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Deconvolution of CYP Induction Mechanisms in HepaRG Nuclear Hormone Receptor Knockout Cells

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Running Title: CYP induction in PXR and CAR knockout HepaRG

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List of abbreviations:

AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CDK, cyclin-dependent kinases; CITCO, 6-(4-Chloro-phenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; cKit, tyrosine-protein kinase KIT; DDI, drug-drug interactions; dKO, double knockout; DMSO, dimethyl sulfoxide; DNA-PK, DNA-dependent protein kinase; EMA, European Medicines Agency; FDA, Food and Drug Administration; ISR, integrated stress response; KO, knockout; MAPK, mitogen-activated protein kinase; MSC, Merck Serono compound; NHR, nuclear hormone

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receptor; PBS -/-, phosphate buffered saline without magnesium and calcium; PHH, primary human hepatocytes; PXR, pregnane X receptor; TRKA, tropomyosin receptor kinase A

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ABSTRACT

Pregnane X receptor (PXR), constitutive androstane receptor (CAR) and PXR/CAR knockout (KO) HepaRG cells, as well as a PXR reporter gene assay were used to investigate the mechanism of CYP3A4 and CYP2B6 induction by prototypical substrates and a group of compounds from the Merck KGaA oncology drug discovery pipeline. The basal and inducible gene expression of CYP3A4 and CYP2B6 of nuclear hormone receptor (NHR) KO HepaRG relative to control HepaRG was characterized. The basal expression of CYP3A4 was markedly higher in the PXR (10-fold) and CAR (11-fold) KO cell lines compared to control HepaRG, whereas inducibility was substantially lower. Inversely, basal expression of CYP3A4 in PXR/CAR double KO (dKO) was low (10-fold reduction). Basal CYP2B6 expression was high in PXR KO (9-fold) cells which showed low inducibility whereas the basal expression remained unchanged in CAR and dKO cell lines compared to control cells. Most of the test compounds induced CYP3A4 and CYP2B6 via PXR and to a lesser extent via CAR. Furthermore, other non-NHR driven induction mechanisms were implicated, either alone or in addition to NHRs. Notably, 5 of the 16 compounds (31%) that were PXR inducers in HepaRG did not activate PXR in the reporter gene assay, illustrating the limitations of this system. This study indicates that HepaRG is a highly sensitive system fit for early screening of CYP induction in drug discovery. Furthermore, it shows the applicability of HepaRG NHR KO cells as tools to deconvolute mechanisms of CYP induction using novel compounds representative for oncology drug discovery.

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SIGNIFICANCE STATEMENT

This works describes the identification of induction mechanisms of CYP3A4 and CYP2B6 for an assembly of oncology drug candidates using HepaRG NHR KO and displays its advantages compared to a PXR reporter gene assay. With this study, risk assessment of drug candidates in early drug development can be improved.

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INTRODUCTION

Risk assessment of CYP induction in early drug development is important as upregulation of CYP enzymes may lead to subtherapeutic levels of the drug itself or co-administrated agents or increased toxicity of the co-administered drug (Chu et al., 2009). Screening and actively designing out molecules with elevated risk for CYP induction prior to development has therefore become an adopted practice within the pharmaceutical industry (Lin et al., 2008).

Induction of CYP enzymes is mainly mediated by nuclear hormone receptors (NHRs). PXR regulates several genes related to the hepatobiliary disposition of drugs, of which CYP3A4 is the most important for drug-drug interactions (DDI). The activity of PXR can be regulated through direct binding to the receptor but also via posttranslational modifications (Lin et al., 2008; Mackowiak & Wang, 2016). CAR is involved in the expression of CYP3A4 but is more relevant for the expression of the CYP2B6 gene. The activity of CAR can also be regulated via direct binding as well as indirectly (Mackowiak & Wang, 2016; Mutoh et al., 2013). The expression of CYP enzymes can also be induced by other transcription factors such as the aryl hydrocarbon receptor (AhR). AhR regulates the expression of CYP1A2. The commonly used positive control for CYP1A2 induction, omeprazole, regulates via an indirect mechanism (Fujii-Kuriyama & Mimura, 2005).

Induction of human CYPs is often missed in preclinical animal models, partially explained by inter-species differences in the ligand-binding domains of CAR and PXR (Timsit & Negishi, 2007). Hence, the use of human cell systems, which recapitulate the human gene regulatory networks and express relevant proteins involved in indirect activation mechanisms of CAR and PXR, such as primary human

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hepatocytes (PHH) or HepaRG appear essential for adequate risk assessment of human DDI.

PHH are regarded as the system of choice for final risk assessment of human CYP induction. PHH are also recommended by regulatory agencies in the development of new chemical entities for such assessments (European Medicines Agency, 2012; Food and Drug Administration, 2020). PHH closely resemble the molecular phenotype of human liver *in vivo*, including physiologically relevant NHR levels. However, they do not proliferate and, while multiple efforts have shown promise to induce proliferation of mature PHH (Oliva-Vilarnau et al., 2020) or hepatic stem cells (Hu et al., 2018; Huch et al., 2015) currently only cell lines offer possibilities for stable genetic modifications. Consequently, cell lines provide an important complement to delineate CYP induction mechanisms. Specifically, the HepaRG cell line has emerged as a valuable model for the study of CYP induction. While their overall transcriptomic signatures and functionality differ from PHH (Bell et al., 2017; Berger et al., 2016; Hart et al., 2010), they are genetically accessible and feature stable and relevant levels of CYP and NHR expression, as well as other liver-specific functions (Kanebratt & Andersson, 2008).

Comparing CYP induction in HepaRG PXR and CAR single or dKO cells provides a powerful setup to delineate induction mechanisms and quantify the contribution of these NHRs. The utility of the single KO has been validated previously with prototypical inhibitors (Williamson et al., 2016). In this study we characterize the use of a dKO cell line, further characterize and discuss the inducibility of these genetically modified cell lines with emphasis on the induction of CYP3A4 and CYP2B6. The aim of this study was to characterize pathways for CYP3A4 and CYP2B6 induction in the

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chemical space representative for typical small molecule drug candidates in oncology using these genetically modified tools.

Initial risk assessment of CYP induction is commonly performed using gene reporter assays. An industry survey revealed, that 64% of respondents routinely used nuclear transactivation assays to assess the enzyme induction potential of new compounds (Chu et al., 2009). These assays are however susceptible to false negative results (Hariparsad et al., 2008) as typically only PXR activation via direct binding can be estimated. Furthermore, it has been established that the correlation between reporter gene systems and more physiologically relevant systems such as PHH is poor, especially regarding Emax calculation (McGinnity et al., 2009). In this study we examined the utility of a PXR reporter gene assay on a set of compounds representative for oncology drug discovery. Additionally, we discuss the shortcomings of this assay system in the context of the data collected from genetically modified HepaRG cells.

