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Thalidomide Increases Human Hepatic Cytochrome P450 3A Enzymes by Direct Activation of the Pregnane X Receptor

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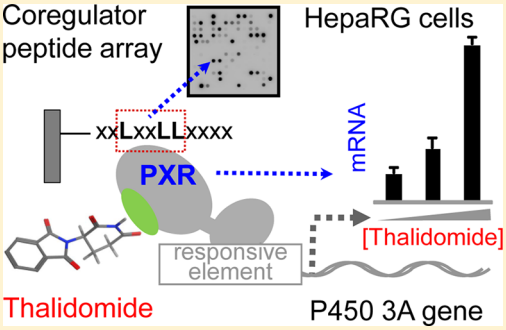
Supporting Information

ABSTRACT: Heterotropic cooperativity of human cytochrome P450 (P450) 3A4/3A5 by the teratogen thalidomide was recently demonstrated by H. Yamazaki et al. ((2013) *Chem. Res. Toxicol. 26*, 486−489) using the model substrate midazolam in various *in vitro* and *in vivo* models. Chimeric mice with humanized liver also displayed enhanced midazolam clearance upon pretreatment with orally administered thalidomide, presumably because of human P450 3A induction. In the current study, we further investigated the regulation of human hepatic drug metabolizing enzymes. Thalidomide enhanced levels of P450 3A4 and 2B6 mRNA, protein expression, and/or oxidation activity in human hepatocytes, indirectly suggesting the activation of upstream transcription factors involved in detoxication, e.g., the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR). A key event after ligand binding is an alteration of nuclear receptor conformation and recruitment of coregulator proteins that alter chromatin accessibility of target genes. To investigate direct engagement and functional alteration of PXR and CAR by thalidomide, we utilized a peptide microarray with 154 coregulator-derived nuclear receptor-interaction motifs and coregulator and nuclear receptor boxes, which serves as a sensor for nuclear receptor conformation and activity status as a function of ligand. Thalidomide and its human proximate metabolite 5-hydroxythalidomide displayed significant modulation of coregulator interaction with PXR and CAR ligand-binding domains, similar to established agonists for these receptors. These results collectively suggest that thalidomide acts as a ligand for PXR and CAR and causes enzyme induction leading to increased P450 enzyme activity. The possibilities of drug interactions during thalidomide therapy in humans require further evaluation.



INTRODUCTION

The sedative drug thalidomide [α-(*N*-phthalimido)glutarimide] was withdrawn in the early 1960s due to its potent teratogenic effects,[1] but it was subsequently approved for the treatment of multiple myeloma.[2,3] Because of the recent emergence of thalidomide as a drug with clinical potential, there is renewed interest in both its toxicity and pharmacological mechanisms. Various hypotheses (for both) have been proposed, including the generation of reactive oxygen species,[4] generation of reactive acylating[5] and arene oxide intermediates,[6] inhibition of angiogenesis,[7] and inhibition of the protein cereblon.[8] Two analogues of thalidomide with increased potency and reduced toxicity, lenalidomide and pomalidomide, have also entered the clinic for the treatment of refractory multiple myeloma.[9]

The teratogenicity of thalidomide is species-specific and occurs in primates but not in rats and mice. The mechanism of action of thalidomide still remains unclear, but it has been shown that the metabolism of thalidomide is important for both teratogenicity and cancer treatment outcome. We previously reported the cooperativity of human P450 subfamily 3A enzymes for thalidomide[10] and 5-hydroxy product formation from thalidomide (Figure 1) mediated by human P450 3A4.[11,12] Also, the second oxidation step involves a reactive intermediate, possibly an arene oxide that can be trapped by GSH to give GSH adducts.[13] Two aspects of *in vivo* drug interaction of thalidomide were reported:[14] an enhanced

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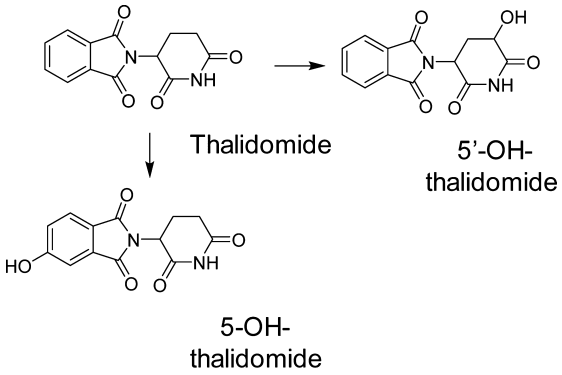

