

Determination of Drug Toxicity Using 3D Spheroids Constructed From an Immortal Human Hepatocyte Cell Line

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Received February 13, 2012; accepted March 20, 2012

Numerous publications have documented that the immortal cells grown in three-dimensional (3D) cultures possess physiological behavior, which is more reminiscent of their parental organ than when the same cells are cultivated using classical two-dimensional (2D) culture techniques. The goal of this study was to investigate whether this observation could be extended to the determination of LD₅₀ values and whether 3D data could be correlated to *in vivo* observations. We developed a noninvasive means to estimate the amount of protein present in a 3D spheroid from its planar area ($\pm 21\%$) so that a precise dose can be provided in a manner similar to *in vivo* studies. This avoided correction of the actual dose given based on a protein determination after treatment (when some cells may have lysed). Conversion of published *in vitro* LC₅₀ data (mM) for six common drugs (acetaminophen, amiodarone, diclofenac, metformin, phenformin, and valproic acid) to LD₅₀ data (mg compound/mg cellular protein) showed that the variation in LD₅₀ values was generally less than that suggested by the original LC₅₀ data. Toxicological analysis of these six compounds in 3D spheroid culture (either published or presented here) demonstrated similar LD₅₀ values. Although *in vitro* 2D HepG2 data showed a poor correlation, the primary hepatocyte and 3D spheroid data resulted in a much higher degree of correlation with *in vivo* lethal blood plasma levels. These results corroborate that 3D hepatocyte cultures are significantly different from 2D cultures and are more representative of the liver *in vivo*.

Key Words: spheroid culture; drug toxicity; planimetry; protein-to-drug ratio; *in vivo/in vitro* correlation; LD₅₀/LC₅₀.

There is an increasing amount of data suggesting that when cells are grown in three-dimensional (3D) environments (in suspension, on scaffolds, etc.), they express a number of physiological characteristics and resemble more closely the native tissue from which they originated than the same cells grown in classical two-dimensional (2D) culture flasks. The reason for this has been suggested to be related to the fact that 3D cell culture allows the cells to develop a more elaborate extracellular matrix and better intercellular communication (Daus *et al.*, 2011; Lin *et al.*, 2006; Loessner *et al.*, 2010), and this

leads to a recovery or maintenance of *in vivo* function (Selden *et al.*, 2000). Three-dimensional (3D) structure has therefore been proposed as the missing link, which will provide *in vitro* models that sufficiently mimic *in vivo* conditions (Griffith and Swartz, 2006; Nelson *et al.*, 2010; Pampaloni *et al.*, 2007).

The data supporting this concept is fragmentary and has come from many sources. Many of the studies have used primary cells and these have shown that 3D culture prolongs crucial liver-like functionality in primary rat (Brophy *et al.*, 2009; Schutte *et al.*, 2011), porcine (Nelson *et al.*, 2010), and human hepatocytes (Tostoes *et al.*, 2012). Spheroid formation maintains cytochrome P450 expression (Adachi *et al.*, 2011) enhances drug efflux activities (Oshikata *et al.*, 2011) and preserves (compared with adult liver) gene expression of several key hepatic markers (including adhesion molecules, transcription factors, metabolic enzymes including cytochrome P450, and transporters) (Sakai *et al.*, 2010). Although most studies have used primary hepatocytes, there are several reports in the literature that illustrate that immortal hepatocytes also can recover crucial liver-like functionality and that this might increase their value for toxicological studies (Elkayam *et al.*, 2006; Selden *et al.*, 2000; Xu *et al.*, 2003a,b). In this report, we have extended these studies, addressed some commonly encountered obstacles and investigated six commonly used drugs using spheroids constructed from immortal human C3A hepatocytes.

There are a number of obstacles that make quantitative toxicology using 3D cultures more complicated than using 2D cultures. The first of these relate to the fact that it is impossible to count the cells in 3D structures. The second is that it is difficult to take a representative portion of a 3D spheroid culture because the culture is “particulate.” Finally, the third problem is that cells in mature 3D cultures grow significantly slower than their counterparts in 2D. The doubling time for the C3A cell line grown in 2D cultures is about 40 h, whereas in 3D, the same cell line has a doubling time of weeks, even though the cells are equally metabolically active.

In order to circumvent these problems, we decided to normalize our data to the actual amount of protein present, in a similar manner as is used in toxicity studies *in vivo* where the animal is treated with an amount of the compound (typically in milligrams) correlated to the weight of the animal (kilograms). Our first challenge was thus to devise a nondestructive means to relate protein amount to the size of the spheroids and determine whether it was accurate enough to use. Once this was achieved, we determined the median lethal dose (LD₅₀) for six common drugs that are often used for toxicity studies to determine whether the values obtained using 3D spheroids could be compared with those obtained *in vitro* in classical studies (using either primary hepatocytes or immortal cell lines) and with *in vivo* observations of lethal toxicity (e.g., from overdose cases).

