Fibrates induce *mdr2* gene expression and biliary phospholipid secretion in the mouse

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Disruption of the murine *mdr2* gene leads to the complete absence of biliary phospholipids. We tested the hypothesis that the increase in biliary phospholipid output induced by fibrates is mediated via induction of the hepatic *mdr2* gene and its encoded product, the P-glycoprotein canalicular flippase. Increased levels of *mdr2* mRNA were observed in the liver of mice treated with different fibrates: ciprofibrate, $660 \pm 155\,\%$ (as compared with control group); clofibrate, $611 \pm 77\,\%$; bezafibrate, $410 \pm 47\,\%$; fenofibrate, $310 \pm 52\,\%$; gemfibrozil, $190 \pm 25\,\%$ ($P < 0.05$ compared with control group). Induction of expression of the *mdr* gene family was specific to the *mdr2* gene. Two- to three-fold increases in P-glycoprotein immunodetection were evident on the canalicular plasma-membrane domain of clofibrate- and ciprofibrate-treated mice. Biliary phospholipid output increased from 4.2 ± 1.2 nmol/min per g of liver in the control group to 8.5 ± 0.6, 7.1 ± 2.9 and 5.8 ± 2.5 in ciprofibrate-, clofibrate- and bezafibrate-treated mice respectively ($P < 0.05$ compared with control group). Moreover, a significant correlation between biliary phospholipid output and the relative levels of *mdr2* mRNA was found ($r = 0.86$; $P < 0.05$). In treated animals, bile flow as well as cholesterol and bile acid outputs remained unchanged. Our findings constitute the first evidence that pharmacological modulation of biliary lipid secretion mediated by fibrates can be related to the overexpression of a specific liver gene product, the *mdr2* P-glycoprotein, and are consistent with the hypothesis that the *mdr2* P-glycoprotein isoform plays a crucial role in the secretion of biliary phospholipid.

INTRODUCTION

The multiple drug-resistance (*MDR*) gene products, named P-glycoproteins (P-gps), are plasma-membrane proteins initially identified through their ability to confer the multidrug-resistance phenotype in mammalian tumour cells [1]. These active transporters couple the hydrolysis of ATP to the translocation of a variety of substrates across biological membranes (for reviews, see refs. [2,3]). The sequence and domain organization of P-gps are typical of the ATP-binding cassette (ABC) superfamily of active transporters found in several prokaryotic and eukaryotic cell types [3–5]. The mammalian P-gps are encoded by a small linked multigene family, two in humans and three in rodents. In humans, the genes are named *MDR1* and *MDR3* (also called *MDR2*) [6,7]. In mice they are named *mdr1* (or *mdr1b*), *mdr2* and *mdr3* (or *mdr1a*) [8–10].

The multidrug-resistance gene family is expressed not only in naturally occurring human tumours and multidrug-resistant tumour cell lines [11], but also in normal mammalian tissues [12,13]. P-gps are usually located on the apical domain of epithelial cells. On the basis of this strategic tissue distribution, it has been proposed that the physiological function of P-gps is to facilitate cellular excretion of xenobiotics and endogenous compounds [13–17].

Analysis of RNA and protein expression has revealed that P-gps are distributed in a tissue-specific and species-conserved manner [8,9], and the *mdr2* P-gp is expressed predominantly in the canalicular plasma-membrane domain of hepatocytes [8,15].

Even though *mdr1* and *mdr2* have 90 % nucleotide sequence identity, overexpression of *mdr2* does not confer the multidrug-resistance phenotype [3]. Until recently, attempts to find a function for the mouse *mdr2* gene and its human homologue had been unsuccessful. Using a knockout approach, it has been shown that the homozygous disruption of the murine *mdr2* P-gp gene leads to the complete absence of biliary phospholipids and to the development of a liver disease characterized by small-bile-duct destruction [16]. These results suggested that the *mdr2* P-gp is a flippase or a phospholipid transporter and has an essential role in the secretion of phospholipid into bile [16]. The evidence that the *mdr2* P-gp is a phosphatidylcholine translocase, or flippase, is that the murine *mdr2* gene has been shown to encode an ATP-dependent phosphatidylcholine-specific phospholipid transporter [18]. The human *MDR3* P-gp may also function as a phospholipid flippase, since the *MDR3* P-gp isoform can promote the transfer of phosphatidylcholine from the inner to the outer leaflet of the plasma membrane in fibroblasts transfected with the human *MDR3* gene [19].

