3

High-Throughput Flow Cytometry for Predicting Drug-Induced Hepatotoxicity

Marion Zanese[1], Laura Suter[2], Adrian Roth[2],
Francesca De Giorgi[1] and François Ichas[1]
[1]*Fluofarma,*
[2]*F. Hoffmann-La Roche,*
[1]*France*
[2]*Switzerland*

1. Introduction

The development of a new drug is a long, expensive and complex process which aims to identify a pharmacologically-active low toxicity drug candidate. Large amount of resources and time are wasted if a drug fails in late stages of development or is withdrawn from the market because of toxicity. Hepatotoxicity in particular is a frequent cause for the failure of a drug to get approved, or for the withdrawal of already marketed medicines (Stevens & Baker, 2009). Current preclinical testing systems lack predictivity and need to be significantly improved in order to allow the identification of potentially hepatotoxic drug candidates, and the safety-based prioritization of compounds early in the development process. A cost-effective identification of compounds with potential liver liabilities in the initial preclinical phase of drug development would undoubtedly reduce the number of drug nonapprovals and withdrawals.

We present here the development of an optimized methodology for predicting drug-induced hepatotoxicity, which could be used early in the drug development process (e.g. during lead optimization), relying on the assessment of multiple cellular readouts by high-throughput flow cytometry.

This methodology is based on the measurement of key intracellular events reflecting the main cellular and metabolic changes occurring in hepatocytes in response to hepatotoxicant exposition (i.e. cytolysis, mitochondrial membrane depolarization, NAD(P)H depletion, ROS production, glutathione (GSH) depletion, and variations in lipid content). Each measurement was optimized in terms of robustness (reproducibility), sensitivity and dynamic range, and when possible multiplexed, in 3 hepatic cellular models: the HepG2 cell line, fresh rat hepatocytes and cryopreserved human hepatocytes.

The HepG2 cell line, derived from a human hepatoma, is probably the most utilized cell line in hepatotoxicological studies. HepG2 cells are attractive because they are cheap, easy to handle and generate reproducible results. However, they are known to have a reduced drug metabolizing activity compared to primary hepatocytes. Fresh primary rat hepatocytes provide a model of fully functional hepatocytes, well suited to flow cytometry experiments,

although the results obtained are not always relevant to human, due to species differences in metabolism and cell biology between rat and man. Primary human hepatocytes are considered the "gold standard" but the reduced viability of frozen human hepatocytes and the significant donor-to-donor variability both in terms of quality and metabolic activity, as well as their restricted availability constitute important limiting factors for their extensive use. Nevertheless, these biological systems with their inherent advantages and flaws are the methods of choice for *in vitro* assessment of hepatotoxicity. The high-throughput flow cytometry approach presented here is applicable to all these biological test systems.

2. Drug testing process

The entire drug testing process (from the seeding of the cells until the flow cytometry analysis) was performed in 96-well plates. Each step of this process was optimized. First the cell density and the medium composition were adjusted for each cell type in order to obtain a confluent monolayer of hepatocytes with high viability.

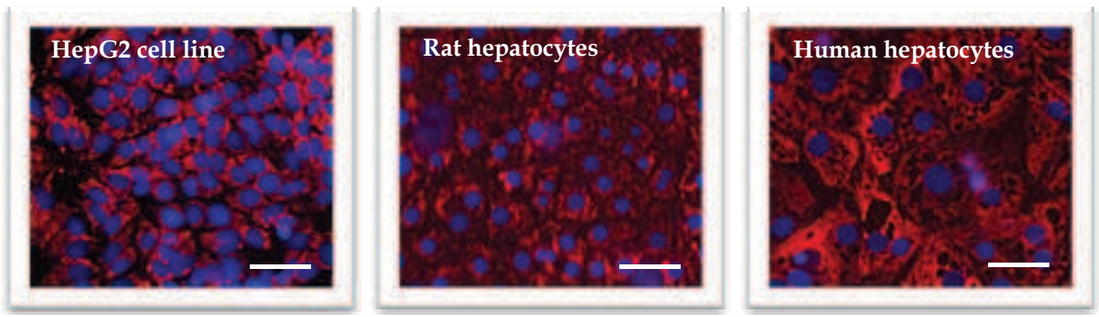


Fig. 1. Hepatic cellular models

HepG2 cells (*left*), freshly isolated primary rat hepatocytes (*middle*), and cryopreserved human hepatocytes (*right*) were observed 72 h post-seeding in 96-well plates. Nuclei (in blue) were stained with Hoechst 33342 and mitochondria (in red) of viable cells were stained with the potentiometric probe TMRM (scale bar = 50µm).

The cell detachment process was adapted to each cellular model and its innocuousness was checked by comparing flow cytometry results on suspended cells with imaging results on adherent cells.

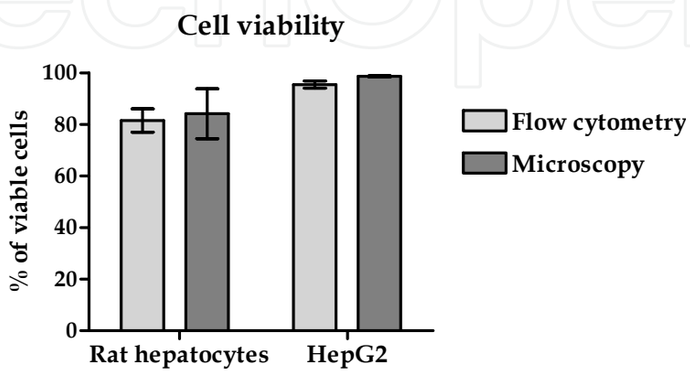


Fig. 2. Innocuousness of the cell detachment process

The cell viability of rat hepatocytes and HepG2 cells after 48 h in culture was assessed on adherent cells in microscopy and after cell detachment in flow cytometry (on suspended cells). Viable cells were identified by staining polarized mitochondria with a potentiometric probe. Results are expressed as the mean ± SD of 3 replicates.

In addition, several probes and staining protocols were tested for each functional assay in order to maximize the dynamic range (signal/noise ratio) of the measurement, and to ensure sufficient signal stability over time (compatible with flow cytometry analysis).

Flow cytometry experiments were performed using a special order BD LSRFortessa™ cell analyzer equipped with 3 excitation sources (355 nm, 488 nm, and 561 nm) and a high-throughput injection module capable of handling 96-well plates. In our customized configuration, the instrument allows the simultaneous detection of up to 10 colors. As the optical filters are removable and interchangeable, this flow cytometer has the flexibility to support a large variety of multicolor flow cytometry assays, which allowed us to multiplex most of the assays we developed. Analysis of flow cytometry outputs was performed using BD FACSDiva software. Typically a few thousands cells per well were analyzed, and the wells in which less than 100 cells could be analyzed were rejected.

Thanks to the extensive optimization of the entire drug testing process, we were able to develop robust and sensitive assays with low variability, in all 3 cellular models. This allowed us to generate accurate data by performing experiments in duplicate (2 replicates per experimental condition). Both the multiplexing of assays and the use of a reduced number of experimental replicates contribute to the high throughput of the assay, required for the rapid testing of numerous compounds in the early stage of drug development.

Although the functional assays we developed were multiplexed, they are presented separately for easier understanding.

