Identification of Gene-selective Modulators of the Bile Acid Receptor FXR*

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BAR is a nuclear bile acid receptor (BAR) (FXR) receptor that regulates gene networks involved in cholesterol and bile acid homeostasis. We have identified two classes of synthetic compounds that differentially modulate BAR activity. The first class activates BAR target genes in the predicted fashion and is 25-fold more potent than endogenous bile acids. The second class, represented by AGN34, antagonizes BAR in transient reporter assays. Surprisingly, this compound acts in a gene-selective manner in vivo: it is an agonist on CYP7A1, an antagonist on IBABP, and is neutral on SHP. These findings indicate that synthetic BAR modulators can be developed to regulate transcription in a gene-specific fashion. Given the ability of BAR to regulate several lipid homeostatic pathways, the identification of gene-selective BAR modulators have important implications for the development of improved cholesterol lowering agents.

Cholesterol is a multifunctional molecule that is essential for a broad array of physiologic processes including membrane biogenesis, caveolae formation, and the distribution of embryonic signaling molecules. It is also as an essential precursor in the synthesis of transcriptionally active lipids including the steroid hormones and oxysterols (1). Although essential, cholesterol is highly insoluble and can form deposits that contribute to a variety of diseases including gallstones and heart disease (2, 3). Indeed, excess circulating cholesterol is a major risk for atherosclerotic heart disease (3, 4). This disease is responsible for nearly 500,000 deaths each year in the United States (42) and is the single largest cause of mortality in industrialized nations. It has been estimated that 10% of the U.S. population would benefit from cholesterol-lowering therapies (5). This has prompted an intense search for therapeutic agents that specifically modulate cholesterol homeostasis.

Cholesterol levels are controlled at a variety of levels including intestinal uptake, endogenous biosynthesis, transport, and elimination. The major pathway for cholesterol elimination is via hepatic conversion of cholesterol into water-soluble bile acids (6) and their subsequent secretion into the gastrointestinal tract. Approximately 95% of the secreted bile acids are recycled via intestinal uptake and are returned to the liver through the portal blood. The remaining 5% of bile acids are eliminated from the gut thereby forcing the liver to replenish these losses by converting as much as 0.5 g of cholesterol to bile acids each day (7). The liver therefore has an enormous capacity to metabolize cholesterol and therapies that target this process have the potential to eliminate cholesterol derived from a variety of sources including diet, synthesis, and atherosclerotic lesions (via the reverse cholesterol transport pathway).

Two metabolic pathways have been identified that convert cholesterol to bile acids (6). In humans, the classic pathway is responsible for at least 90% of all bile acid synthesis. The first and rate-limiting step in this pathway is catalyzed by CYP7A1, a liver-specific cholesterol 7α-hydroxylase. CYP7A1 transcription is strongly repressed by its bile acid end products (8). An important advance in understanding this feedback loop came with the identification of a member of the nuclear receptor superfamily (FXR, NR1H4, hereafter referred to as BAR) that suppresses CYP7A1 transcription in response to endogenous bile acids (9–12). Two bile acid response elements (BAREs) have been identified in the CYP7A1 promoter. However, BAR is unable to bind directly to either element, suggesting an indirect role for BAR in the regulation of CYP7A1 (13).

A mechanism has been proposed whereby BAR induces the negative transcriptional regulator SHP (small heterodimer partner), which in turn represses transcription factors that bind to the CYP7A1 BAREs (14, 15). This mechanism for CYP7A1 repression was suggested based on experiments using transiently overexpressed SHP. Because SHP can repress (16, 17) and/or activate (18) numerous nuclear receptors under these conditions, the SHP-induction model does not account for the specificity by which bile acids regulate gene transcription.

