FIGURE 6-11

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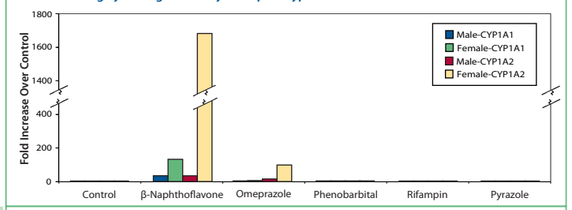

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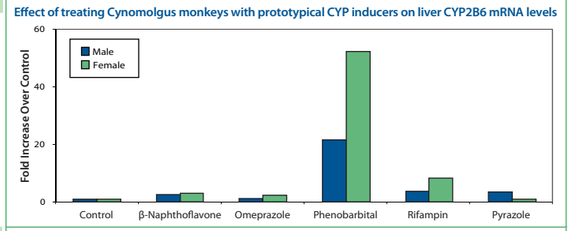

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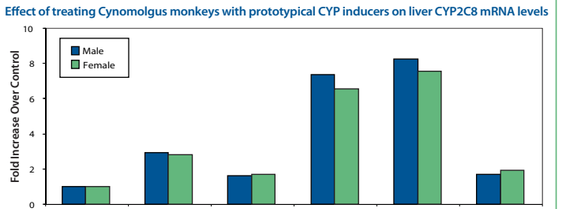

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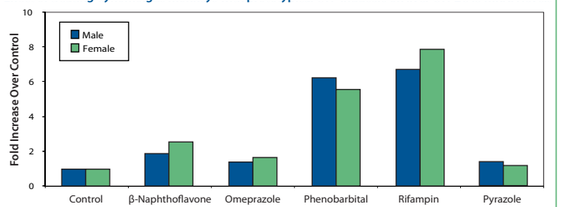

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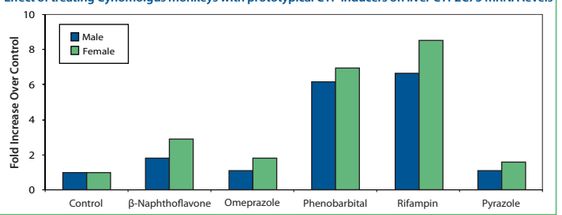

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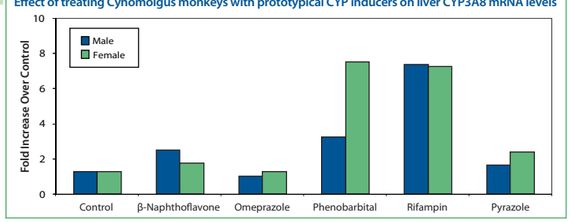

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Induction of Liver and Intestinal Cytochrome P450 (CYP) Enzymes in Male and Female Cynomolgus Monkeys

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ABSTRACT

The objective of the present study was to determine the effects of various prototypical inducers on the activity and mRNA expression of multiple CYP enzymes in monkey liver and intestine.

