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Development of a Multiparametric Cell-based Protocol to Screen and Classify the Hepatotoxicity Potential of Drugs

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Hepatotoxicity is a major reason for drug nonapprovals and withdrawals. The multiparametric analysis of xenobiotic toxicity at the single cells level using flow cytometry and cellular imaging–based approaches, such as high-content screening (HCS) technology, could play a key role in the detection of toxicity and the classification of compounds based on patterns of cellular injury. This study aimed to develop and validate a practical, reproducible, *in vitro* multiparametric cell-based protocol to assess those drugs that are potentially hepatotoxic to humans and to suggest their mechanisms of action. The assay was applied to HepG2 human cell line cultured in 96-well plates and exposed to 78 different compounds for 3 and 24 h at a range of concentrations (1–1000μM). After treatments, cells were simultaneously loaded with five fluorescent dyes showing optical compatibility and were then analyzed with the High-Content Screening Station Scan^R (Olympus). By using the new technology of HCS cell parameters associated with nuclear morphology, plasma membrane integrity, mitochondrial function, intracellular calcium concentration, and oxidative stress, indicative of prelethal cytotoxic effects and representative of different mechanisms of toxicity, were measured at the single cells level, which allows high-throughput screening. This strategy appears to identify early and late events in the hepatotoxic process and also suggests the mechanism(s) implicated in the toxicity of compounds to thereby classify them according to their degree of injury (no injury, low, moderate, and high injury).

Key Words: **hepatotoxicity; drug; screening; mechanism; classification.**

Hepatotoxicity is a major cause of failure in both the clinical and postapproval stages of drug development and poses an important challenge for the pharmaceutical industry (Kaplowitz, 2001; Lee, 2003). Indeed, hepatotoxicity has been acknowledged as a major reason for drug withdrawal based on safety, probably because of the central role that the liver plays in intermediary and energy metabolism, and in the biotransformation of foreign compounds, which accounts for organ vulnerability (Abboud and Kaplowitz, 2007; Park *et al*., 2005a,b). Several molecular mechanisms are primarily involved in drug-induced hepatocyte injury and the way in which intracellular organelles are affected defines the pattern of liver disease (Abboud and Kaplowitz, 2007; Lee, 2003; Park *et al*., 2005a; Russmann *et al*., 2009). Thus, the development and validation of novel preclinical tools that successfully identify drug hepatotoxicity are a paramount need for the pharmaceutical industry.

The aim of applying *in vitro* hepatotoxicity assays during early drug development is to screen large numbers of new molecules and to prioritize them for further testing. *In vitro* assays are already being applied to a large set of marketed drugs that produce hepatotoxicity by multiple mechanisms for the purpose of assessing their correlation with human toxicity (Dambach *et al*., 2005). Current *in vitro* cytotoxicity assays have, in general, a low concordance with human liver toxicity, thus making predictions of the hepatotoxicity potential of new drugs very difficult (O'Brien *et al*., 2006; Xu *et al*., 2004). There are several reasons for the low sensitivity of cell-based hepatotoxicity assays. Among them, one major reason is that most assays evaluate a single endpoint, although hepatotoxicity could be the result of multiple mechanisms, which should be assessed by different parameters. Multiparametric and time-resolved assays are expected to greatly improve the prediction of toxicity as they assess complex mechanisms of toxicity. The application of "omic" strategies to *in vitro* hepatotoxicity enables multiparametric analyses, which may contribute to the development of more accurate and predictive screenings (Amacher, 2010; O'Brien *et al*., 2006). Cytomics may be defined as the cytometry of cellular systems and aims to determine changes in the molecular phenotype of single cells, which can be further related to a given stimulus or injury (Herrera *et al*., 2007). Therefore, the multiparametric analysis of compound toxicity at the level of

individual cells using flow cytometry and cellular imaging–based approaches, such as high-content screening (HCS) methodology, could play a key role in the detection of toxicity and classification of compounds based on their mechanism of action (Abraham *et al.*, 2008; Cosgrove *et al.*, 2009; Jan *et al.*, 2008).

Multiparametric assays integrate the data obtained simultaneously from different indicators of the cell function, which aim to suggest the mechanism of toxic action of a given compound and of prioritizing compounds in drug discovery based on their potential hepatotoxicity to humans. These strategies will enable toxicological profiles of drugs and concordance between *in vitro* and *in vivo* results. More to the point, the present study aimed to develop and validate a feasible, reproducible, multiparametric cell-based protocol to assess the hepatotoxic potential of drugs and to classify them by their mechanism of action using HCS technology. To achieve this aim, a mechanism-based selection of the compounds is of crucial importance. Compounds with well-documented *in vivo* toxicity were selected according to the different mechanisms of hepatotoxicity (i.e., apoptosis, DNA synthesis/genotoxicity, oxidative stress, mitochondrial damage/dysfunction, bioactivation). An exhaustive literature research was conducted to document these compounds' toxicological properties (Gomez-Lechon *et al.*, 2010).

We report herein the assessment and application of an HCS multiparametric cytotoxicity assay that simultaneously measures nuclear morphology, mitochondrial function (transmembrane potential), cell viability, intracellular calcium concentration, and oxidative stress for the initial screening of potential hepatotoxic compounds.

MATERIALS AND METHODS

Materials. Culture media and complements were purchased from GIBCO (Gibco BRL, Paisley, U.K.). Fluorescent probes (E,E)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY 665/676), tetramethyl rhodamine methyl ester (TMRM), and Fluo-4 acetoxymethyl ester (Fluo-4 AM) were acquired from Molecular Probes, Invitrogen (Madrid, Spain). Propidium iodide (PI) and Hoechst 33342 were obtained from Sigma-Aldrich (Madrid, Spain).

Selection of compounds. A total of 66 hepatotoxic compounds were included in the study. Compounds were chosen on the basis of previous information on both their hepatotoxic potential and their described pathways implicated in toxicity (Gomez-Lechon *et al.*, 2010). Additionally, sodium citrate, caffeine, ascorbic acid, acetylcysteine, betaine, dexamethasone, gentamicin, lactose, dimethyl sulfoxide (DMSO), ketotifen, sorbitol, and 3-acetamidophenol (nonhepatotoxic compounds) were used as negative controls (Table 1).

Culture of HepG2 cells. HepG2 cells were cultured in Ham's F-12/Leibovitz L-15 (1:1 vol/vol) (supplemented with 7% newborn calf serum, 50 U penicillin/ml, and 50 μg streptomycin/ml). For subculturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA at 37°C.

Incubation with selected compounds. HepG2 cells were seeded at a density of 5000 cells per well in 96-well microtiter plates and were allowed to grow and equilibrate for 24 h. Then, cells were exposed for 3 and 24 h to four concentrations of chemicals (1, 10, 100, and 1000μM). When the 1000μM concentration could not be achieved because of the solubility limit in the culture medium, the highest possible concentration was used. Each experimental condition was assayed in triplicate wells. The stock solutions of compounds were prepared in DMSO and were conveniently diluted in the culture medium to obtain the desired final concentrations. The final DMSO concentration in the culture medium never exceeded 0.5% (vol/vol), and control cultures were treated with the same amount of solvent.

