13 Assessing the Hepatic Disposition and Toxicity of Xenobiotics Using Primary Hepatocytes

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13.1 INTRODUCTION

When developing a new drug, the goal is to produce therapeutic agents that are safe and effective at treating or preventing diseases. It is estimated that over 10% of drugs fail in clinical trials because of pharmacokinetic (PK) reasons and unexpected drug–drug interactions [1]. As such, the US Food and Drug Administration (FDA) guidelines emphasize the identification of metabolic pathways, relevant major metabolites, and the potential for drug–drug interactions of new chemical entities (NCEs), where metabolism is the principal route of elimination [2]. Consequently, preclinical drug metabolism and drug–drug interaction studies are critical for the development of orally administered drug candidates.

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A number of liver-derived *in vitro* systems, such as slices, subcellular fractions (microsomes), and primary and immortalized hepatocytes, are typically utilized to assess the metabolism and toxicity of new drugs. Microsomes derived from preclinical species and human livers are the screening model of choice for assays based on high throughput drug metabolism, and these are increasingly being replaced or complemented by fresh isolated or cryopreserved primary hepatocytes [3,4]. Before utilizing primary hepatocytes for *in vitro* drug testing, it is helpful to appreciate the key architectural features and molecular factors that determine their unique phenotype *in vivo*. Hepatocytes from adult mammalian liver are highly differentiated epithelial cells and perform a wide variety of functions related to absorption, metabolism, and excretion of drugs and other xenobiotics. The cytoplasm is usually enriched in rough endoplasmic reticulum due to the secretory nature of the cells. There are also numerous areas of smooth endoplasmic reticulum which contain most of the enzymes involved in the metabolism of xenobiotics, especially the cytochrome P450 (P450) and uridinediphospho (UDP)-glucuronosyltransferase enzymes. Fat droplets may appear as inclusions in the cytoplasm, particularly in hepatocytes isolated from donors with a high body mass index (BMI). Hepatocytes are highly polarized cells and exhibit distinct domains on the plasma membrane, specifically the sinusoidal domain on the blood sides and the canalicular domain on the bile side, which is crucial for establishing the vectorial uptake, metabolism, and excretion of xenobiotics from the liver. Within the hepatocyte, there is a polarized localization of cytoplasmic organelles, such as the endoplasmic reticulum, Golgi apparatus, and the cytoskeletal elements, which aids in establishing and maintaining the overall architecture and therefore, functions of the cell [5,6].

Metabolism of drugs and other xenobiotics primarily takes place in the hepatocytes and usually occurs in two phases. Phase I metabolism involves reactions such as oxygenation, oxidation, reduction, dehalogenation and hydrolysis, that expose or introduce functional groups into the lipophilic molecules, thereby making them more hydrophilic and more readily excretable [7]. Phase II metabolism (conjugation enzymes) comprises synthetic reactions in which small endogenous molecules (e.g., glucuronic acid, glutathione, sulfate, glycine, and other amino acids) are added to the functional groups of xenobiotics or their phase I metabolites, thus making them even more polar and readily eliminated. Phase I metabolism does not necessarily precede conjugation and direct phase II metabolism, without prior oxidation/hydroxylation is common. For example, diclofenac is metabolized by direct conjugation of the parent drug as well as oxidation of the aromatic rings, usually followed by conjugation [8]. Similarly, the nonsteroidal anti-inflammatory drug (NSAID) carprofen is directly conjugated to form an ester glucuronide, and this represents the only significant pathway of metabolism [9]. Conjugated metabolites are actively transported out of the hepatocyte by efflux pumps that reside in the basolateral and canalicular (apical) membranes and excreted via bile and urine [7].

Historically, one of the major obstacles to determine the pharmacological and toxicological effects of new drugs on the liver has been the lack of adequate *in vitro* model systems. Therefore, a major goal of pharmaceutical researchers studying drug metabolism and hepatotoxicity has been to develop *in vitro* cell models that simulate *in vivo* conditions. Short-term metabolism and safety studies can be conducted with isolated hepatocytes and liver slices, as the incubation medium, and cells can be easily separated for analysis of drug metabolites [10,11]. When isolated and handled properly, primary hepatocytes from adult liver contain a broad complement of

metabolizing enzymes and transport proteins, organized in a physiologically relevant context and regulated via cellular processes that occur within the liver *in vivo* (e.g., nuclear-hormone-mediated xenosensors) [12].

It must be appreciated, however, that the behavior of the liver is, to some extent, dependent on its anatomy, and this cannot be mimicked by standard suspension culture methods. Cells in suspension inevitably lack various features of the organization of hepatocytes in the intact liver, and they cannot be used for evaluating drug-induced changes in gene expression (i.e., enzyme induction) due to their refractory nature while in suspension [13,14]. Moreover, suspended cells are spherical, entirely covered with microvilli and lack distinct membrane regions such as the sinusoidal versus canalicular domains. In addition, they lack the contact with extracellular matrix or cell–cell contacts (with either other hepatocytes or nonparenchymal cells), which appear to be important for maintaining differentiated function *in vivo* [5,12].

The development of more physiologic hepatocyte culture systems has permitted major advances in the study of hepatic gene regulation and cellular differentiation [14,15]. Many related molecular and biochemical pathways are restored and respond to experimental manipulation only after a period of many days in culture. Therefore, a major advantage of the hepatocyte culture systems compared to other *in vitro* liver preparations is their relative longevity of several days, as compared to hours for perfused liver, liver slices, or suspensions of isolated hepatocytes. Another advantage of primary hepatocyte culture, as compared to freshly isolated suspensions, may be that deficiencies of the latter, resulting from damage of the cell membrane by the collagenase treatment, are resolved during the early stages of culture. It has been suggested that recovery might include restoration of cell polarity, some hormone receptors [14,16,17], rates of protein synthesis [18], restoration of normal intracellular ions and glutathione levels, repair of DNA damaged during cell isolation [19], removal of proteolytic enzymes that may have persistent effects in fresh suspensions [20,21] and removal (as nonattached cells) of any residual damaged hepatocytes.

In addition to the repair of damage, recovery of polarity, and restoration of cell–cell interactions, a preincubation period in monolayer culture before initiating studies may also allow better definition of experimental variables by minimizing effects of prior exposure to hormones or nutrients *in vivo*, especially for primary human hepatocytes [14]. Another special advantage of the hepatocyte culture systems compared to cell suspensions or liver slices is the ease with which cells can be examined using today's high content microscopic imaging techniques, so that structural and subcellular changes induced by exposure to a drug can be readily detected [22]. Likewise, the regulation of cell–cell contacts and the mechanisms involving their re-establishment can be studied in cell culture [5]. Similar studies would be virtually impossible to perform using other *in vitro* model systems.

For applications requiring longer experimental periods, cells stably adapted to a defined *in vitro* environment, or where cell architecture and polarity are likely to be important and more sophisticated culture systems may be required for *in vitro* studies. Moreover, for investigating the hepatotoxic effects of drugs, especially on growth control, changes in gene expression, and bioaccumulation, the ability to maintain functional hepatocytes for many days or even months can be essential. For example, culturing hepatocytes in primary sandwich culture between two layers of extracellular matrix proteins (e.g., collagen type I and Matrigel) dramatically prolongs the longevity of cultures and retains many of the hepatocyte-specific functions [23]. Culturing hepatocytes

on collagen, with and without the overlay, results in morphologically distinct hepatocytes with relatively flattened nonoverlaid cells, while in sandwich culture, hepatocytes form aggregates and retain their cuboidal shape [3]. Sandwich-cultured hepatocytes can form extensive, functional bile canaliculi, while these are minimal in the absence of the overlay [24].

With the increased knowledge of the molecular and cellular factors that dictate hepatocyte structure and function *in vivo*, improved isolation, incubation, and cell culture techniques have greatly expanded the number of applications for hepatocytes during the drug discovery and development process [3,25–27]. In this chapter, several standard approaches are described for utilizing primary hepatocytes in suspension or monolayer culture to address specific issues related to hepatic metabolism, transport, and toxicity.

13.2 METABOLIC STABILITY OF DRUG CANDIDATES

13.2.1 Background

Liver-derived systems, such as microsomes, S9 fractions, and intact hepatocytes are routinely utilized to assess the metabolic stability of drug candidates. The use of intact hepatocytes is advantageous because of the presence of the major drug-metabolizing enzymes, xenobiotic transporters, and relevant cofactors [12]. Compounds can be screened and rank ordered according to *in vitro* intrinsic clearance (CL_{int}) values obtained from hepatocyte-based metabolic stability studies and *in vivo* CL_{int} extrapolated. For some therapeutic areas (e.g. neuropathic pain, Parkinson's disease and osteoporosis), long half-life drugs ($T_{1/2}$) with appropriate target potency and volume of distribution (V_d) are desirable, so that once per day or once per week dosing can be achieved [28,29]. In other instances, a short $T_{1/2}$ is desired as in the case of prodrugs or drugs for insomnia, where it is important to have a short $T_{1/2}$ so that there is no residual drowsiness during the day. Metabolic screening assays enable synthesis of more (or less) stable compounds through structure–activity relationships and prevent the progression of labile compounds prior to more costly *in vivo* studies.

13.2.2 Experimental Approaches

In vitro CL_{int} can be calculated by evaluating metabolite formation or substrate depletion using microsomes and hepatocytes [30,31]. Direct comparisons between these *in vitro* systems indicate that hepatocytes are the superior system. For metabolic stability experiments using the metabolite formation approach, a range of substrate concentrations are employed with a set of protein concentration and incubation time. The Michaelis–Menten parameters V_{max} and K_m are estimated and CL_{int} calculated according to the following equation:

$$CL_{int} = \frac{V_{max}}{K_m}$$

Information regarding metabolites is required when using this method and in some cases, authentic metabolite standards are not available to determine formation rates [30]. Furthermore, linear incubation conditions with respect to protein content and incubation time need to be employed to ensure parent depletion is not greater than 10% [31]. The

substrate depletion approach utilizes a single low concentration assumed to be much lower than the K_m value. Typically, hepatocytes or microsomes are incubated with a substrate concentration of 1 μM and the disappearance of parent compound monitored over several time points. Monoexponential kinetics is assumed as CL_{int} is a function of the first-order rate constant, k. This approach is also referred to as the *in vitro* half-life ($t_{1/2}$) method and the equation used to calculate CL_{int} is

$$CL_{int} = \frac{0.693}{t_{1/2}} \times \frac{V}{N}$$

where $k = 0.693/t_{1/2}$, V is the incubation volume, and N is the number of cells [32,33].

The area under the concentration versus time curve is at times incorporated within similar equations derived from the substrate depletion method [34]. Assuming that the *in vitro* clearance process follows first-order kinetics, CL_{int} is calculated from the ratio of the initial amount of the test compound minus the amount remaining after 2 h of incubation and the corresponding *in vitro* AUC (area under the curve) between 0 and 2 h. In contrast to metabolite formation methodology, this approach is most effective when at least 20% of parent compound is metabolized over the incubation period [31].

The substrate depletion method is simple and amenable to use in an early discovery setting and microsomes or hepatocytes can be used. The advantage of using hepatocytes is that unlike microsomes that only extrapolate biotransformation reactions carried out by P450 enzymes, the contribution of phase II enzymes to metabolism is also assessed [35]. The use of microsomes poses the risk of underestimation of *in vivo* CL_{int} if metabolic elimination routes other than P450s predominate *in vivo*. Also, if active uptake is required for transport of drug to the enzymatic active site, this factor would be missed entirely by the use of microsomes, leading to overestimation of CL_{int}. Alternatively, liver S9 fractions could be utilized, as these contain both microsomes and cytosol. However, S9 fractions lack membranes as well as the multiple cofactors needed for the phase I and phase II reactions, thereby offering limited advantage over microsomes and the disadvantage of adding multiple relatively unstable cofactors [35]. Healthy hepatocytes with the complete cellular machinery including the additional required cofactors and membrane transporters provide the most complete system for conducting metabolic stability measurements.

13.2.3 Metabolism in Fresh and Cryopreserved Suspensions

The availability of healthy cryopreserved hepatocytes has facilitated the use of these cells in routine drug disposition studies by lessening the issues related to availability of fresh tissue. Moreover, these cells have the added advantage of consistency, as hepatocytes isolated from the same liver can be used multiple times, over a period of time [35–37]. We used the substrate depletion method to estimate *in vitro* CL_{int} in freshly isolated human hepatocytes and cryopreserved human hepatocytes processed from the same donor and calculated values in suspensions and monolayers. Our data demonstrated that metabolic activities were statistically similar between freshly isolated and cryopreserved human hepatocytes from the same preparation. We recommend that fresh or cryopreserved hepatocytes may be used for these studies, as these have comparable metabolic capacity. It is important to include the same positive controls across experiments to ensure the integrity of the individual hepatocyte preparation and validity of experimental conditions.

We prepared primary human hepatocytes from three separate tissue samples and used a portion of each preparation immediately after isolation or brief storage (<24 h) in preservation medium or after at least one month of cryopreservation. Both freshly isolated and cryopreserved hepatocytes from each preparation were subsequently characterized for metabolic activity of the major drug-metabolizing enzymes as per the methods shown in Table 13.1. The resulting metabolic activities of fresh and cryopreserved human hepatocytes from the same preparation were comparable, and statistically significant differences were not observed between activities of specific isoforms (Figure 13.1). The observed differences in metabolic activity between the individual hepatocyte preparations are due to interdonor variability and not cryopreservation. These data demonstrate that functional drug-metabolizing enzymes are maintained in

TABLE 13.1 Incubation Conditions for specific activity

Enzyme	Probe Substrate	Concentration (μM)	Incubation Time (min)
CYP1A2	Phenacetin	100	15
CYP2B6	Bupropion	500	15
CYP2C9	Diclofenac	25	15
CYP2C19	S-Mephenytoin	250	30
CYP3A	Testosterone	200	15
UGT	7-Hydroxycoumarin	100	30

Substrate incubations were performed with human hepatocytes in suspension at a density of 0.50×10^6 cells/mL in multi-well plates. Incubations were terminated by freeze on contact or with organic and samples analyzed by HPLC or LC/MS/MS analyses to determine specific activity.

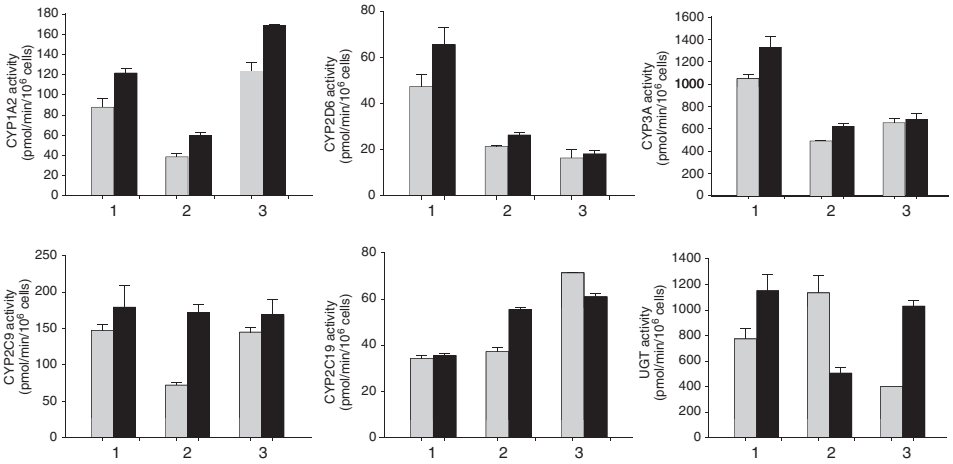


Figure 13.1 Enzyme-specific activities in freshly isolated and cryopreserved human hepatocytes from the same donor. The data represents three separate hepatocyte preparations (1, 2, and 3). The gray bars represent fresh hepatocytes and the black bars cryopreserved hepatocytes from the same preparation. Bars represent mean ± standard deviations of triplicate determinations. Missing error bars are indicative of duplicate determinations. Assay conditions are provided in Table 13.1.

cryopreserved human hepatocytes. Next, we assessed the feasibility of using cryopreserved human hepatocytes for metabolic stability evaluations. Six compounds that covered a range of known metabolic pathways were evaluated in hepatocyte preparations isolated from one donor liver. Phenacetin, midazolam and dextromethorphan were chosen as representative examples of moderate to high turnover compounds and tolbutamide (TLB) and diazepam (DZP) represented compounds belonging to a low turnover class. The substrate benzydamine was chosen as a probe for flavin-containing monooxygenase (FMO) activity to assess non-P450 activity in fresh and cryopreserved human hepatocytes. The intrinsic clearance results for moderate to high turnover compounds were comparable between fresh and cryopreserved suspensions (Table 13.2). In our studies, low turnover substrates proved challenging in short-term suspension studies, as reliable CL_{int} values were not attainable and R^2 values from linear regression analyses were below acceptable ranges (data not shown). Metabolic turnover percentages for TLB and DZP generally did not reach the 20% or greater levels that are essential when using this method [31,38].

