

The Lack of Antitumor Effects of o,p'DDA Excludes Its Role as an Active Metabolite of Mitotane for Adrenocortical Carcinoma Treatment.

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1 **The lack of antitumor effects of o,p’DDA excludes its role as an active**
2 **metabolite of mitotane for adrenocortical carcinoma treatment**

3
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18

19 **Short title:** o,p’DDA is not an active metabolite of mitotane

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24 **Keywords:** Adrenocortical carcinoma, mitotane, o,p’-DDD, o,p’-DDA

25

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27 **Abstract**

28 Mitotane (o,p'DDD) is the most effective treatment of advanced adrenocortical carcinoma (ACC) but
29 its mechanism of action remains unknown. Previous studies suggested that o,p'DDA may represent
30 the active metabolite of mitotane. We aimed at re-evaluating the potential role and pharmacological
31 effects of o,p'DDA. Functional consequences of o,p'DDA exposure were studied on proliferation,
32 steroidogenesis and mitochondrial respiratory chain in human H295R and SW13 adrenocortical cells.
33 Mitotane and its metabolites were quantified using HPLC-UV in these cells treated with o,p'DDD or
34 o,p'DDA and in human adrenal tissues. Dose-response curves up to 300 μ M showed that, as opposed
35 to o,p'DDD, o,p'DDA did not inhibit cell proliferation, nor alter respiratory chain complex IV activity,
36 gene expression nor induce mitochondrial biogenesis, oxidative stress or apoptosis. However, whereas
37 mitotane drastically decreased expression of genes involved in steroidogenesis, o,p'DDA slightly
38 reduced expression of some steroidogenic enzymes yet exerts weak anti-secretory effects only at high
39 doses. While o,p'DDD concentration was significantly reduced by 40% in H295R cell supernatants
40 after 48 h incubation, o,p'DDA levels remained unchanged suggesting that o,p'DDA was not
41 efficiently transported into the cells. O,p'DDA was not detected in cell homogenates or supernatants
42 after 48h exposure to o,p'DDD, consistent with the absence of o,p'DDA production in these
43 models. Finally, unlike o,p'DDD, we found that o,p'DDA content was undetectable in two ACC and
44 one normal adrenal gland of mitotane-treated patients, suggesting a lack of cellular uptake and *in situ*
45 production. Our results demonstrate that o,p'DDD, but not o,p'DDA, induces functional alterations in
46 adrenal cells.

47 **Introduction**

48 Mitotane (o,p'DDD) represents the most effective drug for the treatment of advanced adrenocortical
49 carcinoma (ACC), and is the only drug approved in the advanced setting[1, 2]. Mitotane is prescribed
50 either in monotherapy or associated with platine-based chemotherapy and induces tumor response
51 rates in up to one third of patients[3]. Recently, the first phase III trial performed in advanced ACC
52 demonstrated that combination of mitotane chemotherapy with cisplatin-doxorubicin-epidoxin was
53 more effective in prolonging progression free survival and rate of objective response than the
54 combination of mitotane and streptozotocin[4]. In addition, mitotane is also recommended in the
55 adjuvant setting in ACC patients at high risk of recurrence[5, 6].

56 However, the mechanism of mitotane action remains to be understood. To date, the only predictive
57 factor of tumor response is the plasma mitotane level. Indeed, several studies have reported that a
58 higher response rate correlates to plasma mitotane levels above 14 mg/l [7–11]. Several retrospective
59 studies have also suggested a prolonged survival in case of plasma mitotane levels above 14 mg/l [7,
60 9, 11] but not all [10, 12]. In addition, as neurological toxicities have been found more frequent with
61 mitotane plasma levels above 20 mg/l [13], the recommended therapeutic window is currently between
62 14 and 20 mg/l [2].

63 In a recent study, Hermsenet *al.* analysed for the first time the respective role of plasma mitotane level
64 and its metabolites 1,1-(o,p'-dichlorodiphenyl) acetic acid (o,p'DDA) and o,p'DDE as predictive
65 factors of response in advanced ACC patients. This study concluded that the plasma o,p'DDD level
66 only was associated with tumor response while no major role for its metabolites could be retrieved,
67 questioning the real impact of o,p'DDA as an active metabolite, as previously suggested in preclinical
68 studies.

69 At the time of ingestion, only 35% of o,p'DDD is absorbed from the gastrointestinal tract [14] and
70 o,p'DDA has been first described as the main urinary metabolite of o,p'DDD[15, 16]. O,p'DDA is an
71 acid derivative of o,p'DDD, obtained from two consecutive reactions of β -hydroxylation and
72 dehydrochlorination[17]. The site of o,p'DDA synthesis remains unclear in humans. Martz and Straw
73 and later Pohland et al were able to detect o,p'DDA in hepatic microsomes suggesting an hepatic