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MATERIALS AND METHODS

Materials

Penicillin/Streptomycin, dimethyl sulfoxide (DMSO), CITCO, phenobarbital, insulin, hydrocortisone and rifampicin were purchased from Sigma-Aldrich (St. Louis, MO). Omeprazole and flumazenil were purchased from Cayman Chemicals (Ann Arbor, MI). Williams medium E, QuantiGene Sample Processing kit, QuantiGene Plex Gene Expression kit, GlutaMAX and PBS without calcium and magnesium (PBS -/-) were purchased from Thermo Fisher Scientific (Waltham, MA). Lyophilized collagen I from rat tail was purchased from Roche Diagnostics GmbH (Basel, Switzerland). ADD711 medium supplement was purchased from Biopredic International (Saint-Grégoire, France). The PXR (human) reporter assay kit was purchased from INDIGO Biosciences, Inc. (State College, PA).

Compound selection

Available in-house data from CYP induction studies at different concentrations in HepaRG cells and PHH were sorted using KNIME v4.0.1 from KNIME AG (Zürich, Switzerland). The dataset was collected over approximately a decade and was composed mainly of internal compounds but also key competitor compounds and references. In total it consisted of 109 compounds. Data on CYP3A4 induction were extracted and substances were sorted according to their fold-induction into no, low, medium and high inducers (<2, 2-4.9, 5-24.9 and >25 – fold expression, respectively). High and medium inducers were grouped according to similarities in their chemical structure and physiochemical properties. From each group, compounds were selected resulting in a total set of 16 compounds (Table 1).

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Cell culture

Control HepaRG cells (HepaRG 5F), as well as PXR KO, CAR KO and dKO HepaRG cells were supplied by Sigma-Aldrich. Control, PXR-KO, CAR-KO and dKO HepaRG cells were thawed in pre-induction medium (William's medium E supplemented with additive 711C, GlutaMAX, 100 units/ml penicillin, 100 µg/ml streptomycin) and plated into 96-well collagen I coated plates (Corning, Corning, NY) at a volume of 200 µl/well. The cells were incubated at 37°C in a humidified incubator with 5% CO_2 for 24h. The pre-induction medium was replaced with 200µl/well of recovery medium (pre-induction medium supplemented with 0.5% DMSO). On day 4 medium was replaced with 200µl/well maintenance medium (pre-induction medium supplemented with 1.7% DMSO). Cells were kept in maintenance medium for 17-21 days until full differentiation and medium was renewed twice weekly. Maintenance medium was removed, cells were washed twice with PBS -/- and pre-induction medium was added. After 72 h, cells were incubated with control substances (flumazenil (10µM), omeprazole (25µM), rifampicin (25µM), phenobarbital (750µM) or CITCO (1µM)) or one of the 16 test substances at two concentrations (1µM and 10µM) in serum-free induction medium (William's medium E supplemented with 5 µg/ml insulin, 25 µg/ml hydrocortisone, GlutaMAX, 100 units/ml penicillin, 100µg/ml streptomycin) for 48h with renewal of compounds after 24h. Compounds were dissolved in DMSO and induction medium with a final DMSO concentration of 0.1%.

Gene expression analysis

After the treatment, cells were washed twice with PBS -/- and further processed with the QuantiGene Sample Processing kit for cultured cells according to the

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manufacturer's instructions. Induction of CYP1A2, CYP2B6, CYP2C9, CYP3A4 and CYP2C19 was measured using the QuantiGene Plex Gene Expression Assay kit according to the manufacturer's instructions. For the measurement, three biological replicates were combined, and measurements were performed in triplicates. The assay was run on a Luminex 200 (Luminex Corporation, Austin, TX) instrument.

PXR reporter gene assay

The human PXR reporter assay uses reporter cells which express a hybrid form of human PXR. The PXR DNA binding domain has been replaced by the GAL4 DNA binding domain and GAL4 is used as a reporter gene. The assay was performed according to the manufacturer's instructions. In brief, reporter cells were thawed and transferred into pre-warmed Cell Recovery Medium. 200µl of cell suspension were dispensed per well and cells were pre-incubated for 5 hours at 37°C and 5% CO_2. After pre-incubation, medium was removed, and cells were treated with 200µl of compound in Compound Screening Medium at the concentrations mentioned above. Cells were incubated for 24 hours at 37°C and 5% CO_2. Luciferase Detection Reagent was prepared, media was removed and 100µl of Luciferase Detection Reagent was added to each well. After 5 minutes of incubation at RT, luminescence was measured using the Synergy 2 (BioTek Instruments GmbH, Germany).

Data analysis

Statistical analysis was performed using GraphPad Prism version 8.2.0. Results are shown as mean ± standard deviation (SD) unless stated otherwise. To test for significant differences between CYP induction after treatment with control substances

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in different cell lines, a two-way ANOVA followed by Bonferroni post-test correction was performed. A compound was classified as an inducer, if induction was >2-fold and if it was >20% of the induction observed with the respective control substance as recommended by the FDA (Food and Drug Administration, 2020).

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RESULTS

Basal gene expression of CYP3A4, 2B6 and 1A2

First, we evaluated the effects of NHR KOs on basal CYP expression. The basal CYP3A4 expression was increased by 11-fold and 10-fold in CAR as well as PXR KO cells, respectively, while a reduction was observed in the dKO (10-fold, Table 2, Figure 1B). In contrast to CYP3A4, the basal expression of CYP2B6 was only marginally changed in CAR KO and dKO cells, whereas it was increased by around 9-fold in PXR KO (Figure 1D). Basal CYP1A2 expression decreased by approximately 10-fold in CAR KO cells but was only marginally affected in other KOs (Figure 1F).

Induction of CYP3A4, CYP2B6 and CYP1A2 by prototypical CYP inducers

The PXR ligand rifampicin induced CYP3A4 strongly in control HepaRG cells (20-fold) (Figure 1A). The dual PXR and CAR activator phenobarbital also showed strong induction (17-fold) and the aryl hydrocarbon receptor (AhR) activator omeprazole caused a weaker induction (4-fold). No CYP3A4 induction was observed by the specific CAR activator CITCO. In CAR KO cells the induction pattern was similar with the exception that CITCO marginally induced CYP3A4 expression (2.4-fold). In stark contrast, CYP3A4 induction by rifampicin, omeprazole, phenobarbital and CITCO was strongly reduced in PXR KO and dKO cells (Figure 1A).

CYP2B6 expression in HepaRG control cells was induced by all four prototypical inducers (Figure 1C). Omeprazole and phenobarbital induced CYP2B6 expression in CAR KO cells with similar magnitude compared to controls, whereas rifampicin induction was significantly higher (21-fold, p-value <0.0001). No induction was

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observed using the CAR inducer CITCO as expected. In PXR KO cells, CYP2B6 induction patterns were similar to control cells with the exception that induction by the PXR activator rifampicin was lost. As expected, none of the prototypical compounds induced CYP2B6 in dKO cells.

In contrast to CYP3A4 and CYP2B6, CYP1A2 expression was induced by the AhR ligand omeprazole in all cell lines tested (Figure 1E). Notably, this induction was significantly higher in PXR and CAR KO cells compared to controls (CAR KO: 23-fold, p-value <0.0001; PXR KO: 33-fold, p-value <0.0001). CITCO also induced CYP1A2 in all cell lines but to a lower degree than omeprazole.