To alleviate the possibility of severely reduced viability as a reason for decreased metabolism, cell viability was assessed immediately after thawing and removal of cryopreservation medium (fresh hepatocytes) or postthaw (cryopreserved hepatocytes) and also after a 2 h of incubation at 37°C. Viabilities at the onset of incubations were 77% and 78% for fresh and cryopreserved hepatocytes, respectively. Less than 5% loss in cell viability was observed during the incubation period for fresh hepatocytes, and a 6% decrease in viability was observed in cryopreserved hepatocytes. Our results demonstrate that cryopreserved human hepatocytes are good tools for short-term intrinsic clearance experiments and demonstrate particular suitability for moderate to high turnover compounds. As more drugs are identified as substrates of non-P450 pathways, it is critical to have confidence in the metabolic competency of cryopreserved hepatocytes as an *in vitro* tool for studying these pathways. We have conducted metabolic stability studies in hepatocyte suspensions for up to 4 h in culture. We found that incubating hepatocytes for extended periods of time in suspension cultures (i.e., >4 h) causes unacceptable drops in viability (>20%) and a differential loss of phase II metabolic rates compared to those for phase I reactions.

13.2.4 Metabolism in Cultured Fresh and Cryopreserved Primary Human Hepatocytes

In vitro metabolic stability measurements in suspension hepatocytes often underpredict *in vivo* CL_{int}, particularly for low turnover compounds. For orally administered candidate drugs, prediction of *in vivo* CL_{int} is required for dose estimations required to achieve appropriate PK for target pharmacological activity. In these cases, short-term *in vitro* suspension assays in which the substrate depletion method is used may not be applicable for CL_{int} predictions. Griffin and Houston proposed using monolayer cultures as an alternative to suspensions for estimating intrinsic clearance [39]. In their studies, seven compounds were assessed in rat suspensions and monolayers including low turnover substrates *S*-warfarin and tolbutamide and the data demonstrated that CL_{int} can be effectively measured at below 0.1 μL/min/10^6cells.

We assessed the metabolic stability of six compounds in human hepatocyte monolayers isolated from a single donor liver. The moderate to high turnover compounds

TABLE 13.2 Comparison of Intrinsic Clearance Values Obtained in Freshly Isolated and Cryopreserved Hepatocytes Suspensions and Cultures Processed from the Same Donor Tissue

Substrate	Fresh or Cryopreserved	Hepatocyte Monolayers			Hepatocyte Suspensions			Observed *In Vivo* CL (mL/min/kg)
		CL_{int} *In Vitro* (μL/min/10^6 cells)	CL_{int} Scaled (mL/min/kg)	Predicted CL_H (mL/min/kg)	CL_{int} *In Vitro* (μL/min/10^6 cells)	CL_{int} Scaled (mL/min/kg)	Predicted CL_H (mL/min/kg)	
PHN	Fresh	15.3 ± 1.1	40.3 ± 2.9	13.4 ± 0.3	78.3 ± 8.0	207 ± 21	18.2 ± 0.2	19.6 ± 4.5[a]
	Cryopreserved	9.13 ± 1.17	24.1 ± 3.1	10.9 ± 0.6	54.5 ± 4.8	144 ± 13	17.5 ± 0.2	
MDZ	Fresh	13.0 ± 2.1	34.2 ± 5.5	12.6 ± 0.8	21.5 ± 0.3	56.7 ± 0.8	14.8 ± 0.1	12.0 ± 1.6[b]
	Cryopreserved	6.48 ± 1.29	17.1 ± 3.4	9.16 ± 1.03	13.1	34.6	12.5	7.0 ± 2.1[a]
DXT	Fresh	2.70 ± 1.17	7.12 ± 3.09	5.11 ± 1.81	13.1 ± 1.9	34.7 ± 4.9	12.6 ± 0.7	8.6[c]
	Cryopreserved	2.56 ± 0.66	6.77 ± 1.75	5.01 ± 1.01	8.40 ± 3.42	22.0 ± 9.0	10.2 ± 2.2	
TLB	Fresh	1.88 ± 0.32	4.95 ± 0.85	3.96 ± 0.55	N.R.	N.R.	N.R.	0.21 ± 0.04[a]
	Cryopreserved	0.329 ± 0.146	0.869 ± 0.384	0.829 ± 0.351	N.R.	N.R.	N.R.	
DZP	Fresh	2.47 ± 0.62	6.51 ± 1.64	4.87 ± 0.94	N.R.	N.R.	N.R.	0.37 ± 0.10[a]
	Cryopreserved	0.807 ± 0.067	2.13 ± 0.18	1.92 ± 0.15	N.R.	N.R.	N.R.	
BNZ	Fresh	1.58 ± 0.24	4.18 ± 0.65	3.45 ± 0.45	4.40	11.6	7.33	2.3[d]
	Cryopreserved	2.14 ± 0.68	5.65 ± 1.79	4.36 ± 1.10	5.20	13.7	8.09	

Abbreviations: BNZ, benzydamine; DXT, dextromethorphan; DZP, diazepam; MDZ, midazolam; PB, phenobarbital; PHN, phenacetin; TLB, tolbutamide; N.R. not reported.

Primary human hepatocytes cultures and suspensions were incubated with a low concentration of substrate (0.5 or 1 μM) and intrinsic clearance values determined from linear regression analyses of disappearance of parent versus time profiles. *In vitro* clearances were scaled to predicted hepatic clearance as described within the text.

[a]Stringer *et al*. [46].

[b]Blanchard *et al*. [40].

[c]McGinnity *et al*. [32].

[d]Obach *et al*. [33].

were incubated with the hepatocyte monolayers for up to 8 h and the low turnover compounds tolbutamide and diazepam were incubated for up to 24 h. The cell monolayers revealed no marked change in cell morphology after compound treatment, relative to vehicle control, after overnight incubations. We observed higher turnover with tolbutamide and diazepam in the hepatocyte monolayers as compared to that in suspensions. While *in vitro* CL_{int} values were statistically similar between fresh and cryopreserved monolayers, CL was slightly higher in fresh monolayers for phenobarbital, midazolam, tolbutamide, and diazepam (Table 13.2). This trend was more evident when the values were scaled to *in vivo* CL_{int}. Overall, our data are in line with other published studies comparing CL_{int} in fresh and cryopreserved human hepatocyte suspensions and monolayers [32,34,40,41].

Hepatic clearance (CL_H) can be estimated by three separate established models, each with different perceptions of liver physiology. These include the well-stirred model, parallel tube model, and dispersion model, all of which rely on inherent assumptions [30,42,43]. The fraction of drug unbound (f_u) is incorporated in the models to account for plasma protein binding. The present study utilized the well-stirred model to estimate CL_H with omission of the fraction unbound terms [32]. The following scaling factors were utilized in the equation: human liver weight = 22 g/kg body weight, hepatocellularity = 120×10^6 cells/g of liver, and hepatic blood flow = 20 mL/min/kg [32]. CL_H was compared to clearance observed in clinical studies. Predicted hepatic clearance was within the range (less than twofold) observed for *in vivo* clearance for phenobarbital, midazolam, dextromethorphan, and benzydamine in both fresh and cryopreserved hepatocyte suspensions. *in vivo* clearance for these compounds could also be predicted (less than twofold) from both fresh and cryopreserved hepatocyte monolayers. Interestingly, predictions were overestimated for low turnover compounds in the hepatocyte monolayers. Fresh monolayers overestimated tolbutamide CL by about 19-fold, while cryopreserved monolayers overestimated CL by about fourfold. A similar trend was observed with diazepam predictions where fresh and cryopreserved monolayers produced 13-fold and 5-fold overestimations in CL, respectively. These results are in agreement with a previously published study which reported overestimations of *in vivo* clearance for low clearance compounds using rat hepatocyte monolayers [39].

It is not immediately clear why fresh hepatocyte monolayers produced higher clearance than cryopreserved monolayers, as our prior studies have shown similar metabolic activity. Tolbutamide is metabolized by CYP2C9, while diazepam is metabolized by CYP2C19 and to a lesser extent, CYP3A. The CYP2C9-, CYP2C19-, and CYP3A-specific activities of the hepatocyte preparation used for this study did not differ significantly between the fresh and cryopreserved preparations (Fig. 13.1). If there was a difference in the two preparations in culture, this did not manifest in the predictions obtained for moderate and high turnover compounds. More studies are required with low turnover substrates and additional hepatocyte preparations to better understand the discrepancy between fresh and cryopreserved monolayers observed with tolbutamide and diazepam.

13.2.5 *In Vitro–In Vivo* Correlations

In vitro–in vivo correlation is the real measure of success for any given systematic approach relative to metabolic stability. Ito and Houston [44] reported a ninefold underestimation of *in vivo* CL_{int} for 52 drugs using published *in vitro* CL_{int} data derived

from human liver microsomes. A separate report examined 37 drugs in cryopreserved human hepatocytes from collated sources and reported a 4.5-fold underestimation in predicted clearance [45]. A recent study assessed compounds cleared primarily by glucuronidation in hepatocytes and microsomes. A poor *in vitro–in vivo* correlation was observed in microsomes, even after adding alamethicin and UDPGA in the incubations for Phase II metabolism [46]. In contrast, hepatocytes were proficient at catalyzing the phase II reactions. Overall, the general consensus is that primary hepatocytes predict hepatic clearance within two- to threefold of *in vivo* clearance values and are superior to microsomes for this purpose [35]. Currently, cryopreserved hepatocytes are the favorable model for metabolic stability studies because of the convenience of availability and because the same batch can be used multiple times, lessening concerns related to interdonor variability [35]. These results and others support the utility of cryopreserved human hepatocytes for metabolic stability and other metabolism studies (e.g., metabolic profiling and metabolite identification).

One of the main causes of discordance between *in vitro* and *in vivo* predictions and between laboratories that often goes unrecognized or underappreciated is the quality of the cell preparations—whether freshly isolated or cryopreserved. There are limited industry-wide acceptance criteria or established quality standards for primary human hepatocytes that has gained widespread adoption. As such, we propose the following criteria that can and should be considered whenever selecting a batch of cryopreserved hepatocytes for metabolic stability evaluations:

1. Consider the cellular and biochemical data that is provided with each batch of hepatocytes from the vendor. For example, most providers of hepatocytes include post-thaw viability and cell yield data, as well as the average specific activities for key P450 and Phase II enzymes, which should be within acceptable ranges. Average yields and viabilities vary somewhat between preparations and vendors but minimally they should be between 5.0 and 10.0 million cells per vial and $>80\%$, respectively [38].
2. Information about "viability stability" over time should be provided by or obtained from the vendor to determine the suitability of preparations for suspension incubations. For instance, most metabolic stability studies require incubation times of 1–3 h. As such, it is important that the viability of the hepatocytes stays reasonably stable and does not drop more than 15–20% during that time period.
3. Average intrinsic clearance values of known substrates determined for each batch of cells should also be requested, if not provided. Typical values for low, medium, and high clearance compounds are available in literature and represent some guidance regarding the suitability of cryopreserved hepatocytes for metabolic stability assays.
4. Finally, in the event that accurate clearance values are needed for low turnover compounds, additional data about the attachment efficiency and percent confluence of the corresponding monolayers are the relevant parameters. On the basis of our experience, cryopreserved hepatocytes that exhibit $>70\%$ attachment efficiency after an initial 4–6 h attachment period generally are suitable for metabolic stability studies, with the prerequisite that suitable intrinsic clearance values are obtained for prototypical substrates during the subsequent 12- to 24-h culture period.

13.3 METABOLIC PROFILING AND METABOLITE IDENTIFICATION

13.3.1 Metabolic profiling

Metabolic profiling is the qualitative measure of the parent compound, the metabolites and their intermediates in response to actions by drug-metabolizing enzymes in biological systems. The measurement and interpretation of the metabolite profile from biological samples (typically primary hepatocytes or hepatic microsomes *in vitro*; and urine, serum, bile, or tissue extract, *in vivo*) provide a view of the exposure that a particular species has had, or will have, to the xenobiotic. The metabolism of drugs is a complex process involving one or more phase I and phase II enzyme systems. In discovery, the potential half-life of compounds is ranked based on metabolic stability, whereas in preclinical development, *in vivo* rodent and nonrodent species are used for allometric scaling to extrapolate to the human. Metabolic profiling is very useful in selecting the right preclinical nonrodent species for safety and PK assessments. For this, it is essential to choose the species with metabolites that are similar to what is expected in humans. Reasonably similar metabolites in *in vitro* systems support the relevance of a particular animal species to the assessment of a potential human risk. Moreover, knowledge of differences between a preclinical species and humans (e.g., a toxic metabolite in a particular preclinical species but not in humans) could aid in interpretation of clinical data. If there are major differences in the metabolites between the test species and humans, this would reduce the confidence in these studies as predictors of safety in humans. At the least, the species chosen should have all the major metabolites found in the human *in vitro* samples.

A decade ago, human liver microsomes were routinely used for metabolic profiling comparisons. However, this approach can lead to underprediction of metabolism, as the assumption that phase I oxidation is the major pathway of metabolism of the drug candidate is made. For example, in humans, ethinyl estradiol is predominantly metabolized by phase II metabolism into glucuronide and sulfate conjugates [47]. Human liver microsomes produce primarily the 2-hydroxyl metabolite, while human hepatocytes produce both the conjugated metabolites, similar to what is observed in human plasma samples. Microsomes underpredict ethinyl estradiol metabolism and fail to provide a true pictures of metabolite exposure. Several such studies have demonstrated that while microsomes allow for the evaluation of specific phase I oxidative metabolites, intact fresh isolated or cryopreserved hepatocytes represent a more scientifically relevant experimental system, as these provide a more complete system to assess all possible metabolites. In cases where human hepatocyte samples develop a major metabolite not present in any of the animal cells, that metabolite has to be synthesized and exposed to the preclinical species, so that any potential toxicity can be assessed. These applications demonstrate the utility of hepatocytes for *in vitro* metabolic profiling and identification studies, as these have the capacity to develop phase I and phase II metabolites, similar to those formed *in vivo*.

Metabolic profiling studies are typically conducted over a short time period (2–4 h) using suspension hepatocytes that are placed in a nutrient-rich buffer or media, in a gently shaking, 37°C environment. In most cases, this is an appropriate format to conduct these experiments. In some cases, particularly for low turnover compounds, for example, *S*-warfarin and tolbutamide, this time is not sufficient for metabolism to completely occur. For these studies, we have found that similar to the intrinsic

clearance measurements, it is best to conduct overnight incubations. Since suspension hepatocytes do not thrive for more than 4 h in this environment, attached hepatocytes on a simple collagen substratum should be used, as these allows for a longer incubation and the formation of most of the possible metabolites [48].

13.3.2 Metabolite Identification

Structural identification of metabolites that have been generated by *in vitro* or *in vivo* evaluations is an integral part of preclinical and clinical drug metabolism studies. Literature indicates that the qualitative metabolite pattern of drug candidates obtained *in vitro* generally reflects that found *in vivo* [49]. *In vitro* knowledge of the structures of the major metabolites helps in predictions of the fate of drug candidates and enables the planning of timely strategies in cases where there may be toxicokinetics or hepatotoxicity issues in preclinical species or in the clinic.

The evaluation of all major human metabolites *in vitro* is emphasized in the FDA 2008 guidance "Safety Testing of Drug Metabolites." The guidance recommends safety testing of drug metabolites that (i) account for more than 10% of parent drug AUC in human samples, (ii) are present only in human plasma, or (iii) constitute disproportionately higher levels in human samples as compared to preclinical species. Safety testing includes synthesis of the metabolite and exposure of the metabolite to a chosen preclinical species, followed by the conduct of toxicity studies. Conducting appropriate studies with hepatocytes before clinical evaluations could help in early attrition of drug candidates that have the potential to fail in the clinic due to metabolite-related toxicities.