It is well known that hypolipidaemic drugs such as fibrates induce several genes required for the peroxisomal β-oxidation of long-chain fatty acids, genes of the cytochrome *P*-450 IV family, as well as genes encoding proteins involved in cell growth and cell proliferation [20,21]. The effect of fibrates on *mdr* gene expression has not been reported. Taking advantage of our preliminary observation that fibrates increase biliary phospholipid output in mice, we tested the hypothesis that this effect may be due to the *in vivo* induction of *mdr2* gene expression in the liver.

We found that clofibrate and some of its structurally related compounds increase the steady-state level of hepatic *mdr2* mRNA, its encoded product, and simultaneously stimulate biliary phospholipid output. Our findings constitute the first evidence that pharmacological modulation of biliary lipid secretion, mediated by fibrates, can be related to the overexpression

Abbreviations used: *mdr*, multidrug-resistance gene in mice; P-gp, P-glycoprotein; ABC, ATP-binding cassette; ALP, alkaline phosphatase; mAb, monoclonal antibody.

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of a specific liver gene product, the *mdr2* P-gp, and are consistent with the hypothesis that the *mdr2* P-gp plays a crucial role in the secretion of phospholipids into bile.

EXPERIMENTAL

Animals and diet

Male CF I mice, weighing between 25 and 28 g, were used in these studies. The animals were housed in wire-floored cages, fed *ad libitum* and kept for at least 1 week under the same environmental conditions. All mice received human care as outlined in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 86-23, revised 1986). Control mice were fed for 1 week on a diet containing 28 % casein, 40 % sucrose, 19 % cellulose, 10 % corn oil, 1 % vitamin mix, 0.3 % DL-methionine and 2 % salt mix, prepared according to the recommendations of the American Institute of Nutrition. Experimental groups received a similar diet supplemented with 0.2 % or 0.3 % clofibrate (ICI Pharmaceuticals, Macclesfield, Cheshire, U.K.), 0.005 % ciprofibrate (Winthrop Products, New York, NY, U.S.A.), 0.5 % bezafibrate (Boehringer-Mannheim, Mannheim, Germany), 0.5 % fenofibrate (Bristol Laboratories, Evansville, IN, U.S.A.) or 0.5 % gemfibrozil (Parke-Davis & Co., Morris Plains, NJ, U.S.A.). The different fibrates used were dissolved in ethanol, mixed with the diet and the solvent was evaporated at room temperature under a hood. The doses of clofibrate and its analogues were in the range used in rodents and were selected according to previous studies [22–24]. All mice receiving test compounds had the same estimated daily food consumption as control animals (i.e. 1.5 g).

Bile and liver sampling

On the day of the experiments, mice were anaesthetized with intraperitoneal pentobarbital (45 mg/kg; Nembutal, Abbott Laboratories, North Chicago, IL, U.S.A.) at 08.00–09.30 h. The common bile duct was cannulated with a PE10 polyethylene catheter (Clay-Adams, New York, NY, U.S.A.). Bile was collected for 30 min keeping body temperature between 37 and 37.5 °C. Each group of treated mice was compared with a control group ($n = 3$) from the same litter. After bile sampling, the liver was removed, weighed, rapidly frozen in liquid nitrogen and stored at −70 °C until processing. In some experiments, liver samples were taken for morphological and immunohistochemical analysis. Biliary cholesterol, phospholipids and bile acids were quantified [25], and biliary bile acid pool composition was estimated by HPLC as previously described [26].

Probes

Specific mouse *mdr1*, *mdr2* and *mdr3* partial cDNA probes were prepared using reverse transcriptase PCR as previously described [27]. Murine *mdr*-specific oligonucleotide primers were selected and synthesized (Chiron Corporation, Emeryville, CA, U.S.A.), based on the published cDNA sequences [8–10]. An 18S rRNA partial cDNA probe was also prepared and used in the experiments to confirm comparable RNA loading in Northern-blot experiments. The 18S rRNA cDNA oligonucleotide primers were: upstream, 5′-TAGAGCTAATACATGCCGACG-3′ (163–168); downstream, 5′-TTAATCATGGCCTCAGTTCCG-3′ (1901–1921) [28]. The probes were labelled to high specific radioactivity (1×10^9 d.p.m./μg of DNA) with [α-^{32}P]dCTP (NEN Research Products, Boston, MA, U.S.A.; 3000 Ci/mmol) using the method described previously [29].

