

STUDIES COMPARING IN VIVO:IN VITRO METABOLISM OF THREE PHARMACEUTICAL COMPOUNDS IN RAT, DOG, MONKEY, AND HUMAN USING CRYOPRESERVED HEPATOCYTES, MICROSOMES, AND COLLAGEN GEL IMMOBILIZED HEPATOCYTE CULTURES

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ABSTRACT:

The *in vivo* metabolism of three pharmaceutical compounds, EMD68843, EMD96785, and EMD128130, was compared in fresh and cryopreserved hepatocyte (CPH) suspensions and microsomes from rat, dog, monkey, and human livers and fresh human and rat hepatocyte collagen gel immobilized cultures (GICs). Half of the major *in vivo* metabolites was produced by phase 1 (hydroxylation, oxidation, hydrolysis, *N*-dealkylation) and half by phase 2 metabolism (mostly glucuronidation but also sulfation and glycine conjugation). The identity and percentage of phase 1 and 2 metabolites from each compound produced in hepatocytes compared well with that in each species *in vivo*. Glucuronidation was more extensive in GICs than in CPHs. In contrast, CPHs but not GICs, produced sulfate metabolites. Microsomes (supplemented with NADPH only) produced most of

the phase 1 but no phase 2 metabolites. Metabolism in CPHs was the same as in fresh hepatocyte suspensions. Discrete species differences in metabolism were detected by CPHs and microsomes. Cytochrome P450 and glucuronosyl *S*-transferase contents of CPHs did not account for species differences in the percentage of phase 1 and 2 metabolites or the rate of disappearance of the parent compounds in these cells. These data show a good correlation between major metabolites formed *in vivo* and *in vitro*. CPHs and GICs, unlike microsomes, carried out sequential phase 1 and 2 metabolism. Each *in vitro* system has its own advantages, however, for short-term metabolism studies CPHs may be more useful since they are readily available, easier and quicker to prepare than GICs, and have more comprehensive enzyme systems than microsomes.

Hepatocytes and liver microsomes are routinely used for the prediction of *in vivo* metabolism of drug candidates with the obvious advantage that they are easier to work with than whole animals (Wrighton et al., 1995). These *in vitro* systems should, therefore, reflect *in vivo* metabolism, detect species differences, and predict compound clearance. Few have demonstrated species differences *in vitro* due to the lack of an appropriate compound whose metabolism is unique to one specific species *in vivo*. Notable exceptions include Loxitidine (a potent histamine antagonist), the species-dependent metabolism of which was reflected in fresh rat, dog, and human hepatocyte suspensions (Bayliss et al., 1999) and ketotifen, whose species-dependent metabolism *in vivo* was reproduced in fresh rat, rabbit, and human hepatocytes (Le Bigot et al., 1987). There are other good *in vitro* and *in vivo* correlations using hepatocytes for prediction of metabolism (Oldham et al., 1990; Lacarelle et al., 1991; Lavrijsen et al., 1992; Sandker et al., 1994; Pahernik et al., 1995) and intrinsic

clearance of drugs (Houston, 1994; Carlile et al., 1998; Bayliss et al., 1999). Likewise, microsomes have been used to predict metabolism, clearance, and drug-drug interactions (von Moltke et al., 1994; Worboys et al., 1996; Carlile et al., 1999).

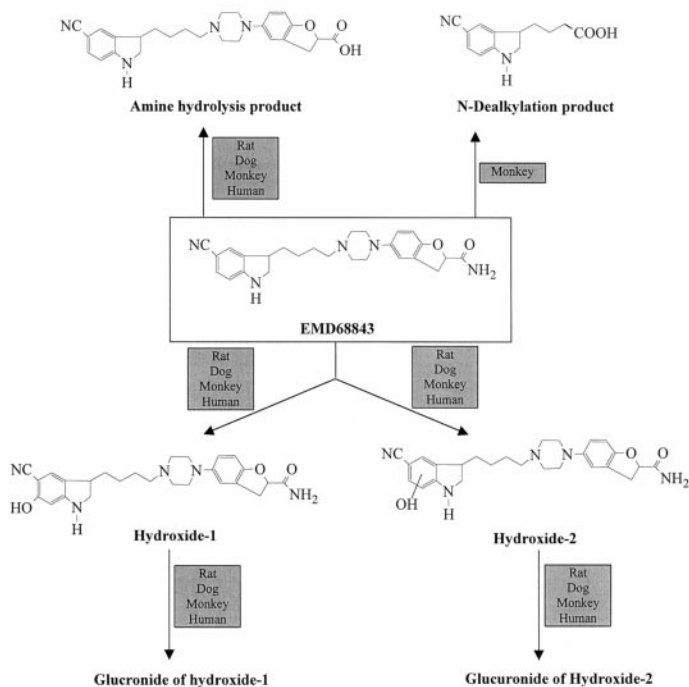
Our current studies are based on the metabolism of three putative pharmaceutical compounds, namely, EMD68843, EMD96785, and EMD128130. EMD68843 is an antidepressant and EMD96785 and EMD128130 have been developed for the treatment of myocardial infarction and schizophrenia, respectively. These compounds were chosen because their *in vivo* metabolism in animals and humans has been extensively studied and established at Merck KGaA (Fig. 1). The phase 1 enzymes involved in the metabolism of all three compounds are cytochrome P450 (CYP¹) 3A4, CYP2D6, CYP2C9, and CYP2C19. Phase 2 metabolism is important in the excretion of all three compounds, especially glucuronidation. Other phase 2 pathways involved are sulfation, and glycine conjugation, important in EMD128130 metabolism. Our aims were to compare the *in vivo* metabolism of these three compounds with that in two hepatocyte test systems, cryopreserved hepatocyte suspensions (CPHs) and fresh hepatocyte gel immobilized cultures (GICs), and in liver microsomes.

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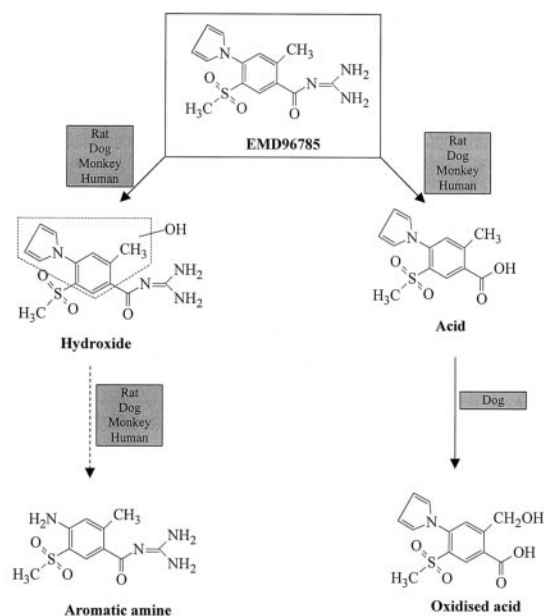
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¹ Abbreviations used are: CYP, cytochrome P450; CPH, cryopreserved hepatocyte; GIC, gel immobilized culture; UGT, UDP-glucuronosyltransferase; CP, cryopreserved; HPLC, high-performance liquid chromatography.

