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Title Page

Di(2-ethylhexyl) phthalate is a highly potent agonist for the human constitutive androstane receptor splice variant, CAR2

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Running Title Page

Running Title: DEHP is a highly potent agonist for the human CAR splice variant, CAR2

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Abbreviations: CAR, constitutive androstane receptor; DEHP, di(2-ethylhexyl) phthalate; PXR, pregnane X receptor

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Abstract

The human constitutive androstane receptor (CAR, CAR1) regulates the expression of genes involved in xenobiotic metabolism in the liver. The CAR gene utilizes multiple alternative splicing events during pre-mRNA processing, thereby enhancing the CAR transcriptome. Previous reports have identified two prominent human CAR variants, CAR2 and CAR3, possessing 4- and 5-amino acid insertions in their ligand binding domains, respectively. Unlike the constitutively active reference form of the receptor, we now demonstrate that CAR2 is a ligand activated receptor and comprises approximately 30% of the reference transcript level in human liver tissues in human hepatocytes. Further, we identify the common plasticizer, di(2-ethylhexyl) phthalate (DEHP), as a highly potent and uniquely selective agonist of CAR2. Results from reporter transactivation and mammalian two-hybrid assays reveal that DEHP activates CAR2 at low nanomolar concentrations, results further supported by analysis of CAR target gene expression in primary human hepatocytes. In addition, comparative genomic analysis show that the typical mouse, rat and marmoset models of DEHP toxicity can not accurately profile potential human toxicity due to these species inability to generate a CAR2-like transcript. The discovery that CAR2 is an ultimate human DEHP receptor identifies a novel pathway modulating human DEHP toxicity with potential clinical implications for a subset of patients undergoing critical care medical interventions.

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Introduction

The constitutive androstane receptor (CAR, NR1I3) is a member of the nuclear receptor superfamily that is expressed primarily in the liver (Baes *et al.*, 1994). In conjunction with the pregnane X receptor (PXR, NR1I2), CAR regulates hepatic genes involved in all three phases of xenobiotic metabolism (Wei *et al.*, 2000). The products of these genes play a role in modulating the metabolism of a variety of pharmaceuticals, such as acetaminophen (Zhang *et al.*, 2002) and cyclophosphamide (Roy *et al.*, 1999), as well as endogenous compounds including thyroid hormone (Maglich *et al.*, 2004), bile acids (Guo *et al.*, 2003) and steroid hormones (Xie *et al.*, 2003).

In human livers, the CAR gene expresses a number of differentially spliced mRNA transcripts, generated through the use of alternative splice acceptor sites during pre-mRNA processing (Auerbach *et al.*, 2003;Savkur *et al.*, 2003;Arnold *et al.*, 2004;Jinno *et al.*, 2004;Lamba *et al.*, 2004). Previous reports from our laboratory have focused on functional analysis of two prominent human CAR variants, termed CAR2 (Auerbach S.S *et al.*, 2007) and CAR3 (Auerbach *et al.*, 2005). Results from these and other studies demonstrated that both CAR2 and CAR3 activate reporters containing response elements derived from the endogenous promoters of the CYP2B6 and CYP3A4 genes (Auerbach *et al.*, 2005;Auerbach S.S *et al.*, 2007). Unlike CAR1, CAR3 functions as a ligand dependent receptor (Auerbach *et al.*, 2005), activating transcription in the presence of the human CAR ligand, CITCO (Maglich *et al.*, 2003). Our recent study of CAR2 suggested that it may have a ligand binding profile that is distinct from both CAR1 and CAR3 (Auerbach S.S *et al.*, 2007).

Based on these data, and results from molecular modeling analyses, we hypothesized that CAR2 possesses distinct biological properties. In this report we now demonstrate that CAR2 is a

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ligand activated receptor that comprises approximately one-third of the total CAR transcriptome in human hepatocytes. Further, we identify the common plasticizer, di(2-ethylhexyl) phthalate (DEHP), as a highly potent and uniquely selective agonist of CAR2. Results from reporter transactivation and mammalian two-hybrid assays reveal that DEHP activates CAR2 at low nanomolar concentrations. These results are further supported by analysis of CAR target gene expression in primary human hepatocytes, where DEHP activation of CAR2 markedly induced the CYP2B6 and CYP3A4 transcripts. Further, using genomic analyses, we demonstrate that the alternative splice site used to generate the CAR2 transcript is not conserved in mice, rats or marmosets – widely used animal models of DEHP toxicity.

DEHP is a plasticizer with an annual production volume approaching 260 million pounds (Kavlock *et al.*, 2006). Human exposure to DEHP is ubiquitous, as it is used in the manufacturing of flexible vinyl found in medical tubing, other medical devices, commercial floorings, food product wraps, and many additional consumer products (Hauser and Calafat, 2005). Concerns regarding the human toxicology of DEHP are prevalent; for example, during medical interventions patients can be exposed to relatively high doses of DEHP that leaches from plastic medical devices into infusate (FDA, 2001). Our finding that DEHP is a potent and selective activator of CAR2 likely has important implications for predictive human toxicity.

In summary, in this report we demonstrate that the naturally occurring NR1I3 splice variant, CAR2, is unique in that: 1) CAR2 is ligand activated, unlike the previously recognized constitutively active reference form of the receptor; 2) CAR2 comprises ~30% of the reference transcript level in human liver tissues; and, 3) CAR2 is selectively activated by low nanomolar concentrations of di(2-ethylhexyl) phthalate (DEHP), levels achieved in human serum and urine of certain patients. Further, these studies yield new mechanistic insight for the toxicological

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effects of DEHP in human liver, findings that are particularly relevant since the conventional rodent and more recent marmoset models for DEHP toxicity do not reflect that of the human DEHP receptor, CAR2.

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Materials and Methods

Chemicals. Di(2-ethylhexyl) phthalate (DEHP, CAS No. 117-81-7), 5α-androstan-3α-ol (androstanol) and dimethyl sulfoxide (DMSO, CAS No. 67-68-5) were purchased from Sigma (St. Louis, MO). 6-(4-Chlorophenyl)imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (CITCO, CAS No. 338404-52-7) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Plasmids. The vectors: CMV2-CAR1, CMV2-CAR2, 3.1-RXRα, 3.1-RXRαLBD, VP16-CAR1LBD, VP16-CAR2LBD, 2B6XREM and 3A4XREM were described previously (Auerbach S.S *et al.*, 2007). The vectors CMV2-CAR3 and VP16-CAR3LBD were also reported previously (Auerbach *et al.*, 2005) as well as the pM-SRC1 clone (Chang *et al.*, 1999). All clones were derived from human genes.

