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Prediction of Liver Injury Induced by Chemicals in Human With a Multiparametric Assay on Isolated Mouse Liver Mitochondria

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Drug-induced liver injury (DILI) in humans is difficult to predict using classical *in vitro* cytotoxicity screening and regulatory animal studies. This explains why numerous compounds are stopped during clinical trials or withdrawn from the market due to hepatotoxicity. Thus, it is important to improve early prediction of DILI in human. In this study, we hypothesized that this goal could be achieved by investigating drug-induced mitochondrial dysfunction as this toxic effect is a major mechanism of DILI. To this end, we developed a high-throughput screening platform using isolated mouse liver mitochondria. Our broad spectrum multiparametric assay was designed to detect the global mitochondrial membrane permeabilization (swelling), inner membrane permeabilization (transmembrane potential), outer membrane permeabilization (cytochrome *c* release), and alteration of mitochondrial respiration driven by succinate or malate/glutamate. A pool of 124 chemicals (mainly drugs) was selected, including 87 with documented DILI and 37 without reported clinical hepatotoxicity. Our screening assay revealed an excellent sensitivity for clinical outcome of DILI (94 or 92% depending on cutoff) and a high positive predictive value (89 or 82%). A highly significant relationship between drug-induced mitochondrial toxicity and DILI occurrence in patients was calculated ($p < 0.001$). Moreover, this multiparametric assay allowed identifying several compounds for which mitochondrial toxicity had never been described before and even helped to clarify mechanisms with some drugs already known to be mitochondriotoxic. Investigation of drug-induced loss of mitochondrial integrity and function with this multiparametric assay should be considered for integration into basic screening processes at early stage to select drug candidates with lower risk of DILI in human. This assay is also a valuable tool for assessing the mitochondrial toxicity profile and investigating the mechanism of action of new compounds and marketed compounds.

Key Words: **mitochondria; hepatotoxicity; DILI; drugs; chemicals; screening; prediction.**

Drug-induced liver injury (DILI) occurrence is a major concern for pharmaceutical companies because it can lead to drug withdrawal from the market being imposed by an agency (Food and Drug Administration [FDA] or European Medicines Agency [EMA]) after a careful risk-benefit assessment, or during phase II or III clinical trials by consensus or by company decision. Actually, the worst scenario involves a postmarketing recall combining serious patient health and company damage, a major loss of income, lawsuits stretching over years, loss of credibility, and deteriorated image in media and medical community. Over a thousand drugs described in the modern pharmacopoeia can induce liver damage with different clinical presentations (Biour *et al.,* 2004; Larrey, 2000). Most cases of DILI are benign, accompanied by slight (or moderate) alterations of plasma parameters such as transaminases and bilirubin, and reversible upon treatment cessation. However, with some hepatotoxic drugs and in some patients, DILI may trigger acute liver failure requiring liver transplantation, or even leading to a fatal outcome (Björnsson, 2009).

DILI is classically considered as either intrinsic or idiosyncratic. Whereas intrinsic DILI is usually dose-related and generally discovered during animal toxicity studies, idiosyncratic DILI is less predictable. Indeed, idiosyncratic hepatotoxicity occurs in some individuals with different genetic and metabolic predispositions, or in individuals exposed to other environmental factors (Begriche *et al*., 2011). It was reported that idiosyncratic DILI represents around 13% of acute liver failure cases in the United States (Ostapowicz *et al*., 2002). However, because drugs inducing intrinsic hepatotoxicity during clinical development rarely reach the market, most cases of DILI can be considered as idiosyncratic (Pessayre *et al*., 2010; Stirnimann *et al.,* 2010). Importantly, both types of DILI can result from mitochondrial toxicity.

Indeed, mitochondrial dysfunction is considered as a key mechanism of DILI (Begriche *et al.,* 2011; Labbe *et al.,* 2008; Pessayre *et al.,* 2010; Russmann *et al.,* 2009), although chemicals can cause hepatotoxicity through other pathways, such as the generation of reactive metabolites and specific immune reactions (Lee, 2003; Russmann *et al*., 2009). All these initial events can have different deleterious consequences for the hepatocytes, thus leading to hepatic cytolysis. Importantly, drug-induced mitochondrial dysfunction may be elicited by a parent drug and/or by reactive metabolites generated through cytochrome P450 (CYP)-mediated metabolism (Begriche *et al.,* 2011; Labbe *et al.,* 2008; Masubuchi *et al.,* 2005). Moreover, it is noteworthy that mitochondrial dysfunction is a generic term including alteration of different metabolic pathways and damage to mitochondrial components. For instance, drugs can (1) impair mitochondrial fatty acid oxidation, electron transfer within the mitochondrial respiratory chain, and the oxidative phosphorylation (OXPHOS) process; (2) deplete the mitochondrial genome by inhibiting the mitochondrial DNA (mtDNA) polymerase γ and/or induce oxidative damage to the mtDNA; and (3) trigger mitochondrial membrane permeabilization, thus inducing the release of mitochondrial proapoptotic proteins into the cytoplasm (Begriche *et al.,* 2011; Fromenty and Pessayre, 1995; Labbe *et al.,* 2008; Lee, 2003; Pessayre *et al.,* 2010; Russmann *et al.,* 2009). In addition, drug-induced blockade of the mitochondrial respiratory chain results in overproduction of reactive oxygen species and lipid peroxidation (Begriche *et al.,* 2011; Berson *et al.,* 1998; Pessayre *et al.,* 2010). Importantly, drug-induced mitochondrial dysfunction can be responsible for cytolytic hepatitis, microvesicular steatosis (Reye-like syndrome), steatohepatitis, liver failure, and even cirrhosis (Begriche *et al.,* 2011; Labbe *et al.,* 2008; Pessayre *et al.,* 2010). Drugs that can induce idiosyncratic DILI through mitochondrial toxicity are, for instance, valproic acid, troglitazone, and antiretroviral drugs such as stavudine and zidovudine (Boelsterli and Lim, 2007; Labbe *et al.,* 2008; Stewart *et al.,* 2010). Finally, it should be stressed that drug-induced mitochondrial toxicity can also involve extrahepatic tissues such as muscles, heart, pancreas, neurons, or kidney, thus eliciting reports on myopathy, rhabdomyolysis, pancreatitis, peripheral neuropathy, or renal dysfunction among others (Dykens and Will, 2007; Gougeon *et al.,* 2004; Igoudjil *et al.,* 2006; Lebrecht *et al.,* 2009; Scatena *et al.,* 2007).

Bearing in mind the serious consequences for patients and pharmaceutical industry, drug-induced mitochondrial dysfunction should be detected early, ideally during screening of potential drug candidates. The development of high-throughput *in vitro* screening techniques could represent a major breakthrough for a rapid selection of safer compounds (Begriche *et al.,* 2011; Berson *et al.,* 1998; Dykens and Will, 2007; Gougeon *et al.,* 2004; Igoudjil *et al.*, 2006; Labbe *et al.,* 2008; Masubuchi *et al.,* 2005; Pessayre *et al.,* 2010). Hence, the main objective of this study was to determine whether DILI could be predicted using a combination of high-throughput *in vitro* screening tests performed on isolated mouse liver mitochondria. To this end, we selected 87 compounds documented for inducing DILI in human and 37 compounds without known clinical hepatotoxicity based on the updated "Hepatox" database (http://hepatoweb.com/hepatox.php).

MATERIALS AND METHODS

Reagents and compounds. Oligomycin A, rotenone, *m*-chlorocarbonylcyanide phenylhydrazone (mClCCP), alamethicin, and other chemicals were purchased either from Sigma Aldrich (Saint-Quentin-Fallavier, France) or from Santa Cruz Biotechnology (Heidelberg, Germany). Cyclosporin A (CsA) was purchased from Tebu Bio SA (Le Perray-en-Yvelines, France).

Purification of mouse liver mitochondria. Liver mitochondria from 6-week-old BALB/cByf female mice (Charles River, Saint-Germain-sur-L'arbresle, France) were isolated and purified by isopycnic density-gradient centrifugation in Percoll, as previously described (Buron *et al.,* 2010; Lecoeur *et al.,* 2004), allowing pure and stable mitochondrial preparations. In previous experiments, we found that parameters measured from mitochondria isolated from female mice showed less interindividual variability than mitochondria from male mice, in particular in response to chemicals (Brenner and Borgne-Sanchez, unpublished data).

Assessment of large amplitude swelling and $\Delta\Psi_m$. Mitochondrial swelling and mitochondrial transmembrane potential ($\Delta\Psi_m$) were evaluated as described previously (Buron *et al.,* 2010) in presence of succinate and rotenone. Calcium ($CaCl_2$; 50μM) and mCICCP (50μM) were used as the 100% baseline for swelling and loss of $\Delta\Psi_m$, respectively. Effective concentration at 20% of the maximal effect (EC_{20}) for these parameters were the drug concentrations leading to 20% of the maximal swelling and 20% of the maximal loss of $\Delta\Psi_m$ after a 30-min incubation, respectively.

