

The Ratio of CAR to PXR Determines the Activity of Guggulsterone against the Cyp2b10 Promoter

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NON-STANDARD ABBREVIATIONS

CAR, constitutive androstane receptor; PXR, pregnane X receptor; PXR-KO, PXR Knockout; SRC1, Steroid Receptor Coactivator 1; Cyp2b10, cytochrome P450 2b10; PCN, pregnenolone 16 α -carbonitrile.

ABSTRACT

Guggulsterone is the active ingredient in gugulipid, an organic extract of the *Commiphora mukul* plant. Gugulipid has been used for nearly 3000 years in Ayurvedic medicine, mainly as a treatment for arthritis. Herbal practitioners currently use gugulipid therapy in conditions as diverse as rheumatism, coronary artery disease, arthritis, hyperlipidemia, acne, and obesity. The active ingredient in gugulipid is guggulsterone, a plant sterol compound recently identified as a pregnane X receptor (PXR, NR1I3) ligand. We show here that guggulsterone treatment represses the expression of cytochrome P450 2b10 (Cyp2b10) gene expression by inhibiting constitutive androstane receptor (CAR, NR1I3) activity in hepatocytes lacking functional PXR (PXR-KO). We also show that PXR-CAR crosstalk determines the net activity of guggulsterone treatment toward Cyp2b10 gene expression. Using mammalian two-hybrid assays we show that treatment with guggulsterone differentially affects protein cofactor recruitment to these two nuclear receptors. These data identify guggulsterone as an inverse agonist of the nuclear receptor CAR. When viewed together with the data showing that PXR and CAR expression is highly variable in different ethnic populations, and that CAR expression is under the control of a circadian rhythm, our data provide important insights into the molecular mechanism of inter-individual variability of drug metabolism. These data, together with the recent resolution of the crystal structures of PXR and CAR, will likely aid in the rational design of more specific CAR inverse agonists that are currently viewed as potential anti-obesity drugs.

Gugulipid is an herbal remedy derived from the gum resin of the *Commiphora mukul* tree. Gugulipid has been used in Ayurvedic medicine mainly to treat arthritis and inflammation. A number of clinical trials performed in India determined that gugulipid treatment produces favorable lipid profiles in patients with high cholesterol (Agarwal et al., 1986; Gopal et al., 1986; Nityanand et al., 1989). This finding has led to the widespread import and use of gugulipid extract in humans as a cholesterol-lowering agent sold in nutrition centers without a prescription in western societies. A recent human clinical trial has raised questions regarding the efficacy of gugulipid extract in lowering serum cholesterol (Szapary et al., 2003). Moreover, our laboratory has recently demonstrated that the use of gugulipid induces the expression and activity of drug-metabolizing cytochrome P450 genes through activation of the nuclear receptor protein PXR in cells (Brobst et al., 2004). The notion that gugulipid produces herb-drug interactions in humans is further supported by the results of a small well-controlled study that revealed significantly reduced ($P < 0.01$) peak plasma concentrations of either diltiazem or propranolol in volunteer patients co-fed one gram of gugulipid in comparison with those that received one of these drugs in combination with a placebo (Dalvi et al., 1994).

The active ingredients in gugulipid are the ketosteroids cis- and trans-4,17(20)-pregnadiene-3,16-dione, also known as E- and Z-guggulsterone, respectively (Tripathi et al., 1984; Tripathi et al., 1988; Beg et al., 1996; Verma et al., 1998). Recent experiments in mice lacking the farnesoid X receptor suggest that guggulsterones lower hepatic cholesterol levels in rodents by antagonizing the activity of this nuclear receptor (Urizar

et al., 2002). Other research suggests that guggulsterone is a selective farnesoid X receptor modulator that differentially regulates the expression of a subset of target genes (Cui et al., 2003). Additional research has demonstrated that guggulsterone interacts with multiple nuclear receptor superfamily members including the estrogen, mineralocorticoid, progesterone, and androgen receptors (Brobst et al., 2004; Burris et al., 2005). Taken together, these data indicate that the molecular basis of guggulipid's biological activity is more complex than previously recognized.

PXR is a xenobiotic sensor that is activated by a large number of structurally diverse compounds (reviewed in (Kliwer, 2003)). CAR was originally identified as xenobiotic sensor that, together with PXR, regulates drug-inducible expression of CYP2B and CYP3A genes in liver (Honkakoski et al., 1998). A number of studies have shown that PXR and CAR share ligands and target genes (Moore et al., 2000; Maglich et al., 2002; Wei et al., 2002). Analysis of the human orthologues of these two cytochrome P450 genes reveals that CAR and PXR compete for the same or overlapping enhancer sequences located in the promoters of these two genes (Xie et al., 2000; Goodwin et al., 2001). More recently, CAR has been distinguished from PXR in that CAR appears to be activated in response to metabolic and nutritional stress, while PXR is not (Maglich et al., 2004). Moreover, CAR plays a role in regulating the expression of genes involved in the metabolism of fatty acids and glucose homeostasis, while PXR does not (Ueda et al., 2002). The finding that activation of CAR in mice lacking PXR (PXR-KO) produces much higher levels of induction of shared target genes in liver suggests that the presence

of PXR might affect the functional regulation of shared CAR-PXR-target genes in vivo (Staudinger et al., 2003).

We show here that both cis- and trans-guggulsterone function as efficacious CAR inverse agonists. Our data reveal that guggulsterone represses Cyp2b10 expression in PXR-KO hepatocytes, but not in wild type hepatocytes. We demonstrate that PXR-CAR crosstalk determines the net effect of guggulsterone treatment towards the Cyp2b10 promoter. Moreover, we show that in the presence of guggulsterone, the ratio of PXR to CAR determines the net activity of these two receptors by regulating the amount of the steroid receptor co-activator protein-1 (SRC-1) associated with either PXR or CAR. Taken together, our studies underscore the complexity of regulation of PXR- and CAR-shared-target gene networks and provide important information on the potential mechanism of inter-individual variability in drug metabolism. These data together with the recently solved crystal structures of PXR and CAR (Watkins et al., 2001; Shan et al., 2004; Suino et al., 2004; Xu et al., 2004) will likely aid in the rational design of more specific CAR inverse agonists, which due to their likely ability to modulate serum thyroid hormone levels are viewed as potential anti-obesity drugs (Maglich et al., 2004; Qatanani et al., 2005).

MATERIALS AND METHODS

Animal Care. Generation of the PXR knockout mice was previously described (Staudinger et al., 2001). All rodents were maintained on standard laboratory chow and were allowed food and water ad libitum. The studies reported here have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Plasmids and Chemicals. The full-length mouse CAR and mouse PXR mammalian expression vectors were described previously. VP16-mPXR was described previously (Ding and Staudinger, 2005). Cyp2b10-Luc was described previously (Rivera-Rivera et al., 2003). VP16-mCAR encodes full-length mouse CAR fused to VP16 (BD Biosciences, Palo Alto, CA). GAL-SRC-1 was a generous gift from Dr. Barry Forman. The SV- β -Gal plasmids are commercially available (Invitrogen, Carlsbad, CA). All compounds were purchased from Sigma except guggulsterone (Steraloids, Newport, RI) and were dissolved as 1000X stocks in DMSO (St. Louis, MO).

Cell Culture and Transient Transfection of CV-1 Cells. CV-1 cells were plated on 96-well plates as described previously. For the Cyp2b10-LUC reporter gene assays, each well was transfected with 20 ng of reporter gene, 5 ng of nuclear receptor expression vector(s) unless otherwise indicated, 40 ng of SV-beta-gal and added with pBluescript to 110 ng of total DNA per well. The mammalian two-hybrid system analysis was

performed as described previously (Ding and Staudinger, 2005). Transfection mixtures contained pFR-LUC (20ng), GAL-SRC-1 (20 ng), VP16-mPXR and/or VP16-CAR (10 ng, unless otherwise indicated), pSV- β -GAL (40 ng), and added with pBluescript to 110 ng of total DNA per well. Twenty-four hr post-transfection, cells were drug-treated for 24 h. The luciferase and β -galactosidase activities were determined using the Dual-Light Kit per the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Northern Blot Analysis. Mouse hepatocytes were isolated using a two-step perfusion. Briefly, the liver was cleared with wash solution (150 ml wash solution is made by mixing 15 ml of 10X HBSS (Gibco), 150 μ l of 0.5 M EGTA, 825 μ l of 1.0 M Glucose (Dextrose) and 134 ml of H₂O) at a flow rate of 16 ml/min for 4 min at 37°C. The liver was digested for 8 min at a flow rate of 8 ml/min at 37°C with digestion solution (150 ml digestion solution is made by mixing 15 ml of 10X HBSS, 225 μ l of 1.0 M CaCl₂, 825 μ l of 1.0 M Glucose, 70-75 mg of collagenase type I (Gibco, 200-300 units/mg. Dissolved in 20 ml of digest medium that does not contain collagenase and then filter-sterilized using 0.45 μ m filter) and 134 ml of H₂O). Then digested liver was put in 10-15 ml of digest solution in a sterile petri dish and cut into small pieces. Hepatocytes were dissociated by pipetting using a 5 ml pipet. The suspension was filtered through a 100 μ m cell strainer (Becton Dickinson, Palo Alto, CA). The cells were pelleted at 93 x g for 5 min at 4°C and washed twice in DMEM supplemented with 5% fetal bovine serum, 100 nM dexamethasone, 1 μ M insulin, and antibiotics. Cell viability was determined using trypan blue staining. Generally, >90% viability was achieved. Mouse hepatocytes were plated in 6-well collagen-coated plates at 8x10⁵ cells/well in DME Medium