Figure 1. Chemical structures of thalidomide, the primary rodent metabolite 5′-hydroxythalidomide (5′-OH-thalidomide), and the primate metabolite 5-hydroxythalidomide (5-OH-thalidomide).

clearance of midazolam and a higher area-under-the-curve of 4-hydroxymidazolam following pretreatment with thalidomide in humanized-liver mice, presumably due to human P450 3A induction. Although induction of total P450 contents by thalidomide in rat livers has been reported,[15] apparently no interaction of thalidomide has been shown with ethinyl estradiol (P450 3A4 substrate and inhibitor) in humans.[16] *In vivo* cooperativity of human P450 3A enzymes was also reported as another aspect, with a higher area-under-the-curve for 1′-hydroxymidazolam following cotreatment with thalidomide in the humanized mice.[14] It is important to evaluate any drug interactions through human P450 enzymes by concomitant thalidomide therapy using the basic research technique.

To clarify the potential of thalidomide, a microarray assay for real-time coregulator−nuclear receptor interaction (MARCoNI) was adopted for investigating P450 induction by thalidomide and 5-hydroxythalidomide, one of the metabolites formed by human P450 subfamily 3A enzymes, plus 5′-hydroxythalidomide, a major product formed by rodents (Figure 1) in the present study. We report here that thalidomide can act as an agonist for pregnane X receptor (PXR) and cause P450 3A enzyme induction.

MATERIALS AND METHODS

Chemicals and Cells. (*R*)-(+)-Thalidomide, rifampicin, dexamethasone, RU486 (Sigma-Aldrich, St. Louis, MO), pomalidomide (Selleck Chemicals, Houston, TX), and GSH transferase (GST)-tagged human ligand-binding PXR and constitutive androstane receptor (CAR) domains (LBD-GST, PV4841, and PV4838, respectively, Invitrogen, Breda, Netherlands) were purchased from the indicated sources. 5-Hydroxy- and 5′-hydroxythalidomide were synthesized as reported previously.[17] Polyclonal anti-human P450 3A4 antibodies were obtained from BD Biosciences (San Jose, CA). Other chemicals and reagents used in this study were obtained from the sources described previously[14] or were of the highest quality commercially available.

Cryopreserved differentiated HepaRG cells (Biopredic International, Rennes, France) and human hepatocytes (BD Biosciences) were plated in collagen-coated 24-well plates according to these manufacturers' instructions. After plating, the differentiated HepaRG cells were maintained for 72 h (at 37 °C, 5% CO_2, v/v) in the general purpose supplement ADD670. At incubation, the medium was aspirated and replaced with a serum-free induction medium, which included induction supplement 650 (ADD650). Thalidomide and its metabolites were added to the medium on each concentration. The medium was replaced every 24 h, and each chemical was added for 48 or 72 h.

Measurements of Catalytic Activities, Protein, and mRNA of P450s in Cells. After incubation with thalidomide and its metabolites, the culture medium was aspirated. The cells were rinsed twice with prewarmed phosphate-buffered saline and were incubated at 37 °C in 5% CO_2 (v/v) with serum-free incubation medium containing midazolam (50 μM) and bupropione (100 μM) for 3 h. After incubation, the reaction was stopped by adding an equal volume of ice-cold CH_3OH containing the internal standard caffeine (100 μM). The samples were centrifuged at 10^4g for 5 min, and the supernatant was collected for metabolite analysis. A Quattro Micro API mass analyzer (Waters, Tokyo, Japan) was used, directly coupled to a Waters LC 2695 system with an octadecylsilane (C_{18}) column (Atlantis, 3 μm, 2.1 mm × 50 mm), with MassLynx NT4.1 software used for data acquisition (Waters). Measurements of 1′- and 4-hydroxymidazolam were performed in the electrospray positive ionization mode as described previously,[14] and 1′-hydroxymidazolam *O*- and *N*-glucuronides were monitored using the multiple reaction monitoring mode of the transition m/z 518 → m/z 324. Bupropione hydroxylation activities were determined using the same LC/MS conditions but with different moleculer mass. The metabolites were quantified using the m/z 342 → 325 transition for hydroxybupropione and the m/z 195 → 138 transition of the internal standard caffeine, respectively.

