Clinically Relevant Cytochrome P450 3A4 Induction Mechanisms and Drug Screening in Three-Dimensional Spheroid Cultures of Primary Human Hepatocytes

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Cytochrome P450 (CYP) 3A4 induction is an important cause of drug–drug interactions, making early identification of drug candidates with CYP3A4 induction liability in drug development a prerequisite. Here, we present three-dimensional (3D) spheroid cultures of primary human hepatocytes (PHHs) as a novel CYP3A4 induction screening model. Screening of 25 drugs (12 known CYP3A4 inducers in vivo and 13 negative controls) at physiologically relevant concentrations revealed a 100% sensitivity and 100% specificity of the system. Three of the in vivo CYP3A4 inducers displayed much higher CYP3A4 induction capacity in 3D spheroid cultures as compared with in two-dimensional (2D) monolayer cultures. Among those, we identified AZD1208, a proviral integration site for Moloney murine leukemia virus (PIM) kinase inhibitor terminated in phase I of development due to unexpected CYP3A4 autoinduction, as a CYP3A4 inducer only active in 3D spheroids but not in 2D monolayer cultures. Gene knockdown experiments revealed that AZD1208 requires pregnane X receptor (PXR) to induce CYP3A4. Rifampicin requires solely PXR to induce CYP3A4 and CYP2B6, while phenobarbital-mediated induction of these CYPs did not show absolute dependency on either PXR or constitutive androstane receptor (CAR), suggesting its ability to switch nuclear receptor activation. Mechanistic studies into AZD1208 uncovered an involvement of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway in CYP3A4 induction that is sensitive to the culture format used, as revealed by its inhibition of ERK1/2 Tyrosine 204 phosphorylation and sensitivity to epidermal growth factor (EGF) pressure. In line, we also identified lapatinib, a dual epidermal growth factor receptor/human epidermal growth factor receptor 2 (EGFR/HER2) inhibitor, as another CYP3A4 inducer only active in 3D spheroid culture. Our findings offer insights into the pathways involved in CYP3A4 induction and suggest PHH spheroids for preclinical CYP3A4 induction screening.

What is the current knowledge on the topic?
✔ Drug interactions are very important clinically, and drug-mediated inhibition or induction of cytochrome P450 (CYP) 3A4 should be avoided. Current in vitro systems for prediction of drug-mediated induction of CYP3A4 in vivo have failed in many cases.

What question did this study address?
✔ We used three-dimensional (3D) spheroid cultures of primary human hepatocytes (PHHs) as a novel CYP3A4 induction screening model and made a validation using known in vivo inducers of CYP3A4. We investigated mechanisms by which drugs indirectly activate pregnane X receptor (PXR) and induce CYP3A4 expression in 3D spheroid culture.

What does this study add to our knowledge?
✔ We show a higher capacity for prediction of CYP3A4 induction in PHH 3D spheroids as compared with two-dimensional (2D) monolayer cultures. We identified an atypical mechanism of CYP3A4 induction caused by indirect activation of PXR linked to the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway.

How might this change clinical pharmacology or translational science?
✔ We propose that 3D spheroid cultures of PHHs should be a more useful and sensitive drug development tool to screen for CYP3A4 induction than the currently used in vitro models and would be a useful asset for mechanistic studies into CYP3A4 induction.
Drug–drug interactions (DDIs) are an important issue that can result in undesired therapeutic responses and adverse drug reactions.1 Many clinically significant DDIs are related to interference with cytochrome P450 (CYP) enzymes, partly due to drug-drug-gene interactions.2,3 CYP3A4 inhibition but also induction is of particular importance as this enzyme metabolizes over half of all clinically used small molecule drugs.4 Addressing the liability of drug candidates to induce CYP3A4 early in drug development is thus necessary to reduce costs and enhance patient safety.