3. Cell death measurements

Cytolysis is a conventional cytotoxicity indicator. Indeed, loss of plasma membrane integrity is a measure of cell death and quantitates a general cytotoxicity that is not necessarily unique to liver. In our experiments, cytolysis is assessed with a fluorescent high affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes but do not cross healthy cell membranes. It is thus very easy to distinguish fluorescent cytolytic cells from non-fluorescent viable cells in flow cytometry.

The loss of the mitochondrial inner transmembrane potential ($\Delta\psi$) maintained by the respiratory chain can be triggered by many different drug-induced toxic mechanisms, leading to cell death either by apoptosis or by necrosis.

Mitochondrial potential can be readily measured with fluorescent cationic dyes (rhodamines, carbocyanines, etc.). The accumulation of these amphiphilic cationic probes is dependent on the mitochondrial potential value, and thus cells with active mitochondria (high $\Delta\psi$) will brightly fluoresce whereas cells in which mitochondria are depolarized (low $\Delta\psi$) will be barely fluorescent.

Cytolysis and mitochondrial transmembrane potential measurements were optimized in the 3 cellular models using acetaminophen (APAP) as a model compound for drug-induced

hepatotoxicity. APAP is widely used for the treatment of pain and fever. Although it is safe and effective at therapeutic levels, the drug causes severe liver injury following overdosing (whether on purpose or by accident) with the potential to progress to liver failure (Tang, 2007). Partly because of its widespread use, APAP hepatotoxicity accounts for more than a third of drug-related acute liver failure cases in the US.

The results obtained in the mitochondrial depolarization assay after 24 h of treatment with acetaminophen are very similar to those obtained in the cytolysis assay. In rat hepatocytes, the EC_{50} is slightly lower in the mitochondrial assay compared to the cytolysis assay, and there is no significant difference between EC_{50} for mitochondrial depolarization and cytolysis in HepG2 cells or human hepatocytes.

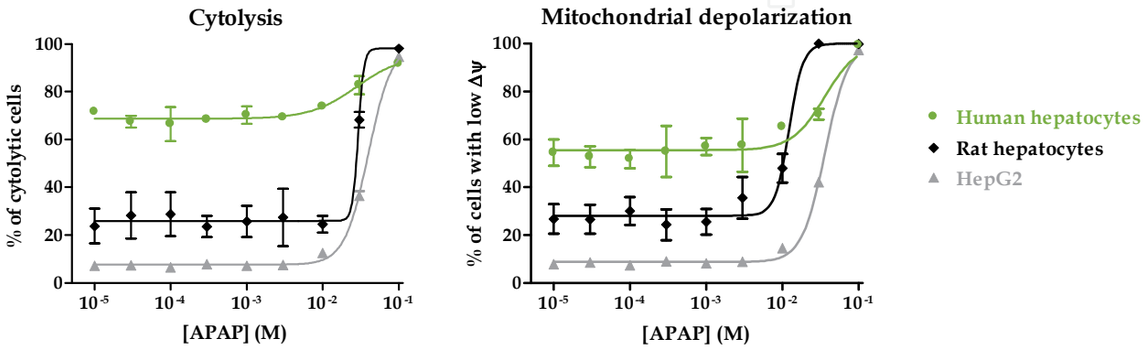


Fig. 3. Acetaminophen-induced cell death in hepatocytes

Primary human hepatocytes, primary rat hepatocytes and HepG2 cells were incubated for 24 h with increasing concentrations of acetaminophen. Cytolysis and mitochondrial membrane depolarization were measured in high-throughput flow cytometry with SYTOX Green and TMRM respectively. Results are expressed as the mean ± SD of 2 replicates. Sigmoidal curve fitting was performed with GraphPad Prism software.

The EC_{50} of the curves representing the percentage of non-viable cells after 24 h of treatment with acetaminophen are very similar from one cell type to another. The 3 cellular models are thus equally sensitive to acetaminophen cytotoxicity. In contrast the signal/noise ratio differs greatly between cellular models. This is the direct consequence of the differences in the cytolysis rate in basal (vehicle-treated) conditions: less than 10% in HepG2 cells, around 25% in primary rat hepatocytes and more than 60% in cryopreserved human hepatocytes. Note that the basal percentage of non viable cells after 48 h in culture (which determines the dynamic range of the cell death assays) varies substantially (from 30% to 80% in our tests) from one batch of cryopreserved human hepatocytes to another (not shown).

More than 80 other drugs were tested and gave similar results in the cytolysis assay and in the mitochondrial depolarization assay, confirming that both are direct indicators of cell death.

Regarding the relationship between *in vitro* cell death induction and clinically observed drug-induced hepatotoxicity, we show here the results obtained with two drugs which are considered non-hepatotoxic, namely metformin and entacapone (Fig. 4).

Metformin, an orally available biguanine derivative, is a cornerstone for the treatment of type 2 diabetes, in particular in overweight and obese people. Metformin is safe, cost effective and remains the first line of diabetes therapy with diet and exercise (Andujar-Plata et al., 2011). The drug may also be used in polycystic ovary syndrome (PCOS), non-alcoholic fatty liver disease (NAFLD) and premature puberty, three other conditions that feature insulin resistance. Although metformin mechanism of action is not well understood, it includes a decrease of hepatic insulin resistance and a change in bile acids metabolism. As expected for a safe drug, no toxicity of metformin was evidenced in our cell death assays (cytolysis and mitochondrial depolarization) after 24 h of treatment, in all three cellular models.

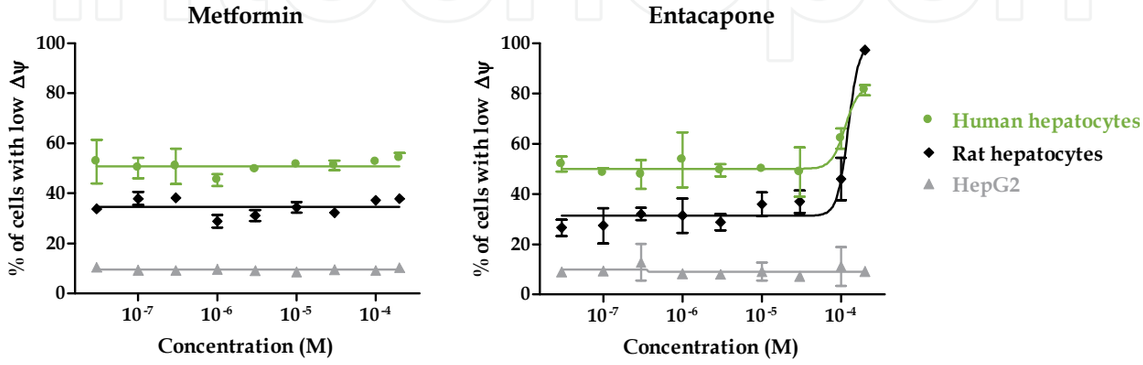


Fig. 4. Assessment of cell death induced by non-hepatotoxic drugs

Primary human hepatocytes, primary rat hepatocytes and HepG2 cells were incubated for 24 h with increasing concentrations of metformin or entacapone. Cell death was quantified in high-throughput flow cytometry by measuring mitochondrial membrane depolarization. Results are expressed as the mean ± SD of 2 replicates. Sigmoidal curve fitting was performed with GraphPad Prism software.