After measurements of drug oxidation activities, the cells were washed with 500 μL of phosphate-buffered saline twice and were collected by centrifugation (at 2×10^3g for 3 min). Cell lysis buffer (30 μL) was added to the suspended cells. After freezing and thawing, the cell supernatants were collected following centrifugation (at 1.1×10^4g for 15 min). Cell lysates were analyzed for protein content using Pierce BCA protein analysis reagent and used for SDS−polyacrylamide gel electrophoresis and immunoblotting with anti-human P450 antibodies. The specific protein P450 3A4/5 was detected by using an ECL-detection kit (GE Healthcare, Buckinghamshire, UK).

After incubation of human hepatocytes or HepaRG cells with thalidomide and its metabolites, total RNA was also extracted with the use of a Qiagen RNeasy mini-kit according to the manufacturer's instructions. Levels of mRNA of human P450 2B6 (Hs04183483_g1) and 3A4 mRNA (Hs01546612_m1) were determined with the use of a TaqMan expression quantitative polymerase chain reaction according to the manufacturer's protocol. The relative P450 expression levels were estimated following normalization for the levels of GAPDH (Hs99999905_m1) in three independent amplifications.

Microarray Assay for Real-Time Analysis of Coregulator−Nuclear Receptor Interaction (MARCoNI). Ligand-modulated interaction of coregulators with PXR and CAR was assessed using a PamChip peptide microarray containing 154 unique coregulator motifs (#88101, PamGene International BV, Den Bosch, Netherlands) as described previously.[18] Briefly, all incubations were performed at 20 °C on a PamStation-96 (PamGene), using two cycles per minute. Assay mixtures contained 5 nM PXR or CAR (glutathione-*S*-transferase-tagged ligand binding domain), 25 nM Alexa488-conjugated GST antibody (A11131, Invitrogen), 0.1 mM thalidomide or its metabolites, dexamethasone, rifampicin, or RU486 in TR-FRET PXR assay buffer or TR-FRET coregulator buffer G (PV4842 and PV4553, respectively, Invitrogen), and 5 mM dithiothreitol. The complete assay mix with solvent only (DMSO, 2% final concentration, v/v), representing nuclear receptors in the apo conformation (no ligand), served as the negative control.

Each array was blocked for 20 cycles with 25 μL of 1% bovine serum albumin (w/v) and 0.01% Tween20 (w/v) in Tris-buffered saline. Next, the blocking buffer was removed by aspiration and each array was incubated for 80 cycles with 25 μL of each assay mix. Subsequently, the unbound ligand/receptor was removed by washing of the arrays with 25 μL of Tris-buffered saline, and finally a .tiff image of each array was acquired by the CCD-camera of the PamStation.

Image Analysis. The fluorescent signal of each spot in each array (representative of nuclear receptor binding to that particular coregulator motif) was quantified using BioNavigator software (PamGene). Each .tiff image (single array) was overlaid with a synthetic grid of spot-sized circles. An algorithm was used to optimize the placement of each circle around its respective spot (actual peptide

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position) on the .tiff image. The median fluorescence within each circle, as well as that in a defined area surrounding the circle, was quantified. For each spot, the median signal minus background was calculated and used for further analysis.

Comparison of Responses to Compounds. Each condition was measured using four technical replicates (arrays) and thus resulted in four binding values for the compound-stimulated (or apo) nuclear receptor binding to a particular coregulator motif. Data were analyzed and visualized using BioNavigator R, version 2.15.3 (The R Foundation for Statistical Computing). Compound-mediated modulation of nuclear receptor binding to each individual coregulator (Modulation Index, MI) was calculated as $\log_{10}$-transformed nuclear receptor binding (fluorescence) in the presence of ligand over that in the presence of solvent only. In addition, we performed Student's *t* test on nuclear receptor binding (apo vs compound-stimulated) to assess the significance of the effect of each compound. Because each array contains 154 unique coregulator motifs, each compound was characterized by a 154-point MI signature. Compound signatures were subjected to hierarchical clustering using Euclidean distance and average linkage. For each receptor, compound (dis)similarities were visualized as a dendrogram of a clustered MI heat map in which the significance of the modulation of each interaction is indicated (*, $p < 0.05$; **, $p < 0.01$; ***: $p < 0.001$).

