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Hepatic cells derived from human skin progenitors show a typical phospholipidotic response upon exposure to amiodarone

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ABSTRACT

Phospholipidosis is a metabolic disorder characterized by intracellular accumulation of phospholipids. It can be caused by short-term or chronic exposure to cationic amphiphilic drugs (CAD). These compounds bind to phospholipids, leading to inhibition of their degradation and consequently to their accumulation in lysosomes. Drug-induced phospholipidosis (DIPL) is frequently at the basis of discontinuation of drug development and post-market drug withdrawal. Therefore, reliable human-relevant *in vitro* models must be developed to speed up the identification of compounds that are potential inducers of phospholipidosis. Here, hepatic cells derived from human skin (hSKP-HPC) were evaluated as an *in vitro* model for DIPL. These cells were exposed over time to amiodarone, a CAD known to induce phospholipidosis in humans. Transmission electron microscopy revealed the formation of the typical lamellar inclusions in the cell cytoplasm. Increase of phospholipids was already detected after 24 h exposure to amiodarone, whereas a significant increase of neutral lipid vesicles could be observed after 72 h. At the transcriptional level, the modulation of genes involved in DIPL was detected. These results provide a valuable indication of the applicability of hSKP-HPC for the quick assessment of drug-induced phospholipidosis *in vitro,* early in the drug development process.

1. Introduction

Drug-induced phospholipidosis (DIPL) is a lipid storage disorder characterized by intracellular accumulation of phospholipids in the affected tissues. It can be caused by short- or long-term administration of cationic amphiphilic drugs, CADs (Halliwell, 1997). There are more than 50 CADs that have been reported to cause phospholipidosis in several tissues in the human body, particularly in the liver and lungs (Reasor, 1989). CADs are classified according to their pharmacological effect. They encompass antibacterials (*e.g.* tetracycline), antiarrythmics (*e.g.* amiodarone), antimalarials (*e.g.* chlorochine), antidepressants (*e.g.* iprindole), and many others (Reasor et al., 2006). All CADs share a high affinity for phospholipids, thereby forming drug-phospholipid particles. The low degradation of these complexes leads to their accumulation in lysosomes (Schmitz and Müller, 1991). This further induces the formation of lamellar bodies (LBs) in the cytoplasm and/or lysosomes of affected cells (Anderson and Borlak, 2006; Schmitz and Müller, 1991). LBs are characterized by multilamellar, concentric whorls with deposition of granular material. The abnormal accumulation of LBs represents the hallmark of DIPL in humans (Anderson and Borlak, 2006). The mechanisms causing the induction of DIPL are still widely debated and may also vary from drug to drug (Anderson and Borlak, 2006). Several hypotheses have been put forward to explain the formation of CADs-phospholipid interactions and the implications of phospholipid accumulation. It is believed that CADs uptake into cells is facilitated by their common chemical structure, which consists of a hydrophobic ring structure and a hydrophilic side chain with a charged cationic amine group (Fig. 1). The non-polar ring increases the ability of the drug to pass through the non-polar layer of the cell membrane.

Abbreviations: CADs, cationic amphiphilic drugs; DIPL, drug-induced phospholipidosis; hSKP, human skin-derived precursors; hSKP-HPC, hSKP-derived hepatic progenitor cells; LB, lamellar bodies; PL, phospholipids; NL, neutral lipids.

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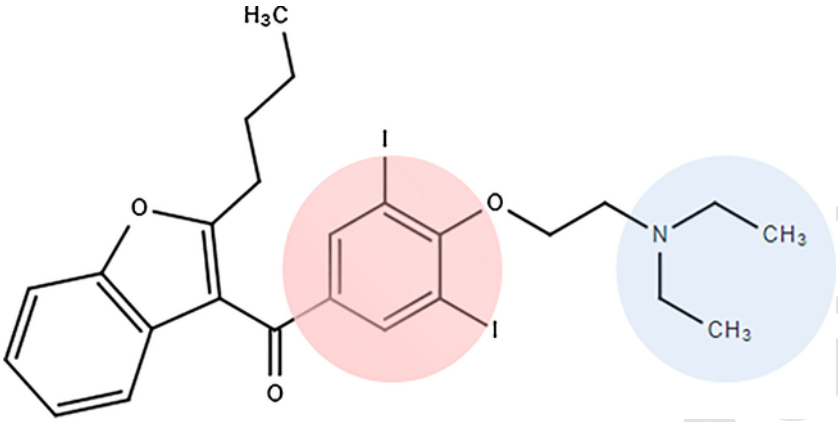

Fig. 1. Molecular structure of amiodarone. Amiodarone consists of a hydrophobic region with aromatic ring structure (red circle) and a hydrophilic region with amine group (blue circle). The non-polar ring increases the ability of the drug to pass through the non-polar layer of the cell membrane. In contrast, the polar chain, with the ionized amine group, promotes permanence of the drug in the membrane, causing the formation of intracellular deposits. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In contrast, the polar chain, with the ionized amine group, promotes permanence in the membrane (Lüllmann et al., 1978). Therefore, when a drug is incorporated, it gradually interacts with the phospholipid bilayer, causing the formation of intracellular deposits (Lüllmann et al., 1978). These CADs-phospholipids complexes are resistant to the degradation of lysosomal hydrolytic enzymes, such as phospholipases, which are involved in the degradation of cellular membranes (Anderson and Borlak, 2006). An additional theory speculates that DIPL may be induced by the suppression of lysosomal phospholipase activity. This could occur either by a direct inhibition of the enzyme by CADs or by an indirect mechanism involving the CADs-phospholipid binding (Reasor et al., 2006). Another explanation for the intracellular entrapment of CADs refers to their "lysosomotropic properties" (Goldman et al., 2009; Shayman and Akira, 2013). Amphiphilic drugs are usually weak bases that, once distributed by passive diffusion through the lysosomal membrane, are ionized in the acidic microenvironment of lysosomes. The continuous accumulation of the drug and conversion to ionized amines leads to a drop of the lysosomal pH, rendering the lysosomal hydrolases inactive (Goldman et al., 2009). Overall, it is assumed that the entrapment of CADs within the lysosome is responsible for the impairment of lysosomal enzyme activity, intracellular trafficking and dysfunction of the phospholipid metabolism (Hoestetler and Matsuzaway, 1981; Schmitz and Müller, 1991). Using a toxicogenomics approach, Sadawa and co-workers identified four main mechanisms of action that affect cellular functions of DIPL, namely (i) inhibition of phospholipase activity; (ii) inhibition of lysosomal enzyme transport; (iii) increased phospholipid biosynthesis; (iv) increased cholesterol biosynthesis (Sawada et al., 2005). It is important to mention that DIPL is often considered as a reversible condition, whereby phospholipid levels return to normal and the typical LBs disappear following cessation of drug administration (Reasor et al., 2006). As such, DIPL may represent a defensive mechanism rather than a cellular toxic response. If so, LBs act as cellular repositories by sequestering drug and undigested drug-phospholipid complexes within the cell in order to restrain any damage elsewhere in the tissue (Shayman and Akira, 2013). Regardless of the reversibility of this condition, data on adverse reactions induced by CADs is poor, rendering the clinical relevance of DIPL unclear (Berridge et al., 2007). Whether phospholipidosis causes hepatocellular injury is still uncertain, but it is speculated that secondary effects caused by increased cellular phospholipids may have serious consequences (Anderson and Borlak, 2006; Dragovic et al., 2016; Guigui et al., 1988; Lewis et al., 1990). Therefore, DIPL is still of concern for the pharmaceutical industry and remains a challenge in the risk assessment policy of public health agencies such as the Food and Drug Administration (FDA) (Berridge et al., 2007).

