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Comparative MO-QSAR studies in various species including man

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Abstract

In the present study it is demonstrated that MO-QSARs (quantitative structure activity relationships based on calculated molecular orbital substrate characteristics) of cytochrome P450-catalysed biotransformation of benzene derivatives obtained in previous studies for Wistar rats, can be extrapolated to other species, including man. First, it was demonstrated that the regioselectivity of the in vivo aromatic hydroxylation of two fluorobenzene derivatives can be quantitatively predicted, on the basis of the calculated density distribution of the reactive π-electrons in the aromatic ring of the fluorobenzene derivative, for all experimental animal species tested. Second, it was investigated whether the preferential site for in vitro aromatic hydroxylation of 3-fluoroaniline could be predicted on the basis of the same calculated parameter. This was done because extrapolation to human systems requires in vitro instead of in vivo experiments. The results obtained indicated that the variation in the regioselectivity of the aromatic hydroxylation of 3-fluoroaniline by liver microsomes from different species, including man, was only a few percent, and was mainly directed by calculated chemical reactivity characteristics of the 3-fluoroaniline substrate. Finally, possibilities for the extrapolation from rat to other species, of the MO-QSAR for the rate of in vitro C4 hydroxylation of a series of aniline derivatives converted in an iodosobenzene-supported microsomal cytochrome P450 system, were investigated. Experiments with liver microsomes from rats, mice, rabbit and man resulted in clear MO-QSARs with correlation coefficients for the relationship between the ln k_{cat} and the E(HOMO) of the aniline substrates that were ≥ 0.97 in all cases. Thus, the results of the present study clearly demonstrate that MO-QSARs previous-

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ly described for Wistar rats can be extrapolated to mice, rabbit, guinea pig and even to man. Regioselectivities obtained and QSAR lines for the rate of conversion plotted against calculated E(HOMO) values of the aniline derivatives are similar for the various species investigated. Altogether, these results strongly support the conclusion that the conversion of the relatively small benzene derivatives in the relatively large and aspecific active sites of the mammalian cytochromes P450, even when derived from various species, are mainly dependent on chemical reactivity parameters of the substrates. Therefore, the results of the present study support the hypothesis that MO-based QSARs obtained in rat for the cytochrome P450 catalysed aromatic hydroxylation of benzene derivatives can provide a basis for prediction of biotransformation pathways in different species, including man.

Keywords: Cytochrome P450; Species differences; Aromatic hydroxylation; Halogenated benzene derivatives; Molecular orbital calculations; MO-QSARs

1. Introduction

Upon exposure of mammals to benzene derivatives these compounds are known to be metabolised in phase I and phase II type biotransformation reactions before they can be excreted from the body, or before they exert their toxic effects [1,2]. Phase I metabolism of substituted benzene derivatives is mediated by the cytochrome P450 enzyme system, which consists of a series of isoenzymes with broad, and partially overlapping substrate specificity. Metabolic patterns resulting from cytochrome P450-catalysed biotransformation may vary with variation in the cytochrome P450 enzyme pattern. This enzyme pattern is dependent on both external and internal factors. Among the internal factors, species differences are known to influence the cytochrome P450 enzyme pattern. Species differences have been reported for both Phase I and Phase II enzymes and can result in variation in both routes and rates of metabolite formation [3–6]. Such species differences, and, as a result, variations in metabolic profiles, may hamper extrapolation of results obtained in biotransformation studies with one type of experimental animal to other animal species, or, ultimately, to man.

Variations in the cytochrome P450 enzyme pattern of the microsomal preparations, due to pretreatment of animals by specific inducers or due to species differences, are well known to be able to affect the outcomes of the biotransformation. For example, the metabolic profile of chlorobenzenes was influenced by the type of cytochrome P450 enzymes present in the microsomal preparations [7,8]. For bromobenzene it has been reported that 3-methylcholanthrene-induced cytochrome P450 patterns result in preferential hydroxylation at the position ortho with respect to the bromine substituent, leading to relatively harmless metabolites, whereas metabolism by a phenobarbital induced cytochrome P450 population results in preferential para hydroxylation of bromobenzene leading to formation of intermediates that induce liver damage [9,10]. In contrast to these results, previous studies demonstrated that for the cytochrome P450-catalysed conversion of relatively small substrates, like fluorinated benzenes or monofluoroanilines, QSARs (quantitative structure activity

relationships) for their conversion by cytochromes P450 could be obtained and the influence of variations in the cytochrome P450 enzyme pattern appeared to be relatively small [11–13]. These QSARs were based on calculated molecular orbital characteristics, and thus, chemical reactivity characteristics of the substrates. It was demonstrated for instance that the actual regioselectivity of the cytochrome P450-catalysed conversion of a series of fluorinated benzenes could be predicted within 6% accuracy and a correlation coefficient of 0.96 on the basis of the calculated reactivity of the various carbon centres in the benzene derivatives for electrophilic attack [11]. In a similar way the preferential site of hydroxylation for a series of monofluoroanilines could be predicted [12]. Moreover, the actual rate of 4-hydroxylation of a series of halogenated aniline derivatives in an iodosobenzene-supported cytochrome P450-catalysed conversion was demonstrated to correlate with the calculated energy of the reactive π-electrons in the aniline substrate, providing another MO-QSAR (QSAR based on calculated molecular orbital characteristics) for a cytochrome P450-catalysed reaction [13].

Thus, for relatively small substrates, chemical reactivity characteristics of the substrates may dominate over the influence of the specific P450 enzymes in determining the regioselectivity of the biotransformation both in vitro and in vivo and also in setting the rate of the reaction in an iodosobenzene-supported system in vitro. This observation would imply that MO-QSARs obtained up to now for cytochrome P450-catalysed reactions using the cytochromes P450 from Wistar rats may be valid for other species as well. The present study was undertaken to investigate whether the MO-QSARs obtained up to now for rat cytochromes P450, are also valid for the prediction of the metabolism of substituted benzene derivatives by other species than rat, and thus, may provide a basis for extrapolation to man. Therefore, in the present study, the validity of QSARs previously described for reactions catalysed by cytochromes P450 from Wistar rats, was investigated for cytochromes P450 obtained from mice, guinea pig, rabbit and also from man.

