APPLICATION NOTE

Tracking the Induction of Five Cytochrome P450 (CYP450) Enzymes using a Platform of Parallel Reporter Assays

Bill Ho, Ph.D., Jeff Johnson, Ph.D., and Maria I. Morano, Ph.D.
Cayman Chemical Company

Key Features

- Induction of five CYP450 enzymes was monitored using Cayman's reverse transfection reporter assay (RTA) technology.
- RTA was applied to express different nuclear receptors individually, or in combination, for a rapid investigation into the molecular mechanisms of CYP450 induction.
- An additive and potentially synergistic effect on CYP3A4 induction was observed after co-activation of PXR and CAR1.

Introduction

Xenobiotics may interact with specific nuclear receptors to modulate the expression levels of the CYP450 drug metabolizing enzymes. The interplay of multiple CYPs may lead to adverse effects on the enzyme's ability to properly metabolize or clear drugs/compounds.[1,2] The constitutive androstane receptor (CAR) and pregnane x receptor (PXR) are two key nuclear receptors that can induce several CYPs, including CYP2B6, CYP3A4, and CYP2C9.[3,4] CAR1 is the main CAR isoform, of more than 20 identified variants, found in hepatocytes.[5] CAR1 shows strong constitutive activity when heterologously expressed in cell lines while two less abundant splice variants, CAR2 and CAR3, display no constitutive activity and can trans-activate CYP2B6 and CYP3A4 upon ligand binding.[6]

To investigate the molecular mechanism of CAR1-mediated CYP induction in hepatocytes, we assembled secreted alkaline phosphatase (SEAP) reporter gene constructs using the native promoter sequences for CYPs. These reporters were co-transfected by reverse transfection into HepG2 cells in various combinations of CARs, PXR, and HNF4α. Reverse transfection complexes with optimized stoichiometries of DNAs were coated on microplates. This approach allowed the direct comparison of reporter activity with different combinations of nuclear receptors without the cell lineage differences associated with stable cell lines. The reporter constructs were functionally verified by expression of PXR or CAR3 in HepG2 cells that were stimulated with their corresponding ligands. As expected, in the presence of CAR1, both CYP2B6 and CYP3A4 reporters exhibited strong constitutive activity that masked the induction effect of the CAR ligand CITCO (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime). The co-expression of PXR and CAR1 drastically suppressed the basal constitutive activities of CAR1 on both reporters, resulting in a greater than 5-fold increase in CITCO induced activation of CAR1 on the CYP2B6 reporter. Although CITCO binding to CAR1 or CAR3 triggered approximately a 2-fold induction of the CYP3A4 reporter, the co-activation of CAR1 and PXR led to a more robust (>11-fold) induction of CYP3A4 than by activation of either nuclear receptor alone, suggesting a synergistic effect.

This study demonstrated the interaction of nuclear receptors in the regulation of gene expression by using the original genomic promoter sequences with multiple native regulatory elements in reporter gene assays.

Methods

Reverse Transfection, CYP450 Induction, and SEAP Reporter Assay

CYP450 SEAP Reporter Constructs

Genomic DNA fragments containing all known critical regulatory elements for CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 gene regulation were amplified by PCR and assembled into SEAP reporter constructs (**Figure 1**). Each reporter construct contained 2-5 kb of genomic fragments that have been mapped to be necessary for induction. The use of the SEAP reporter gene allows sample analysis at multiple time points.

