Drospirenone induces the accumulation of triacylglycerides in the fish hepatoma cell
line, PLHC-1

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Abstract

Drospirenone (DRO) is one of the most commonly used progestins reaching the aquatic environment through wastewater treatment plant effluents. It is a progesterone receptor agonist, and as such, can act primarily in the brain and reproductive organs of fish. In order to better understand and predict its effects, this work evaluates the lipidomic changes induced in PLHC-1 cells after exposure to drospirenone at concentrations below the EC_{10} (1 and 10 μ M) by direct injection of the lipid extracts into a ESI(+/-) Orbitrap mass spectrometer. A significant accumulation of triacylglycerides, particularly long chain ones with unsaturated fatty acid moieties (TGs 46:2, 56:4-7; 58:5-8) and a concomitant decrease of diacylglycerides (DGs 32:1, 34:1-2, 36:1-2, 38:2-4) was observed after 48 h exposure to 10 μ M DRO, which corresponded to an intracellular concentration of 8.3 $\text{ng}\cdot\text{mg}^{-1}$ protein. No significant alteration of PLHC-1 cell lipids was observed following exposure to 1 μ M DRO. EC_{50} for the cytotoxicity of DRO ranged from 105-119 μ M (24 h exposure) to 51-58 μ M (48 h exposure). The study evidences a dysregulation of neutral lipid metabolism and increased TG/DG ratio in fish hepatic cells exposed to DRO.

Keywords: Drospirenone; lipidomics; PLHC-1 cells; mass spectrometry

Introduction

Drospirenone (DRO, 6,7,15,16-dimethylene-3-oxo-17-pregn-4-ene-21,17-carbolactone) is one of the most commonly used contraceptives of the so called fourth generation progestins, which were developed to bind with greater specificity to the progesterone receptor and, unlike previous generations, to lack androgenic or estrogenic actions (Sitruk-Ware, 2000; Fent, 2015). DRO is an analogue of 17α -spironolactone, structurally related to testosterone and with a pharmacologic profile closely related to progesterone, which combines potent progestogenic and antiandrogenic activities. It is included in oral contraceptives and in hormonal replacement therapies in combination with ethinylestradiol or estradiol, respectively (Rapkin and Winer, 2007).

DRO reaches the aquatic environment through the effluents of waste water treatment plants. The number of studies that analyse the occurrence of this compound in the aquatic environment is still limited, but concentrations in the $\text{ng}\cdot\text{L}^{-1}$ range have been reported in effluents and fresh water samples (Avar et al., 2016; Golovko et al., 2018; Šauer et al., 2018); the presence of DRO in sediments and biota is still poorly documented. Some studies have suggested that progestins at the low $\text{ng}\cdot\text{L}^{-1}$ may pose a biological risk to fish (Maasz et al., 2017). As a key initiating event, DRO transactivated the fathead minnow nuclear Progesterone Receptor (PR) and acted as a weak agonist of rainbowfish PR (Ellestad et al., 2014; Bain et al., 2015). Gene expression analyses in brain and ovary of female zebrafish showed a down-regulation of vitellogenin mRNA and an enrichment of genes involved in steroid hormone receptor activity and circadian rhythm (Zucchi et al., 2014). Increased plasma levels of 17-hydroxypregnenolone, 17-hydroxyprogesterone and androstenedione and an up-regulation of *cyp17a1* were detected in juvenile sea bass exposed to $10\text{ ng}\cdot\text{g}^{-1}$ DRO

through the food (Blanco et al., 2016). Zeilinger et al. (2009) reported reduced fertility of fathead minnow exposed to DRO ($6.5 \mu\text{g}\cdot\text{L}^{-1}$), while no effect on reproduction or alteration in plasma steroid concentrations was observed after 21 days exposure to $100 \text{ ng}\cdot\text{L}^{-1}$ DRO (Runnalls et al., 2013).