2. Materials and methods

2.1. Chemicals

Aniline, 2-fluoroaniline, 3-fluoroaniline, 2-chloroaniline, 3-chloroaniline and 2,3-difluoroaniline were purchased from Janssen Chimica (Beerse, Belgium). 1,3-Difluorobenzene was obtained from Fluorochem (Derbyshire, UK). 1,2,4-Trifluorobenzene was purchased from Aldrich Chemie (Steinheim, Germany). The purity of all compounds was >98%. Iodosobenzene was synthesized by the base-catalysed hydrolysis of diacetoxyiodobenzene (Fluka, Switzerland), essentially as described by Saltzman and Sharefkin [14], and added to the incubations from a 20 mM sonicated suspension in 10% DMSO in demineralised water.

2.2. In vivo exposure of different species to 1,3-difluoro- and 1,2,4-trifluorobenzene

Male U inbred, R inbred and Sprague–Dawley (SD) rats (400 g) were exposed to

200 μmol (500 μmol/kg body weight) of the 1,3-difluoro- or 1,2,4-trifluorobenzene, administered in olive oil by oral injection. Male NMRi, C57/BL mice (40 g) were exposed to 20 μmol (500 μmol/kg body weight), male New Zealand White (NZW) rabbit (4000 g) was exposed to 2000 μmol (500 μmol/kg body weight) and male guinea pig (800 g) was exposed to 400 μmol (500 μmol/kg body weight) of the 1,3-difluorobenzene or 1,2,4-trifluorobenzene, administered in olive oil by oral injection. After dosing, 0–24 h and 24–48 h urine samples were collected and stored at −20°C until analysis.

2.3. Analysis of urine samples

Urine samples were analyzed by ^{19}F NMR after 1:1 dilution in 0.2 M potassium phosphate (pH 7.6). Enzyme hydrolysis of urine samples was carried out as described previously [15] using either β-glucuronidase from *Escherichia coli* K12 (Boehringer, Mannheim, Germany) or arylsulphatase/β-glucuronidase from *Helix pomatia* (Boehringer, Mannheim, Germany). Samples were made oxygen free by 4 cycles of evacuation and filling with argon.

2.4. ^{19}F NMR measurements

^{19}F NMR measurements were performed on a Bruker AMX 300 spectrometer as described previously [15–17]. Between 1500 and 20 000 scans were recorded, depending on the concentrations of the fluorine-containing compounds and the signal to noise ratio required. The sample volume was 1.71 ml, containing 100 μl of $^{2}H_2O$ for locking the magnetic field and 10 μl of 8.4 mM 4-fluorobenzoic acid solution added as an internal standard. Chemical shifts are reported relative to $CFCl_3$ [18]. Concentrations of the various metabolites observed in ^{19}F NMR spectra were calculated by comparison of the integrals of their ^{19}F NMR resonances to the integral of the ^{19}F NMR of 4-fluorobenzoic acid, as an internal standard.

2.5. Preparation of microsomes

Microsomes were prepared from the perfused livers of male rats (400 g), male mice (40 g) and male NZW rabbit (4000 g), which were treated with isosafrole (Sigma, St. Louis, MO), 150 mg/kg body weight, using a stock solution 100 mg/ml in olive oil, administered i.p., daily for 3 days. Following homogenisation of the livers in Tris-sucrose buffer (50 mM Tris–HCl, 0.25 M sucrose, pH 7.4) and centrifugation at 10 000 × *g* (20 min), the supernatants were centrifuged for 75 min at 105 000 × *g*. The microsomal pellet was washed once with Tris-sucrose buffer and finally suspended in 0.1 M potassium phosphate pH 7.25 containing 20% glycerol and 1 mM EDTA. The final preparation was immediately frozen in liquid nitrogen and stored at −80°C, until use.

Cytochrome P450 contents were determined as described by Omura and Sato [19]. Protein was assayed by the method of Lowry [20] using bovine serum albumin (Sigma, St. Louis, MO) as the standard.

Microsomes from human livers (labelled A, B, C and D) were prepared in a similar way from specimens obtained from patients who underwent resection of one or two liver segments for metachronous liver metastases of colorectal carcinoma. In all patients preoperative liver function tests were normal. Some characteristics of the patients are given in Table 1. Immediately after resection a representative specimen of normal liver tissue was excised from the resected segment and cooled. All patients had epidural anaesthesia and after introduction (fentanylcitrate, thiopental, vecuroniumbromide) nitrous oxide and isoflurane as anaesthetic agent were applied continuously during the operation. All patients gave permission preoperatively for the use of some liver tissue for scientific purposes.

2.6. Microsomal incubations

Microsomal incubations were carried out at 37°C in 0.1 M potassium phosphate (pH 7.6) containing 1 μM microsomal cytochrome P450 and 0–20 mM (halogenated) aniline added as 1% (v/v) of a stock solution in DMSO. The reaction was started by the addition of NADPH or iodosobenzene (both at 1 mM final concentration) and terminated after 10 min (NADPH) or 1 min (iodosobenzene) by freezing the reaction mixture in liquid nitrogen for ^{19}F NMR measurements or by adding 1.0 ml of the reaction mixture to 0.3 ml 20% trichloroacetic acid (TCA) for the chemical detection of 4-aminophenol. The cytochrome P450-mediated microsomal conversion of the aniline derivatives to their 4-aminophenols was observed to be linear in time for at least 10 min in an NADPH/O_2-supported reaction and for at least 75 s in an iodosobenzene-supported reaction. In order to correct for the chemical reaction between iodosobenzene and the aniline substrates resulting in 4-hydroxylation of the substituted anilines, control incubations were carried out for each substrate concentration in the absence of microsomal cytochrome P450. This correction was 5–40%, depending on the substrate concentration and aniline derivative used.

Conditions for the ^{19}F NMR samples for determination of the regioselectivity of 3-fluoroaniline hydroxylation in an NADPH/O_2 driven reaction were essentially similar using a final volume of 2 ml, 2 μM microsomal cytochrome P450, a 3-fluoroaniline concentration of 10 mM and adding 2 mM ascorbic acid and 0.2 mg/ml of superoxide dismutase (SOD) to prevent autoxidation of the aminophenol metabolites especially during the overnight ^{19}F NMR measurement.