CYP induction patterns vary widely among different species,5 partially explained by differences in the structures of the ligand-binding domains of the nuclear receptors, pregnane X receptor (PXR) and constitutive androstane receptor (CAR).6 A classic example is rifampicin, which is a potent PXR-mediated inducer of CYP3A4 in humans but does not bind mouse PXR and consequently does not induce Cyp3a11, the mouse orthologue of CYP3A4.7 Hence, CYP induction screening evidently requires human-based models. Cultures of primary human hepatocytes (PHHs) are currently the recommended model by regulatory agencies to preclinically screen for CYP induction.8–10 In current practice, two-dimensional (2D) monolayer cultures of PHHs are the choice of culture model to execute CYP induction studies.11 However, PHHs cultured as 2D monolayers rapidly lose important hepatic functions and widespread deregulation of important cellular pathways manifests quickly after seeding.12,13 Among others, basal messenger RNA (mRNA) levels of key nuclear receptors and CYP enzymes rapidly decline already during the first 24 hours of culture.13 The impact of hepatocyte dedifferentiation for CYP induction has not been fully investigated, but raises concerns as to the CYP induction predictive power of PHH 2D monolayers.

A notable example of the current limited in vitro–to–in vivo translational accuracy of CYP induction patterns is the case of AZD1208. This proviral integration site for Moloney murine leukemia virus (PIM) kinase inhibitor candidate, developed for the treatment of acute myeloid leukaemia,14 showed no signs of CYP3A4 induction in vitro, in both hepatoma HepaRG cells and 2D monolayer cultures of PHHs from multiple different donors.15 In clinical trials in humans, however, a time-dependent decline in plasma concentration profiles was observed following multiple dosing of AZD1208, resulting in insufficient bioavailability. As a result, AZD1208 was terminated from development.15,16 Subsequent investigations revealed that AZD1208 induced plasma levels of 4β-hydroxycholesterol, an endogenous biomarker for CYP3A activity, at a similar magnitude as rifampicin.15,16 In vitro studies indicated that phase I metabolism of AZD1208 is predominantly carried out by CYP3A4,15 and due to its CYP3A4 induction capacity in vivo leads to autoinduction of its own metabolism. The reason for lack of evidence of this atypical, yet clinically relevant, mechanism of CYP3A4 induction in the employed 2D cultures of HepaRG cells and PHHs is currently unknown.

In recent years, there has been increasing interest in the development of more physiologically relevant in vitro models with the knowledge that tissue architecture is an important determinant of phenotype.17,18 Various approaches, including three-dimensional (3D) bioprinting,19 organ-on-a-chip,20 and bioreactor cultures,21 have demonstrated prolonged hepatocyte viability and improved hepatic functionality important for drug toxicity testing. We previously developed and characterized a 96-well 3D spheroid model of PHHs cultured in chemically defined, serum-free media.22 In this model, PHHs display molecular phenotypes similar to adult human liver tissue, and crucial hepatocyte functions, such as albumin secretion and drug-metabolizing activities, are stably maintained for several weeks in culture.22,23 These features have demonstrated their use in assessing drug hepatotoxicity, including those that are delayed in onset.22,24–26 Characterization and evaluation of such novel, more complex in vitro liver culture systems for prediction of drug-mediated CYP induction is currently awaited. Here, we present PHH 3D spheroids as a novel sensitive screening platform for clinically relevant CYP3A4 induction and provide novel insights into the mechanisms leading to CYP3A4 induction.

METHODS

Culture and treatment of primary human hepatocyte 3D spheroid cultures
Cryopreserved PHHs (lots SSR, JEL, HJK, S1070T) were obtained from Bio IVT (Westbury, NY, USA) and KaLy-Cell (Plobsheim, France). PHHs were seeded in Corning Costar 96-well ultra-low attachment plates (1,500 cells/well) to form 3D spheroid cultures as previously described.22 During spheroid formation, PHHs were maintained in 100 µL 10% fetal bovine serum–containing PHH medium (William’s E medium supplemented with 2 mM l-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 10 µg/mL insulin, 5.5 µg/mL transferrin, 6.7 ng/mL sodium selenite, and 100 nM dexamethasone; all components obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) or Thermo Fisher Scientific, Stockholm, Sweden). Spheroids were shifted to and maintained in serum-free PHH medium 4–6 days after seeding. Exposures with the test compounds (all obtained from Sigma-Aldrich except AZD1208 which was provided by AstraZeneca (Göteborg, Sweden)) were performed on day 8, and cells were harvested for downstream analyses after 72 hours of drug exposure. All compounds were dissolved in dimethyl sulfoxide (DMSO) with a final maximum concentration of 0.1% DMSO, except acetaminophen, which was dissolved directly in the culture medium.