Similar to metabolic stability and profiling measurements, a much clearer and more complete picture of the metabolites is obtained using hepatocytes versus microsomes. Improved tissue culture methodologies have enabled analysis of biliary metabolites *in vitro* [50]. By culturing primary hepatocytes in a 3D micropatterned collagen gel substrate configuration, Matsui *et al*. were able to quantify bile acids embedded in a microcavity with a diameter of 60–80 μm after directly recovering from the bile canaliculi of hepatocytes.

Previously, structural elucidation was typically performed later in preclinical development; however, over the past decade, this assessment has moved to Discovery in order to reduce later stage attrition. Owing to the larger number of compounds and the tighter time lines in Discovery, there is the need for immediately available and higher throughput methods (e.g., using cryopreserved hepatocytes and LC/MS/MS systems that utilize "smart" software to assist in the acquisition, identification, and structural characterization of metabolites). Although sensitivity can be a factor when using hepatocytes, substrate incubations at higher concentrations (i.e., 10–50 μ M) and longer incubation times (i.e., 2–4 h) can circumvent this potential issue. Mass analyzers including time of flight, triple-stage quadrupole, ion traps, and Fourier transform mass spectrometry are increasingly sensitive. However, mass spectrometry (MS) alone is unable to adequately address all the caveats of structural elucidation. Additional information such as the site of biotransformation can aid structure activity relationship (SAR) to prevent undesirable PK effects when utilized in drug development. Where structural elucidation is needed, wet chemistry techniques combined with MS are useful including chemical derivatization, hydrogen/deuterium exchange, and hydrolysis.

Nuclear magnetic resonance (NMR) spectroscopy is also a valuable tool for structural characterization of metabolites. For example, LC/MS/MS was used to identify metabolites of retigabine in rat, dog, and human urine and other biological excreta [51]. While glucuronide metabolites were discovered in all three species, an additional acetylated metabolite was identified in rats and humans, which was absent in dogs. Using NMR, it was shown that the structures of retigabine metabolites in human and rat were similar across the *in vivo* urine samples and *in vitro* hepatocyte extracts. Similarly, novel metabolites of verapamil were identified in both primary human hepatocytes and human urine using LC/MS/MS and the structures elucidated using NMR [52]. All the above studies support the role of hepatocytes for metabolism studies, particularly for metabolic stability, profiling, and identification.

13.4 INHIBITION

13.4.1 Background

PK drug–drug interactions can occur when one drug alters the metabolism of a coadministered drug. Specifically, the metabolic route(s) of elimination, including most of those occurring via the P450 family of enzymes, can be inhibited by concomitant drug treatment leading to serious clinical drug–drug interactions. P450 inhibition is implicated in a majority of clinically relevant drug–drug interactions [53,54], as a P450 inhibitor can decrease metabolic clearance of a coadministered substrate drug and/or decrease formation of an active metabolite (or formation of a drug from a prodrug), resulting in decreased efficacy. This inhibition can be assessed in preclinical *in vitro* studies using hepatic microsomes and hepatocytes. The increase of plasma concentrations caused by drug interactions can be substantial, for example, the interaction between ketoconazole or itraconazole (CYP3A4 inhibitors) and triazolam (CYP3A4 substrate), which resulted in a 22- to 27-fold increase in triazolam exposure following coadministration with ketoconazole and itraconazole, respectively [55]. Unwanted effects are most obvious and expected when they involve drugs such as warfarin with a narrow therapeutic range resulting in an increase in plasma concentration.

Interactions involving enzyme inhibition have been responsible for the limited development potential, severe dosing restrictions, and even termination of development of many investigational drugs. If these interactions are not identified early in the development process, the result can be exposure to toxic drug levels in some patients, resulting in a black box warning on the product label or withdrawal of the drug from the market. A classic example of a drug interactions is with the nonsedating antihistamine Seldane® (terfenadine) and the common antibiotic erythromycin [56]. Patients treated with terfenadine along with erythromycin accumulated dangerously high blood levels of terfenadine because of the inhibition of CYP3A4 enzymes by erythromycin, resulting in potentially fatal arrhythmia. On the basis of this, the FDA issued a recall of Seldane® in 1997 and subsequently recommended that the potential of an investigative drug to be involved in drug–drug interactions be assessed relatively early in drug development.

Drugs that cause time- or metabolism-based inhibition of CYP450 enzymes may also result in potent, potentially toxic drug interactions. One such compound, mibefradil, a Ca^{2+} channel antagonist for the treatment of hypertension and

chronic stable angina pectoris caused serious drug–drug interactions, including rhabdomyolysis and renal failure with simvastatin, and symptomatic bradycardia with β-blockers [57,58]. Subsequently, mibefradil was found to be a potent inhibitor of CYP3A4, CYP2D6, and P-glycoprotein (P-gp) and a potent time-dependent inhibitor for CYP3A4/3A5 [59–61]. These serious adverse events resulted when mibefradil was coadministered with CYP3A4 substrates, likely because of time-dependent inhibition of CYP3A4 and mibefradil was withdrawn from the market a year after launch. Similarly, rofecoxib, a cyclooxygenase-2-selective NSAID, was withdrawn from clinical use in 2006 because of cardiovascular events, as rofecoxib moderately increased plasma concentrations and effects of theophylline [62] and the *R*-isomer of warfarin [63]. Therapeutic doses of rofecoxib increased plasma concentrations of tizanidine more than 10-fold, suggesting potent inhibition of CYP1A2 [64]. *In vitro* studies demonstrated that rofecoxib was a potent, mechanism-based CYP1A2 inhibitor and provided a mechanistic explanation for the interactions of rofecoxib with CYP1A2 substrates [65].

13.4.2 Experimental Approaches and Data

To understand the potential of drug candidates to cause PK-based drug–drug interactions via inhibition of drug-metabolizing enzymes, *in vitro* screens are employed to determine the extent and type of P450 inhibition. These assays typically use recombinantly expressed P450s, human liver microsomes, and human hepatocytes.

A majority of all enzyme inhibition studies are conducted using microsomes. More recently, the use of human hepatocytes to study P450 reversible and time-dependent inhibition is increasing, as primary hepatocytes provide an *in vitro* environment that closely resembles the human liver. CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 reversible inhibition has been evaluated by known isoform-specific inhibitors in primary human hepatocytes [66]. Cryopreserved hepatocytes from a mixed pool of 10 adult human donors were used. Hepatocytes (2×10^6 cells/mL) were incubated with the test inhibitor and isoform-specific substrates: 50 μ*M* phenacetin (CYP1A2), 50 μ*M* coumarin (CYP2A6), 75 μ*M* tolbutamide (CYP2C9), 50 μ*M* *S*-mephenytoin (CYP2C19), 8 μ*M* dextromethorphan (CYP2D6), 50 μ*M* chlorzoxazone (CYP2E1), and 50 μ*M* testosterone (CYP3A4), for up to 2 h before terminating with organic solvent. The data set demonstrated that 10 μ*M* furafylline (CYP1A2), 50 μ*M* diethyldithiocarbamate (CYP2A6), 1 μ*M* sulfaphenazole (CYP2C9), 10 μ*M* omeprazole (CYP2C19), 1 μ*M* quinidine (CYP2D6), 100 μ*M* 4-methylpyrazole (CYP2E1), and 1 μ*M* ketoconazole (CYP3A4) inhibited metabolite production by 91.8%, 77.6%, 78.9%, 74.7%, 89.9%, 82.7%, 94.9%, respectively. Another report compares K_i values determined in rat microsomes and freshly isolated hepatocytes using six P450 inhibitors (miconazole, fluconazole, ketoconazole, quinine, fluoxetine, and fluvoxamine) [67]. These inhibitors were included in studies utilizing four probe substrates for CYP2C, CYP2D, and CYP3A enzymes (tolbutamide and phenytoin, dextromethorphan, and midazolam, respectively). All analyses were performed under initial rate conditions with respect to incubation time, microsomal protein content, or hepatocyte density. The K_i values determined in this investigation were in good agreement between microsomes and hepatocytes.

Time-dependent P450 inhibition has also been evaluated using cryopreserved human hepatocytes for six structurally diverse compounds known to exhibit time-dependent

inhibition [68]. CYP3A4 activities were determined after incubation with amprenavir, diclofenac, diltiazem, erythromycin, raloxifene, and troleandomycin. Human hepatocytes (0.5×10^6 cells/mL) were preincubated with and without time-dependent inactivators in 48-well plates for 1 h at a final volume of 250 μL. At the end of the preincubation, 200 μL aliquots were transferred to 96-well tubes, centrifuged, and the resulting pellet was washed and resuspended in cell culture medium containing midazolam, the probe substrate. The kinetic parameters (k_{inact} and K_I) associated with the inactivation of CYP3A4 in human hepatocytes were derived. In addition, the kinetic parameters of inactivation (k_{inact} and K_I) were measured using human liver microsomes. In general, this side-by-side comparison indicated that the microsome-based predictions overestimate the inactivation potency observed in hepatocytes for troleandomycin, erythromycin, raloxifene, and amprenavir. The discrepancies are likely related to metabolic stability, nonspecific binding, active membrane transport, or a combination of these factors. Therefore, these factors must be considered when microsomal inactivation parameters are used to predict inhibition-based drug interactions in intact cell systems. Time-dependent P450 inhibition has also been evaluated using human hepatocytes in cultures [69]. P450 activities were determined after incubation with tienilic acid, a prototypical inhibitor of CYP2C9 and erythromycin, troleandomycin, and fluoxetine, prototypical inhibitors of CYP3A4. Human hepatocytes were cultured on six-well collagen-coated plates and treated with varying concentrations of inhibitor (0.1, 1, and 10 μ*M*) for up to 48 h. At several time points during the incubation period, isoform-specific substrates in cassette format were added to each well. Aliquots were removed at various time points after addition of probe substrate, and the samples were quenched by the addition of organic solvent. Phenacetin deethylation, diclofenac 4′-hydroxylation, *S*-mephenytoin 4′-hydroxylation, bufuralol 1′-hydroxylation, and midazolam 1′-hydroxylation were used as selective probe reactions for CYP1A2, 2C9, 2C19, 2D6, and 3A4, respectively. The kinetic parameters (k_{inact} and K_I) associated with the inactivation of these enzymes in human hepatocytes were derived. In addition, the kinetic parameters of inactivation (k_{inact} and K_I) were measured using recombinantly expressed enzymes and human liver microsomes. In a side-by-side comparison, it was noted that the kinetic parameters obtained using human hepatocytes were in good agreement with those values derived using recombinantly expressed enzymes and human liver microsomes [69].

We have developed hepatocyte-based methods for the identification and quantification of reversible and time-dependent inhibition for CYP1A2, 2B6, 2C8, 2C9, 2C19, and 3A4, validating these assays with appropriate positive control inhibitors. Using single concentrations of the inhibitors investigated, 12–73% reversible inhibition was observed with the human hepatocytes (Table 13.3). Time-dependent inhibition in hepatocytes was assessed for a single concentration of inhibitor after a 30-min preincubation with hepatocytes and remaining activity compared to hepatocytes preincubated with vehicle only. At the inhibitor concentrations used, enzyme activities were reduced to 29–69% of control activity (Table 13.4). For CYP1A2, CYP2B6, CYP2C19, and CYP2D6, it was also observed that the 30-min preincubation with vehicle resulted in a reduction in activity of the enzymes as compared to coincubation alone (data not shown). The percentage of inhibition observed after 30-min preincubation with hepatocytes was compared to previous data generated in microsomes at identical inhibitor concentrations (Table 13.4). For all isoforms investigated, the inhibition in hepatocytes was similar to or less than the inhibition observed in human liver microsomes. This reduction in inhibition could be due to the contribution of cell

TABLE 13.3 Percentage of Reversible Inhibition in Cryopreserved Human Hepatocytes When Coincubated with Positive Control Inhibitors

Enzyme	Assay	Chemical Inhibitor	Inhibitor Concentration (μM)	Metabolic Activity in the Presence of Inhibitor (Percentage of Vehicle Control)
CYP1A2	Phenacetin *O*-dethylase	Furafylline	5	57.8 ± 1.7
CYP2B6	Bupropion hydroxylase	Thiotepa	20	53.0 ± 1.2
CYP2C8	Paclitaxel 6α-hydroxylase	Quercetin	10	88.1 ± 3.3
CYP2C9	Diclofenac 4′-hydroxylase	Sulfaphenazole	6.25	37.3 ± 3.2
CYP2C19	(*S*)-Mephenytoin 4′-hydroxylase	Ticlopidine	1	26.8 ± 9.2
CYP2D6	Dextromethorphan *O*-demethylase	Quinidine	0.4	58.6 ± 14.3
CYP3A4/5	Midazolam 1′-hydroxylase	Ketoconazole	0.1	41.6 ± 8.3
CYP3A4/5	Testosterone 6β-hydroxylase	Ketoconazole	0.1	60.2 ± 1.6

TABLE 13.4 Percentage of Time-Dependent Inhibition in Cryopreserved Human Hepatocytes When Preincubated with Positive Control Inhibitors

Enzyme	Substrate	Chemical Inhibitor	Inhibitor Concentration (μM)	Metabolic Activity in the Presence of Inhibitor (Percentage of Vehicle Control)	
				Hepatocytes	HLM
CYP1A2	Phenacetin *O*-dethylase	Furafylline	1	29.3 ± 6.8	11.0–12.9
CYP2B6	Bupropion hydroxylase	Thiotepa	30	35.5 ± 2.1	10.9–12.4
CYP2C8	Paclitaxel 6α-hydroxylase	Phenelzine	100	69.1 ± 6.0	25.4–45.2
CYP2C9	Diclofenac 4′-hydroxylase	Tienilic acid	3	58.3 ± 8.3	5.8–39.5
CYP2C19	(*S*)-Mephenytoin 4′-hydroxylase	Ticlopidine	0.5	39.4 ± 19.6	20.4–36.1
CYP2D6	Dextromethorphan *O*-demethylase	MDMA	10	55.4 ± 6.1	3.3–5.3
CYP3A4/5	Midazolam 1′-hydroxylase	Mifepristone	10	35.1 ± 5.5	5.6–22.7
CYP3A4/5	Testosterone 6β-hydroxylase	Mifepristone	10	45.3 ± 4.5	6.2–9.5

Abbreviations: HLM, human liver microsomes; MDMA, 3,4-methylenedioxymethamphetamine.

membranes and/or uptake/efflux transporters, limiting the access of the inhibitors to the CYP enzymes. We also quantified the time-dependent inhibition of CYP3A4/5 by mifepristone, using midazolam 1′-hydroxylase activity as a marker for CYP3A4/5 activity. The inhibition parameters were determined to be $K_I = 23.1 \pm 2.1\,\mu M$ and $k_{inact} = 0.101 \pm 0.003\,min^{-1}$. We demonstrated that hepatocytes can be used for the determination of reversible and time-dependent inhibition; however, it is difficult to prove that any inhibition observed is a result of metabolic activation. Further work will require a focus on conditions that would optimize a controlled environment for metabolic activation.

In another work reported by Mao *et al*., cryopreserved human hepatocytes were used to compare the accuracy of enzyme inhibition predictions in a protein-free system combined with the fraction unbound in human plasma for inhibitor(s) with those obtained with protein-containing incubations [70]. Hepatocytes were incubated with or without CYP3A, CYP2C9, or CYP2D6 inhibitors at multiple concentrations, in human plasma or tissue culture media. The data indicated a general underprediction when using free systemic plasma concentrations. Hepatocytes suspended in the plasma proved to be a simple, accurate predictor of drug–drug interactions and superior to the protein-free approach [70].

Over the past 10–15 years, hepatocytes in primary culture have become widely recognized as the "gold standard" for predicting *in vivo* P450 induction in humans. In contrast, progress is just now being gained toward understanding the use of hepatocytes in inhibition/drug interaction studies. It is clear that the use of hepatocytes in these studies is advantageous in that the study of both phase I and phase II processes together with drug efflux and/or uptake and cellular accumulation can be assessed. Further studies should focus on further optimization of the experimental conditions and on understanding how the obtained results can be used in *in vitro–in vivo* predictions of drug–drug interactions mediated via P450 inhibition.