MATERIALS AND METHODS

Cell Culture

The immortal human hepatocyte cell line, HepG2/C3A (American Type Culture Collection [ATCC] cat. no. CRL-10741, third passage after receipt from ATCC, Manassas, VA), was thawed from liquid nitrogen storage and left in standard tissue culture conditions (87.5% Dulbecco's Modified Eagle's Medium (D-MEM) (1 g glucose/l) (Gibco, Carlsbad, CA; Cat. no. 31885-023); 1% non-essential amino acids (Gibco; Cat. no. 11140-035); 10% fetal calf serum (FCS) (Sigma, St Louis, MO; Cat. no. F 7524); 0.5% penicillin/streptomycin (Gibco; Cat. no. 15140-122) 1% GlutaMAX (Gibco; Cat. no. 35050-038), 37°C, 5% CO₂ 95% air). To eliminate any effects of storage in liquid nitrogen or thawing, cells were grown for at least three passages before starting the experiments, exchanging the medium every 2–3 days.

Standard 2D Culture Conditions

For experiments with cells grown in traditional tissue culture conditions, we used a customized D-MEM growth medium (87.5% D-MEM (1 g glucose/l) (Gibco; Cat. no. 31885-023); 1% non-essential amino acids (Gibco; Cat. no. 11140-035); 10% FCS (Sigma; Cat. no. F 7524); 0.5% penicillin/streptomycin (Gibco; Cat. no. 15140-122) 1% GlutaMAX (Gibco; Cat. no. 35050-038). Cells were trypsinised (0.05% trypsin/EDTA, Cat. no.: Gibco. 15400-054) for 3 min and sown out into falcon flasks/microtiter plates and cultivated at 37°C, 5% CO₂ 95% air in a humidified incubator. The doubling time for C3A cells grown under these conditions was 76 h. The cells were allowed to recover from the seeding stress and grow for 24 h before any treatment.

Three-dimensional Spheroid Culture Conditions

Preparation of spheroids using AggreWell plates. The C3A cell spheroids have been prepared with use of AggreWell 400 plates (Stemcell Technologies, Grenoble, France; Cat. no. 27845). Before use, the plates were washed twice with growth medium (customized D-MEM). In order to remove all residual air bubbles from the well surface, the plates were prefilled with 0.5 ml of growth medium and centrifuged (3 min with 3000 × g). Cells (1.2 × 10⁶) were added to each well, and the plates were centrifuged (3 min with 100 × g) and left in the AggreWell plate overnight to form spheroids.

Spheroid culture in bioreactors. The spheroids were detached from the AggreWell plates by gently washing the wells with prewarmed growth medium. The detached spheroids were collected into a Petri dish, and the quality of the spheroids checked by microscopy. Compact spheroids were introduced into bioreactors (MC2 Biotek, Hørsholm, Denmark; Cat. no. 010). These bioreactors are specially constructed to be easy to open and close and designed to maintain 100% humidity around the growth chamber. The

spheroids (approximately 300 per bioreactor) were then cultivated at 37°C, 5% CO₂ 95% air in a humidified incubator for a minimum of 21 days, exchanging the medium every 2–3 days (Wrzesinski, 2009).

The day when the cells were transferred into the bioreactor is defined as day 0. An estimated 90% of the medium was changed on day 1 and thereafter three times a week during the differentiation period. To achieve a stable suspension of spheroids, the rotation speed of the bioreactors was initially set between 21 and 23 rpm, and it was adjusted to compensate for the growth of the spheroids (and reached 27–30 rpm on day 21). The density of the spheroid population was regulated by opening the bioreactor and splitting or removing excess spheroids (this usually needed to be performed between day 11 and 14). Before use, the spheroid batch quality was assessed by staining for 3 min with 0.4% Trypan Blue (Gibco; Cat. no. 15250-061). Batches showing greater than 90% viability were accepted.

Microscopy and Planimetry

Photomicrographs were taken using an Olympus IX81 motorized microscope and an Olympus DP71 camera. Images were transferred to the Olympus AnalySiS Docu program (Soft Imaging System) and the “shadow” area of spheroids measured using the “Fitted Polygon Area” function that calculates the planar surface of the spheroids in μm².

Drug Treatment

Acetaminophen (APAP), amiodarone, diclofenac, metformin, phenformin, and valproic acid (VPA) were all purchased from Sigma. Stock solutions were prepared just before use by dissolving the compound in either growth medium or dimethyl sulfoxide (DMSO) and diluted into growth media. A control media was prepared which contained the same concentration of DMSO vehicle without any compound. The maximum final DMSO concentration in all cases where DMSO was needed was 0.01%.

Sixty 21-day-old spheroids (corresponding to approximately 15 million cells or 3 mg protein) were gently pipette (using a cut-tip) into each 10 ml bioreactor before treatment to provide ample material for analysis. To initiate drug treatment, the bioreactor rotation was stopped for 30 s to allow the spheroids to settle to the bottom of the bioreactor. Ninety percent of the media volume was exchanged with media containing the compound at concentrations, which gave the final treatment concentrations given in the figures. Experiments to determine the LD₅₀ were carried out initially using a broad range of drug concentrations (e.g., varying by a factor of 10) followed by two experiments using a narrow concentration range (varying typically by a factor of two). For all drugs used except APAP, each experiment was carried out in duplicate, and adenosine triphosphate (ATP) was measured in technical triplicates for each sample. For APAP, the experiment was carried out in triplicate. The LD₅₀ values presented are thus an average of 18 measurements for APAP ($n = 6$) and 12 for the other 5 compounds ($n = 4$).

