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Identification of HMG-CoA reductase inhibitors as activators for human, mouse and rat constitutive androstane receptor (CAR)

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Running title: HMG-CoA reductase inhibitors are CAR activators in humans

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Abbreviations: CYP, cytochrome P450; CAR, constitutive androstane receptor; PBREM, phenobarbital responsive enhancer module; LBD, ligand-binding domain; hCAR, human CAR; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; mCAR, mouse CAR; prPXRE, proximal PXR response element; PXR, pregnane X receptor: rCAR, rat CAR; XREM, xenobiotic-responsive enhancer module

ABSTRACT

Constitutive active (or androstane) receptor (CAR, NR1I3), a member of the nuclear receptor family, is a major regulator for induction of cytochrome P450 2B (*CYP2B*) genes by phenobarbital. Phenobarbital-like inducer, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), is a potent mouse CAR (mCAR) ligand that has been used to study CAR target genes in mice but does not activate human CAR (hCAR) or rat CAR (rCAR). Although 6-(4-chlorophenyl) imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) was reported to be an hCAR agonistic ligand, activation of hCAR by CITCO in cell-based reporter assay was weak. Therefore, we performed a screening of 50 drugs and chemicals using cell-based reporter assays to identify activators of hCAR. Among them, HMG-CoA reductase inhibitors (cerivastatin, simvastatin, fluvastatin and atorvastatin) enhanced the hCAR-mediated transcriptional activation of PBREM reporter gene by up to 3 fold. Similar activation by HMG-CoA reductase inhibitors was also observed with mouse and rat CARs. On the other hand, pravastatin did not activate hCAR at the concentrations tested (up to 30 μM). The extent of activation by the HMG-CoA reductase inhibitors was stronger than that by CITCO. Cerivastatin, simvastatin, fluvastatin and atorvastatin induced CYP2B6 mRNA in stable hCAR-expressed FLC7 cells but not in original FLC7 cells. Therefore, we concluded that CAR mediates the effects of HMG-CoA reductase inhibitors on the induction of *CYP2B* genes, although HMG-CoA reductase inhibitors also activate pregnane X receptor. HMG-CoA reductase inhibitors such as cerivastatin would be useful to study for elucidating molecular and cellular mechanisms of hCAR.

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Introduction

Constitutive active (or androstane) receptor (CAR, NR1I3), a member of the nuclear receptor family, is a major regulator of hepatic cytochrome P450 (CYP) 2B1 in rats (Muangmoonchai et al., 2001), CYP2B10 in mice (Honkakoski and Negishi, 1997; Kawamoto et al., 1999) and CYP2B6 in humans (Sueyoshi et al., 1999). Treatment of primary hepatocytes with phenobarbital results in the translocation of CAR into the nucleus, where CAR binds to phenobarbital responsive enhancer module (PBREM) located upstream of the *CYP2B* gene as a heterodimer with the retinoid X receptor-α (RXRα, NR2B1) and activates transcription of *CYP2B* genes (Sueyoshi and Negishi, 2001). 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) is a potent mouse CAR (mCAR) ligand that has been used to study CAR target genes in mice but does not activate human CAR (hCAR) or rat CAR (rCAR) (Tzameli et al., 2000; Moore et al., 2000; Poland et al., 1981). Phenobarbital translocates hCAR, mCAR and rCAR into the nucleus and induces CYP2B; however, it is not known whether PB acts as an agonistic ligand for CAR since no direct binding of PB to CAR has been observed (Sueyoshi and Negishi, 2001). 5α-Androstan-3α-ol and clotrimazole bind to hCAR, whereas these compounds are antagonist ligands of hCAR (Forman et al., 1998; Moore et al., 2000). Recently, 6-(4-chlorophenyl) imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) and phenytoin have been reported to be hCAR agonist ligands (Maglich et al., 2003; Wang et al., 2004). However, the activation of hCAR by CITCO or phenytoin in a cell-based reporter assay was negligible. Therefore, the molecular and cellular mechanisms of the hCAR-mediated transactivation remain poorly understood at the present time.

On the other hand, pregnane X receptor (PXR1 NR1I2), a member of the nuclear receptor family, mediates induction of *CYP3A* gene transcription (Bertilsson et al., 1998;

Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). In the presence of a ligand, PXR binds to proximal PXR response element (prPXRE) and distal xenobiotic-responsive enhancer module (XREM) in regulatory regions of the *CYP3A4* gene as a heterodimer with RXRα. Unlike CAR, PXR can be activated by numerous compounds that are known CYP3A4 inducers, including drugs, steroids and environmental chemicals, in cell-based reporter assays (Jones et al., 2000; Moore et al., 2000). Thus, selective chemical tools (e.g., rifampicin) are available to study the function of PXR in humans.

