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Through a glass, darkly? HepaRG and HepG2 cells as models of human phase I drug metabolism

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ABSTRACT

The pharmacokinetic and safety assessment of drug candidates is becoming increasingly dependent upon *in vitro* models of hepatic metabolism and toxicity. Predominant among these is the HepG2 cell line, although HepaRG is becoming increasingly popular because of its perceived closer resemblance to human hepatocytes. We review the functionality of these cell lines in terms of Phase I protein expression, basal cytochrome P450-dependent activity, and utility in P450 induction studies. Our analysis indicates that HepG2 cells are severely compromised: proteomic studies show that they express few key proteins in common with hepatocytes and they lack drug-metabolizing capacity. Differentiated HepaRGs are more hepatocyte-like than HepG2s, but they also have limitations, and it is difficult to assess their utility because of the enormous variability in data reported, possibly arising from the complex differentiation protocols required to obtain hepatocyte-like cells. This is exacerbated by the use of DMSO in the induction protocol, together with proprietary supplements whose composition is a commercial secret. We conclude that, while currently available data on the utility of HepaRG generates a confusing picture, this line does have potential utility in drug metabolism studies. However, to allow studies to be compared directly a standardized, reproducible differentiation protocol is essential and the cell line's functionality in terms of known mechanisms of P450 regulation must be demonstrated. We, therefore, support the development of regulatory guidelines for the use of HepaRGs in induction studies as a first step in generating a database of consistent, reliable data.

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Introduction

The human liver tumor-derived cell lines HepG2 and HepaRG are widely used in academia and industry: HepG2 is well-established in the prediction of hepatotoxicity, including the potential for drug-induced liver injury (Weaver et al. 2017, 2020), while HepaRG has, in recent years, become increasingly popular for studies on xenobiotic metabolism, including the prediction of intrinsic clearance (Lübberstedt et al. 2011; Bonn et al. 2016; Kratochwil et al. 2017). Such widespread dependence upon these cell lines raises the question of how closely they reflect primary human hepatocytes and consequently 'Are these cell lines sufficiently informative to be used for regulatory purposes?'

Immortalized cell lines like HepG2 and HepaRG are intrinsically unlike healthy human hepatocytes, yet their use in drug discovery and development involves assuming that their metabolic activity and gene regulatory networks accurately reflect those of human hepatocytes *in vivo*. We set out to review the ability of these cell lines to represent human hepatic Phase I metabolism with sufficient reliability and reproducibility for research purposes and acceptance by regulators.

In this context, studies that only measure changes in mRNA expression are of limited value because such changes often do not segregate with protein level and functional activities (Buccitelli and Selbach 2020). In the case of P450 induction, it is known that fold changes in mRNA expression frequently fail to accurately reflect changes in enzyme activity (Chu et al. 2009). We, therefore, excluded studies in which expression and/or induction were addressed only at the mRNA level.

In considering HepaRG and HepG2 cells as models of human hepatocyte function, this commentary focused on measurable phenotypic characteristics, particularly enzyme activity, evaluating the cell lines in terms of

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basal activities with diagnostic substrates compared against those of freshly prepared human hepatocytes and inducibility compared against that of cultured human hepatocytes. These characteristics are evaluated in the context of the cell lines' history, genetic characteristics, and protein expression profiles.

Background

The HepG2 cell line, established by Knowles et al. (1980), was soon shown to be capable of activating cyclophosphamide to genotoxic products, resulting in sister chromatid exchanges, and to exhibit some benzphetamine N-demethylation activity (Dearfield et al. 1983). It could hydroxylate 7-ethoxycoumarin, this activity being induced 20- to 30-fold after 3–4 days' exposure to 3-methylcholanthrene (3-MC, 5 μM) (Dawson et al. 1985). No induction was detected using phenobarbital (PB). Subclones with higher P450-dependent activities were subsequently isolated. One of these, HepG2/C3A, exhibited 7-ethoxyresorufin and phenacetin O-deethylase activities consistent with the expression of cytochrome P450 (CYP) 1A2 and responded to 3-MC with up to 40-fold induction (Kelly and Sussman 2000). This cell line was patented (Patent Numbers US5290684A, 1994 and US6653105B2, 2003) and used to develop a commercial fluorescence-based assay for CYP1A2 induction; these patents have expired and the HepG2/C3A cell line is available from the American Type Culture Collection (ATCC HB-8065).

The patent-protected HepaRG cell line (PCT/FR02/02391, 2002) was established from a Grade I hepatocarcinoma growing in an adult female with chronic hepatitis C infection. The resulting cultures comprised bipolar oval cells with the capacity to differentiate when treated with dimethyl sulfoxide (DMSO; ∼2% in cell culture medium) and hydrocortisone (50 μM) (Parent et al. 2004). The resulting hepatocyte-like cells metabolized phenacetin, tolbutamide, dextromethorphan, and nifedipine with specific activities ∼2–10 pmol/min/mg protein (Gripon et al. 2002), while fluorescence-based assays demonstrated 7-ethoxyresorufin O-deethylation (EROD), tolbutamide 4-hydroxylation, testosterone 6β-hydroxylation and chlorzoxazone 6-hydroxyation in differentiated HepaRGs; EROD activity was upregulated by 3-MC (5 μM, 24 h) but testosterone 6β-hydroxylation was not further increased in response to the CYP3A4 inducer rifampicin (25 μM, 72 h) (Aninat et al. 2006).

HepaRGs only exhibit these characteristics in the presence of hydrocortisone and high concentrations of DMSO. When thus treated, most of the cells die; those that survive form small clusters of hepatocyte-like cells which organize themselves into well-delineated trabeculae surrounded by biliary epithelial-like cells. The expression of CYP3A4 in the surviving cells is attributable to the presence of the differentiating agents, which are known to upregulate CYP3A4 (El-Sankary et al. 2000; Xie et al. 2020). This P450 profile is likely to reflect that of inducer-exposed hepatocytes rather than naïve cells, a consideration to keep in mind when evaluating induction studies using HepaRGs. It should also be noted that DMSO inhibits several P450 isoforms including CYP3A4 (Easterbrook et al. 2001).

Genetic profile

Different HepG2 stocks have disturbingly variable chromosome complements: one study specifies the karyotype as 55 compared with the normal human karyotype of 46 XX or 46 XY (Wiśniewski et al. 2016), while others report karyotypes 49–52 XY or 52–78 XY with numerous duplications, deletions, and rearrangements (Wong et al. 2000; Zhou et al. 2019). This may reflect the cell line's evolution over the >40 years since it was established. Many HepG2 stocks do not closely resemble human hepatocytes (Tascher et al. 2019), illustrating the importance of obtaining stocks of any cell line from reputable sources (e.g. the ATCC).

A detailed haplotype-resolved and integrated genome analysis of HepG2s acquired from the Stanford ENCODE Product Center for Mapping of Regulatory Regions provides a detailed karyotype (Zhou et al. 2019). This emphasizes the line's many structural and numerical abnormalities and offers a comprehensive analysis of genomic structural features including single nucleotide variants, copy number and ploidy changes, phased haplotype blocks, CRISPR targets, novel retrotransposon insertions, and structural variants including deletions, duplications, inversions, translocations, and complex rearrangements.

Only limited information on the karyotype of HepaRGs is available. They carry a supernumerary (remodeled) copy of chromosome 7 and only have one intact copy of chromosome 22; a t(12:22) translocation leads to monosomy of 12p (Gripon et al. 2002), but at least part of chromosome 22 must remain diploid because the cells have been genotyped as CYP2D6*2*9, indicating that they carry two copies of *CYP2D6* (Jackson et al. 2016). The CYP2D6*2 group of alleles, which is present in almost every population studied, encodes an enzyme with activity resembling that of the reference haplotype, CYP2D6*1. Its several sub-alleles differ from the reference sequence at

2581 C>T and 4181 G>C, leading to an Arg to Cys substitution at amino acid 296 and a Ser to Thr substitution at amino acid 486 (Nofziger et al. 2020); it is unclear which sub-allele the HepaRG cell line contains. CYP2D6*9 is characterized by a 3 bp deletion commonly described as g.2616 del AAG (rs5030656) leading to the deletion of Lys 281 (Nofziger et al. 2020). Cyp2D6*9 is classified as a reduced function haplotype (https://www.pharmgkb.org/haplotype/PA165948317) because the variant protein it encodes has reduced activity and is expressed at lower levels than the reference haplotype; HepaRGs are therefore predicted to be of CYP2D6 intermediate metabolizer phenotype.