ATP Assay

Cell viability was measured based on their ability to produce ATP. Samples of the hepatocytes grown either in classical culture conditions (in 96-well microtitre plates) or as spheroids (usually 2–6 spheroids per assay point) were collected at appropriate times and transferred to white opaque microtitre plates (Nunc, Roskilde, Denmark; Cat. no. 165306), the volume of growth medium was adjusted to 100 μl. The cells were then lysed with 100 μl of lysis buffer (CellTiter-Glo luminescent cell viability assay, Promega, Fitchburg, WI; Cat. no. G7571) and shaken in the dark for 20 min before the luminescence was measured in a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany) using the following parameters: one kinetic window, 10 measurement cycles with 0.3 s of measurements interval time, 2 s delay per measurement, additional 0.5 s delay per position change (repeated twice for each measured plate). Replicates were performed as described under “Drug Treatment.” The data was normalized with reference to a standard curve for ATP and to the untreated control.

Protein Determination

In order to correlate the shadow area of the spheroid to its protein content, the latter was determined using the fluorescence-based ProStain Protein Quantification Kit (Active Motif, Inc., La Hulpe, Belgium; Cat. no. 15001)

according to the manufacturer's instructions. All data was expressed as a function of the amount of protein present.

RESULTS

Spheroids were initiated by sowing 1.2 million freshly trypsinized human hepatocyte C3A cells into individual wells of an AggreWell plate, centrifuging and leaving them to adhere to each other overnight. Thereafter, they were released into suspension as described and grown in rotating bioreactors for 21 days to reach maturity (Fig. 1A). At various stages, spheroids were selected from the culture, photographed at both $\times 4$ and $\times 10$ magnification and then their protein content determined. Protein determination was carried out on either pairs or quadruplets of spheroids (Fig. 1B). The boundary of each spheroid was identified on the digital images using the "Fitted Polygon Area" function in the program (Fig. 1B) to calculate the planimetric area of each spheroid. This area was determined for at least 170 spheroids at each magnification for both pairs and quadruplicates.

Comparison of the planimetric area and protein content of the spheroids demonstrated a clear correlation with an acceptable SD (of 21%) to be used as a working tool (Fig. 2). Essentially, the results (with similar accuracies) were obtained using either 2 or 4 spheroids or using either $\times 4$ or $\times 10$ optical magnification (not shown) indicating that either magnification or number of spheroids could be used interchangeably.

This noninvasive approach allowed us to estimate and adjust the protein content of the bioreactor before assaying the toxicity of various compounds so that it was possible to treat a predetermined number of cells with a given amount of compound.

In order to assess whether cells in 3D cultures respond in a similar way to treatment with toxic compounds, mature differentiated spheroids were treated with six different compounds and their total cellular ATP content measured as a measure of their viability. The six compounds were selected

to be physiologically diverse so that the conclusions of the analysis would be more representative of toxicity.

Thus, they were selected on the basis that there was suitable LC_{50} data available in the literature; that they were metabolized by different cytochrome P450 enzymes, have different half-lives; and that they had their beneficial effects on different target organs (Table 1).

Mature 21-day-old differentiated spheroids were treated with different concentrations of the compounds and their total cellular ATP content measured as a measure of their viability.

The LC_{50} and LD_{50} results obtained by treating the 3D spheroids with increasing concentrations of APAP are shown in Figure 3. Note that at low APAP concentrations (0.625 and 1.25 mg APAP per mg cellular protein), there was a reproducible stimulation of ATP production. This may be a reflection of the cells attempt to counter the APAP-induced toxicity. Interpolation of the graph indicates that the LD_{50} for APAP in these conditions was about 20 mg APAP/mg cellular protein (LC_{50} 40mM APAP) (Table 2).

We determined the LC_{50} using ATP production in 3D spheroids in a similar manner for five other compounds (Table 2). The results are given both as LD_{50} (mg compound per mg cellular protein [mg/mg]) and as LC_{50} (mM) to facilitate comparison with published data. In the cases of amiodarone and metformin, cellular ATP production was slightly increased at low compound concentrations in a manner similar to that seen for APAP, whereas this was not seen for diclofenac, phenformin, and VPA.

DISCUSSION

There is an increasing amount of evidence showing that when primary cells are grown in 3D culture, they retain physiological attributes for longer compared with when the same cells are grown using classical (2D) cell culture

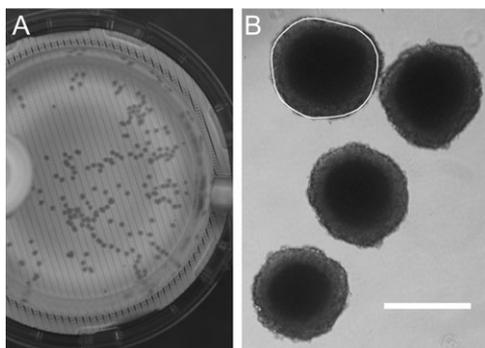


FIG. 1. (A) Photograph of a bioreactor showing 21-day-old spheroids. The lines visible on the gas exchange membrane are 1 mm apart. (B) Enlarged photograph of four spheroids. The line delimiting their area can be seen at various points on their periphery and has been highlighted on the top left spheroid. Scale bar = 0.5 mm.