For decades, toxicity testing of phospholipidosis-inducers has been monitored in animals (Chatman et al., 2009). Yet, besides the ethical constraints associated with animal experimentation, there are some scientific drawbacks using this approach. Due to species differences, the extrapolation of data generated in animals to humans is unreliable and the predictive capacity is very low (Hartung, 2009). At present, histopathological identification of LB detected by transmission electron microscopy (TEM) is the "gold standard" approach to screen phospholipidotic compounds (Reasor, 1989). However, TEM is a time-consuming and expensive technique. For this reason, there is an increasing interest in developing quantitative and cost-effective human-based, accurate methods that could be used early in the drug development process to identify candidate drugs that are potential inducers of DIPL.

Human-based hepatic *in vitro* models have been shown to predict drug-induced liver injury (DILI). These cell models can recapitulate features of human responses and therefore provide relevant information for human safety. In this context, human postnatal stem cells are gaining *momentum* in toxicology. The possibility of isolating stem cells from several tissues and culturing them *in vitro*, as well as the ability to differentiate them into a number of specialized cell types provides an effective tool for drug discovery (Kitambi and Chandrasekar, 2011). hSKP are multipotent stem cells isolated from human skin. These cells have a high self–renewal capacity and plasticity (Toma et al., 2005). Our group has previously demonstrated that human skin precursors (hSKP), cultured in presence of specific hepatic growth factors, can be differentiated towards the hepatic lineage (Rodrigues et al., 2014). After hepatic differentiation, hSKP-derived hepatocyte-like cells (hSKP-HPC) express hepatic progenitor cell markers (EPCAM, NCAM2, PROM1) and adult hepatocyte markers (ALB), as well as key biotransformation enzymes (CYP1B1, FMO1, GSTA4, GSTM3) and influx and efflux drug transporters (ABCC4, ABCA1, SLC2A5) (Rodrigues et al., 2014).

These cells were shown to be able to predict the toxicity of compounds that induce hepatic liver failure, hepatitis B and steatosis (Rodrigues et al., 2016, 2015, 2014). hSKP-HPC exposed to acetaminophen showed a toxicological response similar to that of primary human hepatocytes *in vitro* (Rodrigues et al., 2014). In addition, hSKP-HPC exposed to sodium-valproate showed an excessive intracellular accumulation of lipid droplets. Transcriptomics analyses further revealed that this steatotic response was similar to what was observed in liver samples obtained from patients suffering from steatosis (Rodrigues et al., 2016).

In this study, we focus on the application of hSKP-HPC for DIPL detection. Hepatic differentiated hSKP are exposed to subcytotoxic concentrations of amiodarone, a CAD used in the treatment of cardiac arrhythmias and associated with the occurrence of hepatic phospholipidosis and the formation of LBs (Goldman et al., 1985). Here, we investigate whether DIPL is manifested in hSKP-HPC upon exposure to amiodarone and consequently causes changes at the functional and gene expression levels. The HepG2 cell line is used for comparison since these cells are widely employed in hepatotoxicity studies.

2. Material and methods

2.1. Human skin-derived precursors (hSKP)

hSKP were isolated, under informed consent, from human circumcised foreskin samples of 1- to 6-year-old boys. The cells were isolated as previously described (De Kock et al., 2012). Briefly, small tissue segments were cut in 5 mm^2 pieces and incubated overnight at 4 °C with liberase DH solution (Roche Applied Science). After mechanical and enzymatic dissociation steps, a cell population of approximately 1×10^6 cells per ml was obtained and seeded on 75 cm^2 cell culture flasks (T75) (Falcon™ BD). Cells were cultured as a suspension in growth medium that consisted of Dulbecco's modified Eagle medium (DMEM) + GLUTAMAX/F12 Nutrient Mixture (3:1; Thermo Fisher Scientific) supplemented with 7.3 IU/mL benzyl penicillin (Continental Pharma), 50 mg/mL streptomycin sulfate (Sigma-Aldrich), 2.5 mg/mL fungizone, 2% (v/v) B27 Supplement (Thermo Fisher Scientific), 40 ng/mL basic fibroblast growth factor (FGF) 2, and 20 ng/mL epidermal growth factor (both from Promega). Cells were cultured in suspension at 37 °C in a humidified incubator under an atmosphere of 5% (v/v) CO_2. Every 2–3 days the growth medium was supplemented with B27 Supplement. After 2 weeks of culture, the hSKP spheres were dissociated using 3 ml TrypLE Reagent™ (Thermo Fisher Scientific). The obtained cells were seeded at a density of 0.5–1×10^6 cells/cm^2 in T75 flasks containing growth medium supplemented with 20% (v/v) B27 and 5% (v/v) fetal bovine serum FBS (Hyclone) to allow cell attachment. The growth medium was refreshed after 24 h. Cells were passaged a second time, approximately 7 days later, to obtain sufficient cells. These were collected in a cryoprotectant solution composed of 90% (v/v) FBS and 10% (v/v) dimethylsulfoxide (cell culture grade; Sigma-Aldrich) at a density of 1×10^6 cells/mL and preserved in liquid nitrogen. For all further experiments, three different donor batches from the cryopreserved cell bank were used.

2.2. Hepatic differentiation of human skin-derived precursors (hSKPs-HPC)

Separate experimental runs were performed for three different donors. Cells isolated from each of these donors, were individually differentiated for 24 days to obtain hepatic-like cells from skin stem cells, as previously described (Rodrigues et al., 2014). Briefly, cryopreserved cells were thawed and cultured in growth medium as mentioned above. The cells were trypsinized and seeded on collagen-coated (0.1 mg/mL) T75 cell culture flasks and 24-well plates when 90% confluence was achieved. Basal culture medium (BM) was composed of 3 parts DMEM + GLUTAMAX and 1 part of F12 Nutrient Mixture (both Thermo Fisher Scientific) supplemented with 7.33 IU/mL benzyl penicillin (Continental Pharma), 50 mg/mL streptomycin sulfate (Sigma-Aldrich), 2.5 mg/mL fungizone (Thermo Fisher Scientific), 0.1 Mm L-ascorbic acid (Sigma-Aldrich), 4 mg/L L-nicotinamide (Sigma-Aldrich), 1 mg/mL linoleic acid-albumin (Sigma-Aldrich) and 27.3 mg/mL sodium pyruvate (Sigma-Aldrich). Differentiation was initiated when 100% cell confluence was achieved. During the differentiation protocol growth factors were added sequentially over 24 days, as follows: day 0, BM was supplemented with 50 ng/mL activin A (Thermo Fisher Scientific); day 1, BM was supplemented with 25 ng/mL activin A (Thermo Fisher Scientific), 5 ng/mL FGF4 (Biosource) and 10 ng/mL bone morphogenetic protein (BMP) 4; day 3, BM was supplemented with 10 ng/mL FGF4 (Biosource) and 20 ng/mL BMP4; day 6, BM was supplemented with 5 ng/mL FGF4 (Biosource), 10 ng/mL BMP4, 30 ng/mL hepatocyte growth factor (HGF) (Thermo Fisher Scientific) and 0.5% (v/v) insulin-transferrin-sodium-selenite (ITS) (Sigma-Aldrich); day 9, BM was supplemented with 30 ng/mL HGF (Thermo Fisher Scientific), 0.25% (v/v) ITS (Sigma-Aldrich) and 0.02 μg/mL dexamethasone (Sigma-Aldrich); day 12, BM was supplemented with 20 ng/mL HGF (Thermo Fisher Scientific) and 0.02 μg/mL dexamethasone (Sigma-Aldrich); day 15, 18, and 21, BM was supplemented with 20 ng/mL HGF (Thermo Fisher Scientific), 0.02 μg/mL dexamethasone (Sigma-Aldrich) and 10 ng/mL oncostatin M (Thermo Fisher Scientific). The differentiation protocol was completed at day 24.