Previous studies have demonstrated that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, which are in widespread clinical use for the treatment of hypercholesterolemia, increase the expression of P450s. Kocarek and Reddy (1996) demonstrated that treatment of primary cultures of rat hepatocytes with lovastatin, simvastatin, or fluvastatin increased the levels of CYP2B and CYP3A mRNA and immunoreactive protein. In contrast, pravastatin had little or no effect on the amount of any of the P450s examined in rat hepatocyte cultures. In addition, fluvastatin activated luciferase expression in primary cultures of rat hepatocytes that were transfected with the CYP2B1 reporter plasmid in the presence of cotransfected plasmid expressing mCAR or rCAR (Kocarek and Mercer-Haines, 2002). After the discovery that PXR mediates the effects of many CYP3A-inducing agents, EL-Sankary et al. (2001) demonstrated that simvastatin or lovastatin, but not pravastatin, activated transcription from a CYP3A4 reporter plasmid in transiently transfected HepG2 cells. Furthermore, treatment of primary cultures of human hepatocytes with lovastatin, simvastatin, fluvastatin or atorvastatin increased the levels of CYP3A4 and CYP2B6 mRNA and immunoreactive protein, but treatment with pravastatin did not (Kocarek et al., 2002). Although some HMG-CoA reductase inhibitors could activate human PXR and increase the amounts of CYP3A4 mRNA and immunoreactive protein as described above, it is not known whether activation of hCAR is involved in the induction of transcription of the *CYP2B6* gene

by treatment with HMG-CoA reductase inhibitors.

Therefore, we performed a screening of 50 drugs including HMG-CoA reductase inhibitors, by using cell-based reporter assays to identify hCAR activators. Among them, HMG-CoA reductase inhibitors (cerivastatin, simvastatin, fluvastatin and atorvastatin) enhanced the hCAR-mediated transcriptional activation of the PBREM reporter gene. They also enhanced mCAR- and rCAR-mediated activation.

Materials and Methods

Chemicals. 5α-Androstane-3α-ol (3α̃androstanol) was from Steraloids Inc. (Newport, RI). Atorvastatin, cerivastatin, fluvastatin, pravastatin and simvastatin were kindly provided by Sankyo Co. Ltd. (Tokyo, Japan). 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) was synthesized as described by Kende et al. (1985). 6-(4-Chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) was obtained from BIOMOL (Plymouth Meeting, PA).

Plasmids. The open reading frame of hCAR was amplified from a liver 5'-stretch plus cDNA library (Clontech, Palo Alto, CA) using specific primers (5'-TGA CGT CAT GGC CAG TAG GGA AGA TG-3' and 5'-GAG CAT GGC CTC AGC TGC AGA TCT CC-3'). The PCR fragment was cloned into pGEM-T Easy vector (Promega, Madison, WI). After *EcoR*I digestion, the fragment was ligated into the *EcoR*I site of pTARGET vector (Promega), resulting in pTARGET-hCAR. Correct insert orientation was confirmed by DNA sequence analysis. An mCAR expression plasmid was generated by PCR amplification of C57BL/6Cr mouse hepatic cDNA using specific primers (5'-AGG AGA CCA TGA CAG CTA TGC TAA CAC –3' and 5'-GGG TCT GGG GAA AGG ATG CAA GCC TG-3') and insertion into the *EcoR*I site of pTARGET vector, resulting in pTARGET-mCAR. A human PXR expression vector was generated as described previously (Kobayashi et al., 2004). An rCAR expression vector (pCR3-rCAR) was constructed as described previously (Yoshinari et al., 2001). A chimeric CYP2B6 luciferase repoter plasmid was prepared as follows. A CYP2B6 5'-flanking fragment (-1789 to -1667) containing the PBREM was generated by PCR from human genomic DNA as a template and was cloned into pGL3-promoter vector (Promega) digested with *Nhe*I and *Bgl*II to generate a PBREM-luciferase plasmid (pGL3-PBREM). A chimeric CYP3A4 luciferase reporter plasmid containing XREM and

prPXRE (pGL3-XREM/prPXRE) was generated as described previously (Kobayashi et al., 2004).

Cell Culture and Transfection Assay. FLC7, a human hepatocellular carcinoma cell line, was kindly provided by Dr. Nagamori (Kyorin University, Tokyo, Japan). FLC7 cells (Kawada et al., 1998) were cultured in Dulbecco's modified Eagle's medium/F-12 (DMEM/F12) supplemented with 10% fetal bovine serum. Cells (1.8×10^5/well) were plated in 24-well plates one day before transfection. Plasmid pGL3-PBREM (200 ng/well) was cotransfected with pTARGET-hCAR (20 ng/well) and phRL-TK vector (Promega, 4 ng/well) into FLC7 cells by lipofectAMINE^{TM} reagent and PLUS^{TM} reagent (Invitrogen, Carlsbad, CA). At 4 hours after transfection, the cells were treated with chemicals at the indicated concentrations. Stock solutions of the chemicals were prepared in dimethylsulfoxide (DMSO), and the final concentration of the solvent was 0.1% (v/v). Control cultures received a vehicle (0.1% DMSO) alone. Luciferase reporter activities after 24 hours were measured using a Dual-Luciferase reporter assay system (Promega) and Tuner Designs Luminometer TD-20/20 (Sunnyvale, CA) according to the manufacturer's instructions. The *Renilla* luciferase activity of the control plasmid phRL-TK was used to normalize the results of the firefly luciferase activity of the plasmid pGL3-PBREM.

