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Mitotane Therapy in Adrenocortical Cancer Induces CYP3A4 and Inhibits 5α-Reductase, Explaining the Need for Personalized Glucocorticoid and Androgen Replacement

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Context: Mitotane [1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane] is the first-line treatment for metastatic adrenocortical carcinoma (ACC) and is also regularly used in the adjuvant setting after presumed complete removal of the primary tumor. Mitotane is considered an adrenolytic substance, but there is limited information on distinct effects on steroidogenesis. However, adrenal insufficiency and male hypogonadism are widely recognized side effects of mitotane treatment.

Objective: Our objective was to define the impact of mitotane treatment on *in vivo* steroidogenesis in patients with ACC.

Setting and Design: At seven European specialist referral centers for adrenal tumors, we analyzed 24-h urine samples (n = 127) collected from patients with ACC before and during mitotane therapy in the adjuvant setting (n = 23) or for metastatic ACC (n = 104). Urinary steroid metabolite excretion was profiled by gas chromatography/mass spectrometry in comparison with healthy controls (n = 88).

Results: We found a sharp increase in the excretion of 6β-hydroxycortisol over cortisol ($P < 0.001$), indicative of a strong induction of the major drug-metabolizing enzyme cytochrome P450 3A4. The contribution of 6β-hydroxycortisol to total glucocorticoid metabolites increased from 2% (median, interquartile range 1–4%) to 56% (39–71%) during mitotane treatment. Furthermore, we documented strong inhibition of systemic 5α-reductase activity, indicated by a significant decrease in 5α-reduced steroids, including 5α-tetrahydrocortisol, 5α-tetrahydrocorticosterone, and androsterone (all $P < 0.001$). The degree of inhibition was similar to that in patients with inactivating 5α-reductase type 2 mutations (n = 23) and patients receiving finasteride (n = 5), but cluster analysis of steroid data revealed a pattern of inhibition distinct from these two groups. Longitudinal data showed rapid onset and long-lasting duration of the observed effects.

Conclusions: Cytochrome P450 3A4 induction by mitotane results in rapid inactivation of more than 50% of administered hydrocortisone, explaining the need for doubling hydrocortisone replacement in mitotane-treated patients. Strong inhibition of 5α-reductase activity is in line with the clinical observation of relative inefficiency of testosterone replacement in mitotane-treated men, calling for replacement by 5α-reduced androgens. (***J Clin Endocrinol Metab* 98: 161–171, 2013)**

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Abbreviations: ACC, Adrenocortical carcinoma; An/Et, ratio of androsterone to etiocholanolone; CI, confidence interval; CYP, cytochrome P450; GC, gas chromatography; LDA, linear discriminant analysis; MS, mass spectrometry; 6βOHF/F, ratio of 6β-hydroxycortisol to cortisol; PCA, principal component analysis; Q1, quartile 1; 5α-THB/THB, ratio of 5α-tetrahydrocorticosterone/tetrahydrocorticosterone; 5α-THF/THF, ratio of 5α-tetrahydrocortisol to tetrahydrocortisol.

Adrenocortical carcinoma (ACC) is a rare cancer, with an incidence of one to two cases per million per year and a poor prognosis, mostly due to a high risk of recurrence and limited therapeutic options (1). Mitotane [1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (o,p′-DDD)], an analog of the insecticide dichlorodiphenyltrichloroethane (DDT), has been used in the treatment of ACC since 1959 (2). Mitotane alone or in combination with cytotoxic chemotherapy is now established as the first-line treatment for metastatic ACC (3–8) and is also widely used as adjuvant therapy in patients with apparently complete surgical removal of the primary tumor, especially if considered at high risk of recurrence (9).

Despite the widespread use of mitotane in adrenal cancer, there is limited knowledge regarding the mechanisms underlying its antitumor activity, usually described as adrenolytic, *i.e.* a direct cytotoxic effect on the adrenal cortex (3, 10–12). There is also a paucity of information on distinct effects of mitotane on steroidogenesis, although it has been noted early on as an efficient treatment for Cushing's syndrome (13–16), and in patients with normal adrenal function, mitotane therapy invariably results in adrenal insufficiency. There is *in vivo* evidence of enhanced production of cortisol-binding globulin and SHBG in mitotane-treated patients (17, 18). Notably, glucocorticoid replacement has to be administered in higher doses than usual in the general context of adrenal insufficiency to prevent adrenal crisis (3, 19). Mitotane-induced hypogonadism is frequently observed in male patients (18), but testosterone replacement often lacks clinical efficacy.

We have recently shown that urine steroid metabolomics, *i.e.* the combination of steroid profiling by gas chromatography (GC)/mass spectrometry (MS) and computational data analysis, is a highly promising diagnostic tool for the detection of adrenocortical malignancy (20). Here we investigated the effects of mitotane on *in vivo* steroid production employing urinary steroid metabolomics for the analysis of 24-h urine samples from patients with adrenal cancer receiving mitotane for adjuvant treatment or metastatic disease.

Subjects and Methods

Subjects

The 24-h urine samples from ACC patients were collected between 2006 and 2010 in seven specialist endocrine referral centers participating in the European Network for the Study of Adrenal Tumors (ENSAT; www.ensat.org), with approval of local ethical review boards and after obtaining written informed patient consent. We included 24-h urines from 100 patients (53 women, 47 men; median age 52, range 16–80 yr) with histologically confirmed ACC who provided a total of 127 samples including 46 paired samples. Samples were collected before (ADJ, n = 12) and during (ADJ+M, n = 11) adjuvant mitotane therapy or before (MET, n = 57) and during (MET+M, n = 47) mitotane treatment for metastatic ACC. Samples during mitotane treatment were collected 3–4 months after initiation of therapy, *i.e.* at a time when therapeutic-range plasma mitotane levels (14–20 mg/liter) (21) generally had been achieved. None of the patients on adjuvant therapy had documented recurrence during this initial treatment period, and there were no major changes in tumor burden as documented by imaging in the metastatic group patients. Plasma mitotane levels were available for 50 of the 58 patients on mitotane, all of them measured by HPLC (Lysosafe, Paris, France). Exclusion criteria included pregnancy and exposure to drugs known to induce expression and activity of hepatic cytochrome P450 (CYP) enzymes or to alter steroid secretion in any way, with the exception of glucocorticoid replacement therapy, which was routinely commenced in all mitotane-treated patients.

