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# Induction of human *CYP2C9* by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor (PXR)

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Nonstandard abbreviations: CYP, Cytochrome P450 protein; hRXR, human retinoid X receptor; hCAR, human constitutive androstane receptor; mCAR, mouse constitutive androstane receptor; hPXR, human pregnane X receptor; hGR, human glucocorticoid receptor; CAR-RE, constitutive androstane receptor-responsive element; GRE, glucocorticoid-responsive element; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene , DMSO, dimethylsulfoxide

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## ABSTRACT

Human CYP2C9 is important in the metabolism of numerous clinically used drugs such as the anticoagulant warfarin, the anticonvulsant phenytoin, antidiabetic drugs such as tolbutamide and glipizide, the hypertensive agent losartan, and numerous nonsteroidal anti-inflammatory drugs. Several studies have reported that certain drugs such as rifampicin and phenobarbital induce CYP2C9, but the molecular basis for this induction remains unknown. In the present study, we demonstrate that the human pregnane X receptor (hPXR) mediates induction of *CYP2C9* by the prototype drugs rifampicin, hyperforin (found in St. John's Wart) and phenobarbital. Deletion and mutagenesis studies with luciferase reporter constructs showed that a functional PXR responsive element located –1839/-1824 bp upstream from the translation start site was the primary binding site mediating the rifampicin induction of *CYP2C9*. This site was previously described as a constitutive androstane receptor (CAR) responsive element (CAR-RE). Mutational analysis of 3kb and 12kb *CYP2C9* promoter fragments indicated that this proximal binding site was essential for rifampicin inducibility, although a cooperative effect could be attributed to a second CAR-RE located at -2899/-2883. In summary, we have demonstrated rifampicin induction of *CYP2C9* promoter constructs that is consistent with the magnitude of induction of CYP2C9 protein and mRNA reported *in vivo* and in primary human hepatocytes, and we have identified the cis-element essential for this response. This is the first report to demonstrate that the nuclear receptor PXR mediates induction of *CYP2C9* with rifampicin, PB and hyperforin.

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CYP2C9, the principal member of four human CYP2C enzymes, is one of the most important drug metabolizing P450s in human liver. CYP2C9 is the rate-limiting enzyme in the metabolic clearance of clinically used drugs such as the hypoglycemic agents tolbutamide and glipizide, the anticonvulsant phenytoin, the *S*-enantiomer of the anticoagulant warfarin, and numerous non-steroidal anti-inflammatory drugs such as flurbiprofen, diclofenac, torsemide and ibuprofen (Goldstein et al., 1994; Miners et al., 1998; Goldstein, 2001; Lee et al., 2002). Approximately 16% of clinically used drugs are metabolized by CYP2C9.

Individual variability occurs in the metabolism of CYP2C9 substrates in humans and a principal factor is the presence of genetic polymorphisms in humans (Sullivan-Klose et al., 1996; Goldstein, 2001). Most notably the *CYP2C9*2* and **3* alleles have significantly lower intrinsic clearances of CYP2C9 substrates both *in vivo* and *in vitro* (Lee et al., 2002). Drug induction is another source of variation in the metabolism of CYP2C9 substrates, which may result in drug toxicity or therapy failure.

Studies in primary human hepatocytes have indicated that CYP2C9 mRNA, protein, and catalytic activity are induced by drugs such as rifampicin, hyperforin (the active constituent in St John's Wort), phenobarbital, and dexamethasone (Gerbal-Chaloin et al., 2001; Rae et al., 2001; Raucy et al., 2002; Madan et al., 2003; Watkins et al., 2003). Some clinical studies have also reported that rifampicin treatment consistently enhances the clearance of drugs eliminated by CYP2C9, such as tolbutamide, phenytoin, losartan, *S*-warfarin, and the antidiabetic drugs glyburide and glipizide (Zilly et al., 1975; Kay et al., 1985; Heimark et al., 1987; Williamson et al., 1998; Niemi et al., 2001), suggesting that rifampicin induces CYP2C9 expression *in vivo*.

Induction of CYP genes generally occurs at the transcriptional level and is mediated by certain nuclear receptors including the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) (Waxman, 1999). Many nuclear receptors are ligand dependent, including human PXR, which binds rifampicin, hyperforin, and lithacholic acid among others (Moore et al., 2000a; Moore et al., 2000b; Moore et al., 2000c). In contrast, the prototypical inducer phenobarbital (PB) and many other compounds known to induce *CYP2B* genes via CAR-dependent mechanisms do not appear to directly bind to CAR. Most drugs which induce gene expression via CAR-dependent mechanisms are thought to signal CAR translocation to the nucleus (Zelko et al., 2001; Kawana et al., 2003; Maglich et al., 2003). Both CAR and PXR then heterodimerize with the retinoid-X-receptor (RXR) in the nucleus, bind to various responsive elements within gene promoters, and associate with coactivators/corepressors to regulate gene transcription (Honkakoski, 2003). Additional transcriptional factors such as HNF4$\alpha$ may also be involved in the upregulation of certain P450s by drugs through CAR and PXR (Tirona et al., 2003).

To date, three studies of the *CYP2C9* promoter have been reported and four regulatory elements have been identified at -155bp, -1675bp, -1839bp and - 2899bp from the translation start site. The first site is a HNF4$\alpha$ binding site, which was suggested to influence the hepatic expression of *CYP2C9* (Ibeanu et al., 1995). A site at –1675bp identified as a glucocorticoid responsive element (GRE) interacts with hGR to mediate dexamethasone induction of (Gerbal-Chaloin et al., 2002). Two additional sites in *CYP2C9* (a distal and proximal CAR-RE) were shown to bind hCAR and mCAR *in vitro* and transactivate *CYP2C9* luciferase reporter constructs (a distal and proximal

CAR-RE) (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002). However, there was little evidence of human-relevant drug induction of *CYP2C9* promoter activity by these elements or nuclear factors in these reports. These sites bind CAR and PXR, yet rifampicin treatment produced only a minimal 60% increase in luciferase activity of a reporter construct containing four copies of the proximal CAR-RE in HepG2 cells cotransfected with hPXR. This increase was much lower than the average 4-fold mRNA induction of *CYP2C9* seen in primary human hepatocytes (Raucy, 2003), and no increase was seen with reporter constructs carrying the natural context of the *CYP2C9* promoter containing this element (Gerbal-Chaloin et al., 2002). Therefore, the nuclear factors and transcription factor binding sites regulating rifampicin induction of *CYP2C9* requires further investigation.

