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Abstract

Context: Mitotane (*o,p*'-DDD), the only approved drug for advanced adrenocortical carcinoma (ACC), is a lipophilic agent that accumulates into circulating lipoprotein fractions and high lipid-containing tissues.

Objective: The aim of our study was to evaluate the *in vivo* and *in vitro* biological implication of serum lipoproteins on pharmacological action of mitotane. Distribution and concentration of mitotane were studied in plasma and adrenal tissue samples from mitotane-treated patients. The impact of lipoprotein-bound or free (LP-F) mitotane was analyzed on proliferation and apoptosis of human adrenocortical H295R cells. A retrospective study of ACC patients treated or not with statins was also performed.

Results: *o,p*'-DDD distribution among VLDL, LDL, HDL and lipoprotein-free (LP-F) fractions obtained after ultracentrifugation of 23 plasmas of mitotane-treated patients was widely distributed in each subfraction. A positive correlation was observed between mitotane levels in plasma and in LDL, HDL but also LP-F compartment. Intra-tumor o,p'-DDD concentrations in 5 ACC samples of mitotane-treated patients were found independent of cholesterol transporter expression, scavenger receptors (SrB1) and LDL-Receptors. *In vitro* studies showed significant higher anti-proliferative and pro-apoptotic effects and higher cell and mitochondrial uptake of mitotane when H295R cells were grown in LP-F medium. Finally, retrospective study of an ACC cohort of 26 mitotane-treated patients revealed that statin therapy was significantly associated with a higher rate of tumor control.

Conclusions: Altogether, our *in vitro* and *in vivo* studies provided compelling evidence for a greater efficacy of lipoprotein-free mitotane. ACC patients may thus benefit from therapeutic strategies that aim to increase LP-F mitotane fraction.

Introduction

Mitotane is the only drug approved in advanced adrenocortical carcinoma (ACC) (1). The antitumor clinical impact of mitotane has been shown on both prospective and retrospective studies that found partial response rates in 10 to 33% of patients treated with mitotane alone but also improved overall survival (2,3). Based on these results, mitotane is also recommended as an adjuvant therapy in ACC patients at high risk of recurrence (4–6). In both indications, plasma mitotane monitoring is recommended to look for a therapeutic window of 14-20 mg/L (1). Indeed, several studies have reported higher response rate and/or a prolonged survival in patients with plasma mitotane levels above 14 mg/L (7–11). In addition, neurological toxicities have been described with plasma level above 20 mg/L (11,12).

Mitotane is also known as *o,p*'-DDD, an insecticide-derivative lipophilic drug that accumulates in lipoproteins (13). Dyslipidemia have been observed in mitotane-treated patients but the mitotane-induced dyslipidemic profile differs from one study to another (14–19). The mechanism of mitotane-induced hypercholesterolemia is not fully understood but could be related to an activation of HMGCoA reductase (20) and to an increase in cholesterol and lipoproteins synthesis (14). Little is known about the influence of dyslipidemia on mitotane distribution among lipoproteins and about the influence of this distribution on its anti-tumor efficacy.

Mechanism of mitotane action was poorly understood until recently. Two mitotane metabolites are described: *o,p'*-DDE and *o,p'*-DDA, the latter being described as the main urinary metabolite of *o,p*'-DDD (21). We recently reported evidence that *o,p'*-DDA is unlikely an active metabolite of mitotane (22). Several studies suggested that mitotane could have a mitochondrial effect (23,24), and more specifically, our group demonstrated a mitotane-induced defect in cytochrome c oxidase (complex IV of the mitochondrial respiratory chain) (25).

In the present study, we explored the biologic implication of serum lipoproteins on mitotane pharmacological action using human plasma samples, tissues of mitotane-treated patients and finally *in vitro* on human adrenocortical H295R cells. Altogether, our results showed that lipoprotein-free mitotane appeared to be the most efficient form. Based on these findings, we retrospectively examined

72 the disease control rate of 26 consecutive stage IV-ACC patients treated with mitotane, according to
73 the concurrent use of statins.
74

Patients, material and methods

Patients

Medical files of 70 metastatic ACC patients treated with mitotane, followed between 2007 and 2014 at Gustave Roussy were retrospectively reviewed to study the correlation between occurrence of dyslipidemia or statin therapy (Rosuvastatin not metabolized by Cyp3A4) within the first 3 months of mitotane therapy and neurological toxicity or tumor response. Inclusion criteria were patients with stage IV ACC treated with mitotane after 2007 and the exclusion criteria was the absence of lipid profile available within the first 3 months of mitotane therapy. In each file, the following criteria were recorded: mitotane plasma level, HDL, LDL triglycerides levels (high HDL, high LDL or high triglycerides defined as above 1.5 time the upper value, concurrent use of statins given during at least 3 months of mitotane therapy, presence of neurological toxicities and disease control rate (stabilization and partial response) at 6 months according to RECIST 1.1 (26). An informed consent was obtained from all patients.

Human plasma samples and human adrenal tissues

Twenty-five plasma samples form 20 mitotante-treated ACC patients were taken at Gustave Roussy hospital and thanmade available for this study form the central repository of HRA Phama (Paris, France) to evaluate lipoprotein partitioning in normal and dyslipidemic samples. To analyze the correlation between mitotane concentrations and cholesterol transporter expression, human adrenal tissues were obtained from 6 mitotane-treated patients followed at Gustave Roussy or Bicêtre Hospital. All were ACC but one who underwent bilateral adrenalectomy for an ectopic Cushing syndrome. Tissues were collected while patients underwent surgery for therapeutic reasons. Tissues were lysed in H_2O using a TissueLyser apparatus (Qiagen, Courtaboeuf, France). All patients signed an informed consent.

Human adrenocortical cells and mitochondria isolation

For *in vitro* studies, H295R cells (from passage 2 to 15) were cultured as previously described (25). Media were enriched with 10% fetal calf serum (FCS, control medium) or lipoprotein deficient FCS

(LP-F medium) enriched or not with LDL or HDL subfractions obtained from ultracentrifugation (with a final cholesterol concentration of 30 mg/L, similar as in all media). *O,p'*-DDD (HRA Pharma) and BLT1 (Sigma-Aldrich, St. Louis, MO) were solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and used at indicated concentrations. The percentage of DMSO in culture medium never exceeded 0.1%. Cholesterol (Sigma-Aldrich) and Bovine serum albumin (BSA, Euromedex, Mundelsheim, France) were solubilized in culture medium and used at final concentrations of 9.6 mg/L (low) and 38.4 mg/L (high) for cholesterol and 1.8 mg/L (low) and 3.6 mg/L (high) for BSA.

Mitochondrial fractions were purified and prepared from permeabilized cells using digitonin and percoll as previously described (27).

Lipoprotein isolation from plasma and fetal calf serum by ultracentrifugation

Density gradient ultracentrifugation using iodaxinol (Optiprep®, Sigma Aldrich) was used for the isolation of lipoprotein fractions in plasma samples. A saline solution with HEPES buffer was added to a mixed solution of 60% (m/v) iodixanol in water (d = 1.32 g/mL) and plasma in Optiseal® vials. This final solution was ultracentrifuged at 350,000 g at 16°C for 3 h. Each lipoprotein fraction was collected with syringe and needle systems.

