Available online at www.sciencedirect.com
SCIENCE DIRECT®
Chemico-Biological Interactions 150 (2004) 221–232
Chemico-Biological Interactions
www.elsevier.com/locate/chembioint

In vitro metabolism of carbofuran by human, mouse, and rat cytochrome P450 and interactions with chlorpyrifos, testosterone, and estradiol

Khawja A. Usmani, Ernest Hodgson, Randy L. Rose*

Department of Environmental and Molecular Toxicology, North Carolina State University, P.O. Box 7633, Raleigh, NC 27695, USA

Accepted 25 September 2004
Available online 30 October 2004

Abstract

Carbofuran is a carbamate pesticide used in agricultural practice throughout the world. Its effect as a pesticide is due to its ability to inhibit acetylcholinesterase activity. Though carbofuran has a long history of use, there is little information available with respect to its metabolic fate and disposition in mammals. The present study was designed to investigate the comparative in vitro metabolism of carbofuran from human, rat, and mouse liver microsomes (HLM, RLM, MLM, respectively), and characterize the specific enzymes involved in such metabolism, with particular reference to human metabolism. Carbofuran is metabolized by cytochrome P450 (CYP) leading to the production of one major ring oxidation metabolite, 3-hydroxycarbofuran, and two minor metabolites. The affinity of carbofuran for CYP enzymes involved in the oxidation to 3-hydroxycarbofuran is significantly less in HLM (K_m = 1.950 mM) than in RLM (K_m = 0.210 mM), or MLM (K_m = 0.550 mM). Intrinsic clearance rate calculations indicate that HLM are 14-fold less efficient in the metabolism of carbofuran to 3-hydroxycarbofuran than RLM or MLM. A screen of 15 major human CYP isoforms for metabolic ability with respect to carbofuran metabolism demonstrated that CYP3A4 is the major isoform responsible for carbofuran oxidation in humans. CYP1A2 and 2C19 are much less active while other human CYP isoforms have minimal or no activity toward carbofuran. In contrast with the human isoforms, members of the CYP2C family in rats are likely to have a primary role in carbofuran metabolism. Normalization of HLM data with the average levels of each CYP in native HLM, indicates that carbofuran metabolism is primarily mediated by CYP3A4 (percent total normalized rate (% TNR) = 77.5), although CYP1A2 and 2C19 play ancillary roles (% TNR = 9.0 and 6.0, respectively). This is substantiated by the fact that ketoconazole, a specific inhibitor of CYP3A4, is an excellent inhibitor of 3-hydroxycarbofuran formation in HLM (IC_{50}: 0.31 μM). Chlorpyrifos, an irreversible non-competitive inhibitor of CYP3A4, inhibits the formation of 3-hydroxycarbofuran in HLM (IC_{50}: 39 μM). The use of phenotyped HLM demonstrated that individuals with high levels of CYP3A4 have the greatest potential to metabolize carbofuran to its major metabolite. The variation in carbofuran metabolism among 17 single-donor HLM

* Corresponding author. Tel.: +1 919 515 4378; fax: +1 919 515 7169.
E-mail address: randy_rose@ncsu.edu (R.L. Rose).

doi:10.1016/j.cbi.2004.09.015

samples is over 5-fold and the best correlation between CYP isoform activity and carbofuran metabolism was observed with CYP3A4 ($r^2 = 0.96$). The interaction of carbofuran and the endogenous CYP3A4 substrates, testosterone and estradiol, were also investigated. Testosterone metabolism was activated by carbofuran in HLM and CYP3A4, however, less activation was observed for carbofuran metabolism by testosterone in HLM and CYP3A4. No interactions between carbofuran and estradiol metabolism were observed.

Keywords: Carbofuran; Metabolism; Human liver microsomes; Cytochrome P450; CYP3A4; Testosterone; Interactions

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is the most commonly used carbamate in agriculture and forestry. Carbofuran is a broad spectrum pesticide that kills insects, mites, and nematodes on contact or after ingestion. The mechanism of toxicity is anticholinesterase activity [1], resulting in accumulation of acetylcholine in synapses and subsequent malfunction of the nervous system. In addition to neurotoxic effects, carbofuran has been demonstrated to influence steroid metabolism in mammals [2–4]. Recent epidemiological studies indicate that individuals with high levels of exposure to carbofuran and other carbamate pesticides may have increased risk for lung cancer (C.R. Alavanja, personal communication) and non-Hodgkins lymphoma [5].

The most recent extensive review of carbofuran toxicity and metabolism is that by Gupta [6]. Primary mammalian metabolites include 3-hydroxycarbofuran, 3-ketocarbofuran, 3-ketocarbofuran-7-phenol, and carbofuran phenol. Each of these metabolites is found in the free state and also as sulfate and glucuronide conjugates which are excreted in the urine. In vivo studies involving oral exposure of rats to carbofuran demonstrated that the predominant metabolite in the bile was 3-hydroxycarbofuran glucuronide, a metabolite which may be cleaved to yield a potent anticholinesterase aglycone. Since the enterohepatic cycling of glucuronides involves cleavage of the conjugate in the gut, biliary excretion may actually lead to increased systemic activity of toxic carbofuran metabolites since both carbofuran and 3-hydroxycarbofuran are equally potent cholinesterase inhibitors [6].

Metabolism plays an important role in the determination of pesticide toxicity. Hepatic metabolism of carbofuran in humans has neither been previously investigated in vitro nor have the contributions of CYP isoforms to metabolic pathways been elucidated. An understanding of the metabolic pathways and the varying contributions of specific CYP isoforms involved will enable better understanding of differences in metabolism among individuals as well as among subpopulations and will provide important information relative to metabolic interactions of carbofuran with other chemicals.

Studies of pesticide metabolism in humans can provide important information on differences between humans and laboratory animals in metabolism. Rodent studies have been useful for decades for predicting human health hazards associated with pesticide use. However, studies conducted in experimental animals can sometimes be misleading since human xenobiotic-metabolizing enzymes often differ dramatically from those of experimental animals, rendering such extrapolations of little value. The present in vitro study was designed to: (1) compare the metabolism of carbofuran in human, rat, and mouse liver microsomes; (2) elucidate human CYP isoforms responsible for metabolism of carbofuran; (3) determine potential differences in oxidation activities among individual human liver microsomes; (4) examine potential interactions of carbofuran with endogenous chemicals that are substrates for the same enzymes.

