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Association of mitotane with chylomicrons and serum lipoproteins: practical implications for treatment of adrenocortical carcinoma

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Introduction

Adrenocortical carcinoma (ACC) is a rare tumor with dismal prognosis (see (1, 2) for recent reviews). Mitotane (1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane, *o,p'*-DDD) has been clinically used for treatment of ACC for decades (3) either as monotherapy or in

combination with cytotoxic chemotherapies (4, 5). Only recently our laboratory identified sterol O-acyltransferase 1 (SOAT1) as a key target of mitotane. We have shown that SOAT1 inhibition by mitotane leads to intracellular accumulation of toxic lipids which results in activation

of the endoplasmic reticulum (ER) stress response which in turn results in reduced steroidogenesis and apoptosis (6) of ACC cells.

Mitotane treatment is hampered by the requirement of therapeutic drug monitoring and unfavorable pharmacokinetic properties (7, 8).

Several studies reported an association of mitotane serum concentrations above 14 mg/l with treatment response (9, 10, 11, 12, 13). Since serum concentrations >20 mg/l are associated with more frequent and more severe adverse effects (14), serum concentrations between 14 and 20 mg/l are aimed at (15, 16). However, even with high doses of mitotane for 3 months, concentrations >14 mg/l are achieved in only half of patients (17, 18) and are never reached in some patients. The reasons for this are unknown.

The long time interval required for reaching effective mitotane serum concentrations is directly related to the excessively long elimination half-life of mitotane which approaches up to 6 months (19). Alkyl oxidation of mitotane leads to formation of the inactive metabolite (20) *o,p'*-dichlorodiphenylacetic acid (*o,p'*-DDA) (20), which is present in blood in up to tenfold higher concentrations compared to the parent compound and is excreted in urine (21). In contrast, the second metabolite *o,p'*-dichlorodiphenyldichloroethane (*o,p'*-DDE) is much less abundant (22).

Mitotane has been previously reported to be associated with lipoproteins in serum (23, 24), and it has been suggested that mitotane binding to lipoprotein particles may contribute to the apparent tissue specificity of the adrenal cortex. Since adrenocortical cells require large amounts of cholesterol for steroidogenesis, it appears conceivable that lipoprotein binding might favor entry of mitotane into adrenocortical cells similar to a Trojan horse. Recently, lipoprotein association of mitotane has been studied in more detail (25), and lipoprotein-free mitotane reported to confer cytotoxicity in cell culture disproving this hypothesis. It is also known that mitotane treatment increases serum LDL cholesterol which has been attributed to increased hepatic cholesterol synthesis (26, 27).

Here we report a translational study starting with the analysis of mitotane in the chyle of a 29-year-old mitotane-treated ACC patient to gain insights into mitotane absorption in the intestine. Subsequently, we challenged data published previously by analyzing association of mitotane and metabolites with serum lipoproteins (25) in 14 additional patients. Cell culture assays were used to study the functional consequences of the association of mitotane with chylomicrons and serum lipoproteins.

Subjects and methods

Chemicals

Mitotane for *in vitro* treatment was from ISP Chemical Products (Columbus, OH, USA), *o,p'*-DDD, *o,p'*-DDE and *p,p'*-DDE for HPLC were from Sigma (PESTANAL), and all buffers and solvents from Merck unless otherwise stated.

Iodixanol was supplied as 60% w/v solution (Opti-Prep) by Axis-Shield PLC (Dundee, UK). OptiSeal tubes (3.2 ml) were supplied by Beckman Coulter (Krefeld, Germany).

Triacylglycerol, cholesterol, LDL cholesterol, HDL cholesterol assays, calibrators, and serum lipid control levels I and II were purchased from Roche. All lipid measurements were performed on the Cobas C501 from Roche.

Synthesis of DDA

o,p'-DDA was synthesized as described in [Supplementary data](#), see section on [supplementary data](#) given at the end of this article.

HPLC

Stock solutions of *o,p'*-DDD (250 µg/ml), *o,p'*-DDA (500 µg/ml), *o,p'*-DDE (50 µg/ml), and *p,p'*-DDE (250 µg/ml, internal standard) respectively were prepared in acetonitrile (AcN). Working solutions of *o,p'*-DDD (25 µg/ml), *o,p'*-DDA (50 µg/ml), and *o,p'*-DDE (5 µg/ml) were prepared in AcN and stored at -20 °C. Seven different calibration samples were prepared by accurately spiking 250 µl of 5% BSA (in H₂O) with stock and working solutions to realize calibration ranges of *o,p'*-DDD 0.5–40 µg/ml, *o,p'*-DDA 1.25–100 µg/ml, and of *o,p'*-DDE 0.25–20 µg/ml. A volume of 25 µl *p,p'*-DDE was added to 250 µl aliquots of calibration samples or serum and iodixanol gradient fractions, mixed with 250 µl of 0.1N sodium acetate pH 4.6. In total, 5 µl ascites were used to minimize matrix interferences. Analyte extraction was performed by adding twice 3 ml each of tert-butyl methyl ether for 5 min, followed by centrifugation at 5000 g for 5 min. The organic layer was transferred into glass tubes and evaporated to dryness (40 °C) under a gentle stream of nitrogen. The residue was reconstituted with 125 µl of AcN and diluted with 125 µl H₂O–0.1% trifluoroacetic acid (TFA) adjusted to pH 3.0 with triethylamine (TEA). A volume of 10 µl of this solution was injected into the HPLC system.