Individual lipoprotein subfractions were isolated from fetal calf serum by isopycnic density gradient ultracentrifugation for 48 h at 288,000 g using a Beckman XL70 centrifuge and a SW41 rotor as previously described (28). After centrifugation, gradients were collected from the top of the tubes with an Eppendorf precision pipette in fractions corresponding to LDL subfractions (density < 1.063 g/mL) and HDL sub-fractions (density from 1.063 g/mL to 1.179 g/mL).

Measurements of o,p'DDD in plasma, tissues, cells and mitochondria

Analyses of plasma samples were conducted by high performance liquid chromatography combined to an ultraviolet detection (HPLC-UV) as previously described (22) and analyses of cells and mitochondria samples were conducted by gas chromatography combined to a mass spectrometry (GCMS). All samples were spiked with known amounts of *p,p'*-DDE used as an internal standards (IS) of *o,p*'-DDD measurements. *O,p'*-DDD concentrations were determined through the ratio of their

peak surface area to the peak surface of known concentrations of IS. Concentrations of *o,p*'-DDD in cells and mitochondria are expressed in ng per femtomoles of nuclear or mitochondrial DNA. Nuclear and mitochondrial DNA were extracted from samples using standard techniques and quantified by real-time quantitative PCR using the 18S gene and the cytochrome c oxidase 2 (COX2) gene as nuclear and mitochondrial specific genes, respectively as previously described (25).

Cell proliferation and apoptosis analysis

Cell proliferation tests were performed by using the WST1 assay (Roche, Meylan, France) and apoptosis tests were performed by using the Caspase-Glo 3/7 assay (Promega, Madison, WI) according to the manufacturer's recommendations. Cells were cultured in 96-well plates and treated with 0 to 150 µM *o,p*'-DDD for 24 or 48 h. Optical densities were measured 4 h after addition of WST1 solution (10 µl per well) by spectrophotometry (Viktor, Perkin Elmer, Courtaboeuf, France). Luminescence was measured 1h after addition of Caspase-Glo 3/7 solution (equal volume) by luminometry (Viktor, Perkin Elmer).

Reverse Transcriptase-PCR (RT-PCR) and Quantitative real-time PCR (RT-qPCR)

Total RNAs were extracted from cells with the RNeasy kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. RNA was thereafter processed for RT-PCR as previously described (25). Quantitative real-time PCR (RT-qPCR) was performed using the Fast SYBR® Green Master Mix (Life Technologies) and carried out on a StepOnePlus™ Real-Time PCR System (Life Technologies) as previously described (25). The relative expression of each gene was expressed as the ratio of attomoles of specific gene to femtomoles of 18S rRNA.

Western Blot analysis

Total protein extracts were prepared as previously described (22). Western Blot analyses were performed as described (22). Antibodies used were a rabbit anti-BCL2 antibody (1:500 dilution, Cell Signaling, Saint Quentin en Yvelines, France) with a mouse anti-α-Tubulin antibody (1:10,000 dilution, Sigma-Aldrich) or a rabbit anti-CYP11A1 antibody (1:500 dilution, Sigma-Aldrich) with a

mouse anti-GAPDH antibody (1:10,000 dilution, Sigma-Aldrich). Proteins were visualized with an Odyssey-Fc apparatus (LI-COR).

Statistical Analysis

Results are expressed as means ± SEM of n independent replicates performed in the same experiment or from separated experiments (n). Correlation was tested with a Pearson test. Non-parametric Mann Whitney tests were used when appropriate and differences between groups were analyzed using non-parametric Kruskall-Wallis multiple comparison test followed by a post-test of Dunn's (Prism software, GraphPad, CA). Difference between groups of patients was assessed using the Fisher's exact test. A P value of 0.05 was considered as statistically significant (*P<0.05; **P<0.01; ***P<0.001).

Results

Distribution of *o,p'*-DDD and its metabolites among lipoproteins and lipoprotein-free subfractions in ACC patients plasma samples

The distribution of mitotane and its two major metabolites, *o,p*'-DDE and *o,p*'-DDA, in lipoproteins was evaluated in 20 ACC patients. *O,p*'-DDD, *o,p*'-DDA and *o,p*'-DDE were thus measured with HPLC-UV after ultracentrifugation of plasma samples in VLDL, LDL, HDL and lipoprotein-free (LP-F) subfractions. *O,p*'-DDD was widely distributed among lipoprotein fractions as follows : 34.6 ± 9.9% LP-F (including proteins-bound and free mitotane), 26.3 ± 5.8% HDL, 26 ± 4.6% LDL and 13 ±4.2% VLDL (Figure 1A). The distribution of *o,p*'-DDE among these subfractions favoring LP-F, was as follows (72.9 ± 15.3% LP-F, 17.7 ± 12.2% HDL, 8.2 ±1 0.5% LDL and 1.3 ± 2.7% VLDL) while, in sharp contrast, *o,p*'-DDA was almost exclusively recovered in LP-F fractions (94.6 ± 3.1%, Suppl. Figure S1A and B). No significant difference in *o,p*'-DDD, *o,p*'-DDA or *o,p*'-DDE distribution was observed according to the presence and the degree of dyslipidemia or the plasma mitotane level (data not shown). Of interest, plasma mitotane level correlated with *o,p*'-DDD measured in LP-F fractions (Figure 1B, r^2=0.41; P<0.001) but also in those of HDL and LDL (Figure 1C, r^2=0.76; P<0.001).

Intra-tumor *o,p'*-DDD concentrations and cholesterol transporters expression.

To further explore which LP subfraction might account for mitotane uptake in human ACC tissue, we measured the relative expression of genes encoding for SrB1 (Scavenger B1 receptor, HDL receptor) and LDL-R in six samples of human adrenal tissue collected from one ectopic Cushing's disease and 5 ACC mitotane-treated patients (Table 1). On a case by case analysis, no association was found between *SrB1* or *LDL-R* expression and intra-tumor *o,p*'-DDD concentrations.

Influence of lipoprotein-binding on mitotane efficiency *in vitro*

Impact on cell proliferation and apoptosis

To evaluate the influence of lipoprotein binding on mitotane efficacy in terms of cell proliferation and apoptosis, H295R cells were incubated in different culture conditions containing either HDL lipoproteins (HDL), LDL lipoproteins (LDL) or lacking lipoprotein fractions (lipoprotein-free, LP-F)

and compared to control (FCS). Cell proliferation index was measured at baseline and after incubation with various mitotane concentrations for 48h. Basal cell proliferation was not different between these conditions (Suppl. Figure S2A). Mitotane exerts a dose-dependent anti-proliferative effect in all conditions but was more efficient when cells were cultured in LP-F medium with an IC50 of approximately 40 µM compared to 140 µM under control conditions (Figure 2A) with a left-shift of dose-dependent curve. Apoptosis index as measured by caspase 3/7 assays was significantly higher after a 24h treatment with 100 µM mitotane in LP-F condition compared to LDL, HDL or control conditions (Figure 2B). Furthermore, expression of the anti-apoptotic protein BCL2 was reduced by 100 µM mitotane in LP-F condition but not in others (Suppl. Figure S2B). Altogether, our results clearly indicate that the cytotoxic effects of mitotane are more pronounced in the absence of lipoproteins.