supplemented with 100 nM dexamethasone, 5% FBS, 1 μ M Insulin, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin as described previously. Twelve-sixteen hours after plating, the medium was replaced with maintenance medium {William's E medium supplemented with 100 nM dexamethasone, 100 units/ml penicillin, 100 μ g/ml streptomycin and 1x Insulin-Transferrin-Selenium (Invitrogen, Carlsbad, CA)}. About 48 hr post-plating, the hepatocytes were treated with drugs in maintenance medium for 24 hours. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA (10 μ g/lane) was resolved on 3.7 formaldehyde 1% agarose gel for Northern blot analysis. Blots were hybridized with 32 P-labeled cDNA corresponding to the cDNA sequence for mouse Cyp2b10 (GenBank NM_009998). The 18S ribosomal RNA probe was amplified from liver cDNA using commercially available primers per the manufacturer's instruction (Ambion, Austin, TX).

Real-time Quantitative PCR. One microgram of DNaseI-treated RNA was reverse transcribed using random primers following the manufacturers' instructions (Promega, Madison, WI). Equal amounts of cDNA were used in real-time quantitative PCR reactions using the Cepheid Smart Cycler (Sunnyvale, CA). Reactions included 200 nM fluorogenic probe and 300 nM primers specific for 18S or Cyp2b10. The fluorogenic probe and primer sets were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). BioSearch Technologies (Novato, CA) synthesized the fluorogenic probes. The sequences (5' to 3') for the primers and probes are as follows: Cyp2b10, forward primer

(GACTTTGGGATGGGAAAGAG), fluorogenic probe (FAM-
TAGTGGAGGAACTGCGGAAATCCC-BHQ1), reverse primer
(CCAAACACAATGGAGCAGAT); 18S, forward primer
(CCAGTAAGTGC GGGTCATAA), fluorogenic probe (FAM-
CGATTGGATGGTTTAGTGAGGCC-BHQ1), reverse primer
(GGTTCACCTACGGAAACCTT). Cycling conditions were 95°C for 2 minutes
followed by 45 cycles of 95°C for 15 seconds, 60°C for 15 seconds, 68°C for 15 seconds
using the Cepheid SmartCycler system (Sunnyvale, CA). Fold-induction was calculated
as described (Staudinger et al., 2003).

RESULTS

Repression of Cyp2b10 Expression by Guggulsterone.

To investigate the regulation of Cyp2b10 promoter activity by guggulsterones, we performed a series of reporter gene assays in CV-1 cells. Cells were transiently transfected with the expression vector that encodes mouse CAR together with CAR-responsive luciferase reporter gene Cyp2b10-Luc. This reporter gene contains the PBREM enhancer sequence that was previously shown to be required for activation of Cyp2b10 gene expression by CAR (Rivera-Rivera et al., 2003). Both cis- and trans-guggulsterone repressed the expression of Cyp2b10-luc in the presence of CAR with an efficacy similar to that achieved with androstanol, a prototypical inverse agonist of mouse CAR, and similar results were obtained when the experiment was performed in the presence of the known CAR agonist 1,4 bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (Fig 1A). Full concentration-response analysis of cis-guggulsterone revealed IC₅₀ values of approximately 900 nM and 8.4 μM in the absence and presence of 250 nM TCPOBOP, respectively (Fig. 1B). Moreover, full concentration-response analysis of TCPOBOP demonstrated that addition of 10 μM cis-guggulsterone increased the EC₅₀ value of TCPOBOP against CAR by approximately one order of magnitude (21 nM to 200 nM) (Fig. 1C).

Cellular PXR to CAR Ratio Regulates the Expression of Cyp2b10 Following Treatment with Guggulsterone.

To determine the effect of guggulsterone on Cyp2b10 gene expression in liver cells, we treated primary mouse hepatocytes and performed Northern blot analysis. Androstanol

was used as a positive control for CAR antagonism. Because our previous studies show that guggulsterone is a PXR agonist and to rule out the involvement of PXR, we first performed our experiments in PXR-KO mouse hepatocytes. Both cis- and trans-guggulsterone treatments repressed Cyp2b10 gene expression levels in PXR-KO mouse hepatocytes (Fig. 2A). Surprisingly, treatment with androstanol did not produce repression of Cyp2b10 expression, indicating that the biological activity of androstanol might be more complicated in cultured primary hepatocytes than generally believed. To determine the biological activity of guggulsterones in a normal cellular environment, we also performed similar experiments in wild-type cultured mouse hepatocytes. Unlike in PXR-KO mouse hepatocytes, treatment with cis- and trans-guggulsterone modestly induced the expression levels of Cyp2b10 in WT mouse hepatocytes, and a similar effect was observed following treatment with androstanol (Fig. 2B). To quantitatively determine the effect of guggulsterone on the Cyp2b10 gene expression and to confirm that the repression of Cyp2b10 gene expression by guggulsterone in PXR-KO mouse hepatocytes was mediated by CAR, we also co-treated PXR-KO mouse hepatocytes with guggulsterone and the prototypical rodent CAR agonist TCPOBOP and performed real-time quantitative PCR analysis. As shown in figure 2C, in PXR-KO mouse hepatocytes guggulsterone repressed the basal level of Cyp2b10 as well as the induction of Cyp2b10 by TCPOBOP. Taken together, these data suggest that the presence of PXR compromised the ability of guggulsterones to repress Cyp2b10 gene expression in cultured hepatocytes.

This notion was further confirmed by reporter gene studies in CV-1 cells (Fig. 3A). CV-1 cells were transfected with mouse CAR, mouse PXR, or both CAR and PXR, together with the Cyp2b10-Luc reporter gene. In the presence of PXR alone, both cis- and trans-guggulsterone induced the expression of Cyp2b10-Luc reporter gene. However, in the presence of CAR alone, guggulsterone treatment produced repression of the Cyp2b10-Luc reporter gene. Interestingly, in the presence of both PXR and CAR, the ability of guggulsterone treatment to produce repression was compromised. Moreover, the basal level of reporter gene activity was significantly increased when CAR was present. To further investigate the role that crosstalk between CAR and PXR plays in the regulation of Cyp2b10 by guggulsterone, CV-1 cells were transfected with fixed amount of CAR expression vector (5 ng) and increasing amounts of PXR expression vector (0.25-5 ng), or fixed amount of PXR expression vector (5 ng) and increasing amounts of CAR expression vector (0.25-5 ng) and treated with 10 μ M cis-guggulsterone (Fig. 3B). When the PXR to CAR ratio was high, guggulsterones transactivated the Cyp2b10-Luc promoter, but when the PXR to CAR ratio was low, guggulsterones transrepressed the Cyp2b10-Luc promoter.

Guggulsterone Differentially Regulates the Association of SRC-1 with CAR and PXR.

The interaction between steroid receptor co-activator proteins and nuclear receptors enhances nuclear receptor activity in cells. Because we observed differential effects of guggulsterones on the expression of Cyp2b10 in different cellular environments, we sought to determine whether guggulsterones differentially modulated the strength of CAR-SRC-1 and PXR-SRC-1 protein-protein interaction using the mammalian two-

hybrid system. CV-1 cells were transfected with the expression vector encoding the receptor interacting domain from the nuclear co-activator protein SRC-1 fused to GAL4 DNA-binding domain together with expression vectors encoding either VP16-tagged mouse CAR or VP16-tagged mouse PXR together with the GAL4-responsive luciferase reporter gene pFR-LUC. Similar to androstanol, treatment with guggulsterone recruited VP16-tagged PXR to GAL-SRC1, but displaced VP16-CAR from GAL-SRC1 (Fig. 4A). Interestingly, in the presence of both VP16-CAR and VP16-PXR, treatment of *cis*-guggulsterone modulated the reporter activity in a PXR-CAR ratio dependent manner (Fig. 4B). When cells were transfected with excessive amounts of VP16-CAR, the reporter gene activity was repressed by the treatment with *cis*-guggulsterone, indicating that SRC-1 dissociation from CAR is predominant over its recruitment to PXR. However, as more VP16-mPXR was titrated into this system, the response of the pFR-Luc reporter gene to *cis*-guggulsterone treatment moved from negative to positive in a dose-dependent manner, demonstrating that recruitment of VP16-PXR to GAL-SRC1 became predominant. Interestingly, the presence of higher levels of VP16-PXR produced an increase in basal reporter gene activity.

