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► **To cite this version:**

Ségolène Hescot, Atmane Seck, Maryse Guerin, Florence Cockenpot, Thierry Huby, et al.. Lipoprotein-free mitotane exerts high cytotoxic activity in adrenocortical carcinoma. *Journal of Clinical Endocrinology and Metabolism*, Endocrine Society, 2015, pp.22. <inserm-01175477>

HAL Id: inserm-01175477

<http://www.hal.inserm.fr/inserm-01175477>

Submitted on 10 Jul 2015

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1 **Lipoprotein-free mitotane exerts high cytotoxic activity in adrenocortical carcinoma**

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9

10 **Acknowledgments:** We thank Rita Chadarevian and Michele Resche-Rigon for the helpful discussion

11 **Abbreviated title:** Liprotein-free mitotane is more efficient

12 **Key terms:** adrenocortical carcinoma - mitotane - lipoprotein

13 **Word count:** 3496

14 **Tables and Figures:** 1 table and 5 figures

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17 **Disclosure summary:** S.H. is recipient of a fellowship from HRA Pharma Laboratories (Bourse
18 CIFRE).

19 The authors declare that they have no conflict of interest.

20

21

22 **Abstract**

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

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

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

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

44

45 **Introduction**

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

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

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

68 In the present study, we explored the biologic implication of serum lipoproteins on mitotane
69 pharmacological action using human plasma samples, tissues of mitotane-treated patients and finally
70 *in vitro* on human adrenocortical H295R cells. Altogether, our results showed that lipoprotein-free
71 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

75 **Patients, material and methods**

76 *Patients*

77 Medical files of 70 metastatic ACC patients treated with mitotane, followed between 2007 and 2014 at
78 Gustave Roussy were retrospectively reviewed to study the correlation between occurrence of
79 dyslipidemia or statin therapy (Rosuvastatin not metabolized by Cyp3A4) within the first 3 months of
80 mitotane therapy and neurological toxicity or tumor response. Inclusion criteria were patients with
81 stage IV ACC treated with mitotane after 2007 and the exclusion criteria was the absence of lipid
82 profile available within the first 3 months of mitotane therapy. In each file, the following criteria were
83 recorded: mitotane plasma level, HDL, LDL triglycerides levels (high HDL, high LDL or high
84 triglycerides defined as above 1.5 time the upper value, concurrent use of statins given during at least
85 3 months of mitotane therapy, presence of neurological toxicities and disease control rate (stabilization
86 and partial response) at 6 months according to RECIST 1.1 (26). An informed consent was obtained
87 from all patients.

88

89 *Human plasma samples and human adrenal tissues*

90 Twenty-five plasma samples from 20 mitotane-treated ACC patients were taken at Gustave Roussy
91 hospital and then made available for this study from the central repository of HRA Phama (Paris,
92 France) to evaluate lipoprotein partitioning in normal and dyslipidemic samples. To analyze the
93 correlation between mitotane concentrations and cholesterol transporter expression, human adrenal
94 tissues were obtained from 6 mitotane-treated patients followed at Gustave Roussy or Bicêtre
95 Hospital. All were ACC but one who underwent bilateral adrenalectomy for an ectopic Cushing
96 syndrome. Tissues were collected while patients underwent surgery for therapeutic reasons. Tissues
97 were lysed in H₂O using a TissueLyser apparatus (Qiagen, Courtaboeuf, France). All patients signed
98 an informed consent.

99

100 *Human adrenocortical cells and mitochondria isolation*

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

103 (LP-F medium) enriched or not with LDL or HDL subfractions obtained from ultracentrifugation
104 (with a final cholesterol concentration of 30 mg/L, similar as in all media). *O,p'*-DDD (HRA Pharma)
105 and BLT1 (Sigma-Aldrich, St. Louis, MO) were solubilized in dimethyl sulfoxide (DMSO, Sigma-
106 Aldrich) and used at indicated concentrations. The percentage of DMSO in culture medium never
107 exceeded 0.1%. Cholesterol (Sigma-Aldrich) and Bovine serum albumin (BSA, Euromedex,
108 Mundelsheim, France) were solubilized in culture medium and used at final concentrations of 9.6
109 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.
110 Mitochondrial fractions were purified and prepared from permeabilized cells using digitonin and
111 percoll as previously described (27).