Quantitative analysis was performed on a Hitachi LaChrom Ultra HPLC system (VWR, Darmstadt, Germany) equipped with a L-2160 solvent pump, a L-2455U UV-VIS photodiode array detector, a L-2200 autosampler and a L-2350 column oven. Chromatographic separation of analytes was carried out on ASCENTIS Express C18 column (100×2.1 mM/2.7 μM; SUPELCO) protected by a C18 guard column (4×2 mM ID; Phenomenex, Aschaffenburg, Germany) using a linear gradient A/B 50%-0.5 min-90% B→90% B/3.5 min→50% B in 0.5 min→6.5 min re-equilibration. The mobile phase A consisted 0.1% TFA adjusted to pH 3.0 with TEA and AcN (70:30, v/v solvent A; 10:90, v/v solvent B). The flow rate was 0.2 ml/min, and the column was maintained at 40 °C. Detection was performed at 230 nm.

Retention times for *o,p'*-DDD, *o,p'*-DDA, *o,p'*-DDE, and *p,p'*-DDE (IS) were 8.5, 4.2, 9.9, and 10.8 min respectively (Fig. S1, see section on [supplementary data](#) given at the end of this article).

Patients

A total of 15 ACC patients treated with mitotane including the index case (ten with palliative intention and five as adjuvant treatment) were included in this study. The study was approved by the Ethics Committee of the University of Würzburg (Nos 93/02 and 88/11), and written informed consent was obtained from all patients. Anonymized serum samples from routine laboratory analyses of healthy individuals without mitotane treatment were used to prepare lipoprotein pools not containing mitotane.

Density gradient ultracentrifugation of lipoproteins

Serum samples of the 14 ACC patients were collected during mitotane treatment and stored at 4 °C until density gradient ultracentrifugation.

The procedure for the separation of lipoprotein subclasses using iodixanol originally described by Graham *et al.* (28, 29) was adapted as follows. In total, 1.0 ml serum, 1.24 ml Hepes buffer saline (HBS) buffer (10 mM HEPES; 0.8% NaCl; pH7.4), and 0.54 ml OptiPrep (60% iodixanol) were transferred into a Beckman OptiSeal tube (3.2 ml) and thoroughly mixed by gentle, repeated over-head rotation. Finally, the mixture was carefully overlaid with distilled water to fill the tube. The tubes were housed in a Beckman TLN100 rotor and centrifuged at 350 000 *g* for 0300 h at 15 °C with acceleration program 9 and deceleration program 9 in a Beckman Optima E-max benchtop ultracentrifuge.

Gradients were collected using a Beckman gradient unloader, which pierces the tube bottom, a Watson-Marlow 520S/R precision peristaltic pump, and a Gilson (Villiers, France) fraction collector. A total of 20 fractions, each 0.16 ml, were collected by continuously pumping the gradient from the bottom of the tube to the fraction collector numbering the fractions from top (fraction 1) to bottom (fraction 20). The fractions of the gradient were pooled to obtain the following lipoprotein fractions: VLDL (fractions 1–2), IDL (fractions 3–4), LDL1 (fractions 5–8), LDL2 (fractions 9–11), LDL3 (fractions 12–13), HDL (fractions 14–17), and lipoprotein-depleted serum (fractions 18–20). In each of the lipoprotein fractions triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol were measured on the Cobas c501 (Roche) with the reagent kits from Roche. To isolate chylomicrons, ascites was mixed with HBS and centrifuged for 10 min at 100 000 *g*.

As controls, 14 anonymous samples from individuals without mitotane treatment were fractionated in the same manner and pooled as described.

Cell culture

The adherent variant of the ACC cell line NCI-H295 (NCI-H295R) was cultured as described (30).

Cell-based assays

Viability testing using WST-1 reagent was performed according to the manufacturer's protocol (Roche). To assess the efficacy of mitotane in chyle, NCI-H295 cells have been incubated with medium containing increasing concentrations of mitotane dissolved in ethanol or the equivalent quantity of mitotane from chyle from the index patient. Quantification of mitotane in chyle was performed by HPLC as described above. To assess the impact of lipoproteins on mitotane efficacy, equal quantities of lipids (cholesterol+triglycerides) from the VLDL, LDL2, and HDL2 fractions of pooled control serum samples were added to serum-free cell culture medium, and either diluent, 25 or 50 μM mitotane was added. The viability of the control cells cultivated in serum-free medium and medium containing fetal calf serum was very similar (100±8.3 vs 91.34±4.4%). Cortisol was measured in the supernatant with an Immulite2000 analyzer (Siemens Healthcare Diagnostics, Erlangen, Germany). Quantitative real-time PCR was performed as described (6).