2.3. HepG2 cell culture

The human hepatocellular carcinoma HepG2 cell line originates from the liver tissue of a 15-year-old Caucasian male affected by hepatocellular carcinoma. This cell line has been widely employed in hepatotoxicity studies (Dragovic et al., 2016; Schoonen et al., 2005; Weaver et al., 2017). HepG2 cells (ATCC; clone HB-8065) were grown as monolayers in T75 culture flasks at 37 °C in a humidified incubator under an atmosphere with 5% (v/v) CO_2. The HepG2 culture medium contained DMEM + 4.5 g/L glucose with ultraglutamine I (Lonza), 10% (v/v) FBS (Hyclone), 1% penicillin/streptomycin solution (Thermo Fisher Scientific). When 50% confluence was achieved, cells were passaged using TripLE Reagent™. Culture medium was refreshed every 2–3 days. The experiments were performed with HepG2 cells passaged 3 times after thawing.

2.4. Determination of subcytotoxic concentration of amiodarone

The subcytotoxic concentration of amiodarone in hSKP-HPC was evaluated by a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. Amiodarone was first dissolved in dimethyl sulfoxide (DMSO) and then further diluted in culture medium with a final concentration of 0.5% DMSO. Eight different concentrations of amiodarone ranging from 0.5 μM to 100 μM were prepared by serial dilution. Medium, containing DMSO (0.5%), was used as a control. The cell culture medium with the respective concentrations of amiodarone was refreshed every 24 h. The 72-h IC_{10} value (inhibitory concentration at which 10% of the cells died) was determined by a 4-parameter logistic nonlinear regression analysis of the obtained dose response curves using Masterplex Readerfit 2010 software (Hitashi Solutions, USA). The MTT-assay was performed in triplicate.

2.5. Isolation of total RNA and reverse transcriptase-polymerase chain reaction (qPCR)

After 24 h, exposed and non-exposed cells were collected in RNA lysis buffer solution (Sigma-Aldrich) containing β-mercaptoethanol (100:1). On average, three T75 culture flasks were used per condition. Total mRNA was extracted from all samples using a GenElute Mammalian Total RNA Purification Miniprep kit (Sigma-Aldrich), according to the manufacturer's instructions. The isolated RNA was quantified at 260 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Total mRNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (BioRad, Nazareth, Belgium) followed by cDNA pu-

rification with Genelute PCR clean-up kit (Sigma-Aldrich). Taqman probes and primers specific to the target genes of interest were used to perform qPCR reaction. All samples were processed in duplicate. Each run included two template-free controls and a serial dilution of a pooled cDNA mix from all cDNA samples to estimate the quantitative polymerase chain reaction (qPCR) efficiency. The qPCR reaction mix consisted of 10 µl TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific), 1 µl Assay-on-Demand Mix (Thermo Fisher Scientific), and 2 µl of cDNA in a 20 µl volume adjusted with DNase/RNase-free water. qPCR conditions, using a StepOne Plus system (Thermo Fisher Scientific) were as follows: incubation for 20 s at 95 °C, followed by 40 cycles of 1 s denaturation at 95 °C, and annealing for 20 s at 60 °C. Gene expression of hSKP-HPC (cell line from one donor) and HepG2 was conducted in triplicate.

2.6. qPCR data analysis

The following housekeeping genes were tested: ubiquitin C (*UBC*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hydroxymethylbilane synthase (*HMBS*), 18s ribosomal RNA (*18s*), beta-2-microglobulin (*B2M*) and beta-actin (*ACTB*).

Using geNorm (Biogazelle qbase+, Ghent, Belgium) and MIQE guideline, *UBC* and *B2M* genes were identified as the optimal reference genes for hSKP-HPC (Bustin et al., 2009). *HMBS* and *18s* genes were determined as reference targets in HepG2.

The relative mRNA levels of the gene targets were expressed in fold changes and normalized against the respective controls. The following genes were investigated (Table 1): stearoyl-CoA desaturase 1 *(SCD1)*, fatty acid elongase 6 (*ELOVL6)*, fatty acid synthase (*FASN)* and fatty acid desaturase 2 *(FADS2)*, lanosterol synthase (*LSS*), ATP binding cassette subfamily A member 1 (*ABCA1*), apolipoprotein B (*APOB*), fatty acid translocase (*CD36/FAT),* solute carrier family 2 member 3 (*SLC2A3*), clathrin-associated/assembly/adaptor protein, small 1 (*AP1S1*), phospholipase A group XV (*PLA2G15*), N-acylsphingosine amidohydrolase 1 (*ASAH1*), carnitine palmitoyltransferase 1 (*CPT1A*), acyl-CoA dehydrogenase short/branched chain (*ACADSB*).

2.7. Neutral lipid and phospholipid staining

Upon exposure to amiodarone for 24, 48, 72 h, hSKP-HPC were incubated with HCS LipidTOX™ (Thermo Fisher Scientific) to detect neutral lipids and phospholipids. Exposed and non-exposed cells growing in 24 multiwell plates, were fixed in 4% paraformaldehyde and stained with LipidTOX™ Green neutral lipid dye ($\lambda_{excitation}/\lambda_{emission}$ ~495/505 nm; emission in green) and LipidTOX™ Red phospholipidosis detection agent ($\lambda_{excitation}/\lambda_{emission}$ ~595/615 nm; emission in red) (Thermo Fisher Scientific). VECTASHIELD (Vector Laboratories), containing 4′,6-diamidino-2-phenylindole (DAPI) was used to counterstain the cellular nuclei and for bleaching protection. Fluorescent microscopy (Nikon Eclipse Ti-S) revealed the presence of neutral lipids and phospholipids in green and red channels, respectively.

2.8. Flow cytometry

Flow cytometry analyses (Attune Acoustic Focusing Cytometer, Thermo Fisher Scientific) were conducted in triplicate with hSKP-HPC isolated from one donor. Data were confirmed with hSKP-HPC isolated from two additional donors. The five datasets were included in the data analysis. For each donor, differentiated hSKP-HPC (both control cells and cells exposed to amiodarone for 24, 48 and 72 h), were detached from 24-well plates using TripLE Reagent™ and collected in phosphate buffered saline (PBS). At least four wells were pooled per condition. Different samples of exposed and non-exposed cells were stained with LipidTOX™ Green for neutral lipid dye (1:1000) + Hoechst 33342 (1:1000) or LipidTOX™ Green phospholipidosis detection agent ($\lambda_{excitation}/\lambda_{emission}$ ~495/525 nm) (1:1000) + Hoechst 33342 (1:1000). Cells were gated based on forward and side-scatter gating. A sample of unstained cells was used to set the gate of Hoechst- and LipidTOX-positive cells. Only the measured events that were positive for Hoechst were included in the data analysis.

Table 1
Analysed genes categorized according to their contribution to specific mechanisms of action involved in phospholipidosis.

