Mitotane induces \textit{CYP3A4} expression via activation of the steroid and xenobiotic receptor

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Abstract

Adrenocortical carcinoma (ACC) is a rare disease with an extremely poor prognosis. Mitotane alone or in combination with other cytotoxic drugs is a common therapeutic option for ACC. In addition to its adrenolytic function, mitotane has been known for decades to increase the metabolic clearance of glucocorticoids. It was recently shown that the tyrosine kinase inhibitor sunitinib is also rapidly metabolized in patients treated with mitotane, indicating that mitotane engages in clinically relevant drug interactions. Although the precise mechanism of these interactions is not well understood, cytochrome P450 mono-oxygenase 3A4 (\textit{CYP3A4}) is a key enzyme to inactivate both glucocorticoids and sunitinib. The nuclear receptor steroid and xenobiotic receptor (SXR (NR1I2)) is one of the key transcriptional regulators of \textit{CYP3A4} gene expression in the liver and intestine. A variety of xenobiotics bind to SXR and stimulate transcription of xenobiotic-response elements (XREs) located in the \textit{CYP3A4} gene promoter. In this study, we evaluated the effects of mitotane on SXR-mediated transcription \textit{in vitro} by luciferase reporter analysis, SXR–steroid receptor coactivator 1 (SRC1) interactions, quantitative real-time PCR analysis of \textit{CYP3A4} expression, SXR knockdown, and \textit{CYP3A4} enzyme activity assays using human hepatocyte-derived cells. We found that mitotane activated SXR-mediated transcription of the XREs. Mitotane recruited SRC1 to the ligand-binding domain of SXR. Mitotane increased \textit{CYP3A4} mRNA levels, which was attenuated by SXR knockdown. Finally, we showed that mitotane increased \textit{CYP3A4} enzyme activity. We conclude that mitotane can induce \textit{CYP3A4} gene expression and suggest that mitotane is used cautiously due to its drug–drug interactions.

Key Words

- mitotane
- \textit{CYP3A4}
- SXR
- adrenocortical carcinoma

Introduction

Adrenocortical carcinoma (ACC) is a rare disease with an extremely poor prognosis. The annual incidence is \(~1–2\) per million individuals worldwide. At the time of diagnosis, \(~40–70\%\) of the tumors have already undergone metastatic spread. Even when an apparently radical resection has been performed, eventual local recurrence or distant metastases are often recognized (reviewed in Allolio & Fassnacht (2006)). Mitotane, 1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) ethane (\textit{O,p'-DDD}), is a compound derived from the
insecticide dichlorodiphenyltrichloroethane (DDT), which specifically destroys the adrenal cortex and is able to block cortisol synthesis by inhibiting 11β-hydroxylation and cholesterol side chain cleavage (Hague et al. 1989, Alloio & Fassnacht 2006). In patients with metastatic or progressive disease, medical treatment with mitotane is usually begun. In addition to its action on the adrenal cortex, mitotane also affects hepatic microsomal enzyme induction. High-dose glucocorticoid replacement, a daily dose of 50 mg hydrocortisone or greater, is typically required to prevent adrenal insufficiency (Alloio & Fassnacht 2006). The plasma half-life of dexamethasone is shortened among patients who are treated with mitotane (Robinson et al. 1987). Urinary excretion of 6β-hydroxycortisol, which is an indicator of hepatic microsomal enzyme induction, is increased in patients after mitotane administration (Fukushima et al. 1971).