HCS assay: incubation of fluorescent probes and imaging. Drug-induced liver toxicity was determined by an HCS analysis, which included the following functional endpoints: cell viability, nuclear morphological changes, lipid peroxidation, alterations of mitochondrial membrane potential (MMP), and intracellular calcium concentration.

The Hoechst 33342 nucleic acid stain is a widely used cell-permeant nuclear counterstain that emits blue fluorescence when bound to double-stranded DNA (Crissman *et al.*, 1988). This dye allows a sensitive cell number determination by fluorescence microscopy and is often used to distinguish condensed pyknotic nuclei in apoptotic cells.

Cell viability was determined by PI exclusion. Nonviable cells with compromised membrane permeability were identified by their positive red fluorescence in the nuclei. This allows the direct quantification of cytotoxicity, as well as the exclusion of dead cells from the HCS analysis, thus restricting further functional determinations to the live-cell population in each sample (Juan *et al.*, 1994).

Mitochondrial activity was determined using TMRM, a lipophilic cationic fluorescent probe that freely crosses the plasma membrane and accumulates within mitochondria, depending on their membrane potential (Gomez-Lechon *et al.*, 2008).

Lipid peroxidation, as a measure of oxidative stress damage, can be detected with the lipophilic probe, BODIPY 665/676 dye. This probe exhibits a change in fluorescence after interaction with peroxyl radicals (Naguib, 2000).

Fluo-4 AM is a cell-permeant fluorophore that can be used to measure changes in cytosolic-free calcium concentrations. Fluo-4 AM passively diffuses across the plasma membrane and, once inside the cell, is hydrolyzed, causing the free Fluo-4 to be trapped in the cytosol. Unlike Fluo-3, Fluo-4 offers a brighter emission signal upon binding to Ca^{2+} (Lim *et al.*, 2006).

Following treatment, cells were simultaneously loaded with 1.5 μg/ml Hoechst 33342, 1.5 μg/ml PI, 75 ng/ml TMRM, 0.27 μg/ml Fluo-4 AM, and 1.5 μg/ml BODIPY 665/676. After 30 min of incubation at 37°C with the culture media containing the fluorescent probes, cells were imaged using the Scan^R system (Olympus, Germany), and the InCell 1000 Analyzer system (GE Healthcare) was also employed in some setup experiments. In order to acquire enough cells (> 500) for the analysis, nine fields per well were imaged. The 10× objective was used to collect images for the distinct fluorescence channels. Dyes were excited and their fluorescence was monitored at the excitation and emission wavelengths with appropriate filter settings. In the setting up of the procedure, exposure times were adjusted in order to avoid overlapping emission between different probes. The collected images were analyzed using the Scan^R analysis module that allows the simultaneous quantification of subcellular structures, which are stained by different fluorescent probes and measure the fluorescence intensity associated with predefined nuclear and cytoplasmic compartments.

HCS assay: image analysis. Background correction was applied to all the images before being quantified. Cell count and nuclear morphological alterations were assessed from the Hoechst 33342 staining. The nucleus was defined as the main object using an edge detection algorithm. In order to separate individual cells, segmentation was applied. Cell viability was determined by PI exclusion in the main object. All the measurements were restricted to alive cells. Lipid peroxidation was detected from BODIPY 665/676 fluorescence intensity in the cytoplasm. Cellular MMP was defined as the TMRM fluorescence intensity in punctuate cytosolic regions around the nucleus. Fluo-4 AM intensity was used to measure the changes in cytosolic-free calcium concentrations. An intensity algorithm with a fixed threshold was used to measure TMRM, Fluo-4 AM, and BODIPY fluorescence. Each measure was performed in single cells; the values for the same treatment (e.g., triplicate wells) were averaged and then normalized by the average value from the solvent-treated cells.

TABLE 1
Compounds Selected According to Their Documented Mechanism of Action

Compounds	MW	CAS number	Mechanism of toxicity	References
17a-Ethynylestradiol	296.4	57-63-6	BA, MI	(1, 2)
2,4-Dinitrophenol	184.11	51-28-5	MI	(3, 4)
2-Nitrofluorene	211.22	607-57-8	BA, DN	(5–8)
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	210.25	86270-92-0	BA, DN	(9–11)
Acetaminophen or paracetamol	151.16	103-90-2	AP, BA, OS	(12–19)
Acetylsalicylic acid	330.4	50-78-2	MI, OS	(20–24)
Aflatoxin B1	312.27	1162-65-8	AP, BA, DN, MI	(25–29)
Amikacin	621.63	37517-28-5		(30–32)
Amiodarone HCl	681.8	19774-82-4	MI	(33, 34)
Amitriptyline-(amitriptyline hydrochloride)	313.86	549-18-8	MI, OS	(35–38)
Atropine sulfate	151.2	5908-99-6	BA	(39)
Azathioprine	277.26	446-86-6	MI, OS	(40–44)
Bupropion hydrochloride	176.2	31677-93-7	AP, BA, OS	(45, 46)
Captopril	217.29	62571-86-2	AP	(47–50)
Carbamazepine	780.9	298-46-4	BA, MI	(38, 51–56)
Chloramphenicol	323.13	56-75-7	BA, MI, OS	(57–61)
Chloroquine	515.86	50-63-5	AP, MI, OS	(62–67)
Chlorpromazine	355.33	69-09-0	MI	(68)
Clozapine	326.82	5786-21-0	BA, OS	(69–72)
Colchicine	288.4	64-86-8	OS	(73)
Cumene hydroperoxide	152.19	80-15-9	DN, OS	(74, 75)
Cycloheximide	284.7	66-81-9	AP	(76–78)
Cyclophosphamide monohydrate	279.1	6055-19-2	BA, DN	(14, 17, 26, 55, 79, 80)
Cyclosporine A	1202.61	59865-13-3	MI	(81, 82)
Diclofenac	318.13	15307-79-6	AP, BA, MI	(14, 17, 33, 83, 84)
Didanoside	236.23	69655-05-6	MI	(23, 85, 86)
Digoxin	399.5	20830-75-5	MI	(87)
Doxycycline	512.94	24390-14-5	AP, MI	(88–90)
Erythromycin	733.93	114-07-8	AP, OS	(91–94)
Etoposide	588.56	33419-42-0	AP	(95–97)
Fenofibrate	360.83	49562-28-9	AP, MI	(98, 99)
Fialuridine	372.1	69123-98-4	MI	(33, 100, 101)
Fluoxetin	345.79	56296-78-7	OS	(102)
Flutamide	276.21	13311-84-7	BA, MI	(103)
Galactosamine	281.22	157297-00-2	AP	(104–106)
Glycochenodeoxycholate	471.61	16564-43-5	AP, MI, CA	(107–110)
Imipramine hydrochloride	316.87	113-52-0	AP, MI	(111–114)
Isoniazid	137.14	54-85-3	BA, MI, OS	(33, 115–118)
Lovastatin	404.54	75330-75-5	DN, OS, CA	(119–122)
Malathion	180.2	121-75-5	OS	(123–126)
Maprotiline hydrochloride	313.86	10347-81-6	CA, MI	(127–134)
Menadione	172.18	58-27-5	AP, BA, MI, OS	(135–140)
Mercury II chloride	271.5	7487-94-7	AP, MI, OS	(141–146)
Methotrexate	454.44	59-05-2	DN, OS	(14, 147)
Orphenadrine hydrochoride	305.8	341-69-5	BA	(148, 149)
Paraquat	257.16	1910-42-5	OS	(50, 150–152)
Pentachlorophenol	694.8	87-86-5	BA, OS	(153–157)
Phenobarbital	194.2	50-06-6	MI	(56, 158, 159)
Phenytoin	252.27	57-41-0	BA, MI	(14, 23, 160, 161)
Piperonyl butoxide	338.44	51-03-6	AP, DN	(162–164)
Rifampicine	823	13292-46-1	MI, OS	(165–167)
Rotenone	394.42	83-79-4	AP, MI	(168–171)
Simvastatin	418.57	79902-63-9	AP, MI, CA	(119, 172–175)
Sodium valproate	60.1	1069-66-5	BA, MI, OS	(33)
Stavudine	224.21	3056-17-5	MI	(33, 176)
Tamoxifen	371.51	10540-29-1	CA, MI, OS	(38, 177–180)
Taurolithocholate	505.69	6042-32-6	AP, CA, OS	(181–183)