74 metabolism of o,p'DDD[18, 19]. Quantitative analyses measuring radiolabelled o,p'DDA in various
75 species further revealed minute amounts of o,p'DDA in adrenal mitochondria [20–22]. From these
76 initial observations emerged a first hypothesis that o,p'DDA could be synthesized in the liver or the
77 adrenals and may represent the active metabolite responsible of the cytotoxic effect observed in the
78 adrenal cortex. Others studies suggested that an adrenal metabolic transformation of o,p'DDD into
79 o,p'DDA could be responsible of the adrenolytic effect [23]. Finally, the question on whether
80 o,p'DDA is responsible for antitumor effects of mitotane in ACC patients remains totally unanswered.
81 We have previously described molecular effects of o,p'DDD on cell proliferation, steroidogenesis and
82 respiratory chain activity [24]. The aim of the present study was to investigate the exact role of
83 o,p'DDA, in comparison with o,p'DDD. For this purpose, we first explored *in vitro* moleculareffects
84 of o,p'DDA on cell proliferation, mitochondrial respiratory chain and steroidogenesis. We thus studied
85 o,p'DDA conversion and uptake in human adrenocortical H295R and SW13 cell lines and human
86 adrenal tissue samples.

87 **Materials and Methods**

88 **Human adrenocortical cells and human adrenal tissues**

89 H295R and SW13 cells were cultured in DMEM/HAM'S F-12 medium (PAA, Les Mureaux, France)
90 supplemented with 20 mM HEPES (Life technologies, Saint Aubin, France), antibiotics (penicillin
91 100 IU/ml, streptomycin 100 µg/mL) and 2 mM glutamine (all from PAA). The medium for H295R
92 cell culture was enriched with 10% fetal bovine serum and a mixture of insulin/transferrin/selenium.
93 Both cell lines (from passage 2 to 15) were cultured at 37°C in a humidified incubator with 5% CO₂.
94 Mitotane (supplied by HRA Pharma, Paris, France) and o,p'DDA (Alsachim, Illkirch-Graffenstaden,
95 France) were solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Quentin Fallavier,
96 France) and used at 10 to 300 µM final concentrations. The percentage of DMSO in culture medium
97 never exceeded 0.1%.

98 Tissues were obtained from six patients treated with mitotane. Five ACC patients were followed at
99 GustaveRoussy, while patient 1, followed at Bicêtre Hospital, underwent bilateral adrenalectomy for
100 an ectopic Cushing syndrome due a bronchopulmonary ACTH-secreting tumor. All patients signed an
101 informed consent. Tissues were lysed in H₂O using a TissueLyser apparatus (Qiagen, Courtaboeuf,
102 France).

103

104 **Steroid secretion**

105 Steroid hormones concentrations were measured in the supernatants of H295R cultured cells using
106 radioimmunoassays for 11-deoxycortisol (DiaSource Immunoassays, Louvain-la-Neuve, Belgium) and
107 17-hydroxyprogesterone (MP Biomedicals, Ohio, USA) or chemiluminescent enzyme immunoassay for
108 cortisol (Immulite 2000 XPI, Siemens Healthcare Diagnostics Products, Llanberis, UK). The intra-
109 assay coefficient of variation (CV) were 5.2% and 7.7% at 3.7 and 28.3 ng/ml, and the inter-assay CV
110 were 11.5% and 15.1% at 5.5 and 36.9 ng/ml for 11-deoxycortisol. The intra-assay CV of 17-
111 hydroxyprogesterone radioimmunoassay were 7.8% and 8.3% at 2.7 and 13.2 ng/ml respectively and
112 the inter-assay CV were 9.8% and 12.8% at 12.3 and 22.8 ng/ml. The intra-assay CV of cortisol assay

113 were 6.1% and 5.2% at 33 and 85 ng/ml, and the inter-assay CV were 8.2% and 6.8% at 33 and 85
114 ng/ml.

115

116 **Cell proliferation analysis**

117 Cell proliferation tests were performed by using the WST1 assay (Roche, Meylan, France) according
118 to the manufacturer's recommendations. Cells were cultured in 96-well plates and treated with 10 to
119 300 μ M mitotane or o,p'DDA for 48 h. Optical densities were measured 4 h after addition of WST1
120 solution (10 μ l per well) by spectrophotometry (Viktor, Perkin Elmer, Courtaboeuf, France).

121

122 **Respiratory chain analysis**

123 Respiratory chain activities were measured using spectrophotometric assays. H295R cells were treated
124 with 50 μ M of mitotane (16 mg/l), 50 μ M of o,p'DDA (14 mg/l) or vehicle (DMSO) alone for 48 h
125 and the activity of complex IV (cytochrome c oxidase, COX) was measured in a Cary 50
126 spectrophotometer, as previously described [24]. The activity of Complex IV was normalized to that of
127 citrate synthase activity, as an index of mitochondrial mass.

128

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

130 Total RNAs were extracted from cells with the RNeasy kit (Qiagen, Courtaboeuf, France) according to
131 the manufacturer's recommendations. RNA was thereafter processed for RT-PCR as previously
132 described [24]. Quantitative real-time PCR (RT-qPCR) was performed using the Fast SYBR[®] Green
133 Master Mix (Life Technologies) and carried out on a StepOnePlus[™] Real-Time PCR System (Life
134 Technologies) as previously described [24]. Standards and samples were amplified in duplicate and
135 analyzed from six independent experiments. The ribosomal 18S rRNA was used as the internal control
136 for data normalization. The relative expression of each gene was expressed as the ratio of attomoles of
137 specific gene to femtomoles of 18S rRNA. The primer sequences of the genes analyzed by qRT-PCR
138 are shown in the Supplemental Table 1.