2. Materials and methods

2.1. Chemicals

Carbofuran was purchased from Chem Service Inc. (West Chester, PA). Carbofuran metabolites, 3-hydroxycarbofuran, 3-ketocarbofuran, 3-ketocarbofuran phenol, 3-hydroxycarbofuran phenol, and carbofuran phenol were a gift from FMC (Princeton, NJ). Testosterone, 6β-hydroxytestosterone,

17β-estradiol, 2-hydroxyestradiol were purchased from Steraloids (Newport, RI). HPLC grade acetonitrile and water were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals, if not specified, were purchased from Sigma (St. Louis, MO).

2.2. Liver microsomes, human hepatocytes, and cytochrome P450 isoforms

Rat liver microsomes (RLM) and mouse liver microsomes (MLM) were prepared from adult male Long Evans rats and adult male CD-1 mice (Charles River Laboratories, Raleigh, NC), respectively, according to the method of Cook and Hodgson [7].

Pooled and single-donor human liver microsomes (HLM), human CYP isoforms (CYP1A1, 1A2, 1B1, 2A6, 3A4, 3A5, 3A7, 4A11, 2B6, 2C8, 2C9*1, 2C9*2, 2C9*3, 2C18, 2C19, 2D6*1, 2D6*10, and 2E1), and rat CYP isoforms (CYP3A1, 3A2, 2C6, 2C11, and 2C13) expressed in baculovirus infected insect cells (Sf9) (BTI-TN-5BI-4), were purchased from BD Biosciences (Woburn, MA). Immunoquantitation results of individual CYP isoforms in single-donor HLM samples were provided by GenTest Corporation (Woburn, MA) [8].

Fresh human hepatocyte suspensions were obtained from Vesta Therapeutics (Research Triangle Park, NC). Cell viability was more than 87% measured by the trypan blue exclusion method.

2.3. In vitro carbofuran metabolism

Enzyme kinetic assays for pooled HLM, RLM, and MLM were performed by incubation in 1.5 ml microcentrifuge tubes of serial concentrations of carbofuran (final concentration 12.5–3200 μM) with microsomes. The initial combination of substrate, NADPH regenerating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase) and 100 mM potassium phosphate buffer with 5 mM $MgCl_2$ (pH 7.4) were incubated at 37 °C for 5 min. The reactions were initiated by the addition of ice cold microsomes (protein concentration 1 mg/ml) with gentle mixing followed by incubation for 10 min at 37 °C. To determine metabolic activity of individual HLM, incubations of carbofuran (final concentration 1600 μM) were conducted for 10 min at 37 °C using 1 mg/ml protein concentration. Reactions were terminated by the addition of an equal volume of acetonitrile and vortexing. The control reactions were performed under identical conditions using microsomes prepared from Sf9 cells.

Enzyme kinetic assays using fresh human hepatocytes were performed by incubating serial concentrations of carbofuran (final concentration 200–3200 μM) with the hepatocytes in 1.5 ml microcentrifuge tubes. The initial combinations of substrate and hepatocyte culture media were incubated at 37 °C for 5 min. The reactions were initiated by the addition of ice cold human hepatocytes (0.1 million cells/0.25 ml) with gentle mixing followed by incubation for 30 min at 37 °C. After 29 min the reactions were centrifuged for 1 min to sediment the cells and the supernatants were transferred to 250 μl of acetonitrile. The suspended cells were pooled to prepare S9 fractions. Protein was determined from S9 fractions in order to express the V_{max} based on mg protein.

Metabolic activity assays designed to screen human CYP isoforms for metabolic activity towards carbofuran were performed by incubation with the appropriate substrate (final concentration 1600 μM) with an NADPH regenerating system in specific buffers recommended by the enzyme supplier (BD Biosciences, Woburn, MA). After preincubation at 37 °C for 5 min, the reactions were initiated by the addition of ice cold CYP isoforms (final concentration 50 pmol/ml) with gentle mixing and incubated for 30 min at 37 °C. The control reactions were performed under identical conditions with a Sf9 insect cell control. For the CYP isoforms 1A1, 1A2, 1B1, 2E1, 2C8, 2D6*1, 2D6*10, 3A5, 3A7, 2B6, 2C18, 3A4, and 2C19, and Sf9 cell control, a 100 mM potassium phosphate buffer with 5 mM $MgCl_2$ (pH 7.4) was used. For the CYP isoforms 2C9*1 (Arg_{144}), 2C9*2 (Cys_{144}), 2C9*3 (Leu_{359}), 4A11, and 2A6, a buffer consisting of 100 mM Tris–HCl with 5 mM $MgCl_2$ (pH 7.5) was used.

After screening assays were completed, isoforms determined to have the greatest impact on metabolism were selected for further characterization. Enzyme kinetic assays for human CYP3A4 and 2C19 were performed by incubating serial dilutions of carbofuran (final concentration 25–1600 μM) with 50 pmol/ml of CYP isoform for 10 min as described above. The control reactions were performed under identical conditions using Sf9 cells. Similarly, rat CYP3A1, 3A2, 2C6, 2C11, and 2C13 incubations were performed for

enzyme kinetic assays in CYP-specific buffers recommended by the enzyme supplier (BD Biosciences, Woburn, MA).

To demonstrate the role of CYP3A4 in carbofuran metabolism in HLM (protein concentration 1 mg/ml), a CYP3A4 specific inhibitor, ketoconazole (final concentration 0–10 μM), was added to the reaction mixture simultaneously with 1600 μM carbofuran. Similarly, the ability of chlorpyrifos to inhibit carbofuran metabolism in HLM was also explored. In this case, HLM (1 mg/ml) were pre-incubated with reaction mixtures containing various concentrations of chlorpyrifos (final concentration 0–125 μM) in the presence of an NADPH-generating system. After 5 min the reaction was initiated by the addition of 1600 μM carbofuran. In both cases, the reactions were terminated after 10 min and the supernatants analyzed for products by HPLC.