The hepatic cytochrome P450 enzymes (CYPs) catalyze the 6β-hydroxylation of steroid hormones. CYP mono-oxygenase 3A4 (CYP3A4) is the most abundant CYP expressed in human liver and is involved in the metabolism of approximately one-half of the drugs in clinical use today. Recently, van Erp et al. (2011) reported that mitotane-treated ACC patients exhibited high levels of induced CYP3A4 activity. These authors used orally administered midazolam as a phenotypic probe to determine the activity of CYP3A4. In their pharmacokinetic study using time-course measurement of plasma concentrations, mitotane-treated patients showed an ~18-fold reduced exposure to midazolam (AUCO–12 h) but an ~12-fold increased exposure to its metabolite, 1-hydroxy midazolam. Furthermore, they observed that the tyrosine kinase inhibitor sunitinib, which is a substrate of CYP3A4, was rapidly metabolized in patients treated with mitotane. However, the precise mechanism of CYP3A4 induction by mitotane is not well understood.

The orphan nuclear receptor (NR), steroid and xenobiotic receptor (SXR, also called the pregnane X receptor, NR1I2), is highly expressed in the liver and intestine where it regulates genes that control xenobiotic and endogenous steroid hormone metabolism. SXR binds to xenobiotic-response elements (XREs) located in the promoters of genes that encode phase I CYP enzymes (e.g. CYP3A4), phase II conjugating enzymes (e.g. UDP glucuronosyltransferase 1A1 (UGT1A1)), and phase III drug transporters (e.g. P-glycoprotein/multidrug resistance protein 1 (MDR1)) (Kliwer et al. 2002, Zhang et al. 2008, Zhou et al. 2009). Induction of such drug-metabolizing genes by SXR can cause drug–drug interactions. For example, the antibiotic rifampicin (RFP) is a well-known SXR agonist that reduces plasma concentrations of coadministered drugs metabolized by CYP3A4, such as glucocorticoid derivatives, calcium channel blockers, oral contraceptives, and cyclosporine. In this study, we tested whether mitotane induces CYP3A4 gene expression via SXR activation.

**Materials and methods**

**Materials**

RFP was obtained from Sigma–Aldrich. Mitotane was from Wako Pure Chemical Industries, Ltd (Osaka, Japan), and 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbonyl O-3,4-dichlorobenzyl) oxime (CITCO) was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Cryopreserved HepaRG cells and cryopreserved human hepatocytes of donor A (batch number HEP187186, 58-year-old man) were purchased from Biopredic International (Rennes, France). Cryopreserved human hepatocytes of donor B (lot LMP, 38-year-old female Caucasian) were purchased from In Vitro Technologies, Inc. (Baltimore, MD, USA).

**Plasmids**

Human SXR, GAL4 steroid receptor coactivator 1 (SRC1)-receptor-interacting domain (RID), VP16 SXR-ligand-binding domain (LBD), and VP16 SXR-ΔAF2 LBD expression plasmids were described previously (Takeshita et al. 2006, 2011). The luciferase (LUC) reporter construct, 5X upstream activating sequence (UAS)-thymidine kinase minimum promoter (TK)-LUC (Cohen et al. 2000), and xenobiotic-responsive enhancer module (XREM)–CYP3A4–LUC (Goodwin et al. 1999) were described previously. Plasmid phRL-TK, which contains the RenillaLUC gene with the TK promoter, was purchased from Promega.

**Transient co-transfection experiments**

HepG2 cells were grown in DMEM containing 10% FCS. The serum was stripped of hormones by constant mixing with 10% (w/v) AG1-X8 resin (Bio-Rad) and powdered charcoal before ultrafiltration. Cells were maintained without antibiotics and were transiently transfected using the calcium phosphate coprecipitation method in 24-well plates with 0.5 μg reporter plasmid containing either XREM–CYP3A4–LUC or SXUAS–TK–LUC cDNA and the 0.1 μg human SXR expression plasmid. One hundred nanograms of phRL-TK were used as an internal control.
In some samples, empty mock vectors were added to equalize the concentration of total transfected plasmid. Cells were grown for 24 h in the absence or presence of compounds and then harvested. Cell extracts were analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity of the reporter plasmid was corrected by the Renilla luciferase activity of the control plasmid. The corrected luciferase activities of untreated samples were normalized to the luciferase activities of samples as described in the figure legends. All transfection studies were repeated in triplicate. The results shown are the mean ± s.d. (n = 3).

