Chemico-Biological Interactions 185 (2010) 163–173

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Metabolism of carbosulfan II. Human interindividual variability in its *in vitro* hepatic biotransformation and the identification of the cytochrome P450 isoforms involved

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ARTICLE INFO

Article history:
Received 2 February 2010
Received in revised form 10 March 2010
Accepted 11 March 2010
Available online 20 March 2010

Keywords:
Pesticides
Xenobiotic
Risk assessment
In vitro metabolism
P450
LC–MS

ABSTRACT

This study aims to characterize interindividual variability and individual CYP enzymes involved in the *in vitro* metabolism of the carbamate insecticide carbosulfan. Microsomes from ten human livers (HLM) were used to characterize the interindividual variability in carbosulfan activation. Altogether eight phase I metabolites were analyzed by LC–MS. The primary metabolic pathways were detoxification by the initial oxidation of sulfur to carbosulfan sulfinamide ('sulfur oxidation pathway') and activation via cleavage of the nitrogen sulfur bond (N–S) to give carbofuran and dibutylamine ('carbofuran pathway'). Differences between maximum and minimum carbosulfan activation values with HLM indicated nearly 5.9-, 7.0, and 6.6-fold variability in the k_m, V_{max} and CL_{int} values, respectively. CYP3A5 and CYP2B6 had the greatest efficiency to form carbosulfan sulfinamide, while CYP3A4 and CYP3A5 were the most efficient in the generation of the carbofuran metabolic pathway. Based on average abundances of CYP enzymes in human liver, CYP3A4 contributed to 98% of carbosulfan activation, while CYP3A4 and CYP2B6 contributed 57 and 37% to detoxification, respectively. Significant correlations between carbosulfan activation and CYP marker activities were seen with CYP3A4 (omeprazole sulfoxidation), CYP2C19 (omeprazole 5-hydroxylation) and CYP3A4 (midazolam 1′-hydroxylation), displaying $r^2 = 0.96$, 0.87 and 0.82, respectively. Activation and detoxification pathways were inhibited by ketoconazole, a specific CYP3A4 inhibitor, by 90–97% and 47–94%, respectively. Carbosulfan inhibited relatively potently CYP3A4 and moderately CYP1A1/2 and CYP2C19 in pooled HLM. These results suggest that the carbosulfan activation pathway is more important than the detoxification pathway, and that carbosulfan activation is predominantly catalyzed in humans by CYP3A4.

1. Introduction

Carbosulfan [2,3-dihydro-2,2-dimethylbenzofuran-7-yl(dibutylaminothio) methyl carbamate] is a widely used systemic insecticide with contact and stomach actions. Like other carbamate insecticides, carbosulfan inhibits cholinesterase and is assigned to toxicity class II by WHO [1].

There are reports about the metabolism of carbosulfan in the environment [2,3] and in plants [4]. In mammals three primary metabolites, 3-hydroxycarbofuran, 3-keto-7-phenolcarbofuran, and dibutylamine, were detected by TLC in rat *in vivo* [5]. Moreover, carbofuran and polysulfide derivatives of carbosulfan were detected in rat stomach by TLC [6]. In male and female rats *in vivo*, ten metabolites were identified by TLC and HPLC and major metabolites were confirmed by GC-MS [7]. We have recently described the *in vitro* metabolic pathways of carbosulfan in microsomal hepatic preparations from seven mammalian species including human. The primary metabolic pathways in these *in vitro* studies were the detoxification via the initial oxidation of sulfur to carbosulfan sulfinamide ('sulfur oxidation pathway') and the activation via the cleavage of the nitrogen sulfur bond (N–S) to give carbofuran and dibutylamine ('carbofuran pathway'). Carbofuran was oxidized to 3-hydroxycarbofuran and/or 7-phenolcarbofuran, which were further oxidized to 3-ketocarbofuran or 3-hydroxy-7-phenolcarbofuran, respectively, and finally to 3-keto-7-phenolcarbofuran [8].

The cytochrome P450 (CYP) superfamily comprises a broad class of phase I oxidative enzymes that catalyze many hepatic metabolic processes [9,10]. Recently, a number of papers have been published on the activity of human P450s involved in the metabolism of pesticides [11–21].

CYPs variation within the human population is well known [10] and important for the risk assessment of the chemicals. With

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doi:10.1016/j.cbi.2010.03.024

respect to metabolism, variability in CYP activity is among the primary determinants of variability in biotransformation [22]. Studies investigating the formation of carbosulfan sulfinamide and the carbofuran metabolic pathway by individual cytochrome P450 (CYP) isoforms have been limited. Therefore, the aims of this study were (1) to determine the *in vitro* interindividual variability of enzyme kinetic parameters in carbosulfan bioactivation in a panel of ten human hepatic preparations and (2) to characterize the specific human CYP isoforms involved in carbosulfan biotransformation by the study of kinetic parameters with the appropriate cDNA-expressed isoforms, correlation studies with model CYP substrate activities across the human liver bank, and inhibition experiments with CYP-selective chemical inhibitors and the CYP inhibitions by carbosulfan itself.

2. Materials and methods

2.1. Chemicals

Carbosulfan, (2,3-dihydro-2,2-dimethylbenzofuran-7-yl(dibutylaminothio) methylcarbamate), carbofuran (2,3-dihydro-2,2-dimethylbenzofurany-7-yl methylcarbamate), 3-hydroxycarbofuran (2,3-dihydro-3-hydroxy-2,2-dimethylbenzofuran-7-yl methylcarbamate), 3-ketocarbofuran (2,3-dihydro-3-oxy-2,2-dimethylbenzofuran-7-yl methylcarbamate), 3-keto-7-phenolcarbofuran (2,3-dihydro-2,2-dimethyl-3-oxobenzofuran-7-ol), 3-hydroxy-7-phenolcarbofuran (2,3-dihydro-2,2-dimethylbenzofuran-3,7-diol), and 7-phenolcarbofuran (2,3-dihydro-2,2 dimethylbenzofuran-7-ol) were purchased from ChemService (West Chester, PA). Dibutylamine was purchased from Sigma-Aldrich (Germany) and carbaryl was a kind gift from Agrochem (Eg). Midazolam was a kind gift from F. Hoffman-La Roche (Basel, Switzerland), and omeprazole was a gift from Astra Zeneca (Mölndal, Sweden). HPLC-grade solvents were obtained from Rathburn (Walkerburn, UK) and Labscan (Dublin, Ireland). All other chemicals used were from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Water was freshly prepared in-house with the Simplicity 185 (Millipore S.A., Molsheim, France) water purification system and was UP-grade (ultra pure, 18.2 MΩ).

2.2. Human liver microsomes and cDNA-expressed human P450 enzymes

Human liver samples used in this study were obtained from the University Hospital of Oulu as surplus from organ donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Finland. All liver samples were of Caucasian race including 4 female and 6 male between the ages of 21 and 62. Intracerebral hemorrhage was the primary cause of death. Detailed characteristics of the liver samples are presented in our previous publication [15]. The livers were transferred to ice immediately after the surgical excision and cut into pieces, snap-frozen in liquid nitrogen, and stored at −80 °C. Microsomes were prepared by standard differential ultracentrifugation [23]. The final microsomal pellet was suspended in 100 mM phosphate buffer, pH 7.4. Protein content was determined by the Bradford method [24]. Baculovirus insect cell-expressed human CYPs (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7 and 4A11) were purchased from BD Biosciences Discovery Labware (Bedford, MA).