Entacapone is a catechol-O-methyl transferase (COMT) inhibitor used in the treatment of Parkinson's disease. It is usually administered in conjunction with the dopaminergic agent L-DOPA in order to increase its bioavailability by preventing COMT from metabolizing L-DOPA into 3-methoxy-4-hydroxy-L-phenylalanine (3-OMD). Entacapone is often used in hepatotoxicological studies in parallel with tolcapone, another COMT inhibitor with liver liabilities, to form a model drug pair: "non-liver toxic drug" (entacapone) versus "hepatotoxic drug" (tolcapone). In our assays, entacapone elicited mitochondrial depolarization in primary hepatocytes after 24 h of treatment at the highest tested concentration of 200 µM (Fig. 4). However, no effect was observed in HepG2 cells in the same experimental conditions evidencing differences in susceptibility to entacapone-induced toxicity between the primary cells and the HepG2 cell line. The fact that entacapone induces cell death in primary hepatocytes is not incompatible with the fact that it is a safe drug. Indeed as Paracelsus pointed out 500 years ago, the *dose* makes the *poison,* and all drugs can be toxic at high enough concentrations. What determines whether a drug is safe or not is the difference between the therapeutically active dose and the toxic dose, in other words, the safety margin of the drug.

By measuring mitochondrial depolarization or cytolysis, we could determine the exposure levels that lead to cell death *in vitro*. Using lower doses, we could explore earlier indicators

of toxicity which are more hepato-specific and reflect the initial cellular processes that may lead to cell death.

4. Energetic metabolism and oxidative stress measurements

To improve sensitivity of the assay, we chose to measure NAD(P)H depletion, ROS production and glutathione depletion as earlier indicators of toxicity.

Nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) are two of the most used coenzymes in cellular metabolism. They can exist in two different redox states (reduced and oxidized) that change into each other by accepting or donating electrons. The balance between the oxidized and reduced forms of these coenzymes is an important component of what is called the *redox state* of a cell, a measurement that reflects both the metabolic activity and the health of cells.

Hundreds of enzymes use $NAD(P)^+$ to catalyze reduction-oxidation reactions reversibly. Some of these are among the most abundant and well-studied enzymes participating in energetic metabolism (glycolysis, Krebs cycle, Lynen helix), biosynthesis, degradation, defense against oxidative damage, etc.

The reduced and oxidized forms of these coenzymes have distinct fluorescence characteristics: NADH and NADPH are fluorescent, while their oxidized forms (NAD^+ and $NADP^+$ respectively) are not. Fluorescence signals consistent with NAD(P)H can be measured to monitor cellular activity through redox status. As a cell changes its metabolic activity, the balance between NAD(P)H and $NAD(P)^+$ shifts correspondingly as the reduction-oxidation (redox) state of the cell fluctuates.

In our experiments, cells are measured in suspension and thus there are little effects of absorption and morphology on fluorescence emission. The sole contributors are intracellular fluorophores (in this case, endogenous fluorescence). Autofluorescence signals consistent with NAD(P)H are measured to monitor energetic metabolism through redox status. The use of flow cytometry is of real advantage because NADH has a very short fluorescence lifetime (0.4 nanoseconds) and cannot be easily quantified by fluorescence microscopy.

The NADH quantitation assay is attractive because, as it relies on cell autofluorescence and no staining step is required, it is cheap and quickly performed. In particular we used this assay to characterize batches of cryopreserved human hepatocytes (Fig. 5).

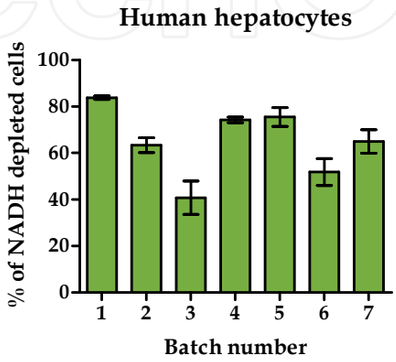


Fig. 5. Characterization of cryopreserved human hepatocytes batches

The quality of 7 different batches (i.e. donors) of human hepatocytes was assessed by measuring NADH depletion in high-throughput flow cytometry (excitation 355 nm – emission 450±20 nm) after 3 days in culture. Results are expressed as the mean ± SD of 2 replicates.

We generated dose-response curves for 25 chemicals, which all showed almost identical NADH depletion and mitochondrial depolarization results. However, a stronger NADH depletion than mitochondrial depolarization was evident for a few chemicals, including Alpha-naphtylisotiocianate (ANIT) (Fig. 6).

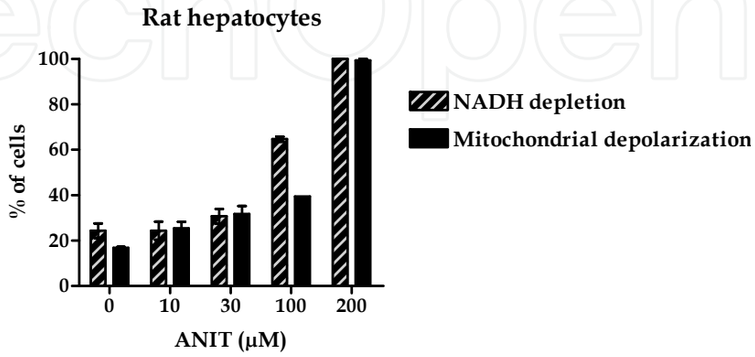


Fig. 6. NADH depletion "precedes" mitochondrial depolarization

Primary rat hepatocytes were incubated for 48 h with the indicated concentrations of ANIT. NADH depletion and mitochondrial membrane depolarization were measured in high-throughput flow cytometry as mentioned above. Results are expressed as the mean ± SD of 2 replicates.

ANIT is a known hepatotoxicant used in rodents to model human intrahepatic cholestasis. Cholestasis is the disruption of bile flow from the liver to the duodenum which leads to the accumulation of bile acids and other bile components in the liver and, ultimately, hepatobiliary toxicity. Cholestasis is often divided into two categories, extrahepatic and intrahepatic, based upon etiology. Extrahepatic cholestasis is the consequence of a mechanical blockage in the duct system (typically observed in patients with gallstones or tumors of the common biliary tract) whereas intrahepatic cholestasis is caused by physiological and pathological factors including genetic defects and chemicals. In rats, a single administration of ANIT induces intrahepatic cholestasis through damage to biliary epithelium cells (Orsler et al., 1999).

Measuring NADH depletion results in only a small improvement in sensitivity compared to cytolysis and mitochondrial depolarization. Perhaps the high correlation between the 3 assays is in part due to the fact that measurements are performed after 24 hours of treatment. Shorter exposures may be needed to increase the differences between these readouts.

Oxidative stress is an important mechanism of drug-induced toxicity. Oxidative stress is characterized by both increased production of oxidants or free radicals, and intracellular macromolecular change due to oxidative injury such as decreased glutathione.

Oxidative stress was first assessed by measuring intracellular levels of reactive oxygen species (ROS). ROS are molecules or ions (e.g. singlet oxygen, superoxides, peroxides,

hydroxyl radical, and hydroperoxides) formed by the incomplete one-electron reduction of oxygen. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However ROS levels can increase dramatically under different conditions of cell stress. The accumulation of these strong oxidants can result in significant damage to cell structures. Among the most important of these are the actions of free radicals on the fatty acid side chains of lipids in the various membranes of the cell, especially mitochondrial membranes (which are directly exposed to the superoxide anions produced during cellular respiration).