Although the mechanisms underlying transrepression by BAR is unclear, it is well known that BAR activates transcription by binding to specific response elements (19, 20) as a heterodimer with the nuclear receptor RXR. Several genes have been identified whose transcription is activated by BAR including SHP, the ileal bile acid-binding protein (IBABP), and the hepatic bile salt export pump (BSEP, ABCB11) (21). These genes are critical for bile acid homeostasis. IBABP is an intra-

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1 The abbreviations used are: CYP7A1, cholesterol 7α-hydroxylase; BAR, bile acid receptor; FXR, farnesoid receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SHP, short heterodimer partner; IBABP, ileal bile acid-binding protein; CDCA, chenodeoxycholic acid; AF2, activation function 2; GRIP, glucocorticoid receptor interacting protein; BARM, bile acid receptor modulator; SERM, selective estrogen receptor modulator; BARE, bile acid response elements; PPAR, peroxisome proliferator-activated receptor; GADPH, glyceraldehyde-3-phosphate dehydrogenase; ER, estrogen receptor; TTNPB, (E)-4-(2-(5,6,7,8-tetrahydro-5,5,8-tetramethyl-2-naphthalenyl)propen-1-yl)benzoic acid.
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EXPERIMENTAL PROCEDURES

Reagents—Full-length proteins were expressed using the pCMX vector (25) and have been previously described (9). Gal-t-RAR is the human RARα ligand binding domain (GI931542, accession number X06614) fused to the C-terminal end of the yeast Gal4 DNA binding domain. Gal-t-RXR has been described (9). BAR AF2m contains a single X06614) fused to the C-terminal end of the yeast Gal4 DNA binding domain. The luciferase reporter constructs were as follows. Mouse IBABP (26), CYP3A4x3 was used for mouse and human PXR and/or phospholipid transfer protein (reviewed in Ref. 21). Regardless of the mechanism, it appears that BAR activation promotes reciprocal effects on cholesterol and triglyceride levels.

Given its critical role in repressing CYP7A1-mediated cholesterol degradation, BAR provides an attractive target for the development of novel cholesterol lowering agents. However, the effects of BAR on other target genes implies that a generalized antagonist would produce undesirable effects including elevations in triglycerides, a lowering of biliary bile acid transport, and/or cholesterol. Therefore, the most desirable therapeutic agents would be gene-selective modulators that selectively regulate a subset of BAR-specific genes. In a search for such compounds we have identified two classes of BAR modulators. The first class include agonists that are ~25-fold more potent than naturally occurring bile acids. These compounds activate BAR and produce the expected regulation pattern on endogenous target genes. Second, we identified AGN34 as a gene-selective BAR modulator (BARM: it acts as an agonist on CYP7A1, an antagonist on IBABP, and is neutral on SHP). These data demonstrate that SHP induction is not required to repress CYP7A1. More significantly, we provide evidence for gene-selective BARMs, a finding with important implications for the treatment and prevention of atherosclerotic heart disease.

RESULTS

Identification of Synthetic BAR Agonists—To begin to identify BAR-specific modulators, we noted that the synthetic retinoid TTNPB (E4-4(2,5,6,7,8-tetrahydro-5,5,8,tetramethyl-2-naphthalenyl)propan-1-yl]benzoic acid) (Fig. 1A) binds to and activates BAR (11, 29). A major limitation in the use of TTNPB as a BAR agonist is that TTNPB is a 1000-fold higher affinity ligand for the retinoic acid receptor (RAR) (30). Thus, it is important to identify TTNPB derivatives that lack activity on RAR.

Analysis of the crystal structure of retinoid-bound RAR indicates that the methyl group in the central isoprene unit (Fig. 1A, TTNPB, shaded area) interacts with critical residues in helix 5 of the RAR ligand binding domain (31). We hypothesized that placement of bulky residues in this position would create a steric hindrance to RAR binding. At the same time, compounds with a bulky moiety in this location might retain activity on BAR as this structure would more closely approximate the cyclohexane ring at the corresponding position of the BAR ligand chenodeoxycholic acid (CDCA) (Fig. 1A). To test this hypothesis, AGN29 and AGN31 were synthesized with trimethylsilyl and n-butyl groups in place of the methyl moiety on the central isoprene unit of TTNPB (Fig. 1A, shaded area). At concentrations of 5 μM, AGN29 (91-fold) and AGN31 (85-fold) were potent and efficacious activators of the BAR-RXR heterodimer (Fig. 1B). By comparison, TTNPB was less efficacious in activating BAR (65-fold) and CDCA, an endogenous BAR ligand, required 20-fold higher concentrations (100 μM) for optimal activity (Fig. 1B). As predicted, AGN29 and AGN31 were both dramatically less effective than TTNPB in activating RAR (Fig. 1C) and had no effect on other nuclear receptors including AR, mouse, and human PXR, ERα, CAR, LXRα, PPARα, PPARγ, PPARδ, VDR, and T3Rβ (data not shown). Thus, unlike the parent ligand TTNPB, AGN29 and AGN31 exhibit significant selectivity for BAR.