2.3. In vitro screening assay of metabolites

The standard incubation mixture contained 150 μM carbosulfan, 0.15 mg pooled liver microsomal protein ($n = 10$), and 1 mM NADPH in a final volume of 200 μl of 0.1 M phosphate buffer (pH 7.4). Carbosulfan was prepared once a week in dimethylsulfoxide (DMSO; final amount in the reaction medium 1.0%). After a 2-min incubation at +37 °C in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany), the reaction was started by adding NADPH. The mixture was incubated at +37 °C for 30 min and the reaction was stopped with 600 μl of ice cold acetonitrile containing an internal standard. All incubations were carried out in triplicate. After centrifugation at 10,000 × *g* for 15 min, the supernatant was collected and stored at −20 °C until analyzed.

To measure the main carbosulfan metabolites of recombinantly expressed CYP enzymes, the standard incubation mixture (200 μl) contained 0.1 M phosphate buffer (pH 7.4), 1 mM NADPH, 100 μM carbosulfan, and recombinantly expressed CYP enzymes (50 pmol CYP *per* ml). Incubations were carried out according to the manufacturer's instructions. Shortly, the reaction was started by adding recombinant enzymes to the preincubated reaction mixture (2 min at +37 °C), mixed gently and incubated for 30 min at +37 °C in an incubator block without agitation. Otherwise, the incubation protocol and analytical method were similar to those for microsomal incubations.

2.4. Chromatography of the carbosulfan metabolites

Samples were centrifuged before analysis for 10 min at 10,000 × *g*. Chromatographic separation was carried out with the Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA). The column used was a Waters Atlantis T3 (2.1 mm × 100 mm, particle size of 3 μm) together with a Phenomenex C18 2.0 mm × 4.0 mm precolumn (Phenomenex, Torrance, CA). The temperature of the column oven was 45 °C. The eluent flow rate was 0.4 ml/min. The eluents used were ultrapure-grade water containing 0.1% acetic acid (A) and methanol (B). A linear gradient elution from 5% B to 75% B in 8 min was applied. Solvent B was thus maintained at 98% for 3 min before re-equilibration (6 min). The total analysis time was 17 min.

2.5. Mass spectrometry

The initial screening of the compounds and accurate mass measurements were carried out using a Micromass LCT (Micromass, Altrincham, UK) time of flight (TOF) mass spectrometer equipped with a Z-Spray ionization source. Detailed information was presented in our previous publication [8].

The quantification (multiple reaction monitoring, MRM) and fragmentation measurements were performed with a Micromass Quattro II triple quadrupole instrument equipped with a Z-spray ionization source. The capillary voltage was 4000 V, and desolvation and source temperatures were 280 and 150 °C, respectively. The collision gas was argon with a CID gas cell pressure of 2.0×10^3 mbar. Nitrogen was used as the drying and nebulizing gas with flow rates of 450 and 15 l/h. The fragmentation reactions monitored (MRM), collision energies, and sample cone voltages for metabolites and the internal standard are presented in Fig. 1. External standards were measured in the beginning, middle, and end of the experiment to ensure the quality of the analysis. The lower limit of quantitation was 1 μM for all compounds. Intraday coefficients of variation were less than 20% throughout the quantitation range of 2.5–300 μM.

2.6. Kinetic parameters

To measure the enzyme kinetic parameters in both the microsomal samples and recombinantly expressed CYP enzymes, the standard incubation mixture contained carbosulfan (final concentrations 2.5–300 μM). Incubation mixtures and methods were the same as mentioned above, except the incubation times were

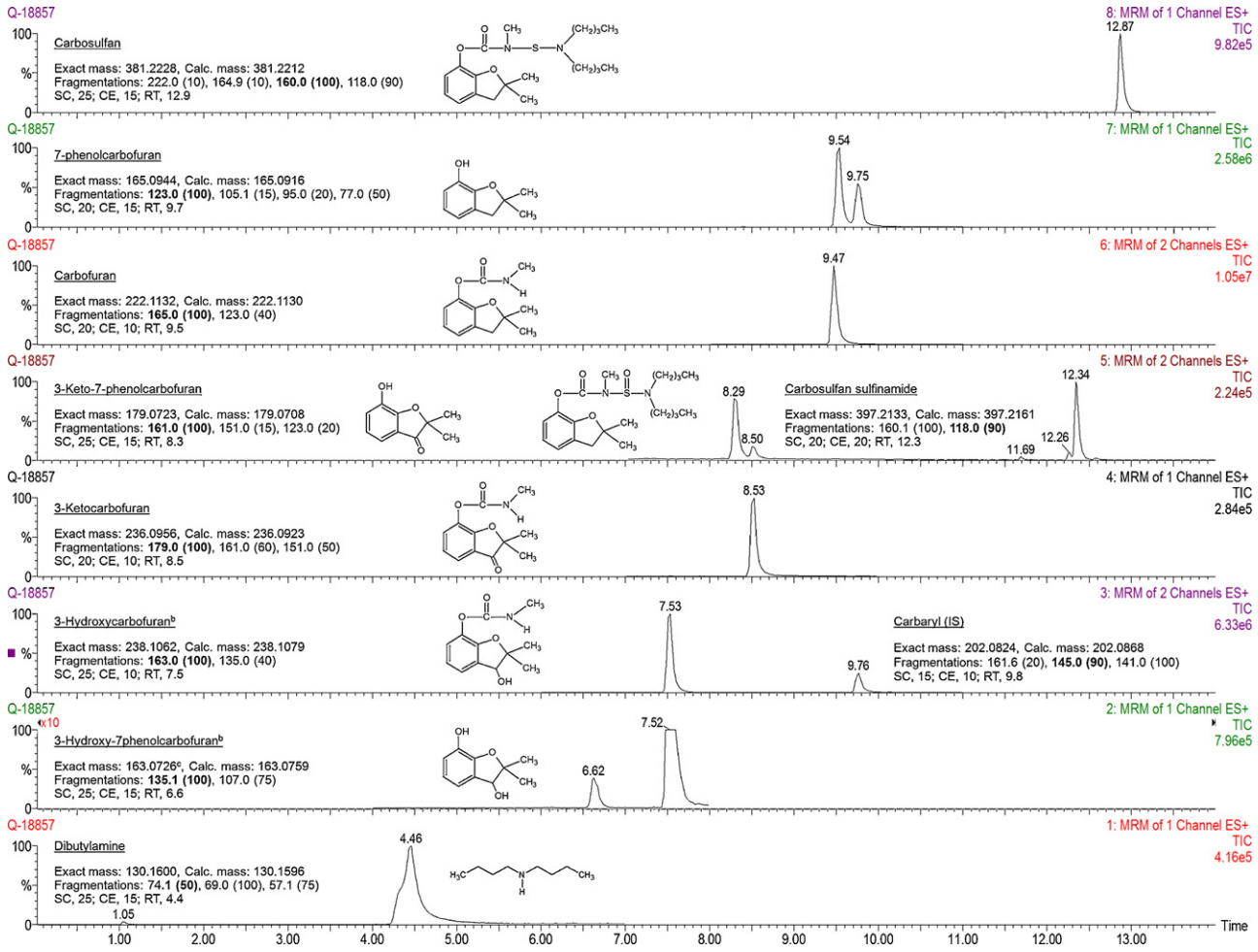

Fig. 1. Extracted mass chromatograms of carbosulfan metabolites formed by *in vitro* incubation with mammalian hepatic microsomes. Analytes, exact masses, fragmentations[a], sample cone voltages (SC), collision energies (CE), and retention times (RT) of analytes used in the measurements by LC–MS are presented.
[a] Fragmentations monitored in the quantification are presented in bold.
[b] Metabolites were quantified as the protonated dehydrated molecule $[M - H_2O + H]+$ due to significant in-source fragmentation.
[c] Exact mass could be measured only from the protonated dehydrated molecule $[M - H_2O + H]+$.