Bioinformatics. Protein sequences were retrieved from the NCBI database. Amino acids 212-348 of the human CAR1 protein (NP_005113.1) were used as the reference sequence and aligned using the blosum matrix of the ClustalW2 program from the EBI website (http://www.ebi.ac.uk/Tools/clustalw2/). Genomic alignments used the UCSC genome browser http://genome.ucsc.edu/ with the mouse CAR gene as the reference sequence.

Cell Culture. Culture conditions for the COS-1 and HepG2 cell lines were described previously (Auerbach S.S *et al.*, 2007), with the exception that the cells were maintained in media containing 8% fetal bovine serum (FBS) and transfected in media containing 8% dextran/charcoal treated FBS (HyClone, Logan, UT). Culturing conditions for primary human hepatocytes have been described (Olsavsky *et al.*, 2007). Available donor information is presented in Table 1. Preparations enriched for hepatocytes were received plated in collagen-coated 6-well culture dishes. Upon arrival the media was removed and replaced with 2 mL of

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fresh media. Matrigel was added along with a 2 mL media change within 48 h of plating. 2 mL changes of media occurred once, 24 h after the addition of matrigel and then again approximately 48 h later. Cells were harvested 24 h subsequently if no treatment was added. For cells that underwent chemical exposures, media was replaced in the morning (72 h post matrigel) and then again ~4 h later with fresh media that included the chemical agents. Cells were harvested 24 h after treatments. If not specified otherwise, all culturing materials were obtained from Invitrogen (Carlsbad, CA).

RNA Isolation and Quantitative PCR Analysis. Total RNA was extracted from the hepatocytes using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol. RNA concentrations were assessed by UV absorbance at 260 nm using a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA) and RNA integrity was ascertained using an Agilent Bioanalyzer (Agilent, Santa Clara, CA). A High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) was used to reverse transcribe RNA into cDNA, according to the manufacturer's instructions. TaqMan® Assays-on-Demand™ Gene Expression assays (Applied Biosystems) were performed according to manufacturer's directions for a 50 µL reaction volume subsequently divided into duplicate 25 µL reactions in a 96-well plate, and run in an Applied Biosystems 7300 Real-Time PCR System. Taqman probes were custom designed to detect the presence of the CAR2 insertion at the Exon 6-7 junction. A multiplex reaction was performed containing the 'REF' assay (CAT#: Hs00901572_g1, Applied Biosystems) and 'CAR2' probe. The 'REF' probe sequence is 5'-AGATGGAGCCCGTGTGGGGTTCCAG-3' (FAM Reporter). The 'CAR2' probe sequence is 5'-TGTATCTCCCACAGTGGG-3' (VIC Reporter). Absolute quantification was determined through the use of a standard curve generated using plasmid DNA containing either the CAR1 (REF) or CAR2 variant. Six point standard curves were used,

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encompassing the range from 2 fg to 6000 fg of target sequence. Applied Biosystems inventoried assays were used for detection of 18S (CAT#. Hs99999901_s1), CYP2B6 (CAT# Hs00167937_g1) and CYP3A4 (CAT#: Hs00604506_m1).

Transient Transfection Assays. The details of the luciferase reporter assays were described previously (Auerbach S.S *et al.*, 2007). Similarly, the mammalian two-hybrid assays were conducted using 40 ng pVP16 expression plasmid, 10 ng pM (GAL4) expression plasmid, 100 ng of pFR-luc reporter and 10 ng pRL-CMV (Auerbach S.S *et al.*, 2007). When the pcDNA3.1 expression plasmid, containing RXR-LBD, was incorporated in to the two-hybrid assay, 10 ng was used. The pM-SRC1 (Chang *et al.*, 1999) vector was substituted in place of the pM-SRC1 RID vector in the mammalian two-hybrid experiments.

Statistical Analysis. Unless stated otherwise, statistics and EC_{50} values were obtained using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA); specific details are provided in the figure legends.

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Results

Mice, rats and marmosets can not generate the CAR2 transcript. Protein sequences were retrieved from the NCBI database and aligned as described under materials and methods. Helix-6 and helix-7 of the CAR ligand-binding domain (Xu *et al.*, 2004) are shown (Figure 1A). The result shows that the rhesus monkey sequence includes a 4 amino acid insertion very similar to CAR2 (shaded boxes) suggesting that the splice variant is conserved across multiple species. To determine if other species could generate similar transcripts a genomic alignment was performed for 3 species of rodents and 6 primates using the UCSC genome browser (Kent *et al.*, 2002), with mouse CAR as the reference sequence (Figure 1B). While the splice acceptor site (AG) for CAR1 is conserved across each species, the CAR2 site is not conserved in marmoset, mouse or rat, indicating that these latter species are incapable of generating a CAR2-like, 4 amino acid insertion protein.

The CAR2 insertion is found in ~30.5% of total CAR transcripts in human hepatocytes. To determine the frequency of the CAR2 insertion we used a TaqMan qPCR approach as described under materials and methods. The frequency of the CAR2 insertion was found to be very consistent across all donors with a minimum of 25.9%, a maximum of 34.3% and an average of 30.5% (Figure 2). These data show that a significant portion of CAR transcripts in hepatocytes contain the CAR2 insertion.

DEHP is a contaminant of fetal bovine serum and a potent agonist of CAR2. Previous studies conducted in our laboratory used media containing untreated fetal bovine serum (FBS). Results from these studies implied that CAR2 was constitutively active, like CAR1, while CAR3 was uniquely ligand-activated (Auerbach *et al.*, 2003;Auerbach *et al.*, 2005;Auerbach S.S *et al.*, 2007). Although no previous reports indicated an effect caused by using dextran/charcoal treated

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FBS for the study of CAR activity, our initial data suggested that CAR2 may possess a unique ligand-binding profile (Auerbach S.S *et al.*, 2007). To examine this property further, we tested charcoal/dextran treated FBS to reduce the likelihood of potential background effects that may be contributed from endogenous compounds.