Induction of CYP3A4 and CYP2B6 in HepaRG cells by test compounds

To evaluate the relative importance of the NHRs CAR and PXR in CYP induction, expression of CYP3A4 and CYP2B6 was measured after treatment with 16 test compounds. All test compounds had been previously shown to induce CYP3A4 expression in control HepaRG by at least 2-fold (data not shown). All compounds, except for MSC2, induced by more than 20% of the rifampicin induction with at least one of the applied concentrations.

The CYP3A4 induction was driven by PXR for more than half of the compounds. These compounds showed induction above 2-fold in the CAR KO but did not induce in the PXR or dKO. (Figure 2A). Most of these compounds also induced CYP2B6 at least 2-fold in the HepaRG control cells. No induction of CYP2B6 was observed in either the PXR KO or dKO HepaRGs for any of the compounds (Figure 2C).

Four compounds were indicated to induce CYP3A4 not only via PXR but also via CAR. All four compounds induced CYP3A4 in the HepaRG control cells, the CAR KO

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as well as the PXR KO by at least 2-fold. Induction was not seen in the dKO (Figure 3A). A similar picture was observed for MSC9 and MSC12 for CYP2B6 expression. MSC8 did not induce in the control cells and MSC13 did not induce in the CAR KO or in the PXR KO (Figure 3C).

Three out of 16 compounds induced CYP3A4 by at least 2-fold in the control cells and in the dKO. MSC11, a selective kinase inhibitor, showed a similar pattern across all cell lines indicating the induction was independent of PXR as well as CAR (Figure 4A). Sunitinib showed clear reduction of CYP3A4 induction in the PXR as well as CAR KO compared to control cells, indicating these NHRs were important to the induction (Figure 4A). Sunitinib also showed induction in the dKO, different from the more kinase selective Sunitinib analogues which did not show significant induction in this cell line (Figure 5). However, one of the analogues, MSC2 did result in CYP3A4 induction in the dKO just above 2-fold, but not in the control cells, making deconvolution of the mechanism difficult. Sunitinib also induced CYP2B6 at least 2-fold in all KOs and control cells, whereas the other compounds did not induce CYP2B6 in the dKO. MSC2 induced CYP2B6 in control cells but not in any of the NHR KOs (Figure 4C).

Comparison of CYP3A4 induction between reporter gene assay and HepaRG cell lines

To evaluate the ability of a PXR reporter gene assay to use as an approximation for direct PXR activation upon drug treatment, all 16 compounds were tested. The reporter gene expression was at least 2-fold higher compared to the DMSO control treatment and 20% of the induction caused by rifampicin for 7 of the compounds.

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Furthermore, four test compounds resulted in at least a 2-fold higher expression of the reporter gene compared to the control treatment. Five out of 16 test compounds (MSC12, MSC6, MSC7, MSC11, MSC10) did not result in any change in reporter gene expression (Figure 6, 7). These compounds did however induce CYP3A4 expression in HepaRG control cells. Further, apart from MSC11, all of these compounds showed decreased induction in the HepaRG PXR KO compared to control cells, indicating a PXR dependent induction mechanism. 11 out of 15 compounds which were classified as PXR or PXR and CAR inducers using the HepaRG KO cell lines, did result in a change in reporter gene expression. From the two compounds which were not classified as PXR inducers, MSC11 did not result in a change of reporter gene expression, whereas MSC2 did (Figure 7). There was no correlation between induction of CYP3A4 in HepaRG control cells and fold-change of PXR activation in the PXR reporter gene assay (Data not shown).

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DISCUSSION

PXR is the most common NHR involved in inducing DDIs through the induction of CYP3A4 (Moore & Kliewer, 2000; Sinz, 2013). CAR has also been demonstrated to contribute to CYP3A4 induction, although it is generally assumed to be more relevant for the induction of CYP2B6 (Faucette et al., 2006; Pascussi et al., 2003). Other pathways, indirectly affecting these NHRs, such as inhibition of the ERK pathway have also been reported to influence CYP induction (Hendriks et al., 2020). In this study we used the ZFN-targeted PXR KO and CAR KO HepaRG cell lines previously characterized by Williamson et al. (2016) as well as the novel PXR/CAR dKO HepaRG cells to deconvolute induction pathways for a set of compounds representative for oncology drug discovery.

Two concentrations (1 and 10 µM) were tested for CYP induction in order to evaluate the DDI risk as applied in drug discovery. Using this approach, it was possible to identify compounds with a CYP induction liability and their tentative mechanisms in a dose dependent manner. This approach does not allow for establishing EC50 and Emax values, required for a more refined assessment of the DDI risk, which is of particular value when taking the therapeutic dose in physiologically based pharmacokinetic modelling into account (Almond et al., 2016). Obtaining estimates of these more refined endpoints is frequently challenging in oncology drug development as many compounds suffer from poor solubility and/or pronounced cell toxicity at the higher concentrations required to establish the Emax.

Reference compounds were used in this study to validate the HepaRG NHR KOs as tools to deconvolute mechanisms of CYP induction (Figure 1). The selective CAR ligand CITCO showed no CYP2B6 induction in CAR KO cells. Since CITCO is only

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activating CAR but not PXR, loss of CYP2B6 induction in CAR KO cells was expected. Induction of CYP3A4 and CYP2B6 was observed using the dual PXR/CAR activator phenobarbital in CAR (9-fold and 14-fold respectively) or PXR KO (3-fold and 15-fold respectively) cells, which is in accordance with literature (Faucette et al., 2007; Hariparsad et al., 2004). In aggregate, these results confirm the ability of phenobarbital to activate both CAR and PXR (Sueyoshi et al., 1999). The selective PXR activator rifampicin (LeCluyse, 2001) showed no induction of CYP3A4 or CYP2B6 in PXR KO cells but retained CYP3A4 induction in dKO cells (3-fold). The finding in the dKO may be explained by relatively low basal CYP3A4 expression in this cell line resulting in an elevated inducibility, thereby increasing the sensitivity to non-NHR dependent pathways (Table 2, Figure 1B). These findings were in general in accordance to previous published work (Li et al., 2015; Williamson et al., 2016) confirming that deconvolution of CYP induction pathways using HepaRG NHR KOs is possible.