Some progestins, particularly those with androgenic properties, are reported to alter blood levels of lipids and to diminish the beneficial effects of estrogens on lipoprotein metabolism (Jiang and Tian, 2017). The natural steroid progesterone was found to stimulate adipogenesis in 3T3-L1 cells (Rondinone et al., 1992). Progesterone also induced the upregulation of lipogenic enzymes in adipose tissue and hyperplasia of the smooth endoplasmic reticulum (SER) in the liver of rats; SER participates in lipid biosynthesis, detoxification processes and calcium regulation, among other functions (Boada et al., 2002; Stelmanska and Swierczynski, 2013). Although DRO was developed to minimize the impact on lipids when used for hormonal replacement therapies, increased levels of TGs and γ -glutamyltransferase, a biomarker of oxidative stress, has been detected in the serum of healthy women using oral contraceptives containing estradiol and DRO (Kowalska et al., 2017). Other studies have reported a marked dose-dependent inhibition of adipose differentiation and TGs accumulation in 3T3-L1 cells through the antagonistic effect of DRO on the mineralocorticoid receptor (Caprio et al., 2011). Nevertheless, despite of these evidences, the role of progestins in the regulation of lipid metabolism is not well established, and so far, information for aquatic vertebrates is lacking.

In recent years, advances in mass spectrometry have accelerated the development of lipidomics and have allowed characterizing the entire lipid content or lipidome of cells and tissues. The application of lipidomics to study the interaction of progestins with cell lipid dynamics will be extremely powerful in detecting and

quantifying changes not only in lipid classes (i.e. TGs), but also in individual lipid molecules, allowing the detection of subtle changes in lipid metabolism and the responsible molecular mechanisms (Han, 2016).

In this work, we applied a lipidomic-based approach to identify changes in the lipidome of the topminnow liver cells PLHC-1 exposed to non-cytotoxic concentrations of DRO for 24 and 48 h. The intracellular concentration of DRO and the concentration in culture medium were determined to improve the sensitivity and accuracy of the in-vitro approach, and to detect the minimum internal concentrations of DRO necessary to alter the lipidome of hepatic cells.

Materials and methods

Chemicals and reagents

Drospirenone was purchased from Sigma-Aldrich (Steinheim, Germany). Alamar Blue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) were from Invitrogen (Life Technologies; Eugene, OR, USA). Fluorescamine was obtained from Sigma (Steinheim, Germany). LC-MS grade water, methanol, dichloromethane and acetonitrile ($\geq 99\%$) were purchased from Merck (Darmstadt, Germany). Formic acid was from J.T. Baker® (Deventer, Netherland). Nitrogen (purity $>99.999\%$) was supplied by Air Liquide (Madrid, Spain). Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Cell culture and cytotoxicity assays

PLHC-1 cells (*Poeciliopsis lucida* hepatocellular carcinoma; ATCC® CRL-2406™) were routinely cultured in supplemented Eagle's Minimum Essential Medium (Gibco, Paisley, UK) at 30°C and 5% CO₂ as described in Pérez-Albaladejo et al. (2016). Experiments were performed in confluent cell monolayers (passage 7-26).

For the assessment of cytotoxicity, cells were seeded at a density of $7.5 \cdot 10^4$ and $6 \cdot 10^4$ cells per well in 96 well-plates and exposed to DRO for 24 and 48 hours, respectively. For the 48 h experiments, the medium was replaced with freshly spiked medium after 24 h exposure. Each treatment was performed in three different plates (six replicates per plate) with cells from different passages. The cytotoxicity of DRO was measured using AB (excitation/emission: 530/590 nm) and CFDA-AM (485/530 nm) as described in Pérez-Albaladejo et al. (2016).

Analysis of DRO in culture medium and cells

Cells were seeded in 24 well-plates at a density of $6 \cdot 10^5$ cells per well, allowed to attach overnight, and exposed to 10 µM DRO for 24h. For the 48 h exposure experiment, cell density at seeding was $4.5 \cdot 10^5$ cells per well, and cells were exposed to 1 and 10 µM DRO, replacing the medium after 24 h with freshly spiked medium. Each treatment was performed in three different plates (3 replicates per plate) with cells from different passages. Solvent control samples consisted in cells grown with culture medium containing 0.5 % DMSO. After exposure, an aliquot of 200 µL of medium was taken from each well, and the three replicates from the same plate were pooled (n=3). Cells were washed with PBS, trypsinized, centrifuged, and the pellets stored at -80 °C under argon atmosphere until extraction. One replicate per plate was used for intracellular analysis (n=3), while the other two wells were used for lipid analysis (n=6).

Intracellular concentration of DRO was measured for cells exposed to the higher concentration (10 μ M).