Culture and treatment of primary human hepatocyte 2D monolayer cultures
Cryopreserved PHHs were seeded in Corning rat tail collagen type I-coated 24-well plates (125,000 cells/well) in 500 µL 10% fetal
bovine serum–containing PHH medium. After cells had attached for two hours, medium was replaced with serum-free PHH medium. Drug exposure studies were started 24 hours after seeding, and cells were harvested for downstream analyses after 48 hours of drug exposure.

**Gene knockdown experiments**

Prior to seeding the cells for spheroid formation, PHHs were mixed with a preincubated mixture of small interfering RNA (siRNA)-lipofectamine RNAiMAX (Thermo Fisher Scientific) complexes dissolved in OptiMEM (Thermo Fisher Scientific). The siRNA constructs used were obtained from Thermo Fisher Scientific: NRII2 (PXR) (s16909), NRII3 (CAR) (s19369), PIM1 (s10527), PIM2 (s21749), and PIM3 (s35946). Final siRNA concentrations were 50 nM per construct. Spheroid formation, cultivation, and drug exposures were otherwise identical to described above.

**Gene expression profiling**

Total RNA was extracted using QIAzol Lysis Reagent (Qiagen, Sollentuna, Sweden). RNA was reverse-transcribed into complementary DNA using SuperScript III reverse transcriptase (Thermo Fisher Scientific). Gene expression analysis was performed by quantitative reverse transcription polymerase chain reaction on a 7500 Fast Real-Time PCR system (Thermo Fisher Scientific) using TaqMan probes obtained from Thermo Fisher Scientific: CYP1A2 (Hs00167927_m1), CYP2B6 (Hs04183483_g1), CYP2C9 (Hs02383631_s1), CYP2D6 (Hs02576168_g1), CYP3A4 (Hs00604506_m1), NRII2 (PXR) (Hs0114267_m1), NRII3 (CAR) (Hs00901571_m1), PIM1 (Hs01065498_m1), PIM2 (Hs00179139_m1), PIM3 (Hs00420511_g1), and TBP (Hs00427620_m1). Data were analyzed using the ΔΔCt method.

**Western blotting**

Proteins were extracted using RIPA buffer containing Roche complete protease and PhosSTOP phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentration was determined by the Bradford assay, and 30 µg of protein was loaded onto a 10% precast SDS-PAGE gel (Bio-Rad, Solna, Sweden). CYP3A4 protein was detected using rabbit antihuman CYP3A4 (PAP 011, 1:5,000; CYPEx, Dundee, UK) and goat antiantibit/HRP (P0448, 1:5,000; Dako, Stockholm, Sweden). The blots were developed using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and visualized using a ChemiDoc Imaging system (Bio-Rad). For analysis of protein expression by DigiWest spheroid pellets (~100 µg protein) were processed and analyzed by NMI TT (Reutlingen, Germany), as previously described.27

**Immunofluorescence**

Spheroids were fixed with 4% formaldehyde in phosphate-buffered saline O/N at 4°C, cryoprotected in 30% sucrose in phosphate-buffered saline overnight (O/N) at 4°C, and embedded in Tissue-Tek O.C.T. compound (Sakura, Alphen aan den Rijn, The Netherlands). Spheroid cryosections (8 µm) were stained for rabbit antihuman CYP3A4 (PAP 011, 1:5,000, CYPEx, UK) using Alexa Fluor 488 donkey antirabbit immunoglobulin G (IgG) antibody (Life Technologies). Slides were mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Fluorescent images were acquired using an LSM710 confocal microscope (Zeiss, Göttingen, Germany).

**CYP3A4 enzyme activity**

PHH spheroids were incubated with a CYP probe substrate mixture containing 10 µM midazolam for 4 hours at 37°C. Subsequently, supernatants were spun and snap-frozen, and formed metabolites (i.e., hydroxymidazolam for CYP3A4) were quantified using liquid chromatography with high resolution mass spectrometry as previously described.23

**Data processing and analysis**

All data analyses, quantifications, and statistical evaluations were done in GraphPad Prism (version 7.04, La Jolla, CA, USA). Image processing was performed using ZEN lite (version 2.1, Zeiss, Göttingen, Germany) and ImageJ (Fiji) (version 1.52s). The sample sizes, and, where appropriate, statistical analyses are described in the figure legends.