HepG2 cells were transfected in media containing either normal FBS or charcoal/dextran treated FBS and reporter assays were conducted for activation of the 2B6XREM reporter by the CAR variants (Figure 3A). In cells that were cultured using media containing the untreated FBS, CAR1 and CAR2 activated the reporter both in the presence and absence of the specific CAR ligand CITCO, while CAR3 was ligand activated as detected previously (Auerbach *et al.*, 2005). Surprisingly, the use of dextran/charcoal-treated FBS resulted in CAR2 activation only in the presence of CITCO, whereas CAR1 and CAR3 were relatively unaffected by the differences in media formulation. This result suggested that FBS contains a specific CAR2 agonist that is removed by dextran/charcoal treatment.

It should be noted that the data in figure 3A were obtained using HepG2 cells, a human hepatoma cell line. We have previously shown that the HepG2 and COS-1 cell lines obtain similar results in our system (Auerbach S.S *et al.*, 2007). In our hands, COS-1 cells generally exhibit a much higher transfection efficiency and consistency than HepG2 cells, therefore further experiments were conducted using the COS-1 cell line.

Using methods to be described elsewhere we identified DEHP as a contaminant of FBS that is efficiently removed by charcoal/dextran treatment. We assessed the ability of both DEHP and CITCO to activate CAR2 using the 2B6XREM reporter (Figure 3B). DEHP (Figure 3C) activated the reporter through CAR2 with an EC50 of 211 nM, more than 10-fold greater than the EC_{50} of CITCO, a highly potent CAR ligand.

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DEHP shows strong specificity for CAR2 when compared to CAR1 and CAR3. Activity of the CAR variants was assessed by 2B6XREM reporter activity after treatment (Figure 4A). CAR1 is active under all conditions tested due to its constitutive nature and ligand-independent nuclear accumulation after over-expression in cell lines. Under normal physiologic conditions CAR1 is sequestered in the cytoplasm and translocates to the nucleus upon activation (Kawamoto *et al.*, 1999). This complex mechanism of CAR1 makes *in vitro* assessment of ligand activation difficult. The CAR3 insertion is predicted not to significantly alter the ligand binding pocket and therefore CAR3 is likely an accurate proxy of CAR1 ligand interactions (Faucette *et al.*, 2007). CAR3 shows only a minimal response to DEHP at a dose of 25.2 µM but is markedly activated by 5 µM CITCO, as previously reported (Auerbach *et al.*, 2005). CAR2 again displayed a strong dose-response to DEHP; no effect is seen in the empty expression vector (CMV2) control group.

Transactivation of target genes by CAR occurs through the recruitment of coactivators when the receptor is in its active conformation. Steroid receptor coactivator 1 (SRC1) has been shown previously to be recruited by CAR1, CAR2 and CAR3 (Moore *et al.*, 2000;Auerbach *et al.*, 2005;Auerbach S.S *et al.*, 2007). To assess the interaction of SRC1 with CAR2 and CAR3 in the presence of DEHP we employed a mammalian two-hybrid assay as described under materials and methods. Both CAR2 and CAR3 interact with SRC1 in the presence of 5 µM CITCO (Figure 4B). CAR2 also shows a dose dependent interaction with SRC1 in response to DEHP, an effect that is not seen with CAR3. No effect resulted when CAR2 or CAR3 were co-transfected with a pM vector that did not include SRC1 (data not shown).

In order to better evaluate the interaction between CAR1 and DEHP we applied the mammalian two-hybrid system used in figure 4B to CAR1. The dynamic range of this system is

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greater than that obtained using the 2B6XREM reporter construct, allowing a more sensitive approach for evaluating physical interactions of chemical entities with the ligand binding pocket of CAR1, as determined by their abilities to compete away the inverse agonist androstanol (Forman *et al.*, 1998). The interaction between the CAR1LBD and SRC1 was measured with and without 10 µM androstanol. In the presence of 10 µM androstanol the reporter activity is approximately 30% of the constitutive activity seen in the absence of androstanol (Figure 4C). When CITCO is titrated into the experiment reporter activity is readily restored, with a clearly pronounced effect at 100 nM. On the other hand, DEHP acts as only a weak competitor of androstanol at 10 µM. Similarly weak competition of androstanol was also observed in separate analysis of mCAR (data not shown). To better illustrate this effect, dose response curves were generated using the data in figure 4C (inset). The result shows an EC50 of 571 nM for CITCO and 299 µM for DEHP. In a previous report, using a CAR-FRET assay, the EC50 for CITCO was published as 49 nM (Maglich *et al.*, 2003), 8.6% of that determined in our competition study. If the competition between CITCO and androstanol and DEHP and androstanol is linear we estimate the EC50 of DEHP for CAR1 to be ~25 µM, an estimate that is consistent with the data obtained for the CAR3 variant (Figure 4A).

DEHP increases CYP2B6 and CYP3A4 transcript levels in primary human hepatocytes.

Hepatocytes were isolated from 3 separate donors and cultured as described under materials and methods. Cells were treated with 100 nM CITCO or increasing concentrations of DEHP for 24 h and total RNA was isolated for use in qPCR analysis for activation of two CAR target genes, CYP2B6 and CYP3A4 (Figure 5). All 3 donors showed an increase in CYP2B6 and CYP3A4 transcripts in response to 50 µM DEHP. Donor H displayed a strong dose-response to DEHP with an increase in transcript seen at a 10 nM dose for both CYP2B6 and CYP3A4. The other

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two donors exhibited more moderate responses to DEHP, although the responses from all donors indicated a dose-response enhancement of transcript levels with DEHP. The more modest responses noted for Donors I and J likely reflect either interindividual differences in effect, or the apparent higher basal level of expression for both CYP2B6 and CYP3A4 existing prior to treatments.

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Discussion

The report of the crystal structure of CAR1 demonstrated that helix-6 and helix-7 line a large portion of the ligand binding pocket, and that a F234A mutation of CAR1 results in loss of the receptor's constitutive activity (Xu *et al.*, 2004). CAR2 contains a 4 amino acid insertion that resides in the highly conserved loop between helix-6 and helix-7 of the ligand binding domain, and lies just 1 amino acid upstream of F234 (Figure 1A). These data provide support for a hypothesis that CAR2 lacks constitutive activity and may possess a unique ligand binding profile. We now demonstrate that, unlike CAR1, CAR2 is a ligand-dependent receptor and that DEHP is a highly sensitive and selective activator of this variant CAR. In separate experiments (DeKeyser et. al., manuscript in preparation), we demonstrated that no other member of the human NR1I subfamily possess a similar affinity for DEHP, establishing a potency of DEHP for CAR2 that is 40-fold greater than that of any other human NR1I receptor.