HepaRGs are homozygous for a poor metabolizer allele of CYP2C9 *(CYP2C9*2*2)*. The *CYP2C9*2* variant (3608 C>T, rs1799853) has an Arg to Lys substitution at amino acid 144 leading to significantly reduced catalytic activity and decreased clearance of CYP2C9 substrates *in vivo* (Zanger and Schwab 2013). Clinical consequences include an increased risk of bleeding during treatment with anticoagulants such as warfarin, and adverse events in patients treated with sulfonylurea anticoagulants and non-steroidal anti-inflammatory drugs. The allele frequency of CYP2C9*2 is 8–13% in European, Central/South American, Near Eastern, and Latino populations but <3% in other populations (Sangkuhl et al. 2021). Only 1–2% of the Caucasian population is homozygous for this variant (Zanger and Schwab 2013).

HepaRGs are also homozygous for CYP3A5*3 (Jackson et al. 2016). This variant (6986 A>C; rs776746) creates a splicing defect in *CYP3A5* intron 3. Leading to reduced expression and enzymic activity, it is associated with reduced metabolism and dose requirements for drugs that are preferentially metabolized by CYP3A5, including tacrolimus and saquinavir (Zanger and Schwab 2013).

The homozygous poor metabolizer origin of HepaRGs for both CYP2C9 and CYP3A5 together with their CYP2D6 heterozygosity (one extensive and one poor metabolizer allele) means that they do not represent the majority of the human population, at least in terms of metabolism mediated by these isoforms.

We are not aware of a publicly available genome sequence for HepaRG, but this information would establish how closely HepaRGs resemble human hepatocytes and is key to the informed interpretation of data obtained with this cell line. A genome sequence would also reveal whether novel mutations acquired during tumor development or subsequent *in vitro* culture are present in P450 promoter regions or transcription factors, e.g. the constitutive androstane receptor (CAR), pregnane X receptor (PXR). Sequencing of multiple cultures would also indicate the rate of genetic drift, as seen in the HepG2 line.

Expression of phase I proteins

Several groups have compared HepG2 and HepaRG proteomes with those of the human liver, either in the form of cultured hepatocytes or tissue biopsies.

Comparing HepG2s, grown either as monolayers or spheroids, with cryopreserved PHHs analyzed as cell suspensions revealed similar findings (Wiśniewski et al. 2016; Hurrell et al. 2018). Most PHH batches clustered separately from HepG2s, which exhibited similar proteomes in 2D and 3D cultures. All P450 isoforms studied (CYPs 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 3A5) were readily identified in PHHs but barely detectable in HepG2s, which expressed a strong cancer cell signature, the proteins found at high abundance being those associated with cell proliferation, including components of the DNA replication, transcription, and translation machinery (Hurrell et al. 2018).

Focusing on Phase I by comparing HepG2 and other cell line microsomes with commercially acquired human liver microsomes supports these conclusions. The levels of 44/101 xenobiotic-metabolizing enzymes, including 19/24 P450s, were below the limit of detection (BLD) in HepG2 microsomes, the overall expression pattern of HepG2 microsomes being distinct from those of the Hep3B and Huh7 cell lines and PHHs (Shi et al. 2018).

In a comparison between differentiated HepaRGs, HepG2s, Upcyte® hepatocytes and cryopreserved primary human hepatocytes (PHHs), Sison-Young et al. (2015) identified a total of 4696 proteins, only 2722 of which were common to all the models evaluated. The overall proteomic profile of HepaRGs was closest to that of cryopreserved PHHs, although these were clearly segregated from the other models examined. This relationship was maintained when the analysis was restricted to 50 Phase I, 51 Phase II, and 27 Phase III proteins. Looking specifically at CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 4A11, 4F11, 4F12, 4V2, and 20A1, the expression of CYP3A4 in HepaRGs was 2.5x that in cryopreserved PHHs, the only case where a P450 was overexpressed in a cell line. Western immunoblotting confirmed that CYP1A2 expression was BLD in both HepaRGs and HepG2s; CYP2D6 was barely detectable in HepaRGs but not at all in HepG2s. Strong expression of CYP2E1 and CYP3A4 was detected in HepaRGs differentiated in house, but not in HepG2s.

Table 1. Proteomic analysis of cytochrome P450 expression in HepaRG cultures, cryopreserved PHHs and human liver biopsies.

	Protein expression (fmol/μg soluble protein)		
Isoform	HepaRG	PHHs	Human liver
CYP1A1	0.01 ± 0.00	0.01–0.25	0.01–0.17
CYP1A2	0.15 ± 0.03	2.0–7.7	2.4–9.0
CYP2B6	0.10 ± 0.02	1.4-22.7	0.3–1.2
CYP2C8	0.57 ± 0.03	10.0–23.2	5.0-8.2
CYP2C9	3.11 ± 0.16	32.0–38.7	9.7–19.1
CYP2C19	1.31 ± 0.07	2.4–6.6	<2.6
CYP2D6	BLQ	5.0–8.6	0.06–7.9
CYP2E1	0.44 ± 0.02	5.1–14.7	2.6–4.6
CYP3A4	2.68 ± 0.14	29.2–93.7	1.8–8.3
CYP3A5	0.11 ± 0.00	0.31–0.63	0.2–3.1

ND: not determined; BLQ: below the limit of quantitation.
Summarized from Supplementary Tables S8–S10 of Hammer et al. (2021). Fresh frozen PHHs from three donors were thawed and prepared for analysis without cultivation or treatment. Five human liver biopsies (analyzed and published previously) were included for comparison. Undifferentiated HepaRGs were obtained from Biopredic International and grown for two weeks in the absence of DMSO then differentiated using 1.0% DMSO (days 1–3) followed by 1.7% DMSO (days 4–14).

HepaRGs are now available in a cryopreserved, predifferentiated format (HPR116), which may help to reduce study-to-study variability. These cryoHepaRGs lack metabolic competence when first thawed but recover if maintained in culture for 7–10 days after thawing; they have been used for the assessment of basal P450-dependent activities (Jackson et al. 2016). Comparing HPR116 with PHHs and HepG2s, Tascher et al. (2019) found marked differences in the abundance of many proteins between HepaRGs and PHHs (1790 proteins) as well as between HepaRGs and HepG2s (1598) and HepG2s and PHHs (1646). While the expression of structural proteins was generally similar HepaRGs and PHHs, there were exceptions, particularly associated with contractility, trafficking, cell-cell communication, polarity, and morphogenesis; however, the majority of Phase I and II proteins exhibited comparable expression. The differences observed were attributed to the HepaRG trans-differentiation process and an altered balance between senescence and proliferation typical of transformed cells.

Hammer et al. (2021) report a detailed evaluation of protein expression in HepaRGs. Targeted proteomic analysis of HepaRGs acquired as undifferentiated stocks and differentiated in house, Wistar rat liver, C57BL/6 mouse liver, PXR/CAR-humanized mouse liver, FRG®-KO mouse liver populated with human hepatocytes and PHHs was compared with historical human liver biopsy results (summarized in Table 1), revealing very low levels of CYPs 1A2, 2B6, 2C8, 2C9, 2D6, and 2E1 in differentiated HepaRGs compared with PHHs. The level of CYP3A4 in HepaRGs resembled that in PHHs, possibly due to the inducing effect of DMSO. CYP2B6 was expressed at levels lower than those in PXR/CAR humanized mouse liver, humanized FRG®-KO mouse liver, and PHHs but resembling those of the equivalent proteins, CYP2B1 and Cyp2b10, in uninduced wild-type Wistar rat and C57BL/6 mouse liver.

One challenge in proteomic analysis of P450s in hepatocytes and cell lines is that little is known about the expression of the key nuclear receptors (NRs) PXR and CAR at the protein level. They are not addressed in the proteomic studies discussed above, and a lack of specific antibodies means that their expression has not been characterized immunochemically, either immunohistologically or by immunoblotting.

Basal P450-dependent activities

Consistent basal enzyme activities comparable to those of the human liver are a key feature of any model of human drug metabolism. In the context of Phase I metabolism, the model's basal P450-dependent activities should resemble those of freshly prepared PHHs.

P450-dependent activities tend to be close to or below the limit of detection in HepG2s, which are sometimes used as a negative control against which to compare other cell types because of their very limited metabolic activity (Kratochwil et al. 2017). Only very occasionally is measurable activity reported, and this is usually not replicated in other studies using the same substrates. Only for CYP3A4 are there several reports of measurable activity, and even then the activities are very low, midazolam 1′-hydroxylation in HepG2s being, at best, ∼100-fold lower than in monolayer cultured PHHs. Overall, therefore, HepG2s do not express measurable basal levels of P450-dependent xenobiotic metabolism.