(A) EMD68843



(B) EMD96785



(C) EMD128130

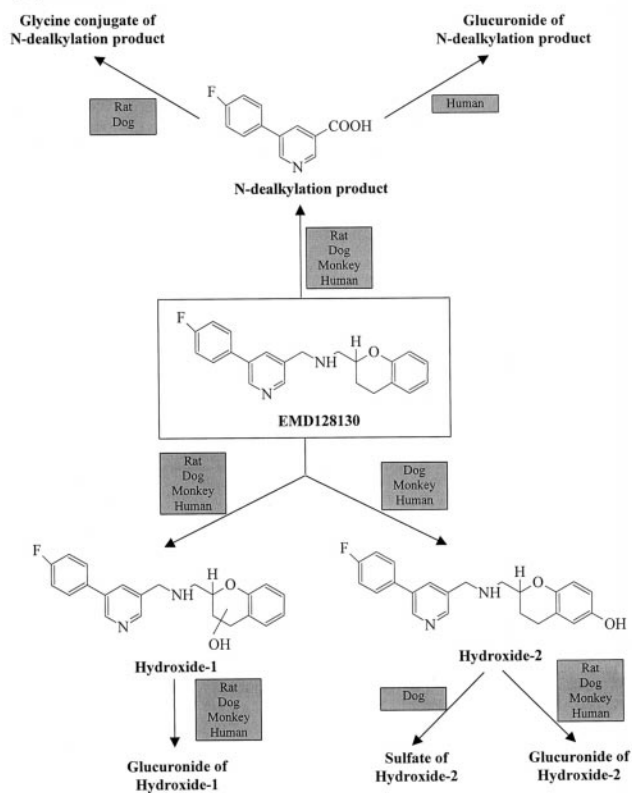


FIG. 1. Structures and the major *in vivo* metabolic pathways of EMD68843 (A), EMD96785 (B), and EMD128130 (C) in the rat, dog, monkey, and human.

Although microsomes are very effective in producing phase 1 metabolites, hepatocytes are more relevant because they not only reflect the phase 1 and 2 metabolism of a compound but also its uptake and excretion across the plasma membrane (Li et al., 1999a,b). Here, we have used both fresh GICs and CPHs to determine metab-

olism. The fresh rat and human GICs used in these studies have been established for a number of years (Koebe et al., 1994), such that the three-dimensional collagen matrix supports metabolic functions for up to 3 weeks. Human GICs have already been used to study the metabolism of a pharmaceutical compound, namely, Pimobendan, a

cardiovascular drug (Pahernik et al., 1995). The rate of metabolism of this compound was stable for 3 weeks, although the amount of its *N*-glucuronide was lower than in vivo. The main disadvantage of fresh human hepatocytes is the sporadic supply of liver donors, although this is partly solved by GIC long-term cultures. Therefore, we have also used CPHs in our metabolism studies. This hepatocyte test system allows for experiment planning and the investigation of compound metabolism in more than one species in 1 day. Previous data using diagnostic substrates have shown that P450 activities in rat, dog, monkey, and human hepatocyte suspensions are not significantly decreased by cryopreservation (Swales et al., 1996a,b; Swales and Utesch, 1998; Steinberg et al., 1999; Hewitt et al., 2000). Phase 2 metabolism was more unstable than phase 1, however, CPHs from all species retained appreciable UDP-glucuronosyltransferase (UGT), sulfotransferase, and glutathione *S*-transferase activities. To confirm that metabolism of the three compounds was not affected by cryopreservation, the metabolism of each compound was determined in fresh rat and dog hepatocytes (EMD96785 was also incubated in fresh monkey hepatocytes).

Materials and Methods

Chemicals. Collagenase A (0.18 U/mg of protein), glucose 6-phosphate, glucose 6-phosphate dehydrogenase, dihydronicotinamide adenine dinucleotide phosphate, oxidized form (NADP) were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Percoll was purchased from Biochrom (Berlin, Germany). Dulbecco's modified Eagle's medium, Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} ($\times 10$), and uridine 5'-diphosphoglucuronic acid were from Life Technologies (Paisley, Scotland or Eggenstein, Germany). Dihydronicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and all other chemicals were from Merck KGaA (Darmstadt, Germany). Test compounds and standards of the metabolites were from Merck KGaA.

Microsome Preparation. Dog and monkey microsomes were purchased from In Vitro Technologies, Baltimore, MD. Human microsomes were purchased from the International Institute for Advancement of Medicine, Exton, PA. Rat microsomes were prepared at the Institute of Pharmacokinetics and Metabolism, Merck KGaA, Grafing, Germany. The livers were excised, washed in isotonic saline, and then blotted to dryness. All subsequent steps were carried out at 4°C. The liver was minced with scissors and then diluted 1:4 (w/v) with 0.02 M Tris buffer, pH 7.4, containing 0.25 M sucrose and 5.5 mM EDTA. The pulp was then homogenized with a Potter (Braun, Melsungen) homogenizer. The homogenate was centrifuged at 13,500g for 10 min, after which, the pellet was discarded. The supernatant was centrifuged at 105,000g for 1 h and the pellet resuspended in 0.05 M Tris buffer, pH 7.4. After a further centrifugation at 105,000g for 1 h, the pellets were resuspended in 0.05 M Tris buffer, pH 7.4, to a density of 0.5 mg of wet weight/ml of buffer. The microsomes were stirred for 45 to 60 min at 4°C prior to storage at -80°C.

Cell Isolation. Hepatocytes were isolated from liver sections from male cynomolgus monkeys (*Macaca fascicularis*, 3–7 kg) and beagle dogs (8–10 months, control animals from separate toxicity studies) and whole livers from Sprague-Dawley rats by a perfusion technique previously described (Swales and Utesch, 1998). Human hepatocytes were isolated and cryopreserved as described by Steinberg et al. (1999) and frozen hepatocytes were transported on dry ice within 24 h and stored in liquid nitrogen until required. The viabilities of rat, dog, and monkey fresh hepatocytes for test compound metabolism were 90 ± 4 , 96 ± 1 , and $95 \pm 2\%$, respectively.