Most of the commercially available probes to monitor ROS production by flow cytometry in living cells are cell-permeant chemicals that undergo changes in their fluorescence spectral properties once oxidized by ROS. Two different probes were used in our assay: dihydroethidium (DHE, also called hydroethidine) and CM-H_2DCFDA (chloromethyl-dichlorodihydrofluorescein diacetate). DHE exhibits blue fluorescence in the cytosol until oxidized by superoxide to 2-hydroxyethidium which intercalates within the DNA staining the cell nucleus a bright fluorescent red. In contrast the nonfluorescent CM-H_2DCFDA is first hydrolyzed to DCFH in the cell by intracellular esterases and DCFH is oxidized to form the highly fluorescent DCF in the presence of ROS such as hydrogen peroxide.

We applied the ROS production assay to many drugs including troglitazone, a thiazolidinedione which was approved in 1997 for the treatment of type 2 diabetes. Troglitazone was an effective antidiabetic drug with a fundamentally new mechanism of action but several cases of liver injury and failure were reported in troglitazone-treated patients. The drug was eventually withdrawn from the market in 2000, after the approval of a newer generation of thiazolidinediones (i.e. rosiglitazone and pioglitazone) with diminished incidence of toxicity. Since then, a significant effort has been made to elucidate the mechanisms underlying troglitazone-induced hepatotoxicity. Possible mechanisms of troglitazone-induced cell injury include the formation and accumulation of toxic metabolites, mitochondrial dysfunction and oxidant stress, ATP depletion and subsequent cell death (Tang, 2007).

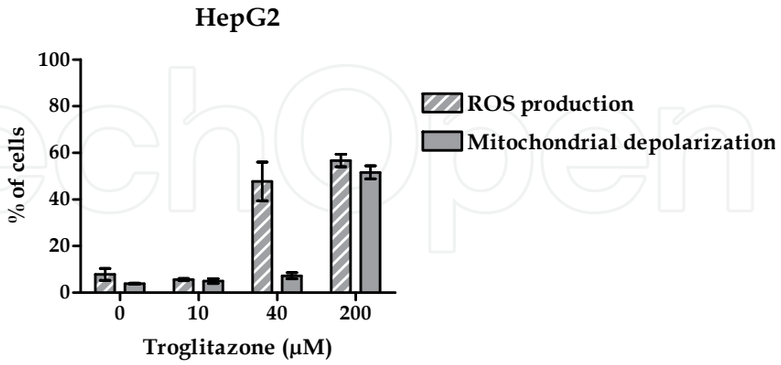


Fig. 7. ROS production "precedes" mitochondrial depolarization

HepG2 cells were incubated for 72 h with the indicated concentrations of troglitazone. ROS production and mitochondrial membrane depolarization were measured in high-throughput flow cytometry as mentioned above. Results are expressed as the mean ± SD of 2 replicates.

As shown in Fig. 7, after 72 h of treatment with troglitazone in HepG2 cells, a significant mitochondrial depolarization could be evidenced but only at the highest tested concentration of 200 µM. In contrast a significant ROS production was detected at a concentration of 40 µM. This pre-lethal assay is thus sensitive enough to detect ROS production at lower troglitazone concentrations than those needed for cytotoxicity.

Oxidative stress was also assessed by quantifying intracellular glutathione (GSH) levels. Glutathione (a tripeptide composed of three amino acids: cysteine, glutamic acid, and glycine) is the most abundant and important nonprotein thiol in mammalian cells. Glutathione plays a major role in the protection of the liver against several hepatotoxicants. Indeed, in addition to its central role in protecting cells of all organs against damage produced by free radicals, glutathione is involved in drug detoxification.

Xenobiotics including drugs are subject to metabolism which most likely acts as a self-defense mechanism of the body. Drugs are metabolized through a complex series of biochemical reactions which are categorized into two major pathways, referred to as Phase I (oxidative reactions) and Phase II (conjugation reactions). This can result in toxification or detoxification (the activation or deactivation respectively) of the chemical. While both toxification and detoxification occur, the major metabolites of most drugs are detoxification products.

During phase I and phase II, drugs are converted into more polar products that can be excreted in the urine or the bile depending on the particular characteristics of the end product. In phase I a variety of enzymes acts to introduce reactive and polar groups into their substrate. These reactions are mainly catalyzed by cytochrome P450 enzymes (often abbreviated as CYPs), amine oxidase, peroxidases, and flavin-containing monooxygenase. The cytochrome P450 superfamily is a large and diverse group of enzymes which catalyze the oxidation of organic substances. CYPs are the major enzymes involved in drug metabolism accounting for about 75% of the total number of different metabolic reactions. A significant side effect of phase I oxidative metabolism is the formation of potentially harmful reactive electrophiles which are mostly neutralized by glutathione that is in turn oxidized to glutathione disulfide (GSSG). Phase 2 reactions, also known as conjugation reactions, are usually detoxificating in nature and consist in the conjugation of phase I metabolites with charged species such as glutathione, sulfate, glycine or glucuronic acid. The conjugates formed are highly hydrophilic, which promotes their excretion. In this process, reduced glutathione levels decrease at the expense of the formation of glutathione conjugates. Thus GSH plays a key role in drug metabolism and depletion of reduced form of glutathione was reported to be a marker of hepatotoxicity (Xu et al., 2004).

In our experiments, reduced glutathione content was assessed with monochlorobimane. This non fluorescent bimane derivative reacts with GSH (but do not react with GSSG) to form a highly fluorescent conjugated product readily quantified in viable cells by flow cytometry.

The measurement of GSH in the 3 cellular models was first optimized using buthionine sulfoximine (BSO) which inhibits GSH neosynthesis thus reducing cellular GSH levels. The quantitation of GSH levels was restricted to non cytolytic cells (Fig. 8) based on cell morphology using the forward and side scatter channels (FSC and SSC) which are related to the cell size and the internal complexity respectively.

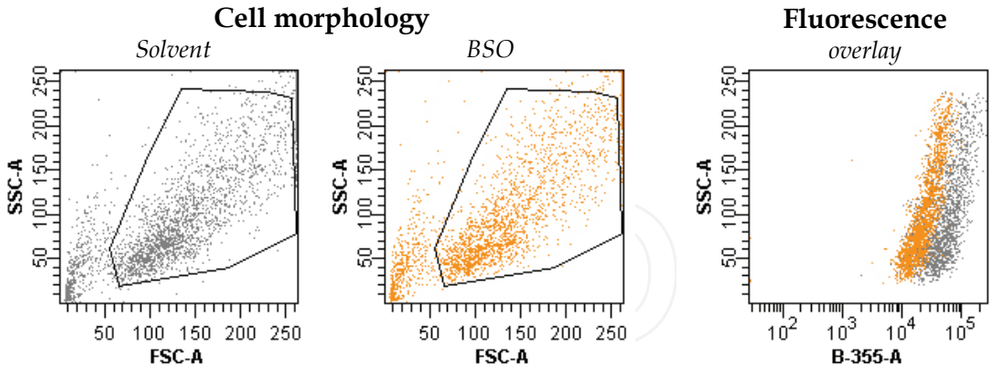


Fig. 8. Analysis of flow cytometry outputs for the GSH depletion assay

Biparametric cell morphology plots show the selection of non-cytolytic HepG2 cells incubated with solvent (*left plot*) or 300 µM BSO (*middle plot*) for 72 h. The decrease in the fluorescence intensity of monochlorobimane staining after treatment with BSO is clearly visible in the overlay of the fluorescence outputs from both conditions (*right plot*): the fluorescence of the non-cytolytic solvent-treated cells (in grey) is more intense than the fluorescence of the non-cytolytic BSO-treated cells (in orange).