Table 2 compares basal P450-dependent activities in HepaRGs with those of PHHs. This comparison should, ideally, be made using freshly prepared PHHs, but in practice most investigators use cryopreserved stocks assayed immediately after thawing (possibly with a brief recovery period in suspension before assay) or cultured for a period before activities are measured. Table 2, therefore, includes studies undertaken using both fresh and cryopreserved cells. It is restricted to studies which (i) address two or more of the key models (HepG2s or HepaRGs grown in monolayer culture, cultured PHHs or PHH suspensions); (ii) measure activities using diagnostic substrates applied directly to intact cells; (iii) use liquid chromatography/mass spectrometry (LC/MS) to quantify either substrate depletion or metabolite production; and (iv) report specific activities (as pmol/min/mg protein or pmol/min/10^6 cells). Studies that do not report specific activities but only

Table 2. Basal P450-dependent activities in HepG2 and HepaRG cultures compared with PHHs.

	Substrate	HepG2	HepaRG	PHH (2D culture)	PHH (suspension)	Study
Activities expressed in pmol/min/mg protein						
CYP1A2	Phenacetin	ND	~6, ~2, ~2, ~3	~7, ~13, ~15	ND	1
		ND	~0.3 to ~0.9; ~0.06 to ~0.4	Mean ~26 ($n = 5$)	Mean ~18 ($n = 5$)	2
		~0.9	ND	~20 at 4 h ~1 at 48 h	ND	3
		0.02 ± 0.03	ND	3.9 ± 2.8	ND	4
		ND	~0.3	~1.8	ND	5
		BLD[1]	7.65, 8.33, 10.2	2.06, 1.94, 4.34	ND	6
		~0.1	~2 to ~4	~7, ~7, ~10	ND	7,8
	Tacrine	1.6	1.8	ND	1.5 (pooled, single batch)	9
CYP2A6	Coumarin	ND	<~0.2; <~0.5	Mean ~24 ($n = 5$)	Mean ~49 ($n = 5$)	2
		BLD	ND	16.3 ± 9.2	ND	4
CYP2B6	Bupropion	ND	~5, ~2, ~1, ~1	~3, ~28, 278	ND	1
		ND	~3 to ~8; <~0.3	Mean ~9 ($n = 5$)	Mean ~11 ($n = 5$)	2
		ND	ND	~1 at 4 h ~0.1 at 48 h	ND	3
		BLD	ND	2.2 ± 3.4	ND	4
		<0.04	0.3	ND	0.7 (pooled, single batch)	9
		ND	~2	Mean ~3.6 Days 1–28	ND	5
		BLD	10.6, 34.4, 50.7	BLD, 1.66, 2.61	ND	6
		~0.6	~3 to ~7	~1, ~3, ~6	ND	7,8
CYP2C8	Amodiaquine	ND	~0.9	~2.4	ND	5
CYP2C9	Diclofenac	ND	~18, ~9, ~13, ~9	~42, ~32, ~72	ND	1
		ND	~3 to ~12; ~0.1 to ~5	Mean ~26 ($n = 5$)	Mean ~66 ($n = 5$)	2
		~0.2	ND	~30 at 4 h ~ 0.3 at 48 h	ND	3
		0.02 ± 0.02	ND	29.3 ± 14.3	ND	4
		<0.04	2.3	ND	3.1 (pooled, single batch)	9
		ND	~5	~6	ND	5
		BLD	3.79, 19.4, 12.5	1.81, 1.84, 3.72	ND	6
		BLD	~30 to ~70	~7, ~10, ~8	ND	7,8
CYP2C19	Mephenytoin	ND	~4 to ~11; ~0.4 to ~4	Mean ~10 ($n = 5$)	Mean ~6 ($n = 5$)	2
		BLD	ND	2.5 ± 2.1	ND	4
		ND	~0.4	~0.8	ND	5
		BLD	2.73, 6.21, 10.3	BLD, 2.49, 0.788	ND	6
CYP2D6	Dextro-methorphan	0.02	ND	5.5 ± 7.9	ND	4
		<0.04	0.2	ND	9.6 ± 0.1 (pooled, 2 batches)	9
		ND	~0.2	~0.5	ND	5
	~0.5	~0.3 to ~ 3	~22, ~11, ~2	ND	7,8	
	Bufuralol	ND	~0.2 to ~1; ~0.02 to ~0.3	Mean ~2 ($n = 5$)	Mean ~3 ($n = 5$)	2
		0.015	2.33, 3.34, 2.21	0.178, 0.908, 0.660	ND	6
CYP2E1	Chlorzoxazone	ND	~0.06 to ~0.3	Mean ~3 ($n = 5$)	Mean ~13 ($n = 5$)	2
		BLD	ND	12.5 ± 7.1	ND	4
CYP3A4	Midazolam	ND	~25, ~10, ~2, ~10	~1, ~37, ~35	ND	1
		ND	~3 to ~18; ~0.3 to ~1.5	Mean ~13 ($n = 5$)	Mean ~ 13 ($n = 5$)	2
		~0.02	ND	~10 at 4 h ~0.1 at 48 h	ND	3
		<0.15	12.4 ± 2.8	ND	32.5 ± 0.7 (pooled, 4 batches)	9
		0.10 ± 0.07	ND	10.1 ± 5.2	ND	4
		ND	~3.1	~2.3	ND	5
		0.225	131, 114, 69.6	0.349, 33.7, 72.6	ND	6
		~0.5	~60	~45, ~43, ~10	ND	7,8
Activities expressed in pmol/min/10^6 cells						
CYP1A2	Phenacetin	ND	2.78 ± 0.728	7.18 ± 7.78	Median = 48 ($n = 212$)[§]	10
		ND	5.00	2.82, 2.85, 9.18 14.9, 3.03, 19.58	ND	11
		ND	4.27 ± 0.459	0.072–40 ($n = 52$) Median = 4.94	1–349; median = 48 ($n = 212$)[§]	12
	Caffeine	BLD	0.153 ± 0.007	0.030 ± 0.003	ND	13
CYP2A6	Coumarin	ND	0.851 ± 0.159	1.03 ± 0.974	ND	10
CYP2B6	Bupropion	ND	17.9 ± 2.21	2.94 ± 2.78	Median = 32.1 ($n = 137$)[§]	10
		ND	1.93 ± 0.0754	0.210–13.1 ($n = 52$) Median = 2.16	1.86–304; median = 32.1 ($n = 137$)[§]	12
	Efavirenz	BLD	0.110 ± 0.007	0.18 ± 0.023	ND	13
CYP2C8	Paclitaxel	ND	0.223 ± 0.035	0.517 ± 0.802	Median = 5.24 ($n = 149$)	10
CYP2C9	Diclofenac	ND	3.94 ± 0.380	5.37 ± 9.54	Median = 88.2 ($n = 187$)	10
		ND	~9 ± 0.7 (Day 0)	~10 ± 7 (Day 0)	~66 ± 14	14

(*continued*)

Table 2. Continued.

	Substrate	HepG2	HepaRG	PHH (2D culture)	PHH (suspension)	Study
			∼6 (Day 7) ∼5 (Day 14)	∼1.5 ± 1.0 (Day 7) ∼0.5 (Day 14)		
	Losartan	BLD	0.037 ± 0.003	0.005 ± 0.001	ND	13
CYP2C19	Mephenytoin	ND	1.52 ± 0.408	2.26 ± 4.71	Median = 13.8 (n = 208)	10
		ND	∼1.1 (Day 0) ∼0.7 (Day 7) ∼1.8 ± 0.4 (Day 14)	∼2.2 ± 2.2 (Day 0) ∼0.4 ± 0.7 (Day 7) ∼0.7 ± 0.7 (Day 14)	∼11.3 ± 1.3	14
	Omeprazole	0.053 ± 0.003	6.17 ± 0.33	0.157 ± 0.013	ND	13
CYP2D6	Dextromethorphan	ND	0.401 ± 0.062	1.18 ± 1.70	Median = 21.1 (n = 24)	10
	Bufuralol	ND	∼2.8 ± 0.3 (Day 0) ∼3.0 ± 0.1 (Day 7) ∼3.2 ± 0.2 (Day 14)	∼2.0 ± 1.6 (Day 0) ∼1.3 ± 1.4 (Day 7) ∼0.7 ± 0.9 (Day 14)	∼20.5 ± 1.8	14
	Metoprolol	BLD	0.230 ± 0.020	0.083 ± 0.003	ND	13
CYP3A4	Testosterone	ND	248 ± 50.9	55.2 ± 49.4	Median = 407 (n = 195)	10
		ND	∼170 ± 14 (Day 0) ∼140 ± 7 (Day 7) ∼140 ± 4 (Day 14)	∼28 ± 30 (Day 0) ∼6 ± 5 (Day 7) ∼6 ± 5 (Day 14)	∼114 ± 10	14
	Midazolam	ND	28.4 ± 2.39	ND	Median = 63.7 (n = 132)[§]	10
		ND	38.3	7.13, 8.93, 12.73 16.7, 2.78, 10.67	ND	11
		ND	6.67 ± 0.392	5.5–20.1	1.54–593 (n = 132); median = 63.7[§]	12
0.213 ± 0.013		28.5 ± 1.50	9.13 ± 0.50	ND	13	

PHH (2D culture) includes PHHs cultured on collagen-coated substrates with or without a Matrigel™ or other overlay. Where specified, PPHs were cultured for 4 – 120 h prior to assay. Approximate values (∼) were presented graphically in the original publications consulted; the values tabulated here have been estimated by eye from printed pdf versions.

