Dual action of oestrogens on the mouse constitutive androstane receptor

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mCAR (mouse constitutive androstane receptor; NR1I3) controls the expression of cytochrome P450 as well as other enzymes involved in drug and steroid metabolism. The high basal activity of mCAR can be modulated by inhibitory steroids related to androstenol and by activating xenobiotic chemicals such as 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene and chlorpromazine. The ability of oestrogens and some other xenobiotics to activate mCAR is not clear. In the present study, co-transfection assays in HEK-293 cells indicated that oestrogens varied in their efficacy to activate mCAR, depending on variation at the steroid D-ring and position of hydroxy groups. In general, oestrogens were weaker activators of mCAR than 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene and chlorpromazine. Also, the induction of CYP2B10 mRNA by oestrogens was less pronounced in mouse

primary hepatocytes. Yeast two-hybrid assays indicated that, unlike androstenol and the established activators, oestrogens attracted both nuclear receptor co-repressors and co-activators to the mCAR ligand-binding domain, thus limiting the extent of mCAR activation. This novel dual action is not limited to oestrogens, but is shared by some xenobiotic CYP2B inducers such as clotrimazole and methoxychlor. These findings offer an alternative explanation for the recently suggested nuclear activation step of mCAR.

Key words: co-activator, co-repressor, HEK-293 cells, hepatocyte, nuclear receptor CAR (constitutive androstane receptor), oestrogen.

INTRODUCTION

Nuclear receptors (NRs) constitute a group of ligand-dependent DNA-binding transcription factors. This superfamily includes the classical steroid and thyroid hormone receptors, retinoid receptors and the so-called orphan receptors, all of which control critical processes in cellular development and homeostasis [1,2]. NRs are also increasingly attractive targets in medicinal chemistry, since new ligands and target genes for orphan receptors have been found recently [3,4] and the functional basis of both NR agonism and antagonism are being unravelled. Agonist binding induces a conformational change in the NR LBD (ligand-binding domain), which leads to physical association of NR co-activators with the LBD [2,5]. This interaction between NR co-activators and the LBD appears to be highly dependent on the orientation of helix 12 at the C-terminus of the NR [6–8]. Co-activators then transmit the signal-to-target gene promoters in a cascade that involves histone acetylation, local opening of the chromatin structure and activation of gene transcription [2,8,9]. In contrast, antagonist binding prevents orientation of helix 12 to the agonistic position and NR co-activators fail to bind. Instead, NR co-repressors are being recruited to the LBD, which results in histone deacetylation, chromatin condensation and suppression of gene transcription [5,9]. In ER β (oestrogen receptor- β), the partial agonist genistein adopts a similar orientation in the ligand-binding pocket as the natural ligand E2 (3,17 β -oestradiol), but fails to orient helix 12 into the agonistic position [7]. Moreover, different NRs appear to have distinct preferences to specific NR co-regulators [5,10]. These findings suggest that structurally variable ligands can induce subtle variations in the NR transcriptional activity.

CAR (constitutive androstane receptor, NR1I3) controls the drug- and xenobiotic-inducible expression of mammalian cytochrome P4502B (*CYP2B*) and other genes through its binding to the PB-responsive enhancer module (PBREM) [11–14]. The most striking feature of CAR is its wide ligand specificity. Uniquely, mCAR (mouse CAR) displays a high basal activity that can be suppressed by androstenol [15]. Structurally various chemicals including 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and chlorpromazine can induce CYP2B10 mRNA and activate PBREM in primary hepatocytes [16,17] and at least TCPOBOP and chlorpromazine can recruit the co-activator SRC1 (steroid receptor co-activator 1) to mCAR [18,19]. However, many known inducers of the CYP2B10 mRNA fail to activate mCAR in co-transfection assays [20]. For instance, the results on activation of mCAR by E2 and clotrimazole disagree [20–22]. During our studies on the specificity of PBREM for NRs [22], we noted that 17 α -ethynyl-E2 was an activator of both full-length and GAL4-LBD-fusion of mCAR, but not of human CAR. These variable results may, in part, stem from different methodologies and cell lines used, but clarification of ligand specificity of mCAR would be valuable for understanding its mechanism of action. These mixed findings prompted us to study further the effects of oestrogens on mCAR. Surprisingly, and in contrast with TCPOBOP and chlorpromazine, oestrogens were found to have both antagonistic and agonistic characters in mCAR binding. Some other xenobiotics, including clotrimazole and methoxychlor, share this dual action. Our results suggest that ligand- and mCAR-dependent CYP2B mRNA expression may vary, depending on the nature of the NR co-regulators recruited. They underscore

Abbreviations used: CAR, constitutive androstane receptor; mCAR, mouse CAR; CMV, cytomegalovirus; CYP, cytochrome P450; E2, 3,17 β -oestradiol; ER, oestrogen receptor; β -gal, β -galactosidase activity; LBD, ligand-binding domain; LUC, luciferase; NR, nuclear receptor; NCoR, NR co-repressor; 2OH-E2, 2-hydroxy-E2; PB, phenobarbital; PBREM, PB-responsive enhancer module; PXR, pregnane X activity receptor; RT, reverse transcriptase; SRC1, steroid receptor co-activator 1; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene.

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the complexity of mCAR signalling and provide an alternative explanation for the recently evoked nuclear activation step of mCAR.

MATERIALS AND METHODS

Chemicals

All steroids were at least 99 % pure and obtained from Steraloids (Newport, RI, U.S.A.). Wy-14643 was purchased from ChemSyn (Lenexa, KS, U.S.A.). All other compounds were obtained from Sigma (St. Louis, MO, U.S.A.). The synthesis of TCPOBOP (> 98 % purity) has been described in [22].