RESULTS AND DISCUSSION

P450 3A Induction by Thalidomide in Human Hepatocytes and HepaRG Cells. Human hepatocytes and HepaRG cells were cultured with thalidomide and its metabolites for 72 h (Figure 2). The midazolam 4- and 1′-hydroxylation activities were increased by treatment with thalidomide but not by its metabolites in human hepatocytes (Figure 2A) and HepaRG cells (Figure 2B). A low formation rate of 1′-hydroxymidazolam *O*-glucuronide was enhanced 3-fold in HepaRG cells (Figure 2C). Similarly, induction of P450 2B-dependent bupropion hydroxylation activity by thalidomide was seen (Figure 2D). Under the separate experiments, P450 3A4 was induced (>2-fold) by a positive control, rifampicin, in the hepatocytes and HepaRG cells (results not shown).

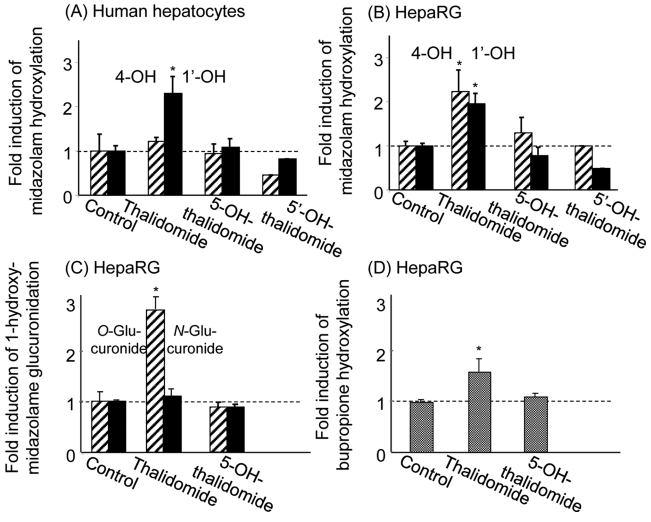

Figure 2. Effects of thalidomide and its metabolites on P450 3A- and 2B6-mediated drug oxidation activities in human hepatocytes and HepaRG cells. Human hepatocytes (A) and HepaRG cells (B–D) were cultured with thalidomide (1.0 mM) and its metabolites for 72 h. Control formation rates of midazolam 1′ (filled bar) and 4 (hatched bar) hydroxylation in human hepatocytes were 0.08 and 8.0 nmol/h/10^6 cells, respectively. Control formation rates of midazolam 1′ (filled bar) and 4 (hatched bar) hydroxylation and 1′-hydroxymidazolam *O* (hatched)- and *N* (filled)-glucuronide formation in HepaRG cells were 0.01, 0.80, 0.01, and 0.005 nmol/h/10^6 cells, respectively. Bupropione hydroxylation activity in the control HepaRG cells was 0.02 nmol/h/10^6 cells. Columns and bars show means and SD values from triplicate determinations. *, $p < 0.05$.

Effects of thalidomide on expression levels of P450 mRNA in human hepatocytes and HepaRG cells were examined after 48 h of incubation (Figure 3). The mRNA levels of P450 3A4 and 2B6 were enhanced following exposure to increasing concentrations of thalidomide (Figure 3). The induction of P450 3A4 mRNA in hepatocytes and HepaRG cells was 2.0- and 4.5-fold, respectively. In contrast, the fold induction of P450 2B6 mRNA in hepatocytes and HepaRG cells was much less (1.2- and 1.4-fold, respectively). These results collectively indicate that thalidomide is an inducer of human P450 3A enzymes but not a potent one, as judged by the catalytic activity, protein expression, and mRNA determinations in human hepatocytes or HepaRG cells.

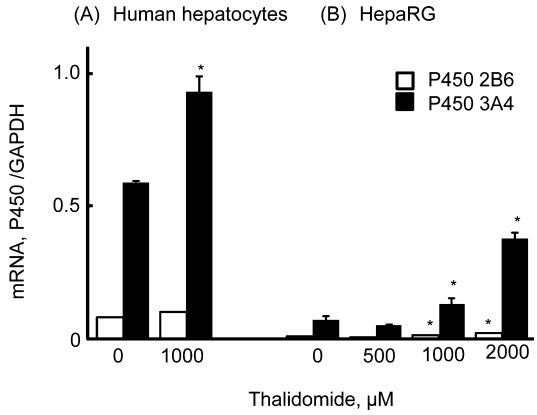

Figure 3. Effects of thalidomide and its metabolites on P450 3A and 2B6 mRNA levels in human hepatocytes and HepaRG cells. P450 mRNA levels were determined after 48 h treatment with thalidomide (Materials and Methods). Columns and bars show the means and SD values for triplicate determinations. *, $p < 0.05$.