13.5 ENZYME INDUCTION

13.5.1 Background

Induction of hepatic drug-metabolizing enzymes can have significant consequences on the PK and toxicity of drugs. Chronic dosing of a compound that is an inducer can increase the expression of drug-metabolizing enzymes and transporters, which often leads to a concomitant increase in the activity of these clearance pathways. Consequently, any coadministered drug that utilizes these same clearance pathways will be more rapidly eliminated, resulting in lower drug concentrations and may become less effective. For example, rifampin can decrease the *in vivo* AUC of coadministered drugs by 70–90% (e.g., when coadministered with (*R*)-verapamil [71]), thereby significantly decreasing efficacy. Induction of P450s has been shown to result in toxicity by increasing reactive metabolite production or the CYP1A-mediated bioactivation of procarcinogens [72]. Induction effects are further complicated, as foods (e.g., grapefruit juice), cultural and lifestyle-related agents (e.g., alcohol and nicotine), and herbal supplements (e.g., St. John's Wort) can also induce enzyme activities. Chronic exposure to inducing agents has been shown to disrupt cellular homeostasis, leading to endocrine disruption, altered inflammatory response, and bone density loss [73–76]. Potent inducers such as rifampin and St John's Wort simultaneously upregulate multiple genes including CYP3A4, CYP2B6, UDP-glucuronosyltransferase (UGTs), and

glutathione-S-transferase (GSTs), as well as multiple transporters such as MDR1 (multidrug-resistant protein 1) and organic anion-transporting polypeptides (OATPs).

In most cases, the inducer directly or indirectly activates a cytosolic receptor complex causing it to translocate to the nucleus of the cell where the complex is converted to an active transcription factor, leading to increased transcription and translation of target genes that possess the corresponding DNA binding sequences in their promoter regions [77]. Although increased levels of these enzyme systems can occur by the stabilization of mRNA and/or proteins (e.g., induction of CYP2E1) [78,79], inductive events that lead to clinically significant changes in the clearance or bioactivation of drugs predominantly occur by a receptor-mediated process that causes increased production of an enzyme or other protein initiated at the level of gene transcription. These clinical effects can be predicted qualitatively *in vitro* using primary hepatocytes in sandwich culture.

In hepatocytes, the principle nuclear receptors, the aryl hydrocarbon receptor (AhR), the pregnane X receptor (PXR), and the constitutive androstane receptor (CAR) control the expression of key P450 enzymes involved in drug metabolism including CYP1A (AhR), CYP2B, CYP3A, and CYP2C subfamilies (PXR and CAR); the Phase II enzymes such as UGTs, glutathione-S-transferases, sulfotransferases; and transporters including MDR1, MRP2 (multidrug-resistance-associated protein 2), OATPs, and organic cation transporters (OCTs). Under appropriate culture conditions, primary hepatocytes retain relevant levels of the major drug-metabolizing enzymes, transporter proteins, nuclear receptors, and transcription factors, while thus far no immortalized or transformed hepatic cell line (e.g., HepG2, Fa2N4, or HepaRG) has been reported to contain the complete complement of enzymes, transporter proteins, and all corresponding receptor signaling cofactors. As such, primary hepatocytes in a sandwich configuration remain the model of choice for *in vitro* induction studies [14].

13.5.2 Experimental approaches

As stated above, under appropriate conditions, primary human hepatocytes express the biochemical and molecular machinery necessary to respond to enzyme inducers much as they would *in vivo* [80–82]. Currently, the most relevant culture model for induction is the sandwich culture system in which freshly isolated or cryopreserved hepatocytes are seeded on collagen, type I and allowed to attach and sandwich with an extracellular matrix overlay, typically hydrogels of collagen or Matrigel® [14,24,83]. This format provides a more "natural" environment for the hepatocytes, which retain liver-like cell morphology and form functional bile canaliculi [24,84,85]. In this culture configuration, the cumulative effects of metabolism, induction, time-dependent inhibition, and metabolite effects replicate the more steady-state-like effects on P450 activity and gene expression [86].

Tissue culture media also influences the morphology, integrity, and cellular function of hepatocytes. Our data indicate that Williams E Medium supports a higher basal CYP3A expression relative to the Dublecco's modified Eagles medium/nutrient mixture Ham's F-12 (DMEM/Ham's F-12) and modified Chee's media and, along with hepatocyte plating density, supports overall cell health and functionality [5]. Adult hepatocytes work best when cultured close to confluence ($\geq$90%) at the time of plating, as the cells redevelop normal epithelial morphology, junctional complexes, and bile canaliculi. When underplated at less than 50% confluence, hepatocytes

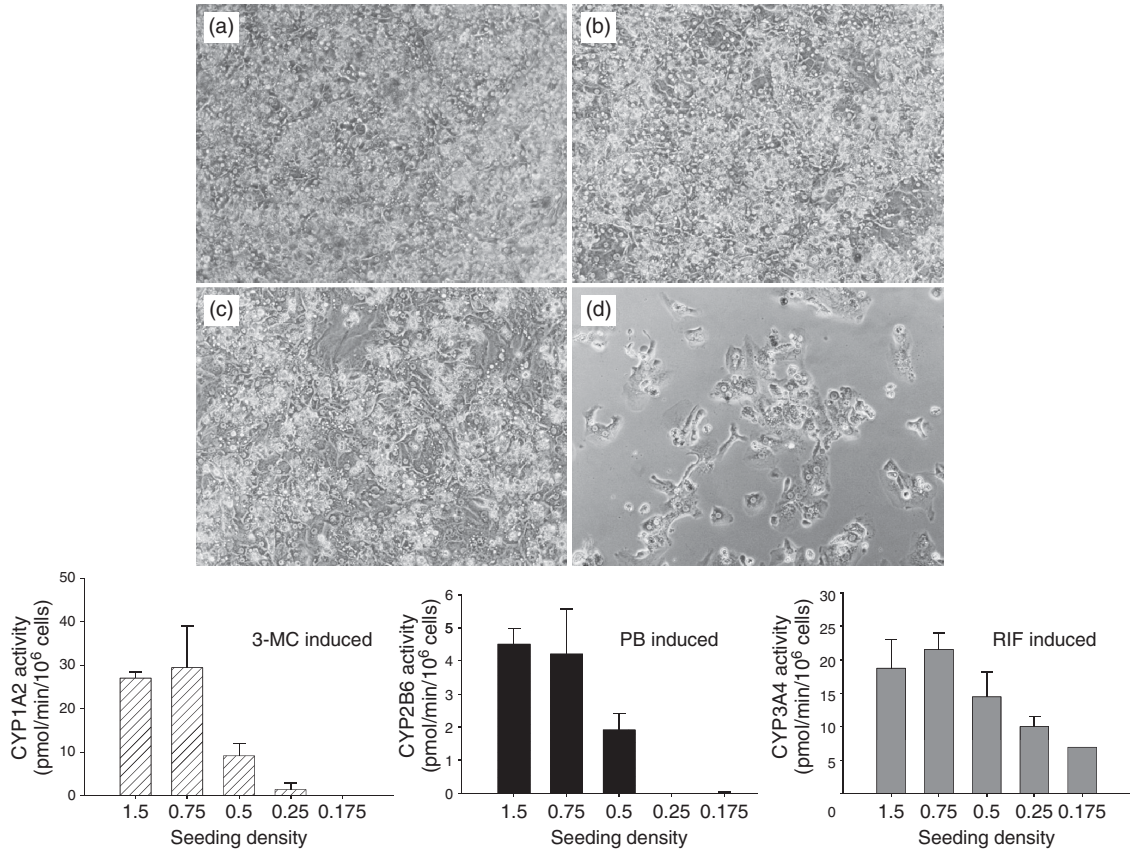


Figure 13.2 Effect of plating density on monolayer integrity and P450 enzyme activity is primary cultures of cryopreserved human hepatocytes. Photomicrographs of cryopreserved human hepatocytes at (a) 150%, (b) 100%, (c) 70%, and (d) 25% confluence. Bar graphs depict representative results of a single induction experiment with primary human hepatocytes cultured at five different seeding densities ($\times 10^5$ cells per well in a 24-well plate; $n = 3$). For the activity assays, hepatocytes were cultured for two days and then treated for three days with the prototypical inducers at the concentrations indicated in Table 13.3.

appear fibroblast-like after 24 h in culture and send out filopodial extensions. These hepatocytes have significantly lower CYP3A4 activity and responsiveness to prototype inducers than those plated at optimal confluence, although PXR levels remain equivalent (Fig. 13.2) [5]. Similar results have been obtained using freshly isolated and cryopreserved hepatocytes from the same donors. CYP1A1/2, CYP2B6, and CYP3A4 activities decreased with lower cell density, even though data was normalized for number of cells.

Fetal bovine serum enhances attachment and is only used for the initial attachment period of 4–6 h, as components in the serum can stimulate cell proliferation and loss of phenotypic expression [12]. Additives such as glucocorticoids (e.g., dexamethasone and hydrocortisone), insulin, selenium, and transferrin aid in the preservation of hepatocyte-specific morphology and function [12]. In most cases, albumin is included in media supplements at a final concentration of ~1 mg/mL, which affects both lipid and drug dispositions. After an initial recovery period in culture (24–48 h) during which hepatocytes are generally refractory to treatment with inducers, primary hepatocytes can be used for induction experiments and are typically incubated for two days with the test compounds when mRNA is the end point and for three days when enzyme activity is to be assessed. Cryopreserved hepatocytes can also be used for induction studies. For reasons not yet fully understood, a percentage of human hepatocyte preparations lose the ability to attach in culture after cryopreservation and thus cannot be used for induction studies. However, the hepatocytes that do attach efficiently and form a confluent monolayer retain the ability to induce, similar to fresh hepatocytes.

For induction assays, it is important to use healthy hepatocytes, appropriate culture conditions (medium and matrix), and positive control inducers at relevant concentrations that maximize inductive response while minimizing potential cytotoxicity, effective vehicle controls, appropriate time in culture, and the right complement of end points to allow effective interpretation of data. Table 13.5 provides a list of recommended positive control inducers based on our practical experience and understanding of various nuclear receptor pathways and their role in P450 transcription. A list of typical probe substrates for each relevant P450 isoform and their working concentrations are also provided.

As mentioned above, one of the most important considerations for achieving optimal outcomes from an *in vitro* induction study is the health and integrity of the hepatocyte monolayer. For example, often a very large fold change in mRNA or enzyme activity (e.g., >100-fold increase or >20- to 30-fold increase in CYP3A mRNA or activity, respectively) is not physiologically relevant to human liver *in vivo* and likely reflects an artificial response because of poor cell health or inappropriate culture conditions, which can result in diminished basal CYP3A expression and other liver-specific functions and regulatory pathways [5]. Conducting studies with compromised cultures with suppressed functionality may lead to misleading results, especially where metabolites of a new drug are the active species or toxic agents. Ideally, monolayers of primary hepatocytes, whether freshly isolated or cryopreserved, should be >80% confluent, show little or no sign of morphological deterioration (Fig. 13.3) over the study period (especially under control conditions), and exhibit at least 2.5-, 5-, and 10-fold increases in enzymatic activity over vehicle control for CYP3A4, CYP2B6, and CYP1A2, respectively, when treated with prototypical inducers [e.g., rifampicin, phenobarbital, and 3-methylcholanthrene (3-MC)]. Notably, induction of CYP3A4 enzyme activity greater than 10-fold for CYP3A4 is unusual and should serve as a red flag,

TABLE 13.5 Nuclear Receptors and the Corresponding Enzymes with the Positive Control Inducers and Substrates Typically Used for *In Vitro* Induction Studies with Human Hepatocytes

Receptor	Enzyme	Inducers (μM)	Substrate (μM)	Incubation (min)	Marker Metabolite
AhR	CYP1A1/2	3-Methylcholanthrene (2) Omeprazole (50) β-Naphthoflavone (25)	Phenacetin	15	Acetaminophen
CAR	CYP2B6	Phenobarbital (1000) CITCO (1) Phenytoin (50)	Bupropion	20	Hydroxybupion
PXR	CYP3A4/5	Rifampin (10)	Testosterone	14	6β-Hydroxytestosterone

Abbreviation: CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-3,4-dichlorobenzyl.

as this is not generally observed *in vivo* and could be due to artificially low basal activities that have resulted from poor cell health.

In addition to cell health, other considerations, such as the choice of vehicle control, positive control inducers, matrix and medium conditions (discussed above), and time of incubation, are also important for optimal results. The choice of vehicle control is often dictated by the solubility of the NCE being assessed for induction potential. Common solvents include dimethylsulfoxide (DMSO), methanol, and acetonitrile for most drugs, or simple isotonic buffers (e.g., phosphate buffered saline (PBS) and Hanks buffered salt solution (HBSS)) and cell culture medium for water-soluble compounds. Care should be taken in choosing the concentration of solvent vehicles, as they can contribute to an elevated baseline P450 expression and reduce the dynamic range of induction, as well as cause overt cytotoxicity at higher concentrations [5,80].

13.5.3 Experimental End Points

Induction of P450 enzyme expression can be assessed by evaluating mRNA, protein, and enzymatic activity. While the FDA 2006 draft guidance recommends evaluating activities of CYP1A1/2 and CYP3A4, a more recent commentary by the FDA recommends the addition of CYP2B6 activity to the screening regimen to account for activators of CAR [87]. The current FDA 2012 draft guidance recommends evaluating mRNA levels of all three enzymes, followed by CYP2C9 in cases where CYP3A4 induction is observed [2]. The newer guidance agrees with Fahmi *et al*. [88] that mRNA evaluation provides more appropriate *in vitro–in vivo* correlations, as the confounding effects of CYP inhibition, especially time-dependent inhibition, are not an issue. When there is no change, or more than a 40% increase in enzyme activity and mRNA expression relative to positive control inducer, the results are easy to interpret. Figure 13.4 depicts typical results with known inducers of CYP3A4, namely, rifampin, phenytoin, dexamethasone, and phenobarbital. In Fig. 13.4a, the results of an induction experiment where all compounds were treated at a final concentration of $10\,\mu M$ are

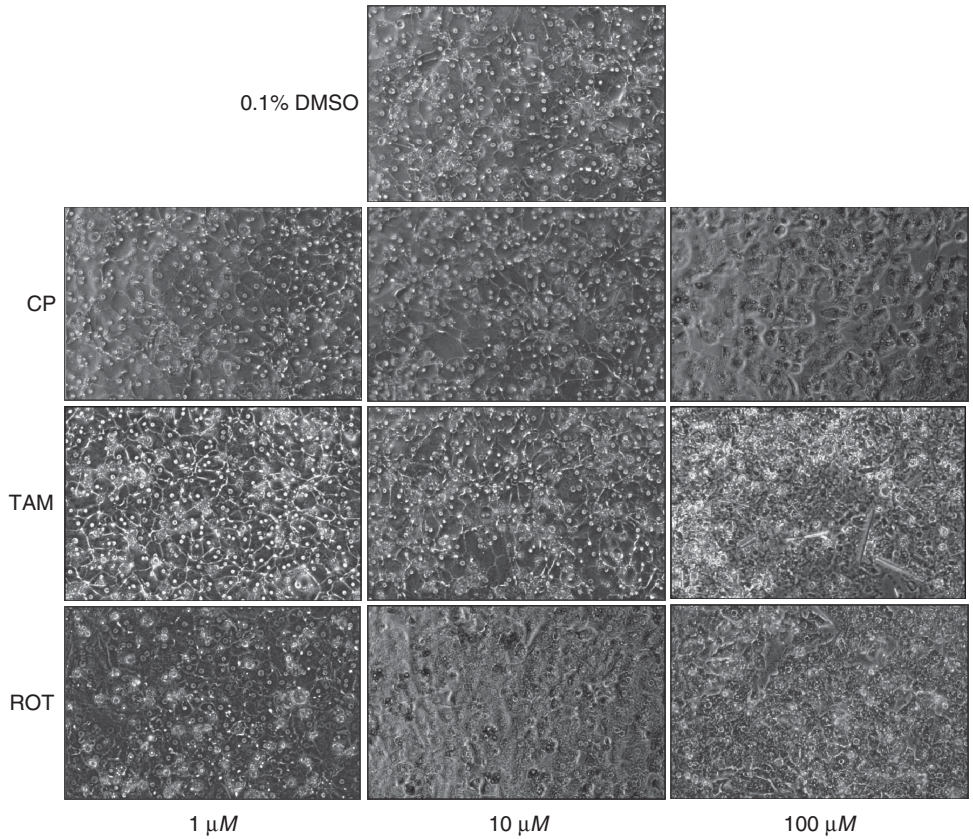


Figure 13.3 Photomicrographs of hepatocytes following drug treatment. Cryopreserved human hepatocytes were treated with vehicle control (0.1% DMSO) or known toxicants such as chlorpromazine (CP), tamoxifen (TAM), or rotenone (ROT) at different concentrations (1, 10, or 100 μ*M*) for 48 h and then assessed for changes in cell morphology using light microscopy.