For the analysis of DRO in culture medium, samples were extracted with ethyl acetate (x3), evaporated to dryness and reconstituted in acetonitrile. DRO was analyzed by high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD). The chromatographic system consisted of an Agilent Technologies 1200 series equipped with a Zorbax Eclipse XDB-C18 column (4.6 $\times$ 150 mm, 5 μ m) (Agilent, USA) protected with a guard column (C18, 4.0 $\times$ 3.0 mm) (Phenomenex, USA) at a flow rate of 1 mL $\cdot$ min⁻¹. The injection volume was 20 μ L. The column was maintained at 30 $^{\circ}$ C and the mobile phase consisted of a gradient of water (A) and acetonitrile (B), which started at 50 % of B and increased to 100 % in 7 min, where was kept for 3 min, and reached initial conditions in 2 min, where it was maintained for 2 more minutes, with a total acquisition time of 14 min. Elution of DRO was monitored at 252 nm with a G1315D DAD model (Agilent 1200 series). Quantification was carried out by preparing external calibration curves in culture medium, which were extracted with ethyl acetate and reconstituted in acetonitrile. The extraction efficiency calculated with an external calibration curve prepared in acetonitrile was of $84 \pm 2\%$ (n=3; 5 μ M DRO).

The intracellular concentration of DRO after 24 and 48 h exposure was determined by UHPLC-MS/MS. Cells were extracted with ethyl acetate (x3) in an ultrasonic bath for 2 min, the extracts taken to dryness under nitrogen and reconstituted in acetonitrile for the analysis. The analysis was performed in an Ultra Acquity LC System, TQ Detector, (Waters, USA) using multiple reaction mode (MRM) as acquisition mode as described in Blanco et al. (2016). To determine the cells protein content, fluorescamine was added to each well and the fluorescence was read at the

excitation/emission wavelengths of 360/460 nm as described by Lorenzen and Kennedy (1993). Bovine serum albumin was used as standard.

Analysis of cell lipidome

Lipids from cells exposed to DRO as described above were extracted in an ultrasonic bath for 2 minutes using ethyl acetate (x3). Extracts were dried and stored at -20°C under argon atmosphere. Lipid extracts were analysed by FIA-Orbitrap-Exactive (Thermo Fisher Scientific, Bremen, Germany) as described in Marqueño et al. (2019). Briefly, 5 µL of the lipid extract conveniently diluted were infused to the instrument with a flow rate of 50 µL·min⁻¹ of methanol:dichloromethane (80:20). Mass spectra were acquired in full scan alternating positive and negative ionization polarity in a single run. LIPID MAPS, exact mass, isotopic distributions, charge, adducts formed, number of ring plus double bond (RDB = 0.5–15), and elements in molecular formula, were used for the identification of the lipid molecules with a maximum permitted mass error fixed at <5 ppm. Mass spectra were processed using Xcalibur 2.1 (Thermo Fisher Scientific, Bremen, Germany). Lipid species were annotated as <lipid class> - <total fatty acyl chain>:<total number of double bonds>, and quantified (pmol·mg⁻¹ protein) relative to an internal standard mix added to the samples before the analysis that consisted of 1,2,3-17:0 TG (100 pmol), 1,3-17:0D5 diacylglyceride (50 pmol), 16:0D31-18:1 phosphatidylcholine (200 pmol), 17:0 lysophosphatidylcholine (50 pmol), 16:0D31-18:1 phosphatidylethanolamine (200 pmol), 16:0D31-18:1 phosphatidylinositol (50 pmol), 16:0D31-18:1 phosphatidylserine (200 pmol). Repeatability, expressed as intra-day relative standard deviation calculated for all lipid standards, was satisfactory (TG: 26%, diacylglycerides (DG): 16%,

phosphatidylcholines (PC): 19%, lysophosphatidylcholines (LPC): 24%,
phosphatidylethanolamines (PE): 23%, phosphatidylinositol (PI): 17%,
phosphatidylserines (PS): 21%).

Statistical analysis

Dose-response curves and concentrations which caused a 50 % decline of cell viability (EC_{50}) were calculated using Sigmaplot 11.0 software. Lipid profiles were analysed with the on-line software Metaboanalyst 4.0 (Xia and Wishart, 2016). Partial least square-discriminant analysis (PLS-DA) was applied to compare control and exposed samples, and to identify the discriminant lipids based on the variable importance in the projection (VIP) parameter. Volcano plots were applied to visualize the significance and the magnitude of the changes detected in the cellular lipidome (fold change ≥ 1.5 ; $p < 0.05$, Student t -test).