mRNA levels. A stable hCAR-transfected FLC7 cell line (FLC7-hCAR) was cotransfected with pTARGET-hCAR expression vector and selected by neomycin resistance. To measure CYP2B6 mRNA level, cDNA prepared from total RNA of FLC7-hCAR cells and FLC7 cells treated with 30 μM of each HMG-CoA reductase inhibitor was subjected to quantitative real-time PCR with ABI prism 7000 (Applied Biosystems, Foster City, CA). The mRNA levels of CYP2B6 were determined by using Human Assays-On-Demand, a gene expression product for CYP2B6. The mRNA levels were normalized against 18S rRNA determined by Pre-Developed TaqMan Assay Reagents for 18S rRNA (Applied Biosystems).

To measure CYP2B6 mRNA levels in HepG2 cells and FLC7 cells, total RNA was prepared from non-treated cells using TRIZOL reagent (Invitrogen). Each cDNA (0.5 μL in 25 μL of reaction mixture) synthesized from 2 μg of RNA was subjected to 40-cycle amplification of CYP2B6 mRNA. The primers (5'-CAC TAT GAG GGA CTT CGG GAT GG-3' and 5'-GCA GAT GAT GTT GGC GGT AAT GG-3') were designed from the reported sequences (accession number NM_000767) across intron 3. PCR reactions were performed using AmpliTaq Gold (Applied Biosystems) at 95°C for 1 min, 60°C for 30s and 72°C for 30s. The amplified DNA (151 bp) was separated on a 3% agarose gel and visualized with staining by ethidium bromide. GAPDH mRNA was amplified as an internal control.

Statistics. Results are expressed as mean ± S.D. Differences between two groups were analyzed using a two-sample *t* test.

Results

Identification of hCAR activators in a cell-based reporter gene assay screening. Fifty drugs, including five HMG-CoA reductase inhibitors, were screened in cell-based reporter gene assays using hCAR and PBREM-reporter gene construct. These drugs consisted of anticonvulsants, antidepressants, antiarrhythmic drugs, NSAIDs, etc. in addition to HMG-CoA reductase inhibitors, which are hydrophobic and mainly metabolized via CYP. Therefore, we considered that these drugs penetrated the cell membrane and were screened in cell-based reporter gene assay. As shown in Figure 1A, compounds that increased hCAR-mediated transcriptional activity at the concentration of 10 μM were cerivastatin (2.8 fold), simvastatin (1.9 fold), atorvastatin (1.8 fold) and fluvastatin (1.7 fold). Temazepam and thioridazine decreased hCAR-mediated transcriptional activity by >50%. The effects of the other 44 chemicals on the hCAR-mediated transcriptional activity were weak or negligible (< 1.5 fold).

hCAR-mediated transcriptional activation by HMG-CoA reductase inhibitors. Since four HMG-CoA reductase inhibitors showed activation of the reporter gene (Fig. 1A), five HMG-CoA reductase inhibitors, including pravastatin, were selected for further analysis. As shown in Figure 1B, cerivastatin significantly increased hCAR-mediated transcriptional activity at the concentration of 1 μM. Simvastatin, fluvastatin and atorvastatin also showed significant activation of the reporter gene at the concentration of 30 μM. The manner of activation of hCAR-mediated transcription by the four HMG-CoA reductase inhibitors was dose-dependent. Maximum induction of hCAR-mediated transcriptional activity by the four HMG-CoA reductase inhibitors at the concentrations tested was 2-4 fold. On the other hand, pravastatin did not show dose-dependent activation of hCAR at the concentrations tested (up to 30 μM). In addition, we tried treating the cells with HMG-CoA reductase inhibitors in the presence of exogenous mevalonate, whereas the results were essentially the same as those

obtained under standard treatment conditions (data not shown).

Repressive effects of androstanol on HMG-CoA reductase inhibitor-induced hCAR-, mCAR- and rCAR-mediated transactivation. In agreement with previous reports (Sueyoshi et al., 1999), expression of the transfected PBREM-reporter gene was activated by cotransfection of the hCAR expression vector in FLC7 cells (Fig. 2A). 3α-Androstanol repressed this hCAR-mediated activation by 45%. 3α-Androstanol also repressed the increase in PBREM-luciferase activity induced by HMG-CoA reductase inhibitors (30 μM). When not only hCAR but also mCAR or rCAR was cotransfected, increase in PBREM-luciferase activity induced by HMG-CoA reductase inhibitors and repression by 3α-androstanol were observed (Fig. 2B and 2C). On the other hand, distinct activation profiles by the potent PB-type inducer TCPOBOP were observed when the hCAR, mCAR or rCAR expression vector was cotransfected with a PBREM luciferase reporter. TCPOBOP elicited a significant induction of the reporter in the presence of mCAR, but induction by TCPOBOP at an equivalent concentration was not evident when hCAR or rCAR was cotransfected.