The inductive capacity of CYP3A4 was significantly reduced in CAR (p-value <0.0001) and PXR (p-value <0.0001) KO cells compared to control cells (Figure 1A). Aside from loss of inductive pathways caused by the KO of NHRs, comparably high basal expression in these cell lines also contributed to a reduced induction by phenobarbital and rifampicin (Table 2, Figure 1B). In contrast, basal CYP3A4 expression was low in dKO cells compared to other cell lines (Table 2, Figure 1B), consequently a modest increase in inductive capacity of CYP3A4 was observed. This was illustrated by the induction of rifampicin in dKO cells while no induction was observed in the PXR KO (Figure 1). In fact, reduction in basal expression caused by KOs resulted in a higher inductive capacity of the affected CYPs. Similar effects have been observed in 3D cultures of PHH and *in vivo* where basal expression of CYP3A4

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has been reported to be substantially higher compared to 2D monolayers and, consequently, the fold-change of induction was much lower (Hendriks et al., 2020). In contrast to HepaRG KO cells, PXR knockdown in PHH spheroids resulted in reduced basal expression of CYP3A4. *In vivo* studies in $PXR^{-/-}$ mice showed conflicting results, Staudinger et al showed increased basal levels of Cyp3a11 mRNA compared to wildtype mice (Staudinger et al., 2001). A similar study by Xie et al showed no effect on Cyp3a11 expression (Xie et al., 2000). Hence, the effects of PXR knockdown may differ between *in vitro* systems and possibly also *in vivo*, the underlying factors for the observed differences remains to be elucidated. Interestingly, CYP2B6 inducibility by PXR activators was increased in CAR KO cells compared to HepaRG controls. However, basal expression of CYP2B6 in CAR KO cells was not reduced compared to control cells (Figure 1D, Table 2). In contrast, knockdown of CAR in PHH spheroids resulted in a reduction of CYP2B6 expression (Hendriks et al., 2020). Further, CYP1A2 induction upon omeprazole treatment was more pronounced in the PXR KO compared to the CAR KO cells. However, the basal expression was lower in CAR KO compared to PXR KO cells. This indicated that basal expression alone was not sufficient to explain the differences seen in the magnitude of induction across the KOs.

CYP2B6 induction was driven by PXR alone or together with CAR. For two compounds (MSC8 and MSC12), deconvolution of the pathway using HepaRG NHR KO cells was difficult, since either no induction or only minor induction was achieved in HepaRG control cells. Sunitinib induced CYP2B6 in the dKO cells by at least 2-fold with one of the applied concentrations. Since basal expression and therefore inductive capacity of CYP2B6 in dKO cells was similar to control cells, this suggests a PXR and CAR independent mechanism. CYP2B6 induction was shown to be

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dependent on kinase activities in rat hepatocytes but the cellular targets are not completely identified yet (Joannard et al., 2000). For the kinase inhibitors sunitinib, CAR involvement was excluded. Other cellular targets of kinases might be the reason for the induction of CYP2B6 in the dKO HepaRG. To identify these targets, further investigations are needed.

PXR has been reported to be responsible for the majority of CYP3A4 induction observed for marketed drugs in *in vitro* systems (Shukla et al., 2011). This study confirmed these findings. The CYP3A4 induction was significantly reduced in PXR KO cells for 15 of the 16 compounds studied. However, 4 compounds (25%) were identified as inducers of CAR in addition to PXR. The fact that all of these were also induced by PXR supports previous reports that CAR often acts as a co-inducer together with PXR (Faucette et al., 2007). CAR as a sole driver for the CYP3A4 induction was previously only observed for a few compounds including CITCO (Maglich et al., 2003; Wang et al., 2004).

Surprisingly, three compounds were found to induce CYP3A4 also in dKO HepaRG cells, suggesting CAR and PXR independent mechanisms (Sunitinib, MSC11, MSC2). MSC11, a relatively selective kinase inhibitor strongly inhibiting tropomyosin receptor kinase A (TRKA), gave similar degrees of induction in all cell lines (Figure 4A). Other marketed TRK inhibitors, like entrectinib and larotrectinib, have been shown to induce CYP3A4 *in vitro* but also inhibit CYP3A4 activity, resulting in a less clinically relevant induction (Food and Drug Administration, 2018, 2019). However, as HepaRG cells do not express TRKA (data accessible at NCBI GEO database (Kohara et al., 2020), accession GSE146023), CYP3A4 induction by MSC11 does not appear to be mediated through TRKA. Given the structural similarity and binding

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mode among the TRKA inhibitors, it is plausible that they also share an interaction with other proteins relevant to the expression of CYP3A4.

Sunitinib induced CYP3A4 in all cell lines but the induction was greatly attenuated in CAR and PXR KO cells suggesting that these NHRs were important in the induction process. Induction was also observed in dKO cells, suggesting additional NHR-independent mechanisms. Interestingly, the close O-methyl analogue, MSC13 and the structurally related compounds (MSC14 and MSC2), with improved kinase selectivity, did not induce CYP3A4 in the dKO (Figure 2-4). Sunitinib is a multi-kinase inhibitor inhibiting more than 50% of the kinome, the differences in kinome selectivity may indicate that one or several kinases contribute to the induction process in this system (Figure 5). Kinase inhibition has been shown to affect CYP3A4 expression previously, albeit by interactions through NHRs via the MAP/ERK pathway (Hendriks et al., 2020). This particular pathway appears less relevant as the NHRs were not present in the dKO but other mechanism related to kinase inhibition may still be of relevance.

MSC2, a selective tyrosine-protein kinase KIT (cKit) inhibitor, did not induce CYP3A4 in control cells. This made further interpretation of the findings in the KO cell lines difficult given the differences in basal expression and inducibility.

Risk assessment of CYP3A4 induction using a PXR reporter gene assay was routinely conducted in pharmaceutical industry (Chu et al., 2009). However, this approach assumes direct binding to PXR to be the main driver of CYP3A4 induction. The 16 compounds in the test-set, representing a typical chemical space of small molecule drug candidates in oncology, highlight some of the issues with this strategy. Only seven (43 %) of the compounds were clearly identified as carrying a CYP3A4

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induction liability defined as >2-fold induction as well induction of at least 20% of rifampicin (European Medicines Agency, 2012; Food and Drug Administration, 2020). In addition, another 4 compounds (25%) showed induction above two-fold but less than 20% of the positive control. The remaining five compounds (31%) showed no signs of induction of CYP3A4 in the PXR reporter gene assay (MSC12, MSC6, MSC7, MSC11 and MSC10, Figure 6 and 7). The reason for the discrepancy between the systems may be explained by multiple factors. Earlier reports have described the correlation between reporter gene assay and more complex systems as poor. Especially a poor correlation to Emax has been reported, resulting in a lower sensitivity of the reporter gene assay (McGinnity et al., 2009, Hariparsad et al., 2008). This may explain why compounds with significant induction in the HepaRG model were not identified in the reporter gene assay. Only direct PXR interaction was quantified in the reporter gene system, this may explain why MSC12 was not detected and MSC8 and MSC9 were close to the detection limit, as these compounds also had a CAR component in their ability to induce CYP3A4. Further, the induction of MSC11 in HepaRG was shown to be independent of direct PXR activation. Hence, detection in the reporter gene assay was not to be expected.