Pathway	Gene symbol	Description	Function
De novo Phospholipid Biosynthesis	*SCD1*	Stearoyl-CoA Desaturase	Synthesis of oleate and palmitoleate (mono-unsaturated long-chain fatty acids) from stearoyl-CoA and palmitoyl-CoA (Song et al., 2011).
	ELOVL6	Fatty Acid Elongase 6	Elongation of mono-unsaturated long-chain fatty acids (Donald, 2012).
	FASN	Fatty Acid Synthase	Synthesis of palmitate (long-chain saturated fatty acids) from acetyl-CoA and malonyl-CoA (Donald, 2012).
	FADS2	Fatty Acid Desaturase 2	Desaturation of fatty to generate poly-unsaturated long-chain fatty acids (Song et al., 2011).
Cholesterol Metabolism	*LSS*	Lanosterol Synthase	Production of lanosterol (intermediate in biosynthesis of cholesterol) (Sakakura et al., 2001).
	ABCA1	ATP Binding Cassette Subfamily A Member 1	Cholesterol efflux transporter (Wang et al., 2001).
Lipid Transport	*CD36/FAT*	Fatty Acid Translocase	Binding to long-chain fatty acids and transport into cells (He et al., 2011).
	APOB	Apolipoprotein B	Carrier of lipids and cholesterol in very low density lipoprotein (VLDL) and transport to all cells (Feingold and Grunfeld, 2015).
	SLC2A3	Solute Carrier Family 2 Member 3	Mediation of glucose transport (César-Razquin et al., 2015).
Lysosomal Activity	*AP1S1*	Clathrin-Associated/Assembly/Adaptor Protein, Small 1	Link of clathrin to receptors in coated vesicles (Zhu et al., 1999).
	PLA2G15	Phospholipase A2 Group XV	Degradation of cellular membranes through hydrolysis of lysophosphatidylcholine to glycerophosphorylcholine and a free fatty acids (Shayman and Akira, 2013).
	ASAH1	*N*-Acylsphingosine Amidohydrolase 1	Degradation of ceramide to sphingosine (Li et al., 1999).
β-Oxidation	*ACADSB*	Acyl-CoA Dehydrogenase, Short/Branched Chain	Dehydrogenation of acyl-CoA in the metabolism of fatty acids (Fromenty et al., 1997).
	CPT1A	Carnitine Palmitoyl Transferase 1A	Mediation of mitochondrial uptake of fatty acids (Fromenty et al., 1997).

2.9. Transmission electron microscopy (TEM)

Electron microscopic detection of intracytoplasmic lamellar bodies is considered the most reliable method for the identification of phospholipidosis. After exposing, hSKP-HPC and HepG2 to 5 μM amiodarone for 72 h, cells were detached by trypsinization with TripLE Reagent™. Next, cells were rinsed in PBS and were fixed with 2.5% glutaraldehyde (Merck) in cacodylate buffer (0.1 M) (Merck) and stored at 4 °C. The cells were then post-fixed with 1% osmium-tetroxide and 2% uranyl acetate, dehydrated through ascending series of ethanol (immersed in propylene oxide for solvent substitution) and embedded in poly/bed 812 Araldite resin (Polysciences). Subsequently, ultrathin sections (0.05 μm) were cut with an ultramicrotome (Reichert-Jung), mounted on a copper grid and contrasted with lead citrate. Electron microscopy was performed using a Tecnai 10 Philips electron microscope and digital images were made using a mega view G2 CCD camera (SIS-company).

2.10. Statistical analysis

Data generated by flow cytometry were expressed as the median fluorescence intensity of non-exposed and exposed hSKP-HPC. Wilcoxon matched paired test (Graph Prism 7.0) was used for statistical analysis and a p-value smaller than .05 was considered statistically significant. Statistical analysis of qPCR data was performed using unpaired *t*-test (Graph Prism 7.0). Gene expression modulations with a p-value smaller than .05 were considered significant. Amiodarone was considered to have a strong effect when gene expression modulation was higher than 2 (fold change ≥ 2 or ≤ -2).

3. Results

3.1. Amiodarone induces accumulation of phospholipids and neutral lipids in hSKP-HPC

The subcytotoxic concentration of amiodarone was determined by exposing hSKP-HPC to a range of amiodarone concentrations for 72 h, followed by an MTT viability test. An IC_{10} value of 5 μM was defined and used in all further experiments. In parallel, HepG2 were exposed to the same concentration of amiodarone which is described to induce phospholipidosis in this cell type (Atienzar et al., 2007; Nioi et al., 2007). The 5 μM concentration is in the same order of magnitude of the plasma concentration of patients taking a therapeutic dosage of amiodarone (2 μM) (Lafuente-Lafuente et al., 2009; Pomponio et al., 2015).

Intracellular accumulation of neutral lipids (NL) and phospholipids (PL), determined by LipidTOX stainings, was observed in hSKP-HPC exposed to IC_{10} amiodarone for 24, 48 and 72 h (Fig. 2). As shown, phospholipid content was pronounced at all time-points (Fig. 2D–F). However, the accumulation of neutral lipid droplets was weak at earlier time points, becoming more visible at 72 h. Although sparse lipid droplets were also observed in control cells, their extent was marginal (Fig. 2A–C).

3.2. Lamellar bodies are detected in hSKP-HPC and not in HepG2 exposed to amiodarone

Morphological evaluation by TEM showed the presence of characteristic lamellar bodies in hSKP-HPC exposed to 5 μM amiodarone for 72 h (Fig. 3B and C). These ultrastructural lesions, have a diameter ranging between 0.2 and 1 μm and are densely packed in the cytoplasm. They consisted of concentric membranes arranged in a fingerprint pattern together with the deposition of granular material. These lesions occurred only in hSKP-HPC exposed to amiodarone. They were absent in the control condition (Fig. 3A). This finding is in line with previous reports in which the detection of intracellular LBs was seen as evidence of DIPL (Anderson and Borlak, 2006). This typical lamellar bodies were not detected in HepG2 cells exposed to amiodarone (Fig. 3E and F). Yet, neutral lipid and phospholipid droplets were observed in these cells.

3.3. hSKP-HPC exposed to amiodarone show accumulation of phospholipids at 24 h and neutral lipid droplets at 72 h

Flow cytometry analysis was performed to quantify the lipid storage of NL and PL. As shown in the histograms, the median fluorescence intensity of NL and PL was measured over 72 h between non-exposed control samples and cells exposed to amiodarone (Fig. 4A and C). With respect to the fluorescence intensity of phospholipids, there was a shift over time in exposed *versus* non-exposed hSKP-HPC (Fig. 4B). At 24 h after exposure, the phospholipid content in exposed cells was significantly higher ($p = .03$) than the control condition. Levels of phospholipids in the exposed cells progressively increased at 48 and 72 h. Yet, this decrease was not significant. On the contrary, the fluorescence intensity of the neutral lipids was strongly augmented at 72 h in amiodarone-exposed cells (Fig. 4D). Neutral lipid intensity in exposed cells did not show any significant change at 24 and 48 h when compared to the controls. After 72 h of exposure, the neutral lipid content raised, resulting in a significant increase ($p = .03$) in amiodarone-treated cells compared to control cells. Overall, the effect of amiodarone resulted in different time-dependent effects with respect to the PL- and NL- content of the cells as shown in Fig. 4E. An elevated PL storage appeared in exposed cells immediately after exposure to the drug and it was maintained over time. However, a modest increment in the content of neutral lipids was observed during the exposure time, with a significant increase detected at 72 h.