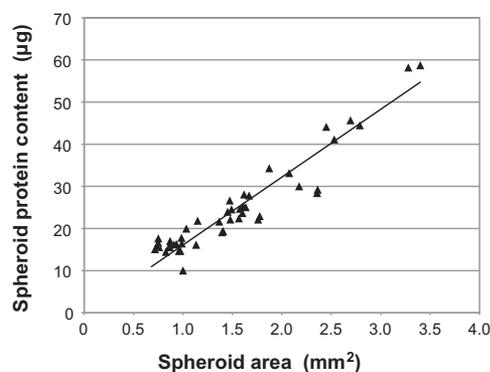


FIG. 2. Correlation between spheroid planar size and their protein content. Spheroids of different ages were photographed and then their protein content determined using the ProStain Protein Quantification Kit. The area of the spheroids was determined using the Olympus AnalySiS Docu Soft Imaging System program. The figure shows the data for groups of four spheroids, photographed with $\times 10$ magnification only. SD was 21% of the average area.

TABLE 1
Selection Characteristics of Compounds Chosen for the Determination of LC₅₀ Using 3D Spheroids

Compound	Metabolic cytochrome (references)	Terminal elimination half-life (h), (Schulz and Schmoldt, 2003)	Target tissue (at therapeutic dose)
APAP	1A2, 2E1, and 3A4 (Hazai <i>et al.</i> , 2002; Manyike <i>et al.</i> , 2000)	3	CNS
Amiodarone	3A4 (Zahno <i>et al.</i> , 2011)	75	Cardiac cells
Diclofenac	2C9 (Mancy <i>et al.</i> , 1999)	1.5	Inflammatory cells
Metformin	3A4 (Choi <i>et al.</i> , 2010)	3	Liver
Phenformin	1A1, 2D6 (Boobis <i>et al.</i> , 1983; Granvil <i>et al.</i> , 2002)	7	Liver
Valproic acid	4B1 (Rettie <i>et al.</i> , 1995)	15	CNS

Note. CNS, central nervous system.

techniques. Despite that immortal cells are transformed and may contain numerous chromosomal aberrations, it appears that they are able to recover at least some of these physiological attributes when grown in 3D, apparently retracing their differentiation pathway.

Growing cells in 3D culture systems has not yet been widely adopted. This is presumably because of the additional challenges in working with 3D cultures that are not present when using the classical 2D culture systems. Three of these challenges are related as follows: to the fact that it is impossible to count the cells in a spheroid; that it is difficult to collect a representative sample from a spheroid culture; and that spheroid cultures are characterized by having very high cell numbers per volume growth media.

In 2D cultures, cells are normally counted during the trypsinization procedure and then sown out and left for a day or two to recover before an analysis is carried out. This provides a fairly accurate estimate of the number of cells (or how much

protein) was used in the analysis (and this estimate can be confirmed by taking an aliquot for protein determination).

In the 3D spheroid cultures described here, typically the cells are counted after trypsinization and then spheroids are initiated and left to mature for 21 days. Because their growth rate falls rapidly during this extended period, it is difficult to estimate how many cells (or how much protein) are present in a spheroid culture. It is also difficult to take a representative aliquot because, by its very nature, the spheroid culture is particulate.

This manuscript describes a simple approach to estimating the number of cells in a spheroid and illustrates that it is important to take the total number of cells into consideration when determining LD₅₀ values (something that is normally done *in vivo* in animal studies but not normally done *in vitro* in cell-based studies when median lethal concentration (LC₅₀) data is produced).

To further investigate the metabolic capacity of human immortal hepatocytes grown in 3D, we have determined the LD₅₀ of six commonly used compounds (APAP, amiodarone, diclofenac, metformin, phenformin, and VPA). In comparing our data with published 24 h toxicity studies, we have not paid

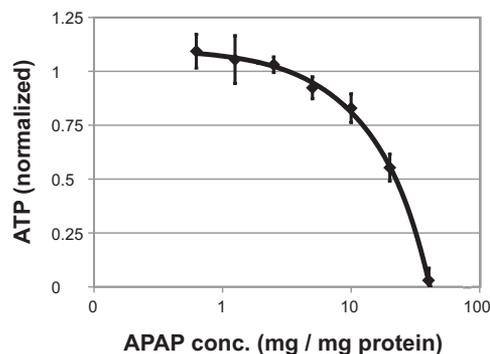


FIG. 3. APAP LD₅₀ determination as measured by the amount of ATP present in the cells. The total number of 21-day-old spheroids in a 10 ml bioreactor was adjusted so that the total cellular protein content was 3 mg. The spheroids were exposed to different concentrations of APAP for 24 h and their ATP content determined by the CellTiter-Glo luminescent cell viability assay from Promega. Data was normalized to a mock-treated control. The experiment was repeated three times, and duplicate samples were collected from each bioreactor.