DNA plasmids

The CMV (cytomegalovirus) promoter-driven GAL4/mCAR-LBD-fusion protein plasmid has been described in [22]. The GAL4/mCAR Δ 8 plasmid lacking eight amino acids in helix 12 that constitute the mCAR AF2 domain [23] was generated by standard PCR methods and was verified by sequencing. The GAL4-dependent reporter UAS₄-tk-luc plasmid (where tk stands for thymidine kinase) was a gift from R. M. Evans (The Salk Institute for Biological Studies, San Diego, CA, U.S.A.), and pCMV β was purchased from BD Clontech (Palo Alto, CA, U.S.A.). The plasmids were purified with Qiagen (Hilden, Germany) columns.

Cell culture, DNA transfection and mCAR assays

HEK-293 cells were cultured in 48-well plates in Dulbecco's modified Eagle's medium devoid of Phenol Red and complemented with 5 % (v/v) delipidated serum (Sigma). The cells were transfected with pCMV β (25 ng/well), CMV-GAL4-mCAR LBD (6.25 ng/well) and UAS₄-tk-luc (12.5 ng/well) by calcium phosphate [22]. After transfection for 4 h, the medium was changed. In the normal mCAR activation assay, the fresh medium contained either the vehicle (0.1 %, v/v) or the test compound at indicated final concentrations (optimum concentrations chosen from preliminary dose-response tests). In mCAR re-activation assay, 2 μ M androsthenol was included in the medium to suppress mCAR activity [22]. As reference compounds for mCAR, we used TCPOBOP and chlorpromazine as specific activators [18,19] and androsthenol as an inhibitor [15]. The cells were lysed 24 h after medium change and then assayed for β -gal (β -galactosidase) and LUC (luciferase) activities [24] using the Victor²™ luminescence and absorbance reader for 96-well plates (PerkinElmer Wallac, Turku, Finland). The normalized LUC activities were from three independent transfections in each experiment. The experiments were repeated two or three times independently with similar results and the results are expressed as means $\pm$ S.D.

Yeast two-hybrid assays

The pGBKT7 and pGADT7 plasmids in the Matchmaker GAL4 System 3 (BD Clontech) were used. Plasmids harbouring the mCAR LBD and the mouse NCoR (NR co-repressor) interaction domain [25] have been described in [22]. The NR interaction domain from mouse SRC1 (residues 549–789) [26] was amplified from liver RNA and inserted between *Nde*I and *Bam*HI sites of the pGADT7 plasmid. All manipulations were performed according to the manufacturer's instructions. Yeast colonies that expressed both mCAR LBD and the partner (NCoR or SRC1) were selected on synthetic dropout medium plates lacking both leucine and tryptophan. Random colonies were amplified and

aliquots of yeast cell suspensions were then treated with vehicle or test chemicals for 3.5 h. The cell density was determined by measuring the attenuation at 595 nm and the cells were then lysed by adding SDS (0.05 % final concentration). Lysates were assayed for β -gal activity [27] and controlled for any turbidity by measuring the absorbance at 570 nm. The normalized β -gal activities (means $\pm$ S.D.) were from two separate experiments, each with three independent treatments.

CYP mRNA expression in mouse primary hepatocytes

Primary hepatocytes from male DBA/2 mice were isolated and incubated on 100 mm culture dishes as described previously [28,29] with the exception that the serum-free culture medium contained 7.5 nM dexamethasone. The plated cells were treated for 24 h with oestrogens (10 μ M), various xenobiotics (2, 10 or 200 μ M), chlorpromazine (10 μ M), TCPOBOP (1 μ M) or the vehicle (0.1 % v/v) in three independent plates. Total RNA was isolated by TRIzol[®] extraction and quantified by measuring the absorbance at 260 nm. A quantitative reverse transcriptase (RT)-PCR method for CYP2B10 mRNA was set up for the ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions. Complementary DNA was synthesized from 2 μ g of total RNA with M-MuLV reverse transcriptase, dNTPs and random hexamer primers. Amplification of cDNA (40 ng in duplicate) was performed with the CYP2B10 primers (5'-GGGAACCTCTTGACAGATG and 5'-CCCAGGTGCACTGTGAA) and internal control β -actin primers (5'-CGTTGCCGGTCCACAC and 5'-AGCCGTTGTGCG-ACGACC) to yield amplicons of 92 and 74 bp respectively. The amplified cDNA was quantified with CYP2B10 (5'-FAM[™]-TCAGCTTCGAGAAAAATATGGCGATG-TAMRA[™]-3') and β -actin (5'-VIC[™]-CACCAGTTCGCCATG-MGB[™]-3') probes. The primers and probes were designed to span exon-intron boundaries with the Primer Express 2.0 program (Applied Biosystems) and to display minimal self-hybridization and dimer formation with the Oligo 6.0 program (Molecular Biology Insights, West Cascade, CO, U.S.A.). The primers and probes were then verified for specificity through BLAST searches and were purchased from Sigma-GenoSys or Applied Biosystems (β -actin probe). Quantification of mRNAs was performed using the comparative threshold cycle number method [30]. CYP2B10 signals were then normalized with the corresponding β -actin signals and the extent of induction (-fold) between treated and vehicle control cells is expressed as means $\pm$ S.D. from three independent treatments for each chemical.

The performance of the quantitative RT-PCR assay was verified by measuring both CYP2B10 and β -actin mRNA levels with an independent RNase protection assay from cells exposed to either the vehicle, TCPOBOP, chlorpromazine, 17 α -ethynyl-E2, 4-oestren-17-one or 2OH-E2 (2-hydroxy-E2). The extent of CYP2B10 mRNA induction was similar in both assays (results not shown).