139

140 **Western Blot analysis**

141 Total protein extracts were prepared from cells lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150
142 mM NaCl, 5 mM EDTA, 30 mM Na pyrophosphate, 50 mM Na fluoride, 1% Triton X-100), and 1X
143 protease inhibitor (Sigma-Aldrich, St. Louis, MO). After protein blotting on an Odyssey nitrocellulose
144 membrane (LI-COR, Lincoln, NE, USA), blots were incubated for 1 h at RT in a blocking buffer (5%
145 fat-free milk in Phosphate-buffer saline PBS with 0.1 % Tween 20) before an overnight incubation at
146 4°C with a rabbit anti-Bcl2 antibody (1:500 dilution, Cell Signaling, Saint Quentin en Yvelines,
147 France) and a mouse anti- α -Tubulin antibody (1:10,000 dilution, Sigma-Aldrich). After extensive
148 washes, blots were incubated with an IRDye 800-conjugated affinity purified anti-rabbit IgG second
149 antibody (1:15,000 dilution, Perbio Science, Brebières, France) and an IRDye 680-conjugated affinity
150 purified anti-mouse IgG second antibody (1:15,000 dilution, Perbio Science) for 1 h at RT. After
151 washes, proteins were visualized with an Odyssey-Fc apparatus (LI-COR). Specific signals for Bcl2
152 were normalized by the infrared fluorescence of α -Tubulin signals as determined by densitometry
153 using the Image Studio software (LI-COR).

154

155 **Measurements of o,p'DDD, o,p'DDA and o,p'DDE metabolites**

156 Analyses were conducted by high performance liquid chromatography combined to an ultraviolet
157 detection (HPLC-UV) on plasma samples of 100 μ l spiked with known amounts of *p,p'*-DDE used as
158 an internal standards (IS) of *o,p'*-DDD and *o,p'*-DDE and *p,p'*-DDA used as an internal standards (IS)
159 of *o,p'*-DDA measurements. *O,p'*-DDD (Mitotane), *o,p'*-DDA and *o,p'*-DDE concentrations were
160 determined through the ratio of their peak surface area to the peak surface of known concentrations of
161 IS. The method was validated in terms of linearity, precision and accuracy, with a threshold detection
162 limits of 2.5 mg/l, 0.1 mg/l and 0.25 mg/l for *o,p'*-DDA, *o,p'*-DDE and *o,p'*-DDD, respectively.

163

164 **Statistical Analysis**

165 Results are expressed as means \pm SEM of n independent replicates performed in the same experiment
166 or from separated experiments (n). Non-parametric Mann Whitney tests were used when appropriate
167 and differences between groups were analyzed using non-parametric Kruskal-Wallis multiple

168 comparison test followed by a post-test of Dunn's (Prism software, GraphPad, CA). A *P* value of 0.05
169 was considered as statistically significant (**P*<0.05; ***P*<0.01; ****P*<0.001).

170 **Results**

171 **Comparative analysis of o,p'DDD and o,p'DDA impact on cell proliferation**

172 We studied the potential anti-proliferative effect of o,p'DDA on H295R and SW13 cells, testing the
173 hypothesis that o,p'DDA might exert antitumor properties. It is well established that circulating
174 o,p'DDA concentrations are about 5 times higher than those of o,p'DDD measured in plasma of
175 patients treated with mitotane[11]. We thus explored functional consequences of high doses of
176 o,p'DDA(50 to 300 μ M corresponding to 14 to 84 mg/l) compared to o,p'DDD on cell proliferation
177 using the colorimetric solution WST1 (Fig. 1). As previously reported, we confirmed that o,p'DDD
178 inhibited proliferation of H295R cells in a dose-dependent manner, with a significant 50% decrease
179 when cells were exposed to 100 μ M for 48 h and a 95% inhibition when treated with 200 and 300 μ M.
180 In sharp contrast, we showed that o,p'DDA, even at highest concentrations corresponding to its
181 plasma circulating levels in patients, had no significant effect on H295R cell proliferation.

182

183 **Comparative effects of o,p'DDD and o,p'DDA on respiratory chain complex IV activity**

184 We have previously shown that mitotane significantly impaired mitochondrial respiratory chain
185 function by selectively inhibiting enzymatic complex IV activity (cytochrome c oxidase, COX) [24].
186 We thus evaluated the effects of o,p'DDA on COX activity in H295R cells by spectrophotometric
187 assays. COX activity remained unaffected after 48 h exposure to 50 μ M of o,p'DDA with a mean
188 activity of 204 ± 13 nmol/min per mg protein, not significantly different from vehicle-treated cells
189 (Fig. 2A). Likewise, activity of citrate synthase (CS), an index of the mitochondrial mass, was not
190 modified in o,p'DDA-treated cells (186 ± 8 nmol/min per mg protein), as compared to vehicle-treated
191 cells (207 ± 6 nmol/min per mg protein). When COX enzymatic activity were normalized to citrate
192 synthase activity (Fig. 2B), o,p'DDA exposure did not modify mitochondrial respiratory chain activity
193 whereas o,p'DDD significantly reduced COX/CS ratio, as anticipated. We also compared the effect of
194 o,p'DDA and o,p'DDD on the steady-state level of mitochondrial DNA-encoded *COX2* transcript
195 (Fig. 2C). While *COX2* expression was significantly reduced by more than 40% upon o,p'DDD
196 treatment, o,p'DDA failed to modify *COX2* mRNA levels. Similar results were obtained with nuclear

197 DNA-encoded *COX4* transcripts (data not shown). Taken together, these results clearly demonstrate
198 that o,p'DDA was ineffective in modulating mitochondrial activity.

199

200 **Consequences of o,p'DDD or o,p'DDA treatment on mitochondrial biogenesis, oxidative stress** 201 **and apoptosis.**

202 In response to mitotane-elicited respiratory chain inhibition, we have previously demonstrated a
203 compensatory increase of mitochondrial biogenesis[24]. We therefore investigated whether or not
204 o,p'DDA was able to induce similar compensatory mechanisms. Thus, expression of the Peroxisome
205 proliferator-activated receptor gamma coactivator 1-related Coactivator (*PRC*), a key regulator of
206 mitochondrial biogenesis, was quantified by RT-qPCR (Fig. 3A). We showed that o,p'DDD exposure
207 induces a 2-fold increase in *PRC* transcript levels whereas treatment with o,p'DDA has no significant
208 effect of *PRC* mRNA levels, consistent with the absence of effect on respiratory chain activity
209 described above. We also evaluated consequences of o,p'DDA treatment compared to o,p'DDD on
210 oxidative stress and apoptosis by studying the expression of *SOD2* encoding superoxide dismutase 2,
211 an enzyme involved in reactive oxygen species (ROS)-induced detoxification and known to be induced
212 during oxidative stress. Steady-state levels of *SOD2* messengers were significantly increased after
213 incubation with o,p'DDD whereas they remained unchanged with o,p'DDA, suggesting that o,p'DDD
214 but not o,p'DDA induces oxidative stress in H295R cells (Fig. 3B). We found similar results in SW13
215 (data not shown). At a 50 μ M concentration, neither o,p'DDD nor o,p'DDA induces apoptosis as
216 revealed by the anti-apoptotic protein B cell lymphoma 2 (*Bcl2*) expression using Western Blot.
217 However, a drastic reduction of *Bcl2* expression was observed with 100 μ M mitotane, such reduction
218 was not detected upon o,p'DDA incubation, suggesting that H295R cells were unaffected by high
219 doses of o,p'DDA(Fig. 3C).

220

221 **Differential effects of o,p'DDD and of o,p'DDA on steroidogenesis**

222 Mitotane might exert dissociated effects with distinct time-course patterns, consisting of an early
223 inhibitory effect on steroidogenesis followed by a later cytolytic effect responsible for adrenal cortex
224 atrophy. To explore the impact of o,p'DDA on steroidogenesis, 17-hydroxyprogesterone,

225 deoxycortisol and cortisol concentrations were measured in the supernatants of steroid-secreting
226 H295R cells. Exposure to 50 μM o,p'DDA failed to inhibit secretion of 17-hydroxyprogesterone (Fig.
227 4A) as compared to the 80% reduction observed following o,p'DDD incubation. However, when used
228 at higher concentrations up to 300 μM , o,p'DDA slightly but significantly reduced deoxycortisol and
229 cortisol production without affecting 17-hydroxyprogesterone secretion of H295R cells (Fig. 4B).
230 Expression of several genes encoding for proteins involved in steroidogenesis was studied by RT-
231 qPCR (Fig. 4C). While 50 μM mitotane significantly induced a 80 to 95% decrease of all genes
232 studied, o,p'DDA failed to modify expression of *StAR* (encoding the transporter of cholesterol into
233 mitochondria) or *CYP11A1* (encoding cholesterol desmolase) but had negligible effect on *HSD3B2*
234 (encoding 3β -hydroxysteroid dehydrogenase), *CYP21* (encoding 21-hydroxylase) and *CYP11B1*
235 (encoding 11β -hydroxylase) consistent with the anti-secretory effects of higher doses of o,p'DDA.

236

237 **Intracellular concentrations of o,p'DDD and o,p'DDA in human adrenocortical cells treated** 238 **with mitotane**

239 We next examined the intracellular distribution of these drugs and investigated the ability of human
240 adrenocortical cells to uptake o,p'DDD. We also addressed the question on whether human
241 adrenocortical H295R and SW13 cells were able to actively convert mitotane into o,p'DDA. To
242 explore these possibilities, H295R and SW13 cells were treated with
243 50 μM of mitotane for 48 h and o,p'DDD and o,p'DDA concentrations were measured in cultured cell
244 supernatants as well as in cell pellets by using HPLC-UV. As shown in Fig. 5A, when H295R cells
245 were exposed to o,p'DDD at a theoretical concentration of 50 μM (measured concentration of 54.4 ± 6.2
246 μM , corresponding to a initial total amount of $325 \pm 49 \mu\text{g}$), a remaining $31.1 \pm 8.7 \mu\text{M}$ concentration
247 ($200 \pm 56 \mu\text{g}$) was recovered in cell supernatants after 48 h, suggesting an approximately 40% cellular
248 uptake of o,p'DDD. Accordingly, we were able to measure a total amount of $79 \pm 5.1 \mu\text{g}$ of mitotane
249 in cell pellets (mean \pm SEM, n= 3 independent experiments) indicating that approximately 25% of
250 o,p'DDD was actively concentrated into H295R cells while 15% of initial o,p'DDD was likely
251 deteriorated or bound to the plate as confirmed by a cell free culture experiment. Similar results were
252 found with SW13 cells (data not shown). Surprisingly, under these experimental conditions, despite a

253 substantial o,p'DDD uptake as measured above, o,p'DDA concentrations were below limit LOQ
254 threshold in both cell homogenates and cell supernatants, indicating that H295R cells were unable to
255 convert o,p'DDD into o,p'DDA and subsequently to secrete the acidic metabolite of mitotane.

256

257

258 **Assessment of o,p'DDA uptake in human adrenocortical H295R and SW13 cells**

259 We next examined the ability of H295R cells to uptake o,p'DDA as compared to o,p'DDD. As shown
260 in Fig. 5B, under experimental conditions in which H295R cells were exposed to 50 μ M of o,p'DDA
261 during 48 h, o,p'DDA concentration measured in cell supernatant was not modified (48.75
262 \pm 0.75 μ M, mean \pm SEM of three independent experiments), and was below LOQ threshold in cell
263 homogenate, suggesting that o,p'DDA was not efficiently transported into H295R cells or was not
264 significantly retained into cell compartment.

265

266 **O,p'DDD, o,p'DDA and o,p'DDE contents in adrenal tissues of mitotane-treated patients**

267 In order to examine the tissue distribution of mitotane and its metabolites in humans, six samples of
268 human adrenal tissue from mitotane-treated patients were collected. Patient 1 underwent bilateral
269 adrenalectomy for an ectopic Cushing syndrome due a bronchopulmonary ACTH-secreting tumor
270 whereas patients 2 to 6 had ACC. Cumulative doses of mitotane, duration of treatment and plasma
271 mitotane levels are reported in Table 1. Patient 2 had a local ACC recurrence with an imaging work-up
272 performed three months later showing a progressive disease according to RECIST criteria [25]. Thus,
273 he received mitotane for 11 months and the last imaging work-up preceding surgery showed a stable
274 disease according to RECIST criteria, defining this patient as responder to mitotane. Patient 3 received
275 mitotane associated with cisplatin and etoposide, his last morphologic work-up before surgery showing
276 a stable disease according to RECIST. Patient 4 and 5 are considered as non responders to mitotane.
277 Indeed, patient 4, presenting with a metastatic ACC, had progressive disease according to RECIST
278 criteria whereas patient 5 presented a local recurrence while he was treated with mitotane as an
279 adjuvant therapy. Finally, evaluation of mitotane response for patient 6 was not possible since she died
280 shortly after surgery without undergoing imaging work-up. We quantified o,p'DDD, o,p'DDA and

281 o,p'DDE contents in adrenal tissue homogenates measured by HPLC-UV assays (Fig. 6). Results of
282 adrenal gland contents of mitotane and its metabolites are reported in Table 1. O,p'DDD was found
283 20- to 40-fold higher in normal adrenal tissue compared to ACC despite lower plasma mitotane level
284 (3.3 mg/l for Cushing's patient vs 20.03, 18.5, 23.7, 14.7 and 4 mg/l for ACC patients). More
285 importantly, in both ACC and a normal adrenal gland of mitotane-treated patients, o,p'DDA was
286 below limit of quantification (LOQ) threshold unlike o,p'DDD.(Fig.6).

287 **Discussion**

288 Mitotane remains a major therapeutic option in ACC patients and no new pharmacological strategy
289 has been proposed for the past 30 years [1, 2]. However, only one third of patients are considered as
290 responders and the only predictive factor of response is plasma mitotane level, which has to be
291 between 14 and 20 mg/l, a therapeutic window reached after a mean of three months of treatment [8,
292 26, 27]. For this reason, elucidation of mitotane mechanism of action constitutes a major field of
293 research. Preclinical studies have suggested that o,p'DDA could be the active metabolite of o,p'DDD.
294 The role of o,p'DDA compound has also been recently reevaluated in ACC patients. In a first study,
295 mean o,p'DDA plasma concentrations measured in mitotane-treated patients were found five times
296 higher than those of o,p'DDD. However, the median time to reach maximal o,p'DDA values was
297 found shorter than for o,p'DDD (11 vs 55 days, respectively), suggesting that o,p'DDA might
298 constitute an earlier predictor of response [27]. In a second study, o,p'DDA plasma level above 92
299 mg/l could be more specific than o,p'DDD above 14 mg/l in the prediction of tumor response but its
300 mean level was not correlated with tumor response [11]. Based on the lack of convincing clinical
301 evidence for a major role of o,p'DDA as an active antitumor metabolite of mitotane in ACC patients,
302 we aimed at reevaluating the exact pharmacological contribution of o,p'DDA by studying its
303 molecular effects *in vitro* on human adrenocortical H295R and SW13 cells and by investigating its
304 adrenal gland distribution in mitotane-treated patients.

305 In order to rule out an antitumor role of o,p'DDA, we compared o,p'DDA and o,p'DDD action on cell
306 proliferation, respiratory chain, mitochondrial biogenesis, oxidative stress, apoptosis and
307 steroidogenesis. To the best of our knowledge, direct functional consequences of o,p'DDA have never
308 been studied before. We first demonstrate that o,p'DDA did not exert any anti-proliferative effect on
309 adrenocortical cells, even at high concentrations up to 300 μ M (84 mg/l) corresponding to mean
310 plasma o,p'DDA levels measured in mitotane-treated patients. Second, functional assays showed that
311 low concentrations such as 50 μ M o,p'DDA had no significant impact on mitochondrial respiratory
312 chain activity and biogenesis, and were not able to induce H295R cell oxidative stress or apoptosis in
313 sharp contrast to o,p'DDD, as previously described [28, 29].

314 We quantified for the first time o,p'DDD and its metabolites in 6 human samples of adrenal tissues.
315 Patients rarely receive mitotane before undergoing surgery that may explain the low number of
316 available samples. We were unable to quantify o,p'DDA metabolite as its concentration was below
317 LOQ, strongly suggesting a lack of *in situ* production and an absence of uptake of circulating
318 metabolite by adrenal glands. Along this line, o,p'DDA was undetectable in human adrenocortical
319 H295R or SW13 cell supernatants and pellets after a 48 h exposure with 50 μ M mitotane providing
320 additional arguments against an active metabolism of o,p'DDD in human adrenocortical cortex.
321 Further studies should be performed in primary cultures of ACC to confirm the findings of the current
322 study. Previous studies identified o,p'DDA in adrenals of bovines, dogs or rats treated with
323 radiolabelled mitotane [19–21] but not all [22]. Of note, in these studies, intra-adrenal radiolabelled-
324 o,p'DDA represented less than 5% of total radioactivity. In contrast, an hepatic production of
325 o,p'DDA has been previously reported, as demonstrated by metabolic conversion of o,p'DDD with
326 hepatic microsomes of dogs, rats or bovines [18, 19]. Similarly, an active hepatic metabolism of
327 o,p'DDD is likely given the mitotane-induced *CYP3A4* expression in the liver [30, 31]. Moreover,
328 elevated plasma mitotane levels were found to be associated with a gene polymorphism of *CYP2B6*
329 encoding a cytochrome known to catalyze the metabolism of o,p'DDT, structurally close to
330 o,p'DDD [32]. Although our study does not rule out a production of o,p'DDA outside the adrenal
331 gland, likely in liver as previously suggested, our results strongly suggest a lack of capture by adrenal
332 cells and therefore a low if any antitumor activity of o,p'DDA.

333 We confirmed an uptake of o,p'DDD by H295R cells [29] and demonstrated that up to 25% of
334 o,p'DDD was measured in cell pellets, suggesting that o,p'DDD itself could be the active compound.
335 Interestingly, we discovered that o,p'DDD content measured into normal human adrenal gland
336 collected after surgery of a mitotane-treated ectopic Cushing patient was much higher than that
337 quantified in five resected ACC, while plasma circulating mitotane levels were much lower in the
338 former patient compared to the latter (see Table 1). These observations strongly support the notion that
339 the rate of mitotane uptake in the adrenal gland might be related to the specific transport processes
340 active in normal adrenocortical cells while weakly expressed or absent in ACC. In ACC, o,p'DDD
341 content was higher in adrenal tissue of the patient presenting with stable disease compared to the

342 adrenal tissues of other patients. However, no conclusion could be drawn from this observation given
343 the small number of samples. Altogether, our experiments bring decisive arguments to exclude that
344 o,p'DDA is the active metabolite responsible for adrenolytic effects of mitotane.

345 In patients, mitotane is known to exert dual effects on adrenal cells including an early inhibition of
346 steroidogenesis and a late destruction of adrenal tissue [33]. Here, o,p'DDA may potentially exert an
347 inhibitory effect of steroidogenesis. Indeed, although o,p'DDA up to 300 μ M did not inhibit 17-
348 hydroxyprogesterone secretion, we showed a slight and significant reduction in the expression of some
349 (*HSD3B2*, *CYP21* and *CYP11B1*) but not all genes encoding steroidogenic enzymes, consistent with
350 the weak inhibition of cortisol secretion at 300 μ M o,p'DDA. Accordingly, it has been reported that
351 the aromatic moiety of the o,p'DDD molecule could be responsible for an anti-steroidogenic effect
352 whereas the aliphatic group of this compound could account for its cytolytic effect as previously
353 suggested [34]. Indeed, o,p'DDD and m,p'DDD which display similar aromatic structures inhibited
354 steroidogenesis while p,p'DDD that exhibits a different aromatic cycle, was unable to inhibit
355 steroidogenesis [35]. Thus, these findings pointed to the importance of the aliphatic group, notably the
356 dihalogenated methane carbon which is critical for the cytotoxic activity [34], yet lacking in the
357 o,p'DDA structure.

358 To conclude, our results indicate for the first time the absence of adrenal cell metabolism and uptake of
359 o,p'DDA and provide *in vitro* evidence that o,p'DDA is very unlikely an effective metabolite of
360 mitotane to control adrenocortical cell proliferation and to impact mitochondrial respiratory chain
361 activity. By contrast, we confirm major interaction of o,p'DDD with adrenal cell functions and
362 proliferation and unambiguously demonstrate a high cellular uptake of o,p'DDD by adrenal cells.
363 Taken together, our results indicate that research on mitotane mechanism of action should focus on
364 o,p'DDD.

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369 **Author contributions**

370 SH, AP, EB and ML designed the study; SH, AS, AHS and SBT performed the experiments; SH, AS,
371 AHS, SBT, EB and ML analyzed the results; AP, SV, ST and JY helped interpret the data and
372 participated in discussions; SH, SV, EB and ML wrote the paper; all the authors have read, revised and
373 approved the manuscript.

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376 **Declaration of interest**

377 SH is recipient of a fellowship from HRA Pharma Laboratories (Bourse CIFRE).

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

379

380

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481

482

483 **Figures legends**

484 **Figure 1**

485 Dose-dependent impact of increasing concentrations of o,p'DDD and o,p'DDA (10-300 μ M) on the
486 proliferation index of human adrenocortical H295R cells after 48 h as determined by WST1 assays.
487 Results are expressed as mean percentage \pm SEM of 6 to 18 independent determinations, values of
488 vehicle-treated cells being arbitrarily set at 100%. O,p'DDD inhibits cell proliferation in a dose-
489 dependent manner whereas o,p'DDA has no significant impact on cell proliferation. * P <0.05 and
490 *** P <0.001, Mann-Whitney U test.

491

492 **Figure 2**

493 Enzymatic activities of complex IV (cytochrome c oxidase, COX) (A) and ratio between complex IV
494 and citrate synthase activities (B) measured in cell homogenates of cells treated for 48 h with 50 μ M
495 of o,p'DDD or o,p'DDA. The enzymatic activities were measured using spectrophotometry as
496 described in the Materials and Methods section. Results are expressed as nmol/min per mg proteins
497 (A) or as percentage of control ratio arbitrarily set at 100%. Each experiment was repeated three times.
498 Expression of *COX2* (C). H295R cells were treated with 50 μ M o,p'DDD or o,p'DDA for 48 h.
499 Relative mRNA expression of each gene was determined using RT-qPCR. Results are means \pm SEM
500 of six different experiments performed in duplicate and are expressed as percentage of the relative
501 expression in vehicle-treated cells, arbitrarily set at 100%. O,p'DDA has no effect on respiratory chain
502 activity or gene expression. * P <0.05 and *** P <0.001, Mann-Whitney U test.

503

504 **Figure 3**

505 Expression of *PRC* (A) and *SOD2* (B). H295R cells were treated with 50 μ M o,p'DDD or o,p'DDA
506 for 48 h. Relative mRNA expression of each gene was determined using RT-qPCR. Results are means
507 \pm SEM of six different experiments performed in duplicate and are expressed as percentage of the
508 relative expression in vehicle-treated cells, arbitrarily set at 100%.

509 Steady-state levels of Bcl2 protein (anti-apoptotic factor) by Western Blot with anti-Bcl2 and anti-
510 tubulin antibodies. H295R cells were treated with 50 or 100 μ M o,p'DDD or o,p'DDA for 48 h.

511 O,p'DDD is able to increase mitochondrial biogenesis, to induce oxidative stress and apoptosis
512 whereas o,p'DDA fails to exert similar effects. * P <0.05 and *** P <0.001, Mann-Whitney U test.

513

514 **Figure 4**

515 Inhibition of 17-hydroxyprogesterone (17-OHP) in the steroid-secreting H295R cells with o,p'DDD
516 but not with o,p'DDA (A). Cells were cultured with 50 μ M mitotane (o,p'DDD) for 48 h and the 17-
517 OHP concentrations were measured in the cell supernatants by radioimmunometric assays. Results are
518 mean percentage \pm SEM. of 3 independent determinations and are expressed as percentage of secretion
519 under basal conditions in vehicle-treated cells; arbitrarily set at 100% (mean 17-OHP secretion was
520 799 ng /48 h /mg protein).

521 Inhibition of 17-hydroxyprogesterone (17-OHP), deoxycortisol and cortisol in the steroid-secreting
522 H295R cells with high doses of o,p'DDA (B). Cells were cultured with 100 or 300 μ M o,p'DDA for
523 48 h and the steroid concentrations were measured in the cell supernatants by radioimmunometric
524 assays. Results are mean percentage \pm SEM. of 3 independent determinations and are expressed as
525 percentage of secretion under basal conditions in vehicle-treated cells, arbitrarily set at 100% (mean
526 deoxicortisol and cortisol secretion were 848 ng/48h/mg protein and 207ng/48h/mg protein
527 respectively).

528 Expression of genes encoding proteins involved in steroidogenesis (C). H295R cells were treated with
529 50 μ M o,p'DDD or o,p'DDA for 48 h. Relative mRNA expression of each gene was determined using
530 RT-qPCR. Results are means \pm SEM of 6 different experiments performed in duplicate and are
531 expressed as percentage of the relative expression in vehicle-treated cells, arbitrarily set at 100%.
532 O,p'DDD inhibits steroid production and gene expressions whereas o,p'DDA has a weaker or no
533 effect. * P <0.05 and *** P <0.001, Mann-Whitney U test.

534

535

536

537 **Figure 5**

538 o,p'DDD concentrations as measured by HPLC-UV in the supernatants of cultured H295R cells before
539 and after 48 h exposure to mitotane as well as in cell pellet after 48 h mitotane treatment (A).
540 Concentrations of o,p'DDD and o,p'DDA as assessed by UV-HPLC in cultured H295R cell
541 supernatant after a 48 h exposure to o,p'DDD or o,p'DDA (B). Results are expressed as mean
542 percentage \pm SEM of the initial o,p'DDD concentration in cell culture medium at time zero (T0). Each
543 experiment was repeated 3 to 5 times. H295R cell uptake represents 40% of initial o,p'DDD yet
544 o,p'DDA was not recovered in the cell pellet whether H295R cells were incubated with o,p'DDD or
545 o,p'DDA. * $P < 0.05$ and ** $P < 0.01$, Mann-Whitney U test.

546

547 **Figure 6**

548 HPLC-UV analysis of o,p'DDD and o,p'DDE in human adrenal tissues: a normal adrenal (A) and an
549 ACC (B), with p,p'DDD as the internal standard. O,p'DDD is higher in normal adrenal compared to
550 ACC. O,p'DDE is undetectable in human adrenal tissues of patients treated with mitotane.
551 HPLC-UV analysis of o,p'DDA in human ACC (C) compared to a point of the range at 25 mg/l (D),
552 with p,p'DDA as the internal standard. O,p'DDA is undetectable in human adrenal tissue of patient
553 treated with mitotane.

554

555

556 **Table 1**

557 **O,p'DDD, o,p'DDA and o,p'DDE concentrations in human adrenal tissues samples of mitotane-**
 558 **treated patients**

559

Patients (#)	Clinicalpresentation	Treatment duration (months)	Cumulative dose (g)	Plasma mitotane level (mg/l)	O,p'DDD (µg per 10mg of tissue)	O,p'DDE (µg per 10 mg of tissue)	O,p'DDA (µg per 10 mg of tissue)
1	Ectopic Cushing	5	225	3.3*	17.2	< LOQ	< LOQ
2	ACC	11	720	20.03**	0.79	0.16	< LOQ
3	ACC	21	1470	18.5**	0.21	< LOQ	< LOQ
4	ACC	8	765	23.7**	0.44	0.11	< LOQ
5	ACC	10	870	14.7**	0.65	< LOQ	< LOQ
6	ACC	2	180	4*	4.1	0.6	< LOQ

560

561 An informed consent was obtained for each patient. O,p'DDD and its metabolites were measured in
 562 tissue homogenates using HPLC-UV analysis.

563 LOQ: limit of quantification.

564 * Plasma mitotane level measured two weeks before surgery

565 ** Mean of 4 to 15 plasma mitotane levels assessed during mitotane treatment and before surgery

566

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570

Supplemental Table 1

Primer sequences of the genes analyzed by RT-qPCR (5' – 3')

Gene	Amplicon	Sensesequene	Antisensesequene
18S	71 pb	GTGCATGGCCGTTCTTAGTTG	CATGCCAGAGTCTCGTTCGTT
StAR	121 pb	GCCACAGACTTCGGGAACAT	AGTAGCCACGTAAGTTTGGTCTTAGAG
Cyp11A1	149pb	CGATTACCGTGGCATCCTCTA	AGGTTGCGTGCCATCTCATAAC
HSD3B2	149 pb	GGAGGAAGCCAAGCAGAAAA	CAGGTGGGTGGAGTTTGATGA
Cyp21	180pb	GGCTGAAGCAGGCCATAGAG	CCACCCCTTGGAGCATGTAG
Cyp11B1	99 pb	GGAGACACTAACCCAAGAGGACAT	ACGTGATTAGTTGATGGCTCTGAA
COX 2	194 pb	TACGGCGGACTAATCTTCAA	CCGGGAATTGCATCTGTTTT
PRC	201 pb	CCACGTACTCAGGGTT	GGTCTAGGGGCCTCTT
SOD2	60 pb	GCAAGGAACAACAGGCCTTA	GTAGTAAGCGTGCTCCCACAC

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572