**Cell culture**

Differentiated HepaRG cells were seeded into collagen I-coated 96-well plates using HepaRG Thawing and Seeding Medium 670 (Biopredic International), according to the manufacturer’s instructions. After 3 days, the culture medium was changed to HepaRG Induction Medium 640 (Biopredic International) containing either mitotane or RFP dissolved in DMSO (final concentration 0.1% (v/v)). The control group received 0.1% DMSO only. Human hepatocytes from donor A were seeded into collagen I-coated 96-well plates using Hepatocyte Seeding Medium 670 (Biopredic International), according to the manufacturer’s instructions. After 24 h, the medium was changed to Hepatocyte Culture Medium (Biopredic International), according to the manufacturer’s instructions. After 24 h, the medium was changed to Hepatocyte Culture Medium (Biopredic International) containing mitotane, RFP, or CITC dissolved in 0.1% DMSO. Human hepatocytes from donor B were cultured in collagen-coated 12-well plates with Lanford medium. After a 24-h incubation, the cells were treated with either mitotane or RFP dissolved in 0.1% DMSO.

**Quantitative real-time PCR (qRT-PCR)**

HepaRG cells and human hepatocytes from donor A were analyzed with Power SYBR Green Cells-to-Ct (Applied Biosystems) using a PE-Applied Biosystems Prism 7700 instrument. For human hepatocytes from donor B, total RNA was prepared using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA was reverse transcribed using random hexamers and TaqMan reagents (Applied Biosystems). With the resulting cDNA as a template, qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers. The following primers were used for CYP3A4: forward primer 5′-CATTCCCTCATCCCCAATTCTTT-GAAGT-3′, reverse primer 5′-CCACTCGGTGTTTTGTTG-3′; for CYP2B6: forward primer 5′-GAAGGACTGCTTCAC-3′; for UGT1A1: forward primer 5′-GAATCAACTGCTTCAC-3′, reverse primer 5′-ACCACAATTCCATGTTCTCCA-3′; for SXR: forward primer 5′-CATGTTCAAAGGCCATCATCAG-3′, reverse primer 5′-ATCTCAGTGACACAGCTCGAA-3′; for CAR (NRII3): forward primer 5′-TGATCAGCTGCAAGAGGAGA-3′, reverse primer 5′-AGGCTAGCAACCTTGCCATA-3′; and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward primer 5′-GGCCTCC-AAGGAGTAAGACC-3′, reverse primer 5′-AGGGGAGATT-CAGTGTGGTG-3′. Results were normalized with GAPDH using the 2**-ΔΔCT** method and expressed relative to the untreated control. Representative results are expressed as the mean ± s.d. (n=3 wells per treatment) of two independent experiments.

**RNAi**

HepaRG cells or human hepatocytes from donor A were cultured in collagen I-coated 96-well plates. The cells were transfected with 5 nM Silencer Select predesigned siRNA (Ambion, Austin, TX, USA) against human SXR (s16909) or human CAR (s19369) or with the negative control siRNA (control No. 1). Introduction of siRNA was carried out using Lipofectamine RNAiMAX (Invitrogen) and the reverse transfection method according to the manufacturer’s instructions. After 24 h, cells were treated with the test compounds. After 48 h, CYP3A4, CYP2B6, and UGT1A1 expressions were determined by qRT-PCR.