Male and female Cynomolgus monkeys were dosed orally (by nasogastric intubation) once daily for four days with saline (vehicle control), β-naphthoflavone (BNF, 50 mg/kg/day), phenobarbital (PB, 25 mg/kg/day), rifampin (RIF, 25 mg/kg/day), pyrazole (200 mg/kg/day) or omeprazole (OMP, 50 mg/kg/day). Microsomal samples from liver and intestine were analyzed for total cytochrome P450 content, cytochrome b_5 content, NADPH-cytochrome *c* reductase activity, 7-ethoxyresorufin *O*-dealkylation (CYP1A1/2), testosterone 16α-hydroxylation and testosterone 16β-hydroxylation (CYP2B17), 4-nitrophenol hydroxylase (CYP2E1), testosterone 2α-hydroxylation and testosterone 6β-hydroxylation (CYP3A8). CYP mRNA levels in liver were determined for CYP1A1, 1A2, 2A24, 2B6, 2C8, 2C43, 2C75, 2E1, 3A8 and 4A11 by RT-PCR. Given the amount of data collected on this project, the primary focus will be CYP mRNA data. Treatment of monkeys with BNF caused significant increases in liver CYP1A1 and CYP1A2 mRNA levels, and little or no increase (< 4-fold) in the mRNA levels of the other enzymes examined. Omeprazole caused increases in liver CYP1A1 and 1A2 mRNA levels, but as a CYP1A mRNA inducer OMP was less effective than BNF. Treatment with BNF caused an increase in CYP1A1 mRNA levels (66- and 240-fold in males and females) and CYP1A2 mRNA levels (51- and 1600-fold in males and females). Treatment with OMP caused an increase in CYP1A1 mRNA levels (2.2- and 5.8-fold in males and females) and CYP1A2 mRNA levels (14.9- and 173-fold in males and females). Similarly, BNF and OMP caused increases in liver microsomal CYP1A1/2 activity. Treatment with BNF caused significant increases in CYP1A activity in both liver and intestine. The increase in liver CYP1A activity by BNF was similar between male and female monkeys (11- and 9.4-fold, respectively) whereas induction of intestinal CYP1A activity by BNF was much lower in male than female monkeys (2.7- and 19-fold, respectively). Though to a smaller extent compared to BNF, OMP also caused increases in microsomal CYP1A1/2 activity (3.7- and 4.3-fold in the liver) and (1.5 and 4.3-fold in intestine) for male and female monkeys, respectively. Treatment with PB caused a large increase in liver CYP2B6 mRNA levels (21.6- and 52.5-fold in males and females, respectively), and caused less pronounced increases in CYP2A24 (5.6- and 7.3-fold), 2C8 (7.3- and 6.5-fold), 2C43 (6.4- and 5.7-fold), 2C75 (6.2- and 7.0-fold), and 3A8 (2.6- and 6.0-fold) mRNA levels in males and females, respectively. PB also caused an increase in liver microsomal CYP2B17 and CYP3A8 activity (3.2- and 2.2-fold, respectively). Rifampin treatment caused an increase in liver CYP2B6 (3.6- and 8.2-fold), 2C8 (8.2- and 7.5-fold), 2C43 (6.9- and 8.1-fold), 2C75 (6.7- and 8.6-fold) and 3A8 (5.9- and 5.8-fold) mRNA levels in males and females, respectively. Except for a small increase (3.5-fold in CYP2B6 and 2.7-fold in CYP4A mRNA levels), pyrazole did not cause an increase in the liver mRNA levels of any of the CYP enzymes examined. Results from this study indicate that BNF and OMP are *in vivo* inducers of CYP1A1 and CYP1A2 in monkeys, while PB and rifampin are inducers of CYP2B, 2C and CYP3A enzymes.

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INTRODUCTION

Evaluation of the potential of drug candidates to cause drug interactions is a requirement of the regulatory drug approval process (Huang *et al.*, 2008). The ability of a drug to inhibit, induce or be a substrate of drug-metabolizing enzymes may be predictive of its ability to affect disposition, efficacy or toxicity when co-administered with other drugs. Cynomolgus monkeys are commonly used to study metabolism of drugs during drug development. The objective of the present study was to determine the effects of known prototypical CYP inducers on the activity and gene expression of various CYP enzymes in monkey liver and intestine.

MATERIALS & METHODS

Animal Treatment and Tissue Collection

Male and female Cynomolgus monkeys *(Macaca fascicularis)* (2-4/treatment/sex) were treated orally (nasogastric intubation) once daily for four consecutive days with vehicle control (saline) or prototypical CYP inducers. The treatment groups included saline (vehicle control), β-Naphthoflavone (50 mg/kg/day) for CYP1A1, phenobarbital (25 mg/kg/day) for CYP2B, pyrazole (200 mg/kg/day) for CYP2E, rifampin (25 mg/kg/day) for CYP3A and omeprazole (400 mg/kg/day) for CYP1A. Following the final day of treatment, animals were anesthetized by intramuscular ketamine and euthanized by intravenous barbiturate overdose. The livers were removed and snap frozen in liquid nitrogen. The upper half of the small intestine (duodenum and jejunum) was collected, placed in chilled saline solution and processed within twenty-four hours of collection.

Microsomal CYP Enzyme Assays

Microsomes were isolated from monkey livers by differential centrifugation based on methods described in Lu and Levin (1972). Microsomal protein concentrations were determined with a BCA Protein Assay Kit (Pierce Chemical Company, Rockford, IL) based on method described by Smith *et al.* (1985).