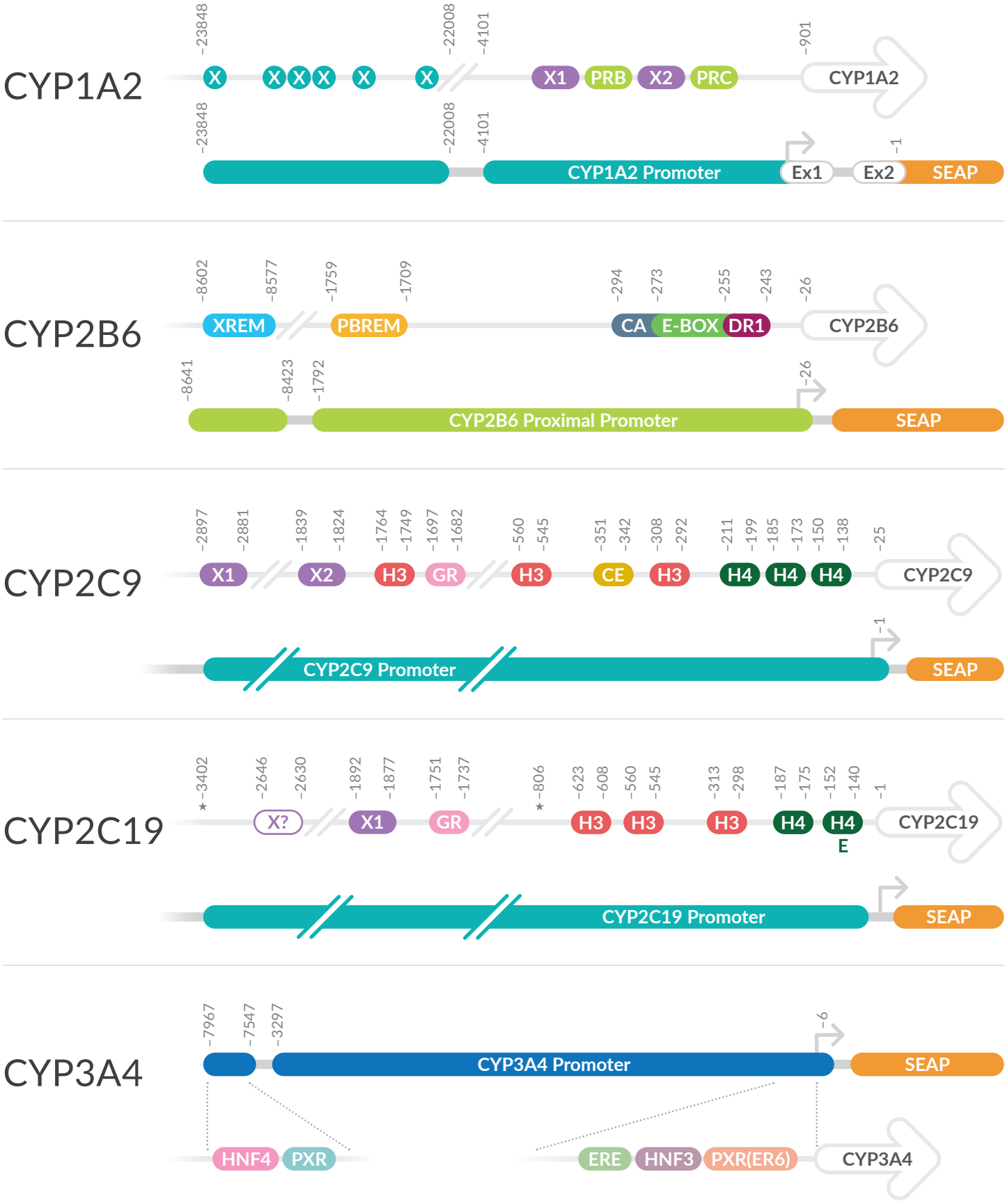

Figure 1. CYP450 SEAP reporter constructs. Xenobiotic response elements are labeled as XREM, X, X1, X2, and PXR. HNF3/H3 and HNF4/H4 are denoted for corresponding hepatic nuclear factor binding sites. Glucocorticoid receptor binding sites are marked with GR in CYP2C9 and CYP2C19. Phenobarbital response element is highlighted as PBREM in CYP2B6.

Reverse transfection plates were coated with transfection complexes containing a SEAP reporter gene supplemented with the necessary transcription factor(s) and nuclear receptor(s). HepG2 cells were added to the plates the day before applying testing compounds. For a more detailed explanation of the reserve transfection assay methodology please see the technical brief titled: User Friendly, No-Maintenance Reporter Assays - The Technology and Utility of Reverse Transfection.

Results

CYP450 Induction Assays

Induction of reporters for five CYP450 genes, CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4, were examined using reverse transfection on HepG2 cells by activating individual nuclear receptors with prototypical small molecule inducers (**Figure 2**).

Induction of the CYP1A2 reporter was monitored up to 96 hours after stimulation of the endogenous aryl hydrocarbon receptor (AHR) by 3-methylcholanthrene (3-MC). A 14-fold increase in reporter activity was already observed at 24 hours with an EC_{50} of 135 nM and a Z' value of >0.7 while the up-regulation persisted throughout the 96 hours of stimulation.

The reporter for CYP2B6 was tested in a CAR3-mediated induction assay. Although a plateau response by CITCO was not achieved due to limited solubility, a strong induction was observed after 24 hours incubation and it reached over 13-fold at 72 hours. With 10 µM CITCO, the Z' value at 72 hours was >0.8.

A highly robust induction of CYP2C9 reporter was observed after the activation of glucocorticoid receptor (GR) with dexamethasone for 24 hours with an EC_{50} of 8.1 nM, and a Z' value of >0.7. The fold of stimulation decreased with prolonged incubation. The CYP2C19 reporter was also examined using a similar approach where hydrocortisone treatment resulted in a smaller but noticeable increase in reporter activity with an EC_{50} of 80 nM and a Z' value well above 0.7 at 72 hours.

The CYP3A4 reporter was tested with PXR supplementation and rifampicin stimulation. At 30 µM, a near-saturated response was obtained. Higher doses resulted in severe cytotoxicity and quenching of the luminescent signal (data not shown). The calculated Z' values were >0.5 at 72 hours and near 0.7 at 96 hours at both 10 µM and 30 µM rifampicin. The inducibility of all five CYP450 reporter constructs was confirmed.