The present study examines the mechanism of CYP2C9 induction using *CYP2C9* promoter-constructs in HepG2 cells cotransfected with PXR and ligands rifampicin, hyperforin, and phenobarbital. We used deletion constructs and site-directed mutagenesis to identify the elements involved in induction. The proximal CAR-RE was identified as the essential PXR responsive element within a 12 kb *CYP2C9* promoter construct to mediate PXR dependent induction of *CYP2C9* by rifampicin, hyperforin, and phenobarbital.

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## MATERIAL AND METHODS

### Chemicals:

DMSO, rifampicin, hyperforin, phenobarbital and other common reagents were purchased from Sigma Aldrich (St. Louis, MO). Phenobarbital was dissolved in cell culture medium and stored at -20°C. Rifampicin and hyperforin were dissolved in DMSO. Cell culture media was purchased from Invitrogen (Carlsbad, CA). Desalted oligonucleotides were purchased from Genosys, Inc. (The Woodlands, Tx). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). All other reagents were of the highest grade available.

### Transient transfection constructs

A 3kb region of the 5'-flanking promoter of *CYP2C9* from a previous study (Ferguson et al., 2002) was excised and inserted into fresh pGL3-Basic vector called *CYP2C9*-3k. This construct was used to produce deletion and mutational constructs. The *CYP2C9*-3k plasmid was double digested with *Nhe I* and either *Stu I* or *Hind III* to progressively delete the inserted promoter fragment *CYP2C9*-3k from the 5' end. The adhesive ends were blunted with Klenow Fragment (New England Biolabs, Beverly, MA) and self-ligated to produce two deletion constructs: *CYP2C9*-1.9kb and *CYP2C9*-1.5kb. A human *CYP2C9* PAC clone (Incyte Genomics, Palo Alto, CA) was digested with *Nhe I* and *Pst I* to obtain an additional 11 kb of the 5'-flanking promoter region of *CYP2C9* (-12096 bp to –988 bp). After gel purification, the recovered DNA fragment was inserted into linearized *CYP2C9*-3k/pGL3-Basic that had been linearized by double digestion with *Nhe* I and *Pst* I to yield wild type *CYP2C9*-12k.

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pSG5-hPXR was kindly provided by Steve Kliewer (GlaxoSmithKline)(Kliewer et al., 1998). (XREM)-*3A4*-362/+53 was obtained from Brian Goodwin (Goodwin et al., 1999).

**Cell culture and transfection**

HepG2 cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and antibiotics at 37°C under 5% $CO_2$. Luciferase constructs and receptor constructs (or empty vectors, 100 ng of each) were combined with 10ng of internal control pRL-TK, then mixed with Effectene transfection reagents (QIAGEN, Valencia, CA), and transfected into HepG2 cells 12-24 hours after seeding into 24-well plates (1-1.5 x $10^5$ cells per well). Twenty-four hours later, medium was replaced, and drugs were added in the appropriate concentrations. Drugs were incubated with the cells for 24-36 hr, followed by dual luciferase assays (Promega, Madison, WI). Firefly luciferase activities were normalized to Renilla Luciferase readings to calculate promoter activity.

**Site-directed mutagenesis**

The pGL3-Basic construct including -3 kb of the *CYP2C9* promoter region was used as the template for site-directed mutagenesis (QuickChange Site-directed mutagenesis, Stratagene, La Jolla, CA) to prepare *CYP2C9*-3k/-2899m, *CYP2C9*-3k/-1839m and *CYP2C9*-3k/dmut, respectively. The forward primers utilized for mutagenesis are as follows (hexamer half-sites are indicated by bold capital letters and mutated nucleotides are underlined): Distal CAR-RE mutation: 5'gtacaacacaaagaa**GCTTGC**ctaca**TAAACT**atg3', Proximal CAR-RE-mutation: 5'c**CAAACT**cttc**TCTGGT**ctcaatctagtcaac3'. DNA sequencing was performed for all constructs to verify the mutations and to assure that no spurious mutations occurred.

To insert the mutated proximal CAR-RE in the 12 kb promoter luciferase construct, *Kpn I* and *Pst I* were used to excise an 11 kb 2C9 promoter fragment (from a *Kpn I* site in the vector to –988 bp in the promoter) from the wt *CYP2C9*-12k construct. This fragment was subcloned into pUC19. A region of the promoter from *Dra III* (-2750 bp) to *Pst I* (-988 bp) containing the proximal CAR-RE was replaced by the corresponding *CYP2C9* fragment from the *CYP2C9*-3k/-1839m construct containing the mutated proximal CAR-RE. This strategy was used to avoid a second *Dra III* site in the pGL3-Basic vector. The resulting construct was double-digested with *Kpn I* and *Pst I*. The 11 kb of the *2C9* promoter fragment possessing the mutated CAR-RE was reinserted into *Kpn I* and *Pst I* linearized *CYP2C9*-3k, resulting in the desired *CYP2C9*-12k/-1839m construct.

**Gel shift assays**

Human RXR and hPXR were synthesized *in vitro* using the TNT Quick-Coupled In Vitro Transcription Translation System (Promega, Madison, WI), following the manufacturer's protocol. Klenow Fragment (New England Biolabs, Beverly, MA) was employed to incorporate $^{32}$P-dCTP at the 5' ends of the double-stranded oligonucleotides. Approximately 70,000 cpm of labeled probe was incubated with or without synthesized nuclear receptors in a 10 µl binding reaction containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% (v/v) glycerol, 50 mM NaCl and 1 µg of non-specific competitor poly (dI-dC) (Sigma, St. Louis, MO). In parallel reactions, specific cold competitors or specific hRXRα antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added to the mixture before the addition of proteins. After 20 min incubation at room temperature, 7.5 µl of the reaction mixture was

loaded onto a 5% non-denaturing polyacrylamide gel for electrophoresis in 0.5X TBE buffer for 2 hours at 150V. The gels were dried and exposed to film. The following are the sequences of the oligonucleotides used as probes, wild type or mutated specific cold competitors (hexamer half-sites are indicated by bold capital letters and mutated nucleotides are underlined):

CAR-RE of *CYP2C9*-prox-wt: 5'-ctagac**CAAACT**cttc**TGACCT**ct-3'

CAR-RE of *CYP2C9*-prox-mut: 5'-ctagc**CAAACT**cttc**T<u>CTGG</u>T**ctg-3'

CAR-RE of *CYP2C9*-distal-wt: 5'- ctagaa**TGAACC**ctaca**TAAACT**at-3'

CAR-RE of *CYP2C9*-distal-mut: 5'-gtacaacacaaagaa**<u>G</u>GG<u>G</u>ACC**ctaca**TAAACT**atg.

**Statistical analysis**

Analysis of variance procedures were used to assess the significance of differences between activity of constructs and effects of cotransfection with hPXR and various drug treatments, and the interaction between these factors. A variance-stabilizing logarithmic transformation was used in these analyses. Pairwise comparisons were made by Fisher's Least Significant Difference (LSD) test.

