

# Meclizine Is an Agonist Ligand for Mouse Constitutive Androstane Receptor (CAR) and an Inverse Agonist for Human CAR

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The constitutive androstane receptor (CAR, NR1I3) is a key regulator of xenobiotic and endobiotic metabolism. The ligand-binding domains of murine (m) and human (h) CAR are divergent relative to other nuclear hormone receptors, resulting in species-specific differences in xenobiotic responses. Here we identify the widely used antiemetic meclizine (Antivert; Bonine) as both an agonist ligand for mCAR and an inverse agonist for hCAR. Meclizine increases mCAR transactivation in a dose-dependent manner. Like the mCAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, meclizine stimulates binding of steroid receptor coactivator 1 to the murine receptor *in vitro*. Meclizine administration to

mice increases expression of CAR target genes in a CAR-dependent manner. In contrast, meclizine suppresses hCAR transactivation and inhibits the phenobarbital-induced expression of the CAR target genes, cytochrome p450 monooxygenase (CYP)2B10, CYP3A11, and CYP1A2, in primary hepatocytes derived from mice expressing hCAR, but not mCAR. The inhibitory effect of meclizine also suppresses acetaminophen-induced liver toxicity in humanized CAR mice. These results demonstrate that a single compound can induce opposite xenobiotic responses via orthologous receptors in rodents and humans. (*Molecular Endocrinology* 18: 2402–2408, 2004)

DETOXIFICATION OF BOTH potentially harmful foreign compounds and endogenous toxic products is a critical function of the liver (1). A family of phase I enzymes, particularly the cytochrome p450 monooxygenases (CYPs), initially modifies such compounds and provides the first line of defense. The phase II conjugating enzymes, including UDP-glucuronosyltransferases and glutathione-S-transferases (GSTs), add bulky polar adducts such as glucuronic acid and glutathione to facilitate excretion. More recently, it has become clear that a number of transmembrane transporters at both the basolateral and apical faces of the hepatocyte are also integral components of this detoxification network (2).

This process is regulated by two members of the nuclear receptor superfamily, CAR (constitutive androstane receptor NR1I3) and PXR (pregnane X receptor, NR1I) (1, 3–5). CAR mediates the well-studied induction of detoxifying enzymes and transporters in response to phenobarbital (PB) and other structurally diverse xenobiotic

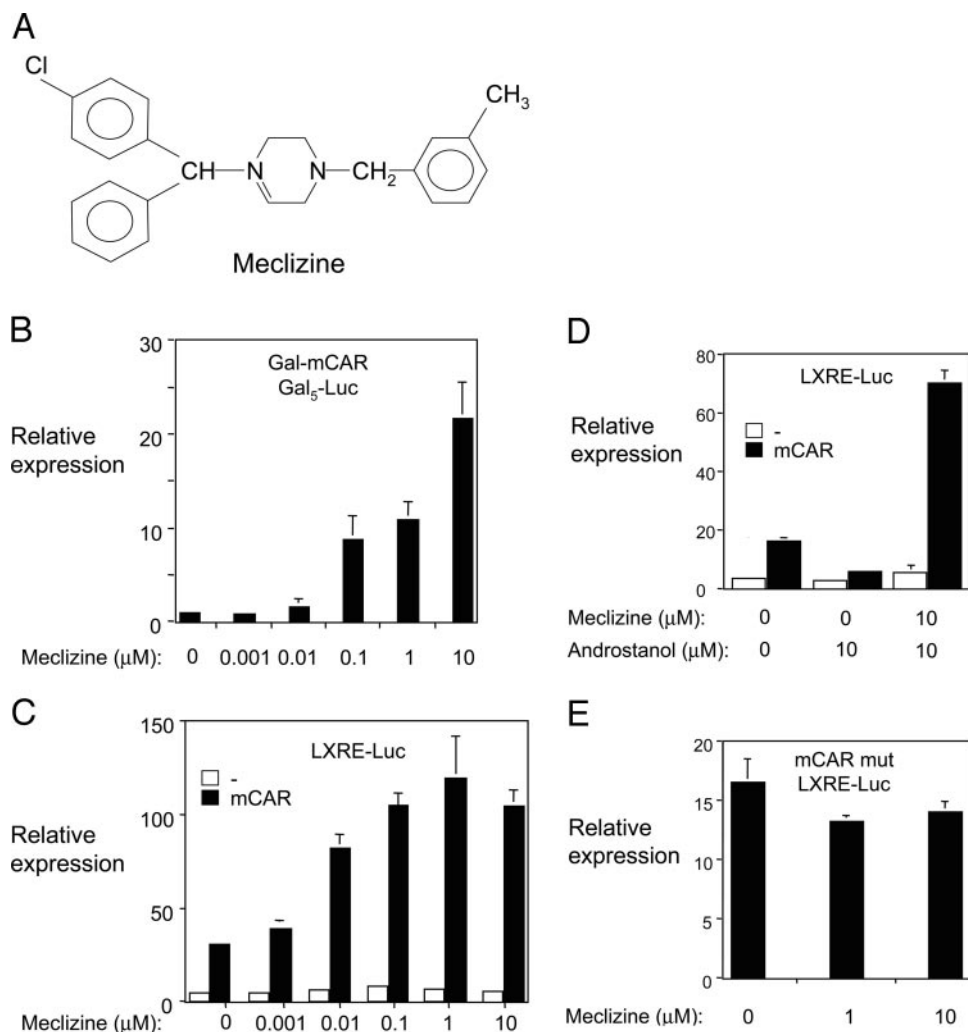
compounds termed “PB-like inducers” (6–8). PXR induces expression of an overlapping but distinct set of target genes in response to other xenobiotics. Thus, these xenosensors coordinate responses to diverse xenobiotics (9–11). They also function in response to endogenous toxic products. PXR activation promotes detoxification of the toxic bile acid lithocholic acid (12, 13), and both CAR and PXR have been associated with clearance of bilirubin (14–17).