As a consequence of their wide spread use and environmental prevalence, there has been a great deal of concern noted for potential human toxicity resulting from exposure to phthalates, concerns that have been focused primarily on reproductive toxicity, stemming from the anti-androgenic properties of DEHP. A recent report from the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) underscores the issues over potential adverse effects of DEHP on the developing male reproductive tract, with infants, particularly those who have faced critical illness with associated medical device interventions, at the greatest risk (Kavlock *et al.*, 2006). It has been suggested that blood phthalate levels in patients undergoing critical care procedures may approach micromolar concentrations (McKee, 2004), with DEHP levels of 1.19 µg/mL reported in cord blood (Latini *et al.*, 2003). DEHP exposures in males may compromise sperm quality and quantity (Kavlock *et al.*, 2006;Hoppin,

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2003;Duty *et al.*, 2005) and disrupt circulating levels of thyroid hormone (Meeker *et al.*, 2007). Notably, the metabolism of thyroid hormone is enhanced through CAR regulation (Maglich *et al.*, 2004). Further, the human CYP2B6 and CYP3A4 enzymes metabolize testosterone (Imaoka *et al.*, 1996). It is interesting to speculate that phthalate levels in certain tissues and perhaps hepatocytes may actually exceed circulating blood levels, implying that CAR2 may be activated by DEHP at even lower blood concentrations than achieved in critical care interventions, and that CAR1 or CAR3 may additionally take on a target role under such circumstances.

Mono(ethylhexyl) phthalate (MEHP), a major metabolite of DEHP, has been implicated as a mediator of the reproductive and developmental toxicity associated with DEHP (Heindel and Powell, 1992;Kavlock *et al.*, 2006). We tested MEHP as a CAR2 activator, but even at 10 μM this agent exhibited only weak activity in our *in vitro* reporter assays (data not shown), results that further support our conclusion that the DEHP parent compound itself is the most active modulator of CAR2. It is noteworthy that most of the previous conclusions regarding phthalate toxicology (Kavlock *et al.*, 2006) have been drawn from high dose studies in rodent or marmoset models, species that are not capable of generating a CAR2-like transcript (Figure 1B). Further, it has been suggested that DEHP is metabolized to MEHP to a lesser degree in primates compared to rodents (Rusyn *et al.*, 2006;Ito *et al.*, 2005). In light of these considerations, the potential role of CAR2 as a mediator of the developmental and reproductive toxicities associated with DEHP in humans warrants further investigation.

In this report, we demonstrate that the CAR2 transcript comprises ~30% of the CAR reference level in human hepatocytes (Figure 2), and that exposure to DEHP induces transcription of the CAR target genes, CYP2B6 and CYP3A4. In particular, one donor exhibited a DEHP response at a dose that likely activates CAR2 exclusively (Figure 5). The discovery that

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low levels of DEHP can induce xenobiotic metabolism in human hepatocytes may have immediate clinical relevance for some patients, especially those receiving large amounts of intravenous fluids or blood transfusions, as well as medications that have contraindications associated with CAR target genes.

Further, the results of our study strongly imply that CAR2 has the capacity to interact with a set of ligands that are highly distinct from that of other nuclear receptors. This finding will likely serve to redirect future evaluation of drug and chemical safety data and should set new directions underscoring the importance of CAR in human biology, including for the emerging role of CAR as a regulator of hepatic energy and lipid metabolism (Konno *et al.*, 2008;Masson *et al.*, 2008).

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Footnotes

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Legends for Figures

Figure 1. Rodent and primate alignments of the amino acid and genomic nucleotide sequences flanking the CAR2 insertion. (A) The protein alignment of helix-6 and helix-7 of the ligand-binding domain. Protein sequences were retrieved from the NCBI database. Amino acids 212-348 of the human CAR1 protein (NP_005113.1) were used as the reference sequence and aligned using the blosum matrix of the ClustalW2 program from the EBI website (http://www.ebi.ac.uk/Tools/clustalw2/). (B) CAR genomic alignment based on the mouse CAR gene using the UCSC genome browser. The intron 6, exon 7 junction is shown (relative to human CAR). The splice acceptor sites that are used to generate CAR1 or CAR2 are boxed.

Figure 2. Frequency of the CAR2 insertion in primary human hepatocytes. TaqMan qPCR was performed on total RNA obtained from 7 primary human hepatocyte cultures. Hepatocytes were plated into collagen-coated 6-well culture dishes and cultured for approximately 6 days before harvesting. Quantification of each variant was determined with standard curves generated from plasmid DNA DNA containing either the CAR1 or CAR2 coding sequence. The data are expressed as a % of total transcript, defined as the sum of the REF and CAR2 probes divided by the individual probe of interest. The error bars indicate the range based on the standard deviation of the technical replicates using the 'square root of sum-of-squares' technique.

Figure 3. Differential activation of the CAR variants in response to dextran/charcoal treated FBS and activation of CAR2 by DEHP. All transfections included the 3.1-RXRα expression vector, the 2B6XREM reporter and the pRL-CMV vector for normalization of transfection efficiency. Data are represented as normalized luciferase values and each data point represents the mean (+/-

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S.D.) of four separate transfections. (A) HepG2 cells were transfected with CMV2-CAR1, CMV2-CAR2 or CMV2-CAR3 in media containing 8% FBS or 8% charcoal/dextran treated FBS and then treated for 24 h. (B) COS-1 cells were transfected with CMV2 or CMV2-CAR2 and treated for 24 h in media containing 8% charcoal/dextran treated FBS. The inset shows the CAR2 data plotted as a dose-response curve. The DMSO group defined the 0% response and the 10 μM DEHP group was set to 100% response. (C) Chemical structure and properties of DEHP. *Denotes data points that were significantly different from their respective DMSO control as determined by unpaired two-tailed t-test ($p<0.0001$). **Denotes data points that were significantly different from their respective DMSO control as determined by ANOVA in combination with a Dunnett's multiple comparison post test ($p<0.01$). #Denotes that the difference in the LogEC50 values was statistically significant as determined by F-test ($p<0.0001$). (D) DEHP concentrations ascertained using quantitative mass spectroscopy.