Intracellular uptake of mitotane and mitochondrial impact

To examine whether the nature of LP fractions affects mitotane uptake, *o,p*'-DDD concentrations were measured in cell pellets after 48h of 50 µM exposure in different conditions. Intracellular *o,p*'-DDD concentrations, measured by the sensitive GC-MS technique and normalized to nuclear DNA were at least 3 fold higher in cells cultured in LP-F medium than in other media (Figure 3A). The intracellular mitotane was mostly recovered in the mitochondrial fraction (89.2 ± 3.6% of *o,p*'-DDD) whereas only 10.8 ± 3.6% of the measured intracellular *o,p*'-DDD was in the cytosolic fraction (data not shown). Intra-mitochondrial *o,p*'-DDD concentration was 15 higher (90.26 ± 7.66 ng/ atomol of mt DNA) in cells grown in LP-F condition compared to other culture conditions, highly suggestive of a better cellular and thus mitochondrial uptake of mitotane in the absence of lipoproteins (Figure 3B). We next studied the expression of genes encoding proteins involved in oxidative phosphorylation or steroidogenesis by RT-qPCR in *o,p*'-DDD treated cells under HDL, LDL, LP-F and control conditions. In LP-F medium, mitotane strongly inhibited *COX2* expression (encoded by the mitochondrial mtDNA for the subunit 2 of cytochrome c oxidase or respiratory chain complex IV, Figure 3C) but also *StAR* and *CYP11A1* involved in steroidogenesis (Suppl. Figure S3A and B). LP-F condition also led to a drastic reduction in CYP11A1 protein expression in H295R cells after a 48h

224 treatment with 50 µM mitotane (Suppl. Figure S3C). Collectively, our findings provide additional
225 evidence that lipoprotein-free mitotane was the most efficient leading to alter cellular functions.
226
227 *Effect of BLT1 treatment and cholesterol saturation*
228 BLT1 is a powerful but not fully specific inhibitor of SrB1 and thus a pharmacological inhibitor of
229 cellular lipoprotein uptake. SrB1 also participates to cellular efflux of lipophilic molecules such as
230 cholesterol or vitamin E and very likely mitotane. *O,p*'-DDD displays a more potent anti-proliferative
231 activity on H295R cells with a left-shift dose-dependent in the presence of BLT1 and a proliferation
232 index at 7.5 ± 3.8% in BLT1-treated cells compared to 69.5 ± 4.3% in control cells ($p<0.01$) at 100
233 µM mitotane concentration for 48h. Likewise, mitotane-treated cells exhibited a higher apoptotic
234 capacity in the presence of BLT1, as revealed by the reduced expression of the anti-apoptotic protein
235 BCL2 (Figure 4B). This was accompanied by an significant increase in intracellular *o,p*'-DDD
236 concentration when lipoproteins receptors were inhibited with BLT1 (Figure 4C). Free cholesterol was
237 added into the culture medium to saturate lipoproteins in an attempt to reduce lipoprotein-bound
238 mitotane through cholesterol exchange, thus potentially enhancing LP-F mitotane bioavailability.
239 Cholesterol supplementation in the medium dose-dependently enhanced mitotane cytotoxic effects,
240 confirming the key role of LP-F mitotane *in vitro* (Figure 4D).
241
242 *Efficiency of unbound mitotane in vitro*
243 To examine the relative contribution of free and protein-bound mitotane on H295R cell proliferation
244 or apoptosis, the impact of mitotane was compared in FCS-free medium, devoid of protein and
245 lipoproteins, supplemented with increasing concentrations of bovine-serum albumin (BSA) and in LP-
246 F media. Consistently, *o,p*'-DDD was more efficient in inhibiting cell proliferation in protein-free
247 medium while BSA supplementation dose-dependently impaired mitotane efficiency (Figure 4E).
248 Moreover, relative caspase activity was 4-fold higher in mitotane treated cells when incubated in the
249 absence of protein than in the control medium (Figure 4F). Altogether, our results demonstrate that the
250 most potent activity *in vitro* was achieved with free mitotane.
251

Statin therapy and disease control rate in stage IV-ACC patients

To translate these observations into the clinic, we retrospectively collected data from ACC patients followed at our institution. Stage IV-ACC patients under mitotane therapy initiated between September 2007 and January 2014 were included. Twenty-six patients had a mean plasma mitotane level of 16.7 ± 9.2 mg/L (13.2 ± 9.4 mg/L at 1 month, 17.8 ± 12.2 mg/L at 3 month and 18.4 ± 8.5 mg/L at 6 month). Among them, 16 (61.5%) experienced hypertriglyceridemia, 15 (57.7%) an increase in HDL cholesterol or 8 (30.8%) an increase in LDL cholesterol level under mitotane. Eleven patients (42.3%) were treated with statins (introduced at least 3 months before RECIST evaluation). Neurological toxicity was reported in 8 out of 26 patients (30.8%) at 1, 3 and/or 6 months. According to RECIST criteria, (Response Evaluation Criteria in Solid Tumors) disease control rate (DCR) at 6 months was 46.2% (12 out of 26 patients) including 4 patients that experienced a partial response. A significant positive association was found between the use of statin therapy and DCR at 6 months: 67% or 33% DCR was observed in patients treated or not with statins ($p<0.05$, Fisher's exact test, Figure 5). No association was found between dyslipidemia and neurological toxicity or tumor control or statins therapy and neurological toxicity.

Discussion

As mitotane remains the most effective treatment of advanced ACC, many efforts are done to better understand its mechanism of action (2). Its lipophilic properties lead to a distribution of *o,p*'-DDD in lipoproteins (13) and a storage in adipose tissue (29). Given that mitotane content in lipoprotein fractions has been assumed to play a role in drug distribution in tissues (30), we therefore evaluate the potential role in its antitumor activity using different materials: ACC patients' plasma samples, human adrenal cortex tissues and human adrenocortical H295R cells.

The partitioning of mitotane and its metabolites in lipoproteins was found to differ strongly. Indeed, whereas *o,p*'-DDD and o,p'DDE were equally distributed among different fractions (vLDL, LDL, HDL and LP-F subfractions), *o,p*'-DDA was entirely recovered with protein fractions, consistent with its hydrophilic properties. In our 23 plasma samples originating from 20 ACC patients, we found a correlation between plasma *o,p*'-DDD levels and its corresponding lipoprotein contents but more importantly between circulating mitotane concentrations and its distribution in LP-F subfraction. These findings raise the question of the relative contribution of mitotane-free versus bound lipoprotein fractions in the pharmacological action of *o,p*'-DDD (30). To further explore this question, expression of genes encoding for lipoprotein receptors (SrB1 and LDLR) were studied and compared to intra-tissue *o,p*'-DDD concentrations of mitotane-treated patients' adrenals. In this small number of tissue samples owing to the low incidence of ACC, no relationship was observed between adrenal cortex *o,p*'-DDD content and lipoprotein receptor expression, suggesting no predominant impact of mitotane-bound lipoproteins on tissue mitotane uptake.