The induction of xenobiotic metabolism by these receptors is generally a protective response, but generation of toxic metabolites can sometimes produce deleterious effects. One particular example of this is the role of CAR in acetaminophen metabolism (18). Overdoses of acetaminophen cause significant hepatotoxicity and potentially fatal centrilobular necrosis (19, 20). CAR can be activated by high doses of acetaminophen, which induces expression of CYP3A11, CYP1A2, and GSTP1 and increases both the production of a toxic acetaminophen metabolite and the depletion of hepatic glutathione. Thus, acetaminophen induces its own toxicity via a pathway mediated by CAR (18). In the mouse, this pathway can be blocked by the CAR inverse agonist ligand, androstanol, thereby preventing acetaminophen toxicity. However, androstanol has no effect on human CAR (hCAR). No similarly potent inverse agonists have been described for the human receptor, although clotrimazole has been shown to have a modest inhibitory effect (21).

Meclizine is a histamine receptor-blocking drug used to treat nausea and motion sickness and the active ingredient in the prescription drugs Antivert and Bonine.

Abbreviations: ALT, Alanine aminotransferase; CAR, constitutive androstane receptor; CYP, cytochrome p450 monooxygenase; GST, glutathione-S-transferase; LBD, ligand-binding domain; LXR, liver X receptor; LXRE, LXR response element; MRP2, multidrug resistance-associated protein; PB, phenobarbital; PXR, pregnane X receptor; SRC-1, steroid receptor coactivator; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene.

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**Fig. 1.** Activation of mCAR by Meclizine

A, Structure of meclizine. B, HepG2 cells were cotransfected with expression for Gal4 alone or a Gal4-mCAR ligand binding domain fusion plus the Gal4 reporter pG5E1b-luciferase (Luc) reporter, with or without increasing concentrations of meclizine (Mec) as indicated. C, HepG2 cells were cotransfected with expression plasmids for vector alone or full-length of mCAR and the LXRE-thymidine kinase (TK)-Luc reporter, with or without increasing concentrations of meclizine. D, Cells were transfected as in panel C, but treated with solvent, 10 μM androstanol alone, or plus 10 μM meclizine. E, HepG2 cells were cotransfected with an expression vector for full-length of mCAR carrying the two mutations in panel E and the LXRE-TK-Luc reporter, and incubated with solvent (–), 1 μM meclizine, or 10 μM meclizine.

We have found that meclizine is a xenobiotic inducer in mice that functions as a mouse CAR agonist ligand. Unexpectedly, the same compound is shown to be an inverse agonist for hCAR. In a humanized CAR transgenic mouse, meclizine can inhibit acetaminophen-induced liver toxicity. Thus, a single compound can have opposite effects on orthologous receptors in different mammalian species.