Protein fractions were taken from the treated cells as cell-lysates after the evaluation of catalytic function shown in Figure 2A and B. Induction of P450 3A protein by 1.0 mM thalidomide treatment (but not by the metabolites) 2.0-fold in both human hepatocytes and HepaRG cells was confirmed by immnoblotting with anti-human P450 3A antibodies (Figure S1, Supporting Information). These results are comparable with enhancement of catalytic activity (Figure 2A,B) and mRNA level (Figure 3) in human hepatocytes and HepaRG cells.

Thalidomide-Induced Modulation of CAR and PXR Affinity for Coregulator Proteins. Ligand binding by a nuclear receptor allosterically induces conformational changes and regulates interaction with coregulator proteins. To test whether thalidomide was able to modulate CAR or PXR affinity, we applied a microarray assay for real-time coregulator–nuclear receptor interaction (MARCoNI). In this assay, a set of coregulator proteins is represented by its nuclear receptor-interacting (CoR)NR-box motifs by means of immobilized peptides. A GST-tagged ligand binding domain of each receptor was incubated on the array in the presence or absence of each compound. Receptor binding to each motif was detected using a fluorescently labeled GST antibody, resulting in a 154-point binding profile for each condition. These binding profiles were converted into response profiles by calculating the

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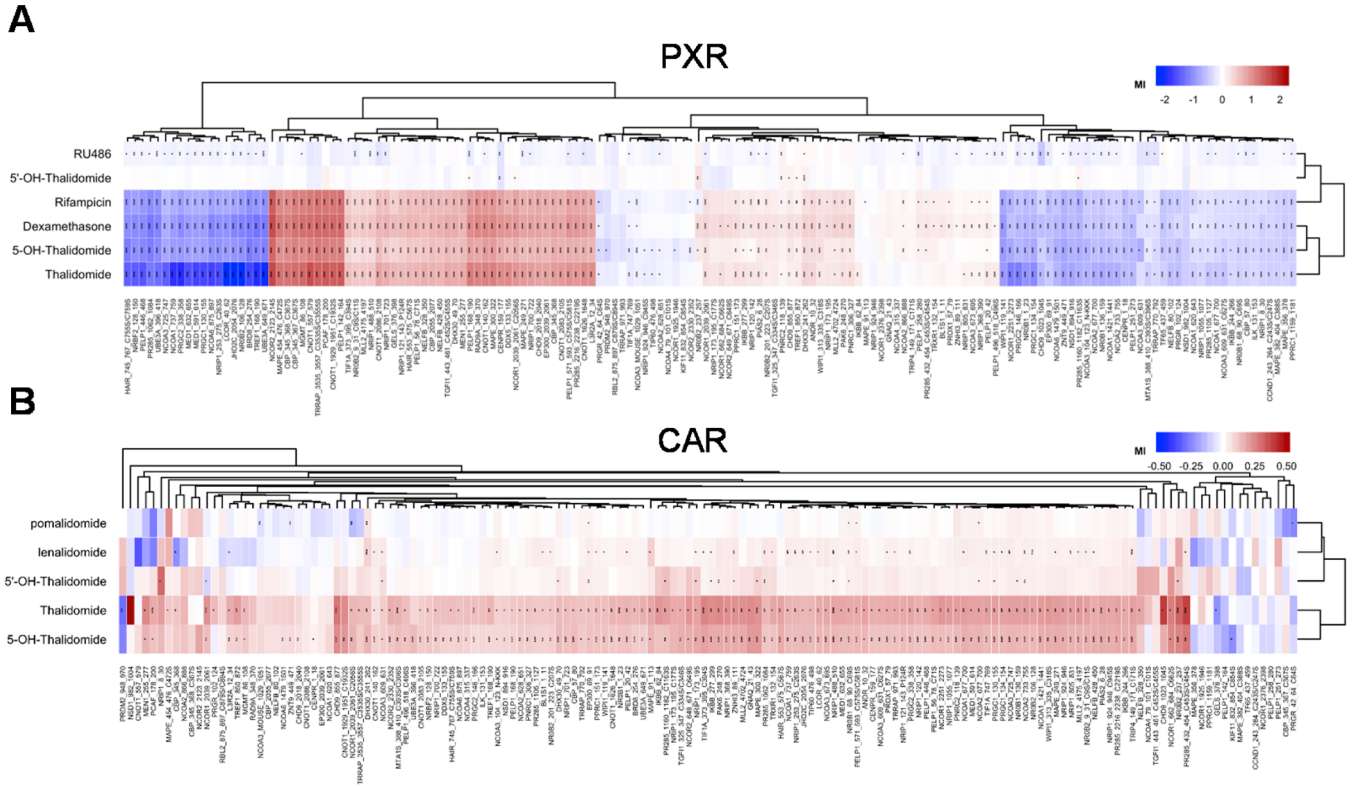