Determination of cytochrome* c *release. Cytochrome *c* release was evaluated as described previously (Buron *et al.,* 2010) using an ELISA kit (R&D Systems, France). Treatment with 20 μg/ml alamethicin, a peptide able to form channels in membranes, was used as the 100% baseline. EC_{20} for this parameter was the drug concentration inducing 20% of the maximal cytochrome *c* release after a 30-min incubation.

Measurement of oxygen consumption. Oxygen consumption was monitored as previously described (Will *et al.,* 2006), with some modifications. Briefly, isolated mitochondria (100 μg proteins) were incubated with drug in buffer containing 250mM sucrose, 30mM K_2HPO_4, 1mM EGTA, 5mM $MgCl_2$, 15mM KCl, and 1 mg/ml bovine serum albumin supplemented with respiratory substrates and 50 nM MitoXpress, an oxygen-sensitive phosphorescent dye (LUXCEL, Cork, Ireland). Mitochondrial respiration was measured in the presence of 1.65mM ADP (state 3 of mitochondrial respiration) and with substrates for complex I (5mM malate and 12.5mM glutamate) or complex II (25mM succinate). Rotenone (2μM), a specific inhibitor of complex I, was also added for the assessment of mitochondrial respiration with succinate. Oxygen consumption was then measured in real time for 60 min at 37°C in 96-well plates using a spectrofluorimeter (Tecan Infinite 200; λExcitation 380 nm; λEmission 650 nm). Rotenone (2μM) and oligomycin A (1μM) were used as 100% baseline for complex I and complex II inhibition, respectively. The areas under curve were used for calculations. To calculate the activation, the untreated mitochondria were taken as 0% activation. EC_{20} was the drug concentration causing 20% of the maximal inhibition or activation of oxygen consumption. For some fluorescent or colored compounds ($n = 17$) interfering with the MitoXpress probe, oxygen consumption through complex I and complex II was measured using a Clark electrode as described previously (Buron *et al.,* 2010).

Selection of parameters to detect drug-induced mitochondrial toxicity. Drugs can induce mitochondrial toxicity by altering mitochondrial membrane permeability and/or inhibiting the respiratory chain (Begriche *et al*., 2011; Labbe *et al*., 2008; Pessayre *et al*., 2010). These events can be rapidly studied on isolated mouse liver mitochondria by assessing the mitochondrial swelling, loss of $\Delta\Psi_m$, cytochrome *c* release, and oxygen consumption (i.e., respiration). Swelling and loss of $\Delta\Psi_m$ were comonitored by real-time spectrofluorimetry (Figs. 1A and 1B). Calcium (Ca^{2+}) was used as positive control inducing a massive mitochondrial swelling (Fig. 1A, left) and loss of $\Delta\Psi_m$ (Fig. 1B, left) through mitochondrial permeability transition pore (mPTP) opening with subsequent cytochrome *c* release (Fig. 1C, left). Importantly, the specific mPTP inhibitor CsA inhibited the Ca^{2+}-induced swelling (Fig. 1A, right), loss of $\Delta\Psi_m$ (Fig. 1B, right), and cytochrome *c* release (Fig. 1C, right). The OXPHOS uncoupler mClCCP was used to decrease the $\Delta\Psi_m$ in a mPTP-independent manner (Fig. 1B, right). Alamethicin was chosen as a positive control inducing a massive cytochrome *c* release (Fig. 1C). Besides mitochondrial membrane integrity, we also assessed the oxygen consumption with respiratory substrates oxidized by complex I (malate + glutamate) (Fig. 1D) or complex II (succinate) (Fig. 1E). The oxygen sensitive probe MitoXpress was used for most compounds for a rapid screening by spectrofluorimetry (Will *et al*., 2006).

Statistical analysis. In this study, data related to mitochondrial toxicity were only those obtained from our multiparametric assay, and thus data from the literature were not considered for statistical analysis. The relationship between mitochondriotoxicity (yes/no) and hepatotoxicity (yes/no) was assessed using a χ^2-test. Sensitivity, specificity, and predictive values of mitochondriotoxicity in term of hepatotoxicity were calculated as below:

$$\text{Sensitivity (Se)} = \frac{\text{No. of mitochondriotoxic and hepatotoxic compounds}}{\text{No. of hepatotoxic compounds}}$$

$$\text{Specificity (Sp)} = \frac{\text{No. of nonmitochondriotoxic and nonhepatotoxic compounds}}{\text{No. of nonhepatotoxic compounds}}$$

$$\text{Positive predictive value} = \frac{\text{No. of mitochondriotoxic and hepatotoxic compounds}}{\text{No. of mitochondriotoxic compounds}}$$

$$\text{Negative predictive value} = \frac{\text{No. of nonmitochondriotoxic and nonhepatotoxic compounds}}{\text{No. of nonmitochondriotoxic compounds}}$$

RESULTS

Multiparametric Screening of 124 Compounds, Mainly Drugs, on Isolated Mouse Liver Mitochondria

We measured the ability of numerous hepatotoxic (n = 87) and nonhepatotoxic (n = 37) compounds to induce mitochondrial toxicity (Table 1). Compounds able to induce liver injury were selected within the library created by Biour *et al*. (2004) and the corresponding updated "Hepatox" database (http://hepatoweb.com/hepatox.php). These 124 compounds were all tested for their ability to induce swelling, loss of $\Delta\Psi_m$, cytochrome *c* release, or an inhibition of the succinate-driven state 3 oxygen consumption. Compounds not altering these four parameters were then evaluated for their ability to inhibit oxygen consumption in the presence of malate and glutamate. For all these parameters, EC_{20} were determined in comparison with the 100% baseline obtained with their respective positive controls. It is noteworthy that some compounds (e.g., carbamazepine, erlotinib, lidocaine, saquinavir, spectinomycin, and zidovudine) increased oxygen consumption with malate/glutamate and/or succinate. Nevertheless, these compounds were considered as toxic for mitochondria. Indeed, enhanced oxygen consumption often reflects an increased mitochondrial entry of protons, which can be elicited by OXPHOS uncoupling, or by a global loss of inner mitochondrial membrane integrity (Begriche *et al.,* 2011; Fromenty and Pessayre, 1995; Labbe *et al.,* 2008).

Correlation Analysis Between Mitochondrial Toxicity and DILI in Human

Two different approaches were used to ascertain drug-induced mitochondrial toxicity (Table 2). In the first approach, a drug was considered as toxic for mitochondria if, for at least one of the five mitochondrial parameters, the EC_{20} was ≤ 100 × C_{max} (maximal plasma concentration), a cutoff currently used for safety assessment in pharmaceutical industry (Dykens *et al.,* 2008). Because C_{max} was not available for several compounds selected in this study, we used a second approach for which a compound was considered as toxic for mitochondria if the EC_{20} was ≤ 200µM for at least one of the five mitochondrial parameters. We indicated for each hepatotoxicant (Table 2A) the proposed (or suspected) mechanisms of toxicity, if any, and whether DILI was detected in animals. For nonhepatotoxic compounds (Table 2B) the occurrence of other organ toxicities was reported.

Using the first definition of mitochondrial toxicity (100 × C_{max}), only 114 compounds were taken into account (Table 3A). Interestingly, 81 of the 86 (94%) compounds able to induce DILI were found to be toxic for mitochondria, demonstrating a very high sensitivity. In contrast, 36% of the compounds not inducing DILI were found to be toxic for mitochondria. This difference was statistically significant and showed a significant relationship between mitochondrial toxicity and DILI ($p < 0.001$). With the second approach (EC_{20} ≤ 200µM), all the 124 compounds were included (Table 3B) and 80 of the 87 (92%) compounds known to induce DILI were found to be toxic for mitochondria. However, 49% of the compounds not inducing DILI were found to be toxic for mitochondria. This difference was also statistically significant ($p < 0.001$). Thus, by using two different definitions of mitochondrial liability, our study showed a highly significant relationship between toxicity on isolated mouse liver mitochondria and DILI in human.

