Species differences in the response of liver drug metabolizing enzymes to EMD 392949 in vivo and in vitro

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Abbreviations: bw, body weight; CYP, cytochrome P450; DME, drug metabolizing enzyme; DMEM, Dulbecco’s Minimum Essential Medium; EMD, EMD 392949; EROD, 7-ethoxyresorufin-O-deethylase; BROD, 7-benzyloxyresorufin-O-debenzylase; FCS, fetal calf serum; nt, nucleotide(s); PPAR, peroxisome proliferator-activated receptor; PROD, 7-pentoxyresorufin-O-depentylase; SD, standard deviation; TLDA, TaqMan®Low Density Array; UGT, UDP-glucuronosyl-transferase; v/v, volume per volume.
Abstract

Induction of drug metabolizing enzymes (DMEs) is highly species-specific and can lead to drug-drug interaction and toxicities. In this series of studies we tested the species-specificity of the anti-diabetic drug development candidate and mixed peroxisome proliferator-activated receptor (PPAR)α/γ agonist EMD 392949 (EMD) with regards to the induction of gene expression and activities of DMEs, their regulators and typical PPAR target genes. EMD clearly induced PPARα target genes in rats in vivo and in rat hepatocytes but lacked significant induction of DMEs, except for cytochrome P450 (CYP) 4A. CYP2C and 3A were consistently induced in livers of EMD-treated monkeys. Interestingly, classic rodent peroxisomal proliferation markers were induced in monkeys after 17 but not after a 4-week treatment, a fact also observed in human hepatocytes after 72 h but not 24 h of EMD treatment. In human hepatocyte cultures, EMD showed similar gene expression profiles and induction of CYP activities as in monkeys, indicating that the monkey is predictive for human CYP induction by EMD. In addition, EMD induced a similar gene expression pattern as the PPARα agonist fenofibrate in primary rat and human hepatocyte cultures. In conclusion, these data showed an excellent correlation of in vivo data on DME gene expression and activity levels with results generated in hepatocyte monolayer cultures, enabling a solid estimation of human CYP-induction. This study also clearly highlighted major differences between primates and rodents in the regulation of major inducible CYPs, with evidence of CYP3A and CYP2C inducibility by PPARα agonists in monkey and humans.
Introduction

The liver is the major site of biotransformation of xenobiotics and biotransformation is divided into three main phases: activation (phase I), conjugation (phase II) and drug transport (phase III). Phase I reactions, including microsomal cytochrome P450 (CYP) dependent oxidation pathways and phase II reactions like UDP-glucuronosyl transferase (UGT) dependent conjugation, are involved in detoxification and elimination of endogenous and exogenous substances, formation of pharmacologically active drugs from pro-drugs but also generation of toxic metabolites (Parkinson, 2001).

Exposure to drugs, occupational and industrial chemicals or environmental pollutants can lead to either the induction or the inhibition of biotransformation (Coecke et al., 2006). Due to their inducibility, drug metabolizing enzymes (DMEs) such as CYPs can be involved in various side effects such as profound endogenous hormonal disturbances, increased liver weight, drug-drug interactions and exacerbated toxic effects. Therefore, evaluation of the inducing potential of a given chemical on these enzymes is invaluable for human safety assessment (Madan et al., 2003).

Due to major species differences, both in the catalytic activities and regulation of this group of enzymes, the evaluation of a compound’s effect can be accurately performed only with human tissue (Silva et al., 1998). During the past decade, primary cultures of isolated human hepatocytes have proven to be a valuable model to study the inducing potential of drugs on different CYP isozymes (e.g., LeCluyse et al., 2000 and 2005; Richert et al., 2003 and 2006; Hewitt et al., 2007). Major families of inducers have been identified and transcription factors involved in specific induction pathways have been discovered (Waxman, 1999), such as the arylhydrocarbon...
receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR) and peroxisome proliferator-activated receptor α (PPARα).

PPARs are nuclear receptors that control a variety of genes involved in several pathways of lipid metabolism (Devergne and Wahli, 1999). In man, PPARα and PPARγ are important regulators of lipid and lipoprotein metabolism, cellular differentiation and glucose homeostasis. PPARα mainly acts on lipid and lipoprotein catabolism genes, predominantly in the liver (e.g., β-oxidation of fatty acids) whereas PPARγ plays an active role in the regulation of lipid storage and contributes to insulin action. Consequently, the development of PPARα/γ agonists represents an opportunity to produce tailored compounds that can treat both perturbation of lipid metabolism and insulin resistance (Harrity et al., 2006; Staels and Fruchart, 2005).

EMD 392949 (EMD) is a new chemical entity activating both PPARα and PPARγ, and has been shown to ameliorate hyperglycemia and hyperinsulinemia in db/db mice (unpublished data). By combining the pharmacological properties of a PPARα and a PPARγ activator, EMD would be an ideal candidate for the treatment of “metabolic syndrome” and type 2 diabetes.

Recently, drug-drug interactions have been observed in humans after administration of PPARα ligands such as fenofibrate. This is most probably related, at least in part, to CYP induction (Prueksaritanont et al., 2005). The aim of the present study was therefore to compare the effects of EMD administration on DME regulators and DME mRNA expression in conjunction with related monooxygenase activities in different animal species and man.
Materials and Methods

Chemicals and Reagents

Chemicals used in this study were obtained from Sigma-Aldrich (St. Quentin-Fallavier, France) and reagents for cell culture were from Invitrogen (Cergy Pontoise, France) unless stated otherwise. Cell culture plastics were purchased from Becton Dickinson (Grenoble, France). EMD 392949 ((S)-4-o-tolylsulfanyl-2-(4-trifluormethyl-phenoxy)-butyric acid, batch 00195C0, > 95% purity) was from Merck Santé (France).

Animals and treatment

All animal experiments were approved by the local authorities and were conducted in compliance with the principles of Good Laboratory Practice (GLP) of the OECD, the EU and the FDA GLP regulations 21 CFR Part 58 as well as the local animal welfare regulations.

In a rat toxicity study, a group of 3 male HsdCpb:WU Wistar rats with a mean age of 7 weeks at the beginning of treatment, were dosed for 13 weeks with EMD 392949 by daily oral (gavage) administration of 0 (control, 0.25 % aqueous hydroxypropyl methylcellulose), 3 or 100 mg/kg body weight (bw). Approximately 1 hour after the last treatment the animals were sacrificed and portions of livers were immediately frozen in liquid nitrogen until required. The study was performed by Merck KGaA (Germany).