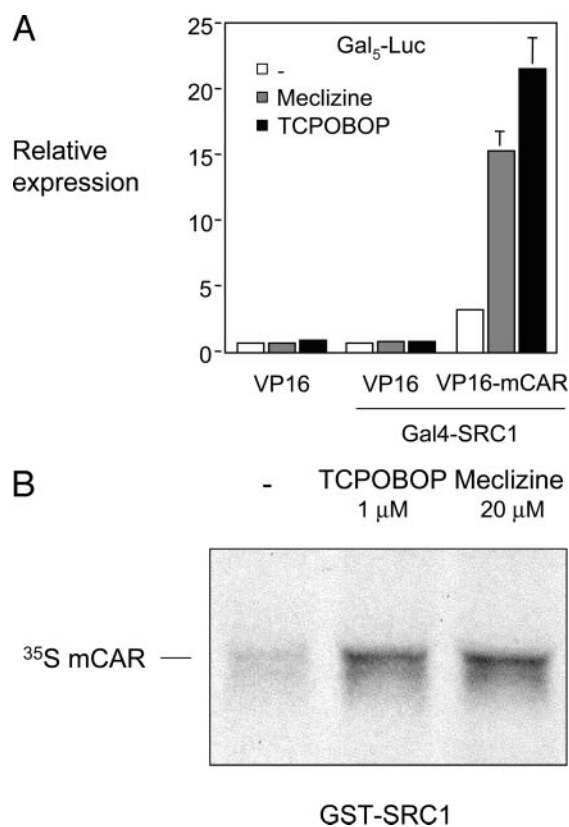
## RESULTS

### Meclizine Is a Mouse CAR Agonist

The Gen-Plus library of 960 bioactive compounds (MicroSource Discovery System, Inc., Gaylordsville,

MD) was screened in high throughput for compounds with effects on transactivation directed by fusions of the Gal4 DNA-binding domain and the ligand-binding domains (LBDs) of murine or hCAR (Gal-mCAR and Gal-hCAR). Meclizine (Fig. 1A) was identified as a CAR modulator.

Transient transfections using either Gal4-mCAR with a reporter containing Gal4 binding sites (Fig. 1B) or a full-length mCAR and a previously characterized liver X receptor-response element (LXRE) reporter (Fig. 1C), which shows robust responses to agonist activation relative to constitutive activity (7), confirmed that meclizine is a potent activator of mCAR. The meclizine concentration required for half-maximal induction of full-length mCAR transactivation ( $EC_{50}$ ) is

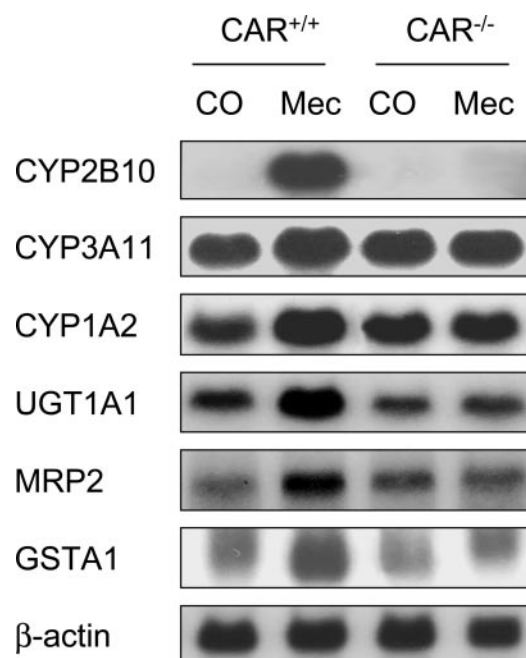


**Fig. 2.** Meclizine Induces Coactivator Binding to mCAR. A, HepG2 cells were cotransfected with expression vectors for Gal4-SRC-1 RID and VP16 or VP16-mCAR-LBD fusion proteins, together with the pG5E1b-Luc reporter, and treated with solvent, or meclizine (10 μM) or TCPOBOP (250 nM) as indicated. B, <sup>35</sup>S-labeled mCAR was incubated with beads carrying a GST-SRC-1 receptor-interacting domain fusion protein in the presence of solvent (control), 1 μM TCPOBOP (TC), or 20 μM meclizine (Mec). Specifically bound proteins were eluted and resolved by SDS-PAGE.

approximately 25 nM, comparable to that of the previously described specific mCAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (7).