TABLE 2
LD₅₀ and LC₅₀ Values for Six Common Compounds as Determined by Treating 21-day-old 3D Spheroids for 24 h and Then Measuring ATP Production

Compound	LD ₅₀ (mg compound/mg cellular protein)	LC ₅₀ (mM)	SD (%)
Acetaminophen (APAP)	20	40	20.1
Amiodarone	0.6	0.26	12.7
Diclofenac	2.8	2.6	13.8
Metformin	2.76	5	14.5
Phenformin	0.02	0.03	8.9
Valproic acid (VPA)	10	18	8.5

Note. *n* = 6 for APAP and *n* = 4 for the five other compounds.

great attention to the different methods used for determining the LC₅₀ because assay differences were shown to be minimal for VPA treatment of rat hepatocytes grown in sandwich cultures (Kiang *et al.*, 2010, 2011) and for tamoxifen treatment of HepG2 cells (Riss and Moravec, 2004), and no clear correlation emerged between assay method and LC₅₀ obtained when analyzing the data (see below).

There are numerous LC₅₀ studies of APAP carried out on human, rat, mouse primary, or immortal cells. Published LC₅₀ values vary considerably from 0.21 to 28.2mM for human hepatocytes (Jemnitz *et al.*, 2008; Lin *et al.*, 2012; Riches *et al.*, 2009; Ullrich *et al.*, 2009); from 7.6 to 25mM for rat hepatocytes (Jemnitz *et al.*, 2008; Lewerenz *et al.*, 2003; Wang *et al.*, 2002); from 3.8 to 20mM for mouse hepatocytes (Jemnitz *et al.*, 2008; Moon *et al.*, 2010; Toyoda *et al.*, 2012); and from 0.14 to 29.8mM for cell lines (Huh-7 (Lin *et al.*, 2012), HCC-T (Lin *et al.*, 2012), C3A (Flynn and Ferguson, 2008), and HepG2 (Miyamoto *et al.*, 2009b; Wang *et al.*, 2002). Initial inspection of LC₅₀ data presented here suggested that the 3D spheroids were more resistant to APAP toxicity because an APAP concentration of about 40mM was necessary to reach 50% lethality. Spheroids contain many more cells per culture volume than is normally used in 2D cultures, and one would expect that many cells can metabolize and detoxify a given dose of APAP faster than few cells. Therefore, in order to compare the results obtained here with literature values, published LC₅₀ values (millimolar) were converted to LD₅₀ values (milligram APAP per milligram cellular protein or mg/mg). This conversion involved making assumptions in a few cases where full details were not included in the original publication. These assumptions were that: all cells (human, mouse, rat; primary or immortal) contain 0.2 ng soluble protein per cell; culture vessels were 90% confluent (e.g., that a well in a 96-well plate contained 30,000 cells); cells were 100% viable; before treatment, primary cells do not propagate but immortal cell lines do; immortal cell lines have a 40 h doubling time; and that during the treatment with compound, no cells propagate. Since the

amount of drug present is also dependent on the reaction volume (i.e., doubling the volume doubles the amount of drug present), when not otherwise stated, we assumed that the culture vessels used in the published literature contained a convenient working minimum volume (e.g., plates with 96, 48, 24, 12, and 6 wells have working volumes of 0.1, 0.5, 0.75, 1.4, and 2.7 ml, respectively). These assumptions correspond to the normal practice used in many of the cited reports. In some cases, the LC₅₀ value was interpolated from the appropriate figure in the reference.

A comparison of the LC₅₀ (mM) and the corresponding LD₅₀ (mg/mg) are shown in Figure 4A for APAP. Data based on human hepatocytes are indicated with a square (■), rat hepatocytes with a triangle (▲), mouse hepatocytes with a diamond (◆), human cell lines with a cross (×), rat cell lines with a plus (+), and 3D spheroid cultures with a circle (●). Each data point is indicated together with a number, and the reference is given in the figure legend.

The LD₅₀ for APAP presented here (point 35 in Fig. 4A, 20 mg/mg) using 3D spheroids constructed from C3A cells, corresponds well with data obtained with spheroids made from HepG2 cells, where the culture flask was swirled to construct the spheroids (LD₅₀ 10 mg/mg) (Xu *et al.*, 2003a). It also corresponds reasonably well with the average of the published data for human hepatocytes (average LD₅₀ = 31 mg/mg) but corresponds less well when HepG2 cells were grown in classical 2D culture (average LD₅₀ 55 mg/mg). The observation that the LD₅₀ value for the 3D spheroids is lower than that measured using primary hepatocytes or using HepG2 cells grown in 2D cultures suggests that APAP permeability into the spheroid is not a limitation.