For comparison, we employed 24-h urine samples of 88 healthy controls (62 females, 26 males, age range 18–60 yr). In addition, for the assessment of 5α-reductase activity, we also compared the results with 24-h urine samples from patients with inactivating mutations in *SRD5A2* encoding 5α-reductase type 2 (n = 23) and patients treated with the 5α-reductase type 2 inhibitor finasteride (n = 5).

GC/MS urinary steroid metabolome analysis

Measurement of 24-h urinary steroid metabolite excretion was carried out by GC/MS as previously described (20). In summary, free and conjugated steroids were extracted from urine by solid-phase extraction. Steroid conjugates were enzymatically hydrolyzed, reextracted, and chemically derivatized to form methyloxime trimethyl silyl ethers. GC/MS analysis of the urine samples was carried out on an Agilent (Santa Clara, CA) 5973 instrument operating in selected-ion-monitoring (SIM) mode. This achieved sensitive and specific detection and quantification of 32 selected steroid metabolites chosen to include important representatives of steroid groups such as androgen metabolites, glucocorticoid metabolites, mineralocorticoid metabolites, and

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3β-hydroxy-Δ (5) steroid metabolites (for details of the steroid metabolite profile see Ref. 20).

After analysis of the entire profile, we calculated substrate metabolite to product metabolite ratios to assess the effects of mitotane on the *in vivo* net activity of distinct steroidogenic enzymes. This included 5α-reductase indicated by the ratio of 5α-tetrahydrocortisol to tetrahydrocortisol (5α-THF/THF), ratio of androsterone to etiocholanolone (An/Et), and ratio of 5α-tetrahydrocorticosterone/tetrahydrocorticosterone (5α-THB/THB). As a measure of the activity of the major drug-metabolizing enzyme CYP3A4, we calculated the ratio of 6β-hydroxycortisol to cortisol (6βOHF/F) (22). Total steroid output was calculated as the sum of all quantified steroid metabolites with the exception of glucocorticoid metabolites because these also reflected exogenously administered glucocorticoid replacement. Total when used in this paper relates to the targeted compounds measured, which are dominant metabolites of hormonal steroids and their precursors. It does not include a multitude of minor metabolites.

Statistical analysis

Diagnostic ratios were presented as median and interquartile ranges [quartile 1 (Q1)–Q3]. In a first analysis, we considered all samples to be independent and employed nonparametric Kruskal-Wallis test and Dunn's *post hoc* test to detect significant differences of individual ratios among the treatment groups. To take the paired nature of a subset of samples into account, we provided a separate analysis of the data employing Wilcoxon signed rank test. These analyses were carried employing SigmaPlot (Systat Software Inc., Chicago, IL).

Furthermore, we analyzed the influence of mitotane on 5α-reductase activity by performing multivariate analyses of the ratios reflecting 5α-reductase activity, An/Et, 5α-THF/THF, and 5α-THB/THB. We performed principal component analysis (PCA) and linear discriminant analysis (LDA) (23) to generate two-dimensional representations of the data. Prior to analysis, the values of the ratios were log-transformed and normalized to zero mean and unit variance. The generated scatter plots allowed identification of clusters of similar ratio profiles. These analyses were done using the software MATLAB (Mathwork Inc., Natick, MA).

Finally, we analyzed the association between plasma mitotane levels and the values of the steroid ratios indicative of 5α-reductase and CYP3A4 activities by computing Spearman's rank correlation coefficient, thereby accounting for the lack of normal distribution of the data. This analysis was carried out employing MATLAB.

Results

Mitotane down-regulates overall steroidogenesis

First, we analyzed total steroid excretion to assess whether mitotane has an impact on the initial steps of steroidogenesis, namely CYP11A1 activity converting cholesterol to pregnenolone. For this analysis, we disregarded active glucocorticoid metabolites because the mitotane-treated patients invariably received hydrocortisone replacement therapy, which prevented a comprehensive assessment of endogenous glucocorticoid production. Comparing the remainder of total steroid excretion, we found that mitotane led to a significant down-regulation of overall steroidogenesis in metastatic ACC patients, as documented by a significant decrease in the sum of total androgen and mineralocorticoid metabolites (Fig. 1A and Table 1). This down-regulation was significant for the larger group of metastatic ACC patients, decreasing both androgen and mineralocorticoid excretion to the level found in healthy controls, but failed to reach significance for the smaller adjuvant therapy group (Fig. 1A and Table 1).

Of note, we found that the excretion of the 11-deoxycortisol metabolite tetrahydro-11-deoxycortisol did not differ when comparing steroid excretion before and after the initiation of mitotane therapy (Fig. 1B and Table 1), indicating that mitotane had no effect on 11β-hydroxylase activity, which converts 11-deoxycortisol to cortisol.