**CYP3A4 activity**

To determine the effects of test compounds on CYP3A4 activity in HepaRG cells, we used the P450-Glo CYP3A4 Assay with Luciferin-IPA (Promega) according to the manufacturer’s instructions. Briefly, HepaRG cells were grown in HepaRG Induction Medium 640 containing mitotane or RFP dissolved in 0.1% DMSO. Cell culture medium without cells served as the background control. After a 48-h treatment, cells were incubated with 3 μM Luciferin-IPA in HepaRG Induction Medium 640 for an additional 60 min. An aliquot of the medium was then combined with an equal volume of the luciferin detection reagent in a white luminometer plate, and the luminescence was read. The remaining cells were assessed using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega) to estimate the number of cells in each well, and CYP3A4 activity was normalized to cell number. Results are expressed as the mean ± s.d. (n=3).
Statistical analysis

Groups were compared by the Mann–Whitney $U$ test; $P<0.05$ was considered significant in all cases. Statistical analysis was carried out with StatView 5.0 software for Macintosh (SAS Institute, Cary, NC, USA).

Results

To determine whether mitotane stimulates SXR-mediated transcription of the CYP3A4 gene, transient transfection assays were performed with a reporter plasmid, XREM–CYP3A4–LUC, containing the enhancer (nucleotides $-7836$ to $-7208$) and promoter (nucleotides $-362$ to $+53$) regions of CYP3A4 to drive luciferase gene expression (Goodwin et al. 1999) in HepG2 cells. A previous study identified four putative SXR-response elements within the XREM; three of the four elements cooperatively promote RFP induction by SXR (Goodwin et al. 1999). As shown in Fig. 1, mitotane, as well as RFP, increased transcription of the CYP3A4 gene in a dose-dependent manner, although the magnitude of the activation by mitotane was less than that of RFP. Co-transfection of the human SXR plasmid further enhanced transcriptional activation, consistent with previous reports of the relative low expression of endogenous SXR in this cell line (Zucchini et al. 2005, Naspinski et al. 2008). This reporter assay suggests that mitotane binds to SXR as a ligand and then stimulates CYP3A4 transcription.

Transcriptional activation by NRs is mediated by ligand-dependent interactions with coactivators, including p160 of the NR coactivator family (SRC1, also called NCOA1; TIF2, also called SRC2, GRIP1, or NCOA2; and TRAM1, also called SRC3, p/CIP, AIB1, ACTR, RAC3, or NCOA3) (Smith & O’Malley 2004). To examine the ligand-induced interactions between SXR and the p160 coactivators, we employed one representative of p160, SRC1, in mammalian two-hybrid assays. The RID of SRC1 was fused to the DNA-binding domain of GAL4 (GAL4 SRC1-RID), and the LBD of human SXR was fused to the transactivation domain of VP16 (VP16 SXR-LBD). These constructs were co-transfected with a reporter plasmid containing five copies of a GAL4 UAS in HepG2 cells (Fig. 2). GAL4 SRC1-RID interacted with VP16 SXR-LBD in the presence of mitotane as well as RFP in a dose-dependent manner. The C-terminal region of the LBD (AF-2) is conserved among NRs, and deletion or point mutations in this region impair transcriptional activation without changing either ligand- or DNA-binding affinities. Ligands of NRs induce major conformational changes in the AF-2 region so that ligand-bound receptors interact with coactivators (Smith & O’Malley 2004). When the C-terminal truncated AF2 mutant VP16 SXR-ΔAF2 was used, neither RFP nor mitotane increased luciferase activity, suggesting that mitotane-bound SXR recruits coactivators in an AF2-dependent manner.

To determine whether mitotane induces CYP3A4 expression in the liver, HepaRG cells (Fig. 3A, B, and C; Kanebratt & Andersson 2008) and cryopreserved human hepatocytes of two donors (donor A: Fig. 3D, E, and F; donor B: G, H, and I) were treated with either RFP or mitotane for 24 or 48 h. The mRNA levels were then determined by qRT-PCR. As shown in Fig. 3A, D, and G, both mitotane and RFP increased CYP3A4 mRNA levels in these human hepatocyte-derived cells. Although mitotane had a smaller effect than RFP, apparent CYP3A4 induction by mitotane was observed at concentrations exceeding $10 \mu$M. In addition, both RFP and mitotane increased mRNA levels of CYP2B6 (Fig. 3B, E, and H) and UGT1A1 (Fig. 3C, F, and I). Interestingly, the effect of mitotane on CYP2B6 mRNA levels was higher than that of RFP in HepaRG cells (Fig. 3B) and donor A hepatocytes (Fig. 3E).
Mitotane causes drug–drug interactions