TABLE 1—*Continued*

Compounds	MW	CAS number	Mechanism of toxicity	References
t-Butyl hydroperoxide	90.12	75-91-2	OS	(184–186)
Tetracycline hydrochloride	480.9	64-75-5	MI	(19, 23, 187)
Thioacetamide	75.13	62-55-5	AP, BA, OS	(188–191)
Ticlopidine	300.25	53885-35-1	BA, OS	(103, 192)
Tilorone	483.47	27591-69-1	OS	(193, 194)
Troglitazone	441.54	97322-87-7	BA, CA	(14, 18, 195–198)
Verapamil hydrochloride (±)	491.1	152-11-4	BA	(199, 200)
Warfarin	308.33	81-81-2	MI	(15, 201–203)
Zidovudine (Retrovir)	10 mg/ml	399024-19-2	MI, OS	(204–206)
Nonhepatotoxic compounds				
3-Acetamidophenol	151.16	621-42-1		(15, 207)
Acetylcysteine	163.19	616-91-1		(15)
Ascorbate	198.11	134-03-2		(15, 207)
Betaine	422.62	19223-55-3		(15)
Caffeine	281.4	58-08-2		(15, 208)
Citrate (trisodium dihydrate)	294.1	6132-04-3		(15, 209)
Dexamethasone	392.46	50-02-2		(15, 207)
DMSO	78.13	67-68-5		(15)
Gentamicin	50 mg/ml	1405-41-0		(209, 210)
Ketotifen	4255	34580-14-8		(15, 207, 209)
Lactose	342.3	63-42-3		(15)
Sorbitol	182.17	50-70-4		(15, 207)

Note. AP, apoptosis; BA, bioactivation; CA, calcium homeostasis; CAS, chemical abstracts service; DN, DNA synthesis; MI, mitochondrial impairment; MW, molecular weight; OS, oxidative stress. Bibliographic references in supplementary data.

Analysis of HCS data. The minimal effective concentration (MEC) was defined as the lowest concentration to produce a significant change ($p \leq 0.05$) in all the parameters analyzed when compared with the control (solvent-treated cells). For all the compounds and studied parameters, a variation of at least 20% in fluorescence intensity or in the corresponding morphological parameter in relation to untreated cultures was considered. It was also defined the toxicity risk (TR) for each compound as the MEC/peak concentration in plasma (C_{max}) ratio at 3 and 24 h treatment.

In order to compare the degree of injury, the level of change for each parameter at 3 and 24 h was studied isomolarly (100μM). Four different scores of damage were established according to the level of variation when compared with control cells: 0 (no variation or variation lower than 20%); 1 (variation ± 20–40%); 2 (variation ± 40–60%); and 3 (variation ± 60–100%). Because calcium levels changed within a different range, other levels were established (for more information, see the legend of Supplementary table 1). Significant differences were seen among the different levels of toxicity ($p \leq 0.05$). The sum of each individual score resulted in the total level of toxicity for each compound and was defined as its degree of injury. From this calculation, an injury scale was established: high (> 15), moderate (6–15), low (1–5), and no injury. When this first analysis resulted in a value equal to 0 (nontoxic), a subsequent analysis was performed at the highest concentration (1000μM or the maximum concentration permitted by the solubility limit in the culture medium). From these data, new information about the degree of injury was obtained.

Assessment of predictivity. Specificity was defined as the proportion of nontoxic drugs testing negative defined as TN/(TN + FP) where TN is the number of nontoxic drugs testing negative, and FP is the number of nontoxic drugs testing positive. Sensitivity was calculated as the proportion of toxic drugs testing positive TP/(TP + FN) where TP is the number of toxic compounds testing positive, and FN is the number of toxic drugs testing negative.

Statistical analysis. Each experimental procedure was performed in at least three independent cell preparations. The statistical analysis of the data was performed using ANOVA followed by Bonferroni or Student's *t*-test. A *p* value below 0.05 was considered statistically significant.

RESULTS

HCS Assessment of Drug-Induced Hepatotoxicity

The assay done for the screening of drug-induced hepatotoxicity and the implicated mechanisms is summarized in Figure 1. Up to 50 different parameters can be measured simultaneously in a single well using our HCS approach by multiplexing five different dyes. After an initial analysis, the best parameters covering a wide spectrum of the mechanisms implicated in drug-induced hepatotoxicity were chosen. The final selected parameters were: cell viability (obtained from the calculation of the number of live cells under each condition), morphological nuclear changes (obtained by integrating the nuclear texture and compactness data), MMP (obtained from TMRM fluorescence), the intracellular calcium concentration (calculated from Fluo-4 AM fluorescence), and oxidative stress (estimated as the lipid peroxidation obtained from the quantification of BODIPY 665/676 fluorescence). Then, 78 compounds were studied at four concentrations (1, 10, 100, and 1000μM) after two incubation periods (3 and 24 h).