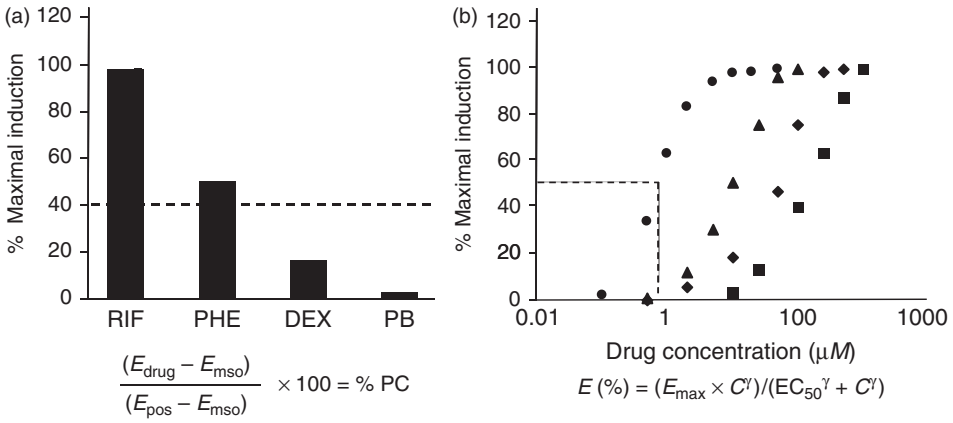


Figure 13.4 Representative induction responses for CYP3A4 by prototypical inducers in human hepatocyte cultures. Hepatocytes were maintained for two days in culture without treatment followed by three consecutive days of treatment with (a) a single concentration ($10\,\mu M$) or (b) multiple concentrations of rifampin (RIF), dexamethasone (DEX), phenytoin (PHN), or phenobarbital (PB). Hepatocytes from each treatment group were then harvested and microsomal CYP3A4 activity was determined using testosterone as substrate [71].

represented as a percentage of the positive control, which in this case was rifampin. At a single concentration, the compounds can be rank ordered according to their potency as an inducer and those causing an effect at or above 40% relative to the positive control would be designated for further evaluation.

However, it is important to note that all these agents cause significant induction of liver enzymes *in vivo* and that not all of them were tested at clinically relevant concentrations. As such, when tested over a broader range of concentrations in order to derive the entire concentration–response profile, it is clear that all the agents have the potential to cause significant induction of CYP3A4 (Fig. 13.4b). This underscores the importance of choosing the appropriate number and range of concentrations that are clinically relevant when conducting an *in vitro* study. The 2006 FDA guidance on drug–drug interactions recommended conducting activity assays and testing at concentrations equivalent to and an order of magnitude greater than the predicted or observed maximum plasma concentrations (C_{max}) of a NCE. The 2012 guidance recommends using multiple test compound concentrations and mRNA analysis as an end point to avoid the confounding effects of concomitant inhibition. Bear in mind that protein in the medium (e.g., albumin) can impact the free fraction of a drug and, therefore, using "total" drug concentrations often serve as a better guide when determining the relevant potency of an induction effect.

Determining the induction potential of test drugs is more complicated in cases where a decrease in enzyme activity is observed, which may or may not be accompanied by a drop in mRNA expression of the corresponding enzyme. There are three possible explanations for a decrease in enzyme activity after chronic treatment: (i) enzyme inhibition (as mentioned above), (ii) gene suppression, or (iii) cytotoxicity [89]. In the first case, enzyme inhibition can mask the induction response if a compound is both an inhibitor and an inducer of the same enzyme system(s). For example, the endothelin A receptor

antagonist CI-1034 does not cause significant induction of CYP3A4 activity in primary human hepatocyte cultures after three days of exposure. However, immunoblots and real-time polymerase chain reaction (PCR) analysis reveal significant increases in CYP3A4 mRNA and protein expression. CI-1034 was subsequently found to be a time-dependent (mechanism-based) inhibitor of CYP3A4. Notably, CI-1034 PK were linear in the clinical trials, even though this compound was metabolized predominantly by CYP3A4 [85]. The second cause of drug-induced decreases in P450 activity is when suppression of P450 gene expression occurs at the transcriptional level. A classical example of this effect occurred when children with a fever experienced central nervous system (CNS) toxicity after theophylline administration [90]. It has since been shown that proinflammatory cytokines downregulate many hepatic P450 genes, which appears to be mediated through the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway [91]. It is worth noting that the clinical manifestation of enzyme suppression can be similar to that of enzyme inhibition. As in the case described above, the cytokine-induced suppression of CYP1A2 activity in the infected children caused a decrease in theophylline clearance, which in turn led to increased blood levels of the drug and subsequent CNS toxicity.

The induction of P450s by prototypical inducers can also be attenuated at the transcriptional level, as is the case with CYP1A1 induction by tetrachloro-*p*-dibenzodioxin (TCDD) in HepG2 cells in the presence of microtubule-disrupting agents. The marked induction of CYP1A1 gene expression that normally occurs is blocked by colchicine and nocodazole because these agents disrupt microtubule-mediated signaling pathways and arrest the G2/M cell cycle [92]. Similarly, phenobarbital-induced CYP2B1 expression in primary rat hepatocytes is attenuated by docosahexaenoic acid. This effect occurs at the level of the nuclear receptor, as CAR accumulation in the nuclear fraction is decreased with a concomitant increase in the cytosolic fraction [93]. An earlier study determined that attenuation of CAR translocation and decreased subsequent binding to response elements in the *CYP2B1* promoter are involved in this chemical-induced down-regulation [94]. In the final case of drug-induced reduction in P450 enzymatic activity, the decreased enzyme activity can be caused by the onset of cytotoxicity. A number of compounds that are known to be potent P450 enzyme inducers produce "bell-shaped" concentration–response curves because of the fact that they begin to cause cytotoxicity at higher concentrations. For instance, both rifampicin and troglitazone exhibit this behavior when administered over prolonged periods to primary hepatocytes at concentrations of more than $50\,\mu M$ [82]. Accordingly, it is crucial to routinely conduct morphological assessments and, when a loss in enzyme activity is observed, to assess cytotoxicity in the monolayers using standard approaches such as cellular ATP content or lactate dehydrogenase (LDH) leakage.

When evaluating induction of P450 enzymes by a compound that is also a mechanism-based inhibitor, there may be no significant change in the activity of an individual isoform because of offsetting inhibition and induction, even though the compound is a nuclear receptor activator. In these cases, it is helpful to also evaluate the mRNA or protein concentrations to aid in the interpretation of results. P450 mRNA levels will not be affected by classical inhibition mechanisms that affect enzyme activity; however, they will decrease because of cytotoxicity or gene suppression. In most of these cases, the clinical end point is very difficult to predict. An example is the endothelin A receptor antagonist CI-1034, a compound metabolized predominantly by CYP3A4 that is also a metabolism-dependent inhibitor of this

enzyme. Analysis of enzyme activity in primary cultures after three days of treatment revealed no change in activity, while Western immunoblots revealed an increase in CYP3A4 immunoreactive protein and real-time PCR analysis demonstrated increases in CYP3A4 mRNA similar to that induced by rifampicin [85]. Interestingly, clinical data revealed linear PK, perhaps indicating that neither the inhibition nor the induction was significant *in vivo* or possibly, these processes negated each other. While this experimental end point could not be predicted *in vitro*, it is helpful to know the inhibition potential of a compound before conducting *in vivo* induction studies, so that appropriate study designs can be planned.

On the basis of our own experience and the reports of others, we recommend that an *in vitro* induction study minimally include (i) CYP1A, CYP2B6, and CYP3A enzymatic activity measurements; (ii) corresponding mRNA or protein determinations; (ii) a cytotoxicity end point (e.g., ATP content); and (iv) microscopic evaluation to monitor cell and monolayer integrity before and at the termination of an experiment.

13.5.4 *In Vitro/In Vivo* Correlations

It is now routine to consider a response of $\geq$40% of the positive control activity *in vitro* as an indication of potential *in vivo* induction [95]. Many inducers produce a sigmoidal concentration–response profile (Fig. 13.4b) that is characteristic for a particular compound. The relevant characteristics to assess induction potential are the potency of the response (EC_{50}, effective concentration at half-maximal response), the efficacy (E_{max}, maximum effect or response), the no observable effect level (NOEL) or the highest no-effect concentration, and the relative shape of the concentration–response curve [96].

Similar to predictions of enzyme inhibition, a C_{max}/EC_{50} ratio of >1 indicates a likely inducer *in vivo* and a C_{max}/EC_{50} ratio of 0.1–1 indicates possible induction, while a C_{max}/EC_{50} ratio of <0.1 indicates low potential for induction *in vivo*. Figure 13.5 demonstrates a simulated study with three compounds for which a $C_{max} < 1\,\mu M$ would predict no induction potential, while a C_{max} of $8\,\mu M$ would indicate significant induction for drug X and moderate induction for drugs Y and Z. Table 13.6 depicts the utility of using EC_{50} values for CYP3A4 induction and how the C_{max}/EC_{50} ratio relates to the predicted CYP3A4 induction potential. For example, while troglitazone and rosiglitazone have similar EC_{50} values for CYP3A4 induction, troglitazone is a potent clinical inducer [82,97,98] while rosiglitazone has no significant clinical induction effects [99]. Because of their differential selectivity for the therapeutic target (i.e., peroxisome proliferator-activated receptor (PPAR)), rosiglitazone is prescribed at a lower dose and the C_{max} is correspondingly lower and significantly below its EC_{50} value for PXR activation. As such, it can be important to understand and compare the selectivity and the therapeutic index between the on- and off-target effects of drug candidates. The lowest concentration at which the induction response occurs (NOEL) should also be considered [100].

According to the latest FDA guidance document on drug–drug interactions [2], the decision to conduct an *in vivo* drug–drug interaction study for an investigational drug as an enzyme inducer should be based on quantitative analysis of both *in vitro* and clinical PK data. Such analysis can be performed by a variety of algorithms and models including basic models, mechanistic static models, and more comprehensive dynamic models, for example, physiologically based pharmacokinetic (PBPK) models.

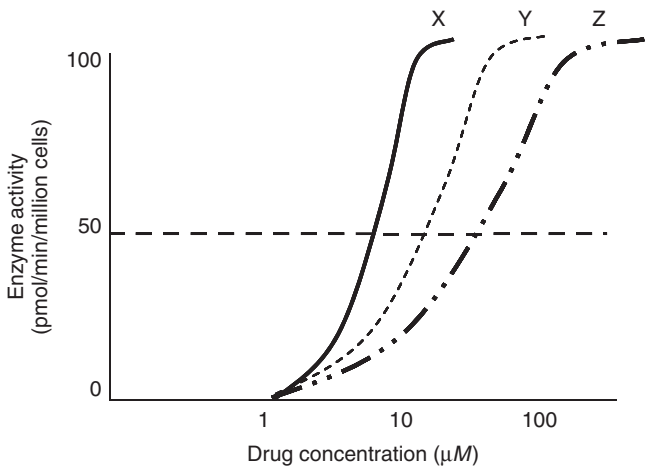


Figure 13.5 Simulated induction profiles of three hypothetical compounds (X, Y, and Z). Three distinct induction profiles were simulated to illustrate potential compound differences in potency (EC_{50}) while exhibiting similarities in efficacy (E_{max}) and NOEL.

TABLE 13.6 Relationship between *In Vitro* Potency and the Prediction of *In Vivo* Induction using the EC_{50} and Therapeutic Plasma Concentrations[a]

Compound	EC_{50} (μM)	C_{max}/C_{ss} (μM)	[I]/EC_{50}	Induction Potential
Rifampin	0.8	14	17.5	Likely
Carbamazepine	0.8	25	28	Likely
Phenobarbital	125	40–180	0.3–1.5	Likely
Phenytoin	25	80	3.2	Likely
Troglitazone	3–5	7	2.3	Likely
Avasimibe	0.2	1–6	5–30	Likely
Rosiglitazone	5–10	0.3–1.2	0.06–0.12	Possible
Simvastatin	0.14	0.024	0.17	Possible
Lovastatin	1–5	0.008	0.008–0.02	Unlikely
Clotrimazole	1–5	topical	(inhibition)	Unlikely
Nifedipine	8	0.008	0.001	Unlikely

[a]Ref. 101.

Basic models have been predominantly used because they are simple and practical. These models are conservative, but in some cases, they eliminate the need for later clinical investigations of drug–drug interaction potential. For example, the cutoff value to decide whether further *in vivo* investigation of a drug as an inducer is needed is generally calculated based on the fold induction over a negative or vehicle control (e.g., fourfold or higher) or the percentage of induction relative to an appropriate positive control (e.g., $\geq$40% of a positive control) [88,101].

As suggested before, comparison of induction response curves is more complex than simply determining any one of these parameters alone [101]. Whatever method is used to calculate the potential induction risk *in vivo*, once a compound is found to be positive in an *in vitro* hepatocyte induction assay, drug interaction clinical trials are generally warranted, but it is rare that these results would stop the development

of a compound. However, attrition sometimes occurs if the compound showed potent induction effects, especially if these were expected and interfered with cotherapies. Often multiple options are pursued when considering the implications of a positive *in vitro* induction response. Most often extra drug–drug interaction (DDI) studies are requested in the clinical trials (recommended by the FDA) or other PK properties of the compound are assessed to determine the overall induction potential. For example, some of the other properties considered were protein binding, clearance, and bioavailability, all of which influence the induction response *in vivo*. Of course, another option which is often pursued is that the induction data is provided back to the discovery chemists in an attempt to minimize or eliminate the inductive properties of new molecules.

13.6 HEPATOBILIARY TRANSPORT

13.6.1 Background

Transporters in the hepatocyte basolateral and apical membrane are responsible for carrier-mediated processes involved in the uptake, biliary secretion, and systemic reabsorption of xenobiotics, their metabolites, and endogenous compounds. Hepatic clearance is a function of transporter and drug-metabolizing-enzyme-mediated processes. Hepatic clearance starts with transport into hepatocytes (phase 0) by passive diffusion through the cell membrane or by active transport. In hepatocytes, transport is mediated by basolateral membrane organic anion transporters (OATs) including OAT2 and OAT5; OATP1A2; OATP1B1; OATP1B3; OATPB2B1; the organic cation transporter OCT1; and the sodium taurocholate cotransporting polypeptide (NTCP). All these transporters are retained in the membranes of primary sandwich-cultured hepatocytes (Figure 13.6). Phase III is transporter-mediated elimination of the intact drug and metabolites (if any) from the hepatocytes via ATP-dependent apical or basolateral membrane efflux transporters and in some cases, bidirectional organic anion or cation transporters. Apical membrane hepatobiliary transporters involved in xenobiotic transport include the MDRs such as MDR1, MRP1, MRP2, the breast cancer resistance protein (BCRP), and the bile salt efflux pump bile salt export protein (BSEP) [102]. These efflux transporters are maintained in primary hepatocytes in sandwich culture after two to three days of plating. Drugs that are eliminated unchanged via the bile may undergo enterohepatic circulation and may be reabsorbed from the intestine [103]. The BSEP mediates the efflux of conjugated and unconjugated bile salts into the bile, and inhibition of this transporter by a drug could result in hepatotoxicity [104]. Multiple transporters mediate the excretion of compounds from hepatocytes back into systemic circulation via basolateral membrane efflux transporters (phase III). As shown in Fig. 13.6, the outwardly directed efflux transporters include the ATP-binding cassette (ABC) proteins MRP1 and MRP3. Thus, multiple membrane transporters in hepatocytes work in concert with the drug-metabolizing enzymes to mediate hepatic drug clearance. These transporters play a key role in drug clearance and may contribute to drug–drug interactions.