Cryopreservation and Thawing of Cells. Hepatocytes were frozen as described by Swales and Utesch (1998) and stored for up to 2 years in liquid nitrogen. Cells were thawed in a stepwise manner and washed in suspension buffer (an amino acid-containing Krebs-Henseleit buffer, pH 7.6; Utesch et al., 1992). The cells were subjected to Percoll purification by centrifuging a mixture of cell suspension (32 ml) and Percoll solution (18 ml for rat, dog, and monkey, 16 ml for human hepatocytes [1 part Hanks' balanced salt solution ($\times 10$):9 parts Percoll]) at 150g for 20 min (4°C). After a further wash in suspension buffer, the viability and cell density were measured by trypan blue exclusion. The viabilities of thawed and Percoll-purified rat, dog, monkey, and

human hepatocytes were $86 \pm 4\%$ ($n = 8$), $89 \pm 6\%$ ($n = 8$), $89 \pm 7\%$ ($n = 9$), and $88 \pm 10\%$ ($n = 7$), respectively.

Incubation of Test Compounds with Hepatocyte Suspensions. Rat, dog and monkey hepatocytes (fresh and CP) were diluted in suspension buffer to 10^6 total (viable and nonviable) cells/ml and human hepatocytes were diluted to 0.3 to 0.5×10^6 total cells/ml. The cell suspensions were incubated for up to 4 h with the test compounds [dissolved in water (EMD96785) or dimethyl sulfoxide (EMD128130 and EMD68843)] at the highest nontoxic concentrations: 120 μM (EMD96785) and 20 μM (EMD68843 and EMD128130). At appropriate times, an aliquot (1 ml in duplicate) of cell suspension was removed and mixed with 4 ml of acetonitrile (EMD68843 and EMD128130) or methanol (EMD96785). The protein was removed by centrifugation at 2000g for 10 min, after which, the supernatant was dried under a stream of nitrogen gas and reconstituted in 100 μl of the appropriate HPLC mobile phase.

Incubation of Test Compounds with Microsomes. Microsomes (0.5–1 mg/ml) from each species were diluted into phosphate buffer, pH 7.4, containing 1.25 mM MgCl_2 , 2.5 mM glucose 6-phosphate, 0.7 units/ml glucose 6-phosphate dehydrogenase, 2.5 mM nicotinamide, and 125 μM NADP. The reaction was started by the addition of test compound (6 μM EMD68843, 100 μM EMD96785, and 1–700 μM EMD128130) and terminated at times up to 1 h (for EMD68843 and EMD128130) or 2 h (for EMD96785) by addition of 450 μl of the reaction mixture to 2 ml of ice-cold ethanol. The ethanol was evaporated under a stream of nitrogen gas and the dried sample reconstituted in 150 μl of HPLC mobile phase.

Incubation of Test Compounds with Gel Immobilized Cultures of Fresh Rat and Human Hepatocytes. Rat and human hepatocytes were isolated and cultured by, and according to, Koebe et al. (1994). The viability of human and rat hepatocytes was 93 ± 2 and $90 \pm 3\%$, respectively. Dog and monkey hepatocytes were not used in this culture system. The cells were cultured by gel immobilization in Dulbecco's modified Eagle's medium, supplemented with insulin (125 mU/ml), hydrocortisone (60 ng/ml), penicillin (100U/ml), gentamycin (100 $\mu\text{g}/\text{ml}$), and fetal calf serum (5%, v/v). The hepatocytes were cultured for 3 days before the addition of test compounds (2–20 μM EMD68843, 20 μM EMD96785, and 20 μM EMD128130). Metabolism was terminated at 0, 2, 4, 8, and 24 h of incubation, the medium was separated from the cells and each were analyzed for metabolites separately.

In Vivo Metabolite Profiling. The metabolism and excretion of [^{14}C]EMD68843, [^{14}C]EMD96785, and [^3H]EMD128130 were studied in Wistar rats, beagle dogs, and cynomolgus monkeys. Single i.v. doses were 1, 1, and 0.5 mg/kg for [^{14}C]EMD68843, [^{14}C]EMD96785, and [^3H]EMD128130, respectively. Single p.o. doses were 1, 1, and 2.5 mg/kg for [^{14}C]EMD68843, [^{14}C]EMD96785, and [^3H]EMD128130, respectively. Concentrations of radioactivity were measured by liquid scintillation counting. Metabolites were determined in urine, feces, bile, and plasma up to 48 h after treatment (0–4, 0–8, 0–24, and 0–48 h). For human metabolism profiles, 80 mg of unlabeled EMD68843, 25 mg of [^{14}C]EMD96785, and 10 mg of [^{14}C]EMD128130 (50 μCi) were given to healthy male volunteers. Metabolites were determined in urine, feces, and plasma up to 144 h after application.

Metabolite Analysis. HPLC analysis of metabolites from all compounds was carried out at Merck KGaA, Darmstadt, or Institute of Pharmacokinetics and Metabolism, Merck, Grafing, according to the methods developed and validated by Merck. Authentic standards were obtained as follows: EMD68843 metabolite standards = EMD68843 hydrolysis product (mass 442), EMD68843-*N*-dealkylation product (mass 228), EMD68843 hydrolysis product (mass 442); EMD96785 metabolite standards = EMD96785-amine (mass 270), EMD96785-acid (mass 279), EMD96785-acid-oxide (mass 295); EMD128130 metabolite standard = EMD128130-hydroxide (mass 364), glycine conjugate of EMD128130 *N*-dealkylation product (mass 274). Glucuronides were identified by glucuronidase hydrolysis and subsequent appearance of phase 1 metabolites. The metabolites formed from each EMD compound in all hepatocytes and microsomes were elucidated and their structures identified by liquid chromatography-mass spectrometry/mass spectrometry.

Statistics. Results were compared using the Student's unpaired *t* test or the Dunnett's *t* test. A value of $P < 0.05$ was considered significant. *n* refers to the number of different hepatocyte preparations from different animals.