Statistical analysis

Patients were divided into high and low lipid groups according to the median of their total lipid (cholesterol + triglyceride) serum concentration. Statistical analyses were made using Prism 6.0 (GraphPad, La Jolla, CA, USA). Statistical differences between groups were calculated using either Fisher's *t*-test or ANOVA non-parametric test (Kruskal–Wallis) depending on the number of data sets. Correlation analyses were carried out using the non-parametric Spearman *r* correlation test. All error bars show s.d. Box plots indicate mean values and quartiles, and the whiskers represent the minimal and maximal values.

Results

Case description

A 29-year-old female ACC patient was admitted to the hospital for disease staging after chemotherapy with gemcitabine, capecitabine, and mitotane (Table 1, index case) (31). The patient complained about abdominal discomfort and abdominal swelling. An 18FDG–PET–CT revealed abundant ascites (Fig. 1A and B). Paracentesis was performed and 4.1 l opaque ascites were removed. Cytology showed abundant mast cells and lymphocytes without detection of tumor cells. Chemistry demonstrated 48 550 µg/ml triglycerides consistent with the clinical diagnosis of chylous ascites.

In the absence of detectable peritoneal carcinosis and gross liver metastases, we suspected misplaced pedicle screw implants to be causative of chylous ascites keeping in mind the close proximity of Th11 screws to central abdominal lymph vessels. Vertebra L1 had been replaced and spondylolysis of Th11–L3 performed due to ACC metastasis to L1 (Fig. 1A, B, C and D). The chylomicron fraction isolated by ultracentrifugation contained 179 840 µg/ml triglycerides and 54 400 µg/ml cholesterol (Fig. 1E).

Mitotane was discontinued, and the patient was placed on a fat-reduced diet with medium-chain fatty acid supplementation. This resulted in nearly complete remission of ascites until the next tumor evaluation 3 months thereafter.

Mitotane and its metabolites are abundant in chylomicrons

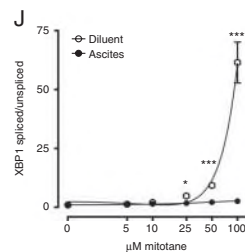
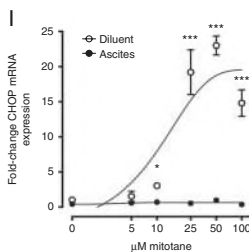
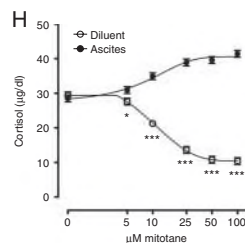
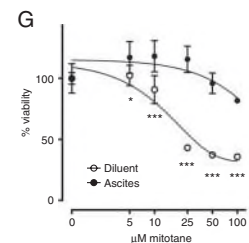
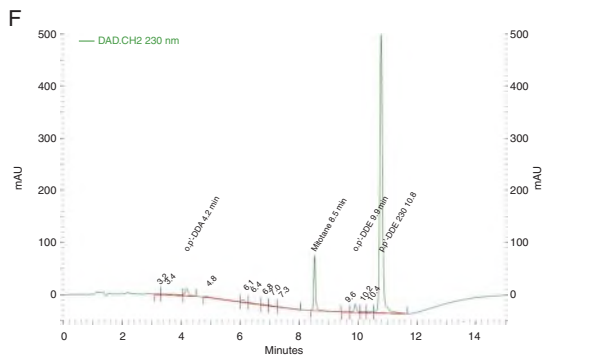
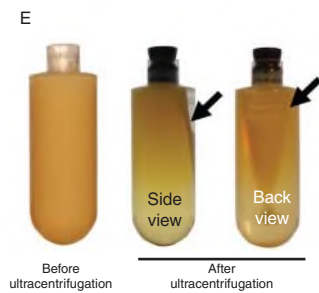
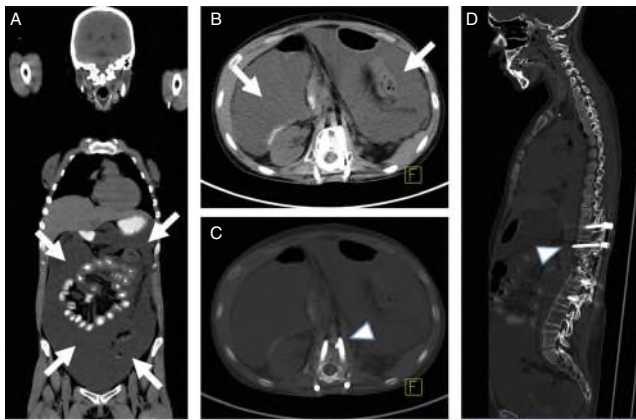
We employed HPLC to quantify mitotane and its metabolites *o,p'*-DDA and *o,p'*-DDE in chyle. Strikingly, we detected 197 mg/l mitotane, 53 mg/l *o,p'*-DDA, and 51 mg/l *o,p'*-DDE in the ascites specimen (Fig. 1F). At the time of paracentesis, the serum level of mitotane in this patient was 5.5 mg/l.

Mitotane bound to chylomicrons has reduced activity in NCI–H295 cells

To determine the biological activity of chylomicron-associated mitotane, we made use of the NCI–H295 ACC cell line. Equal amounts of chylomicron-bound mitotane

Table 1 Patient characteristics. The patient with chylous ascites is termed 'index case'.