Figure 4. Compound-induced modulation of PXR (A) and CAR (B) interaction with coregulator proteins. Each row shows the ligand-mediated $\log_{10}$-fold modulation of nuclear receptor binding (modulation index, MI) to each of the coregulator motifs on the array. Significance of the modulation of each interaction is indicated ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). (Dis)similarity between compounds or coregulator motifs, as determined by hierarchical clustering (Euclidean distance and average linkage) is summarized in the dendrograms. Red, positive response; blue, negative response. 5′-OH-Thalidomide and 5′-hydroxythalidomide; 5-OH-thalidomide and 5-hydroxythalidomide.

compound-induced $\log_{10}$-fold modulation of binding vs control (solvent only).

Two positive control agonists for PXR activation (Figure 4A), dexamethasone and rifampicin, clearly modulate coregulator interaction, reflected by significant and robust attraction (red) and repulsion (blue) of particular subsets of coregulator motifs. Alternatively, RU486, another PXR agonist, shows only moderate repulsion of coregulators, which suggests an alternative mechanism of action. To emphasize the (dis)similarity in response profiles (and mechanisms of action) between different compounds, we applied hierarchical clustering analysis. Interestingly, thalidomide and its human metabolite 5-hydroxythalidomide also strongly modulated PXR−coregulator interaction in a manner highly similar to that of dexamethasone and rifampicin, with some small differences in individual interactions. Thalidomide even displayed superior displacement efficacy of a series of motifs (extreme left) compared to the reference agonists, a feature that appears to be lost following conversion to 5-hydroxythalidomide. Alternatively, the rodent metabolite 5′-hydroxythalidomide seemed to lack virtually all of the modulating effect on this (human) receptor. Similarly, thalidomide and the human, but not rodent, metabolite similarly modulate coregulator interaction of human CAR, with slightly higher efficacy for thalidomide (Figure 4B). Recently, we reported that pomalidomide was oxidized by human liver microsomes and P450s 2C19, 3A4, and 2J2 to the corresponding phthalimide ring-hydroxylated product.[19] In addition, we tested the analogues lenalidomide and pomalidamide, which displayed weak or absence of modulation. It has been recently reported that many ligands for inducing CAR are indirect, not directly binding ligands, but actually ligands for the EGF receptor.[20]

In the present study, we investigated the induction of P450 3A enzymes to fully understand the metabolic activation of thalidomide. Interestingly, there was little effect of 5′-hydroxythalidomide. The autoinduction by thalidomide of human P450 3A should be associated with PXR, one of the members of the steroid hormone nuclear receptor family, revealed by a novel microarray assay for real-time analysis of coregulator−nuclear receptor interaction (MARCoNI, Figure 4). Heterodimers (e.g., RXR) with PXR could regulate the expression of human P450 enzymes and/or some other enzymes such as GSTs and UDP-glucuronosyltransferase. These drug-metabolizing enzymes could have some impact on the clearance of thalidomide in humans. The apparent functional drug oxidation activities mediated by P450 3A4 and 3A5 *in vitro* and *in vivo* could be differentially observed in combination with induction and cooperativity of P450 3A4 and/or P450 3A5. In conclusion, thalidomide was capable of binding to PXR directly and induced transcriptional regulation of human P450 3A gene. Drug interactions of thalidomide should be evaluated with the knowledge that the drug and its human metabolite are P450 inducers.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Effects of thalidomide on P450 3A protein detected with anti-human P450 3A antibody in human hepatocytes and HepaRG cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

○N.M. and R.v.B. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CAR, constitutive androstane receptor; MARCoNI, a microarray assay for real-time coregulator-nuclear receptor interaction; PXR, pregnane X receptor

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