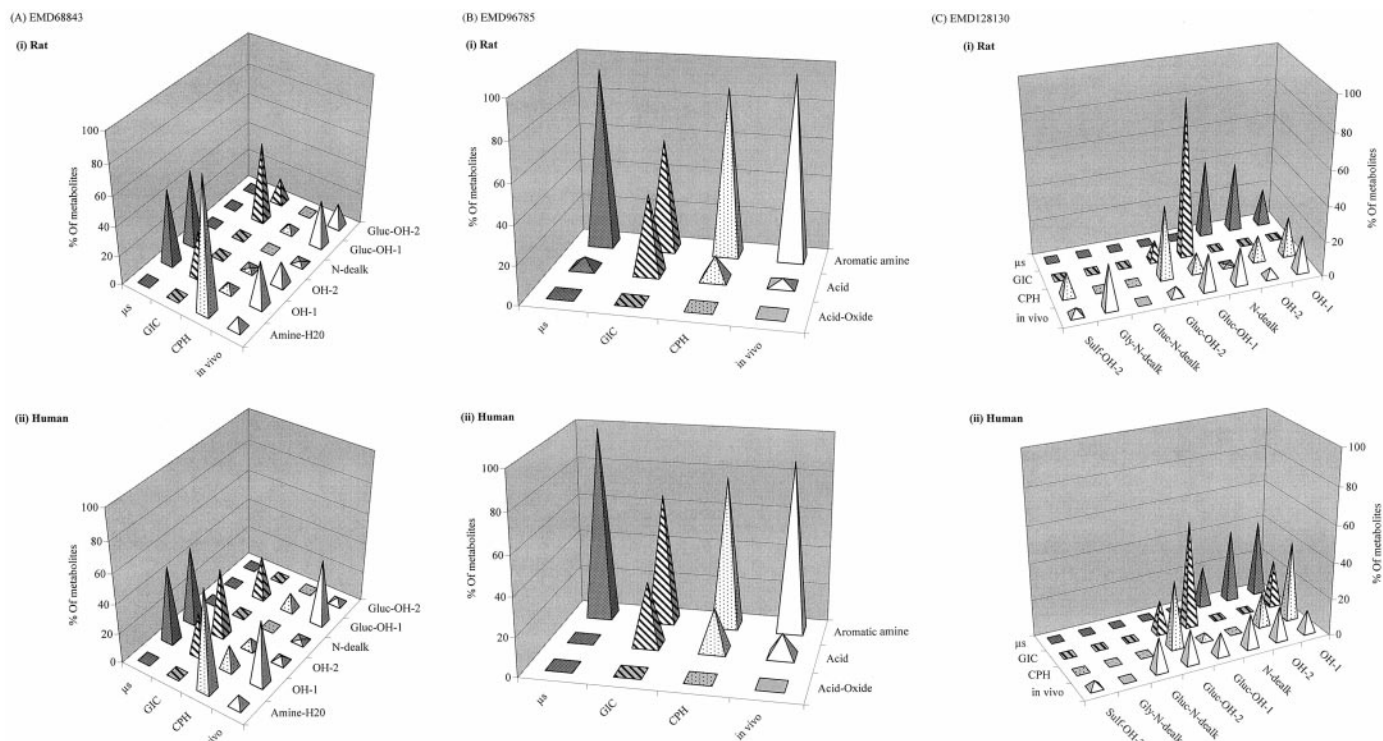


FIG. 2. Comparison of the percentage of metabolites formed in the rat and human in vivo.

Comparison of the percentage of metabolites formed in the rat and human in vivo (□) and in CPHs (▨), GICs (▩), and microsomes (▧), ($n > 3$, mean, error bars are not shown). μ s, microsome; Amine-H2O, amine hydrolysis product; OH-1, hydroxide-1; OH-2, hydroxide-2; N-dealk, *N*-dealkylation product; Gluc-OH-1, glucuronide of hydroxide-1; Gluc-OH-2, glucuronide of hydroxide-2; Sulf-OH-2, sulfate of hydroxide-2; Gly-N-dealk, glycine conjugate of *N*-dealkylation product; Gluc-N-dealk, glucuronide of *N*-dealkylation product.

Results

Metabolism of EMD68843. Figure 1A shows the major in vivo metabolic pathways involved in the metabolism of EMD68843. The majority of this compound was hydroxylated and subsequently glucuronidated in each species. Figure 2A and Table 1A show a comparison between in vivo and in vitro metabolism of EMD68843 with respect to the percentage of each metabolite in each species (dog and monkey graphs are not shown, for which no GIC data were available). Hydroxide-1 is a major in vivo metabolite in all four species (30–43% of the metabolites), and was always produced by CPHs, GICs, and microsomes [4–16% (CPHs), 28–31% (GICs), and 42–50% (microsomes) of the metabolites in all species tested]. Hydroxide-2 was less predominant and accounted for 15, 8, 3, and 4% of the metabolites in vivo in the rat, dog, monkey, and human, respectively. This metabolite comprised 1, 11, 4, and 6% of the metabolites in rat, dog, monkey, and human CPHs, respectively. This metabolite was not detected in rat GICs but accounted for 44% of the metabolites in human GICs. Hydroxide-2 represented 50% of the metabolites in rat, dog, and human microsomes and 42% of the metabolites in monkey microsomes. In vivo, both hydroxides appear partly as glucuronides, which are excreted in the bile (25–38% of the metabolites). Rat GICs produced both glucuronides, which were 53 and 16% of the metabolites. Rat, dog, and monkey CPHs produced only the glucuronide formed from the major hydroxide-1 (5–11% of the metabolites). The second glucuronide, excreted in humans in vivo (15% of the metabolites), was not produced by either human CPHs or human GICs. Microsomes, which were not supplemented with uridine 5'-diphosphoglucuronic acid, produced no glucuronides. EMD68843 is also *N*-dealkylated in vivo, which was a major metabolite in monkeys only (22% of the metabolites). This metabolite was produced only in

monkey microsomes and not rat, dog, or human microsomes. Monkey CPHs did not produce this metabolite. Of less importance was the amine hydrolysis product, which accounted for approximately 8% of the metabolites in vivo. This metabolite was also produced in CPHs but in much higher amounts (90, 71, 80, and 68% of the metabolites produced in rat, dog, monkey, and human CPHs, respectively). In contrast, neither GICs nor microsomes produced this metabolite.