Our results indicated positive predictive values of 89 (81/91) and 82% (80/98) depending on the definition of mitochondrial toxicity (Table 3). Thus, any compound found to induce mitochondrial toxicity with our multiparametric assay would have a high probability for inducing DILI in human. We also calculated the negative predictive values that provided a reasonable threshold for correct clinical outcome with 78 (18/23) and 73% (19/26) depending on cutoff (Table 3). Specificity calculated as 64 or 51% depending on cutoff was modest, probably due to sample size and involvement of mitochondrial toxicity

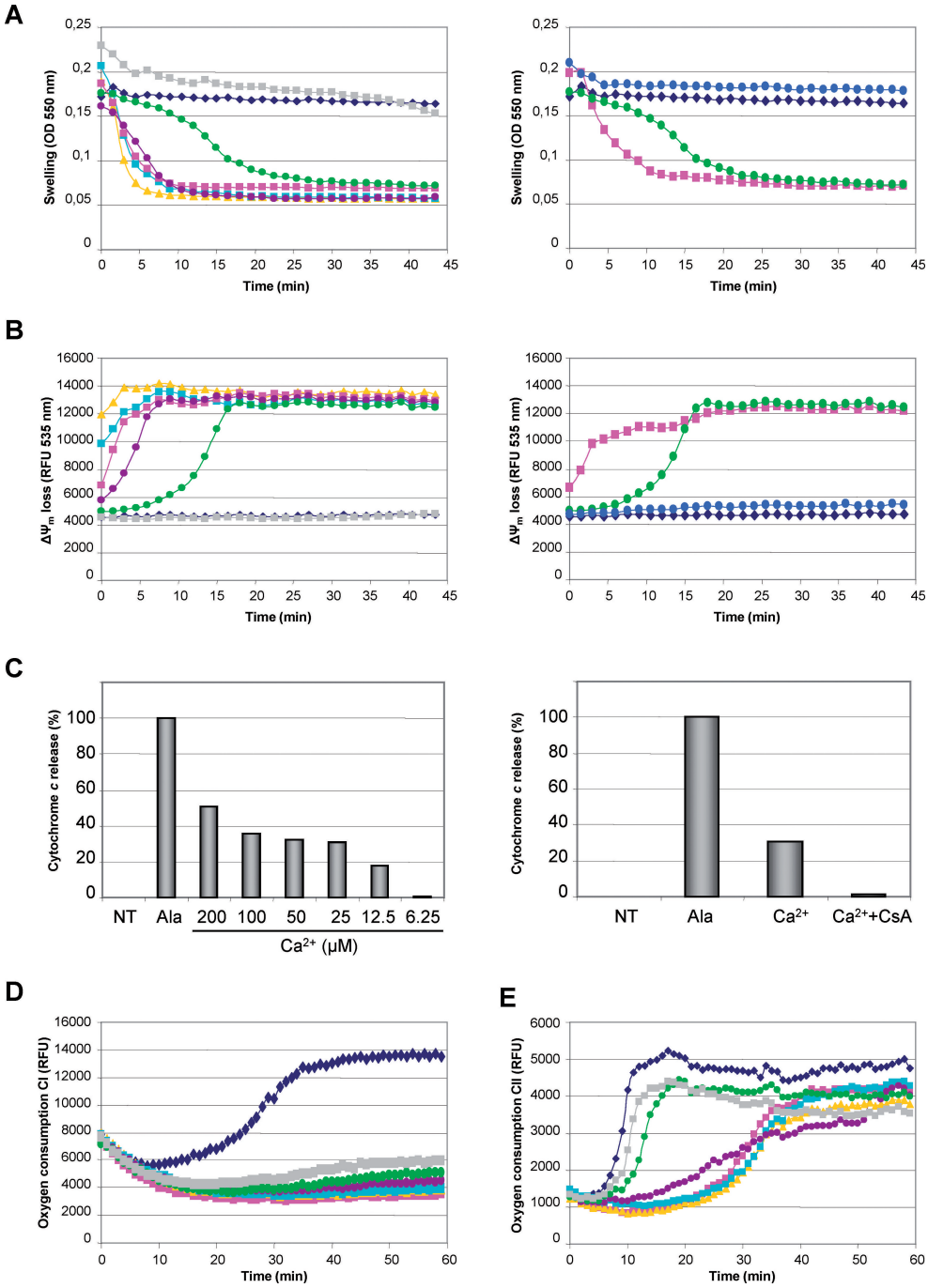

FIG. 1. Selected parameters used to detect mitochondrial alterations. (A) Mitochondrial swelling. Left panel: mitochondria isolated from mouse liver were untreated (◆) or treated with increasing Ca^{2+} concentrations before evaluation of mitochondrial swelling (▲200, ■100, ■50, ●25, ●12.5, and ■6.25μM). Right panel: massive mitochondrial swelling induced by 50μM Ca^{2+} (■) was taken as 100% baseline. Preincubation of mitochondria with 10μM CsA (●) fully inhibited mitochondrial swelling induced by 12.5μM Ca^{2+} (●), thus giving a curve similar to untreated mitochondria (◆). (B) Loss of $\Delta\Psi_m$. Left panel: loss of $\Delta\Psi_m$ was simultaneously measured in real time in presence of increasing Ca^{2+} concentrations (same symbols as left panel of Fig. 1A). Right panel: collapse of $\Delta\Psi_m$ induced by 50μM mClCCP (■) was taken as 100% baseline. Preincubation with 10μM CsA (●) fully inhibited the collapse of $\Delta\Psi_m$ induced by 12.5μM Ca^{2+} (●), thus giving a curve similar to untreated mitochondria (◆). (C) Cytochrome *c* release. Left panel: supernatants of Ca^{2+}- and alamethicin-treated mitochondria were subjected to ELISA assays to assess cytochrome *c* release. Massive cytochrome *c* release obtained with 20 μg/ml alamethicin was used as 100% baseline. Right panel: preincubation with 10μM CsA almost fully inhibited the cytochrome *c* release induced by 25μM Ca^{2+}. (D) Mitochondrial respiration through complex I. Oxygen consumption in the presence of malate, glutamate, and ADP was measured without (◆) or with rotenone (■2, ▲1, ■0.5, ●0.25, ●0.125, and ■0.062μM). The massive oxygen consumption inhibition caused by 2μM rotenone was taken as 100% baseline. (E) Mitochondrial respiration through complex II. Oxygen consumption in the presence of succinate, ADP, and rotenone was measured without (◆) or with oligomycin A (■1, ▲0.5, ■0.25, ●0.125, ●0.062, and ■0.031μM). The massive oxygen consumption inhibition caused by 1μM oligomycin A was taken as 100% baseline.