Induction mechanisms of MSC6, MSC7 and MSC10 appear more complex as the HepaRG KO systems indicated strong induction driven by PXR alone, yet they were not detected in the reporter gene assay. These findings may relate to metabolites that activate PXR and consequently induce CYP3A4. Desai et al. showed that the tamoxifen metabolite 4-hydroxytamoxifen, is a very potent activator of PXR, while tamoxifen itself is only a weak activator (Desai et al., 2002). However, the fact that close analogues to MSC6 and MSC7 in the same chemical series showed induction (Table 1) speaks against this explanation. Another explanation could be that this

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group of compounds act via indirect mechanism on PXR via systems not expressed in the reporter gene assay. Especially, binding to other sides than the ligand-binding domain and the phosphorylation status of PXR, might be responsible for PXR activation (Mackowiak & Wang, 2016). Such indirect activation is not captured by the PXR reporter assay used in this study nor in standard PXR binding assays. Both systems exclusively detect binding to the ligand-binding domain. Zhu et al. compared a binding assay with a transactivation assay and found that some compounds showed tight binding but low transactivation (Zhu et al., 2004). Hence, further complexities may arise translating binding data to function. In fact, the compounds tested in this study might bind to PXR. However, binding alone would not be captured by HepaRG KO cells. Therefore, the results from the HepaRG KO cells were compared to the functional readout of a PXR reporter assay. A PXR reporter gene assay with full length NHRs may detect also indirect PXR activators which was demonstrated by Pinne et al. with several kinase inhibitors (Pinne et al., 2017). However, the signaling of indirect activation via kinases may require more physiological systems. Mitogen-activated protein kinase/extracellular signal-regulated kinase pathway inhibitors was for example reported to only be detected in spheroids (Hendriks et al., 2020). In addition, for all compounds, it is possible that the compounds classified as false negatives in the reporter gene assay would have shown signs of induction at higher concentrations provided adequate solubility.

Overall, drug mediated CYP induction is compound specific and mechanistically complex. Thus, the use of simplified reporter assays that measure direct PXR activation exclusively harbors the inherent risk of failing to detect other pathways. PXR and/or CAR KO HepaRG provide a new tool to identify the contribution of these NHRs to CYP induction. Furthermore, the use of dKO cells allows the identification of

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compounds which induce CYPs independent of CAR and PXR. The detection of such pathways may trigger follow-up investigations to determine the exact mechanism of induction. This study indicates that such interactions may be of special relevance in the oncology space as the mechanism of action frequently goes via the kinome or other pathways central to cell proliferation. Finally, we conclude that NHR KO HepaRG cells provide an accurate and scalable complement to increase the understanding of CYP induction mechanisms.

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AUTHORSHIP CONTRIBUTION

Participated in research design: Hewitt, Petersson, Georgi and Preiss

Conducted experiments: Preiss and Rui

Performed data analysis: Petersson, Lauschke, Badolo, Preiss and Rui

Wrote or contributed to the writing of the manuscript: Petersson, Lauschke, Badolo, Georgi, Thompson and Preiss

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FOOTNOTES

Lena C. Preiss, Ruoqi Liu, Philip Hewitt, David Thompson, Carl Petersson, Lassina Badolo and Katrin Georgi were employed either by Merck KGaA or its one of its subsidiaries when this study was conducted. Some of the products/cell lines discussed in this publication are sold by a subsidiary of Merck KGaA, Millipore Sigma.

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FIGURE LEGENDS

Figure 1 Basal expression and induction response following treatment with control substances of HepaRG cells. CYP3A4 (A), CYP2B6 (C) and CYP1A2 (E) induction in HepaRG PXR, CAR and dKO cells was compared to HepaRG control cells after treatment with 25µM omeprazole, 25µM rifampicin, 750µM phenobarbital or 1µM CITCO. Basal expression of CYP3A4 (B), CYP2B6 (D) and CYP1A2 (F) are shown as fold-change compared to control cells. Results are expressed as mean + SD. ns $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ Two-Way ANOVA test compared to control cells.

Figure 2 Relative and basal expression of CYP3A4 (A, B) and CYP2B6 (C, D) in control HepaRG (red bars), CAR KO HepaRG (green bars), PXR KO HepaRG (blue bars) and dKO HepaRG (pink bars) of PXR activators. Values were normalized to the housekeeping genes HPRT1 and TFRC. Cells were treated with 1µM (light color) or 10 µM (dark color) of test compound. The black dotted line marks the 2-fold cut off, the light gray dotted line 20% of the control treatment. Results are expressed as mean + SD.

Figure 3 Relative and basal expression of CYP3A4 (A, B) and CYP2B6 (C, D) in control HepaRG (red bars), CAR KO HepaRG (green bars), PXR KO HepaRG (blue bars) and dKO HepaRG (pink bars) of PXR and CAR activators. Values were normalized to the housekeeping genes HPRT1 and TFRC. Cells were treated with 1µM (light color) or 10 µM (dark color) of test compound. The black dotted line marks

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the 2-fold cut off, the light gray dotted line 20% of the control treatment. Results are expressed as mean + SD.

Figure 4 Relative and basal expression of CYP3A4 (A, B) and CYP2B6 (C, D) in control HepaRG (red bars), CAR KO HepaRG (green bars), PXR KO HepaRG (blue bars) and dKO HepaRG (pink bars) of compounds with other mechanisms involved. Values were normalized to the housekeeping genes HPRT1 and TFRC. Cells were treated with 1µM (light colour) or 10 µM (dark colour) of test compound. Data could not be obtained for the 10µM treatment with Sunitinib. The black dotted line marks the 2-fold cut off, the light gray dotted line 20% of the control treatment. Results are expressed as mean + SD.

Figure 5 Sunitinib and its structural analogues. Chemical structures and waterfall plots of target inhibition in % for sunitinib, MSC14, MSC13 and MSC2 are shown. Waterfall plots were generated using the Reaction Biology Kinase Waterfall Charts version 1.31 (Merck KGaA).

Figure 6 Relative PXR activation upon treatment with 10µM flumazenil, 25µM omeprazole, 25µM rifampicin, 750µM phenobarbital or 1µM CITCO and 1µM or 10µM of 17 test compounds. PXR activation was measured using a PXR reporter gene assay and results are expressed relative to activation upon treatment with 0.1% DMSO. Compounds were categorized into three groups according to the results in HepaRG NHR KO cells.

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Figure 7 Venn diagram of MSCs inducing CYP3A4 at 10μM through CAR (green circle), PXR (blue circle) or other mechanisms (purple circle) or activating PXR in the PXR reporter gene assay (grey circle). Induction mechanisms (CAR, PXR, others) were classified using HepaRG NHR KO cells. *deconvolution of induction mechanism difficult

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Table 1 Compound list including compound ID, physicochemical properties (logP, pKa, ion class, target and NHR activation according to CYP3A4 induction in HepaRG NHR KO cells.