Mitotane induces CYP3A4 activity and glucocorticoid inactivation

After the initiation of mitotane treatment, 6βOHF/F showed a significant increase in both patients with metastatic disease and patients receiving adjuvant therapy (Fig. 1, C and D, and Table 1 and Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org), which was due to large increases in 6β-OHF excretion, indicative of a highly enhanced rate of inactivation of cortisol to 6βOHF, a conversion predominantly catalyzed by CYP3A4 (24–26). Before mitotane treatment, 6βOHF represented only 1.3% (ADJ; Q1–Q3 1.0–2.6%) and 1.8% (MET; Q1–Q3 0.8–4.2%), respectively, of total measured glucocorticoid metabolite excretion. By contrast, during mitotane treatment, 63.8% (ADJ+M; Q1–Q3 48.9–70.2%) and 52.5% (MET+M; Q1–Q3 38.8–69.1%), respectively, of measured glucocorticoids were excreted as 6βOHF (Fig. 1E), suggesting rapid inactivation of exogenously administered hydrocortisone in the mitotane-treated patients.

To exclude a significant contribution of the concurrent hydrocortisone replacement on the induction of CYP3A4 observed in the mitotane-treated patients, we also studied the percentage of 6βOHF as part of total glucocorticoid excretion in patients with adrenal insufficiency, specifically 30 patients on a regular-dose hydrocortisone replacement (10–30 mg/d) and 10 patients who received 400 mg hydrocortisone during the 24-h period of urine collection. Results revealed that 6βOHF on regular hydrocortisone dose did not differ from healthy controls, whereas high-dose hydrocortisone slightly increased the median percentage of 6βOHF to just under 5% (Fig. 1E), confirming an only very minor contribution of hydrocortisone to the observed effect.

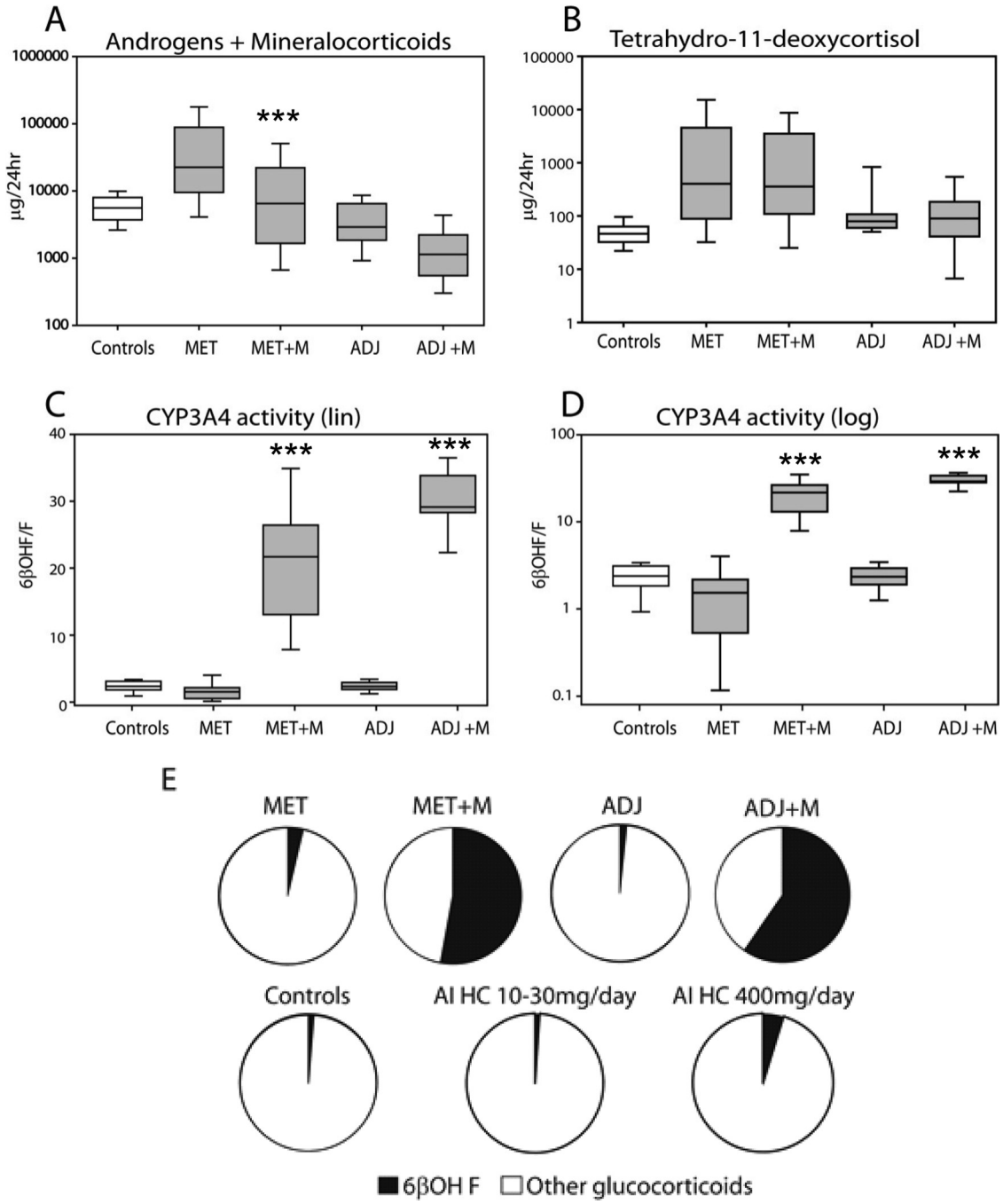

FIG. 1. Comparison of steroid concentrations and ratios in patients before and during mitotane treatment for metastatic ACC (MET; MET+M) and patients before and during adjuvant mitotane therapy (ADJ; ADJ+M) in comparison with healthy controls. A, Total androgen and mineralocorticoid metabolite excretion; B, excretion of the 11-deoxycortisol metabolite tetrahydro-11-deoxycortisol; C and D, linear scale and log scale representations of 6βOHF/F that is reflective of CYP3A4 activity; E, percentage of total glucocorticoid metabolites excreted as 6βOHF in ACC patients before and during mitotane treatment in comparison with healthy controls (HC) and with patients with adrenal insufficiency (AI) on hydrocortisone replacement with 10–30 mg/24 h (n = 30) and 400 mg/24 h (n = 10). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for comparison of MET *vs.* MET+M and ADJ *vs.* ADJ+M, respectively.