Drugs Classification According to Their Mechanism of Action

Representative images of untreated cells (control) and those cells treated with known hepatotoxins (amiodarone, flutamide, and tert-butyl hydroperoxide) are shown in Figure 2. All the compounds were assessed using the HCS approach over 3- and 24-h exposure periods. The MEC values obtained for each cell

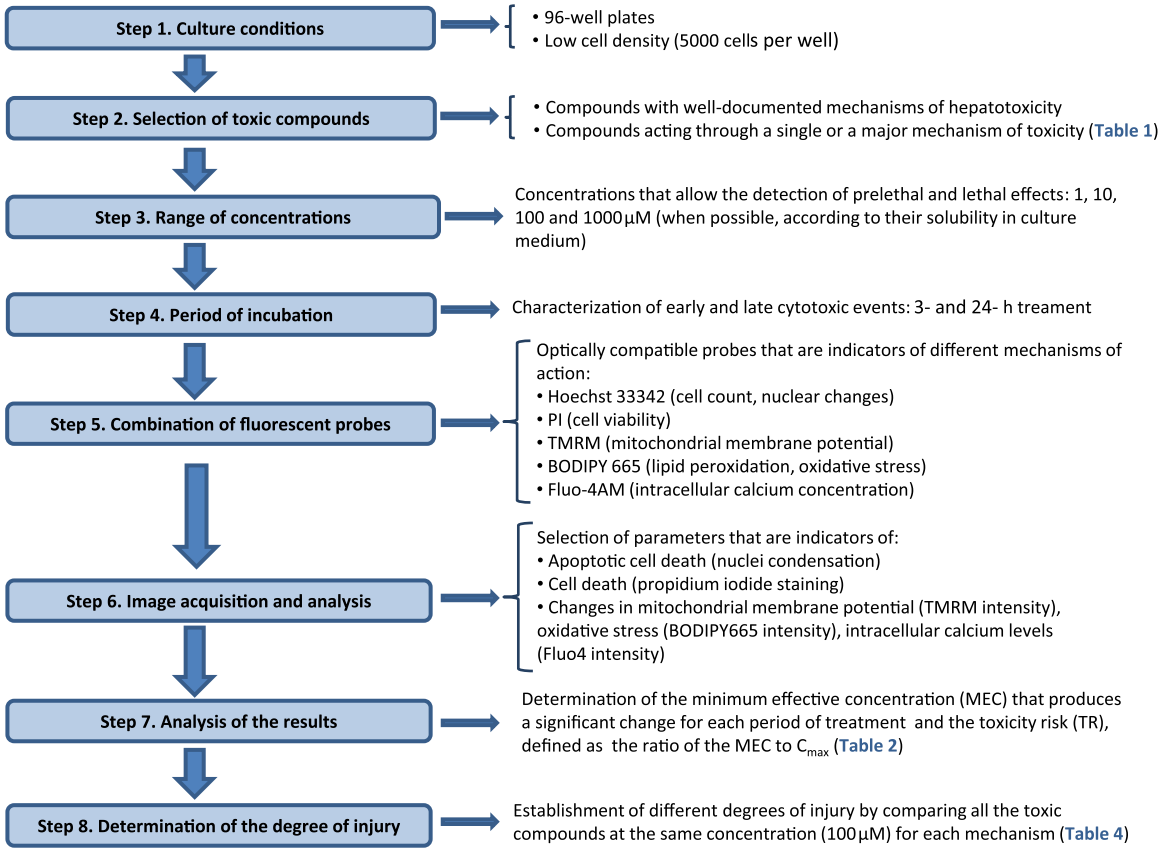

FIG. 1. Scheme of the different steps of the HCS strategy for the initial screening of hepatotoxic compounds. The different development phases of the HCS system with the definition of the considered criteria and final steps.

mechanism parameter and each incubation period are represented in Table 2. Thus, by using a multiparameter approach, a greater insight into the mechanistic action of a compound can be gained over single parameter assays. According to the score system created from the MEC, the main mechanism implicated in toxicity for each compound may be outlined. Representative dose responses in different parameters are shown in Figure 3. Additionally, the TR for each compound and period of incubation were calculated when information from C_{max} was available (Table 2).

Mitochondrial impairment was observed after treatment with several compounds. Both hyperpolarization (i.e., sodium valproate) (Bachmann *et al.*, 2009) and depolarization (i.e., rotenone, amiodarone) (Donato *et al.*, 2012) were observed in accordance with the drug studied and the chemical concentration. The most sensitive effects were observed after rotenone and tamoxifen (Isenberg and Klaunig, 2000; Tuquet *et al.*, 2000) treatment because already they showed a significant decrease in TMRM fluorescence at 1µM and 3 h of treatment.

On the other hand, increased lipid peroxidation, as a measure of oxidative stress, was observed when HepG2 cells were exposed to those compounds that are known to produce oxidative stress (i.e., tamoxifen or chloroquine) (Jamshidzadeh *et al.*, 2007; Lee *et al.*, 2000) after 3 and 24 h of treatment.

Calcium homeostasis alters in response to many cellular injuries. Although it is not specific of a particular mechanism, elevation of intracellular calcium is an indicator of deleterious effects on cells (Dong *et al.*, 2006; Gao *et al.*, 2006; Kedderis, 1996). For this reason, it was studied and significant alterations were observed after the treatment of several drugs. At the lowest dose, tamoxifen and lovastatin brought about a significant increase in intracellular calcium concentration. Regarding nuclear changes, etoposide and captopril, known to produce apoptotic cell death (De la Iglesia Inigo *et al.*, 2009; Ohtsu *et al.*, 1998), induced significant nuclear morphological alterations both after 3 and 24 h treatment.

The mechanism detected at the lowest concentration was defined as the main mechanism (bold, denoted in Table 2). Our findings are in agreement with the mechanism of hepatotoxicity reported in the literature (endpoints reported in the literature were italicized in Table 2), although additional mechanisms were also detected.

Predictivity of the HCS Assay

The specificity of the HCS test was 92% because 11 of the 12 tested nonhepatotoxic drugs (which included nontoxic and nonhepatotoxic compounds) assessed did not produce significant changes in any of the parameters studied.