Microsomal incubations were conducted in duplicate at 37 ± 1°C in 200-µL incubation mixtures containing potassium phosphate buffer (50 mM), $MgCl_2$ (3 mM) (all but 4-nitrophenol, where $MgCl_2$ was replaced with 1 mM ascorbic acid), EDTA (1 mM), an NADPH-generating system (1 mM NADP, 5 mM glucose-6-phosphate, 1 Unit/mL glucose-6-phosphate dehydrogenase, and marker substrate at the final concentrations indicated. The testosterone incubations also contained 1 µM *N,N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (4-MA) (Sonderfan and Parkinson, 1988). Reactions were started by addition of the NADPH-generating system, and were stopped after 10 or 30 minutes by the addition of acetonitrile containing the appropriate internal standard. Precipitated protein was removed by low-speed centrifugation, and aliquots of the supernatant fractions were analyzed by HPLC/MS/MS or spectrophotometrically.

The *O*-dealkylation of 7-ethoxyresorufin was measured by the fluorimetric method of Burke *et al.* (1996), with minor modifications (Pearce *et al.*, 1996; Dutton *et al.*, 1989).

Metabolites were quantified by reference to a standard calibration curve based on back calculation of a weighted (1/x), linear, least-squares regression. The regression fit was based on the peak ratio of the analyte to internal standard calculated from calibration standard samples, which were prepared from authentic metabolite standards. For HPLC/MS/MS, peak areas were integrated with Applied Biosystems/MDS SCIEX (Foster City, CA) Analyst® data system.

CYP mRNA Analysis

Messenger RNA (mRNA) levels in liver were determined by RT-PCR with an Applied Biosystems 7300 Real Time PCR sequence detection system. The relative quantity of the target cDNA compared with that of the control cDNA (18s rRNA) was determined by the ΔΔCT method (Applied Biosystems User Bulletin #2). Relative quantification measures the change in mRNA expression in a test sample relative to that in a control sample (*e.g.*, DMSO). This method assumes that the efficiency of the target amplification and the efficiency of the endogenous control amplification are approximately equal. Calculations were as follows:

1. $\Delta C_t = C_t$ (target) – C_t (endogenous control)
2. $\Delta\Delta C_t = \Delta C_t$ (treated sample) – ΔC_t (untreated control)
3. Fold change in expression = $2^{-\Delta\Delta C}t$

Data Processing

Data were processed and graphed with a validated, custom software program for Microsoft Excel (Office 2003). Individual rates of reaction from like treatment groups were averaged, and for those groups with n ≥ 3, standard deviations were determined.

RESULTS

Enzyme Activity and mRNA Expression

The effects of treating monkeys with prototypical CYP inducers on liver and intestinal CYP activity are shown in **Table 1** and **Figures 1-5**. The effects of treating monkeys with prototypical CYP inducers on intestinal CYP mRNA expression are shown in **Table 2** and **Figures 6-11**.

Treatment with β-Naphthoflavone (BNF) caused significant increases in 7-ethoxyresorufin *O*-dealkylation (CYP1A activity) in both liver and intestine. The increase in CYP1A activity in the liver was similar between male and female monkeys (11- and 9.4-fold, respectively) whereas the increase in intestinal CYP1A activity was lower in male than female monkeys (2.7-versus 19-fold, respectively). Omeprazole (OMP) also induced CYP1A activity in liver (3.7- and 4.3-fold) and intestine (1.5 and 4.3-fold) in male and female monkeys, respectively. But as a CYP1A inducer, OMP was less effective than BNF. BNF and OMP induced CYP1A1 and CYP1A2 mRNA levels, but OMP was less effective than BNF, and fold increases were greater in female than in male monkeys. Also, monkey liver had greater expression of CYP1A2 mRNA than CYP1A1 mRNA.