[§]Identical values appear to be from the same historical study. ND: Not determined. BLD: Values reported as zero are tabulated as BLD.

Studies 1-14, itemised in the right-hand column of the table, are as follows:

[1]Kanebratt and Andersson (2008a). For HepaRG, the first value is in the presence of 2% DMSO, the other three are in its absence.

[2]Anthérieu et al. (2010). The text refers to pmol/h/mg protein, but the values are graphed as pmol/min/mg protein and these values have been used here. For HepaRG, the first range of values is in the presence of 2% DMSO, the second is in its absence.

[3]Ulvestad et al. (2013). Note that the results are graphed on a logarithmic scale, so it is difficult to derive accurate values.

[4]Tolosa et al. (2016). For human hepatocytes, n = 4 - 6.

[5]Kvist et al. (2018). PHHs were a single ten-donor pool.

[6]Yokoyama et al. (2018). Presents results from one batch of HepG2s, three batches of HepaRGs and three individual cultures of hepatocytes. Mean ± SD is presented in the paper, but these appear to be technical replicates rather than independent experiments.

[7]Seo et al. (2019). HepG2 values (n = 1) were assayed on Day 1; HepaRG values (n = 8) on Days 1-28. Differentiation period was from Days 15-28.

[8]Seo et al. (2020). HepaRG values (n = 1) were assayed on Day 3.

[9]Kratochwil et al. (2017). HepG2s (single value) assayed on Day 4; HepaRGs (single value) on Day 7.

[10]Jackson et al. (2016). CryoHepaRGs were assayed on Day 10 after thawing.

[11]Vermet et al. (2016), values converted from nmol/h/10^6 cells.

[12]Ramaiahgari et al. (2017).

[13]Berger et al. (2016), values converted from pmol/h/50,000 cells.

[14]Lübberstedt et al. (2011).

make internal comparisons between two systems may provide additional supportive evidence but are not tabulated because their results cannot be compared with those of other investigators. Furthermore, assay data are often presented in graphical rather than tabular form making it impossible to compare studies statistically; however, some qualitative conclusions can be drawn (Table 3).

Even when all four criteria are met there is enormous variability both within and between the reported data. Regarding individual human preparations, this could be partly due to the biological variation but variation within a particular cell line is likely to be due to the use of different culture systems, differentiation protocols, and assay methodologies; indeed, different activity values have been reported in a single study depending upon which of two different substrate cocktails was used (Anthérieu et al. 2010).

P450-dependent activities are generally higher in differentiated HepaRGs than in HepG2s, even the HepG2/C3A subclone (Nelson et al. 2017), and can resemble those in PHHs cultured in monolayer format for at least 48 h. When compared with primary hepatocyte suspensions, however, both HepaRGs and monolayer cultured PHHs exhibit much lower, sometimes barely detectable, activities. The only exception is that HepaRGs consistently exhibit CYP3A4-dependent activities at least as high as in monolayer cultured PHHs and sometimes even higher, whether measured as midazolam 1′-hydroxylation or testosterone 6β-hydroxylation; indeed, this is the only case in which a P450-dependent activity observed in a cell line resembles that of PHH

Table 3. Summary of basal P450-dependent activities in HepG2 and HepaRG cultures.

	HepG2	HepaRG
CYP1A2	Phenacetin O-deethylation activities and paraxanthine production from caffeine tend to be below or close to the limit of detection.[3,4,6,7,13] One study found phenacetin O-deethylation activity resembling that of PHHs 48 h after plating, although PHH activity was much higher 4 h after plating.[3]	Phenacetin O-deethylation activities usually resemble those of PHHs in monolayer culture,[1,5,6,8,10–12] but considerably lower than historical values for PHHs in suspension.[10,12] Tacrine hydroxylation is reportedly similar in HepG2s, HepaRGs and a single batch of PHHs.[9] Paraxanthine production from caffeine may be slightly higher in HepaRGs than in monolayer cultures of PHHs.[13]
CYP2A6	Coumarin hydroxylase activity is reportedly BLD.[4]	Coumarin hydroxylation activity reportedly resembles that in monolayer cultured PHHs.[10]
CYP2B6	Only one study reports measurable bupropion hydroxylation activity;[7] three others report activities BLD.[4,6,9] Efavirenz 8′-hydroxylation is also reportedly BLD.[13]	Bupropion hydroxylation activities tend to be similar to/slightly higher than those in monolayer cultures of PHHs,[1,5,6,8–10,12] but well below the medians reported for fresh PHH suspensions.[10,12] Efavirenz 8′-hydroxylation activities reportedly resemble those of monolayer cultured PHHs.[13]
CYP2C8	No reports found.	Activities toward amodiaquine and paclitaxel are reportedly slightly lower than those in monolayer cultured PHHs[5,10] and much lower than in PHH suspensions.[10]
CYP2C9	Diclofenac 4′-hydroxylation and oxidation of losartan are usually close to or below the limit of detection.[3,4,6,7,9,13]	Even though HepaRGs are of CYP2C9 poor metabolizer origin, diclofenac 4′-hydroxylation activities resembling those of monolayer PHHs, but considerably lower than those in PHH suspensions, have been reported.[1,5,6,8–10,14] Comparability between HepaRGs and monolayer cultured PHHs may result from loss of activity in the PHHs rather than the presence of active CYP2C9 in HepaRGs. Losartan oxidation activity, though low in both cases, is reportedly slightly higher in HepaRGs than in monolayer cultured PHHs.[13]
CYP2C19	S-mephenytoin hydroxylation is reportedly BLD.[4,6] Omeprazole 5-hydroxylation has been reported at a level about 1/3 of that in monolayer cultured PHHs.[13]	Similar S-mephenytoin hydroxylation activities have been reported in HepaRGs and monolayer cultured PHHs.[5,6,10,14] When compared with suspension PHHs the activities reported were considerably lower in both HepaRGs and monolayer cultured PHHs than in PHH suspensions.[10,14] Omeprazole 5-hydroxylation activity has been reported at a level about 40x that in monolayer cultured PHHs.[13]
CYP2D6	Low dextromethorphan O-demethylation in has been reported.[4,9] Metoprolol hydroxylation activity is reportedly BLD.[13] Values reported for bufuralol 1′-hydroxylation in HepG2s differ by >30-fold.[6,7]	Measurable dextromethorphan O-demethylation, metoprolol hydroxylation and bufuralol 1′-hydroxylation activities have been reported in numerous studies.[5,6,8–10,13,14] These often resemble those in monolayer cultured PHHs, though when compared to the activity of PHH suspensions the HepaRG activities are much lower.[9,10,14]
CYP2E1	Chlorzoxazone 6-hydroxylation is reportedly BLD.[4]	No reports found. Jackson et al. (2016)[10] could not quantify CYP2E1 activity in HepaRGs because of the inhibitory effect of DMSO on this isoform.
CYP3A4	Midazolam 1′-hydroxylation varies between very low values[3,4,9] and activities which are readily measurable but still ∼100-fold lower than in monolayer cultured PHHs.[6,7,13]	Relatively high CYP3A4 activities are reported, whether measured as midazolam 1′-hydroxylation or testosterone 6β-hydroxylation.[1,5,6,8–14] These are usually at least as high as in monolayer cultured PHHs and often considerably higher;[6,10,11,13,14] indeed, this is the only case in which a P450-dependent activity observed in a cell line is in a similar range to that in PHH suspensions,[9,10,12,14] although the HepaRG activities still tend to be toward the lower end of the range when large numbers of PHH preparations are included.[10,12]

[1–14]As in Table 2.

suspensions. Even in this case, however, HepaRG activities tend to be toward the lower end of the range when large numbers of PHH batches are included (Jackson et al. 2016; Ramaiahgari et al. 2017). In general, therefore, apart from CYP3A4, HepaRGs exhibit at best moderate P450-dependent activities compared with PHHs, even allowing for the considerable variability between PHH cultures.