Figure 4. Transactivation and coactivator recruitment by CAR1, CAR2 and CAR3 in response to DEHP. All transfections were done in COS-1 cells using media containing 8% charcoal/dextran treated FBS and included the 3.1-RXRα expression vector (A) or the 3.1-RXRαLBD expression vector (B&C) as well as the pRL-CMV vector for normalization of transfection efficiency. Data are represented as normalized luciferase values and each data point represents the mean (+/- S.D.) of four separate transfections. (A) Cells were transfected with the 2B6XREM reporter and CMV2 or CMV2-CAR1, 2 or 3 and treated for 24 h. (B) Cells were transfected with pFR-Luciferase, pM-SRC1 and VP16-CAR2LBD or VP16-CAR3LBD expression vectors and treated for 24 h. (C) Cells were transfected with pFR-Luciferase, pM-SRC1 and VP16-CAR1LBD expression vector and treated for 24 h. The inset shows the + androstanol data plotted as a dose-

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response curve. A 100% response was defined as the reporter activity in the presence of 5 µM CITCO (without androstanol) and a 0% response was defined as the reporter activity in the presence 10 µM androstanol (without CITCO or DEHP). *Denotes data points that were significantly different from their respective DMSO control as determined by ANOVA in combination with a Dunnett's multiple comparison post test ($p<0.01$). #Denotes that the difference in the LogEC50 values was statistically significant as determined by F-test ($p<0.0001$).

Figure 5. Relative levels of CYP2B6 and CYP3A4 transcript in primary human hepatocytes after treatment with DEHP or CITCO. Hepatocytes were plated into collagen-coated 6-well culture dishes and cultured for approximately 5 days before being treated for 24 h after which they were harvested for TaqMan qPCR analysis. All Data have been normalized to the expression of 18s RNA and are presented as the fold change in transcript relative to the DMSO treated group. The error bars indicate the range based on the standard deviation of the technical replicates using the 'square root of sum-of-squares' technique.

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Tables

Table 1: Hepatocyte Donor Information

Donor	Age	Gender	Race	Donor	Age	Gender	Race
A	0.75	M	Caucasian	F	3	F	Caucasian
B	57	M	Caucasian	G	61	M	Caucasian
C	49	M	Caucasian	H	60	F	Unknown
D	61	M	Caucasian	I	69	F	Caucasian
E	63	M	Caucasian	J	57	F	Caucasian

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Figure 1

A.

```
CAR1_human   LQTQNFLCGPLRYTIEDGARVG----FQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMAL
CAR2_human   LQTQNFLCGPLRYTIEDGARVSPTVGFQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMAL
CAR_chimp    LQTQNFLCGPLRYTIEDGARVG----FQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMAL
CAR_rhesus   LQTQNFLCGPLRYTIEDAARVSPAVGFQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMAL
CAR_mouse    LQTENFFCGPLCYKMEDAVHAG----FQYEFLESILHFHKNLKGLHLQEPEYVLMAATAL
CAR_rat      LQTQNFFCGPLCYKMEDAVHVG----FQYEFLELIIHFHKTLKRLQLQEPEYALMAAMAL
             ***:**:**** *.:**..:..    ** ****.:***.*:*:******.*:**
```

Helix-6 Helix-7

B.

chr1: 173147420 173147430 173147440 173147450 173147460 173147470

---> GAGCAATGGCCAGGAGGCTCCTCAGTCTACCATCTCTCACAGCAGGCTTCCAGTACGAGTTTTGGAGTCGAT

UCSC Gene Predictions Based on RefSeq, UniProt, GenBank, and Comparative Genomics

Nr1i3 A G F Q Y E F L E S I

Nr1i3 A G F Q Y E F L E S I

Nr1i3

30-Way Multiz Alignment & Conservation

Mammal Cons

Gaps

```
Mouse      GAGCAATGGCCAGGAGGCTCCTCAGTCTACCATCTCTCACAG A G F Q Y E F L E S I
Rat        GAGCAATGGCCAGGAGGCTGCTCAGCCCACCATCTCTCACAG V G F Q Y E F L E L I
GuineaPig  GTAAGGTGACTTGGAG--TCCTAAATCCAGTGTCTCTCACAG V G F Q R D F L E L L
Human      GTCAGGTGACCTAGAGGCTCCTAATCCTAGTATCTCCCACAG V G F Q V E F L E L L
Chimp      GTCAGGTGACCTAGAGGCTCCTAATCCTAGTATCTCCCACAG V G F Q V E F L E L L
Rhesus     GTCAGGTGACCTAGAGGCTCCTAATCCTAGTATCTCCCGCAG V G F Q V E F L E L L
Orangutan  GTCAGGTGACCTAGAGGCTCCTAATCCTAGTATCTCCCGCAG V G F Q V E F L E L L
Marmoset   GTCAGGTGACCTAGAGACTCCTAATCCTGGTGTCTCCCACAG V G F Q V E F L E L L
Bushbaby   GTCTGGTGACCTGGAGGCTCCTAAAACTAGTATCTCCCACAG V G F E V E F L E L L
```