Results

The cytotoxicity of DRO in PLHC-1 cells after 24 and 48 h exposure is shown in Figure S1 (supplementary info). Effective concentrations causing a 50 % decline in cell viability (EC_{50}) for CFDA-AM and Alamar Blue were 119 ± 3 and 102 ± 2 μ M, respectively after 24 h exposure. EC_{50} significantly decreased after 48 h exposure to DRO (58 ± 4 and 51 ± 1 μ M, respectively). Estimated EC_{10} were of 42 and 23 μ M after 24 and 48 h exposure, respectively. Concentrations below the EC_{10} (1 and 10 μ M) were selected to investigate the effect of DRO in cellular lipids.

ROS production reached a maximum of 1.5-fold increase in cells exposed to 200 μ M DRO for 15 min; no induction of ROS was observed at concentrations below 10 μ M DRO. Basal levels of ROS were almost achieved after 120 min of exposure. (Figure S2, Supplementary info).

The analysis of DRO in culture medium evidenced good stability and availability of the compound under the assay conditions. After 24 h exposure, 9.1 ± 0.2 μ M of DRO was detected dissolved in culture medium (10 μ M nominal concentration), while for the 48 h exposure experiment, concentrations of DRO of 0.7 ± 0.1 and 9.0 ± 0.4 μ M were measured, which were well in agreement with the nominal concentrations (1 and 10 μ M). Consequently, EC₅₀ values calculated using nominal and experimental concentrations were not significantly different.

The intracellular concentration of DRO was determined for cells exposed to 10 μ M DRO. After 24 h exposure, intracellular concentration was 9.5 ± 2.4 ng·mg⁻¹ protein, while a concentration of 8.3 ± 1.1 ng·mg⁻¹ protein was measured after 48 h exposure. The bioconcentration factor (BCF) calculated as the ratio between the concentration of DRO in the cells (in ng·mL⁻¹; assuming a cellular volume of 0.001 mL) and the concentration in the culture medium (ng·mL⁻¹), was 0.23-0.25, after 24-48 h exposure, which indicates no significant bioaccumulation of DRO.

The analysis of lipids in PLHC-1 cells allowed the identification of 154 lipid species. The most abundant lipid subclasses were PCs (45%) followed by PEs (22%) and plasmalogen/plasmanyln PEs (PE-P/PE-Os; 11%). Additionally, 17 fatty acids (FA 16:0, 16:1, 18:0, 18:1, 18:2, 20:0, 20:1, 20:2, 20:3, 20:4, 22:0, 22:2, 22:3, 22:4, 22:5, 22:6, 24:0) were identified.

Statistical analysis revealed differences between the lipid pattern of control and exposed cells. PLS-DA scores plot showed a segregation between the lipidome of

control cells and those exposed to 10 μ M DRO for 24 h (65.4% of variance explained, $R^2 = 0.84$, $Q^2 = 0.49$) and 48 h (57.3 % of variance explained, $R^2 = 0.97$, $Q^2 = 0.81$) (Figure 1A-C). A segregation was also observed for the lipidome of cells exposed to 1 μ M DRO for 48 h and control cells ($R^2 = 0.79$; $Q^2 = 0.08$) (Figure 1B). However, the large discrepancy between R^2 and Q^2 for this experimental condition clearly indicated an overfitting of the model and a low predictability. For cells exposed to 10 μ M DRO for 24 h, 61 VIPs > 1.0 were generated. The hierarchical cluster analysis showed an accumulation of TGs in the cells exposed to DRO (TGs 46:1-2, 48:1-3, 52:5, 54:3-4-5, 56:3-4-5-6, 58:5-6-7-8), but not a good grouping of the control and exposed samples (Fig. 2A). In contrast, when cells were exposed to 10 μ M DRO for 48 h, the PLS-DA analysis generated 51 VIPs > 1.0 ; and the hierarchical cluster analyses perfectly assembled the samples in two groups that corresponded to control and exposed cells (Fig. 2B). Cells exposed to DRO showed an accumulation of TGs (52:4-5, 54:4-5-6, 56:4-5-6-7) with a relatively high number of double bonds, and a down-regulation of some DGs (30:0, 32:0-1, 34:0-1-2, 36:1-2, 38:2-3-4). Consequently, the ratio TG/DG increased from 29.5 ± 3.5 (control cells) to 43.3 ± 5.0 after exposure to 10 μ M DRO.

