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 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 mt dysfunction has never been established. We examined the functional consequences of mitotane exposure on proliferation, steroidogenesis, and mt respiratory chain, biogenesis and morphology, 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 of 50 μM (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 mt proteins involved in steroidogenesis (*STAR*, *CYP11B1*, and *CYP11B2*). In both H295R and SW13 cells, 50 μM 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 (MT-CO2) and nuclear DNA-encoded COX4 (COX4I1) subunits. In contrast, the activity and expression of respiratory chain complexes II and III were unaffected by mitotane treatment. Lastly, mitotane exposure enhanced mt biogenesis (increase in mtDNA content and *PGC1α* (*PPARGC1A*) expression) and triggered fragmentation of the mt network. Altogether, our results provide first evidence that mitotane induced a mt respiratory chain defect in human adrenocortical cells.

Key Words

- adrenocortical carcinoma
- mitotane
- *o,p'*-DDD
- mitochondria
- cytochrome *c* oxidase

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Introduction

Adrenocortical carcinoma (ACC) is a rare disease affecting two patients per million people per year, representing <0.1% of all cancer cases. ACC prognosis is poor with <15% of patients surviving 5 years or more once metastases are diagnosed (Icard *et al*. 2001, Assie *et al*. 2007, Fassnacht & Allolio 2009, 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 & 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 (Haak *et al*. 1994, Baudin *et al*. 2001, Malandrino *et al*. 2010, Wangberg *et al*. 2010, Hermsen *et al*. 2011). 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 (mt) effects have been reported. Kaminsky *et al*. (1962) observed swollen mitochondria in the adrenal cortex of mitotane-treated dogs by electron microscopy. Subsequently, Martz & Straw (1977) 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. Mitotane metabolism seems to involve two successive reactions of β-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 mt 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*. 2012). More recently, Stigliano *et al*. (2008) 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. Interestingly, some of these regulated proteins were mt 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 mt inner membrane. Together with the ATP synthase complex, it performs an essential mt 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, Fedeles *et al*. 2011, Scatena 2012).

The aim of this study was to evaluate the functional consequences of mitotane exposure on mt 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 mt 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 DMEM/HAM'S F-12 (PAA, Les Mureaux, France) supplemented with 20 mM HEPES (Invitrogen, Life Technologies), antibiotics (penicillin 100 IU/ml and 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 passages 2–15) were cultured at 37 °C in a humidified incubator with 5% CO_2. Mitotane (supplied by HRA Pharma, Paris, France) dissolved in DMSO was added to cell cultures at final concentrations of 10–100 μM; the therapeutic plasma mitotane level is 50 μM (approximately 14 mg/l).

Cell proliferation analysis

Cell proliferation was studied in Celltiter 96 assays (Promega) according to the manufacturer's recommendations. Cells were cultured in 96-well plates and treated

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with 10–100 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 Celltiter solution per well.

Cortisol and 17-hydroxyprogesterone secretion

The cortisol and 17-hydroxyprogesterone (17-OH-progesterone) concentrations in H295R culture supernatants were determined by radioimmunometric assays using polyclonal antibodies (anti-cortisol: Orion Diagnostica, Spectria, Espoo, Finland; anti-17-OH-progesterone: MP Biomedical, Solon, OH, USA). The intra- and interassay 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-OH-progesterone 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 and quantitative real-time PCR

Total RNA was extracted from tissues or cells with the RNeasy Kit (Qiagen) according to the manufacturer's recommendations. RNA was thereafter processed for reverse transcriptase-PCR (RT-PCR) as described previously (Martinerie *et al.* 2011). Quantitative real-time PCR (qRT-PCR) was performed using the Fast SYBR Green Master Mix (ABI, Applied Biosystems) and carried out on a StepOnePlus Real-Time PCR System (Applied Biosystems) as described previously (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 Supplementary Table 1, see section on supplementary data given at the end of this article.

