and monkey.

6.3. Comparative studies within the CYP2B

In humans the only representative of this family is CYP2B6, the expession of which is less than 1% of the total hepatic CYP pool [60] with more than two order of magnitude interindividual variation in bupropion hydroxylation velocity [61]. Other possible substrates (used in other species, such as mouse, rat, rabbit, dog, monkey and human [1] are phenoxazone derivatives, 7-ethoxycoumarin, 6-aminochrysene, 3-methoxy-4-aminoazobenzene and nicotine [60-63].

In other species than man several active forms are expressed: rat, 2B1, 2B2; rabbits 2B4, 2B5; dog 2B11 and one 2B form in monkey with a high testosterone hydroxylation activity [26]. For instance, in beagle dogs a 13-fold increase in pentoxyresorufin O-dealcylase activity after phenobarbital induction was seen [7].

However, no comparative study using the same validated methodology has been carried out to resolve species-dependent differences in catalytic characteristics of CYP2B enzymes with the above mentioned substrates. Therefore, no clear ranking between species can be carried out.

6.4. Comparative studies within the CYP2C family

CYP2C family comprises about 20% of total hepatic P450s in humans and four representatives of 2C enzymes have been characterized (2C8, 2C9, C18, 2C19) with >80% identical amino acid sequences. Marker activity for polymorphically expressed CYP2C8 in humans is paclitaxel 6-hydroxylation [64]. Based on enzyme classification or substrate specificity studies no such counterpart has been identified experimental animals.

Marker activities for CYP2C9 are S-warfarin 7-hydroxylation and tolbutamide methyl hydroxylation reactions [65], for CYP2C19 S-mephenytoin 4-hydroxylation and tolbutamide methyl hydroxylation [66].

In animals a huge species-dependent differences within this family can be observed (Table 7). The lowest catalytic mephenytoin 4'-hydroxylase determinations were recorded in minipigs [3]. In rats eight CYP2C forms are expressed with remarkable gender, growth hormone and other circadial rythm-linked factors affecting their expression [67-69]. For example, based on *in vitro* assays and antibody inhibition experiments the N-oxidation of irsogladine is catalysed by CYP2C proteins in rat, dog and monkey microsomes but no significant metabolism was obtained by human microsomes or recombinant CYP2C9 protein [70].

As a recent example of species-dependent CYP2C differences is metabolism of (-)-verbenone. In the rat, the male-specific CYP2C11 was a major enzyme in (-)-verbenone 10-hydroxylation by untreated rat livers, and CYP2B1 catalyzed this reaction in liver microsomes of phenobarbital-treated rats. A female-specific enzyme, CYP2C12 did not catalyze (-)verbeno-

ne 10-hydroxylation. Human recombinant CYP2A6 and CYP2B6 catalyzed (-)verbenone 10-hydroxylation at Vmax values of 15 and 21 nmol/min/nmol P-450 with apparent Km values of 16 and 91 μM. Rat CYP2A1 and 2A2 did not catalyze (-)-verbenone 10-hydroxylation at all [71].

In rabbits 2C enzyme profile is even more complicated; nine forms (2C1, 2C2, 2C3, 2C4, 2C5, 2C14, 2C15, 2C16, 2C30) have been identified (www.icgeb.org/~p450srv/P450Nom_Animals_2.html#CYP2C). In rabbit CYP2C protein(s) catalyses testosterone 6β-hydroxylation. Obviously multiplicity of CYP2C forms in rabbits is compensatory to replace the wide catalytic flexibility of CYP3A4 in human liver.

S-mephenytoin has been used as a probe for human CYP2C19. Interspecies comparison (mouse, rat, rabbit, dog, monkey, human) demonstrated that microsomal 4-hydroxylation of R-enantiomer was 2 to 6 times higher in rabbits, dogs and rats compared to S-enantiomer hydroxylation rates. Preferential, a human-like, S-mephenytoin 4-hydroxylation was obtained only with monkeys [72]. Representative examples concerning CYP2C enzymatic activities in different species is shown in Table 6.

Due to multiple genes in animals CYP2C proteins have obviously taken some stake holding properties which are linked CYP3A4 in man. Mo-

TABLE 6

CYP2C associated mephenytoin 4'-hydroxylation in different species and laboratories

Species	*Ref. [5]*	*Mephenytoin 4'-hydroxylation [3] pmol/min/mg protein*
Human	39±23	19.8m; 9.6 f
Monkey	144±26	23 m; 19.7 f
Dog	106±13	3.2 m; 5 f
Guinea pig	139±17	ND
Rat	74±15	1.6 m; 0.6 f
Minipig	ND	< 0.4 m; < 0.3 f

ND, not determined; f, female; m, male

reover, the sex-dependent expression CYP2C proteins in several species complicate interpretation of any animal CYP2C data across the species and to humans. The low catalytic mephenytoin 4'-hydroxylation may decrease the value of minipig in CYP2C linked metabolic studies.