We then explored the role of mitotane binding to each lipoprotein subfraction or lipoprotein-free mitotane on its cytotoxic effect in human adrenocortical H295R cells. We provided evidence that mitotane exerts a more efficient anti-proliferative and pro-apoptotic action when cells are grown in LP-F medium suggesting that lipoprotein-bound mitotane is not the most potent pharmacological vehicle.

In the present study, we also confirm that mitochondrion is a critical target of mitotane action since most intracellular mitotane was recovered within the mitochondrial compartment, consistent with our previous results (25). Herein, we further demonstrated that LP-F mitotane was more effectively

captured by mitochondria and more efficient in inhibiting the respiratory chain activity. Experiments using BLT1, an SrB1 receptor inhibitor, or cholesterol saturation, that both favour mitotane action through its LP-F fraction, comfort the prominent pharmacological role of free mitotane by showing an increased efficiency of mitotane when added to the culture medium. These results do not exclude that SrB1 could be involved in mitotane efflux. Van Slooten first suggested that albumin-bound mitotane might be responsible for at least the neurological side effects observed in patients (12). We further evaluated the impact of albumin on *o,p*'-DDD toxicity in H295R cells, and unambiguously demonstrated that free mitotane exerts the most efficient pharmacological properties.

Altogether, our results indicate that free mitotane induces the most potent cytotoxic effects, questioning the precise molecular mechanism of its transmembrane transport and yet excluding that intracellular transport of lipoproteins might play a major role in the adrenal specificity of mitotane action.

In ACC patients, a variable delay of several weeks between mitotane initiation and anti-tumorigenic effect is well described in the literature (2). Consistent with our results demonstrating that free mitotane might constitute the active form of the drug, this delay may correspond to the time required to fully saturate circulating lipoproteins in patients' plasma. To further explore the implication of our *in vitro* results in humans, and based on clinical and biochemical data collected from a cohort of ACC, mitotane-treated patients, we examined mitotane plasma levels, lipid profile as well as neurological toxicity and clinical responses according to RECIST at 1, 3 and 6 months after mitotane initiation. Among the 26 patients included, 20 (76.9%) had dyslipidemia under mitotane therapy, including isolated hypertriglyceridemia, hypercholesterolemia (HDL and/or LDL) or both, in accordance to previous reports (16–19). More interestingly, we found that patients who received statins (42.3%), presented with a better tumor control including stable disease and partial responses. We hypothesize that statins, through a reduction of plasma lipoproteins levels could lead to an increased free mitotane ratio. Despite the hypothesis of van Slooten who suggested that albumin-bound mitotane could be responsible for neurological toxicity, we didn't find any association between LDL, HDL or statins and neurological side effects. Our study has clear limitation including the small sample size, the heterogeneity in patients' follow-up and plasma collections and the uncontrolled nature of the design

inherent to a the retrospective data collection. These preliminary data should be further confirmed and the question on whether patients could benefit from statins is presently being addressed through an ongoing prospective study (MITOLIPO Study).

In sum, we provide strong evidence that *o,p*'-DDD unbound to lipoprotein fractions is more efficient *in vitro* and that patients could benefit strategies that aim to increase LP-F mitotane fraction. The potential role of dyslipidemia and statin therapy on mitotane effects will be further explored in a prospective study.

Figures Legends

Figure 1

Distribution of *o,p*'-DDD in lipoproteins (VLDL, LDL and HDL) and in the lipoprotein-free (LP-F) subfraction obtained from ultracentrifugation of 23 plasma samples of 20 ACC patients (A) *O,p*'-DDD measured by UV-HPLC, is expressed as percentage of the sum of *o,p*'-DDD measurements in each subfraction.

Correlation between plasma mitotane level and *o,p*'-DDD measured in LP-F (B) or HDL and LDL (C), all levels are expressed in mg/L. Plasma mitotane levels significantly correlate to LP-F mitotane ($P<0.001$, $r^2=0.41$), to HDL mitotane ($P<0.001$; $r^2=0.76$) and to LDL mitotane ($P<0.001$; $r^2=0.76$, Pearson test).

Figure 2

(A) Dose-dependent impact of increasing concentrations of *o,p*'-DDD (0-200 µM) on the proliferation index of H295R cells after 48 h culture in control, LP-F, LDL or HDL conditions as determined by WST1 assays. Results are expressed as mean percentage ± SEM of 6 to 24 independent determinations, values of vehicle-treated cells being arbitrarily set at 100%.

(B) Relative caspase activity of H295R cells cultured in control, LP-F, LDL and HDL conditions before and after 24h treatment with 100 µM *o,p*'-DDD as determined by Caspase-Glo 3/7 assays. Results are expressed as mean arbitrary units ± SEM of 6 to 24 independent determinations. **P<0.01, Mann-Whitney U test.

Figure 3

O,p'-DDD concentrations as measured by GC-MS in H295R cell pellets (A) and mitochondrial pellets (B) after 48h exposure to 50 µM mitotane in control, LP-F, LDL and HDL conditions. Results are expressed as mean percentage ± SEM of 3 to 6 independent determinations, values of control-cultured cells being arbitrarily set at 100%. **P<0.01, Mann-Whitney U test.

(C) Relative mRNA expression of *COX2* determined by RT-qPCR. H295R cells were treated with 50 µM *o,p*'-DDD for 48h. Results are expressed as mean percentage ± SEM of 6 different experiments

359 performed in duplicate, values of vehicle-treated cells being arbitrarily set at 100%. ***P<0.001,
360 Mann-Whitney U test.
361
362 **Figure 4**
363 (A) Dose-dependent impact of increasing concentrations of *o,p*'-DDD (0-200 µM) on the proliferation
364 index of H295R cells after 48 h culture in the absence or presence of 5 µM BLT1 as determined by
365 WST1 assays. Results are expressed as mean percentage ± SEM of 6 independent experiments
366 performed in duplicate, values of vehicle-treated cells being arbitrarily set at 100%.
367 (B) Steady-state levels of BCL2 protein (anti-apoptotic factor) by Western blot with anti-BCL2 and
368 anti-tubulin antibodies. H295R cells were cultured in the absence or presence of 5 µM BLT1 and
369 treated with 50 µM *o,p*'-DDD for 48h.
370 (C) *O,p*'-DDD concentrations as measured by GC-MS in H295R cell pellets after 48h exposure to 50
371 µM mitotane in the absence or presence of 5 µM BLT1. Results are expressed as mean percentage ±
372 SEM of 6 independent determinations, values of control-cultured cells being arbitrarily set at 100%.
373 **P<0.01, Mann-Whitney U test.
374 (D) Dose-dependent impact of increasing concentrations of *o,p*'-DDD (0-200 µM) on the proliferation
375 index of H295R cells after 48 h culture without or with 96 mg/L (low) or 384 mg/L (high) cholesterol
376 as determined by WST1 assays. Results are expressed as mean percentage ± SEM of 6 independent
377 experiments performed in duplicate, values of vehicle-treated cells being arbitrarily set at 100%.
378 (E) Dose-dependent impact of increasing concentrations of *o,p*'-DDD (0-200 µM) on the proliferation
379 index of H295R cells after 48 h culture in control or LP-F conditions or with 1.8 mg/L (low) or 3.6
380 mg/L (high) bovine serum albumin (BSA) as determined by WST1 assays. Results are expressed as
381 mean percentage ± SEM of 6 independent experiments performed in duplicate, values of vehicle-
382 treated cells being arbitrarily set at 100%.
383 (F) Relative caspase activity of H295R cells cultured in the presence or absence of fetal calf serum
384 (FCS) before and after 24h treatment with 100 µM of *o,p*'-DDD as determined by Caspase-Glo 3/7
385 assays. Results are expressed as mean arbitrary units ± SEM of 6 independent determinations.
386 **P<0.01, Mann-Whitney U test.