2.4. Interactions with testosterone and estradiol

The effect of testosterone and estradiol on carbofuran metabolism was assayed in HLM (protein concentration 1 mg/ml), or human CYP3A4 (final P450 content 50 pmol/ml) by co-incubation of carbofuran (final concentration 100, 400, and 1600 μM) with testosterone (final concentration 0–200 μM) or estradiol (final concentration 0–200 μM) for 10 min. The effect of carbofuran on testosterone 6β-hydroxylation and 2-hydroxyestradiol was assayed in HLM (protein concentration 1 mg/ml), or human CYP3A4 (final P450 content 50 pmol/ml) by co-incubation of testosterone (final concentration 4, 20, and 100 μM) or estradiol (final concentration 20 and 100 μM) with carbofuran (final concentration 0–1600 μM) for 10 min. Metabolic effects of carbofuran and testosterone or carbofuran and estradiol on each others metabolism were determined by monitoring 3-hydroxycarbofuran and 6β-hydroxytestosterone or 2-hydroxyestradiol levels by HPLC, respectively. Testosterone and its metabolites were separated using the HPLC method described by Usmani et al. [9]. Estradiol and its metabolites were separated using the HPLC method described by Suchar et al. [10].

All reactions were terminated by the addition of equal volumes of acetonitrile and vortexing. After 5 min of centrifugation at 15,000 rpm in a microcentrifuge, the supernatants were analyzed for concentrations by HPLC. The protein concentrations and incubation times used in the assays were found to be in the linear range in preliminary experiments. No metabolites were detected when incubations were carried out in the absence of an NADPH-generating system.

2.5. Analysis of carbofuran metabolites by HPLC

Metabolites were analyzed using a Shimadzu HPLC system (Kyoto, Japan). This Shimadzu HPLC system consisted of one pump (LC-10ATVP), autoinjector (SIL-10ADVP), UV–vis detector (SPD-10AVVP), system controller (SCL-10AVP), four-position solvent selection valve (FCV-10ALVP) and a degasser (DGU-14A). Chromatography software was CLASS-VP version 4.3. Carbofuran and its metabolites were separated using a mobile phase consisting of A (100% water) and B (100% acetonitrile). A gradient system was initiated at 20% solvent B and increased linearly in the following manner: 60% of solvent B in 0–10 min, 60% of solvent B in 10–12 min, 20% of solvent B in 12 and 13 min, and maintained at 20% of solvent B for 15 min. The flow rate was 1.0 ml/min. Metabolites were separated with a Synergi column (Synergi 4 μ, 150 mm × 4.6 mm, MAX-RP, 80A; Phenomenex, Rancho Palos Verdes, CA) at a wavelength of 280 nm. The injection volume was 50 μl. Using this gradient, carbofuran and its metabolites were well separated with baseline separation. Under these conditions, the retention times for carbofuran, 3-hydroxycarbofuran, unknown carbofuran metabolite, 3-ketocarbofuran phenol, and 3-ketocarbofuran were 5.7, 6.7, 7.7, and 8.2 min, respectively. The limit of detection for carbofuran metabolites was 0.05 μM. The concentrations of metabolites were obtained through extrapolation of peak area from a standard curve.

2.6. Enzyme kinetics calculations

All values were expressed as mean ± S.E.M. ($n = 3$ determination). The apparent K_m and V_{max} parameters were calculated using a nonlinear regression analysis program (SigmaPlot Enzyme Kinetic Module, SigmaPlot software, Inc., Chicago, IL). These K_m and V_{max} values were then used to calculate the intrinsic clearance value (K_m/V_{max}). The percent total

normalized rates (% TNR) were determined as described by Rodrigues [11]. This is done by multiplying the rate of hydroxylation (nmol/nmol CYP/min) of each isoform by the nominal specific content (nmol CYP/mg protein) of the corresponding CYP form in native human liver microsomes to derive the normalized rate (NR) for each isoform. The nominal specific content utilized in these determinations was derived from a pool of liver microsomes ($N = 12$) phenotyped by GenTest Corp. [11]. Since subsequent data indicated that CYP2B6 levels may have been high in this population, we used a value of 0.0207 nmol/mg protein derived from a different pool of 12 individuals phenotyped by GenTest Corp. [8]. The NR values obtained were then summed and the % TNRs were determined for each isoform.

3. Results

3.1. Enzyme kinetics of carbofuran metabolism in liver microsomes and human hepatocytes

In vitro incubations of carbofuran with HLM, RLM, and MLM resulted in the production of one major and two minor metabolites (Fig. 1). The major metabolite of carbofuran incubations in microsomal samples was 3-hydroxycarbofuran, while the minor metabolites included 3-keto-7-phenol and one unidentified minor metabolite. Further investigations involving incubations of 3-hydroxycarbofuran with HLM demonstrated that it is metabolized by CYPs to a minor metabolite, 3-ketocarbofuran, which is immediately converted non-enzymatically to 3-keto-7-phenol. Incubations of 3-ketocarbofuran in the presence and absence of the NADPH-generating system showed no difference in the production of 3-keto-7-phenol (data not shown). For the purposes of kinetic calculations, rates of 3-hydroxycarbofuran and 3-keto-7-phenol were combined, however, significant difference in kinetics were not observed when calculated in the absence of 3-keto-7-phenol (data not shown).

Although metabolite formation among these species was not qualitatively different, there were significant quantitative differences with mice and rats producing significantly greater quantities of metabolites than humans. These differences in metabolism are reflected by the kinetic data which demonstrates that HLM have lower affinity (higher K_m) and lower intrinsic clearance rate than RLM and MLM (Table 1). Incubations of carbofuran with human hepatocytes showed similar K_m but lower V_{max} values than those observed using HLM (Table 1).

3.2. Carbofuran metabolism by human CYP isoforms

A screen of several CYP isoforms to investigate the oxidation of carbofuran was conducted (Fig. 2). Among 15 different CYP isoforms and three different polymorphic variants investigated, only CYP2A6, 4A11, 2C18, 2D6*10, and 2E1 did not have detectable activity toward carbofuran. All other CYP isoforms were active in generating 3-hydroxycarbofuran, although the extent of metabolism varied widely among isoforms. CYP3A4, 1A2, and 2C19 were among the most active in carbofuran metabolism. Normalization of the data with respect to average CYP isoform levels in native HLM indicated that metabolism was primarily mediated by CYP3A4 (% TNR = 77.5), although CYP1A2 and 2C19 play ancillary roles (% TNR = 9.0 and 6.0, respectively; Fig. 3).