6.5. Comparative studies within the CYP2D

Due to clinically relevant interactions with several crucial drugs, such as cardiovascular and psychotrophic drugs, and a well-known pharmacogenetic polymorhism, this form in humans is under special focus. If a drug candidate demonstrates interactive potential towards this protein it may be a reason to discard the molecule from further development.

Humans have only one catalytically active polymorphically expressed form CYP2D6. It represents about 4% of total P450 content in the human liver. In rats four genes (2D1-4) have been identified but, for instance, their overall amount in rat's hepatic total P450 pool is still unresolved. Moreover, catalytic characteristics or responses to chemical inhibitors of rat CYP2D forms have not been studied in details.

In a comparative study [3] using bufuralol 1-hydroxylase activity as a probe for the enzyme remarkable species-dependent differences were observed. The species were divided into two groups; dog and man having similar enzyme kinetics and quinidine inhibition profiles compared to the rest tested: mouse, rat, rabbit, micropig and monkey [3].

Interestingly, rabbit genome does not contain any gene corresponding to human CYP2D6 [73]. Representative examples concerning CYP2D enzymatic activities in different species is shown in Table 7.

In primates several CYP2D proteins have been identified. For instance, from Japanese monkey liver a 497 amino acid protein (CYP2D29) shared 96, 91 and 88 % homology with human CYP2D6, cynomolgus monkey CYP2D17 and marmoset monkey [74]. CYP2D29 content was considerably higher in Japanese monkey liver microsomes than CYP2D6 in humans. Km values of bufuralol 1-hydroxylations were comparable between CYP2D6 and 2D29 but the Km value for debrisoquine hydroxylation was much lower in Japanese monkey CYPD29 compared to human CYP2D6 [74].

TABLE 7

CYP2D associated bufuralol 1'-hydroxylation in different species and laboratories

Species	*Ref. [5]*	*Bufuralol 1'-hydroxylation [3] (pmol/min/mg protein)*
Human	20±14	151 m, 87 f
Monkey	471±58	1167 m; 955 m
Dog	12±2	191 m; 260 f
Guinea pig	29±14	ND
Rat	743±74	1021 m; 596 f
Minipig	ND	653 m; 638 f

ND, not determined; f, female; m, male

Based on the date above the most human-like CYP2D responses can be obtained by dog microsomes. The use of pig or minipig microsomes or hepatocytes as a denominator for CYP2D linked reactions is still under debate.

6.6. Comparative studies within the CYP2E

According to the study of Court et al (1997) [75], based on the chlorzoxazone 6-hydroxylation activity determinations, CYP2E1 is quite well conserved throughout the species. A single-enzyme Michaelis-Menten model was obtained for cat, cow, dog, horse, human, mouse and rabbit microsomal samples but a two-enzyme model was obtained for ferret, monkey, pig and rat. Moreover, diethyldithiocarbamate was a potent mechanism-based inhibitor of 6-OH- chlorzoxazone formation in all species studied. [75]. However, the results from Bogaards et al. [3] did not support this conclusion in which 10-fold species-dependent differences in the chlorzoxazone 6-hydrolation were observed, and an anti rat CYP2E1 antibody did not inhibit chlorzoxazone 6-hydrolase activity in micropig microsomes [3]. However, a monoclonal antibody against rat CYP2E1 detects a CYP2E counterpart in immunohistochemistry [40]. In conclusion, this suggests that pig may not be the optimal animal model for CYP2E1

mediated reactions. More comparative studies with substrates, diagnostic inhibitors and antibodies will be needed to resolve this discrepancy.

6.7. Comparative studies within the CYP3A

Testosterone 6β-hydroxylation reaction has been used as a marker for CYP3A4 in human tissues. CYP3A4 and rat 3A12 share 79% amino acid sequence similarity but still - based on antibody studies - their active sites seem to be substantially different. As an example is the metabolism of dexamethasone in mouse, rat, quinea pig, hamster and human liver microsomes *in vitro*. Remarkable qualitative and quantitative species-dependent differences were observed. Potency of ketoconazale inhibition varied according to species so that it cannot be regarded as a selective inhibitor of CYP3A enzymes in all species [76]. On the other hand nifedipine hydroxylation and testosterone 6β-hydroxlylation activities are comparable between man and minipig but inhibition potency of nifedipine oxidation by triactyloleandromycin was considerably lower in minipig microsomes than in human samples [33]. In dogs testosterone 6β-hydroxylase has been reported to be one eight of that obtained by human microsomes [29]. Representative examples concerning CYP3A enzymatic activities in different species and substrates are shown in Table 8.