As only viable cells are analyzed, the obtained dynamic range is high in all tested cell types (Fig. 9), even in cryopreserved human hepatocytes. After 24 hours of treatment with 300 µM BSO, a complete depletion in GSH is observed in all cellular models. However the EC_{50} of glutathione depletion is lower in HepG2 cells than in primary hepatocytes revealing differences in kinetics of GSH depletion between the cell line and the primary cells.

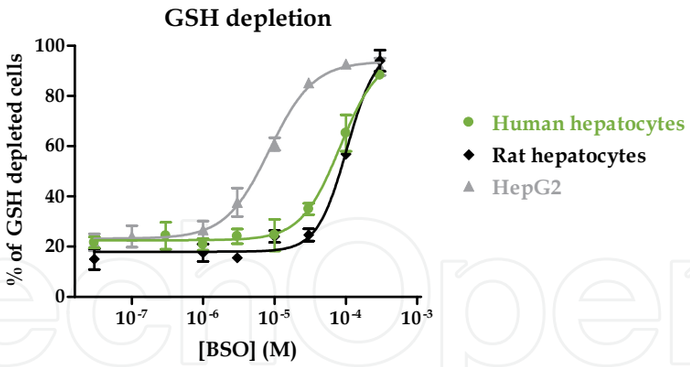


Fig. 9. Optimization of the GSH depletion assay with BSO

Primary human hepatocytes, primary rat hepatocytes and HepG2 cells were incubated for 24 h with increasing concentrations of BSO. GSH depletion was measured in high-throughput flow cytometry as mentioned above. Results are expressed as the mean ± SD of 2 replicates. Sigmoidal curve fitting was performed with GraphPad Prism software.

Acetaminophen hepatotoxicity is mediated by the formation of NAPQI (N-acetyl-p-benzoquinone imine), a reactive metabolite produced by a minor APAP clearance pathway mainly catalyzed by CYP2E1. Because of its chemical reactivity, NAPQI undergoes a conjugation with GSH. When large quantities of APAP are metabolized, the amount of

hepatic GSH is not sufficient to detoxify NAPQI. Glutathione pools are depleted and the reactive metabolite accumulates and binds to critical mitochondrial proteins ultimately causing cell death. Intracellular events resulting in hepatocyte death may include: disturbance of cellular calcium homeostasis, mitochondrial oxidative stress, collapse of mitochondrial membrane potential, decreased ATP synthesis, DNA fragmentation, and cytolysis (Tang, 2007).

After 24 hours of treatment with 10 mM acetaminophen, a significant proportion of GSH-depleted cells is observed in primary hepatocytes (from rat and human). However no GSH depletion is detected in the HepG2 cell line (Fig. 10).

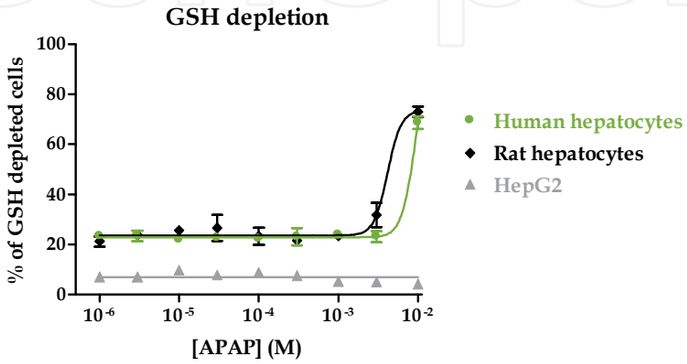


Fig. 10. Acetaminophen-induced GSH depletion in hepatocytes

Primary human hepatocytes, primary rat hepatocytes and HepG2 cells were incubated for 24 h with increasing concentrations of APAP. GSH depletion was measured in high-throughput flow cytometry as mentioned above. Results are expressed as the mean ± SD of 2 replicates. Sigmoidal curve fitting was performed with GraphPad Prism software.

Acetaminophen-induced glutathione depletion is a CYP-dependent mechanism and HepG2 cells are known to have a reduced metabolic activity. The absence of glutathione depletion in the cell line is thus probably due to a reduced or inexistent formation of NAPQI. But interestingly acetaminophen is as toxic in HepG2 cells as it is in primary cells, as far as cell death is concerned (Fig. 3). Therefore the intracellular events leading to cell death in response to acetaminophen exposure appear to be different in HepG2 cells and primary hepatocytes, suggesting that a "non canonical" mechanism of action is involved in APAP-induced cell death in the cell line.

5. Lipid content measurement

Drug-induced liver injury encompasses a large spectrum of lesions, some of which are the consequence of steatosis and phospholipidosis.

Drugs are known to be able to induce steatosis, an abnormal accumulation of neutral lipids which can lead to liver failure. When fat accumulates, lipids are primarily stored as triglycerides. Steatosis is believed to result from an imbalance between hepatic free fatty acids inflow and triglyceride synthesis and excretion. Steatosis most often occurs in the liver which is the primary organ of lipid metabolism, although it may occur in any organ, such as the kidneys, heart, and muscles. The risk factors associated with steatosis are varied, and

include diabetes mellitus, hypertension and obesity. Macrovesicular steatosis, characterized by the presence of large lipid vesicles that displace the nucleus of hepatocytes, is the most common form of fatty degeneration, and may be caused by oversupply of lipids due to obesity, insulin resistance, or alcoholism. Microvesicular steatosis, the accumulation of multiple small lipid droplets, can be caused by several diseases including Reye's syndrome and hepatitis D. Fat accumulation is not necessarily a pathological condition and drug-induced steatosis is often reversible.

Prolonged exposure to certain drugs can cause macrovesicular steatosis, a benign hepatic lesion. Nevertheless chronic macrovesicular steatosis can evolve in certain cases into steatohepatitis (a liver inflammation resulting from steatosis) and ultimately into cirrhosis. Moreover, in a few patients, some drugs induce microvesicular steatosis which can potentially lead to liver failure with fatal consequences.

One of the major mechanisms involved in drug-induced liver steatosis is the inhibition of beta-oxidation (degradation of long chain fatty acids) either by direct inhibition of mitochondrial beta-oxidation enzymes or by sequestration of the cofactors involved in this metabolic pathway. Other drugs eventually reduce beta-oxidation as a result of mitochondrial dysfunction (because the oxidized cofactors NAD+ and FAD which are produced by mitochondrial respiration are needed for beta-oxidation).

Mitochondrial dysfunction plays a key role in the pathophysiology of steatohepatitis. Indeed, respiratory chain deficiency results in decreased ATP formation and increased ROS generation. The combination of decreased beta-oxidation (resulting in lipid accumulation) and increased ROS generation (resulting in lipid peroxidation and release of aldehydic derivatives with detrimental effects on hepatocytes) is an important mechanism of drug-induced steatohepatitis.

Phospholipidosis is a lysosomal storage disorder characterized by the excess accumulation of phospholipids in cells. The mechanisms of drug-induced phospholipidosis involve trapping or selective uptake of the phospholipidosis-inducing drugs within the lysosomes and acidic vesicles of affected cells. Drug trapping is followed by a gradual accumulation of drug-phospholipid complexes within the internal lysosomal membranes. The increase in undigested materials results in the abnormal accumulation of multi-lammellar bodies (myeloid bodies) in tissues. Many cationic amphiphilic drugs, including anti-depressants and cholesterol-lowering agents, are reported to cause drug-induced phospholipidosis in animals and humans.

Phospholipidosis is often accompanied with various associated toxicities in the liver. It does not *per se* constitute frank toxicity but is reportedly predictive of drug or metabolite accumulation in affected tissues (Xu et al., 2004).

