ORIGINAL ARTICLE

Lipoprotein-Free Mitotane Exerts High Cytotoxic Activity in Adrenocortical Carcinoma

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Context: Mitotane (*o,p'*-DDD), the only approved drug for advanced adrenocortical carcinoma (ACC), is a lipophilic agent that accumulates into circulating lipoprotein fractions and high-lipid-containing tissues.

Objective: The aim of our study was to evaluate the in vivo and in vitro biological implication of serum lipoproteins on pharmacological action of mitotane. Distribution and concentration of mitotane were studied in plasma and adrenal tissue samples from mitotane-treated patients. The effect of lipoprotein-bound or lipoprotein-free (LP-F) mitotane was analyzed on proliferation and apoptosis of human adrenocortical H295R cells. A retrospective study of patients with ACC treated or not with statins was also performed.

Results: *o,p'*-DDD distribution among very low-density lipoprotein, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and LP-F fractions obtained after plasma ultracentrifugation of 23 of mitotane-treated patients was widely distributed in each subfraction. A positive correlation was observed between mitotane levels in plasma and in LDL, HDL, but also LP-F compartment. Intratumor *o,p'*-DDD concentrations in five ACC samples of mitotane-treated patients were found to be independent of cholesterol transporter expression, scavenger receptors, and LDL receptors. In vitro studies showed significant higher antiproliferative and proapoptotic effects and higher cell and mitochondrial uptake of mitotane when H295R cells were grown in LP-F medium. Finally, retrospective study of an ACC cohort of 26 mitotane-treated patients revealed that statin therapy was significantly associated with a higher rate of tumor control.

Conclusions: Altogether, our in vitro and in vivo studies provided compelling evidence for a greater efficacy of LP-F mitotane. Patients with ACC may thus benefit from therapeutic strategies that aim to increase LP-F mitotane fraction. **(*J Clin Endocrinol Metab* 100: 2890–2898, 2015)**

Mitotane (*o,p*'-DDD) is the only drug approved for advanced adrenocortical carcinoma (ACC) (1). The antitumor clinical effect of mitotane has been shown on both prospective and retrospective studies that found partial response rates in 10–33% of patients treated with mitotane alone but also improved overall survival (2, 3). Based on these results, mitotane is also recommended as an adjuvant therapy in patients with ACC at high risk of recurrence (4–6). In both indications, plasma mitotane monitoring is recommended to look for a therapeutic win-

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Abbreviations: ACC, advanced adrenocortical carcinoma; COX2, cytochrome *c* oxidase 2; DCR, disease control rate; FCS, fetal calf serum; GCMS, gas chromatography combined with mass spectrometry; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LP-F, lipoprotein free; mtDNA, mitochondrial DNA; *o,p'*-DDD, mitotane; RECIST, Response Evaluation Criteria In Solid Tumors; SrB1, Scavenger B1 receptor; VLDL, very low-density lipoprotein.

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dow of 14–20 mg/L (1). Indeed, several studies have reported higher response rate and/or a prolonged survival in patients with plasma mitotane levels greater than 14 mg/L (7–11). In addition, neurological toxicities have been described with plasma level greater than 20 mg/L (11, 12).

Mitotane is also known as *o,p*'-DDD, an insecticide-derivative lipophilic drug that accumulates in lipoproteins (13). Dyslipidemia have been observed in mitotane-treated patients but the mitotane-induced dyslipidemic profile differs from one study to another (14–19). The mechanism of mitotane-induced hypercholesterolemia is not fully understood but could be related to an activation of 3-hydroxy-3-methyl-glutaryl-CoA reductase (20) and to an increase in cholesterol and lipoproteins synthesis (14). Little is known about the influence of dyslipidemia on mitotane distribution among lipoproteins and about the influence of this distribution on its antitumor efficacy.

Mechanism of mitotane action was poorly understood until recently. Two mitotane metabolites are described: *o,p*'-DDE and *o,p*'-DDA, the latter being described as the main urinary metabolite of *o,p*'-DDD (21). We recently reported evidence that *o,p*'-DDA is unlikely an active metabolite of mitotane (22). Several studies suggested that mitotane could have a mitochondrial effect (23, 24), and more specifically, our group demonstrated a mitotane-induced defect in cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain) (25).

In the present study, we explored the biologic implication of serum lipoproteins on mitotane pharmacological action using human plasma samples, tissues of mitotane-treated patients, and finally, in vitro on human adrenocortical H295R cells. Altogether, our results showed that lipoprotein-free (LP-F) mitotane seemed to be the most efficient form. Based on these findings, we retrospectively examined the disease control rate of 26 consecutive patients with stage IV ACC treated with mitotane, according to the concurrent use of statins.

Patients, Materials, and Methods

Patients

Medical files of 70 patients with metastatic ACC treated with mitotane, followed between 2007 and 2014 at Gustave Roussy, were retrospectively reviewed to study the correlation between occurrence of dyslipidemia or statin therapy (Rosuvastatin not metabolized by Cyp3A4) within the first 3 months of mitotane therapy and neurologic toxicity or tumor response. Inclusion criterion was patients with stage IV ACC treated with mitotane after 2007 and, the exclusion criterion was the absence of lipid profile available within the first 3 months of mitotane therapy. In each file, the following criteria were recorded: mitotane plasma level, HDL, low-density lipoprotein (LDL), triglycerides levels (high HDL, high LDL, or high triglycerides defined as greater than 1.5 times the upper value), concurrent use of statins given during at least 3 months of mitotane therapy, presence of neurologic toxicities, and disease control rate (stabilization and partial response) at 6 months according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 (26). Informed consent was obtained from all patients.

Human plasma samples and human adrenal tissues

Twenty-five plasma samples from 20 mitotane-treated patients with ACC were taken at Gustave Roussy hospital and then made available for this study from the central repository of HRA Pharma (Paris, France) to evaluate lipoprotein partitioning in normal and dyslipidemic samples. To analyze the correlation between mitotane concentrations and cholesterol transporter expression, human adrenal tissues were obtained from six mitotane-treated patients followed at Gustave Roussy or Bicêtre Hospital. All had ACC but one underwent bilateral adrenalectomy for an ectopic Cushing's syndrome. Tissues were collected while patients underwent surgery for therapeutic reasons. Tissues were lysed in H_2O using a TissueLyser apparatus (QIAGEN). All patients signed an informed consent.

Human adrenocortical cells and mitochondria isolation

For in vitro studies, H295R cells (from passage 2–15) were cultured as previously described (25). Media were enriched with 10% fetal calf serum (FCS; control medium) or lipoprotein-deficient FCS (LP-F medium) enriched or not with LDL or HDL subfractions obtained from ultracentrifugation (with a final cholesterol concentration of 30 mg/L, similar as in all media). *o,p*'-DDD (HRA Pharma) and BLT1 (Sigma-Aldrich) were solubilized in dimethylsulfoxide (Sigma-Aldrich) and used at indicated concentrations. The percentage of dimethylsulfoxide in culture medium never exceeded 0.1%. Cholesterol (Sigma-Aldrich) and BSA (Euromedex) were solubilized in culture medium and used at final concentrations of 9.6 mg/L (low) and 38.4 mg/L (high) for cholesterol and 1.8 mg/L (low) and 3.6 mg/L (high) for BSA.

Mitochondrial fractions were purified and prepared from permeabilized cells using digitonin and percoll as previously described (27).

Lipoprotein isolation from plasma and fetal calf serum by ultracentrifugation

Density gradient ultracentrifugation using iodaxinol (Optiprep, Sigma-Aldrich) was used for the isolation of lipoprotein fractions in plasma samples. A saline solution with HEPES buffer was added to a mixed solution of 60% (m/v) iodixanol in water (d = 1.32 g/mL) and plasma in Optiseal vials. This final solution was ultracentrifuged at 350 000 g at 16°C for 3 hours. Each lipoprotein fraction was collected with syringe and needle systems.

Individual lipoprotein subfractions were isolated from FCS by isopycnic density gradient ultracentrifugation for 48 hours at 288 000× *g* using a Beckman XL70 centrifuge and a SW41 rotor as previously described (28). After centrifugation, gradients were collected from the top of the tubes with an Eppendorf precision pipet in fractions corresponding to LDL subfractions (density $<$ 1.063 g/mL) and HDL subfractions (density, 1.063–1.179 g/mL).

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Measurements of *o,p'*-DDD in plasma, tissues, cells, and mitochondria

Analyses of plasma samples were conducted by HPLC combined with HPLC-UV detection as previously described (22) and analyses of cells and mitochondria samples were conducted by gas chromatography combined with mass spectrometry (GCMS). All samples were spiked with known amounts of *p,p*'-DDE used as an internal standards of *o,p*'-DDD measurements. *o,p*'-DDD concentrations were determined through the ratio of their peak surface area to the peak surface of known concentrations of internal standards . Concentrations of *o,p*'-DDD in cells and mitochondria are expressed in nanograms per femtomoles of nuclear or mitochondrial DNA (mtDNA). Nuclear and mtDNA were extracted from samples using standard techniques and quantified by RT-qPCR using the 18S gene and the cytochrome *c* oxidase 2 (COX2) gene as nuclear- and mitochondrial-specific genes, respectively as previously described (25).

Cell proliferation and apoptosis analysis

Cell proliferation tests were performed by using the WST1 assay (Roche) and apoptosis tests were performed by using the Caspase-Glo 3/7 assay (Promega) according to the manufacturer's recommendations. Cells were cultured in 96-well plates and treated with 0–150μM *o,p*'-DDD for 24 or 48 hours. Optical densities were measured 4 hours after addition of WST1 solution (10 μL per well) by spectrophotometry (Viktor, PerkinElmer). Luminescence was measured 1 hour after addition of Caspase-Glo 3/7 solution (equal volume) by luminometry (Viktor, PerkinElmer).

RT-PCR and real-time qPCR

Total RNAs were extracted from cells with the RNeasy kit (QIAGEN) according to the manufacturer's recommendations. RNA was thereafter processed for RT-PCR as previously described (25). real time -qPCR was performed using the Fast SYBR Green Master Mix (Life Technologies) and carried out on a StepOnePlus Real-Time PCR System (Life Technologies) as previously described (25). The relative expression of each gene was expressed as the ratio of attomoles of specific gene to femtomoles of 18S rRNA.

Western blot analysis

Total protein extracts were prepared as previously described (22). Western blot analyses were performed as described (22). Antibodies used were a rabbit anti-BCL2 antibody (1:500 dilution, Cell Signaling) with a mouse anti-α-tubulin antibody (1: 10,000 dilution, Sigma-Aldrich) or a rabbit anti-CYP11A1 antibody (1:500 dilution, Sigma-Aldrich) with a mouse anti-GAPDH antibody (1:10,000 dilution, Sigma-Aldrich). Proteins were visualized with an Odyssey-Fc apparatus (LI-COR).

Statistical analysis

Results are expressed as means ± SEM of n independent replicates performed in the same experiment or from separated experiments (n). Correlation was tested with a Pearson test. Nonparametric Mann-Whitney *U* tests were used when appropriate and differences between groups were analyzed using nonparametric Kruskall-Wallis multiple comparison test followed by a post test of Dunn's (Prism software, GraphPad). Difference between groups of patients was assessed using the Fisher's exact test. $P = .05$ was considered statistically significant (*, $P < .05$; **, $P < .01$; ***, $P < .001$; see figures).

Results

Distribution of *o,p'*-DDD and its metabolites among lipoproteins and LP-F subfractions in plasma samples of patients with ACC

The distribution of mitotane and its two major metabolites, *o,p*'-DDE and *o,p*'-DDA, in lipoproteins was evaluated in 20 patients with ACC. *o,p*'-DDD, *o,p*'-DDA, and *o,p*'-DDE were thus measured with HPLC-UV after ultracentrifugation of plasma samples in very low-density lipoprotein (VLDL), LDL, HDL, and LP-F subfractions. *o,p*'-DDD was widely distributed among lipoprotein fractions as follows : 34.6 ± 9.9% LP-F (including protein-bound and -free mitotane), 26.3 ± 5.8% HDL, 26 ± 4.6% LDL, and 13 ± 4.2% VLDL (Figure 1A). The distribution of *o,p*'-DDE among these subfractions favoring LP-F was as follows (72.9 ± 15.3% LP-F, 17.7 ± 12.2% HDL, 8.2 ± 1 0.5% LDL, and 1.3 ± 2.7% VLDL) whereas, in sharp contrast, *o,p*'-DDA was almost exclusively recovered in LP-F fractions (94.6 ± 3.1%; Supplemental Figure 1, A and B). No significant difference in *o,p*'-DDD, *o,p*'-DDA, or *o,p*'-DDE distribution was observed according to the presence and the degree of dyslipidemia or the plasma mitotane level (data not shown). Of interest, plasma mitotane levels correlated with *o,p*'-DDD measured in LP-F fractions (Figure 1B; $r^2 = 0.41$; $P < .001$) but also in those of HDL and LDL (Figure 1C; $r^2 = 0.76$; $P < .001$).

Intratumor *o,p'*-DDD concentrations and cholesterol transporters expression

To further explore which LP subfraction might account for mitotane uptake in human ACC tissue, we measured the relative expression of genes encoding for scavenger B1 receptor (SrB1; HDL receptor) and LDL-R in six samples of human adrenal tissue collected from one ectopic Cushing's disease and five mitotane-treated patients with ACC (Table 1). On a case-by-case analysis, no association was found between *SrB1* or *LDL-R* expression and intratumor *o,p*'-DDD concentrations.

Influence of lipoprotein-binding on mitotane efficiency in vitro

Effect on cell proliferation and apoptosis

To evaluate the influence of lipoprotein binding on mitotane efficacy in terms of cell proliferation and apoptosis, H295R cells were incubated in different culture conditions containing either HDL, LDL, or LP-F and compared with control (FCS). Cell proliferation index was measured

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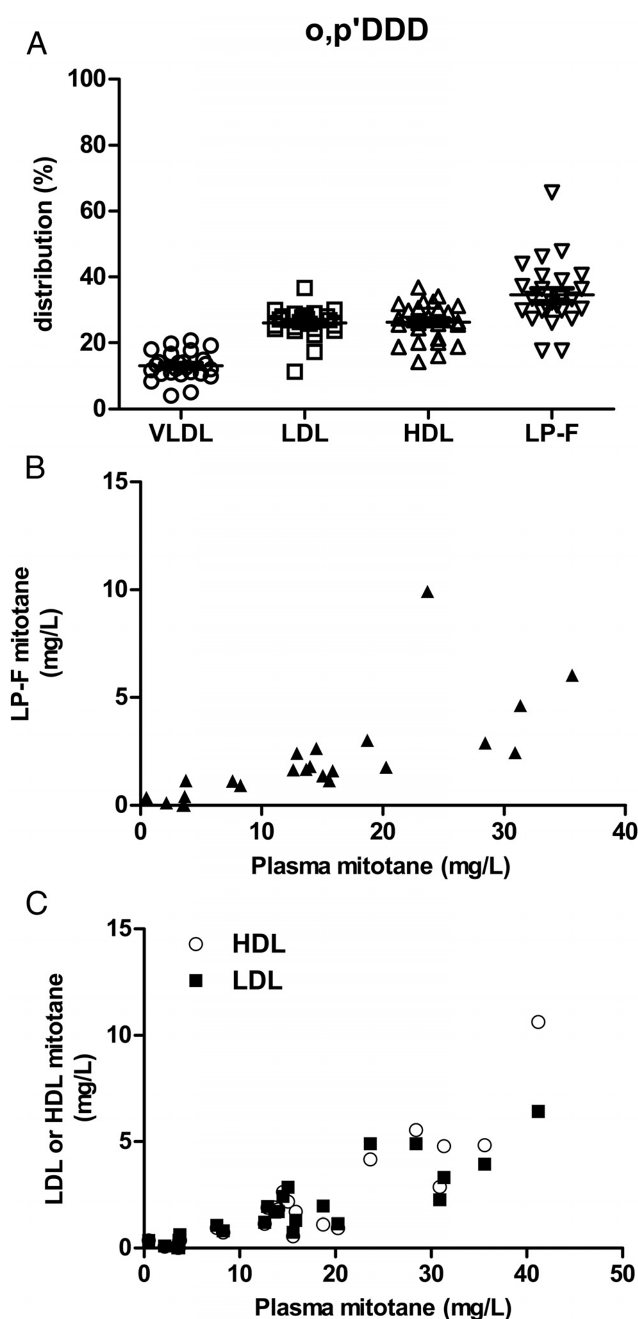

Figure 1. Distribution of *o,p'*-DDD in lipoproteins (VLDL, LDL, and HDL) and in the LP-F subfraction obtained from ultracentrifugation of 23 plasma samples of 20 ACC patients (A) *o,p'*-DDD measured by UV-HPLC, is expressed as percentage of the sum of *o,p'*-DDD measurements in each subfraction. Correlation between plasma mitotane level and *o,p'*-DDD measured in LP-F (B) or HDL and LDL (C), all levels are expressed in mg/L. Plasma mitotane levels significantly correlate with LP-F mitotane ($P < .001$; $r^2 = 0.41$), with HDL mitotane ($P < .001$; $r^2 = 0.76$), and with LDL mitotane ($P < .001$; $r^2 = 0.76$, Pearson test).

at baseline and after incubation with various mitotane concentrations for 48 hours. Basal cell proliferation was not different between these conditions (Supplemental Figure 2A). Mitotane exerts a dose-dependent antiproliferative effect in all conditions but was more efficient when cells were cultured in LP-F medium with an IC_{50} of approximately 40μM compared with 140μM under control conditions (Figure 2A) with a leftshift of dose-dependent curve. Apoptosis index as measured by caspase 3/7 assays was significantly higher after a 24-hour treatment with 100μM mitotane in LP-F condition compared with LDL, HDL, or control conditions (Figure 2B). Furthermore, expression of the antiapoptotic protein BCL2 was reduced by 100μM mitotane in LP-F condition but not in others (Supplemental Figure 2B). Altogether, our results clearly suggest that the cytotoxic effects of mitotane are more pronounced in the absence of lipoproteins.

Intracellular uptake of mitotane and mitochondrial effect

To examine whether the nature of LP fractions affects mitotane uptake, *o,p*'-DDD concentrations were measured in cell pellets after 48 hours of 50μM exposure in different conditions. Intracellular *o,p*'-DDD concentrations, measured by the sensitive GCMS technique and normalized to nuclear DNA were at least 3-fold higher in cells cultured in LP-F medium than in other media (Figure 3A). The intracellular mitotane was mostly recovered in the mitochondrial fraction (89.2 ± 3.6% of *o,p*'-DDD) whereas only 10.8 ± 3.6% of the measured intracellular *o,p*'-DDD was in the cytosolic fraction (data not shown). Intramitochondrial *o,p*'-DDD concentration was 15-fold greater (90.26 ± 7.66 ng/atomol of mtDNA) in cells grown in LP-F condition compared with other culture conditions, highly suggestive of a better cellular and thus mitochondrial uptake of mitotane in the absence of lipoproteins (Figure 3B). We next studied the expression of genes encoding proteins involved in oxidative phosphorylation or steroidogenesis by RT-qPCR in *o,p*'-DDD-treated cells under HDL, LDL, LP-F, and control conditions. In LP-F medium, mitotane strongly inhibited *COX2* expression (encoded by the mitochondrial mtDNA for the subunit 2 of cytochrome *c* oxidase or respiratory chain complex IV; Figure 3C) but also *StAR* and *CYP11A1* involved in steroidogenesis (Supplemental Figure 3, A and B). LP-F condition also led to a drastic reduction in CYP11A1 protein expression in H295R cells after a 48-hour treatment with 50μM mitotane (Supplemental Figure 3C). Collectively, our findings provide additional evidence that LP-F mitotane was the most efficient leading to alter cellular functions.

Effect of BLT1 treatment and cholesterol saturation

BLT1 is a powerful but not fully specific inhibitor of SrB1 and thus a pharmacological inhibitor of cellular lipoprotein uptake. SrB1 also participates to cellular efflux

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Table 1. *SrB1* and *LDL-R* Gene Expression, Plasma, and Tissue Concentrations of *o,p'*-DDD in Human Adrenal Tissue Samples of mitotane-Treated Patients

Patient, No.	Clinical Presentation	SrB1 (mRNA)	LDL-R (mRNA)	*o,p'*-DDD Tissue	Plasma Mitotane Level, mg/L
1	EC	33.86	1.46	53.75	3.3[a]
2	ACC	2.62	0.03	2.47	20.03[b]
3	ACC	0.33	0.02	0.66	18.5[b]
4	ACC	3.61	0.18	1.48	23.7[b]
5	ACC	20.42	1.31	2.01	14.7[b]
6	ACC	4.50	0.21	12.81	4[a]

Informed consent was obtained for each patient and LDL-R expression in adrenal tissue samples was measured by RT-qPCR after DNA extraction and are expressed as attomol per attomol of GAPDH as described in Patients, Materials, and Methods. *o,p'*-DDD concentration was measured in tissue homogenates and plasma using HPLC-UV analysis and are expressed in nmol/mg of tissue or in mg/L, respectively. EC, Ectopic Cushing's syndrome.

[a] Plasma mitotane level measured 2 weeks before surgery.

[b] Mean, 4–6 plasma mitotane levels assessed during treatment.

of lipophilic molecules such as cholesterol or vitamin E and very likely mitotane. *o,p*'-DDD displays a more potent antiproliferative activity on H295R cells with a left-shift dose-dependent in the presence of BLT1 and a proliferation index at 7.5 ± 3.8% in BLT1-treated cells compared with 69.5 ± 4.3% in control cells ($P < .01$) at 100μM mitotane concentration for 48 hours. Likewise, mitotane-treated cells exhibited a higher apoptotic capacity in the presence of BLT1, as revealed by the reduced expression of the antiapoptotic protein BCL2 (Figure 4B). This was accompanied by an significant increase in intracellular *o,p*'-DDD concentration when lipoproteins receptors were inhibited with BLT1 (Figure 4C). Free cholesterol was added into the culture medium to saturate lipoproteins in an attempt to reduce lipoprotein-bound mitotane through cholesterol exchange, thus potentially enhancing LP-F mitotane bioavailability. Cholesterol supplementation in the medium dose-dependently enhanced mitotane cytotoxic effects, confirming the key role of LP-F mitotane in vitro (Figure 4D).

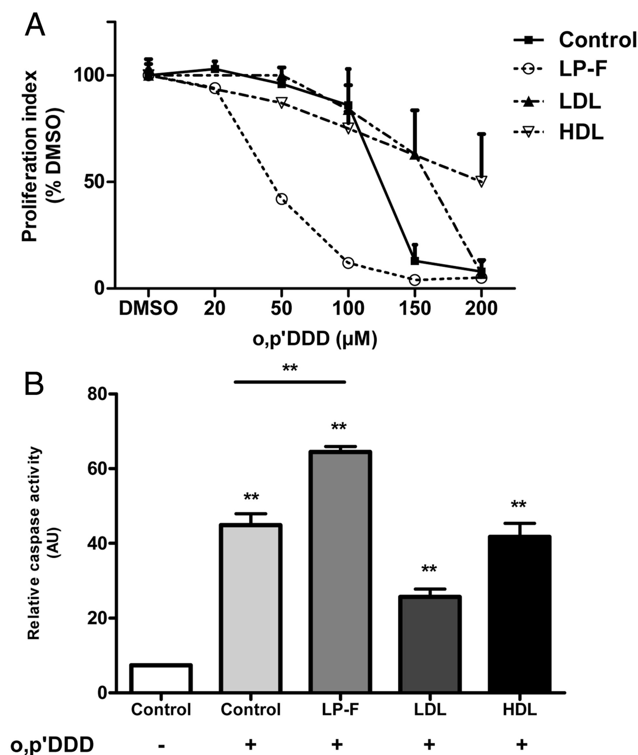

Figure 2. A, Dose-dependent effect of increasing concentrations of *o,p'*-DDD (0–200μM) on the proliferation index of H295R cells after 48 h culture in control, LP-F, LDL, or HDL conditions as determined by WST1 assays. Results are expressed as mean percentage ± SEM of six to 24 independent determinations, values of vehicle-treated cells being arbitrarily set at 100%. B, Relative caspase activity of H295R cells cultured in control, LP-F, LDL, and HDL conditions before and after 24-h treatment with 100μM *o,p'*-DDD as determined by Caspase-Glo 3/7 assays. Results are expressed as mean arbitrary units ± SEM of six to 24 independent determinations. **, $P < .01$, Mann-Whitney *U* test.

Efficiency of unbound mitotane in vitro

To examine the relative contribution of free and protein-bound mitotane on H295R cell proliferation or apoptosis, the effect of mitotane was compared in FCS-free medium, devoid of protein and lipoproteins, supplemented with increasing concentrations of BSA and in LP-F media. Consistently, *o,p*'-DDD was more efficient in inhibiting cell proliferation in protein-free medium whereas BSA supplementation dose-dependently impaired mitotane efficiency (Figure 4E). Moreover, relative caspase activity was 4-fold higher in mitotane-treated cells when incubated in the absence of protein than in the control medium (Figure 4F). Altogether, our results demonstrate that the most potent activity in vitro was achieved with free mitotane.

Statin therapy and disease control rate in patients with stage IV ACC

To translate these observations into the clinic, we retrospectively collected data from patients with ACC followed at our institution. Patients with stage IV ACC receiving mitotane therapy initiated between September

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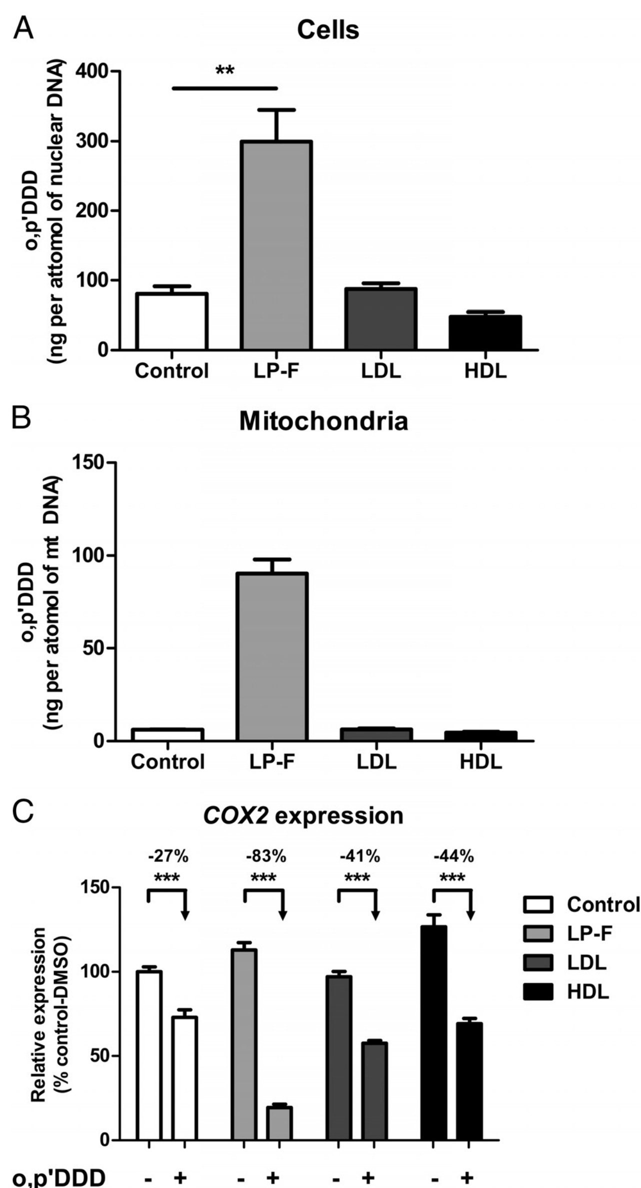

Figure 3. *o,p*′-DDD concentrations as measured by GCMS in H295R cell pellets (A) and mitochondrial pellets (B) after 48-h exposure to 50μM mitotane in control, LP-F, LDL, and HDL conditions. Results are expressed as mean percentage ± SEM of three to six independent determinations, values of control-cultured cells being arbitrarily set at 100%. **, $P < .01$, Mann-Whitney *U* test. C, Relative mRNA expression of *COX2* determined by RT-qPCR. H295R cells were treated with 50μM *o,p*′-DDD for 48 h. Results are expressed as mean percentage ± SEM of six different experiments performed in duplicate, values of vehicle-treated cells being arbitrarily set at 100%. ***, $P < .001$, Mann-Mann-Whitney *U* test.

2007 and January 2014 were included. Twenty-six patients had a mean plasma mitotane level of 16.7 ± 9.2 mg/L (13.2 ± 9.4 mg/L at 1 mo, 17.8 ± 12.2 mg/L at 3 mo, and 18.4 ± 8.5 mg/L at 6 mo). Among them, 16 (61.5%) experienced hypertriglyceridemia, 15 (57.7%) an increase in HDL cholesterol, or wight (30.8%) an increase in LDL cholesterol level under mitotane. Eleven patients (42.3%) were treated with statins (introduced at least 3 mo before RECIST evaluation). Neurologic toxicity was reported in eight of 26 patients (30.8%) at 1, 3, and/or 6 months. According to RECIST criteria, disease control rate (DCR) at 6 months was 46.2% (12 of 26 patients) including four patients who experienced a partial response. A significant positive association was found between the use of statin therapy and DCR at 6 months: 67% or 33% DCR was observed in patients treated or not with statins ($P < .05$, Fisher's exact test; Figure 5). No association was found between dyslipidemia and neurologic toxicity or tumor control or statins therapy and neurologic toxicity.

Discussion

Given that mitotane remains the most effective treatment of advanced ACC, many efforts are made to better understand its mechanism of action (2). Its lipophilic properties lead to a distribution of *o,p*'-DDD in lipoproteins (13) and a storage in adipose tissue (29). Given that mitotane content in lipoprotein fractions has been assumed to play a role in drug distribution in tissues (30), we therefore evaluated the potential role in its antitumor activity using different materials: Plasma samples of patients with ACC, human adrenal cortex tissues, and human adrenocortical H295R cells.

The partitioning of mitotane and its metabolites in lipoproteins was found to differ strongly. Indeed, whereas *o,p*'-DDD and *o,p*'-DDE were equally distributed among different fractions (VLDL, LDL, HDL, and LP-F subfractions), *o,p*'-DDA was entirely recovered with protein fractions, consistent with its hydrophilic properties. In our 23 plasma samples originating from 20 patients with ACC, we found a correlation between plasma *o,p*'-DDD levels and its corresponding lipoprotein contents but more importantly between circulating mitotane concentrations and its distribution in LP-F subfraction. These findings raise the question of the relative contribution of mitotane-free vs -bound lipoprotein fractions in the pharmacological action of *o,p*'-DDD (30). To further explore this question, expression of genes encoding for lipoprotein receptors (SrB1 and LDL receptor) were studied and compared with intratissue *o,p*'-DDD concentrations of mitotane-treated patients' adrenals. In this small number of tissue samples owing to the low incidence of ACC, no relationship was observed between adrenal cortex *o,p*'-DDD content and lipoprotein receptor expression, suggesting no predominant effect of mitotane-bound lipoproteins on tissue mitotane uptake.

We then explored the role of mitotane binding to each lipoprotein subfraction or LP-F mitotane on its cytotoxic

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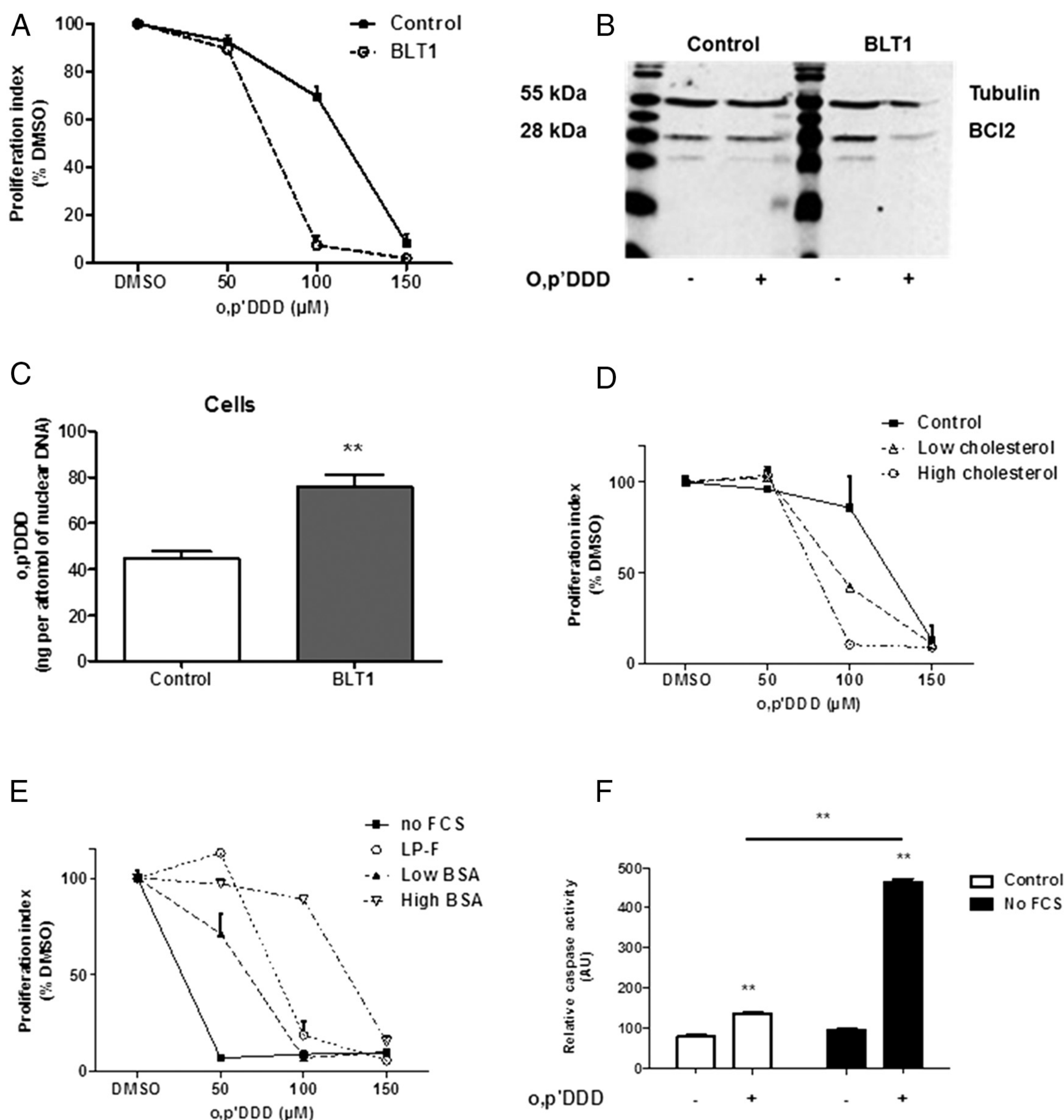

Figure 4. A, Dose-dependent effect of increasing concentrations of *o,p'*-DDD (0–200μM) on the proliferation index of H295R cells after 48 h culture in the absence or presence of 5μM BLT1 as determined by WST1 assays. Results are expressed as mean percentage ± SEM of six independent experiments performed in duplicate, values of vehicle-treated cells being arbitrarily set at 100%. B, Steady-state levels of BCL2 protein (antiapoptotic factor) by Western blot with anti-BCL2 and antitubulin antibodies. H295R cells were cultured in the absence or presence of 5μM BLT1 and treated with 50μM *o,p'*-DDD for 48 h. C, *o,p'*-DDD concentrations as measured by GCMS in H295R cell pellets after 48 h exposure to 50μM mitotane in the absence or presence of 5μM BLT1. Results are expressed as mean percentage ± SEM of six independent determinations, values of control-cultured cells being arbitrarily set at 100%. **, $P < .01$, Mann-Whitney *U* test. D, Dose-dependent effect of increasing concentrations of *o,p'*-DDD (0–200μM) on the proliferation index of H295R cells after 48 h culture without or with 96 mg/L (low) or 384 mg/L (high) cholesterol as determined by WST1 assays. Results are expressed as mean percentage ± SEM of six independent experiments performed in duplicate, values of vehicle-treated cells being arbitrarily set at 100%. E, Dose-dependent effect of increasing concentrations of *o,p'*-DDD (0–200μM) on the proliferation index of H295R cells after 48 h culture in control or LP-F conditions or with 1.8 mg/L (low) or 3.6 mg/L (high) BSA as determined by WST1 assays. Results are expressed as mean percentage ± SEM of six independent experiments performed in duplicate, values of vehicle-treated cells being arbitrarily set at 100%. F, Relative caspase activity of H295R cells cultured in the presence or absence of FCS before and after 24-h treatment with 100μM of *o,p'*-DDD as determined by Caspase-Glo 3/7 assays. Results are expressed as mean arbitrary units ± SEM of six independent determinations. **, $P < .01$, Mann-Whitney *U* test.

effect in human adrenocortical H295R cells. We provided evidence that mitotane exerts a more efficient antiproliferative and proapoptotic action when cells are grown in LP-F medium, suggesting that lipoprotein-bound mitotane is not the most potent pharmacological vehicle.

In the present study, we also confirm that mitochondrion is a critical target of mitotane action given that most intracellular mitotane was recovered within the mitochondrial compartment, consistent with our previous results (25). Herein, we further demonstrated that LP-F mitotane

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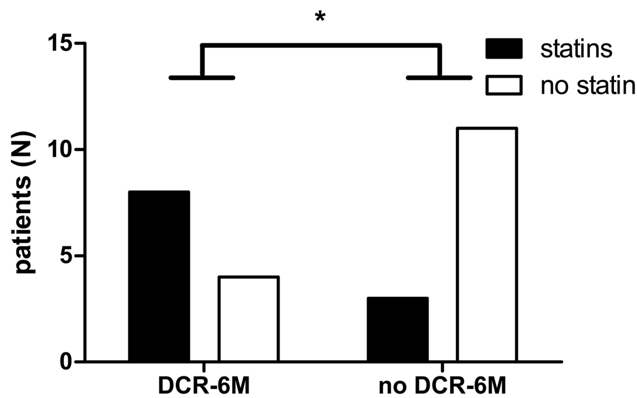

Figure 5. Association between statin therapy and disease control rate at 6 mo (DCR-6M) in a cohort of 26 ACC patients under mitotane. Disease control rate is defined by partial response or stable disease according to RECIST criteria. *, $P < .05$, Fisher exact test.

was more effectively captured by mitochondria and more efficient in inhibiting the respiratory chain activity. Experiments using BLT1, an SrB1 receptor inhibitor, or cholesterol saturation, that both favor mitotane action through its LP-F fraction, comfort the prominent pharmacological role of free mitotane by showing an increased efficiency of mitotane when added to the culture medium. These results do not exclude that SrB1 could be involved in mitotane efflux. Van Slooten et al (12) first suggested that albumin-bound mitotane might be responsible for at least the neurologic adverse effects observed in patients. We further evaluated the effect of albumin on *o,p*'-DDD toxicity in H295R cells and unambiguously demonstrated that free mitotane exerts the most efficient pharmacological properties.

Altogether, our results suggest that free mitotane induces the most potent cytotoxic effects, questioning the precise molecular mechanism of its transmembrane transport and yet excluding that intracellular transport of lipoproteins might play a major role in the adrenal specificity of mitotane action.

In patients with ACC, a variable delay of several weeks between mitotane initiation and antitumorigenic effect is well described in the literature (2). Consistent with our results demonstrating that free mitotane might constitute the active form of the drug, this delay may correspond with the time required to fully saturate circulating lipoproteins in patients' plasma. To further explore the implication of our in vitro results in humans, and based on clinical and biochemical data collected from a cohort of mitotane-treated patients with ACC, we examined mitotane plasma levels, lipid profile, neurologic toxicity, and clinical responses according to RECIST at 1, 3, and 6 months after mitotane initiation. Among the 26 patients included, 20 (76.9%) had dyslipidemia under mitotane therapy, including isolated hypertriglyceridemia, hypercholesterolemia (HDL and/or LDL) or both, in accordance with previous reports (16–19). More interestingly, we found that patients who received statins (42.3%) presented with a better tumor control including stable disease and partial responses. We hypothesize that statins, through a reduction of plasma lipoproteins levels, could lead to an increased free mitotane ratio. Despite the hypothesis of van Slooten et al (12), who suggested that albumin-bound mitotane could be responsible for neurologic toxicity, we did not find any association between LDL, HDL, or statins and neurologic adverse effects. Our study has clear limitations including the small sample size, the heterogeneity in patients' followup, and plasma collections and the uncontrolled nature of the design inherent to a the retrospective data collection. These preliminary data should be further confirmed and the question on whether patients could benefit from statins is presently being addressed through an ongoing prospective study (MITOLIPO study).

In summary, we provide strong evidence that *o,p*'-DDD unbound to lipoprotein fractions is more efficient in vitro and that patients could benefit from strategies that aim to increase LP-F mitotane fraction. The potential role of dyslipidemia and statin therapy on mitotane effects will be further explored in a prospective study.