Table 1
Some characteristics of the patients from whom liver specimens were obtained

Patient	Sex	Age	Histology liver	Alcohol	Smoking	Medication
A	Male	63	Steatosis ±	2U/day	No	No
B	Male	67	Normal	4U/day	No	No
C	Female	66	Steatosis ± Fibrosis+	No	No	No
D	Female	60	Steatosis+	No	No	No

2.7. Chemical detection of 4-aminophenol

Halogenated 4-aminophenols were determined essentially by the method of Brodie and Axelrod [21]. In short, to 1 ml of TCA-precipitated supernatant 100 μl of phenol reagent (5% phenol in 2.5 M NaOH) and 200 μl of 2.5 M Na_2CO_3 were added. After 60 min at room temperature the absorbance at 630 nm was measured. Halogenated 4-aminophenols were quantified using the values of molar extinction coefficients at 630 nm for their corresponding halogenated 4-aminophenol-derived indophenols reported by Cnubben et al. [13].

2.8. Kinetic analysis

The apparent Michaelis constant K_m (mM) for the aniline substrates and the apparent maximum reaction rate k_{cat} (nmol of halogenated 4-aminophenol per nmol of cytochrome P450 per minute) at infinite aniline concentration, for the 4-hydroxylation of the aniline derivatives by the microsomal cytochrome P450 were determined by fitting the data to the standard Michaelis-Menten equation $V = V_{max}[S]/(K_m+[S])$ using the program of KaleidaGraph, version 2.0.2. (Abelbeck Software). In spite of its multistep reaction cycle, it appeared possible to analyze the kinetics of the cytochrome P450-catalysed 4-hydroxylation of the various anilines by Michaelis-Menten kinetics, the correlation coefficient r of the fits being greater than 0.97 in all cases.

2.9. Molecular orbital parameters

Molecular orbital parameters needed for the various comparisons of experimental data to calculated molecular orbital reactivity parameters were taken from the literature [11–13] and were calculated using the AM1 Hamiltonian [22]. The parameters used were either the reactivity of the π-electrons characterised by their calculated energy of the highest occupied molecular orbital (E(HOMO)), or the calculated density distribution of the reactive π-electrons in the aromatic ring. This density distribution was calculated from the density distribution of the HOMO and the HOMO–1 (i.e. first occupied orbital at the highest energy level below the HOMO) as described by Fukui et al. [23].

3. Results

3.1. QSARs for the in vivo aromatic ring hydroxylation of fluorinated benzenes

To investigate whether the MO-QSAR for the prediction of the regioselectivity of the in vivo aromatic ring hydroxylation of fluorinated benzenes can be extrapolated from Wistar rats to other species, the regioselectivity of the aromatic hydroxylation of 1,3-difluorobenzene and 1,2,4-trifluorobenzene was determined in U and R inbred rats and SD rats, in two mice strains, in a rabbit strain and in the guinea pig. As an example, Fig. 1 shows the ^{19}F NMR spectra of the arylsulphatase/β-