mtDNA quantification

mtDNA quantification was performed on total DNA extracted from tissues or cells using standard techniques. DNA was quantified by qPCR using the cytochrome *c* oxidase 2 (*COX2* (*MT-CO2*)) gene on the mtDNA as a target gene as described previously (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, 24, 48, or 72 h, and the activity of four mt respiratory complexes – complex I (NADH–ubiquinone oxidoreductase), complex II (succinate–ubiquinone oxidoreductase), complex III (ubiquinone–cytochrome *c* oxidoreductase), and complex IV (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 mt mass. Complex I assays were performed on purified mt fractions and prepared from permeabilized cells as described previously (Chretien *et al.* 2003).

BN-PAGE analysis

Mitochondria and OXPHOS complexes were isolated from cultured cells using 2% (W/V) digitonin and analyzed as described (Nijtmans *et al.* 2002*a*,*b*). 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 MABs (Mitosciences, Mundolsheim, France) raised against the complex I subunit GRIM19, 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 ECL (Pierce, Rockford, IL, 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).

mt morphology

Cells were seeded at subconfluence on a glass coverslip and incubated for 24–48 h in the presence or absence of 50 μM mitotane, briefly rinsed with warm PBS, and 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±s.e.m. of *n* independent replicates performed in the same experiment or from *n* separated experiments. Differences between groups were

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analyzed using nonparametric Kruskal–Wallis ANOVA followed by Dunn's multiple comparison test or nonparametric Mann–Whitney *U* 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 μM *o,p*′-DDD significantly reducing the proliferation rate of H295R by 45% and that of SW13 cells by 30% (Fig. 1A and B). The anti-proliferative effect of mitotane was also time dependent, 100 μM mitotane inhibiting the proliferation of H295R cells by 18% after 24 h and by 70% after 72 h. Subsequent experiments were performed using 50 μM 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 hormone concentrations in the culture supernatant of H295R cells. Exposure to 50 μM 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 mt effectors of steroidogenesis by qRT-PCR. Mitotane significantly decreased the expression of such genes: *STAR*, which encodes the STAR that transports cholesterol into mitochondria, the first rate-limiting step for the intra-mt steroidogenic pathway (80% inhibition after 48 h; Fig. 2A); cholesterol desmolase (*CYP11A* (*CYP11A1*)), 3β-hydroxysteroid dehydrogenase (*HSD3B2*); 11β-hydroxylase (*CYP11B1*), which catalyzes 11-deoxycorticosterone and 11-deoxycortisol transformation into corticosterone and cortisol respectively (75% inhibition; Fig. 2B); and aldosterone synthase (*CYP11B2*), the last intra-mt 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.

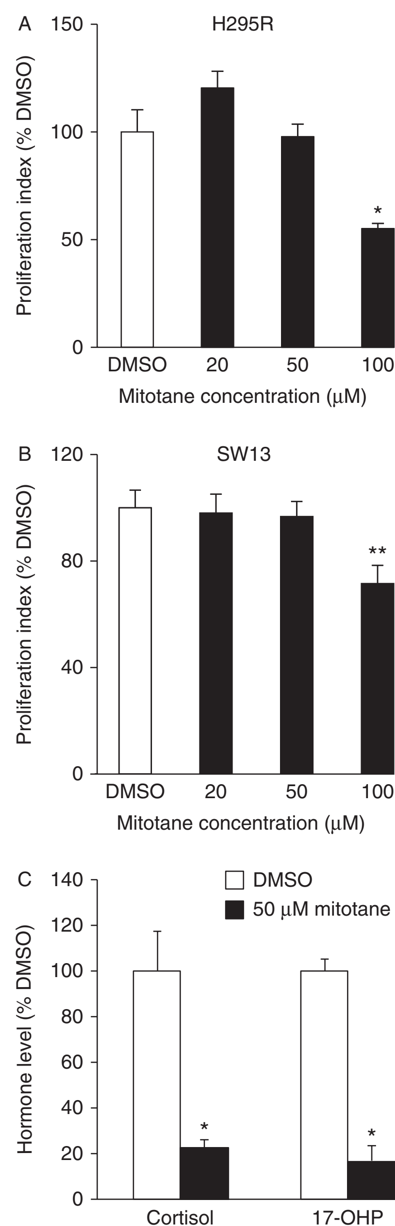

Figure 1
Dose-dependent inhibition of the proliferation index of human adrenocortical H295R (A) and SW13 (B) cells in response to increasing concentrations of mitotane (0–100 μM) after 48 h, as determined by Celltiter assay (See Materials and methods section). Results are expressed as the mean percentage ± S.E.M. of 12 independent determinations of the expression measured in untreated cells, arbitrarily set at 100%. *$P<0.05$ and **$P<0.01$, Mann–Whitney *U* test. Proliferation was affected in a dose-dependant manner in both cell lines. Inhibition of cortisol and 17-hydroxyprogesterone secretions by the steroid-secreting H295R cells (C). Cells were cultured with 50 μM mitotane for 48 h and the steroid concentrations were measured in the cell supernatants by radioimmunometric assays. Results are means ± S.E.M. of four independent determinations and are expressed as percentage of secretion under basal conditions (mean cortisol and 17-OHP secretions were 157 and 358 ng/48 h per mg protein per well respectively). Steroid hormone secretion was significantly inhibited by 80% after mitotane exposure.

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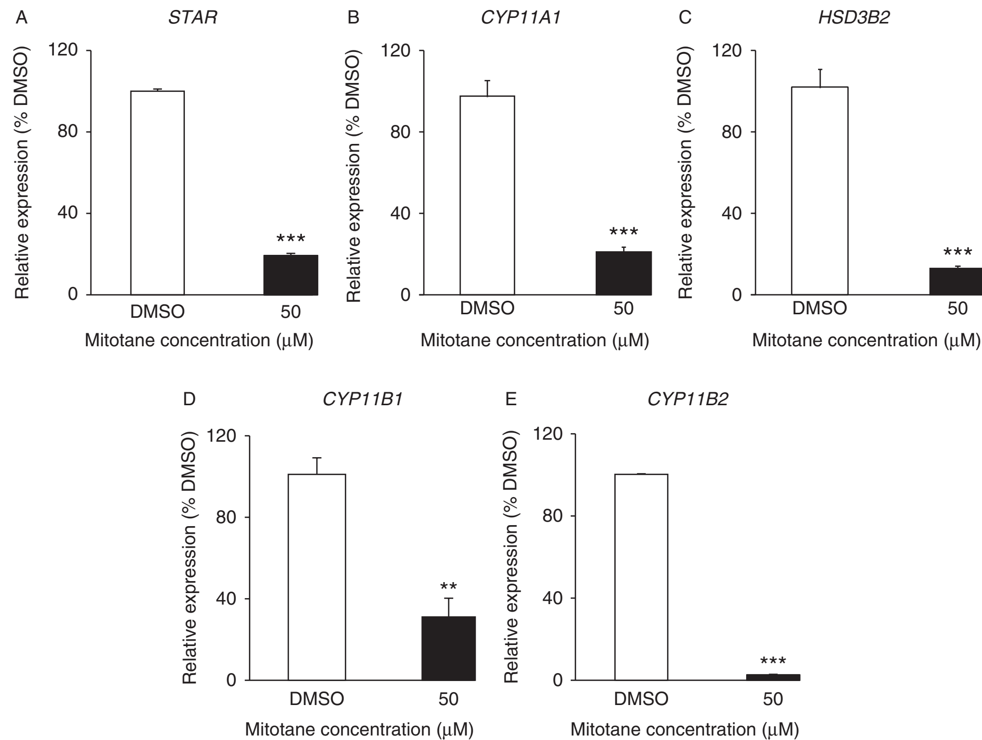

Figure 2
Expression of genes encoding mitochondrial and cytoplasmic proteins involved in steroidogenesis. H295R cells were treated with 50 μM mitotane for 48 h. Relative mRNA expression of (A) *STAR*, (B) *CYP11A*, (C) *HSD3B2*, (D) *CYP11B1*, and (E) *CYP11B2* was determined using qRT-PCR. Results are means ± S.E.M. of four 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. $^{**}P<0.01$ and $^{***}P<0.001$, Mann–Whitney *U* test.

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 mt respiratory complexes in H295R and SW13 cells treated with vehicle (DMSO) or 50 μM mitotane during 48 h (Table 1). Citrate synthase activity, belonging to the mt citric acid cycle, was used as an index of the mt mass. Its activity was very high in H295R cells (299 ± 22 nmol/min per mg protein; $n=12$) but lower in SW13 cells (159 ± 11 nmol/min per mg protein; $n=8$), suggesting that H295R cells have a greater mt population than SW13 cells consistent with their important steroidogenic capacity. However, citrate synthase activity was not affected by mitotane exposure (260 ± 31 nmol/min per mg protein in H295R and 135 ± 7 nmol/min per mg protein in SW13 cells).

Both H295R and SW13 mitotane-treated cells exhibited a significant COX (or complex IV) defect of ~50% after 48 h while complex II (succinate–ubiquinone oxidoreductase) appeared unaffected (Table 1). Complex III (ubiquinol–cytochrome *c* oxidoreductase) activity remained unchanged in H295R cells and was slightly reduced in SW13 cells after mitotane treatment (Table 1), but this decrease was not confirmed after normalization to citrate synthase activity (Table 1). Complex I (NADH–ubiquinone oxidoreductase) activity can only be reliably

Table 1 Mitochondrial respiratory activities.

Conditions	H295R cells Vehicle	H295R cells 50 μM mitotane	SW13 cells Vehicle	SW13 cells 50 μM mitotane
Citrate synthase	299 ± 22	260 ± 31	159 ± 11	135 ± 7
Complex I	8.3 ± 0.7	3.6 ± 1.1*	14.4	7.5
Complex II	55 ± 8	43 ± 9	37 ± 3	32 ± 1
Complex III	95 ± 14	86 ± 26	89 ± 6	55 ± 10*
Complex IV	276 ± 15	153 ± 19†	283 ± 53	127 ± 37†
CII/CS	0.28 ± 0.03	0.21 ± 0.03	0.26 ± 0.02	0.30 ± 0.04
CIII/CS	0.29 ± 0.03	0.36 ± 0.07	0.68 ± 0.07	0.68 ± 0.10
CIV/CS	1.05 ± 0.08	0.52 ± 0.02‡	0.78 ± 0.08	0.56 ± 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 ± S.E.M. of 6–12 independent experiments, expressed as nmol/min per mg protein. Ratio between complex II or complex III or complex IV:citrate synthase (CS) activities is also presented. $^{*}P<0.05$, $^{\dagger}P<0.01$, and $^{\ddagger}P<0.001$ with nonparametric Mann–Whitney *U* test.

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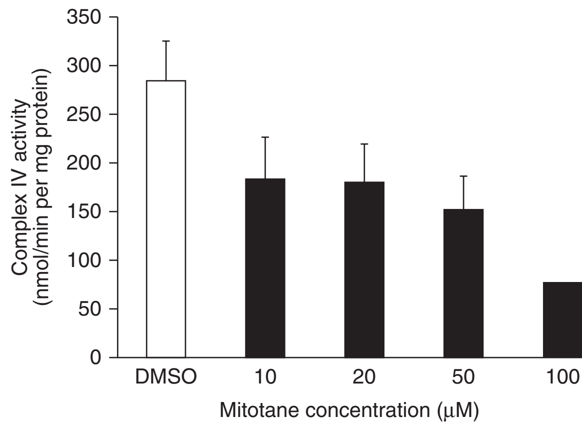