TABLE 8

CYP3A associated enzymatic activities (erythromycin N-demethylation, testosterone 6ß hydroxylation and nifedipine oxidation) in different species

Species	*Erythromycin N-demethylation*	*Tβ-OH*	*Nifedipine oxidation*
		(pmol/min/mg protein)	
Human	244±212	5307 m; 8997 f	605±691
Monkey	586±101	11941 m; 10764 f	3826±131
Dog	144±70	1488 m;1656 f	456±126
Guinea pig	500±65	ND	2990±815
Rat	930±164	3396 m; 378 f	820±187
Minipig	ND	4852 m; 4116 f	ND

Erythromycin N-demethylation [5]; testosterone 6b hydroxylation [3]; nifedipine oxidation [36]; ND, not determined.

7. CYP ENZYMES AND TESTOSTERONE HYDROXYLATIONS

Testosterone 6β-hydroxylation reaction is generally used for CYP3A determinations in man. Moreover, numerous other testosterone hydroxylation reactions are catalyzed by the other CYP enzymes. Table 9 summarizes briefly major testosterone hydroxylation products and CYP forms mainly catalyzing the reactions according to species. It can be seen that depending on the species several unresolved activities exists. Further analysis with rat, pig, rabbit, dog and human hepatocytes has demonstrated [36] that according to the total qualitative and quantitative steroid hydroxylation profile the "best-fit" animal models are pig and dog. Total testosterone metabolite profile was in those species mostly "human-like" both quantitatively and qualitatively. In rat and rabbit hepatocytes remarkable deviation was observed. Unfortunately, monkey microsomes or hepatocytes were not included in the study.

TABLE 9

Testosterone hydroxylation reactions and the main catalyzing CYP forms in microsomal fractions from man, rat, rabbit, dog and pig

Testosterone Hydroxylation	*Man*	*Rat*	*Rabbit*	*Dog*	*Pig*
15β	3A4	3A1	?	?	?
6β	3A4	1A1/2	2C3, 3A6	3A12	3A
7α	ND	2A1	ND	ND	ND
16α	?	2B1,2C11	2B4	2C21	?
16β	2C9	2B1	?	3A12	?
2α	ND	2C11	ND	3A12	ND
2β	3A4	ND	?	3A12	?
Formation of androstenedione	Isomerase	2B1,2C11	?	?	?

(??) activity detectable, but the CYP form catalysing the reaction has not been identified. ND, not detectable; (modified by the author from [32, 36]; and based on the references in the article and www.xenotechllc.com)

8. CONCLUDING REMARKS

According to the present date the selection of optimal model to mimic metabolism in man based on CYP enzymes only is not an easy task. As a general remark CYP1A and CYP2E associated activities and responses are more generalized that those of other CYPs. Especially wide variation in catalytic responses is within the CYP2C proteins. They are expressed in multiple forms and some of those are under sex hormone or growth hormone regulation. Obviously all the data obtained by rat is relevant but still it remains uncertain weather it is the optimum model for man. Via transgenic and knock- out mouse models the importance of mouse pathologists and overall understanding of mouse xenobiotic metabolizing enzymes has increased. Still, characterization of the xenobiotic-metabolizing pathways in mouse is far form the details reported for rat. Pig or minipig, is an interesting alternative for rodents in toxicological studies but yet their documentation in terms of CYP, for instance, and scientific basis is far from the traditional rodent ones. Especially warranty should be considered if CYP2C, CYP2D or 2E are involved in the metabolism of a compound under investigation. Hamster and rabbit seem to be far from any validated model for CYP panel. On the basis of the testosterone metabolizing CYP activity profiles dog and pig seem to be relevant species to mimic CYP enzymes in man.

In conclusion: More comparative studies with validated methodology are needed to resolve species-dependent differences in catalytic characteristics; inhibitions with different antibodies, reaction kinetics with "diagnostic" substrates and chemical inhibitors *e.t.c.* To this aim a global "interspecies CYP test panel tool box" should be established and systematically evaluated in order to facilitate comparisons of the results obtained in different laboratories and species, and, especially in the interpretation the obtained result to man.

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