Figure 5

Association between statin therapy and disease control rate at 6 months (DCR-6M) in a cohort of 26 ACC patients under mitotane. Disease control rate is defined by partial response or stable disease according to RECIST criteria. * P <0.05, Fisher exact test.

Legends to the Supplemental Figures

Figure S1

Distribution of mitotane's metabolites *o,p*'-DDE (A) and *o,p*'-DDA (B) in lipoproteins (vLDL, LDL and HDL) and in the lipoprotein-free (LP-F) subfraction obtained from ultracentrifugation of 23 plasma samples of ACC patients. *O,p*'-DDE and *o,p*'-DDA are measured by UV-HPLC and expressed as percentage of the sum of *o,p*'-DDD measurements in each subfraction.

Figure S2

(A) Proliferation index of H295R cells cultured in control, LP-F, LDL or HDL conditions for 48h as determined by WST1 assays. Results are expressed as mean percentage ± SEM of 6 to 24 independent determinations, values of control-cultured cells being arbitrarily set at 100%.

(B) Steady-state levels of BCL2 protein (anti-apoptotic factor) by Western blot with anti-BCL2 and anti-tubulin antibodies. H295R cells were cultured in control, LP-F, LDL or HDL conditions and treated with 50 µM *o,p*'-DDD for 48h.

Figure S3

Relative mRNA expression of *StAR* (A) and *CYP11A1* (B) determined by RT-qPCR. H295R cells were treated with 50 µM *o,p*'-DDD for 48h. Results are expressed as mean percentage ± SEM of 6 different experiments performed in duplicate, values of vehicle-treated cells being arbitrarily set at 100%. ***P<0.001, Mann-Whitney U test.

(C) Steady-state levels of CYP11A1 protein by Western blot with anti-CYP11A1 and anti-GAPDH antibodies. H295R cells were cultured in control, LP-F, LDL or HDL conditions and treated with 50 µM *o,p*'-DDD for 48h.

Bibliography

1. **Berruti A, Baudin E, Gelderblom H, Haak HR, Porpiglia F, Fassnacht M, Pentheroudakis G.** Adrenal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* 2012;23 Suppl 7:vii131–138.

2. **Baudin E.** Adrenocortical Carcinoma. *Endocrinol Metab Clin North Am* 2015;in press.

3. **Fassnacht M, Terzolo M, Allolio B, Baudin E, Haak H, Berruti A, Welin S, Schade-Brittinger C, Lacroix A, Jarzab B, Sorbye H, Torpy DJ, Stepan V, Schteingart DE, Arlt W, Kroiss M, Leboulleux S, Sperone P, Sundin A, Hermsen I, Hahner S, Willenberg HS, Tabarin A, Quinkler M, de la Fouchardière C, Schlumberger M, Mantero F, Weismann D, Beuschlein F, Gelderblom H, Wilmink H, Sender M, Edgerly M, Kenn W, Fojo T, Müller H-H, Skogseid B.** Combination chemotherapy in advanced adrenocortical carcinoma. *N. Engl. J. Med.* 2012;366(23):2189–2197.

4. **Berruti A, Fassnacht M, Baudin E, Hammer G, Haak H, Leboulleux S, Skogseid B, Allolio B, Terzolo M.** Adjuvant therapy in patients with adrenocortical carcinoma: a position of an international panel. *J. Clin. Oncol.* 2010;28(23):e401–402; author reply e403.

5. **Else T, Williams AR, Sabolch A, Jolly S, Miller BS, Hammer GD.** Adjuvant therapies and patient and tumor characteristics associated with survival of adult patients with adrenocortical carcinoma. *J. Clin. Endocrinol. Metab.* 2014;99(2):455–461.

6. **Terzolo M, Angeli A, Fassnacht M, Daffara F, Tauchmanova L, Conton PA, Rossetto R, Buci L, Sperone P, Grossrubatscher E, Reimondo G, Bollito E, Papotti M, Saeger W, Hahner S, Koschker A-C, Arvat E, Ambrosi B, Loli P, Lombardi G, Mannelli M, Bruzzi P, Mantero F, Allolio B, Dogliotti L, Berruti A.** Adjuvant mitotane treatment for adrenocortical carcinoma. *N. Engl. J. Med.* 2007;356(23):2372–2380.

7. **Haak HR, Hermans J, van de Velde CJ, Lentjes EG, Goslings BM, Fleuren GJ, Krans HM.** Optimal treatment of adrenocortical carcinoma with mitotane: results in a consecutive series of 96 patients. *Br. J. Cancer* 1994;69(5):947–951.

8. **Baudin E, Pellegriti G, Bonnay M, Penfornis A, Laplanche A, Vassal G, Schlumberger M.** Impact of monitoring plasma 1,1-dichlorodiphenildichloroethane (o,p'DDD) levels on the treatment of patients with adrenocortical carcinoma. *Cancer* 2001;92(6):1385–1392.

9. **Malandrino P, Al Ghuzlan A, Castaing M, Young J, Caillou B, Travagli J-P, Elias D, de Baere T, Dromain C, Paci A, Chanson P, Schlumberger M, Leboulleux S, Baudin E.** Prognostic markers of survival after combined mitotane- and platinum-based chemotherapy in metastatic adrenocortical carcinoma (ACC). *Endocr Relat Cancer* 2010. doi:10.1677/ERC-09-0341.

10. **Wängberg B, Khorram-Manesh A, Jansson S, Nilsson B, Nilsson O, Jakobsson CE, Lindstedt S, Odén A, Ahlman H.** The long-term survival in adrenocortical carcinoma with active surgical management and use of monitored mitotane. *Endocr. Relat. Cancer* 2010;17(1):265–272.

11. **Hermsen IG, Fassnacht M, Terzolo M, Houterman S, den Hartigh J, Leboulleux S, Daffara F, Berruti A, Chadarevian R, Schlumberger M, Allolio B, Haak HR, Baudin E.** Plasma concentrations of o,p'DDD, o,p'DDA, and o,p'DDE as predictors of tumor response to mitotane in adrenocortical carcinoma: results of a retrospective ENS@T multicenter study. *J. Clin. Endocrinol. Metab.* 2011;96(6):1844–1851.

12. **Van Slooten H, Moolenaar AJ, van Seters AP, Smeenk D.** The treatment of adrenocortical carcinoma with o,p'-DDD: prognostic implications of serum level monitoring. *Eur J Cancer Clin Oncol* 1984;20(1):47–53.

13. **Gebhardt DO, Moolenaar AJ, van Seters AP, van der Velde EA, Gevers Leuven JA.** The distribution of o,p'-DDD (mitotane) among serum lipoproteins in normo- and hypertriglyceridemia. *Cancer Chemother. Pharmacol.* 1992;29(4):331–334.