In a monkey ex vivo CYP induction study (performed by Covance, UK), a group of 3 male Cynomolgus monkeys (Macaca fascicularis), which were at least 24 months old, were treated by daily oral (gavage) dosing with 0 (vehicle control: 0.25 % aqueous hydroxypropyl methylcellulose), 30, 100 or 300 mg/kg bw/day EMD for 4 weeks. On the day of necropsy,
approximately 1 hour after the last treatment, portions of livers were immediately frozen in liquid nitrogen until required.

In a monkey toxicity study (performed by MDS Pharma Services, France), a group of 3 male Cynomolgus monkeys with an age of 26 to 33 months at the beginning of treatment were treated daily by oral (gavage) administration of 0 (vehicle control: 0.25 % aqueous hydroxypropyl methylcellulose), 15 or 150 mg/kg bw/day EMD for 17 weeks. At the end of the treatment the animals were sacrificed and portions of livers were frozen in liquid nitrogen until use.

**Source of human livers**

Liver samples were taken from patients undergoing liver resection for different pathologies (Table 1). All experimental procedures were performed in compliance with French law and regulations after approval by the National Ethics Committee (France). Informed consent was obtained from all patients for the use of liver tissue for research purposes.

**Hepatocyte isolation**

Rat hepatocytes were isolated from male Wistar rat livers by a two-step collagenase perfusion method as previously described (Viollon-Abadie et al., 2000).

Human hepatocytes were isolated based on a modification of a two-step collagenase digestion method, according to a recently described protocol (Richert et al., 2004; LeCluyse et al., 2005).

**Hepatocyte culture and treatment**

Rat and human hepatocytes were plated in 60 mm dishes at a density of 3.5 x 10^6 cells per dish, or in 6-well BD BioCoat™ plates at a density of 1.5 x 10^6 cells per well in 3 ml or 2 ml
attachment medium, respectively, and cultured under a CO$_2$/air (5%/95%) humidified atmosphere at 37°C. Attachment medium consisted of DMEM medium containing 5% fetal calf serum, 50 mg/l gentamycin, 4 mg/l insulin and 10$^{-5}$ mol/l hydrocortisone. After a 24-hour attachment period, the medium was discarded and replaced by incubation medium, consisting of DMEM medium, supplemented with 50 mg/l gentamycin, 4 mg/l insulin and 10$^{-5}$ mol/l hydrocortisone and containing 0 (vehicle control: 0.1% (v/v) DMSO), 30, 100 µM EMD or 100 µM fenofibrate (five dishes for each group). Every day, medium in all dishes was renewed.

**Microsome preparation**

**Liver microsomes**

The livers (n=3 per species and dose level) were thawed in ice-cold 50 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, scissor-minced and homogenized. Microsomal suspensions were prepared by differential centrifugation as described previously (Richert et al., 2002). Briefly, liver homogenates were sonicated and centrifuged for 20 min at 9,000x g and 4°C. Supernatant fractions were collected and centrifuged for 60 min at 100,000x g and 4°C. The resulting microsomal pellets were resuspended in 80-120 µl of 0.25 M sucrose. The protein concentration of each sample was determined by the Lowry assay (Lowry et al., 1951). The microsomes were snap-frozen and stored at -80°C until evaluated.

**Hepatocyte microsomes**

At the end of the 72 h incubation period, cells from dishes were harvested for microsome preparation. Culture dishes within individual treatment groups were scraped, pooled and frozen at -80°C as previously described (Richert et al., 2004; LeCluyse et al., 2005). After thawing of cell homogenates, microsomes were prepared by differential centrifugation as described above. The
protein concentration of each sample was determined by the bicinchoninic acid protein assay kit, according to the manufacturer’s instructions (Sigma-Aldrich, St. Quentin-Fallavier, France) and bovine serum albumin was used as a standard.

**Microsomal enzyme activity assays**

Microsomal activities of 7-ethoxyresorufin O-deethylase (EROD, CYP1A), 7-pentoxyresorufin O-depentylase or 7-benzyloxyresorufin O-debenzylase (PROD/BROD, CYP2B), bupropion-hydroxylase (CYP2B), testosterone 6β- and 16β-hydroxylases (CYP3A and CYP2B, respectively) and lauric acid 12-hydroxylases (CYP4A) were determined as previously described (Richert et al., 2002; Robertson et al., 2000; Faucette et al., 2000; Okita et al., 1991).

**Acyl-CoA oxidase (ACOX) enzyme activity**

ACOX activity was measured in rat and monkey liver samples. Fifty mg portions of frozen livers were homogenized for 30 sec on ice in 1 ml sucrose solution (10% (w/v), 3 mM imidazole, pH 7.4) using a rotor stator homogenizer. Liver homogenates were frozen in liquid nitrogen and stored at -80°C until analyzed. After thawing on ice and centrifugation (10 min, 4°C, 7,000x g), the protein concentration was determined in the supernatants using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as standard. Palmitoyl-CoA oxidase activity was determined in supernatants according to a modification of a previously described method (Ammerschlaeger et al., 2004; Small et al., 1985). Absorption was recorded at 502 nm for 4 min every 12 seconds.
mRNA preparation and analysis

_Liver mRNA_

Frozen portions of rat or monkey livers were fragmented in liquid nitrogen. Pieces of 40 - 100 mg were immersed in TRI Reagent™ (Sigma, Taufkirchen, Germany) and immediately homogenized for 45 sec on ice using a rotor stator homogenizer. Total RNA was isolated following the TRI Reagent™ standard protocol provided by the manufacturer. RNA pellets were dissolved in nuclease-free water and stored at -80°C until further use.

_Hepatocyte mRNA_

At the end of the incubation periods (24 h and 72 h), each well was rinsed twice with ice cold PBS and 500 µl of TRI Reagent™ was added to each well. Cells were scraped and the three wells from individual treatment groups were pooled. Total RNA was isolated following the TRI Reagent™ standard protocol provided by the manufacturer. RNA pellets were dissolved in nuclease-free water and stored at -80°C until further use.