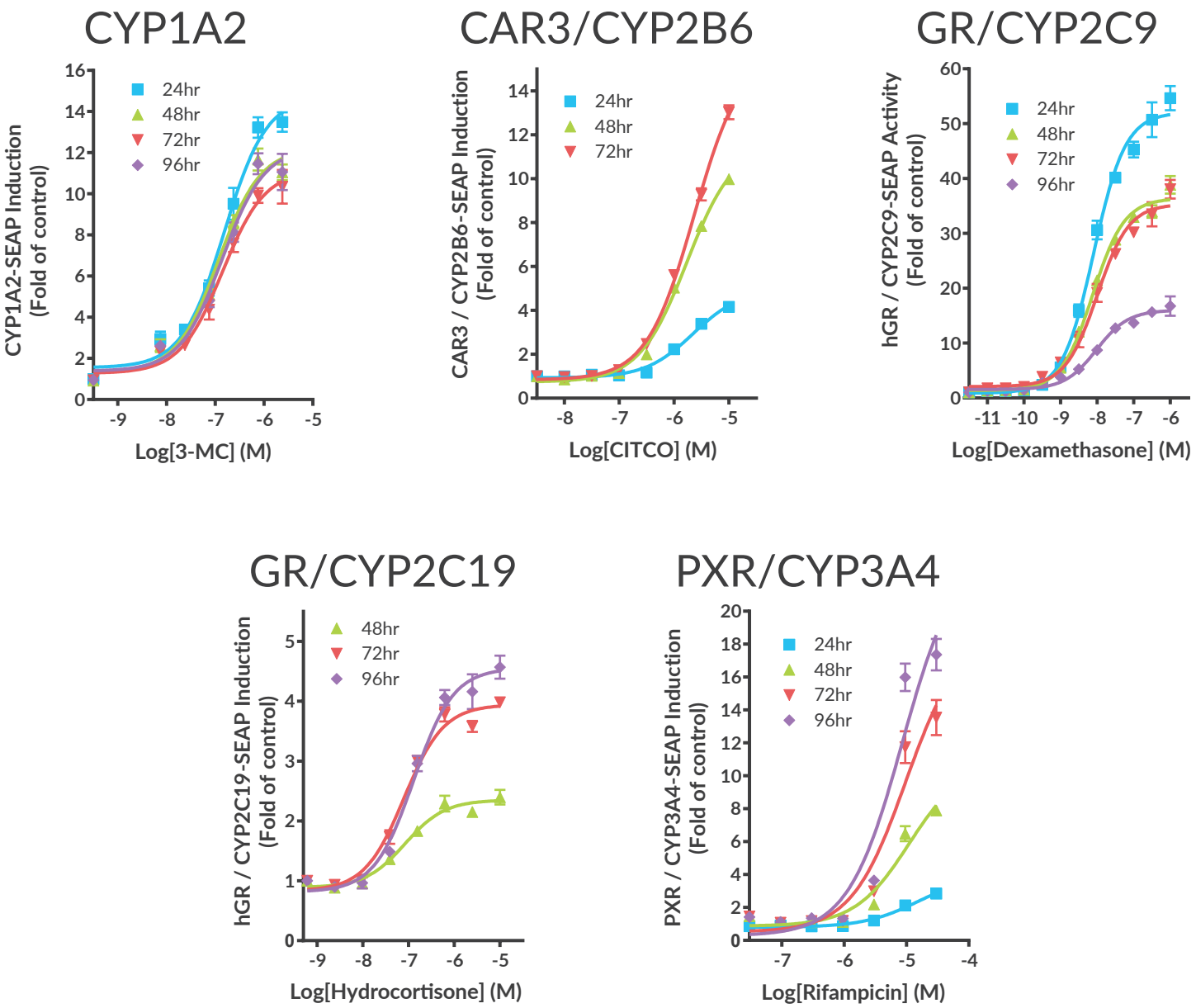

Figure 2. Induction assays for CYP1A2, CAR3/CYP2B6, GR/CYP2C9, hGR/CYP2C19, and PXR/CYP3A4 reporter genes.

CAR1 Effect on CYP450 Reporter Gene Expression

CAR has been reported to be the major modulator for CYP2B6 and, to a lesser extent, for CYP3A4. It has also been implicated to mediate small inductions in CYP2C9, CYP2C19, and CYP1A1/2. The effects of CAR1 expression on the activations of these reporters were evaluated after 72 hour incubation with 10 µM CITCO or control medium. The constitutive activity of CAR1 was observed when it was co-transfected with each of the five CYP450 reporter constructs resulting in significant elevation of reporter activities (**Figure 3**). The smallest upregulation by CAR1 was on the CYP1A2-SEAP where the basal reporter activity was increased by only 1.3-fold. It was also noted that an endogenous mechanism mediated a stimulatory effect of CITCO in the absence of CAR supplementation leading to a 1.5-fold higher reporter activity. Such CITCO effect was detected on top of the constitutive activity by CAR1. The strongest effect of CAR1 was observed with the CYP2B6-SEAP reporter where the basal reporter activity was augmented by more than 12-fold. The constitutive activity of CAR1 on the CYP3A4-SEAP reporter was not as

prominent as on CYP2B6-SEAP, however, both CYP2B6-SEAP and CYP3A4-SEAP reporters exhibited a 2-fold CAR1-dependent CITCO effects. In addition, CAR1 also exhibited a 6-fold increase in basal reporter gene activities of CYP2C9-SEAP and CYP2C19-SEAP but with no further induction by the addition of CITCO. The CITCO effect on CYP2C19 reporter in the absence of CAR1 was not examined here but it was shown to have no effect in a separate experiment (data not shown).