112

113 *Lipoprotein isolation from plasma and fetal calf serum by ultracentrifugation*

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

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

124

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

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

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

136

137 *Cell proliferation and apoptosis analysis*

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

145

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

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

153

154 *Western Blot analysis*

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

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

161 *Statistical Analysis*

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

168 **Results**

169 **Distribution of *o,p'*-DDD and its metabolites among lipoproteins and lipoprotein-free** 170 **subfractions in ACC patients plasma samples**

171 The distribution of mitotane and its two major metabolites, *o,p'*-DDE and *o,p'*-DDA, in lipoproteins
172 was evaluated in 20 ACC patients. *O,p'*-DDD, *o,p'*-DDA and *o,p'*-DDE were thus measured with
173 HPLC-UV after ultracentrifugation of plasma samples in VLDL, LDL, HDL and lipoprotein-free (LP-
174 F) subfractions. *O,p'*-DDD was widely distributed among lipoprotein fractions as follows : $34.6 \pm$
175 9.9% LP-F (including proteins-bound and free mitotane), $26.3 \pm 5.8\%$ HDL, $26 \pm 4.6\%$ LDL and 13
176 $\pm 4.2\%$ VLDL (Figure 1A). The distribution of *o,p'*-DDE among these subfractions favoring LP-F, was
177 as follows ($72.9 \pm 15.3\%$ LP-F, $17.7 \pm 12.2\%$ HDL, $8.2 \pm 10.5\%$ LDL and $1.3 \pm 2.7\%$ VLDL) while,
178 in sharp contrast, *o,p'*-DDA was almost exclusively recovered in LP-F fractions ($94.6 \pm 3.1\%$, Suppl.
179 Figure S1A and B). No significant difference in *o,p'*-DDD, *o,p'*-DDA or *o,p'*-DDE distribution was
180 observed according to the presence and the degree of dyslipidemia or the plasma mitotane level (data
181 not shown). Of interest, plasma mitotane level correlated with *o,p'*-DDD measured in LP-F fractions
182 (Figure 1B, $r^2=0.41$; $P<0.001$) but also in those of HDL and LDL (Figure 1C, $r^2=0.76$; $P<0.001$).

183

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

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

190

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

192 *Impact on cell proliferation and apoptosis*

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

196 and compared to control (FCS). Cell proliferation index was measured at baseline and after incubation
197 with various mitotane concentrations for 48h. Basal cell proliferation was not different between these
198 conditions (Suppl. Figure S2A). Mitotane exerts a dose-dependent anti-proliferative effect in all
199 conditions but was more efficient when cells were cultured in LP-F medium with an IC50 of
200 approximately 40 μ M compared to 140 μ M under control conditions (Figure 2A) with a left-shift of
201 dose-dependent curve. Apoptosis index as measured by caspase 3/7 assays was significantly higher
202 after a 24h treatment with 100 μ M mitotane in LP-F condition compared to LDL, HDL or control
203 conditions (Figure 2B). Furthermore, expression of the anti-apoptotic protein BCL2 was reduced by
204 100 μ M mitotane in LP-F condition but not in others (Suppl. Figure S2B). Altogether, our results
205 clearly indicate that the cytotoxic effects of mitotane are more pronounced in the absence of
206 lipoproteins.