CAR2 CAR1

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Figure 2

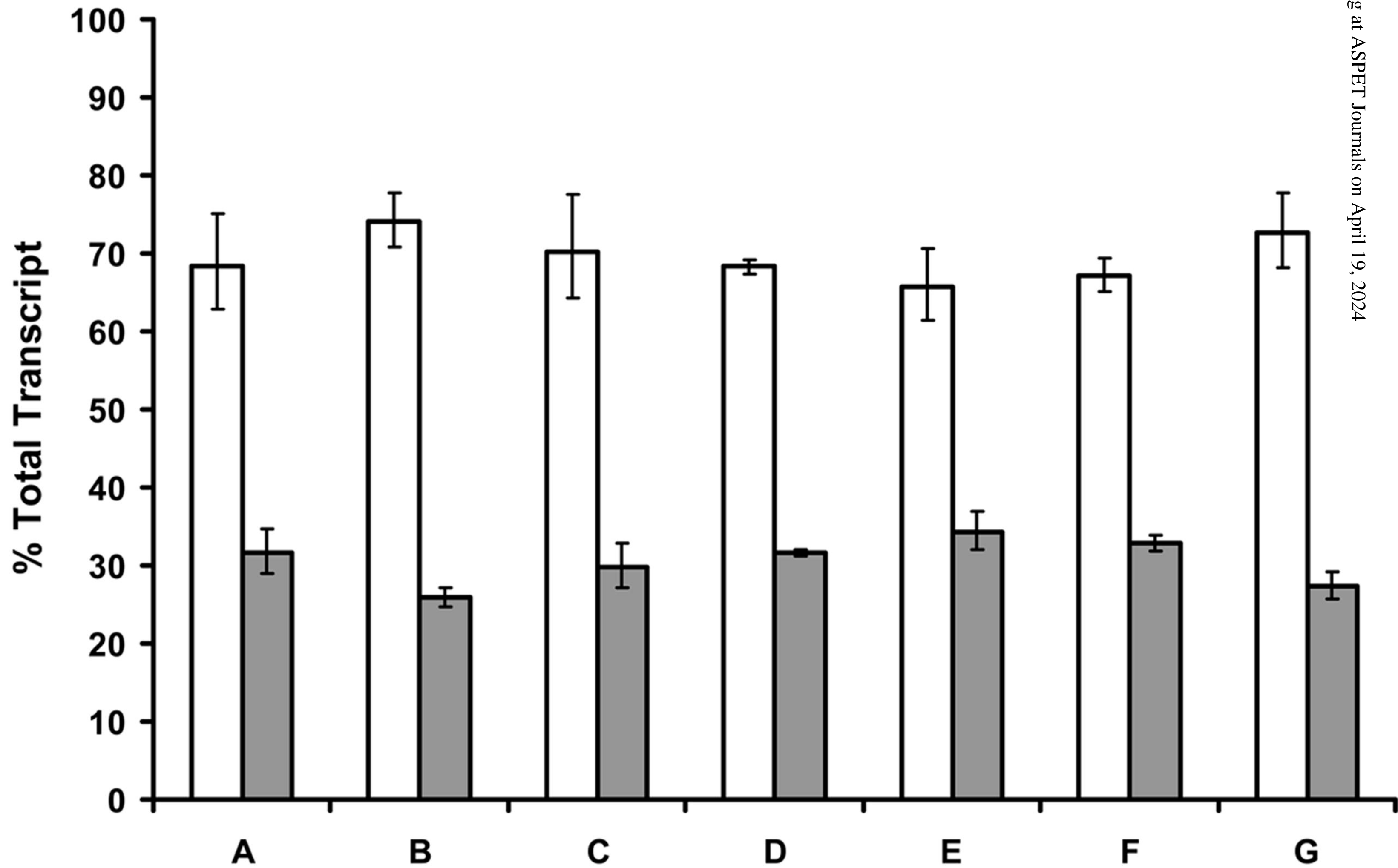

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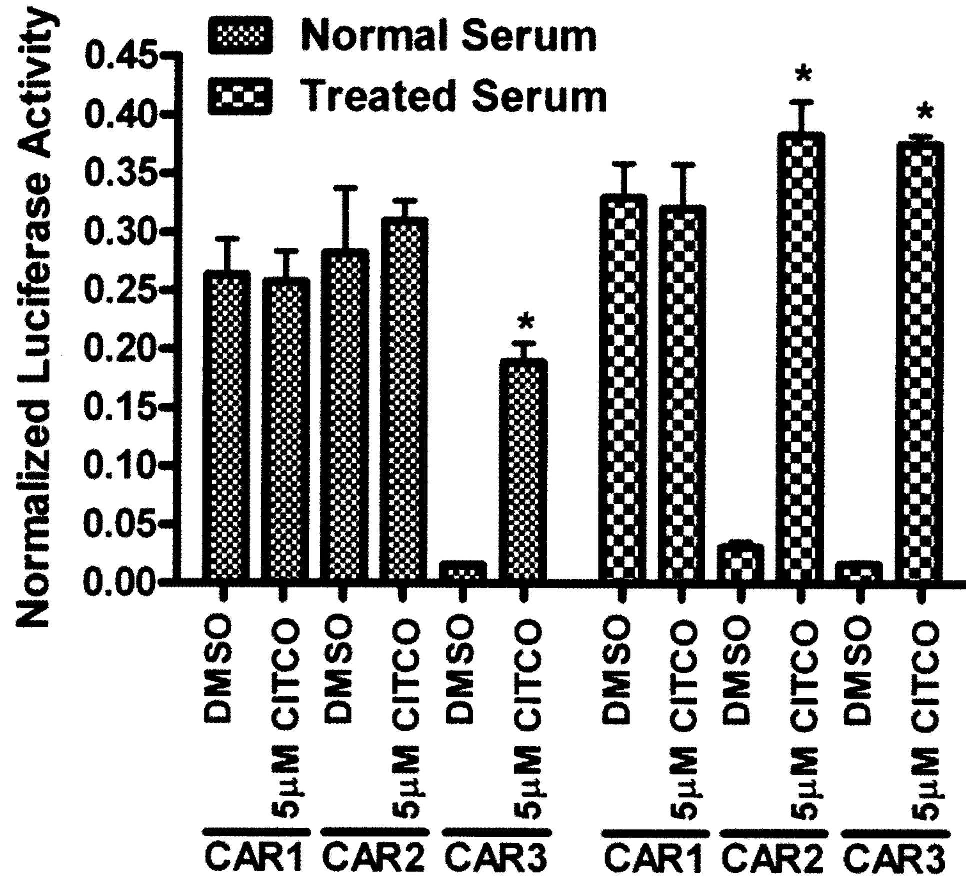
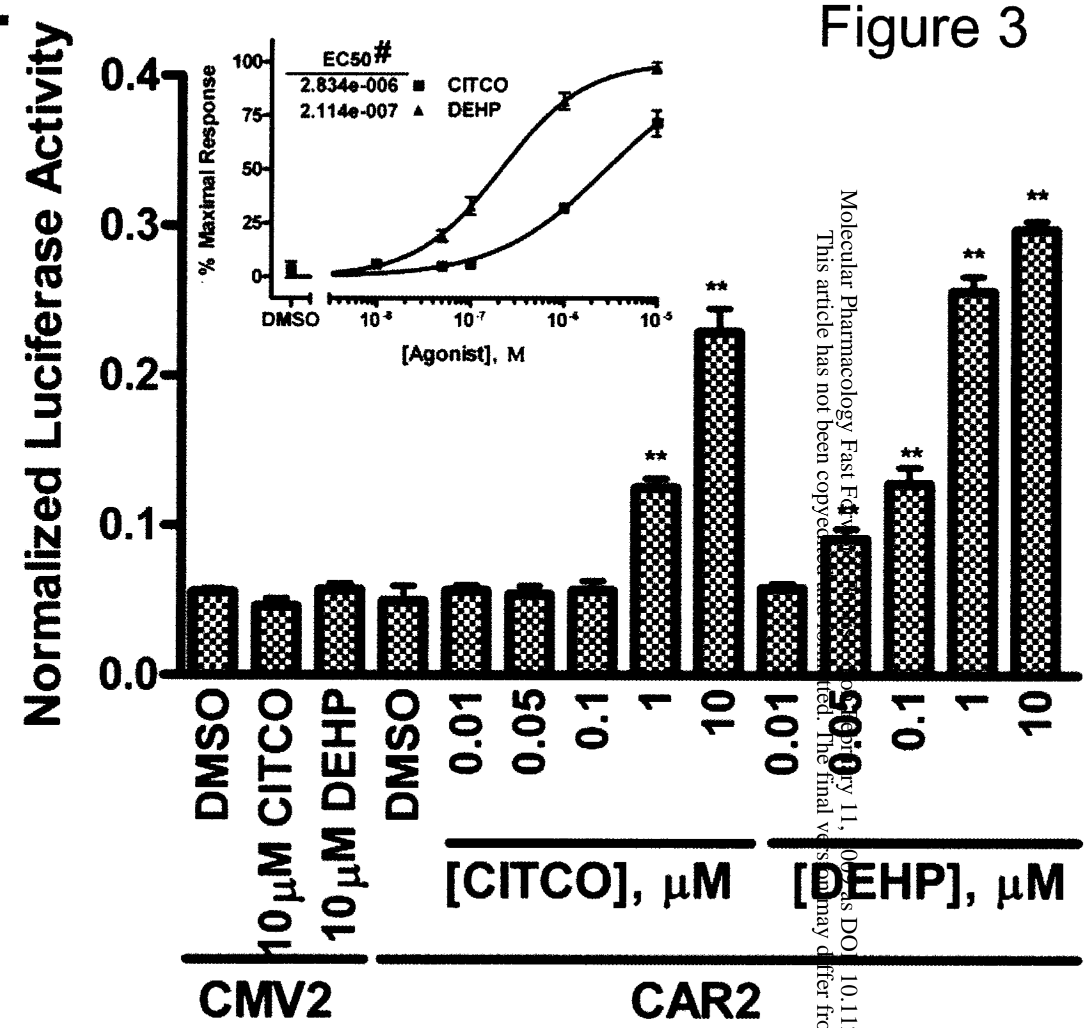