Table 1
Kinetic parameters for ring hydroxylation of carbofuran by pooled HLM, RLM, MLM, human cytochrome P450 isoforms, and rat cytochrome P450 isoforms

	K_m	V_{max}	V_{max}/K_m
Liver microsomes			
HLM	1.974 ± 0.020	3.30 ± 0.2	1.70
RLM	0.207 ± 0.009	5.50 ± 0.1	26.6
MLM	0.551 ± 0.019	13.3 ± 0.2	24.1
Human hepatocytes	2.066 ± 0.055	0.64 ± 0.1	0.31
Human P450s			
CYP3A4	0.742 ± 0.170	40.3 ± 4.3	54.3
CYP1A2	0.238 ± 0.027	22.0 ± 0.8	92.3
CYP2C19	0.199 ± 0.033	18.1 ± 0.9	90.8
Rat P450s			
CYP3A1	0.514 ± 0.025	53.8 ± 1.1	105
CYP3A2	0.751 ± 0.065	106 ± 4.6	141
CYP2C6	0.091 ± 0.005	13.3 ± 0.2	146
CYP2C11	0.224 ± 0.006	31.7 ± 0.3	142
CYP2C13	0	0	0

Values are expressed as mean ± S.E. ($n = 3$ determinations). K_m is expressed as mM. V_{max} is expressed as nmol/mg protein/min for liver microsomes and human hepatocytes. V_{max} is expressed as nmol/nmol isoforms/min for human and rat P450s. V_{max}/K_m is expressed as μl/mg protein/min for liver microsomes. V_{max}/K_m is expressed as μl/nmol isoforms/min for human and rat P450s.

Kinetic studies of carbofuran incubation by CYP3A4, 1A2, and 2C19 demonstrated that CYP3A4 has ca. 3-fold lower affinity (K_m) than CYP1A2 and 2C19 (Table 1). Kinetic studies with rat CYPs, CYP3A1, 3A2, 2C6, and 2C11, indicated that rat CYP3A1 and 3A2 had similar K_m's as their human counterpart, CYP3A4, while rat CYP2C6 and 2C11 had much lower K_m's than rat CYP3A1 and 3A2 (Table 1).

3.3. Carbofuran metabolism in single-donor HLM

Carbofuran metabolism was analyzed in 17 single-donor HLM. Differences in carbofuran metabolism among individuals varied by as much as 5-fold (Fig. 4). Phenotype data based on metabolic activities of these individuals was used to derive correlations between CYP isoforms content and carbofuran metabolic activity. CYP3A4 was the isoform with the best correlation

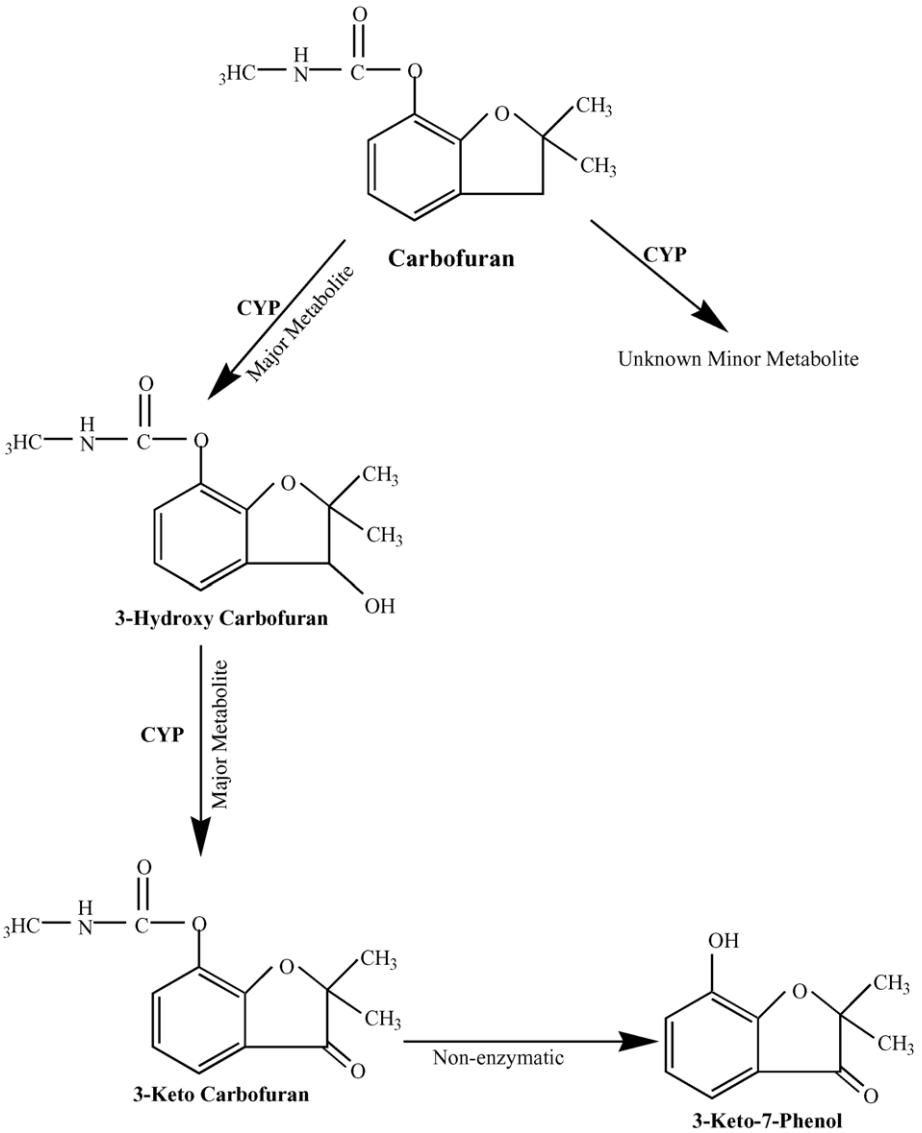

Fig. 1. CYP-dependent metabolism of carbofuran.