*mRNA analysis*

Quality and concentration of total RNA were determined using the NanoDrop spectrophotometer (Kisker, Steinfurt, Germany) and the Agilent Bioanalyzer 2100 applying the Total RNA Nano Assay (Agilent Technologies, Waldbronn, Germany) according to the manufacturer’s protocols.
cDNA synthesis and analysis

Five microgram of total RNA were reverse transcribed to cDNA using random hexamer primers with the “Transcriptor first strand cDNA synthesis kit” (Roche, Mannheim, Germany) according to the protocol provided by the manufacturer. cDNA quality and concentration were determined using the Agilent Bioanalyzer 2100 applying the mRNA Pico Assay (Agilent Technologies, Waldbronn, Germany).

Real-time PCR

Real-time PCR analysis was essentially performed as described by Tuschl and Mueller (2006). Briefly, single gene real-time PCR primers and probes were delivered as “TaqMan® Gene Expression Assays” (Applied Biosystems, Darmstadt, Germany) for the rat and human genes listed in Table 2. Assays targeting human genes were also applied to analyze mRNA isolated from cynomolgus monkey liver samples. The amplicon sequences for the human assays in Tables 2 and 3 were used to search for sequence similarities in homologous genes of cynomolgus monkey (Macaca fascicularis) or other non-human primate species. The BLAST (Altschul et al. 1990) results are shown in Table 4. Real-time PCR was performed on Applied Biosystems ABI Prism 7000 Sequence Detection System with ABI Prism 7000 SDS Software 1.0. Two nanogram cDNA were used per reaction and 18S ribosomal RNA (rRNA) control (#4310893E, Applied Biosystems, Darmstadt, Germany) was used for normalization. Reactions were performed in triplicate for each sample. Analysis of gene expression values was performed using the efficiency-corrected comparative CT method. Gene expression ratios were calculated using the following formula:

\[ R = \left( \frac{E_{\text{target}}}{E_{18S}} \right)^{\frac{\Delta CT_{\text{target}} \text{(control--sample)}}{\Delta CT_{18S} \text{(control--sample)}}} \]
**TaqMan® Low Density Array**

In addition to single gene real-time PCR measurements, “TaqMan® Low Density Arrays” (TLDA; Applied Biosystems, Darmstadt, Germany) were used to analyze rat, human and monkey mRNA. Fifty nanogram cDNA were used per sample and loaded into a single sample loading port. Tables 3 and 5 list human and rat genes with the corresponding gene expression assays present on the respective TLDAs. Assays targeting human genes were also applied to analyze mRNA isolated from *Macaca fascicularis* liver samples (see above). Thermal cycling and fluorescence detection was performed on Applied Biosystems ABI Prism 7900HT Sequence Detection System with ABI Prism 7900HT SDS Software 2.1. Analysis of gene expression values was performed using the efficiency-corrected comparative CT method (see above).

**Statistical Analysis**

Statistical significance of alterations in enzyme activity or gene expression was analyzed using Origin® Software (OriginLab Corporation, Northampton, MA, USA). An ANOVA with Tukey’s post-hoc test was applied to analyze each experimental group. Statistical analysis was not employed on human hepatocyte data, since each donor is presented individually and no mean value of biological replicates was calculated. Statistically significant results are labeled with capital letters (p-values < 0.01) or lower case letters (p < 0.05) in Figures 1-4 and Table 6. The letter a/A stands for significantly different from control and b/B for significantly different from control and other dose(s) labeled with b/B or c/C. The letter c/C indicates no significant difference from control but significant difference from other dose labeled with c/C or b/B.
Results

**Effects of EMD 392949 on hepatic xenobiotic metabolizing enzymes following repeated in vivo administration in rats and monkeys**

Male Wistar rats were treated orally with EMD 392949 (EMD) at 0, 3 or 100 mg/kg bodyweight (bw) per day for 13 weeks. At the end of the treatment period, livers were assessed for mRNA expression of various DMEs, relevant nuclear receptors and transcription factors (Figure 1A), as well as for selected microsomal CYP-dependent monooxygenase activities (Figure 1B). Following repeated oral administration of EMD, neither Cyp1A2 mRNA expression nor Cyp1A specific EROD monooxygenase activity were affected at 3 mg/kg/day but were decreased with statistical significance at the high dose of 100 mg/kg/day. The latter effect was associated with a slight decrease, although not statistically significant, in the abundance of AhR mRNA, the main regulator of Cyp1A expression. Cyp2B mRNA expression was strongly increased at both doses, but didn’t reach statistical significance due to strong interindividual variation in the magnitude of induction. Moreover, the related PROD monooxygenase-dependent activity was moderately and significantly increased. Cyp3A mRNA expression and activity were increased about 2-fold at 3 mg/kg while at 100 mg/kg this effect had disappeared at the enzyme activity level and was even reduced at the gene expression level. In addition, MDR1 gene expression was significantly repressed by EMD. While PXR mRNA abundance was almost unchanged, there was a significant elevation of about 2-fold in CAR expression at both dose levels. At 100 mg/kg/day EMD, a strong and dose-dependent increase (> 15-fold) in Cyp4A activity was observed, along with a 6-fold induction of corresponding Cyp4A3 mRNA, both being highly significant and typical features of PPARα activation. In line with these
observations, we observed significant and dose-dependent inductions of acyl-CoA oxidase (ACOX), carnitine-palmitoyl transferase (CPT1a) and PPARα mRNAs by EMD. ACOX enzyme activity was also markedly and significantly increased by EMD treatment in rats (Table 6). Overall, these findings strongly confirm that EMD is a potent PPARα agonist in the rat.

We then compared the effects of EMD observed in rats with those in monkeys. In an ex vivo CYP induction study, male cynomolgus monkeys were treated by daily oral dosing with 0, 30, 100 or 300 mg/kg EMD for 4 weeks. At the end of the treatment period, livers were assessed for mRNA expression of various DMEs, their regulators and typical PPAR target genes (Figure 2A), as well as for selected microsomal CYP dependent monooxygenase activities (Figure 2B). EMD strongly and significantly decreased CYP1A2 mRNA in a dose-dependent manner, again correlating with a reduction in AhR expression especially at the highest dose tested. Additionally, CYP2B6 mRNA was significantly repressed. In contrast, CYP2C9 was slightly induced at 30 mg/kg EMD while CYP3A4 and CYP4A were induced only at 300 mg/kg. Enzyme activities of CYP2B6, CYP3A and CYP4A were moderately increased (maximum of 2-fold) while CYP1A dependent EROD activity was almost unchanged or weakly decreased at all three dose levels.