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## RESULTS

### Rifampicin induces *CYP2C9* promoter transactivation

When a 3kb *CYP2C9* luciferase reporter construct was transfected into HepG2 cells, cotransfection with human PXR (hPXR) increased luciferase activity 2.5-fold ($p < 0.0001$) and treatment of cells with 10 μM rifampicin produced a further 3- fold increase over DMSO vehicle controls ($p< 0.0001$). As expected, rifampicin produced a strong activation of a *CYP3A4* positive control (XREM)-*3A4*-362/+53 reporter construct in HepG2 cells cotransfected with hPXR (~30-fold). Neither PXR nor rifampicin had any effect on activity of the empty pGL3-Basic control construct.

### Functional evaluation of the putative PXR binding sites

Two deletion constructs of the *CYP2C9* promoter were examined to initially localize the cis element(s) that mediate the rifampicin induction (Figure 2A). As shown in Figure 2B, rifampicin increased luciferase activity of both the *CYP2C9*-3k and *CYP2C9*-1.9 k constructs when the cells were co-transfected with hPXR ($p < 0.0001$), although the activity of 3kb construct was greater than that of the 1.9kb construct ($p<0.0001$). In contrast, the *CYP2C9*-1.5k construct did not respond to hPXR and was not induced by rifampicin. Therefore, the rifampicin responsive element appeared to be located between –3kb to –1.5kb of the *CYP2C9* promoter region.

Within this region, two CAR-binding elements have been identified -2899bp and -1839bp from the *CYP2C9* translation start site (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002). To evaluate their contributions to rifampicin induction of *CYP2C9*-3k, these two CAR-REs were then mutated individually or in combination within the *CYP2C9*-3k luciferase construct (Fig. 3 A). The resulting mutant constructs were transfected into

HepG2 cells, followed by treatment with10 μM rifampicin, and assayed for luciferase activity. As shown in Figure 3B, the wild-type *CYP2C9*-3k promoter construct showed the highest response to rifampicin in cells cotransfected with PXR, while the mutation of the distal CAR-RE decreased the response by ~30% ($p < 0.005$). When the proximal CAR-RE was mutated alone or in combination with the distal element, the response to rifampicin in cells cotransfected with PXR was almost abolished. These results indicate that the proximal CAR-RE (-1839/-1824) of the *CYP2C9* promoter plays an essential role in hPXR mediated rifampicin induction, although the distal element may play a cooperative role to produce maximal induction.

To further examine the direct binding of these two CAR-REs to hPXR, *in vitro* synthesized hPXR and hRXR were mixed with labeled oligonucleotides in electrophoretic mobility shift assays. As shown in Figure 4, both elements formed specific complexes with the hPXR/hRXR dimers, which were competed out by 20- fold excess of unlabeled wild type probes but not by the same amount of the mutated cold competitors. When specific antibody against hRXR was included, supershifted complexes formed with both elements. Furthermore, the specific complex formed by the proximal element was stronger than the complex formed by the distal one.

**Drug responses of *CYP2C9***

We then investigated whether hPXR and the responsive elements identified above also mediate the induction of *CYP2C9* by other drugs, such as phenobarbital and hyperforin. As shown in Figure 5A, 0.5 mM phenobarbital produced a 2.5-fold induction of the *CYP2C9*-3k promoter construct when HepG2 cells were cotransfected with hPXR ($p < 0.0001$). This response was abolished when the proximal CAR-RE was mutated,

while mutation of the distal element had a minimal effect on this induction ($p < 0.05$). Hypeforin (0.2 nM) induced the activity of the *CYP2C9*-3k construct approximately 2.5-fold ($p < 0.0001$, Fig. 5B). Mutation of the proximal element essentially abolished induction (no significant increase observed after hyperforin treatment, $p = 0.2862$, Fig. 5B), while mutation of the distal element had a much smaller effect on induction ($p<0.05$).

**The proximal CAR-RE functions as an essential PXR responsive element of *CYP2C9***

To determine whether any additional distal regulatory elements within the *CYP2C9* promoter play a role in rifampicin induction, we compared the ability of rifampicin to activate 3kb and 12kb *CYP2C9* promoter fragments in parallel transfections in HepG2 cells cotransfected with hPXR. Rifampicin induced activity of both the *CYP2C9*-12kb and *CYP2C9*-3kb fragments in cells cotransfected with hPXR ($p < 0.0001$) (Fig. 6). Importantly, induction of the *2C9*-12kb promoter was no greater than that of the *2C9*-3kb promoter. Moreover, mutation of the proximal CAR-RE within the context of the either the *CYP2C9*-3k and *CYP2C9*-12k promoter luciferase reporter constructs completely abolished rifampicin induction in cells cotransfected with hPXR.

## DISCUSSION

Clinical studies have shown that rifampicin increased the clearance of a number of CYP2C9 substrates *in vivo* in man (Zilly et al., 1975; Kay et al., 1985; Heimark et al., 1987; Williamson et al., 1998; Niemi et al., 2001). Moreover, rifampicin, hyperforin, and phenobarbital induce CYP2C9 protein and mRNA 2 to 5-fold in primary human hepatocytes (Gerbal-Chaloin et al., 2001; Rae et al., 2001; Raucy et al., 2002; Madan et al., 2003; Watkins et al., 2003). Previous studies identified two CAR binding sites in the human *CYP2C9* promoter located –2899 bp and –1839 bp from the *CYP2C9* translation start site (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002). Gel-shift assays showed that these sites bound both CAR and PXR, but *CYP2C9* promoter luciferase constructs were activated by hCAR to a much greater extent than hPXR. However, studies investigating the *CYP2C9* inducibility via these two elements were inconclusive. Androstenol repressed mouse CAR activation of the *CYP2C9* promoter, and the mouse CAR ligand TCPOBOP (1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene) derepressed this activation. However, in the absence of a suitable cell model for studying hCAR, there was no conclusive evidence that hCAR could mediate induction of *CYP2C9* by phenobarbital or other drugs.

Rifampicin has been shown to be a potent ligand for hPXR (Moore et al., 2000c). Our present results show that the nuclear receptor PXR mediates the induction of human *CYP2C9* by rifampicin, and the effects of hPXR were mediated via the two elements previously described as putative CAR binding sites (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002). Both deletion analyses and mutation of the putative CAR binding sites at –1839 and –2899 demonstrated that these two sites were completely

responsible for the induction of *CYP2C9* by rifampicin. These data are consistent with the cross-talk reported between CAR and PXR for the PXR-responsive ER6 and DR3 in *CYP3A* genes and the DR4 motifs in *CYP2B* (Xie et al., 2000; Sueyoshi et al., 2001). In the present study, the proximal site of *CYP2C9* was essential for rifampicin induction, while the distal site appeared to play a cooperative role to produce maximum induction. The data from gel shift assays in this study support with our transfection results, showing that both elements bind hPXR, but the proximal the proximal element forms a much stronger complex than the distal element.