Treatment with phenobarbital (PB) caused an increase in liver microsomal testosterone 16β-hydroxylation (CYP2B activity) in male (3.2-fold) and female (2.3-fold) monkeys, and an increase in liver microsomal coumarin 7-hydroxylation (CYP2A activity) in male (4.6-fold) and female (1.6-fold) monkeys. In contrast to rat, testosterone 16α-hydroxylation was not a selective marker for liver microsomal CYP2B activity in the monkey, in agreement with reports by Bullock *et al.* (1995). Treatment with PB caused a large increase in liver CYP2B6 mRNA levels (21.6- and 52.5-fold in males and females, respectively) (**Figure 7),** and caused less pronounced increases in CYP2A24 (5.6- and 7.3-fold), CYP2C8 (7.3- and 6.5-fold), CYP2C43 (6.4- and 5.7-fold), CYP2C75 (6.2- and 7.0-fold), and CYP3A8 (2.6- and 6.0-fold) mRNA levels in males and females, respectively. CYP2A activity was undetectable or below the limits of quantification in intestinal microsomes (data not shown).

Rifampin (RIF) induced microsomal testosterone 6β-hydroxylation (CYP3A activity) in liver (male, 2.6-fold; female, 2.5-fold) and intestine (male, 3.7-fold; female, 4.6-fold). RIF treatment caused an increase in liver CYP3A8 (5.9- and 5.8-fold), CYP2B6 (3.6- and 8.2-fold), CYP2C8 (8.2- and 7.5-fold), CYP2C43 (6.9- and 8.1-fold), and CYP2C75 (6.7- and 8.6-fold) mRNA levels in males and females, respectively.

Pyrazole induced liver microsomal 4-nitrophenol hydroxylation (CYP2E activity) in male (4.1-fold) and female (4.9-fold) monkeys, and also increased intestinal CYP3A activity (3.2-fold) and CYP4A activity (3.6-fold) in female monkeys. Except for a small increase (3.5-fold in CYP2B6 and 2.7-fold in CYP4A mRNA levels), pyrazole did not cause an increase in the liver mRNA levels of any of the CYP enzymes examined.

Microsomal UGT activity toward triiodothyronine and thyroxine, and 4-methyl-umbelliferone glucuronidation was also measured. Treatment of monkeys with prototypical CYP inducers did not induce UGT activity (data not shown).

CONCLUSION

The results of this study indicate that β-Naphthoflavone (BNF) and omeprazole (OMP) are *in vivo* inducers of CYP1A1 and CYP1A2 in monkeys, whereas phenobarbital (PB) and rifampin (RIF) are inducers of CYP2B, CYP2C and CYP3A enzymes. In both liver and intestine at the dosages tested, BNF was a more effective inducer of CYP1A than OMP, PB was the most effective inducer of CYP2B, whereas RIF was the most effective inducer of CYP3A.

TABLE 1

Effect of treating Cynomolgus monkeys with prototypical CYP inducers on liver and intestinal microsomal enzyme activity: Fold-increase

		7-Ethoxyresorufin *O*-dealkylation (CYP1A)		Coumarin 7-hydroxylation (CYP2A)		Testosterone 16β-hydroxylation (CYP2B)		4-Nitrophenol hydroxylation (CYP2E)		Testosterone 16β-hydroxylation (CYP3A)		Lauric acid 12-hydroxylation (CYP4A)	
	Treatment	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver	β-Naphthoflavone	11	9.4	0.31	0.40	0.89	0.57	0.90	0.78	0.64	0.58	0.47	0.62
	Omeprazole	3.7	4.3	0.69	1.1	1.1	1.0	0.94	1.0	0.79	1.0	0.62	1.3
	Phenobarbital	1.7	3.4	4.6	1.6	3.2	2.3	1.6	1.5	1.2	2.2	0.80	1.4
	Rifampin	2.6	2.7	1.0	0.86	1.7	1.6	1.3	1.1	2.6	2.5	0.99	1.1
	Pyrazole	2.0	3.2	0.56	1.1	1.2	1.0	4.1	4.9	0.88	1.6	0.67	0.75
Intestine	β-Naphthoflavone	2.7	19	NA	NA	NA	NA	NA	NA	0.60	0.76	0.32	0.64
	Omeprazole	1.5	4.3	NA	NA	NA	NA	NA	NA	0.74	0.91	0.45	0.66
	Phenobarbital	0.17	NA	NA	NA	NA	NA	NA	NA	1.3	2.5	0.44	1.3
	Rifampin	0.25	0.35	NA	NA	NA	NA	NA	NA	3.7	4.6	0.39	1.2
	Pyrazole	NA	0.45	NA	NA	NA	NA	NA	NA	1.5	3.2	0.81	3.6

NA: Not applicable due to analyte peak not detected or rates lower than the limit of quantification.