To confirm that mitotane induction of CYP3A4 in human hepatocyte-derived cells requires SXR, we evaluated the effects of SXR knockdown on mitotane-induced gene expression in HepaRG cells (Fig. 4A). After a 24-h transfection with control or SXR-specific siRNA, the cells were treated with 10 μM RFP, 10 μM mitotane, or DMSO (vehicle) for 48 h, and gene expression was analyzed by qRT-PCR. Transfection of gene-specific siRNAs reduced SXR mRNA levels by 74% (data not shown). Similar to results observed in HepaRG cells, SXR knockdown in donor A hepatocytes markedly reduced mitotane-induced expression of CYP3A4 (Fig. 4D) but not CYP2B6 (Fig. 4E), whereas RFP-induced expression of both genes was suppressed. In agreement with the previous reports, CITCO demonstrated preferential induction of CYP2B6 (Fig. 4E, control siRNA) over CYP3A4 (Fig. 4D, control siRNA). As expected, CAR siRNA but not SXR siRNA abrogated CYP2B6 induction by CITCO (Fig. 4E). Similarly, CAR knockdown significantly reduced mitotane-induced CYP2B6 expression (from 5.3- to 1.6-fold) (Fig. 4E), indicating that CAR is a key regulator of mitotane-induced CYP2B6 expression. Both SXR and CAR knockdown reduced mitotane-induced UGT1A1 expression (Fig. 4F). Taken together, our results suggest that mitotane induction of CYP3A4 is primarily mediated by SXR, mitotane induction of CYP2B6 is primarily mediated by CAR, and mitotane induction of UGT1A1 is mediated by both SXR and CAR.

To determine the effects of mitotane on CYP3A4 enzymatic activity in HepaRG cells, we used an assay kit containing Luciferin-IPA. This substrate is metabolized specifically by CYP3A4 to release luciferin, which can be quantified by luminescence through its reaction with ATP-luciferase. HepaRG cells were treated for 48 h with different concentrations of RFP or mitotane. Mitotane increased CYP3A4 activity in a dose-dependent manner, although the magnitude of mitotane-induced CYP3A4 activity was lower than that induced by RFP (Fig. 5).

Discussion

In patients with ACC in whom surgical cure is not possible, mitotane, either as a single agent or in combination with other traditional cytotoxic chemotherapies, is generally administered. Adverse effects of mitotane, such as gastrointestinal distress, neurological disturbances, hepatotoxicity, and adrenal insufficiency, are well known. Although it has been known for decades that mitotane increases the metabolic clearance of glucocorticoids, drug–drug interactions had not been clearly documented until the recent report by...
van Erp et al. (2011). Our results indicate that mitotane can activate SXR and possibly contribute to the increase in CYP3A4.

A wide variety of xenobiotics can bind to SXR as ligands and stimulate SXR-mediated transcription. X-ray crystallographic studies of the LBD of human SXR revealed that SXR contains a relatively large ligand-binding pocket compared with other NRs (Watkins et al. 2001). In addition, the crystal structure of SXR with one of its ligands, SR12813, revealed that a single drug molecule could be bound in the LBD in three distinct orientations (Watkins et al. 2001). The flexibility of the LBD likely enables SXR to recognize a wide range of xenobiotics, including mitotane. It is noteworthy that DDT, from which mitotane is derived, has been reported to be a potent activator of human SXR as well (Medina-Diaz & Elizondo 2005, Medina-Diaz et al. 2007). Thus, mitotane-bound SXR recruits p160 coactivators to induce CYP3A4 gene expression.