TABLE 1
Effects of the Compounds on Each Parameter of Mitochondrial Toxicity

Compound	Therapeutic class	Route of administration	Swelling EC_{20} µM	$\Delta\Psi_m$ loss EC_{20} µM	Cyto *c* EC_{20} µM	O_2 cons CII EC_{20} µM	O_2 cons CI EC_{20} µM	C_{max} µM
Acetaminophen	Analgesic	IV, PO, R	> 200	> 200	> 400	348.5	> 400	130
Acetylsalicylic acid	NSAID	PO	> 800	335.5	> 200	> 800	149.8	1650
Alpidem	Anxiolitic	PO	ND	83.7	394.7	25.6	29.6	0.3
Amantadine	Antiviral	PO	> 800	261.2	> 400	> 400	> 400	1.7
Ambroxol	Expectorant	PO	> 400	> 200	> 400	> 400	> 200	0.48
Amiodarone	Antiarythmic	IV, PO	ND	2.6	< 50	45.92	ND	0.81
Amoxicillin	Antibiotic	IM, IV, PO	> 400	> 400	> 400	90.8*	188.8*	14.1
Ampicillin	Antibiotic	IM, IV	> 400	> 400	> 400	> 400	161.2*	8.42
Antipyrine	NSAID	A, PO	> 400	300.0	> 400	> 400	> 400	92.5
Arsenic trioxide	Anticancer	IV	> 200	237.9	> 200	< 50	0.9	0.17
Atorvastatin	Hypolipidemic	PO	5.6	4.3	< 50	44.5	ND	0.06
Biotin	Nutritive agent	IM, IV, PO	> 400	> 400	> 400	> 400	> 400	< 1
Bisacodyl	Antihypertensive	PO, R	> 400	312.4	> 400	50.9	52.5	0.15
β-Estradiol	Hormone	C, IM, PO, V	ND	> 200	> 200	> 200	> 200	0.0006
Bupivacaine	Local anesthetic	IS	> 800	258.2	> 800	60.6	> 800	0.7
Busulfan	Anticancer	IV, PO	> 800	483.1	> 400	169.8	ND	4.9
Butein	Anticancer	IP	> 200	> 200	> 200	29.6	ND	ND
Caffeine	Analgesic	PO	> 400	> 400	> 400	> 400	> 400	42
Capsaicin	Topical analgesic	C	> 200	275.0	> 200	15.0	15.7	0.06
Carbamazepine	Anticonvulsivant	PO	> 200	66.9	> 400	53.4	170.8*	6.43
Cefixime	Antibiotic	PO	> 400	> 400	> 400	41.8	216.8	7.29
Chlorambucil	Anticancer	PO	> 200	> 200	> 200	138.7	140.9	1.97
Ciprofloxacin	Antibiotic	IV, PO	> 400	> 400	> 400	195.0*	ND	16
Clodronate	Antihypercalcemic	IV, PO	> 400	> 400	> 400	> 400	227.2	2.77
Clotrimazole	Antifungal	V	ND	23.9	> 800	2.9	ND	1.02
Coumarin	Anticoagulant	C, PO	> 200	0.9	> 100	ND	ND	1.25
Curcumin	Phytotherapy	PO	> 200	> 200	> 200	> 200	> 200	1.75
Dapsone	Antibiotic	PO	> 400	> 400	> 400	> 400	205.5	5.6
Dasatinib	Anticancer	PO	127.0	20.8	167.7	200.4	42.8*	0.23
Daunorubicin	Anticancer	IV	< 6.25	ND	47.2	12.8	10.9	78
Dexamethasone	Glucocorticoid	A, O, PO	> 200	> 200	> 200	> 200	> 200	0.23
Diazoxide	Antihypertensive	PO	> 200	> 200	> 200	4.9	ND	151.73
Diclofenac	NSAID	C, IM, PO	> 800	137.9	> 200	9.1	29.8	4.2
Diflunisal	NSAID	IV, PO	> 200	17.9	> 200	9.8	ND	495
Dipyrone	Analgesic	IV, PO	> 200	354.4	> 200	136.0	107.3	34.5
Disulfiram	Antialcoholism	PO	> 400	12.6	> 400	< 100	ND	5.4
Doxorubicin	Anticancer	PO	85	8.9	23.3	15.9	ND	0.2
D-penicillamine	Immunosuppressor	IV	> 400	> 400	> 400	> 800	> 800	53.62
Econazole	Antifungal	C, V	30.5	13.3	298.7	4.2	ND	540
Erlotinib	Anticancer	PO	ND	195.9	ND	328.8	17.4*	13.79
Erythromycin	Antibiotic	C, IV, PO	> 400	> 400	> 400	293.7	27.9	11
Ethyl paraben	Preservative	C, PO	> 400	297.5	> 400	293.1	47.8	ND
Folic acid	Vitamin	IV, PO	> 800	276.2	> 400	> 400	213.0	3.4
Fluconazole	Antifungal	PO	> 400	512.7	> 400	> 400	186.6	21.94
Flufenamic acid	NSAID	PO	> 200	42.5	> 200	1.7	ND	46
Flutamide	Antiandrogen	PO	> 800	27.0	> 400	ND	ND	6
Gallic acid	Antioxidant	PO	> 200	> 200	> 200	103.7	153.9	2
Gefitinib	Anticancer	PO	63.6	9.6	50.5	269.6	ND	0.72
Genistein	Anticancer	PO	> 200	150.6	> 200	81.3	ND	1.84
Gentamicin	Antibiotic	IM, IV, O	> 800	49.0	> 200	> 200	ND	13
Glimepiride	Antidiabetic	PO	ND	94.6	> 800	16.6	ND	0.5
Gossypol	Anticancer	PO	> 10	1.0	> 10	30.3	ND	1.99
Hyperforin	Antidepressant	PO	> 10	0.1	> 10	ND	ND	0.23
Ibuprofen	NSAID	C, PO	> 200	355.9	> 200	170.1	132.1	250
Imatinib	Anticancer	PO	ND	25.7	163.6	ND	ND	2.71
Imipramine	Antidepressant	PO	274.4	74.8	> 400	75.5*	18.3*	0.6
Indomethacin	NSAID	O, PO, R	> 800	443.2	> 200	25.2	ND	6
Isoniazide	Antibiotic	PO	> 800	504.6	> 400	59.8	ND	40
Ketoconazole	Antifungal	C	> 400	243.0	> 400	> 400	2.9	7
Lamivudine	Antiretroviral	PO	> 400	347.2	ND	160.7	317.4	17

TABLE 1—*Continued*

Compound	Therapeutic class	Route of administration	Swelling EC_{20} µM	$\Delta\Psi_m$ loss EC_{20} µM	Cyto *c* EC_{20} µM	O_2 cons CII EC_{20} µM	O_2 cons CI EC_{20} µM	C_{max} µM
Lidocaine	Local anesthetic	A, B, O	> 800	339.5	> 800	> 400	188.2*	36
Lonidamine	Anticancer	IV, PO	660.0	138.1	> 800	18.9	ND	23.6
Lovastatin	Hypolipidemic	PO	71.5	43.6	118.2	4.4	6.2	0.01
Lumiracoxib	NSAID	PO	> 200	148.8	> 800	26.3	12.0	22.47
Manganese chloride	Nutritive agent	IV, PO	> 800	> 800	> 800	> 400	> 400	0.053
Mefenamic acid	NSAID	PO	> 400	49.7	> 200	10.1	ND	15.74
Mercaptopurine	Anticancer	PO	> 400	406.9	> 400	130.8	131.8*	1
Metformin	Antidiabetic	PO	> 400	388.4	> 800	> 400	351.8	4.5
Methimazole	Antithyroid	PO	> 400	> 400	ND	193.8	368.6	9.2
Methyldopa	Antihypertensive	PO	> 400	> 400	ND	> 800	63.1	11
Methyl paraben	Preservative	C, PO	> 200	> 200	> 200	> 200	94.8	ND
Mitomycin C	Antineoplastic antibiotic	IV	ND	> 200	> 200	4.9	ND	7.1
Mitoxantrone	Anticancer	IV	ND	7.00	> 400	ND	ND	1.9
Molsidomine	Antianginal	PO	> 400	> 400	> 400	> 200	> 200	0.31
Naloxone	Analgesic	IV	> 400	298.7	> 400	233*	> 400	0,00047
Nelfinavir	Antiretroviral	PO	ND	6.3	> 200	< 25	ND	6
Nicergoline	Anti-ischemic	PO	169.8	82.6	> 200	57.0*	42.7*	0.0002
Nicotine	Smoking deterrent	C, PO	> 400	> 400	> 400	> 400	312	0.09
Nifuroxazide	Antibiotic	PO	> 400	388.2	> 400	61.5	3.7	ND
Nilotinib	Anticancer	PO	ND	11.5	> 400	ND	71.4*	3.6
Nimesulide	NSAID	PO	> 200	10.1	> 200	< 25	ND	15
Nitrofurantoin	Antibiotic	PO	> 800	442.5	> 400	232.3	8.7	6
Novobiocin	Antibiotic	PO	289.5	351.6	> 400	88.2	173.5	1
Oxybutynine	Spasmolytic	PO	302.8	178.3	> 400	172.8	115.4	0.17
Pargyline	Stimulant laxative	PO	> 400	243	> 400	> 400	206.8	0.3
Perhexiline	Antianginal	PO	14.8	3.2	< 25	88.4	87.7	0.28
Phenylbutazone	NSAID	C	> 400	140.2	ND	11.4	ND	438
Phloroglucinol	Antispasmodic	IM, IV, PO	> 800	> 800	> 400	> 400	> 400	ND
Piroxicam	NSAID	IM, PO	> 200	101.7	> 200	224.5	6.6	5
Pravastatin	Hypolipidemic	PO	> 200	> 200	> 200	5.0	ND	0.10
Propyl paraben	Preservative	C, PO	> 800	162.8	> 800	63.0	28.4	ND
Propylthiouracil	Antithyroid	PO	> 800	391.5	> 400	66.9*	15.8*	42
Pyrazinamide	Antibiotic	PO	> 400	> 400	> 400	107.5	190.9	325
Ranitidine	Antiulcer agent	IM, IV, PO	> 400	368.1	ND	> 400	97.3	3.99
Resveratrol	Antiaging	PO	> 200	395.4	> 200	7.7	ND	0.18
Riboflavin	Vitamin	IV, PO	672.3	ND	> 400	264.6	182.8	0.0039
Rifampicin	Anti-infectious	IV, PO	> 800	> 800	> 200	124.1	ND	9
Ritonavir	Antiretroviral	PO	ND	24.8	ND	35.5	ND	7.07
Roxithromycin	Antibiotic	PO	310.0	244.8	> 400	104.9	ND	13.14
Saccharin	Sweetening agent	PO	> 400	> 400	> 400	> 400	179.4	147.37
Salicylic acid	NSAID	C, O	> 400	304.3	> 200	354.4	120.9	604.63
Saquinavir	Antiretroviral	PO	ND	10.3	> 400	21.1*	30.4*	0.37
Simvastatin	Hypolipidemic	PO	173.2	76.5	> 200	1.6	ND	0.02
Sorafenib	Anticancer	PO	ND	0.5	> 400	283.4	ND	5.60
Spectinomycin	Antibiotic	IM	> 400	> 400	> 400	185.9*	118.2*	322.97
Streptomycin	Antibiotic	IM, IV	417.5	78.8	ND	> 400	ND	52
Sucrose	Antiasthenic	PO	> 800	> 800	> 800	> 800	> 800	ND
Sulfamethoxazole	Antibiotic	IV, PO	> 400	> 400	> 400	> 400	> 400	159.9
Sulindac	NSAID	PO	> 200	> 200	> 200	> 400	35.6	19
Sumatriptan	Antiheadaches	N, PO, SCI	> 400	248.3	> 400	> 400	> 400	0.04
Sunitinib	Anticancer	PO	ND	23.2	ND	222.8	ND	70.8
Tamoxifen	Anticancer	PO	9.0	2.9	7.7	ND	ND	0.40
Taurine	Antiasthenic	IV	> 800	> 800	> 800	> 800	> 800	ND
Terbinafine	Antifungal	C, PO	20.6	12.5	> 50	ND	ND	4
Ticlopidine	Antiplatelet	PO	58.9	50.5	ND	ND	ND	7.06
Tolcapone	Anti-Parkinson	PO	> 400	3.9	> 400	ND	ND	27.81
Tolfenamic acid	NSAID	PO	335.3	7.1	> 200	3.7	ND	4.16
Tramadol	Analgesic	IV, PO	> 800	263.8	> 400	> 400	238.7*	1.9
Troglitazone	Antidiabetic	PO	> 200	3.4	> 400	3.9	6.0	6.60
Troxerutin	Antihemoroid	PO	> 400	> 400	> 400	39.3	170.5	0.004
Valproic acid	Antiepileptic	IV	> 800	267.2	> 800	> 400	44.9	902