RESULTS

Responses and specificity of mCAR to ligands

Activation of mCAR in the absence of androsthenol (Figure 1A, mCAR) indicated that TCPOBOP and chlorpromazine could increase mCAR activity by 2.5–3.5-fold in mammalian HEK-293 cells. Androsthenol inhibited mCAR by more than 90 %, whereas the PXR (pregnane X receptor)-specific activator RU486 was a moderate inhibitor (30 % decrease) and peroxisome

Figure 1 Ligand-specific activation of mCAR and its dependence on helix 12

(A, B) HEK-293 cells were transfected with the indicated expression plasmids for mCAR, mCAR Δ 8 or control GAL4 and reporter plasmids. The transfected cells were treated with vehicle (open bar), 1 μ M TCPOBOP (hatched-down), 10 μ M chlorpromazine (hatched-up), 10 μ M androstamol (black), 10 μ M RU486 (vertical stripes) or 10 μ M Wy-14643 (horizontal stripes). The chemicals were added in the absence of androstamol (A, normal mCAR assay) or in the presence of 2 μ M androstamol (B, re-activation assay). The treated cells were then assayed for LUC and β -gal activities. The normalized activity obtained for mCAR and vehicle was set at 100 (A) or unity (B). Results are the means $\pm$ S.D. from three repeated experiments with three independent transfections. *Statistically significant differences ($P < 0.05$) compared with the respective vehicle control. (C, D) The same compounds were assayed in the yeast two-hybrid system for their ability to enhance the interaction of mCAR or mCAR Δ 8 with either SRC1 (C) or NCoR (D). Control incubations were made with yeast cells containing only one interacting partner (Control, grey bars). The results shown are normalized β -gal activities with means $\pm$ S.D. from two independent experiments, each performed in triplicate. *Statistically significant differences ($P < 0.05$) compared with the respective vehicle control.

proliferator-activated receptor- α ligand Wy-14643 had no effect. When the same assay was performed with mCAR Δ 8 lacking helix 12 (Figure 1A, mCAR Δ 8), the basal activity was decreased by approx. 90% to control levels obtained without the mCAR (Figure 1A, Control). Mouse CAR activators RU486 or Wy-14643 had no effect on mCAR Δ 8 activity, whereas androstamol treatment resulted in a further 35% decrease in activity. Thus, the activation of mCAR is specific and both the high basal and chemical-induced activities are dependent on the presence of helix 12. In the re-activation assay performed in the presence of 2 μ M androstamol, TCPOBOP and chlorpromazine re-activated the androstamol-suppressed mCAR by 20–30-fold, whereas androstamol, RU486 and Wy-14643 had no effect (Figure 1B, mCAR). None of the chemicals could activate mCAR Δ 8 (Figure 1B, mCAR Δ 8).

The strong basal activity of mCAR is also reproduced in the SRC1 interaction assay (Figure 1C, cf. mCAR: vehicle with Control: SRC1 only). TCPOBOP and chlorpromazine further enhanced the association of mCAR and SRC1 by 1.8–2.3-fold,

Figure 2 Modulation of mCAR activity by oestrogens

Mouse CAR activity was measured in normal mCAR assay (A), re-activation assay (B) and in yeast two-hybrid assays with SRC1 (C) and NCoR (D) as in Figure 1. The cells were treated with vehicle (open bar), reference compounds [1 μ M TCPOBOP (hatched-down), 10 μ M chlorpromazine (hatched-up) and 10 μ M androstamol (black)] or 10 μ M of the indicated chemicals (grey). The results are means $\pm$ S.D. from two or three independent experiments, each performed in triplicate. *Statistically significant differences ($P < 0.05$) compared with the respective vehicle control.

whereas androstamol and Wy-14643 had no significant effect and RU486 decreased it by approx. 20%. Removal of helix 12 completely abolished the interaction with SRC1 (Figure 1C, mCAR Δ 8), decreasing it to background levels obtained with yeast cells containing only one of the interacting partners (Figure 1C, Control). In the NCoR interaction assay, the basal mCAR-mediated activity was quite low, and neither of the mCAR activators, RU486 or Wy-14643, elicited a response. Only androstamol was effective in recruiting this co-repressor (Figure 1D, mCAR). Removal of helix 12 did not decrease the ability of mCAR to interact with NCoR in response to androstamol (Figure 1D, mCAR Δ 8). These results suggest that the strong inhibition of mCAR by androstamol is mediated by the attraction of co-repressors, which do not require the presence of helix 12. Thus, TCPOBOP and chlorpromazine are agonists for mCAR, whereas androstamol has an antagonistic character in both types of assays.

Activation of mCAR by oestrogens

The activation profiles of 13 chemicals that are structurally related to E2 or known partial agonists or antagonists for ER [31–33] were then measured, along with the reference compounds TCPOBOP, chlorpromazine and androstamol. It is known that HEK-293 cells do not express ERs, making them a good assay platform for oestrogenic compounds [33,34]. Among the oestrogens (Figure 2A, grey bars), 10 μ M E2, 17 α -ethinyl-E2 and oestrone activated

mCAR, but their responses were significantly weaker (1.4–2.6-fold) than those of TCPOBOP or chlorpromazine (3–3.5-fold; hatched bars). 4-Oestren-17-one, 2OH-E2, 6 β OH-E2 and genistein also showed variable increases in mCAR activity (1.5–3-fold), whereas oestratrien-3-ol was a weak inhibitor. Other compounds tested did not have any significant effect. Preliminary dose–response studies (0.3–30 μ M) indicated that maximal effects of oestrogens were reached at the concentration of 10 μ M, with EC₅₀ values $\leq$ 1 μ M (results not shown). To gain a better view of their activation potential, the same compounds were assayed in the presence of 2 μ M androsthenol (Figure 2B). Efficient re-activation of androsthenol-suppressed mCAR (6-fold) took place with all activators identified in Figure 2(A). Compounds which displayed no significant effects or inhibition in Figure 2(A) were also rather inactive in this re-activation assay. The only exceptions were that epi-E2 (17 α -oestradiol) was a weak activator (similar to 2OH- and 6 β OH-E2) and that genistein was a weaker activator ($\leq$ 2-fold) than anticipated from the normal mCAR assay. Overall, these results indicate that various oestrogens have effects on mCAR that range from moderate inhibition to apparent inactivity to strong activation.