PID	Sex	Age (years)	ENSAT stage	Treatment intention	Serum mitotane (mg/l)	Serum triglycerides (µg/ml)	Serum cholesterol (µg/ml)	Sum serum cholesterol + triglycerides (µg/ml)
Index case	F	29.1	4	Palliative	5.1	1230	820	2050
19	M	54.7	2	Adjuvant	12.1	1160	3493	4653
20	F	75.9	3	Adjuvant	20.1	1213	4197	5410
22	M	44.0	4	Palliative	16.6	802	3096	3898
23	F	37.1	4	Palliative	6.6	678	1862	2540
31	M	46.4	4	Palliative	7.7	891	3174	4065
32	M	52.7	2	Adjuvant	12.5	1618	1877	3495
34	F	50.4	4	Palliative	25.1	739	3003	3742
38	M	69.7	4	Palliative	8.1	2498	2040	4538
40	M	54.5	4	Palliative	14.3	2024	2310	4334
42	M	63.5	4	Palliative	20.2	3779	2755	6534
43	M	63.1	4	Palliative	17.0	743	1876	2619
46	M	57.3	2	Adjuvant	2.3	531	2512	3043
52	M	54.8	3	Adjuvant	3.4	1504	2959	4463
55	M	50.6	4	Palliative	5.2	994	1954	2948
Median						1160	2512	3898
Normal range						740–1720	1300–2200	



from the index patient and mitotane diluted in ethanol were added to cell culture medium, and cell viability was assessed by WST-1 assay. We found the EC₅₀ after 24 h to be 15.4 μ M for free mitotane and >100 μ M for chylomicron-bound mitotane (Fig. 1G). Accordingly, free mitotane but not chylomicron-associated mitotane led to inhibition of cortisol synthesis by more than fourfold (from 1.45- to 0.35-fold compared to baseline).

ER stress, the underlying molecular effector mechanism of both impaired steroidogenesis and apoptosis, was strongly activated by free mitotane. ER stress marker C/EBP homologous protein (CHOP) mRNA was induced 19.2 \pm 3.2-fold, 23.0 \pm 1.3-fold, and 14.8 \pm 1.9-fold at 25, 50, and 100 μ M respectively. At variance, chylomicron-associated mitotane did not induce any changes of CHOP mRNA expression (Fig. 1J). Similarly, activation of X-box binding protein (XBP1) mRNA splicing indicative of ER stress was observed with free mitotane (4.8 \pm 0.1-fold, 9.3 \pm 0.1-fold, and 61.5 \pm 8.7-fold with 25, 50, and 100 μ M

Figure 1

Analysis of the index patient. Coronal (A), axial (B, C), and sagittal (D) slices of CT displayed in both soft tissue (A, B) and bone (C, D) windows are given. The 29-year-old patient was referred for re-staging. Note important quantities of ascites in the abdomen (A, B; arrows). Misplaced pedicle screws in close proximity to the chyle cistern were the likely cause of the chylous ascites (C, D; arrowheads). No intravenous contrast agent was administered due to chronic kidney disease caused by nephrectomy and previous platinum-based chemotherapy (5). Ultracentrifugation (E) proved ascites to contain chylomicrons (arrows) which float on buffer. (F) HPLC of chyle provided evidence of *o,p'*-DDA (retention time 4.2 min), mitotane (8.5 min), and *o,p'*-DDE (9.9 min). *p,p'*-DDE (10.8 min) served as internal standard. Viability of NCI-H295 cells (G) was significantly less reduced when equal amounts of mitotane associated with chylomicrons (closed symbols) were used compared with mitotane dissolved in ethanol (open symbols). Cortisol secretion of NCI-H295 cells (H) exposed to equal amounts of mitotane associated with chylomicrons (closed symbols) was less reduced compared to mitotane dissolved in ethanol (open symbols). Expression of ER stress marker gene CHOP (I) and XBP1 mRNA splicing (J) was not increased in NCI-H295 cells exposed to mitotane associated with chylomicrons (closed symbols) but strongly increased in cells exposed to equal concentrations of mitotane in ethanol (open symbols). **P*<0.05 and ****P*<0.001.

mitotane respectively) but not with mitotane bound to chylomicrons ($P < 0.001$).

The majority of circulating mitotane is bound to lipoproteins

We next investigated the distribution of mitotane and its metabolites in serum lipoproteins in 14 ACC patients treated with mitotane (see Table 1 for patient characteristics) by subjecting serum samples to LipoDens density gradient ultracentrifugation. Patients were classified as high (> 12.3 mg/l) vs low mitotane (< 12.3 mg/l) based on the median serum value and no significant difference in total triglycerides (1180 ± 663 $\mu\text{g/ml}$ vs 1560 ± 1094 $\mu\text{g/ml}$; $P = 0.5$; Fig. 2A), and total cholesterol (2571 ± 650 $\mu\text{g/ml}$ vs 2731 ± 816 $\mu\text{g/ml}$; $P = 0.9$; Fig. 2B) was observed.

In the VLDL fraction, $21.7 \pm 21.4\%$ of total mitotane (Fig. 2C) was found, $1.9 \pm 0.8\%$ in IDL, and $8.9 \pm 5.5\%$ in LDL1, $18.9 \pm 9.6\%$ in LDL2, $10.1 \pm 4.0\%$ in LDL3, and $26.3 \pm 13.0\%$ in HDL2 lipoprotein fractions. Only $12.3 \pm 5.5\%$ were present in the lipoprotein-depleted fraction. In contrast *o,p'*-DDA (Fig. 2D) was mostly present in the HDL2 fraction ($21.9 \pm 6.9\%$) and in the lipoprotein-depleted fraction ($68.1 \pm 8.8\%$). *o,p'*-DDE (Fig. 2E)

distribution was similar to mitotane (VLDL: $16.4 \pm 14.3\%$; IDL: $2.1 \pm 1.6\%$; LDL1: $7.9 \pm 4.2\%$; LDL2: $14.2 \pm 5.5\%$; LDL3: $9.3 \pm 2.9\%$; HDL2: $21.2 \pm 8.6\%$; and lipoprotein-depleted: $28.7 \pm 6.7\%$). We next determined concentrations of mitotane and lipids in individual lipoprotein classes and examined the impact of lipid composition on mitotane binding to individual lipoproteins. Mitotane content of lipoproteins showed a positive correlation with both cholesterol ($r = 0.77$; 95% CI of regression curve = $0.67-0.84$; Fig. 2F) and triglyceride content ($r = 0.59$; 95% CI = $0.45-0.71$; Fig. 2G). Best correlation was obtained for the sum value of triglycerides and cholesterol ($r = 0.83$; 95% CI = $0.75-0.88$; Fig. 2H). Accordingly, relative abundance of mitotane in cholesterol and triglyceride-rich VLDL particles was higher in the group of patients with high total cholesterol and triglyceride serum concentrations ($32.0 \pm 25.0\%$ vs $11.4 \pm 11.1\%$; $P < 0.05$; Fig. 2C; low lipid group: blue symbols; high lipid group: red symbols).

A moderate correlation was found for total mitotane and lipoprotein free mitotane (Fig. 2I) ($r = 0.68$; 95% CI = $0.22-0.89$). Total serum lipids inversely correlated with lipoprotein free mitotane ($r = -0.33$; 95% CI = $-0.74-0.26$; Fig. 2J).

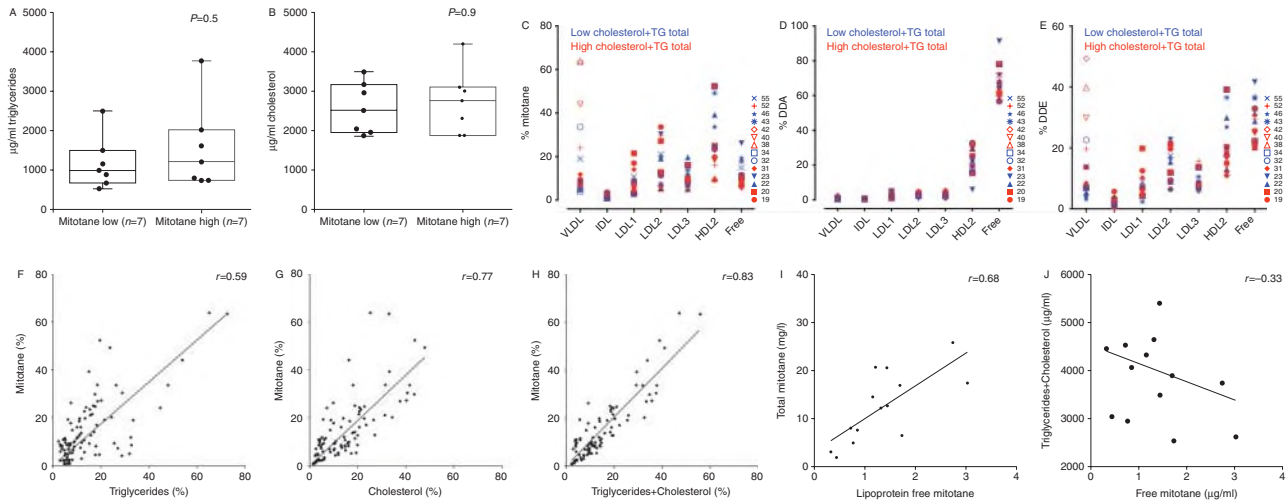


Figure 2

Lipoprotein association of mitotane and its metabolites. Serum triglycerides (A) and serum cholesterol (B) did not differ significantly between patients with low (left) and high (right) mitotane serum concentrations. Lipoprotein binding of mitotane (C), *o,p'*-DDA (D), *o,p'*-DDE (E) in patients with high serum lipids (triglycerides and cholesterol, red), and low serum lipids (blue) reveals differential association of mitotane and *o,p'*-DDE with lipoproteins, whereas *o,p'*-DDA is mostly present in

lipoprotein-depleted and HDL2 fractions. Lipoprotein association of mitotane correlates with cholesterol (F) and triglycerides (G) in the respective fraction. Best correlation is obtained with combined cholesterol and triglycerides (H). Lipoprotein-free mitotane concentration correlates with total mitotane serum concentration (I) and negatively correlates with serum cholesterol and triglyceride concentration (J).