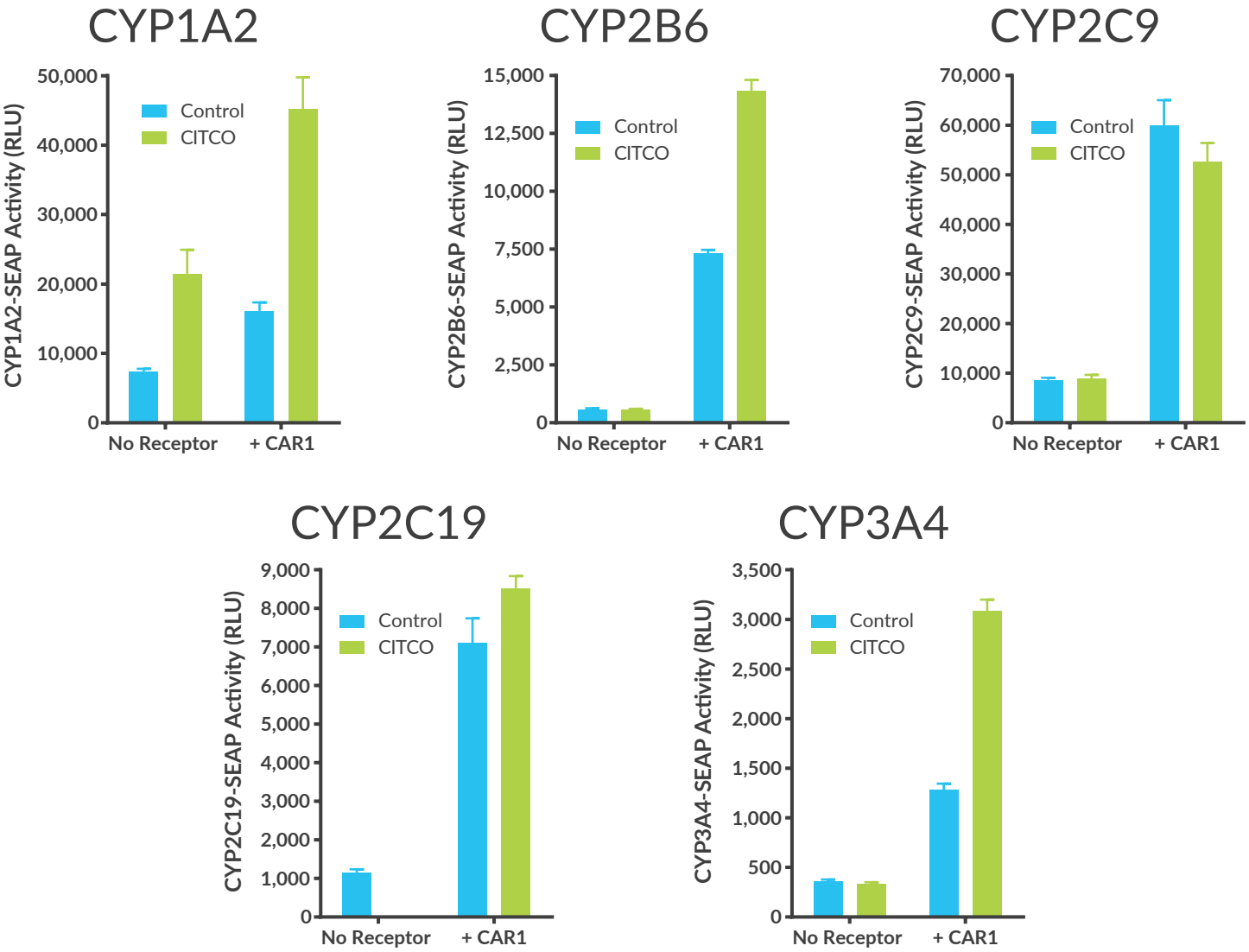

Figure 3. Constitutive activity of CAR1 when co-transfected with CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 reporter constructs.

PXR-Mediated Modulation of CYP450 Reporter Gene Expression

In contrast to the constitutive activity of CAR1, the expression of PXR resulted in a 40% to 80% suppression of basal activity of all the CYP450 reporters, presumably due to competition between the promoters for the transcription factors (**Figure 4**). Without supplementation of PXR, 10 µM rifampicin did not show any effect on reporter gene activities driven by CYP2B6, CYP2C9, CYP2C19, and CYP3A4 promoters, but a small decrease in CYP1A2-SEAP. With the expression of PXR, rifampicin produced strong inductions of CYP2B6-SEAP and CYP3A4-SEAP reporters and moderate inductions of CYP2C9-SEAP and CYP2C19-SEAP reporters, whereas such an effect on the CYP1A2-SEAP reporter was not statistically significant.