Potency of cerivastatin for CAR activation compared with that of CITCO. As shown in Figure 3, hCAR has been shown to transactivate the PBREM reporter gene in the absence of any exogenous ligands. In addition, CITCO, a hCAR-specific ligand, enhanced the hCAR-mediated transcriptional activation of PBREM reporter (by ~1.3 fold) at a concentration of 10 μM as reported by Juinno et al. (2004). On the other hand, the effect of an HMG-CoA reductase inhibitor, cerivastatin, at the concentration of 10 μM on hCAR-mediated transcriptional activity was much greater than that of CITCO.

hCAR-mediated induction of CYP2B6 mRNA in human hepatoma cell line. To assess the hCAR-mediated induction of CYP2B6 mRNA by HMG-CoA reductase inhibitors, HMG-CoA reductase inhibitors were treated to hCAR-expressing cells derived from FLC7 cells (FLC7-hCAR). Unlike HepG2 cells, FLC7 cells constitutively express CYP2B6 mRNA

(Fig. 4A). However, no inductive effect of HMG-CoA reductase inhibitors on CYP2B6 mRNA levels was observed in FLC7 cells. In contrast, CYP2B6 mRNA levels in FLC7-hCAR were increased by cerivastatin, simvastatin, fluvastatin and atorvastatin but not by pravastatin (Fig.4B).

Effects of HMG-CoA reductase inhibitors on hPXR-mediated transcriptional activities. To compare the effects of HMG-CoA reductase inhibitors on transactivation of hCAR with those of human PXR, a human PXR expression vector was cotransfected with the PBREM-reporter gene, and a reporter gene assay was performed. Except for atorvastatin and pravastatin, the HMG-CoA reductase inhibitors significantly enhanced the expression of the PBREM-reporter gene (Fig. 5A). The extents of the activation at the concentration of 30 μM were about 3 fold. On the other hand, when XREM/prPXRE-reporter gene construct was used, atorvastatin (12.2 fold) in addition to cerivastatin (29.4 fold), simvastatin (26.6 fold) and fluvastatin (39.8 fold) also enhanced the expression of the XREM/prPXRE-reporter gene (Fig. 5B).

Discussion

The present study showed that HMG-CoA reductase inhibitors, cerivastatin, simvastatin, fluvastatin and atorvastatin were effective in activating hCAR in a cell-based reporter gene assay using human hepatocellular carcinoma FLC7 cells. In addition, the increase in the level of CYP2B6 mRNA induced by these HMG-CoA reductase inhibitors was shown to be regulated by hCAR. These findings were supported by the following results. Since the expression of CAR and PXR in FLC7 cells is negligible (data not shown), no effect by treatment with HMG-CoA reductase inhibitors on CYP2B6 mRNA level was observed in FLC7 cells. In contrast, significant induction of CYP2B6 mRNA by treatment with HMG-CoA reductase inhibitors was detected in stable hCAR-expressing FLC7 cells (termed FLC7-hCAR, Fig. 4B). CITCO has been reported to be a selective hCAR ligand and to activate hCAR (Maglich et al., 2003). However, the extent of transactivation by CITCO in a reporter gene assay was weak (Fig. 3), although induction by CITCO of CYP2B6 *in vivo* was strong. On the other hand, phenobarbital and phenytoin, which induce CYP2B6 *via* hCAR (Sueyoshi et al., 1999; Wang et al., 2004), do not enhance hCAR-mediated transcriptional activity in a cell-based reporter gene assay. The lack of an effective activator of hCAR in a cell-based reporter gene assay makes the characterization of hCAR difficult. The present study suggested that HMG-CoA reductase inhibitors might provide us with useful tools to study the function of CAR in humans.

In this study, FLC7 cells were used for the reporter gene assay. Our preliminary study showed that the mRNA levels of CAR in FLC7 cells were much lower, but the heterodimerization partner RXRα is expressed in sufficient amounts. In addition, the activation of mCAR by TCPOBOP and the deactivation of hCAR and mCAR by androstanol observed in FLC7 cells

(Fig. 2) are in agreement with previously reported results in HepG2 cells (Moore et al., 2000). Therefore, it is considered that the assay system using FLC7 cells is a valid model for examination of CAR-mediated activation or deactivation.

TCPOBOP is a potent mCAR ligand that has been used for identification of CAR target genes, but does not activate hCAR or rCAR in a cell-based reporter gene assay (Fig. 2). In contrast, phenobarbital activates CAR of any species, but phenobarbital does not bind to the receptor. It is well known that CAR is translocated from the cytoplasm to the nucleus after treatment with phenobarbital (Kawamoto et al., 1999). Although it has not been determined whether HMG-CoA reductase inhibitors directly bind to CAR, HMG-CoA reductase inhibitors could activate hCAR, rCAR and mCAR at least in cell-based reporter gene assay (Fig. 2). The availability of activators for hCAR, rCAR and mCAR will enable direct comparison of their similarity and difference in the transactivation mechanism.