Responses of NR co-regulators to oestrogens

The yeast two-hybrid system has been successfully used for studying ligand-dependent interactions of ER α and ER β with NR co-regulators in response to a large number of oestrogens, other steroids and xenobiotics [27,35–37]. When the oestrogens were assayed for ligand-dependent interaction of mCAR with SRC1 in yeast (Figure 2C), we found that E2, 17 α -ethynyl-E2, oestrone and 4-oestren-17-one displayed a stronger activity (3-fold enhancement) than TCPOBOP or chlorpromazine (approx. 2-fold). Epi-, 2OH-, 4OH- and 6 β OH-E2 showed responses similar to chlorpromazine, whereas oestratrien-3-ol, oestriol and 4OH-tamoxifen had a weak activating tendency that was not statistically significant. The isoflavonoids were inactive. These results indicate that oestrogens can elicit activation of mCAR by a co-activator-dependent mechanism. However, oestrogens appear to be stronger activators than the classical mCAR ligand TCPOBOP in the SRC1 assay, whereas the opposite is true in the normal mCAR assay (Figure 2A). Similarly, although epi-E2 and 4OH-E2 can clearly recruit SRC1, they did not activate mCAR in the normal mCAR assay. Only the re-activation assay (Figure 2B) could identify them as weak activators. In the NCoR assay (Figure 2D), both TCPOBOP and chlorpromazine had very little, if any, activity. However, most oestrogens, including epi-E2 and 4OH-E2, enhanced the interaction of mCAR with NCoR significantly to 15–50% of the androsthenol response. Weak responses (< 5%) were recorded only for 2OH-, 6 β OH- and 16 α OH-E2. The isoflavonoids and 4OH-tamoxifen were inactive. Thus many oestrogens have both agonistic and antagonistic characters in mCAR binding. Owing to this dual effect, oestrogens appeared to be weaker activators of mCAR than the pure agonist TCPOBOP in HEK-293 cells.

Responses of mCAR to other xenobiotics

To see whether the dual action on mCAR was limited to oestrogens, we tested other xenobiotics that have been shown to increase expression of rat or mouse CYP2B isoforms, albeit to a lesser extent than the prototype inducers PB (phenobarbital) or TCPOBOP. The antimycotic clotrimazole [38], the pesticide methoxychlor [39], the peripheral benzodiazepine receptor ligand PK11195 [40] and the antihyperlipidemic drug clofibrate [41] had

Figure 3 Modulation of mCAR activity by selected xenobiotic CYP2B inducers

Mouse CAR activity was measured in normal mCAR assay (A), re-activation assay (B) and in yeast two-hybrid assays with SRC1 (C) and NCoR (D) as in Figure 1. The cells were treated with vehicle (open bar), 1 μ M TCPOBOP (hatched-down), 10 μ M 17 α -ethynyl-E2 (stippled), 10 μ M androsthenol (black), 2 μ M clotrimazole, 200 μ M clofibrate, 10 μ M methoxychlor or 10 μ M PK11195 (grey). Results are means $\pm$ S.D. from two independent experiments, each performed in triplicate. *Statistically significant differences ($P < 0.05$) compared with the respective vehicle control.

only moderate or marginal effects in the normal mCAR assay (Figure 3A). However, they reproducibly activated androsthenol-suppressed mCAR by 1.8–6.8-fold in the re-activation assay (Figure 3B), thus demonstrating the better sensitivity of this type of assay. In the SRC1 assay (Figure 3C), methoxychlor enhanced mCAR-SRC1 interaction by 2.9-fold, followed by clofibrate (2.8-fold), PK11195 (2.2-fold) and clotrimazole (2.0-fold). In the NCoR assay (Figure 3D), PK11195 elicited a remarkable 50% response, whereas clotrimazole, methoxychlor and clofibrate were less active (7–36% response) in comparison. These results show that the dual action on mCAR is not limited to oestrogens, but shared by a number of xenobiotics known to induce CYP2B forms in rats or mice.

Induction of hepatic CYP2B10 mRNA by oestrogens and xenobiotics

To assess the effects of oestrogens and xenobiotics in another *in vivo* system, we measured the response of CYP2B10 mRNA in an established mouse primary hepatocyte culture and compared them with reference inducers TCPOBOP and chlorpromazine (Figure 4). TCPOBOP (1 μ M) and chlorpromazine (10 μ M) increased CYP2B10 mRNA by 6.5- and 3.6-fold respectively, compared with control hepatocytes treated with vehicle. A quantitatively equal response (6.4- and 4.0-fold) was seen in an RNase protection assay that was used to verify the quantitative RT-PCR method (results not shown). The response of TCPOBOP was also similar to that previously observed with PB in the same culture system [28,29]. Among oestrogens, 17 α -ethynyl-E2, E2, oestrone, 4-oestren-17-one and 6 β OH-E2 induced CYP2B10 mRNA by more than 2-fold, whereas epi-E2 increased it by 64%. Oestratrien-3-ol, 2OH-E2, 4OH-E2, 16 α OH-E2 and 4OH-tamoxifen were without any significant effect. Daidzein and genistein had a small, statistically insignificant tendency (15–20%) to decrease CYP2B10 mRNA expression. Finally, all xenobiotics tested except clofibrate proved to be moderate inducers of CYP2B10 mRNA (2.2–2.6-fold). These results

Figure 4 Modulation of hepatic CYP2B10 mRNA expression by oestrogens

The cultured mouse hepatocytes were treated with vehicle (0.1%, v/v), TCPOBOP (1 μ M), chlorpromazine (10 μ M), indicated oestrogens (10 μ M), selected xenobiotics (2 μ M clotrimazole, 200 μ M clofibrate, 10 μ M methoxychlor and 10 μ M PK11195) or were left untreated for 24 h. Total RNA samples (2 μ g) were reverse-transcribed and 40 ng of cDNA samples were analysed in duplicate for expressions of CYP2B10 and β -actin mRNAs with the quantitative RT-PCR [30]. The experiments were repeated on three different incubations and results are expressed as means $\pm$ S.D. The extent of induction (-fold) is defined by comparing CYP2B10/ β -actin signal ratios with that of vehicle control. *Statistically significant differences ($P < 0.05$) compared with hepatocytes treated with vehicle (set at 100%).

indicate that oestrogens at high doses and certain xenobiotics are inducers of *Cyp2b10* gene expression, but their induction potential *in vivo* is decreased in comparison with the xenobiotic inducer TCPOBOP. It should be noted that the rank correlation between CYP2B10 mRNA levels and the more sensitive mCAR re-activation assay results was excellent (Spearman $r^2 = 0.89$, $N = 19$), whereas comparison with the normal activation assay gave a less perfect agreement (Spearman $r^2 = 0.66$, $N = 19$).