ID	logP	pKa	Ion Class	Target	Structural Group	NHR Activation
MSC1	4.5	13.7	N	Lipid synthesis	A	PXR
MSC2	2.7	-	N	cKit	D	Others
MSC3	3.7	14.7	N	Lipid synthesis	A	PXR
MSC4	3.9	14.7	N	Lipid synthesis	A	PXR
MSC5	3.7	13.7	N	Lipid synthesis	A	PXR
MSC6	2.2	14.9	N	DNA-PK	E	PXR
MSC7	3.6	14.5	N	cKit	D	PXR
MSC8	3.9	14.5	N	Lipid synthesis	B	CAR + PXR
MSC9	3.9	13.7	N	Lipid synthesis	A	CAR + PXR
MSC10	3.3	14	N	Lipid synthesis	B	PXR
MSC11	2.1	-	N	ISR (Kinase)	G	Others
MSC12	2.3	11.2	N	Lipid synthesis	A	CAR + PXR
MSC13	3.9	9.2	B	cKit	C	CAR + PXR
MSC14	3.8	-	N	cKit	D	PXR
MSC15 (Sunitinib)	4.2	9.2	B	cKit	C	CAR + PXR; Others
MSC16	2.9	-	N	DNA repair	F	PXR

N = neutral, B = basic; [1] chemical series 1 within the target group, [2] chemical series 2 within the target group

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Table 2 Relative gene expression of CYP3A4, CYP2B6 and CYP1A2 in HepaRG control cells, CAR KO, PXR KO and dKO cells. Expression is normalized to the housekeeping genes HPRT1 and TFRC and mRNA levels are shown relative to control cells. Data is shown as means of three biological replicates ± SD.

	Control	**CAR KO**	**PXR KO**	**dKO**
CYP3A4	1.0	11.5 ± 1.7	10.0 ± 1.9	0.1 ± 0.09
CYP2B6	1.0	0.9 ± 0.2	8.7 ± 0.3	1.2 ± 0.4
CYP1A2	1.0	0.1 ± 0.05	0.6 ± 0.3	0.9 ± 0.2