DISCUSSION

Guggulipid extract has been used in Ayurvedic medicine to treat various diseases ranging from dyslipidemia to inflammation for thousands of years and is also gaining popularity in Western societies for their putative cholesterol-lowering properties. The stereoisomers and ketosteroids *cis*- and *trans*-guggulsterone, respectively, are the main ingredients in guggulipid. Besides lipid-lowering effects, guggulsterone also has been shown to have thyroid-stimulating activity in rats (Tripathi et al., 1984; Tripathi et al., 1988). Although the mechanism is not clear, it is suggested to be different from that of TSH and is probably not mediated through the pituitary.

Like its cousin PXR, CAR was originally believed to be a xenobiotic sensor that regulates the expression of xenobiotic responsive genes following xenobiotic insults. However, recent work suggests that, unlike PXR, CAR plays an important role in energy homeostasis (Ueda et al., 2002; Maglich et al., 2004). Treatment with the synthetic CAR ligand TCPOBOP produces lower serum levels of thyroid hormones in wild-type mice but not in CAR knockout mice (Maglich et al., 2004). Twenty-four hour fasting also produces decreases in serum levels of both triiodothyronine (T3) and T4 in wild-type mice but not in CAR-KO mice. The decrease in thyroid hormone levels in wild-type mice is associated with induction of *Ugt1a1*, *Sultn*, *Sult1a1* and *Sult2a1* gene expression (Maglich et al., 2004; Qatanani et al., 2005). These genes encode TH-metabolizing enzymes, and induction of these genes by fasting and CAR ligands is totally absent in CAR knockout mice. More importantly, CAR knockout mice lost over twice as much

weight as their wild-type littermates when both were placed on a 40% caloric restriction diet for 12 weeks (Maglich et al., 2004). In light of these data, our results suggest that some of the thyroid-stimulating and lipid-burning activity of guggulsterone observed in rats might be mediated through antagonism of CAR.

Although Wei et al., clearly demonstrated the repressive effect of androstanol on the induction Cyp2b10 gene expression by TCPOBOP in vivo in mice (Wei et al., 2002), it remains unknown how androstanol treatment affects the basal level of Cyp2b10 in vivo. Although we did not observe a significant repressive effect of androstanol on the induction of Cyp2b10 by TCPOBOP in cultured primary mouse hepatocytes (data not shown), guggulsterone did repress Cyp2b10 gene expression in the absence as well as presence of TCPOBOP (Figure 2). There are several potential reasons underlying this discrepancy. First, in our cultured mouse hepatocyte system, all compounds were used at 10 μ M, which are likely very different from the concentrations achieved in vivo during experiments performed by Wei et al. It is possible that the repression of the induction of Cyp2b10 by androstanol requires a relatively low concentration of TCPOBOP and a high concentration of androstanol. Such possibility is currently under investigation in our lab. Second, although androstanol and guggulsterone have similar activities against PXR and CAR, the regulation of Cyp2b10 gene expression by these two compounds might involve distinct mechanisms. Finally, it is possible that some important hepatic factors are missing in cultured hepatocytes and these factors are necessary to mediate the repressive effect of androstanol on Cyp2b10 gene expression but are not required for guggulsterone to repress Cyp2b10 gene expression.

While the observed repression of Cyp2b10 expression in PXR-KO mouse hepatocytes produced by guggulsterone treatment is consistent with their function as inverse agonists of CAR, guggulsterone did not repress Cyp2b10 expression in wild-type mouse hepatocytes. This is likely due to the activation of PXR. This observation is also consistent with the effects of the known inverse CAR agonist, androstanol, in our culture system.

Because PXR and CAR gene expression exhibits a high degree of inter-individual variability (Lamba et al., 2004a; Lamba et al., 2004b), and because CAR expression exhibits circadian rhythms (Kanno et al., 2004), we decided to investigate how the ratio of PXR to CAR affects the biological activity of guggulsterone on the Cyp2b10 promoter. Our reporter gene studies clearly demonstrate that the ratio of PXR to CAR determines the effect of guggulsterone treatment on the expression of the Cyp2b10 promoter. The differential modulation of the effect of guggulsterones on Cyp2b10 promoter by the ratio of PXR to CAR is illustrated in the model in figure 5. When PXR expression is dominant when compared with CAR, the net effect of guggulsterones on Cyp2b10 promoter is positive. On the other hand, when CAR expression is dominant when compared with PXR, the net effect is negative. Therefore, compounds or drugs with guggulsterone-like activity can induce very different response in different people or even in the same person at different times of the day.

There are at least two classes of ligands that regulate the activity of CAR through two distinct mechanisms. The first class of CAR ligand is represented by phenobarbital, which activates CAR through a cytoplasm-to-nucleus translocation mechanism and does not involve direct binding to CAR (Kawamoto et al., 1999). On the other hand, TCPOBOP and androstanol represent a second class of CAR modulators that regulate the activity of CAR by modulating CAR-cofactor interactions and involve direct binding of these compounds to CAR (Forman et al., 1998; Tzamei et al., 2000; Maglich et al., 2003). Our mammalian two-hybrid data reveal that guggulsterone treatment reduces CAR-SRC-1 interaction, which suggests that guggulsterone modulates CAR activity, likely by direct binding and displacing the SRC1 co-activator protein in a manner similar to androstanol.

During the preparation of this manuscript, the crystallographic structure of CAR was published (Shan et al., 2004; Suino et al., 2004; Xu et al., 2004). Our studies, together with the crystal structures of PXR and CAR, will provide important information on the regulation of PXR and CAR by direct ligands and will therefore help in the rational design of specific CAR ligands. Such compounds are currently viewed as having a high potential as anti-obesity drugs due to their likely ability to modulate serum thyroid hormone levels.

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References

Agarwal RC, Singh SP, Saran RK, Das SK, Sinha N, Asthana OP, Gupta PP, Nityanand S, Dhawan BN and Agarwal SS (1986) Clinical trial of gugulipid--a new hypolipidemic agent of plant origin in primary hyperlipidemia. *Indian J Med Res* 84:626-634.

Beg M, Singhal KC and Afzaal S (1996) A study of effect of guggulsterone on hyperlipidemia of secondary glomerulopathy. *Indian J Physiol Pharmacol* 40:237-240.

Brobst DE, Ding X, Creech KL, Goodwin B, Kelley B and Staudinger JL (2004) Guggulsterone activates multiple nuclear receptors and induces CYP3A gene expression through the pregnane X receptor. *J Pharmacol Exp Ther* 310:528-535.

Burris TP, Montrose C, Houck KA, Osborne HE, Bocchinfuso WP, Yaden BC, Cheng CC, Zink RW, Barr RJ, Hepler CD, Krishnan V, Bullock HA, Burris LL, Galvin RJ, Bramlett K and Stayrook KR (2005) The hypolipidemic natural product guggulsterone is a promiscuous steroid receptor ligand. *Mol Pharmacol* 67:948-954.

Cui J, Huang L, Zhao A, Lew JL, Yu J, Sahoo S, Meinke PT, Royo I, Pelaez F and Wright SD (2003) Guggulsterone is a farnesoid X receptor antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. *J Biol Chem* 278:10214-10220.

Dalvi SS, Nayak VK, Pohujani SM, Desai NK, Kshirsagar NA and Gupta KC (1994) Effect of gugulipid on bioavailability of diltiazem and propranolol. *J Assoc Physicians India* 42:454-455.

Ding X and Staudinger JL (2005) Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. *Biochem Pharmacol* 69:867-873.

Forman BM, Tzamelis I, Choi HS, Chen J, Simha D, Seol W, Evans RM and Moore DD (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* 395:612-615.

Goodwin B, Moore LB, Stoltz CM, McKee DD and Kliewer SA (2001) Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol* 60:427-431.

Gopal K, Saran RK, Nityanand S, Gupta PP, Hasan M, Das SK, Sinha N and Agarwal SS (1986) Clinical trial of ethyl acetate extract of gum gugulu (gugulipid) in primary hyperlipidemia. *J Assoc Physicians India* 34:249-251.

Honkakoski P, Zelko I, Sueyoshi T and Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* 18:5652-5658.

Kanno Y, Otsuka S, Hiromasa T, Nakahama T and Inouye Y (2004) Diurnal difference in CAR mRNA expression. *Nucl Recept* 2:6.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* 19:6318-6322.

Kliwer SA (2003) The nuclear pregnane X receptor regulates xenobiotic detoxification. *J Nutr* 133:2444S-2447S.

Lamba JK, Lamba V, Yasuda K, Lin YS, Assem M, Thompson E, Strom S and Schuetz E (2004a) Expression of constitutive androstane receptor splice variants in human tissues and their functional consequences. *J Pharmacol Exp Ther* 311:811-821.

Lamba V, Yasuda K, Lamba JK, Assem M, Davila J, Strom S and Schuetz EG (2004b) PXR (NR1I2): splice variants in human tissues, including brain, and identification of neurosteroids and nicotine as PXR activators. *Toxicol Appl Pharmacol* 199:251-265.