TABLE 1—*Continued*

Compound	Therapeutic class	Route of administration	Swelling EC_{20} µM	$\Delta\Psi_m$ loss EC_{20} µM	Cyto *c* EC_{20} µM	O_2 cons CII EC_{20} µM	O_2 cons CI EC_{20} µM	C_{max} µM
Vinblastine	Anticancer	IV	185.7	25.7	> 400	5.6	102.3*	0.13
Ximelagatran	Anticoagulant	PO	> 400	535.7	> 800	85.6	341.8*	0.45
Zidovudine	Antiretroviral	IV, PO	> 200	416.1	> 200	> 800	242.0*	4

Note. Selected compounds ($n = 124$) were tested for their ability to induce swelling, loss of $\Delta\Psi_m$, cytochrome *c* release, and inhibition of mitochondrial respiration through complexes I and II. EC_{20} was calculated using GraphPad Prism 4. EC_{20} with an asterisk (*) indicates drug-induced acceleration of oxygen consumption. Results are means of two to three independent experiments (SD < 10%). The C_{max} found in the literature (PubMed) or databases (Pharmapendium, RxList, and ToxNet) are indicated. For each compound the therapeutic class and the usual route of administration are indicated (data from the Vidal and DrugBank databases). A, auricular; B, buccal; C, cutaneous; IM, intramuscular; IP, intraperitoneal; IS, intraspinal; IV, intravenous; N, nasal; ND, not determined; O, ophthalmic; PO, per os; R, rectal; SCI, subcutaneous injection; V, vaginal.

in other tissues. Interestingly, the false-negative group (i.e., nonmitochondriotoxic but hepatotoxic compounds) showed a low percentage (6 or 8%), which is encouraging in a screening assay contrary to the false-positive group (i.e., mitochondriotoxic but nonhepatotoxic compounds), which was much higher (36 or 49%).

Mitochondrial Toxicity of Compounds Already Known to Have Deleterious Effects on Mitochondria

In this study, we confirmed the mitochondrial toxicity of several chemicals mainly compounds for which such deleterious effect had already been demonstrated in different experimental models including rodent liver, primary cultured hepatocytes, and isolated mitochondria. This is, for instance, the case for alpidem, diclofenac, perhexiline maleate, and the highly debated troglitazone (Table 1; Fig. 2).

Alpidem was described to accelerate Ca^{2+}-induced mPTP, uncouple OXPHOS, and selectively inhibit the respiratory complex I on isolated rat liver mitochondria (Berson *et al.,* 2001). In this study, this anxiolytic drug induced $\Delta\Psi_m$ loss (EC_{20} = 83.7µM) and inhibited mitochondrial respiration for concentrations lower than 30µM, thus confirming a significant mitochondrial toxicity (Table 1; Fig. 2A).

The nonsteroidal anti-inflammatory drug (NSAID), diclofenac, was shown to uncouple OXPHOS and induce mPTP opening on isolated rat mitochondria (Masubuchi *et al.,* 2002). However, it is noteworthy that in this study, the assessment of mPTP opening was performed in the presence of Ca^{2+}. Interestingly, we found that diclofenac was unable to induce mitochondrial swelling and cytochrome *c* release (Table 1; Fig. 2B). Because our assay was carried out without Ca^{2+} pulse, these data suggest that diclofenac-induced mPTP opening occurs only when mitochondria are loaded with Ca^{2+}. We demonstrated a clear inhibition of mitochondrial respiration through complex I (EC_{20} = 29.8µM) and complex II (EC_{20} = 9.1µM) with subsequent $\Delta\Psi_m$ loss (Table 1; Fig. 2B).

Several studies demonstrated that perhexiline maleate can induce mitochondrial dysfunction by different mechanisms, including OXPHOS uncoupling and inhibition of different enzymes involved in the mitochondrial respiratory chain and fatty acid oxidation (Deschamps *et al.,* 1994; Kennedy *et al.,* 1996). In our study, we confirmed that perhexiline maleate inhibited mitochondrial respiration when substrates gave their electrons to complexes I and II (Table 1; Fig. 2C). Moreover, we discovered that perhexiline maleate induced mitochondrial swelling and cytochrome *c* release when very low concentrations were used (Table 1; Fig. 2C), indicating that this antianginal drug can seriously disturb the mitochondrial membrane integrity.

The antidiabetic troglitazone has been reported to impair mitochondrial function by different mechanisms including mPTP opening and mtDNA damage (Masubuchi *et al.,* 2006; Okuda *et al.,* 2010; Rachek *et al.,* 2009). However, as already mentioned for diclofenac, troglitazone-induced mPTP opening occurred in conditions of Ca^{2+} pulse (Masubuchi *et al.,* 2006; Okuda *et al.,* 2010). In this study, troglitazone was unable to trigger mitochondrial swelling and cytochrome *c* release (Table 1; Fig. 2D), thus indicating that Ca^{2+} preloading is mandatory for troglitazone-induced mPTP opening. Nevertheless, we demonstrated that troglitazone inhibited mitochondrial respiration and induced $\Delta\Psi_m$ loss when relatively low concentrations were used.

Mitochondrial Toxicity of Compounds Not Already Described to Induce Mitochondrial Dysfunction

This study allowed disclosing for the first time the mitochondrial toxicity of several drugs, such as lumiracoxib, nilotinib, and saquinavir (Table 1, Fig. 3). Lumiracoxib and saquinavir were reported to cause liver injury (http://hepatoweb.com/hepatox.php), but the mechanisms underlying this hepatotoxicity had not been elucidated. Further investigations will be required to determine whether lumiracoxib- and saquinavir-induced impairment of the respiratory chain (Table 1, Fig. 3) is indeed a key mechanism leading to liver injury. Although nilotinib hepatotoxicity has not been reported so far, this anticancer drug can induce moderate to severe cardiotoxicity (Brauchli *et al.,* 2010; Orphanos *et al.,* 2009).