Two of the main regulators of DMEs, the nuclear receptors PXR and CAR, were regulated in an opposite direction. There was a weak reduction in PXR expression whereas CAR was slightly induced at 30 mg/kg. The transcription factor HNF1α was induced at 300 mg/kg. ACOX, a hallmark marker of peroxisome proliferators in rodents, was repressed at the mRNA level by EMD (Figure 2A) but no significant change in ACOX enzyme activity was noted compared to the vehicle treated control (Table 6).

In a second study, cynomolgus monkeys were treated orally with 0, 15 or 150 mg/kg/day for 17 weeks followed by a 4-week recovery period for a group of the high-dose animals. Livers
were assessed for mRNA expression as described above (Figure 3). Similar to the 4-week study, CYP1A2 was significantly repressed after 17 weeks of treatment, but only at the high dose. In contrast to the repression of CYP2B6 in the short-term study, there were only minor changes after 17 weeks. A very distinct increase in CYP2C9, CYP3A4 and MDR1 mRNA was observed after 17 weeks (Figure 3) that was less apparent in the 4-week study (Figure 2A). Interestingly, CYP4A11 was weakly but notably induced, in line with a distinct induction of ACOX and PPARα (Figure 3), although ACOX enzyme activity was not increased (Table 6). Contrary to the 4-week study (Figure 2A), mRNA levels for the transcription factors HNF1α, AhR and PXR but not CAR were higher after 17 weeks of treatment (Figure 3). At the end of the recovery period, most of the gene expression changes were significantly reversed.

**In vivo vs. in vitro effects of EMD 392949 on hepatic xenobiotic metabolizing enzymes in rats**

For comparison of *in vivo* with *in vitro* effects, male Wistar rat hepatocytes were treated with EMD at 0, 30 or 100 µM for 24 h and 72 h. The doses were chosen based on peak plasma concentrations observed in the rat toxicity study (peak plasma concentrations were in the range of 30-470 µM) and on PPARα/γ activity *in vitro* (3-100 µM; data not shown). Fenofibrate was included as a reference PPARα activator. After 24 h and 72 h incubation with the compounds, hepatocyte cultures were assessed for mRNA expression of DMEs, transcription factors and PPAR marker genes as described above (Figure 4A and 4B). Additionally, selected microsomal CYP activities were tested after 72 h of treatment with EMD (Figure 4C).

After 24 h and 72 h treatment, the effects of EMD on mRNA expression were very similar to that of fenofibrate, especially at the corresponding dose of 100 µM, where there was no statistically significant difference between both compounds’ profiles. The changes in gene
expression after \textit{in vitro} treatment of cultured rat hepatocytes with EMD were altogether equivalent to those observed after \textit{in vivo} administration (Figure 1A), for all genes measured. The same was true for the effects of EMD on microsomal monooxygenase activities: decrease of Cyp1A dependent activity, slight increase of Cyp3A dependent activity and a strong increase in Cyp4A dependent activity observed after \textit{in vitro} treatment (Figure 4C); although only Cyp4A activity was significantly changed. Overall, the gene expression profiles, as well as the DME activities, were in agreement with the observations after \textit{in vivo} treatment with EMD (Figure 1).

\textbf{Effects of EMD 392949 on hepatic xenobiotic metabolizing enzymes in human hepatocytes}

Finally, we assessed EMD for its CYP inducing capacity in human hepatocytes to allow an extrapolation to humans. Fresh human hepatocytes from 3 different donors (Table 1) were treated with EMD at 0, 30 or 100 µM for 24 h and 72 h and cultures were assessed for mRNA expression (Figures 5-7). Again, fenofibrate was included as a reference PPAR\textalpha activator. From one donor (donor 3; see Table 1) microsomal CYP activities were measured after 72 h treatment (Figure 7C).

The effects of EMD on gene expression were comparable to that of fenofibrate, although not as similar as seen in rat hepatocytes (Figure 4A and 4B). Depending on the donor and on the gene of interest, effects were maximal after 24 h or 72 h of treatment. CYP1A1 was consistently repressed after 24 h and induced after 72 h treatment, especially at 100 µM EMD, in all three donors. Similar to the results in monkeys (Figure 3), AhR was induced by EMD and to a lesser extent by fenofibrate. CYP1A2 mRNA expression was decreased by the treatment at both time-points in hepatocytes from 2 out of the 3 donors but was induced after 72 h in donor 3 (Figure 7B). In hepatocytes from donor 3, CYP1A1/2-dependent EROD activity was not affected by EMD treatment (Figure 7C).
A distinct increase in CPT1a, a typical PPARα marker in human hepatocytes (Ammerschlaeger et al., 2004), was detected in all 3 human hepatocyte cultures at 24 h and 72 h. PPARα was weakly induced by EMD and fenofibrate similar to the effects on AhR (Figures 5–7). Interestingly, CYP4A11 and ACOX mRNA were more strongly induced after 72 h (Figures 5–7), indicating a delayed induction of these classic rodent PPARα markers. This finding was confirmed by a 2-fold increase in CYP4A dependent lauric acid hydroxylase activity in donor 3 (Figure 7C).

Strikingly, CYP2C8, CYP3A4 and MDR1 were consistently and strongly induced on the mRNA level (Figures 5-7). Furthermore, CYP3A activity was distinctively and dose-dependently increased (Figure 7C) in hepatocytes from donor 3, confirming the gene expression data. The mRNA expression of PXR and CAR - regulators of CYP3A and/or 2C - were not consistently deregulated. However, there was a slight repression of PXR and CAR by EMD in the majority of cases.
Discussion

In regulatory animal toxicity and toxicokinetic studies the drug development candidate EMD showed marked species-specific differences in its kinetic properties: exposure after repeated dosing was not dose-proportional in cynomolgus monkeys, whereas these effects were only minor in rats (unpublished data). These observations are indicative of induction of metabolism of the parent drug predominantly in monkeys. To better characterize the species-specific properties of EMD with respect to DME induction, in particular CYPs, we compared its effect on specific DME mRNA expression and activity. In addition, the expression of major regulators of DMEs in vivo in rats and monkeys and in rat hepatocytes was studied. We then further investigated how these effects might translate to human beings by using primary human hepatocyte cultures.