Maglich JM, Parks DJ, Moore LB, Collins JL, Goodwin B, Billin AN, Stoltz CA, Kliwer SA, Lambert MH, Willson TM and Moore JT (2003) Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes. *J Biol Chem* 278:17277-17283.

Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT and Kliewer SA (2002) Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol* 62:638-646.

Maglich JM, Watson J, McMillen PJ, Goodwin B, Willson TM and Moore JT (2004) The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. *J Biol Chem* 279:19832-19838.

Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL and Kliewer SA (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* 275:15122-15127.

Nityanand S, Srivastava JS and Asthana OP (1989) Clinical trials with gugulipid. A new hypolipidaemic agent. *J Assoc Physicians India* 37:323-328.

Qatanani M, Zhang J and Moore DD (2005) Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. *Endocrinology* 146:995-1002.

Rivera-Rivera I, Kim J and Kemper B (2003) Transcriptional analysis in vivo of the hepatic genes, Cyp2b9 and Cyp2b10, by intravenous administration of plasmid DNA in mice. *Biochim Biophys Acta* 1619:254-262.

Shan L, Vincent J, Brunzelle JS, Dussault I, Lin M, Ianculescu I, Sherman MA, Forman BM and Fernandez EJ (2004) Structure of the murine constitutive androstane receptor complexed to androstenol: a molecular basis for inverse agonism. *Mol Cell* 16:907-917.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* 98:3369-3374.

Staudinger JL, Madan A, Carol KM and Parkinson A (2003) Regulation of drug transporter gene expression by nuclear receptors. *Drug Metab Dispos* 31:523-527.

Suino K, Peng L, Reynolds R, Li Y, Cha JY, Repa JJ, Kliewer SA and Xu HE (2004) The nuclear xenobiotic receptor CAR: structural determinants of constitutive activation and heterodimerization. *Mol Cell* 16:893-905.

Szapary PO, Wolfe ML, Bloedon LT, Cucchiara AJ, DerMarderosian AH, Cirigliano MD and Rader DJ (2003) Guggulipid for the treatment of hypercholesterolemia: a randomized controlled trial. *Jama* 290:765-772.

Tripathi YB, Malhotra OP and Tripathi SN (1984) Thyroid stimulating action of Z-guggulsterone obtained from *Commiphora mukul*. *Planta Med*:78-80.

Tripathi YB, Tripathi P, Malhotra OP and Tripathi SN (1988) Thyroid stimulatory action of (Z)-guggulsterone: mechanism of action. *Planta Med* 54:271-277.

Tzamelis I, Pissios P, Schuetz EG and Moore DD (2000) The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. *Mol Cell Biol* 20:2951-2958.

Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari CA, Lehmann JM and Negishi M (2002) Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. *Mol Pharmacol* 61:1-6.

Urizar NL, Liverman AB, Dodds DT, Silva FV, Ordentlich P, Yan Y, Gonzalez FJ, Heyman RA, Mangelsdorf DJ and Moore DD (2002) A natural product that lowers cholesterol as an antagonist ligand for FXR. *Science* 296:1703-1706.

Verma N, Singh SK and Gupta RC (1998) Simultaneous determination of the stereoisomers of guggulsterone in serum by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 708:243-248.

Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA and Redinbo MR (2001) The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science* 292:2329-2333.

Wei P, Zhang J, Dowhan DH, Han Y and Moore DD (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response.

Pharmacogenomics J 2:117-126.

Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS and Evans RM (2000) Reciprocal activation of xenobiotic response genes by nuclear receptors

SXR/PXR and CAR. *Genes Dev* 14:3014-3023.

Xu RX, Lambert MH, Wisely BB, Warren EN, Weinert EE, Waitt GM, Williams JD, Collins JL, Moore LB, Willson TM and Moore JT (2004) A structural basis for constitutive activity in the human CAR/RXRalpha heterodimer. *Mol Cell* 16:919-928.

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FIGURE LEGENDS

Fig. 1. Guggulsterones Repress CAR Activity in Cyp2b10-Luc Reporter Gene

Assays. (A) CV-1 cells were transfected with the expression vector for mouse CAR and the Cyp2b10-Luc reporter gene. Cells were treated with vehicle (Veh, 0.1% DMSO) or 10 μ M of the following compounds: cis, cis-guggulsterone; trans, trans-guggulsterone; An, androstanol both in the absence and presence of 250 nM TCPOBOP. (B) CV-1 cells were transfected as in (A) and treated with increasing concentrations of cis-guggulsterone both in the absence and presence of 250 nM TCPOBOP. (C) Transfected CV-1 cells were treated with increasing concentrations of TCPOBOP both in the absence and presence of 10 μ M cis-guggulsterone. All cells were treated for 24 h. The data represent the mean of replicates \pm SEM (n=4) and are normalized against β -galactosidase activity, and are expressed as fold induction over vehicle control.

Fig. 2. Guggulsterone Treatment Represses Cyp2b10 Gene Expression in PXR-KO

Mouse Hepatocytes but not in Wild-type Mouse Hepatocytes. (A) PXR-KO cultured primary mouse hepatocytes were treated with vehicle (Veh, 0.1% DMSO) or 10 μ M of the following compounds: cis, cis-guggulsterone; trans, trans-guggulsterone; An, androstanol. (B) Wild-type primary mouse hepatocytes were treated as in (A). All cells were treated for 24 h before RNA isolation. Total RNA (10 μ g) was used for Northern blot analysis. The blots were probed sequentially with 32 P-labeled fragments of Cyp2b10 and 18S. (C) PXR-KO cultured primary mouse hepatocytes were treated with vehicle, 10 μ M of cis-guggulsterone, 10 μ M of TCPOBOP or both for 24 h. RNA was subjected to

real-time quantitative PCR analysis to determine the relative expression levels of Cyp2b10 as described in materials and methods. The data are normalized to 18S levels and are expressed as average values (n=3) \pm the standard error of the mean.

Fig. 3. Regulation of Cyp2b10-Luc by Guggulsterone is Dependent on the Ratio of PXR to CAR. (A) CV-1 cells were transfected with expression vectors for mouse CAR, mouse PXR or both and the Cyp2b10-Luc reporter gene. Cells were treated for 24 h with vehicle (Veh, 0.1% DMSO) or 10 μ M of the following compounds: cis, cis-guggulsterone; trans, trans-guggulsterone; An, androstanol. (B) CV-1 cells were transfected with the Cyp2b10-Luc reporter gene and the indicated amounts of expression vectors encoding mouse CAR and mouse PXR. Transfected cells were treated with 10 μ M cis-guggulsterone for 24 h. The data represent the mean of replicates \pm SEM (n=4) and are normalized against β -galactosidase activity, and are expressed as reporter gene activity.

Fig. 4. Differential Modulation of CAR-SRC-1 and PXR-SRC-1 Interactions by Guggulsterones. (A) Transient transfection of CV-1 cells was performed as described in materials and methods. Twenty-four hr post-transfection, CV-1 cells were treated with vehicle (Veh, 0.1% DMSO) or 10 μ M of the following compounds: cis, cis-guggulsterone; trans, trans-guggulsterone; An, Androstanol. (B) CV-1 cells were transfected with the pFR-LUC reporter gene, the GAL-SRC-1 expression vector and the indicated amounts of expression vectors for VP16-mCAR and VP16-mPXR. Transfected

cells were treated with 10 μ M cis-guggulsterone for 24 h. The data represent the mean of replicates \pm SEM (n=4) and are normalized against β -galactosidase activity, and are expressed as reporter gene activity.

Fig. 5. Model of the PXR to CAR Ratio-dependent Regulation of the Cyp2b10 Promoter by Guggulsterones. Guggulsterone recruits the co-activator SRC-1 to PXR and displace SRC-1 from CAR. The ratio of PXR to CAR determines the ratio of the number of SRC-1-occupied nuclear receptors to the number of SRC-1-absent nuclear receptors on the Cyp2b10 promoter in the presence of guggulsterone. When PXR is predominant over CAR, the net effect of guggulsterone on Cyp2b10 promoter is positive, leading to transactivation of Cyp2b10 gene. However, if CAR expression is predominant, the net effect of guggulsterone on the Cyp2b10 promoter becomes negative, thereby leading to transrepression of the Cyp2b10 gene.