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References

1. **Berruti A, Baudin E, Gelderblom H, Haak HR, Porpiglia F, Fassnacht M, Pentheroudakis G.** Adrenal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol.* 2012;23 (Suppl 7):vii131–vii1138.
2. **Baudin E.** Adrenocortical Carcinoma. *Endocrinol Metab Clin North Am* 2015;44(2):411–434.
3. **Fassnacht M, Terzolo M, Allolio B, et al.** Combination chemotherapy in advanced adrenocortical carcinoma. *N Engl J Med.* 2012; 366(23):2189–2197.
4. **Berruti A, Fassnacht M, Baudin E, et al.** Adjuvant therapy in patients with adrenocortical carcinoma: A position of an international panel. *J Clin Oncol.* 2010;28(23):e401–e402; author reply, e403.
5. **Else T, Williams AR, Sabolch A, Jolly S, Miller BS, Hammer GD.**

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Adjuvant therapies and patient and tumor characteristics associated with survival of adult patients with adrenocortical carcinoma. *J Clin Endocrinol Metab*. 2014;99(2):455–461.
6. **Terzolo M, Angeli A, Fassnacht M, et al.** Adjuvant mitotane treatment for adrenocortical carcinoma. *N Engl J Med*. 2007;356(23):2372–2380.
7. **Haak HR, Hermans J, van de Velde CJ, et al.** Optimal treatment of adrenocortical carcinoma with mitotane: Results in a consecutive series of 96 patients. *Br J Cancer*. 1994;69(5):947–951.
8. **Baudin E, Pellegriti G, Bonnay M, et al.** Impact of monitoring plasma 1,1-dichlorodiphenildichloroethane (*o,p*'DDD) levels on the treatment of patients with adrenocortical carcinoma. *Cancer*. 2001;92(6):1385–1392.
9. **Malandrino P, Al Ghuzlan A, Castaing M, et al.** Prognostic markers of survival after combined mitotane- and platinum-based chemotherapy in metastatic adrenocortical carcinoma (ACC). *Endocr Relat Cancer*. 2010;17(3):797–807.
10. **Wängberg B, Khorram-Manesh A, Jansson S, et al.** The long-term survival in adrenocortical carcinoma with active surgical management and use of monitored mitotane. *Endocr Relat Cancer*. 2010;17(1):265–272.
11. **Hermsen IG, Fassnacht M, Terzolo M, et al.** Plasma concentrations of *o,p*'DDD, *o,p*'DDA, and *o,p*'DDE as predictors of tumor response to mitotane in adrenocortical carcinoma: Results of a retrospective ENS@T multicenter study. *J Clin Endocrinol Metab*. 2011;96(6):1844–1851.
12. **van Slooten H, Moolenaar AJ, van Seters AP, Smeenk D.** The treatment of adrenocortical carcinoma with *o,p*'-DDD: Prognostic implications of serum level monitoring. *Eur J Cancer Clin Oncol*. 1984;20(1):47–53.
13. **Gebhardt DO, Moolenaar AJ, van Seters AP, van der Velde EA, Gevers Leuven JA.** The distribution of *o,p*'-DDD (mitotane) among serum lipoproteins in normo- and hypertriglyceridemia. *Cancer Chemother Pharmacol*. 1992;29(4):331–334.
14. **Maher VM, Trainer PJ, Scoppola A, Anderson JV, Thompson GR, Besser GM.** Possible mechanism and treatment of *o,p*'DDD-induced hypercholesterolaemia. *Q J Med*. 1992;84(305):671–679.
15. **Gebhardt DO, Moolenaar AJ, van Seters AP.** Mitotane (*o,p*'-DDD) administration raises the serum level of high density lipoprotein (HDL) in normotriglyceridemia. *Horm Metab. Res*. 1993;25(4):240–241.
16. **Terzolo M, Pia A, Berruti A, et al.** Low-dose monitored mitotane treatment achieves the therapeutic range with manageable side effects in patients with adrenocortical cancer. *J Clin Endocrinol Metab*. 2000;85(6):2234–2238.
17. **Allolio B, Hahner S, Weismann D, Fassnacht M.** Management of adrenocortical carcinoma. *Clin Endocrinol (Oxf)*. 2004;60(3):273–287.
18. **Daffara F, De Francia S, Reimondo G, et al.** Prospective evaluation of mitotane toxicity in adrenocortical cancer patients treated adjuvantly. *Endocr Relat Cancer*. 2008;15(4):1043–1053.
19. **Shawa H, Deniz F, Bazerbashi H, et al.** Mitotane-induced hyperlipidemia: A retrospective cohort study. *Int J Endocrinol*. 2013;2013:624962.
20. **Stacpoole PW, Varnado CE, Island DP.** Stimulation of rat liver 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity by *o,p*'-DDD. *Biochem Pharmacol*. 1982;31(5):857–860.
21. **Moy RH.** Studies of the pharmacology of *o,p*'DDD in man. *J Lab Clin Med*. 1961;58:296–304.
22. **Hescot S, Paci A, Seck A, et al.** The lack of antitumor effects of *o,p*'DDA excludes its role as an active metabolite of mitotane for adrenocortical carcinoma treatment. *Horm Cancer*. 2014;5(5):312–323.
23. **Kaminsky N, Luse S, Hartroft P.** Ultrastructure of adrenal cortex of the dog during treatment with DDD. *J Natl Cancer Inst*. 1962;29:127–159.
24. **Poli G, Guasti D, Rapizzi E, et al.** Morpho-functional effects of mitotane on mitochondria in human adrenocortical cancer cells. *Endocr Relat Cancer*. 2013;20(4):537–550.
25. **Hescot S, Slama A, Lombès A, et al.** Mitotane alters mitochondrial respiratory chain activity by inducing cytochrome *c* oxidase defect in human adrenocortical cells. *Endocr Relat Cancer*. 2013;20(3):371–381.
26. **Eisenhauer EA, Therasse P, Bogaerts J, et al.** New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur J Cancer*. 2009;45(2):228–247.
27. **Chretien D, Bénit P, Chol M, et al.** Assay of mitochondrial respiratory chain complex I in human lymphocytes and cultured skin fibroblasts. *Biochem Biophys Res Commun*. 2003;301(1):222–224.
28. **Guérin M, Bruckert E, Dolphin PJ, Turpin G, Chapman MJ.** Fenofibrate reduces plasma cholesteryl ester transfer from HDL to VLDL and normalizes the atherogenic, dense LDL profile in combined hyperlipidemia. *Arterioscler Thromb Vasc Biol*. 1996;16(6):763–772.
29. **Cueto C, Brown JH, Richardson AP.** Biological studies on an adrenocorticolytic agent and the isolation of the active components. *Endocrinology*. 1958;62(3):334–339.
30. **Pohland RC, Counsell RE.** The role of high density lipoproteins in the biodistribution of two radioiodinated probes in the rat. *Toxicol Appl Pharmacol*. 1985;77(1):47–57.

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