Comparison of either all the data or of the data from human, rat, or mouse hepatocytes or HepG2 showed that the maximum ratio between the highest and lowest published LC₅₀ values were usually smaller when the data was expressed as LD₅₀ values (Table 3). For example, the highest LC₅₀ concentration described in the literature for APAP is 29.75mM using HepG2 cells (Wang *et al.*, 2002) and the lowest is 0.137mM using Huh-7 cells (Lin

TABLE 3
Variability of the Published Data for APAP Toxicity in Different Test Systems Comparing the Published LC₅₀ Data (Expressed as mM) With Its Converted LD₅₀ Values (Expressed as mg APAP per mg Cellular Protein)

	Total, n = 17		Human Hepatocytes, n = 4		Rat Hepatocytes, n = 3		Mouse Hepatocytes, n = 2		HepG2 Cell Line, n = 2		Other Cell Lines (any species), n = 4		3D Spheroids ^a , n = 2	
	mM	mg/mg	mM	mg/mg	mM	mg/mg	mM	mg/mg	mM	mg/mg	mM	mg/mg	mM	mg/mg
Maximum ratio ^b	218	20	136	8.5	3.3	3.3	5.3	6.9	10	3.1	147	12	1.5	1.9
Average (mM or mg/mg) ^c	13	31	17	30	16	32	10	34	16	55	5	23	34	15
Stds ^c	11	22	12	21	9	17	9	23	19	39	10	21	9	7
Stds % ^c	83	70	71	69	57	52	88	69	116	72	186	91	26	45

Note. This comparison is made on the basis of the literature cited in Figure 4A.

^aAveraged data of (Xu *et al.*, 2003a) and data presented here.

^bMaximum ratio: the ratio between the highest and the lowest published LC₅₀ values.

^cThe average, SD, and percentage SD of all published LC₅₀ data within the group defined by the column heading.