207

208 *Intracellular uptake of mitotane and mitochondrial impact*

209 To examine whether the nature of LP fractions affects mitotane uptake, *o,p'*-DDD concentrations were
210 measured in cell pellets after 48h of 50 μ M exposure in different conditions. Intracellular *o,p'*-DDD
211 concentrations, measured by the sensitive GC-MS technique and normalized to nuclear DNA were at
212 least 3 fold higher in cells cultured in LP-F medium than in other media (Figure 3A). The intracellular
213 mitotane was mostly recovered in the mitochondrial fraction ($89.2 \pm 3.6\%$ of *o,p'*-DDD) whereas only
214 $10.8 \pm 3.6\%$ of the measured intracellular *o,p'*-DDD was in the cytosolic fraction (data not shown).
215 Intra-mitochondrial *o,p'*-DDD concentration was 15 higher (90.26 ± 7.66 ng/ atomol of mt DNA) in
216 cells grown in LP-F condition compared to other culture conditions, highly suggestive of a better
217 cellular and thus mitochondrial uptake of mitotane in the absence of lipoproteins (Figure 3B). We next
218 studied the expression of genes encoding proteins involved in oxidative phosphorylation or
219 steroidogenesis by RT-qPCR in *o,p'*-DDD treated cells under HDL, LDL, LP-F and control
220 conditions. In LP-F medium, mitotane strongly inhibited *COX2* expression (encoded by the
221 mitochondrial mtDNA for the subunit 2 of cytochrome c oxidase or respiratory chain complex IV,
222 Figure 3C) but also *StAR* and *CYP11A1* involved in steroidogenesis (Suppl. Figure S3A and B). LP-F
223 condition also led to a drastic reduction in CYP11A1 protein expression in H295R cells after a 48h

224 treatment with 50 μ M 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 $7.5 \pm 3.8\%$ in BLT1-treated cells compared to $69.5 \pm 4.3\%$ in control cells ($p < 0.01$) at 100
233 μ M 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

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

253 To translate these observations into the clinic, we retrospectively collected data from ACC patients
254 followed at our institution. Stage IV-ACC patients under mitotane therapy initiated between
255 September 2007 and January 2014 were included. Twenty-six patients had a mean plasma mitotane
256 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
257 mg/L at 6 month). Among them, 16 (61.5%) experienced hypertriglyceridemia, 15 (57.7%) an increase
258 in HDL cholesterol or 8 (30.8%) an increase in LDL cholesterol level under mitotane. Eleven patients
259 (42.3%) were treated with statins (introduced at least 3 months before RECIST evaluation).
260 Neurological toxicity was reported in 8 out of 26 patients (30.8%) at 1, 3 and/or 6 months. According
261 to RECIST criteria, (Response Evaluation Criteria in Solid Tumors) disease control rate (DCR) at 6
262 months was 46.2% (12 out of 26 patients) including 4 patients that experienced a partial response. A
263 significant positive association was found between the use of statin therapy and DCR at 6 months:
264 67% or 33% DCR was observed in patients treated or not with statins ($p < 0.05$, Fisher's exact test,
265 Figure 5). No association was found between dyslipidemia and neurological toxicity or tumor control
266 or statins therapy and neurological toxicity.

267 **Discussion**

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

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

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

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

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

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

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

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

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

330

331 **Figures Legends**

332 **Figure 1**

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

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

341

342 **Figure 2**

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

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

351

352 **Figure 3**

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

357 (C) Relative mRNA expression of *COX2* determined by RT-qPCR. H295R cells were treated with 50
358 μM *o,p'*-DDD for 48h. Results are expressed as mean percentage \pm 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 μ M) on the proliferation
364 index of H295R cells after 48 h culture in the absence or presence of 5 μ M BLT1 as determined by
365 WST1 assays. Results are expressed as mean percentage \pm 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 μ M BLT1 and
369 treated with 50 μ M *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 μ M mitotane in the absence or presence of 5 μ M BLT1. Results are expressed as mean percentage \pm
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 μ M) 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 \pm 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 μ M) 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 \pm 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 μ M of *o,p'*-DDD as determined by Caspase-Glo 3/7
385 assays. Results are expressed as mean arbitrary units \pm SEM of 6 independent determinations.
386 **P<0.01, Mann-Whitney U test.

387 **Figure 5**

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

391

392 **Legends to the Supplemental Figures**

393 **Figure S1**

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

398

399 **Figure S2**

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

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

406

407 **Figure S3**

408 Relative mRNA expression of *StAR* (A) and *CYP11A1* (B) determined by RT-qPCR. H295R cells
409 were treated with 50 μ M *o,p'*-DDD for 48h. Results are expressed as mean percentage \pm SEM of 6
410 different experiments performed in duplicate, values of vehicle-treated cells being arbitrarily set at
411 100%. ***P<0.001, Mann-Whitney U test.