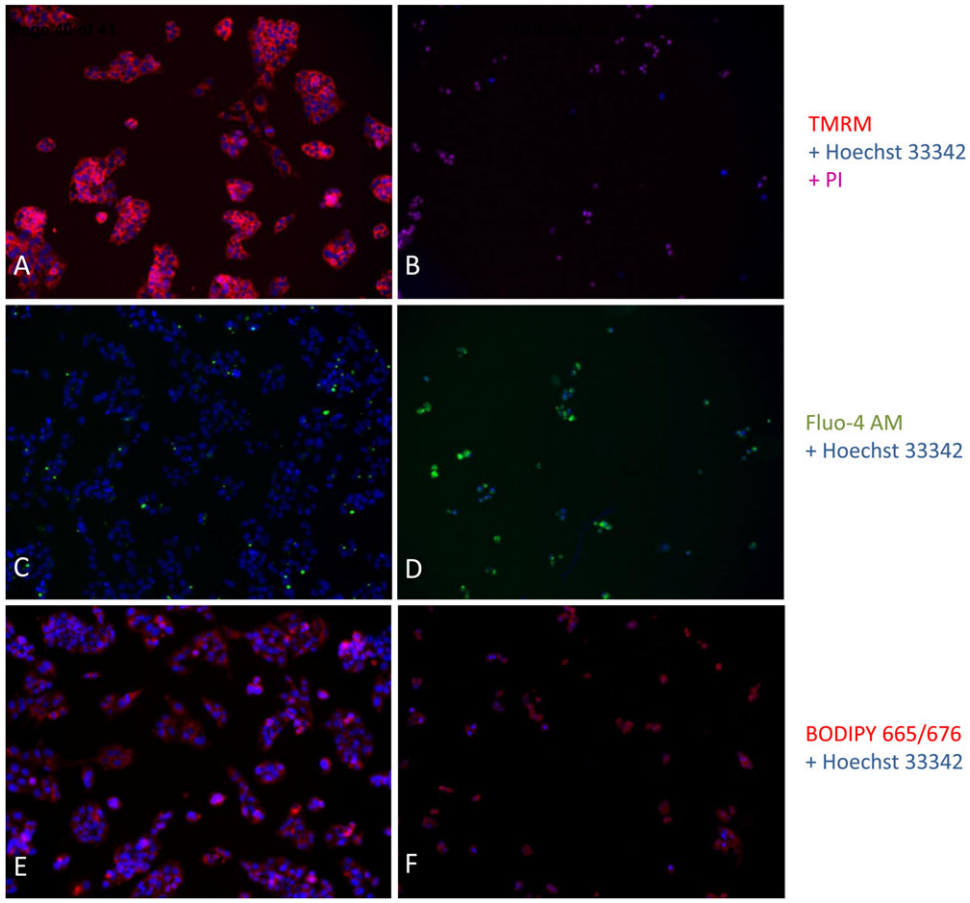

FIG. 2. Representative images of the HCS analysis for the prediction of drug-induced hepatotoxicity. Vehicle-treated (A, C, and E), exposed to 100μM of amiodarone (B) or flutamide (D) or tert-butyl hydroperoxide (t-BHP; F), HepG2 cells are shown. Fluorescence of TMRM (to detect changes in MMP, red) (A, B) or Fluo-4 AM fluorescence (to study alterations in intracellular calcium concentration, green) (C, D) or BODIPY 665/676 staining (to detect lipid peroxidation as a measure of oxidative stress) (E, F) is exemplified. Nuclei were identified by Hoechst 33342 staining (blue) in all the images. Amiodarone caused significant mitochondrial depolarization, flutamide led to an increase in the intracellular calcium concentration, and t-BHP induced significant oxidative stress. Untreated cells (control) showed no significant changes.

The sensitivity for the detection of changes in MMP, oxidative stress, intracellular calcium concentration, and nuclear changes was 83, 97, 100, and 63%, respectively. If at least one of the mechanisms implicated in compound-induced toxicity was considered, a sensitivity of 90% was obtained. Moreover, without considering a specific mechanism, only significant changes in any of the studied parameters, the value was 94% (Table 3).

Determination of Degree of Injury

In order to compare isomolarly the degree of injury of all the test compounds, we compared the different effects at 100μM after 3 and 24 h of incubation. Statistically different scores were created according to the level of the alteration (the group formation criterion is described in the Materials and Methods section), and then a value was assigned to each parameter (Supplementary table 1). The addition of all the values for each parameter and incubation period was calculated, and four distinct groups were created according to their level of damage. From this analysis, the degree of injury for each compound studied was defined (Table 4). Thus, for example, tamoxifen can be defined as producing a severe injury, whereas aflatoxin B1 may be considered to produce a moderate damage, and chloramphenicol causes low injury.

Twenty-six compounds scored 0 on the toxicity scale obtained after studying the effects at 100μM. For these compounds, we also analyzed effects at a higher concentration (up to 1000μM) to determine whether they produce any alteration and the level of this change (Supplementary table 2). Eleven of them showed toxic effects at this concentration and were also defined as toxic compounds, although their level of injury was assumed to be lower than the other compounds because they produced significant toxic effects at 1000μM, but not at 100μM (Table 5).

DISCUSSION

The pharmaceutical industry is strongly interested in establishing screening systems and mechanistic models to

TABLE 2
Cytotoxic Effects of Tested Compounds: Minimal Effective Concentration and Toxicity Risk