13.6.2 Transporter-mediated drug interactions

Similar to the drug-metabolizing enzymes, drug–drug interaction involving hepatic membrane transporters can occur by competition for the same substrate-binding site of

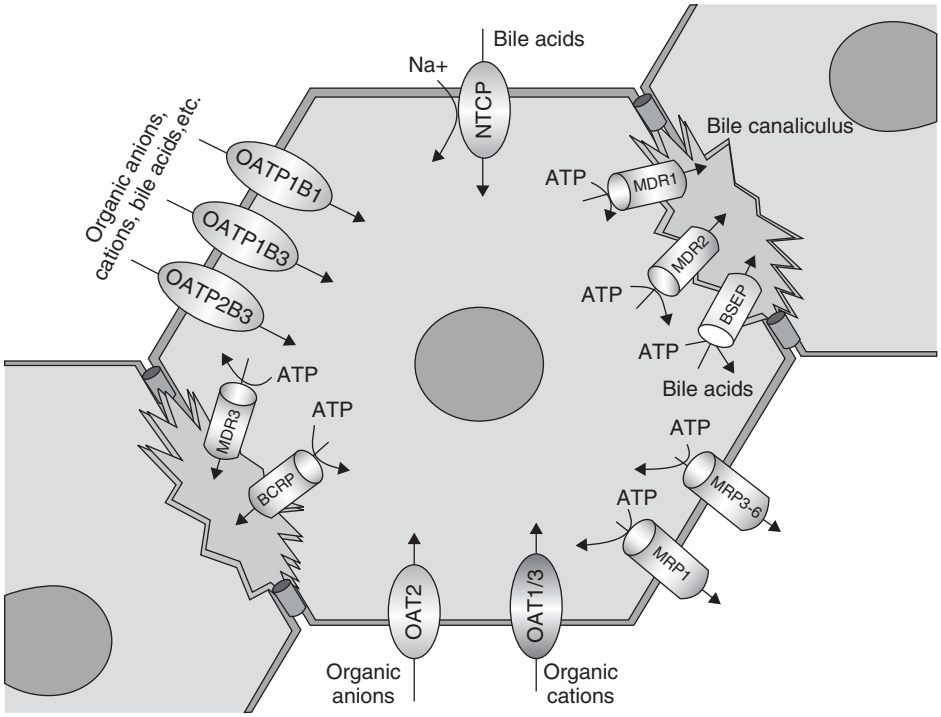


Figure 13.6 Schematic of uptake and efflux xenobiotic transporters in human hepatocytes.

the transporter, or tight or allosteric binding leading to inhibition of transporter activity, or by change in expression level of transporters. This has the potential to alter the blood concentration–time profiles of drugs, leading to elevated levels of a coadministered compound. Evaluating the substrate potential of a drug candidate for hepatic transporters *in vitro* is beneficial when these organs are the drug target. For example, the hepatitis C drugs α-interferon and *S*-acyl-2-thioethyl esters or the HMGCoA inhibitors must achieve adequate concentrations in the liver for pharmacological activity. Evaluating potential substrates of efflux transporters also aids in predictions of drug clearance when compounds are predominantly eliminated into the bile, for example, atorvastatin. Evaluating drug interactions with transporters *in vitro* during drug discovery can lead to improved predictions of clinical drug–drug interactions. Hepatic transporters are now implicated in adverse interactions that were earlier attributed to the drug-metabolizing enzymes. *In vitro* studies with mibefradil revealed CYP3A4 inhibition potential, but the clinical outcome was significantly larger than what was predicted from the *in vitro* studies [105]. A few years later, mibefradil was found to inhibit P-gp as well [106], and the additive effect of this interaction explained the underprediction of the effect on PK of coadministered therapeutics. Similarly, the effect of grapefruit juice on fexofenadine PK, which was initially attributed to CYP3A4, was later reported to involve the inhibition of OATP-mediated fexofenadine uptake [107]. Digoxin, which has been used as a P-gp substrate in both *in vitro* and *in vivo* studies and is the FDA probe substrate of choice for both preclinical and clinical drug interactions studies, is now

reported to be a substrate for hepatic OATP transporters as well, and there are ongoing discussions on the appropriateness of this compound as a P-gp substrate. Other examples of adverse drug interactions attributed to hepatic transport proteins involve the statins, as coadministration of gemfibrozil with pravastatin, simvastatin, lovastatin, and cerivastatin significantly increases statin AUC and C_{max} [108,109]. Inhibition of the basolateral membrane hepatic uptake transporter OATP1B1 by gemfibrozil contributes to these drug interactions [110,111]. While the above clinical transporter-mediated drug interactions were unexpected, with current strategies, *in vitro* transporter assays can be used to identify the transport proteins involved in drug disposition, thereby aiding drug interaction predictions, so that fewer adverse events occur in the clinic. Some of these methodologies are discussed in the following section.

In addition to drug–drug interactions, hepatic transporters also play a role in hepatotoxicity. Drug-induced hepatotoxicity is a major problem in drug development and there is growing evidence that inhibition of bile acid transporters is a contributing mechanism of liver toxicity [112]. Multiple apical and basolateral membrane hepatic transporters work in concert to transport bile acids and xenobiotics from blood to bile via the hepatocytes, and inhibition of any of these processes could result in adverse events. Hepatotoxicity is a significant cause of compound attrition. Several drugs (bosentan, troleandomycin, troglitazone, glyburide, tacrine, and erythromycin) have exhibited no or minimal hepatotoxicity in preclinical *in vivo* experiments and resulted in significant increases in liver function enzymes in clinical trials, or in the market when exposed to a wider patient population. Drug-mediated inhibition of active canalicular transport of biliary components is a contributing cause of this hepatotoxicity [104,113]. Inhibition of the apical membrane bile transporter BSEP has been shown to result in cholestasis and hyperbilirubinemia [84,114], and there is evidence that inhibition of OATP1B1 can result in hyperbilirubinemia [115]. MRP4 may play an important role in minimizing hepatotoxicity, as this transporter is upregulated when BSEP is downregulated [116].

In many cases, drugs that cause cholestasis are preferentially eliminated (>50%) via the biliary pathway, and this hepatotoxicity is likely associated with drug-mediated inhibition of active canalicular transport of bile components [113,114]. Since the transporters that participate in biliary drug elimination also transport endogenous bile components, the potential for inhibition of bile acid and drug efflux from the liver could result in an increase in drug and bile acids retained in the liver over time. Many of these drugs have now been found to be substrates for active liver-transporter-mediated uptake and efflux into the bile canaliculi using primary hepatocytes [84,112,117]. Table 13.7 lists some of the major hepatic transporters involved with hepatotoxicity and drug–drug interactions. In an earlier study using six macrolide antibiotics, we showed that drugs with greater hepatotoxic risk are stronger inhibitors of taurocholate transport in cultured human hepatocytes [84]. In addition to primary hepatocytes, hepatocytes in suspension can also be used to evaluate hepatic transport.

13.6.3 Experimental approaches

13.6.3.1 Hepatic Couplets. The hepatocyte couplets are consist of two hepatocytes with a distinct bile canalicular space between these. This model allows for the evaluation of drug uptake and biliary elimination. The advantage of the couplets is that these are polarized and have a sinusoidal membrane interacting with the media/buffer and an apical bile canaliculus, which is the sealed lumen between the two adjacent

TABLE 13.7 Major Human Hepatic Transport Proteins and Known Substrates and Inhibitors

Transporter	Gene Name	Substrates	Inhibitors
NTCP	SLC10A1	Taurocholate, glycocholate, taurochenodeoxycholate and tauroursodeoxycholate, estrone-3-sulfate, BSP	Rifampin, rifamycin SV, glibenclamide, cyclosporin A, indomethacin, ritonavir, saquinavir, efavirenz, troglitazone
OATP1B1	SLCO1B1	Bile acid, BSP, DHEAS, DPDPE, E217βG, estrone-3-sulfate, ouabain, bilirubin, bilirubin glucuronides, pravastatin, rosuvastatin, atorvastatin, rifampin, olmesartan, bosentan, fexofenadine, methotrexate	Estrone-3-sulfate, gemfibrozil, methotrexate, ketoconazole, cyclosporin A, rifampin, rifamycin SV, clarithromycin, indinavir, ritonavir, CI-1034
OATP1B3	SLCO1B3	bile acids, BSP, DHEAS, digoxin, DPDPE, E217βG, estrone-3-sulfate, ouabain, bilirubin, rifampin, pravastatin, rosuvastatin, pitavastatin, telmisartan, fexofenadine, olmesartan, bosentan, nafcillin, β-lactam antibiotics	cyclosporin A, rifampin, ketoconazole, clarithromycin, ritonavir, verapamil, T-3095, T-3157, CI-1034
OATP2B1	SLCO2B1	Benzylpenicillin, BSP, DHEAS, estrone 3-sulfate, rosuvastatin, atorvastatin	Cyclosporin A, estrone-3-sulfate, methotrexate, CI-1034
BSEP	ABCB11	Taurocholate, glycocholate, taurochenodeoxycholate, tauroursodeoxycholate and pravastatin	Bosentan, glyburide, troglitazone, rifampin, rifamycin SV, glibenclamide, cyclosporin A, ritonavir, saquinavir, efavirenz, rosiglitazone

Refs 84,85,87,102,114, and 118–123.

hepatocytes [124]. The application of couplets is limited because the substrate must contain a fluorescent chromophore to be quantitated. While the data obtained from these studies is valuable, isolating and working with hepatic couplets requires skill and is very low throughput, further accounting for the limited transporter studies with this model over the past decade.

13.6.3.2 Primary Hepatocytes. Hepatocytes in suspension, attached to tissue culture dishes or in primary sandwich culture are good models of hepatic clearance, uptake, efflux, biliary excretion, transporter-mediated drug interactions, induction, and hepatotoxicity. The contribution of transporter-mediated uptake to hepatic CL was recognized when H_{CL} was consistently underpredicted for many series of chemotypes using just metabolic stability for the calculations. Factoring in transporter-mediated hepatic uptake

along with metabolic clearance using hepatocytes in suspension improved these predictions [125]. From our experience, hepatic uptake via a particular transporter can be effectively assessed with single-transfected cell lines (e.g., HEK-293 cells transfected with OATP1B1 or OATP1B3 or OATP2B1), yet this system is less effective in extrapolating H_{CL} because of the significant differences between transporter types and content in the cell lines and in hepatocytes [126]. Therefore, plated and suspension hepatocytes have become the system of choice for quantitative information on hepatic drug uptake.

13.6.3.3 Suspension Hepatocytes. Hepatocytes in suspension can be used for uptake experiments shortly after isolation from the liver, or for up to 24 h, if cells are kept in the appropriate buffer on ice. Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, with minor changes likely due to the absence of significant numbers of other hepatic cell types, for example, Kupffer cells and cholangiocytes [127]. Hepatic uptake studies typically measure the rate of appearance of substrate into cells after a relatively short incubation period, in most cases from 10 s to 3 min. After incubation, hepatocytes are centrifuged in microfuge tubes through an oil layer (that does not allow passage of the buffer and traps lipophilic compound that may be on the outer cell membrane) into a layer of 2 N NaOH or KOH or cesium chloride, for digestion [128]. This bottom layer along with the digested trapped hepatocytes is counted or analyzed by MS. This methodology has provided robust, mechanistic data that can successfully predict the *in vivo* clearance of drugs [39]. Cryopreserved hepatocytes can be successfully used for these studies, and there is evidence to suggest that transporter gene expression in human hepatocytes in suspension is not affected by cryopreservation [127]. In this study, gene expression for primary human hepatocytes after cryopreservation was similar to the liver of origin and included transport, lipid metabolism, phase II metabolism, protein processing, degradation, and recycling. This indicates that both freshly isolated and cryopreserved hepatocytes can be used for transporter-mediated uptake studies.

We have evaluated uptake in multiple lots of cryopreserved human hepatocytes and found up to a 10-fold difference between donors. For example, taurocholate CL was typically between 12 and 48 μL/min/million cells, digoxin CL varied from 0.6 to 4.5 μL/min/million cells, and estradiol-17β-glucuronide (E217βG) CL varied from 0.5 to 5 μL/min/million cells (Fig. 13.7). We also prepared five donor pools of cryopreserved human hepatocytes. This protocol includes rapidly thawing out the hepatocytes, pooling cells from five different preparations, subjecting these to a percoll gradient to remove dead cells and refreezing these in liquid nitrogen as per our standard cryopreservation procedure. Since these cells undergo two freezing steps, we were uncertain how well the membrane transporters continued to function. We studied uptake in this preparation and compared the data to standard cryopreserved cells and also to literature data. As shown in Fig. 13.7, the pooled cells could be used adequately to assess membrane transport of taurocholate, E217βG, and digoxin.

13.6.3.4 Primary Culture. Hepatocytes cultured on plastic culture dishes or on rigid collagen dedifferentiate and rapidly lose hepatic transport activity and other liver-specific functions. Owing to this, transporter-mediated uptake studies utilizing hepatocytes cultured in this configuration should be conducted as soon as possible, either right after attachment (~4 h before plating) or within 24 h of plating.

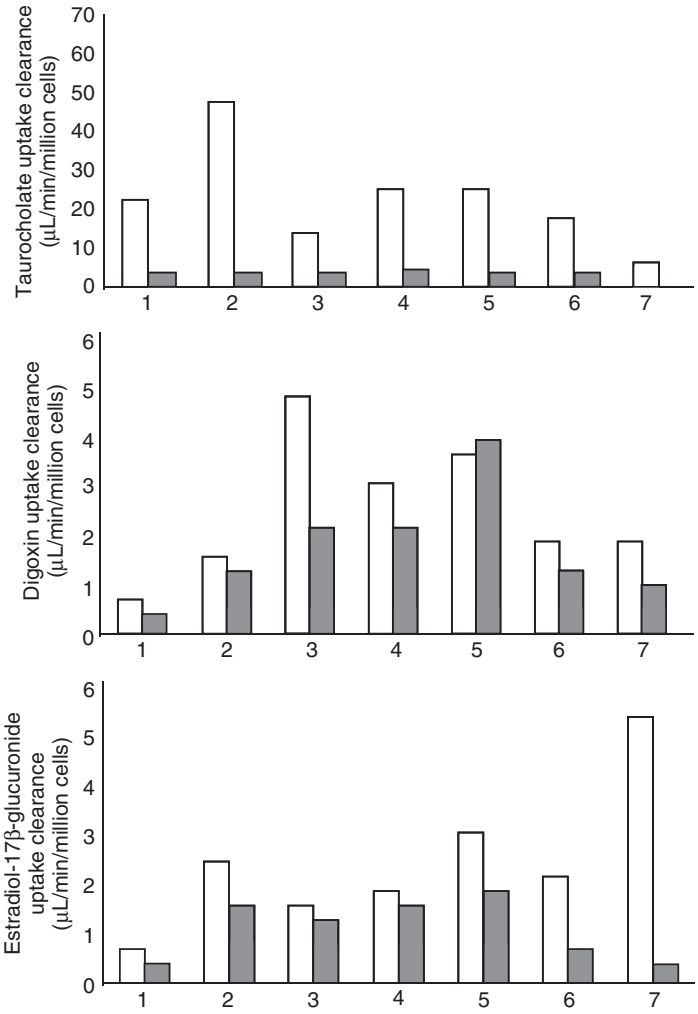


Figure 13.7 Uptake clearance in cryopreserved primary hepatocytes in suspension. For these experiments, either single-cryopreserved (1–5) or pooled cryopreserved (6–7) hepatocytes were used in suspension. Hepatocytes were thawed and resuspended in a buffer, and uptake of the prototypical substrates (1 μ*M* each) of BSEP (taurocholate), OATP transporters (estradiol-17β-glucuronide), and OATP1B3 (digoxin) is assessed by the oil spin method. Data represent a single experiment for each lot conducted in triplicate.

In the sandwich culture configuration, hepatocytes polarize, retain membrane transporters and drug-metabolizing enzymes (albeit at varying concentrations over time), and establish functional bile canaliculi [24,84]. The bile canaliculi formed in sandwich culture (Fig. 13.8) have morphological and biochemical characteristics similar to those observed *in vivo*, including microvilli in the bile canaliculi, localization of function-specific drug-metabolizing enzymes and relocation of the major uptake and efflux transporter to the appropriate cell membrane [129,130]. Using this model, apical and basolateral membrane transport of a drug and metabolites can be evaluated.