Thus, the variations in both neutral lipids content and polar lipids (phospholipids) content may be early indicators of drug-induced hepatotoxicity. Various probes are available for use in intact cells that either titrate the lipid pool of interest or get accumulated as a lipid mimetic. We developed a proprietary probe formulation and detection methodology (lipotracker) that allows the simultaneous quantitation of neutral lipids and polar lipids (quantitation service available at www.fluofarma.com). The ratio of neutral lipids content upon polar lipids content at the single cell level is directly generated by the flow cytometry platform. This lipid ratio can be used to normalize cell size and dye uptake and provides a

reliable and precise measurement. As it has a very low variability it allows the detection of slight variations in lipid content. By using FSC and SSC outputs, lipid content is only measured in intact (non-cytolytic) cells, as it is the case for the glutathione depletion assay.

The lipid content assay was first optimized with valproic acid (2-n-propylpentanoic acid), a drug commonly prescribed worldwide in the treatment of epilepsy and in the control of several types of seizures affecting both children and adults. The mechanism of the antiepileptic action of VPA involves the regional changes in the concentration of the neurotransmitter gamma-aminobutyric acid (GABA). VPA is well tolerated by the vast majority of patients but it can induce severe and sometimes fatal hepatotoxicity that is characterized by microvesicular steatosis and most likely results from beta-oxidation inhibition.

A prerequisite to beta-oxidation is the uptake of fatty acids by mitochondria. Short- and medium-chain fatty acids may pass through the mitochondrial membrane directly, whereas long-chain fatty acids are transported across the mitochondrial membrane by a CoA/carnitine-dependent carrier system. Through several mechanisms, valproic acid inhibits the CoA/carnitine-dependent transport of fatty acids resulting in their accumulation in the cytoplasm. Besides a few valproic acid metabolites are suspected to directly inhibit specific enzyme(s) in the beta-oxidation pathway (Tang, 2007).

Consistent with its known steatotic potential, VPA induces an increase in the lipid ratio, and this increase is identical in the 3 studied cell types after 24 h of treatment (Fig. 11).

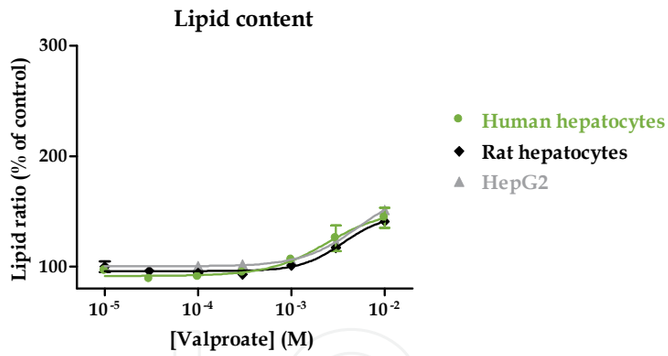


Fig. 11. Valproate-induced variations in lipid content after short exposure

Primary human hepatocytes, primary rat hepatocytes and HepG2 cells were incubated for 24 h with increasing concentrations of VPA. Variations in lipid content were assessed by measuring the single cell lipid ratio (neutral lipids / polar lipids) in high-throughput flow cytometry as mentioned above. Results are expressed as a percentage of the control value (solvent-treated cells) and correspond to the mean ± SD of 2 replicates. Sigmoidal curve fitting was performed with GraphPad Prism software.

In contrast, with a longer exposure to VPA, the observed effects are significantly different from one cellular model to another (Fig. 12).

Primary hepatocytes (from human and rat) and HepG2 cells were incubated with increasing concentrations of VPA for 48 h or 72 h respectively. Variations in lipid content were assessed by measuring the single cell lipid ratio (neutral lipids / polar lipids) in high-throughput

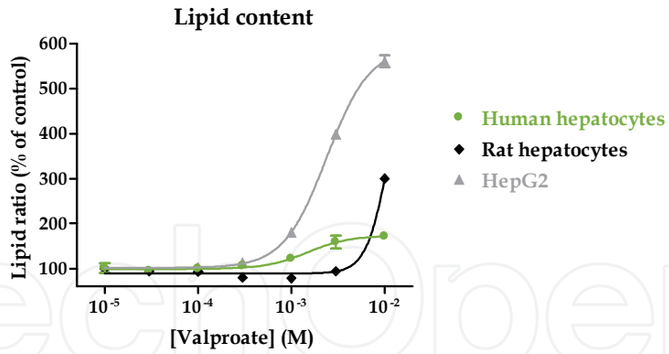


Fig. 12. Valproate-induced variations in lipid content after long exposure

flow cytometry as mentioned above. Results are expressed as a percentage of the control value (solvent-treated cells) and correspond to the mean ± SD of 2 replicates. Sigmoidal curve fitting was performed with GraphPad Prism software.

The accumulation of neutral lipids (evidenced by an increase in lipid ratio) is much more pronounced in HepG2 cells than in primary hepatocytes, with human hepatocytes exhibiting the smallest amplitude of variation. This is probably due to the fact that the basal content in neutral lipids (in vehicle-treated condition) varies considerably between cell types (i.e. the lipid ratio is higher in primary hepatocytes than in HepG2 cells).

Variations in neutral lipid content and polar lipid content were also examined separately to corroborate that the observed VPA-induced augmentation in lipid ratio corresponds to an increase in neutral lipids (and not a decrease in phospholipids content). The same experiment was performed using amiodarone, an antiarrhythmic drug which induces both phospholipidosis and steatosis (Fig. 13).

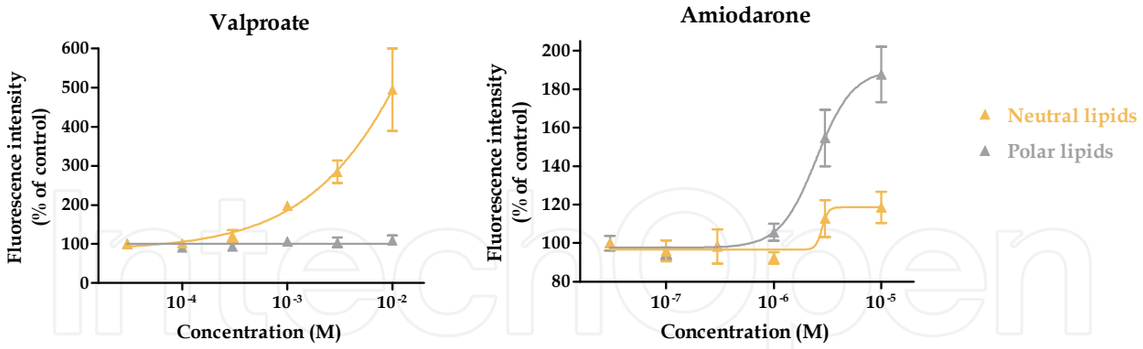


Fig. 13. Steatosis and phospholipidosis in HepG2 cells

HepG2 cells were incubated for 72 h with increasing concentrations of valproate or amiodarone. The fluorescence intensities of the neutral lipids and the polar lipids stainings were quantified in high-throughput flow cytometry as mentioned above. Fluorescence intensity is expressed as a percentage of the control value (solvent-treated cells). Data are given as mean ± SD of 2 replicates. Sigmoidal curve fitting was performed with GraphPad Prism software.