	3-h Treatment					24-h Treatment							
Compounds	V	NC	MMP	OS	CA	V	NC	MMP	OS	CA	C_{max} (μM)	TR 3 h	TR 24 h
17a-Ethynylestradiol	—	—	—	—	**250**	100	—	***100***	**100**	**100**	0.001	370,500	148,200
2,4-Dinitrophenol	—	**250**	***250***	**250**	—	250	250	—	**100**	—	NA	NA	NA
2-Nitrofluorene	—	*1000*	—	**100**	**100**	—	***1000***	—	—	**1000**	NA	NA	NA
3-Acetamidophenol	—	—	—	—	—	—	—	—	—	—	NA	NA	NA
Acetaminophen	—	—	—	—	—	1000	—	—	***1000***	—	130	> 7.69	7.69
Acetylcysteine	—	—	—	—	—	—	—	—	—	—	1900	> 0.52	> 0.52
Acetylsalicylic acid	—	—	—	—	—	1000	—	—	***1***	1000	1650	> 0.61	0.00061
Aflatoxin B1	—	—	—	—	**100**	1	***1***	*500*	**1**	100	0.000003	33,184,910	331,849
Amikacin	—	—	—	—	—	—	—	—	—	—	34.30	> 29.15	> 29.15
Amiodarone HCl	10	1000	***10***	**10**	**10**	10	100	***10***	**10**	**10**	2.20	4.55	4.55
Amitriptyline	1000	—	*1000*	*1000*	**100**	1000	—	***1***	*1000*	**1**	0.19	526.32	5.26
Ascorbic acid	—	—	—	—	—	—	—	—	—	—	NA	NA	NA
Atropine sulfate	—	—	—	**1000**	**1000**	100	—	—	**1000**	**1000**	NA	NA	NA
Azathioprine	1000	—	—	—	**100**	1000	**1000**	***1000***	***1000***	**1000**	0.34	294	2941
Betaine	—	—	—	—	—	—	—	—	—	—	940	> 1.06	> 1.06
Bupropion	—	—	—	—	**1000**	1000	—	—	***1000***	**1000**	0.50	2000	2000
Caffeine	—	—	—	—	—	—	—	—	—	—	42	> 23.81	> 23.81
Captopril	—	***1***	—	—	—	—	***1***	1000	—	—	4.00	0.25	0.25
Carbamazepine	—	—	—	**1000**	**1000**	—	**1**	***1***	—	—	5.28	189.54	0.19
Chloramphenicol	—	—	—	—	—	100	—	—	***100***	—	57	> 17.54	1.75
Chloroquine	100	—	*100*	***10***	100	10	*100*	***10***	*100*	**10**	0.48	20.83	20.83
Chlorpromazine	100	—	***100***	**100**	**100**	1	—	*100*	**1**	10	0.50	200	2
Citrate	—	—	—	—	—	—	—	—	—	—	NA	NA	NA
Clozapine	300	—	300	*300*	**100**	100	—	**100**	***100***	**100**	1.1	91.74	91.74
Colchicine	—	1	100	—	—	1	**1**	**1**	***1***	**1**	0.02	62.5	62.5
Cumene hydroperoxide	100	—	1000	***100***	**100**	100	—	**100**	***100***	**100**	NA	NA	NA
Cycloheximide	—	—	—	—	**1000**	1	***1***	—	**1**	**1**	NA	NA	NA
Cyclophosphamide	—	***1000***	—	—	—	—	—	—	—	—	143	6.99	> 6.99
Cyclosporine A	—	—	***100***	200	200	10	—	***1***	10	10	0.20	500	5
Dexamethasone	—	—	—	—	—	—	—	—	—	—	0.23	> 4347	> 4347
Diclofenac	—	***1000***	—	1000	—	1000	—	—	**1000**	**1000**	4.20	238.10	238.10
Didanoside	—	—	—	—	—	—	—	***10***	1000	100	9.23	> 108	1.08
Digoxin	—	**1**	—	—	500	1	—	***1***	**1**	**1**	0.005	189.79	189.79
DMSO	—	—	—	—	—	—	—	—	—	—	< 1000	> 1	> 1
Doxycycline	—	—	***1000***	—	**1000**	—	—	***1000***	**1000**	**1000**	8.77	114.03	114.03
Erythromycin	—	—	1000	—	**100**	10	***100***	—	***100***	**100**	11	9.09	0.91
Etoposide	1000	***1***	1000	1000	1000	1	***1***	1000	**1**	100	17	0.06	0.06
Fenofibrate	1000	—	**1000**	**1000**	—	10	*1000*	*1000*	**10**	1000	25	40	040
Fialuridine	—	—	**500**	—	—	500	—	***100***	500	500	1	500	100
Fluoxetin	100	100	100	*100*	**10**	10	100	100	***10***	100	0.93	10.75	10.75
Flutamide	—	100	*1000*	—	**10**	10	—	***100***	**100**	**100**	6	1.67	1.67
Galactosamine	—	—	—	—	—	—	—	—	—	—	NA	NA	NA
Gentamicin	—	—	—	—	—	—	—	—	—	—	83.3	> 12	> 12
Glycochenodeoxycholate	—	—	—	—	—	—	—	—	***100***	*1000*	NA	NA	NA
Imipramine	100	*1000*	***100***	**100**	**100**	1	***10***	*100*	100	**10**	0.47	212.77	2.13
Isoniazid	—	—	***100***	*1000*	—	—	—	—	—	—	40	2.5	> 25
Ketotifen	—	—	—	—	—	1000	—	—	—	**1000**	0.0001	NA	NA
Lactose	—	—	—	—	—	—	—	—	—	—	NA	NA	NA
Lovastatin	—	—	—	—	**1**	1	*100*	100	***1***	**1**	0.01	100	100
Malathion	—	—	—	—	—	1000	—	—	***1000***	—	NA	NA	NA
Maprotiline	10	—	***10***	1000	***10***	10	—	***1***	10	*10*	0.16	62.64	6.26
Menadione	100	***10***	*1000*	*1000*	100	100	—	*100*	*100*	**10**	4.65	2.15	2.15
Mercury II chloride	1000	***1000***	***1000***	***1000***	**1000**	100	***1000***	***1000***	***1000***	**1000**	NA	NA	NA
Methotrexate	1000	—	—	**1000**	—	1000	—	—	***1000***	—	0.40	2500	2500
NNK	—	—	—	—	—	—	—	—	—	**100**	NA	NA	NA
Orphenadrine	1000	—	**1000**	**1000**	**1000**	1000	**10**	1000	1000	100	0.49	2038.67	20.39
Paraquat	1000	—	—	***1000***	—	10	100	10	*100*	**1**	NA	NA	NA
Pentachlorophenol	—	**1000**	—	—	**1000**	1000	1000	1000	***10***	1000	NA	NA	NA
Phenobarbital	—	—	—	—	—	—	—	—	—	**1000**	174	> 5.75	5.75
Phenytoin	500	—	—	—	—	500	—	***500***	—	—	5.48	91.24	91.24

TABLE 2—*Continued*

Compounds	3-h Treatment					24-h Treatment					C_{max} (μM)	TR 3 h	TR 24 h
	V	NC	MMP	OS	CA	V	NC	MMP	OS	CA			
Piperonylbutoxide	100	*1000*	—	**100**	1000	100	***1000***	**1000**	**1000**	**1000**	NA	NA	NA
Rifampicine	—	—	—	—	**250**	250	—	*250*	***100***	250	9.30	26.90	10.76
Rotenone	—	*500*	***1***	500	10	1	***1***	***1***	10	10	50	0.02	0.02
Simvastatin	500	—	***100***	500	**100**	10	*100*	*10*	10	**1**	0.02	5000	50
Sodium valproate	—	—	—	—	—	1000	—	***1***	*1000*	—	481	> 2.08	0.0021
Sorbitol	—	—	—	—	—	—	—	—	—	—	NA	NA	NA
Stavudine	—	—	—	—	—	—	—	—	—	—	3.62	> 276	> 276
Tamoxifen	**1**	—	***1***	***1***	**1**	10	—	*10*	*10*	***1***	0.27	3.70	3.70
Taurolithocholate	—	—	**100**	***100***	*1000*	—	—	—	—	***1000***	NA	NA	NA
Tert-butyl hydroperoxide	100	**100**	**100**	***100***	**100**	10	—	**10**	***10***	**10**	NA	NA	NA
Tetracycline HCl	—	—	—	**1000**	**1000**	—	—	*1000*	**100**	1000	14.20	70.42	7.04
Thioacetamide	—	—	—	—	—	—	—	—	—	—	NA	NA	NA
Ticlopidine	1000	**1000**	**1000**	***1000***	**1000**	1	100	1000	***1***	1000	7.09	140.96	0.14
Tilorone	250	—	—	***250***	**250**	100	—	—	***100***	**100**	NA	NA	NA
Troglitazone	—	—	—	—	***300***	—	—	**100**	—	***100***	6.39	46.97	15.66
Verapamil	—	—	**100**	1000	1000	1000	—	**1000**	—	**1000**	0.44	227.27	2273
Warfarin	—	—	***1000***	—	—	—	—	—	—	—	7.00	142.86	> 142
Zidovudine	350	—	—	—	—	350	—	***100***	***350***	**350**	4.77	73.38	20.96

Note. CA, intracellular calcium concentration; NA, not applicable; NC, nuclear changes; OS, oxidative stress; V, viability. In bold the MEC that indicates a significant change. Italicized text highlights those endpoints that were described in the literature for each compound.