In clinical usage, it is recommended that mitotane blood levels be maintained between 14 and 20 µg/ml (i.e. 43.7–62.5 µM) to achieve tumor regression and avoid adverse effects. Mitotane, at all measured endpoint levels, significantly increased CYP3A4 activity. Although our in vitro assays may not appropriately reflect the in vivo situations of patients treated with mitotane, the concentration of mitotane used in our in vitro studies is certainly achievable pharmacologically in vivo.
hypoprothrombinemic effects of warfarin, a substrate of CYP2C9, during mitotane treatment (Cuddy & Loftus 1986). In this study, we showed that mitotane induces CYP2B6 and UGT1A1 as well as CYP3A4 in human liver-derived cells. CYP2B6 contributes 2–10% of total hepatic CYP content and 3–12% of drug metabolism capacity (Wang & Tompkins 2008). Known substrates of CYP2B6 include the anticancer drugs cyclophosphamide and ifosfamide, antiretroviral drugs nevirapine and efavirenz, anesthetic drugs propofol and ketamine, the synthetic opioid methadone, and the anti-Parkinson drug selegiline (Wang & Tompkins 2008). UGT1A1, one of the most extensively characterized UGT isoforms, is an enzyme of the glucuronidation pathway, which transforms a diverse range of lipophilic xenobiotics and endogenous compounds (e.g. drugs, environmental chemicals, carcinogens, vitamins, bilirubin, steroid hormones, thyroid hormones, and glycolipids) into water-soluble, excretable metabolites (Tolson & Wang 2010). Therefore, CYP2B6 and UGT1A1 possess important functions in human drug metabolism.

Results of our knockdown experiments suggest that CAR participates in mitotane-induced CYP2B6 and UGT1A1 activation, whereas mitotane-induced CYP3A4 activation appears to be largely explained by SXR. CAR and SXR regulate an overlapping set of xenobiotic-metabolizing genes, including genes that encode several CYP enzymes (i.e. CYP3A4, CYP2B6, CYP2Cs, and CYP2A6), UGTs (i.e. UGT1A1, UGT1A6, and UGT1A9), and the drug transporter MDRI (Tolson & Wang 2010). By contrast to most NRs, CAR displays high constitutive activity in the absence of agonist binding but can also be directly activated by ligands (e.g. CITCO) or indirectly activated (e.g. by phenobarbital). In liver cells, CAR is

Figure 4
Mitotane induces CYP3A4 expression in human liver cells by activating SXR. HepaRG cells (A, B, and C) were transfected with control siRNA or SXR siRNA and treated with 10 μM RFP, 10 μM mitotane, or 0.1% DMSO (vehicle control) for 48 h. Human hepatocytes from donor A (D, E, and F) were transfected with control siRNA, SXR siRNA, or CAR siRNA and then treated with 10 μM RFP, 10 μM mitotane, 1 μM CITCO, or 0.1% DMSO (vehicle control) for 48 h. Expression levels of CYP3A4 (A and D), CYP2B6 (B and E), and UGT1A1 (C and F) were analyzed by qRT-PCR, normalized to GAPDH, and expressed relative to the DMSO control. Results are expressed as mean ± S.D. (n = 3).
advanced and metastatic ACC treatment (the FIRM-ACT study) showed that the combination regimen of mitotane and etoposide, doxorubicin, and platinum (EDP) had a higher response rate than that of the combination of mitotane and streptozocin, even though both etoposide and doxorubicin are substrates of CYP3A4 (Fassnacht et al. 2012). Additionally, no one has shown that EDP toxicity is decreased when used in combination with mitotane. Therefore, therapeutic drug monitoring is needed to clarify this issue, which may in turn improve chemotherapy outcomes for patients with ACC (Kroiss et al. 2011).

In conclusion, our in vitro study shows that mitotane induces CYP3A4 gene expression via the activation of SXR, explaining the many clinical observations of the drug–drug interactions caused by mitotane. Careful therapeutic drug monitoring may therefore lead to improved outcomes and prognoses for patients with ACC.

**Declaration of interest**

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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