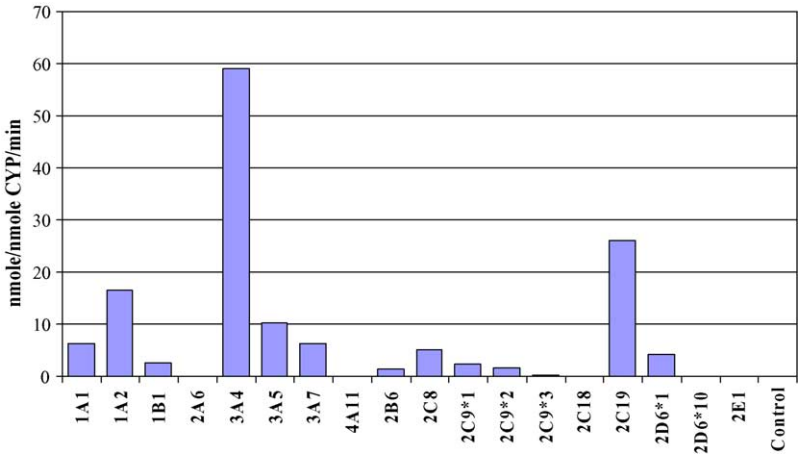

Fig. 2. Metabolism of carbofuran by human CYP isoforms (nmol product/nmol CYP/min). Columns represent the mean of two separate determinations.

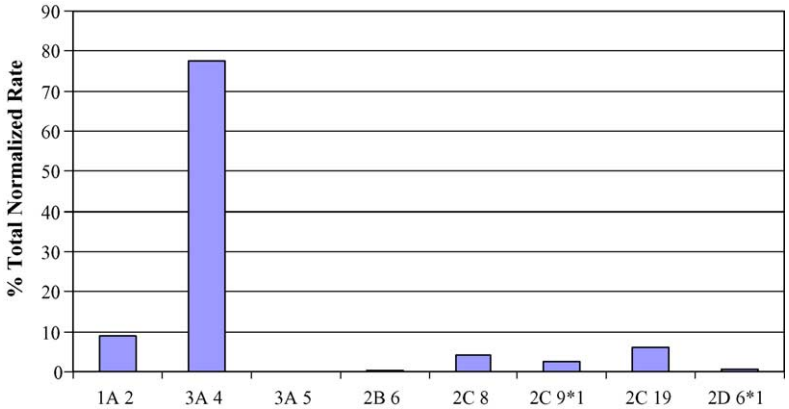

Fig. 3. Percent total normalized rates (% TNR) based on relative hepatic abundance of CYP-mediated metabolism of carbofuran.

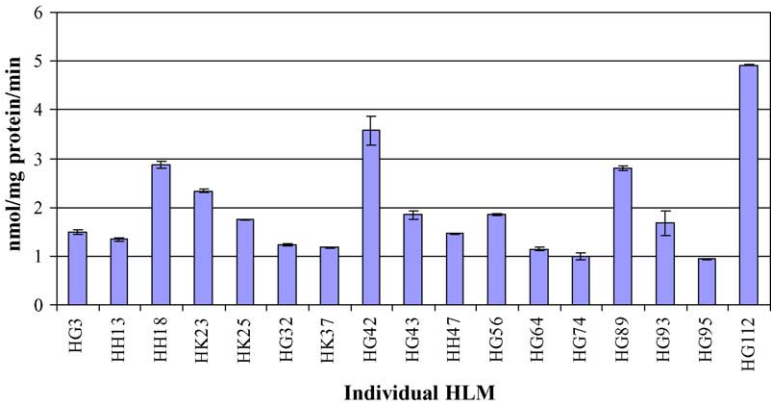

Fig. 4. Hydroxylation of carbofuran by single-donor human liver microsomes. Columns represent the mean of three separate determinations and error bars represent S.E.M.

($r^2 = 0.96$). All other correlations between carbofuran hydroxylation activity and specific CYP isoforms were less than 0.40.

3.4. Inhibition of carbofuran metabolism in HLM by ketoconazole and chlorpyrifos

The role of CYP3A4 in the metabolism of carbofuran was verified by co-incubations of ketoconazole, a specific CYP3A4 inhibitor, with carbofuran and HLM. Ketoconazole, a competitive inhibitor of CYP3A4, inhibited carbofuran hydroxylation with an IC_{50} value of 0.31 μM (Fig. 5). Chlorpyrifos, an irreversible non-competitive inhibitor of CYP3A4, inhibited carbofuran hydroxylation with an IC_{50} value of 39 μM (Fig. 5). However, neither ketoconazole nor chlorpyrifos inhibited carbofuran metabolism completely even at some of the highest concentrations (Fig. 5).

3.5. Metabolic interactions of carbofuran and testosterone or estradiol in HLM and CYP3A4

Co-incubations of various concentrations of testosterone with increasing concentrations of carbofuran in pooled HLM resulted in increased 6β-hydroxytestosterone production at all three testosterone substrate concentrations tested (Fig. 6). The greatest level of activation was observed at the lowest testosterone concentration (4 μM) in combination with a 1600 μM concentration of carbofuran (ca. 30-fold). Incubations of 100 μM testosterone were not as readily activated by increasing carbofuran concentrations. With CYP3A4 preparations, the activation of testosterone metabolism by carbofuran followed a similar pattern but lower levels of activation were observed as compared with HLM (Fig. 6). The greatest level of activation was observed at the lowest testosterone concentration (4 μM) in combination with a 1600 μM concentration of carbofuran (ca. 9.6-fold).

In contrast with the activation of testosterone metabolism by carbofuran, co-incubations of various concentrations of carbofuran with increasing concentrations of testosterone in pooled HLM and CYP3A4 did not result in increased metabolism of carbofuran to 3-hydroxycarbofuran except at the highest concentrations of testosterone (Fig. 7). Similarly, no activation or inhibition of varying concentrations of estradiol on increasing concentrations of carbofuran or varying concentrations of carbofuran on increasing concentrations of estradiol were observed (data not shown).

4. Discussion

The predominant metabolite of carbofuran in HLM, RLM, MLM, and human hepatocytes is 3-hydroxycarbofuran. The enzyme kinetic studies indicated that K_m values for carbofuran metabolism in HLM and human hepatocytes are ca. 9.5-fold and 3.6-fold lower than RLM and MLM, respectively. The mean metabolic intrinsic clearance rates, as estimated by V_{max}/K_m, indicate that RLM and MLM metabolize carbofuran ca. 15-fold more efficiently than HLM, indicating that, in general, humans are not as active as rodents in carbofuran metabolism. This study suggests that studies conducted in rodents for human risk assessment may be misleading due to species differences.