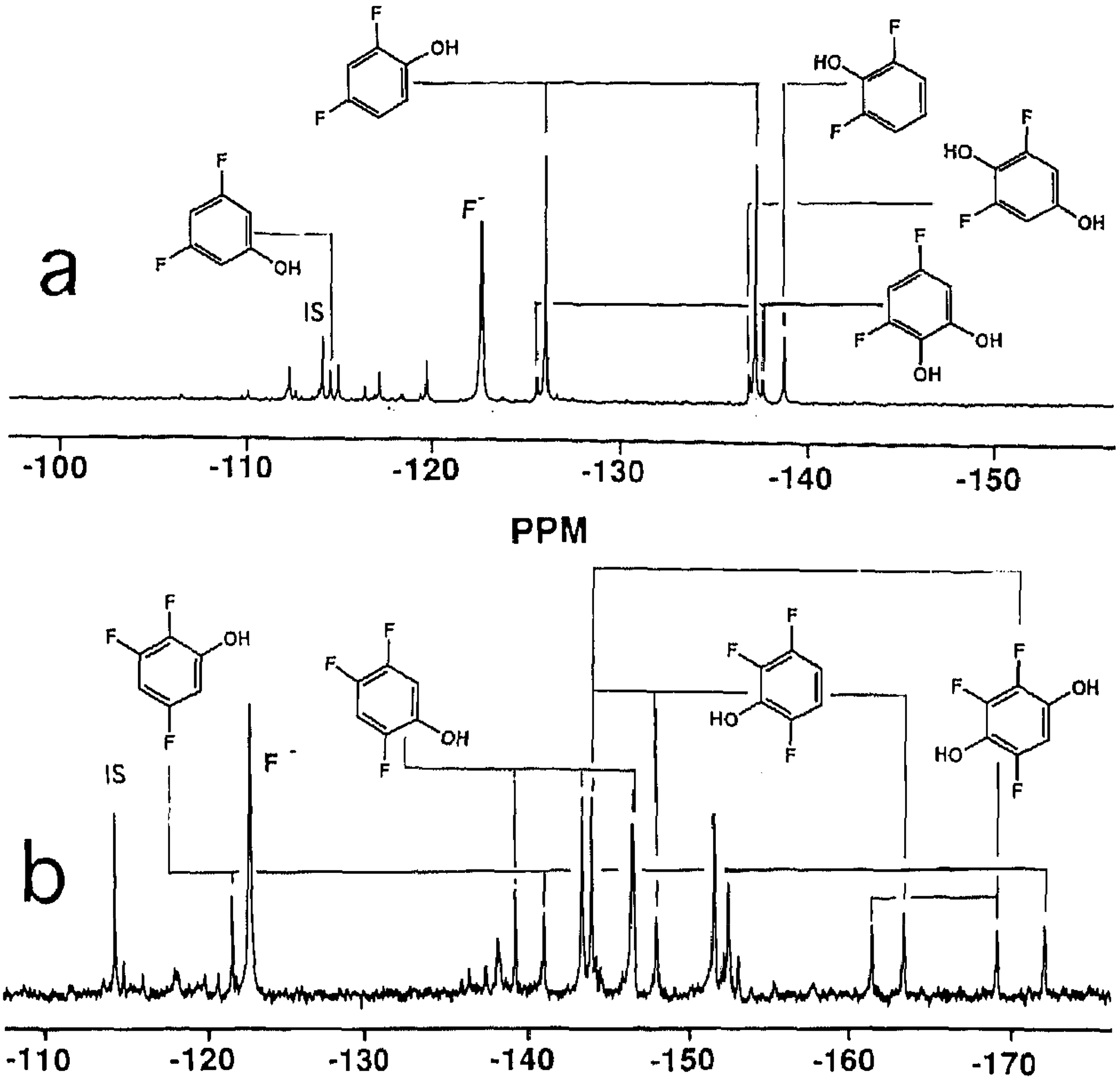

Fig. 1. ^{19}F NMR spectra of the arylsulphatase/β-glucuronidase-treated 24 h urine of NMRi mice exposed to (a) 1,3-difluorobenzene and (b) 1,2,4-trifluorobenzene. The resonances of the hydroxylated metabolites were identified as previously described [11]. The resonance marked IS is from the internal standard 4-fluorobenzoic acid.

glucuronidase-treated urine samples of NMRi mice exposed to 1,3-difluorobenzene (Fig. 1a) or 1,2,4-trifluorobenzene (Fig. 1b). The ^{19}F NMR spectra of urine from Wistar rats exposed to these fluorobenzenes were previously reported [11]. Results obtained from ^{19}F NMR analysis of urine samples of the various animals exposed to 1,3-difluorobenzene or 1,2,4-trifluorobenzene are summarised in Tables 2 and 3, respectively. For both 1,3-difluorobenzene and 1,2,4-trifluorobenzene all urinary metabolite patterns contain large amounts (>48% of total fluorine containing metabolites) of phenolic metabolites. Formation of some ortho hydroxylated phenol (catechol) from 1,3-difluorobenzene is observed in all species, the relative amount being slightly higher in rabbit than in the other species (Table 2). This observation is accompanied by a slightly lower amount of monophenols in the urine of 1,3-difluorobenzene-exposed rabbits compared to the urine from the other species ex-

Table 2
Phenolic metabolite pattern in urine of different species exposed to 1,3-difluorobenzene as determined by ^{19}F NMR analysis of the arylsulphatase/β-glucuronidase-treated 24 h urine of exposed animals

Species	Phenolic metabolites (% of total fluorine containing aromatic metabolites)				Regioselectivity observed at carbon centers
	Phenols	2-Hydroxyphenol	4-Hydroxyphenol	Total	C2:C4/6:C5
Wistar rat[a]	46.5	2.1	n.o.[b]	48.6	0.12:0.82:0.06
SD rat	60.8	6.0	n.o.[b]	66.8	0.09:0.85:0.06
U inbred rat	55.2	4.4	n.o.[b]	59.6	0.11:0.84:0.05
R inbred rat	53.7	4.9	n.o.[b]	58.6	0.11:0.84:0.05
C57/BL mouse	61.3	3.7	2.7	67.7	0.11:0.84:0.05
NMRi mouse	66.3	5.5	3.3	75.1	0.10:0.85:0.05
NZW rabbit	42.1	11.7	n.o.[b]	53.8	0.12:0.79:0.09
Guinea pig	65.4	7.8	n.o.[b]	73.2	0.13:0.81:0.06

[a]Data were taken from the literature [11].
[b]n.o. means not observed in the ^{19}F NMR spectrum based on the known position of the resonance of 2,6-difluoro-4-hydroxyphenol at −137.1 ppm [11].

posed to 1,3-difluorobenzene. Catechol formation from 1,2,4-trifluorobenzene is not observed (Table 3). Formation of para-hydroxylated phenols (hydroquinones) is observed only in mice for both fluorobenzenes. Some resonances in the ^{19}F NMR spectrum of 1,3-difluoro- and 1,2,4-trifluorobenzene remain unidentified. Since these unidentified peaks do not represent primary halogenated phenolic metabolites, they were not investigated in detail in this study.

Table 3
Phenolic metabolite pattern in urine of different species exposed to 1,2,4-trifluorobenzene as determined by ^{19}F NMR analysis of the arylsulphatase/β-glucuronidase-treated 24 h urine of exposed animals

Species	Phenolic metabolites (% of total fluorine containing aromatic metabolites)			Regioselectivity observed at carbon centers
	Phenols	4-Hydroxyphenol	Total	C3:C5:C6
Wistar rat[a]	48.6	n.o.[b]	48.6	0.25:0.51:0.24
D inbred rat	53.7	n.o.[b]	53.7	0.34:0.46:0.20
U inbred rat	51.9	n.o.[b]	51.9	0.31:0.49:0.20
Black mouse	59.6	6.3	65.9	0.31:0.50:0.19
NMRi mouse	55.3	7.8	63.1	0.30:0.50:0.20
NZW rabbit	69.6	n.o.[b]	69.6	0.29:0.44:0.27
Guinea pig	67.8	n.o.[b]	67.8	0.31:0.42:0.27

[a]Data were taken from the literature [11].
[b]n.o. means not observed in the ^{19}F NMR spectrum based on the known position of the resonances of 2,3,5-trifluoro-4-hydroxyphenol at −144.4, −162.2 and −169.7 ppm [11].

Most interesting for the objective of the present study, however, are the regioselectivities of the aromatic hydroxylation also presented in Tables 2 and 3. The results obtained show that conversion of 1,3-difluorobenzene and 1,2,4-trifluorobenzene by various species results in similar regioselectivity for the aromatic hydroxylation. The regioselectivities observed in all species closely match the regioselectivity predicted on the basis of the calculated reactivity of the various carbon centers for an electrophilic attack, i.e. C2:C4/6:C5 = 0.16:0.74:0.10 for 1,3-difluorobenzene and C3:C5:C6 = 0.19:0.60:0.21 for 1,2,4-trifluorobenzene [11].