DISCUSSION

Mechanisms and assays of mCAR activation

It is now well-established that the mCAR agonists TCPOBOP and chlorpromazine increase the association of mCAR with co-activators of the p160 family such as SRC1 and TIF2 (transcriptional intermediary factor 2) [18,19,42]. On the other hand, the inverse agonists androstenol and androstanol appear to decrease moderately SRC1 or TIF2 interactions [15,43] and, more significantly, increase recruitment of co-repressors such as NCoR and SMRT (silencing mediator for retinoic acid receptor and thyroid-hormone receptor) to the mCAR LBD [22,42]. In the present study, we have shown for the first time that oestrogens differ from the above chemicals since they can enhance interactions of mCAR with both the co-activator SRC1 and the co-repressor NCoR. Since the activation of mCAR by any chemical is the net effect of its association with available co-activators and co-repressors in mammalian cells, it is now evident why oestrogens appear to be weaker activators than TCPOBOP in two different experimental settings, in mammalian co-transfection assays and as inducers of CYP2B10 mRNA in mouse primary hepatocytes. Also, owing to the same dual effect, several xenobiotics that are moderate CYP2B inducers *in vivo* were not recognized as activators by the normal mCAR assay. These different net effects are demonstrated in Table 1 where SRC1 and NCoR results are used to predict the effects of oestrogens and xenobiotics after mCAR activity and then correlated with the actual data shown in Figures 2 and 3. This rather crude prediction can distinguish between mCAR

Table 1 Prediction of oestrogen- and xenobiotic-dependent mCAR activities

Responses of oestrogens and xenobiotics, measured with the SRC1 and NCoR assays (Figures 2C, 2D, 3C and 3D), are coded by graded descriptors: strong (+++), medium (++), weak (+) and none (0) (100–70, 70–30, 30–10 and <10% of maximum observed in each assay respectively). First, vehicle background values were subtracted from the NCoR and SRC1 values of the test chemicals and then the percentage value for the chemical in the NCoR assay was subtracted from the corresponding value in the SRC1 assay. The resulting percentage difference was then scored according to the same grading scheme to yield the 'predicted effect' on mCAR activation. Compounds with negative percentage differences (NCoR > SRC1) were classified as inhibitors (–). Effects on mCAR activity and CYP2B10 expression measured with the assays (Figures 2A, 2B, 3A, 3B and 4) are coded in the same way.

Chemical name	SRC1 assay	NCoR assay	Predicted effect	Effect in the activation assay	Effect in the re-activation assay	Effect on CYP2B10 mRNA
E2	+++	++	++	+	++	++
17 α -Ethinyl-E2	+++	++	++	++	++	++
Oestrone	+++	+	+++	++	++	++
Epi-E2	++	++	0	0	+	+
Oestratrien-3-ol	+	++	–	–	0	0
4-Oestren-17-one	+++	+	+++	+++	+++	++
Oestriol	+	0	0	0	0	0
2OH-E2	++	0	++	+	+	0
4OH-E2	++	++	0	0	0	0
6 β OH-E2	+	0	+	+	+	++
Daidzein	0	0	0	0	0	0
Genistein	0	0	0	+	0	0
4OH-tamoxifen	+	0	+	0	0	0
Clofibrate	++	++	+	0	0	0
Clotrimazole	++	0	+	+	+	+
Methoxychlor	++	+	++	0	0	+
PK11195	++	++	–	0	0	+

inhibitors and activators and even rank activators reasonably well: ten out of 17 compounds were accurately predicted. The extent of activation was somewhat overestimated for six compounds. Barring any metabolism of the ligand, this overestimation might be due to the fact that our prediction is based on only a single co-regulator partner (SRC1 and NCoR), whereas mammalian cells express a much larger variety of co-regulators contributing to the net effect of mCAR activity. Only one compound, namely genistein, was misclassified as inactive. It is not known whether the weak mCAR activation observed is due to any protein kinase inhibition by genistein.

Owing to the more robust responses, the re-activation assay in the presence of androstenediol may be more sensitive in detecting weak and/or moderate activators than the normal mCAR assay that is compromised by high basal mCAR activity. In fact, the better correlation of mCAR re-activation data with CYP2B10 mRNA levels ($r^2 = 0.89$ versus $r^2 = 0.66$) points to this possibility. Using the same prediction scheme also for mCAR re-activation, the CYP2B10 mRNA induction potential was accurately predicted for 12 compounds, slightly overestimated for two chemicals (2OH-E2 and 4-oestren-17-one) and underestimated for three compounds (Table 1). Among these, 2OH-E2 is easily metabolized by the catechol *O*-methyl transferase [44], thus decreasing its level in hepatocytes. The underestimated chemicals methoxychlor, PK11195 and 6 β OH-E2 very closely missed the 10 or 30% grade limits for mCAR re-activation. Therefore, in principle, such activation assays seem useful for the identification of mCAR ligands, although more studies are needed to define a proper androstenediol concentration and a lower threshold for re-activation that can distinguish real *in vivo* CYP2B inducers. Even though we do not have a direct measure of the receptor affinity, we feel that the functional response (activation versus inhibition) would be more important here. The estimated EC₅₀ values for mCAR activation and CYP2B10 mRNA induction seem to match reasonably well among different chemicals [16–18,20,24], indicating that activation response may be a useful approximation of the receptor affinity. For structurally similar ligands of PXR, there is a good correspondence between these parameters [14].