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

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Figure 1

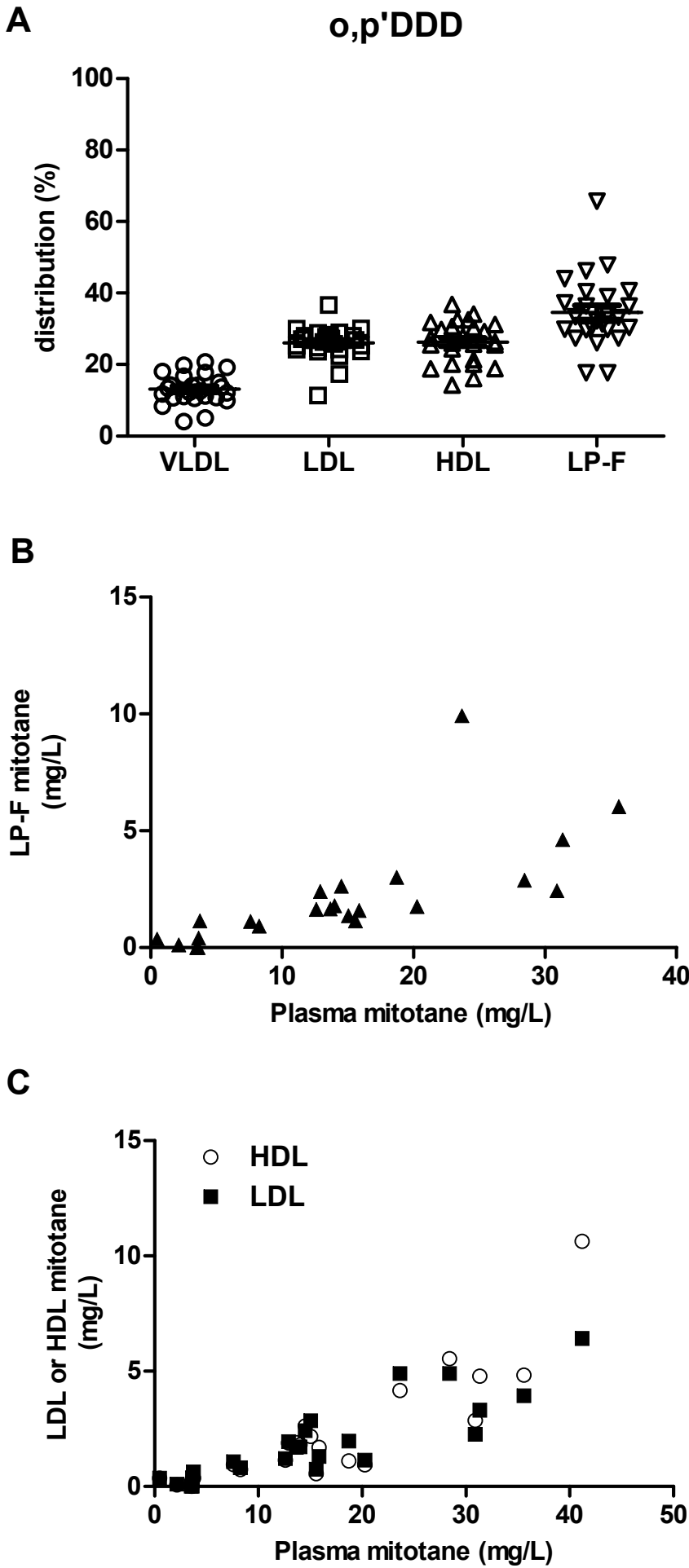


Figure 2

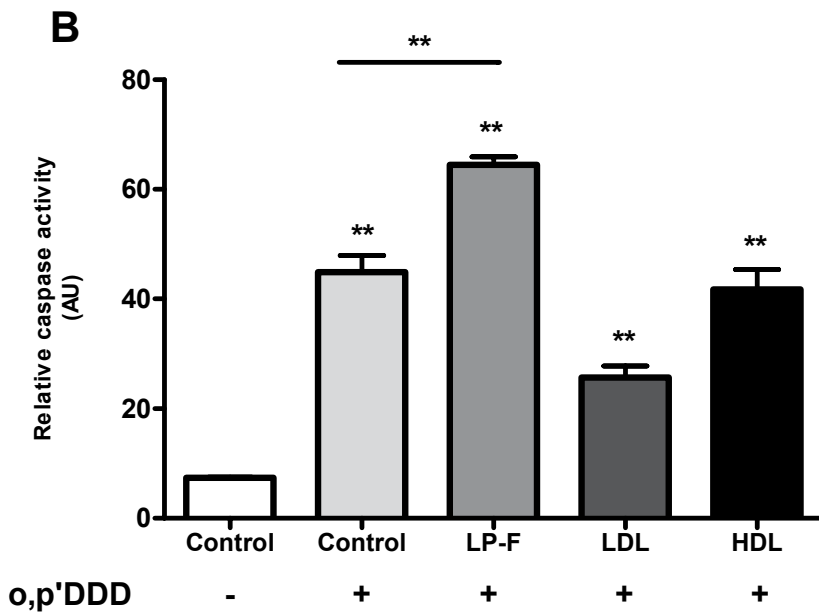