3.4. Amiodarone affects the modulation of phospholipidosis-associated genes in hSKP-HPC

Fourteen genes, potentially involved in the development of phospholipidosis, were investigated in hSKP-HPC and HepG2 cells. Exposure to amiodarone for 24 h induced the modulation of several genes in hSKP-HPC, whereas no important changes were observed in HepG2 cells (Fig. 5; Supplementary Table S1). The genes associated with the biogenesis of phospholipids were found to be strongly overexpressed in hSKP-HPC exposed to amiodarone. As such, stearoyl-CoA desaturase 1 *(SCD1)* (5.1-fold increase, $p = .0003$), fatty acid elongase 6 (*ELOVL6)* (2.2-fold increase, $p = .005$), fatty acid synthase (*FASN)* were highly upregulated (2.9-fold increase, $p = .008$). Fatty acid desaturase 2 *(FADS2)* was also found significantly upregulated (2.2-fold increase, $p = .02$). Also, two genes related to cholesterol metabolism were significantly modulated in hSKP-HPC. Indeed, the expression of lanosterol synthase (*LSS*) was upregulated (2.2-fold increase, $p = .02$) while that of the ATP binding cassette subfamily A member 1 (*ABCA1*) was downregulated (1.9-fold change, $p = .04$).

No significant change was observed in the expression of fatty acid translocase (*CD36/FAT)* and solute carrier family 2 member 3 (*SLC2A3*) mainly accounting for fatty acid and glucose transport, respectively. Yet, a downregulation was found for apolipoprotein B (*APOB*) (1.6-fold decrease, $p = .02$), which plays a role in the secretion of triglycerides from the cells.

A significant upregulation of lysosomal clathrin-associated/assembly/adaptor protein small 1 (*AP1S1*) and phospholipase A2 group XV (*PLA2G15*) genes was observed (both 2.3-fold increase, $p = .01$). Yet,

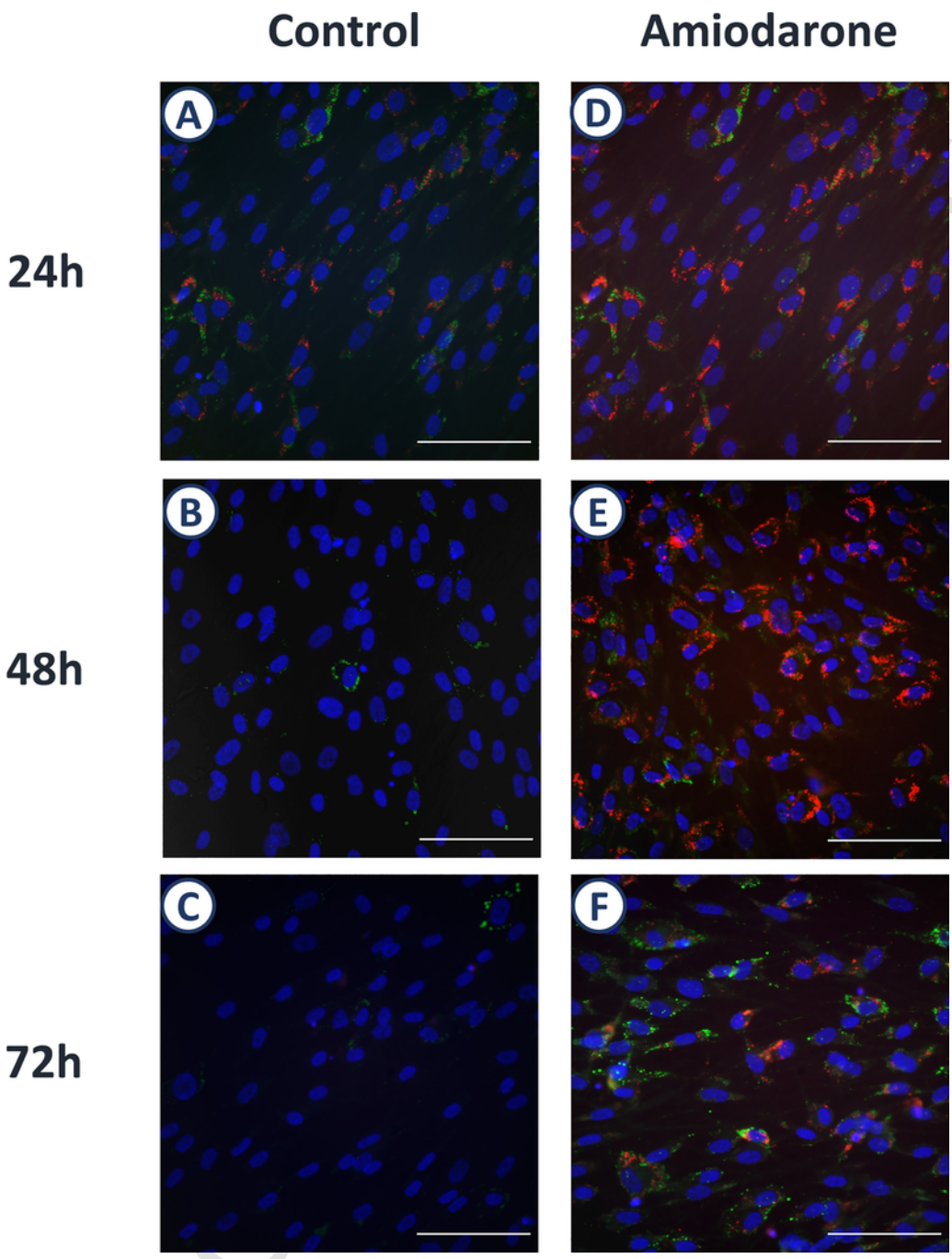

Fig. 2. Intracellular accumulation of neutral lipids and phospholipids by LipidTOX staining. LipidTox Green and LipidTOX Red fluorescence staining at 24, 48 and 72h in non-exposed and amiodarone-exposed hSKP-HPC. Phospholipids (in red) and lipid vesicles (in green) accumulate intracellularly in amiodarone-exposed cells (D–F). The presence of both phospholipids and neutral lipids is marginal in non-exposed cells (A–C). The images are representative of minimum 4 independent experiments. Scale bar indicates 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

no change of *N*-acylsphingosine amidohydrolase 1 (*ASAH1*) was induced by amiodarone.

Although a slight increase of carnitine palmitoyltransferase 1 (*CPT1A*) was observed (1.5-fold increase, p = .03), suggesting a higher fatty acid uptake by mitochondria, no increased β-oxidation was found when evaluating the acyl-CoA dehydrogenase (ACADSB) gene. Based on these findings, a schematic representation of the main phospholipidosis mechanisms triggered in hSKP-HPC is presented in Fig. 6. The gene expression profile of HepG2 cells was only marginally affected by amiodarone. In these cells, a very modest downregulation of *FASN* (1.4-fold decrease, p = .005), *FADS2* (1.4-fold decrease, p = .02), *LSS* (1.5-fold decrease, p = .02), *CD36* (1.5-fold decrease, p = .01), *ABCA1* (1.3-fold decrease, p = .02), *SLC2A3* (1.2-fold, p = .04), *PLA2G15* (1.2-fold decrease, p = .01) and *CPT1A* (1.3-fold decrease, p = .04) was observed. Other genes were not modulated.

