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# Mitotane alters mitochondrial respiratory chain activity by inducing cytochrome c oxidase defect in human adrenocortical cells

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## Abstract

Mitotane, 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o,p'*-DDD) is the most effective medical therapy for adrenocortical carcinoma but its molecular mechanism of action remains poorly understood. Although mitotane is known to have mitochondrial (mt) effects, a direct link to mitochondrial dysfunction has never been established. We examined the functional consequences on proliferation, steroidogenesis, and mitochondrial respiratory chain, biogenesis and morphology, of mitotane exposure in two human adrenocortical cell lines, the steroid-secreting H295R line and the non-secreting SW13 line. Mitotane inhibited cell proliferation in a dose- and a time-dependent manner. At the concentration 50 $\mu$ M (14 mg/L), which corresponds to the threshold for therapeutic efficacy, mitotane drastically reduced cortisol and 17-hydroxyprogesterone secretions by 70%. This was accompanied by significant decreases in the expression of genes encoding mitochondrial proteins involved in steroidogenesis (*STAR*, *CYP11B1*, *CYP11B2*). In both H295R and SW13 cells, 50 $\mu$ M mitotane significantly inhibited (50%) the maximum velocity of the activity of the respiratory chain complex IV (cytochrome c oxidase, COX). This effect was associated with a drastic reduction in steady-state levels of the whole COX complex as revealed by Blue Native PAGE and reduced mRNA expression of both mtDNA-encoded COX2 and nuclear DNA-encoded COX4 subunits. In contrast, the activity and expression of respiratory chain complexes II and III were unaffected by mitotane treatment. Lastly, mitotane exposure enhanced mitochondrial biogenesis (increase in mtDNA content and *PGC1 $\alpha$*  expression) and triggered fragmentation of the mitochondrial network. Altogether, our results provide first evidence that mitotane induced a mitochondrial respiratory chain defect in human adrenocortical cells.

## Introduction

Adrenocortical carcinoma (ACC) is a rare disease affecting 2 patients per million people per year, representing less than 0.1% of all cancer cases. ACC prognosis is poor with less than 15% of patients surviving 5 years or more once metastases are diagnosed (Assie, et al. 2007; Fassnacht and Allolio 2009; Icard, et al. 2001; Lughezzani, et al. 2010).

Mitotane, 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o,p'*-DDD) is a synthetic derivative of an insecticide. It acts selectively on the adrenal cortex where it has a cytotoxic effect and impairs steroidogenesis (Bergenstal and Dao 1953). Mitotane is a part of the reference treatment of advanced ACC (Berruti 2012; Fassnacht, et al. 2012). Indeed, it remains the single most effective drug, inducing a partial response in up to one third of the treated patients (Baudin, et al. 2011). Several retrospective studies have shown that plasma mitotane levels above 14 mg/L are associated with a higher partial response rate and improve overall survival (Baudin, et al. 2001; Haak, et al. 1994; Hermsen, et al. 2011; Malandrino, et al. ; Wangberg, et al.). The current recommendation to achieve optimal benefit over risk ratio in patients with unresectable ACC is to maintain plasma mitotane levels between 14 and 20 mg/L (Berruti 2012).

Mitotane's molecular mechanisms of action remain largely unknown, although mitochondrial effects have been reported. Kaminsky *et al* observed swollen mitochondria in the adrenal cortex of mitotane-treated dogs by electron microscopy (Kaminsky, et al. 1962). Subsequently, Martz *et al* suggested that metabolic transformation of *o,p'*-DDD into the active metabolite *o,p'*-DDA occurs in mitochondria and is catalyzed by an unknown cytochrome P450 (Martz and Straw 1977). Mitotane metabolism seems to involve two successive reactions of  $\beta$ -hydroxylation and dehydrochlorination, leading to production of free radicals that could potentially result in apoptosis (Cai, et al. 1995). Critical steps of mitotane's inhibitory effects on steroidogenesis may occur in mitochondria possibly involving CYP11A1, a mitochondrial enzyme that catalyzes the transformation of cholesterol to pregnenolone (Cai, et al. 1997). Elevated levels of 11-deoxycortisol and 11-deoxycorticosterone in mitotane-treated patients suggest that mitotane may affect CYP11B1, which is responsible for cortisol synthesis (Asp, et al.). More recently, Stigliano *et al* showed by proteomic analysis of H295R cells that expression of proteins involved in stress response, energy metabolism and tumorigenesis was greatly altered by

mitotane exposure (Stigliano, et al. 2008). Interestingly, some of these regulated proteins were mitochondrial components, even though a direct impact on their synthesis and/or stability has not been clearly demonstrated. The functional consequences of mitotane on respiratory chain expression and activity have not yet been examined. The respiratory chain consists of four multienzymatic complexes located in the mitochondrial inner membrane. Together with the ATP synthase complex, it performs an essential mitochondrial function, generating the vast majority of cellular ATP synthesis, while reducing molecular oxygen into water. It is a major source of free radicals in most cells and its function is tightly linked to apoptosis balance. The respiratory chain has been shown to be the target of several pharmacological compounds including non-steroidal anti-inflammatory drugs, antiretrovirals, and chemotherapy agents (Viengchareun, et al. 2007)(Fedele, et al. ; Scatena 2012).

The aim of the present study was to evaluate the functional consequences of mitotane exposure on mitochondrial oxidative phosphorylation (OXPHOS) in human adrenocortical steroid-secreting H295R and non-secreting SW13 cells, both derived from human ACC. We used complementary experimental approaches including spectrophotometric assays, Western Blot, quantitative PCR, and mitochondrial morphological analysis to explore how mitotane affects mediators of steroidogenesis and respiratory chain activity.

## **Materials and methods**

### **Cell culture and treatment**

H295R and SW13 cells were cultured in Dulbecco's modified Eagle's medium/Ham's medium (DMEM/HAM'S F-12, PAA, Les Mureaux, France) supplemented with 20 mM HEPES (Invitrogen, Life technologies, Saint Aubin, France), antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/mL) and 2 mM glutamine. 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<sub>2</sub>. Mitotane (supplied by HRA Pharma, Paris, France) dissolved in dimethyl sulfoxide (DMSO) was added to cell cultures at final concentrations of 10 to 100 µM; the therapeutic plasma mitotane level is 50 µM (14 mg/L).

### **Cell proliferation analysis**

Cell proliferation was studied in Celltiter 96 assays (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Cells were cultured in 96-well plates and treated with 10 to 100 µM mitotane for 24, 48 or 72 h. Absorbance was measured by photometry (Viktor, Perkin Elmer, Courtaboeuf, France) 1 h after addition of 20 µL of Celltiter solution per well.

### **Cortisol and 17-OH-progesterone secretion**

The cortisol and 17-hydroxyprogesterone concentrations in H295R culture supernatants were determined by radioimmunoassays, using polyclonal antibodies (anti-cortisol: Orion Diagnostica, Spectria, Espoo, Finland; anti-17-hydroxyprogesterone: MP Biomedical, Solon, OH, USA). The intra- and inter-assay coefficients of variation of the cortisol were, respectively, 4.5% and 5.5% at 22 µg/L and 4.2% and 4.3% at 269 µg/L, with a detection limit of 5 µg/l while those of the 17-hydroxyprogesterone assay were 7.8% and 12% at 0.92 ng/mL and 8.3% and 9.8% at 4.3 ng/mL with a detection limit of 0.02 ng/mL.

### **Reverse Transcriptase-PCR (RT-PCR) and Quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from tissues or cells with the RNeasy kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. RNA was thereafter processed for RT-PCR as previously described (Martinerie, et al. 2011). Quantitative real-time PCR qRT-PCR was performed using the Fast SYBR<sup>®</sup> Green Master Mix (ABI, Applied Biosystems, FosterCity, CA, USA) and carried out on a StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems) as previously described (Martinerie et al. 2011). Standards and samples were amplified in duplicate and analyzed from three independent experiments. The internal control for data normalization was the ribosomal 18S rRNA. The relative expression of each gene is expressed as the ratio of attomoles of the specific gene to femtomoles of 18S rRNA. The primer sequences of the genes analyzed by qRT-PCR are shown in the Supplemental Table.

### **mtDNA quantification**

Mitochondrial DNA quantification was performed on total DNA extracted from tissues or cells using standard techniques. DNA was quantified by qPCR using the *COX2* gene on the mtDNA as a target gene as previously described (Viengchareun et al. 2007). Results were expressed as relative expression of *COX2* normalized with the nuclear *18S* gene.

### **Respiratory chain analysis**

Respiratory chain activities were measured using spectrophotometric assays. H295R and SW13 cells were treated with mitotane or vehicle (DMSO) alone for various periods of time, 24h, 48 h or 72 h and the activity of four mitochondrial respiratory complexes — complex I (NADH-ubiquinol oxidoreductase), complex II (succinate-ubiquinol oxidoreductase), complex III (ubiquinol-cytochrome c oxidoreductase), and complex IV (cytochrome c oxidase, or COX) — were measured in a Cary 50 spectrophotometer (Rustin, et al. 1994). Assays of complexes II, III, and IV were performed on cell homogenates, and their activities normalized to citrate synthase activity, as an index of mitochondrial mass. Complex I assays were performed on purified mitochondrial fractions, prepared from permeabilized cells as previously described (Chretien, et al. 2003).

### **BN-PAGE analysis**

Mitochondria and oxidative phosphorylation (OXPHOS) complexes were isolated from cultured cells using 2% (W/V) digitonin and analysed as described (Nijtmans, et al. 2002a) (Nijtmans, et al. 2002b). Fifteen micrograms of solubilized OXPHOS proteins were loaded on a 4-16% gradient acrylamide non-denaturing gel (Invitrogen). After electrophoresis, proteins were transferred to a PVDF membrane. Immunoblotting was performed with monoclonal antibodies (Mitosciences, Mundolsheim, France) raised against the complex I GRIM19 subunit, the 70 kDa complex II subunit, the complex III subunit core2 and the complex IV subunit COX1. Peroxidase-conjugated anti-mouse IgG secondary antibodies were added and the signal was generated using enhanced chemiluminescence ECL (Pierce, Rockford, USA). Membranes were scanned using the Odyssey infrared imaging system and images were processed with the Image Studio Software (LI-COR Biosciences, Lincoln, NE, USA).

### **Mitochondrial morphology**

Cells were seeded at subconfluence on a glass coverslip and incubated for 24-48 h in the presence or absence of 50  $\mu$ M mitotane, briefly rinsed with warm PBS, then fixed in 3% paraformaldehyde in PBS. Mitochondria were labeled with antibodies against COX2 subunit as described (Agier, et al. 2012).

### **Statistical Analysis**

Results are expressed as means  $\pm$  SEM of n independent replicates performed in the same experiment or from n separated experiments. Differences between groups were analyzed using non-parametric Kruskal Wallis ANOVA followed by Dunn's multiple comparison test or non-parametric Mann Whitney test as appropriate. The significance level was  $p < 0.05$ .

## Results

### Mitotane treatment reduces human adrenocortical H295R and SW13 cell proliferation

Proliferation index was calculated using the colorimetric solution Celltiter 96. Exposure to mitotane for 48 h inhibited the proliferation of H295R and SW13 cells in a dose dependent manner, 100  $\mu$ M *o,p'*-DDD significantly reducing the proliferation rate of H295R by 45% and that of SW13 cells by 30% (Fig. 1A and 1B). The anti-proliferative effect of mitotane was also time-dependent, 100  $\mu$ M mitotane inhibiting of H295R cells proliferation by 18% after 24 h and by 70% after 72 h. Subsequent experiments were performed using 50  $\mu$ M mitotane to minimize the drug's potential cytotoxic effects.

### Effect of mitotane on steroidogenesis in H295R cells

To confirm the ability of mitotane to inhibit hormone secretion, we measured several steroid hormones concentrations in the culture supernatant of H295R cells. Exposure to 50  $\mu$ M mitotane for 48 h significantly reduced the secretion of both cortisol and 17-OH-progesterone about 80% by H295R cells (Fig. 1C). Other steroid hormones such as aldosterone were undetectable in culture supernatants under these experimental conditions.

To address the mechanisms underlying this decreased steroid secretion, we analyzed the expression of genes that encode mitochondrial effectors of steroidogenesis by qRT-PCR. Mitotane significantly decreased the expression of such genes: *STAR*, which encodes the Steroidogenic Acute Regulatory protein that transports cholesterol into mitochondria, the first rate limiting step for the intramitochondrial steroidogenic pathway (80% inhibition after 48 h, Fig. 2A); *CYP11A* (cholesterol desmolase), *HSD3B2* (3 beta hydroxysteroid dehydrogenase); *CYP11B1* (11 $\beta$  hydroxylase) which catalyzes 11-deoxycorticosterone and 11-deoxycortisol transformation into corticosterone and cortisol respectively (75% inhibition, Fig. 2B); and *CYP11B2* (aldosterone synthase), the last intra-mitochondrial enzymatic step in aldosterone synthesis (97%, inhibition, Fig. 2C). The mitotane-induced inhibition of steroid secretion observed in H295R cells therefore appeared to be due to decreased expression of the steroidogenic enzymes.

### **Effect of Mitotane on the respiratory chain**

The impact of mitotane on respiratory chain activity was evaluated by spectrophotometric assays of the activities of the four mitochondrial respiratory complexes in H295R and SW13 cells treated with vehicle (DMSO) or 50  $\mu$ M mitotane during 48 h (Table I). Citrate synthase activity, belonging to the mitochondrial citric acid cycle, was used as an index of the mitochondrial mass. Its activity was very high in H295R cells ( $299 \pm 22$  nmol/min/mg prot; n= 12) but lower in SW13 cells ( $159 \pm 11$  nmol/min/mg prot; n= 8) suggesting that H295R cells have a greater mitochondrial population than SW13 cells consistent with their important steroidogenic capacity. However, citrate synthase activity was not affected by mitotane exposure ( $260 \pm 31$  nmol/min/mg prot in H295R and  $135 \pm 7$  nmol/min/mg prot in SW13 cells).

Both H295R and SW13 mitotane-treated cells exhibited a significant cytochrome c oxidase (COX or complex IV) defect of approximately 50% after 48 h while complex II (succinate-ubiquinone oxidoreductase) appeared unaffected (Table I). Complex III (ubiquinol-cytochrome c oxidoreductase) activity remained unchanged in H295R cells and was slightly reduced in SW13 cells after mitotane treatment (Table I) but this decrease was not confirmed after normalization to citrate synthase activity (Table I). Complex I (NADH-ubiquinone oxidoreductase) activity can only be reliably measured on purified mitochondrial fractions due to the presence of numerous non-mitochondrial NADH oxidase activities in cell homogenates but its activity was greatly decreased after exposure to mitotane in both human adrenocortical cell lines after exposure to mitotane (Table I). Altogether, our results demonstrate that mitotane selectively inhibits some but not all respiratory chain complexes.

The effect of mitotane on complex IV was concentration-dependent, as shown in Fig. 3, with an IC50 calculated at approximately 67  $\mu$ M mitotane (linear regression test;  $y=92.862-0.583x$ ,  $r^2= 0.97$ ). This mitotane concentration corresponds to the therapeutic plasma threshold predictive of efficacy in clinical practice (Baudin et al. 2001; Haak et al. 1994).

To examine whether *o,p'*-DDD might directly affect the enzymatic activity of complex IV, we measured cytochrome c oxidase activity on cell homogenates incubated with increasing concentrations of mitotane. Under these conditions, we demonstrated that mitotane dose-dependently decreased

complex IV activity with an IC50 of approximately 133  $\mu$ M (linear regression test;  $y=100.2-0.3749x$ ,  $r^2= 0.96$ ) (Fig. 4). This IC50 in the cell homogenate system is twice as high as the IC50 observed when whole cells were treated for 48 h indicating that mitotane exerts both direct and indirect inhibitory effects on cytochrome c oxidase activity. Our results strongly suggested that mitotane inhibits enzymatic activity directly but presumably inhibits the expression of the enzyme. We therefore studied the expression of cytochrome c oxidase at both the mRNA and protein level. The cytochrome c oxidase complex consists of 13 subunits, three of which, including COX2, are encoded by the mitochondrial genome while the remaining 10 subunits, including COX4, are encoded by nuclear genes. We observed that the steady-state levels of mitochondrial and nuclear DNA-encoded COX2 and COX4 transcripts in both H295R and SW13 cells were drastically decreased (by 70%) in H295R cells after exposure to 50  $\mu$ M mitotane for 48 h (Fig. 5A and 5B). Similar results were obtained in SW13 cells (data not shown).

We analyzed the whole respiratory chain complexes by Blue Native PolyAcrylamide Gel Electrophoresis (BN-PAGE). Immunoblotting with antibodies directed against a component of each mitochondrial complexes revealed that Mitotane exposure for 48 h induced a 45-70% decrease in the steady-state expression of complex IV and complex I proteins while the abundance of complexes II and III appeared unchanged (Fig. 5C and 5D). These data were fully consistent with the decreased enzymatic activities described above (Table I). Altogether, our results demonstrate that mitotane has deleterious consequences by acting at the mRNA and protein level to impair respiratory chain expression and function.

To evaluate the possibility that mitotane has direct toxic effects on the mitochondrial DNA, we quantified mitochondrial DNA by qPCR. As illustrated on Fig. 6A, the mitochondrial/nuclear DNA ratio was unaffected by exposure of low or moderate doses of mitotane for 48 h. However, this ratio increased significantly after treatment with 100  $\mu$ M mitotane and with longer exposure times (e.g 50  $\mu$ M mitotane for 72 h), suggesting the presence of a compensatory response of mitochondrial biogenesis (Fig. 6B). To further explore this hypothesis, we quantified the expression of *PGC1 $\alpha$*  (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), a transcriptional coactivator considered a key regulator of mitochondrial biogenesis. *PGC1 $\alpha$*  mRNA expression was slightly but

significantly induced by 50  $\mu$ M mitotane treatment for 48 h, suggesting activation of transcriptional response (Fig. 6C). Furthermore, no mtDNA mutations or deletions were found by sequencing mtDNA from mitotane-treated cells.

### **Effect of mitotane on mitochondrial morphology**

Finally, to get an integrated evaluation of the mitotane-induced mitochondrial respiratory chain defect, we analyzed the mitochondrial morphology by immunocytochemistry using an antibody against COX2. Treatment with 50 $\mu$ M mitotane induced drastic morphological alterations in the mitochondria of adrenocortical cells. In the absence of treatment, the mitochondrial compartment appeared as a highly interconnected tubular network with a filamentous appearance after exposure to the drug but exhibited a more punctiform pattern, consistent with mitochondrial fragmentation (Fig. 7).

## Discussion

Even though combination of mitotane and cisplatin-based chemotherapy has been recently shown to clinically improve the overall survival in advanced adrenocortical carcinoma (Fassnacht et al. 2012), mitotane remains the single most active pharmacological option for the management of ACC, as recognized in recent recommendations (Berruti 2012). However, its mechanism of action still remains unclear. In the present study, we addressed the question on the mitochondrial effects of mitotane on two different human adrenocortical cell lines derived from human ACC aiming at identifying potential molecular targets of the drug. Attempts to perform similar experiments on primary human adrenocortical carcinoma cells have been so far unsuccessful.

We found that at optimal therapeutic concentrations (50  $\mu$ M i.e 4-20 mg/L), mitotane drastically altered mitochondrial function in both steroid-secreting and non-secreting adrenocortical cell lines derived for human ACC. Mitotane inhibited steroid hormone production and secretion which was accompanied by a reduction in steady-state mRNA levels of genes encoding mitochondrial proteins involved in steroidogenesis pathways. More importantly, we demonstrated for the first time that exposure to 50  $\mu$ M mitotane significantly impairs the mitochondrial respiratory chain. Mitotane exposure also stimulated mitochondrial biogenesis and altered mitochondrial morphology in adrenocortical cells.

It is well established that the *in vivo* anti-proliferative efficacy of mitotane depends on its circulating plasma level (Baudin et al. 2001). However, its pharmacokinetic profile with a unmet need for improved bioavailability and its metabolic conversion constitute potential limitations (Scheingart 2007). It has been suggested that the metabolic transformation of *o,p'*-DDD is carried out in the adrenal mitochondria, the first enzymatic step being catalyzed by an unknown P450 cytochrome-mediated hydroxylase leading to an adrenolytic effect (Cai et al. 1995; Martz and Straw 1977). In accordance to previous studies, we confirmed that mitotane inhibits steroidogenesis reducing cortisol and 17-hydroxyprogesterone secretions by 70% (Scheingart, et al. 1993; Stigliano et al. 2008). Mitotane exposure also decreased mRNA levels of *STAR*, the cholesterol carrier into the mitochondria, as well as *CYP11A*, *CYP11B1* and *CYP11B2*, three mitochondrial enzymes involved in cortisol and aldosterone biosynthesis, respectively. However, the degree and extent of mitotane-induced repression

of genes involved in steroidogenesis seem to vary greatly between studies (Asp et al. ; Lin, et al. 2012; Zsippai, et al. 2012), supporting mitochondria as a main target of the drug's action.

The mitotane transformation into active acylchlorine metabolites that takes place in the mitochondria of adrenal gland is believed to be responsible for cell toxicity and may explain the selective adrenolytic effect of the drug (Cai et al. 1995; Lindhe, et al. 2002). This hypothesis awaits further confirmation at the clinical level (Hermsen et al. 2011). At variance with the hepatic microsomal transformation of mitotane by CYP3A4 (Kroiss, et al. 2011; van Erp, et al. 2011), which is likely responsible for the pharmacokinetic interaction whereby mitotane reduces plasma levels of sunitinib (Fassnacht et al. 2012; Kroiss, et al. 2012), it has been suggested that CYPc11 or CYP11B1 could be involved in tissue-specific and compartment-selective mitotane metabolism (Lindhe et al. 2002; Lund and Lund 1995). Although CYP11B1 may catalyze the initial hydroxylation step of mitotane (Cai et al. 1995; Lindhe et al. 2002; Lund and Lund 1995), its direct involvement in mitochondrial dysfunction is very unlikely given that SW13 cells, which do not express CYP11B1, were similarly affected by mitotane treatment. In any case, the relationship between the potential hepatic metabolism of mitotane and its adrenal effect remains currently unknown. For instance, it remains to be established whether intra-mitochondrial transformation of mitotane into *o,p'*-DDA and *o,p'*-DDE compounds has deleterious consequences on OXPHOS. However, preliminary results from our laboratory reveal the presence of active mitotane uptake into H295R cells, suggesting that intracellular accumulation of mitotane and/or one of its metabolites may account for its cytotoxic effects.

Given that most enzymatic steps of steroid hormone biosynthesis take place in the mitochondria and that mitotane inhibits steroidogenesis, we examined whether mitotane impedes mitochondrial respiratory chain function. Interestingly, in both H295R and SW13 cells, OXPHOS analyses indicated that mitotane induced a significant and selective decrease in the maximum velocity of COX activity, whereas complex II and III activities were unaltered. Mitotane has both direct and indirect inhibitory effects on COX: direct inhibition of the enzymatic activity was revealed in our experiments on cell homogenate incubation with *o,p'*-DDD but the drug also inhibited expression of the enzyme at both the mRNA and protein level. Inhibition of gene expression was observed for both the mtDNA-

encoded COX2 and the nuclear DNA-encoded COX4 subunits. Immunoblotting provided additional support for a reduction in steady-state COX protein expression. Concomitantly, normal activity and expression of respiratory chain complexes II and III or of citrate synthase, a Krebs cycle enzyme, suggest that mitotane caused selective enzymatic disruption rather than global mitochondrial damage, as initially proposed (Kaminsky et al. 1962).

Herein, we confirm the adrenolytic effect of mitotane by showing that mitotane exposure leads to a time- and concentration-dependent reduction of adrenocortical cell numbers. Interestingly, this was accompanied by enhanced mitochondrial biogenesis, as demonstrated by increased mtDNA content and *PGC1 $\alpha$*  expression, reminiscent of a cellular compensation mechanism in response to the respiratory chain defect. This adaptive pathway, combining increased mitochondrial mass, increased DNA copy level and impaired OXPHOS, has already been reported in mitochondrial myopathies caused by mtDNA mutations (Srivastava, et al. 2009). However, no mtDNA mutations or deletions were found by sequencing mtDNA from mitotane-treated cells. Of particular interest, mitotane exposure also triggered morphologic fragmentation of the mitochondrial network, which could be related to disequilibrium between mitochondrial fission and fusion (Chen and Chan 2010). It is well established that the integrity of mitochondrial outer and inner membranes is required for respiratory chain activity (Chen, et al. 2010; Liesa, et al. 2009) and presumably steroidogenesis (Duarte, et al. 2012). It is not known however whether mitochondrial fragmentation has a direct relationship with or a causal role in genotoxic stress and apoptosis.

In summary, our results show that mitotane alters mitochondrial respiratory chain activity in human adrenocortical cells, notably by inducing a cytochrome c oxidase defect. Further studies are needed to examine whether and how such mitotane-induced mitochondrial dysfunction translates into adrenolytic and antitumor effects on human ACC (Costa, et al. 2011).

**Abbreviations:**

ACC: Adrenocortical carcinoma; ATP: Adenosine triphosphate; COX: Cytochrome c oxidase; DNA: Deoxyribose nucleic acid; DMSO: dimethyl sulfoxide; mRNA: messenger Ribonucleic acid; mt: Mitochondrial; NADH: reduced Nicotinamide adenine dinucleotide; *o,p*'-DDD: 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; OXPHOS: oxidative phosphorylation; PGC1 $\alpha$ : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; qPCR: quantitative Polymerase chain reaction; rRNA: ribosomal Ribonucleic acid; ROS: Reactive oxygen species; STAR: Steroidogenic Acute Regulatory protein.

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**Author contributions:**

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

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**Disclosure:**

All authors have no disclosure except Dr Rita Chadarevian who is an employee of HRA Pharma.

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## Figure Legends:

### Figure 1

Dose-dependent inhibition of the proliferation index of human adrenocortical H295R (A) and SW13 (B) cells in response to increasing concentrations of mitotane (0  $\mu$ M to 100  $\mu$ M) after 48 h, as determined by Celltiter assay (See Material and Methods section). Results are expressed as the mean percentage  $\pm$  SEM of 12 independent determinations of the expression measured in untreated cells, arbitrarily set at 100%. \* $p$ <0.05, \*\*  $p$ <0.01, Mann Whitney test. Proliferation was affected in a dose-dependant manner in both cell lines.

Inhibition of cortisol and 17 hydroxy-progesterone secretions by the steroid-secreting H295R cells (C). Cells were cultured with 50  $\mu$ M of mitotane for 48 h, and the steroid concentrations were measured in the cell supernatants by Radioimmunometric assays. Results are means  $\pm$  SEM of 4 independent determinations and are expressed as percentage of secretion under basal conditions (mean cortisol and 17OHP secretions were 157 and 358ng/48 h/mg protein per well, respectively). Steroid hormone secretion was significantly inhibited by 80% after mitotane exposure.

### Figure 2

Expression of genes encoding mitochondrial and cytoplasmic proteins involved in steroidogenesis. H295R cells were treated with 50  $\mu$ M of mitotane for 48 h. Relative mRNA expression of *STAR* (Steroidogenic Acute Regulatory protein), *CYP11A*, *HSD3B2*, *CYP11B1* and *CYP11B2* was determined using qRT-PCR. Results, are means  $\pm$  SEM of 4 different experiments performed in duplicate and are expressed as the percentage of the relative expression in DMSO-treated cells, arbitrarily set at 100%. Mitotane drastically inhibits gene expression \*\*  $p$ <0.01, \*\*\*  $p$ <0.001 Mann Whitney test.

### Figure 3

Dose-dependent inhibition of Cytochrome c oxidase (Complex IV, COX) activity in H295R cells by mitotane. H295R cells were treated for 48 h with increasing concentrations of mitotane (0  $\mu$ M to 100

$\mu\text{M}$ ) and the COX activity was measured by spectrophotometry as described in Material and methods section. Results are expressed as nmol/min/mg prot. Each point represents the mean of 3-6 independent determinations. Mitotane inhibits Complex IV activity in a concentration-manner with a calculated IC50 at 58  $\mu\text{M}$ .

#### Figure 4

Dose-dependent inhibition of cytochrome c oxidase (COX) activity by mitotane. COX activity was measured by spectrophotometry in cell homogenates of H295R cells incubated with increasing concentrations of mitotane (0  $\mu\text{M}$  to 100  $\mu\text{M}$ ) for 48 h (black circles, *living cell incubation*) or in cell homogenates simultaneously exposed to increasing concentrations of mitotane (0  $\mu\text{M}$  to 200  $\mu\text{M}$ ) immediately added before the enzymatic assay (Black squares *homogenate incubation*). Each experiment was repeated two to six times. Results are expressed as the percentage of control COX activity measured in the absence of mitotane arbitrary set at 100%. Mitotane directly inhibits Complex IV activity with an IC50 at 133  $\mu\text{M}$  (*homogenate incubation*), while the IC50 of mitotane was calculated at 67  $\mu\text{M}$  for the inhibitory effects on treated cells (*living cell incubation*).

#### Figure 5

Inhibition of the expression of the respiratory chain genes and proteins by mitotane.

The steady-state levels of messenger RNA encoding for the mitochondrial DNA-encoded *COX2* (A) and the nuclear encoded *COX4* (B) were measured by RT-qPCR. The expression of both *COX2* and *COX4* transcripts was drastically reduced after exposure to 50  $\mu\text{M}$  mitotane for 48 h. Results are expressed as the mean percentage  $\pm$  SEM of 4 independent determinations performed in duplicate of the expression measured in untreated cells, arbitrarily set at 100%. \* $p < 0.05$ , \*\*\*  $p < 0.001$  Mann Whitney test.

Steady-state levels of respiratory chain whole complexes were analyzed by BN-PAGE followed by Western Blot with anti-GRIM 19 (a subunit of complex I), anti-70 kDa (a subunit of complex II), anti-core2 (a subunit of complex III) and anti-COX1 (a subunit of complex IV) (C). Band intensities were quantified by ImageJ software revealing that mitotane reduced the steady-state of both the complex I

and complex IV were reduced by 45-70% but had no effect on complex II or complex III expression (D). Results are expressed as the mean percentage  $\pm$  SEM of 4 independent determinations of the expression measured in untreated cells, arbitrarily set at 100%. \* $p < 0.05$ , Mann Whitney test.