RNA extraction

Total cellular RNA was extracted from liver using the guanidinium isothiocyanate/phenol method [30]. The quantity and purity of the RNA were estimated spectrophotometrically and the integrity was checked on 1 % agarose gel containing 2.2 M formaldehyde. Samples in which the rRNAs appeared to be intact were used in the experiments.

Northern-blot analysis

Total cellular RNA was separated by electrophoresis on horizontal 1 % agarose gels containing 2.2 M formaldehyde and transferred to nylon filters (NEN Research Products) [31]. Quantity, integrity and efficiency of transfer of RNAs were checked by staining the 18S and 28S rRNA bands with ethidium bromide before and after transferring the RNA from the gels to the membranes. The filters were baked for 2 h at 80 °C and prehybridized in 0.1 ml/cm² of a solution containing 1 M NaCl, 1 % SDS, 10 % dextran sulphate and 100 μg/ml salmon sperm DNA. Prehybridization was carried out at 65 °C for 2 h. The hybridization was performed overnight at 65 °C in the same solution containing the labelled cDNA probes. After hybridization, the filters were washed once at 65 °C in 0.2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) with 0.1 % SDS and twice with 0.1 × SSC/0.1 % SDS at 65 °C.

Analysis of the autoradiograph was performed using Kodak X-Omat AR film at −70 °C. Densitometric analysis of the autoradiograph was performed using a CS-9000 scanning densitometer (Shimadzu, Kyoto, Japan) and the relative content of *mdr2* mRNA was expressed as arbitrary densitometric units.

Western-blot analysis

Western-blot analysis was performed using proteins isolated from liver plasma-membrane fractions enriched in the bile canalicular domain. These fractions were prepared from control and treated animals as previously described [32,33]. In brief, thin liver slices were added to chilled buffer (300 mM mannitol, 5 mM EGTA, 18 mM Tris/HCl and 0.1 mM PMSF at pH 7.4). The slices were homogenized using a Polytron apparatus (Kinematica, Littau, Switzerland). The homogenate was centrifuged at 48 000 ***g*** for 30 min. The resulting pellet was resuspended in the same buffer as above containing 15 mM $MgCl_2$; precipitation was followed by centrifugation for 30 min at 2445 ***g*** to obtain the canalicular-membrane-enriched fraction. Protein concentration was determined by the Lowry assay with BSA as standard [34]. We determined alkaline phosphatase (ALP) activity in homogenate and canalicular membranes by measuring the production of *p*-nitophenol from *p*-nitrophenyl phosphate by monitoring A_{405} as previously described [33]. Canalicular-membrane proteins (25 μg) were suspended in 30 μl of electrophoresis buffer containing 0.5 % SDS and 10 % glycerol. Electrophoresis in 8 % polyacrylamide gel was carried out as described by Laemmli [35], without heating the samples. Proteins were then transferred electrophoretically to nitrocellulose filters. For immunoblotting, the nitrocellulose membranes were blocked with 5 % skimmed milk in TBS-T buffer (20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 0.05 % Tween 20) for 1 h at room temperature and then incubated in the same solution for 2 h with 1 μg/ml C219 monoclonal antibody (C219 mAb) (Signet Laboratories, Dedham, MA, U.S.A.) [36]. The membranes were washed four times with TBS-T buffer. All washing and incubation steps were performed at room temperature.

After washing, the binding of the C219 mAb was visualized

using the enhanced chemiluminescence procedure (Amersham International, Amersham, Bucks., U.K.).

Immunohistochemical analysis

For immunohistochemistry, we followed the protocol described previously [17]. Frozen livers were sectioned at 5 μm thickness, air-dried and fixed in acetone for 10 min at room temperature. After preincubation with 10 % normal rabbit serum, sections were incubated for 1 h with C219 mAb (16 μg/ml). A streptavidin-biotin–horseradish peroxidase procedure (Dako Corporation, Carpinteria, CA, U.S.A.) was used for detection of the primary antibody. After two rinses at room temperature in PBS, slices were incubated with biotinylated rabbit anti-mouse IgG antibody (diluted 1:1000) for 30 min, followed by three rinses at room temperature with PBS. The slices were then incubated with the streptavidin–biotin–peroxidase complex for 30 min, rinsed and incubated for 10–15 min in a 3-amino-9-ethylcarbazole solution. A light haematoxylin counterstain was used. As a control for background staining, parallel incubations were performed without primary antibody.