Metabolism of EMD96785. Figure 1B shows the major in vivo metabolic pathways involved in the metabolism of EMD96785. Figure 2B and Table 1B show a comparison between the in vivo and in vitro metabolism of this compound in each species. The percentage of the three major metabolites produced in vivo was mirrored by that seen in all three in vitro systems. By far the major metabolite of EMD96785 in all species was the aromatic amine which, in vivo, accounted for 30 to 60% of the dose (77–92% of the total metabolites) in all four species. This was also the major metabolite in CPHs (67–97% of the metabolites in all four species), microsomes (57–100% of the metabolites in all four species), and GICs (59 and 67% of the metabolites in rat and human GICs, respectively). The acid metabolite, formed from aromatic methyl oxidation of the parent compound, comprised 8 to 12% of the in vivo metabolites produced in all four species. Both hepatocyte systems produced this metabolite (3–26% of the metabolites in CPHs from all four species and 41 and 33% of the metabolites in rat and human GICs, respectively). This metabolite was detected in rat (6% of the metabolites) and dog (43% of the metabolites) microsomes but not in monkey or human microsomes. The acid-oxide was an in vivo metabolite unique to the dog (15% of the metabolites). Dog CPHs produced this metabolite (7% of the metabolites), whereas dog microsomes did not.

TABLE 1

Metabolism profiles of EMD68842, EMD96785, and EMD128130 in the dog and monkey in vivo, in CPHs, and in microsomes

Values are expressed as the percentage of the total metabolites formed (mean, $n = 3$ for each system and species; S.D. values shown only for CPHs, all of which were measured after a 4-h incubation).

	Metabolite	In Vivo	CPHs	Microsomes			
EMD68843	Dog	Amine-H ₂ O	8 ^a	71 ± 9	0 ^b		
		OH-1	38 ^a	4 ± 2	50 ^b		
		OH-2	8 ^a	11 ± 6	50 ^b		
	Monkey	<i>N</i> -dealk	1 ^a	7 ± 0	0 ^b		
		Gluc-OH-1	38 ^a	7 ± 6	0 ^b		
		Gluc-OH-2	8 ^a	0 ± 0	0 ^b		
		Amine-H ₂ O	6 ^a	80 ± 7	0 ^b		
		OH-1	33 ^a	9 ± 3	42 ^b		
		OH-2	3 ^a	4 ± 6	42 ^b		
		<i>N</i> -dealk	22 ^a	0 ± 0	17 ^b		
EMD96785	Dog	Gluc-OH-1	33 ^a	7 ± 4	0 ^b		
		Gluc-OH-2	3 ^a	0 ± 0	0 ^b		
		Aromatic amine	77 ^c	67 ± 17	57 ^b		
	Monkey	Acid	8 ^c	26 ± 16	43 ^b		
		Acid oxide	15 ^c	7 ± 7	0 ^b		
		Aromatic amine	88 ^c	97 ± 11	100 ^b		
		Acid	12 ^c	3 ± 1	0 ^b		
		Acid oxide	0 ^c	0	0 ^b		
		EMD128130	Dog	OH-1	18 ^d	39 ± 5	45 ^e
				OH-2	12 ^d	12 ± 7	23 ^e
<i>N</i> -dealk	36 ^d			0.1 ± 0.1	32 ^e		
Gluc-OH-1	18 ^d			12 ± 8	0 ^e		
Gluc-OH-2	12 ^d			28 ± 12	0 ^e		
Gluc- <i>N</i> -dealk	0 ^d			0 ± 0	0 ^e		
Gly- <i>N</i> -dealk	0 ^d			0 ± 0	0 ^e		
Sulf-OH-1	5 ^d			9 ± 2	0 ^e		
Monkey	OH-1			12 ^d	20 ± 12	55 ^e	
	OH-2		18 ^d	9 ± 3	16 ^e		
	<i>N</i> -dealk		36 ^d	0 ± 0	29 ^e		
	Gluc-OH-1		12 ^d	17 ± 3	0 ^e		
	Gluc-OH-2		18 ^d	36 ± 4	0 ^e		
	Gluc- <i>N</i> -dealk		0 ^d	0	0 ^e		
	Gly- <i>N</i> -dealk		0 ^d	0	0 ^e		
	Sulf-OH-1	<5 ^d	19 ± 6	0 ^e			

^a After a 24-h incubation.

^b After a 2-h incubation.

^c After a 4-h incubation.

^d After a 120-h incubation.

^e After a 0.5-h incubation.

Metabolism of EMD128130. Figure 1C shows the major in vivo pathways and metabolites involved in the metabolism of EMD128130. There are three main in vivo phase 1 metabolites produced in all four species, namely, the *N*-dealkylation product and two hydroxides of the chromane ring. Figure 2C and Table 1C show the amount of metabolites formed in vivo and in vitro in each species. The *N*-dealkylation product accounted for approximately 20% of the total in vivo metabolites in the rat and human and 36% of the total in vivo metabolites in the dog and monkey. In rat, human, dog, and monkey microsomes, it accounted for 42, 18, 32, and 29% of the metabolites, respectively. In contrast, the *N*-dealkylation product accounted for less than 1% of the metabolites in rat and dog CPHs. Monkey and human CPHs and rat and human GICs did not *N*-dealkylate EMD128130. The *N*-dealkylation product is further conjugated in vivo with glycine in the rat and dog only and with glucuronic acid in humans only. Microsomes and hepatocytes from these species did not produce either conjugate. The two chromane ring hydroxides were universally excreted in vivo (between 25 and 30% of the metabolites in all four species). Likewise, CPHs and microsomes from all species produced these hydroxides. Together, these two hydroxides comprised 35, 51, 29, and 54% of the total metabolites in

rat, dog, monkey, and human CPHs, respectively. In rat, dog, monkey, and human microsomes both hydroxides accounted for 58, 68, 71, and 79% of the total metabolites, respectively. Neither hydroxide was detected in rat GICs and only one was detected in human GICs (19% of the total metabolites); these were mostly evident as their corresponding glucuronides. The glucuronides were major metabolites in vivo in all species (25–30% of the metabolites) and were also major metabolites in CPHs (52, 40, 53, and 35% of the metabolites in rat, dog, monkey, and human CPHs, respectively). These glucuronides were the only two metabolites formed in rat GICs and accounted for 75% of the metabolites in human GICs. One hydroxide was also sulfated in vivo but was considered a major metabolite in the dog only. This phase 2 metabolite was also produced by rat, dog, and monkey (but not human) CPHs. Neither rat nor human GICs produced the sulfate conjugate, although this was only a minor pathway in these species (less than 5% of the dose).

Metabolism in Fresh and Cryopreserved Hepatocytes. Figure 3A shows the amount of metabolites (as a percentage of the dose) produced in fresh and CP rat and dog hepatocyte suspensions incubated with EMD68843. The overall pattern of metabolites was not altered by cryopreservation, in that the amine hydrolysis product was still 10 to 16% of the dose, compared with the other metabolites, each of which constituted less than 0.5% (rat) and 3% (dog) of the dose. Notably, dog CPHs produced more phase 1 and phase 2 metabolites than fresh cells ($P < 0.05$) but this was only a difference of about 2% of the dose.