20 min for microsomal samples and 30 min for rCYPs. Samples were analyzed by LC–MS-MS. The kinetic parameters V_{max} and k_m were calculated using Prism 5.0 (GraphPad Software, Inc., San Diego, CA) by nonlinear regression. These values were used to calculate the intrinsic clearance value (V_{max}/k_m). All results are expressed as mean ± standard error for three replicates. In the standard experimental conditions used for carbosulfan metabolism, the reaction rate of carbosulfan metabolites formation was linear at least up to 0.15 mg of microsomal protein/ml and 30 min incubation time.

2.7. Correlation with model CYP substrate activities

A bank of ten livers was used to assess the metabolism of carbosulfan in individual livers as well as to correlate the activities with model CYP substrate activities. A correlation was performed between the formation of carbosulfan metabolites and each CYP activity across the human liver bank. Model substrate reactions used for correlations were the same as those used in the inhibition studies below. For all data points the mean of duplicate incubations were used. Bivariate linear Pearson's correlation coefficients (r^2) were calculated between metabolite formations and model activities in livers. The software program Prism 5.0 (GraphPad Software, Inc., San Diego, CA) was used for data analysis.

2.8. Inhibition of in vitro metabolism of carbosulfan by CYP3A4-selective inhibitor

The inhibitory effects of known CYP3A4 isoform-selective inhibitors on the formation of carbofuran and carbosulfan sulfinamide were evaluated. Formation rates of metabolites were determined from the reaction mixtures incubated in the presence or absence of ketoconazole 100 μM. The incubation conditions were as described above.

2.9. Inhibition assays

Ethoxyresorufin-O-deethylation (EROD) (CYP1A1/2), penthoxyresorufin-O-depenthylation (PROD) (CYP2B), 7-ethoxycoumarin-O-deethylation (ECOD) (multiple CYPs; CYP2A6 predominant) and coumarin-7-hydroxylase (COH) (CYP2A6) assays were analyzed fluorometrically. EROD and PROD activities were determined with the method of Burke et al. [25], and ECOD was analyzed using the method of Greenlee and Poland [26]. COH activity was analyzed as described previously in detail in Raunio et al. [27]. The other CYP assays were analyzed by HPLC, and these included bupropion hydroxylation (CYP2B6) [28], amodiaquine de-ethylation (CYP2C8) [29], tolbutamide methylhydroxylation (CYP2C9) [30], dextromethorphan O-demethylation (CYP2D6) [31], chlorzoxazone 6-hydroxylation (CYP2E1) [32], midazolam

hydroxylation (CYP3A4) [33], and omeprazole 5-hydroxylation (CYP2C19) and sulfoxidation (CYP3A4) [34]. The instrumentation and incubation conditions used to assess the enzyme activities have been described previously in detail by Abass et al. [16].

Inhibition interactions were also determined with the help of the n-in-one assay described in detail by Turpeinen et al. [35] and Tolonen et al. [36]. Each inhibition mixture contained 0.5 mg microsomal protein/ml, 0.1 M phosphate buffer (pH 7.4), 1 mM NADPH, and all ten probe substrates. The amounts of metabolites were analyzed with LC–MS-MS. The CYP-specific model reactions are presented in Table 3.

The enzyme activities in the presence of carbosulfan were compared with the control incubations into which only solvent was added. The IC_{50} values for inhibitors (concentration causing 50% reduction of control activity) were determined from duplicate incubations by linear regression analysis from the plot of the logarithm of inhibitor concentration versus percentage of the activity remaining after inhibition using MicroCal Origin 6.0 (MicroCal Software, Inc., Northampton, MA).

3. Results

3.1. Identification of carbosulfan metabolites produced in vitro by human liver microsomes

In our previous *in vitro* study with microsomes from several mammalian species [8], eight carbosulfan metabolites were detected from the extracted mass chromatograms and seven of them were identified with the help of reference standards. In human liver microsomes, seven of these eight metabolites were identified, while 7-phenolcarbofuran was detected only in rabbit liver microsomes in our previous study [8]. The unidentified metabolite could be either a sulfonamide derivative or a hydroxylated carbosulfan. Either of the metabolites or their derivatives has been detected by TLC in two different studies. 3-hydroxycarbosulfan has been identified as a minor metabolite by TLC in rats in one study [7] and sulfone derivatives of carbosulfan have been detected in two separate rat studies [5,7]. In addition, the carbosulfan sulfoxide metabolite has been characterized with an unknown method in human, mouse, and rat liver microsomes [37]. In the ion source of a mass spectrometer, hydroxy metabolites of both carbofuran and 7-phenolcarbofuran produced protonated dehydrated molecules. There is no reason why this should not also happen to the corresponding carbosulfan hydroxy metabolite. Based on previous reports and missing dehydration of the unidentified metabolite, we have assumed that it is carbosulfan sulfinamide even though fragmentation of the analytes also gives some indication of the hydroxylation of carbosulfan. For the exact identification of this unidentified metabolite, more experimental work should be performed. Carbosulfan metabolites, their exact masses, fragments produced in MS-MS, retention times, and analytical parameters are presented in Fig. 1.

Three of the eight metabolites produced significant in-source fragmentation, which could cause errors in the measurements without chromatographic separation. Carbofuran in particular, which produces some in-source fragmentation to 7-phenolcarbofuran, appears in the same chromatograph as an actual 7-phenolcarbofuran metabolite. Because their retention time differs by only 0.2 min, a very large carbofuran peak could occasionally make the detection of the small 7-phenolcarbofuran peak difficult.

3.2. Kinetic parameters of carbosulfan activation in individual liver microsomal samples

Interindividual variability in the biotransformation kinetics for carbosulfan was investigated in hepatic microsomes from ten donors using a wide concentration range (2.5–300 μM) of carbosulfan. Metabolites were quantified by triple quadrupole mass spectrometry and kinetics were calculated for the carbofuran metabolic pathway, since the active chemical moiety is the most relevant for chemical risk assessment. Carbosulfan biotransformation followed Michaelis–Menten kinetics as demonstrated by Eadie-Hofstee plots (*V* versus *V/S*).

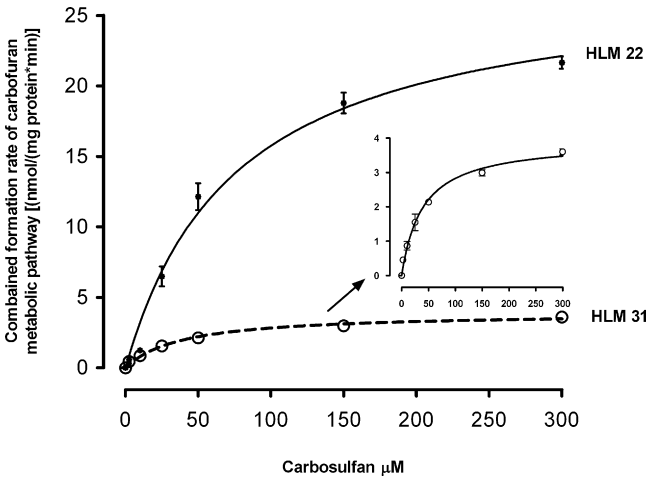

Fig. 2. Combined formation rates of carbofuran metabolic pathway illustrated with HLM22 and HLM31. Details of the experimental conditions were described under materials and methods. Results are expressed as nmol/(mg protein*min) and represent the mean ± S.D. on three independent determinations.