GC/MS analysis of urine also showed a major increase in the excretion of normally minor metabolites formed through CYP3A4 activity but not selected for quantitation in this study, notably 6α- and 1β-hydroxylated metabolites of tetrahydrocortisone and the cortolones. We did not find an effect of mitotane on the steroid ratios reflective of 11β-hydroxysteroid dehydrogenase type 1 or 2 activity.

Mitotane inhibits 5α-reductase activity and androgen activation

Introduction of mitotane therapy resulted in a highly significant decrease of several steroid metabolite ratios reflective of systemic 5α-reductase activity (Fig. 2 and Table 1 and Supplemental Table 1). The degree of inhibition of 5α-reductase activity appeared to be similar to that

TABLE 1. Sums of steroid metabolites and steroid metabolite ratios in healthy controls and patients with adrenocortical cancer receiving treatment for metastatic disease (MET) or in the adjuvant setting (ADJ) before and after initiation of mitotane therapy (+M)

	Controls (n = 88)	MET (n = 57)	MET+M (n = 47)	ADJ (n = 12)	ADJ+M (n = 11)
Total steroid excretion (steroid metabolites 1–19 without active glucocorticoid metabolites 20–32)					
Median	5,579	22,468	6,494	2,908	1,137
Q1–Q3	3,702–7,982	9,470–88,311	1,660–22,130	1,849–6,472	548–22,208
P_1		<0.0001	1.0	0.60	<0.01
P_2			<0.0001		0.97
Tetrahydro-11-deoxycortisol (THS)–11-deoxycortisol metabolite					
Median	47	402	356	79	90
Q1–Q3	33–63	88–4539	108–3515	60–107	41–184
P_1		<0.0001	<0.0001	0.14	0.74
P_2			1.0		1.0
6β-OHF/F − CYP3A4 activity					
Median	2.38	1.53	21.7	2.34	29.1
Q1–Q3	1.83–3.11	0.53–2.17	13.0–26.4	1.90–2.93	28.3–33.8
P_1		0.77	<0.0001	1.00	<0.0001
P_2			<0.0001		<0.0001
5α-THF/THF − 5α-reductase activity					
Median	0.70	0.58	0.02	0.34	0.02
Q1–Q3	0.52–0.95	0.18–4.54	0.01–0.05	0.26–0.93	0.01–0.03
P_1		1.00	<0.0001	1.00	<0.0001
P_2			<0.0001		<0.01
An/Et − 5α-reductase activity					
Median	0.88	0.45	0.20	0.50	0.20
Q1–Q3	0.67–1.21	0.22–0.79	0.13–0.34	0.41–0.82	0.14–0.53
P_1		<0.0001	<0.0001	0.25	<0.0001
P_2			<0.01		0.53
5α-THB/THB − 5α–reductase activity					
Median	2.01	1.27	0.01	1.52	0.01
Q1–Q3	1.48–2.66	0.49–3.22	0.00–0.33	0.95–2.36	0.00–0.03
P_1		0.20	<0.0001	1.00	<0.0001
P_2			<0.0001		<0.01

For the calculation of differences in total steroid excretion, glucocorticoids were excluded because mitotane therapy requires concurrent hydrocortisone replacement, which obscures any differences in endogenous glucocorticoid secretion. Results are presented as median and the range of the 25–75th percentile (Q1–Q3). Statistical analysis was performed employing Kruskal-Wallis nonparametric testing and Dunn's *post hoc* test. P_1, Comparison *vs.* controls; P_2, comparison of MET *vs.* MET+M and ADJ *vs.* ADJ+M, respectively. ADJ, Before adjuvant mitotane therapy; ADJ+M, during adjuvant mitotane therapy; MET, before mitotane treatment for metastatic ACC; MET+M, during mitotane treatment for metastatic ACC.

observed in patients receiving treatment with the established 5α-reductase type 2 inhibitor finasteride (n = 5) and patients with inactivating 5α-reductase type 2 (*SRD5A2*) mutations (n = 25) (Fig. 2 and Table 2). However, of note, the 5α-THB/THB ratio was more significantly inhibited by mitotane than observed in finasteride-treated or *SRD5A2* mutant patients (Fig. 2 and Table 2).

To examine the pattern of inhibition of 5α-reductase in further detail, we carried out cluster analysis employing both LDA and PCA; for this analysis, we considered all three ratios reflective of 5α-reductase activity and selected the patients receiving mitotane in the adjuvant setting to exclude any influence of tumor-related steroid production. Visualization of the data convincingly demonstrated strongly overlapping clustering of finasteride-treated patients and *SRD5A2* mutant patients, who both have selective loss of 5α-reductase type 2 activity (Fig. 3A), indicative of similar ratio profiles in these two groups. By contrast, patients receiving mitotane are clearly separate in a second cluster (Fig. 3A). These findings were confirmed by an independent cluster analysis employing LDA (Fig. 3B).

Longitudinal studies

We analyzed the longitudinal course of the steroid ratios indicative of CYP3A4 activity (Fig. 4A) and 5α-reductase activity (Fig. 4, B and C) in five patients receiving adjuvant mitotane therapy for adrenocortical cancer. Results demonstrate a rapid onset of the effects of mitotane on the enzymatic activities of CYP3A4 and 5α-reductase, with the full extent of the effect already documented shortly after initiation of mitotane treatment (Fig. 4, A–C).

