Mitotane reduces human and mouse ACTH-secreting pituitary cell viability and function

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Abstract

Medical therapy for Cushing's disease (CD) is currently based on agents mainly targeting adrenocortical function. Lately, pituitary-directed drugs have been developed, with limited efficacy. Mitotane, a potent adrenolytic drug, has been recently investigated for the treatment of CD, but the direct pituitary effects have not been clarified so far. The aim of our study was to investigate whether mitotane may affect corticotroph function and cell survival in the mouse pituitary cell line AtT20/D16v-F2 and in the primary cultures of human ACTH-secreting pituitary adenomas, as an *in vitro* model of pituitary corticotrophs. We found that in the AtT20/D16v-F2 cell line and in primary cultures, mitotane reduces cell viability by inducing caspase-mediated apoptosis and reduces ACTH secretion. In the AtT20/D16v-F2 cell line, mitotane reduces *Pomc* expression and blocks the stimulatory effects of corticotropin-releasing hormone on cell viability, ACTH secretion, and *Pomc* expression. These effects were apparent at mitotane doses greater than those usually necessary for reducing cortisol secretion in Cushing's syndrome, but still in the therapeutic window for adrenocortical carcinoma treatment. In conclusion, our results demonstrate that mitotane affects cell viability and function of human and mouse ACTH-secreting pituitary adenoma cells. These data indicate that mitotane could have direct pituitary effects on corticotroph cells.

Key Words

- Cushing's disease
- mitotane
- corticotropin-releasing hormone
- pituitary function
- POMC

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Introduction

Cushing's disease (CD), characterized by hypercortisolism due to excessive secretion of ACTH by the pituitary gland, is a rare disease with an incidence between 0.7 and 2.4 cases per million per year (Patil *et al.* 2008). Currently, the treatment of choice for patients with CD is surgery, but late recurrence occurs in 5–20% of patients (Atkinson *et al.* 2005). Although a variety of treatments are

available, pituitary irradiation is a good option for aggressive CD that fails to respond to surgery, invades the cavernous sinus, or relapses following an initial remission, but the treatment is associated with important side effects (Mahmoud-Ahmed & Suh 2002). Laparoscopic bilateral adrenalectomy can be safely and effectively employed to treat CD but needs permanent glucocorticoid

supplementation (Porgiglia *et al.* 2004). Several medical therapies, including steroidogenic inhibitors, centrally acting agents, and glucocorticoid receptor inhibitors, are currently being used or investigated as a potential treatment for CD (Biller *et al.* 2008, Feelders *et al.* 2010), but their efficacy was found to be unpredictable (Tritos & Biller 2012). Recently, a retrospective study has highlighted the efficacy of mitotane therapy in CD treatment (Baudry *et al.* 2012). Mitotane (*o,p'*-DDD), a derivative of the insecticide dichlorodiphenyltrichloroethane, has been widely used for treatment of advanced (unresectable, metastatic, or relapsed) adrenocortical carcinoma (ACC; Bergenstal *et al.* 1960, Young *et al.* 1973, Hogan *et al.* 1978, Lughezzani *et al.* 2010) and is increasingly used in adjuvant settings (Fassnacht *et al.* 2012). Mitotane concentrations are associated with both efficacy and toxicity (Haak *et al.* 1994, Terzolo *et al.* 2000) and blood levels ≥ 14 mg/l predict ACC tumor response (Haak *et al.* 1994, Hermesen *et al.* 2011). A concentration range between 14 and 20 mg/l (corresponding to 44–62 μ M) is considered as the ACC therapeutic window (Terzolo *et al.* 2000, Lee 2007, Hermesen *et al.* 2011), while lower doses of mitotane have been demonstrated to control hypercortisolism in the settings of CD (Baudry *et al.* 2012). The latter effect is usually ascribed to the adrenolytic action of mitotane, but a central inhibitory action on corticotrophs has never been investigated. We previously demonstrated that mitotane reduces both secretory activity and cell viability of pituitary TSH-secreting mouse cells (Zatelli *et al.* 2010), suggesting a possible direct effect on pituitary cells. Therefore, the aim of our study was to investigate whether mitotane may affect corticotroph function and cell survival *in vitro*.