TABLE 2A
Mitochondrial Toxicity of the 124 Selected Compounds

Compound	Mitotox		Known or suspected mechanism(s)	Animal hepatotox
	100 × C_{max}	200µM		
Acetaminophen	Y	N	RM, OS, M	Y
Acetylsalicylic acid	Y	Y	OS, M, Apop	
Alpidem	Y	Y	RM, OS, M	
Amiodarone	Y	Y	OS, M, SL	N
Amoxicillin	Y	Y	OS	N
Ampicillin	Y	Y	M	Y
Arsenic trioxide	Y	Y	M, Apop	N
Atorvastatin	Y	Y	IM, M, Apop	N
Bupivacaine	Y	Y	M, OS	N
Busulfan	Y	Y	OS	Y
Carbamazepine	Y	Y	RM, M	Y
Cefixime	Y	Y		Y
Chlorambucil	Y	Y	OS	
Ciprofloxacin	Y	Y	OS	N
Clotrimazole	Y	Y		Y
Coumarin	Y	Y	RM, OS	
Dapsone	Y	N	RM, OS	N
Dasatinib	Y	Y		N
Daunorubicin	Y	Y	OS	Y
Dexamethasone	N	N	SL, M	N
Diclofenac	Y	Y	RM, OS, M, Apop, IM	Y
Diflunisal	Y	Y	RM, M	N
Dipyrone	Y	Y		
Disulfiram	Y	Y	OS, M	N
Doxorubicin	Y	Y	OS, Apop	N
D-penicillamine		N	IM	N
Econazole	Y	Y		N
Erlotinib	Y	Y	RM	Y
Erythromycin	Y	Y	IBST, RM	N
Fluconazole	Y	Y		Y
Flufenamic acid	Y	Y	M	N
Flutamide	Y	Y	RM, M	N
Gefitinib	Y	Y	RM	Y
Gentamicin	Y	Y	M, OS	N
Glimepiride	Y	Y		N
Gossypol	Y	Y	M	
Ibuprofen	Y	Y	M	N
Imatinib	Y	Y		Y
Imipramine	Y	Y	RM, M	N
Indomethacin	Y	Y	M	N
Isoniazid	Y	Y	IM, RM, OS	N
Ketoconazole	Y	Y	RM, OS, M	N
Lamivudine	Y	Y	M	N
Lidocaine	Y	Y	M	N
Lonidamine	Y	Y	M, Apop	
Lovastatin	N	Y	M	N
Lumiracoxib	Y	Y	RM	
Mefenamic acid	Y	Y	M	N
Mercaptopurine	N	Y	OS, M, DNA Syn	N
Metformin	Y	N	M	Y
Methimazole	Y	Y	RM, OS	N
Methyldopa	Y	Y	IM, RM	N
Mitomycin C	Y	Y	RM, OS, DNA Syn	N
Mitoxantrone	Y	Y	RM, DNA Syn	N
Nelfinavir	Y	Y	SL, OS	Y
Nimesulide	Y	Y	M, RM, OS	N
Nitrofurantoin	Y	Y	IM, OS, M	N
Novobiocin	Y	Y	DNA Syn, M	N
Perhexiline	Y	Y	M	N
Phenylbutazone	Y	Y	M	N
Piroxicam	Y	Y	M	N
Pravastatin	Y	Y		Y
Propylthiouracil	Y	Y	IM	N
Pyrazinamide	Y	Y	OS	N
Ranitidine	Y	Y		N
Rifampicin	Y	Y	IBST, OS	Y
Ritonavir	Y	Y	Apop, SL	Y
Roxithromycin	Y	Y		N
Saccharin	Y	Y		
Saquinavir	Y	Y	SL	N
Simvastatin	Y	Y	Apop, M	N
Sorafenib	Y	Y	Apop	N
Spectinomycin	Y	Y		
Streptomycin	Y	Y	M	
Sulindac	Y	Y	IBST, OS, M	N
Sunitinib	Y	Y	Apop	N
Tamoxifen	Y	Y	M, RM, OS, SL	N
Terbinafine	Y	Y	RM	Y
Ticlopidine	Y	Y	RM, IM	Y
Tolcapone	Y	Y	M, RM	N
Tolfenamic acid	Y	Y	M	
Tramadol	N	N		N
Troglitazone	Y	Y	RM, M, OS, IBST, Apop	Y
Valproic acid	Y	Y	M, RM	N
Vinblastine	Y	Y	Apop	N
Ximelagatran	N	Y	IM	N
Zidovudine	Y	N	M	N
Compared to human	concordance:	94%	92%	
	error rate:	6%	8%	
Compared to animal	concordance:	100%	90%	
	error rate:	0%	10%	

DISCUSSION

Drug-induced mitochondrial dysfunction can cause several types of hepatotoxicity including cytolytic hepatitis, microvesicular steatosis, steatohepatitis, liver failure, and even cirrhosis (Begriche *et al.,* 2011; Labbe *et al.,* 2008; Pessayre *et al.,* 2010). Furthermore, by inducing mitochondrial liability, compounds can also damage other tissues, such as skeletal muscles, heart, and pancreas. Thus, mitochondrial toxicity should be detected as early as possible during drug development, and ideally during the steps of hit selection and lead optimization. In this context, we showed that a combination of high-throughput *in vitro* screening tests performed on isolated mouse liver mitochondria allowed predicting whether a drug can be potentially harmful for the human liver. Indeed, we found a strong correlation between mitochondrial dysfunction as assessed with our selected tests and

TABLE 2B

Compound		Mitotox 100 × C_{max}	Mitotox 200μM	Other organ toxicity
Amantadine		N	N	Heart
Ambroxol		N	N	
Antipyrine		Y	N	
Biotin		N	N	
Bisacodyl		N	Y	
β-Oestradiol		N	N	Pancreas
Butein			Y	
Caffeine			N	Reproduction
Capsaicin		N	Y	Skin
Clodronate		Y	N	Gastro intestinal
Curcumin		N	N	
Diazoxide		Y	Y	Kidney, pancreas (*)
Ethyl paraben			Y	Reproduction (*)
Folic acid		Y	N	Gastro intestinal, CNS
Gallic acid		Y	Y	
Genistein		Y	Y	Reproduction
Hyperforin		Y	Y	
Manganese chloride		N	N	
Methyl paraben			Y	Reproduction (*)
Molsidomine		N	N	
Naloxone		N	N	Heart
Nicergoline		N	Y	
Nicotine		N	N	
Nifuroxazide			Y	Pancreas
Nilotinib		Y	Y	Heart (*)
Oxybutynine		N	Y	Brain
Pargyline		N	N	
Phloroglucinol		N	N	
Propyl paraben			Y	Reproduction (*)
Resveratrol		Y	Y	
Riboflavin		N	Y	
Salicylic acid		Y	Y	Kidney
Sucrose			N	Kidney
Sulfamethoxazole			N	Pancreas
Sumatriptan		N	N	
Taurine			N	
Troxerutin		N	Y	
Compared to human	concordance:	64%	51%	
	error rate:	36%	49%	

Note. The compounds are listed for their ability to induce (Y) or not (N) mitochondrial toxicity on isolated mouse liver mitochondria. Mitochondrial toxicity was ascertained by two approaches using a different cutoff (100 × Cmax or 200μM). Blanks mean that the mitochondrial toxicity related to Cmax could not have been determined because the Cmax value was not found in the literature or databases. (A) Hepatotoxicants in human according to Biour *et al*. (2004) and to the updated "Hepatox" database (http://hepatoweb.com/hepatox.php) with indication of known (or suspected) mechanisms of DILI and detection of hepatotoxicity in animals during preclinical studies. (B) Nonhepatotoxicants in human with indication of known toxicity to other organs where (*) indicates known (or suspected) mitochondrial toxicity. Apop, apoptosis; DNA Syn, DNA synthesis; IBST, inhibition of bile salt transport; IM, immune-mediated; OS, oxidative stress; M, mitochondrial; RM, reactive metabolites; SL, stimulation of lipogenesis. Blanks are data not found in the databases or literature. The concordance and error rate between mitochondrial toxicity in our assay and human hepatotoxicity, and/or animal hepatototoxicity are indicated for each cutoff.

TABLE 3
Relationship Between Mitochondrial Toxicity and the Occurrence of DILI in Human

A.

Number (%) cutoff at 100 × C_{max}		Hepatotoxic compound		*p* value
		Yes $n = 86$	No $n = 28$	
Mitochondriotoxic compound	Yes	81 (94%)	10 (36%)	< 0.001 (χ^2)
	No	5 (6%)	18 (64%)	
		Predictive positive value: 89%		
		Predictive negative value: 78%		

B.

Number (%) cutoff at 200μM		Hepatotoxic compound		*p* value
		Yes $n = 87$	No $n = 37$	
Mitochondriotoxic compound	Yes	80 (92%)	18 (49%)	< 0.001 (χ^2)
	No	7 (8%)	19 (51%)	
		Predictive positive value: 82%		
		Predictive negative value: 73%		

Note. Results of the χ^2-test are reported with the two approaches used to establish mitochondrial toxicity induced by chemicals (A) ascertained with the 100 × C_{max} cutoff and (B) ascertained with the 200μM cutoff. The *p* values are indicated for both conditions.

hepatotoxicity in humans independently of the type of liver damage. Furthermore, our study unveiled mitochondrial dysfunction with some hepatotoxic compounds for which such a liability had not been previously reported. Finally, these multiparametric assays clarified the mechanisms of mitochondrial toxicity for some compounds already known to induce mitochondrial dysfunction.