3.2. Regioselectivity for the in vitro aromatic hydroxylation of 3-fluoroaniline

In addition to extrapolation to other experimental animals than the Wistar rat, it is of interest to investigate whether the regioselectivity obtained for the aromatic hydroxylations catalysed by cytochromes P450 from Wistar rats can be extrapolated to man. As, for obvious ethical reasons, the in vivo regioselectivity of the aromatic hydroxylation can not be investigated in man, in vitro experiments with 3-fluoroaniline were performed. 3-Fluoroaniline was used for these in vitro studies instead of the fluorobenzenes because the rate of its conversion is higher and therefore results in metabolite patterns that can be quantified by ^{19}F NMR analysis. Fig. 2 presents the ^{19}F NMR spectrum of an incubation of 3-fluoroaniline with human liver microsomes. Table 4 presents the regioselectivity derived from this and additional ^{19}F NMR spectra obtained for incubations with various microsomal preparations. The results presented clearly demonstrate that the regioselectivity of the 3-fluoroaniline hydroxylation observed with microsomes from different species,

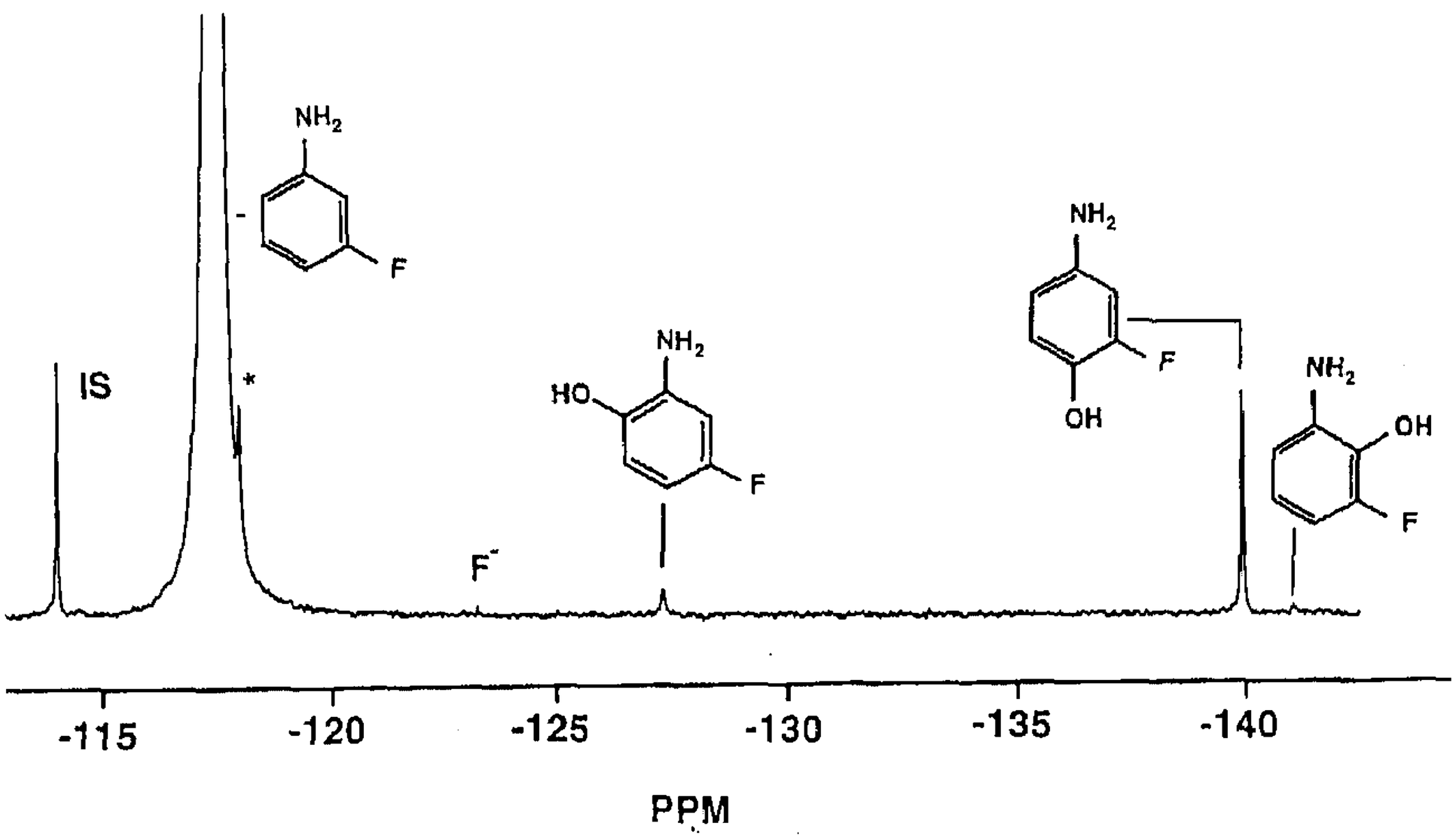

Fig. 2. ^{19}F NMR spectrum of an incubation of 3-fluoroaniline with microsomes from human liver. Metabolites were identified as previously described [12,16]. The resonance marked IS is from the internal standard 4-fluorobenzoic acid. The resonance marked with an asterisk is also present in blank incubation without NADPH.

Table 4
The regioselectivity for the in vitro aromatic ring hydroxylation of 3-fluoroaniline in microsomal incubations from different species as determined by ^{19}F NMR

Species	Regioselectivity observed at the carbon centers C2:C4:C5:C6
Wistar rat[a]	0.05:0.84:0.00:0.11
NMRi mouse	0.05:0.80:0.00:0.15
	0.06:0.77:0.00:0.17
NZW rabbit	0.06:0.84:0.00:0.10
	0.06:0.85:0.00:0.09
Human batch A	0.04:0.76:0.00:0.20
batch B	0.06:0.79:0.00:0.15
batch C	0.08:0.78:0.00:0.14
batch D	0.05:0.86:0.00:0.09

[a]Data were taken from the literature [12].

and also with different human liver microsomal preparations, vary by only a few percent. Qualitatively, the regioselectivity observed follows the calculated density distribution for an electrophilic attack on the reactive π-electrons of 3-fluoroaniline. This frontier electron density distribution for an electrophilic attack is 0.20:0.47:0.05:0.36 for C2:C4:C5:C6 [12]. These MO results qualitatively explain that C5 is not hydroxylated, that C6-hydroxylation is favoured over C2-hydroxylation and that C4 is the preferential site for hydroxylation of 3-fluoroaniline. Apparently, the type of cytochromes P450 present in the microsomal preparation, and thus, the species from which the P450 preparation is derived, is not a main factor influencing the regioselectivity of the hydroxylation of 3-fluoroaniline.