Currently available information on the role of hCAR in the regulation of human *CYP2B6* gene expression is limited. Accumulated evidence suggests that the human *CYP2B6* gene is also regulated in a PXR-dependent manner. The majority of known PXR activators are capable of potently inducing CYP2B6 in primary human hepatocyte cultures, including those that activate hPXR only (rifampicin, hyperforin, SR12813), those that activate both hPXR and hCAR (phenobarbital) or those that selectively activate hCAR (phenytoin). The present study showed that cerivastatin, simvastatin, fluvastatin and atorvastatin enhanced the PBREM reporter gene mediated by hCAR (Fig. 1B) and the XREM/prPXRE reporter gene mediated by hPXR (Fig. 5B). Furthermore, Kocarek et al. (2002) reported that simvastatin, fluvastatin and atorvastatin increased the amount of CYP2B6 and CYP3A mRNA in primary human hepatocytes. Therefore, it was thought that cerivastatin, simvastatin, fluvastatin and

atorvastatin are capable of activating both hCAR and hPXR and of inducing CYP2B6 in primary human hepatocyte cultures.

Among the HMG-CoA reductase inhibitors studied, only pravastatin did not enhance the hCAR-mediated expression of the PBREM reporter gene (Fig. 1B). Due to the hydrophilic nature of pravastatin, the amount of cellular uptake by diffusion is small. In human hepatocytes, organic anion transporting peptide-C (OATP-C) plays a predominant role in the uptake of some HMG-CoA reductase inhibitors such as pravastatin (Nakai et al., 2001). However, HepG2 cells, a frequently used cell model of human hepatocytes, have been immonohistochemically demonstrated to express no OATP-C (Nakai et al., 2001). Therefore, an OATP-C expression vector was cotransfected in FLC7 cells with hCAR and a reporter vector as a preliminary study. However, hCAR-mediated transcriptional activity was not induced by pravastatin even in the presence of OATP-C (data not shown). Kocarek et al. (2002) reported that treatment with pravastatin had no effect on the mRNA level of either CYP2B6 or CYP3A4 in human primary hepatocytes, although pravastatin treatment did produce the expected increase in HMG-CoA reductase mRNA level. Therefore, it appears that pravastatin was not an activator of hCAR in either human primary hepatocytes or human hepatoma cells.

The present screening in cell-based reporter gene assays using hCAR and PBREM-reporter gene construct indicated that temazepam and thioridazine decreased hCAR-mediated transcriptional activity (Fig. 1A). 3α-Androstanol and antimycotic clotrimazole have been reported to deactivate hCAR through direct interactions with LBD of hCAR (Moore et al., 2000). Although the further studies to clarify dose-responsibility and direct interaction with hCAR of temazepam and thioridazine are needed, these drugs may also deactivator or

antagonist of hCAR.

In conclusion, cerivastatin, simvastatin, fluvastatin and atorvastatin enhanced hCAR-mediated transcriptional activity in FLC7 cells and induced the expression of CYP2B6 mRNA in hCAR-expressing FLC7 cells. The activation of hCAR by these HMG-CoA reductase inhibitors in a cell-based reporter gene assay is more effective than that by a specific hCAR ligand, CITCO, although these HMG-CoA reductase inhibitors also activated hPXR. Therefore, HMG-CoA reductase inhibitors are thought to be useful tools to study the function of hCAR in a cell-based reporter gene assay using FLC7 cells.

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Legends of figures

Fig. 1. Effects of fifty drugs, including HMG-CoA reductase inhibitors, on hCAR-mediated transactivation (A) and dose-dependency of HMG-CoA reductase inhibitors on hCAR-mediated transactivation (B).

Constructs pTARGET-hCAR (20 ng/well) and pGL3-PBREM (200 ng/well) were transiently transfected into FLC7 cells. Cells were treated with 10 μM of each drug (A) or with 0.1, 0.3, 1, 3, 10 and 30 μM of each of the HMG-CoA reductase inhibitors (B) for 24 hr. Control cells were treated with 0.1% DMSO (vehicle). Data are expressed as means of duplicate transfections (A) or means ± S.D. of at least three experiments (B). $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with vehicle control. Atorvastatin (ATV, 1), cerivastatin (CRV, 2), fluvastatin (FLV, 3), simvastatin (SMV, 4), pravastatin (PRV, 5), amitriptyline (6), clomipramine (7), desipramine (8), imipramine (9), maprotiline (10), mianserin (11), fluoxetine (12), ketanserin (13), phenelzine (14), alprazolam (15), clonazepam (16), diazepam (17), fludiazepam (18), flunitrazepam (19), flurazepam (20), midazolam (21), temazepam (22), triazolam (23), carbamazepine (24), diphenylhydantoin (25), ethotoin (26), 5-(4-hydroxymethyl)-5-phenylhydantoin (27), R-mephenytoin (28), S-mephenytoin (29), ethosuximide (30), amobarbital (31), barbital (32), barbituric acid (33), cyclobarbital (34), 5-ethyl-5-(p-hydroxyphenyl)-barbituric acid (35), thioridazine (36), haloperidol (37), mequitazine (38), dextromethorphan (39), dopamine (40), lidocaine (41), mexiletine (42), 1,7-dimethylxanthine (43), hypoxanthine (44), theophylline (45), indoleaceic acid (46), indomethacin (47), ketoprofen (48), mefenamic acid (49) and sulindac (50) were used in this study.