As expected from its pharmacological activity (unpublished data), EMD markedly induced Cyp4A dependent lauric acid ω-hydroxylation activity and related mRNA as well as genes from the fatty-acid β-oxidation pathways in Wistar rats. This was also observed after in vitro exposure of rat hepatocytes to EMD or fenofibrate, a prototypical PPARα agonist, consistent with the known effects of PPARα ligands in rodents (for a review see Johnson et al., 2002). Interestingly, PPARα was induced in vivo but not in vitro in rats. This lack of PPARα induction by peroxisome proliferators in vitro has been previously reported by our laboratory (Ammerschlaeger et al., 2004).

In line with the well documented species-specific actions of PPARα agonists in vivo (e.g., Richert et al., 1996; Johnson et al., 2002) and in vitro (e.g., Ammerschlaeger et al., 2004; Perrone et al., 1998), the induction of CYP4A and ACOX mRNA expression were much less pronounced in monkey than in rat livers. In fact, these PPARα markers were repressed (in the case of ACOX)
in the 4-week monkey study, but after treatment for 17 weeks, a notable induction of ACOX, CYP4A and PPARα was detectable. This indicated a time-dependent induction of these typical rodent peroxisome proliferative genes in non-human primates.

Treatment of male Wistar rats with EMD suppressed Cyp1A1/2 metabolic activity and Cyp1A2 related mRNA. This was also observed after treatment of male rat hepatocytes with EMD or fenofibrate. These observations are in line with reports on Cyp1A1/2 metabolic activity suppression in rats after fenofibrate administration (Shaban et al., 2004). These authors concluded that this effect was PPARα dependent due to an inhibitory effect on AhR function. In the present study we actually provide evidence that AhR mRNA expression was repressed by EMD in rats and by EMD and fenofibrate in rat hepatocytes. Male cynomolgus monkeys also responded by decreases in CYP1A related activity and mRNA expression after repeated EMD treatment. However, AhR expression was slightly induced after long-term treatment with EMD but not after 4 weeks. This suggests a different long-term regulation of CYP1A in non-human primates.

The most striking differences between rats and monkeys included the consistent induction of CYP2C, CYP3A and MDR1 mRNAs in monkey but repression and/or marginal effects on these DMEs in rats in vivo and in vitro. CYP2B was also regulated in an opposite manner in monkeys and rats. The present results supported the assumption that EMD is an inducer of CYP2C and 3A in monkeys but not in rats. In monkeys, PXR, the major regulator of CYP3A (Reschly and Krasowski, 2006), was induced after treatment with EMD for 17 weeks indicating that PXR induction leads to increased expression and activity of CYP3A and/or CYP2C. In contrast, CAR (Reschly and Krasowski, 2006), another important CYP regulator, was not induced in monkeys after 17 weeks but only slightly after 4 weeks of treatment. Contrary to the effects in monkeys, Cyp2C mRNA expression was repressed in rat livers by EMD and in rat hepatocytes, by both
EMD and fenofibrate treatment, correlating well with the known effects of PPARα agonists on Cyp2C in rats (Fan et al., 2004). Cyp3A mRNA and activity were only marginally affected in rats, consistent with the minor effects on PXR expression. CAR was induced by EMD, correlating with strong increases in Cyp2B mRNA expression in rats in vivo and in vitro. It has been previously reported that PPARα agonists have the potential to induce Cyp2B and lead to the suppression of 2C11 in rats, both on protein and mRNA levels (Shaban et al., 2005); further confirming that EMD is a potent PPARα agonist in rats. In monkeys, CYP2B6 was repressed after 4 weeks and remained unchanged after 17 weeks. Contrasting effects on CYPs and their regulators in monkeys compared to rats indicate major differences in the mechanisms of regulation of CYPs in non-human primates compared to rats.

The present study showed an excellent correlation between the in vivo effects of EMD on rat livers and the in vitro effects on cultured rat hepatocytes in terms of specific CYP induction, which is in line with our previous results obtained from PPARα ligands (Richert et al., 1996; Goll et al., 1999). Our results further confirmed that primary cultures of hepatocytes can be considered as the gold standard for DME induction studies in vitro (Richert et al., 2003; Castell et al., 2006; Tuschl and Mueller 2006). We therefore extended the evaluation of the response of CYPs and nuclear receptor expression to fenofibrate and EMD to primary cultures of human hepatocytes.

EMD and fenofibrate induced CPT1a in all 3 human hepatocyte cultures, suggesting that EMD activated PPARα in human hepatocytes, which is in accordance with previous observations (Richert et al., 2003; Raucy et al., 2004; Ammerschlaeger et al., 2004). The slightly increased PPARα mRNA levels in human hepatocyte cultures further support this assumption, although there was considerable variation between donors. The classic rodent PPARα markers CYP4A and ACOX were also induced in human hepatocytes and in most cases this was strongest after 72
h treatment. Interestingly, induction of ACOX and CYP4A in monkey livers was only apparent after long-term treatment with EMD (see above). Taken together, the results in human and non-human primates - species that are in general refractory to peroxisome proliferation - suggest that a minor induction of peroxisome proliferation markers may occur in these species after prolonged exposure. Nevertheless, it has to be stressed here that the maximum levels of induction after EMD or fenofibrate treatment were only about 2- to 4-fold in human hepatocytes or monkey livers compared to 8- to 80-fold in rat hepatocytes. This is consistent with the well established difference in susceptibilities of human vs. rodent hepatocytes (Richert et al., 2003). The present results thus further corroborated that PPARα ligands, including EMD, although effective in human hepatocytes and monkey livers, are much less powerful inducers of the peroxisomal fatty acid metabolism pathways in primates than in rodents.