Materials and methods

Reagents

Mitotane (Supelco, Bellefonte, PA, USA) was resuspended in absolute ethanol. Therefore, control cells have been incubated in culture medium containing 0.1% ethanol in all experiments. All reagents were purchased from Sigma if not otherwise indicated.

Cell culture

The mouse ACTH-secreting pituitary adenoma cell line, AtT20/D16v-F2, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line was maintained in DMEM (Invitrogen) supplemented

with 10% horse serum (HS) (LGC Standards, Milano, Italy) and antibiotic antimycotic (EuroClone, Milano, Italy) at 37 °C in 5% CO₂, as described previously (Gentilin *et al.* 2013). Before each experiment, cells were incubated in 0.5% HS medium for 48 h followed by a 24-h incubation in 10% HS medium.

The human thyroid follicular epithelial cell line, Nthy-ori 3-1 (ECACC, Salisbury, UK), was maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (LGC Standards) and antibiotic antimycotic (EuroClone) as described previously (Caselli *et al.* 2012).

The human endometrial stromal cell line, T-HESC (ATCC), was maintained in DMEM/F12 (Invitrogen) supplemented with 1.5 g/l sodium bicarbonate, 1% ITS+Premix, 500 ng/ml puromycin, and 10% charcoal/dextran-treated FBS (LGC Standards).

The human kidney cell line, 4/5 (generously provided by Dr Gianluca Aguiari, Department of Biomedical and Specialty Surgical Sciences, University of Ferrara), was maintained in DMEM (Invitrogen) supplemented with 10% FBS (LGC Standards) and antibiotic antimycotic (EuroClone).

Tissue collection and primary culture

The liver sample was derived from a patient diagnosed with liver hyperplasia and operated on at the University of Ferrara (Section of Endocrinology and Institute of Surgery). The pituitary adenoma samples derived from five patients were operated on for ACTH-secreting pituitary adenomas at the Department of Neurosurgery, Ospedale Maggiore – Bellaria, Bologna, Italy. Tissues were collected following the guidelines of the local committee on human research and immediately minced in RPMI-1640 medium (Invitrogen) under sterile conditions. Primary cultures were then prepared as described previously (Zatelli *et al.* 2006, Martínez-Fuentes *et al.* 2011). Informed consent of the patients was obtained for disclosing clinical investigation and performing the *in vitro* study.

Viable cell number assessment

Variations in cell number were assessed by the ATPlite assay (PerkinElmer, Waltham, MA, USA), as described previously (Zatelli *et al.* 2007). Briefly, the cells were seeded at 9×10^3 cells/well in 96-well white plates and then exposed to test substances. After incubation time, substrate solution was added directly to the cell culture plates

at room temperature. The plates were shaken at 700 r.p.m. for 2 min and then measured for luminescent output (relative light units (RLU)) by Victor³ 1420 Multilabel Counter (PerkinElmer). Results are expressed as mean value $\pm$ S.E.M. percent cell viability vs vehicle-treated control cells in five independent experiments in six replicates.

Caspase activity

Caspase activity was measured using the Caspase-Glo 3/7 assay (Promega) following the manufacturer's instruction as described previously (Tagliati *et al.* 2010). Results are expressed as mean value $\pm$ S.E.M. percent RLU vs vehicle-treated control cells in five independent experiments in six replicates.

ACTH secretion

ACTH secretion was evaluated by measuring mouse ACTH immunoreactivity in the conditioned culture medium with the ACTH 'Ultra Sensitive' lumELISA kit (Calbiotech, Spring Valley, CA, USA). Hormone assays were performed in duplicate after appropriate sample dilutions. The sensitivity was <1 pg/ml at the 95% confidence limit. Intra- and interassay CV were 6 and 8.7% respectively. The assay results were normalized by cell number, as determined from the ATPlite assay. Results are expressed as the mean value $\pm$ S.E.M. percent ACTH concentration vs vehicle control cells in seven experiments in duplicate.

Pomc gene expression

Total RNA from treated cells was extracted with TRIzol reagent (Invitrogen) and treated with RNase-free DNase (Promega), as previously reported (Minoia *et al.* 2012). The Experion automated electrophoresis system (Bio-Rad) was used to determine the concentration and integrity of RNA samples. Only samples with RNA quality index >9 were

reverse transcribed using the first-strand cDNA synthesis kit (Invitrogen) following the manufacturer's instructions.

Pomc expression evaluation was performed by relative quantitative real-time PCR (QRT-PCR). All QRT-PCRs were conducted with the TaqMan gene expression assay (Applied Biosystems), run on Applied Biosystems 7700 ABI Prism thermal cycler, and analyzed with the SDS 1.9 Software (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase was identified as the most stable reference gene from a set of five tested candidate housekeeping genes (Table 1) by the geNorm software, version 3.4 (Vandesompele *et al.* 2002). Relative expression ratio of *Pomc* mRNA (assay ID Mm00435874_m1; Applied Biosystems) was calculated by applying the method described by Pfaffl (2001). Results are expressed as mean value $\pm$ S.E.M. percent *Pomc* expression vs vehicle-treated control cells from at least five independent experiments in five replicates.

Statistical analysis

Results were expressed as $\pm$ S.E.M. and analyzed statistically using Student's *t*-tests to evaluate individual differences between means. Differences were considered significant at $P < 0.05$.

Results

Effects of mitotane on basal and corticotropin-releasing hormone-induced AtT20/D16v-F2 cell viability

In order to determine the effects of mitotane on corticotroph cell viability, the latter was assessed in AtT20/D16v-F2 after 6- and 24-h treatment without or with increasing mitotane concentrations (10–100 μ M, corresponding to plasma levels of 3.2–32 mg/l). As shown in Fig. 1A, after 6 h, mitotane significantly reduced cell viability at 100 μ M (-29% ; $P < 0.01$); after 24 h, mitotane significantly

Table 1 Primers and probe for candidate housekeeping genes

Gene	Primers and probe
Cyclophilin	Primer reverse 5'-AAACAGCTCGAAGGAGACGC-3' Primer forward 5'-ACCGTGTCTTCGACATCACG-3' Probe 5'-6' FAM-ATGACGAGCCCTTGGG-MGB-3'
Ribosomal L37	Primer reverse 5'-CAGCTGCCCTCTTGGGTTT-3' Primer forward 5'-CCGAGATTCAACATGGATT-3' Probe 5'-6' FAM-TGAGGGAACAACGCC-MGB-3'
18S	4319413E TaqMan endogenous control; Applied Biosystems
<i>Gadph</i>	4352339E TaqMan endogenous control; Applied Biosystems
β -Actin	4352341E TaqMan endogenous control; Applied Biosystems

Figure 1

Effects of mitotane on mouse and human ACTH-secreting pituitary adenoma cell viability. (A) AtT20/D16v-F2 cells were incubated for 6 (white bars) and 24 h (black bars) with 10–100 μM mitotane; control cells were treated with vehicle solution. Cell viability was assessed in at least five independent experiments with six replicates each and is expressed as the mean value $\pm$ s.e.m. percent cell viability vs vehicle control cells. * $P < 0.05$ and ** $P < 0.01$ vs vehicle control cells. (B) AtT20/D16v-F2 cells were incubated for 6 h with 10–100 μM mitotane without or with 100 nM CRH; control cells were treated with vehicle solution. Cell viability was assessed in at least five independent experiments with six replicates each and is expressed as the mean value $\pm$ s.e.m. percent cell viability vs vehicle control cells. * $P < 0.05$ and ** $P < 0.01$ vs vehicle control cells. # $P < 0.05$ and ## $P < 0.01$ vs 100 nM CRH-treated cells. (C) Human ACTH-secreting pituitary adenoma primary cultures were incubated for 24 h with 10–100 μM mitotane; control cells were treated with vehicle solution. The graph represents the average of cell viability measurements of five primary cultures. Data were evaluated independently with six replicates each and were expressed as the mean value $\pm$ s.e.m. percent cell viability vs vehicle control cells. ** $P < 0.01$ vs vehicle control cells.

reduced cell viability at concentrations ≥ 40 μM, from 8% ($P < 0.05$) to 45% vs control ($P < 0.01$).

Time-course experiments showed that treatment with 100 nM corticotropin-releasing hormone (CRH) significantly induced cell viability after incubation up to 6 h by $\sim 20\%$ ($P < 0.05$; data not shown). No effect was observed for longer incubation intervals. As the cells were incubated with CRH without any treatment renewal, we hypothesized that CRH will be degraded at long exposure time; therefore, experiments employing CRH were performed with a 6-h incubation time.

To investigate whether mitotane may influence CRH-induced AtT20/D16v-F2 cell viability, the latter was measured after a 6-h incubation with CRH alone or in combination with increasing mitotane concentrations (10–100 μM). As shown in Fig. 1B, CRH induced a significant ($P < 0.05$) increase in AtT20/D16v-F2 cell viability (+25%), which was significantly reduced by mitotane at all concentrations (from 12 to 50% when compared with CRH-treated cells).

Effects of mitotane on human ACTH-secreting pituitary adenoma cell viability

In order to determine the effects of mitotane on human ACTH-secreting pituitary adenoma cell viability, the latter was assessed in human primary cultures from ACTH-secreting pituitary adenomas after 24-h treatment without or with increasing mitotane concentrations (10–100 μM, corresponding to plasma levels of 3.2–32 mg/l). As shown in Fig. 1C, after 24 h, mitotane significantly reduced cell viability at concentrations ≥ 40 μM, from 15% ($P < 0.01$) to 55% vs control ($P < 0.01$).

Effects of mitotane on cell viability of thyroid, endometrial, renal, and liver cells

In order to determine the specificity of mitotane effects, cell viability was assessed in NTHY-ori 3-1, T-HESC, 4/5 cell lines, and in a liver primary culture after 24-h treatment without or with increasing mitotane concentrations (10–100 μM, corresponding to plasma levels of 3.2–32 mg/l). As shown in Fig. 2, mitotane did not significantly modify the viability of any investigated cell line.

Effects of mitotane on AtT20/D16v-F2 apoptosis and CRH influence

To investigate whether mitotane reduces cell viability by activating apoptosis, caspase 3/7 activity was measured in

AtT20/D16v-F2 cells incubated for 6 and 24 h in the presence of increasing mitotane concentrations (10–100 μ M). As shown in Fig. 3A, mitotane significantly induced apoptosis at 40–100 μ M after both 6 h (from 48 to 434%; $P<0.01$) and 24 h (from +83 to +763%; $P<0.01$)

Figure 2

Effects of mitotane on cell viability of thyroid, endometrial, renal, and liver cells. Cells were incubated for 24 h with 10–100 μ M mitotane; control cells were treated with vehicle solution. Nthy-ori (A), T-HESC (B), and 4/5 (C) cell viability was assessed in at least five independent experiments with six replicates each. Liver primary culture cell viability (D) was assessed in six replicates. Cell viability was expressed as the mean value $\pm$ s.e.m. percent cell viability vs vehicle control cells.

when compared with vehicle-treated control cells. We observed that, after 6 h, at concentrations <100 μ M mitotane significantly induced caspase activation but did not affect cell viability, suggesting that a short exposure time does not compromise the viability of corticotroph cells but commits them to apoptosis.

To investigate the influence of CRH on mitotane-induced apoptosis, caspase 3/7 activity was measured in AtT20/D16v-F2 cells incubated for 6 h with CRH alone or in combination with increasing mitotane concentrations. As shown in Fig. 3B, CRH did not significantly affect basal caspase 3/7 activity but completely blocked the proapoptotic effects of mitotane at 40 μ M. However, the inhibitory effect of CRH on mitotane-induced caspase activation was lost partially at 60 μ M and completely at 100 μ M mitotane.

Effects of mitotane on human ACTH-secreting pituitary adenoma primary culture apoptosis

To investigate whether mitotane reduces cell viability in human ACTH-secreting pituitary adenoma primary cultures by activating apoptosis, caspase 3/7 activity was measured after incubation for 24 h in the presence of increasing mitotane concentrations (10–100 μ M). As shown in Fig. 3C, mitotane significantly induced apoptosis at 40–100 μ M (from +60 to +620%; $P<0.01$) when compared with vehicle-treated control cells.

Effects of mitotane on basal ACTH secretion

To determine the effects of mitotane on ACTH secretion, ACTH levels were assessed in conditioned medium from AtT20/D16v-F2 cells treated for 6 and 24 h with increasing mitotane concentrations (10–100 μ M). As shown in Fig. 4A, mitotane significantly reduced basal ACTH secretion after a 6-h treatment at both 80 and 100 μ M (–65 and –87% respectively; $P<0.01$). After 24 h, mitotane significantly reduced ACTH secretion at ≥ 60 μ M (from 40 to 96%).

ACTH levels were also assessed in conditioned medium from human ACTH-secreting pituitary adenoma primary cultures treated for 24 h with increasing mitotane concentrations (10–100 μ M). As shown in Fig. 4B, mitotane significantly reduced basal ACTH secretion at ≥ 60 μ M (from 35 to 94%; $P<0.01$).

Effects of mitotane on CRH-stimulated ACTH secretion

To evaluate ACTH secretory response to CRH *in vitro*, AtT20/D16v-F2 cells were incubated for 0, 2, 5, 10, 15, and

30 min with CRH at 100 nM, a concentration at which CRH is known to induce ACTH secretion in these cells (Strowski *et al.* 2002). ACTH was then assayed in the condition medium. As shown in Fig. 5A, CRH significantly

Figure 3

Effects of mitotane on caspase activity. (A) AtT20/D16v-F2 cells were incubated for 6 (white bars) and 24 h (gray bars) with 10–100 μM mitotane; control cells were treated with vehicle solution. Caspase 3/7 activity was measured in at least five independent experiments in six replicates and is expressed as percent mean value $\pm$ S.E.M. caspase 3/7 activity vs vehicle-treated control cells. $**P < 0.01$ vs vehicle-treated control cells. (B) AtT20/D16v-F2 cells were treated for 6 h with 10–100 μM mitotane in the presence of 100 nM CRH; control cells were treated with vehicle solution. Caspase 3/7 activity was measured in at least five independent experiments in six replicates and is expressed as percent mean value $\pm$ S.E.M. caspase 3/7 activity vs vehicle-treated control cells. $**P < 0.01$ vs vehicle-treated control cells. $##P < 0.01$ vs 100 nM CRH-treated cells. (C) Human ACTH-secreting pituitary adenoma primary cultures were incubated for 24 h with 10–100 μM mitotane; control cells were treated with vehicle solution. The graph represents the average of caspase 3/7 measurements of five primary cultures. Data were evaluated independently with six replicates each and were expressed as the mean value $\pm$ S.E.M. percent cell viability vs vehicle control cells. $**P < 0.01$ vs vehicle control cells.

Figure 4

Effects of mitotane on ACTH secretion. (A) AtT20/D16v-F2 cells were incubated for 6 (white bars) and 24 h (gray bars) with 10–100 μM mitotane; control cells were treated with vehicle solution. ACTH levels were measured in conditioned medium by ELISA in seven independent experiments in duplicate and are expressed as the mean value $\pm$ S.E.M. percent ACTH secretion vs vehicle control cells. $**P < 0.01$ vs vehicle control cells. (B) Human ACTH-secreting pituitary adenoma primary cultures were incubated for 24 h with 10–100 μM; control cells were treated with vehicle solution. ACTH levels were measured in conditioned medium by ELISA in independent experiments with six replicates each. The graph represents the average of ACTH measurements from five primary cultures. Data were expressed as the mean value $\pm$ S.E.M. percent cell viability vs vehicle control cells. $**P < 0.01$ vs vehicle control cells.

induced ACTH secretion up to 15 min, reaching the peak value after 2 min (+80%; $P < 0.01$ vs time point 0).

To determine whether mitotane affects CRH-induced ACTH secretion by AtT20/D16v-F2 cells, the cells were treated with 10–100 μM mitotane for 6 and 24 h. The medium was then removed and cells were stimulated with 100 nM CRH or vehicle for 2 min; then, ACTH concentration was evaluated. As shown in Fig. 5B, basal and CRH-induced ACTH secretion was higher after 6 h when compared with 24-h vehicle incubation. Pre-incubation for 6 h with mitotane at ≥ 40 μM significantly reduced both basal and CRH-induced ACTH secretion, which was completely suppressed at ≥ 80 μM mitotane. Pre-incubation for 24 h with mitotane at ≥ 10 μM significantly reduced both basal and CRH-induced ACTH secretion, which was completely suppressed at ≥ 80 μM mitotane.

Figure 5

Effects of mitotane on CRH-induced ACTH secretion. (A) AtT20/D16v-F2 cells were incubated for 0, 2, 5, 10, 15, and 30 min with 100 nM CRH. Medium was collected and ACTH concentration was assessed by ELISA. Data were evaluated independently in at least seven experiments in duplicate and are expressed as the mean value $\pm$ s.e.m. pg/ml. ** $P < 0.01$ vs vehicle control cells. (B) AtT20/D16v-F2 cells were incubated for 6 and 24 h in culture medium supplemented with 10–100 μ M mitotane and then stimulated with 100 nM CRH or vehicle for 2 min. 0 = vehicle control cells treated with CRH or vehicle for 2 min. Data were evaluated independently in seven experiments in duplicate and are expressed as the mean value $\pm$ s.e.m. percent ACTH secretion vs vehicle control cells. (C) Human ACTH-secreting pituitary adenoma primary cultures were incubated for 24 h in culture medium supplemented with 40–60 μ M mitotane and then stimulated with 100 nM CRH or vehicle. The graph represents the average of ACTH secretion measurements of five primary cultures. Data were evaluated independently with six replicates each and were expressed as the mean value $\pm$ s.e.m. percent ACTH secretion vs vehicle control cells.

To determine whether mitotane affects CRH-induced ACTH secretion by human ACTH-secreting pituitary adenoma primary cultures, the cells were treated with 40–60 μ M mitotane for 24 h and then stimulated with 100 nM CRH or vehicle for 2 min; then, ACTH

concentration was evaluated. As shown in Fig. 5C, CRH induced ACTH secretion by primary cultured cells (+40%; $P < 0.01$); pre-incubation with mitotane at 40–60 μ M significantly ($P < 0.01$) reduced both basal and CRH-induced ACTH secretion.

Effects of mitotane on *Pomc* mRNA expression and CRH influence

To determine the effects of mitotane on *Pomc* mRNA expression, *Pomc* mRNA levels were assessed in AtT20/D16v-F2 cells treated for 6 h with 10–100 μ M mitotane. As shown in Fig. 6, mitotane significantly ($P < 0.01$) reduced basal *Pomc* mRNA expression by $\sim 70\%$ at all concentrations tested. On the contrary, 100 nM CRH significantly ($P < 0.01$) induced *Pomc* mRNA expression (+100% vs vehicle control cells), an effect completely counteracted by mitotane at all concentrations tested.

Discussion

Our study provides for the first time evidence that mitotane has a direct and important action on human ACTH-secreting pituitary adenoma primary cultures and on the AtT20/D16v-F2 cell line, whose function may be greatly and quickly compromised. This inhibitory effect is, at least in part, due to direct inhibition of corticotroph cell viability, which is reduced by mitotane already after 6 h at high doses in AtT20/D16v-F2 cells and after 24 h at the

Figure 6

Effects of mitotane on *Pomc* expression. AtT20/D16v-F2 cells were treated for 6 h without or with 10–100 μ M mitotane in the presence or in the absence of 100 nM CRH; control cells were treated with vehicle solution. *Pomc* expression was assessed by QRT-PCR. Results are expressed as mean value $\pm$ s.e.m. percent *Pomc* expression vs vehicle-treated control cells from at least five independent experiments in five replicates. ** $P < 0.01$ vs vehicle control cells. ## $P < 0.01$ vs cells treated with 100 nM CRH.

concentrations corresponding to the ACC therapeutic window in both mouse and human corticotroph cells. These data indicate that exposure time may have a differential impact on corticotroph viability, in agreement with a previous report indicating that mitotane reduces cell viability of a TSH-secreting pituitary cell line in a similar fashion (Zatelli *et al.* 2010). In addition, the evidence that caspase activation precedes cell viability reduction after mitotane treatment, supports the hypothesis that mitotane rapidly commits corticotroph cells to apoptosis which, in turn, after a long exposure time, results in cell viability reduction. On the other hand, mitotane does not affect cell viability of the endocrine cell line NTHY-ori 3-1, indicating that mitotane cytotoxic effects are not generalized to endocrine cells. Moreover, the viability of non-endocrine cells, such as T-HESC and 4/5 cell lines and liver primary culture, is not influenced by the drug, supporting the hypothesis that mitotane acts rapidly with a specific effect at the pituitary level.

Previous clinical reports showed that in CD patients, mitotane, owing to its adrenolytic action, is highly effective in the long-term suppression of hypercortisolism (Luton *et al.* 1979, Schteingart *et al.* 1980), at concentrations lower than those employed for ACC treatment (Gross *et al.* 2007, Murao *et al.* 2010, Baudry *et al.* 2012), but central inhibitory actions have been overlooked.

Recently, Baudry *et al.* (2012) reported that in 24.5% of CD patients treated with mitotane as first-line treatment and with initial negative magnetic resonance imaging, a pituitary adenoma became apparent during or at the end of the treatment. These results may be explained by the anti-cortisolic effects exerted by mitotane at the doses employed in this study, corresponding to ~20–30 μM , which are approximately twofold lower than those employed in our experiments. Indeed, in their hands, mitotane significantly lowered cortisol levels in the majority of patients, with a consequent disappearance of negative feedback at the pituitary level. The latter allowed the increase in ACTH plasma levels and the visualization of a pituitary adenoma in 12 patients (Baudry *et al.* 2012), similar to what happens in Nelson's syndrome. Of these patients, ten had been operated on, but the authors do not disclose pathological characteristics of the adenomas, such as ki-67 (Fusco *et al.* 2008), which may be helpful in understanding the direct effects of mitotane at pituitary level. By contrast, in our experimental settings, we observed a reduction in corticotroph function and cell viability at higher mitotane concentrations ($\geq 40 \mu\text{M}$), suggesting that direct pituitary effects may become apparent only in the presence of drug concentrations in

the ACC therapeutic window. This hypothesis is further strengthened by the evidence that, upon (low-dose) mitotane withdrawal, ~70% of patients showed recurrence of hypercortisolism (Baudry *et al.* 2012), suggesting a lack of central effect when mitotane plasma level is $< 40 \mu\text{M}$. Our data show that higher mitotane doses impair corticotroph function and viability and therefore might be effective in controlling the disease at the pituitary level and in preventing recurrence after withdrawal. Clinical studies are necessary to clarify this issue.

The hypophysiotropic hormone CRH (Florio *et al.* 2007) has been previously demonstrated to significantly stimulate AtT20/D16v cell proliferation (van Wijk *et al.* 1995). Our results show that CRH induces cell viability only in the short term, suggesting a protective rather than a proliferative effect of this peptide on corticotroph cells. In keeping with our results, Lezoualc'h *et al.* (2000) indicated CRH as an endogenous protective neuropeptide against oxidative cell death in addition to its function in the HPA system. In addition, our data show that mitotane dose dependently reduced the stimulatory effects of CRH on mouse corticotroph cell viability. These data suggest that ACTH-secreting cells may become refractory to physiological stimuli, such as CRH, after exposure to mitotane. Moreover, we observed that the inhibitory effects of mitotane on human and mouse ACTH-secreting pituitary adenoma cell viability are, at least in part, due to apoptosis induction, as previously reported in other experimental models (Pushkarev *et al.* 2007, Zatelli *et al.* 2010). In our settings, caspase activation is prompted quickly and persists for at least 24 h. We also observed that, after 6 h, at concentrations $< 100 \mu\text{M}$, mitotane significantly induced caspase activation but did not affect cell viability, suggesting that a very short exposure time does not compromise the viability of corticotroph cells but commits them to apoptosis.

As already noted, mitotane, at concentrations lower than those reached *in vivo*, is able to reduce steroids and TSH secretion in adrenocortical and pituitary TSH-secreting cell lines respectively (Stigliano *et al.* 2008, Zatelli *et al.* 2010). Our findings support the evidence that mitotane affects pituitary function, also reducing the secretory activity of corticotroph cells. Mitotane inhibits ACTH secretion at high concentrations (80–100 μM) after 6 h of incubation in AtT20/D16v-F2 cells and at $\geq 60 \mu\text{M}$ after 24 h of exposure in human and mouse ACTH-secreting pituitary adenoma cells. This indicates, as previously suggested, that mitotane has a toxic effect on corticotroph function, providing further support to the use of this drug for persistent and refractory CD (Kawai

et al. 1999, Baudry *et al.* 2012). A recent study reported that plasma mitotane concentrations >8.5 mg/l (corresponding to 24 μ M) are sufficient to achieve CD control (Baudry *et al.* 2012). On the contrary, in our settings, mitotane reduced ACTH secretion at a concentration of 60 μ M, suggesting that a greater exposure time may be needed to affect pituitary hormonal secretion.

In patients treated with adjuvant mitotane following complete ACC removal, ACTH levels are often non-significantly increased as it should be expected by mitotane-induced inhibition of adrenal steroidogenesis (Daffara *et al.* 2008). Furthermore, both Takamatsu *et al.* (1981) and Kawai *et al.* (1999) suggested that mitotane had an effect on corticotrophs, reporting that mitotane maintenance therapy may be a good treatment option for persistent and intractable CD. These findings support the hypothesis that mitotane impairs ACTH secretion and has a direct action on corticotroph cells when employed at high concentrations (i.e. those used for ACC).

Our results also show that AtT20/D16v-F2 cells respond to CRH stimulation with a rapid and significant increase in ACTH secretion. The stimulatory effect of CRH, however, is completely blunted by concentrations of mitotane ≥ 40 μ M after 6 h of exposure and at lower concentrations after 24 h. These data indicate that mitotane rapidly affects the capability of adrenocorticotroph cells to respond to physiological stimuli, in keeping with the results obtained on cell viability. The anti-secretory effects of mitotane on corticotroph cells are further supported by data from primary cultures, wherein CRH stimulatory effect is completely abolished by mitotane at the concentrations corresponding to the ACC therapeutic window. These results are in line with evidence that mitotane strongly inhibits TRH-induced TSH secretion, supporting the hypothesis that this drug acts rapidly and profoundly with a generalized effect at the pituitary level. This hypothesis is further strengthened by evidence that, after 6 h, mitotane reduces *Pomc* expression independently of the concentration and regardless of CRH stimulation. Indeed, in keeping with the report by Aoki *et al.* (1997), CRH significantly induces *Pomc* expression, but, in our hands, it is not capable of rescuing cell function from mitotane toxic effects. Our data indicate that the effects of mitotane on ACTH secretion are not completely dependent on the effects on *Pomc* expression. Incubation with mitotane reduces short-term ACTH spontaneous release, suggesting that mitotane, besides affecting *Pomc* gene transcription, may impair ACTH release, also influencing secretory mechanisms.

In conclusion, our results demonstrate that mitotane reduces cell viability and function of mouse and human pituitary ACTH-secreting adenoma cells, suggesting a direct pituitary effect of mitotane. In addition, we provide evidence for a higher sensitivity of corticotroph cells to mitotane, as other cell lines originating from tissues different from pituitary (and also of non-endocrine lineage) are not sensitive to the inhibitory effects of mitotane during short-time exposure.

However, the promising *in vitro* data showing efficacy of mitotane in CD at the doses used for ACC within 24 h must be confirmed *in vivo* for a longer period of time in clinical trials, balancing the efficacy of ACTH secretion reduction with the development of side effects.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. The NIH statement does not apply.

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