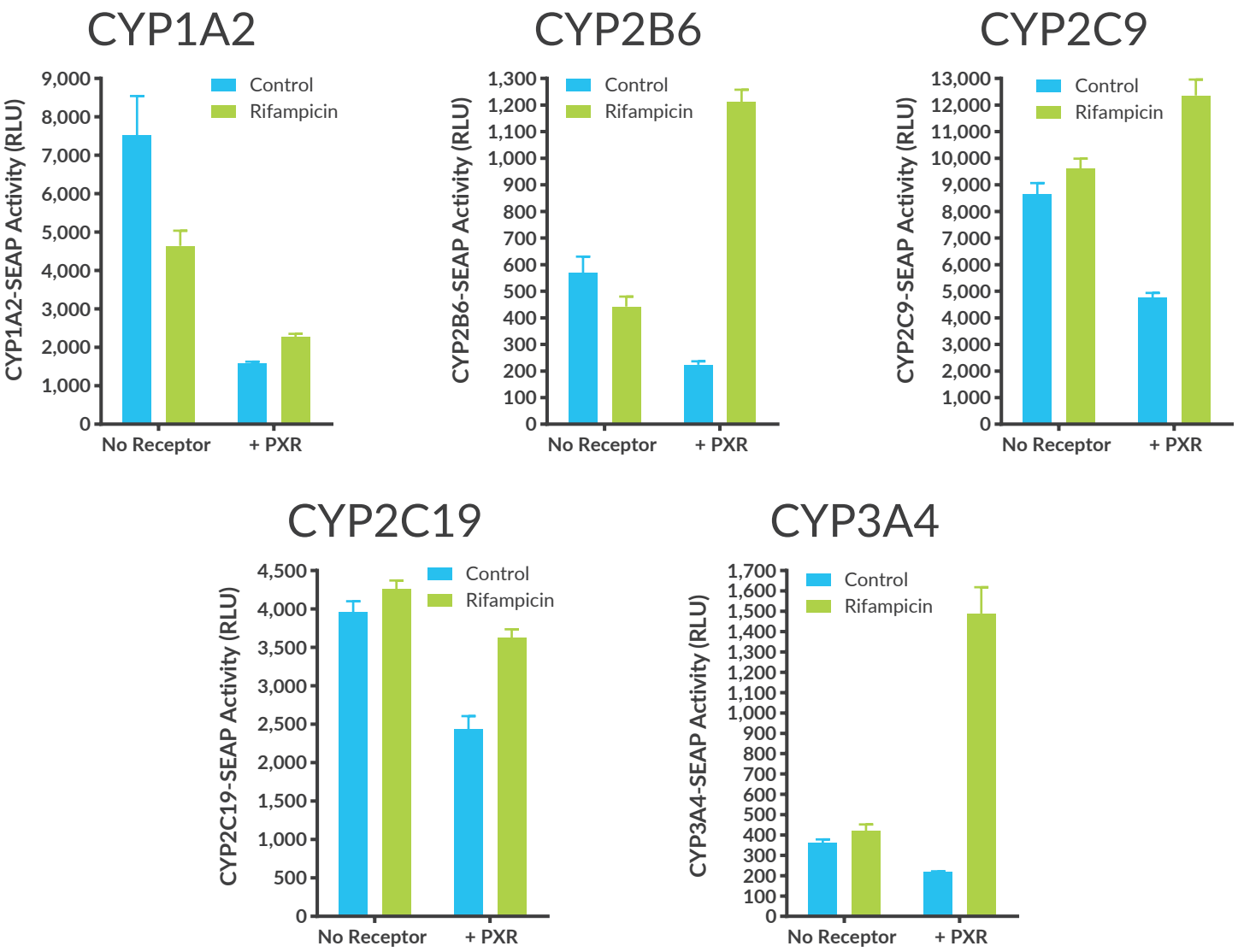

Figure 4. Induction of CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 reporter activity when co-transfected with PXR in the presence of 10 µM rifampicin.

PXR Uncovers CAR1-Mediated CYP450 Induction

The effect of PXR co-expression on CAR1 constitutive activity was examined in several CYP450 reporter assays (**Figure 5**). Without PXR supplementation, the basal activities of CYP1A2-SEAP, CYP2B6-SEAP, CYP2C9-SEAP, and CYP3A4-SEAP elevated by CAR1 were slightly suppressed by rifampicin. Compared to the 40% to 80% reduction in the basal reporter gene activities by PXR, as shown in Figure 4, the suppressive effect of PXR on the CAR1-elevated reporter activity was much stronger. In contrast, the co-expression of CAR1 did not noticeably impact PXR-mediated CYP2B6 and CYP3A4 reporter gene inductions by rifampicin.

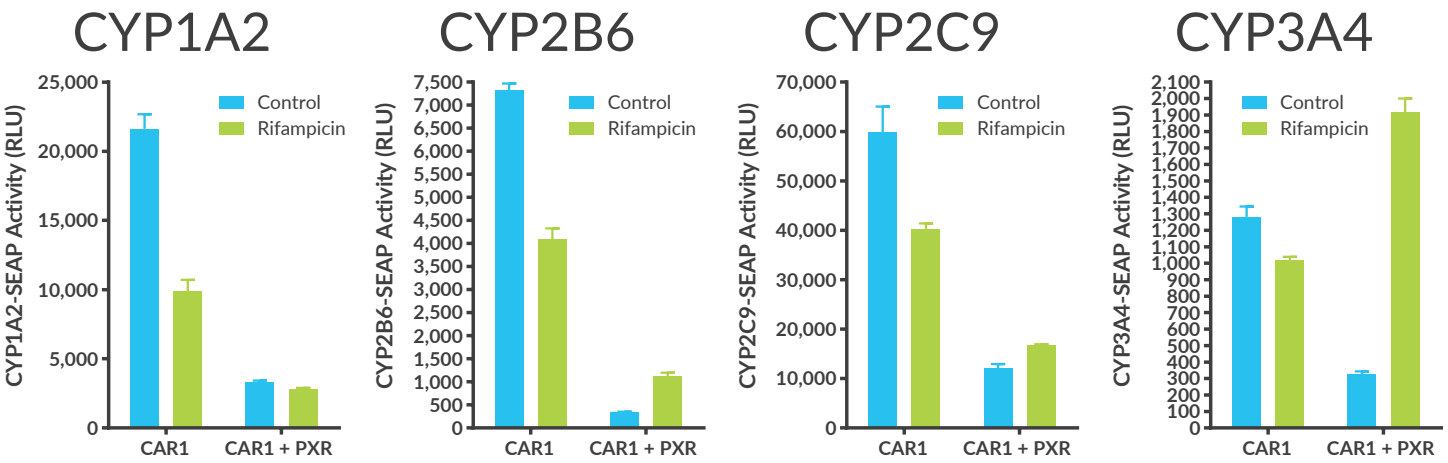

Figure 5. PXR co-expression suppresses CAR1 constitutive activity in CYP1A2, CYP2B6, CYP2C9, and CYP3A4 reporter assays.