RXR-specific ligands such as LG268 are known to activate the BAR-RXR heterodimer (Fig. 1B), however, we have previously established that this occurs via the RXR subunit and not via BAR (9). Because TTNPB derivatives were originally used to identify RXR-specific ligands (30), we tested the ability of AGN29 and AGN31 to activate RXR. Both compounds activated RXR at the same concentrations required to activate BAR-RXR heterodimers (5 μM) and were inactive at lower doses (data not shown). Although AGN29 and AGN31 activate RXR, their action is distinct from that of LG268. For example, when compared with LG268, AGN29 and AGN31 preferentially activate BAR-RXR heterodimers whereas LG268 preferentially activates RXR (compare Fig. 1, B and D).

To further explore the molecular properties of AGN29 and AGN31 we examined their effects on coactivator recruitment in vitro. The primary function of nuclear receptor ligands is to induce a conformation change that facilitates recruitment of
transcriptional coactivator proteins via the AF2 transactivation domain (32). Previous coactivator recruitment assays have demonstrated that bile acids and TTNPB bind directly to BAR (9, 11). We used an electrophoretic mobility shift assay (9) to determine whether AGN29 and AGN31 also serve as BAR ligands. Thus, BAR-RXR heterodimers were formed on a [32P]-labeled BAR response element and incubated with a peptide containing the receptor interaction domains of the coactivator GRIP1. As expected, both AGN29 and AGN31 effectively re-recruited the BAR ligand CDCA. (Fig. 2, lanes 1–3). AGN29 also recruited the BAR ligand CDCA. (Fig. 2, lane 4) but was less effective than AGN29 and AGN31, a finding that parallels the transactivation data (Fig. 1, B and D). To explore the subunit requirements of these compounds, we asked whether the BAR or RXR AF2 was required for these interactions. Mutation of the BAR AF2 resulted in loss of activity for both AGN29 and AGN31 whereas mutation of RXR AF2 had no effect (Fig. 2A, lanes 5–7 and 9–11). In contrast, recruitment by LG268 required the AF2 domain of RXR as well as BAR (Fig. 2A, lanes 8 and 12). Thus, whereas AGN29 and AGN31 can activate an isolated RXR subunit (Fig. 1D), the recruitment of Fig. 2A demonstrates that BAR is essential for the activity of AGN29 and AGN31 in the context of the BAR-RXR heterodimer.

To determine the relative potency of AGN29 and AGN31, transfected cells were treated with increasing doses of AGN29 and AGN31 (Fig. 2B). The half-maximal effective concentrations (EC50) for AGN29 and AGN31 are ~2 μM compared with >50 μM for CDCA. Similarly, using the coactivator recruitment assay, AGN29 and AGN31 produced a dose-dependent increase in the GRIP1-containing complex (Fig. 2C). The concentration of AGN29 and AGN31 that promoted GRIP recruitment closely paralleled the potency of these compounds in transfection assays (Fig. 2B). These results indicate that AGN29 and AGN31 are not only BAR ligands but are >25-fold more potent than their endogenous counterparts.

Synthetic Agonists Regulate BAR Target Genes—BAR regulates transcription of specific target genes in the liver (CYP7A1, SHP) and intestine (IBABP, SHP) (reviewed in Ref. 21). This prompted us to test the effect of AGN29 and AGN31 on gene expression in HepG2 hepatoma cells and in differentiated intestinal Caco-2 cells. Both cell lines were treated for 24 h with optimal concentrations of AGN29, AGN31 (10 μM), or CDCA (100 μM). Like CDCA, AGN29 and AGN31 strongly induced SHP and IBABP expression in Caco-2 cells (Fig. 3A). Similarly, these compounds acted as agonists in HepG2 cells and produced the expected induction of CYP7A1 and repression of CYP7A1 (Fig. 3B). AGN29 and AGN31 had no effect on expression of BAR (data not shown) or the GAPDH internal control (Fig. 3). These data demonstrate that AGN29 and AGN31 are agonists on endogenous target genes.