Most studies of P450-dependent metabolism in HepaRGs only measure activities at a single time after the initiation of differentiation, usually in comparison with monolayer cultured PHHs also assayed at a single time after plating. Such studies are limited because cell lines and primary cultures are dynamic systems: for example, P450-dependent activities drop by 10- to 100-fold when PHHs are assayed *in situ* after 4- or 48-hr adhesion time (Ulvestad et al. 2013).

Only one study has explicitly considered the time course of P450-dependent metabolic capability in HepaRGs, addressing three timepoints during the differentiation process ('Days 0, 7, and 14,' equivalent to days 14, 21, and 28 after shipping) compared with cultured PHHs over the same time course starting on day 3 of culture (Lübberstedt et al. 2011). In both culture systems CYP1A2 activity (measured as EROD) was stable over this culture period, being slightly lower in

HepaRGs than cultured PHHs but much less variable. The activity was much higher than that of cryopreserved PHHs assayed immediately after thawing, raising the possibility that the activity detected may reflect artefactual upregulation of CYP1A1 and/or 1A2 due to dedifferentiation in culture. HepaRGs and monolayer cultured PHHs exhibited similar CYP2C9, CYP2C19, and CYP2D6 activities (diclofenac 4-hydroxylation, S-mephenytoin hydroxylation, and bufuralol 1-hydroxylation) on day 0; these activities were, however, much lower than those in cryopreserved PHHs assayed in suspension immediately after thawing. Over 14 days of differentiation CYP2C9, 2C19, and 2D6 activities in HepaRGs were maintained or increased slightly, whereas activities in monolayer cultured PHHs declined rapidly so that by the end of the differentiation period the activities in HepaRGs were generally higher than those in cultured PHHs. In contrast with all the other P450-dependent activities, CYP3A4 activity (testosterone 6β-hydroxylation) was considerably higher in HepaRGs than in either cultured PHHs or cryopreserved PHHs assayed in suspension, activity in the latter being higher than in cultured PHHs but still lower than in HepaRGs.

It is important to remember that the presence of DMSO is essential to the maintenance of P450-dependent activities in HepaRGs. Removal of DMSO from the culture medium alters P450 expression and activity within 24 h (Kanebratt and Andersson 2008a), although it is unclear whether this is due to loss of the P450-inducing effect of DMSO or general dedifferentiation of the cells in the absence of its differentiating stimulus.

Regulation of P450 expression and activity

The regulation of P450-dependent metabolism, a primary function of hepatocytes, influences both xenobiotic metabolism and susceptibility to hepatotoxins. The most important pathways of P450 regulation are mediated by the PAS domain aryl hydrocarbon receptor (AhR) and the NRs PXR and CAR.

A wide range of high and low-affinity ligands can activate AhR, initiating a pathway that has been conserved through evolution and is functional in most mammalian cell types as well as in tumor cell lines. The activity of this pathway, exemplified by the induction of CYP1A1/1A2 by polycyclic aromatic hydrocarbons, therefore does not reflect a differentiated hepatocyte phenotype.

Regulation *via* PXR occurs in several tissues including the liver and gastrointestinal tract (Koutsounas et al. 2013). The mechanism of action of PXR resembles that of conventional NRs, such as the steroid hormone receptors, except that, instead of binding a narrow range of ligands with very high affinity, it binds a wide range of ligands with relatively low affinity (Stanley et al. 2006). This pattern of ligand responsiveness may have evolved to allow PXR to deal with the many xenobiotics to which organisms are exposed.

The liver-specific receptor CAR can be activated both by agonists [e.g. 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO)] and inverse agonists (e.g. androstanol) which inhibit its function. Other inducing agents, including PB, regulate CAR by indirect mechanisms involving protein phosphorylation and dephosphorylation (McMahon et al. 2019; Negishi et al. 2020). It is important that studies involving functional analysis of CAR investigate both pathways of regulation.

Studies of P450 induction in cultured liver cells have been driven by the need for reliable models to predict drug-drug interactions, especially those which occur because of PXR/CAR-mediated induction of CYP3A4 and CYP2B6. Most studies addressing the induction of CYP2B6 and CYP3A4 at the enzymatic level have been conducted in HepaRGs, sometimes in comparison with PHHs.

This literature is difficult to interpret because of study-to-study inconsistencies in the induction protocols used: when undertaking this review we were unable to identify any two studies in which the same inducing agent, concentration, and duration of exposure were used. In addition, many investigators do not report the basal expression levels against which fold inductions are calculated; the fold induction which is considered meaningful is often not defined; and the activities measured are often close to the limit of detection of the assays used, meaning that even induced activities may not be pharmacologically significant. Furthermore, many studies are conducted at only one concentration of the inducing agent, meaning that a dose-response curve cannot be constructed and making it even more difficult to compare studies. Finally, it is important to note that PXR/CAR ligand-binding affinities do not necessarily predict maximal induction ratios (Stanley et al. 2006; Scheer et al. 2008).

P450 induction in HepG2 cultures

P450 protein expression in HepG2s exhibits only limited responsiveness to P450 inducing agents. Consistent with their origin from a hepatoblastoma, they respond to AhR agonists with upregulation of immunochemically detectable CYP1A2, phenacetin O-deacetylation, and EROD; Choi et al. (2015) also report slight induction

Table 4. Studies comparing induction of CYP2B6 and CYP3A4-dependent activities in PHHs and HepaRG cultures.

CYP3A4 Inducing agent	Induction protocol and substrate	PHH	HepaRG
Phenobarbital	200 μM, 48 h, midazolam[15]	ND	6.2 ± 2.4-fold
	Various concentrations, 72 h, midazolam[16]	ND	Minimal at 100 μM ~2 to ~15-fold at 300 μM ~5 to ~51-fold at 1000 μM
	500 μM, 72 h, midazolam[17]	2.97, 2.12 and 6.08-fold Three donors (2 M, 1 F)	36.6.-fold
	1.5 mM, 24–48 h, testosterone[18]	~1.2-fold and ~1.3-fold (24 h)	9-fold (24 h) 10-fold (48 h)
Carbamazepine	Various concentrations, 72 h, midazolam[16]	ND	~2 to ~3-fold at 30 μM ~1 to ~7.5-fold at 100 μM ~1 to ~22-fold at 300 μM
Rifampicin	4 μM, 48 h, midazolam[15]	ND	7.9 ± 2.9-fold
	Various concentrations, 72 h, midazolam[16]	ND	Minimal at 0.1 μM ~2 to ~10-fold at 0.3 μM ~7 to ~24-fold at 1 μM ~9 to ~42-fold at 3 μM
	10 μM, 72 h, midazolam[2]	ND	~6 to ~21-fold on various days of culture
	(7 values with each of two substrate cocktails)		
	25 μM, 72 h, midazolam[17]	3.72, 2.08 and 10.6-fold Three donors (2 M, 1 F)	42.4-fold
	20 μM, 48 h, midazolam[19]	ND	3.9-fold (2 D cultures) 9.0-fold (tethered spheroids)
	20 μM, 72 h, midazolam[13]	4.7-fold (2 D cultures) 7.2-fold (3 D cultures)	7.9-fold
	50 μM, 48 h, testosterone[20]	ND	3.8-fold (day 30); 2-fold (day 44); 2.2-fold (day 58)
	20 μM, 72 h, testosterone[14]	5.2 ± 3.3-fold	2.8 ± 0.5-fold
	Various concentrations, 72 h, testosterone[10]	ND	~2.5-fold at 0.1 μM ~8.3-fold at 1 μM
~14-fold at 10 μM			
Hyperforin	Various concentrations, 72 h, testosterone[10]	ND	~ 2-fold at 10 nM ~7-fold at 100 nM ~7-fold at 1 μM
CYP2B6			
	Inducing agent	PHH	HepaRG
CITCO	Various concentrations, 72 h, bupropion[10]	ND	~1.6-fold at 2.7 μM ~2.7-fold at 25 μM ~3.3-fold at 222 μM.
Phenobarbital	200 μM, 48 h, bupropion[15]	ND	6.2 ± 4.3-fold
	1mM, 72 h, bupropion[2]	ND	~8 to ~45-fold on various days of culture
	(7 values with each of two substrate cocktails)		
	1.5 mM, 24–48 h, bupropion[18]	~4-fold and ~3.2-fold (24 h)	~ 13-fold (24 h) ~ 13-fold (48 h)
	500 μM, 72 h, bupropion[17]	12.8 and 8.29-fold Two donors (1 M, 1 F)	8.66-fold 2/3 vehicle controls were BLQ
	1 mM, 48 h, bupropion[19]	ND	1.9-fold (2 D cultures) 3.2-fold (tethered spheroids)
	Various concentrations, 72 h, bupropion[10]	ND	~0.7-fold at 4 μM ~1.2-fold at 37 μM
~3.7-fold at 333 μM.			
Phenytoin	40 μM, 48 h, bupropion[15]	ND	3.4 ± 2.1-fold
Rifampicin	4 μM, 48 h, bupropion[15]	ND	5.9 ± 2.4-fold
	25 μM, 72 h, bupropion[17]	8.27 and 2.50-fold Two donors (1 M, 1 F)	2.7-fold
	20 μM, 72 h, efavirenz[13]	3.1-fold (2 D cultures) 2.7-fold (3 D cultures)	4.0-fold