Figure 1

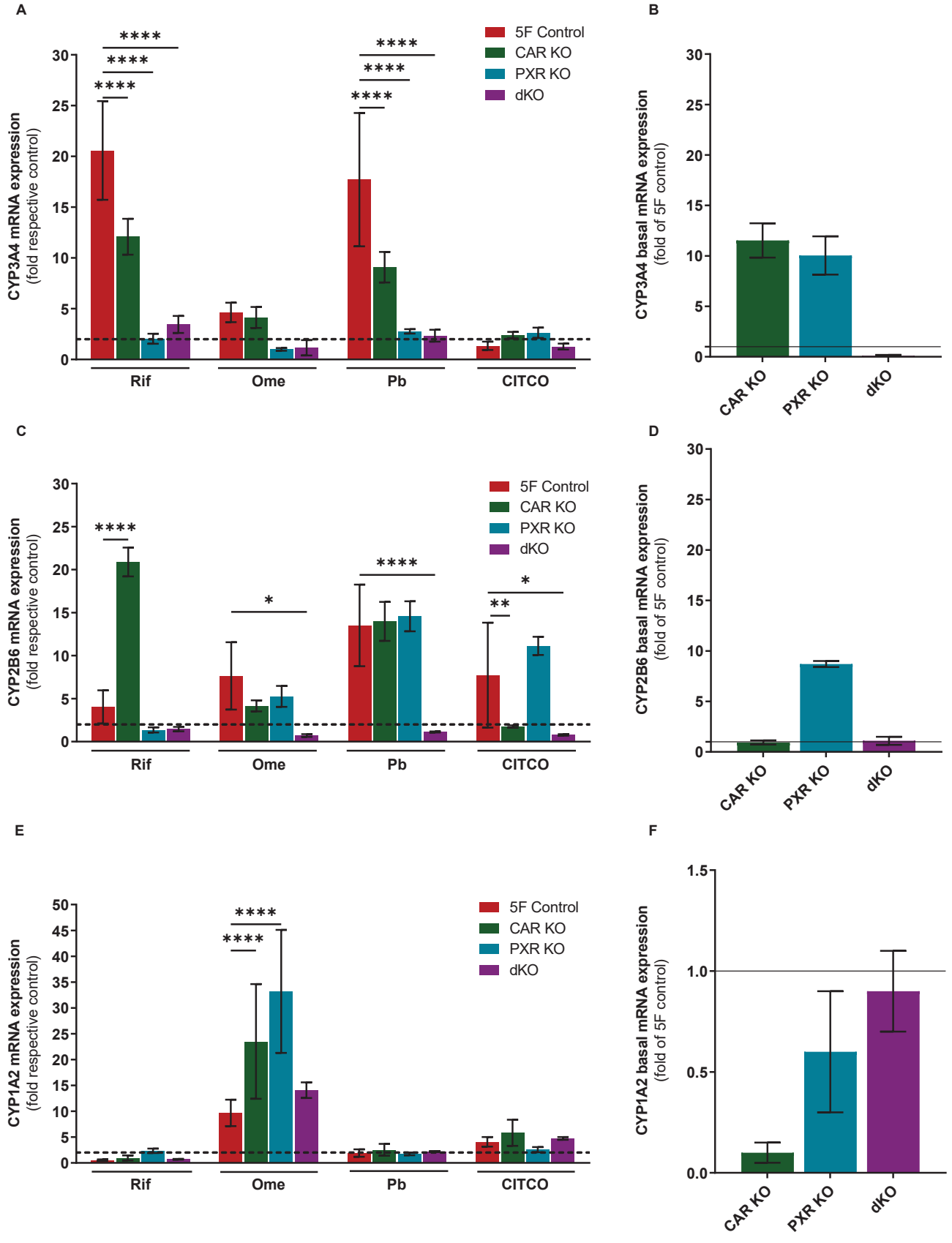

Figure 2

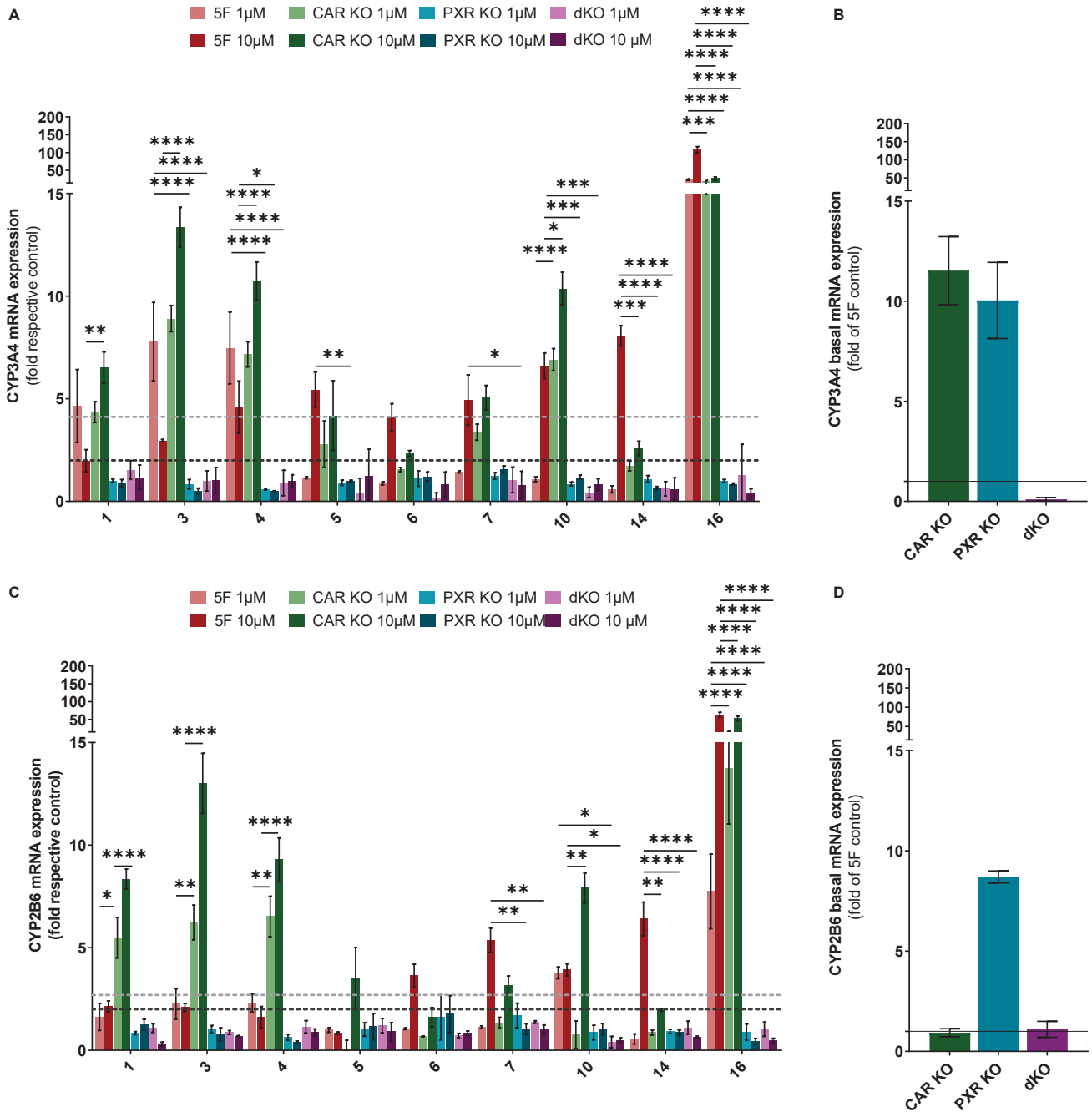

Figure 3

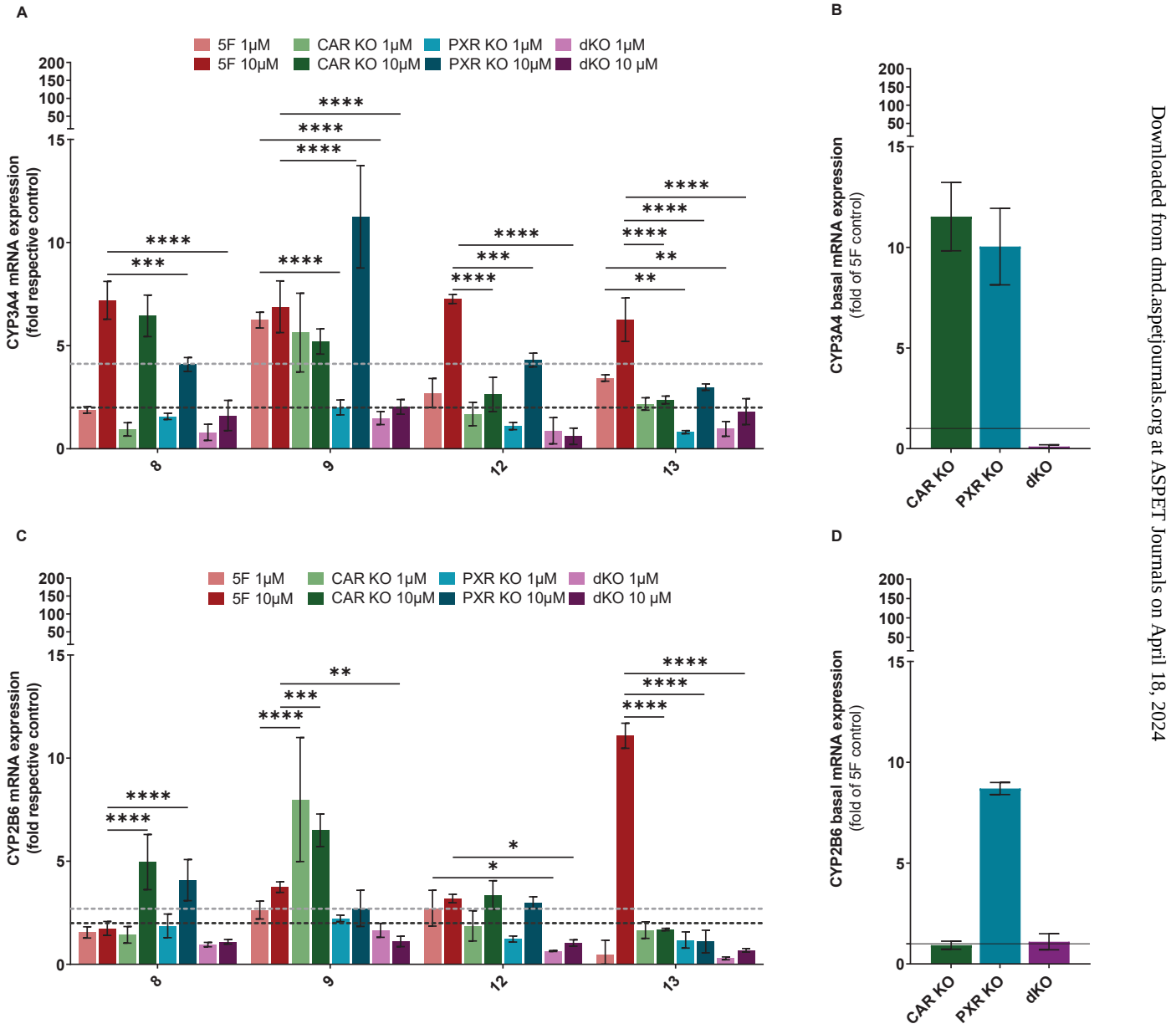