3.3. QSARs for the rate of in vitro conversion of halogenated aniline derivatives in an iodosobenzene-supported cytochrome P450-catalysed C4-hydroxylation

In a final set of experiments it was investigated whether the MO-QSAR describing a correlation between the rates of conversion of a series of aniline derivatives in an iodosobenzene-supported cytochrome P450-mediated C4-hydroxylation and the energy of the reactive π-electrons of these aniline derivatives [13], can be extrapolated to other species than the Wistar rat. To allow comparison of the data of the present study to those previously reported, isosafrole pretreatment of the animals was performed before preparing the microsomes for the present in vitro studies. Previous studies demonstrated aniline hydroxylation in an NADPH/O_2 driven microsomal reaction to be highest with microsomes from isosafrole pretreated rats [13,16].

Table 5 presents the k_{cat} values obtained for the C4-hydroxylation of a series of (halogenated) anilines by liver microsomes from isosafrole-pretreated rats, rabbit and mice. It also presents k_{cat} values obtained for an iodosobenzene-driven micro-

Table 5
Apparent k_{cat} (maximum velocity in nmol converted per nmol of cyt P450 per min) for the iodosobenzene-supported microsomal cytochrome P450-catalysed C4-hydroxylation of aniline derivatives with liver microsomes from different species. Values presented are the mean ± standard error of the mean (n = 2–4)

Substrate	k_{cat} (min^{-1})			
	Wistar rats	NZW rabbit	NMRi mice	Human (batch A and B)
Aniline	115.8 ± 21.6	111.4 ± 32.2	124.7 ± 22.2	223.3 ± 77.5
2-Chloroaniline	104.8 ± 10.5	95.9 ± 24.4	75.6 ± 15.4	164.1 ± 53.6
2-Fluoroaniline	74.1 ± 10.0	55.1 ± 5.1	58.9 ± 20.2	132.9 ± 55.7
3-Chloroaniline	36.5 ± 5.2	28.7 ± 1.3	26.8 ± 3.2	69.2 ± 13.7
3-Fluoroaniline	41.1 ± 3.8	30.8 ± 3.8	29.6 ± 2.6	60.4 ± 5.1
2,3-Difluoroaniline	15.5 ± 1.0	15.1 ± 0.0	15.7 ± 0.0	18.8 ± 0.0

somal system derived from human liver. It appears from the data presented in Table 5, that k_{cat} values for C4-hydroxylation of aniline derivatives obtained using microsomes from various species vary at most two-fold. Furthermore, the results presented in Table 5 show considerable differences in k_{cat} values for the C4-hydroxylation of the different aniline derivatives.

In Fig. 3 the natural logarithm of the k_{cat} values for the iodosobenzene-supported C4-hydroxylation of the (halogenated) anilines is plotted against the E(HOMO) values calculated for the aniline derivatives (Table 6). These E(HOMO) values of the aniline derivatives are of importance for their reactivity in the electrophilic attack by the high-valent-iron-oxo cytochrome P450 intermediate. The results presented in Fig. 3 clearly demonstrate that MO-QSARs are obtained not only for rat, but also for mice, rabbit and even human microsomal systems. The correlation coefficients for the QSARs describing the relation between the ln k_{cat} values and the E(HOMO) values are $r = 0.97$ for rats, $r = 0.98$ for mice, $r = 0.97$ for rabbit and $r = 0.99$ for human microsomes.

Besides, Fig. 3 clearly shows that the QSARs obtained for the various species are similar. These similar QSAR lines obtained for different species indicate that the relative change in the rate of conversion with a change in the reactivity of the aniline substrate is about the same for all species.

4. Discussion

In the present study it was investigated whether MO-QSARs (quantitative structure activity relations based on molecular orbital parameters) described previously for aromatic hydroxylation reactions of halogenated benzene derivatives catalysed by cytochromes P450 from Wistar rats can be extrapolated to cytochrome P450 reac-