Studies on induction in PHHs are only included if they include comparisons with HepaRG, HepG2 or both. Inclusion criteria were the same as for Table 2 except that two studies which deviated slightly were included. In one of these[17] microsomal preparations were used for the activity assays instead of intact cells and in the other[20] the detection method used was HPLC/UV rather than LC/MS. These deviations, which affect both the numerator and denominator of the induction ratio, were deemed to be unlikely to have affected fold induction values. Approximate values (~) were presented graphically in the original publications consulted; the values tabulated here have been estimated by eye from printed pdf versions.

[1–14]As in Table 2.

[15]Kanebratt and Andersson (2008b).

[16]Kaneko et al. (2009).

[17]Gerets et al. (2012); activities measured in microsomal preparations.

[18]Turpeinen et al. (2009).

[19]Wang et al. (2015).

[20]Jossé et al. (2008).

(about 2-fold) of bupropion hydroxylase by PB (1 mM for 48 h) and testosterone 6β-hydroxylase activity by both PB (1 mM for 48 h) and rifampicin (50 μM for 48 h), respectively, but it is unclear against what basal activities this induction was measured, making it difficult to compare their results with those of other investigators. Others have found expression levels of CYP3A4 which were barely detectable by immunoblotting and no evidence of induction by CITCO or rifampicin in HepG2s (Harmsen et al. 2008).

Only two studies addressing PXR- and CAR-mediated induction in HepG2s met our inclusion criteria. Phenobarbital had no effect on midazolam 1′-hydroxylation, which was 1.1× control following treatment at 500 μM for 72 h (Gerets et al. 2012). Similarly, rifampicin had very little inductive effect in HepG2s: Berger et al. (2016) report marginal induction of omeprazole metabolism (1.3-fold) and midazolam 1′-hydroxylation (1.3 and 1.7-fold, respectively, after 72 h at 20 μM (Berger et al. 2015), while Gerets et al. (2012) report no change in midazolam 1′-hydroxylation in microsomal preparations after 72 h at 25 μM. In each of these studies, rifampicin and PB had readily detectable effects on PHHs and HepaRGs (Table 4). At the present time the mechanism of the reduced CAR activity in HepG2 cells is unknown; it may be due to genetic or epigenetic changes which attenuate the signaling cascade(s) involved.

CYP3A4 induction in HepaRG cultures

Table 4 summarizes studies addressing the induction of midazolam 1′-hydroxylation (CYP3A4) by rifampicin in HepaRGs, which indicate fold induction values resembling those in PHHs. It is not possible to evaluate the relationship between fold induction and rifampicin concentration from these experiments, but a dose-response study using four concentrations of rifampicin provides some evidence for concentration dependence (Kaneko et al. 2009). There was, however, wide batch-to-batch variability. In some batches the induction response tailed off at higher concentrations; thus, the EC_{50} (4.3×10^{-3} mM) was outside the concentration range over which induction was determined to be dose-dependent. Kanebratt and Andersson (2008b) found HepaRGs to be much more sensitive to rifampicin, deriving an EC_{50} of 1.2×10^{-4} mM (36-fold lower) for induction of midazolam 1′-hydroxylation; this may reflect the fact that their study did not include concentrations at which the inductive response tailed off. Fold induction of testosterone 6β-hydroxylation in HepaRGs exhibited signs of dose-responsiveness with rifampicin induction but plateaued above 100 nM with hyperforin (Jackson et al. 2016).

Several studies have also considered the induction of CYP3A4 *via* indirect activation of CAR. The fold values obtained in single-dose studies using PB are again highly variable (Table 4). This lack of consistency makes it difficult to evaluate how PB induction of CYP3A4 in HepaRGs compares to that in PHHs, especially as many studies do not report the basal activities against which fold induction is being calculated.

Kaneko et al. (2009) characterized the effects of different concentrations of PB and carbamazepine on the induction of CYP3A4. There was enormous batch-to-batch variation in the response: one of the four batches tested responded strongly to both PB and carbamazepine whereas another exhibited practically no induction with either of these compounds even at the highest doses tested. The EC_{50}s derived from these data were 28.7 mM for PB and 3.8 mM for carbamazepine, based on experiments in which hypothetical maximal induction was never reached; the uncertainty underlying these values is reflected in the coefficients of variation (88.6% for PB and 198.3% for carbamazepine).

Taken together, these studies suggest dose-dependent induction of CYP3A4 by indirect activators of CAR, but the widely variable results highlight concerns about batch-to-batch variability in HepaRGs.

CYP2B6 induction in HepaRG cultures

In studies addressing the effects of CAR activators on CYP2B6-mediated activities in HepaRGs, neither CITCO nor phenytoin had more than a slight effect on bupropion hydroxylation, while the effects of PB were extremely variable. These data suggest that the function of CAR is, at best, compromised in HepaRGs (Table 4). The changes observed do not reflect observations in patients treated with CAR activators, such as PB and phenytoin, which are known to cause significant changes in drug exposure (Khoo et al. 1980).

Studies using rifampicin suggest that CYP2B6 can be regulated to a limited extent by PXR in HepaRGs; where a comparison was made the values for HepaRGs were in the same range as those detected in PHHs (Table 4), although the very low constitutive expression of CYP2B6 in HepaRGs should be borne in mind. When background expression is very low, even large fold changes are unlikely to lead to measurable changes in functional activity.

One approach to circumvent the activity of CAR in HepaRGs is to engineer the cells to constitutively overexpress CAR. Van der Mark et al. (2017) achieved this

Table 5. Rifampicin-induced changes in P450-dependent activities in PHHs and HepaRG cultures.

Isoform	Substrate	PHH	HepaRG
CYP1A2	Caffeine	No induction after 72 h at 20 μM in 2 D cultures; marginal induction in 3 D cultures (2.1-fold).[13]	No induction after 72 h at 20 μM (1.1-fold).[13]
CYP2B6	Bupropion	Using microsomal preparations, 8.27 and 2.50-fold induction of bupropion hydroxylation in PHHs from two donors (1 M, 1 F) after 72 h at 500 μM.[17]	Some induction of bupropion hydroxylation is reported: 5.9 ± 2.4-fold after 48 h at 4 μM and 2.7-fold after 72 h at 25 μM.[15,17]
	Efavirenz	3.2-fold induction after 72 h at 20 μM in monolayer cultures; 2.7-fold in 3 D cultures.[13]	4.0-fold induction after 72 h at 20 μM.[13]
CYP2C9	Losartan	Marginal, if any, induction (1.4-fold) after 72 h at 20 μM in 2 D cultures; slightly greater induction in 3 D cultures (2.6-fold).[13]	No induction after 72 h at 20 μM (1.1-fold).[13]
	Diclofenac	Marginal induction (1.6 ± 0.7-fold) after 72 h at 20 μM.[14]	3.4 ± 0.4-fold induction after 72 h at 20 μM.[14]
CYP2C19	Omeprazole	4.2-fold induction after 72 h at 20 μM in 2 D cultures; induction doubled in 3 D cultures (8.3-fold).[13]	4.1-fold induction after 72 h at 20 μM.[13]
	Mephenytoin	Marginal, if any, induction (1.3 ± 0.1-fold) after 72 h at 20 μM.[14]	Marginal, if any, induction (1.2 ± 0.2-fold) after 72 h at 20 μM.[14]
CYP2D6	Metoprolol	Marginal, if any, induction (1.4-fold) after 72 h at 20 μM in both 2 D cultures and 3 D cultures.[13]	5.7-fold induction after 72 h at 20 μM.[13]
CYP3A4	Midazolam	Using microsomal preparations, 3.72-fold, 2.08-fold and 10.6-fold induction is reported in PHHs from three donors (2 M, 1 F) treated for 72 h at 25 μM.[17] 4.7-fold induction in 2 D cultures and 7.2-fold is reported in 3 D cultures after 72 h at 20 μM.[13]	Most studies report moderate induction: • 7.9 ± 2.9-fold after 48 h at 4 μM;[15] • ∼6 to ∼21-fold after 72 h at 10 μm;[2] • 3.9-fold in 2 D cultures and 9.0-fold in tethered spheroids after 48 h at 20 μM;[19] • 7.9-fold after 72 h at 20 μM.[13] • Using microsomal preparations, 42.4-fold after 72 h at 25 μM).[17] A dose-response experiment indicates induction up to ∼24-fold at 1 μm and up to ∼42-fold at 3 μM, though there was considerable batch-to-batch variability.[16]
	Testosterone	5.2 ± 3.3-fold induction after 72 h at 20 μM.[14]	2.0, 2.2 and 3.8-fold induction after 48 h at 50 μM in cells following various periods in culture.[20] 2.8 ± 0.5-fold induction after 72 h at 20 μM.[19] A dose-response experiment indicates ∼2.5-fold induction after 72 h at 0.1 μM, ∼8.3-fold at 1 μM and ∼25-fold at 10 μM.[10]