Figure 3
Dose-dependent inhibition of cytochrome *c* oxidase (complex IV, COX) activity in H295R cells by mitotane. H295R cells were treated for 48 h with increasing concentrations of mitotane (0–100 μM) and the COX activity was measured by spectrophotometry as described in the Materials and methods section. Results are expressed as nmol/min per mg protein. Each point represents the mean of three to six independent determinations. Mitotane inhibits complex IV activity in a concentration-dependant manner with a calculated IC_{50} at 58 μM.

measured on purified mt fractions due to the presence of numerous non-mt 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 1). 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 IC_{50} calculated at ~67 μM 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 (Haak *et al.* 1994, Baudin *et al.* 2001).

To examine whether *o,p*′-DDD might directly affect the enzymatic activity of complex IV, we measured COX 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 IC_{50} of ~133 μM (linear regression test; $y=100.2-0.3749x$, $r^2=0.96$; Fig. 4). This IC_{50} in the cell homogenate system is twice as high as the IC_{50} observed when whole cells were treated for 48 h, indicating that mitotane exerts both direct and indirect inhibitory effects on COX 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 COX at both the mRNA and protein levels. The COX complex consists of 13 subunits, three of which, including COX2, are encoded by the mt genome while the remaining ten subunits, including COX4 (COX4I1), are encoded by nuclear genes. We observed that the steady-state levels of mt 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 μM mitotane for 48 h (Fig. 5A and B). Similar results were obtained in SW13 cells (data not shown).

We analyzed the whole respiratory chain complexes by blue native PAGE (BN-PAGE). Immunoblotting with antibodies directed against a component of each mt 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 D). These data were fully consistent with the decreased enzymatic activities described earlier (Table 1). 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 mtDNA, we quantified mtDNA by qPCR. As illustrated in Fig. 6A, the mt:nuclear DNA ratio was unaffected by exposure of low or moderate doses of

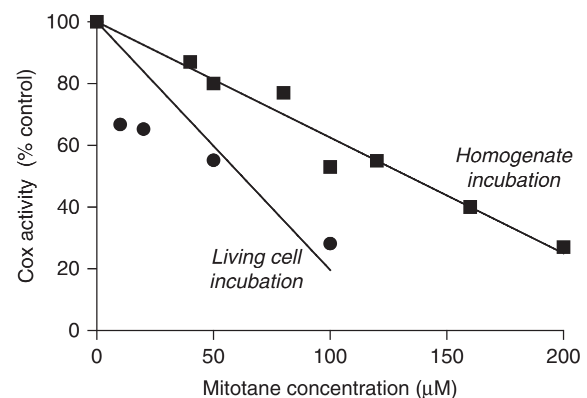

Figure 4
Dose-dependent inhibition of cytochrome *c* oxidase (COX) activity by mitotane. COX activity was measured by spectrophotometry in cell homogenates of H295R cells incubated with increasing concentrations of mitotane (0–100 μM) for 48 h (black circles, living cell incubation) or in cell homogenates simultaneously exposed to increasing concentrations of mitotane (0–200 μM) 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 IC_{50} at 133 μM (homogenate incubation), while the IC_{50} of mitotane was calculated at 67 μM for the inhibitory effects on treated cells (living cell incubation).

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mitotane for 48 h. However, this ratio increased significantly after treatment with 100 μM mitotane and with longer exposure times (e.g. 50 μM mitotane for 72 h), suggesting the presence of a compensatory response of mt biogenesis (Fig. 6B). To further explore this hypothesis, we quantified the expression of peroxisome proliferator-activated receptor gamma coactivator 1α (*PGC1α* (*PPARGC1A*)), a transcriptional coactivator considered a key regulator of mt biogenesis. *PGC1α* mRNA expression was slightly but significantly induced

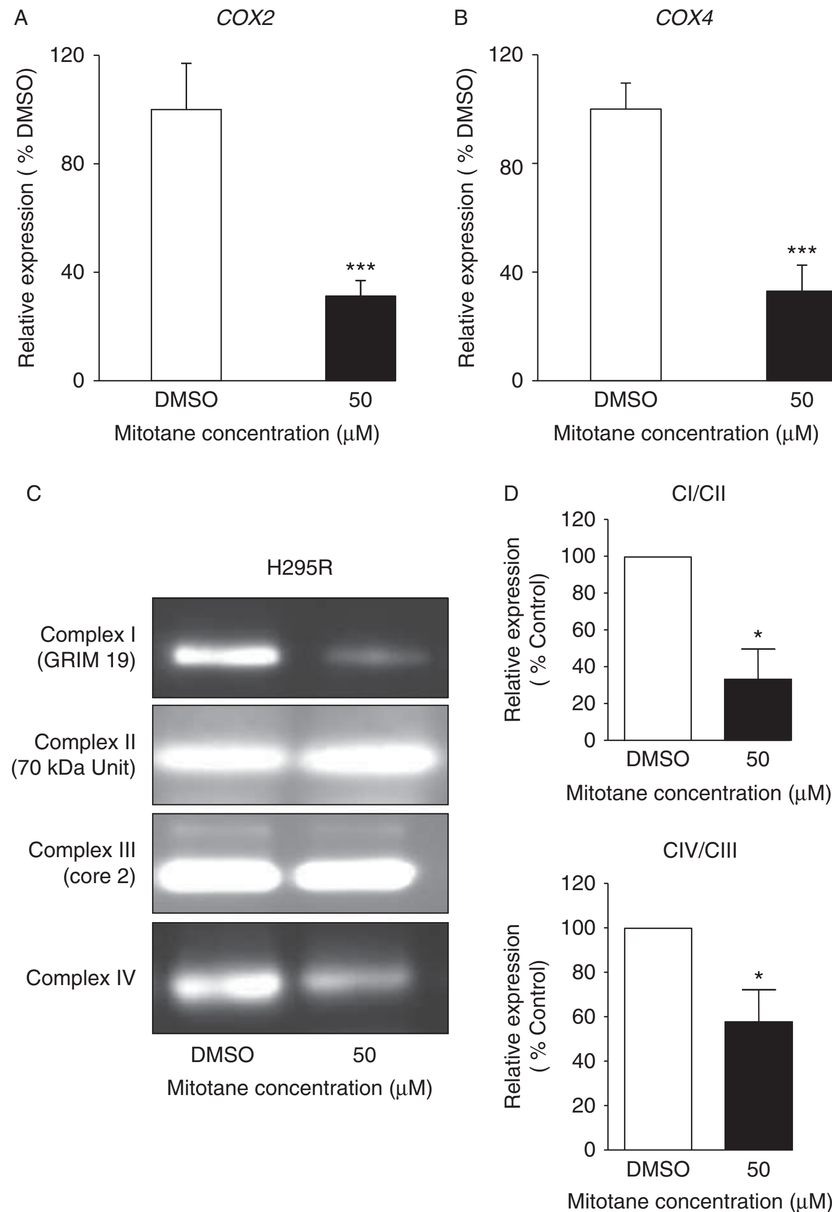

Figure 5
Inhibition of the expression of the respiratory chain genes and proteins by mitotane. The steady-state levels of mRNA encoding for the mitochondrial DNA-encoded *COX2* (A) and the nuclear DNA-encoded *COX4* (B) were measured by RT-qPCR. The expression of both *COX2* and *COX4* transcripts was drastically reduced after exposure to 50 μM mitotane for 48 h. Results are expressed as the mean percentage±s.e.m. of four independent determinations performed in duplicate of the expression measured in untreated cells, arbitrarily set at 100%. $*P<0.05$ and $***P<0.001$, Mann–Whitney *U* 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 the complex IV and were reduced by 45–70% but had no effect on complex II or complex III expression (D). Results are expressed as the mean percentage±s.e.m. of four independent determinations of the expression measured in untreated cells, arbitrarily set at 100%. $*P<0.05$, Mann–Whitney *U* test.