In agreement with the PLS-DA results, volcano plots showed a significant accumulation of TGs of 46, 56 and 58 carbon atoms containing from 4 to 8 double bonds, after both, 24 and 48 h exposure (fold change ≥ 1.5 ; $p < 0.05$) (Fig. 3). The magnitude of the changes is comparable in the two studied scenarios, ranging from 1.5 to 3-fold, but with greater significance after 48 h exposure. In addition, an increase of LPC 18:1, PSs (38:3, 40:7), and PI 40:4 were observed after 24 h exposure, while PE-P 40:6 decreased after 48 h exposure. Figure 4 depicts the TG profile in PLHC-1 cells and the quantitative changes experienced after 48 h exposure to 10 μ M DRO. TGs of 56 and

58 carbon atoms and 4 to 8 double bonds were significantly increased. Overall, this represents a 20% increase in the total TG content of exposed cells.

Discussion

This work evidences the potential of DRO to alter the lipid composition of liver cells and consequently, elicit biological impacts in fish beyond those previously described. DRO induced the accumulation of TGs, mostly long chain ones with a high number of double bonds, and a concomitant depletion of DGs in liver cells. From a quantitative point of view, total TG content increased from 820 ± 132 to 1225 ± 128 pmol/ 10^5 cells after 24 h exposure; and from 949 ± 40 to 1122 ± 36 pmol/ 10^5 cells after 48 h.

At present, it is uncertain whether the change in the lipid profile and the accumulation of TGs in liver cells is indicative of lipid associated toxicity or if, on the contrary, may play a beneficial role. Although, no information is available for fish, TG species with more than four double bonds were high in obesity resistance mice, showing that differences in the degree of saturation of TG species in the liver affect the individual response to high-fat-diet (Nam et al., 2015). Additionally, the increase of TGs could serve to store polyunsaturated FAs as a protective measure against peroxidation (Ackerman et al., 2018), but this is unlikely, considering the relatively low levels of ROS generated after 15 min exposure to DRO (Fig. 2S). On the other hand, the increase of the TG/DG ratio revealed increased metabolism of DGs to TGs in DRO exposed cells, and it is an index of hepatic steatosis (Puri et al., 2007). Liver steatosis, which is defined as an excessive accumulation of TGs and other fats inside liver cells, has been described in fish after handling stress and after dietary exposure to endocrine

288 disrupters that can alter lipid homeostasis by interacting with nuclear receptors signaling
289 pathways (Naour et al., 2017; Carnevali et al., 2017).

290 Although, an increasing number of studies report that xenobiotics, including
291 organotins, alkylphenols, phthalates, fibrates and others, alter lipid homeostasis in fish
292 (Capitão et al., 2017), no such information is available for progestins, apart from a
293 laboratory study, where *Rutilus rutilus* exposed to 500 ng·L⁻¹ of a mixture of progestins
294 (DRO, progesterone and levonorgestrel) for 42 days showed no significant changes in
295 TG serum levels (Maasz et al., 2017). Thus, this is the first report of a lipid disruptive
296 effect of DRO in fish liver cells. The functional consequences of an increase of only
297 TGs with long chain highly unsaturated fatty acid moieties (TGs 56:4-7; 58:5-8) are
298 unknown and should be further investigated.

299 The biological action of progestins is primarily determined by its interactions
300 with steroid receptors, and although DRO is devoid of estrogenic, androgenic,
301 glucocorticoid and mineralocorticoid activity, studies carried out in murine preadipocyte
302 cell lines and primary cultures of human preadipocytes exposed to DRO reported an
303 antiadipogenic effect that was attributed to the antagonistic effect of DRO on the
304 mineralocorticoid receptor (Caprio et al., 2011). More recently, Louw-du Toit et al.
305 (2016) showed that DRO modulates the synthesis of steroids in the human
306 adrenocortical carcinoma cell line H295R by increasing the synthesis of progesterone
307 and 17-hydroxyprogesterone and reducing the synthesis of androgens and
308 glucocorticoids. Similarly, exposure to DRO changes plasmatic levels of steroids in
309 juvenile sea bass by decreasing the concentrations of 17-hydroxypregnenolone, 17-
310 hydroxyprogesterone and androstenedione after 30 days exposure to 10 ng·g⁻¹ DRO
311 through the diet (Blanco et al., 2016). Thus, although further research is needed, one
312 may hypothesize that DRO and other endocrine disrupters, by modulating the synthesis

of androgens and other endogenous steroids may interfere with lipid and TG homeostasis as described by Damsteegt et al (2016), who revealed androgens as major regulators of TG physiology in eels.