CYP3A and CYP2C, the major drug metabolizing CYPs in humans, were strongly induced by fenofibrate and EMD in human hepatocytes. The induction of activity and mRNA expression was comparable to that seen in vivo in monkeys, indicating that monkeys are - in this particular case - predictive for the CYP induction of EMD. An induction in CYP3A4 and CYP2C in human hepatocytes by PPARα agonists has been previously shown for clofibrlic acid (Richert et al., 2003; Prueksaritanont et al., 2005). In addition, MDR1 that is also regulated by PXR and correlates well with CYP3A4 expression (Reschly and Krasowski, 2006) was induced by EMD in monkey and human but not in rats. Taken together, this indicates that PPARα agonists in general may be CYP3A inducers, an assumption that should be confirmed by analysis of a broader variety of PPARα agonists. The marginal effect on Cyp3A and repression of Cyp2C11 in rat hepatocytes, both by fenofibrate and EMD treatment, compared to the strong CYP2C8/9 and CYP3A4 induction in monkey livers and human hepatocytes highlights the marked difference
between rodents and primates in the regulation of these CYPs. PXR was not induced by EMD or fenofibrate in human hepatocytes what is in agreement with previous studies which showed that fibrates failed to activate human PXR in a reporter gene assay (Prueksaritanont et al., 2005). As CYP3A4 and CYP2C8 expression can also be mediated by the glucocorticoid receptor and CAR (Dvorak et al., 2003; Sugatani et al., 2004, Faucette et al., 2006), it is possible that the latter pathway could be involved in the induction of these enzymes. Further work is necessary to explore this possibility.

In summary, we confirmed in the present study the well established differences in typical PPARα activities between rodent and non-rodent species. More interestingly, we have discovered, to our knowledge for the first time, that PPARα agonists are able to significantly induce CYP4A and ACOX in monkeys after extended treatment duration. An even more important finding was the observation that CYP2C and CYP3A mRNAs were strongly induced in monkey livers and human hepatocytes while repressed in rat livers. In conclusion, these data show an excellent correlation between in vivo data on gene expression and activity level of DMEs with results generated in hepatocyte monolayer culture, enabling a reliable estimation of human CYP-induction by EMD. This study also clearly highlighted major differences between primates and rodents in the regulation of all major inducible liver CYPs, with evidence of CYP3A and CYP2C inducibility by PPARα agonists in monkey and humans.
Acknowledgements

We are indebted to Drs. Bernhard Ladstetter and Peter-Jürgen Kramer (Merck Serono) for supporting this study and Dr. Phil Hewitt (Merck Serono) for editing the manuscript. We thank Drs. Francis Cotard and Gilles Chavernac (Merck Serono) for providing pharmacological data on EMD, MDS Pharma Services (France) and Covance (UK) for performing the monkey studies and Dr. Peter Tempel (Merck Serono) for performing the rat study. We also thank Jean-Philippe Guenzi for technical assistance in performing the experiments.
References


Footnotes

Supported by ECVAM grant 19471-2002-05-F1 ED ISP FR.

Lysiane Richert and Gregor Tuschl contributed equally to this work.

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Legends for figures

Figure 1

A Gene expression and B CYP enzyme activity in liver samples from male rats dosed with 3 or 100 mg/kg bw EMD 392949 per day for 13 weeks. Shown are values of fold regulation relative to the untreated control. Bars illustrate mean values from 3 individual samples with standard deviation (SD). Please note that the positive as well as the negative y-axis shows a break. Absolute enzyme activities [pmol/min/mg protein] of untreated controls (B) were as follows (mean ± SD): EROD (Cyp1A) 40 ± 10; PROD (Cyp2B) 3 ± 0; Testosterone 6β-Hydroxylase (Cyp3A) 443 ± 126; Lauric acid 12-Hydroxylase (Cyp4A) 339 ± 104. Capital letters (p-values < 0.01) or lower case letters (p < 0.05) indicate statistical significance. The letter a/A stands for significantly different from control and b/B for significantly different from control and other dose(s) labeled with b/B or c/C. The letter c/C indicates no significant difference from control but significant difference from other dose labeled with c/C or b/B.
**Figure 2**

Gene expression and CYP enzyme activity in liver samples from male monkeys dosed with 30, 100 (B only) or 300 mg/kg bw EMD 392949 per day for 4 weeks. Shown are values of fold regulation relative to the untreated control. Bars illustrate mean values from 3 individual samples with standard deviation (SD). Please note that the negative y-axis in (A) shows a break. Absolute enzyme activities [pmol/min/mg protein] in untreated controls (B) were as follows (mean ± SD): EROD (CYP1A) 387 ± 93; Testosterone 16β-Hydroxylase (CYP2B) 444 ± 82; Testosterone 6β-Hydroxylase (CYP3A) 4950 ± 2300; Lauric acid 12-Hydroxylase (CYP4A) 1980 ± 631. Capital letters (p-values < 0.01) or lower case letters (p < 0.05) indicate statistical significance. The letter a/A stands for significantly different from control and b/B for significantly different from control and other dose(s) labeled with b/B or c/C. The letter c/C indicates no significant difference from control but significant difference from other dose labeled with c/C or b/B.
Figure 3

Gene expression analysis of liver samples from male monkeys dosed with 15 or 150 mg/kg bw EMD 392949 per day for 17 weeks followed by a 4 week treatment-free recovery phase. Shown are values of fold regulation relative to the untreated control. Bars illustrate mean values from 3 individual samples with standard deviation. Please note that the positive as well as the negative y-axis shows a break. Capital letters (p-values < 0.01) or lower case letters (p < 0.05) indicate statistical significance. The letter a/A stands for significantly different from control and b/B for significantly different from control and other dose(s) labeled with b/B or c/C. The letter c/C indicates no significant difference from control but significant difference from other dose labeled with c/C or b/B.
Figure 4

A and B Gene expression and C enzyme activity analysis of primary male rat hepatocyte cultures treated with 30 or 100 µM EMD 392949 or 100 µM fenofibrate for 24 h (A) and 72 h (B and C). Shown are values of fold regulation relative to the untreated control. Bars illustrate mean values from 3 different hepatocyte preparations with standard deviation (SD). Please note that the positive y-axis in (A) shows a break. Absolute enzyme activities [pmol/min/mg protein] of untreated controls (C) were as follows (mean ± SD): EROD (Cyp1A) 27 ± 1; BROD (Cyp2B) 3 ± 3 Testosterone 6β-Hydroxylase (Cyp3A) 70 ± 16; Lauric acid 12-Hydroxylase (Cyp4A) 3368 ± 708. Capital letters (p-values < 0.01) or lower case letters (p < 0.05) indicate statistical significance. The letter a/A stands for significantly different from control and b/B for significantly different from control and other dose(s) labeled with b/B or c/C. The letter c/C indicates no significant difference from control but significant difference from other dose labeled with c/C or b/B.