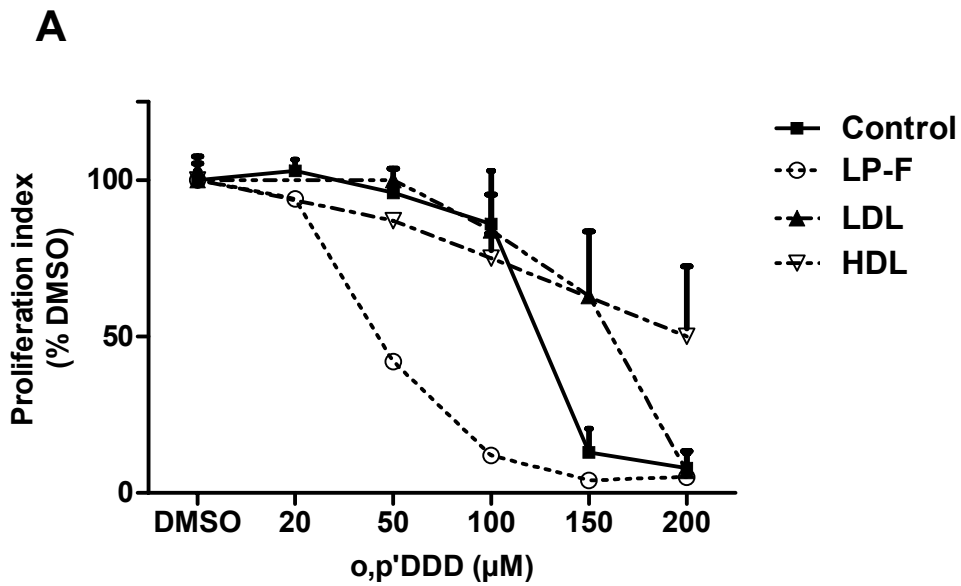
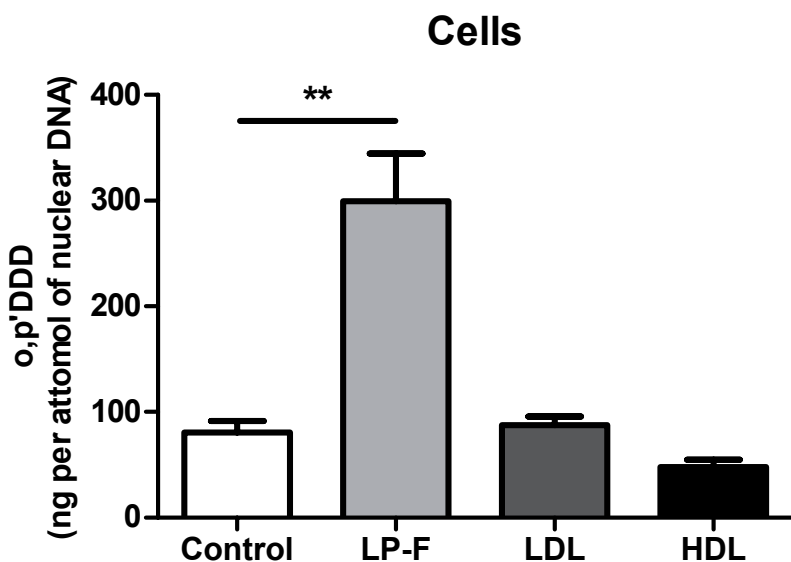
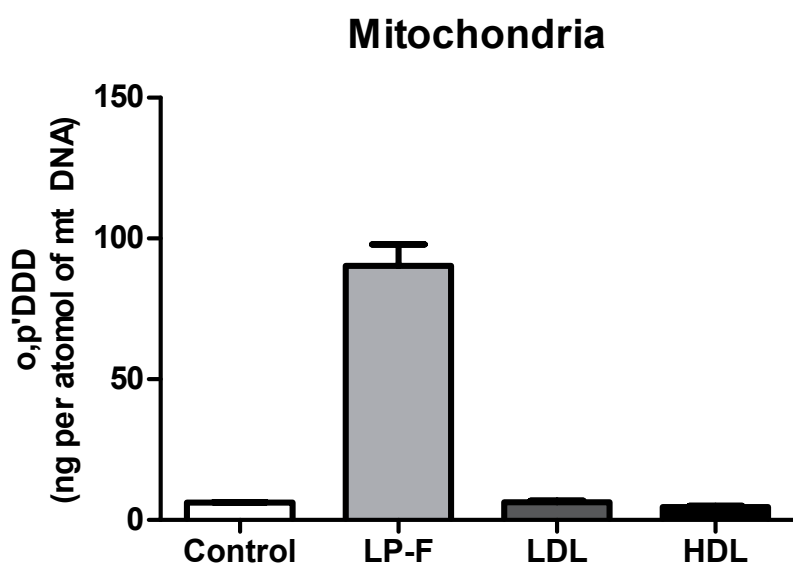