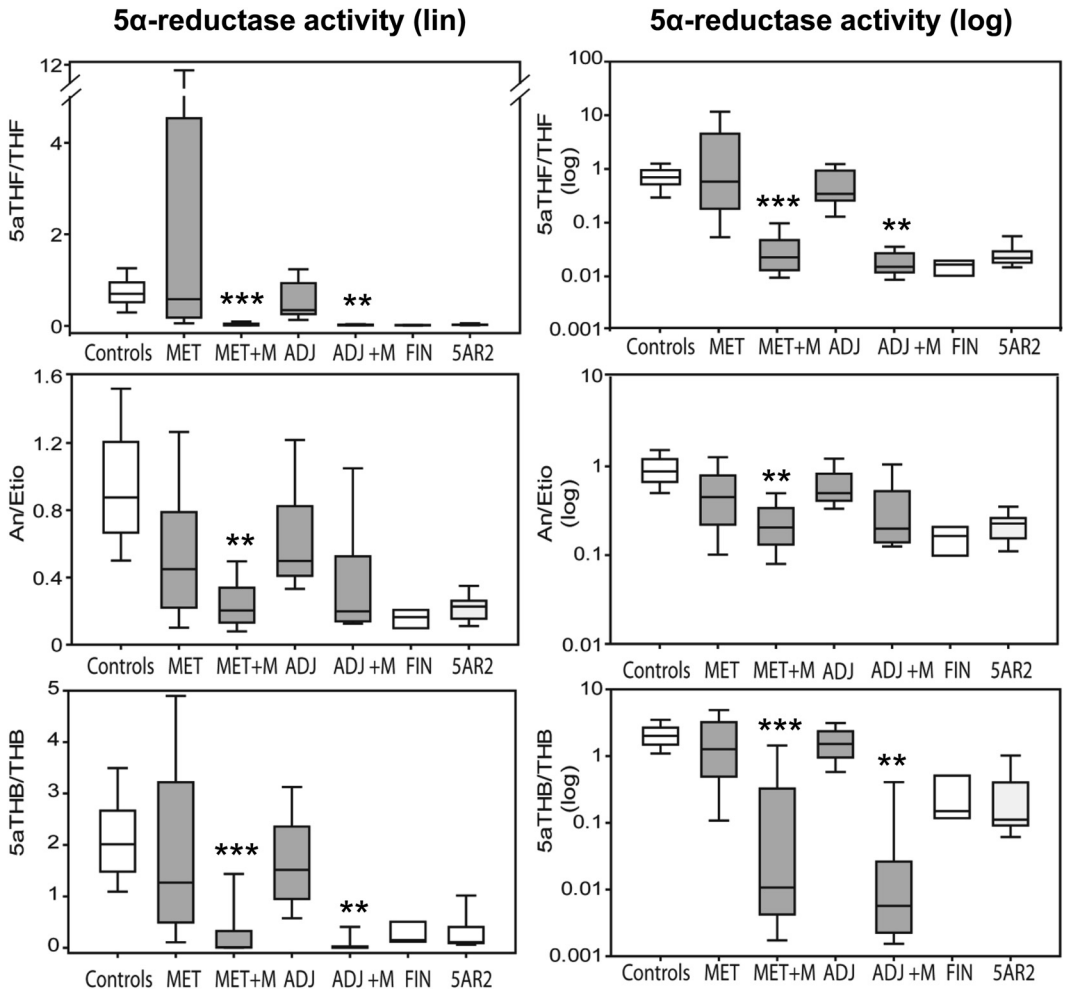

FIG. 2. Comparison of steroid ratios indicative of 5α-reductase activity (5αTHF/THF, An/Et, and 5αTHB/THB) represented in linear and log scale for healthy controls, patients with metastatic ACC before (MET) and during mitotane treatment (MET+M), and patients after apparently complete removal of ACC before (ADJ) and during adjuvant mitotane treatment (ADJ+M). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for comparison of MET *vs.* MET+M and ADJ *vs.* ADJ+M, respectively. 5AR2, 5α-reductase type 2; FIN, finasteride.

We had the opportunity to document the diagnostic steroid ratios in one patient throughout 2 yr of adjuvant mitotane treatment followed by 2 yr of posttreatment observation (Fig. 4D). Plasma mitotane levels oscillated within the suggested therapeutic range (14–20 mg/liter) throughout the treatment period and only became undetectable 1 yr after the last administration of mitotane. Concurrently, the steroid ratios indicative of CYP3A4 and 5α-reductase activity started to recover but had not returned to pretreatment levels even 2 yr after the end of treatment (Fig. 4D), suggestive of long-lasting effects.

Plasma mitotane levels and observed effects on steroidogenesis

We analyzed the correlation between circulating plasma mitotane levels and the severity of CYP3A4 induction and 5α-reductase inhibition, respectively (Supplemental Fig. 1). This revealed a significant correlation between plasma mitotane levels and the induction of CYP3A4 [r = 0.328, 95% confidence interval (CI) = 0.055–0.556, P = 0.02] but not with the steroid ratios indicative of 5α-reductase activity (r = −0.053, 95% CI = −0.327–0.229, P = 0.71 for 5αTHF/THF; r = 0.106, 95% CI = −0.178–0.373, P = 0.46 for An/Et) (Supplemental Fig. 1). Significant effects on the enzymatic activities were already observed at very low plasma mitotane levels and clearly below the suggested therapeutic range of 14–20 mg/liter (21) (Supplemental Fig. 1). These findings are in line with the above-described observation that urine metabolite excretion showed the full effects as early as 1–2 months after initiation of mitotane treatment (Fig. 4, A–C), when in most instances, plasma mitotane levels would not have reached the therapeutic range.