Mitotane efficacy can be reduced by co-incubation with lipoproteins

To examine the impact of lipoproteins on mitotane efficacy, NCI-H295 cells were exposed to 25 and 50 μM mitotane and increasing quantities of lipids from isolated mitotane-free human lipoproteins (Fig. 3). As expected, mitotane treatment in diluent significantly reduced

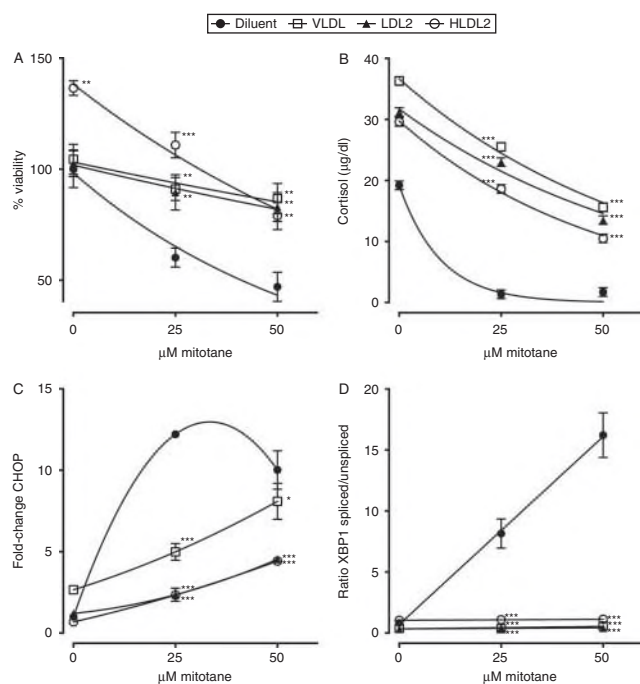


Figure 3

In vitro efficacy of lipoprotein-associated and lipoprotein-free mitotane. Normal mitotane-free lipoprotein fractions (VLDL, LDL2, and HDL2) were added to 25 and 50 μM mitotane and viability (A), cortisol secretion (B), CHOP mRNA expression (C), and XBP1 mRNA splicing in NCI-H295 cells measured. Addition of VLDL (open square) and LDL2 (black triangle) increased viability compared to equal concentrations of mitotane in ethanol. Addition of HDL2 (open circle) increased viability compared to solvent control, but addition of mitotane decreased viability proportionally more than LDL2 and VLDL. Similarly, inhibition of cortisol secretion by mitotane was alleviated through addition of lipoproteins (B). CHOP expression (C) likewise reduces by lipoproteins compared to lipoprotein-free mitotane. The decrease of CHOP expression at 50 μM lipoprotein-free mitotane compared to 25 μM has been observed previously and may be associated with feedback inactivation through downstream events. XBP1 mRNA splicing, an early event of ER-stress, is completely abolished when lipoproteins are added. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

viability of NCI-H295 cells after 6 h treatment to $60.2 \pm 4.3\%$ and $47.0 \pm 6.6\%$ for 25 and 50 μM respectively (Fig. 3A). In contrast, addition of VLDL particles rescued cell viability to $91.0 \pm 5.2\%$ and $86.8 \pm 6.8\%$ for 25 and 50 μM of baseline ($P < 0.05$ each). Similarly, addition of LDL2 particles rescued cell viability to $89.5 \pm 7.9\%$ and $82.9 \pm 6.5\%$ for 25 and 50 μM of baseline ($P < 0.05$ each). Of note, HDL2 alone increased cell viability compared to other lipoproteins and cell culture medium to $136.5 \pm 3.3\%$ (compared to solvent control; $P < 0.05$), but addition of mitotane suppressed cell viability even more strongly than co-incubation with VLDL and LDL2 particles. Similarly, mitotane alone but not mitotane co-incubated with lipoproteins inhibited cortisol secretion in the supernatant (viability 25 μM mitotane: $7.0 \pm 0.3\%$ and 50 μM mitotane $8.9 \pm 0.7\%$ vs an average viability of $68.9 \pm 5.5\%$ and $40.6 \pm 4.4\%$ respectively, $P < 0.001$, after lipoprotein addition) (Fig. 3B). Cortisol production in the presence of lipoproteins was generally higher, as the lipoproteins represent an external source of substrate cholesterol for steroidogenesis. The role of HDL, LDL, and VLDL in promoting adrenal steroidogenesis has been quite well established (32, 33, 34, 35).

We next studied the impact of added lipoproteins on mitotane-induced ER stress gene expression and found CHOP expression after co-incubation with mitotane to be reduced from 12.2 ± 0.2 -fold (25 μM mitotane) to 4.9 ± 0.5 -fold (VLDL added), 2.3 ± 0.4 -fold (LDL2 added), and 2.3 ± 0.1 -fold (HDL2 added). XBP1 mRNA splicing was completely blunted after co-treatment with lipoproteins (25 μM mitotane: 8.2 ± 1.2 -fold; VLDL added: 0.4 ± 0.02 -fold; LDL2 added: 0.4 ± 0.04 -fold; HDL2 added: 1.1 ± 0.04 -fold).

Discussion

Mitotane is routinely administered orally for adjuvant and palliative treatment of ACC. The physicochemical properties of mitotane are characterized by a high degree of lipophilicity. It is current clinical practice to advice patients to take oral mitotane together with fat-containing food such as whole-milk yogurt in order to improve resorption and increase the likelihood of achieving therapeutic drug concentrations. In this study, we exploited the availability of chyle in an ACC patient treated with mitotane to study association of mitotane and its metabolites with lipoproteins.

To unambiguously prove that the ascites removed from the index patient contained chyle, we performed ultracentrifugation to isolate chylomicrons. The high content of triglycerides and cholesterol in the floating fraction after ultracentrifugation confirmed the diagnosis

of chylous ascites. The precise cause of ascites could not decisively be clarified. However, it is likely that the physical activity of the patient (horse riding) and the known misplacement of pedicle screws in the Th11 vertebra led to injury of large abdominal lymph vessels. During follow-up in this patient, near complete remission was achieved by conservative treatment.

We found chyle to contain large amounts of mitotane indicating that mitotane absorption from the intestinal lumen largely occurs as a by-load of nutrient lipids and more specifically long-chain triglycerides. We were surprised to also detect in the chyle significant amounts of both *o,p'*-DDA and *o,p'*-DDE, the two best described mitotane metabolites. *o,p'*-DDA concentrations are known to be up to tenfold higher than the parent substance in serum, whereas *o,p'*-DDE is less abundant (12). Rat liver microsomes have been shown to be capable of mitotane transformation to the inactive metabolite *o,p'*-DDA (36, 20). Circumstantial evidence of mitotane transformation in the liver of humans is suggested by its strong inducing effect on hepatic cytochrome P450 enzymes (37, 38, 39, 40). To our knowledge, intestinal metabolism of mitotane has not yet been described, and the origin of *o,p'*-DDE in serum is not precisely known. Therefore, our finding of *o,p'*-DDA and *o,p'*-DDE in chyle suggests that mitotane biotransformation may occur already during resorption in the intestine. The mechanisms underlying the differences in relative abundance of *o,p'*-DDA and *o,p'*-DDE in serum vs chyle can not be clarified from this data. On the one hand, beyond intestinal mitotane metabolism, hepatic and potentially extra-hepatic metabolism of mitotane may contribute to the overall high abundance of *o,p'*-DDA, since the amount of *o,p'*-DDA recovered from the chyle was only around a quarter of that of mitotane and not tenfold higher as most often found in the serum of mitotane-treated patients. On the other hand, *o,p'*-DDE levels were similar to those of *o,p'*-DDA in chyle, while in patients' serum, it is ten times less than that of mitotane. This suggests that mitotane metabolism in the gut may be directed more toward production of *o,p'*-DDE. On the other hand, *o,p'*-DDE might be further metabolized in organs such as the liver, resulting in only trace amounts present in the serum. As a third alternative, the relatively small proportion of circulating mitotane vs *o,p'*-DDA may be attributable in part to diffusion into lipid-rich tissues. We cannot exclude at this point that *o,p'*-DDA and *o,p'*-DDE undergo an enterohepatic circulation; hence, chyle would be enriched in *o,p'*-DDA, resulting from hepatic transformation. However, no significant quantities of *o,p'*-DDA have

been reported in feces (21, 41). Conversely, intestinal microsomes are highly active in xenobiotic transformation and CYP3A-mediated metabolism is prevalent in intestinal mucosa (42, 43). Therefore, intestinal exposure to mitotane may lead to enzyme induction and metabolic transformation already during absorption. Inhibition of intestinal (and hepatic) CYP3A enzymes may therefore shorten the time interval to reach therapeutic serum levels of mitotane (44). There are already inhibitors of CYP3A4 used in the clinic like ritonavir or cobicistat, which are pharmacokinetic enhancers of protease inhibitors (45, 46). Our observation is limited by the availability of only one suitable case. Therefore, animal studies – preferably in non-rodents (47) – are required to confirm our finding.

Mitotane association with lipoproteins was reported earlier (23), and mitotane-induced increase of serum lipids is frequent (48).

In our study, serum lipids were similar between patients with high and low mitotane concentrations precluding selection bias. We found the vast majority of mitotane to be bound to lipoproteins with only $12.3 \pm 5.5\%$ lipoprotein-free mitotane. Distribution of mitotane between serum lipoproteins was variable and depended on the lipid status of the patient. A narrow correlation was observed between lipoprotein-associated mitotane and total cholesterol + triglyceride concentration in the VLDL fraction. A similar distribution was seen for *o,p'*-DDE. In contrast, *o,p'*-DDA was preferably detected in HDL2 and lipoprotein-depleted fraction. Hence, lipoprotein binding most likely is a result of the different lipophilicity of the parent compound and metabolites rather than specific binding to lipoprotein constituents (so, mitotane and *o,p'*-DDE might just be having a higher affinity for lipids while *o,p'*-DDA is not lipophilic).

This is in good agreement with data by Gebhardt *et al.* (23) but not completely identical to the very recent data by Hescot *et al.* (25). In the latter paper, serum lipids were not measured; therefore, it could be that relatively low overall lipid and especially triglyceride levels in the study population may have led to relative high lipoprotein-free mitotane levels. Our patient samples cover a range of physiological and elevated lipid concentrations potentially explaining this difference. Moreover, care was applied in our study to avoid freeze–thaw cycles which are known to potentially impair lipoprotein integrity. It is unclear whether this was the case in the work by Hescot *et al.* Of note, we found free mitotane to correlate with total serum mitotane and to inversely correlate with total cholesterol + triglyceride concentrations, suggesting that lipoproteins constitute a circulating pool of mitotane in

the serum. From a pharmacological perspective, our combined clinical data can be interpreted in several ways: one possibility is that mitotane bound to chylomicrons, or their remnants enters hepatocytes and is incorporated in triglyceride-rich VLDL and cholesterol-rich LDL and HDL particles. Alternatively, mitotane may be exchanged between lipoproteins passively which is likely given the close correlation of mitotane and lipid content of lipoproteins. We cannot exclude however that shuttling proteins contribute to exchange of mitotane and its metabolites between the different lipoproteins.

Human adrenocortical cells mainly rely on LDL cholesterol for steroid synthesis, but HDL cholesterol is a relevant source of cholesterol as well (49). Therefore, it is tempting to speculate that mitotane enters adrenocortical cells using HDL and/or LDL particles as a Trojan horse. However, our *in vitro* data employing the NCI-H295 model cell line contradict this hypothesis. Chylomicron-associated mitotane failed to impair viability and reduce steroidogenesis even at high concentrations. Given the correlation of mitotane and combined cholesterol and triglyceride concentration, we decided to add lipoproteins to defined concentrations of mitotane and assess the consequences at gene expression level and in terms of viability and steroidogenesis. By normalizing for lipid content, we found that VLDL and LDL particles alleviated the effect of mitotane on cell viability. HDL particles alone increased viability. HDL particles are known to be taken up by adrenocortical cells through the scavenger receptor B1 (49) and promote cell growth and proliferation by activation of the PI3 kinase/Akt pathway (50). However, a proportional decrease of viability was seen when mitotane was added. The notion of blunted response to mitotane in the presence of lipoproteins was even more clear when steroid hormone production and transcriptional up-regulation of ER stress markers CHOP and XBP1 mRNA splicing, two early steps during mitotane action, were assessed (6). Together these data provide compelling evidence that lipoprotein-associated mitotane is less active in reducing cell viability compared to unbound mitotane and likely functions as a buffer or sink which may also prevent mitotane efficacy *in vivo* in some cases.

The data presented here have significant clinical consequences. First, patients with a disposition toward low serum lipids may be unable to achieve total serum concentrations of mitotane deemed effective. Conversely, patients with severe hypertriglyceridemia – as sometimes observed during mitotane treatment for unknown reasons – are likely to have overall higher mitotane serum concentrations with albeit lower lipoprotein-free

mitotane. This is consistent with the clinical experience that excessively high mitotane blood concentrations are tolerated in hyperlipidemia without significant side effects. Efficacy of mitotane in these patients has not yet been studied systematically. Secondly, our data together with the study by Hescot *et al.* (25) put into question the current threshold of 14 mg/l of total mitotane in serum that has been demonstrated to be associated with superior treatment response. It may improve patient care to instead quantify lipoprotein-free mitotane in order to better balance toxicity and efficacy.

Clinically, two obvious consequences remain to be investigated: we cannot, at present, finally argue for or against a particular diet for patients taking mitotane. Increased dietary lipids may be expected to increase total serum lipids but fail to increase actual lipoprotein-free mitotane concentration. A pharmacokinetic study of lipoprotein-free mitotane concentration in a fasting state vs taken with a meal could solve this issue. Pharmacologic lowering of triglycerides and cholesterol may be another approach to increase the levels of lipoprotein-free mitotane. Though, we have reported that – at low mitotane concentrations – hydroxymethylglutaryl-CoA reductase inhibitors counteract cytotoxic effects of mitotane on adrenal cortex cells by lowering concentrations of free intracellular cholesterol (6) which induces lipotoxic ER stress. Moreover, it remains to be demonstrated that administration of lipid-lowering drugs indeed increases lipoprotein-free mitotane.

In conclusion, we found that the intestine likely contributes to metabolic transformation of mitotane and incorporation into chylomicrons. We confirm previous data indicating that lipoprotein-free mitotane is the active form of the drug. Lipoproteins decrease the bioavailability of active mitotane. A better understanding of the impact of food and co-administration of lipid-lowering drugs on lipoprotein-free mitotane concentrations is necessary to improve patient care. We additionally propose a prospective clinical trial to study disease response in relation to total and lipoprotein-free mitotane concentrations.

Declaration of interest

M Fassnacht received a lecture fee from HRA Pharma and has been a co-investigator of an HRA Pharma sponsored clinical trial on the pharmacokinetics of mitotane.

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