Previously, Gerbal-Chaloin et al. (Gerbal-Chaloin et al., 2002) used an artificial luciferase construct with four copies of the proximal CAR-RE inserted upstream of SV40 promoter to test whether this CAR-RE mediates rifampicin induction of *CYP2C9*. This construct was not activated by hPXR in HepG2 cells, but a small 1.6-fold induction by rifampicin was observed in cells cotransfected with PXR. They were unable to observe rifampicin induction of the intact promoter. In our experiments, much stronger hPXR activation and induction by rifampicin were observed within the natural context of *CYP2C9* promoter constructs that harbor a single copy of the proximal CAR-RE. This suggests that the context of the element within the promoter is important to evaluate induction by CAR/PXR activators. An example of such an interaction has been reported for a HNF-4 binding site immediately upstream of two PXR/CAR responsive elements in the distal XREM of *CYP3A4* that affects the response to PXR (Tirona et al., 2003).

Hyperforin, an active component of the herbal extract St John's Wort, is a high affinity ligand for PXR (Moore et al., 2000c) and activates the promoters of *CYP3A4* and *CYP2B6* through activation of PXR and specific PXR cis-elements (Wentworth et al.,

2000; Goodwin et al., 2001; Watkins et al., 2003). The PXR-mediated activation of *CYP2C9* by 0.2 nM hyperforin is consistent with the high affinity of this compound as a ligand for PXR. Induction studies in human primary hepatocytes demonstrated that phenobarbital induces CYP2C9 (Gerbal-Chaloin et al., 2001; Raucy et al., 2002; Madan et al., 2003), yet the molecular mechanism of induction remained elusive. Previous studies of the induction of *CYP2C9* by phenobarbital addressed its known ability to act as a CAR activator (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002). hCAR mediated induction has been difficult to study due to the inherent nuclear localization of hCAR in cell lines and its high constitutive activity. However, phenobarbital has been reported to upregulate *CYP3A4* via PXR (Luo et al., 2002), and one study suggests it is a weak agonist of PXR (Moore et al., 2000c). Alternatively, one cannot exclude the possibility that phenobarbital acts by recruiting co-activators to PXR. The present study shows that phenobarbital induction of *CYP2C9* is mediated by hPXR. Although we cannot rule out a role for hCAR, the magnitude of the PXR mediated promoter activation by phenobarbital is consistent with magnitude of induction of CYP2C9 protein in human hepatocytes (Gerbal-Chaloin et al., 2001; Rae et al., 2001; Raucy et al., 2002; Madan et al., 2003; Watkins et al., 2003).

Recently, distal drug responsive elements have been identified in the promoters of certain P450 genes, such as the XREM localized at –7.8kb of the *CYP3A4* promoter and XREM at –8.5kb of the *CYP2B6* promoter (Goodwin et al., 1999; Wang et al., 2003). These distal elements bind PXR and synergize with the proximal element to achieve full rifampicin induction. To determine whether possible distal regulatory elements within *CYP2C9* promoter play a role in rifampicin induction, we compared

induction of a 12kb *CYP2C9* luciferase promoter construct with that of a *CYP2C9*-3kb promoter construct. The two promoter constructs were induced equally by rifampicin. Moreover, mutation of the proximal CAR-RE abolished the induction with either the 3kb and 12 kb *CYP2C9* luciferase reporter constructs by rifampicin, suggesting that it is unlikely that additional distal PXR elements play a major role in induction of *CYP2C9*.

In conclusion, inducers of *CYP2C9* such as rifampicin, hyperforin, and phenobarbital act via PXR. Rifampicin induction of *CYP2C9* appears to be mediated primarily via an essential proximal PXR responsive element at –1839 from the translation start site of *CYP2C9*. A second distal CAR/PXR responsive element at –2899 may play a smaller cooperative role. Comparison of –12kb and –3kb promoter constructs suggests that it is unlikely that more distal sites play major roles in the induction of *CYP2C9*.

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**Figure Legends**

**Fig. 1.** Rifampicin induces transactivation of *CYP2C9* promoter through hPXR in HepG2 cells. 100 ng of luciferase reporter constructs were combined with 2 ng of internal control pRL-TK and transiently transfected into HepG2 cells with or without cotransfected pSG5-hPXR. Twenty-four hours after transfection, cell media was refreshed, and cells were treated for 36 hours with 0.1% DMSO or 10 μM rifampicin (RIF) and assayed for luciferase activity. Values represent the means ± the standard error (SE) of three independent transfections. [a] hPXR cotransfection significantly increased luciferase activity compared to the empty pSG5 control, $p < 0.0001$. [b] Rifampicin treatment significantly increased luciferase activity over hPXR cotransfected controls, $p < 0.0001$.

**Fig. 2.** Deletion of *CYP2C9* promoter to localize rifampicin responsive element(s). *CYP2C9* promoter constructs (A) were transfected into HepG2 cells along with pSG5-hPXR expression plasmids or empty pSG5 vector. Cells were treated for 36 hours with drugs (0.1% DMSO vehicle or 10 μM rifampicin) and assayed for luciferase activity (B). Drug responses were normalized to pSG5 empty vector without drug treatment (ND). Values represent the mean ± SE of three independent transfections. [a] In cells cotransfected with hPXR, rifampicin treatment significantly increased luciferase activity of the 3 kb and 1.9 kb constructs over DMSO controls ($p<.0.0001$). [b] Rifampicin treatment increased luciferase activity to a greater extent in cells transfected with the *2C9*-3k construct than cells transfected with the *2C9*-1.9k construct ($p < 0.0001$).

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**Fig. 3.** Identification of the hPXR responsive element in the *CYP2C9*-3k mediating rifampicin induction. (A) Wild type *CYP2C9*-3k and mutants investigated. Bold characters indicate consensus hexamer half-sites of two CAR-REs with mutated bases underlined. X indicates the mutated CAR-RE within the *CYP2C9-3k* promoter reporters. (B) Transfections were performed in HepG2 cells with the combination of internal control pRL-TK and wild type *CYP2C9*-3k or mutants, respectively. Luciferase activity was analyzed 36 hours after treatment with10 μM rifampicin. ND: No drug treatment. Values represent the means ± SE of three independent experiments. [a] In cells cotransfected with hPXR, rifampicin treatment significantly increased luciferase activity of the *2C9*-3k and *2C9*-3k/-2899m constructs, $p < 0.0001$. [b] The response of the *2C9*-3k/-2899m construct to rifampicin was 30% lower than that of the *2C9*-3k construct ($p < 0.005$). Mutation of the proximal element alone (the *2C9*-3k/-1839m construct) alone or in combination with the distal element (*2C*9-3k/dmut) eliminated the response to rifampicin.