The current model of *Cyp2b10* gene activation by mCAR suggests that mCAR translocates from the cytoplasm into the nucleus after exposure to inducers that include TCPOBOP, chlorpromazine, E2 and oestrone [20,45]. A step of nuclear activation for mCAR has often been evoked in recent reviews [12,46,47] to explain the frequent accumulation of mCAR in the nucleus without a parallel increase in CYP2B10 mRNA. Since the Ca²⁺/calmodulin-dependent kinase inhibitor KN-62 seems to repress partially CYP2B10 mRNA induction and does not affect mCAR translocation in TCPOBOP-exposed hepatocytes, it has been suggested that a nuclear kinase may further activate ligand-bound mCAR, so as to allow interactions with co-activators and, ultimately, transcription of the *Cyp2b10* gene. However, as yet, there is no physical evidence that mCAR is being phosphorylated to support this suggestion. In the light of dual actions of oestrogens and xenobiotics presented here, another possibility to be considered is that many chemicals may bind to mCAR and elicit its translocation. However, depending on the NR co-regulators these chemicals prefer to recruit to the mCAR LBD, they may or may not activate the *Cyp2b10* gene to varying degrees. This alternative hypothesis can thus explain the presence of apparently inactive nuclear mCAR.

Relatively large doses of E2 and oestrone (approx. 1 μ M) have been reported to increase the expression of CYP2B10 mRNA in mouse hepatocyte spheroid cultures and this induction was not reversed by the ER antagonist tamoxifen [48]. In another hepatocyte culture system [20], both E2 and oestrone at 10 μ M

elicited nuclear translocation of mCAR to similar levels as TCPOBOP, but displayed a low, approx. 2-fold induction of CYP2B10 mRNA, compared with a 9-fold increase seen with TCPOBOP; these changes are close to those observed here. The apparent discrepancy between the moderate CYP2B10 induction and the strong mCAR translocation by the oestrogens has not been commented upon [20]. Moreover, human CYP2B6 mRNA was up-regulated by 100-fold by 10 nM E2 in the human breast cancer cell line ZR75-1 [49]. It is not known if this effect could be reversed by tamoxifen or whether these cells could express human CAR. Considering the low dose used and the fact that E2 and 17 α -ethynyl-E2 are not activators for human CAR [20,22], the induction of CYP2B6 could probably be mediated by an ER.

Structure–activity correlation of oestrogens

Potent agonists of ER α were also strong activators of mCAR. Structural changes at the D-ring such as lack of orientation of the C-17 hydroxy group (oestratrien-3-ol, epi-E2) or 16 α -hydroxylation (oestriol) drastically impaired mCAR activation and interaction with SRC1, whereas similar changes decreased ER α binding only moderately [31,32]. An aromatic A-ring is a prerequisite for ER α binding [32,33], but not essential for mCAR activation: the response to 4-oestren-17-one, an analogue of oestrone, is almost equal to that of TCPOBOP. Among the hydroxylated E2 metabolites, 2OH-E2 and 6 β OH-E2 have appreciable mCAR activity, whereas 4OH-E2 and oestriol are weakly active. In contrast, oestriol and the catechol oestrogens are modest ligands for ER α , but 6 β OH-E2 is practically inactive [31]. These changes in mCAR activity are mostly explained by decrease in SRC1 recruitment and are modified by a dramatic loss of the NCoR activity for the hydroxylated E2 metabolites. Only 4OH-E2 retained NCoR activity comparable with E2. Finally, the partial ER agonists daidzein and genistein [33] and the ER antagonist 4OH-tamoxifen have no major effect on mCAR. These results indicate that even though mCAR can be activated by oestrogens, the activation profile of mCAR is different from that of ER α .

The ligand-binding cavity in ER α [6] appears smaller than the cavity of the recent three-dimensional model of mCAR (approx. 450 Å³ versus 1150 Å³; 1 Å = 0.1 nm) [42]. The large cavity of mCAR, with fewer potential protein–ligand interactions to contribute to the binding energy of small ligands, may explain why high concentrations of oestrogens are required for mCAR but not for ER α activation (μ M versus nM). Similarly, larger ligands tend to have a better binding affinity for PXR, which may indicate a better fulfilment of all potential protein–ligand interactions than is possible with smaller PXR ligands [50]. When the amino acids critical for E2 binding in ER α are compared with the corresponding mCAR residues (Figure 5), the residues in ER α , which are connected to the 17 β -OH group (His-524) and surround the D-ring (Ile-424, Gly-521 and Leu-525), correspond to the mCAR residues Glu-339, Phe-248, Tyr-336 and Leu-340 that are of equal size if not larger and also of similar hydrophobicity. This suggests a tight packing around the steroid D-ring in mCAR. In addition, Glu-339 can function as a hydrogen-bond acceptor, similar to the function of His-524 in ER α [6]. In contrast, residues in ER α that are essential for interactions with the 3-hydroxy group (Glu-353 and Arg-394) and the aromatic A-ring (Phe-404) are matched by mCAR residues Met-178, Thr-219 and Gly-230 respectively, which are less efficient in producing analogous interactions with E2. Therefore a less restricted binding environment for the A-ring in mCAR may be present. These comparisons suggest that if mCAR and ER α bind E2 in sufficiently similar orientations, it is easier to understand the present findings that mCAR is more sensitive

Figure 5 Comparison of ER α -binding site with the corresponding mCAR residues

The E2-binding pocket of ER α , hydrogen-bonding (arrows) and hydrophobic (double-headed arrows) interactions between E2 and important ER α residues are taken from the ER α X-ray structures [6]. The mCAR residues (given in parentheses) were identified using NR structural alignment information available at <http://www.gprc.org/NR/>

to modifications of the D-ring and less sensitive to those of the A-ring.

However, it is known that human PXR and mCAR LBDs have approx. 35 % sequence identity and similar sizes of ligand-binding cavities [42,51]. PXR can bind several chemicals including the mCAR activator E2 [52]; therefore, PXR and mCAR may share structural features in binding their ligands. The PXR ligand SR12813 can bind, by using different sets of residues, in three different orientations within the PXR LBD [51]. This raises the possibility that a small ligand such as E2 may also bind to mCAR LBD in several distinct modes. Potentially, the functional response of each mode may differ, with one binding mode promoting interactions with the co-activator and another with the co-repressor. These hypotheses can be tested in future by crystallization and site-directed mutagenesis of mCAR.

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REFERENCES

- Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. et al. (1995) The nuclear receptor superfamily: the second decade. *Cell* (Cambridge, Mass.) **83**, 835–839
- Aranda, A. and Pascual, A. (2001) Nuclear hormone receptors and gene expression. *Physiol. Rev.* **81**, 1269–1304
- Kliewer, S. A., Lehmann, J. and Willson, T. M. (1999) Orphan nuclear receptors: shifting endocrinology into reverse. *Science* **284**, 757–760
- Chawla, A., Repa, J. J., Evans, R. M. and Mangelsdorf, D. J. (2001) Nuclear receptors and lipid physiology: opening the X-files. *Science* **294**, 1866–1870
- Rosenfeld, M. G. and Glass, C. K. (2001) Coregulator codes of transcriptional regulation by nuclear receptors. *J. Biol. Chem.* **276**, 36865–36868
- Brzozowski, A. M., Pike, A. C. W., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Öhman, L., Greene, G. L., Gustafsson, J.-Å. and Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* (London) **389**, 753–758
- Pike, A. C. W., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J.-Å. and Carlquist, M. (1999) Structure of the ligand-binding domain of oestrogen receptor β in the presence of a partial agonist and a full antagonist. *EMBO J.* **18**, 4608–4618
- Bourguet, W., Germain, P. and Gronemeyer, H. (2000) Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. *Trends Pharmacol. Sci.* **21**, 381–388
- Xu, L., Glass, C. K. and Rosenfeld, M. G. (1999) Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Dev.* **9**, 140–147
- Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletcher, R. J. and Yamamoto, K. R. (1998) Structure and specificity of nuclear receptor–coactivator interactions. *Genes Dev.* **12**, 3343–3356
- Honkakoski, P., Zelko, I., Sueyoshi, T. and Negishi, M. (1998) The orphan nuclear receptor CAR–retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the *CYP2B* gene. *Mol. Cell. Biol.* **18**, 5652–5658
- Sueyoshi, T. and Negishi, M. (2001) Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. *Annu. Rev. Pharmacol. Toxicol.* **41**, 123–143
- Handschin, C., Podvínec, M., Stöckli, J., Hoffman, K. and Meyer, U. A. (2001) Conservation of signalling pathways of xenobiotic-sensing orphan nuclear receptors, chicken xenobiotic receptor, constitutive androstane receptor, and pregnane X receptor, from birds to humans. *Mol. Endocrinol.* **15**, 1571–1585
- Willson, T. M. and Kliewer, S. A. (2002) PXR, CAR and drug metabolism. *Nature Rev. Drug Discov.* **1**, 259–266
- Forman, B. M., Tzameli, I., Choi, H. S., Chen, J., Simha, D., Seol, W., Evans, R. M. and Moore, D. D. (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR- β . *Nature* (London) **395**, 612–615
- Honkakoski, P., Moore, R., Washburn, K. and Negishi, M. (1998) Activation by diverse xenobiotics of the 51-base pair phenobarbital-responsive enhancer module in the *CYP2B10* gene. *Mol. Pharmacol.* **53**, 597–601
- Sueyoshi, T., Kawamoto, T., Zelko, I., Honkakoski, P. and Negishi, M. (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human *CYP2B6* gene. *J. Biol. Chem.* **274**, 6043–6046
- Tzameli, I., Pissios, P., Schuetz, E. G. and Moore, D. D. (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
- Wei, P., Zhang, J., Dowhan, D. H., Han, Y. and Moore, D. D. (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J.* **2**, 117–126
- Kawamoto, T., Kakizaki, S., Yoshinari, K. and Negishi, M. (2000) Estrogen activation of the nuclear orphan receptor CAR (constitutive active receptor) in induction of the mouse *Cyp2b10* gene. *Mol. Endocrinol.* **14**, 1897–1905
- Moore, L. B., Maglich, J. M., McKee, D. D., Wisely, B., Willson, T. M., Kliewer, S. A., Lambert, M. H. and Moore, J. T. (2002) Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Mol. Endocrinol.* **16**, 977–986
- Mäkinen, J., Frank, C., Jyrkkärinne, J., Gynther, J., Carlberg, C. and Honkakoski, P. (2002) Modulation of mouse and human phenobarbital-responsive enhancer module by nuclear receptors. *Mol. Pharmacol.* **62**, 366–378
- Choi, H. S., Chung, M., Tzameli, I., Simha, D., Lee, Y. K., Seol, W. and Moore, D. D. (1997) Differential trans-activation by two isoforms of the orphan nuclear hormone receptor CAR. *J. Biol. Chem.* **272**, 23565–23571
- Honkakoski, P., Jääskeläinen, I., Kortelahti, M. and Urtti, A. (2001) A novel drug-regulated gene expression system based on the nuclear receptor constitutive androstane receptor CAR. *Pharmaceut. Res.* **18**, 146–150
- Hu, X. and Lazar, M. A. (1999) The CoNR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature* (London) **402**, 93–96
- Oñate, S. A., Tsai, S. Y., Tsai, M.-J. and O'Malley, B. W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354–1357
- Nishikawa, J., Saito, K., Goto, J., Daikaya, F., Matsuo, M. and Nishihara, T. (1999) New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmacol.* **154**, 76–83
- Salonpää, P., Kottari, S., Pelkonen, O. and Raunio, H. (1997) Regulation of CYP2A5 induction by porphyrinogenic agents in mouse primary hepatocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **355**, 8–13
- Viitala, P., Posti, K., Lindfors, A., Pelkonen, O. and Raunio, H. (2001) Cyclic AMP-mediated upregulation of CYP2A5 in mouse hepatocytes. *Biochem. Biophys. Res. Commun.* **280**, 761–767
- Bustin, S. A. (2002) Quantification of mRNA using real-time reverse-transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* **29**, 23–39
- Anstead, G. M., Carlson, K. E. and Katzenellenbogen, J. A. (1997) The estradiol pharmacophore: ligand structure–estrogen receptor binding affinity relationships and a model for the receptor binding site. *Steroids* **62**, 268–303
- Kuiper, G. G. J. M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. and Gustafsson, J.-Å. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β . *Endocrinology* **138**, 863–870
- Kuiper, G. G. J. M., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B. and Gustafsson, J.-Å. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology* **139**, 4252–4263