As expected, VPA caused an increase in neutral lipid content with no effect on polar lipid content whereas amiodarone elicited an increase in phospholipids and a smaller rise in

neutral lipid content. These results are in accordance with published data reporting that although amiodarone induces both phospholipidosis and steatosis, the accumulation of phospholipids appears after a shorter exposure than those required to provoke an accumulation of neutral lipids (Antherieu et al., 2011).

The lipid assay was subsequently applied to tetracycline, a broad-spectrum antibiotic which inhibits protein synthesis by binding to the 30S subunit of microbial ribosomes. Tetracycline hepatotoxicity seems related to the use of large doses and unlike most other antibiotics, is predictable and reproducible in animal models. With normal low oral doses, tetracycline only rarely causes liver injury. Intravenous or large oral doses of tetracycline induce microvesicular steatosis most likely by direct inhibition of mitochondrial beta-oxidation enzyme(s) (Donato et al, 2009). In our assay, variations in lipid content are detected in cells incubated with sub-cytotoxic concentrations of tetracycline (Fig. 14).

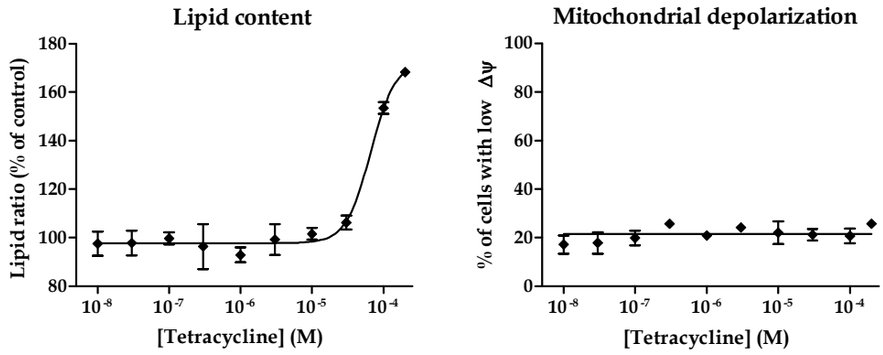


Fig. 14. Variations in lipid content "precede" mitochondrial depolarization

Primary rat hepatocytes were incubated for 48 h with increasing concentrations of tetracycline. Variations in lipid content were assessed by measuring the single cell lipid ratio and results are expressed as a percentage of the control value (solvent-treated cells). Both lipid content and mitochondrial membrane depolarization assays were performed in high-throughput flow cytometry as mentioned above. Results are expressed as the mean ± SD of 2 replicates. Sigmoidal curve fitting was performed with GraphPad Prism software.

Our optimized lipid assay can thus be useful for detecting variations in lipid content with a high sensitivity (thanks to the use of a lipid ratio), and for determining drugs mechanism of action (steatosis and/or phospholipidosis).

6. Idiosyncratic DILI prediction

Although most of the toxic candidate compounds are screened out during preclinical safety studies, each year several new drugs do not get approval or are withdrawn from the market because their toxicity is detected only in late clinical phases or in postmarketing evaluation. Idiosyncratic drug-induced liver injury (DILI) refers to severe (and potentially fatal) hepatic reactions with a low frequency of occurrence (<0.1%), that do not occur in most patients at any dose of the drug, and typically have a delayed onset of weeks or months after initial exposure. Examples of drugs withdrawn from the market because of idiosyncratic DILI include troglitazone and alpidem. In order to assess whether the assays we developed could prove useful for the identification of drugs with an idiosyncratic hepatotoxic potential, we

tested pairs of compounds which are related in chemical structure and mechanism of action but show marked differences in hepatotoxic potential.

First we tested troglitazone and rosiglitazone, two antidiabetic drugs that belong to the thiazolidinedione family. As mentioned above, troglitazone was withdrawn from the market because of idiosyncratic DILI and was replaced by newer thiazolidinediones (including rosiglitazone) with diminished incidence of hepatotoxicity. In HepG2 cells, both drugs produced ROS although a marked effect was obtained with 40 µM of troglitazone whereas 200 µM of rosiglitazone were required to induce an equivalent ROS production (Fig. 15). These results are consistent with published data reporting that 50 µM troglitazone induced ROS production in another human hepatocytes cell line (Shishido et al., 2003). Cell death was only evidenced at the highest tested dose of troglitazone, and a similar GSH depletion was observed with both drugs. In addition, alterations in lipid content were much more pronounced with troglitazone than with rosiglitazone.

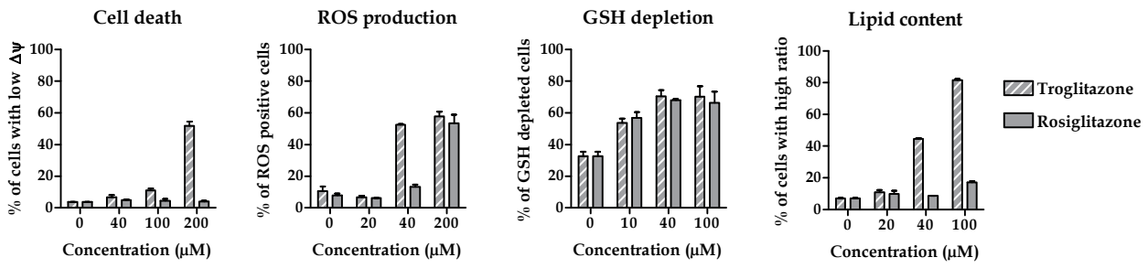


Fig. 15. Multiparametric assessment of troglitazone and rosiglitazone effects in HepG2 cells

HepG2 cells were incubated for 72 h with the indicated concentrations of troglitazone and rosiglitazone. Cell death, ROS production, GSH depletion and lipid content were assessed in high-throughput flow cytometry as mentioned above. Results are expressed as the mean ± SD of 2 replicates.

Our assays were also applied to one pair of drugs from the imidazopyridine class, namely alpidem (a "DILI positive" compound) and zolpidem (a "DILI negative" compound). Although alpidem is related to the better known sleeping medication zolpidem, it does not produce sedative effects at normal doses and was thus used specifically for the treatment of anxiety. Alpidem was released in France in 1991 but was withdrawn from the market a few years later because several cases of severe hepatitis had been reported. The test of these drugs in HepG2 cells (Fig. 16) revealed that neither alpidem nor zolpidem induced ROS production, even at high concentrations. In contrast, as previously reported in rat hepatocytes, both drugs reduced GSH content, with alpidem exhibiting more pronounced effects than zolpidem (Berson et al., 2001). Cell death was apparent only after treatment with alpidem, but not zolpidem. Finally, a very significant increase in lipid ratio was measured at low sub-cytotoxic concentrations of alpidem (2 µM) whereas no effect was observed with equimolar concentrations of zolpidem.

HepG2 cells were incubated for 72 h with the indicated concentrations of alpidem and zolpidem. Cell death, ROS production, GSH depletion and lipid content were assessed in high-throughput flow cytometry as mentioned above. Results are expressed as the mean ± SD of 2 replicates.