[1–19]As in Tables 2–4; this table summarizes a subset of the studies presented in Table 4.

using a lentiviral vector encoding CAR driven by the murine phosphoglycerate kinase 1 promoter. HepaRG-CAR cells exhibited increased activity of both CAR target and non-target P450s and increased clearance of warfarin and prednisolone, although mRNA induction ratios following treatment with omeprazole or CITCO were unchanged. Interestingly, CYP3A4 mRNA induction by rifampicin was ablated in HepaRG-CAR cells, consistent with the idea that CYP3A4 is fully induced in HepaRGs without induction. HepaRG-CAR cells are presented as a more physiologically relevant model for studies on differentiation and metabolism in this cell line without the need for exposure to DMSO.

Effects of rifampicin on other P450-dependent activities in HepaRGs

Various studies have looked more widely at the effects of rifampicin on P450-dependent activities in HepaRGs, considering activities beyond those mediated by CYP2B6 and CYP3A4. The activities reported generally resembled those in PHHs, although in a few cases greater fold induction values were observed in HepaRGs (Table 5). This reflects one of the limitations of cultured cells which express low basal levels of particular P450 isoforms: even where high induction ratios are reported the resulting enzyme level or activity may still be very low. This may also help to explain why the pattern of induction seen for a particular P450 often depends upon the substrate used to measure its activity: thus, marginal, if any, induction of CYP2C19 is observed in either PHHs or HepaRGs when measured as mephenytoin 4-hydroxylation (Lübberstedt et al. 2011), but around 4-fold induction is observed using omeprazole as substrate (Berger et al. 2016).

Use of NR-knockout HepaRGs to elucidate induction mechanisms

Knockout (KO) approaches have been used to characterize the contribution of PXR to the induction of CYP2B6 and CYP3A4 mRNAs in HepaRGs. Note that this

Table 6. Cytochrome P450 mRNA expression and induction in Nuclear Receptor Knockout HepaRGs.

		PXR^{-} CAR^{+}	PXR^{-} CAR^{-}	PXR^{+} CAR^{-}
CYP3A4	Basal mRNA expression	Increased	Decreased	Increased
	Rifampicin induction	No induction	No induction	As control
CYP2B6	Basal mRNA expression	Increased	As control	As control
	Rifampicin induction	No induction	No induction	Increased

Summarized from Preiss et al. (2021).

section is an exception to our policy of including only phenotypically anchored results: protein expression and enzyme activity are rarely if ever, evaluated in studies of this kind.

A zinc-finger targeted PXR-KO HepaRG cell line has been shown to be refractory to rifampicin induction and has greatly decreased sensitivity to CYP3A4 induction by dual activators of PXR and CAR (PB, phenytoin, and carbamazepine), possibly because CYP3A4 is already maximally upregulated in this model. The slight residual activity seen with these agents was thought to be due to upregulation of CYP3A4 *via* CAR; consistent with this, the small CYP3A4 inductive response to CITCO was maintained in the PXR-KO, although this is difficult to reconcile with evidence suggesting that HepaRGs lack CAR functionality. Artemisinin did not upregulate CYP3A4 in PXR-KO cells, suggesting that artemisinin is PXR-specific for CYP3A4 induction in this context, although it did exhibit some activity in CYP2B6 induction (Williamson et al. 2016).

A study using PXR-KO, CAR-KO, and double PXR/CAR-KO HepaRGs (Preiss et al. 2021) is summarized in Table 6. The PXR-KO yielded results consistent with those in PXR-KO mice, in which CYP3A expression (Cyp3a11 in mice) undergoes compensatory upregulation, with no scope for further induction in response to rifampicin (Scheer et al. 2008), and as expected no induction occurred in the CAR-KO cultures treated with CITCO. However, basal CYP3A4 mRNA expression was decreased rather than increased in the double PXR/CAR-KO, and a mixture of effects was seen in CAR-KOs and PXR/CAR-KOs, possibly because CAR function is already compromised in HepaRGs, making the effects of a knockout difficult to interpret. In this study mRNA levels were expressed relative to those in control HepaRGs, in which levels of expression may have been very low, so one cannot conclude absolute values from the results. Models of this kind facilitate the study of PXR/CAR-independent mechanisms of induction, although it is impossible to be sure that such pathways function in HepaRGs in the same way as in hepatocytes.

HepaRGs have also been used in knockdown studies to elucidate the role of other signaling pathways in the regulation of CAR and PXR. The role of WNT/β-catenin signaling in P450 regulation has been studied using the natural WNT ligand WNT3a and siRNA-mediated depletion of β-catenin. Thomas et al. (2015) showed that β-catenin is required for induction of downstream P450 genes by AhR, PXR and CAR, whereas it opposes induction by PPARα, while Chen et al. (2018) performed gene knockdowns of CAR, PXR, hepatocyte nuclear factor 1α (HNF1α) and hepatocyte nuclear factor 4α (HNF4α) on P450 expression in HepaRGs. Knocking down PXR or CAR had little effect on the expression of other transcription factors but reduced the basal and induced expression of CYP2B6 and CYP3A4. Knocking down HNF1α or HNF4α upregulated AhR but decreased the expression of PXR, CAR, and many P450s, possibly *via* effects on PXR and CAR. Further studies are needed to resolve the mechanisms involved.

One potential complicating factor in the interpretation of induction studies using HepG2s and HepaRGs is the lack of information on key regulatory pathways in these cell lines. Without a complete description of the cell lines' regulatory phenotypes, one cannot be sure whether the relatively low inducibility observed is due to the inability of the inducing agents to access receptors, failures in downstream signaling pathways, or flaws in the cells' xenobiotic-metabolizing machinery (e.g. low levels of P450 oxidoreductase or essential cofactors).

Regulatory acceptance

The wide variability in cell culture, differentiation, and induction protocols reported across the HepG2 and HepaRG literature have, historically, made it very difficult to compare the results reported by different research groups, calling into question the utility of these cell lines for regulatory use. In addition, *in vitro* induction studies using PHHs, HepaRG and HepG2s commonly do not make direct comparisons with induction *in vivo*; when they do the results obtained often fail to reflect induction ratios observed in human volunteers. Berger et al. (2016), for example, treated fifteen healthy volunteers with rifampicin (600 mg/day for 7 days) and calculated clearance ratios of probe drugs to assess the extent of P450 induction. They found that

PHHs cultured in 3 D format best reflected *in vivo* induction; the results in HepaRGs resembled those in 3 D PHHs but correlated less well with induction in human volunteers *in vivo*.

The need for a consistent approach to *in vitro* induction studies has been recognized and various regulatory agencies have initiated programs aimed at the development of standardized protocols for the prediction of *in vivo* induction using PHHs and HepaRGs. The European Center for the Validation of Alternative Methods, in collaboration with the EU Reference Laboratories, has developed and validated protocols for induction studies in cryopreserved human PHHs and HepaRGs (Bernasconi et al. 2019). Comparing pharmaceuticals for which human *in vivo* induction data exist with BNF, PB, and rifampicin, they concluded that cryopreserved PHHs and HepaRGs respond appropriately to the three canonical inducing agents and most blinded test items when induction is assessed using a cocktail of phenacetin, bupropion, and midazolam and metabolites are quantified by LC/MS. The best outcome was for induction of CYP3A4, which was correctly predicted in every case except for artemisinin. The HepaRG method was subsequently transferred to the Organization for Economic Co-operation and Development (OECD) Test Guideline development program (https://tsar.jrc.ec.europa.eu/test-method/tm2009-14); however, the OECD decided against taking the PHH method forward (https://tsar.jrc.ec.europa.eu/test-method/tm2009-13).