4. Discussion

At present, many important aspects of DIPL remain unknown. For instance, it is not certain whether, DIPL causes hepatoxicity *per se* or triggers adverse consequences. Despite the fact that DIPL does not

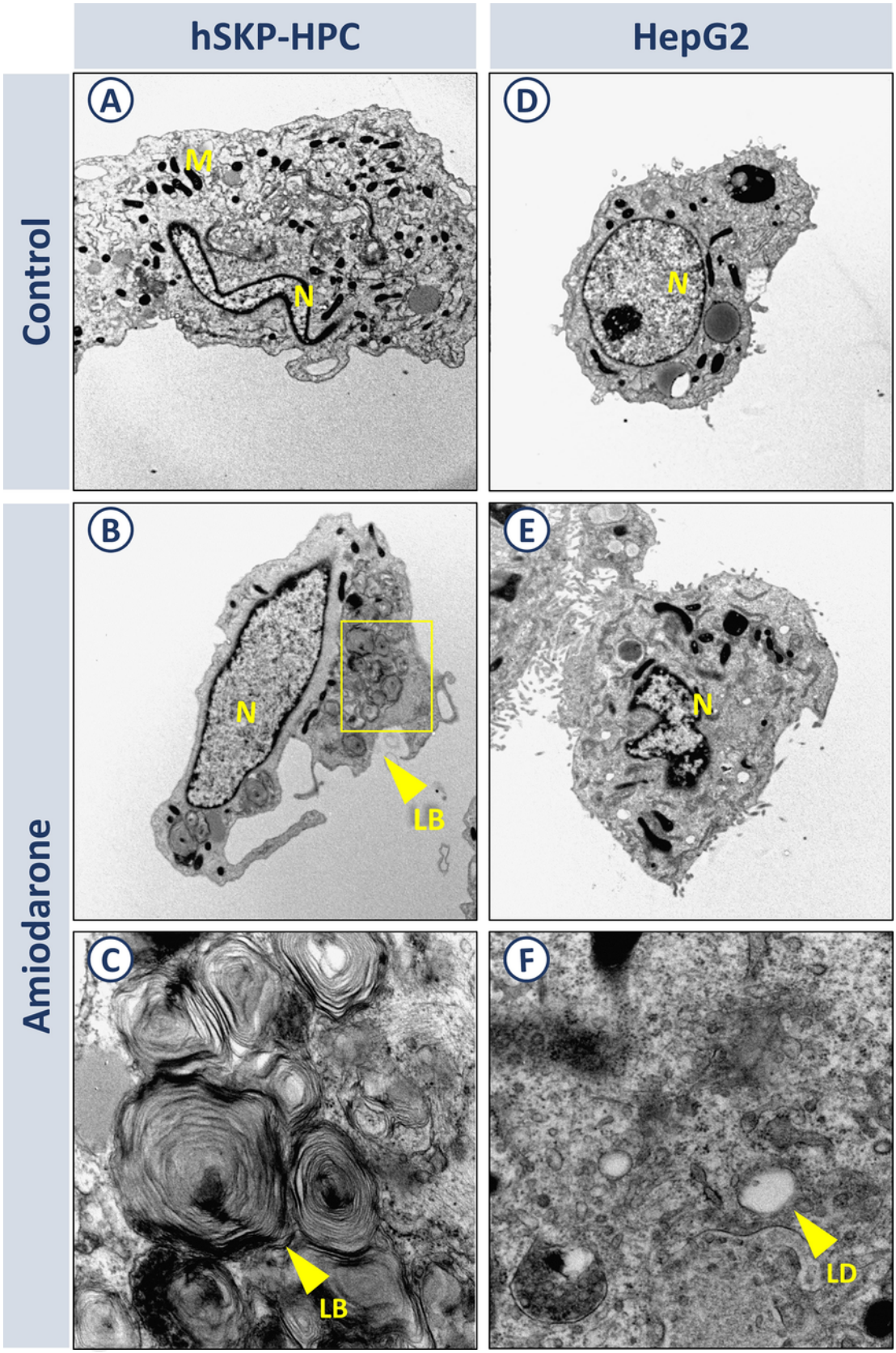

Fig. 3. Transmission electron microscopy in both non-exposed and amiodarone-exposed hSKP-HPC and HepG2. hSKP-HPC (A) and HepG2 cells (D) controls display normal intracellular structures such as the nucleus and mitochondria. Lamellar bodies (LB) can be detected in hSKP-HPC incubated with amiodarone (5 μM) for 72 h (B, C). HepG2 cells do not display these structures (E, F). Magnification 3700× (A, B, D, E); magnification 2400× (C, F).Abbreviations: LD, lipid droplets; LB, lamellar bodies; M, mitochondrion; N, nucleus. The images are representative of 3 independent experiments.

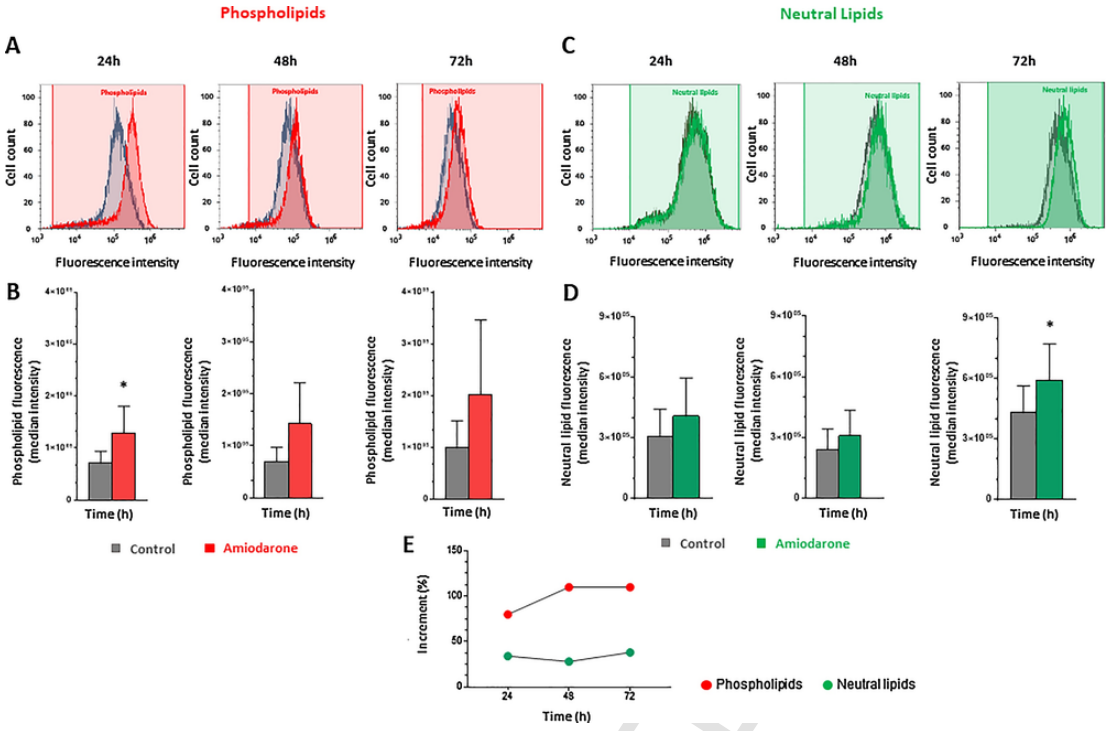

Fig. 4. Flow cytometric analysis of amiodarone-exposed and non-exposed hSKP-HPC for the quantification of phospholipids (PL) and neutral lipids (NL). Histograms represent lipid dye phospholipid-positive cells (in red), neutral lipid-positive cells (in green) in amiodarone-exposed *versus* non-exposed hSKP-HPC (in grey) (A, C). Pronounced accumulation of phospholipids is detected at 24 h, whereas accumulation of neutral lipids significantly increases at 72 h (B, D). Increase over time of PL and NL normalized to the respective controls (E). The data are expressed as median fluorescence intensity ± SEM of five independent experiments (Wilcoxon matched paired test). Significant value = *($p \leq .05$). The histograms are representative of one experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