14. **Maher VM, Trainer PJ, Scoppola A, Anderson JV, Thompson GR, Besser GM.** Possible mechanism and treatment of o,p'DDD-induced hypercholesterolaemia. *Q. J. Med.* 1992;84(305):671–679.

15. **Gebhardt DO, Moolenaar AJ, van Seters AP.** Mitotane (o,p'-DDD) administration raises the serum level of high density lipoprotein (HDL) in normotriglyceridemia. *Horm. Metab. Res.* 1993;25(4):240–241.

16. **Terzolo M, Pia A, Berruti A, Osella G, Alì A, Carbone V, Testa E, Dogliotti L, Angeli A.** Low-dose monitored mitotane treatment achieves the therapeutic range with manageable side effects in patients with adrenocortical cancer. *J. Clin. Endocrinol. Metab* 2000;85(6):2234–2238.

17. **Allolio B, Hahner S, Weismann D, Fassnacht M.** Management of adrenocortical carcinoma. *Clin. Endocrinol. (Oxf)* 2004;60(3):273–287.

18. **Daffara F, De Francia S, Reimondo G, Zaggia B, Aroasio E, Porpiglia F, Volante M, Termine A, Di Carlo F, Dogliotti L, Angeli A, Berruti A, Terzolo M.** Prospective evaluation of mitotane toxicity in adrenocortical cancer patients treated adjuvantly. *Endocr. Relat. Cancer* 2008;15(4):1043–1053.

19. **Shawa H, Deniz F, Bazerbashi H, Hernandez M, Vassilopoulou-Sellin R, Jimenez C, Habra MA.** Mitotane-induced hyperlipidemia: a retrospective cohort study. *Int J Endocrinol* 2013;2013:624962.

20. **Stacpoole PW, Varnado CE, Island DP.** Stimulation of rat liver 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity by o,p'-DDD. *Biochem. Pharmacol.* 1982;31(5):857–860.

21. **Moy RH.** Studies of the pharmacology of o,p'DDD in man. *J. Lab. Clin. Med.* 1961;58:296–304.

22. **Hescot S, Paci A, Seck A, Slama A, Viengchareun S, Trabado S, Brailly-Tabard S, Al Ghuzlan A, Young J, Baudin E, Lombès M.** The lack of antitumor effects of o,p'DDA excludes its role as an active metabolite of mitotane for adrenocortical carcinoma treatment. *Horm Cancer* 2014;5(5):312–323.

23. **Kaminsky N, Luse S, Hartroft P.** Ultrastructure of adrenal cortex of the dog during treatment with DDD. *J. Natl. Cancer Inst.* 1962;29:127–159.

24. **Poli G, Guasti D, Rapizzi E, Fucci R, Canu L, Bandini A, Cini N, Bani D, Mannelli M, Luconi M.** Morpho-functional effects of mitotane on mitochondria in human adrenocortical cancer cells. *Endocr. Relat. Cancer* 2013. doi:10.1530/ERC-13-0150.

25. **Hescot S, Slama A, Lombès A, Paci A, Remy H, Leboulleux S, Chadarevian R, Trabado S, Amazit L, Young J, Baudin E, Lombès M.** Mitotane alters mitochondrial respiratory chain activity by inducing cytochrome c oxidase defect in human adrenocortical cells. *Endocr. Relat. Cancer* 2013;20(3):371–381.

26. **Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J.** New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur. J. Cancer* 2009;45(2):228–247.

27. **Chretien D, Bénit P, Chol M, Lebon S, Rötig A, Munnich A, Rustin P.** Assay of mitochondrial respiratory chain complex I in human lymphocytes and cultured skin fibroblasts. *Biochem. Biophys. Res. Commun.* 2003;301(1):222–224.

28. **Guérin M, Bruckert E, Dolphin PJ, Turpin G, Chapman MJ.** Fenofibrate reduces plasma cholesteryl ester transfer from HDL to VLDL and normalizes the atherogenic, dense LDL profile in combined hyperlipidemia. *Arterioscler. Thromb. Vasc. Biol.* 1996;16(6):763–772.

29. **Cueto C, Brown JH, Richardson AP.** Biological studies on an adrenocorticolytic agent and the isolation of the active components. *Endocrinology* 1958;62(3):334–339.

30. **Pohland RC, Counsell RE.** The role of high density lipoproteins in the biodistribution of two radioiodinated probes in the rat. *Toxicol. Appl. Pharmacol.* 1985;77(1):47–57.