TABLE 2

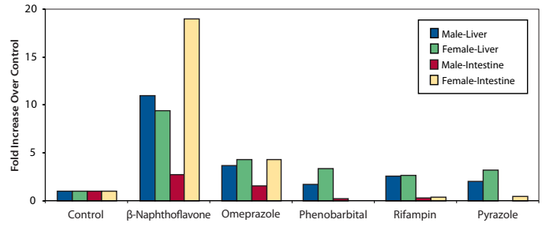
Effect of treating Cynomolgus monkeys with prototypical CYP inducers on liver CYP mRNA levels: Fold-increase

		CYP1A1		CYP1A2		CYP2A24		CYP2B6		CYP2C8		CYP2C43		CYP2C75		CYP2E1		CYP3A8		CYP4A11	
	Treatment	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver	β-Naphthoflavone	66	240	51	1600	1.2	2.0	2.6	2.9	2.9	2.8	1.9	2.6	1.8	2.9	2.4	2.1	2.0	1.4	2.1	3.6
	Omeprazole	2.2	5.8	14.9	173	0.80	1.4	1.1	2.4	1.6	1.7	1.4	1.7	1.1	1.8	1.0	0.90	0.80	1.0	1.2	1.5
	Phenobarbital	1.1	0.19	0.2	0.57	5.6	7.3	21.6	52.5	7.3	6.5	6.4	5.7	6.2	7.0	0.80	1.6	2.6	6.0	1.0	2.2
	Rifampin	0.62	1.1	0.27	0.3	1.7	2.0	3.6	8.2	8.2	7.5	6.9	8.1	6.7	8.6	1.4	0.60	5.9	5.8	2.4	1.7
	Pyrazole	0.68	0.75	0.06	1.6	1.0	0.48	3.5	1.0	1.7	1.9	1.4	1.2	1.1	1.6	0.54	0.93	1.3	1.9	1.4	2.7

FIGURES 1-5

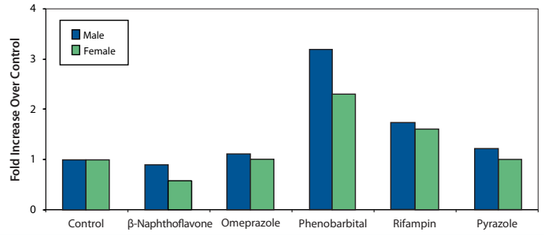
1

Effect of treating Cynomolgus monkeys with prototypical CYP inducers on liver and intestinal microsomal 7-ethoxyresorufin *O*-dealkylase (CYP1A) activity



2

Effect of treating Cynomolgus monkeys with prototypical CYP inducers on liver microsomal testosterone 16β-hydroxylase (CYP2B) activity



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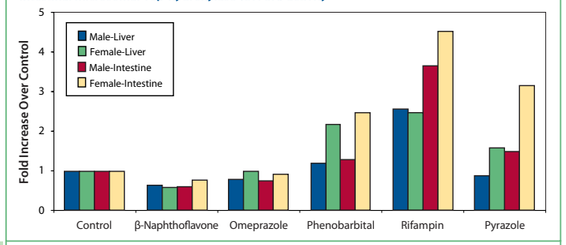
Effect of treating Cynomolgus monkeys with prototypical CYP inducers on liver and intestinal microsomal 16β-hydroxylase (CYP3A) activity



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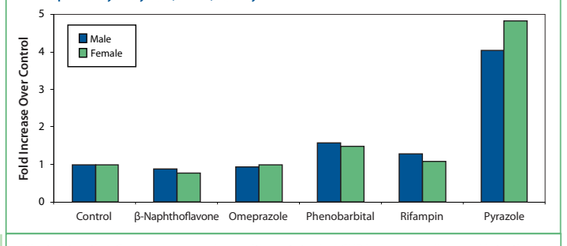
Effect of treating Cynomolgus monkeys with prototypical CYP inducers on liver microsomal 4-nitrophenol hydroxylase (CYP2E) activity



5

Effect of treating Cynomolgus monkeys with prototypical CYP inducers on liver and intestinal microsomal lauric acid 12-hydroxylase (CYP4A) activity