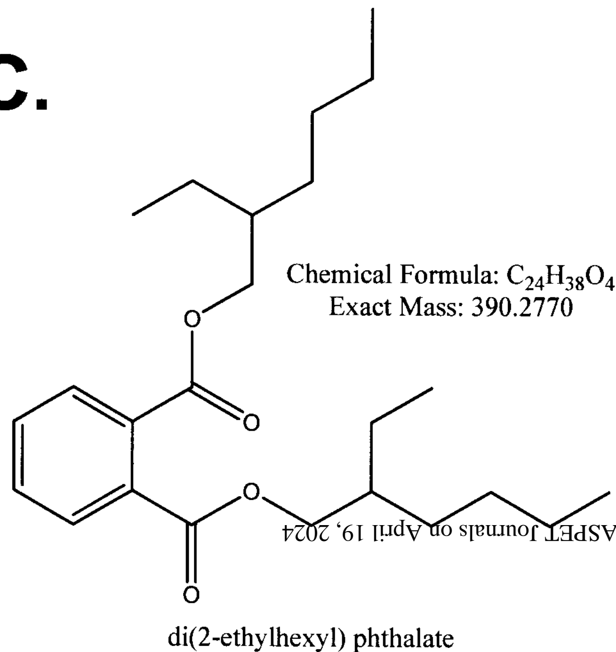
Figure 3







D.

Sample	[DEHP], μM	[+/-], μM
Normal Serum	5.7	0.7
Treated Serum	N/D	N/D

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Figure 4

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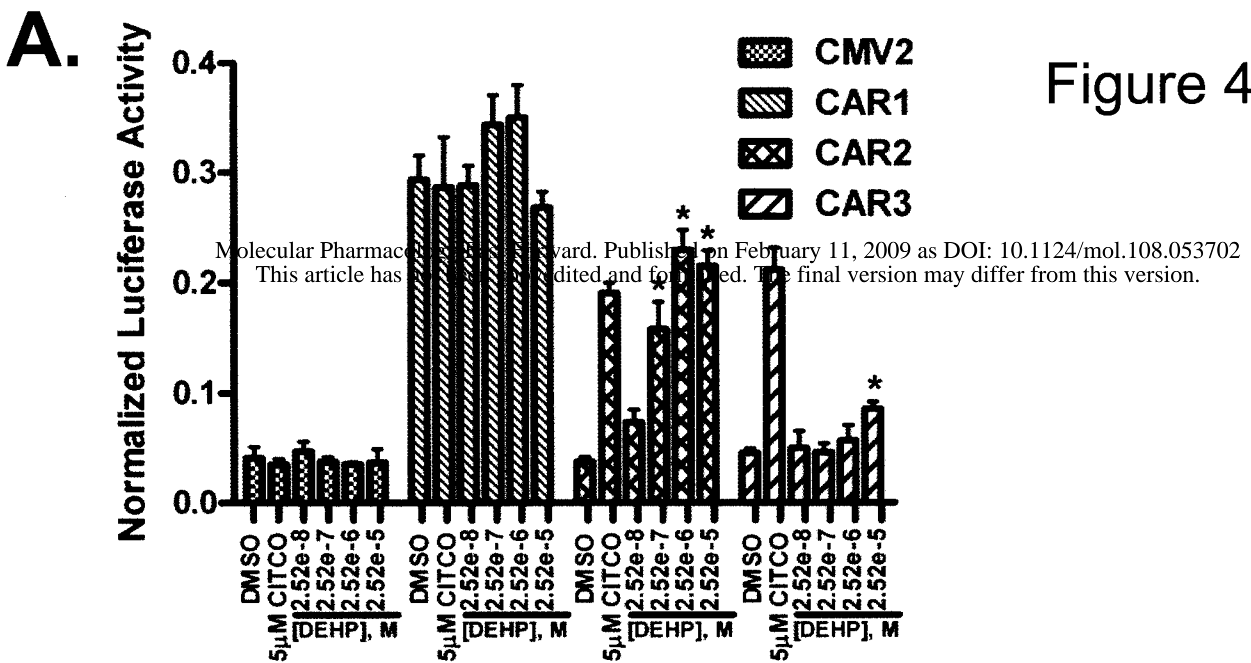