**Fig. 4.** Binding of hPXR to the distal and proximal CAR-REs of *CYP2C9***.** Identical amounts of radiolabeled double-stranded oligonucleotides containing either the distal or proximal CAR-REs of *CYP2C9* were individually mixed with *in vitro* synthesized hRXR or hPXR alone, or with combinations of hPXR/hRXR at room temperature for 20 minutes, followed by the 5% PAGE electrophoresis separation. Twenty-fold molar excess of unlabeled cold competitors (CC) including wild type (w) and mutant (m) probes were added in the parallel binding reactions to compete out specific complexes indicated by small arrows. Two micrograms of polyclonal antibodies (Ab) to hRXRα

were used in parallel experiments to produce supershift bands indicated by the thick arrows.

**Fig. 5.** Human PXR mediates induction of *CYP2C9* by phenobarbital (PB) and hyperforin. HepG2 cells were transfected with wildtype or mutant *CYP2C9*-3k, or (XREM)-*3A4*-362/+53 positive control plasmid respectively, along with hPXR vector versus empty vector pSG5. Twenty-four hours after transfection, 0.5mM of PB (A) or 0.2 nM of hyperforin (B) were added. After 36 hours of incubation with drugs, cells were lysed and assayed for luciferase activity. Data were normalized to values obtained with empty vector and vehicle alone. Values represent means ± SE of three independent analyses. In cells cotransfected with hPXR, drug treatment significantly increased luciferase activity over vehicle controls, [a], $p < .0.0001$; [b], $p < 0.05$. The *CYP2C9*-3k/-1839m construct did not respond to phenobarbital or hyperforin in cells cotransfected with hPXR.

**Fig. 6.** The proximal CAR-RE is the essential hPXR-mediated rifampicin responsive element within the 12kb of *CYP2C9* promoter. HepG2 cells were cotransfected with *CYP2C9*-3k or *CYP2C9*-12k luciferase reporter constructs possessing the wild type or mutated proximal CAR-RE along with empty pSG5 expression plasmid or hPXR. Twenty-four hours following transfection, cells were treated with 10 μM rifampicin, and cells were lysed at 36 hours for luciferase activity assays. Data were normalized to the vehicle treatment. Values represent means ± the SE of three independent analyses. [b]Rifampicin treatment significantly increased luciferase activity over hPXR-cotransfected controls, $p < 0.0001$.

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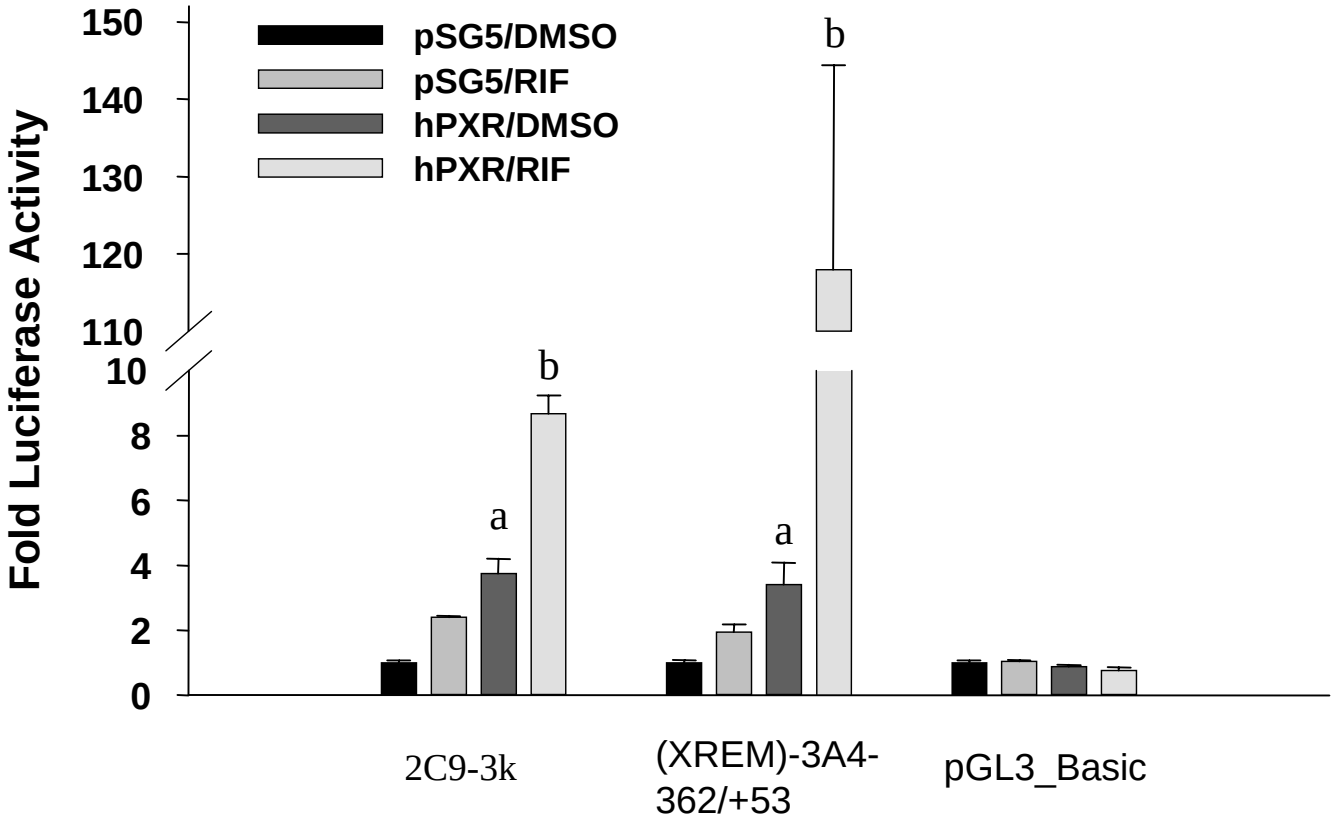