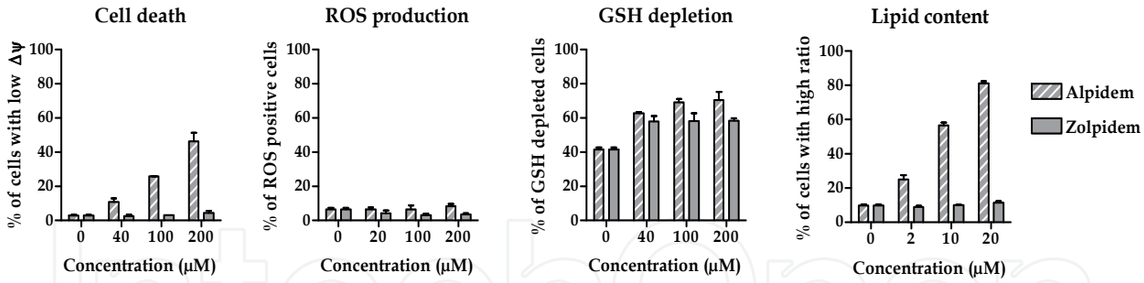


Fig. 16. Multiparametric assessment of alpidem and zolpidem effects in HepG2 cells

All together these data show that the tested drug pairs have very different profiles in our assays. In particular alterations in lipid content seem to be a good indicator of the idiosyncratic hepatotoxic potential of a drug. Our tests could certainly be of great value in preclinical studies, in particular for safety-based prioritization of compounds.

7. Conclusion

Thanks to an extensive optimization, high quality measurement of endpoints could be obtained in all 3 hepatic cellular models: the HepG2 cell line, freshly isolated rat hepatocytes and cryopreserved human hepatocytes. The good quality of these measurements, and in particular the low experimental variability, allowed the detection of small variations in the measured parameters. Combined with the use of early intracellular indicators of hepatotoxicity, we could detect cell alterations at much lower concentrations than those needed for gross cytotoxicity. Moreover these measurements could be performed at high throughput with a flow cytometry platform by multiplexing the assays in 96-well plates and using only 2 replicates per experimental condition.

As expected with a cell line, HepG2 cells generated reproducible results with very low variability. For all the assays we developed, the results obtained with these cells also had an extended dynamic range, indicative of the excellent technical performance of the described assays. However, probably in part due to their reduced drug metabolizing activity, the response of these cells to certain compounds differed from the one obtained in primary hepatocytes. Therefore HepG2 cells are not the most adequate cellular model to elucidate drug toxicity mechanism of action. However, these cells have prove useful for particular studies such as the analysis of drug-induced alterations in lipid content (steatosis and phospholipidosis), and the prioritization of compounds based on their potential to cause liver injury.

Measurements performed with primary cells have a higher variability (in comparison with HepG2 cells) but which is still quite low and compatible with the use of duplicates. In primary hepatocytes, the dynamic range of certain assays is also reduced compared to HepG2 cells, in particular for human hepatocytes. This is largely due to the low basal viability (in untreated conditions) of cryopreserved human hepatocytes, after several days in culture. As a result, although human hepatocytes theoretically represent the best cellular model, the use of frozen hepatocytes with a reduced viability and the lot-to-lot variability constitute limiting factors for their utilization in large scale screening studies. These cells appear to be more suited to mechanistic studies.

As a cellular model for liver toxicity studies, freshly isolated rat hepatocytes represent a good compromise in terms of cell availability and handling, quality of the results (experimental variability, dynamic range and reproducibility), and hepato-specific differentiation and functionality.

In summary, the individual assays we developed can provide insights into the underlying mechanism of action of drug-induced hepatotoxicity and we are currently evaluating whether combinations of these assays could be routinely used early in the drug development process for the prediction of acute hepatotoxicity or the prioritization of compounds based on their potential to cause idiosyncratic liver toxicity.

8. Acknowledgement

The authors would like to thank Lucie Balaguer and Florian Simon for generating experimental data, and Loic Cerf for project management.

9. References

Andújar-Plata, P.; Pi-Sunyer, X. & Laferrère, B. (2011). Metformin effects revisited. *Diabetes research and clinical practice*, DOI:10.1016/j.diabres.2011.09.022

Anthérieu, S.; Rogue, A.; Fromenty, B.; Guillouzo, A. & Robin MA. (2011). Induction of vesicular steatosis by amiodarone and tetracycline is associated with up-regulation of lipogenic genes in HepaRG cells (2011). *Hepatology*, Vol. 53, No. 6, (June 2011), pp. 1895-1905, DOI: 10.1002/hep.24290

Berson, A.; Descatoire, V.; Sutton, A.; Fau, D.; Maulny, B.; Vadrot, N.; Feldmann, G.; Berthon, B.; Tordjmann, T. & Pessayre D. (2001). Toxicity of alpidem, a peripheral benzodiazepine receptor ligand, but not zolpidem, in rat hepatocytes: role of mitochondrial permeability transition and metabolic activation. *The Journal of pharmacology and experimental therapeutics*, Vol. 299, No. 2, pp. 793-800, ISSN: 0022-3565

Donato, MT.; Martínez-Romero, A.; Jiménez, N.; Negro, A.; Herrera, G.; Castell, JV.; O'Connor, JE. & Gómez-Lechón, MJ. (2009). Cytometric analysis for drug-induced steatosis in HepG2 cells. *Chemico-biological interactions,* Vol. 181, No. 3, pp. 417-423

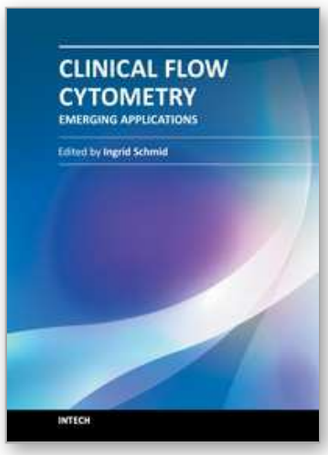
Orsler, DJ.; Ahmed-Choudhury, J.; Chipman, JK.; Hammond, T. & Coleman, R. (1999). ANIT-induced disruption of biliary function in rat hepatocyte couplets. *Toxicological sciences*, Vol. 47, No. 2, pp. 203-210

Shishido, S.; Koga, H.; Harada, M.; Kumemura, H.; Hanada, S.; Taniguchi, E.; Kumashiro, R.; Ohira, H.; Sato, Y.; Namba, M.; Ueno, T. & Sata, M. (2003). Hydrogen peroxide overproduction in megamitochondria of troglitazone-treated human hepatocytes. *Hepatology*, Vol. 37, No. 1, pp. 136-147

Stevens, JL. & Baker TK. (2009). The future of drug safety testing: expanding the view and narrowing the focus. *Drug discovery today*, Vol. 14, No. 3/4, pp. 162-167

Tang, W. (2007). Drug metabolite profiling and elucidation of drug-induced hepatotoxicity. *Expert opinion on drug metabolism and toxicology*, Vol. 3, No. 3, pp. 407-420, ISSN 1742-5255

Xu, JJ.; Diaz, D. & O'Brien, PJ. (2004). Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. *Chemico-biological interactions,* Vol. 150, No. 1, pp. 115-128



Clinical Flow Cytometry - Emerging Applications

Edited by M.Sc. Ingrid Schmid

ISBN 978-953-51-0575-6
Hard cover, 204 pages
Publisher InTech
Published online 16, May, 2012
Published in print edition May, 2012

"Clinical Flow Cytometry - Emerging Applications" contains a collection of reviews and original papers that illustrate the relevance of flow cytometry for the study of specific diseases and clinical evaluations. The chapters have been contributed by authors from a wide variety of countries showing the broad application and importance of this technology in medicine. Examples include chapters on autoimmune disease, cancer, and the evaluation of new drugs. The book is intended to give newcomers a helpful introduction, but also to provide experienced flow cytometrists with novel insights and a better understanding of clinical cytometry.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen