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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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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Short title: o,p'DDA is not an active metabolite of mitotane

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

Word count: 4052

Abstract

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

Introduction

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

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

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

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

metabolism of o,p'DDD[18, 19]. Quantitative analyses measuring radiolabelled o,p'DDA in various species further revealed minute amounts of o,p'DDA in adrenal mitochondria [20–22]. From these initial observations emerged a first hypothesis that o,p'DDA could be synthesized in the liver or the adrenals and may represent the active metabolite responsible of the cytotoxic effect observed in the adrenal cortex. Others studies suggested that an adrenal metabolic transformation of o,p'DDD into o,p'DDA could be responsible of the adrenolytic effect [23]. Finally, the question on whether o'p,DDA is responsible for antitumor effects of mitotane in ACC patients remains totally unanswered.

We have previously described molecular effects of o,p'DDD on cell proliferation, steroidogenesis and respiratory chain activity [24]. The aim of the present study was to investigate the exact role of o,p'DDA, in comparison with o,p'DDD. For this purpose, we first explored *in vitro* molecularеffects of o,p'DDA on cell proliferation, mitochondrial respiratory chain and steroidogenesis. We thus studied o,p'DDA conversion and uptake in human adrenocortical H295R and SW13 cell lines and human adrenal tissue samples.

Materials and Methods

Human adrenocortical cells and human adrenal tissues

H295R and SW13 cells were cultured in DMEM/HAM'S F-12 medium (PAA, Les Mureaux, France) supplemented with 20 mM HEPES (Life technologies, Saint Aubin, France), antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/mL) and 2 mM glutamine (all from PAA). The medium for H295R cell culture was enriched with 10% fetal bovine serum and a mixture of insulin/transferrin/selenium. Both cell lines (from passage 2 to 15) were cultured at 37°C in a humidified incubator with 5% CO_2. Mitotane (supplied by HRA Pharma, Paris, France) and o,p'DDA (Alsachim,Illkirch-Graffenstaden, France) were solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Quentin Fallavier, France) and used at 10 to 300 µM final concentrations. The percentage of DMSO in culture medium never exceeded 0.1%.

Tissues were obtained from six patients treated with mitotane. Five ACC patients were followed at GustaveRoussy, while patient 1, followed at Bicêtre Hospital, underwent bilateral adrenalectomy for an ectopic Cushing syndrome due a bronchopulmonary ACTH-secreting tumor. All patients signed an informed consent. Tissues were lysed in H_2O using a TissueLyser apparatus (Qiagen, Courtaboeuf, France).

Steroid secretion

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

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 ng/ml.

Cell proliferation analysis

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

Respiratory chain analysis

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

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[24]. 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 [24]. Standards and samples were amplified in duplicate and analyzed from six independent experiments. The ribosomal 18S rRNA was used as the internal control for data normalization. The relative expression of each gene was expressed as the ratio of attomoles of specific gene to femtomoles of 18S rRNA. The primer sequences of the genes analyzed by qRT-PCR are shown in the Supplemental Table 1.

Western Blot analysis

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

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

Analyses were conducted by high performance liquid chromatography combined to an ultraviolet detection (HPLC-UV) on plasma samples of 100 µl spiked with known amounts of *p,p*'-DDE used as an internal standards (IS) of *o,p*'-DDD and *o,p*'-DDE and*p,p*'-DDAused as an internal standards (IS) of *o,p*'-DDA measurements. *O,p'*-DDD (Mitotane), *o,p*'-DDA and *o,p*'-DDE concentrations were determined through the ratio of their peak surface area to the peak surface of known concentrations of IS. The method was validated in terms of linearity, precision and accuracy, with a threshold detection 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.

Statistical Analysis

Results are expressed as means ± SEM of n independent replicates performed in the same experiment or from separated experiments (n). 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). A *P* value of 0.05 was considered as statistically significant (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In order to examine the tissue distribution of mitotane and its metabolites in humans, six samples of human adrenal tissue from mitotane-treated patients were collected. Patient 1underwent bilateral adrenalectomy for an ectopic Cushing syndrome due a bronchopulmonary ACTH-secreting tumor whereas patients 2 to 6 had ACC. Cumulative doses of mitotane, duration of treatment and plasma mitotane levels are reported in Table 1. Patient 2 had a local ACC recurrencewith an imaging work-up performed three months later showing a progressive disease according to RECIST criteria [25]. Thus, he received mitotane for11 months and the last imaging work-up preceding surgery showed a stable disease according to RECIST criteria, defining this patient as responder to mitotane. Patient 3 received mitotane associated withcisplatin and etoposide, his last morphologic work-up before surgery showing a stable disease according to RECIST. Patient 4 and 5 are considered as non responders to mitotane. Indeed, patient 4, presentingwith a metastatic ACC,had progressive disease according to RECIST criteria whereas patient 5 presented a local recurrence while he was treated with mitotane as an adjuvant therapy. Finally, evaluation of mitotaneresponse for patient 6 was not possible since she died shortly after surgery without undergoing imaging work-up. We quantified o,p'DDD, o,p'DDAand

o,p'DDE contents in adrenal tissue homogenates measured by HPLC-UV assays (Fig. 6). Results of adrenal gland contents of mitotane and its metabolites are reported in Table 1. O,p'DDD was found 20- to 40-fold higher in normal adrenal tissue compared to ACC despite lower plasma mitotane level (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 importantly, in both ACC and a normal adrenal gland of mitotane-treated patients, o,p'DDA was below limit of quantification (LOQ) threshold unlike o,p'DDD.(Fig.6).

Discussion

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

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

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

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

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

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

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

Fundings

This workwassupported by the Institut National de la Santé et de la Recherche Médicale (Inserm) and Université Paris-Sud. SH is recipient of a fellowship from HRA Pharma Laboratories (Bourse CIFRE).

Author contributions

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

Acknowledgements

The authors are indebted to HRA Pharma for supporting this research.

Declaration of interest

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

The authors declare that they have no conflict of interest.

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Figures legends

Figure 1

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

Figure 2

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

Figure 3

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

Steady-state levels of Bcl2 protein (anti-apoptotic factor) by Western Blot with anti-Bcl2 and anti-tubulin antibodies. H295R cells were treated with 50 or 100 μM o,p'DDD or o,p'DDA for 48 h. O,p'DDD is able to increase mitochondrial biogenesis, to induce oxidative stress and apoptosis whereas o,p'DDA fails to exert similar effects. **P*<0.05 and ****P*<0.001, Mann-Whitney *U* test.

Figure 4

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

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

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

Figure 5

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

Figure 6

HPLC-UV analysis of o,p'DDD and o,p'DDE in human adrenal tissues: a normal adrenal (A) and an ACC (B), with p,p'DDD as the internal standard. O,p'DDD is higher in normal adrenal compared to ACC. O,p'DDE is undetectable in human adrenal tissues of patients treated with mitotane.

HPLC-UV analysis of o,p'DDA in human ACC (C) compared to a point of the range at 25 mg/l (D), with p,p'DDA as the internal standard. O,p'DDA is undetectable in human adrenal tissue of patient treated with mitotane.

Table 1

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

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

An informed consent was obtained for each patient. O,p'DDD and its metabolites were measured in tissue homogenates using HPLC-UV analysis.
LOQ: limit of quantification.
* Plasma mitotane level measured two weeks before surgery
** Mean of 4 to 15 plasma mitotane levels assessed during mitotanetreatment and before surgery

Supplemental Table 1

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

Gene	Amplicon	Sensesequence	Antisensesequence
18S	**71 pb**	**GTGCATGGCCGTTCTTAGTTG**	**CATGCCAGAGTCTCGTTCGTT**
StAR	**121 pb**	**GCCACAGACTTCGGGAACAT**	**AGTAGCCACGTAAGTTTGGTCTTAGAG**
Cyp11A1	**149pb**	**CGATTACCGTGGCATCCTCTA**	**AGGTTGCGTGCCATCTCATAC**
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**