**RESULTS**

PHH spheroids detect CYP3A4 inducers in vivo at clinically relevant concentrations

Here, we set out to test the sensitivity and specificity for in vivo CYP3A4 inducers of our previously described PHH 3D spheroid model.22 Based on clinical reports, we tested a panel of 24 test compounds: 11 drugs with clear evidence of inducing CYP3A4 in humans in vivo and 13 drugs with no CYP3A4 induction reports (Table S1). The experimental setup to screen for CYP3A4 induction in PHH spheroids is shown in Figure 1a. PHHs spontaneously aggregated into a compact, single spheroid of reproducible size per well in 96-well ultra-low attachment plates 4–6 days after seeding. Screening of the test compounds was initiated 8 days post seeding at physiologically relevant concentrations (at or below 1 × maximum plasma concentration (Cmax) Table S1) in serum-free medium. After a single 72-hour exposure, CYP3A4 mRNA induction was evaluated under the differently tested conditions. The prototypical inducer rifampicin most strongly induced CYP3A4 with an almost fivefold mRNA increase compared with control, a magnitude of change very similar to the reported CYP3A4 activity increase (fivefold) in humans in vivo following rifampicin exposure.28 (Figure 1b). All other positive compounds induced CYP3A4 expression at least twofold compared with control and with magnitudes similar to rifampicin (Figure 1b). All test compounds not reported to induce CYP3A4 in vivo did not induce CYP3A4 more than twofold, a criterion for classifying a compound negative for CYP3A4 induction as recommended by regulatory agencies.9,10 (Figure 1c).

We next benchmarked these findings obtained in 3D spheroid culture to the conventionally used 2D collagen monolayer culture system using PHHs from the same donor. A schematic of the experimental setup used for CYP3A4 induction screening in this culture system is shown in Figure 2a, in line with current laboratory practice and recommendations by regulatory agencies.8,10,11 PHHs were seeded in collagen-coated 24-well culture plates. After cell attachment, PHHs were maintained in serum-free medium and allowed to recover for 24 hours. Screening of the test compounds was initiated at day 1 post seeding at the same concentrations as used for PHH spheroids (1 × Cmax), and after a single 48-hour exposure, CYP3A4 mRNA induction was evaluated. This time we observed strikingly different and much more varied CYP3A4 induction responses for the different CYP3A4-inducing drugs (Figure 2b), while, like in 3D spheroid culture, the...
non-CYP3A4-inducing drugs did not induce CYP3A4 in 2D monolayer culture (Figure 2c).

Rifampicin is considered the gold standard to induce CYP3A4 and is used as a marker to relate and to further interpret the relevance of the extent of CYP3A4 induction of test compounds as recommended by regulatory agencies. Firstly, we observed that the magnitude of CYP3A4 induction exerted by rifampicin was much higher in 2D monolayer culture (35-fold) compared with the reported fold induction in vivo (fivefold) or as we previously observed in 3D spheroid culture (compare Figures 1b and 2b). We next plotted the induction capacities of the tested CYP3A4-inducing drugs relative to the rifampicin CYP3A4 induction (Figure 3a). While in 3D spheroid culture a much more homogeneous response of the extent of CYP3A4 induction was observed with all compounds inducing at levels >25% relative to rifampicin, the relative induction was much more variable in 2D monolayer culture. Most notably, phenobarbital and probenecid at Cmax levels reached induction levels of more than 50% relative to rifampicin in 3D spheroid culture, while in 2D monolayer culture induction responses were at or below 20%. The US Food and Drug Administration (FDA) recommends classifying a compound as positive for CYP3A4 induction based on both a ≥ twofold mRNA increase and a response ≥ 20% of the response of the positive control (rifampicin).10 Based on the latter recommendation, the 3D spheroid model predicted the CYP3A4-inducing capacities with 100% sensitivity and specificity, while in 2D monolayer culture, two compounds (phenobarbital and phenytoin) would have been falsely flagged as negative (80% sensitivity and 100% specificity) at 1xCmax levels (Figures 1b,c, 2b,c, 3a).

We next sought to explain the differences in the observed fold changes of CYP3A4 induction relative to control cells in the two different culture systems. Evaluation of the absolute CYP3A4 mRNA levels revealed that basal (noninduced) CYP3A4 mRNA levels were drastically reduced in 2D monolayer culture (0.55%) compared with the levels observed in 3D spheroid culture, which were previously shown to closely resemble in vivo levels (Figure 3b). Indeed, plotting of the absolute CYP3A4 mRNA levels under CYP3A4-inducing conditions revealed drastic differences in the absolute levels of CYP3A4 mRNA between the two culture systems with several orders of magnitude (Figure 3c), thus providing an explanation for the artificially high fold changes observed in 2D monolayer culture. Combined, our results reveal an unanticipated variability in CYP3A4 induction patterns in PHHs, dependent on the culture condition.

AZD1208 induces CYP3A4 exclusively in PHHs cultured as 3D spheroids but not in the corresponding 2D monolayer cultures

AZD1208, a drug candidate for acute myeloid leukemia, was recently terminated from development due to unexpected auto-induction of CYP3A4 in the clinic.15,16 Preclinical models, including immortalized human liver cell lines and 2D monolayer
cultures of multiple different PHH donors, failed to predict the CYP3A4 induction potential of AZD1208. Therefore, we questioned whether our more physiologically relevant PHH 3D spheroid model could identify the potent CYP3A4 induction by AZD1208 as was observed in humans in vivo.

AZD1208 (1 μM, 0.2×Cmax) potently induced CYP3A4 mRNA at 3.5-fold compared with control and at levels very similar to rifampicin (Figure 4a). We then directly compared the ability of AZD1208 to induce CYP3A4 in 2D monolayer cultures of the same PHH donor. Indeed, in line with the previous observations, AZD1208 failed to induce CYP3A4 mRNA above the twofold threshold (1.8-fold) in 2D monolayer culture (Figure 4b). Correlation of the CYP3A4 induction levels relative to rifampicin revealed that AZD1208 in 3D spheroid culture induced CYP3A4 by 78%, while in 2D monolayer culture this was 3% (Figure 4c). In line with induction of CYP3A4 mRNA, CYP3A4 protein was also clearly induced by AZD1208 in 3D spheroid culture at levels comparable to rifampicin as determined by both Western blotting (Figure 4d) and immunofluorescence (Figure 4e). In agreement with observations at the mRNA level, CYP3A4 protein was not induced by AZD1208 in 2D monolayer cultures from the same PHH donor, while rifampicin, as expected, did induce CYP3A4 (Figure 4d). CYP3A4 protein was also functionally induced in 3D spheroid culture at levels comparable to rifampicin as assessed by mass spectrometric analysis of hydroxymidazolam formation from midazolam (Figure 4f). Titration of AZD1208 in 3D spheroid culture revealed a typical concentration-dependent CYP3A4 induction pattern that stabilized at 1 μM (Figure 4g).

We next evaluated whether AZD1208 also induced other CYPs. CYP2B6 mRNA was induced at levels similar to CYP3A4 (3.5-fold), and a twofold induction for CYP2C9 was observed, while expression of CYP1A2 and CYP2D6 was unaffected (Figure 4h).

AZD1208-mediated CYP induction is dependent on the pregnane X receptor

We next sought to address which nuclear receptors are involved in the AZD1208-mediated CYP3A4 and CYP2B6 induction observed in PHH spheroids. To this end, we performed knockdown experiments using siRNA constructs targeting the two most important nuclear receptors: PXR (NR1I2) and CAR (NR1I3). A schematic of the experimental setup is given in Figure 5a. We first validated our knockdown approach by evaluating mRNA levels for PXR and CAR at the day of CYP induction analysis. Knockdown efficiencies were 75% and 82% for PXR and CAR,
respectively, while no compensatory effect on expression of the respective other nuclear receptor was observed (Figure 5b). Under these two knockdown conditions, we observed that PXR knockdown reduced the basal CYP3A4 expression levels by 60%, while CYP2B6 expression was unaffected (Figure 5c). CAR knockdown did not affect basal CYP3A4 mRNA levels, while here CYP2B6 basal expression was reduced by more than 90% (Figure 5c). We then evaluated the effect of PXR and CAR knockdown on CYP3A4 induction by these compounds. The CYP2B6 inductive capacity of AZD1208 and rifampicin was drastically reduced upon PXR knockdown, whereas phenobarbital-mediated CYP2B6 induction was reduced, but not abolished, under these conditions (Figure 5e). Remarkably, under CAR knockdown conditions, AZD1208, rifampicin and phenobarbital all retained the capacity to induce CYP2B6 (Figure 5e). The artificially large extent of CYP2B6 induction by AZD1208 and rifampicin upon knockdown of CAR is explained by the observation that CAR knockdown reduced basal CYP2B6 expression substantially (Figure 5c), leading to a higher-fold induction upon stimulation with inducers, similar to what we observed in 2D monolayer culture.
Involvement of the MAPK/ERK pathway in AZD1208-mediated CYP3A4 induction