### **Figure 6**

Stimulation of mitochondrial biogenesis by mitotane. Mitochondrial DNA was quantified by qPCR in H295R cells treated for 48 h (A) or 72 h (B) with increasing concentrations of mitotane. Higher mitotane concentrations (100  $\mu$ M) and longer time exposure (72 h) increased mitochondrial/nuclear DNA ratio. Increased expression of *PGC1 $\alpha$*  (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) in H295R cells were treated with 50  $\mu$ M of mitotane for 48 h (C). Results are expressed as the mean percentage  $\pm$  SEM of 4 independent determinations performed in duplicate of the expression measured in untreated cells, arbitrarily set at 100%. \* $p < 0.05$ , \*\*\*  $p < 0.001$  Mann Whitney test.

### **Figure 7**

Morphological changes in the mitochondrial compartment network of H295R cells treated with 50  $\mu$ M mitotane for 48 h. Cells were fixed and immunostained with anti-COX2 antibody (green) and counterstained with DAPI (blue). Under mitotane exposure, the filamentous morphology of the mitochondrial compartment observed in vehicle-treated cells (DMSO) displayed a punctiform pattern, indicative of mitochondrial fragmentation.

**Table I: Mitochondrial respiratory activities**

Conditions	H295R cells		SW13 cells	
	Vehicle	50 $\mu$ M Mitotane	Vehicle	50 $\mu$ M Mitotane
Citrate synthase	299 $\pm$ 22	260 $\pm$ 31	159 $\pm$ 11	135 $\pm$ 7
Complex I	9.4	5.6	14.4	7.5
Complex II	55 $\pm$ 8	43 $\pm$ 9	37 $\pm$ 3	32 $\pm$ 1
Complex III	95 $\pm$ 14	86 $\pm$ 26	89 $\pm$ 6	55 $\pm$ 10*
Complex IV	276 $\pm$ 15	153 $\pm$ 19**	283 $\pm$ 53	127 $\pm$ 37**
CII/CS	0.28 $\pm$ 0.03	0.21 $\pm$ 0.03	0.26 $\pm$ 0.02	0.30 $\pm$ 0.04
CIII/CS	0.29 $\pm$ 0.03	0.36 $\pm$ 0.07	0.68 $\pm$ 0.07	0.68 $\pm$ 0.10
CIV/CS	1.05 $\pm$ 0.08	0.52 $\pm$ 0.02***	0.78 $\pm$ 0.08	0.56 $\pm$ 0.01*

Enzymatic activities were measured in cell homogenates with the exception of complex I, which was measured on purified mitochondrial fractions; values are mean  $\pm$  SEM of 6-12 independent experiments, expressed as nmol/min/mg prot. Ratio between Complex II or Complex III or ComplexIV/Citrate synthase (CS) activities are also presented.\*  $p < 0.05$  \*\* $p < 0.01$  \*\*\* $p < 0.001$  with non-parametric Mann Whitney test.

## Supplemental Table

Primer sequences of genes analyzed by qRT-PCR

<b>Gene</b>	<b>Size</b>	<b>Sense sequences</b>	<b>Antisense sequences</b>
<i>18S</i>	71 pb	GTGCATGGCCGTTCTTAGTTG	CATGCCAGAGTCTCGTTCGTT
<i>COX 2</i>	194 pb	TACGGCGGACTAATCTTCAA	CCGGGAATTGCATCTGTTTT
<i>COX 4</i>	143 pb	TGGATGAGAAAGTCGAGTTG	CTTCTGCCACATGATAACGA
<i>StAR</i>	121 pb	GCCACAGACTTCGGGAACAT	AGTAGCCACGTAAGTTTGGTCTTAGAG
<i>Cyp11B1</i>	99 pb	GGAGACACTAACCCAAGAGGACAT	ACGTGATTAGTTGATGGCTCTGAA
<i>Cyp11B2</i>	144 pb	AGGCCCTGTGGTCACTTATC	CAACACTCGCTGCTTGAACAA
<i>PGC1<math>\alpha</math></i>	159 pb	AAGAGCGCCGTGTGATTTAT	TCACAGGTATAACGGTAGGT