Figure 5

A and B Gene expression analysis of primary human hepatocyte cultures (donor 1) treated with 30 or 100 µM EMD 392949 or 100 µM fenofibrate for 24 h (A) and 72 h (B). Shown are values of fold regulation relative to the untreated control. Bars illustrate mean values from triplicate measurements with standard deviation. Please note that the negative y-axis in (A) and the positive y-axis in (B) show a break.
Figure 6

A and B Gene expression analysis of primary human hepatocyte cultures (donor 2) treated with 30 or 100 µM EMD 392949 or 100 µM fenofibrate for 24 h (A) and 72 h (B). Shown are values of fold regulation relative to the untreated control. Bars illustrate mean values from triplicate measurements with standard deviation.

Figure 7

A and B Gene expression and C enzyme activity analysis of primary human hepatocyte cultures (donor 3) treated with 30 or 100 µM EMD 392949 or 100 µM fenofibrate for 24 h (A) and 72 h (B and C). Shown are values of fold regulation relative to the untreated control. Bars illustrate mean values from triplicate measurements with standard deviation (SD). Absolute enzyme activities [pmol/min/mg protein] for untreated controls (C) were as follows (mean ± SD): EROD (CYP1A) 22 ± 2; Bupropion-Hydroxylase (CYP2B) 1.5 ± 0.5; Testosterone 6β-Hydroxylase (CYP3A) 97 ± 2; Lauric acid 12-Hydroxylase (CYP4A) 624 ± 90.
Table 1: Characteristics of human liver donors

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<tr>
<th>Donor No.</th>
<th>Date of isolation</th>
<th>Gender</th>
<th>Age</th>
<th>Pathology</th>
<th>Cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10032005</td>
<td>female</td>
<td>57</td>
<td>Alveolar Echinococcosis</td>
<td>85%</td>
</tr>
<tr>
<td>2</td>
<td>09062005</td>
<td>male</td>
<td>49</td>
<td>Renal metastasis</td>
<td>76%</td>
</tr>
<tr>
<td>3</td>
<td>23062005</td>
<td>male</td>
<td>65</td>
<td>Colorectal metastasis</td>
<td>85%</td>
</tr>
</tbody>
</table>
Table 2: Genes analyzed by single-gene real-time PCR, their respective Taqman® Gene Expression Assay numbers and GenBank Accession numbers.

<table>
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<tr>
<th>Species</th>
<th>Gene name</th>
<th>Gene description</th>
<th>Taqman® Assay No.</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
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<tr>
<td>Rat</td>
<td>HNF1α</td>
<td>transcription factor 1, Tcf1</td>
<td>Rn00562020_m1</td>
<td>NM_012669.1</td>
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<tr>
<td>Rat</td>
<td>PPARα</td>
<td>peroxisome proliferator activated receptor alpha</td>
<td>Rn00566193_m1</td>
<td>NM_013196.1</td>
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<tr>
<td>Rat</td>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
<td>Rn00565750_m1</td>
<td>NM_013149.2</td>
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<tr>
<td>Rat</td>
<td>Cyp2B1</td>
<td>cytochrome P450, family 2, subfamily b, polypeptide 1</td>
<td>custom*</td>
<td>AJ_320166.1, U30327.1</td>
</tr>
<tr>
<td>Rat</td>
<td>Cyp3A1</td>
<td>cytochrome P450, family 3, subfamily a, polypeptide 23/ polypeptide 1</td>
<td>Rn01640761_gH</td>
<td>NM_013105.2</td>
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<tr>
<td>Rat</td>
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<td>Rn00561753_m1</td>
<td>NM_012623.2</td>
</tr>
<tr>
<td>Human</td>
<td>HNF1α</td>
<td>transcription factor 1 (TCF1), hepatic nuclear factor (HNF1)</td>
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<td>NM_000545.4</td>
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<td>Human</td>
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<td>NM_001001928.2,</td>
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<td></td>
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<td>NM_005036.4</td>
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<td>aryl hydrocarbon receptor</td>
<td>Hs00169233_m1</td>
<td>NM_001621.3</td>
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<td>NM_005122.2</td>
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<tr>
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<td>Hs00167961_m1</td>
<td>NM_000778.2</td>
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<tr>
<td>Human</td>
<td>MDR1</td>
<td>ATP-binding cassette, sub-family B (MDR/TAP), member 1</td>
<td>Hs00184500_m1</td>
<td>NM_000927.3</td>
</tr>
</tbody>
</table>

* TaqMan® primers and probe were synthesized by Applied Biosystems (Foster City, CA, USA). Sequences (all 5' → 3') TaqMan® probe:

CCCACAGACAAATCT, forward primer GAGTTCTTCTCCTGGGTTCCTGAAAT, reverse primer ACAATATGGCAATGTAATCGAGGAT.
Table 3: Human genes analyzed by Taqman<sup>®</sup> Low Density Arrays, their respective Taqman<sup>®</sup> Gene Expression Assay numbers and GenBank Accession numbers.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene description</th>
<th>Taqman&lt;sup&gt;®&lt;/sup&gt; Assay No.</th>
<th>GenBank Accession No.</th>
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<td>PXR</td>
<td>nuclear receptor subfamily 1, group I, member 2</td>
<td>Hs00243666_m1</td>
<td>NM_033013.1, NM_022002.1, NM_003889.2</td>
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<tr>
<td>CYP1A1</td>
<td>cytochrome P450, family 1, subfamily a, polypeptide 1</td>
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<tr>
<td>CYP1A2</td>
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<td>NM_000761.3</td>
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<td>CYP2B6</td>
<td>cytochrome P450, family 2, subfamily b, polypeptide 6</td>
<td>Hs00167937_g1</td>
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<tr>
<td>CYP2C8</td>
<td>cytochrome P450, family 2, subfamily c, polypeptide 8</td>
<td>Hs00258314_m1</td>
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<td>CYP2C9</td>
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<td>NM_000771.2</td>
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<tr>
<td>CYP3A4</td>
<td>cytochrome P450, family 3, subfamily a, polypeptide 4</td>
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<tr>
<td>MDR1</td>
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<td>ACOX1</td>
<td>acyl-Coenzyme A oxidase 1, palmitoyl</td>
<td>Hs00244513_m1</td>
<td>NM_004035.5</td>
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<tr>
<td>CPT1a</td>
<td>carnitine palmitoyltransferase 1a</td>
<td>Hs00157079_m1</td>
<td>NM_001876.2</td>
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**Table 4:** BLAST results for the amplicon sequences of the listed human Taqman® Gene Expression Assays. Shown are homologies to Macaca fascicularis or other non-human primate species.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Taqman® Assay No.</th>
<th>Sequence identity</th>
<th>Species</th>
<th>homologous gene</th>
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<tbody>
<tr>
<td>HNF1α</td>
<td>Hs00167041_m1</td>
<td>100% (44/44 nt)</td>
<td>Macaca mulatta</td>
<td>XM_001089567.1 PREDICTED: similar to transcription factor 1, hepatic</td>
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<tr>
<td>PPARα</td>
<td>Hs00231882_m1</td>
<td>97% (143/147 nt)</td>
<td>Macaca mulatta</td>
<td>NM_001033029.1 Macaca mulatta peroxisome proliferator-activated receptor alpha (PPARA), mRNA</td>
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<tr>
<td>AhR</td>
<td>Hs00169233_m1</td>
<td>98% (103/105 nt)</td>
<td>Macaca mulatta</td>
<td>XM_001103903.1 PREDICTED: aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>PXR</td>
<td>Hs00243666_m1</td>
<td>97% (65/67 nt)</td>
<td>Macaca fascicularis</td>
<td>AB169411.1 testis cDNA, clone: QtsA-19884, similar to human nuclear receptor subfamily 1, group 1, member 2 (NR1I2), transcript variant 1, mRNA, RefSeq: NM_003889.2</td>
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<tr>
<td>CAR</td>
<td>Hs00231959_m1</td>
<td>97% (82/84 nt)</td>
<td>Macaca mulatta</td>
<td>NM_001032896.1 constitutive androstane receptor (CAR) AY116212.1 CAR complete cds</td>
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<td>CYP1A1</td>
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<td>Macaca fascicularis</td>
<td>D17575.1 MACCP450 mRNA for cytochrome P-450, complete cds</td>
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<td>CYP1A2</td>
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(Table 4 continued)

<table>
<thead>
<tr>
<th>Gene name</th>
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<td>96% (48/50 nt)</td>
<td>Macaca fascicularis</td>
<td>S53046.1 cytochrome P-450 2C liver, mRNA</td>
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<td>CYP2C9</td>
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<td>DQ074805.1 CYP2C75 mRNA, complete cds /</td>
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<td></td>
<td></td>
<td>95% (141/148 nt)</td>
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<td>DQ074806.1 CYP2C43 mRNA, complete cds</td>
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<td>CYP3A4</td>
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<td></td>
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<td></td>
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<td>MDR1</td>
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<td>ACOX1</td>
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<td>(CPT1A), mRNA</td>
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This article has not been copyedited and formatted. The final version may differ from this version.
Table 5: Rat genes analyzed by Taqman® Low Density Arrays, their respective Taqman® Gene Expression Assay numbers and GenBank Accession numbers.

<table>
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<th>Gene name</th>
<th>Gene description</th>
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<tr>
<td>PXR</td>
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<tr>
<td>CAR</td>
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<td>NM_031559.1</td>
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Table 6: ACOX enzyme activity in liver samples from rat and monkey in vivo studies (see Material and Methods). Fold induction compared to each control (set as 1) is given in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>ACOX enzyme activity [nmol/(min*mg protein)]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>rat 13-wk</strong></td>
<td></td>
</tr>
<tr>
<td>0 mg/kg/day (control)</td>
<td>2.02 ± 1.32</td>
</tr>
<tr>
<td>3 mg/kg/day EMD</td>
<td>3.96 ± 1.84 c (1.96x)</td>
</tr>
<tr>
<td>100 mg/kg/day EMD</td>
<td>8.67 ± 1.90 A, b (4.29x)</td>
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<td><strong>monkey 4-wk</strong></td>
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<td>0 mg/kg/day (control)</td>
<td>2.68 ± 0.65</td>
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<tr>
<td>30 mg/kg/day EMD</td>
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<tr>
<td>300 mg/kg/day EMD</td>
<td>2.79 ± 1.32 (1.04x)</td>
</tr>
<tr>
<td><strong>monkey 17-wk</strong></td>
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</tr>
<tr>
<td>0 mg/kg/day (control)</td>
<td>1.75 ± 1.19</td>
</tr>
<tr>
<td>15 mg/kg/day EMD</td>
<td>1.67 ± 0.25 (-1.05x)</td>
</tr>
<tr>
<td>150 mg/kg/day EMD</td>
<td>0.82 ± 0.62 (-2.13x)</td>
</tr>
<tr>
<td>0 mg/kg/day (control) (recovery)</td>
<td>0.96 ± 0.64</td>
</tr>
<tr>
<td>150 mg/kg/day EMD (recovery)</td>
<td>1.46 ± 0.19 (1.52x)</td>
</tr>
</tbody>
</table>

c, A, b Capital letters (p-values < 0.01) or lower case letters (p < 0.05) indicate statistical significance. The letter A stands for significantly different from control and b for significantly different from control and other dose(s) labeled with b or c. The letter c indicates no significant difference from control but significant difference from other dose labeled with c or b.
Figure 1

A

B

-3

-2

0

+1

+2

+3

+4

+5

+6

+7

+8

+9

+10

+11

+12

+13

+14

+15

EMD 3 mg/kg

EMD 100 mg/kg

HNF1α

PPARα

AhR

PXR

CAR

Cyp1A1

Cyp1A2

Cyp2B1

Cyp2C

Cyp3A1

Cyp4A3

ACOX1

CPT1a

EROD (Cyp1A)

PROD (Cyp2B)

Testosterone 6β-Hydroxylase (Cyp3A)

Lauric acid 12-Hydroxylase (Cyp4A)

fold change

fold change

figure 1
Figure 4

A. Graph showing fold change for various genes with treatments of EMD 30 μM, EMD 100 μM, and Fenofibrate 100 μM.

B. Graph showing fold change for various genes with treatments of EMD 30 μM and EMD 100 μM.

C. Bar chart showing fold change for EROD (Cyp1A1), BRD (Cyp2B), Testosterone 6β-Hydroxylase (Cyp3A), and Lauric acid 12-Hydroxylase (Cyp4A).
A

![Graph A](image)

B

![Graph B](image)

figure 6