Fig. 2. Repressive effects of 3α-androstanol on hCAR (A) -, mCAR (B) - and rCAR (C)

-mediated transactivation by HMG-CoA reductase inhibitors.

Construct pGL3-PBREM (200 ng/well) was transiently transfected with pTARGET-hCAR, pTARGET-mCAR or pCR3-rCAR (each 20 ng/well) into FLC7 cells. Cells were treated with 0.2% DMSO (vehicle), 30 μM of each of the HMG-CoA reductase inhibitors or 250 nM of TCPOBOP in the presence or absence of 10 μM of 3α-androstanol for 24 hr. Data are expressed as means ± S.D. of at least three experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with vehicle control. $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger}p < 0.05$ compared with data in the absence of 3α-androstanol.

Fig. 3. Comparison of cerivastatin (CRV) and CITCO for hCAR-, mCAR- and rCAR-mediated transactivation of PBREM-luciferase gene. Construct pGL3-PBREM (200 ng/well) was transiently transfected with or without pTARGET-hCAR, pTARGET-mCAR or pCR3-rCAR (each 20 ng/well) into FLC7 cells. Cells were treated with 0.2% DMSO (vehicle), 10 μM of CITCO or 10 μM of cerivastatin for 24 hr.

Fig. 4. (A) RT-PCR analysis of CYP2B6 mRNA in HepG2 cells and FLC7 cells. Total RNAs were prepared from two different cell types, HepG2 cells and FLC7 cells, and were subjected to RT-PCR and 3% agarose gel electrophoresis. DNA bands were visualized by ethidium bromide. (B) hCAR-mediated induction of CYP2B6 mRNA in FLC7 cells. Cells (FLC7-hCAR cells) were treated with 0.1% DMSO (vehicle) or 30 μM of each of the HMG-CoA reductase inhibitors for 24 hr. Total RNA was extracted as described in *Materials and Methods* and was subjected to real-time PCR. Relative mRNA abundance is normalized for 18S-rRNA. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with vehicle control.

Fig. 5. Effects of HMG-CoA reductase inhibitors on hPXR-mediated activation of

PBREM-luciferase gene (A) and XREM/prPXRE-luciferase gene (B).

Constructs pTARGET-hPXR (10 ng/well) and pGL3-PBREM (200 ng/well) or pGL3-XREM/prPXRE (200 ng/well) were transiently transfected into FLC7 cells. Cells were treated with 0.1% DMSO (vehicle), 30 μM of each of the HMG-CoA reductase inhibitors or 5 μM of rifampicin (rif) for 24 hr. Data are expressed as means ± S.D. of at least three experiments. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ compared with vehicle control.

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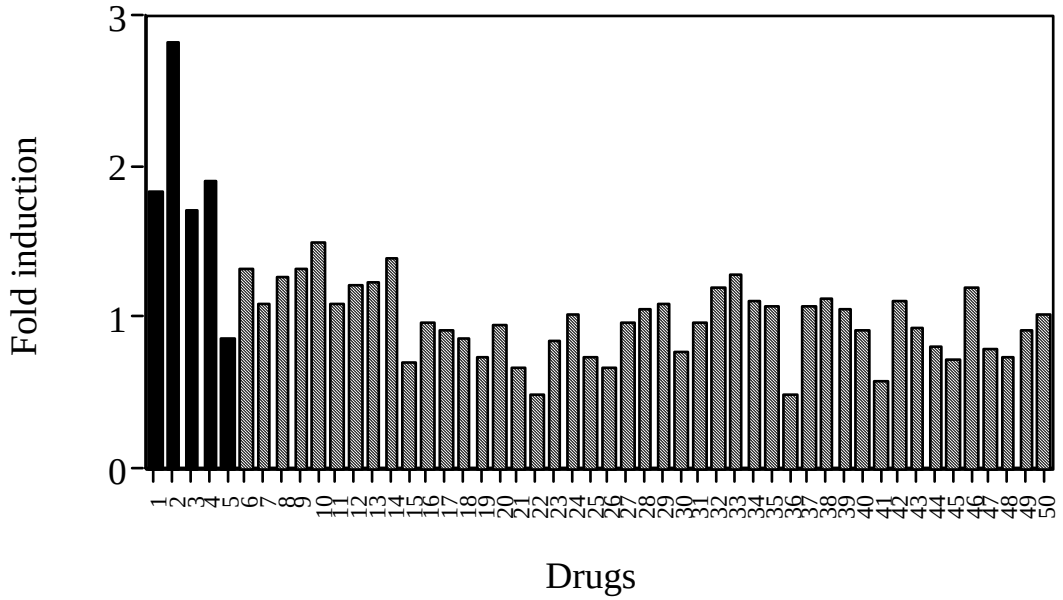
Figure 1