- 34 Kahlert, S., Nuedling, S., van Eickels, M., Vetter, H., Meyer, R. and Grohe, C. (2000) Estrogen receptor α rapidly activates the IGF-1 receptor pathway. *J. Biol. Chem.* **275**, 18447–18453
- 35 Nishihara, T., Nishikawa, J. I., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S. and Utsumi, H. (2000) Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *J. Health Sci.* **46**, 282–298
- 36 Masuyama, H., Hiramatsu, Y., Kunitomi, M., Kudo, T. and MacDonald, P. N. (2000) Endocrine disrupting chemicals phthalic acid and nonylphenol activate pregnane X receptor-mediated transcription. *Mol. Endocrinol.* **14**, 421–428
- 37 Lee, H. S., Miyauchi, K., Nagata, Y., Fukuda, R., Sasagawa, S., Endoh, H., Kato, S., Horiuchi, H., Takagi, M. and Ohta, A. (2002) Employment of the human estrogen receptor β ligand-binding domain and co-activator SRC1 nuclear receptor-binding domain for the construction of a yeast two-hybrid detection system for endocrine disrupters. *J. Biochem.* **131**, 399–405
- 38 Lubet, R. A., Dragnev, K. H., Chauhan, D. P., Nims, R. W., Diwan, B. A., Ward, J. M., Jones, C. R., Rice, J. M. and Miller, M. S. (1992) A pleiotropic response to phenobarbital-type enzyme inducers in the F344/NCr rat: effects of chemicals of varied structure. *Biochem. Pharmacol.* **43**, 1067–1078
- 39 Li, H. C. and Kupfer, D. (1998) Mechanism of induction of rat hepatic CYP2B and CYP3A by the pesticide methoxychlor. *J. Biochem. Mol. Toxicol.* **12**, 315–326
- 40 Yamada, H., Matsuki, Y., Yamaguchi, T. and Oguri, K. (1999) Effect of a ligand selective for peripheral benzodiazepine receptors on the expression of rat hepatic P450 cytochromes: assessment of the effect *in vivo* and in a hepatocyte culture system. *Drug Metab. Dispos.* **27**, 1242–1247
- 41 Gerhold, D., Lu, M., Xu, J., Austin, C., Caskey, C. T. and Rushmore, T. (2001) Monitoring expression of genes involved in drug metabolism and toxicology using DNA microarrays. *Physiol. Genomics* **5**, 161–170
- 42 Dussault, I., Lin, M., Hollister, K., Fan, M., Termini, J., Sherman, M. A. and Forman, B. M. (2002) A structural model of the constitutive androstane receptor defines novel interactions that mediate ligand-independent activity. *Mol. Cell. Biol.* **22**, 5270–5280
- 43 Min, G., Kemper, J. K. and Kemper, B. (2002) Glucocorticoid receptor-interacting protein 1 mediates ligand-independent nuclear translocation and activation of constitutive androstane receptor *in vivo*. *J. Biol. Chem.* **277**, 26356–26363
- 44 Yager, J. D. and Liehr, J. G. (1996) Molecular mechanisms of estrogen carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **36**, 203–232
- 45 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
- 46 Kakizaki, S., Yamamoto, Y., Ueda, A., Moore, R., Sueyoshi, T. and Negishi, M. (2003) Phenobarbital induction of drug/steroid-metabolizing enzymes and nuclear receptor CAR. *Biochim. Biophys. Acta* **1619**, 239–242
- 47 Yamamoto, Y., Kawamoto, T. and Negishi, M. (2003) The role of the nuclear receptor CAR as a coordinate regulator of hepatic gene expression in defense against chemical toxicity. *Arch. Biochem. Biophys.* **409**, 207–211
- 48 Nemoto, N. and Sakurai, J. (1995) Glucocorticoid and sex hormones as activating or modulating factors for expression of Cyp2b-9 and Cyp2b-10 in the mouse liver and hepatocytes. *Arch. Biochem. Biophys.* **319**, 286–292
- 49 Soulez, M. and Parker, M. G. (2001) Identification of novel oestrogen receptor target genes in human ZR75-1 breast cancer cells by expression profiling. *J. Mol. Endocrinol.* **27**, 259–274
- 50 Ekins, S., Mirny, L. and Schuetz, E. G. (2002) A ligand-based approach to understanding selectivity of nuclear hormone receptors PXR, CAR, FXR, LXR α , and LXR β . *Pharmaceut. Res.* **19**, 1788–1800
- 51 Watkins, R. E., Wisely, G. B., Moore, L. B., Collins, J. L., Lambert, M. H., Williams, S. P., Willson, T. M., Kliewer, S. A. and Redinbo, M. R. (2001) The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science* **292**, 2329–2333
- 52 Moore, L. B., Parks, D. J., Jones, S. A., Bledsoe, R. K., Consler, T. G., Stimmel, J. B., Goodwin, B., Liddle, C., Blanchard, S. G., Willson, T. M. et al. (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J. Biol. Chem.* **275**, 15122–15127

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