To further investigate the PXR effect on the down-regulation of CAR1 constitutive activity, various levels of PXR cDNA were titrated into the transfection complex containing a constant amount of CAR1 cDNA in the same expression vector (**Figure 6**). This experiment was conducted with the CYP2B6-SEAP reporter, which exhibited the strongest CAR1 constitutive activity. With a CAR1 to PXR ratio of as little as 4:1, the constitutive activity was suppressed nearly 75% (**Figure 6A**). The suppression of PXR cDNA in the transfection complex with a 4:4 ratio of CAR1 to PXR abolished the constitutive activity due to CAR1 expression. The suppressive effect of PXR was specific to the basal constitutive activity of CAR1 which amplified the CITCO-stimulated induction mediated by CAR1 (**Figure 6B**). In other words, in the presence of PXR, the CYP2B6-SEAP reporter assay with CAR1 becomes a very robust assay to test for potential inducers.

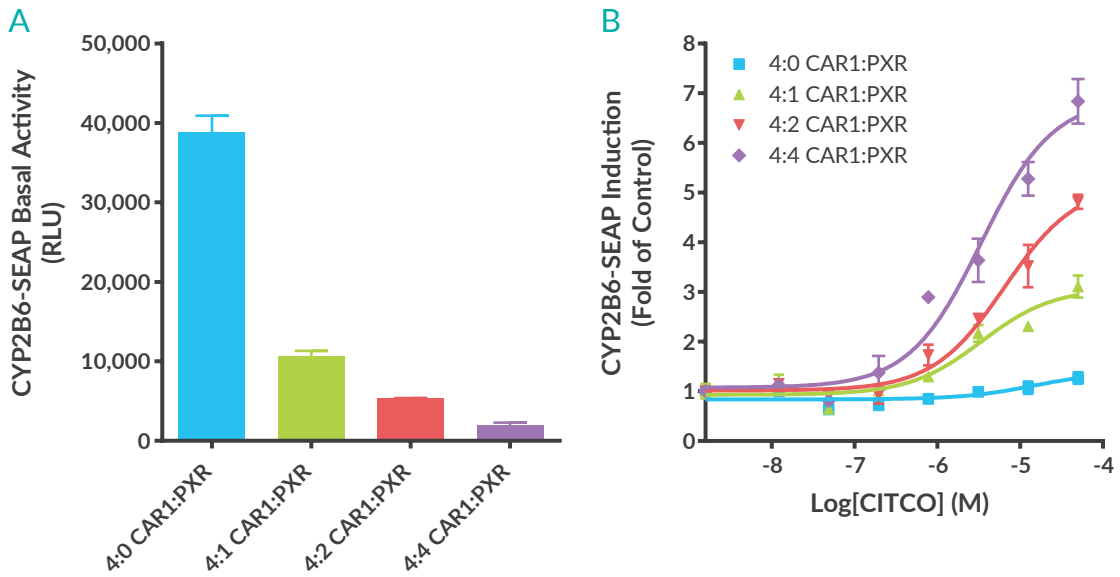

Figure 6. A) PXR suppression of CAR1 constitutive activity at variable concentrations of PXR cDNA. B) CITCO-induced activity of CAR1 at variable concentrations of PXR cDNA.

Interactions between Activated PXR and CAR1 Receptors

The interaction between PXR and CAR1 activation was further examined on both CYP2B6-SEAP (**Figure 7**) and CYP3A4-SEAP (**Figure 8**) reporter gene assays where stronger inductions by the activation of each nuclear receptor were revealed. The stoichiometry of DNA mixtures in reverse transfection complexes was modified to detect the activation of both nuclear receptors. When supplemented with only PXR, 10 µM of rifampicin only produced a 2-fold induction of CYP2B6-SEAP. A smaller increase in CYP2B6-SEAP reporter activity by 1 µM CITCO was also observed in the absence of exogenous CAR. When the cells were incubated with both 10 µM rifampicin and 1 µM CITCO, a nearly 4-fold induction was exhibited. In the presence of CAR1, there was no significant induction of reporter by rifampicin or CITCO due to the high level of constitutive activity (38.81 ± 1.85-fold at 48 hours). In the presence of PXR + CAR1, the response to rifampicin was much lower than with PXR alone, while the CITCO effect on CYP2B6 induction became much more prominent (>5-fold) than with either nuclear receptor supplement separately. Co-incubation of rifampicin and CITCO did not produce further induction.