Statistical analysis

A non-paired Student's *t* test program was used for comparison of differences between means.

RESULTS

Effect of fibrates on *mdr2* gene expression in liver

First, we studied the effect of fibrates on the expression of *mdr2* gene. A Northern-blot analysis of *mdr2* mRNA levels in liver of mice fed on a casein diet containing 0.2 % or 0.3 % clofibrate showed a specific hybridization signal of about 4.5 kb corresponding to *mdr2* mRNA (Figure 1). The steady-state levels of *mdr2* mRNA were clearly greater in livers of treated mice than in livers of control mice. To confirm comparable RNA loading in each lane, the filter was also hybridized with an 18S rRNA cDNA. The time course of clofibrate-mediated induction of *mdr2* gene was also analysed by a Northern-blot experiment in which livers of clofibrate-fed mice were removed at 1, 2, 3, 5 and 7 days. The increase in *mdr2* mRNA level was observed as early as 24 h and a steady-state level of mRNA was reached at 48 h after the mice ate clofibrate (results not shown).

To assess the effect of clofibrate on the other members of the *mdr* gene family, a Northern blot containing total RNA isolated from the livers of control and clofibrate-fed mice was prepared and hybridized to *mdr1* and *mdr3* cDNA probes. Figure 2 shows that clofibrate induced only the *mdr2* gene. These results show that the clofibrate-mediated induction of the *mdr* gene family was specific for the *mdr2* gene in the mouse liver.

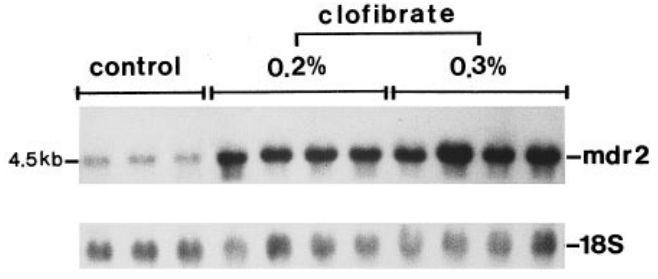

Figure 1 Effect of clofibrate on *mdr2* gene expression in the murine liver

Total RNA (10 μg) isolated from liver of control ($n = 3$) and clofibrate-fed animals ($n = 4$) was hybridized by Northern blot to a radiolabelled *mdr2* cDNA probe (upper panel). 18S rRNA is shown in the lower panel as a loading control of RNA.

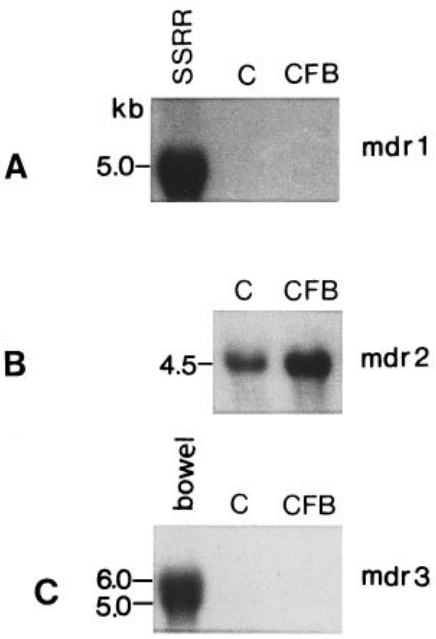

Figure 2 Effect of clofibrate on the expression of the *mdr* gene family

The expression of the *mdr* gene family was analysed by Northern blot using reverse transcriptase-PCR-generated cDNA probes as described in the Materials and methods section. For *mdr1* gene expression (**A**), 10 μg of total RNA extracted from liver of control (C) and 0.3 % clofibrate-fed animals (CFB) were electrophoresed through a formaldehyde-denatured 1 % agarose gel, transferred to nylon membrane and hybridized to the *mdr1* (*mdr1b*) radiolabelled cDNA probe. Suprarenal gland (SSRR), a tissue with a high *mdr1* mRNA content, was used as a positive control of mouse *mdr1* hybridization. For *mdr2* gene expression (**B**), 10 μg of total RNA extracted from liver (control and clofibrate-treated mice) was blotted and hybridized to the *mdr2* radiolabelled cDNA probe. For *mdr3* gene expression (**C**), similar amounts of RNA extracted from liver (control and clofibrate-fed mouse) were prepared in a Northern-blot analysis and hybridized to the *mdr3* (*mdr1a*) radiolabelled cDNA probe. Small bowel, a tissue with a high *mdr3* mRNA content, was used as a positive control of mouse *mdr3* hybridization.