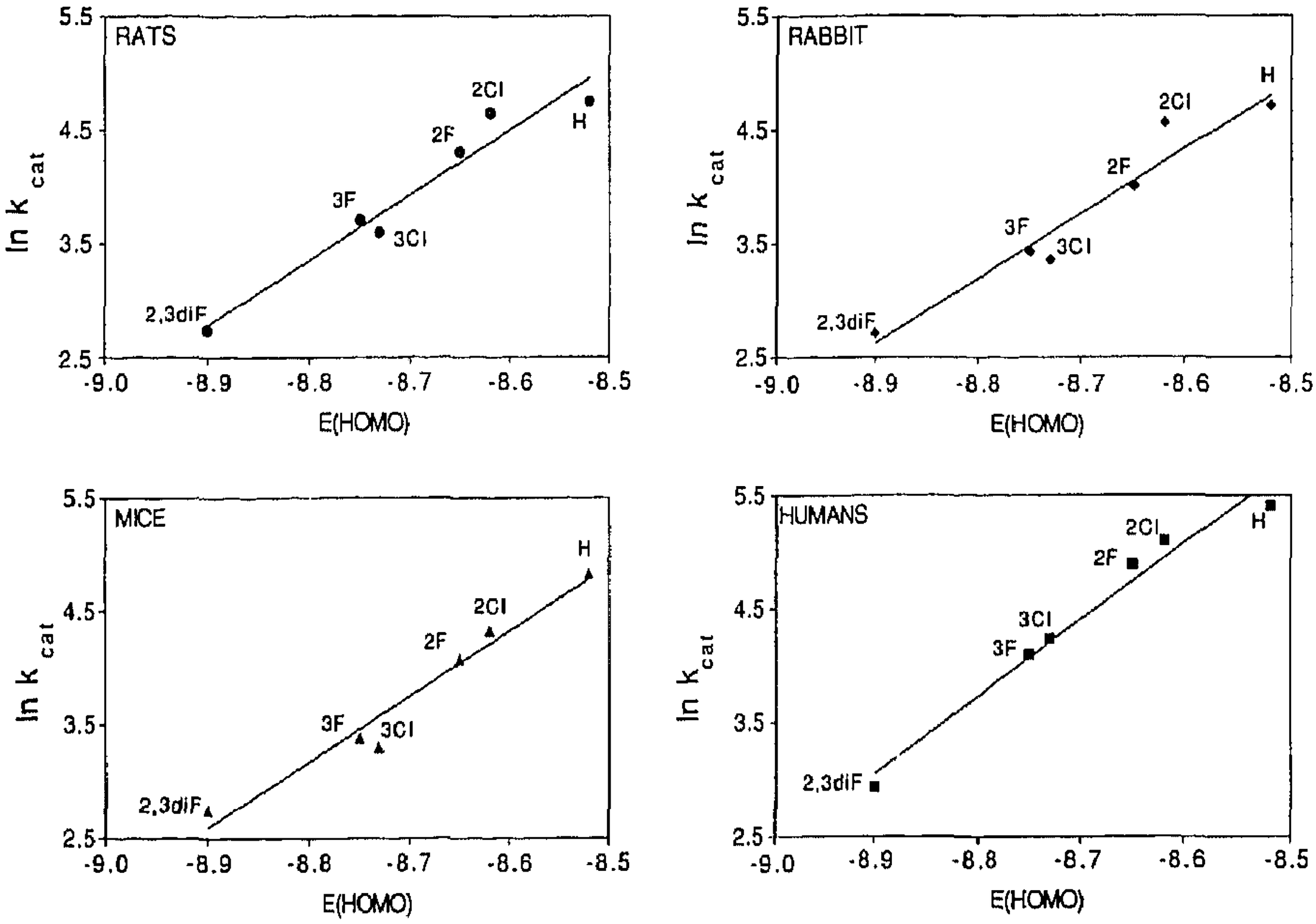

Fig. 3. Relationship between the ln k_{cat} for 4-hydroxylation of a series of halogenated anilines in an iodosobenzene-supported microsomal P450 conversion and the E(HOMO) of these substrates for rats ($r = 0.97$), mice ($r = 0.98$), rabbit ($r = 0.97$) and human ($r = 0.99$) liver microsomes. k_{cat} is expressed in min^{-1}, E(HOMO) is expressed in eV. Theoretically, k_{cat} should be multiplied by h/kT to make the parameter dimensionless before calculating the natural logarithm. However, because this would result in a change of the y-axis values by a constant factor, this theoretically appropriate correction was omitted and the natural logarithm of k_{cat} was plotted.

tions of other species. Because these MO-QSARs describe relationships between experimental parameters and a calculated reactivity parameter of the P450 substrate, it might be foreseen that such MO-QSARs obtained for one species may be valid for other species including man. Previous results with differently induced Wistar rat microsomal preparations as well as with different induced rats metabolising substrates in vivo, already demonstrated that a change in the cytochrome P450

Table 6
Frontier orbital characteristics of the aniline derivatives calculated using the AM1 Hamiltonian

Substrate	E(HOMO) (eV)
Aniline	−8.52
2-Chloroaniline	−8.62
2-Fluoroaniline	−8.65
3-Chloroaniline	−8.73
3-Fluoroaniline	−8.75
2,3-Difluoroaniline	−8.90

enzyme pattern did not influence the regioselectivity of the aromatic hydroxylation of relatively small substrates like fluorinated benzene derivatives or monofluoroanilines [11,12]. These results imply that chemical characteristics of the substrate instead of the types of cytochromes P450 involved, are of major importance in determining the outcomes of the catalysis. The present study was undertaken to provide further support for this hypothesis, and especially to investigate whether this MO-QSAR approach would indeed form a basis for extrapolation of biotransformation results from one species to another including man.

First, the regioselectivity of the in vivo hydroxylation of two model fluorinated benzenes with three discernable sites for aromatic hydroxylation (1,3-difluorobenzene and 1,2,4-trifluorobenzene), was tested in three additional rat strains, in two mice strains, in a rabbit strain and in guinea pig. Second, the regioselectivity of the in vitro 3-fluoroaniline hydroxylation by mice, rabbit and human microsomes was determined and compared to the results obtained with rat microsomes. Finally, it was investigated whether the MO-QSAR for the rate of conversion of a series of aniline derivatives in an iodosobenzene-supported cytochrome P450-catalysed C4-hydroxylation can be extrapolated to studies with mice, rabbit and human microsomes.

The results obtained demonstrate that for all three biotransformation QSARs the results obtained with mice, guinea pig, rabbit and even human systems were similar to those obtained for the rat system. The regioselectivity of the in vivo aromatic hydroxylation of 1,3-difluorobenzene and 1,2,4-trifluorobenzene could be predicted on the basis of the calculated electron density distribution for an electrophilic attack by the activated high-valent-iron-oxo species of the cytochromes P450. Thus, in in vivo studies with mice, guinea pig, other rat strains and rabbit, this MO-QSAR for the prediction of the regioselectivity of the fluorobenzene hydroxylation was valid, indicating that the chemical reactivity of the various sites in the fluorinated benzenes, rather than a stereoselective influence of the active site of the cytochromes P450, determines the regioselectivity of the aromatic hydroxylation. This observation implies that the regioselectivity of the hydroxylation of these fluorinated benzenes in man can be expected to be similar. The similar regioselectivity observed in the present study for the in vitro hydroxylation of 3-fluoroaniline by microsomes from experimental animals or man, supports this view. Furthermore, the qualitative correlation between the regioselectivity predicted for 3-fluoroaniline on the basis of the calculated reactivity of the various carbon centres for an electrophilic attack and the regioselectivity actually observed, also supports this conclusion.