A



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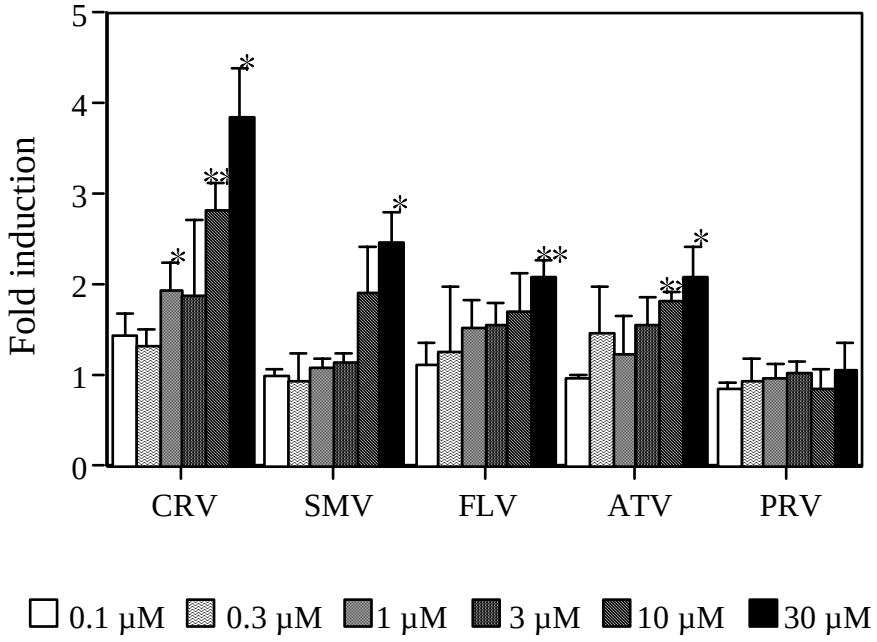