Figure 3

A



B



C

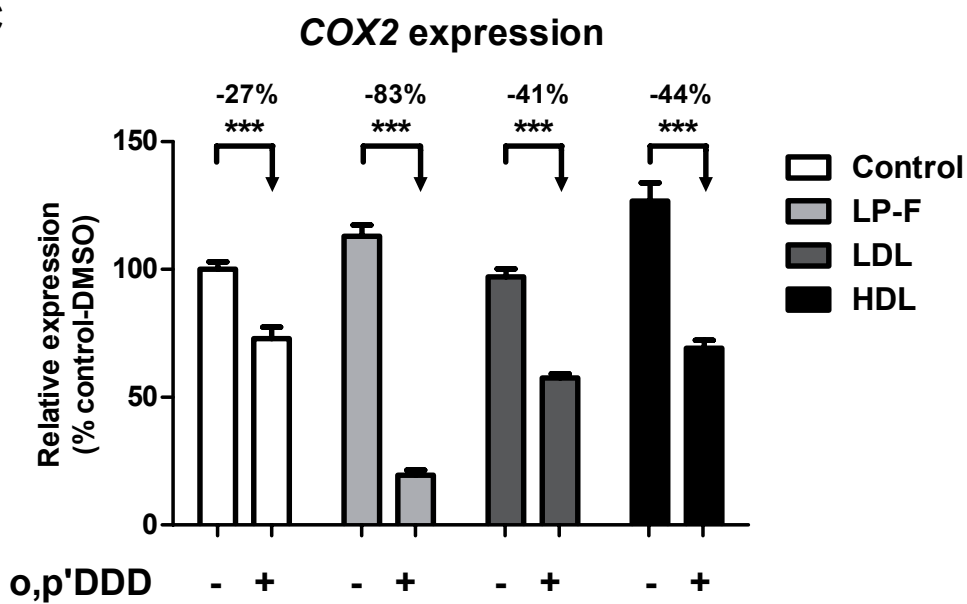


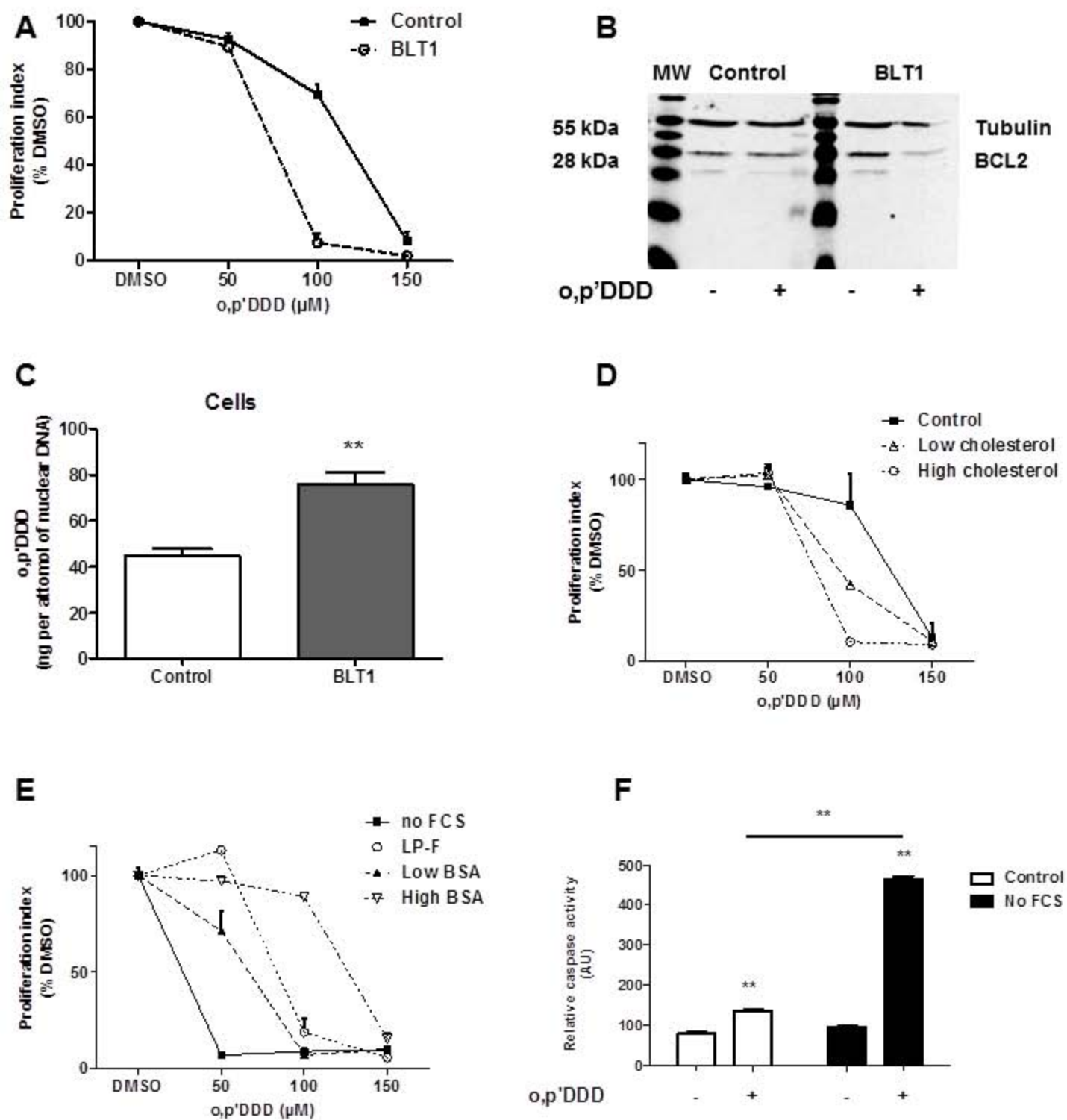
Figure 4

Figure 5