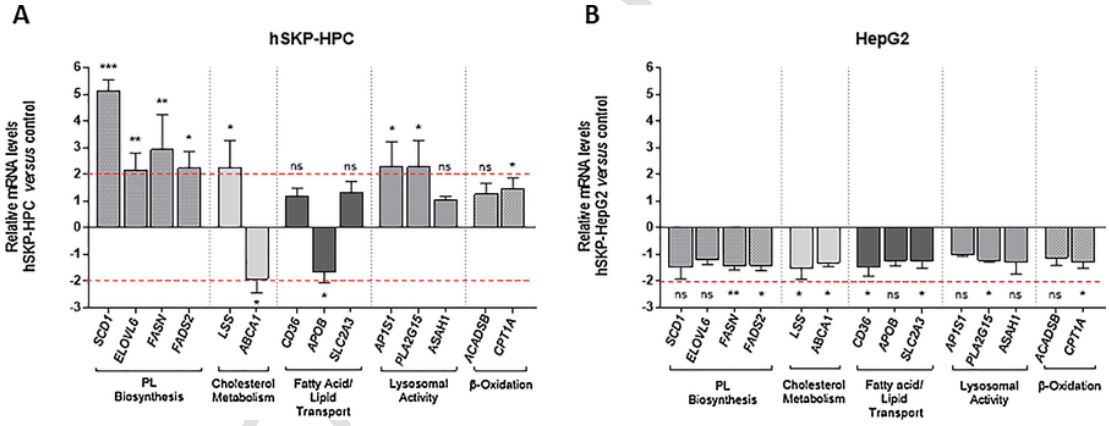

Fig. 5. Relative mRNA levels of specific genes in amiodarone-exposed hSKP-HPC and HepG2 *versus* respective controls. The values are expressed as mean ± SD of triplicate samples. Abbreviations: ns, unpaired student *t*-test with $p > .05$; *: unpaired student *t*-test with ($p \leq .05$); **: unpaired student *t*-test with $p \leq .01$; ***: unpaired student *t*-test with ($p \leq .001$). Gene symbols are described in Table 1.

show directly signs of toxicity in humans, secondary consequences such as integrity and maintenance of cellular homeostasis in the tissues of interest have not been ascertained yet (Chatman et al., 2009). Breakdown of lysosomes due to excessive phospholipid accumulation, could result in seepage of proteolytic enzymes into the cytoplasm and subsequent induction of cellular injuries (Nonoyama and Fukuda, 2008). Also, the similarity between DIPL and the genetic lysosomal storage disease of Niemann-Pick type C may influence the risk management strategies of regulatory authorities and pharmaceutical industry (Chatman et al., 2009). As stated earlier in a report by Chatman et al., phospholipidosis will probably not preclude final registration, but it may adversely affect labelling and marketing of new pharmaceutical products (Chatman et al., 2009). For these reasons, pharmaceutical companies monitor and possibly discontinue the development of drug candidates that could induce phospholipidosis. Currently, there are no predictive human-based methods for monitoring potential DIPL. Animal models are most often used, but besides ethical issues, they are not predictive for the human situation (Bouvier d'Yvoire et al., 2012; Nonoyama and Fukuda, 2008). Therefore, methods based on human cells or tissue represent a good opportunity to monitor for phospholipidosis during the preclinical phase of drug development.

Human primary hepatocytes represent a relevant *in vitro* tool because cells can be isolated from different donors, thus, offering the opportunity to evaluate variable drug responses related to different genetic backgrounds. However, their use is largely hampered by scarce availability, high cost, low quality of the resected tissue and loss of hepatic characteristics when the cells are cultured. Often, the success of isolation of good quality hepatocytes is influenced by fat liver content

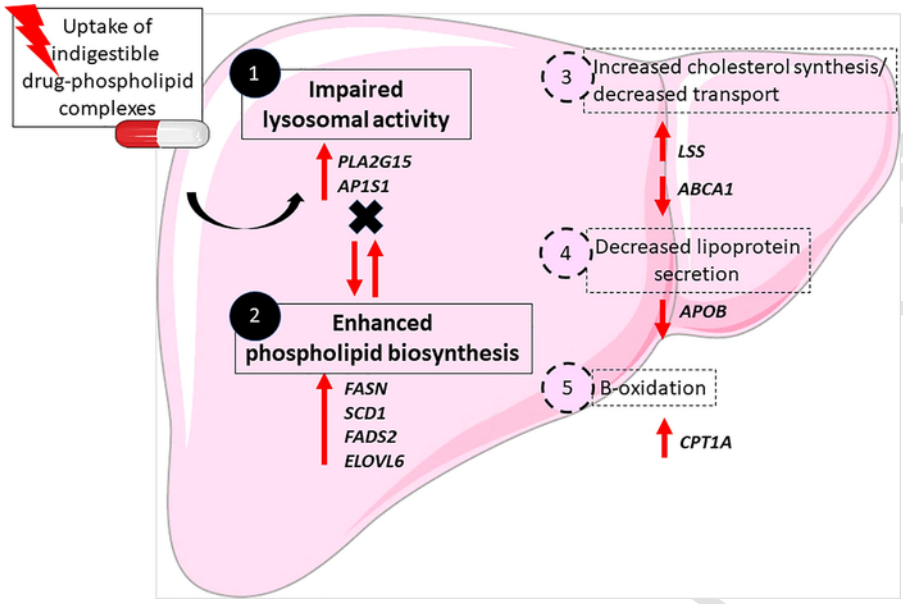

Fig. 6. Proposed molecular mechanisms in the development of phospholipidosis in hSKP-HPC. (1) Due to increasing uptake of indigestible drug-phospholipid complexes into the cell, the expression of phospholipase A2, as well as the transporter *AP1S1*, are augmented to counteract the phospholipid intracellular overload. Continuous accumulation of the cationic amphiphilic drug further causes inhibition of lysosomal enzymes. (2) Enhancement of phospholipid biosynthesis-related genes causes accumulation of undegraded lipids. (3) Increased cholesterol metabolism and decreased lipid transport could be indirectly involved in phosholipidosis. (4) Decreased secretion of VLDL could induce accumulation of neutral lipids. (5) B-oxidation could be involved as an adaptive mechanism due to excessive lipid accumulation. Solid boxes represent events with relevant evidence of being involved in phospholipidosis. Dashed boxes represent events that are possibly involved in phospholipidosis.

and liver damage of the donor coupled with ischemic events occurring during *in vivo* surgery and tissue transport. In addition, one of the most problematic disadvantages of human primary hepatocytes is the drastic decrease in hepatic characteristics over time. Up to now, the *in vitro* investigation of DIPL was mainly based on using human hepatocellular carcinoma cell lines such as HepG2 and HepaRG (Anthérieu et al., 2011; Atienzar et al., 2007; Donato and Gómez-lechón, 2012; Nioi et al., 2007; Sawada et al., 2005). Yet, these cells have several constraints associated with their malignant origin and abnormal genetic background and the fact that they do not recapitulate hepatocyte functionality adequately (LeCluyse et al., 2012). In addition, each of these cell lines is isolated from a single donor. Therefore, they do not represent the population diversity.