Figure 3B shows the amount of metabolites (as a percentage of the dose) produced in fresh and CP rat, dog, and monkey hepatocytes incubated with EMD96785. The amount of aromatic amine produced was significantly higher ($P < 0.05$) in rat and dog CPHs than in fresh cells. CP dog hepatocytes also produced more of the acid and its corresponding oxide than fresh hepatocytes. However, the amine was further metabolized to six other minor phase 1 and 2 metabolites (amalgamated and denoted in the figure as “others”; not shown in Fig. 1), which were decreased upon cryopreservation. In contrast, the amount of each metabolite was the same in fresh and CP monkey hepatocytes.

Figure 3C shows the amount of metabolites (as a percentage of the dose) produced in fresh and CP rat and dog hepatocytes incubated with EMD128130. The pattern of metabolism was the same in fresh and CP rat and dog hepatocytes. Both fresh and CP hepatocytes produced the main in vivo metabolite, the *N*-dealkylation product (always less than 0.2% of the dose, 1% of the total metabolites) but not its two glycine and glucuronide conjugates. The lack of the glucuronide conjugate is consistent with in vivo data. Notably, more of the two hydroxides (and in one case its glucuronide) was produced in CPHs compared with fresh hepatocytes, although the differences were marginal. The sulfate conjugate was produced by both fresh and CPHs. Again, there was a small but significantly higher ($P < 0.05$) percentage of this metabolite in CP dog hepatocytes than in fresh cells.

Rates of Compound Metabolism in Vitro. Table 2 shows the amount of parent compound metabolized by each in vitro system. The rate of EMD68843 and EMD128130 metabolism over 4 h was higher in GICs and microsomes than in fresh and CPH suspensions. However, equivalent amounts of EMD96785 were metabolized by GICs, fresh hepatocyte suspensions, and CPHs (6–20%). Dog, monkey, and human microsomes also consumed a low percentage of EMD96785 (16–27%), in comparison with the other two compounds. The rate of metabolism of all three compounds by CPHs was similar to that in fresh hepatocyte suspensions. Hepatocytes and microsomes (taken as an average of all four species) showed that EMD128130 was gener-

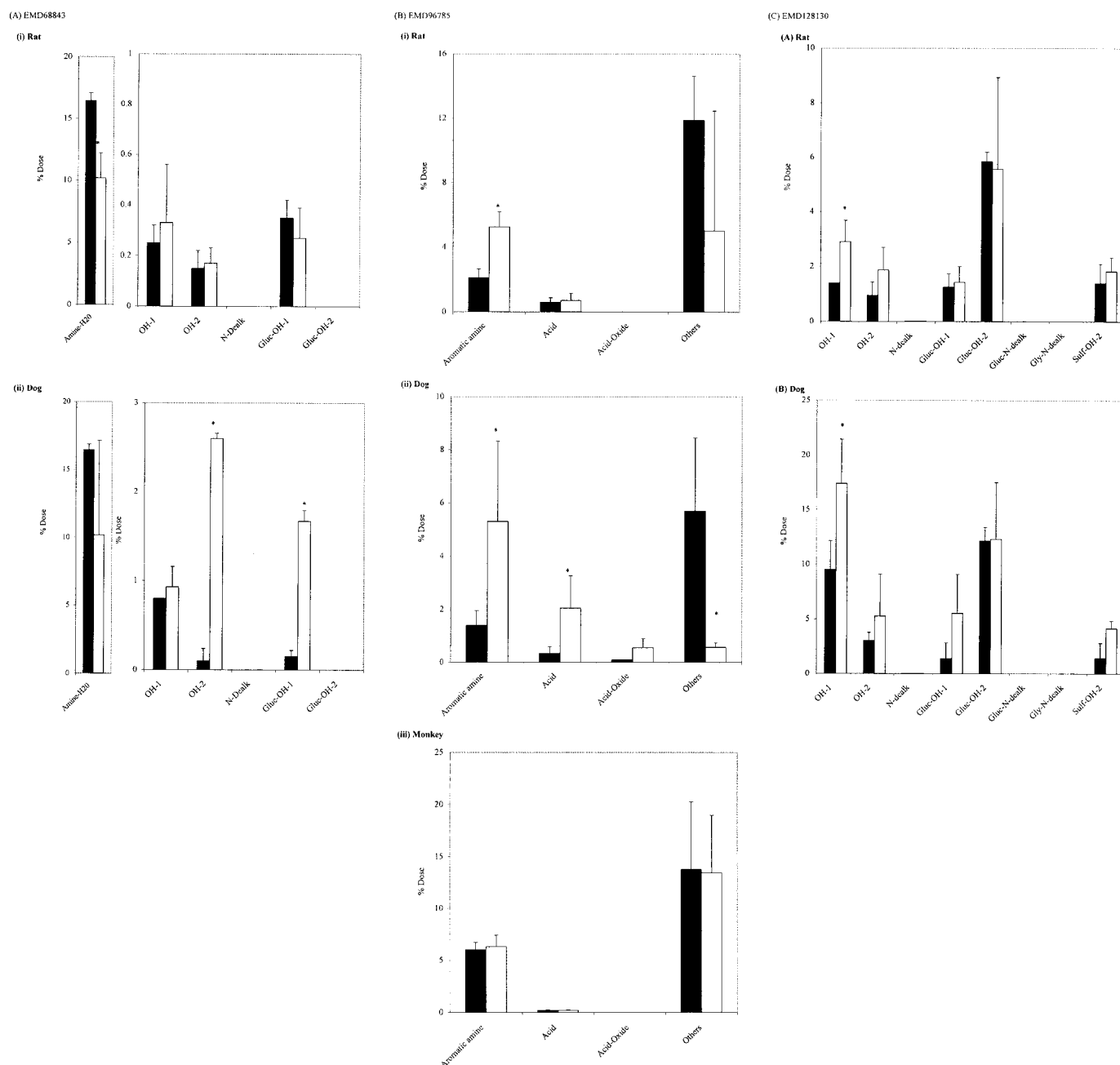


FIG. 3. Comparison of the metabolism of EMD68843 (A), EMD96785 (B), and EMD128130 (C) in fresh (■) and CP (□) rat and dog hepatocytes (mean \pm S.D., $n = 3-7$).

*, denotes significantly different from the fresh value ($P > 0.05$). Abbreviations as shown in Fig. 2.

ally the most rapidly metabolized of the three compounds, whereas EMD96785 metabolism was the slowest.