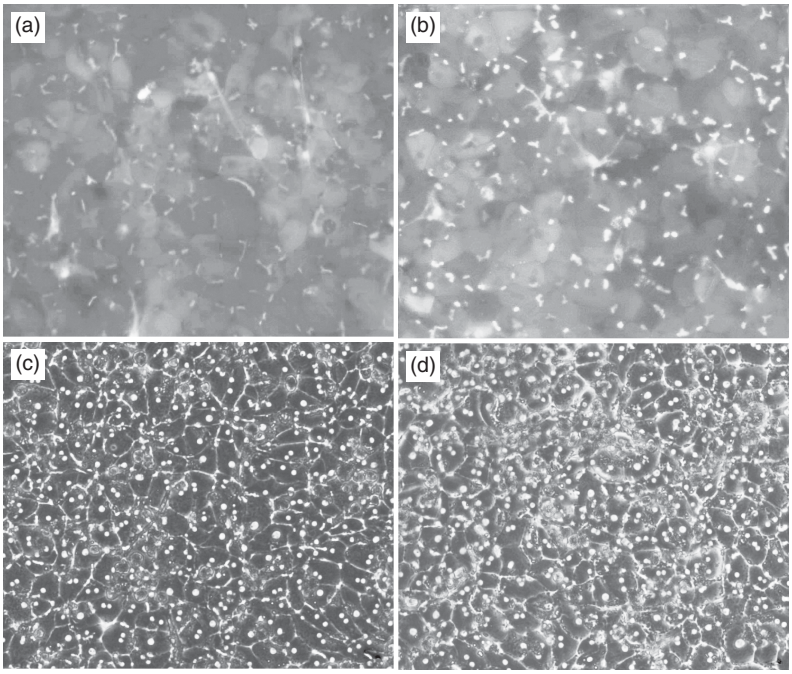


Figure 13.8 Effect of cyclosporine on bile salt transport into hepatic canalicular spaces. Carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA) staining in the presence (a and c) and absence (b and d) of 10 μM cyclosporine. (a) and (b) are fluorescence images and (c) and (d) are phase contrast images at 10 × magnification. Bile canaliculi are formed in sandwich culture and CDFDA is transported into the hepatocytes and out to the bile via active transport mechanisms. Cyclosporine is an inhibitor of these transporters and prevents the transport of CDFDA into the canalicular spaces.

Sandwich-cultured hepatocytes have been used to evaluate MDR1-, MRP2-, MRP3-, and BSEP-mediated hepatobiliary disposition and hepatotoxic potential of drugs successfully [84,129,130].

13.6.3.5 Cryopreserved Hepatocytes. In sandwich culture, cryopreserved hepatocytes preserve the drug-metabolizing enzymes, nuclear receptors, and major uptake and biliary transporters [131,132] and are used for transporter studies as well. Hepatocytes can also be used for evaluating induction of transporters by drugs, aiding in the prediction of drug disposition and potential drug–drug interactions [133,134]. When cryopreserved and cultured appropriately, hepatocytes in sandwich culture reveal good morphology with distinct bile canaliculi. The function of multiple uptake (OATPs and NTCP) and efflux (MRP2, MRD1, and BSEP) transporters was found to be similar to fresh hepatocytes [135]. We have evaluated biliary excretion in multiple preparations of human hepatocytes and compared these with fresh hepatocytes and demonstrated essentially similar values (Figs. 13.9 and 13.10).

13.6.3.6 Small interference RNA (siRNA/RNAi). At this time, there are no specific inhibitors of hepatic transporters. For this reason, when conducting studies in hepatocytes, the contribution of a specific transporter to the uptake or efflux of a drug

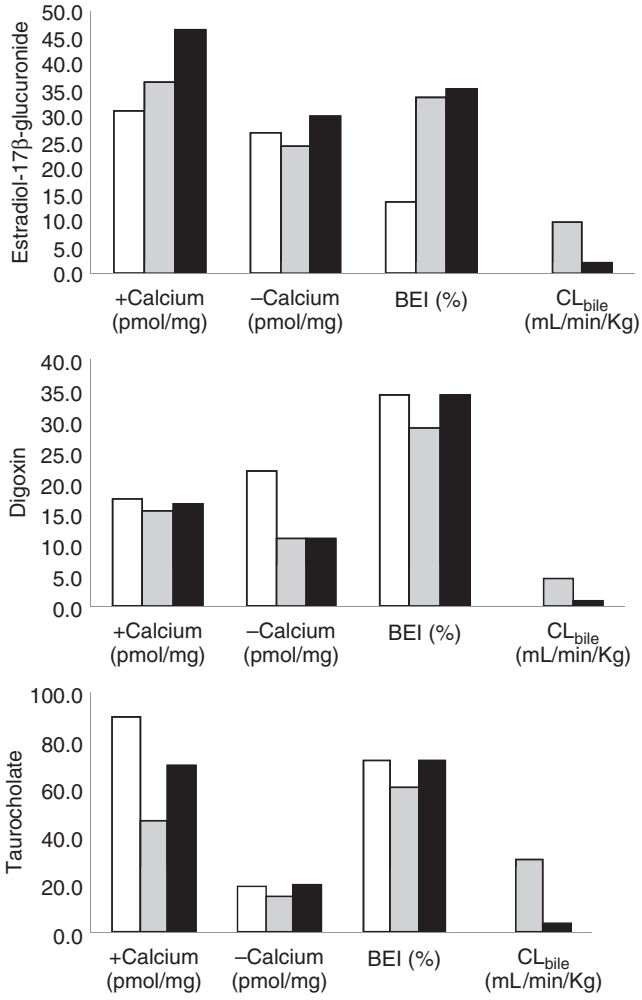
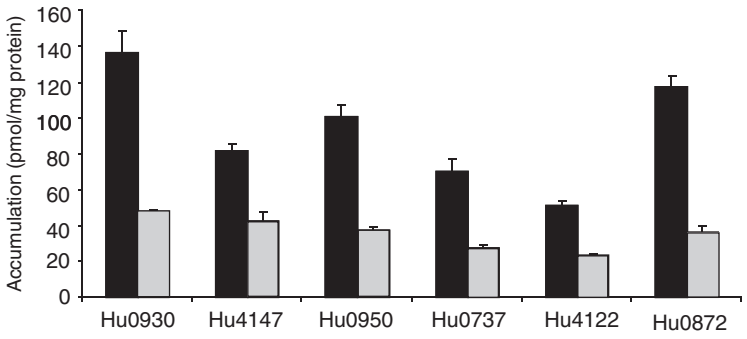


Figure 13.9 Biliary efflux in three preparations of freshly isolated sandwich-cultured human hepatocytes. Human hepatocytes were placed in primary sandwich culture for six days and uptake and biliary efflux of taurocholate, estradiol-17β-glucuronide, and digoxin monitored. Biliary clearance and the biliary clearance index were calculated. BEI, biliary excretion index.

candidate cannot be evaluated using probe substrates or chemical inhibitors. When the contribution of a single transporter needs to be evaluated, small interference RNA (siRNA) could provide the data required. This involves specifically knocking out the mRNA of interest by introducing antisense RNA into hepatocytes, causing degradation of the corresponding complementary mRNA in a gene-dependent manner, followed by loss of the target protein. siRNA has been used successfully to assess transporter activities between wild-type cells and those in which the transporter of interest has been silenced [136,137]. In sandwich-cultured rat hepatocytes, the mrp2 and mrp3 proteins have been specifically knocked down, one at a time using siRNA and resulted in 45–60% decreases in the biliary excretion index of carboxydichlorofluorescein (CDF)



Donors	Accumulation (pmol/mg of protein)				BEI (%)	CL_{bile}
	+ Calcium	SEM	– Calcium	SEM	Mean	Mean
Hu0930	136.5	12.18	47.71	1.2	65.1	69.9
Hu4147	81.7	4.190	42.87	4.900	47.5	30.6
Hu0950	100.9	6.390	37.14	1.800	63.2	50.2
Hu0737	70.2	6.810	27.69	1.820	60.6	33.5
Hu4122	51.5	2.130	23.54	0.450	54.3	22.0
Hu0872	117.3	5.940	36.27	3.390	69.1	63.8

Figure 13.10 Taurocholate accumulation in six preparations of freshly isolated sandwich-cultured human hepatocytes. Human hepatocytes were placed in primary sandwich culture for five to six days and uptake and biliary efflux of taurocholate monitored. Biliary clearance and biliary clearance index were calculated. Filled bars are hepatocytes incubated in a physiological buffer containing the divalent cations Ca^{2+} and Mg^{2+}; stippled bars are hepatocytes incubated in the absence of Ca^{2+} and Mg^{2+}. BEI, biliary excretion index; SEM, mean standard error.

[136]. In another study, the efflux transporter Bcrp was knocked out using Ad-si01Bcrp in rat hepatocyte monolayers and the contribution of this transporter to the biliary excretion of some drugs were evaluated [137]. We have conducted preliminary experiments with cryopreserved human hepatocytes in our laboratory and have successfully knocked down OATP1B1, OATPB1B3, and OATP2B1 mRNA one at a time without affecting the other two transporters (Fig. 13.11).

13.6.4 *In Vitro*/*In Vivo* Correlations

To predict potential transporter-mediated drug interactions, the degree of inhibition can be calculated as per the method of Shitara *et al*. [138,139]. If the test compound is a substrate for hepatic transporters, *in vitro* drug–drug interaction studies with the NCE and likely coadministered therapeutics, known to be the inhibitors of the specific transporter pathway(s), can be conducted to calculate the degree of inhibition in an appropriate transporter system. If historic or experimentally derived *in vitro* data indicates that the test compound is an inhibitor for one or more hepatic transporters, *in vitro* drug–drug interaction studies with the test compound and likely coadministered therapeutics, known to be substrate of the specific transporter pathway(s), can be conducted. The degree of interaction (R) can be estimated by the following equation.

$$R = 1 + \frac{\text{fu} \times I_{\text{in,max}}}{K_{\text{i}}}$$

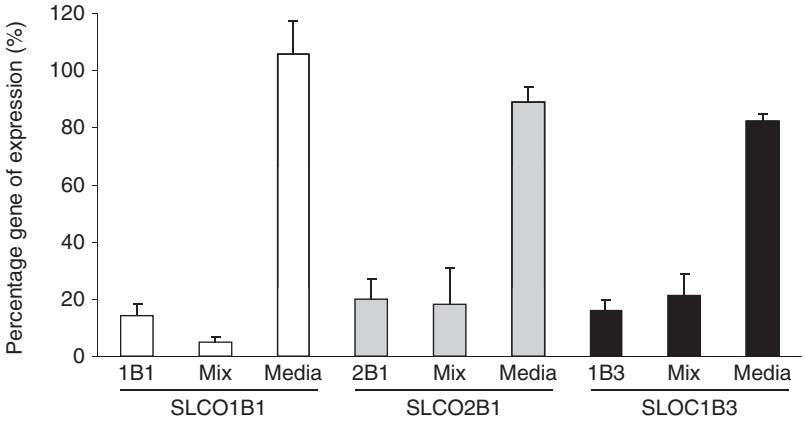


Figure 13.11 siRNA suppression of OATP1B1, OATP2B1, and OATP1B3 in primary human cryopreserved hepatocytes from a single donor. Cryopreserved human hepatocytes were thawed and plated on collagen-coated plates and allowed to attach for 5 h before siRNA transfection. Stealth Select 3 RNAi™ sets for OATP1B1, OATP2B1, and OATP1B3 were used. Lipofectamine RNAiMax™ was complexed with Stealth Select siRNA™, and cells were exposed to siRNA overnight. mRNA was isolated 48-h post transfection and cDNA synthesis and real-time PCR with EXPRESS One-Step Superscript® qRT-PCR Universal. Data is represented as percentage of nontreated cells.

$I_{in,max}$ is the unbound inhibitor concentration (C_{max}) at the entry point of the organ (e.g., in the hepatic portal vein for the liver or the renal artery for the kidney), fu the blood unbound fraction, and K_i the inhibition constant. In most cases, an IC_{50} value is utilized instead of the K_i. The potential for drug–drug interaction is minimal if the calculated R value is close to one and increases as the value of R decreases. These *in vitro* studies can identify potential inhibitors and/or substrates to use in clinical drug–drug interaction studies. The contribution of the inhibition of the transporter-mediated hepatic uptake of cerivastatin by cyclosporine A was used to explain clinical PK interactions between these drugs. Further studies from this laboratory [140] showed the effect of cyclosporine A on the *in vivo* disposition of cerivastatin in rats could be explained using primary rat hepatocytes. *In vitro* and *in vivo* correlation analysis revealed that this PK interaction could be quantitatively explained by the inhibition of transporter-mediated hepatic uptake.

13.7 HEPATOTOXICITY

13.7.1 Background

Drug-mediated hepatotoxicity in humans can be categorized as hepatocellular, cholestatic, and mixed hepatocellular/cholestatic [141]. Hepatotoxicity is the most common single adverse event resulting in product label warnings, failure to market or withdrawal after reaching larger patient populations. Halothane was one of the first compounds to be withdrawn from the market (early 1960s) because of the hepatotoxicity and caused massive centrilobular liver necrosis and hepatic failure [142]. In this, and most other cases, the failure of preclinical animal studies to predict human drug-induced liver toxicity has been attributed to interspecies differences,

some of which are not yet characterized [143]. In attempts to better understand these events, many *in vitro* and *in vivo* models have been developed.

13.7.2 Experimental Approaches

Before the development of successful *in vitro* model systems, researchers relied exclusively on preclinical *in vivo* animal models for predicting human toxicity. While there has been limited success with *ex vivo* isolated/perfused animal livers, researchers have come to the logical conclusion that the best indicator of human hepatotoxicity is to use human cells. Human liver slices have been successfully used over the past two decades for biochemical and histochemical characterization of cellular damage [144,145]. An advantage of liver slices in estimating hepatotoxicity is the integral cellular complement and connections [146]. Limiting factors to this technology, however, include the diffusional barrier that obstructs substrate access to the metabolizing enzymes, as well as oxygen depletion in the slice's inner core [147]. These variables can significantly affect data interpretation in toxicity studies.

The use of isolated human primary and immortalized hepatocytes has removed the limitations associated with liver slices. Since immortalized cells divide, they can be placed in primary culture and utilized over multiple passages. Some view this as an advantage over using fresh primary cells, which are dedifferentiated and can only be used a single time. However, because of the genetic shift associated with repeated passage, most immortalized liver cells lack metabolizing and transporting capabilities, which play a significant role in drug-induced liver toxicity [148]. For this reason, immortalized cell lines may be less or more susceptible to drug-induced toxicity than primary hepatocytes and therefore less predictive of *in vivo* human hepatotoxicity [149,150]. The newer-generation hepatoma cell line HepaRG expresses mRNA encoding various nuclear receptors, P450 enzymes, and phase II enzymes in similar levels as in three- to five-day human primary hepatocyte cultures and may thus serve as an adequate model for early cytotoxicity screening [151].

The optimization of isolating, freezing, thawing, and culturing primary human hepatocytes has resulted in human hepatocytes gaining acceptance as models for cytotoxicity and genotoxicity [83,152]. While both fresh and cryopreserved hepatocytes are used successfully for studying hepatotoxicity, the issues of availability and reproducibility with fresh hepatocytes can be overcome by using cryopreserved cells [153]. Furthermore, interspecies differences can be evaluated simultaneously *in vitro* using cryopreserved hepatocytes from multiple species. Cryopreserved hepatocytes can be characterized for their metabolic capabilities and this information can be incorporated into bioactivation-related hepatotoxicity studies [14]. For example, cytotoxicity studies have demonstrated that CYP2D6-mediated bioactivation of 3,4-methylenedioxymethamphetamine (Ecstasy) forms a metabolite that is more than 100-fold more toxic than the parent compound in primary hepatocytes [154]. Depending on the type/duration of toxicity, one must consider using cell suspensions for short-term toxicity (up to 5–6 h incubations) versus plated hepatocytes for long-term toxicity. Composition of the tissue culture media can also affect the response of isolated hepatocytes to cytotoxic agents. Medium plays a role in preserving liver cell function, and it can also interact with the test drug or its metabolites [83]. Since many compounds bind to proteins, the addition of proteins to the culture medium can greatly decrease toxicity induced by a chemical [155].

13.7.3 *In Vitro* Hepatotoxicity Assays Used in Primary Hepatocytes

Cytotoxicity is a cascade of cellular events that begins with early intracellular changes in homeostatic mechanisms. Necrosis is estimated to be nearly 50% of all drug-related hepatic damage and is associated with membrane leakage, cell swelling, decrease in ATP utilization, and nuclear disintegration [156]. Another major distinction of necrosis is the association of influx of inflammatory cells which then release reactive oxygen species. Conversely, apoptosis is an active process, associated with cell shrinkage, nuclear fragmentation, increased mitochondrial function, pinocytosis, and the formation of apoptotic bodies [157]. Therefore, choosing the appropriate combination of apoptotic and necrotic end points is necessary so that toxicity is not missed.