Human mesenchymal stem cells might contribute to setting up an accurate *in vitro* platform for hepatotoxicity testing (Davila et al., 2004; Rodrigues and De Kock, 2014). Our laboratory previously developed an efficient hepatic differentiation protocol. At the end of hepatic differentiation, hSKP-derived hepatocyte-like cells co-express liver progenitor markers and adult markers and can predict hepatotoxicity *in vitro* (Rodrigues et al., 2016, 2014). Skin stem cells are mesenchymal stem cells which can be extensively expanded *in vitro*. These cells are highly proliferative, assuring a steady availability, while maintaining the capacity to differentiate in hepatic-like cells after thawing. In addition, since they can be derived from different genetic backgrounds, these cells can be used to investigate individual differences in drug susceptibility. Furthermore, the easy isolation from human waste tissues implies absence of invasiveness of the procedure together with significant economic advantages.

In the current study, postnatal stem cells retrieved from human skin of different donors are used to assess DIPL *in vitro*. Differentiated hSKP-HPC were exposed to amiodarone at a concentration (5 μM) of the same order of magnitude as that found in plasma of patients under amiodarone treatment (2 μM) (Lafuente-Lafuente et al., 2009; Pomponio et al., 2015). Hereby, a physiological relevant condition was mimicked *in vitro*. In the current *in vitro* setting the effects of amiodarone could also be monitored in time, allowing the evaluation of their dynamic characteristics.

Over a 72-h period exposure, amiodarone induced increased levels of phospholipids starting at 24 h and this effect persisted during the full exposure time. On the contrary, accumulation of neutral lipid vesicles, which is associated with the manifestation of steatosis, was only prominent at 72-h.

The detection of lipid vesicles is associated to hepatic steatosis in humans after chronic administration of amiodarone (Anderson, 2008). Evidence of intracellular storage of phospholipids could be related to acute effects induced by amiodarone while occurrence of microvesicular steatosis could be due to chronic exposure (Anthérieu et al., 2011). Consistent with the literature, the expression of phospholipid biosynthesis-related genes was triggered in hSKP-HPC (Fu et al., 2012; Nioi et al., 2007; Sawada et al., 2005; Song et al., 2011). Key genes of lipid metabolism, involved in the synthesis, elongation and desaturation of fatty acids, were significantly upregulated in exposed hSKP-HPC (Donald, 2012). Lysosomal-related genes were partially perturbed, as indicated by an upregulation of *AP1S1* and *PLA2G15*. *AP1S1,* a candidate marker for phospholipidosis, is *in vitro* reported to be downregulated by amiodarone (Nioi et al., 2007; Sawada et al., 2005). This gene encodes for the transport of lysosomal enzymes between the trans-Golgi network and the lysosomal compartment (Zhu et al., 1999). *PLA2G15*, also known as LPLA2, is a phospholipase which is localized in lysosomes and is involved in the degradation of cellular membranes (Hiraoka et al., 2006; Schaloske and Dennis, 2006). It is assumed that inhibition of phospholipase activity, confirmed by upregulation of phospholipid-degradation related genes is the primary mode of action responsible for DIPL (Sawada et al., 2005). Interestingly, none of the previous studies on DIPL in human cancer cell lines, showed gene expression modulation of this phospholipase. It is possible that, activation of *PLA2G15* in hSKP-HPC occurs in response to phospholipid accumulation to clear these intracellular deposits and inhibition of the phospholipase activity may occur afterwards.

As expected, *ASAH1* gene was not modulated in hSKP-HPC. *ASAH1 is* considered a candidate marker for DIPL and encodes for a lysosomal

enzyme that hydrolases ceramide to sphingosine (Li et al., 1999; Sawada et al., 2005). Limited expression of *ASAH1* in response to a low dose of amiodarone was in line with previous studies, whereas higher toxic concentrations were able to upregulate the expression of this gene (Nioi et al., 2007). Cholesterol metabolism and lipid transport were considered to indirectly prompt DIPL (Sawada et al., 2005). Cholesterol binds to phospholipids to regulate the composition of cellular membranes. Processes such as synthesis of cholesterol and its efflux transport outside the cell involve *LSS* and *ABCA1* genes, respectively (Sakakura et al., 2001; Wang et al., 2001). Suppression of *ABCA1* expression together with overexpression of *LSS* observed in hSKP-HPC also correlated with previous findings (Anderson and Borlak, 2006). It is assumed that a defective exit of cholesterol may further worsen the lipid storage inside the cell, thus contributing to phospholipidosis. We investigated whether alterations of mitochondrial β-oxidation could contribute to DIPL (Fromenty et al., 1997). However, only a modest modulation of *CPT1A* was detected and *ACADSB* transcript levels were absent in hSKP-HPC. Additionally, among the genes involved in the transport of lipids, *CD36* and *SLC2A3* were not induced in hSKP-HPC, whereas a tendency to a decreased expression of *APOB* could be seen (César-Razquin et al., 2015; Feingold and Grunfeld, 2015; He et al., 2011). Consequently, it is likely that lipid vesicles are retained in hSKP-HPC. Compared to hSKP-HPC, a distinct gene expression profile was observed when HepG2 cells were exposed to amiodarone. In this cell line only very moderate modulation of gene expression was observed. Among the markers for phospholipidosis, *ASAH1* and *AP1S1* genes were not significantly modulated. On the contrary, downregulation of *SLC2A3* transporter was in accordance with previously published results (Nioi et al., 2007; Sawada et al., 2005). Only a slight downregulation of *ABCA1* and *CPT1A* expression could be found in HepG2, comparable with the results in hSKP-HPC. However, in contrast with the findings in hSKP-HPC, the expression of phospholipase (*PLA2G15)* was downregulated. Concerning lipid transport, downregulation of *CD36* was observed but no change of *APOB* could be detected. Also, some genes important for phospholipid synthesis *(SCD1, ELOVL6)* did not show any significant change in HepG2. In addition, the expression of two PL synthesis-related genes (*FASN, FADS2*) and *LSS* (associated to cholesterol metabolism) was significantly downregulated in HepG2 cells. These results were different from those previously reported in HepG2 (Nioi et al., 2007; Sawada et al., 2005). A possible explanation could be found in the existence of a high variability among HepG2 sub-clone lines cultured in different laboratories (Hewitt and Hewitt, 2004; van Pelt et al., 2003). Overall, amiodarone showed at the gene expression level a stronger effect in hSKP-HPC cells compared to HepG2. In particular, genes involved in lysosomal activity and biosynthesis of new phospholipids, initiating DIPL events, are strongly induced in hSKP-HPC.

In conclusion, this study shows that hepatic cells derived from human skin progenitors exhibit typical phospholipidosis characteristics upon exposure to a DIPL-inducer. The cells retain intracellular phospholipids, form lamellar bodies and show alterations at the transcriptional level that elucidate the molecular mechanisms leading to DIPL. Although only one compound was tested, this is a proof of concept study that demonstrates the potential applicability of hSKP-HPC in an *in vitro* platform for screening DIPL-inducers. Clearly, additional phospholipidotic inducers should be further tested to confirm the robustness of this model to investigate DIPL.

The induction of phospholipidosis upon exposure to other clinically relevant cationic amphiphilic drugs such as for example fluoxetine, perhexiline and dieethylaminoethoxyhexestrol, which share similar mechanisms of action with amiodarone, is expected to induce a comparable response in hSKP-HPC (Satapathy et al., 2015).

Conflicts of interest

No competing financial interest exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxlet.2017.11.014.

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