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Figure 2

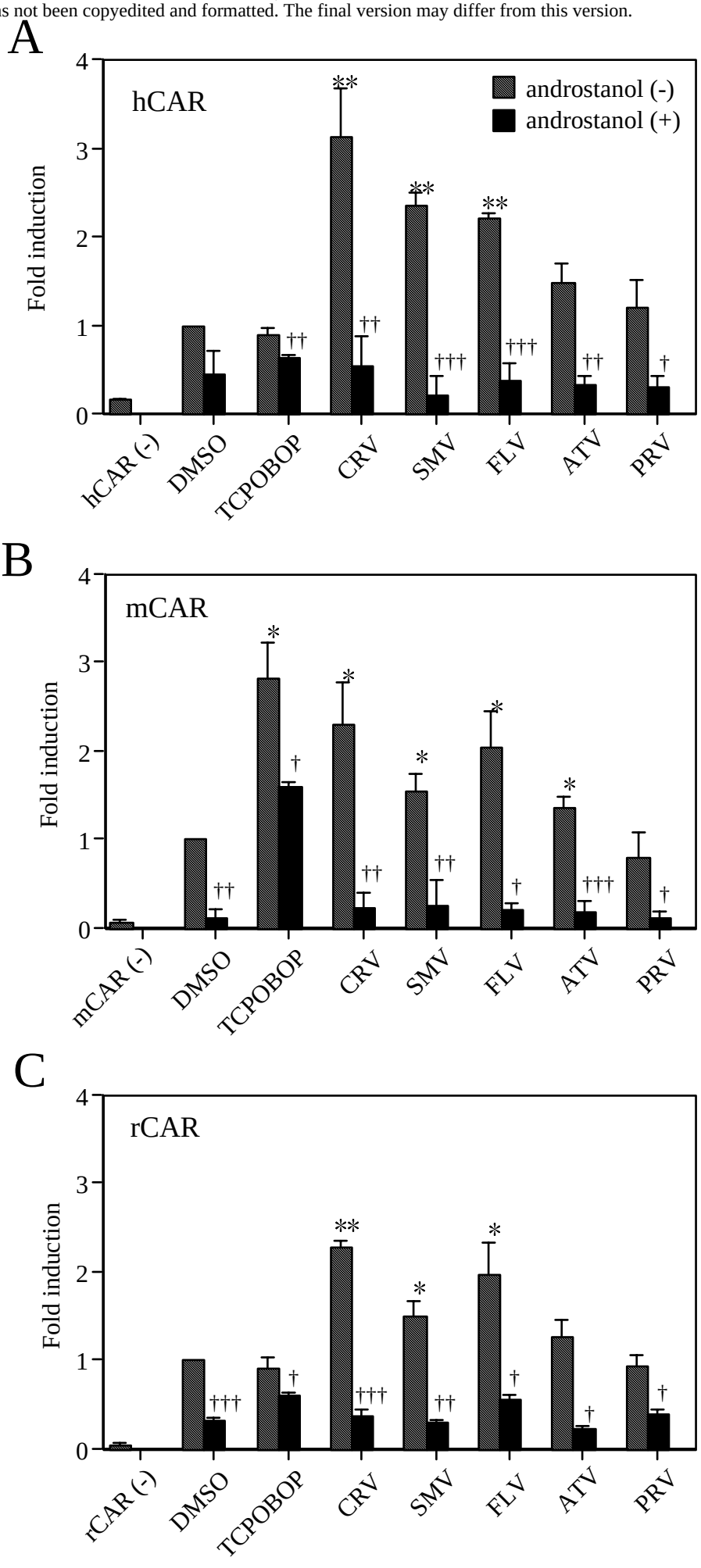

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Figure 3

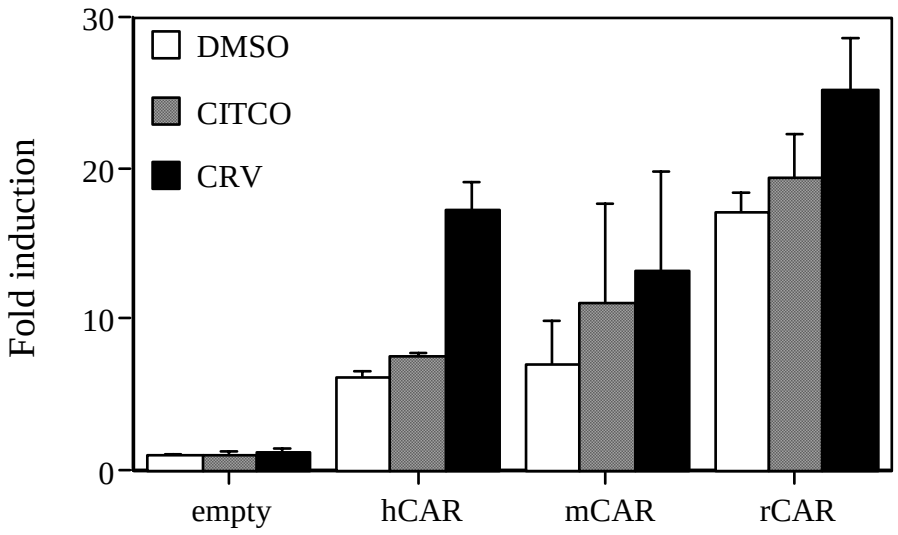

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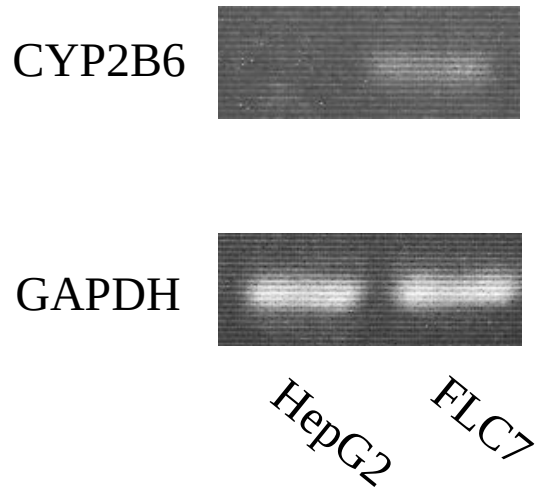
Figure 4

A



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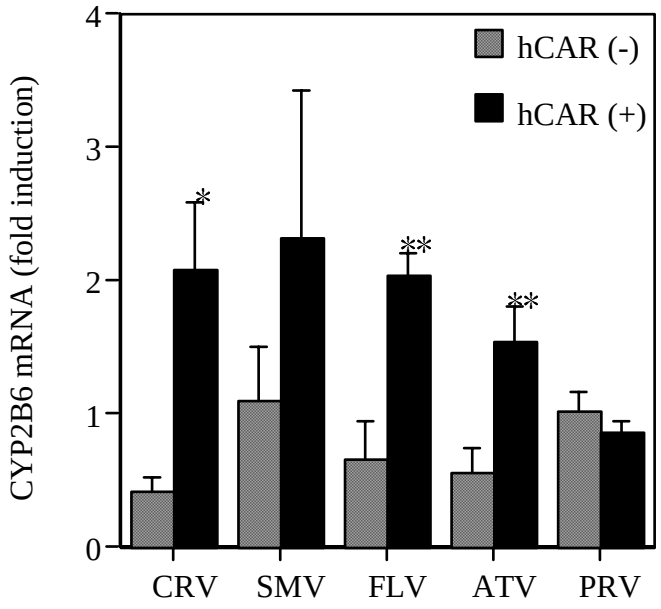

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Figure 5