Because fibrate analogues show marked potency differences on the hypolipidaemic effect and peroxisome proliferation in rodent liver [21,37], we further studied the effect of clofibrate analogues on *mdr2* gene expression. The relative level of *mdr2* mRNA was estimated by densitometric analysis of Northern-blot membranes and expressed as a percentage of the level found in control mice. Clofibrate, in two different doses, and all the analogues tested induced *mdr2* gene expression in the liver, but the clofibrate analogues had a markedly different ability to induce *mdr2* gene expression at the doses used. Clofibrate induced the greatest *mdr2* gene expression (660 % of control), and gemfibrozil the lowest (190 % of control) (Table 1).

Expression of P-gp in the mouse liver

Western-blot analysis was performed to determine whether increased hepatic *mdr2* gene expression was followed by increased expression of its encoded product, the P-gp. The C219 mAb used in these experiments reacts with an epitope common to all isoforms of the P-gp family. Enrichment of the specific activities of ALP, which is a marker enzyme of the bile canalicular-membrane domain, from liver homogenate to isolated mem-

Table 1 Effect of clofibrate analogues on *mdr2* gene expression

Groups of mice were fed for 1 week with the indicated amount of the following clofibrate analogues: ciprofibrate, bezafibrate, fenofibrate and gemfibrozil. Total RNA was extracted from the liver, separated by electrophoresis and hybridized to the mouse radiolabelled *mdr2* cDNA probe. The level of *mdr2* mRNA was estimated by densitometric analysis of the resulting autoradiograph and expressed as a percentage of that found in control animals. The values represent means ± S.D. $^*P < 0.05$ for treated group compared with control group.

Drug	Amount in diet (%)	*n*	*mdr2* mRNA (% of control value)
Control		22	100 ± 35
Ciprofibrate	0.005	4	660 ± 155*
Clofibrate	0.3	5	611 ± 77*
Clofibrate	0.2	8	516 ± 120*
Bezafibrate	0.5	7	410 ± 47*
Fenofibrate	0.5	5	310 ± 52*
Gemfibrozil	0.5	5	190 ± 25

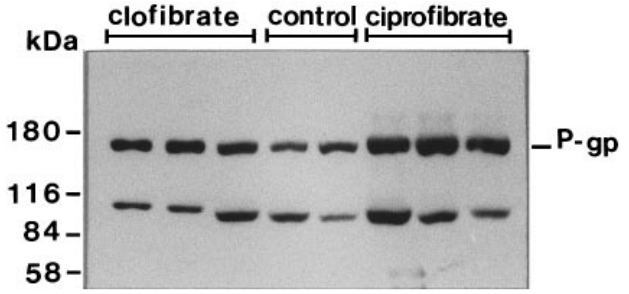

Figure 3 Effect of clofibrate and ciprofibrate on P-gp expression in the mouse liver

Proteins (25 μg) isolated from liver plasma-membrane fractions enriched in bile canalicular domain of control ($n = 2$), 0.3% clofibrate-treated ($n = 3$) and 0.005% ciprofibrate-treated ($n = 3$) mice were subjected to SDS/PAGE and transferred to a nitrocellulose filter. P-gp was immunodetected using the C219 mAb. Sigma molecular-mass markers (kDa) are indicated on the left of the immunoblot.

branes was measured. The specific activities of ALP were enriched by 47-fold in the control group, 37-fold in clofibrate-fed mice and 39-fold in ciprofibrate-fed mice ($n = 2$ in each group). The relative specific activities of ALP in plasma-membrane fractions of mice were similar to the relative specific activities of ALP found in canalicular-domain-enriched plasma-membrane fractions described previously in rats [33]. A molecular-mass range between 155 and 170 kDa was estimated for the immunoreactive P-gp protein (Figure 3). The relative content of P-gp, expressed in arbitrary densitometric units was: control, 1.0 ± 0.2; clofibrate-treated, 1.8 ± 0.2; and ciprofibrate-treated, 2.6 ± 0.5 ($P < 0.05$ for each treated group compared with control group). The additional 95 kDa species detected by C219 mAb is probably a previously reported proteolytic fragment [15,38].