Human hepatocytes showed similar K_m but 5.5-fold lower V_{max} values than those shown in HLM. A possible explanation for the difference in V_{max} might involve the differences between transport mechanisms between microsomes and hepatocytes. Alternatively there may be additional biomolecules within the hepatocyte environment that may compete for metabolic cofactors. Incubations of carbofuran with human hepatocytes produced the same metabolites as those observed in HLM, no phase II enzyme activities were observed. Preliminary studies to examine possible differences in the mass balance for substrate and metabolites between microsome and hepatocyte preparations did not indicate substantial differences. However, future studies involving hepatocytes may include incubation times longer than 30 min to produce phase II metabolic products.

These studies provide strong evidence that CYP3A4 is the predominant isoform responsible for formation of 3-hydroxycarbofuran from carbofuran. Of the 15 isoforms and 3 polymorphic variants screened for activity, only CYP3A4, 1A2, and 2C19 significantly metabolized carbofuran. Intrinsic clearance values among these isoforms tended to rank similarly to affinity determinations. However, the in vivo clearance of carbofuran would be expected to be dependent upon the relative abundance of each CYP isoform. Thus, CYP1A2 and

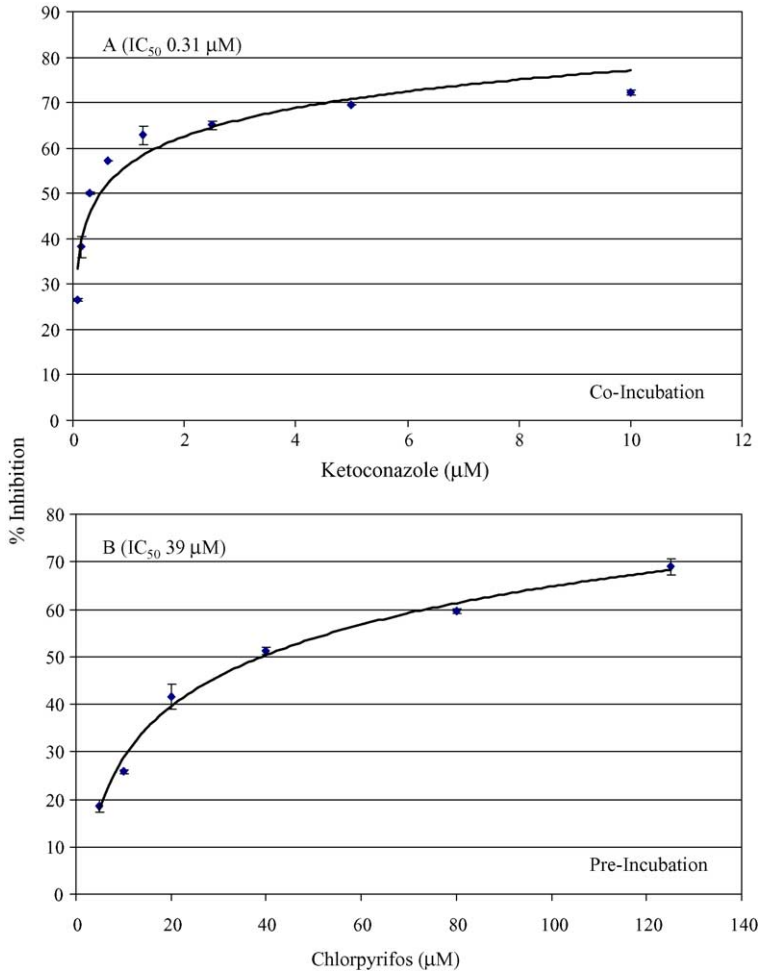

Fig. 5. Inhibition profile of HLM-dependent carbofuran metabolism by ketoconazole (A) and chlorpyrifos (B).

2C19, each of which the produce 3-hydroxycarbofuran, most likely have minor roles in carbofuran clearance in liver tissues because of their lower expression (% TNR = 9.0 and 6.0, respectively). In contrast, CYP3A4 with similar intrinsic clearance rates would contribute substantially to the metabolism of carbofuran (% TNR = 77.5) because of its high relative abundance in liver tissue. The value of this type of analysis in determining the contribution of various isoforms to overall metabolic activity was substantiated by the high correlation observed with CYP3A4 activity in the 17 single-donor HLM samples.

Further verification of the importance of CYP3A4 in the metabolism of carbofuran was obtained by demonstrating 50% inhibition of 3-hydroxycarbofuran production in HLM by 0.31 μM ketoconazole, a specific CYP3A4 inhibitor. Chlorpyrifos, an irreversible non-competitive inhibitor of CYP3A4 [8], also inhibited the metabolism of carbofuran to 3-hydroxycarbofuran in HLM with 50% inhibition occurring at a concentration of 39 μM. In both cases, carbofuran inhibition occurred rapidly at lower concentrations until 60% inhibition was achieved, after which it was difficult to achieve further inhibition. This is due to the fact that these chemicals inhibited CYP3A4 but had lesser effects on the other isoforms involved in carbofuran metabolism.

Human CYP3A4 is one of the most abundant drug-metabolizing CYP isoforms in human liver and, on