We then set out to identify mechanisms upstream of nuclear receptor activation by which AZD1208 exerts its CYP induction that may account for the observed differences between 2D vs. 3D cultivation of PHHs to detect clinically relevant CYP induction. AZD1208 was designed for treatment of acute myeloid leukemia by pan-PIM kinase (mainly PIM1) inhibition. 14

Figure 4 PHH spheroids detect AZD1208-mediated CYP3A4 induction. (a,b) CYP3A4 induction of AZD1208 and rifampicin in PHHs maintained in (a) 3D spheroid culture or (b) 2D monolayer culture relative to the respective DMSO controls. (c) The extent of CYP3A4 induction exerted by AZD1208 relative to rifampicin in 3D spheroid culture and 2D monolayer culture. (d) CYP3A4 protein expression upon AZD1208 (AZ) and rifampicin (RIF) exposure in 3D spheroid culture and 2D monolayer culture as determined by Western blotting. Note that different exposure settings were used for the 2D and 3D experiments and therefore cannot be comparatively assessed. (e) Immunofluorescence staining for CYP3A4 upon AZD1208 and rifampicin exposure in PHH spheroids. (f) CYP3A4 enzyme activity in 3D spheroid culture upon AZD1208 and rifampicin exposure as determined by the formation of hydroxymidazolam from midazolam. (g) Concentration dependency of AZD1208-mediated CYP3A4 induction in 3D spheroid culture. (h) Evaluation of CYP1A2, CYP2B6, CYP2C9, and CYP2D6 mRNA expression upon AZD1208 exposure relative to control and in relation to its extent of CYP3A4 induction in 3D spheroid culture. (i) AZD1208 and rifampicin CYP3A4 induction responses in 3D spheroid cultures from three different PHH donors. For all panels with quantitative data the means ± SEM of n ≥ 3 biologically independent experiments are shown (except for (i) where CYP3A4 induction in donor 3 was evaluated in a single experiment). For (d) and (e) representative blots and images from multiple independent experiments and spheroids are shown. 2D, two-dimensional; 3D, three-dimensional; Ctrl, control; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; mRNA, messenger RNA; PHH, primary human hepatocyte.

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Therefore, we first evaluated whether its CYP induction liability was related to PIM inhibition. Simultaneous knockdown of PIM1-3 revealed no changes in CYP3A4 induction patterns exerted by AZD1208 (or rifampicin and phenobarbital), excluding a direct effect related to its pharmacological activity (Figure S2).

Since direct nuclear receptor ligands can normally be identified in 2D monolayer cultures of PHHs, we hypothesized that AZD1208 may function as an indirect inducer resulting in CYP3A4 induction through a pathway active in PHH that is sensitive to the different culture systems used. Posttranslational modifications, such as phosphorylation, have been implicated to indirectly impact nuclear receptor activity.30 To assess whether AZD1208 may function through altering phosphorylation statuses of implicated proteins, we employed the DigiWest high-throughput Western blot approach.27 After screening of over 80 putative target candidates (Table S2), we noticed that AZD1208, but not rifampicin or phenobarbital, inhibited the phosphorylation of ERK1/2 at Tyr204 and subsequently validated this observation by in-house Western blotting (Figure 6a). To assess whether this inhibition was relevant to its mechanism of CYP induction, we performed coexposures with epidermal growth factor (EGF), the upstream effector of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway. EGF drastically repressed basal CYP3A4 mRNA levels (Figure 6b), whereas CYP2B6 levels were strikingly unaffected by the presence of EGF (Figure 6c). Upon EGF coexposure, AZD1208 lost the ability to induce CYP3A4, but its capacity to induce CYP2B6 was unchanged (Figure 6b,c). In contrast, CYP3A4 induction by rifampicin and phenobarbital was much less affected by the EGF pressure, while similar to AZD1208 its CYP2B6 induction capacity was unaltered (Figure 6b,c). Further strengthening our findings, we identified lapatinib, a dual epidermal growth factor receptor/human epidermal growth factor receptor 2 (EGFR/HER2) inhibitor,31 as a CYP3A4-inducing compound only active in 3D spheroids but not in 2D monolayer culture (Figure 6d).