Figure 1A

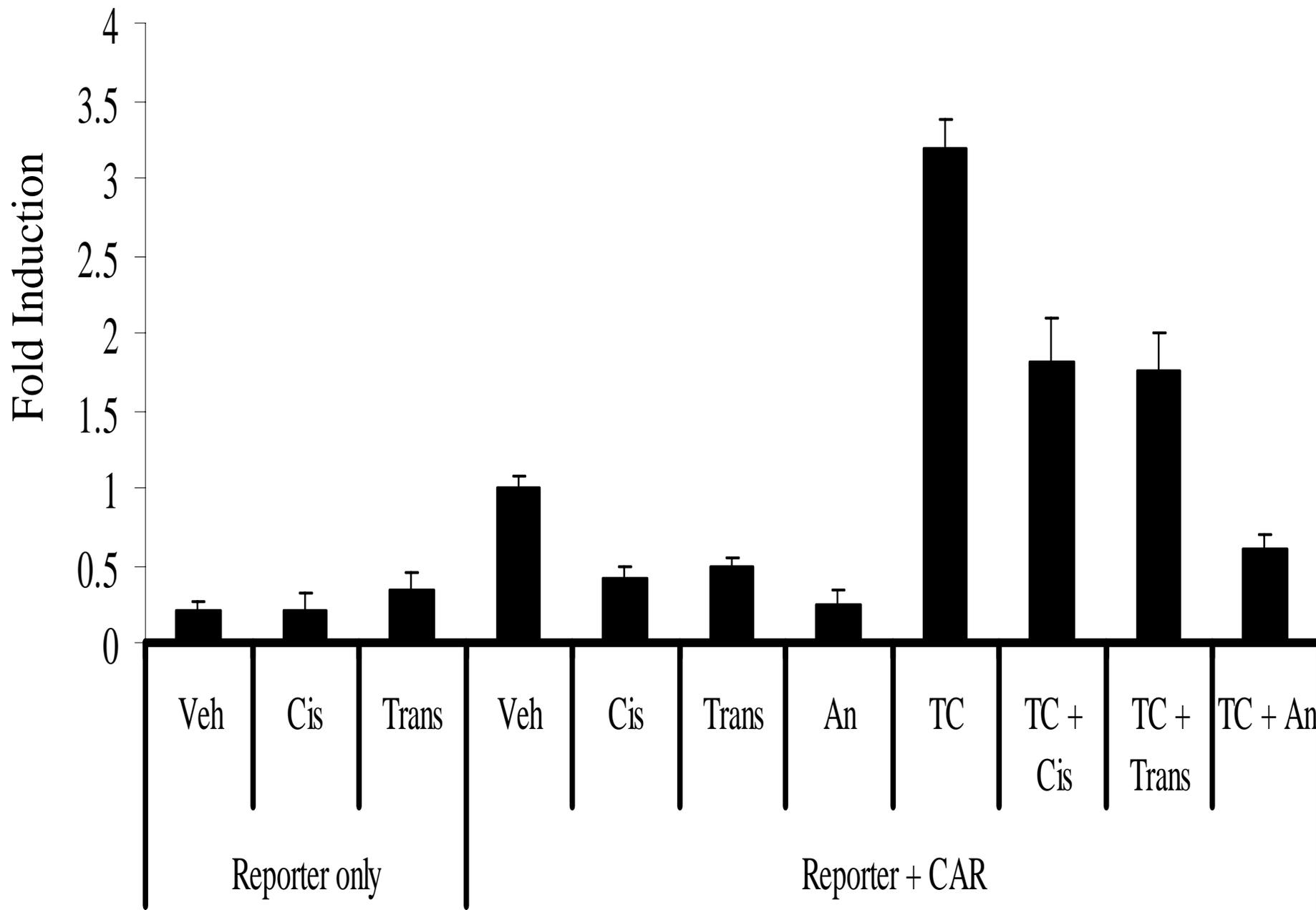


Figure 1B

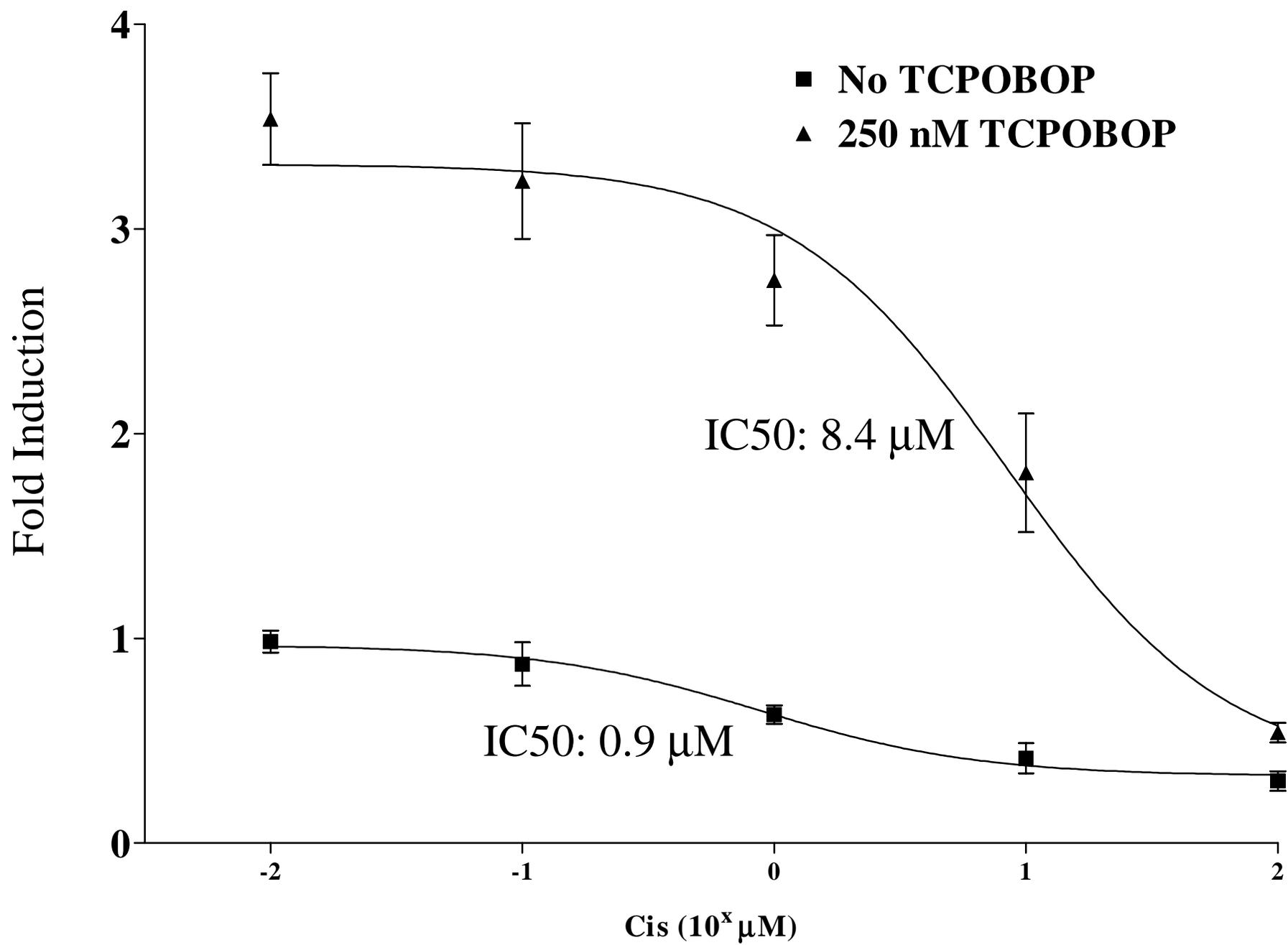


Figure 1C