Cytotoxic effects for DRO were observed at concentrations of 102-119 μM and 51-58 μM after 24 and 48 h exposure, respectively. These are high concentrations, with no environmental relevance. As no discrepancies between nominal and experimental concentrations of DRO in culture medium were observed, we can assure that no significant losses of the compound due to binding to serum proteins or plastic wells occurred, and that there was a good bioavailability of the compound to the cells. Moreover, our results evidenced the uptake of DRO by the cells, with no significant bioconcentration factor ($\text{BCF} < 1$). In fact, only a 0.09 to 0.14% of the compound was detected in the cells under our assay conditions. These results are well in agreement with previous studies that showed no significant bioaccumulation of DRO in fish (Blanco et al, 2016).

Additionally, the alteration of the cells lipidome was observed at a concentration of 9 μM DRO in culture medium ($\sim 3.3 \mu\text{g}\cdot\text{mL}^{-1}$), which is several orders of magnitude higher than the environmental concentrations (Avar et al., 2016; Golovko et al., 2018). Nonetheless, this exposure resulted in intracellular concentrations of 8.4 to 9.5 $\text{ng}\cdot\text{mg}^{-1}$ protein. Considering that the entrance of DRO in the aquatic environment through waste water treatment plants is constant, it is likely that long-term exposures to low doses of DRO could lead to a similar internal concentration in the liver of exposed fish, and consequently, significant dysregulation of the lipidome of hepatic cells. However, in the aquatic environment, fish are not exposed to single compounds, but to mixtures of pollutants, and the alteration of liver lipidome of wild fish will be the result of a combination of different exposures to xenobiotics and natural factors (nutrition,

reproduction). Thus, the very particular lipidic fingerprint detected for DRO in PLHC-1 cells will probably not be observed in wild fish. In a recent study, we analyzed the lipidome of the skeletal muscle of fish in a Mediterranean river and the lipid profiles of fish sampled at the polluted sites, exposed to complex mixtures of endocrine disruptors of urban and industrial origins, were indicative of oxidative stress and deregulation of cholesterol homeostasis, but could not be directly associated to a single contaminant or class of contaminants (Marqueño et al., 2019b).

Overall, this study shows a significant accumulation of TGs with a concomitant decrease of DGs in PLHC-1 cells, and detects the minimal internal concentration of DRO necessary to produce this alteration (low ng/mg protein range). These results contribute to better understand and predict the toxicity of DRO in fish.

Acknowledgements

Anna Marqueño acknowledges a pre-doctoral fellowship BES-2015-074842. Work financed by Ministerio de Ciencia e Innovación, under the project CGL2014-52144-P.

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Figure 1. PLS-DA score plots show the differences between the lipidome of exposed (n = 6-9) and control cells (n = 6-7). Cells exposed to A) 10 μ M DRO for 24 h; B) 1 μ M DRO for 48 h; C) 10 μ M DRO for 48 h.

Figure 2. Dendrograms depicting the clustering between samples and lipid species for cells exposed to 10 μ M DRO for 24 h (A) and 48 h (B). Levels of the lipids are colour coded denoting up-regulation (red) or down-regulation (blue).

Figure 3. Volcano plots evidencing differences in the lipid profile of control cells and cells exposed to 10 μ M DRO for 24 h (A) and 48 h (B). Significantly altered lipids (fold change ≥ 1.5 ; $p < 0.05$) indicated in red.

Figure 4. Increased concentration of triacylglycerides ($\text{pmol}\cdot\text{mg}^{-1}$ protein) in PLHC-1 cells after exposure to 10 μ M DRO for 48 h. The box extends from 25th to 75th percentiles; the horizontal bar within the box is the median and whiskers represent 5-95th percentiles. *Significantly altered lipid species ($p < 0.05$).

Figure 1

Figure 2.

A)

B)

Figure 3.

A)

B)

Figure 4.