Detailed data for kinetics parameters are shown in Table 1. Individual 24, HLM24, exhibited the highest affinity, corresponding to the lowest k_m (12.8 μM), while individual 22 had the lowest, corresponding to the highest k_m (75.9 μM). Individual 22 had the highest capacity, corresponding to the highest V_{max} (27.7 nmol/(mg protein min)), while it was vice versa for individual 31 who had lowest capacity, corresponding to the lowest V_{max} (3.9 nmol/(mg protein min)) (Fig. 2). HLM24 showed the highest CL_{int} rate, whereas HLM31 displayed the lowest rate (670.8 and 101.1 μl/(mg protein min), respectively).

3.3. Interindividual variability in the formation rates of distal carbofuran metabolites

Since reliable enzyme kinetic parameter estimates could not be obtained for more distal metabolites of carbofuran when using carbosulfan as a substrate, metabolite formation rates were used for interindividual comparisons (Fig. 3). In some of the individual human hepatic microsomes, the amounts of 3-keto-7-phenolcarbofuran and 3-hydroxy-7-phenolcarbofuran formed were below the limits of quantification. Among ten human liver microsomes examined for carbosulfan metabolism, HLM31 had the lowest 3-hydroxycarbofuran formation rates, while HLM22 and HLM28 exhibited the highest formation rates. The 3-hydroxycarbofuran formation rate varied from 1.0 to 14.9 nmol/(mg protein min) at 300 μM carbosulfan, displaying a mean value of 5.9 nmol/(mg protein min) and 15.7-fold variation. The rates of 3-ketocarbofuran formation were 0.02–1.1 nmol/(mg protein min), displaying a mean value of 0.3 nmol/(mg protein min) and 73-fold variation. HLM31 displayed the lowest formation rate, while HLM28 had the highest rate.

Since HLM31 had the lowest 3-hydroxy- and 3-ketocarbofuran formation rates, their distal metabolites, 3-hydroxy- and 3-keto-7-phenolcarbofuran, were not detected at any carbosulfan concentrations. HLM28 had the highest activity of 3-hydroxy-7-

Table 1
Kinetic parameters of carbofuran metabolic pathway obtained with ten human liver microsomes.[a].

Human liver microsomes	V_{max} (nmol/(mg protein min))	k_m (μM)	CL_{int} (μl/(mg protein min))
HLM20	6.8 ± 0.5	17.3 ± 5.6	391.2
HLM21	8.7 ± 0.5	33.1 ± 6.9	264.4
HLM22	27.7 ± 1.6	76.0 ± 11.5	364.8
HLM23	6.2 ± 0.4	16.4 ± 3.8	380.7
HLM24	8.6 ± 1.0	12.8 ± 6.6	670.8
HLM28	26.8 ± 1.4	63.9 ± 9.4	420.1
HLM29	11.5 ± 0.8	29.3 ± 7.4	394.2
HLM30	11.6 ± 0.8	24.5 ± 6.4	472.9
HLM31	3.9 ± 0.2	38.8 ± 4.9	101.1
HLM32	7.5 ± 0.9	21.1 ± 9.8	353.9
Average			381.4
Variation (fold)[b]	7.10	5.93	6.64
Interindividual variability (fold)[c]		1.76	

[a] Each value represents the mean ± std. error of three determinations. Combined formation rates of all the metabolites of the carbofuran pathway were used for the calculation of kinetic parameters.
[b] Differences between the maximum and minimum observed values.
[c] Interindividual variability represents fold differences between mean and the highest value in toxicokinetics as defined by Renwick and Lazarus [58].

phenolcarbofuran and 3-keto-7-phenolcarbofuran formations (2.6 and 0.2 nmol/(mg protein*min)).

3.4. Identification of the P450 isoforms involved in the metabolism of carbosulfan

To determine which enzymes were responsible for the formation of carbosulfan metabolites, carbosulfan was incubated with individual human cDNA-expressed P450 isoforms, (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and 4A11) and NADPH, and the results are shown in Fig. 4. Among the isoforms tested, the role of the CYP3A subfamily appeared to be the most active one to metabolize carbosulfan. Among the CYP2C subfamily members, CYP2C8, CYP2C18, and CYP2C19 were associated with the carbofuran metabolic pathway, whereas all tested CYP2C subfamily members were involved in carbosulfan sulfinamidation. CYP1A1 and CYP2B6 mediated both carbosulfan metabolic pathways. CYP1B1 and CYP2E1 did not have detectable activity toward carbosulfan metabolism.

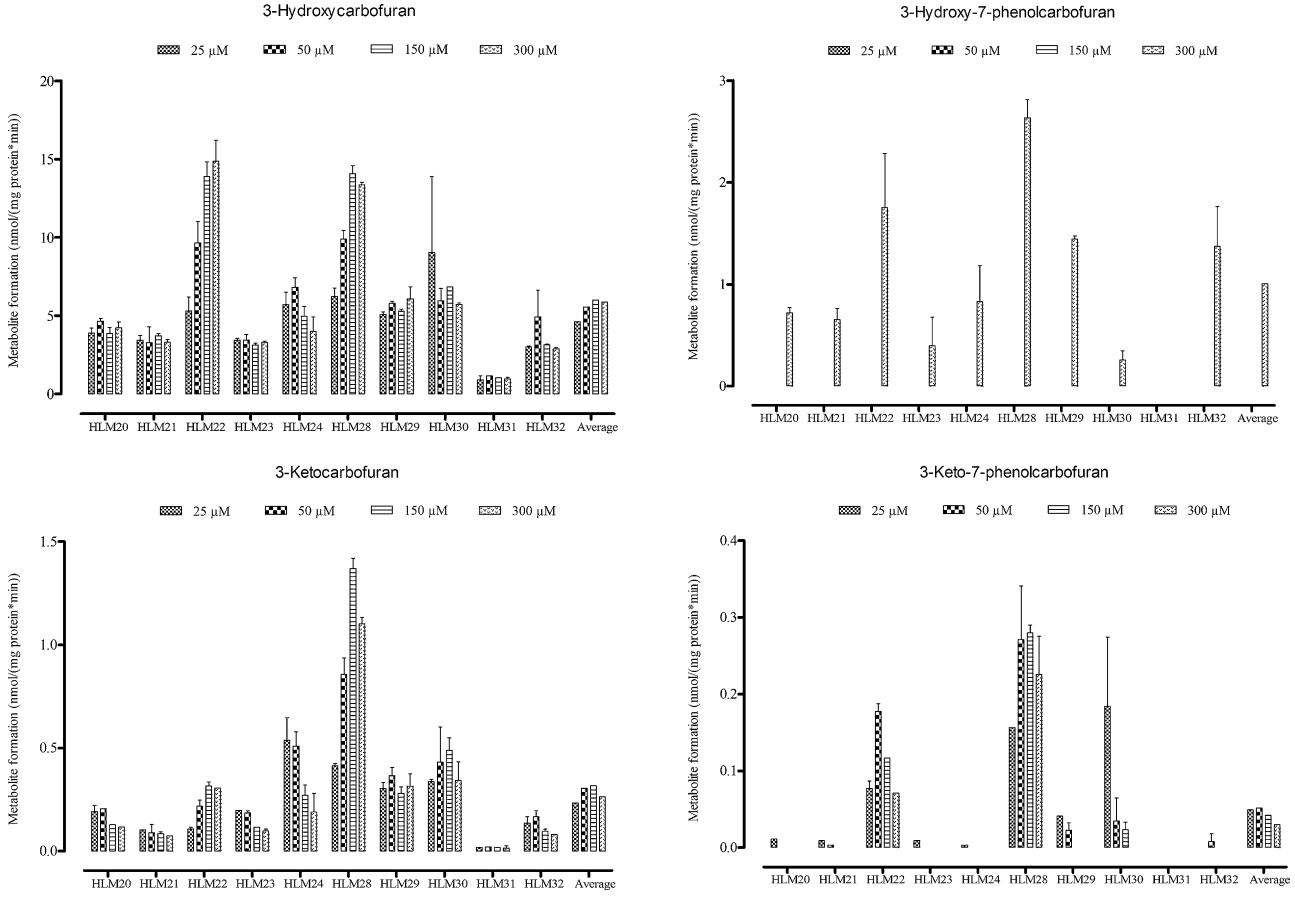

Fig. 3. Interindividual variability in the formation of carbosulfan metabolites. Human liver microsomes, HLM, (0.15 mg protein/ml) were incubated in various carbosulfan concentrations in a 100 mM phosphate buffer (pH 7.4) for 20 min. Columns represent the mean of three separate determinations and the error bars represent S.D.