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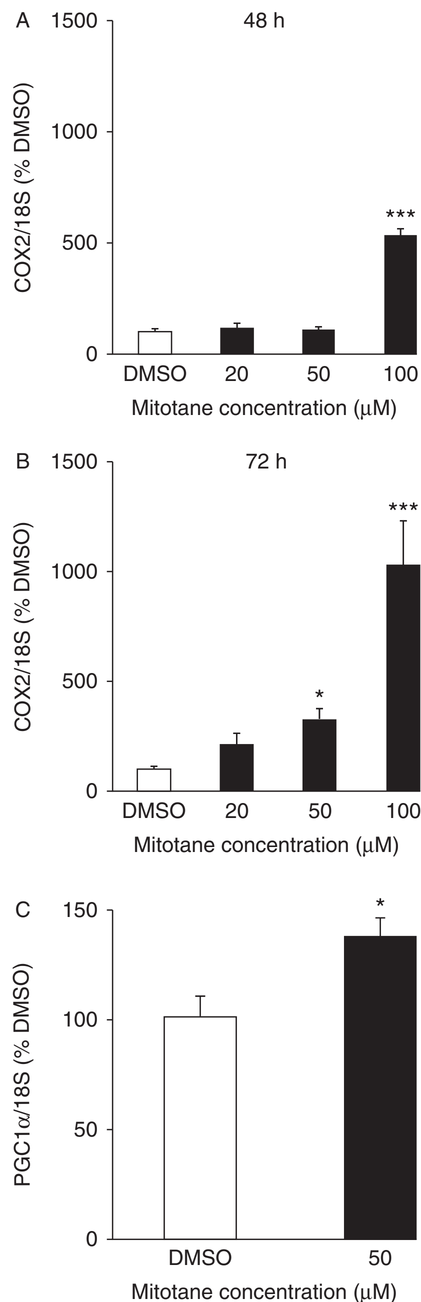

Figure 6
Stimulation of mitochondrial biogenesis by mitotane. Mitochondrial DNA was quantified by qPCR in H295R cells treated for 48 h (A) or 72 h (B) with increasing concentrations of mitotane. Higher mitotane concentrations (100 μM) and longer time exposure (72 h) increased mitochondrial:nuclear DNA ratio. Increased expression of peroxisome proliferator-activated receptor gamma coactivator 1α (*PGC1α*) in H295R cells was treated with 50 μM mitotane for 48 h (C). Results are expressed as the mean percentage ± s.e.m. of four independent determinations performed in duplicate of the expression measured in untreated cells, arbitrarily set at 100%. *$P<0.05$ and ***$P<0.001$, Mann–Whitney *U* test.

by 50 μM 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 mt morphology

Finally, to get an integrated evaluation of the mitotane-induced mt respiratory chain defect, we analyzed the mt morphology by immunocytochemistry using an antibody against COX2. Treatment with 50 μM mitotane induced drastic morphological alterations in the mitochondria of adrenocortical cells. In the absence of treatment, the mt compartment appeared as a highly interconnected tubular network with a filamentous appearance. However, after exposure to the drug the compartment exhibited a more punctiform pattern, consistent with mt 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 ACC (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 this study, we addressed the question on the mt 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 ACC cells have been so far unsuccessful.