Finally, it was demonstrated that the relationship between the natural logarithm for the k_{cat} for iodosobenzene-supported microsomal C4 hydroxylation of a series of aniline derivatives and the energy of the reactive π-electrons of these derivatives can be obtained not only for liver microsomes from Wistar rats as previously described [13] but also for liver microsomes from mice, from rabbit and even from human liver. This MO-QSAR indicates that in the iodosobenzene-supported cytochrome P450-catalysed aniline C4-hydroxylation the actual electrophilic attack of the high-valent-iron-oxo P450 intermediate on the reactive π-electrons of the aniline substrate is the rate limiting step in catalysis. The 2-fold variation in the various

k_{cat} values observed when comparing results from human liver microsomes with those obtained for rats, rabbit or mice microsomes, is the only significant species-dependent difference observed in the present study. Nevertheless, the QSAR lines describing the relationship between the ln k_{cat} for the conversion of the various aniline derivatives and their E(HOMO) are similar for all species investigated. This implies that the relative influence of a change in the chemical reactivity of an aniline derivative on its rate of conversion is about the same for all species.

Altogether, the results of the present study strongly support the conclusion that the conversion of the relatively small benzene derivatives in the relatively large and aspecific active sites of the mammalian cytochromes P450, are mainly dependent on chemical reactivity parameters of the substrates. Therefore, the results of the present study support the hypothesis that MO-based QSARs obtained in rat can provide a basis for prediction of biotransformation pathways in different species, including man.

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References

[1] R.N. Armstrong, Enzyme-catalyzed detoxication reactions: mechanisms and stereochemistry, CRC Crit. Rev. Biochem., 22 (1987) 39–88.

[2] I.G. Sipes and A.J. Gandolfi, Biotransformation of toxicants, in: M.O. Amdur, J. Doull and C.D. Klaassen (Eds.), Casarett and Doull's Toxicology. The Basic Science of Poisons, 4th edn, Pergamon, Oxford, 1991, pp. 64–98.

[3] R.T. Williams, Species variations in the pathways of drug metabolism, Environ. Health Perspect., 22 (1978) 133–138.

[4] R. Kato, Characteristics and differences in the hepatic mixed function oxidases of different species, Pharmac. Ther., 6 (1979) 41–98.

[5] J. Caldwell, Problems and opportunities in toxicity testing arising from species differences in xenobiotic metabolism, Toxicol. Lett., 64/65 (1992) 651–659.

[6] E. Dybing and H.S. Huitfeldt, Species differences in carcinogen metabolism and interspecies extrapolation, IARC Sci. Publ., 116 (1992) 501–522.

[7] H.G. Selander, D. Jerina and J.W. Daly, Metabolism of chlorobenzene with hepatic microsomes and solubilised cytochrome P450 systems, Arch. Biochem. Biophys., 168 (1975) 309–321.

[8] J.J.P. Bogaards, B. van Ommen, C.R. Wolf and P.J. van Bladeren, Human cytochrome P450 enzyme selectivities in the oxidation of chlorinated benzenes, Toxicol. Appl. Pharmacol., 132 (1995) 44–52.

[9] S.S. Lau, G.D. Abrams and V.G. Zannoni, Metabolic activation and detoxification of bromobenzene leading to cytotoxicity, J. Pharmacol. Exp. Ther., 214 (1980) 703–708.

[10] T.J. Monks and S.S. Lau, Reactive intermediates and their toxicological significance. Review paper, Toxicology, 52 (1988) 1–53.

[11] I.M.C.M. Rietjens, A.E.M.F. Soffers, C. Veeger and J. Vervoort, Regioselectivity of cytochrome P450 catalyzed hydroxylation of fluorobenzenes predicted by calculated frontier orbital substrate characteristics, Biochemistry, 32 (1993) 4801–4812.

[12] N.H.P. Cnubben, J. Vervoort, C. Veeger and I.M.C.M. Rietjens, Study on the regioselectivity and mechanism of the aromatic hydroxylation of monofluoroanilines, Chem.-Biol. Interact., 85 (1992) 151–172.

[13] N.H.P. Cnubben, S. Peelen, J.W. Borst, J. Vervoort and I.M.C.M. Rietjens, Molecular orbital-based quantitative structure activity relationship for the cytochrome P450-catalysed 4-hydroxylation of halogenated anilines, Chem. Res. Toxicol., 7 (1994) 590–598.

[14] H. Saltzman and J.G. Sharefkin, Iodosobenzene, in: Organic Syntheses, Collect. Vol. V, John Wiley & Sons, New York, 1973, pp. 658–659.

[15] I.M.C.M. Rietjens and J. Vervoort, A new hypothesis for the mechanism for cytochrome P450 dependent aerobic conversion of hexahalogenated benzenes to pentahalogenated phenols, Chem. Res. Toxicol., 5 (1992) 10–19.

[16] I.M.C.M. Rietjens and J. Vervoort, Microsomal metabolism of fluoroanilines, Xenobiotica, 19 (1989) 1297–1305.

[17] J. Vervoort, P.A. De Jager, J. Steenbergen and I.M.C.M. Rietjens, Development of a ^{19}F NMR method for studies on the in vivo and in vitro metabolism of 2-fluoroaniline, Xenobiotica, 20 (1990) 657–670.

[18] V. Wray, Fluorine nuclei in fluorobenzene derivatives, in: G.A. Webb (Ed.), Annual Reports on NMR Spectroscopy, Vol. 14, Academic Press, London, 1983, p. 252.

[19] T. Omura and R. Sato, The carbon monoxide pigment of liver microsomes, I. Evidence for its haemoprotein nature, J. Biol. Chem., 239 (1964) 2370–2378.

[20] O.H. Lowry, N.J. Rosebrough, A.L. Farr and A.J. Randall, Protein measurements with Folin phenol reagent, J. Biol. Chem., 193 (1951) 2370–2378.

[21] B.B. Brodie and J. Axelrod, The estimation of acetanilide and its metabolic products, aniline, *N*-acetyl *p*-aminophenol and *p*-aminophenol (free and conjugated) in biological fluids and tissues. J. Pharmacol. Exp. Ther., 94 (1948) 22–28.

[22] M.J.S. Dewar, E.G. Zoebisch, E.F. Healy and J.J.P. Stewart, AM1: a new general purpose quantum mechanical molecular model. J. Am. Chem. Soc., 107 (1985) 3902–3909.

[23] K. Fukui, T. Yonezawa, C. Nagata and H. Shingu, Molecular orbital theory of orientation in aromatic, heteroaromatic and other conjugated molecules, J. Chem. Phys., 22 (1954) 1433–1442.