Table 2
Kinetic parameters for the formation of carbosulfan metabolites obtained with human recombinant P450s.[a].

P450 isoforms	V_{max} (nmol/(nmol P450 min))	k_m (μM)	CL_{int} (μl/(nmol P450 min))	Relative contribution[b]
Carbofuran metabolic pathway[c]				
CYP1A1	53.5 ± 5.6	14.4 ± 7.3	3709.6	n.d.
CYP1A2	42.8 ± 9.3	68.2 ± 15.1	626.8	0.22
CYP2A6	92.2 ± 18.8	178.3 ± 42.4	516.9	0.60
CYP2B6	31.0 ± 6.9	40.9 ± 15.9	757.9	0.27
CYP2C8	61.7 ± 9.5	106.4 ± 25.4	580.3	0.45
CYP2C18	84.7 ± 5.6	174.9 ± 32.7	484.4	n.d.
CYP2C19	72.9 ± 5.6	80.8 ± 21.6	902.8	0.41
CYP3A4	816.1 ± 70.8	29.9 ± 10.4	27330.9	98.1
CYP3A5	133.5 ± 10.3	7.6 ± 1.5	17528.9	n.d.
CYP3A7	40.2 ± 3.1	10.2 ± 3.4	3952.8	n.d.
Carbosulfan sulfinamide metabolic pathway				
CYP1A1	29.2 ± 3.7	38.6 ± 18.6	756.4	n.d.
CYP2B6	26.9 ± 3.6	28.0 ± 15.4	961.4	36.8
CYP2C19	11.6 ± 0.6	93.4 ± 16.0	123.7	6.0
CYP3A4	25.3 ± 3.1	170.5 ± 57.3	148.4	57.2
CYP3A5	39.9 ± 2.5	29.7 ± 10.6	1346.3	n.d.
CYP3A7	5.6 ± 0.3	30.5 ± 8.0	185.2	n.d.

n.d., not determined.
[a] V_{max} and k_m values represent means ± S.E. of three determinations.
[b] Average human hepatic microsomal protein amounts of P450 enzymes are taken from Rostami-Hodjegan et al. [38].
[c] Combined formation rates of all the metabolites of the carbofuran pathway were used for the calculation of kinetic parameters.

3.5. Enzyme kinetic analysis

Based on the preliminary screening assays described above (Figure 4), detailed kinetic analyses were performed for carbosulfan with active cDNA-expressed human P450s. Results of these analyses are presented in Table 2. Combined formation rates of all the metabolites of the carbofuran pathway were used for kinetic calculations. Derivations of the k_m and V_{max} values for each isoform allowed the calculations of intrinsic clearances. According to the obtained kinetic values for the carbofuran pathway, the performance of the CYP3A subfamily was the highest. CYP3A4, CYP3A5, and CYP3A7 showed the highest affinity besides CYP1A1, corresponding to the lowest k_m, whereas CYP2A6 and CYP2C18 had the lowest affinity, corresponding to the highest k_m. CYP3A4 and CYP3A5 had the highest capacity, while CYP2B6 had the lowest. The catalytic efficiency (CL_{int}) values illustrated that CYP3A4, CYP3A5, and CYP3A7 were the most efficient CYP isoforms for carbosulfan biotransformation via the carbofuran metabolic pathway (27331, 17529 and 3953 μl/(nmol P450 min), respectively), whereas CYP2C18 was the least efficient (484.4 μl/(nmol P450 min)).

In the case of the carbosulfan sulfinamide metabolic pathway, two members of the CYP3A subfamily, CYP3A5 and CYP3A7, had the highest affinity besides CYP2B6, while CYP3A4 had the lowest. The V_{max} value for CYP3A5 was the highest (39.9 nmol/(mg protein min)), whereas the V_{max} of CYP3A7 was the lowest (5.6 nmol/(nmol P450 min)). V_{max}/k_m values illustrated that CYP3A5, CYP2B6, and CYP1A1 were the most efficient rP450s for carbosulfan transformation to carbosulfan sulfinamide (1346.3, 961.4, 756.4 μl/(nmol P450 min), respectively), whereas CYP2C19 was the least efficient (123.7 μl/(nmol P450 min)).

The contributions of the P450 isoforms studied were determined taking into account the average human hepatic microsomal protein amounts of the main P450s [CYP1A2, 52; CYP2A6, 36; CYP2B6, 11; CYP2C8, 24; CYP2C9, 73; CYP2C19, 14; CYP2D6, 8; CYP2E1, 61; and CYP3A4, 111 pmol/mg microsomal protein] [38] and the actual intrinsic clearance values for various P450s. The relative contributions of each P450 enzyme were calculated and are shown in Table 2. CYP3A4 was the main isoform responsible for the carbofuran and carbosulfan sulfinamide metabolic pathways (98.1 and 57.2%, respectively). In addition, CYP2B6 and CYP2C19 were among the key enzymes that catalyze carbosulfan sulfinamide formation (36.8 and 6.0%, respectively).

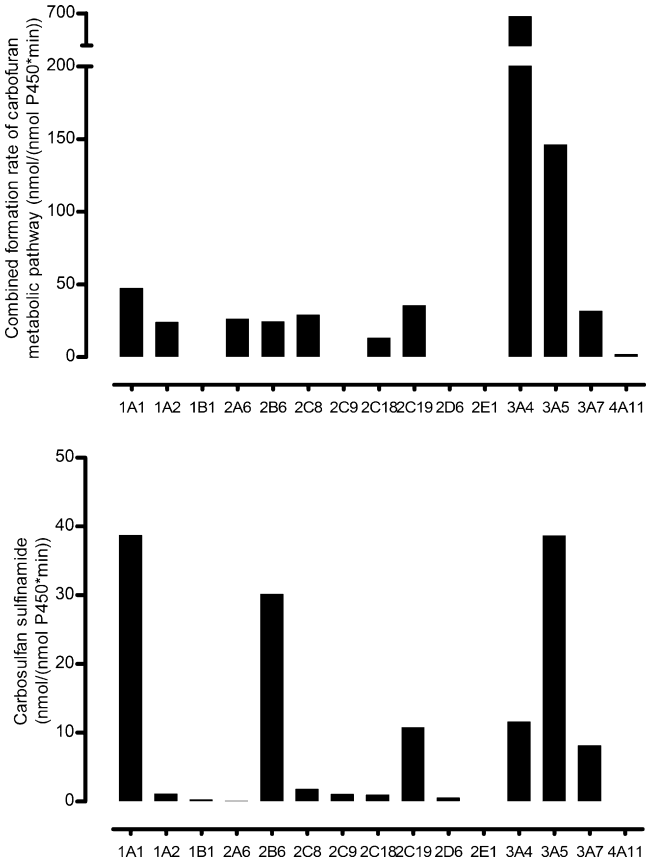

Fig. 4. Combined formation rates of the carbofuran metabolic pathway and carbosulfan sulfinamide formations by human P450s. Results are expressed as the metabolite formation rates of duplicate samples.