Preclinical toxicological tests should have high positive predictive value and sensitivity. In this study, as far as hepatotoxicity was concerned, the positive predictive value of our high-throughput *in vitro* screening tests was over 82%, whereas sensitivity was excellent (> 92%). This indicates that the risk of DILI is expected to be high with new chemical entities affecting one (or several) of our selected mitochondrial parameters. From Table 2A, it appears that about 90% of human hepatotoxicants for which DILI was not detected during preclinical animal studies were found to be mitochondriotoxic in our assays. This was, for instance, the case for amiodarone, ketoconazole, and perhexiline, which showed mitochondrial toxicities using our testing platform. The concordance between mitochondrial toxicity measured in our assays and animal hepatotoxicity was 100% for the cutoff related to C_{max} and 90% for the second cutoff. This study indicates that our platform could allow a better predictivity compared with classical animal studies used during preclinical development. This is in keeping with studies reporting that the concordance between hepatotoxicity detected in animal studies and that observed in clinical practice is around 50% (Greaves *et al*., 2004; Olson *et al*., 2000). However, such valuable information on mitochondrial toxicity could

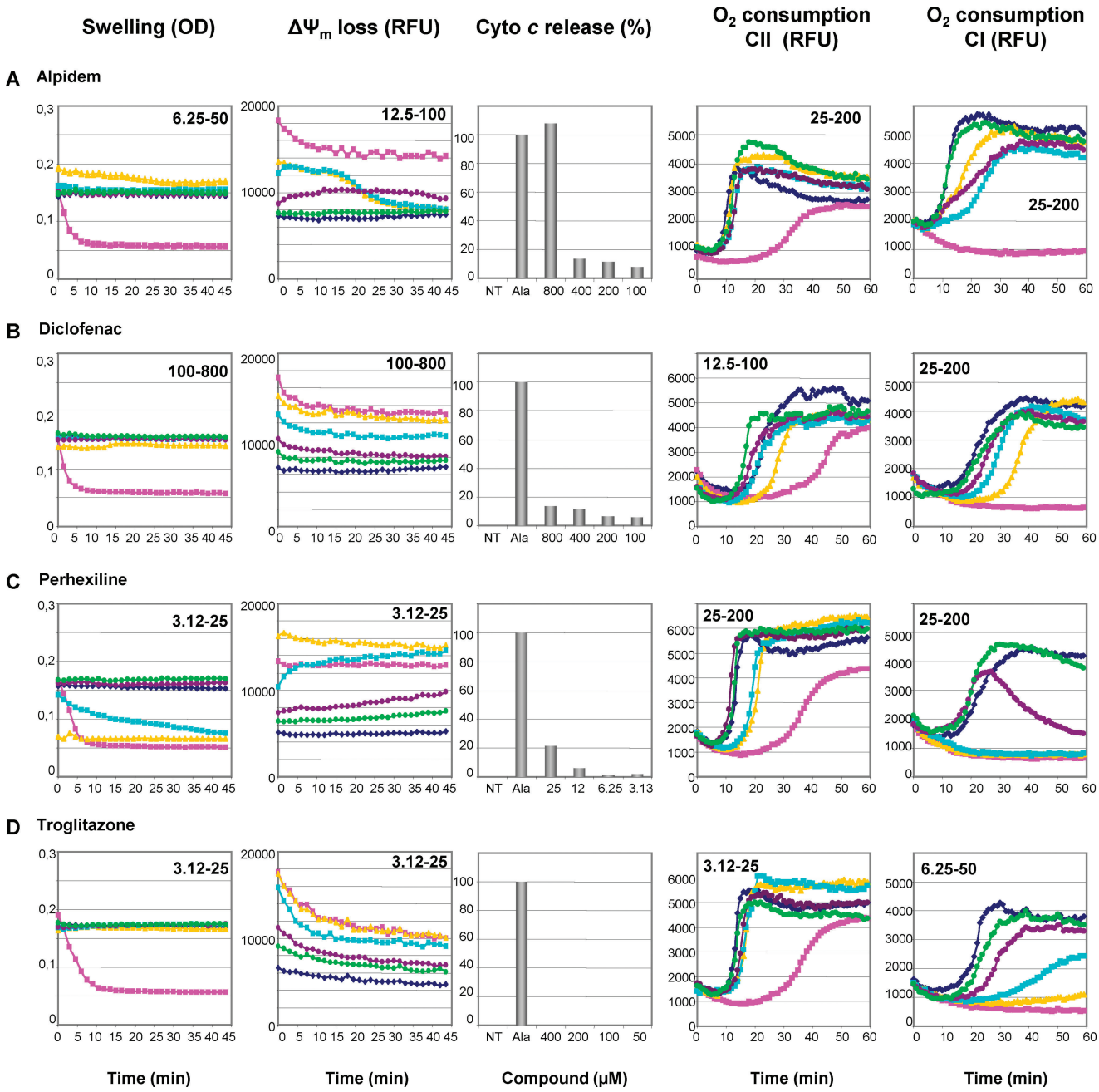

FIG. 2. Mitochondrial toxicity of compounds already described to cause mitochondrial dysfunction. Isolated mouse liver mitochondria were untreated (◆) or treated with increasing concentrations (●, ●, ■, and ▲; range indicated on the graphs) of alpidem, diclofenac, perhexiline maleate, and troglitazone, or with positive controls (■). Mitochondrial swelling, loss of $\Delta\Psi_m$, cytochrome *c* release, and inhibition of oxygen consumption were thus assessed as described in Materials and Methods and Figure 1.

not be used by itself as a decision-making go–no go strategy, but rather as a tool for ranking and prioritizing compounds in order to select safer candidates for subsequent *in vivo* preclinical safety investigations. Moreover, detection of mitochondrial toxicity with compounds of high pharmacological interest should prompt pharmaceutical companies to perform additional investigations using other *in vitro* studies or appropriate animal models (Boelsterli and Hsiao, 2008; Labbe *et al.*, 2008). Hence, in order to select drug candidates, each pharmaceutical company should consider the potential medical benefits of the selected compounds and their toxicological profiles determined during preclinical safety studies. For instance, some nucleoside analogs, such as stavudine (d4T) and zidovudine (AZT), are currently key components of the highly active antiretroviral therapy despite the occurrence of mitochondrial toxicity in a significant

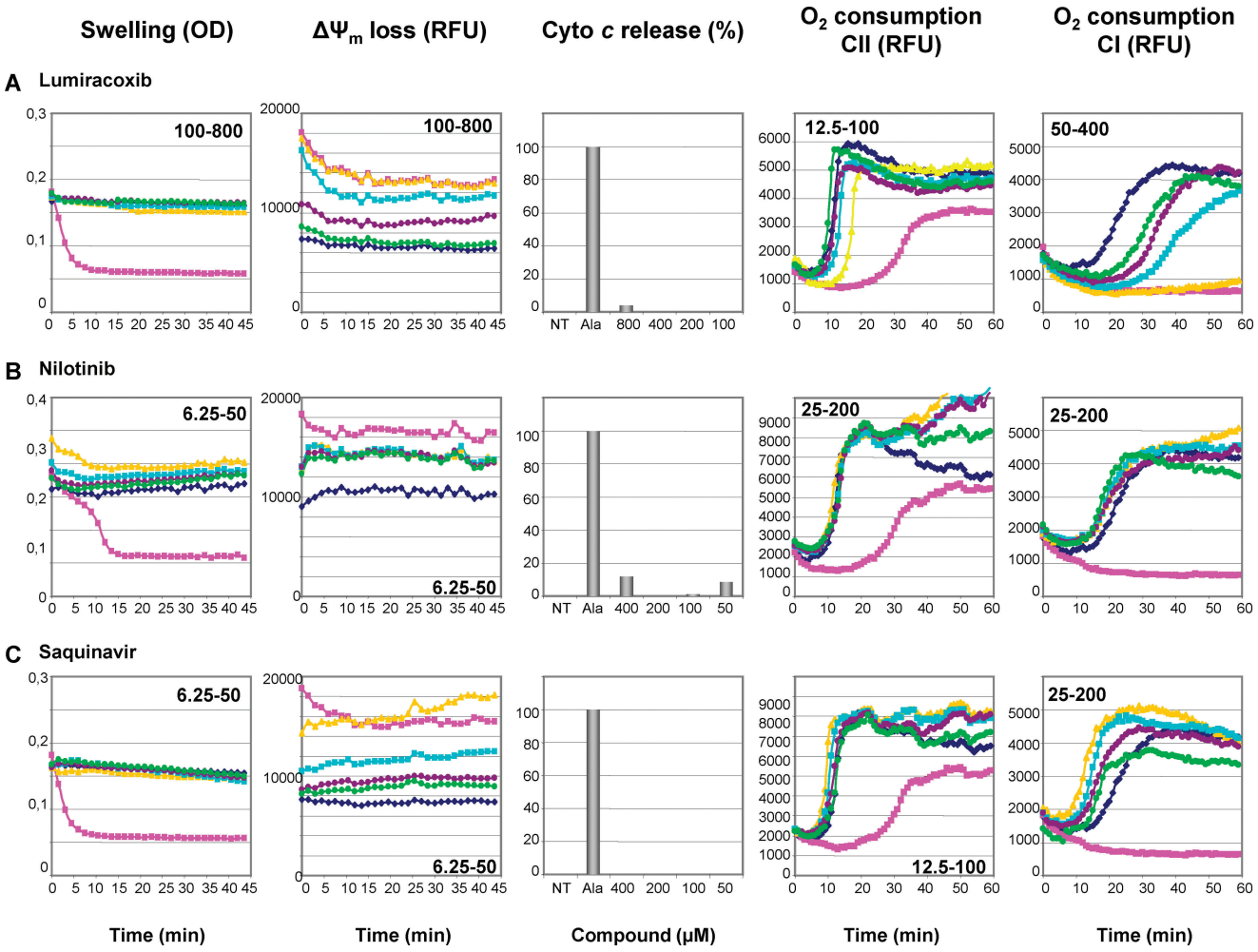

FIG. 3. Mitochondrial toxicity of drugs with no previously reported mitochondrial liability. Isolated mouse liver mitochondria were untreated (◆) or treated with increasing concentrations (●, ●, ■, and ▲; range indicated on the graphs) of lumiracoxib, nilotinib, and saquinavir, or with positive controls (■). Mitochondrial swelling, loss of $\Delta\Psi_m$, cytochrome *c* release, and inhibition of oxygen consumption were thus assessed as described in Materials and Methods and Figure 1.

number of treated patients (Begriche *et al.,* 2011; Fromenty and Pessayre, 1995; Labbe *et al.,* 2008).