Transactivation by mCAR can be blocked by inverse agonist ligands such as androstanol (22). The agonist TCPOBOP competes with such inverse agonists for this inhibitory effect (7). Meclizine also reversed the inhibitory effect of androstanol on CAR activity (Fig. 1D), indicating that it also functions as an mCAR agonist. Two previously described mutations in the CAR ligand binding domain (F161L; I164A) block both the agonist activity of TCPOBOP and the inverse agonist activity of androstanol, but do not affect ligand independent transactivation (7). The resistance of the mutant mCAR LBD to the stimulatory effect of meclizine (Fig. 1E) supports the conclusion that meclizine is an mCAR agonist ligand.

A mammalian two-hybrid approach was used to test the effects of meclizine on coactivator recruitment. As expected for an agonist, meclizine increased the interaction between mCAR and steroid receptor coactivator 1 (SRC-1) (Fig. 2A), although it was not as effective as



**Fig. 3.** Meclizine Induces Expression of CAR Target Genes in Mice

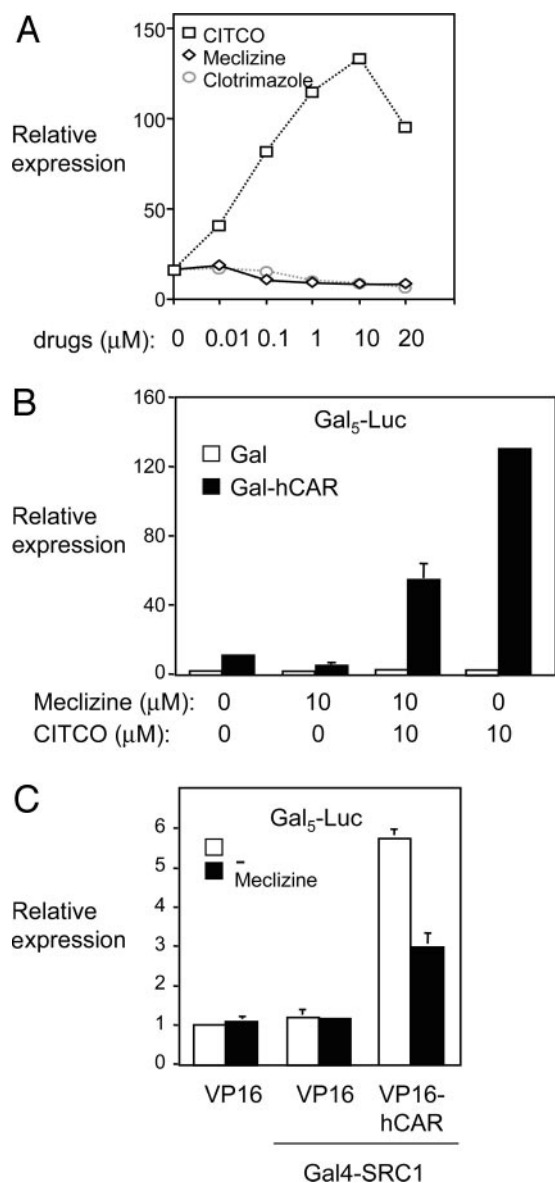
Wild-type or CAR null mice (n = 3) were injected ip once a day with either the corn oil (CO) vehicle or meclizine for 3 d. Liver RNA was prepared, and equivalent amounts of RNA from three individuals were pooled and hybridized with different probes as indicated. UGT1A1, UDP-glucuronosyltransferase-1A1.

TCPOBOP. Meclizine also enhanced the *in vitro* interaction of a GST-SRC-1 fusion with <sup>35</sup>S-labeled mCAR (Fig. 2B).

Wild-type and CAR null mice (8) were used to test the ability of meclizine to induce characteristic xenobiotic responses in a CAR-specific manner (Fig. 3). Meclizine strongly increased expression of a number of previously identified CAR target genes in wild-type mice, including those involved in acetaminophen-induced hepatotoxicity, e.g. CYP1A2 and CYP3A11 (18) and bilirubin metabolism, e.g. UDP-glucuronosyltransferase-1A1 (UGT1A1), multidrug resistance-associated protein 2 (MRP2), and GSTA1 (14). These responses were completely lost in CAR null mice. Overall, we conclude that meclizine is a specific agonist ligand for mouse CAR, both *in vitro* and *in vivo*.