3.6. Correlation analysis

Quantification of carbosulfan metabolism *in vitro* by ten individual human liver microsomes was performed with 150 μM carbosulfan and a 20-min incubation time. Combined formation

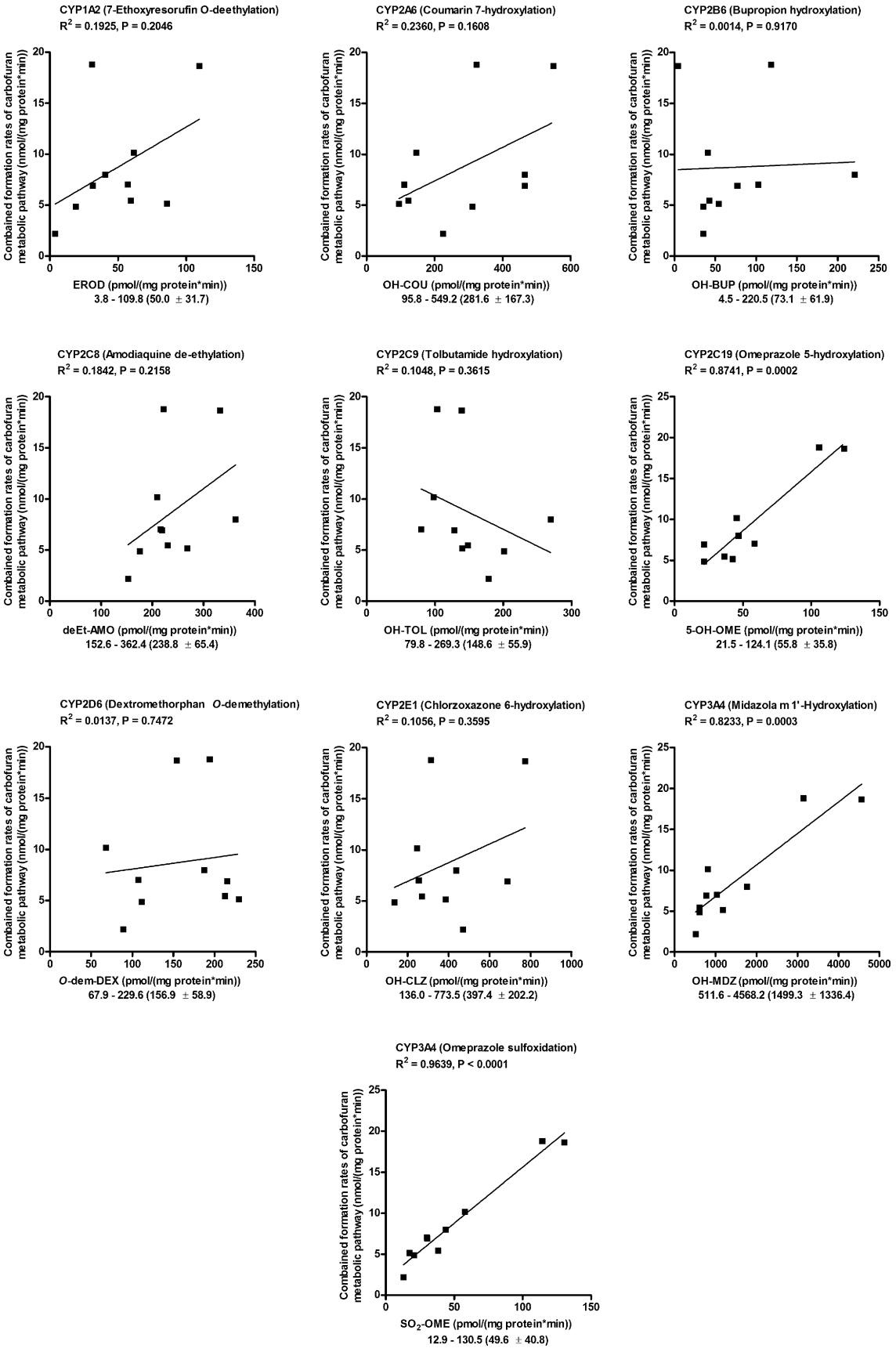

Fig. 5. Correlation of combined formation rates of the carbofuran metabolic pathway with CYP-mediated activities measured in a panel of 10 HLM with 150 μM carbosulfan. CYP marker activities (range of values and mean ± S.D.), on the *X* axis, are expressed as pmol/(mg protein*min) and formation rates of the carbofuran metabolic pathway, on the *Y* axis, are expressed as nmol/(mg protein*min). Activities are the mean of duplicate determinations. r^2 is the correlation coefficient.

rates of all the metabolites of the carbofuran pathway varied from 2.2 to 18.8 nmol/(mg protein min), indicating approximately 8.6-fold variation and a mean value of 8.7 nmol/(mg protein min). The rates of carbosulfan sulfinamide formation in the ten different human liver microsomes were 0.6–1.4 nmol/(mg protein min), displaying 2.6-fold variation and a mean value of 1.0 nmol/(mg protein min).

Rates derived from individual P450 enzymes exhibited considerable variability among ten liver samples. The specific activities of P450 isoform-catalyzed reactions in microsomes from ten human livers and their correlations with combined formation rates of all the metabolites of the carbofuran metabolic pathway were studied (Figure 5). High and significant correlations between the carbofuran metabolic pathway and CYP marker activities were seen with CYP3A4 (omeprazole sulfoxidation), CYP2C19 (omeprazole 5-hydroxylation), and CYP3A4 (midazolam 1′-hydroxylation) with correlation coefficients (r^2) of 0.96, 0.87, and 0.82, respectively. Combined formation rates of the carbofuran metabolic pathway by human liver microsomes did not correlate significantly with other specific activities of P450 isoforms ($r^2 < 0.24$). Negative correlation was found with CYP2C9-specific model activities (tolbutamide hydroxylation).

No correlations were observed between the production of carbosulfan sulfinamide metabolite by different human liver microsomes and the specific activities of P450 isoform-catalyzed reactions in microsomes from ten human livers.

3.7. Inhibition of carbosulfan metabolism by ketoconazole

Different carbosulfan concentrations were incubated with the CYP3A4 isoform-specific inhibitor, ketoconazole, in pooled human liver microsomes. As shown in Figure 6, ketoconazole inhibited formation rates of the carbofuran pathway by 90–97% and to a lesser extent carbosulfan sulfinamide formation (47–94%). The results suggest that CYP3A4 has a major role in carbosulfan metabolism at different carbosulfan concentrations.

3.8. Inhibitory interactions of carbosulfan with different human liver P450s

The effects of carbosulfan on CYP-selective activities were determined in pooled human liver microsomes. The IC_{50} values for various CYP-associated activities are collected in Table 3. Carbosulfan inhibited some CYP enzymes with high affinity. The lowest IC_{50} values for CYP3A4, midazolam 1′-hydroxylation and omeprazole sulfoxidation, being 11.2 and 23.8 μM, respectively.

Moderate values of 58.3 and 61.9 μM were observed for 7-ethoxyresorufin *O*-deethylation (CYP1A1/2) and omeprazole 5-hydroxylation (CYP2C19), respectively. All the other values for CYP2A6, CYP2B, CYP2B6, CYP2C8, CYP2C9, CYP2D6, and CYP2E1 were higher than 100 μM, indicating very low or absent affinity.