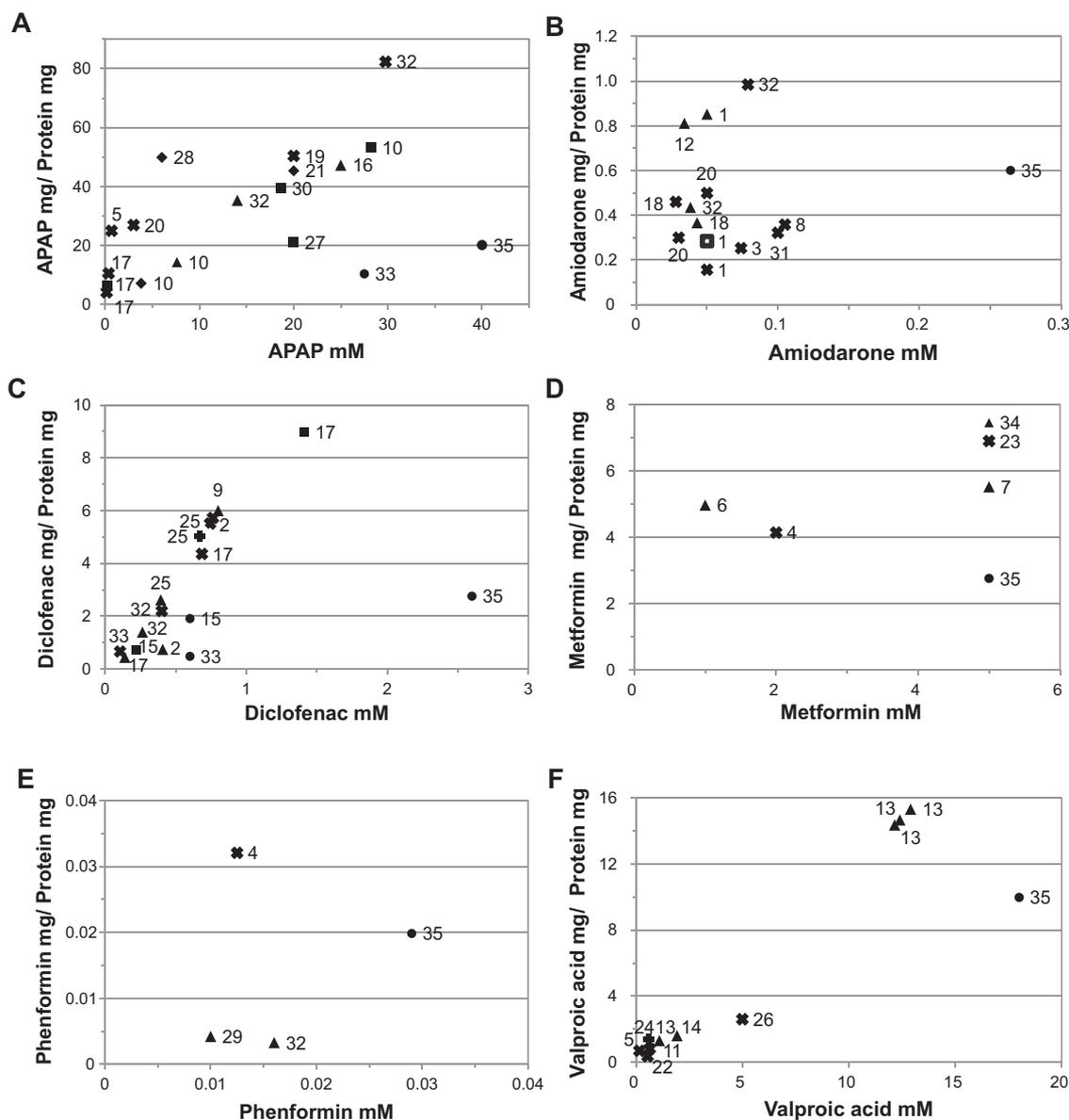


FIG. 4. Comparison of the published LC₅₀ values for (A) APAP, (B) Amiodarone, (C) Diclofenac, (D) Metformin, (E) Phenformin, and (F) Valproic acid with their corresponding LD₅₀ values (millimolar compound concentration or as milligrams compound per milligram cellular protein). Squares (■) indicate studies based on human hepatocytes; open square (□) rhesus monkey; triangles (▲), rat hepatocytes; diamonds (◆), mouse hepatocytes; circles (●), spheroids; crosses (×), HepG2 cell line; and pluses (+), other cell lines. The numbers refer to published references as follows: 1, (Bhandari *et al.*, 2008); 2, (Bort *et al.*, 1998); 3, (Donato *et al.*, 2009); 4, (Dykens *et al.*, 2008); 5, (Flynn and Ferguson, 2008); 6, (Foretz *et al.*, 2010); 7, (Fulgencio *et al.*, 2001); 8, (Golli-Bennour *et al.*, 2012); 9, (Gomez-Lechon *et al.*, 2003); 10, (Jemnitz *et al.*, 2008); 11, (Ji *et al.*, 2010); 12, (Kaufmann *et al.*, 2005); 13, (Kiang *et al.*, 2010); 14, (Kingsley *et al.*, 1983); 15, (Lauer *et al.*, 2009); 16, (Lewerenz *et al.*, 2003); 17, (Lin *et al.*, 2012); 18, (McCarthy *et al.*, 2004); 19, (McGill *et al.*, 2011); 20, (Miyamoto *et al.*, 2009a); 21, (Moon *et al.*, 2010); 22, (Neuman *et al.*, 2001); 23, (Okamoto *et al.*, 2009); 24, (Phillips *et al.*, 2003); 25, (Ponsoda *et al.*, 1995); 26, (Rauchenzauner *et al.*, 2008); 27, (Riches *et al.*, 2009); 28, (Toyoda *et al.*, 2012); 29, (Ubl *et al.*, 1994); 30, (Ullrich *et al.*, 2009); 31, (Waldhauser *et al.*, 2008); 32, (Wang *et al.*, 2002); 33, (Xu *et al.*, 2003a); 34, (Zhou *et al.*, 2001); and 35, data presented in this report.

et al., 2012), giving a maximum ratio of 218. When converted to LD₅₀, the data from these two publications are much closer, namely 82.32 and 4.13 mg/mg, giving a maximum ratio of only 20. This reduction in variation was usually true within species and within cell lines and was usually true when the percentage SD of the average values were compared (Table 3). This illustrates that the assumptions that were made to convert LC₅₀

to LD₅₀ did not prejudice the data and that in fact, shows that there is a higher degree of agreement in the published data than initially meets the eye.

The published data for the other five compounds were also converted to LD₅₀ values and is shown in Figures 4B–F. As with APAP, it can be seen that the LC₅₀ concentration was usually higher for the 3D spheroids (data from this report is shown as

point “35” in Fig. 4) than LC_{50} concentrations reported in the literature. Once again, this difference disappeared when the LC_{50} is expressed as LD_{50} underlining the importance of taking the number of cells into account when describing toxicity values.

Most LD_{50} studies of amiodarone have been carried out using either rat hepatocytes or HepG2 cells, although one study used rhesus monkey hepatocytes (open square in figure 4B) (Bhandari *et al.*, 2008). All the data fell within the narrow range of 0.16–0.98 mg/mg (Fig. 4B).

Xu *et al.* (2003a,b) constructed spheroids from both rat hepatocytes and HepG2 cells (separately) and found insignificant differences in their responses to APAP and diclofenac (the latter in glucose free, galactose-containing medium). He concluded that both models were predictive for chemical-induced hepatic cytotoxicity. The diclofenac LD_{50} data presented here (2.8 mg/mg) match well with their observations (0.5 and 1.9 mg/mg for HepG2 and rat hepatocytes, respectively) (Fig. 4C) (Xu *et al.*, 2003a,b).

Because of the effects of metformin on glucose metabolism, only studies, which investigated metformin's toxicity using alternative carbon sources (e.g., galactose [Dykens *et al.*, 2008] or pyruvate [Okamoto *et al.*, 2009]), were used for data analysis. Once again, conversion to LD_{50} showed that the data reported here were in close agreement with published values in the literature (Fig. 4D).

The spheroid LD_{50} obtained for phenformin was in good agreement with published values, midway between that seen for the rat hepatocytes (points 29 and 32) and the HepG2 data (point 4 in Fig. 4E).