### Meclizine Is an Inverse Agonist for hCAR

In contrast with the results with mCAR but in agreement with the initial screening results, meclizine repressed hCAR transcriptional activity by approximately 50% in a dose-dependent manner (Fig. 4A). The magnitude of this suppression was comparable to the previously described effect of clotrimazole (21), but the EC<sub>50</sub> for the suppression was roughly 10-fold lower for meclizine. As expected, the hCAR agonist CITCO (6-(4-chlorophenyl)imidazo[2-1-b][1,3]thiazole-



**Fig. 4.** Suppression of hCAR Activity by Meclizine

A, HepG2 cells were cotransfected with an expression vector for a Gal4-hCAR ligand binding domain fusion plus the Gal4 reporter pG5E1b-luciferase (Luc) reporter, with or without increasing concentrations of CITCO, meclizine, or clotrimazole, as indicated. B, HepG2 cells were cotransfected with expression vectors for Gal4, the Gal-hCAR fusion and the Gal4 receptor plus meclizine and/or CITCO, as indicated. C, HepG2 cells were cotransfected with expression vectors for Gal4-SRC-1 receptor interacting domain and VP16 or VP16-hCAR-LBD fusion proteins, together with the pG5E1b-Luc reporter, and treated with solvent or meclizine (20  $\mu$ M) as indicated.

5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (23) was a potent stimulator, but its effect was inhibited by an equimolar concentration of meclizine (Fig. 4B). The specificity of this inhibition was tested using Gal4 fusions to the LBDs of a number of other nuclear receptors. Meclizine did not activate any of these receptors,

including either human or rodent versions of the related xenobiotic receptor PXR, or peroxisomal proliferator-activated receptor- $\alpha$ , peroxisomal proliferator-activated receptor- $\gamma$ , liver X receptor (LXR) $\alpha$ , retinoic X receptor- $\alpha$ , vitamin D receptor, farnesoid X receptor, and estrogen receptor- $\alpha$  (data not shown). Meclizine also had no inhibitory effect on ligand-dependent transactivation by these receptors except for a 30–40% decrease in estrogen-dependent transactivation by estrogen receptor- $\alpha$ .

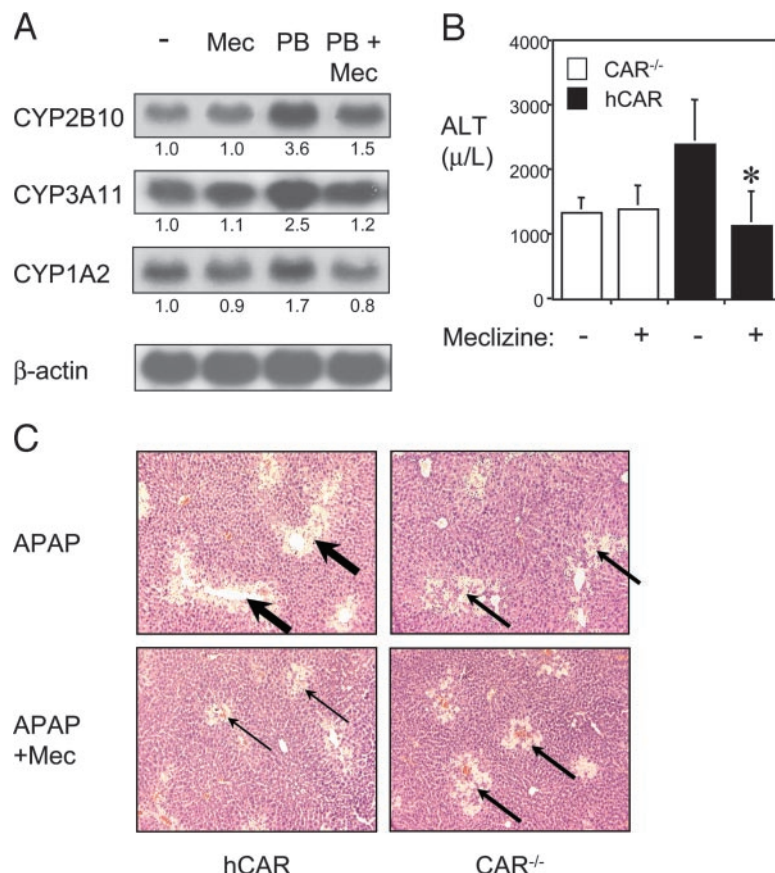
Inverse agonists of CAR (22) and other nuclear receptors (24, 25) inhibit coactivator binding. This was tested using meclizine in a mammalian two-hybrid assay (Fig. 4C). At relatively high concentrations (20  $\mu$ M), the drug specifically decreased the interaction of hCAR with SRC-1 by about 2-fold.

The inhibitory effect of meclizine on hCAR transactivation of endogenous target genes was tested using primary hepatocytes isolated from mice expressing human, but not mouse CAR (18). Meclizine had no effect on basal expression of the CAR target genes CYP2B10, CYP3A11, and CYP1A2 in these hepatocytes. As expected, however, they were induced by the hCAR activator PB, and this response was blocked by meclizine (Fig. 5A).

Activation of CAR has been associated with acetaminophen-induced liver toxicity, which can be blocked in mice using the specific murine inverse agonist androstanol (18). Because the results of cell-based experiments predicted that it should behave as an inverse agonist in the humanized mouse, we next asked whether meclizine inhibition of hCAR could inhibit acetaminophen-induced toxicity. The humanized CAR mice or CAR null mice were treated with a relatively high dose of acetaminophen (500 mg/kg) that induces severe liver toxicity, followed 1 h later by an injection of meclizine (100 mg/kg) or solvent control. Consistent with previous results (18), the humanized CAR mice showed significantly increased liver toxicity compared with CAR knockout mice, indicating that hCAR activation increases acetaminophen-induced toxicity. However, meclizine treatment significantly decreased liver toxicity in these humanized mice, as demonstrated by both alanine aminotransferase (ALT) measurements and liver histology analysis (Fig. 5C). Thus, we conclude that meclizine functions as a specific inverse agonist ligand for hCAR.

## DISCUSSION

The results described here identify meclizine, a histamine H1 receptor antagonist used for the treatment of nausea and vomiting due to a variety of emetic stimuli including motion sickness and pregnancy (26–28), as both an agonist for mCAR and an inverse agonist for hCAR. This demonstration that a single compound can have opposite effects on a single receptor in different species provides an extreme example of the



**Fig. 5.** Suppression of hCAR Function by Meclizine

A, Primary hepatocytes from humanized CAR animals were isolated and cultured in William's medium E in the presence of solvent (-), 10  $\mu$ M meclizine (Mec), 2 mM PB, or combination of these two compounds for 24 h. Total cell RNA was prepared, and 15  $\mu$ g of each RNA sample were used for Northern hybridization. Numbers indicate relative mRNA expression as determined by densitometry and normalization to the level of  $\beta$ -actin expression. B, CAR null (-/-) or humanized CAR mice were given a 500 mg/kg dose of acetaminophen (APAP) by ip injection, either with (+) or without (-) an additional injection of meclizine (100 mg/kg) 1 h later. ALT levels measured 24 h later are shown as average  $\pm$  SD (\*,  $P < 0.05$ ;  $n = 4-5$  per treatment group). C, Liver sections from the same animals treated as indicated were stained with hematoxylin and eosin. Arrows indicate necrotic areas; arrow thickness indicates degree of apparent damage.

many species differences that have been observed in responses to xenobiotics. Such opposite responses would be quite unexpected for most nuclear receptor superfamily members but are consistent with the significant sequence divergence of CAR in mammals. Similar sequence variation is observed with PXR, which also shows significant differences in xenosensor function in humans, rodents, and other species (29). It is an interesting question whether such variations represent an evolutionary adaptation to different environmental exposures of different species, or simply limitations in the effectiveness of selective pressures imposed on a receptor designed to respond to diverse signals.

The identification of meclizine as a mCAR agonist provides a new tool for studies of CAR function. It may be particularly useful for kinetic studies because, in contrast to the metabolically very stable TCPOBOP, meclizine can be cleared from the liver. This results in rapid loss of CYP2B10 expression after cessation of

meclizine treatment (data not shown). In addition, we have observed differences in the responses of CAR target genes to various inducers. For example, TCPOBOP strongly induces both CYP2B10 and CYP3A11 expression, but chlorpromazine induces only the former (10). The current results suggest that MRP2 shows a more robust response to meclizine than that previously described for TCPOBOP (14). It will be interesting to carefully compare the responses induced by distinct activators of CAR and other xenobiotic receptors.