The negative predictive value of our screening tests ranged between 73 and 78%. Accordingly, the lack of mitochondrial dysfunction observed with a new chemical entity cannot exclude the possible occurrence of subsequent liver injury in human. A probable explanation is that mechanisms other than mitochondrial dysfunction can be involved in DILI, such as specific immune reactions and impairment of mitochondria-independent lipid homeostasis (e.g., reduced VLDL secretion or enhanced *de novo* lipogenesis) (Begriche *et al.,* 2011; Lee, 2003; Russmann *et al.,* 2009).

If the false-negative group was very low (6 or 8%), the false-positive group was much higher (36 or 49%). This rather high level of false-positive results can be explained by the fact that drugs can be toxic to other organs through mitochondrial toxicity (Table 2B). Other reasons for detecting false positives could be attributed to the selected cutoff concentrations (which can be adjusted to the pharmacological efficient concentrations), or to the detection of transient and reversible mitochondrial dysfunction that will have no detrimental effect on liver integrity.

Because our screening tests are performed on isolated mitochondria, it can be argued that they cannot detect mitochondrial toxicity induced by CYP-generated reactive metabolite(s). However, it is noteworthy that Kamel *et al.* (2012) recently showed that a short incubation of isolated mitochondria with sumatriptan was sufficient to metabolize 30% of the compound. This is most probably due to the presence of some CYPs within mitochondria such as CYP2E1 (Robin *et al*., 2002), CYP1A2, and CYP2D6 (Buron and Borgne-Sanchez, unpublished data). Thus, it is possible that reactive metabolite(s) could be generated with some drugs during the assay and that the observed mitochondrial dysfunction resulted from the parent drug or from a CYP-generated toxic metabolite. It is noteworthy that, in this study, one-third of the investigated chemicals (with

identified mechanism of action) are metabolized into reactive metabolites (Table 2A). Interestingly, all these compounds were found mitochondriotoxic in our assay. Although further investigations will be needed, these data suggest that for some drugs mitochondrial toxicity could occur via the generation of reactive metabolite(s). It is noteworthy that besides parent drugs, metabolites can be also tested for their mitochondrial liability with our assay provided they can be synthesized in stable forms. Finally, in contrast to CYP-generated reactive metabolites, our screening tests on isolated mitochondria will be unable to detect some mitochondrial alterations requiring repeated and long-term exposure, such as mtDNA depletion (Begriche *et al*., 2011; Fromenty and Pessayre, 1995; Lebrecht *et al*., 2009). Although this mechanism of mitochondrial toxicity could be uncommon, this issue underscores the need to use other *in vitro* models, such as hepatoma cell lines, whenever long-term toxicity should be investigated.

Mitochondrial toxicity had already been described for numerous hepatotoxic compounds selected in our research program. However, our study allowed disclosing mitochondrial toxicity with some compounds for which such a detrimental effect had not been previously reported. For instance, we showed that lumiracoxib (a NSAID removed from the market for hepatotoxicity) strongly inhibited the oxygen consumption through respiratory chain complexes I and II (Table 1, Fig. 3A). We also found that the antiretroviral protease inhibitors nelfinavir, ritonavir, and saquinavir collapsed the $\Delta\Psi_m$ and altered the mitochondrial respiration for concentrations lower than 50μM (Table 1). Because the reduced mitochondrial respiration and $\Delta\Psi_m$ have been reported in cells treated with indinavir (Jiang *et al.,* 2007; Viengchareun *et al.,* 2007), our investigations suggest that mitochondrial toxicity could be common to the whole class of protease inhibitors. Moreover, we showed that low concentrations of the tyrosine kinase inhibitors gefitinib, imatinib, nilotinib, and sunitinib reduced the $\Delta\Psi_m$ (Table 1), although mitochondrial dysfunction has been reported only for gefitinib and sunitinib (Höpfner *et al.,* 2004; Kerkela *et al.,* 2009; Will *et al.,* 2008). In addition to the three antiretroviral protease inhibitors and the six tyrosine kinase inhibitors that are all hepatotoxic but one (nilotinib), we carried out investigations with other therapeutic classes that included more compounds. For instance, 16 antibiotics and 15 NSAIDs were studied, but only two drugs were not hepatotoxic in each of these classes. Thus, the low number of drugs not inducing DILI in these therapeutic classes precluded any conclusion regarding the performance of our assay for drugs belonging to the same therapeutic class but differing in their potential hepatotoxicity. Much more drugs should be studied in order to address this important issue.

Our study also permitted to clarify the mechanisms of mitochondrial toxicity for a few compounds already known to induce mitochondrial dysfunction. For instance, we established that low concentrations of flufenamic acid and tolfenamic acid inhibited the succinate-driven mitochondrial respiration and lowered the $\Delta\Psi_m$ (Table 1). However, although the latter effect is most probably due to OXPHOS uncoupling (Jordani *et al.,* 2000; Li *et al.,* 2009; McDougall *et al.,* 1983), inhibition of the respiratory chain has not been described so far with these anti-inflammatory agents. Further investigations will be needed in order to determine whether this novel mechanism plays a major role in flufenamic- and tolfenamic acid-induced hepatotoxicity. We also demonstrated that several paraben derivatives impaired the mitochondrial respiration, especially by inhibiting the glutamate/malate-driven respiration (Table 1). Previous investigations have only reported paraben-induced mPTP opening in the presence of Ca^{2+} (Nakagawa and Moore, 1999). Interestingly, paraben-induced liver abnormalities have recently been reported in rodents (Vo *et al.,* 2010). Thus, long-term exposure to parabens may cause liver toxicity in human and may represent a major health problem, regarding their widely spread use.

Several parameters were selected in this work in order to assess mitochondrial function because chemicals can be toxic for liver mitochondria by different mechanisms. However, it is currently not possible to tell which parameters could be considered as a primary marker because any significant disturbance of each of the selected parameters could have severe consequences on mitochondrial function and hepatocyte viability. For instance, whereas cytochrome *c* release can induce apoptosis, the loss of transmembrane potential can impair ATP production and lead to necrosis (Labbe *et al.,* 2008; Pessayre *et al.,* 2010). Moreover, from the current knowledge, it is also impossible to tell whether drugs inducing more than one parameter would induce DILI more frequently. Finally, it is important to underline that the different selected parameters can be interdependent. For instance, drugs impairing oxygen consumption can also disturb the transmembrane potential. Thus, more investigations are required to determine whether one particular mitochondrial parameter is better than the others in order to predict DILI.

In this study, hepatotoxicity was determined using the "Hepatox" database (Biour *et al*., 2004; http://hepatoweb.com/hepatox.php), which includes compounds inducing different types of injury, such as cytolysis, cholestasis, and steatosis. Even if numerous investigations showed that mitochondrial dysfunction plays a predominant role in hepatic cytolysis and steatosis (Begriche *et al.,* 2011; Fromenty and Pessayre, 1995; Labbe *et al.,* 2008; Lee, 2003; Pessayre *et al.,* 2010; Russmann *et al.,* 2009), no subclasses were done in this study. Indeed, the limited number of compounds within each DILI subgroup precluded a valid statistical analysis. In contrast, mitochondrial toxicity may not be a major mechanism involved in drug-induced cholestasis, although this issue will deserve further investigations because many bile salt transporters function in an ATP-dependent manner (Pellicoro and Faber, 2007). We plan to study additional compounds to determine whether our screening tests could provide a significant positive predictive value for one or several types of DILI.

In conclusion, our multiparametric assay performed on isolated mouse liver mitochondria may be an interesting tool for screening drug candidates and helpful to select safer compounds for further development. Indeed, our tests for screening drug-induced mitochondrial toxicity can provide rapid and valuable information that can be used to determine the potential ability of drug candidates to induce DILI. Moreover, this multiparametric assay can represent an appealing tool to decipher the mechanisms whereby marketed drugs induce dire side effects related to mitochondrial dysfunction.

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