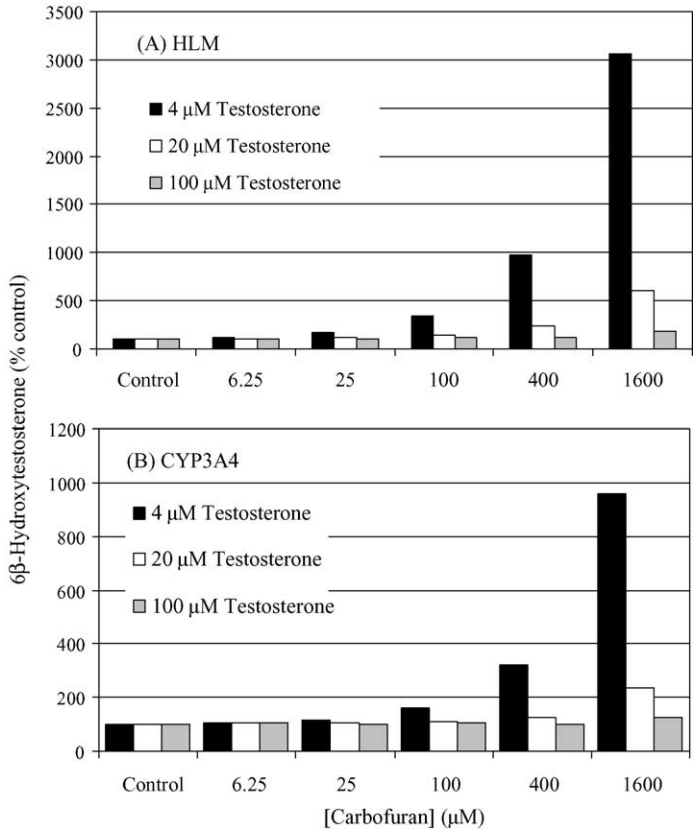

Fig. 6. The effects of carbofuran on 6β-hydroxytestosterone formation in HLM (A) and CYP3A4 (B). Data presented is based on the percent of 6β-hydroxytestosterone formation when compared to control with each data point representing the mean of two separate determinations.

average, accounts for approximately 40% of the total CYP in human liver [12]. In contrast to the human CYP3A subfamily, the rat CYP3A subfamily is not abundantly expressed in rat liver. In rats, the CYP2C subfamily appears to be the predominant CYP subfamily [13]. Although, many CYP3A substrates in humans are likely to be CYP3A substrates in rats, several human CYP3A substrates such as nifedipine, exhibit overlapping substrate specificity with CYP2C family members in the rat [14]. It has been emphasized that, for CYP3A substrates in humans, differences in clearance between humans and rats serve as a good predictors of CYP2C involvement in the rat [14]. While 3-hydroxycarbofuran formation is largely mediated by CYP3A4 in humans, it appears to be largely dependent on CYP2C in male rats. The involvement of CYP2C in 3-hydroxycarbofuran formation is likely to explain differences we found in this study between human and rat data. The data from this study demonstrated that caution is required in extrapolating rat metabolic data to humans for effective human risk assessment studies.

CYP3A4 is one of the most important and abundant isoforms in human liver and has broad substrate specificity. CYP3A4 not only metabolizes xenobiotics but is also responsible for the metabolism of endogenous compounds, such as steroid hormones including testosterone and estradiol [9,15]. Because carbofuran is predominantly metabolized by CYP3A4, it could potentially interact metabolically with endogenous CYP3A4 substrates, such as testosterone and estradiol. The usual interaction between two different substrates for the same enzyme is competitive inhibition. But because the K_m values for carbofuran and testosterone are so widely different

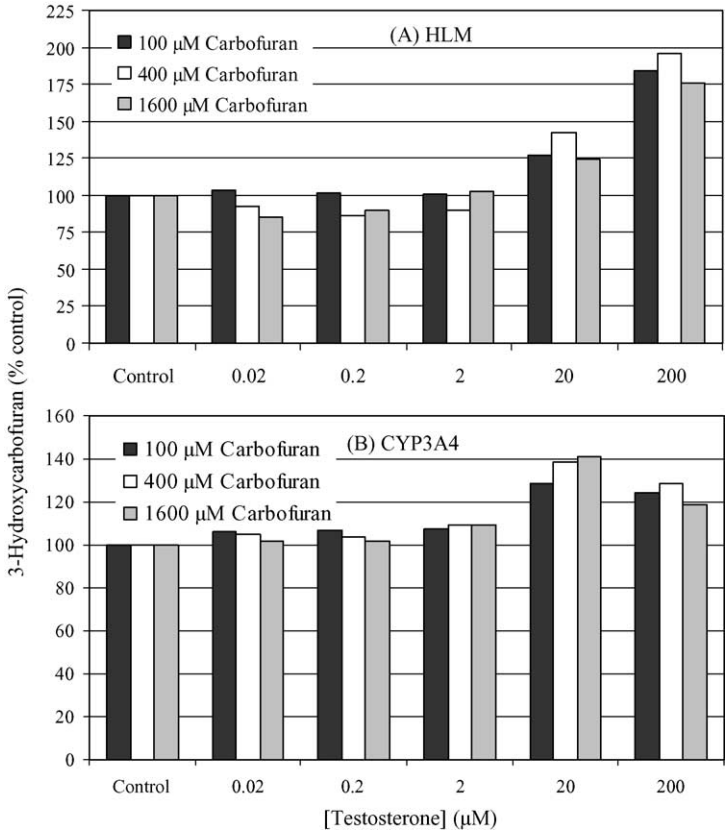

Fig. 7. The effects of testosterone on 3-hydroxycarbofuran formation in HLM (A) and CYP3A4 (B). Data presented is based on the percent of 3-hydroxycarbofuran formation when compared to control with each data point representing the mean of two separate determinations.

(K_m = 742 and 108 μM for carbofuran and testosterone, respectively) interactions between these substrates may be greatly complicated due to the allosteric characteristics of CYP3A4 [9,16,17]. In addition, little is known about the active site topology of CYP3A4, although it is generally recognized that the active site of this enzyme has the capacity to accommodate large molecules and even more than one substrate [18]. Multi-site kinetic models for the interaction of testosterone and xenobiotics in CYP3A4 have demonstrated that xenobiotics in most cases inhibited testosterone metabolism or vice versa [18–23]. However, it is also known that α-naphthoflavone heterotropically stimulates the metabolism of progesterone and testosterone [24,25]. The interaction we observed between carbofuran and testosterone indicates that increasing concentrations of carbofuran activated testosterone metabolism at low testosterone concentrations by as much as 30-fold in HLM and 9.6-fold in CYP3A4. In contrast, high concentrations of testosterone in HLM and CYP3A4 resulted in less than a 2-fold activation of carbofuran metabolism.

In summary, the present investigation demonstrated that human liver microsomes appear to have lower rates of carbofuran metabolism than those obtained from rodent livers. A screen of human CYP isoforms demonstrated that CYP3A4 is the major enzyme responsible for carbofuran metabolism. Individuals with varying levels of CYP3A4, regardless of gender, are more or less active in their metabolism of carbofuran. Interactions between carbofuran and testosterone display activation of testosterone metabolism by carbofuran.