To assess whether the increased expression of *mdr2* mRNA and its encoded product resulted in changes in the pattern of distribution of P-gp in liver, immunohistochemical analysis of liver tissue sections was also performed. By light microscopy, the immunoreactive signal was visible as anastomosing networks of branched channels between adjacent cells predominantly located at the canalicular domain of the plasma membrane of hepatocytes. In treated mice, stronger P-gp immunostaining was

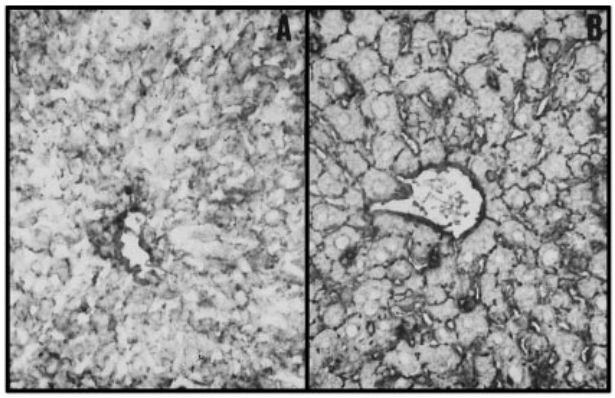

Figure 4 Immunohistochemical analysis of P-gp in the mouse liver

Immunohistochemistry was performed on liver sections of control (left) and 0.005% ciprofibrate-treated (right) mouse liver using C219 mAb. Staining indicates binding of C219 mAb as visualized by a streptavidin–biotin–peroxidase method. Light haematoxylin counterstaining was used. Magnification × 290.

observed, indicating that the newly synthesized P-gp had been incorporated on the canalicular domain of liver cells (Figure 4).

Effect of fibrates on biliary lipid secretion

Mice consumed the different diets equally well, and all groups increased weight similarly during the week (mean increase 26.2%; range 21.6–29.3%). As expected, a substantial increase in liver weight was a consistent finding in mice treated with peroxisome proliferators (Table 2).

Biliary phospholipid output increased in treated mice given all drugs except fenofibrate and gemfibrozil (Table 2). Bile flow and bile acid output were unchanged in the experimental groups. In addition, the profile of the relative bile acid composition, determined by HPLC in clofibrate- and ciprofibrate-treated mice, was similar to that found in control mice (results not shown). The phospholipid/bile salt molar ratio increased in ciprofibrate- and clofibrate-treated mice from 0.11 in control to 0.22 and 0.19 respectively (Table 2). The overall results of these experiments demonstrated that biliary phospholipid output increased independently of the rate of bile acid output and was not related to changes in the biliary bile acid pool composition.

Remarkably, we observed a significant correlation between biliary phospholipid output and the relative levels of *mdr2* mRNA in fibrate-treated mice ($r = 0.86$; $P < 0.05$) (Figure 5).

DISCUSSION

We report the novel effect of fibrates on *mdr2* gene expression and its encoded P-gp in the mouse liver. We found that fibrates increase the steady-state level of *mdr2* mRNA, and this biological effect seems to be specific for the *mdr2* gene, as we did not observe induction of the two other members of the *mdr* gene family. In addition, we observed that the most active inducers of *mdr2* gene expression were ciprofibrate and clofibrate. These findings are in agreement with previous studies that have compared the hepatic effect of fibrates on peroxisome proliferation in rodent liver [21,37]. It is well known that ciprofibrate and clofibrate are the most active peroxisome proliferators, and therefore our observations suggest that the structural requirements for production of hepatic peroxisome proliferation are also required for *mdr2* gene induction. Interestingly, one of the

Table 2 Effect of hypolipidaemic peroxisome proliferators on bile composition

CF I mice were fed for 1 week on a control diet supplemented with fibrates, and bile was collected for 30 min. Bile flow, cholesterol, phospholipid and bile-salts output were quantified as described in the text. The values are means ± S.D. The results of the six control groups ($n = 3$ mice in each group) were pooled. Differences that are significant are indicated: *$P < 0.0005$; †$P < 0.005$; ‡$P < 0.05$. ND, Not determined.