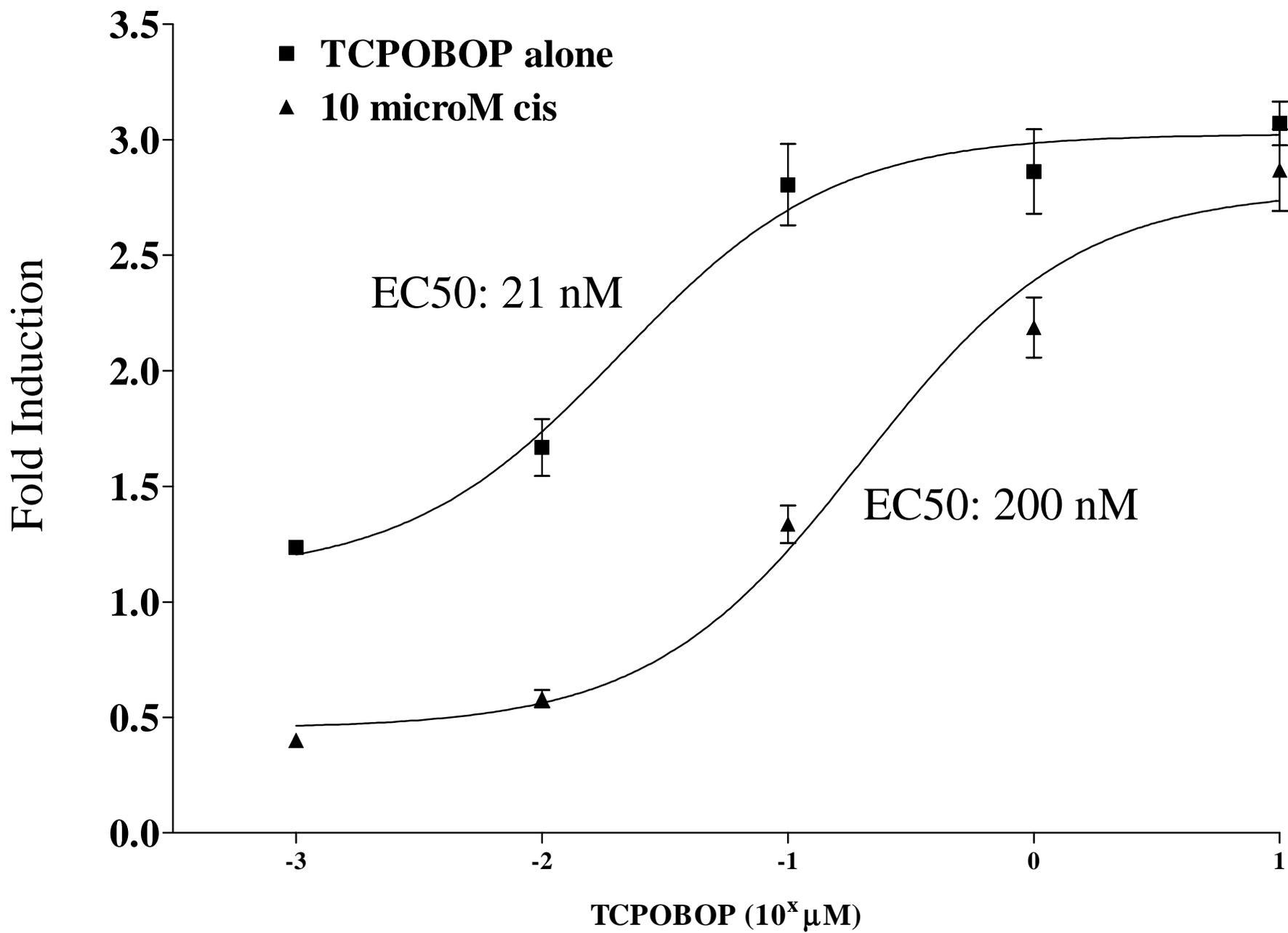


Figure 2A

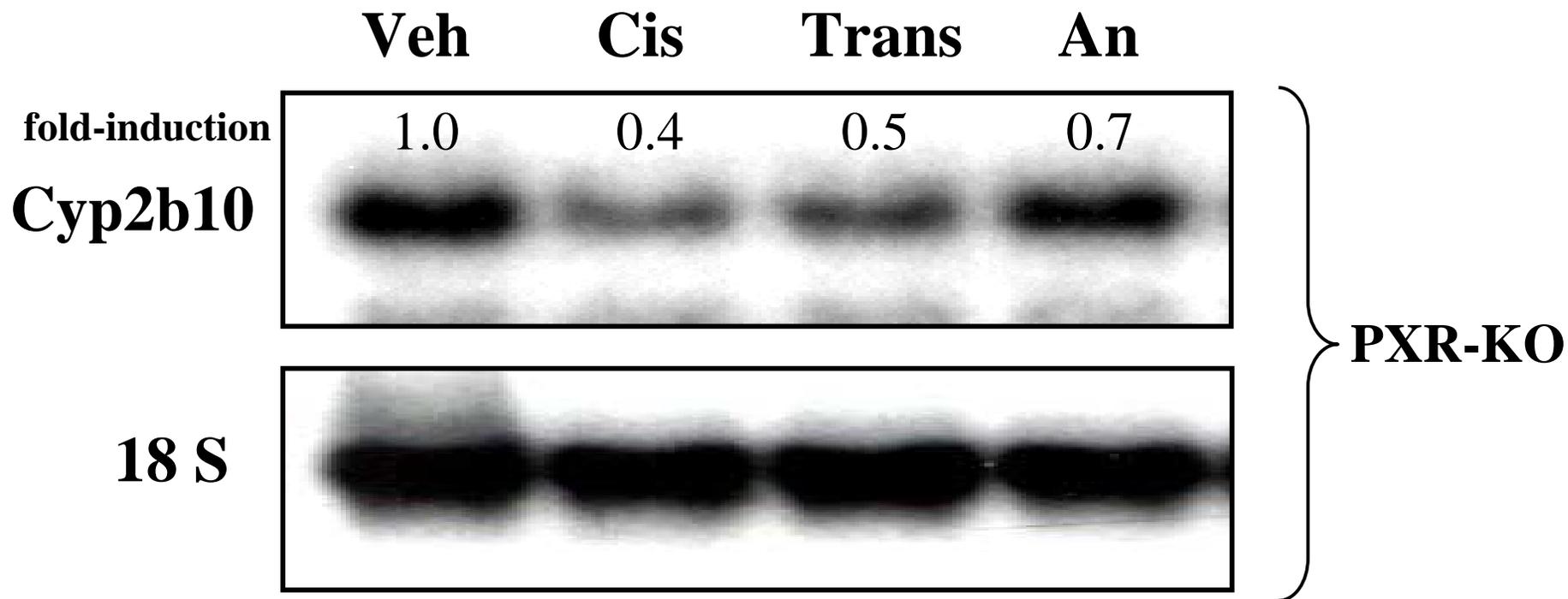


Figure 2B

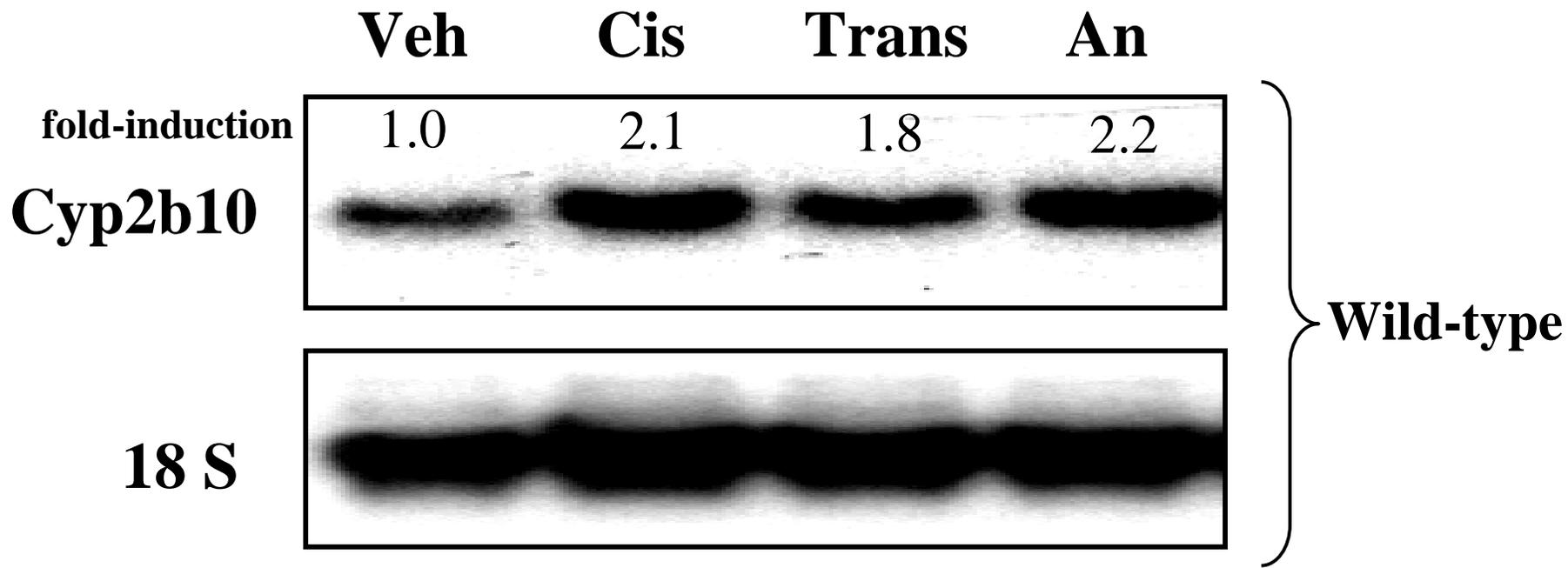