Briefly, the same inhibition values were obtained with a cocktail assay for CYP1A2, CYP2C19, and CYP3A4. Moreover, moderate inhibition with CYP2C9 and CYP2D6 was observed only with the

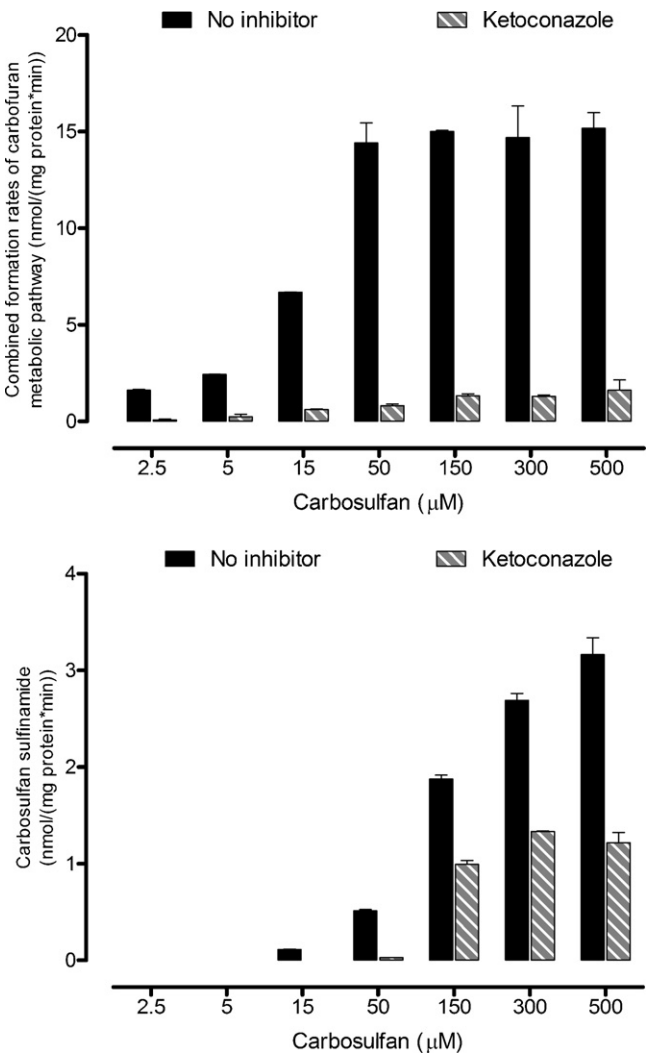

Fig. 6. Effect of CYP3A4 isoform-selective inhibitor, ketoconazole, on the carbofuran metabolic pathway and carbosulfan sulfinamide formation by HLM. Carbosulfan was incubated with pooled HLM in the presence of 100 μM ketoconazole. Columns represent the means of two separate determinations and the error bars represent S.D.

Table 3
The carbosulfan IC_{50} values of different CYPs using pooled human liver microsomes with single substrate and n-in-one assays.

CYP	Substrate	Reaction	IC_{50} (μM)	
			Single substrate assay	n-in-one assay
1A1/2	7-ethoxyresorufin	*O*-deethylation	58.3	–
1A2	Melatonin	6-hydroxylation	–	93.0
2A6	Coumarin	7-hydroxylation	>100	>100
2B	7-pentoxyresorufin	*O*-dealkylation	>100	>100
2B6	Bupropion	Hydroxylation	>100	>100
2C8	Amodiaquine	de-ethylation	>100	93.7
2C9	Tolbutamide	Methylhydroxylation	>100	16.9
2C19	Omeprazole	5-hydroxylation	61.9	16.3
2D6	Dextromethorphan	*O*-demethylation	>100	25.0
2E1	Chlorzoxazone	6-hydroxylation	>100	>100
3A4	Midazolam	1'-hydroxylation	11.2	20.1
3A4	Omeprazole	Sulfoxidation	23.8	18.3
3A4	Omeprazole	3-hydroxylation	–	23.4

cocktail assay. All the other values were higher than 100 μM in both the single substrate and cocktail assays.

4. Discussion

Carbosulfan is metabolized via two metabolic pathways ([8]; this study): (1) carbosulfan undergoes the initial oxidation of sulfur to carbosulfan sulfinamide ('sulfur oxidation pathway') and (2) the major route is the cleavage of the nitrogen sulfur bond (N–S) to give carbofuran and dibutylamine ('carbofuran pathway'). During the present investigation, seven metabolites produced by human liver microsomes were identified using LC/MS/MS and six of them were verified with comparison to reference standards. The carbofuran metabolic pathway contains products (carbofuran, 3-hydroxy-carbofuran, 3-ketocarbofuran), which are more toxic than the parent carbosulfan [5,39,40]. An acute exposure to carbofuran inhibits the action of acetylcholinesterase (AChE) in nerve cells and may cause transient endocrine disruption with increased levels of progesterone, cortisol, and estradiol and decreased testosterone levels [41]. Repeated exposure to carbofuran has adverse chronic effects on a broad spectrum of nervous system functions [42] and may cause serious reproductive problems. Thus, the toxicity of carbosulfan is dependent on its biotransformation to carbofuran and its metabolites. Metabolic intrinsic clearance rates obtained in pooled human liver microsomes and six mammalian liver microsomes indicated that carbosulfan is activated to the carbofuran metabolic pathway more efficiently than it is detoxified to carbosulfan sulfinamide [8]. The CL_{int} values (μl/(mg protein min)) of the carbofuran metabolic pathway were 55- and 11-fold higher than those of the carbosulfan sulfinamide metabolic pathway (data not shown) for individuals HL24 and HL31, representing roughly the highest and the lowest metabolic rates, respectively. These differences suggest that the carbofuran metabolic pathway (activation pathway) is the most important metabolic pathway in human liver.

Human liver microsomes display quite large interindividual differences in all carbosulfan metabolic pathways. Differences between the maximum and minimum observed values in ten individual liver microsomes indicated 5.9-, 7.0-, and 6.6-fold variability in the k_m, V_{max} and CL_{int} values for the carbofuran metabolic pathway, respectively. The up to 7-fold interindividual variability in the rate of carbosulfan activation, as determined by the difference between the lowest and highest value, could be the significant determinant of the toxicity effects. There will be substantial differences in toxic metabolite formation even if different individuals are exposed to the same quantity of carbosulfan. High metabolizers might be at higher risk for carbosulfan-related toxicity. There are large interindividual differences in the expression levels and catalytic activities of CYP enzymes in humans, and these variations sometimes lead to different susceptibilities of humans to the pharmacological and toxicological actions of drugs, toxic chemicals, and carcinogens [10,43].

The relatively large interindividual variability in carbosulfan bioactivation, and partially also in detoxification, seems to be due to the preponderance of carbosulfan metabolism by CYP3A subfamily members, especially by CYP3A4 (see below for further discussion). This became especially apparent in our study on the correlation between specific P450 isoform-catalyzed reactions in microsomes from ten human livers and the combined formation rates of all the metabolites of the carbofuran metabolic pathway. High correlations were observed with CYP3A4 and CYP2C19. The individuals with the highest levels of CYP3A4 (HLM22 and HLM28) had the highest V_{max},of carbofuran pathway and the reverse was true for an individual (HLM31) who had the lowest levels of CYP3A4 isoform. These results on correlation analysis together with other experiments on recombinant enzymes collectively suggest that CYP3A subfamily members play a dominant role in the interindividual variability of carbosulfan activation. Moreover, it is possible that those individuals with a high CYP3A4 and CYP2C19 content may be more susceptible to carbosulfan toxicity.