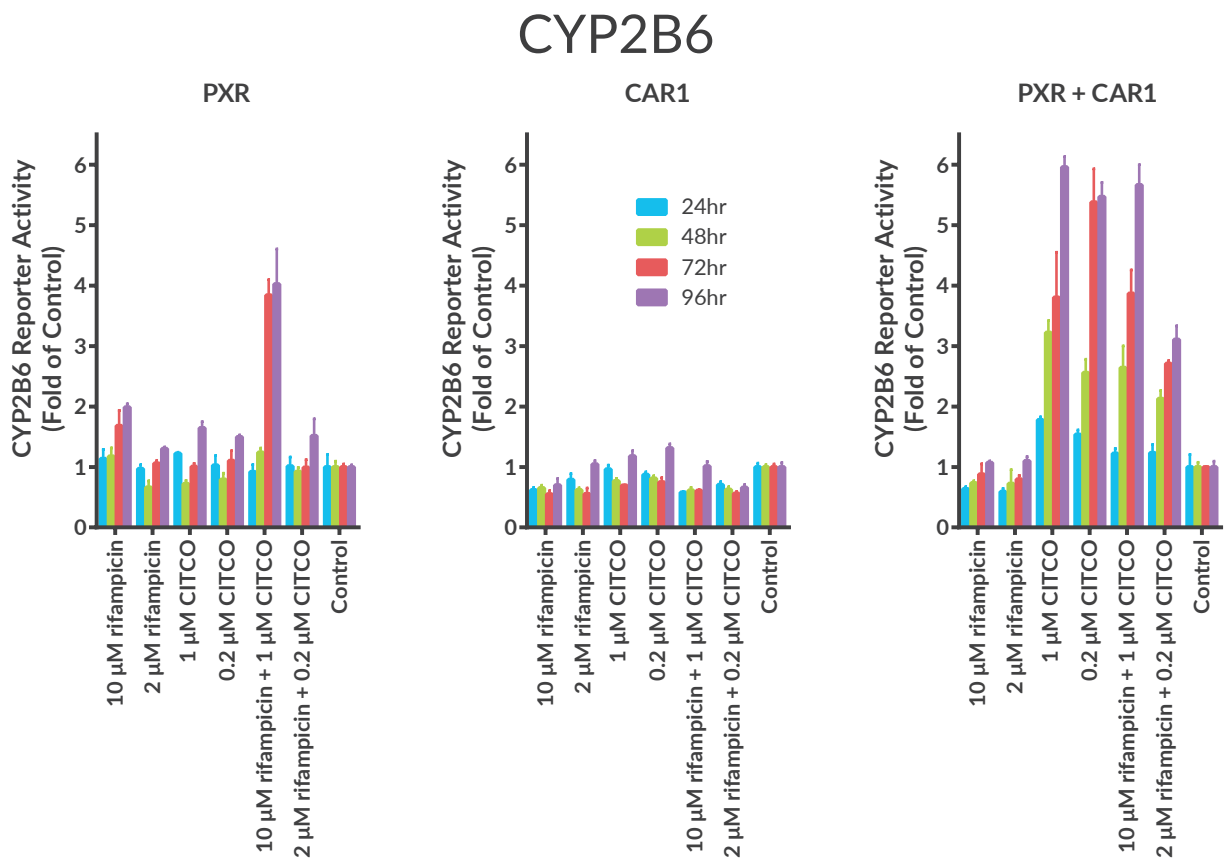

Figure 7. PXR and CAR1 interactions in a CYP2B6-SEAP reporter gene assay.

Similar to the CYP2B6-SEAP reporter experiment above, the effect of PXR-CAR1 interaction on CYP3A4 induction was also examined. With supplementation of PXR alone, 10 µM of rifampicin produced a nearly 4-fold induction of the reporter activity while CITCO resulted in a very small induction without CAR1 supplementation. In the presence of CAR1, the constitutive activity led to a 10.18 ± 1.82-fold increase in basal activity; however, a 2- to 3-fold further induction by CITCO was also observed. With the co-expression of PXR and CAR1, the basal activity dropped below the no receptor control level (data not shown).

Nevertheless, the response to either rifampicin or CITCO alone was not affected by the co-expression of the other nuclear receptor (PXR or CAR1). In contrast, the co-stimulation of both nuclear receptors with 1 µM CITCO and 10 µM rifampicin produced an 11.42 ± 0.53-fold induction at 96 hours revealing a potentially synergistic effect.