Figure 2C

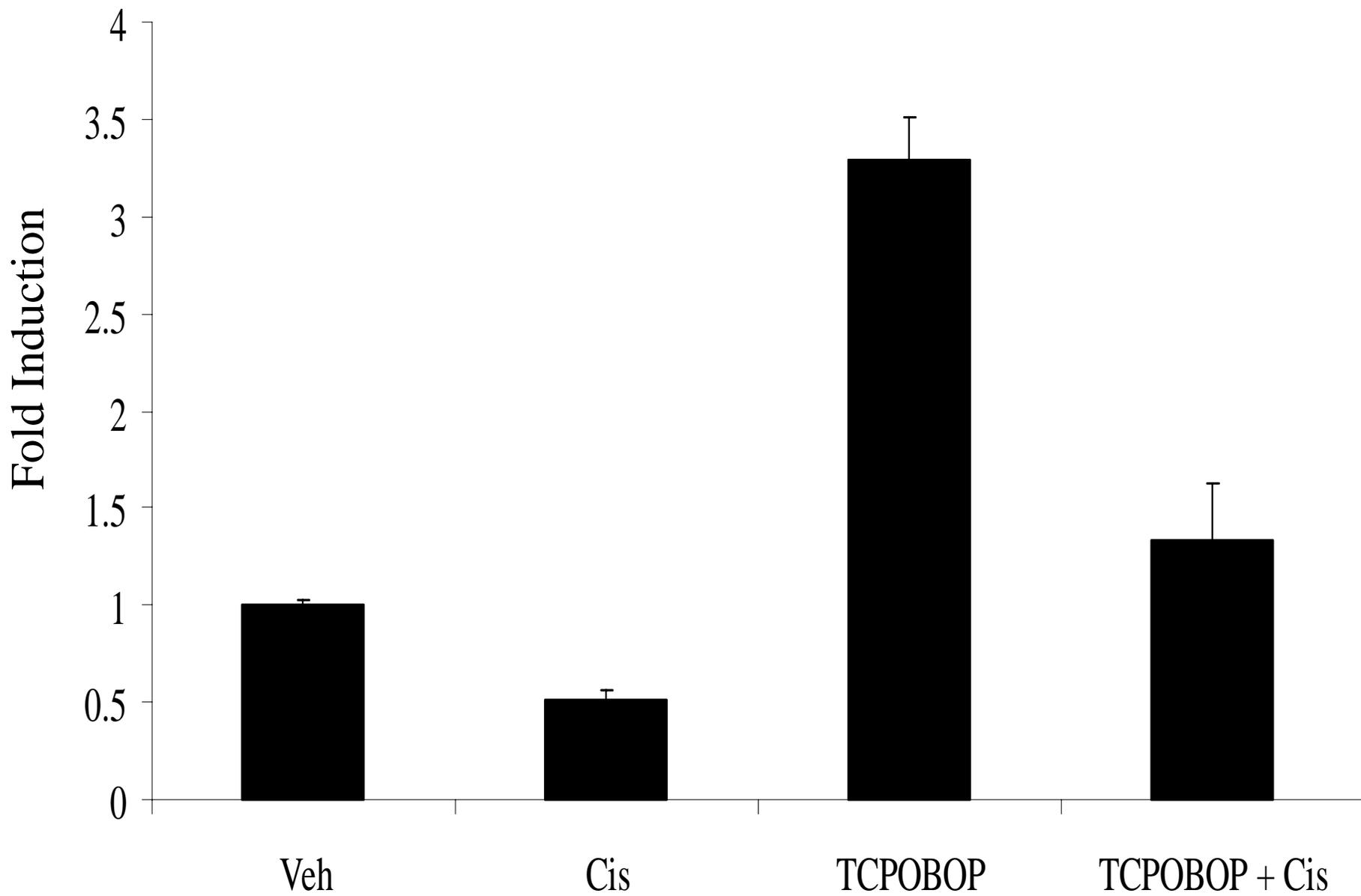


Figure 3A

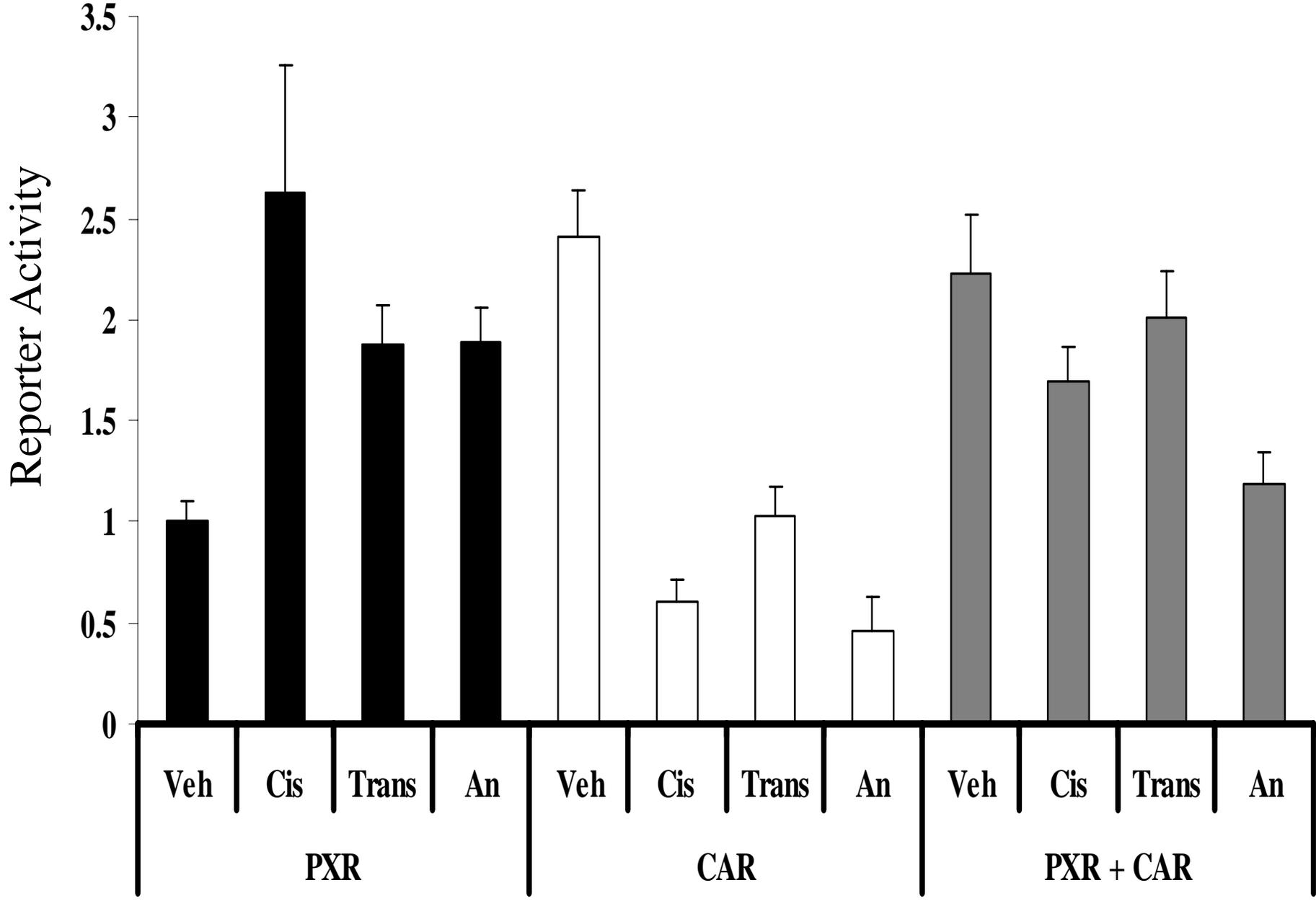


Figure 3B

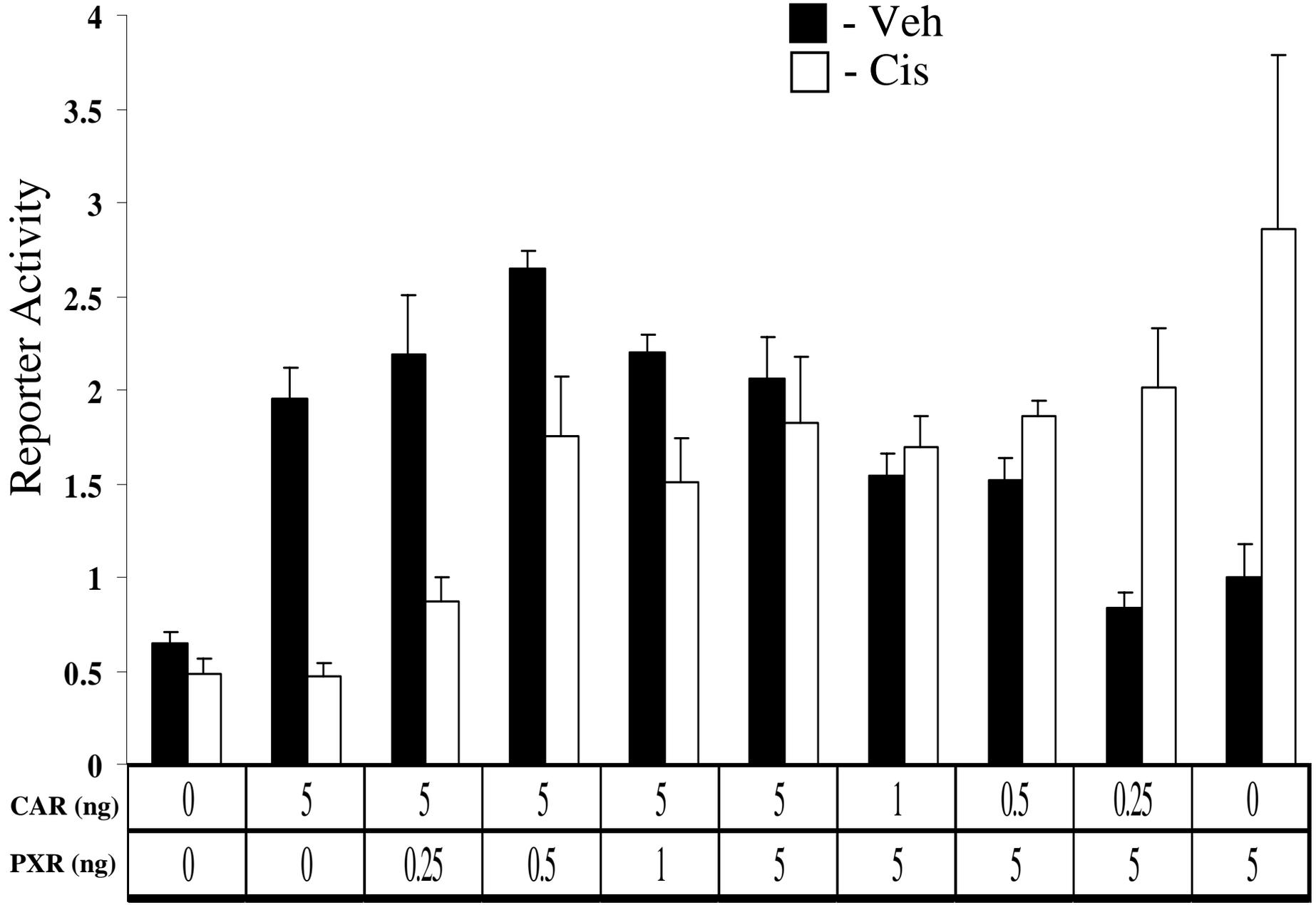