Light microscopy can be used to observe the morphological effects on cell shape, accumulation of vacuoles and lipid droplets, blebbing, and cell attachment/detachment. When necrosis occurs in hepatocytes *in vivo*, the associated plasma membrane leakage can be detected biochemically by assaying plasma (or serum) for liver cytosol-derived enzymes, including LDH and transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT). These three enzymes are used clinically as biomarkers of hepatic damage and are measured *in vitro* in primary hepatocyte cultures to assess for the potential for clinical hepatotoxicity. Figure 13.12 illustrates the concentration-dependent LDH leakage following 48-h exposure of three classical hepatotoxicants: chlorpromazine (CP), tamoxifen (TAM), and rotenone (ROT). Significant LDH leakage was detected following exposure to 100 μM CP, 100 μM TAM, and 10 and 100 μM ROT. Sometimes, detrimental changes in cell morphology precede membrane leakage. Figure 13.3 is a morphological assessment of these treatments. While 1 μM ROT caused minimal LDH leakage, cell imaging via light microscopy shows the beginning signs of toxicity, that is, increased intracellular granularity and diminished brightness of the nuclei. Visual toxicity was detected in all treatment groups that caused LDH leakage. We recommend that cell morphology always be assessed when using cell-based toxicity assays. LDH leakage might sometimes miss the earlier stages of toxicity and an additional toxicity end point is ATP determination. The utilization of ATP energy

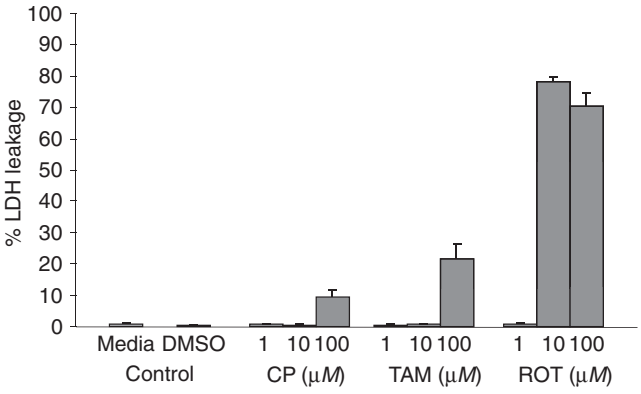


Figure 13.12 Hepatotoxicity as measured by LDH leakage. Cryopreserved human hepatocytes were thawed and plated using CellzDirect complete thawing, plating, and maintenance media. Hepatocytes were treated with multiple concentrations of known cytotoxicants chlorpromazine (CP), tamoxifen (TAM), and rotenone (ROT) for 48 h before assessing LDH leakage.

is significantly decreased as result of necrosis, and there are simple, quick bioluminescence assays available, which are based on luciferase's requirement for ATP in emitting light [158].

Tetrazolium salts such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) are widely used for detecting redox potential of hepatocytes in cytotoxicity assays. These assays are based on the principle that continued cell growth maintains a reduced environment, while inhibition of growth maintains an oxidized environment. Reduction related to growth causes these tetrazolium salt REDOX (reduction–oxidation) indicators to change from the oxidized form to the reduced form. Following reduction, these water-soluble, colorless compounds form uncharged, brightly colored but nonfluorescent formazans, which can be quantified by standard spectrophotometric techniques. Newer generations of tetrazolium salts, such as XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium), and Presto Blue, have been determined more sensitive and less labor intensive than MTT [159–161].

Biochemically, apoptotic cells are distinguished from necrosis by fragmentation of the genome and cleavage or degradation of specific cellular proteins. Early stage apoptosis involves the breaking apart and fragmentation of nuclear chromatin, followed by degradation of the nuclear envelope and nuclear blebbing. These processes result in the formation of micronuclei, which can be easily visualized through staining. Nucleic acid stains such as Hoechst 33342 are readily taken up by cells during the initial stages of apoptosis, whereas cell-impermeant dyes such as propidium iodide and ethidium bromide are excluded [162]. Later stages of apoptosis are accompanied by an increase in membrane permeability, which allows propidium iodide to enter cells [163]. Detection of DNA fragmentation using electrophoresis has been found to be one of the most reliable methods for detecting apoptosis [164]. DNA extracted from apoptotic cells, separated by gel electrophoresis, and stained with ethidium bromide reveals a characteristic ladder pattern of low molecular weight DNA fragments [165]. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assays are commonly used for detecting DNA nicks in apoptotic cells. Once the cells are fixed, DNA strand breaks can be detected *in situ* with an avidin or streptavidin conjugate [166]. Additional apoptosis assays are based on protease activities. Another characteristic of the early stages of hepatic apoptosis is the disruption of active mitochondria. These events include changes in the membrane potential and alterations to mitochondrial oxidation–reduction potential [167]. Fluorescent mitochondrial stains such as calcein and rhodamine 123 provide easy and reliable detection of the loss of mitochondrial membrane potential that occurs during apoptosis [168,169].

Depletion of protein thiols by alkylation or oxidation is an important initial event in the development of drug-induced hepatotoxicity. Glutathione-depleting agents such as diethylmaleate and paracetamol have demonstrated the role of glutathione in the detoxifying process through addition of and by measurement of binding of metabolites to cellular proteins [170]. These metabolites can lead to the production of reactive oxygen species and are associated with idiosyncratic toxicity, especially in the liver. Hepatocytes have the capacity to eliminate these reactive oxygen species, but in some situations, such a capacity is limited and oxygen free radicals can interact with cellular proteins, lipids, or nucleic acids. The detection of these reactive metabolites in *in vitro* systems such as hepatocytes can be used to assess the potential for toxic response

such as adduct formation and immune system intervention. Some compounds can mediate toxicity directly (e.g., paracetamol), while others trigger adaptive immune responses in susceptible patients (e.g., halothane and dihydralazine) [171]. Human hepatocyte cultures have been used for predictions of immunoallergic hepatitis (e.g., clometacin) [172]. Other toxic phase II metabolites such as acyl-glucuronide and acyl-CoA thioester conjugates of carboxylic acid drugs have been studied extensively since several NSAIDs, for example, zomepirac, benoxaprofen, and ibufenac are associated with hepatotoxicity [173].

DNA adducts can also cause mutations and induce chromosome or chromatic-level aberrations [174,175], and this can be estimated in primary hepatocytes by the unscheduled DNA synthesis (UDS) method, which requires small numbers of cells and is accepted by testing and regulatory agencies [176]. Interestingly, the UDS response is usually quite similar in human and rat hepatocytes, whereas other species show marked differences in their responses [177].

13.7.4 Future *In Vitro* Toxicity Models

Newer *in vitro* approaches based on understanding how nuclear receptors and gene signaling play a role in hepatotoxicity are being developed [178]. One major goal is to develop an entire panel of functional toxicity assays (e.g., apoptosis, oxidative stress, and mitochondrial function) and gene expression targets. Sophisticated databases have been developed to analyze data across multiple donor preparations and identify the "firing sequence" of toxicants across the functional assays and gene targets. This technology will allow for clustering of new drugs/chemicals in the hepatocyte-derived database to predict toxicity potential and probe mode-of-action relative to well-characterized "toxicants." For several years, microarrays have distinguished toxic and nontoxic drugs (i.e., troglitazone vs. rosiglitazone and pioglitazone) by measuring changes in protein expression levels in hepatocytes [179]. However, limations in throughput and sensitivity of these more traditional approaches often neglect to incorporate full concentration- and time-related responses. Newer approaches, such as US Environmental Protection Agency (EPA)-driven "ToxCast" allow for standardizing primary hepatocyte data analysis across donors and profiling chemical response with *in vivo* end points [180,181]. Gene-to-gene correlations can provide insight into previously unknown regulation as well as validate an assay by confirming correlations known in current literature.

There is evidence that hepatocytes in monoculture are not predictive of toxicological responses such as fibrosis and cytokine-mediated cytotoxicity. Nonparenchymal cells, including Kupffer, stellate, and sinusoidal endothelial cells, contribute only 6.5% to the liver volume but 40% to the total number of hepatic cells. Many hepatocyte functions are regulated by substances released from neighboring nonparenchymal cells [182,183] (e.g., Kupffer cells) and secrete potent mediators of the inflammatory response (reactive oxygen species, eicosanoids, nitric oxide, carbon monoxide, tumor necrosis factor-α (TNFα), and other cytokines), thereby controlling the early phase of liver inflammation [178]. These enzyme and cytokine mediators may significantly affect liver-metabolizing enzymes, as well as damage hepatocytes. For example, clinical studies have shown that interleukin-2 (IL-2, a cytokine currently used to enhance immune responses in cancer and HIV patients) causes significant suppression of CYP3A4 [184]. While hepatocyte monocultures failed to predict this phenomenon, it was reproduced in Kupffer

cell/hepatocyte cocultures, concluding that the IL-2-mediated suppression is modulated indirectly through Kupffer cells [183]. Similarly, stellate cell/hepatocyte cocultures have been used to predict hepatotoxicity. In the normal liver, stellate cells store vitamin A, control turnover of extracellular matrix, and regulate the contractility of sinusoids. However, damage to hepatocytes under toxic conditions, such as drug-induced hepatitis, alcohol consumption, and biliary obstruction, activates transformation of quiescent stellate cells into myofibroblast-like cells that play a key role in the development of hepatic fibrosis [185]. These findings have shown that hepatocyte cocultures with nonparenchymal cells better represent liver physiology, and these represent the future of *in vitro* hepatotoxicology research.

13.8 FDA DRAFT GUIDANCE

FDA issued a draft drug–drug interaction guidance in 2012 that recommends evaluating potential interactions between an investigational new drug and the most likely to be coadministered drugs, during drug development. This document provides guidance on identifying the principal routes of elimination, the contribution of enzymes and transporters to drug disposition, and characterization of mechanisms of drug–drug interactions.

13.8.1 Drug-Metabolizing Enzymes

The enzymes recommended for evaluation are CYP1A2, 2B6, 2 C8, 2 C9, 2 C19, 2D6, and 3A and UGTs. The UGTs mentioned in the document are UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15. If these enzymes do not explain metabolism of a compound, additional enzymes such as CYP2A6, CYP2J2, CYP4F2, CYP2E1, or non-CYP enzymes including monoamine oxidase (MAO), flavin monooxygenase (FMO), xanthine oxidase (XO) and alcohol/aldehyde dehydrogenase should be evaluated on a case-by-case basis. Recombinant enzymes are suggested for determination of the specific enzymes involved in metabolizing a drug candidate, microsomes for the inhibition assays, and primary hepatocytes for the induction evaluations.

13.8.1.1 Inhibition Studies. The decision tree for metabolism-based drug–drug interaction studies recommends evaluating *in vitro* metabolism for the major drug-metabolizing enzymes using human tissues. If the test compound is a substrate of an enzyme that is responsible for $\geq$25% of its systemic clearance, clinical studies need to be conducted with strong inhibitors and a significant interaction would trigger studies in subjects with different genotypes and clinical studies with less strong inhibitors that are likely to be coadministered. In cases where multiple enzymes cumulatively are responsible for $\geq$25% of the systemic clearance of an investigational drug, the potential of complex DDI should be evaluated. When an interaction is observed, clinical studies are to be conducted with the most sensitive, specific substrates. Positive results would trigger additional studies with substrates that are likely to be coadministered to patients and when appropriate, mechanistic modeling used to make dosage adjustment recommendations. When glucuronidation is responsible $\geq$25% of total metabolism, *in vitro* studies with UGT recombinant enzymes should be conducted. These will help plan clinical studies for UGT1A1

using specific inhibitors such as atazanavir, or with more general inhibitors such as probenecid.

The potential for drug interactions with metabolites that are present at ≥25% of parent drug AUC is also recommended. The guidance also suggests that metabolic drug–drug interactions be explored for investigational drugs that are not eliminated significantly by metabolism, as these drugs could inhibit or induce a coadministered drug's metabolic pathway. An emphasis has been placed on model-based evaluations of interactions, including basic model for initial assessments to mechanistic models such as PBPK modeling. Total test drug concentrations are to be used for all calculations.

13.8.1.2 Induction. FDA suggests evaluating CYP1A2 (positive control: omeprazole), CYP2B6 (positive control: phenobarbital), and CYP3A (positive control: rifampicin), using mRNA *in vitro* and if CYP3A4 induction is observed, CYP2C9 (positive control: rifampicin), using ≥3 separate human hepatocyte preparations. If the increase in mRNA is greater than predefined threshold values using relevant positive and negative controls, or if the calculated R value is less than $1/1 \cdot I$ (i.e., 0.9), then the investigational drug is likely a CYP inducer and area under the curve ratio (AUCR) of a sensitive probe substrate should be calculated using a mechanistic static model. Positive results would determine the need for clinical evaluations with appropriate probe substrates. Total test drug concentrations are to be used for all calculations.

13.8.2 Transporters

The guidance recommends that all investigational drugs are evaluated *in vitro* using transfected cell lines, to determine the substrate potential for the efflux transporters P-gp and BCRP and the uptake transporters OATP1B1 and OATP1B3, when the hepatic pathway is significant (≥25% of total clearance), and the renal transporters OAT1, OAT3, and OCT2, when renal active secretion is ≥25% of total clearance. All investigational small molecule drug candidates are to be evaluated for potential inhibition using known substrates for P-gp (e.g., digoxin), BCRP (e.g., rosuvastatin), OATP1B1/OATP1B3 (statins), OAT1 and OAT3 (e.g., methotrexate and tenofovir), and OCT2 (e.g., metformin). For inhibition studies, total test drug concentrations are to be used for calculations of efflux and hepatic transporters and free drug concentrations for the renal transporters.

13.9 SUMMARY

One of the greatest challenges for pharmaceutical scientists is the accurate prediction of *in vivo* behavior of new compounds from *in vitro* data. The prospect of accurately predicting a drug's metabolic clearance, organ toxicity, and drug–drug interaction potential *in vivo* is still an evolving process. However, our current scientific understanding of the biological processes that dictate overall drug disposition and toxicity has greatly improved our ability to assess the likelihood that a particular drug will cause serious adverse effects or drug–drug interactions *in vivo* [85,89,186]. The pharmacodynamic effects of therapeutic agents cannot be predicted accurately without consideration of the respective PK and toxicokinetics. A number of biological and physiological parameters are involved in determining the total disposition of a particular compound and therefore

will partially determine the exposure of drug to a particular enzyme, transporter, or nuclear receptor site [187,188]. It is important to always compare the *in vitro* concentrations for all metabolism and transporter-based assays with target potency to understand the selectivity. One aspect we have not discussed in this chapter is protein binding. This is because we have yet to figure out when *in vitro* protein binding significantly affects *in vivo* predictions. Continued efforts to define and model these parameters and their impact on drug disposition *in vivo* are needed for proper management of patient drug therapies.

In conclusion, many initial concerns regarding the use of primary hepatocytes for predicting the hepatic clearance, major metabolites, hepatotoxicity, and potential drug interactions of NCEs have been addressed. Primary hepatocytes represent a tremendously versatile preclinical tool for evaluating the overall hepatic disposition of drugs in humans. The response of human hepatocyte cultures to treatment with various compounds (both positive and negative modulators) reflects that observed *in vivo* with regard to both enzyme specificity and potency of the effect. In addition, primary cultures of human hepatocytes have the distinct advantage of exhibiting species-specific profiles and other related responses. However, because of our ever-expanding knowledge of the biological processes involved in overall drug disposition and the creation of more physiologic-based culture platforms, we are faced with a corresponding challenge (and expectation) to improve the accuracy of our *in vivo* predictions as well as the speed at which studies are performed. As such, our future endeavors must find ways to meet the current and future demands that face the pharmaceutical and chemical industries in this new millennium of drug discovery and development.

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ABBREVIATIONS

ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
AUC	Area under the Curve
BCRP	Breast Cancer Resistance Protein
BSEP	Bile Salt Export Protein
CL_H	Hepatic Clearance
CL_{int}	Intrinsic Clearance
CP	Chlorpromazine
E217βG	Estradiol-17β-Glucuronide
ID	Identification
LC-MS	Liquid Chromatography–Mass Spectrometry
LDH	Lactate Dehydrogenase

MDR	Multidrug-Resistant Protein
MRP	Multidrug-Resistance-associated Protein
MS	Mass Spectrometry
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2*H*-Tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NMR	Nuclear Magnetic Resonance
NSAID	Nonsteroidal Anti-Inflammatory Drug
NTCP	Sodium Taurocholate Cotransporting Polypeptide
OAT	Organic Anion Transporter
OATP	Organic Anion-Transporting Polypeptide
OCT	Organic Cation Transporter
P450	Cytochrome P450
PK	Pharmacokinetics
ROT	Rotenone
$T_{1/2}$	Half-Life
TAM	Tamoxifen
XTT	2,3-Bis (2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino) Carbonyl]-2*H*-Tetrazolium Hydroxide

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