Fig. 1

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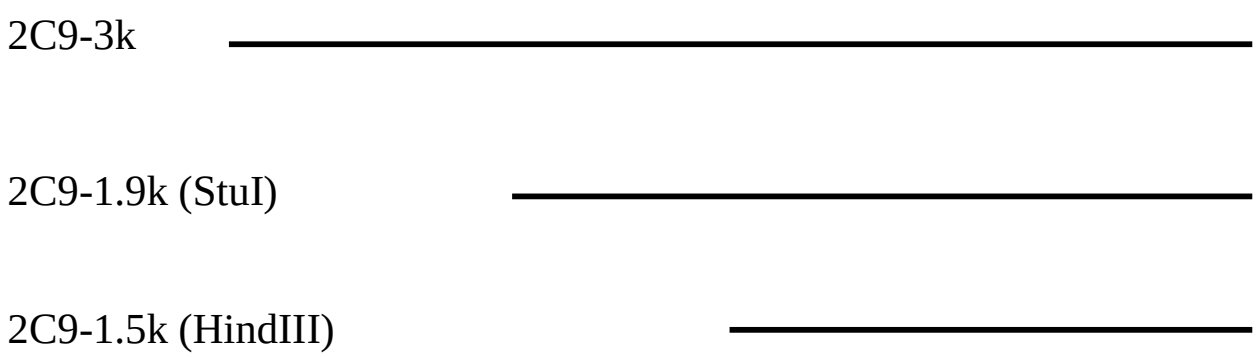
A

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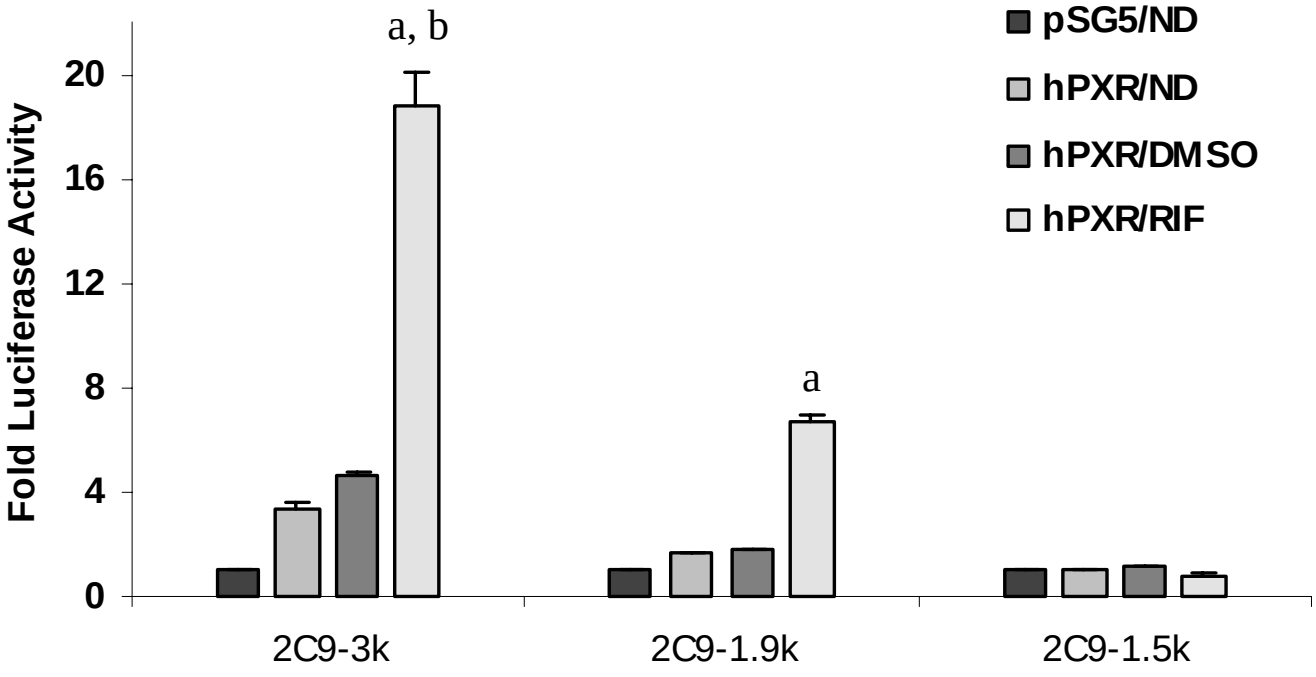


**Fig. 2.**

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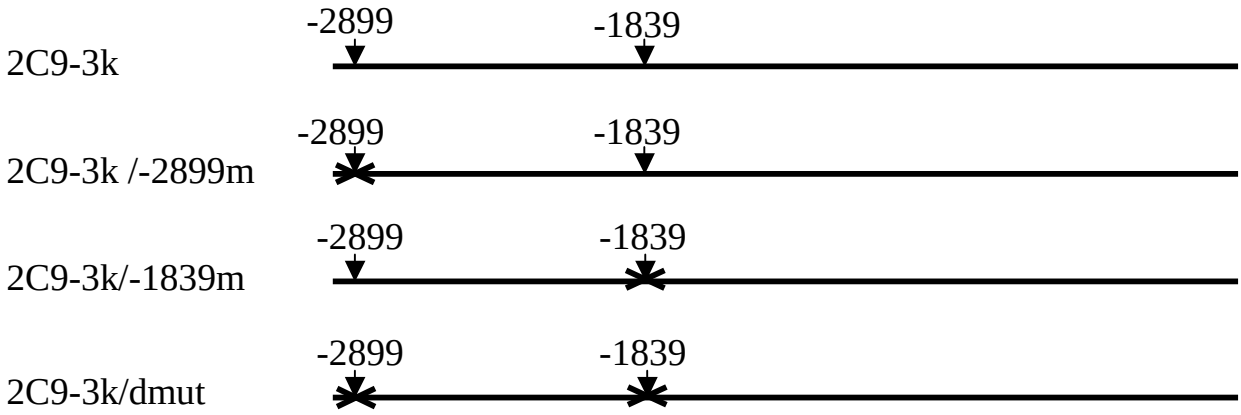
A