Kinetic characterization showed that carbosulfan metabolism to carbosulfan sulfinamide and the carbofuran metabolic pathway were one-phasic; in other words, it can be described as involving one active site or several sites with similar enzyme kinetic characteristics. Actually, our studies on a large set of recombinant P450 enzymes indicated that several enzymes were able to metabolize carbosulfan. The carbofuran pathway was the preferable pathway based on values of kinetic parameters obtained with active recombinant CYPs. Four CYP isoforms, CYP1A1, CYP3A4, CYP3A5, and CYP3A7 were involved in the carbofuran pathway with high affinity. Additionally, CYP2C19 and CYP2B6 displayed some activity towards carbofuran formation. However, it is difficult to separate the involvement of individual CYPs in the carbofuran metabolic pathway leading to the formation of more distal carbofuran metabolites, and further studies are required to clarify the individual contribution of the isoforms involved in minor metabolite formations. Nevertheless, there is strong evidence from earlier studies that CYP3A4 is the predominant isoform responsible for formation of 3-hydroxycarbofuran, when starting from carbofuran as the substrate [13].

CYP3A5, CYP2B6, and CYP1A1 were active in carbosulfan sulfinamide formation, whereas CYP3A7, CYP3A4 and CYP2C19 were less active. All CYPs involved in carbosulfan sulfinamide formation were participating also in the carbofuran metabolic pathway, but relative efficiencies differed considerably: CYP2B6 had a preference for the carbosulfan sulfinamide pathway, whereas CYP1A1, CYP2C19, CYP3A4, CYP3A5, and CYP3A7 were much more active in the carbofuran pathway (4.9-, 7.3-, 184.2-, 13.0-, and 21.3-fold more efficient, respectively).

The relative importance of individual isoforms to *in vitro* and *in vivo* clearance is dependent upon the relative abundance of each isoform. Thus, intrinsic clearance values measured with individual cDNA-expressed enzymes have to be normalized with respect to the human hepatic microsomal P450 isoenzyme concentrations. The relative contribution of CYP3A4 to carbosulfan metabolism was the highest because of both high intrinsic clearance and average amount. Although CYP1A1 had the highest intrinsic clearance value for the carbofuran metabolic pathway, its contribution is probably negligible because the amount of CYP1A1 in the human liver is very small [9]. Based on the average human hepatic microsomal protein amounts of the CYP3A subfamily (155 pmol/mg microsomal protein) [38], the CYP3A subfamily contributes to a superior degree to the carbofuran and carbosulfan sulfinamide metabolic pathways (99.2 and 96.3%), respectively.

CYP2B6 had a higher affinity (k_m = 28.0 μM) for carbosulfan sulfinamide formation than CYP3A subfamily members (CYP3A5, k_m = 29.7; CYP3A7, 30.5 and CYP3A4, 171 μM). However, the calculation of relative contributions of CYPs confirmed the role of the CYP3A subfamily in the carbosulfan sulfinamide metabolic pathway. CYP2B6 contributes with 0.7% to carbosulfan sulfinamide formation compared to CYP3A subfamily members.

The observation of the preponderance of CYP3A4 in carbosulfan metabolism, which was based on the average abundances of CYP enzymes in human liver microsomes, was further confirmed by the correlation studies (see above) and by the observation that the *in vitro* microsomal metabolism of carbosulfan was inhibited by ketoconazole, a selective inhibitor for CYP3A4/5 [10,43]. Our results showed that ketoconazole significantly inhibited the carbofuran metabolic pathway *in vitro*. Furthermore, carbosulfan inhibited relatively potently the CYP3A4-selective enzyme activity in pooled human liver microsomes and moderately CYP1A1/2 and CYP2C19. Inhibitory interactions studies supported the role of CYP3A4 in car-

bosulfan metabolism as CYP3A subfamily members were the P450 isoforms with relatively low k_m values for carbosulfan metabolism.

Although the CYP3A subfamily, CYP3A4, 3A5, and 3A7, represents about 30% of the total hepatic P450 content and is considered the most important CYP subfamily in the biotransformation of xenobiotics [10,44,45], relative abundances of its members are highly different. CYP3A5 exists in only 10-20% of humans and its content is equivalent to only about 0.2% of the total P450 amount [46,47]. The amount of CYP3A7 is very low in adult human livers and is mainly expressed in embryonic, fetal, and newborn livers, where it is the predominant CYP form [48,49]. Thus, CYP3A4 is probably the most important CYP enzyme and it has been shown that it has a vital role in pesticide metabolism, as demonstrated here for carbosulfan, and furthermore it is involved in the metabolism of almost all currently tested pesticides. However, chemical structure influences which CYPs mediate the specific reaction, i.e activation or detoxification. CYP3A4 activate and detoxify parathion, diazinon and chlorpyrifos while it is mainly involved in carbaryl detoxification to 4-hydroxycarbaryl [50–53]. In our previous study [8], the catalytic efficiency of the carbofuran metabolic pathway (CL_{int}) for minipig, monkey, and human liver microsomes displayed similar high values, while rat, rabbit, mouse, and dog liver microsomes had lower values. In view of the current results that CYP3A subfamily members are the main enzymes responsible for carbosulfan activation, our findings are in agreement with our earlier comparative *in vitro* study of hepatic drug metabolism of six experimental animal species and human, in which presumably CYP3A-mediated midazolam α-hydroxylase and omeprazole sulfoxidase activities in human and monkey liver microsomes were relatively high, while activities of rat and rabbit liver microsomes were much lower [54].

Human data are needed for quantitative toxicokinetics comparisons between individuals or between animals and humans [55,56]. The use of chemical-specific toxicological data instead of default assessment factors, whenever possible, was proposed by the International Program on Chemical Safety (IPCS) [57]. Our data concerning the metabolism of carbosulfan in animals and humans have significant implications for the calculation of chemical-specific adjustment factors (CSAFs). The uncertainty factor for human variability in toxicokinetics was 1.76-fold, as defined by Renwick and Lazarus [58] as a variation between the mean and the highest value. This suggested that interindividual variability, for the carbosulfan active chemical moiety, in toxicokinetics is within the standard applied factor for interindividual extrapolation in toxicokinetics. Moreover, we have shown previously that interspecies differences in toxicokinetics are within the standard applied factor for species extrapolation in toxicokinetics. However, it should be kept in mind that these data are restricted to metabolic data from human and animal liver preparations.

In conclusion, in this study we have identified, by LC/MS/MS, seven metabolites produced by human liver microsomes and six of them were verified with comparison to reference standards. The carbofuran metabolic pathway (activation pathway) is the major metabolic pathway in human liver microsomes and there is marked (up to 7-fold) interindividual variability in the carbosulfan activation. We have provided strong evidence that carbosulfan activation is predominantly catalyzed by CYP3A subfamily members. First, the recombinant human CYP3A4 was the major enzyme involved in the metabolism. Second, the formation rate of carbofuran pathway metabolites correlated significantly with the activity of CYP3A in a panel of human liver microsomes. Third, the carbofuran metabolic pathway was potently inhibited by ketoconazole, a strong inhibitor of CYP3A. Fourth, carbosulfan relatively potently inhibited CYP3A4 activity in pooled human liver microsomes. The data demonstrated that metabolism of carbosulfan may interfere with other substrates for the CYP3A family. The inhibitory interactions might be of significance at least in those occupational situations where workers are exposed to the higher pesticide concentrations.

Conflicts of interest

None of the authors has a conflict of interest related to this study.

Acknowledgements

This work was funded by a Ministry of Education-supported position from the Finnish Graduate School in Toxicology (ToxGS) and by grants from The Academy of Finland and The Finnish Granting Agency for Technological Research and Innovation (TEKES) and supported by grants from the Agriculture Foundation of Economic Association in the Oulu province and Orion-Farmos Research Foundation.

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