Acknowledgments

This project was supported in part by NIOSH grant OH07551-ECU.

References

[1] T.R. Fukuto, Mechanism of action of organophosphorus and carbamate insecticides, Environ. Health Perspect. 87 (1990) 245–254.
[2] M.P. Donovan, L.G. Schein, J.A. Thomas, Effects of pesticides on metabolism of steroid hormone by rodent liver microsomes, J. Environ. Pathol. Toxicol. 2 (1978) 447–454.
[3] J.S. Cranmer, D.L. Avery, R.R. Grady, J.I. Kitay, Postnatal endocrine dysfunction resulting from prenatal exposure to carbofuran, diazinon, or chlordane, J. Environ. Pathol. Toxicol. 2 (1978) 357–369.
[4] T. Suzuki, Y. Nakagawa, K. Tayama, K. Yaguchi, T. Suga, Toxicity and effects of 2,6-di-tert-butyl-4-methylphenyl N-methylcarbamate (terbutol) on hepatic cytochrome P450 in F344 rats, Arch. Toxicol. 75 (2001) 555–561.
[5] T. Zheng, S.H. Zahm, K.P. Cantor, D.D. Weisenburger, Y. Zhang, A. Blair, Agricultural exposure to carbamate pesticides and risk of non-hodgkin lymphoma, Occup. Environ. Med. 43 (2001) 641–649.
[6] R.C. Gupta, Carbofuran toxicity, J. Toxicol. Environ. Health 43 (1994) 383–418.
[7] J.C. Cook, E. Hodgson, Induction of cytochrome P-450 by methylenedioxyphenyl compounds: importance of the methylene carbon, Toxicol. Appl. Pharmacol. 68 (1983) 131–139.
[8] Immunoquantitation of cytochrome P450, Catalog, GenTest Corporation, Woburn, MA, 1999–2000, p. 85.
[9] K.A. Usmani, R.L. Rose, E. Hodgson, Inhibition and activation of the human liver microsomal and human cytochrome P4503A4 metabolism of testosterone by deployment-related chemicals, Drug Metab. Dispos. 31 (2003) 384–391.
[10] L.A. Suchar, R.L. Chang, R.T. Rosen, J. Lech, A.H. Conney, High-performance liquid chromatography separation of hydroxylated estradiol metabolites: formation of estradiol metabolites by liver microsomes from male and females rats, J. Pharmacol. Exp. Ther. 272 (1995) 197–206.
[11] A.D. Rodrigues, Integrated cytochrome P450 reaction phenotyping, attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes, Biochem. Pharmacol. 57 (1999) 465–480.
[12] J.M. Lehmann, D.D. McKee, M.A. Watson, T.M. Willson, J.T. Moore, S.A. Kliewer, The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions, J. Clin. Invest. 102 (1998) 1016–1023.
[13] K.C. Cheng, J.B. Schenkman, Purification and characterization of two constitutive forms of rat liver microsomal cytochrome P-450, J. Biol. Chem. 257 (1982) 2378–2385.
[14] D.A. Smith, Species differences in metabolism and pharmacokinetics: are we close to an understanding? Drug Metab. Rev. 23 (1991) 355–373.
[15] A.J. Lee, M.X. Cai, P.E. Thomas, A.H. Conney, B.T. Zhu, Characterization of the oxidative metabolites of 17β-estradiol and estrone formed by 15 selectively expressed human cytochrome P450 isoforms, Endocrinology 144 (2003) 3382–3398.
[16] T. Shimada, F.P. Guengerich, Evidence for cytochrome P450NF, the nifedipine oxidase, being the principle enzyme involved in the bioactivation of aflatoxins in human liver, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 462–465.
[17] C.A. Lee, S.H. Kadwell, T.A. Kost, C.J. Serabjit-Singh, CYP3A4 expressed by insect cells infected with a recombinant baculovirus containing both CYP3A4 and human NADPH-cytochrome P450 reductase is catalytically similar to human liver microsomal CYP3A4, Arch. Biochem. Biophys. 319 (1995) 157–167.
[18] M. Shou, J. Grogan, J.A. Mancewicz, K.W. Krauaz, F.J. Gonzalez, H.V. Gelboin, K.R. Korzekwa, Activation of CYP3A4: evidence for the simultaneous binding of two substrates in a cytochrome P450 active site, Biochemistry 33 (1994) 6450–6455.
[19] H. Nakamura, H. Nakasa, I. Ishii, N. Ariyoshi, T. Igarashi, S. Ohmori, M. Kitada, Effects of endogenous steroids on CYP3A4-mediated drug metabolism by human liver microsomes, Drug Metab. Dispos. 30 (2002) 534–540.
[20] K.E. Kenworthy, S.E. Clarke, J. Andrews, J.B. Houston, Multisite kinetic models for CYP3A4: simultaneous activation and inhibition of diazepam and testosterone metabolism, Drug Metab. Dispos. 29 (2001) 1644–1651.
[21] P. Lu, Y. Lin, A.D. Rodrigues, T.H. Rushmore, T.A. Baillie, M. Shou, Testosterone, 7-benzyloxyquinoline, and 7-benzyloxy-4-trifluoromethyl-comurin bind to different domains within the active site of cytochrome P4503A4, Drug Metab. Dispos. 29 (2001) 1473–1479.
[22] W.R. Wang, D.J. Newton, N. Liu, W.M. Atkins, A.Y.H. Lu, Human cytochrome P-4503A4: in vitro drug–drug interaction patterns are substrate-dependent, Drug Metab. Dispos. 28 (2000) 360–366.
[23] J. Tang, K.A. Usmani, E. Hodgson, R.L. Rose, In vitro metabolism of fipronil by human and rat cytochrome P450 and its interactions with testosterone and diazepam, Chem.-Biol. Interact. 147 (2004) 319–329.
[24] G.E. Schwab, J.L. Raucy, E.F. Johnson, Modulation of rabbit and human hepatic cytochrome P450 catalyzed steroid hydroxylation by α-naphthoflavone, Mol. Pharmacol. 33 (1988) 493–499.
[25] G.R. Harlow, J.R. Halpert, Analysis of human cytochrome P4503A4 cooperativity: construction and characterization of a site-directed mutant that displays hyperbolic steroid hydroxylation kinetics, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 6636–6641.