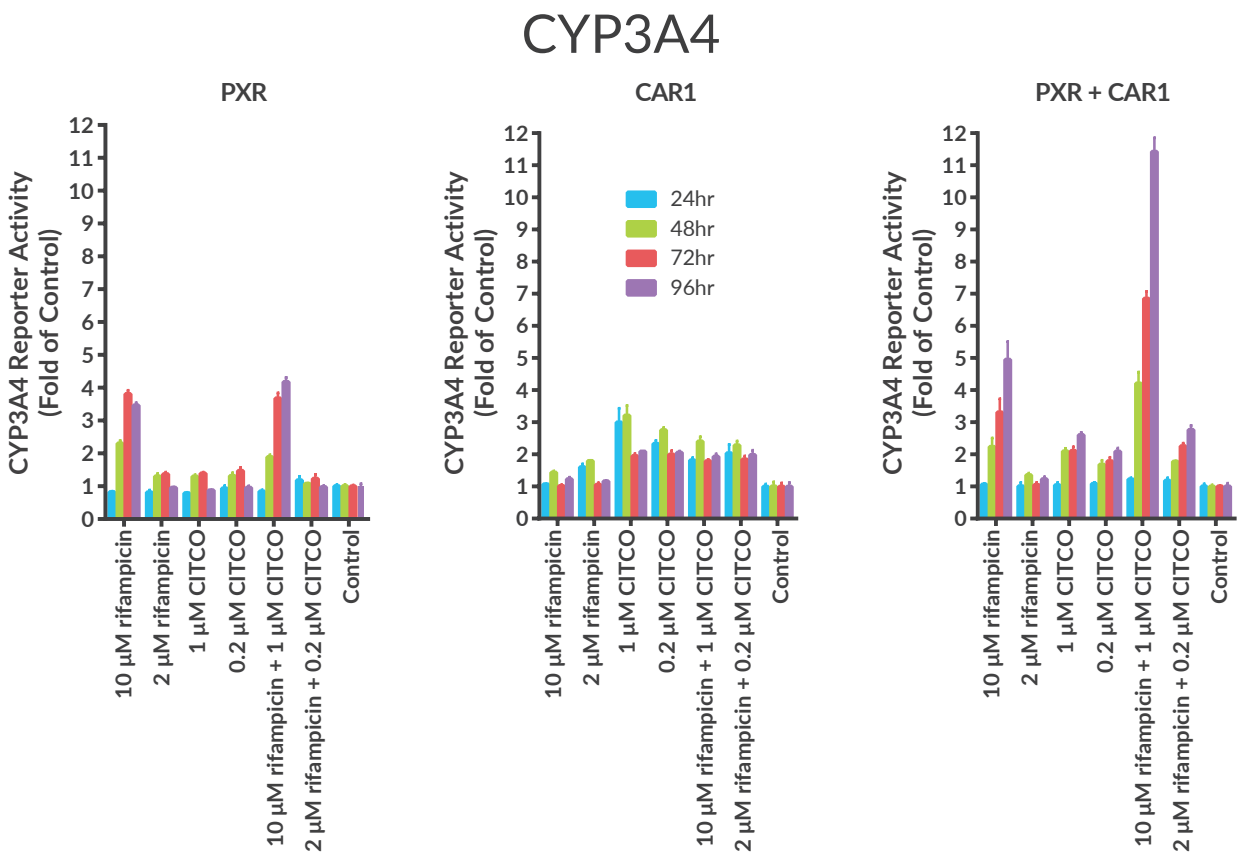

Figure 8. PXR and CAR1 interactions in a CYP3A4-SEAP reporter gene assay.

Conclusions

In conclusion, Cayman's reverse transfection technology resulted in superior co-transfection efficiency and reproducibility in the CYP450 induction reporter assay. This platform is flexible and allows rapid assessment of compounds in CYP450 gene induction by evaluating activation of different transacting factors, expressed individually or in combination, at multiple time points.

In HepG2 cells, CAR1 expression showed a strong constitutive activity in CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 reporter gene assays. The constitutive activity of CAR1 can be suppressed by co-expression of PXR. As a result, the co-expression of PXR in the CAR1/CYP2B6-SEAP assay enhanced the ligand response by CAR1 and allowed the screening of inducers with the major CAR isoform. Moreover, the co-activation of PXR and CAR1 produced an additive, and potentially synergistic, effect on CYP3A4 induction.

References

1. Sinz, M., Wallace, G., and Sahi, J. *AAPS J.* **10(2)**, 391-400 (2008).
2. Polasek, T.M., Lin, F.P.Y., Miners, J.O., *et al. Br. J. Clin. Pharmacol.* **71(5)**, 727-736 (2011).
3. Pelkonen, O., Turpeinen, M., Hakkola, J., *et al. Arch. Toxicol.* **82(10)**, 667-715 (2008).
4. Tompkins, L.M. and Wallace, A.D. *J. Biochem. Mol. Toxicol.* **21(4)**, 176-181 (2007).
5. Anderson, L.E., Dring, A.M., Hamel, L.D., *et al. Toxicol. Lett.* **202(2)**, 148-154 (2011).
6. Molnár, F., Küblbeck, J., Jyrkkärinne, J., *et al. Drug Metabol. Drug Interact.* **28(2)**, 79-93 (2013).

Reverse Transfection Assays Available from Cayman

Item No.	Product Name
600670	CYP1A1/2 Induction Reporter Assay Kit
600680	CYP2B6 Induction Reporter Assay Kit
601120	CYP2C9 Induction Reporter Assay Kit
601380	EP_2 Receptor (human) Reporter Assay Kit*
601390	EP_4 Receptor (human) Reporter Assay Kit*
600410	EP_4 Receptor (rat) Activation Assay Kit (cAMP)
601190	FFAR1 (GPR40) Reporter Assay Kit
601200	FFAR4 (GPR120) Reporter Assay Kit
600180	Melanocortin-3 Receptor Reporter Assay Kit
600190	Melanocortin-4 Receptor Reporter Assay Kit
600240	Orexin 1 Receptor Reporter Assay Kit
600250	Orexin 2 Receptor Reporter Assay Kit
601440	TGR5 (GP-BAR1) Reporter Assay Kit

*Also available for rat homologs (Item Nos. 600340 and 600350)

View a complete list of our reverse transfection assays at www.caymanchem.com

Cayman Contract Research Services

Our team of experts can design and run a customized screening project utilizing reverse transcription assay technology. Don't see your target of interest? Let us build a customized reverse transfection assay catered to your particular experimental plan.

Contact us at contractresearch@caymanchem.com or visit www.caymanchem.com/services