DISCUSSION
Our results indicate that 3D spheroid cultures of PHHs detect CYP3A4 induction with 100% sensitivity and 100% specificity.
based on screening of a panel of 25 drugs. In a direct comparison with conventional 2D monolayer cultures, we found several inducers being much more potent CYP3A4 inducers in 3D spheroid culture at the clinically relevant concentrations tested. We furthermore provide direct evidence for an in vivo–relevant CYP3A4 inducer (AZD1208) that can only be identified as a CYP3A4 inducer in PHHs when cultured as 3D spheroids but not in 2D monolayer culture. These discrepancies further highlight the limited in vitro–to–vivo translatability of CYP3A4 induction studies. The working model for CYP3A4 induction as revealed in this study using 3D spheroid cultures of PHHs is shown in Figure 6. AZD1208 (AZ) induces CYP3A4 via indirect nuclear receptor activation. AZ inhibits phosphorylation of ERK1/2 at Tyr204 and its CYP3A4 induction potential was drastically inhibited by EGF coexposure. Through gene knockdown experiments AZD1208-mediated CYP3A4 induction showed a predominant dependency on PXR, while CAR knockdown reduced the extent of induction. The dashed line of AZD1208 to PXR indicates that potentially also additional pathways beyond the MAPK/ERK pathway are involved that contribute to its PXR-mediated CYP3A4 induction capacity. CYP3A4 induction by rifampicin (RIF) was found to solely rely on PXR, while phenobarbital (PB) showed no specific dependency on either PXR or CAR under knockdown of each single nuclear receptor. The CYP3A4 induction responses of both rifampicin and phenobarbital were largely resistant to EGF coexposure in contrast to that of AZD1208. EGF by itself drastically reduces basal CYP3A4 mRNA levels. An open question remains as to how the downstream effector, ERK1/2, represses the activity of PXR, and possibly CAR. In line with the indication of the MAPK/ERK pathway in CYP3A4 induction, lapatinib (LAP), a dual EGFR/HER2 inhibitor with higher affinity for HER2, was also found to induce CYP3A4. Both AZD1208 and lapatinib were exclusively identified as CYP3A4 inducers in PHHs cultured as 3D spheroids, but not in 2D monolayer culture of PHHs from the same donor, suggesting a culture format-sensitive CYP3A4 induction response related to the MAPK/ERK pathway. For (a) representative blots from multiple biologically independent experiments are shown. For (b–d) the means ± SEM of n ≥ 3 biologically independent experiments are shown. 2D, two-dimensional; 3D, three-dimensional; CAR, constitutive androstane receptor; Ctrl, control; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGFR/HER2, epidermal growth factor receptor/human epidermal growth factor receptor 2; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; mRNA, messenger RNA; ns, not significant; PBREM, phenobarbital-responsive enhancer module; PHH, primary human hepatocyte; PXR, pregnane X receptor; XREM, xenobiotic-responsive enhancer module. Two-sided unpaired t-tests; *P < 0.05; **P < 0.01; ***P < 0.001.
Instead, we found that phenobarbital was also largely resistant to EGF coexposure. While the induction capacity in PHa HepaRG cell line. Indeed, CYP induction responses have been reported to vary widely between different species, and our results provide further evidence for important species-specific differences as well as demonstrate important differences in CYP induction responses of PHHs dependent on the in vitro culture format conditions.

While most evidence for indirect nuclear receptor activation has been reported for CAR, indirect PXR activation has been observed through interfering with components of signaling pathways that influence its phosphorylation status, including protein kinases A and C, albeit in mouse hepatocytes. Our results point towards an important role of the MAPK/ERK pathway in mediating CYP3A4 induction that is sensitive to 2D vs. 3D culturing of PHHs. Firstly, we found AZD1208, whose CYP3A4 induction capacity could only be identified in 3D spheroid culture, to inhibit phosphorylation of ERK1/2 at Tyr204 in PHH spheroids. Secondly, coexposures of AZD1208 with EGF drastically repressed its CYP3A4 inductive capacity in PHH spheroids.

Finally, we identified lapatinib, a dual EGFR/HER2 inhibitor, as another CYP3A4 inducer active only in 3D spheroid culture but not in 2D monolayer culture. While there are no clinical reports on lapatinib-related drug–drug interactions, our results warrant its potential for CYP3A4 induction. The MAPK/ERK signaling pathway plays a prominent role in cellular programs like proliferation and differentiation. Seeding and cultivation of PHHs in 2D monolayer culture induces drastic overactivation of the MAPK/ERK pathway, resulting in hepatocyte dedifferentiation and features of epithelial-to-mesenchymal transition, while much milder activation is observed in PHHs maintained in sandwich culture, indicating that activity of this pathway in PHHs strongly depends on the culture conditions. Thus, these previous indications may provide an explanation for why CYP3A4 inducers, at least in part relying on signaling via the MAPK/ERK pathway, display different CYP induction profiles in PHHs cultured as 2D monolayers as compared with 3D spheroid cultures.

While direct nuclear receptor activators, such as rifampicin, can robustly induce CYP3A4 in 2D monolayer cultures of PHHs, much less is known about the predictivity of this system to identify CYP3A4 inducers functioning through indirect mechanisms of nuclear receptor activation. Our findings with regard to AZD1208 and lapatinib demonstrate important discrepancies in the CYP3A4 induction predictive power between 3D spheroid cultures and 2D monolayer cultures of PHHs. In addition, comparing the extent of CYP3A4 induction of the tested panel of in vitro CYP3A4 inducers revealed (i) in vivo–relevant levels of CYP3A4 induction in 3D spheroid culture but not in 2D monolayer culture, and (ii) a much more varied response between compounds in 2D monolayer culture compared with in 3D spheroid culture. A possible explanation for this discrepancy is that both direct and indirect mechanisms of nuclear receptor activation may determine the CYP3A4 induction capacity of certain compounds. In 3D spheroid culture, PHHs closely resemble the in vivo hepatocyte phenotypes, in contrast to in 2D monolayer culture, where PHH transcriptomes already 24 hours after seeding are highly divergent. The activity of and crosstalk between signaling pathways in 3D spheroid culture is therefore arguably more likely to reflect in vivo conditions and as a result allow the detection of drug-dependent activation of indirect mechanisms of nuclear receptor activation leading to CYP3A4 induction that require complex signaling pathway activities.

In current practice, pharmaceutical companies predominantly rely on 2D monolayer cultures of liver cell lines or PHHs to assess the CYP induction liability of novel drug candidates. The case of AZD1208 that resulted in withdrawal of this compound only in the clinical phase of development due to the lack of identification of its CYP3A4 induction liability in vitro demonstrates the shortcomings of this model. Our results provide evidence for important differences between 2D and 3D cultivation of PHHs that impact on their predictive value of drug-mediated CYP induction responses of clinical relevance. The PHH 3D spheroid model presented here may constitute a novel and highly relevant preclinical asset in drug development to screen for CYP3A4 inducers approximating the level...
of induction in vivo with improved sensitivity to detect atypical mechanisms of CYP3A4 induction. Further validating and benchmarking of the 3D spheroid model by screening an array of PHH donors, evaluating CYP3A4 protein and enzyme activity responses, and performing ring trials, will be needed before routine use in drug development.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the Clinical Pharmacology & Therapeutics website (www.cpt-journal.com).

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CONFLICT OF INTEREST

M.I.-S. and V.M.L. are cofounders and owners of HepaPredict AB. All other authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

D.F.G.H. and M.I.-S. wrote the manuscript. D.F.G.H., S.U.V., T.S., B.C.J., I.J., T.B.A., V.M.L., and M.I.-S. designed the research. D.F.G.H., S.B.J., T.S., S.C.S., Å.N., S.U., and M.K. performed the research. D.F.G.H., S.U.V., and T.S. analyzed the data.

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