**2C9 DR4-proximal:** -1839 ac**CAAACT**cttc**TGACCT**ct

-1839 ac**CAAACT**cttc**T<u>CTGG</u>T**ct

**2C9 DR5-Distal:** -2899 aa**TGAACC**ctaca**TAAACT**at

-2899 aa**<u>GCTTG</u>C**ctaca**TAAACT**at

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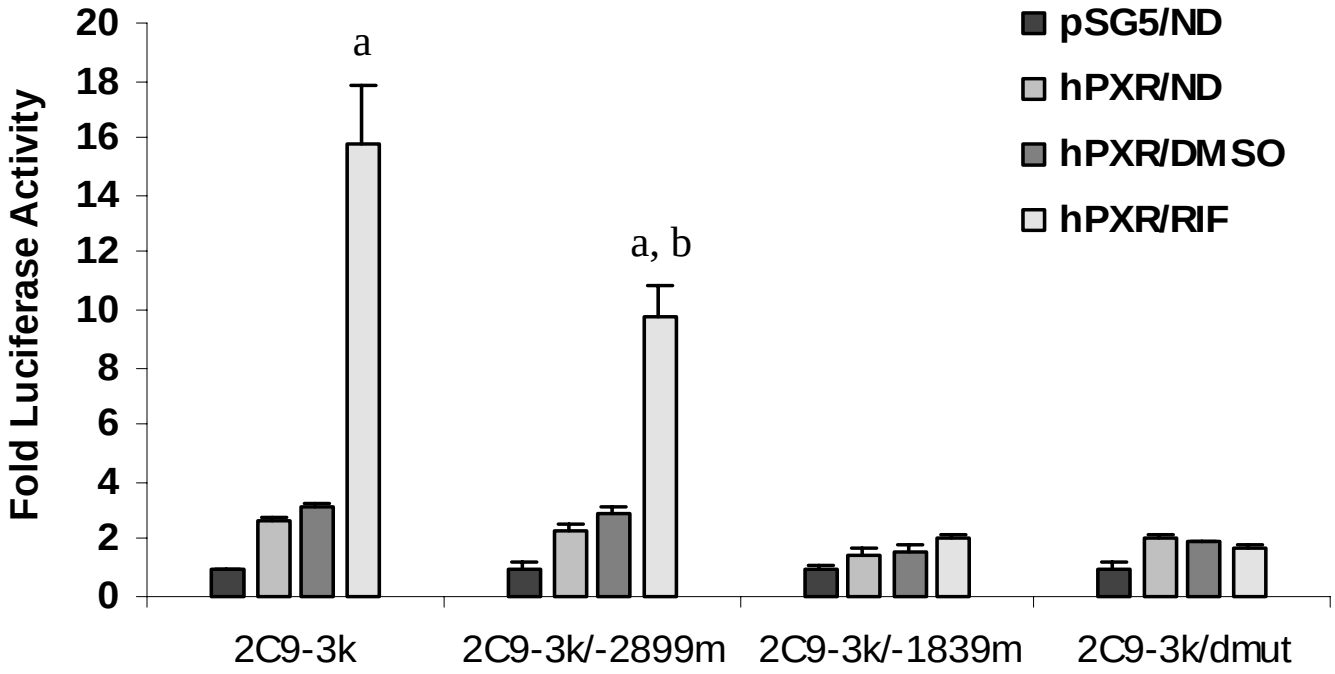


**Fig. 3.**

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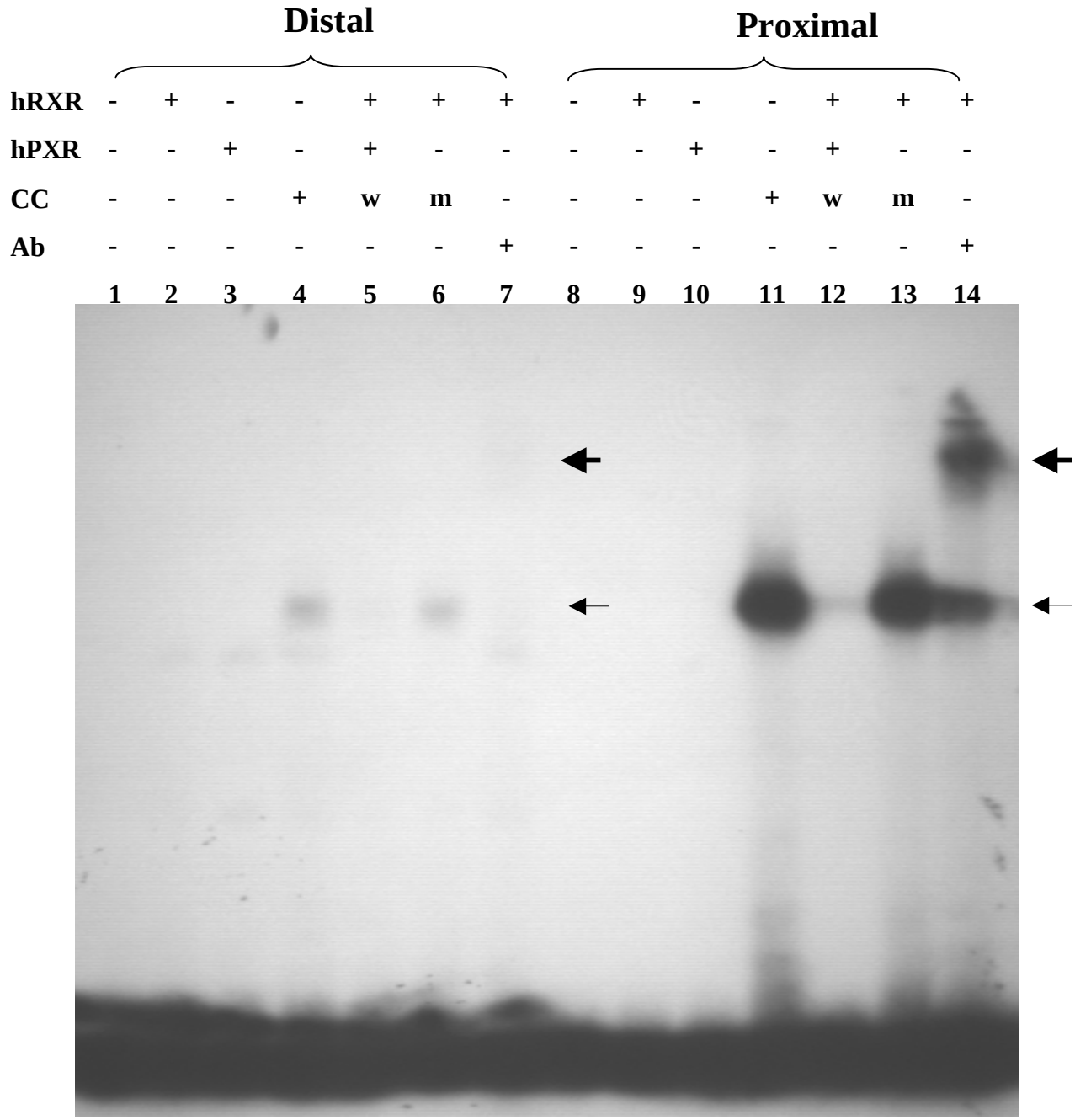