The identification of meclizine as a hCAR inverse agonist raises the question of whether any *in vivo* effects of the antiemetic, particularly potential effects on activity of other drugs, can be attributed to inhibition of CAR activity. This could be of particular importance because meclizine and related antiemetics are used to treat the nausea associated with acute acetaminophen toxicity, and the results described here and previously (18) show that inhibition of either mCAR

or hCAR protects against acetaminophen toxicity in appropriate mouse models. However, we are not aware of any evidence for similar beneficial effects of meclizine in humans.

More broadly, inhibition of CAR activity in humans by meclizine could be expected to decrease drug metabolism and thereby increase the half-life and potential activity or toxicity of other coadministered agents. Meclizine, which causes drowsiness by itself, does increase the effects of other sedatives including ethanol and PB, but this is thought to be due to its effects on the central nervous system rather than on decreased clearance of the other agents (30). General drug-drug interactions associated with suppression of drug metabolism are not included as potential side effects and do not appear to be a major consequence of meclizine treatment, although it remains possible that more subtle effects could have eluded attention.

The apparent lack of effects of meclizine on drug metabolism in humans may be due to the fact that the therapeutic dose is approximately 100-fold lower than the dose used in mice. It is possible that higher doses could elicit such effects in humans, including the beneficial effects on acetaminophen toxicity observed in humanized mice. However, side effects from antihistamine or other activities of meclizine, particularly central nervous system effects (31), could obviously limit such potential applications. It may be necessary to identify a more specific hCAR inverse agonist to effectively block CAR activity in patients. Meclizine provides an appropriate starting point for such efforts.

## MATERIALS AND METHODS

### RNA Analysis

Total liver RNA was prepared using Trizol (Invitrogen, Rockville, MD) according to the manufacturer's instructions. Equivalent amounts of RNA from three to four mice were pooled and 15  $\mu\text{g}$  of the mixture were used for Northern blot analysis using previously described cDNA probes (14, 18).

### Primary Hepatocyte Culture

Mouse primary hepatocytes were prepared and maintained in William's media E (Invitrogen) supplemented with 10  $\mu\text{g}/\text{ml}$  insulin (Sigma, St. Louis, MO) and  $10^{-7}$  M triamcinolone acetonide as described elsewhere (32). Cells were treated with 2 mM PB (Sigma), 10  $\mu\text{M}$  meclizine (Sigma), or both for 24 h. Total RNA was isolated using the RNeasy mini kit (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions and 15  $\mu\text{g}$  RNA was used for Northern blot analysis.

### Animal Treatment

Mice were housed in a pathogen-free animal facility under standard 12-h light, 12-h dark cycle and fed standard rodent chow and water *ad libitum*. Humanized CAR and CAR knockout animals (18) were treated with acetaminophen (500 mg/kg) by ip injection. After 1 h, mice received an additional injection of the corn oil vehicle or meclizine (100 mg/kg). Blood was collected 24 h later and serum was prepared for

measurement of ALT as previously described (8). ALT assays were carried out by the clinical laboratory at Methodist Hospital (Houston, TX). Livers were removed and subjected to staining with hematoxylin and eosin as described (18). All animal experimentation described was conducted in accord with accepted standards of humane animal care.

### Cell Culture and Transfection

HepG2 cells were cultured in 24-well dishes with DMEM supplemented with 10% charcoal-stripped calf serum. Cells were transfected using calcium phosphate with 100 ng of receptor expression vectors, 300 ng of luciferase reporter plasmids, and 100 ng of pSV2- $\beta$ -galactosidase as internal control of transfection efficiency as described elsewhere (10). Drugs were added 12 h after transfection, and cells were incubated for an additional 24 h. The cell lysate was assayed for luciferase activity and normalized to  $\beta$ -galactosidase activity.

### Protein-Protein Interaction

GST protein purification and coactivator assays were carried out as described previously (7). A GST-SRC-1 receptor interaction domain fusion protein was bound to glutathione-Sepharose beads and incubated with [ $^{35}\text{S}$ ]methionine-labeled CAR protein in the presence or absence of 1  $\mu\text{M}$  TCPOBOP or 20  $\mu\text{M}$  meclizine. The beads were extensively washed, and the bound proteins were eluted with 15 mM glutathione, resolved by SDS-PAGE, and visualized by autoradiography.

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