Figure 1

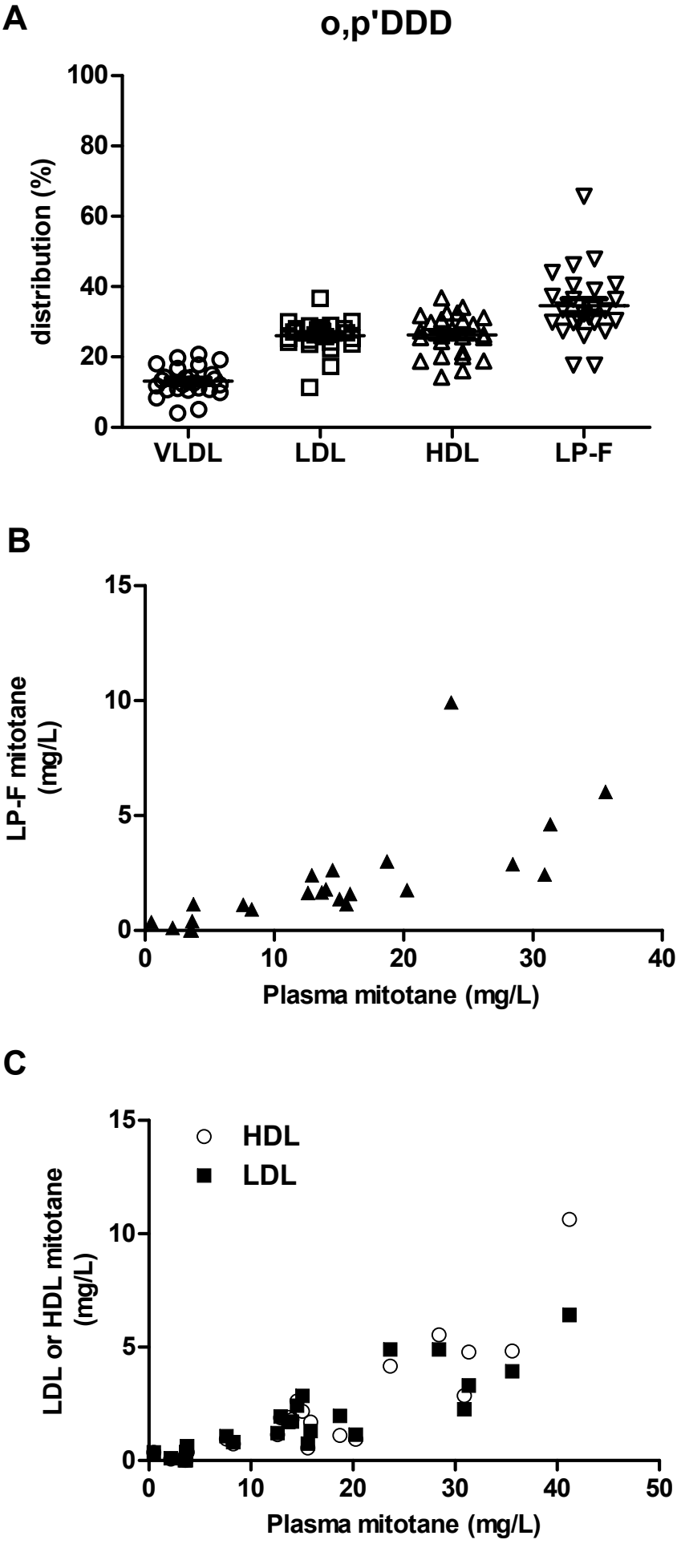

A
o,p'DDD
distribution (%)
100
80
60
40
20
0
VLDL
LDL
HDL
LP-F
B
LP-F mitotane (mg/L)
15
10
5
0
0
10
20
30
40
Plasma mitotane (mg/L)
C
HDL
LDL
LDL or HDL mitotane (mg/L)
15
10
5
0
0
10
20
30
40
50
Plasma mitotane (mg/L)

Figure 2

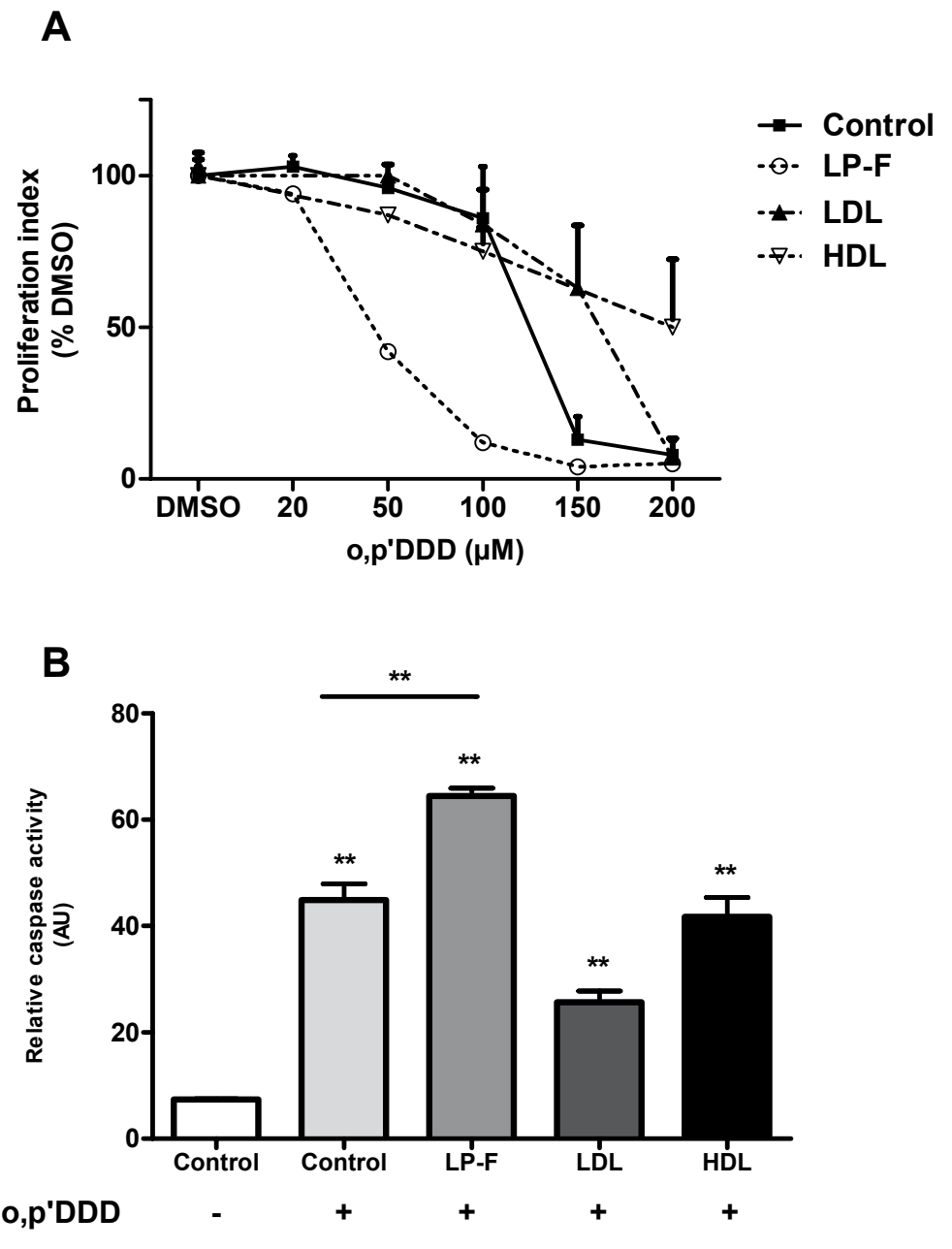

A
Proliferation index (% DMSO)
100
50
0
DMSO
20
50
100
150
200
o,p'DDD (μM)
Control
LP-F
LDL
HDL
B
Relative caspase activity (AU)
80
60
40
20
0
**
**
**
**
**
Control
Control
LP-F
LDL
HDL
o,p'DDD
-
+
+
+
+