**Fig. 4.**

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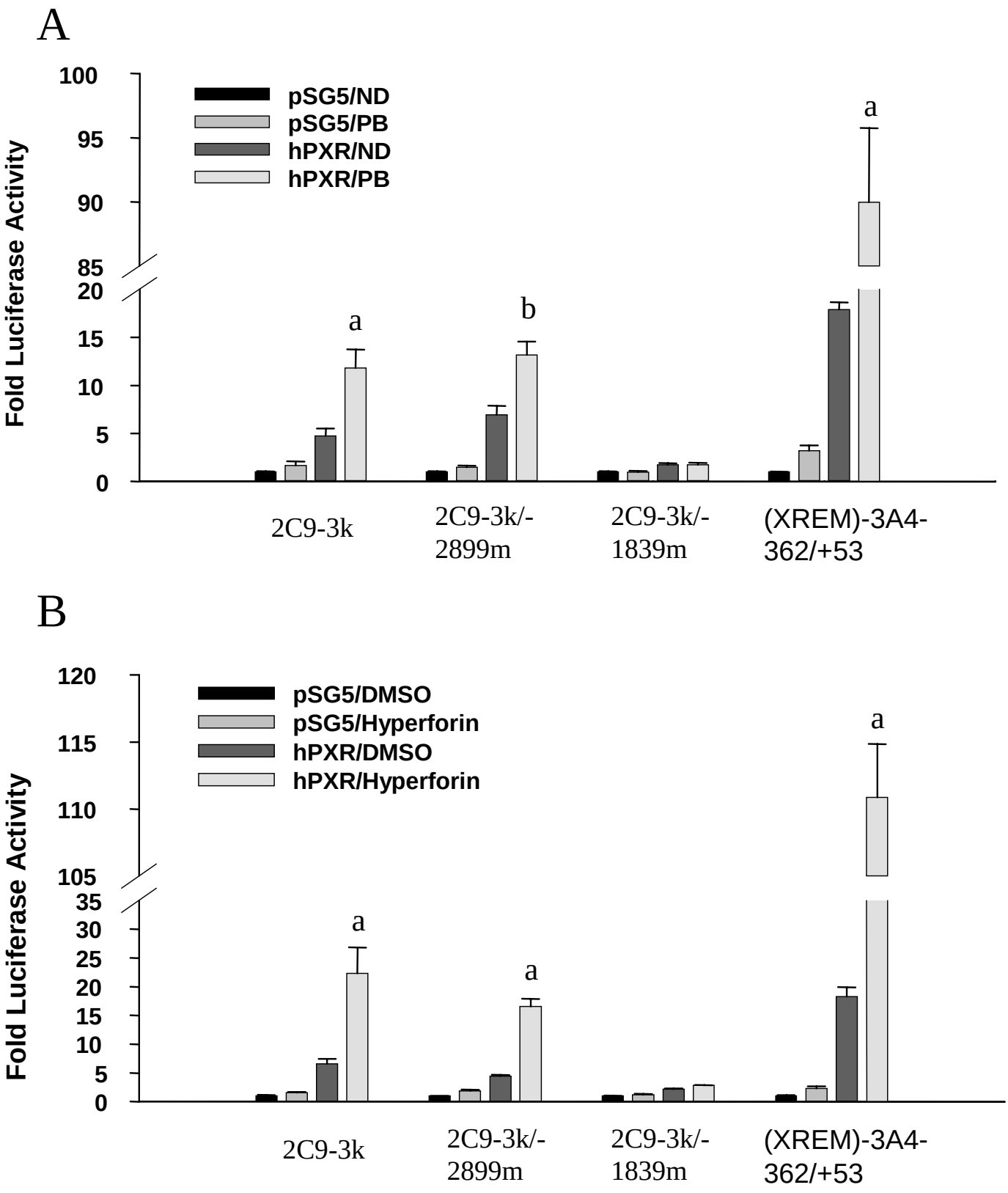


**Fig. 5.**

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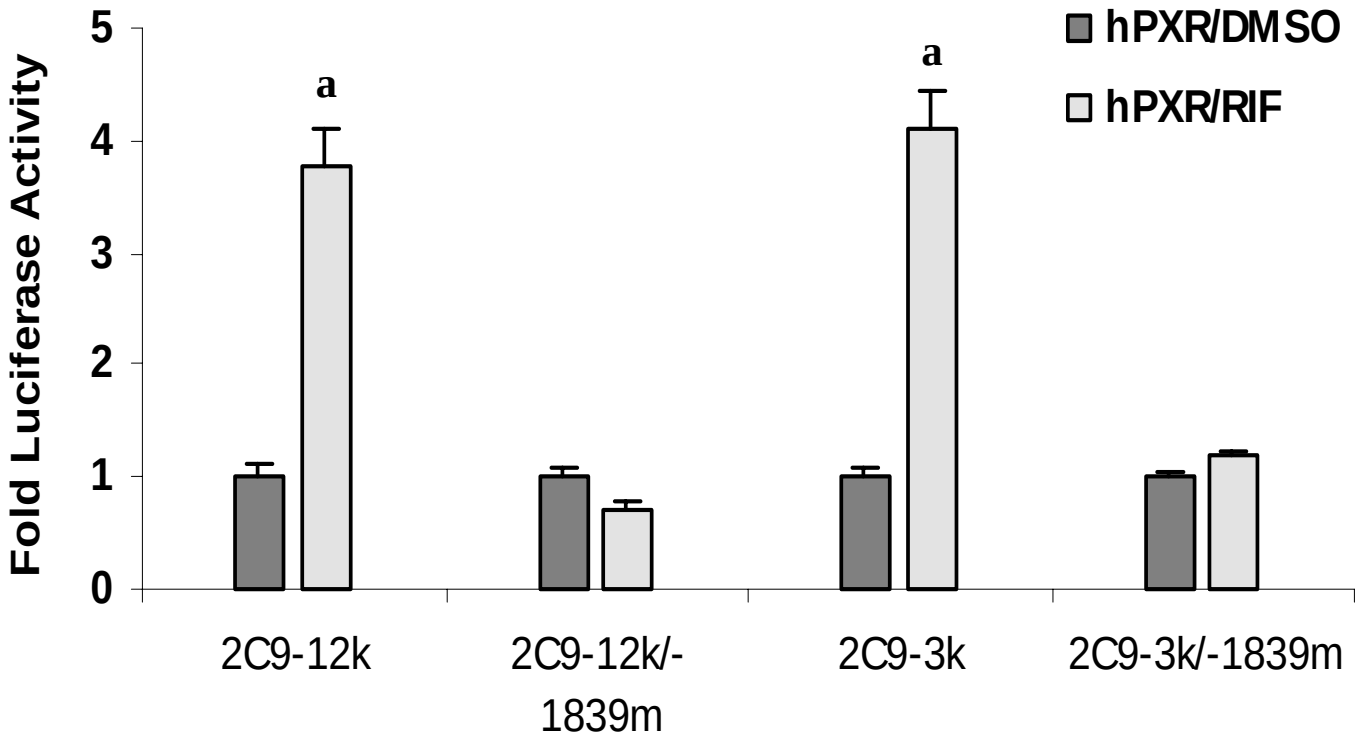


**Fig. 6.**