Drug	Amount in diet (%)	*n*	Liver weight (g)	Bile flow (μl/min per g of liver)	Biliary lipid output (nmol/min per g of liver)			Phospholipids
					Phospholipid	Cholesterol	Bile salts	Bile salts
Control		18	1.3 ± 0.2	1.0 ± 0.4	4.2 ± 1.2	1.1 ± 0.4	38.0 ± 18.1	0.11
Ciprofibrate	0.005	4	2.6 ± 0.4*	1.0 ± 0.2	8.5 ± 0.6*	1.5 ± 0.4	39.0 ± 14.0	0.22
Clofibrate	0.3	6	2.0 ± 0.3†	0.9 ± 0.2	7.1 ± 2.9†	1.1 ± 0.6	37.8 ± 5.1	0.19
Clofibrate	0.2	8	2.0 ± 0.3*	0.8 ± 0.2	6.1 ± 2.3†	0.9 ± 0.2	33.5 ± 18.8	0.18
Bezafibrate	0.5	8	2.4 ± 0.5*	1.1 ± 0.2	5.8 ± 2.5‡	1.2 ± 0.2	35.4 ± 12.0	0.16
Fenofibrate	0.5	5	2.5 ± 0.8*	1.1 ± 0.3	5.3 ± 3.3	1.6 ± 0.2	39.4 ± 7.0	0.13
Gemfibrozil	0.5	4	2.6 ± 0.4*	0.7 ± 0.3	3.3 ± 2.4	ND	ND	ND

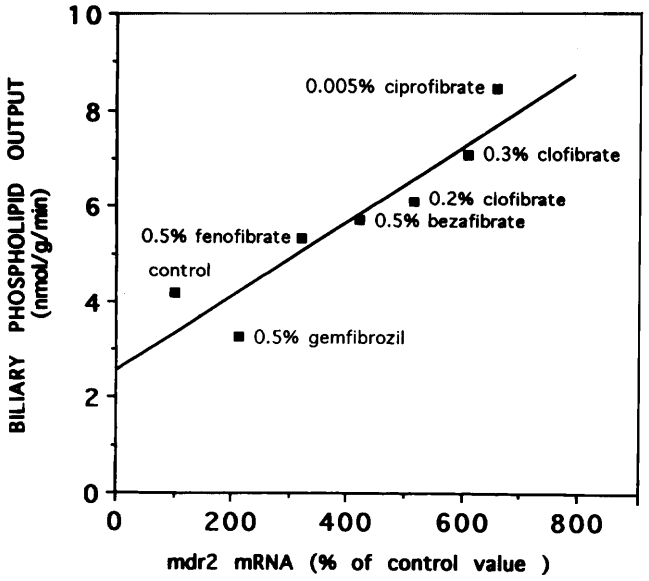

Figure 5 Relationship between biliary phospholipid output and relative level of *mdr2* mRNA in mouse liver: effect of fibrates

Biliary phospholipid output, expressed in nmol/min per g, shown in Table 2, and the relative hepatic content of *mdr2* RNA in fibrate-treated mice, expressed as a percentage of control value, were plotted using simple linear-regression analysis ($r = 0.86$; $P < 0.05$).

major integral membrane proteins of the liver peroxisomes, the 70 kDa peroxisomal-membrane protein, is also a member of the ABC superfamily of active transporters that is induced by peroxisome proliferators in rat liver [39].

Hypolipidaemic fibrates are peroxisome proliferators that promote the activation of genes encoding key metabolic enzymes in peroxisomes, microsomes and mitochondria as well as genes encoding proteins involved in cell growth and cell proliferation [21]. The mechanisms by which peroxisome proliferators regulate gene expression are not completely understood and it has been postulated that fibrates are able to modulate specific gene transcription through the activation of transcription regulatory factors called peroxisome proliferator-activated receptors (for reviews see refs. [21,40]). It has also been proposed that peroxisomal-protein-encoding genes and other genes may be regulated by common mechanisms [21]. The increased hepatic *mdr2* mRNA levels found in the present study could result from increased gene transcription, mRNA stabilization or both. In fact, both transcriptional and post-transcriptional mechanisms are involved in *mdr* gene regulation in rodent cell lines treated with several xenobiotics [3,41]. Further studies are required to define the molecular mechanisms of fibrate-mediated over-expression of the *mdr2* gene.