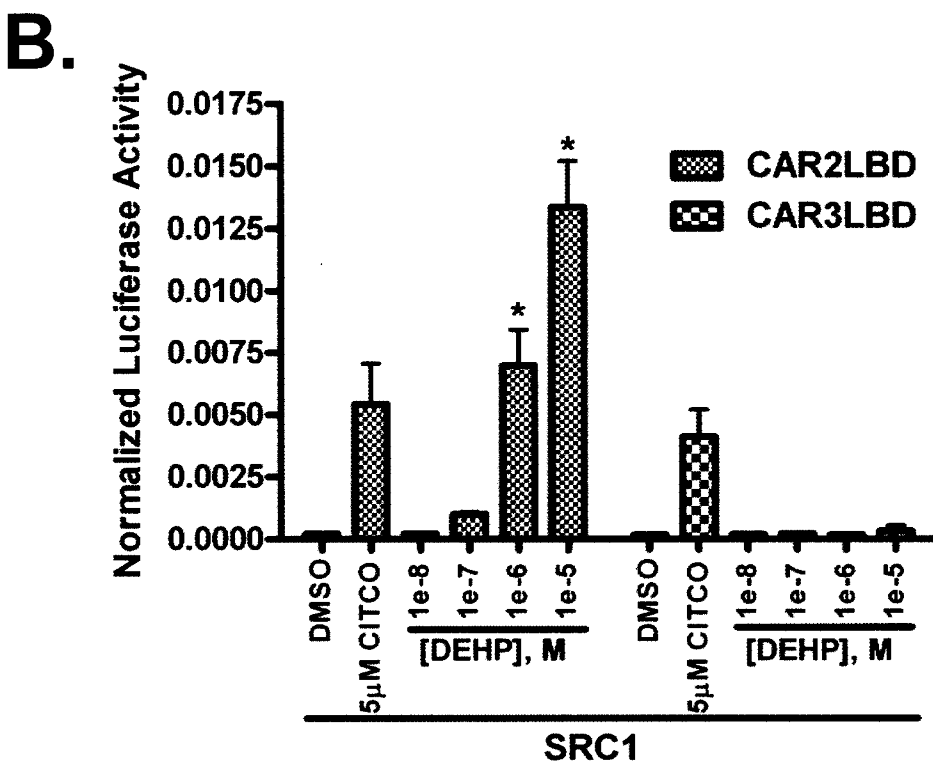

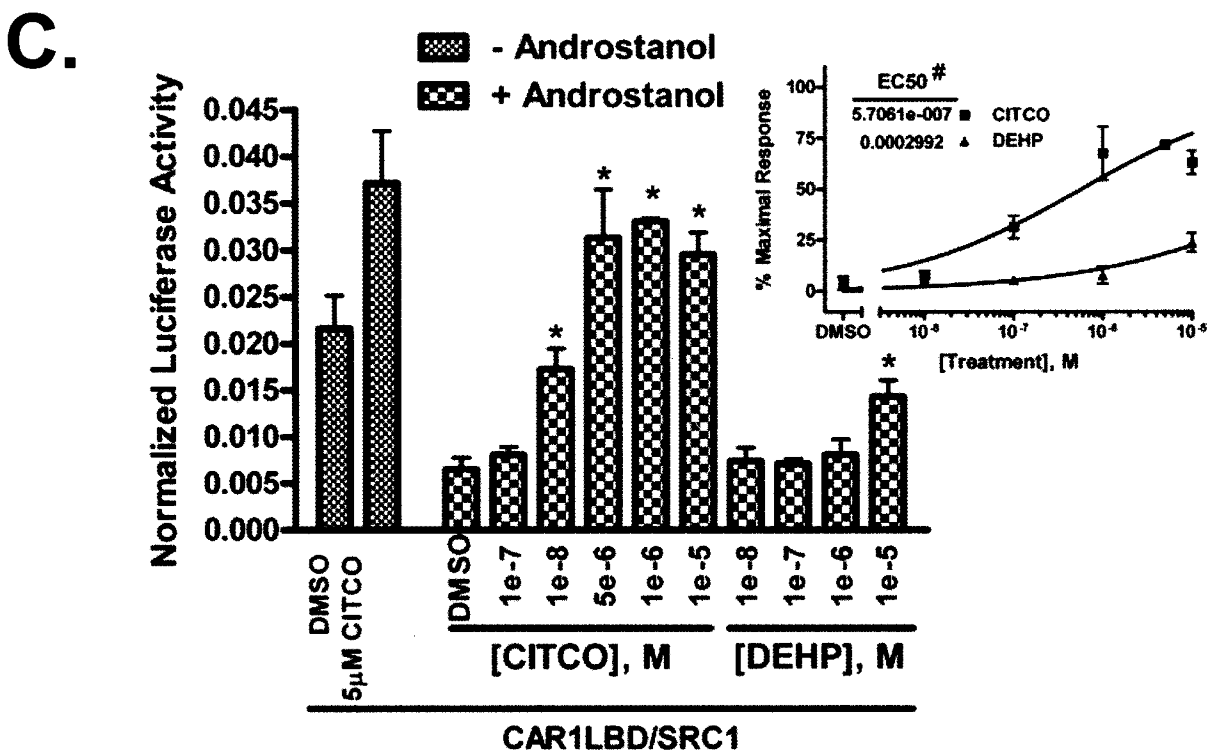

Figure 5