Identification of a BAR Antagonist—We next explored the possibility that retinoid derivatives might serve as BAR antagonists. Indeed, using the transfection assay described above, AGN34 (Fig. 4A, 1 μM) was identified as a compound that effectively blocked transcriptional activation by the endogenous CDCA ligand (50 μM) (Fig. 4B). AGN34 had no effect on basal reporter activity (Fig. 4B). AGN34 also failed to antagonize the nuclear receptors AR, HNF4α CAR, LXR, mouse and human PXR, PPARα, PPARγ, PPARδ, and VDR and exhibited only minimal activity on ERα, TRβ2, and BAR (Fig. 4C). These data demonstrate that AGN34 is a selective antagonist of BAR activity.

AGN34 Is a Direct and “Trans-antagonist”—Because AGN34 possesses a partial retinoid-like structure, we examined the effect of this compound on the retinoid receptors RAR and RXR. AGN34 was a weak antagonist of RAR and other receptors that utilize RXR as an obligate heterodimeric partner (Fig. 4C). However, AGN34 was an effective antagonist of RXR homodimers (Fig. 4C). This prompted us to compare the activity of AGN34 on BAR-RXR and RXR complexes. Dose-response analysis demonstrated that AGN34 antagonized both receptor complexes with a half-maximal inhibitory concentration (IC50) of <10 nM (Fig. 5, A and C). Thus, the ability to antagonize RXR and BAR at similar doses suggest that AGN34 acts to trans-
incubated with [20-
firm that AGN34 acts directly on the RXR subunit. RXR was
DNA-based recruitment assays were performed by mixing GST-GRIP, a
AGN29 and AGN31 recruit coactivator via the AF2 domain of BAR.
300 nM LG268.
ment by AGN29 and AGN31. Recruitment assays were performed as in
CAGN31. Transfections were performed as in Fig. 1
subunit.
agonize bile acid-activated BAR
AGN34 can trans-agonize BAR
 jedtsehen the possibility that it could have additional activities via the BAR
antagonize BAR in transfection assays (Fig. 5D). Addition of
AGN34 resulted in a displacement of coactivator from RXR
homodimers (Fig. 5C, top panel, lanes 1 and 2). Addition of
AGN34 resulted in a displacement of coactivator from RXR
homodimers with an IC_{50} of ~30 nM (Fig. 5C, top panel, lanes
3–10). In contrast, low doses of AGN34 (~30 nM) resulted in
only a partial displacement of coactivator from BAR (Fig. 5C,
bottom panel, lanes 1–5). Full coactivator displacement and
reappearance of the coactivator-free heterodimer required con-
centrations greater than 300 nM (Fig. 5C, bottom panel, lanes
6–10). Similar results were seen using a mutated RXR with
decreased ligand binding affinity (data not shown) suggesting
that AGN34 functions via BAR at these high doses. This
biphasic pattern of coactivator displacement in vitro further
demonstrates that AGN34 antagonizes BAR-RXR via both
subunits.

The ability to trans-agonize BAR-RXR with RXR
antagonists raises the possibility that previously characterized RXR
antagonists such as LG754 (34) might generally serve as
BAR-RXR antagonists. However, LG754 did not effectively
agonize BAR in transfection assays (Fig. 5D) and only dis-
placed coactivator from agonist-occupied BAR-RXR het-
erodimers at very high concentrations (3 μM) (Fig. 5E).

antagonize bile acid-activated BAR-RXR by binding to the RXR
subunit.

We used an in vitro radioligand displacement assay to con-
firm that AGN34 acts directly on the RXR subunit. RXR was
incubated with [20-methyl-3H]9-cis-retinoic acid and binding
was measured in the presence of increasing concentrations of
unlabeled AGN34. We found that AGN34 associates with RXR
with a binding constant of ~2 nM (Fig. 5B). The high in vitro
affinity of AGN34 for RXR closely matches its IC_{50} for both
RXR and BAR-RXR heterodimers. These findings confirm that
AGN34 can trans-agonize BAR-RXR via the RXR subunit.
CYP7A1-mediated conversion of cholesterol to bile acids is the main route for elimination of cholesterol from the body (6). Interventions that enhance CYP7A1 activity are expected to provide a powerful approach for treating hypercholesterolemia.

**DISCUSSION**

CYP7A1-mediated conversion of cholesterol to bile acids is the main route for elimination of cholesterol from the body (6). Interventions that enhance CYP7A1 activity are expected to provide a powerful approach for treating hypercholesterolemia.

**FIG. 4. Identification of a BAR-specific antagonist.** A, chemical structure of AGN34. B, AGN34 prevents CDCA-mediated activation of BAR. Transfections were performed as in Fig. 1B using CDCA (50 μM) with or without AGN34 (1 μM). C, AGN34 is a BAR-specific antagonist. CV-1 cells were transfected with the indicated receptors and corresponding reporter constructs (see “Experimental Procedures”). The cells were then treated with receptor-specific ligands alone or with 1 μM AGN34. Specific ligands were as follows: human AR (1 μM dihydrotestosterone), mouse PXR (10 μM pregnenolone 16α-carbonitrile), human FXR (10 μM rifampicin), ERα (100 nM 17β-estradiol), human LXRα (30 μM hydroxocholic acid methyl ester), mouse PPARγ (1 μM rosiglitazone), mouse PPARδ (1 μM carbaprostacyclin), human α1,25-dihydroxyvitamin D3 (100 nM VDR), human T3Rβ (100 nM triiodothyronine), and endogenous RARβ (100 nM Am580). No agonist ligand was required for the constitutively active receptors CAR and HNF4; for these receptors, fold repression represents the effect of AGN34 on constitutive activity.

**FIG. 5. AGN34 is an antagonist of BAR and RXR.** A, dose response analysis for antagonism of BAR and RXR. Transfections were as described in the legend to Fig. 1B using Gal-1-RXR and UAS,3x TK-Luc or BAR, RXR, and IBABP IR-1x3 TK-Luc. After transfection, cells were treated with 50 nm LG754 (Gal-1-RXR) or 50 μM CDCA (BAR-RXR) and increasing doses of AGN34. B, AGN34 is an RXR ligand. RXR was incubated with 10 nM [20-3H]9-cis-RA and increasing concentrations of AGN34 (30-1,25-dihydroxyvitamin D3). The cells were then treated with 1 μM LG754. The luciferase activity was normalized to the β-galactosidase internal control. C, AGN34 reverses coactivator recruitment. For RXR, coactivator displacement assays were performed by mixing in vitro translated RXR (0.6 μL), 75 ng of purified GST-GRIP, and a 32P-labeled IBABP IR-1 probe with agonist alone (2 μM AGN31) or agonist with increasing concentrations of AGN34. D, AGN34 is a more effective antagonist than LG754. Transfections were performed as described in the legend to Fig. 1B and cells were treated with 50 μM CDCA alone or in combination with 1 μM AGN34 or 1 μM LG754. The luciferase reporter activity was normalized to the β-galactosidase internal control.
and atherosclerosis. Indeed, adenoviral mediated overexpression of CYP7A1 is sufficient to reduce plasma low density lipoprotein concentrations by ~60–75% (35). The most commonly used cholesterol-lowering drugs are the statin class of cholesterol synthesis inhibitors. These agents are nonspecific in that they inhibit the synthesis of both cholesterol and its biologically active precursors. In addition, inhibitors of cholesterol synthesis cannot eliminate pre-existing cholesterol that arises from dietary or other sources. Therefore, an urgent need exists for additional therapeutic strategies. Indeed, coronary arterial disease remains the leading cause of death in industrialized societies and 36-million Americans require cholesterol-lowering therapies (5).

It has been well established that CYP7A1 transcription is strongly repressed by its bile acid end products. Although bile acid-mediated repression is conserved in a variety of mammalian species, this pathway is particularly sensitive in humans (36). Drugs that antagonize bile acid-mediated repression of CYP7A1 would be particularly useful in stimulating cholesterol elimination in humans. A key advance in elucidating the molecular events underlying CYP7A1 repression was the demonstration that the nuclear receptor BAR suppresses CYP7A1 transcription in response to endogenous bile acids (9–12). In principle, a BAR-specific antagonist would prevent CYP7A1 repression thereby facilitating further cholesterol catabolism. Whereas BAR has potent effects on CYP7A1, this receptor plays a broader role in regulating lipid homeostasis. For example, BAR activation lowers triglycerides (23, 24) and stimulates expression of genes involved in biliary bile acid secretion. Thus, a generalized BAR antagonist has the potential to induce serious side effects including cholestasis and hypertriglycerideremia. An attractive means to bypass these effects would be the identification of antagonists whose activities are limited to a subset of target genes.

We describe two novel BAR agonists: AGN29 and AGN31. These compounds are 25-fold more potent than naturally occurring ligands and resulted in the expected activation (IB-ABP, SHP) or repression (CYP7A1) of BAR-target genes. AGN29 and AGN31 are derived from TTPNB, a synthetic retinoid that is a ligand for both BAR and RAR (11, 29, 30). In contrast, AGN29 and AGN31 are BAR-selective ligands in that they have weak activity on RAR and fail to activate other nuclear receptors. Structure-activity studies suggest that their weak activity on RAR results from the placement of bulky functional groups on the central isoprene unit. Indeed, analysis of the crystal structure of retinoid-bound RAR indicates that these bulky residues would clash with critical residues in helix 5 of the RAR ligand binding domain (RARγ Met272) (31).

Interestingly, AGN29 and AGN31 are unique among BAR ligands in that they also activate RXR, the heterodimeric partner required for the formation of an active BAR complex. Thus, AGN29 and AGN31 function through both subunits of the BAR-RXR heterodimer. This prompted us to examine whether other retinoid-like compounds might antagonize the BAR complex. Using transient transfection and in vitro coactivator recruitment assays, we identified AGN34 as an extremely potent compound that antagonized BAR-RXR at concentrations as low as 10 nM. Antagonism at these low concentrations resulted from direct binding to the RXR subunit and subsequent reversal of agonist-induced coactivator recruitment (Fig. 5C). Because coactivator recruitment requires the AF2 transactivation domain of BAR (Fig. 2A), the ability to displace coactivator while binding to RXR demonstrates that AGN34 is an allosteric inhibitor of BAR. Although RXR functions as an obligate partner for many receptors, the effect of AGN34 was specific to BAR (Fig. 4C) and other RXR antagonists were not effective at antagonizing BAR (Fig. 5D). This mode of specific “trans-antagonism” via a partner receptor has not been previously described among the nuclear receptor superfamily.

Although AGN34 antagonizes BAR in transient reporter assays, this compound functions as a gene-selective modulator in vivo: it acts as an agonist on CYP7A1, an antagonist on IBABP, and is neutral on SHP. This divergent pattern of regulation is reminiscent of the activity of selective estrogen receptor (ER) modulators or SERMs (37). These compounds elicit an array of biological effects that are either estrogenic or antiestrogenic depending on the tissue. For example, SERMs such as tamoxifen and raloxifene are used for the prevention and treatment of breast cancer by virtue of their ability to antagonize ER in the breast. In contrast, tamoxifen and raloxifene act as ER agonists in other tissues. These unexpected activating properties have significant advantages as ER agonists have beneficial effects on bone density and plasma lipoprotein levels. By analogy to the SERMs, AGN34 represents a selective BARM. The ability to regulate BAR in a gene-specific fashion suggests that future compounds may be identified that selectively enhance cholesterol elimination without promoting negative effects such as hypertriglyceridermia or cholestasis.

Whereas the divergent activities of SERMs have been appreciated for over 2 decades the molecular mechanisms that underlie their action are only now being elucidated (reviewed in Ref. 38). ER regulates transcription either by binding to specific response elements or indirectly by tethering to other promoter-bound transcription factors. In both cases ER agonists recruit transcriptional coactivators to its targeted promoters. However, in breast cancer cells where coactivator proteins are more highly expressed, tamoxifen act as an antagonist by recruiting corepressors. In endometrial cells the same drug acts as an agonist by recruiting more highly expressed coactivators to ER-tethered promoters. Thus, the direction of SERM activity...
is determined in a combinatorial fashion by at least three factors: the conformation of the ligand-receptor complex, the promoter context, and the relative levels of expression of specific coactivator and corepressor proteins.

Like their SERM counterparts, the future development of BARMs will benefit from a more complete description of the mechanisms by which BAR regulates gene transcription. Direct binding sites for BAR have been identified in the promoters of certain target genes (e.g. IBABP and SHP) (21) but it remains unclear which coactivator proteins and/or transcription factors are utilized by BAR for in vivo regulation of these genes. In the case of transrepression, several negative BARs have been identified in the CYP7A1 gene but BAR does not interact directly with these elements (13). Instead, it had been proposed that transrepression occurs by BAR-mediated stimulation of SHP, which in turn represses transcription at the negative BARE (14, 15). Our findings demonstrate that alternative mechanisms by which BAR regulates gene transcription. Di-

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