For VPA (Fig. 4F), there was very close agreement between the average of the rat hepatocyte published data (9.5 mg/mg) (from Kiang *et al.* [2010]; Kingsley *et al.* [1983] and Kiang *et al.* [2011]) and the spheroid data (10 mg/mg) and poor correlation to the averaged cell line data (1.1 mg/mg).

Thus, in general, there is reasonably good agreement between the data sets, whether cell line, primary hepatocyte, 2D, or 3D culture. However, it remains unclear as to whether

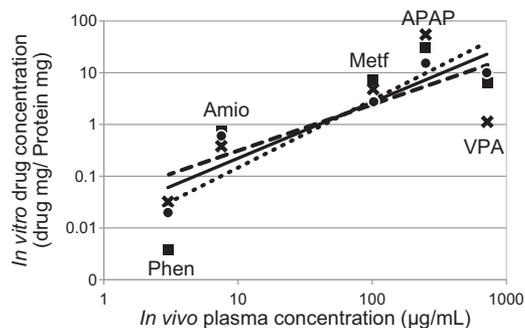


FIG. 5. Correlation of *in vivo* plasma concentrations ($\mu\text{g/ml}$) which induce either comatose condition or lethal with LD_{50} concentrations (expressed as mg compound/mg cellular protein). Squares (■) indicate studies based on hepatocytes (human, rat, or mouse); circles (●), spheroids; crosses (×), HepG2 cell lines. APAP, Acetaminophen; Amio, Amiodarone; Metf, Metformin; Phen, Phenformin; VPA, Valproic acid. There is insufficient data to include other comparisons. No published *in vivo* lethal plasma concentration could be found for diclofenac.

3D cultures are superior to 2D cultures. Therefore, in order to benchmark the data and evaluate its relevance to assessing human toxicity *in vivo*, the data was compared with published plasma concentrations ($\mu\text{g/ml}$) which either produced a comatose state or were lethal *in vivo* in man (Pollak and Shafer, 2004; Schulz and Schmoldt, 2003) (Fig. 5). Although there are numerous reasons why one should not expect a close correlation (the most obvious reason being that the primary or immortal cells represent only one type of cell, whereas the whole organism is constructed of many), the plasma concentration is best measurement for *in vivo* toxicity available.

In making the comparison to the published *in vivo* plasma concentrations, only data based on human hepatocytes were used in the APAP correlation because it has been shown that humans and rodents respond differently to APAP (Jemnitz *et al.*, 2008). Inclusion of all the hepatocyte data would have had a minimal detrimental effect on the correlation (Table 3). For all other compounds, data from human and rodents were treated as equivalent. In addition, it is known that different cell lines exhibit different abilities to metabolize drugs (e.g., diclofenac metabolism in rat primary hepatocytes, rat FaO cells and human HepG2 cells [Ponsoda *et al.*, 1995], and APAP metabolism in human hepatocytes, Huh-7. and HCC-T cells [Lin *et al.*, 2012]). Therefore, the comparisons with conventional 2D culture were restricted to studies that used the human hepatocarcinoma cell line HepG2. LD_{50} data from other 3D spheroid studies (APAP and diclofenac) (Xu *et al.*, 2003a) were included and pooled with the data presented here and used in the *in vitro/in vivo* comparison shown in Figures 4 and 5. No published *in vivo* lethal plasma concentration data could be found for diclofenac. Based on the LD_{50} data presented here in Table 2, the correlation in Figure 5 would suggest that the lethal plasma concentration *in vivo* would be approximately 60 $\mu\text{g/ml}$, close to the toxic range of 50–60 $\mu\text{g/ml}$ given by Schulz and Schmoldt (2003).

Comparison of the HepG2 cell line, *in vitro* data with the *in vivo* lethal plasma concentration data resulted in a poor correlation (Fig. 5, dashed line, R^2 correlation coefficient 0.55), and the inclusion of all of the cell-line-derived data would have reduced the correlation further (not shown). The corresponding comparison of primary hepatocytes with the *in vivo* data gave a much better correlation (Fig. 5, dotted line, R^2 0.75) and concurs with the use of primary human hepatocytes for toxicity determination. The comparison of 3D spheroid data with the *in vivo* data also showed a reasonably good correlation (Fig. 5, solid line, R^2 0.86). The fact that 3D spheroids appeared to correlate better than primary hepatocytes may be due in part to the fact that the primary hepatocyte data were collected in different labs and in part that different assays were used to determine the LD_{50} values and so the actual degree of correlation has to be treated with caution.

In conclusion, there are significant differences in the LD_{50} determined using immortal cells grown in classical 2D culture techniques when compared with 3D spheroids. Spheroids constructed from immortal human hepatocytes appear equally

useful as primary human hepatocytes for the determination of LD₅₀ *in vitro*.

FUNDING

MC2 Biotek, Hørsholm, Denmark; University of Southern Denmark.

ACKNOWLEDGMENTS

The authors would like to thank Signe Marie Andersen, Kira Eyd Joensen, and Jacob Bastholm Olesen for expert technical support in growing and maintaining spheroid cultures and performing some of the assays. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. The authors declare that there are no conflicts of interest.

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