Discussion

This study documented comprehensive *in vivo* evidence for a strong inhibition of 5α-reductase activities and a

TABLE 2. Steroid metabolite ratios reflecting systemic 5α-reductase activity in patients receiving mitotane for metastatic adrenocortical cancer (MET+M) and adjuvant therapy in adrenal cancer (ADJ+M) in comparison with patients treated with the 5α-reductase type 2 inhibitor finasteride (FIN) or patients with inactivating mutations in the gene encoding 5α-reductase type 2 (*SRD5A2*)

Steroid ratio reflective of 5α-reductase activity	MET+M (n = 49)	ADJ+M (n = 11)	FIN (n = 5)	*SRD5A2* (n = 25)
5α-THF/THF				
Median	0.02	0.02	0.02	0.02
Q1–Q3	0.01–0.05	0.01–0.03	0.01–0.02	0.02–0.03
P_1			0.25	1.0
P_2			1.0	0.52
An/Et				
Median	0.20	0.20	0.16	0.23
Q1–Q3	0.13–0.34	0.14–0.53	0.10–0.21	0.16–0.267
P_1			0.79	1.0
P_2			0.75	1.0
5α-THB/THB				
Median	0.01	0.01	0.15	0.14
Q1–Q3	0.00–0.33	0.00–0.03	0.12–0.51	0.09–0.50
P_1			0.35	<0.01
P_2			0.05	<0.01

Results are presented as median and the range of the 25–75th percentile (Q1–Q3). Statistical analysis was performed employing Kruskal-Wallis nonparametric testing and Dunn's *post hoc* test. P_1, Comparisons *vs.* MET+M; P_2, comparisons *vs.* ADJ+M. ADJ, Before adjuvant mitotane therapy; ADJ+M, during adjuvant mitotane therapy; MET, before mitotane treatment for metastatic ACC; MET+M, during mitotane treatment for metastatic ACC.

significant induction of hepatic CYP3A4/5 activities in mitotane-treated patients, with an obvious and important impact on the requirements for glucocorticoid and androgen replacement during mitotane therapy.

Evidence for alterations of cortisol metabolism and a link to hepatic enzyme activity was documented shortly after the introduction of mitotane for the treatment of adrenal cancer in 1959 (2). In 1964, two groups reported on altered cortisol metabolism resulting in increased urinary excretion of 6βOHF in guinea pig (27) and humans (28), respectively. Following up on these reports, two groups documented increased metabolism of pentobarbital (29) and hexobarbital and cortisol (30) by mitotane, postulating the induction of microsomal drug-metabolizing liver enzymes as the underlying cause. Work in the late 1980s demonstrated that the major drug-metabolizing enzyme CYP3A4 and to a lesser degree also CYP3A5 were the enzymes responsible for 6β-hydroxylation in liver and kidney (24–26). Subsequently, the urinary 6βOHF/F has been widely implemented as a relative measure of CYP3A4/5 *in vivo* activity and several studies demonstrated a 4- to 7-fold increase in 6βOHF excretion in patients treated with rifampicin (25) or anticonvulsants (31). This study analyzing 24-h urine samples from 127 patients collected before and during mitotane treatment demonstrated a 10- to 15-fold increase in 6βOHF/F excretion, establishing mitotane as one of the strongest inducers of CYP3A4 activity. This convincingly corroborates a recent report on accelerated midazolam metabolism in four mitotane-treated patients suggestive of induction of CYP3A4 (and CYP3A5) activity (32).

Early reports on hepatic enzyme induction by mitotane and also the invariable finding of highly increased cortisol-binding globulin levels during mitotane treatment (17) suggested an increased dose requirement for glucocorticoid replacement in mitotane-induced adrenal insufficiency. However, this has been widely recognized only in recent years, after a number of reports on adrenal crisis during mitotane treatment covered only with standard glucocorticoid replacement doses (19, 33). Consequently, the perceived rate of reported gastrointestinal toxicity during mitotane treatment has declined over recent years, because many of these signs and symptoms may have been reflective of incipient adrenal crisis. However, currently, there is no uniformly agreed dose regimen for glucocorticoid replacement during mitotane therapy and reported doses have varied widely (17, 18), with lower doses often reported as associated with a high incidence of vomiting and severe fatigue (18, 34). Our findings provide for the first time a reliable quantification of 6β-hydroxylation by mitotane, documenting the rapid excretion of 55–65% of glucocorticoids in the form of 6βOHF.

Of note, CYP3A4/5 are responsible for not only the conversion of cortisol to 6βOHF but also for the 6β-hydroxylation of testosterone. Hypogonadism in mitotane-treated men initially manifests with a decreased free androgen index due to significantly increased SHBG levels that cannot be

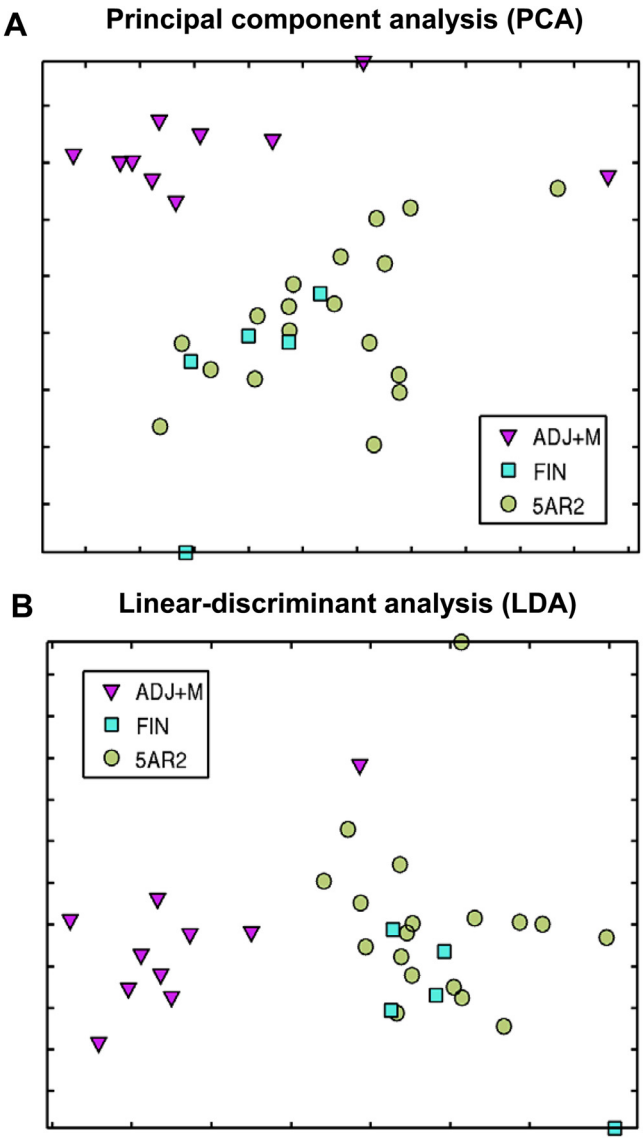

FIG. 3. Cluster analysis of the inhibitory effect of mitotane on 5α-reductase activities comparing patients receiving adjuvant mitotane treatment (ADJ+M) to patients treated with the 5α-reductase type 2 inhibitor finasteride (FIN; n = 5) and patients with inactivating 5α-reductase type 2 (5AR2) mutations (*SRD5A2*; n = 25). Each patient is characterized by the three ratios reflective of systemic 5α-reductase activities (5α-THF/THF, An/Et, and 5α-THB/THB). PCA (A) and LDA (B) were employed to generate a two-dimensional representation of each patient. When employing PCA, the first principal component explained 42% of the total data variance, whereas the second principal component explained 32%.

compensated for by up-regulated testosterone production as documented by increased total testosterone and LH levels (35). With time, gonadal testosterone production exhausts itself and circulating testosterone levels drop, accompanied by clinical manifestations of low testosterone including erectile dysfunction. However, testosterone replacement is often clinically ineffective and is complicated by an increased rate of gynecomastia (18).

This study has yielded comprehensive evidence for a strong inhibition of 5α-reductase activities by mitotane. Importantly, the strong inhibition of 5α-reductase has significant consequences for androgen bioactivity, because the conversion of testosterone to the most potent androgen, 5α-dihydrotestosterone, will be greatly reduced. Consequently, this may result in enhanced conversion of testosterone to 17β-estradiol by widespread CYP19A1 (P450 aromatase) activity, which could explain the high incidence of gynecomastia in mitotane-treated patients. The lack of conversion of testosterone to 5α-dihydrotestosterone also represents a logical explanation for the frequent clinical observation of relative inefficiency of testosterone replacement with regard to erectile dysfunction. Our computational analysis of the ratios of 5α- to 5β-reduced steroids revealed a distinct pattern of global 5α-reductase inhibition by mitotane compared with patients treated with a selective 5α-reductase type 2 inhibitor or patients with inactivating 5α-reductase type 2 mutations. These results could indicate preferential inhibition of 5α-reductase type 1 by mitotane. 5α-Reductase inhibition could also have beneficial consequences in the context of androgen-producing ACC, where it would be likely to help ameliorate the clinical manifestations of androgen excess.

A number of early studies addressed the impact of mitotane on adrenal steroidogenesis, reporting inhibitory effects of mitotane on 11β-hydroxylase, 3β-hydroxysteroid dehydrogenase, and 18-hydroxylase activities (36–39). However, *in vivo* studies by labeled isotope infusion were very limited in numbers, whereas *in vitro* studies were somewhat limited in their methodological approach. In our study, we found no evidence for distinct enzyme inhibition other than the above described strong inhibition of 5α-reductase activities and the induction of CYP3A4/5. Specifically, there was no change in 11-deoxycortisol metabolite excretion, rendering a significant change in 11β-hydroxylase (CYP11B1) enzymatic activity highly unlikely. However, we observed a down-regulation of overall steroidogenesis as quantified by the sum of total androgen and mineralocorticoid excretion; glucocorticoid metabolites were excluded for that analysis as altered due to the mandatory exogenous hydrocortisone replacement in mitotane-treated patients. These findings could indicate an inhibition of CYP11A1, *i.e.* P450 side-chain cleavage enzyme, as previously described *in vitro* (40), which would result in decreased conversion of cholesterol to pregnenolone and thus a decreased substrate entry flow into the steroidogenic pathways. This could contribute to the hypercholesterolemia that is a widely documented side effect of mitotane treatment and that has been previously suggested to be due to increased cholesterol synthesis as a consequence of mitotane-induced up-regulation of 3-hydroxy-3-methyl-glutaryl-coenzyme A-reductase activity (41, 42).

Our study provides a quantifiable measure for the strong induction of CYP3A4/5 activities by mitotane,

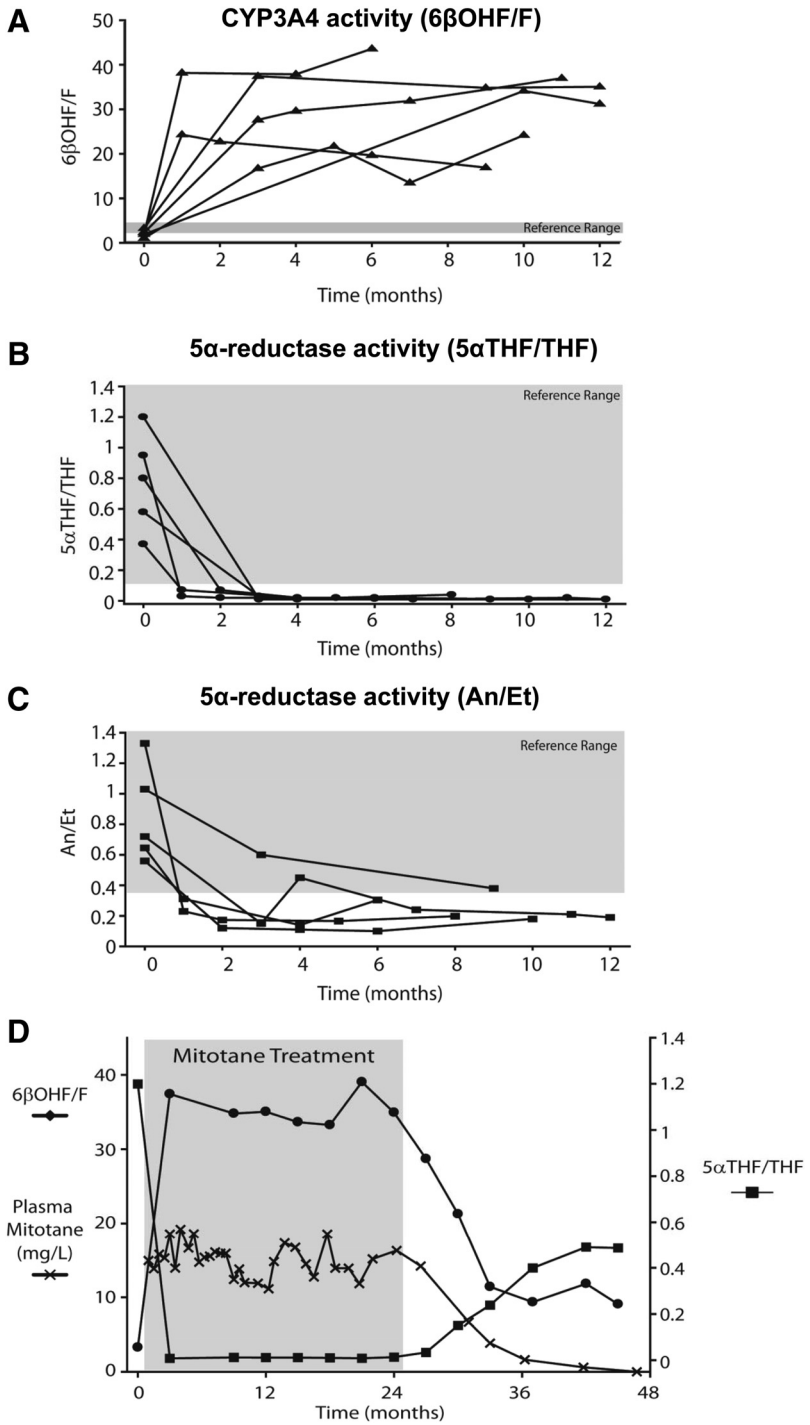

FIG. 4. Longitudinal course of CYP3A4 induction and 5α-reductase inhibition during the first 12 months of adjuvant mitotane therapy in six patients (A) and five patients (B and C) and CYP3A4 and 5α-reductase activities in relation to plasma mitotane levels in a patient during 24 months of adjuvant mitotane treatment and another 24 months of posttreatment follow-up (D).

which has the clinically most relevant consequences, including potential drug interactions in mitotane-treated patients, nicely summarized in a recent review (43). This has an impact not only on drugs needed for the treatment of mitotane-related side effects but, importantly, also on antitumor drugs including tyrosine kinase and mammalian target of rapamycin inhibitors and also chemotherapeutic agents included in the current first-line treatment for metastatic adrenal cancer (44).

Our findings of strong and long-lasting inhibition of 5α-reductase and induction of CYP3A4/5 refine our understanding of the requirements for steroid replacement therapy in mitotane-treated patients. Our observation of a very rapid induction of CYP3A4 by mitotane suggests that lower glucocorticoid replacement doses, such as 25 mg cortisone acetate (18) equivalent to 15 mg hydrocortisone (45), may soon become inadequate and contribute to the gastrointestinal toxicity observed during the first months of mitotane treatment. Thus, we suggest that glucocorticoid replacement in mitotane-treated patients should be initiated and maintained with at least double the dose normally used in primary adrenal insufficiency, *i.e.* 40–50 mg hydrocortisone (equal to 75 mg cortisone acetate) rather than 20–25 mg hydrocortisone (equal to 37.5 mg cortisone acetate) per day. Dexamethasone should be avoided because it exerts a strong CYP3A4/5-inducing effect (46) that is likely to result in even more rapid inactivation. Whether urinary 6βOHF excretion can be used as a guide for dose adjustment will have to be examined by prospective studies. At present, the appropriateness of glucocorticoid replacement during mitotane therapy largely relies on clinical assessment and plasma ACTH measurements after the morning hydrocortisone dose, with increased levels suggestive of glucocorticoid underreplacement. Importantly, we should consider the use of 5α-reduced androgens, including synthetic androgens, for androgen replacement in mitotane-induced male hypogonadism, which may prove more effective and less prone to unwanted side effects than testosterone replacement therapy. Pregnancy needs to be added to the list of contraindications for mitotane therapy, and patients should have safe contraception in place because the strong inhibition of 5α-reductase activity would have a major impact on sexual differentiation, with a high likelihood of disordered sex development in the male fetus.

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