A draft OECD Test Guideline, *Determination of cytochrome P450 (CYP) enzyme activity induction using differentiated human hepatic cells* was issued in October 2019 (OECD 2019). This defines a protocol for induction studies in human derived-metabolically competent hepatic test systems (e.g. cryopreserved differentiated HepaRGs) and highlights key considerations, including the use of DMSO at concentrations below 0.1% in assay medium to avoid interference with PXR (presumably after the completion of the differentiation process, since this usually requires the use of ~2% DMSO). It recommends the use of enzyme activity measurements, rather than mRNA upregulation, as the parameter by which to quantify induction, noting that:

> The measurement of functional CYP enzyme activity induction (i.e., catalytic activity) is considered more informative and relevant for chemical risk assessment than measurement of mRNA, since correlations between the CYP-selective activity and the specific CYP mRNA level are frequently poor or lacking.

This problem has been reported by users of *in vitro* induction assays in the pharmaceutical industry, who report that they sometimes observe increases in mRNA expression without corresponding upregulation of enzyme activity (Chu et al. 2009), particularly when a test item is both an inducer and an inhibitor. Even where induction is observed at both the mRNA and protein level, mRNA results tend to overestimate maximal induction (E_{max}) values by up to 10-fold, sometimes more, especially in the case of time-dependent inhibitors of CYP3A4, such as troleandomycin, verapamil, and ritonavir (McGinnity et al. 2009).

The test protocol, referring to Kanebratt and Andersson (2008b), classifies a test chemical with $\geq$2-fold CYP enzyme activity induction compared to negative control as an *in vitro* positive inducer, resembling the detection limits of 1.6-fold against a water vehicle control and 1.7-fold against 0.1% DMSO determined by Kaneko et al. (2009). It also specifies quality assurance criteria along with a panel of chemicals to be used to demonstrate technical proficiency before attempting to characterize induction by test compounds.

The US Federal Drug Administration (USFDA) will accept induction data obtained using PHHs or immortalized cell lines. No preference is expressed as to which cell line should be used; the only explicit mention of HepaRGs in this guidance is a citation of normalization methods for the prediction of *in vitro* CYP3A4 induction (Vermet et al. 2016); instead, the guidance focuses on the use of well-characterized individual batches of cryopreserved PHHs from at least three donors. In terms of the endpoints used, 'Acceptable study endpoints include mRNA levels and/or enzyme activity levels using a probe substrate.' A 2-fold change in mRNA expression is considered acceptable (subject to other criteria having been met, including justification of the test system used); however, no fold induction level is specified for cases where enzyme activity, rather than mRNA expression, is measured.

This guidance may be helpful in standardizing induction studies using HepaRGs; however, Kaneko et al. (2009) note that they were unable to predict the inducing potential of PB, carbamazepine, sulfinpyrazone, or phenytoin using the methodology recommended in a previous (2006) version of the USFDA guidance.

Both the draft OECD Guideline and the USFDA guidance specify 2-fold induction as being indicative of potential drug-drug interaction liabilities; however, it is important to note that the informativeness of criteria, such as 'fold induction over vehicle control' or '% of positive control' is dependent upon the basal expression level, the magnitude of the positive response and the variability of the results: 40% of a 5-fold positive control response would likely be within the background variability of the assay (Chu et al. 2009). It is also, of

course, critical to relate any observations *in vitro* to likely exposure levels *in vivo*.

Evaluation

The HepG2 cell line has a primitive hepatocyte phenotype consistent with its hepatoblastoma origin (Knowles et al. 1980) and its proteome resembles that of human fetal hepatocytes rather than adult cells (Rowe et al. 2013). This is reflected in its expression of higher levels of CYP3A7 than CYP3A4/5 and its CYP1A profile, which favors the expression of CYP1A1 over that of CYP1A2 (Ulvestad et al. 2013). Consistent with its origin, it retains some AhR functionality and responds robustly to CYP1A inducing agents, but it is essentially refractory to PXR and CAR ligands. Thus, while HepG2s are recognized as being well-characterized and widely used in drug metabolism and toxicology studies, their low levels of CYP3A4 and lack of response to known CYP3A4 inducing agents limit their utility in regulatory studies, such as those aimed at the prediction of drug-drug interactions (Chu et al. 2009).

HepaRGs, when fully differentiated, bear some resemblance to adult human hepatocytes. The group which established them considers them to be representative of PHHs with the advantage of being reproducible over several passages, with functional activities which are maintained for several weeks at the confluence and can be modulated by manipulating the culture conditions (Anthérieu et al. 2010). Other investigators, however, have struggled to detect P450-dependent activities in these cells. The overall picture of their functionality is confusing and contradictory.

The need to culture HepaRGs in the presence of DMSO to maintain the differentiated phenotype raises concerns. The fact that the differentiation process requires a complex series of treatments and replatings, as well as the use of slightly different protocols by different investigators, has created huge study-to-study variability. Furthermore, DMSO is cytotoxic at the concentrations used; indeed, the original description of HepaRG cell differentiation notes that the majority of the cells die in the presence of 2% DMSO and it is only the small number of surviving cells that go on to differentiate (Gripon et al. 2002). The fact that DMSO alters gene expression in ways that have not been fully characterized also raises concerns; HepaRGs exhibit a biochemical profile that is markedly different from that of PHHs with respect to glucose homeostasis, lactate and urea production, galactose and sorbitol elimination, and albumin expression (Lübberstedt et al. 2011). Finally, DMSO is both an inducer of P450 expression and an inhibitor of P450 activity; indeed, it is recognized that the apparent poor responsiveness of HepaRGs to rifampicin induction may be a consequence of CYP3A4 expression having already been maximally induced by DMSO (Aninat et al. 2006; Lübberstedt et al. 2011). HepaRGs may therefore overestimate the contribution of CYP3A4 to hepatic drug metabolism while simultaneously underestimating induction by drugs that act *via* NRs, particularly PXR (Kanebratt and Andersson 2008a).

HepaRGs are widely considered to be a good tool for induction studies; however, they have numerous limitations, particularly wide batch-to-batch variability associated with the need to differentiate each batch before use (Kaneko et al. 2009), although the availability of pre-differentiated cryoHepaRGs goes some way toward overcoming this problem. The fact that the expression of P450 enzymes and NRs, as well as responsiveness to enzyme inducers, in this cell line varies so markedly in response to changes in media composition and culture conditions severely hampers its utility for induction studies, especially those conducted for regulatory purposes (Chu et al. 2009). Better transparency concerning the composition of the proprietary induction media and supplements which are promoted for use with this cell line would be of considerable assistance in overcoming this problem. The availability of DMSO-free differentiation media for HepaRGs (Wang et al. 2019) may help to resolve this issue if adopted and used according to a publicly available, standardized protocol. The use of HepaRG derivatives which can differentiate in the absence of DMSO (e.g. HepaRG-CAR) represents an alternative strategy, although having been further engineered such models are arguably even more unlike PHHs than are HepaRGs themselves.

Conclusions

Based on the available data neither HepG2 nor HepaRG is fully reflective of the drug-metabolizing capabilities of human hepatocytes *in vitro*, let alone *in vivo*. Indeed, no single *in vitro* model can accurately reflect the diverse array of outcomes required to mimic the hepatic function *in vivo* (Chu et al. 2009; Hurrell et al. 2018). Apart from the issues highlighted in this review, including the need for clear guidelines as to the necessary characterization of each batch of cells and consistent protocols for induction and activity measurements, it must be recognized that single-origin cell lines cannot represent other aspects of drug disposition including metabolism in the gastrointestinal tract, delivery to the hepatocyte *via* the portal circulation and factors

affecting subsequent elimination. The best way to use these cell lines, therefore, is in combination with other models, such as mice humanized for CAR, PXR, and various P450s (Henderson et al. 2019), which allow induction capacity *in vivo* to be assessed and pharmacokinetic/pharmacodynamic relationships to be characterized *in vivo* and extrapolated to man. The value of humanized models for mechanistic studies has been recognized for more than a decade (Chu et al. 2009); they provide essential information about systemic effects, a dimension which cell culture models cannot offer.

Disclosure statement

CRW is not involved in the development of immortalized cell lines for use in drug metabolism studies but is involved in the use of humanized mouse models for predicting pathways of P450-mediated metabolism and DDIs in man. LAS is an independent Consultant in Investigative Toxicology but also holds a non-stipendiary appointment as an Associate of the School of Applied Sciences, Edinburgh Napier University. This review was prepared under a consultancy agreement with the University of Dundee.

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