The increase in *mdr2* mRNA levels was associated with higher expression of P-gp in the liver of clofibrate- and ciprofibrate-treated mice shown by Western-blot analysis using canalicular-membrane proteins. Immunohistochemical analysis extended these findings, showing a stronger immunodetection of P-gp at the canalicular domain of hepatocytes of clofibrate-treated mice, suggesting that the newly synthesized P-gp was incorporated at the canalicular domain of liver cells. The use of the C219 mAb, which reacts with an epitope common to all isoforms of the P-gp family, did not allow us to define the specific isoform that was induced in response to ciprofibrate or clofibrate. However, we found that these drugs increased *mdr2* mRNA levels only and therefore our findings strongly suggest that the *mdr2* P-gp was the induced isoform.

Clofibrate and ciprofibrate increased the steady-state level of *mdr2* mRNA and its encoded P-gp in the canalicular membrane and concomitantly increased the secretion of phospholipid into bile. Moreover, a significant correlation was found between the levels of hepatic *mdr2* mRNA in fibrate-treated mice and biliary phospholipid secretion. Therefore our findings are consistent with the novel hypothesis that the *mdr2* P-gp plays an important role in the process of biliary phospholipid secretion through the canalicular membrane of hepatocytes and is a determinant of the amount of phospholipid available for bile secretion.

One of the major determinants of the amount of phospholipid and cholesterol secreted into the bile is the rate of biliary bile acid secretion in all species [42]. Although liver peroxisomes are the major site of bile acid synthesis, there is no increase in the conversion of bile acid intermediates into cholic acid after clofibrate treatment [21,43]. Both the increased biliary phospholipid/bile salt molar ratio and the unmodified biliary bile salt secretion observed in ciprofibrate- and clofibrate-treated mice indicate that the enhancement of biliary phospholipid output was determined by a bile salt-independent mechanism. Interestingly, our findings are in line with recent observations that have shown in heterozygous (+/−) mice for *mdr2* gene disruption that, at various bile-salt-output rates, phospholipid secretion was 30–50% lower than in wild-type mice (+/+) and was negligible in homozygous (−/−) mice, indicating that *mdr2*

P-gp exerts crucial control over biliary phospholipid secretion [44].

Several dietary or pharmacological manipulations may modify and uncouple the rate of biliary phospholipid secretion from bile acid secretion [26,45]. We have previously shown that colchicine selectively induces the *mdr2* gene and its encoded P-gp in mouse liver [27], even though colchicine, like other agents that affect microtubular polymerization, greatly reduce biliary lipid secretion [46]. The only other known compound able to modulate the *mdr2* gene is α-naphthyl isothiocyanate, a xenobiotic that induces the gene expression in monkeys but not in rats, and the effect of this agent on biliary phospholipid secretion has not been studied. [47]. Therefore the present study represents the first evidence that a pharmacological modulation of biliary phospholipid secretion can be related to the overexpression of the canalicular *mdr2* Pgp.

The effect of fibrates on the pleiotropic response of peroxisome proliferation appears to be species-specific since, although fibrates induce peroxisome proliferation in the mouse, rat and hamster, there is no evidence for this effect in primates [48,49]. These differences may explain why in man, in contrast with our findings in mouse (Table 2), fibrate derivatives increase the cholesterol concentration of bile and decrease the bile acid concentration of bile, resulting in an increase in the cholesterol saturation level [50]. Turley et al. [51] have shown in rats that clofibrate causes a non-significant increase in biliary phospholipid output, without any change in bile flow or bile acid or cholesterol output [51].

Recent evidence supports the concept that native biliary cholesterol and phospholipid represent important cytoprotective factors for hepatocyte and biliary epithelial cells against bile acid-induced damage [33]. Theoretically, biliary phospholipid deficiency may have a role in hepatobiliary disorders characterized by cholangiocyte destruction [16]. In this context, the development of pharmacological or dietary models for modulation of human *MDR2* gene stimulating phospholipid output into bile may have important therapeutic implications in some cholestatic liver diseases.

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