We found that at optimal therapeutic concentrations (50 μM, i.e. 14–20 mg/l), mitotane drastically altered mt 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 mt proteins involved in steroidogenesis pathways. More importantly, we demonstrated for the first time that exposure to 50 μM mitotane significantly impairs the mt respiratory chain. Mitotane exposure also stimulated mt biogenesis and altered mt 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 an unmet need for improved bioavailability

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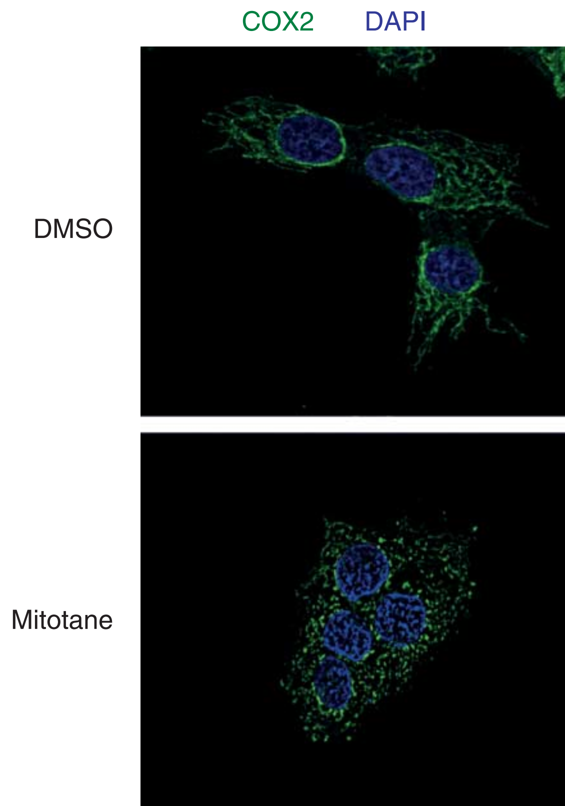

Figure 7
Morphological changes in the mitochondrial compartment network of H295R cells treated with 50 μM mitotane for 48 h. Cells were fixed and immunostained with anti-COX2 antibody (green) and counterstained with DAPI (blue). Under mitotane exposure, the filamentous morphology of the mitochondrial compartment observed in vehicle-treated cells (DMSO) displayed a punctiform pattern, indicative of mitochondrial fragmentation.

and its metabolic conversion constitute potential limitations (Schteingart 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 (Martz & Straw 1977, Cai *et al.* 1995). In accordance with previous studies, we confirmed that mitotane inhibits steroidogenesis reducing cortisol and 17-OH-progesterone secretions by 70% (Schteingart *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 mt 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.* 2012, 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 (van Erp *et al.* 2011, Kroiss *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 (Lund & Lund 1995, Lindhe *et al.* 2002). Although CYP11B1 may catalyze the initial hydroxylation step of mitotane (Cai *et al.* 1995, Lund & Lund 1995, Lindhe *et al.* 2002), its direct involvement in mt dysfunction is very unlikely given that SW13 cells, which do no 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-mt 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 mt 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 levels. 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 mt damage, as initially proposed (Kaminsky *et al.* 1962).

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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 mt biogenesis, as demonstrated by increased mtDNA content and *PGC1α* expression, reminiscent of a cellular compensation mechanism in response to the respiratory chain defect. This adaptive pathway, combining increased mt mass, increased mtDNA copy level and impaired OXPHOS, which has already been reported in mt 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 mt network, which could be related to disequilibrium between mt fission and fusion (Chen & Chan 2010). It is well established that the integrity of mt outer and inner membranes is required for respiratory chain activity (Liesa *et al*. 2009, Chen *et al*. 2010) and presumably steroidogenesis (Duarte *et al*. 2012). It is not known, however, whether mt fragmentation has a direct relationship with or a causal role in genotoxic stress and apoptosis.

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

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0368.

Declaration of interest

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

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Author contribution statement

S H, A S, E B, and M L designed the study; S H, A S, A L, S T, and M L performed the experiments and analyzed the results; A L, A P, H R, R C, S B, and J Y helped interpret the data and participated in discussions; and S H, E B, and M L wrote the paper; all the authors have read, revised, and approved the manuscript.

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