Figure 4A

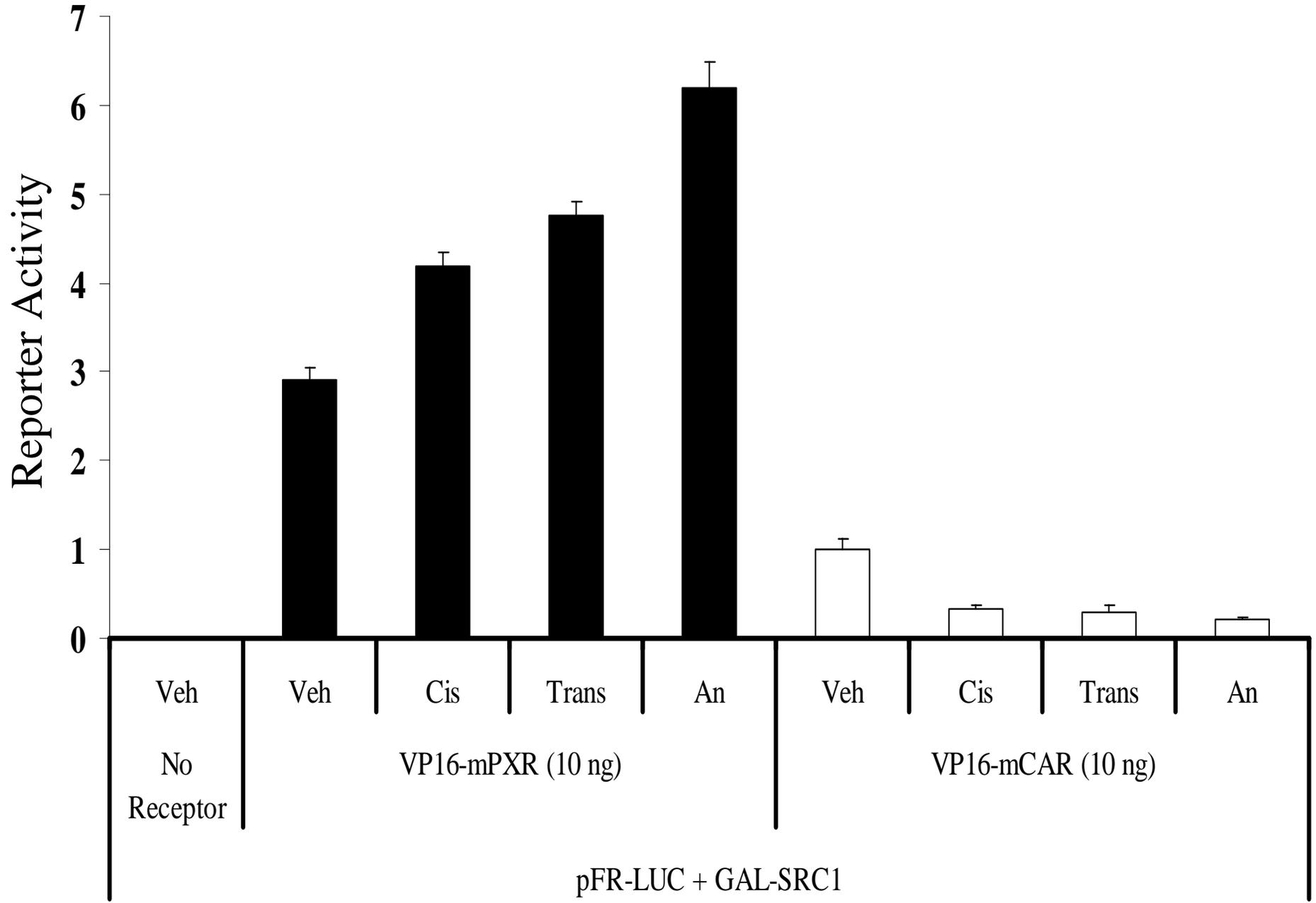


Figure 4B

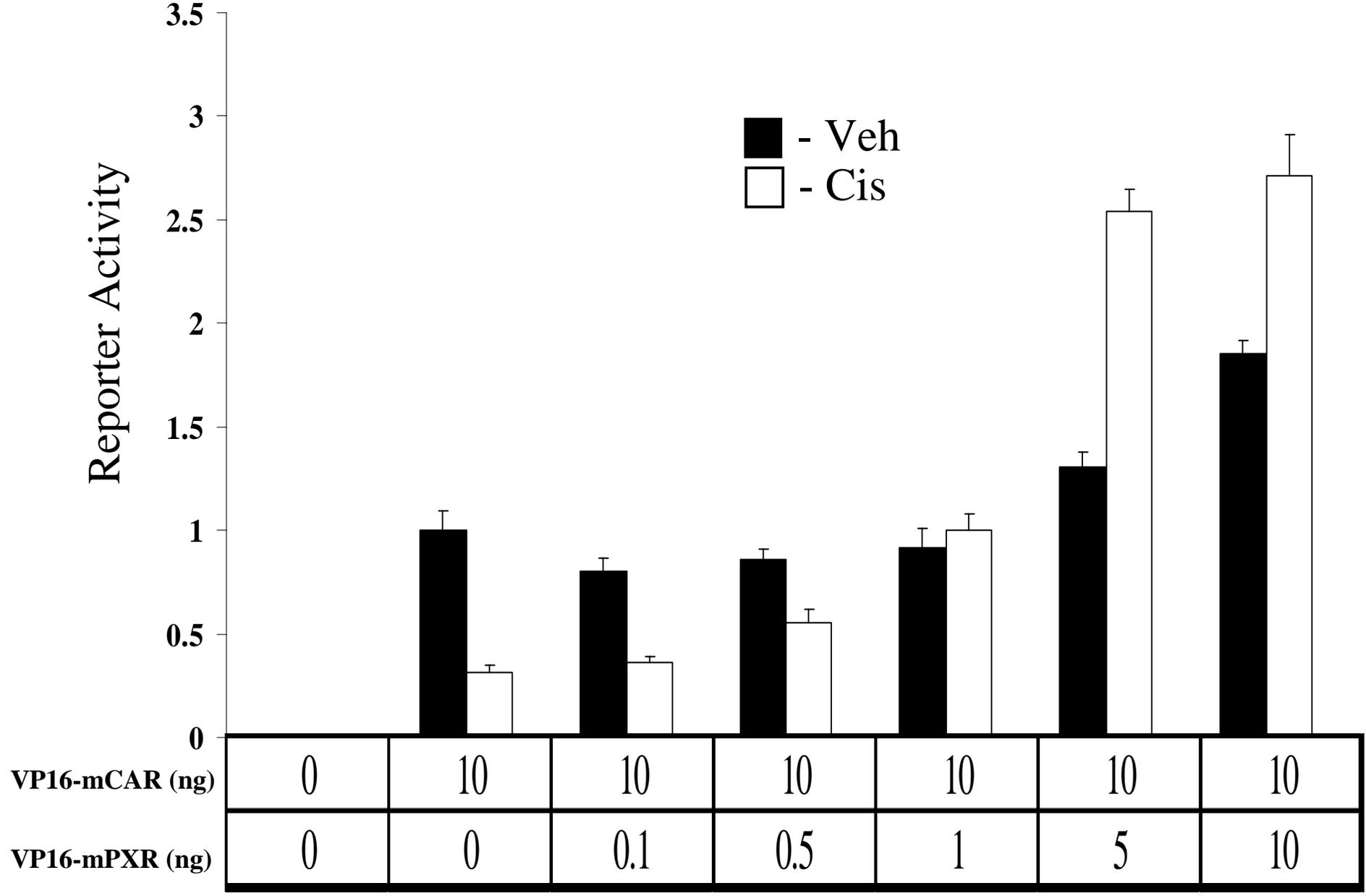


Figure 5