Discussion

The need for in vivo:in vitro correlations for drug screening is ever increasing but, more so, is the need for the in vitro systems to be more physiologically relevant. Hepatocytes have the obvious advantage of being intact cells with physiological concentrations of enzymes and cofactors. Classical studies comparing in vitro systems have used diagnostic substrate metabolism; however, we wanted to know how well GICs, CPHs, and microsomes could reflect the in vivo metabolism of pharmaceutical drugs currently in development at Merck KGaA.

Distinct species differences in metabolism could not be clearly

shown by any of the in vitro systems because some metabolites, although not major, were also produced in varying amounts in all four species in vivo and in vitro (data not shown). However, a number of discrete species differences were detected. For example, the acid-oxide product of EMD96785 was only detected in appreciable amounts in vivo in the dog (up to 10% of the dose) (Table 2) and was also produced in CPH dog hepatocytes but not dog liver microsomes. The *N*-dealkylation product of EMD68843 was specific to monkeys in vivo and in monkey microsomes. However, this species difference was not detected by GICs or CPHs.

There was a good in vivo:in vitro comparison of compound metabolism in rat and human GICs. With the exception of EMD96785, the amount of parent compound metabolized was always higher than in CPHs and, in some cases, microsomes (Table 1). The number of

TABLE 2

A comparison of the percentage of the dose metabolized in fresh hepatocyte suspensions, CPHs, GICs, and microsomes

Values expressed as the percentage of parent compound consumed [mean, $n = 3$ for each system and species, except for fresh dog hepatocytes ($n = 1$)].

	Fresh H	CPH ^a	GIC ^a	Microsomes ^b
EMD68843				
Rat	29	25	100	53
Dog	12	22	N.D.	49
Monkey	N.D.	13	N.D.	78
Human	N.D.	3	44	52
EMD96785				
Rat	15	11	6	65
Dog	7	8	N.D.	16
Monkey	N.D.	20	N.D.	27
Human	N.D.	7	7	26
EMD128130				
Rat	11	14	59	56 ^c
Dog	35	45	N.D.	72 ^c
Monkey	N.D.	24	N.D.	80 ^c
Human	N.D.	32	100	44 ^c

Fresh H, fresh hepatocyte suspensions; N.D., not done.

^a After 4-h incubation.

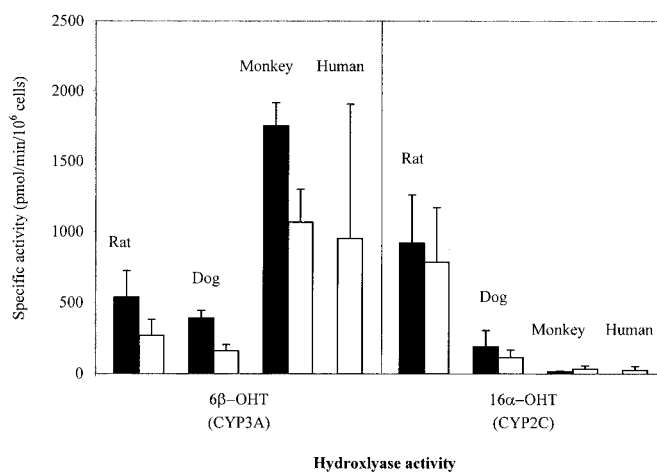
^b After 2-h incubation.

^c After 1-h incubation.

hepatocytes per incubation of GICs was 3-fold higher than CPHs but this did not account for the differences in the rates of metabolism. Moreover, this confirms that enzyme activities, per milligram of microsomal protein, are higher in intact cells than in microsomes (Wortelboer et al., 1990). The extent of glucuronidation in GICs was noteworthy since some phase 1 metabolites (notably hydroxides) were not detected in GIC incubations because they were completely converted to the corresponding glucuronide. This was also observed in the metabolism of acetaminophen in human GICs, the increased UGT activity of which may be a result of increased protein synthesis and indicative of a de-differentiated state (Koebe et al., 1994). Likewise, Kern et al. (1997) observed a sustained increase in the glucuronidation of *p*-nitrophenol in rat and human three-dimensional sandwich cultures. However, in the case of EMD128130, its two hydroxides are excreted in the bile only as their glucuronides and then hydrolyzed in the intestine and excreted in the urine as mainly the unconjugated hydroxide. Thus, the GICs may be a better system to predict metabolites that do not undergo enterohepatic recirculation.

Despite the high rate of metabolism in GICs, a number of metabolites were not produced by these cells. The EMD128130 *N*-dealkylation product, present as the unconjugated or the conjugated forms, was absent from both rat and human GIC incubations. This pathway was, however, evident in rat and dog CPHs, although only in small amounts. In contrast, this metabolite was always produced by liver microsomes from all species in significant amounts. Phase 2 conjugation is important in the excretion of many xenobiotics. All three compounds were conjugated to mainly glucuronides, however, sulfation and glycine conjugation were also important routes of *in vivo* metabolism. The latter two reactions could never be reproduced in microsomes, since these are not microsomal enzymes (Gregus et al., 1996; Falany, 1997). The EMD128130 sulfate was lacking in GICs, which could be attributed to either a loss of sulfotransferase per se or the sulfate cofactor (Koebe et al., 1994). In contrast, CPHs from rats, dogs, and monkeys were all able to sulfate the EMD128130 hydroxide, which was a major metabolite in the dog. The lack of glycine conjugation of the EMD128130 *N*-dealkylation product in hepatocytes (GICs, fresh and CPHs) is noteworthy, since this is a major metabolite detected in the bile of rats and dogs. Freshly isolated rat hepatocytes have been reported to produce glycine conjugates of other compounds, representing between 25 and 99% of the metabolites

(A) Phase 1 activities: Testosterone hydroxylases



(B) Phase 2 activities: Hydroxycoumarin conjugation

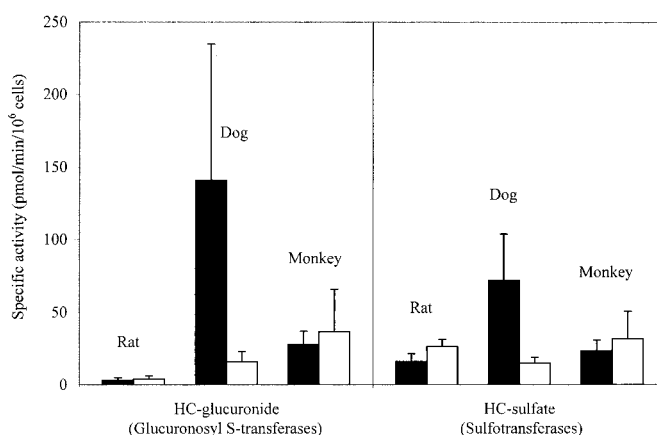


Fig. 4. Phase 1 [A, testosterone hydroxylase (OHT) activities] and phase 2 [B, hydroxycoumarin (HC) conjugations] in fresh (■) and CP (□) rat, dog, monkey, and human hepatocyte suspensions.