detect hepatotoxicity early in the drug development process (Suter, 2006). In order to cut development costs, it is essential that safety of new compounds is assessed early, in a short time and at a reasonable cost. Single endpoint toxicity assays may not provide adequate information for selecting compounds. When they are applied to early toxicity screening, there is a real probability of false positive or negative results being high. However, when two or more endpoints are monitored at several exposure concentrations, the data obtained are considerably more reliable. In fact, it has been previously demonstrated that conventional cytotoxicity assays are sometimes in poor concordance with human toxicity, show low sensitivity to detect adverse cellular effects, and provide a modest mechanistic understanding of toxicity effects (Abraham *et al*., 2008;

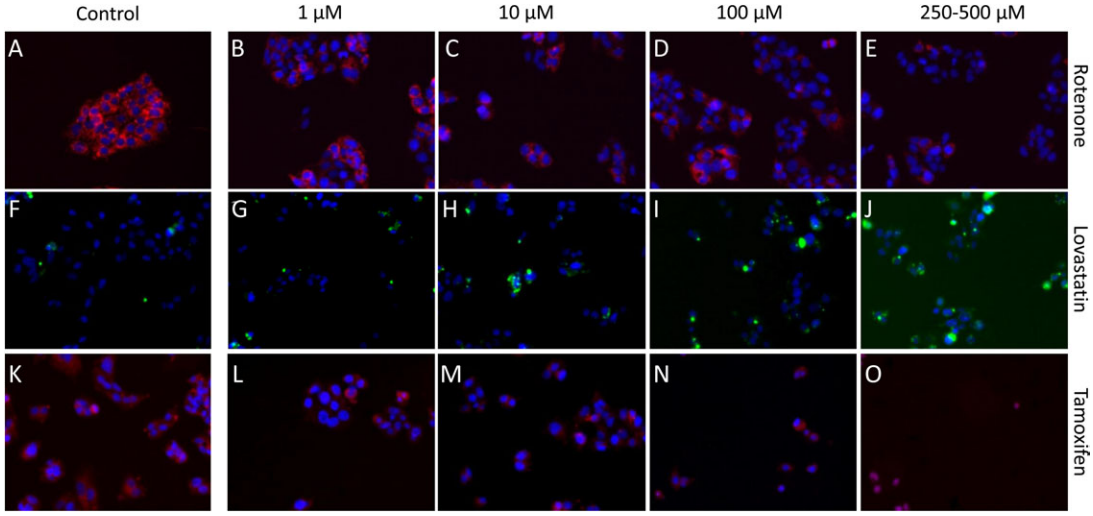

FIG. 3. Representative images of the dose-response effects of compounds assessed by HCS. The vehicle-treated cells (A, F, and K), or exposed for 3 h to rotenone (B–E), lovastatin (G–J), or tamoxifen (L–O), are shown. Nuclei were detected by Hoechst 33342 (blue) staining in all the images. Rotenone produced a dose-response decrease in TMRM fluorescence (red), whereas lovastatin induced an increase in the intracellular calcium levels detected by the increase in Fluo-4 AM fluorescence intensity (green). A dose-dependent reduction in BODIPY 665/676 fluorescence, indicative of oxidative stress induction, was observed after treatment with tamoxifen (red). The maximum concentration for tamoxifen and rotenone was 500 and 250μM for lovastatin.

TABLE 3
Sensitivity of the HCS Test

Predictive mechanism of toxicity	Sensitivity
General hepatotoxicity	94
*At least one mechanism	90
Nuclear changes	63
MMP	83
Oxidative stress	97
Intracellular calcium concentration	100

Note. Sensitivity was calculated as TP/(TP + FN) for each mechanism. *It was considered positive when at least one of the reference mechanisms for a compound was detected. General hepatotoxicity considered significant changes in any of the studied parameters without taking into account the agreement with the reference.

O'Brien *et al*., 2006). Conversely, those methods that provide an early assessment of specific toxicological mechanisms before the onset of the late stages of nonspecific cell death should, theoretically, have greater predictive power (Cosgrove *et al*., 2009; Dykens *et al*., 2008; Noor *et al*., 2009; Xu *et al*., 2008). Additionally, throughput is an important consideration when assessing the practicality of an assay to be used for screening compounds in drug discovery. By combining a well-defined model (human liver–derived cell-proliferating HepG2) and high-content imaging technology, we have developed an *in vitro* testing approach that is capable of identifying many hepatotoxicants and outlining the mechanisms implicated in their toxicity. One of the main advantages of the system is its speed (single incubation with appropriately combined fluorescent probes) and the use of attached cells, which enables the study of morphological changes.

TABLE 4
Degree of Injury After Analyzing Data of 100μM for Each Parameter, Compound, and Period of Incubation

Degree of injury	Compounds
No injury	3-Acetamidophenol, acetaminophen, acetylcysteine, amikacin, ascorbic acid, betaine, bupropion, caffeine, citrate, cyclophosphamide, dexamethasone, DMSO, diclofenac, doxycycline, galactosamine, gentamicin, ketotifen, lactose, malathion, methotrexate, phenobarbital, phenytoin, sorbitol, stavudine, thioacetamide, and warfarin
Low injury	2,4-Dinitrophenol, 2-nitrofluorene, acetylsalicylic acid, amitriptyline, atropine sulfate, azathioprine, captopril, carbamazepine, chloramphenicol, didanoside, fenofibrate, fialuridine, glycochenodeoxycholate, isoniazid, mercury chloride II, NNK, orphenadrine, pentachlorophenol, rifampicine, sodium valproate, taurolithocholate, tetracycline HCl, ticlopidine, troglitazone, verapamil, and zidovudine
Moderate injury	17a-Ethinylestradiol, aflatoxin B1, clozapine, colchicine, cumene hydroperoxide, cycloheximide, cyclosporine A, digoxin, erythromycin, etoposide, flutamide, lovastatin, menadione, paraquat, piperonyl butoxide, simvastatin, and tilorone
High injury	Amiodarone, chloroquine, chlorpromazine, fluoxetin, imipramine, maprotiline, rotenone, tamoxifen, and t-butyl hydroperoxide

TABLE 5
Degree of Injury at 1000μM of Compounds That Did Not Exhibit Toxicological Effects at 100μM

Degree of injury	Compounds
No injury	3-Acetamidophenol, acetylcysteine, amikacin, ascorbic acid, betaine, caffeine, citrate, dexamethasone, DMSO, galactosamine, gentamicin, lactose, sorbitol, stavudine, and thioacetamide
Low injury	Acetaminophen, cyclophosphamide, malathion, ketotifen, methotrexate, phenobarbital, phenytoin, and warfarin
Moderate injury	Bupropion, diclofenac, and doxycycline

We first developed a database of drugs and chemicals with their mechanisms of action (Table 1). The compounds were representative of the different mechanisms implicated in early cytotoxicity, such as mitochondrial dysfunction, oxidative stress, or apoptotic cell death. Thus, optically compatible probes were selected to cover a wide range of mechanisms.