Figure 3

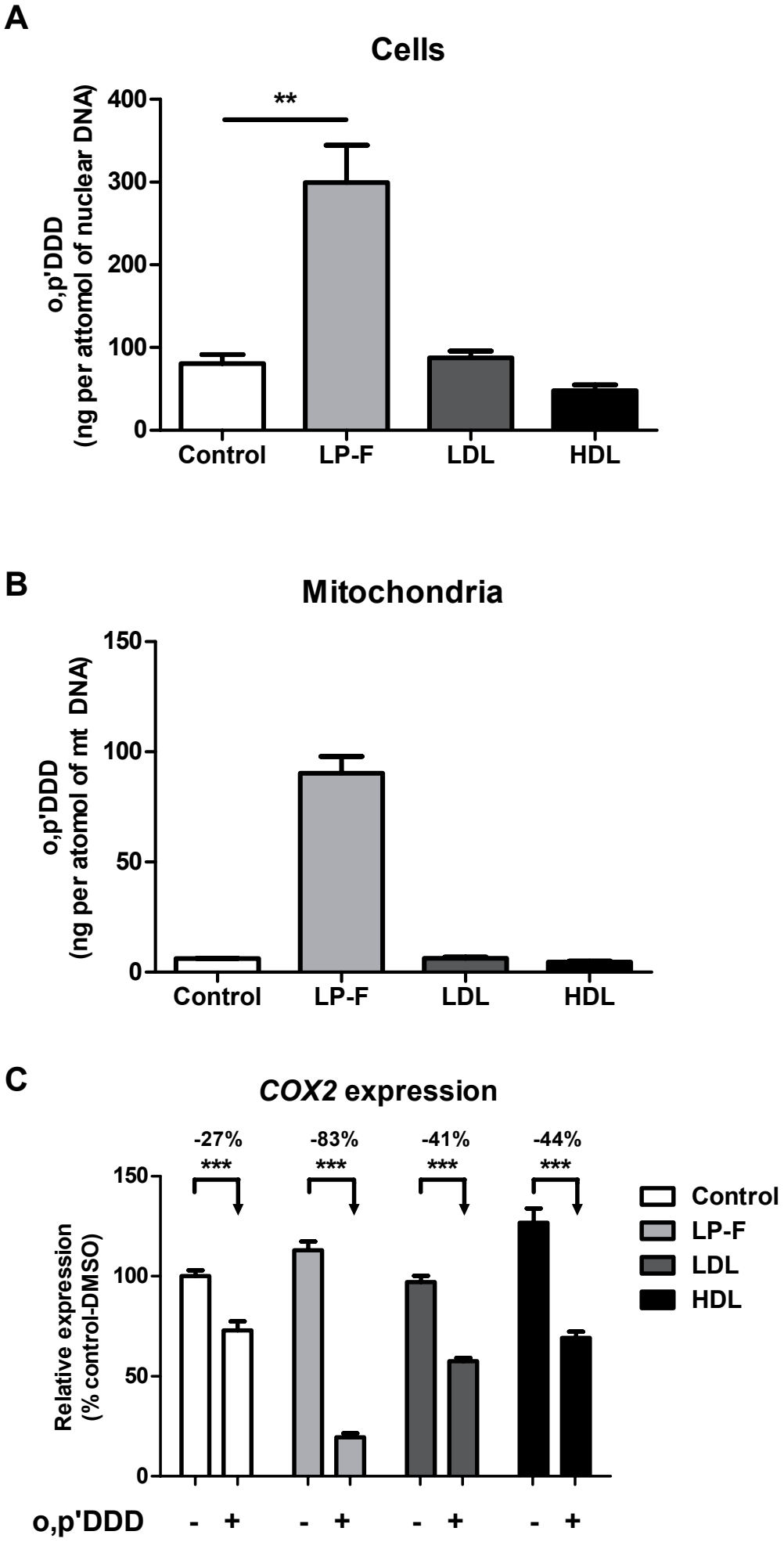
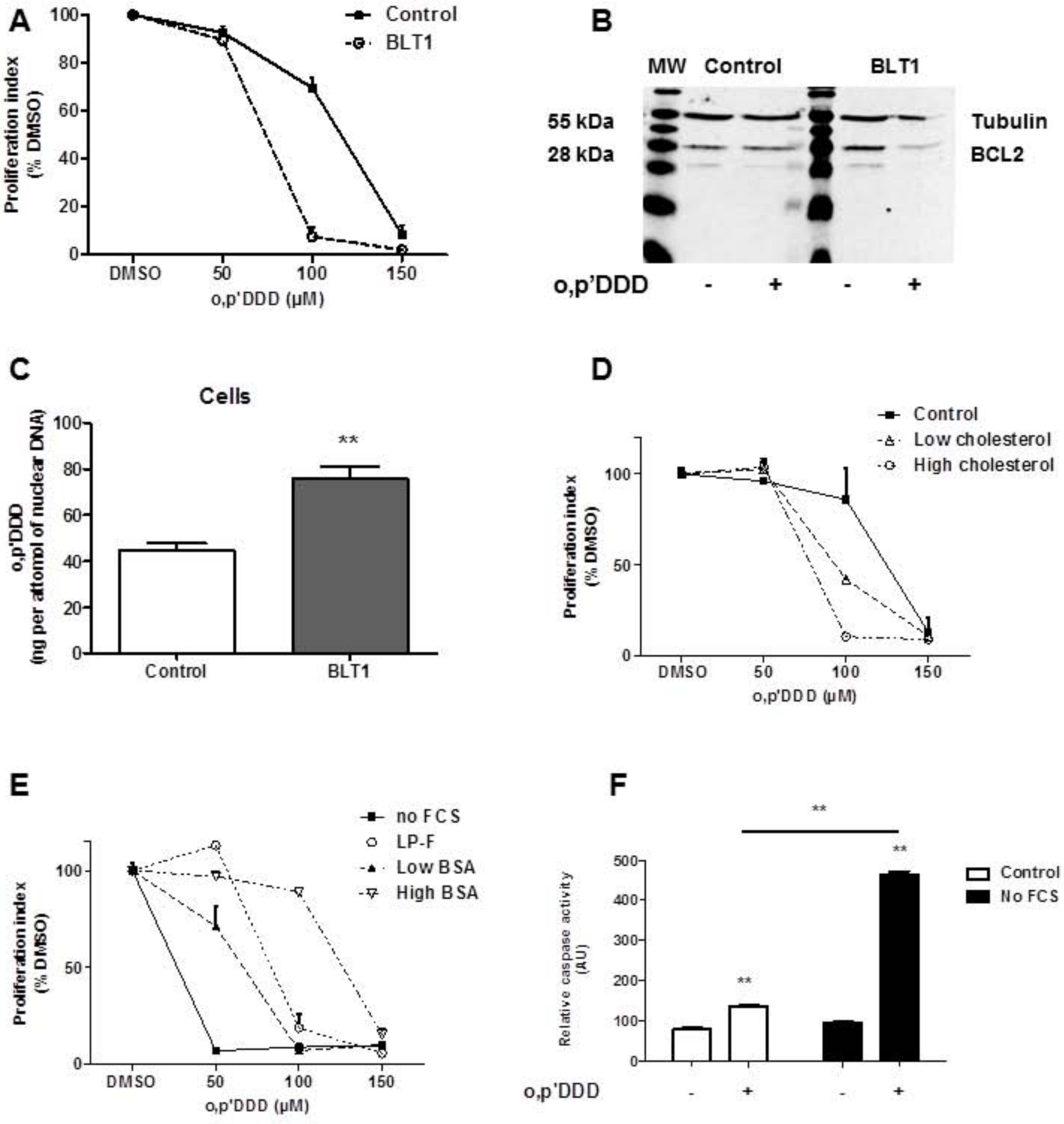

A
Cells
o,p'DDD
(ng per attomol of nuclear DNA)
400
300
200
100
0
**
Control
LP-F
LDL
HDL
B
Mitochondria
o,p'DDD
(ng per atomol of mt DNA)
150
100
50
0
Control
LP-F
LDL
HDL
C
COX2 expression
-27%
-83%
-41%
-44%
***
***
***
***
Relative expression
(% control-DMSO)
150
100
50
0
Control
LP-F
LDL
HDL
o,p'DDD
- + - + - + - +

Figure 4


A
Control
BLT1
Proliferation index (% DMSO)
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80
60
40
20
0
DMSO
50
100
150
o,p'DDD (µM)
B
MW
Control
BLT1
55 kDa
28 kDa
Tubulin
BCL2
o,p'DDD
-
+
-
+
C
Cells
o,p'DDD (ng per attomol of nuclear DNA)
**
Control
BLT1
D
Control
Low cholesterol
High cholesterol
Proliferation index (% DMSO)
o,p'DDD (µM)
E
no FCS
LP-F
Low BSA
High BSA
Proliferation index (% DMSO)
o,p'DDD (µM)
F
**
Relative caspase activity (AU)
Control
No FCS
o,p'DDD

Figure 5