Data was taken from Swales and Utesch (1998), Hewitt et al. (2000), and N. Hewitt, unpublished data. Values are mean \pm S.D., $n = 3$.

(Galle et al., 1989; Peters, 1993), suggesting a certain capability of hepatocytes to carry out this reaction. Further studies are needed to establish whether a lack of glycine conjugation is a general phenomenon in CPHs and GICs or whether this is specific for this compound.

CPHs from all four species also reflected the *in vivo* metabolism of the compounds, via a number of reactions, including hydrolysis, *N*-dealkylation, oxidation, hydroxylation, glucuronidation, and sulfation. Although not the most important metabolite, the hydrolysis product of EMD68843, a major metabolite in humans, was produced by CPHs from all four species but, notably, not by microsomes or GICs. However, the extensive hydrolysis of EMD68843 in CPHs offsets the percentage of other major metabolites away from that seen *in vivo*. The reason for the excessive production of the hydrolysis product in CPHs is unclear, however, it cannot be attributed to nonspecific hydrolysis, since this would have also occurred in microsomal and GIC incubations.

Figure 4 shows the species differences in the main phase 1 and 2 enzyme activities involved in the metabolism of the three compounds in fresh and CPHs (Swales and Utesch, 1998; Hewitt et al., 2000). Of these, only phase 2 activities in dog hepatocytes were markedly decreased by cryopreservation. Human hepatocytes have similar testosterone and UGT activities to fresh cells (Hengstler et al., 2000).

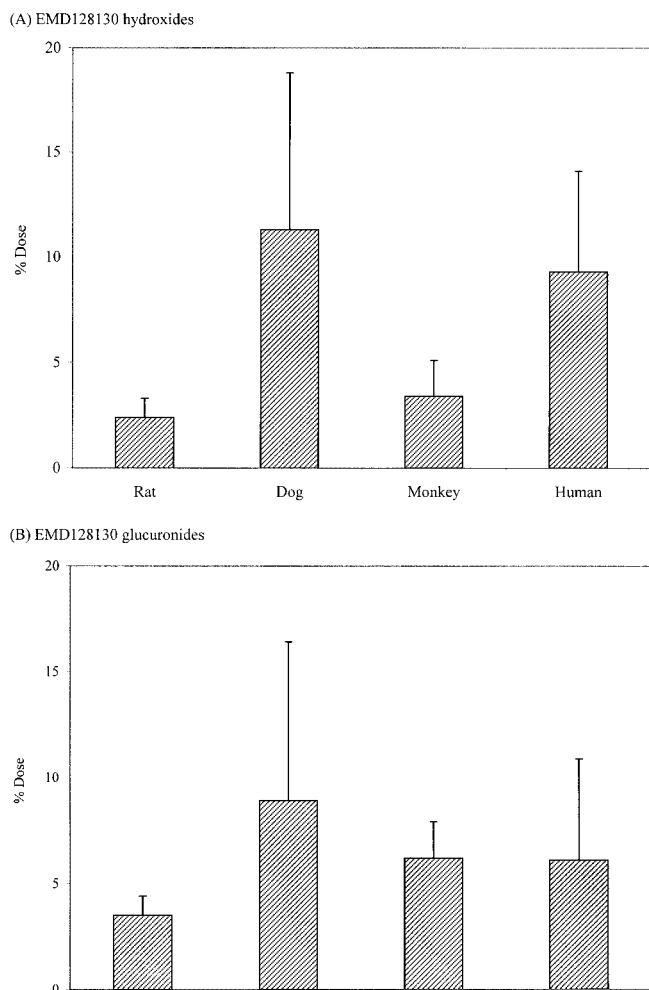


FIG. 5. Comparison of the percentage of the total of EMD128130 hydroxides (A) and glucuronides (B) formed in rat, dog, monkey, and human CPHs.

Values are expressed as percentage of dose, mean \pm S.D., $n = 3$.

Despite the loss of phase 2 activities, dog hepatocytes still produced similar, if not more, amounts of EMD128130 glucuronides to the other three species (Fig. 5B). Indeed, a comparison of fresh and CPH incubations showed that cryopreservation did not compromise the ability of CP dog hepatocytes to metabolize any of the compounds (which may be attributed to the involvement of multiple UGT isoforms in the dog). Moreover, the species differences in phase 1 and 2 activities, determined by diagnostic substrate metabolism, did not reflect species differences in the phase 1 and 2 metabolism of any of the three compounds studied. For example, a comparison of testosterone 6 β -hydroxylase activities in the different species suggests that monkey and human hepatocytes would produce the most hydroxides, however, the consumption of EMD128130 and the production of its hydroxides was highest in CP dog hepatocytes (Fig. 5A), which contained the lowest CYP3A activity [and CYP2D6 and CYP2C9/2C19 (N. Hewitt, unpublished data)]. This suggests either the contribution of other CYP isoforms in the metabolism of this compound in dog CPHs or that this compound is a better substrate in dog CPHs than in rat, monkey, or human CPHs. UGT activity in fresh dog hepatocytes was much higher than in CP dog hepatocytes and CPHs from the other three species [Fig. 4, human hydroxycoumarin UGT activity is similar to that of CP rat hepatocytes (Li et al., 1999b)] but the extent of glucuronidation of EMD128130 hydroxides was similar in all hepatocytes (Figs. 3C and 5).

In summary, *in vitro* incubations with CPHs, microsomes, and GICs can be used to study the major *in vivo* metabolites of pharmaceutical drugs such as EMD68843, EMD96785, and EMD128130. Substrate consumption was higher in GICs and microsomes than in CPHs but the percentage of each metabolite in all three *in vitro* systems could still be compared with *in vivo* data. Distinct species differences in metabolism were detected by microsomes and CPHs. However, CPHs and GICs can both carry out sequential phase 1 and 2 metabolism, giving them an advantage over microsomes. Human GICs, mainly due to the unpredictable supply of livers, may be more suited to induction and mechanistic studies. CPHs have the advantage of being quicker, easier, and more readily available to use than GICs and can, therefore, be adapted for physiologically relevant high-throughput screening in metabolism and drug-drug interaction studies.

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