While developing our toxicity assay, four fixed concentrations and two incubation periods were selected. The concentrations were established to cover a wide range that would allow detecting significant changes induced by drugs. In fact, at 100μM, the 79% of the drugs described as hepatotoxic were detected, whereas at the highest concentration, the 94% of them were identified as toxic. We are aware that the IC50s of the studied compounds vary considerably; however, our aim was to create a standardized system for the preclinical screening of new compounds for which, obviously, no toxicity information is available; thus, a fixed range of concentrations would enable us to compare an unknown drug with well-known compounds on the basis of its degree of injury. Moreover, the TR, defined as the MEC/C_{max} ratio, should help to understand the significance of the cytotoxic signals (O'Brien *et al*., 2006). Even though cytotoxicity assays cannot predict unequivocally human hepatotoxicity, the knowledge derived from mechanism-based assays could facilitate the prioritization and/or the search of alternatives early in preclinical development. Moreover, for unknown drugs or industrial chemicals, the results of the HCS test and other considerations such as the

chemical category or chemical analogy could be used to define safe exposure limits.

Different mechanisms have been defined as being implicated in drug-induced hepatotoxicity, such as mitochondrial impairment, oxidative stress, and apoptotic or necrotic cell death (Park *et al*., 2005a; Russmann *et al*., 2009). Moreover, other mechanisms (steatosis, cholestasis, and phospholipidosis) have also been implicated in hepatotoxicity (Gomez-Lechon *et al*., 2010; Xu *et al*., 2004). Our system is a fast approach, which allows the detection of early events in the hepatotoxic process (Table 2). The different mechanisms involved in hepatotoxicity are highly related, and an initial cell alteration could trigger other events, all of them contributing to cell damage. Oxidative stress, imposed when reactive oxygen species (ROS) generation exceeds the antioxidant protection, is produced by compounds that undergo repeated oxidation/reduction cycles or that can either produce free radicals or are chemically activated by them (Jaeschke *et al*., 2002). An excess of ROS can damage lipids, proteins or DNA, thus inducing lipid peroxidation, altered Ca^{2+} homeostasis, or mitochondrial dysfunction (Labbe *et al*., 2008). Cytosolic-free Ca^{2+} plays a fundamental role in the control of membrane permeability, and mitochondrial Ca^{2+} influences mitochondrial respiration (Dong *et al*., 2006). Moreover, Ca^{2+} activates proteases and endonucleases, and enhances ROS formation, thus leading to necrosis or apoptosis (Grattagliano *et al*., 2009). The mitochondrion is a frequent target of hepatotoxic drugs with immediate effects on cell integrity and function (Kass, 2006). Loss in MMP can result in an imbalance of energy status; induce ROS formation (Grattagliano *et al*., 2009) and/or impairment in Ca^{2+} homeostasis (Dong *et al*., 2006). Moreover, the release of certain mitochondrial proteins is essential in the induction and execution of caspase-dependent and caspase-independent apoptosis (Kass, 2006).

The sensitivity of the multiparametric assay was calculated according to compounds' bibliographic information (Table 3). The test successfully detected changes in MMP, intracellular calcium concentration, and oxidative stress. DNA damaging agents and/or apoptotic compounds showed the lowest sensitivity (63%). However, if one considers compounds that only produce hepatotoxicity due to apoptosis there was a sensitivity of 100%. In this sense, additional panels or studies (e.g., caspase activation) could be added to the study.

Hepatotoxicity results obtained by applying our HCS system are supported by the literature. In fact, a global sensitivity of 90% was obtained if only at least one mechanism is considered; however, the assay failed to detect 10% of them. This could be partially due to either the cellular model's incomplete metabolic competence and/or the inability to detect chronic effects, such as cholestasis or steatosis. However, the fact that most bioactivable hepatotoxins were identified (i.e., aflatoxin B1, acetaminophen, or clozapine) argues against metabolic competence being significantly limiting (O'Brien *et al*., 2006). HepG2 cells have been extensively used for the prediction of toxicity (Schoonen *et al*., 2005a,b) because their human liver origin and the expression of relevant drug metabolism enzymes, including some phase II enzymes (Liu *et al*., 2009; Rodriguez-Antona *et al*., 2002). However, it is well known that HepG2 cells have low-CYPs levels (Aoyama *et al*., 2009; Donato *et al*., 2008; Hewitt and Hewitt, 2004; Wilkening *et al*., 2003). Therefore, alternative strategies, such as transfection with adenoviruses encoding for desirable CYPs (Aoyama *et al*., 2009; Tolosa *et al*., 2011; Vignati *et al*., 2005), addition of liver S9 fractions containing drug-metabolizing enzymes (Liu *et al*., 2009), and use of HepaRG cells (Pernelle *et al*., 2011) or primary hepatocytes (Xu *et al*., 2008), should be applied to further study hepatotoxicants that require biotransformation by CYPs. In spite of these limitations, the HepG2 cell line is suitable for a high-throughput strategy because they are an available and feasible model.

In order to understand the significance of the HCS results, the degree of change and the number of parameters affected should be considered. Although diverse compounds can impair a specific cell function, the level of this damage could significantly differ. For this reason, a score system to detect the distinct degrees of injury was firstly created by comparing all the compounds at 100μM and then using a higher concentration for those compounds that remained undetected after the first analysis. From these data, 92% specificity was achieved. 3-Acetamidophenol, acetylcysteine, ascorbic acid, betaine, caffeine, citrate, dexamethasone, DMSO, gentamicin, lactose, and sorbitol, previously defined as nonhepatotoxic (Donato *et al*., 2009; O'Brien *et al*., 2006; Takakusa *et al*., 2008; Xu *et al*., 2008), had no toxicity at 1000μM, thus confirming that they are suitable negative controls for use in an assay being applied to unknown chemicals. However, although ketotifen, reported as nontoxic compound (Donato *et al*., 2009), did not produce significant changes at 100μM, it was toxic at 1000μM which coincides with the results published by O'Brien *et al*. (2006). By applying this analysis, amikacin, previously reported as hepatotoxic, is also defined as a nontoxic compound; this could be due to the fact that amikacin is a phospholipidosic compound, and toxicity could be detected after a longer period. Because our system aims to detect early cytotoxic events, chronic toxicity should also be considered to determine the toxicity of a new drug, although another kind of analysis should be done (Cosgrove *et al*., 2009). On the other hand, we also identified galactosamine, stavudine, and thioacetamide as nontoxic compounds. Because these compounds have an IC50 > 1.5mM (Biagini *et al*., 2006; Donato *et al*., 2011; Wang *et al*., 2002), higher concentrations should be used to define their toxic potential. In this sense, a wider range of concentrations should be considered for those new compounds that may score 0 in our HCS test.

In summary, we have designed an HCS system that allows early simple screening of compounds to indicate both the mechanism(s) implicated in their toxicity and their degree of injury. The cellular imaging technology described herein is

mainly powerful for identifying the mechanisms implicated in hepatotoxicity and may be used as a prioritization tool in drug development. It seems to exhibit high specificity and can be adapted by using other cell types and/or other fluorescent probes.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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