Figure 4

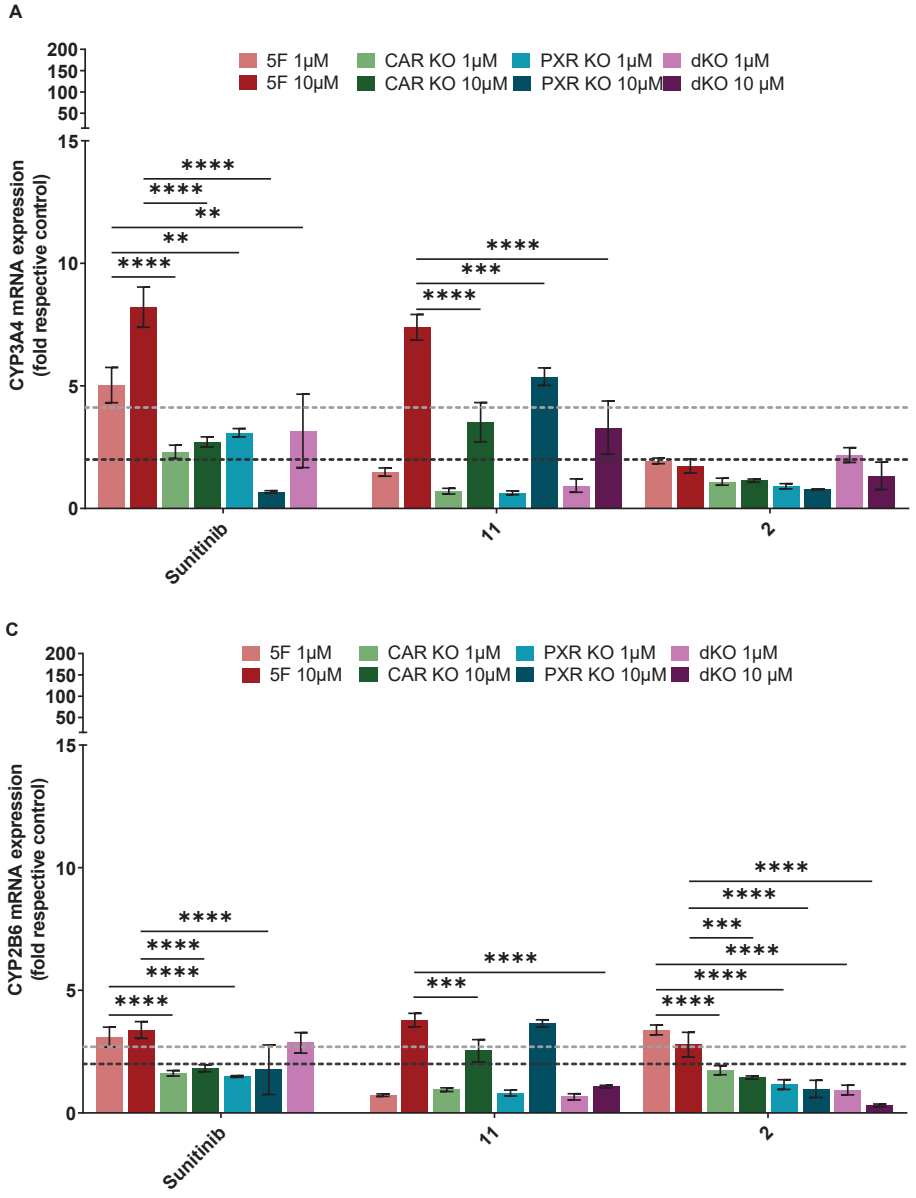

Figure 5

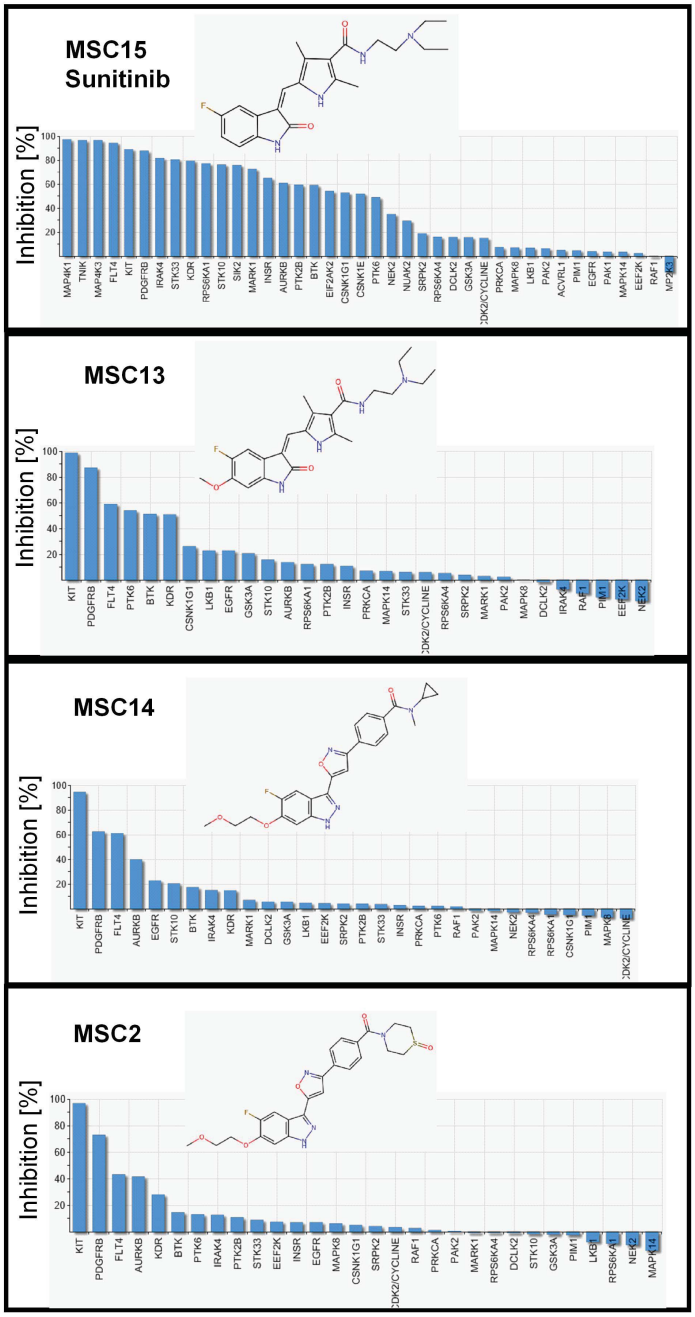

Figure 6

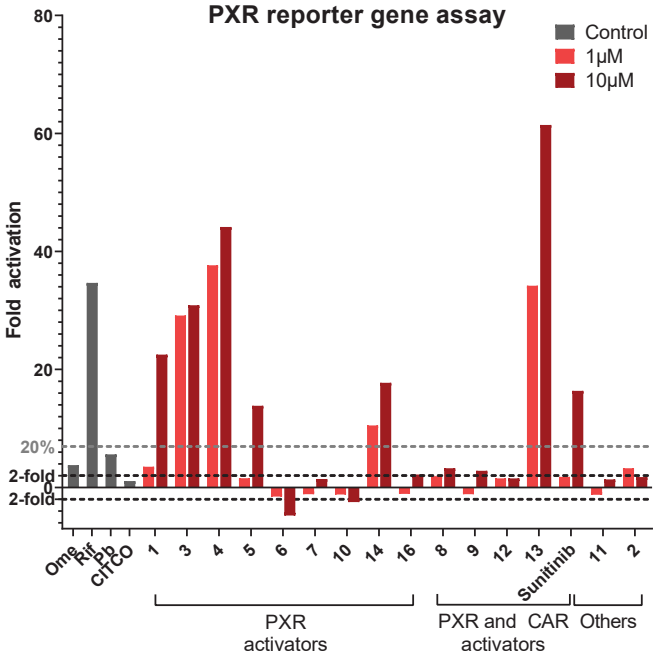

Figure 7