LIVER X RECEPTOR INHIBITION POTENTIATES MITOTANE INDUCED ADRENOTOXICITY IN ACC

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Abbreviations:
ACC: Adrenocortical Carcinoma
LXR: Liver X Receptor
FC: Free Cholesterol
ER: Endoplasmic Reticulum
SOAT1: Sterol-O-acyl-transferase 1
CE: Cholesteryl Esters
27HC: 27-Hydroxycholesterol
CYP27A1: Cytochrome P450 Family 27 Subfamily A Member 1
ABCA1: ATP-Binding Cassette Protein A-1
ABCG1: ATP-Binding Cassette Protein G-1
ApoA1: Apolipoprotein A1
HDL: High Density Lipoprotein
StAR: Steroidogenic Acute Regulatory (Protein)
DN: Dominant Negative
Adrenocortical carcinoma (ACC) is a rare aggressive malignancy with a poor outcome largely due to limited treatment options. Here, we propose a novel therapeutic approach through modulating intracellular free cholesterol via the liver X receptor alpha (LXRα) in combination with current first line pharmacotherapy, mitotane.

H295R and MUC-1 ACC cell lines were pretreated with LXRα inhibitors in combination with mitotane. In H295R, mitotane (20, 40, 50µM) induced dose-dependent cell death, however, in MUC-1 this only occurred at a supratherapeutic concentration (200µM). LXRα inhibition potentiated mitotane-induced cytotoxicity in both cell lines. This was confirmed through use of the CompuSyn model which showed moderate pharmacological synergism and was indicative of apoptotic cell death via an increase in annexinV and cleaved-caspase 3 expression. Inhibition of LXRα was confirmed through downregulation of cholesterol efflux pumps ABCA1 and ABCG1, however, combination treatment with mitotane attenuated this effect. Intracellular free cholesterol levels were associated with increased cytotoxicity in H295R ($r^2=0.5210$) and MUC-1 ($r^2=0.9299$) cells. While both cell lines exhibited similar levels of free cholesterol at baseline, H295R were cholesterol ester rich whereas MUC-1 were cholesterol ester poor.

We highlight the importance of LXRα mediated cholesterol metabolism in the management of ACC, drawing attention to its role in the therapeutics of mitotane sensitive tumours. We also demonstrate significant differences in cholesterol storage between mitotane sensitive and resistant disease.
Adrenocortical carcinoma (ACC) is a rare, aggressive malignancy with an incidence of 2-5 per million and which carries a poor prognosis due to local invasion or distant metastases at the time of diagnosis in the majority of cases (Fassnacht et al., 2013, Libe, 2019). Complete surgical resection (R0) is the only curative therapy (de Reynies et al., 2009, Kerkhofs et al., 2013). Yet, for those on whom R0 resection is achieved, disease recurs in 50-85% (Pommier and Brennan, 1992, Terzolo et al., 2007). Mitotane is the only currently licensed pharmacotherapy for adjuvant use or for recurrent disease, and remains the most effective drug (alone or in combination with platinum based chemotherapy regimens) in preventing and treating recurrence (Terzolo et al., 2007, Haak et al., 1994, Terzolo et al., 2013, Fassnacht et al., 2018). Mitotane’s use is limited by its narrow therapeutic window whereby at serum concentrations below 14mg/L, it demonstrates poor efficacy while at concentrations >20mg/L, it is associated with unacceptable toxic effects ((Terzolo et al., 2013, Hermsen et al., 2011). There is significant clinical need to improve the therapeutic options in managing persistent or recurrent ACC and in this context the current study investigates therapeutic strategies to enhance the efficacy and tolerability of mitotane.

Mitotane has been used for 50 years in the management of adrenocortical neoplasm (Montgomery and Welbourn, 1965) yet its adrenotoxic mechanism remains incompletely understood (Poli et al., 2013, Hescot et al., 2013, Lehmann et al., 2013). Sbiera et al demonstrated that mitotane-induced adrenotoxicity is associated with intracellular free cholesterol (FC) accumulation causing endoplasmic reticulum (ER) stress (Sbiera et al., 2015). The authors also demonstrated that mitotane-induced FC accumulation is associated with inhibition of sterol-O-acyltransferase-1 (SOAT-1) which usually converts FC to inert cholesteryl esters (CE) for intracellular storage. The adrenal cytotoxic effects of SOAT-1 inhibition have also been demonstrated by LaPensee et al through the use of the selective inhibitor ATR-101(LaPensee et al., 2016). Consequently, this lipotoxic mechanism mediated by intracellular FC accumulation is considered significant, or even predominant in terms of mitotane-induced adrenotoxicity.
We theorised that the lipotoxic effects of mitotane on ACC cells could be enhanced by targeting complementary mechanisms to that of SOAT-1 inhibition, which would further increase intracellular FC. Liver X Receptor alpha (LXRα) inhibition represents one such mechanism. LXRα is a nuclear receptor, sensitive to intracellular FC and represents the predominant transcriptional mechanism mediating reverse cholesterol transportation to the liver (Kennedy et al., 2005, Wang et al., 2007, Costet et al., 2000). At rest, LXRα is bound to its nuclear response element and held in an inactive state by co-repressor molecules. Upon ligand activation, it recruits co-activator proteins to dimerise with the retinoid X receptor (RXR), and in turn transcribes regulatory proteins for cholesterol efflux, namely ABCA1 and ABCG1. These deliver excessive intracellular FC to circulating Apolipoprotein (Apo) A1 or High density lipoprotein (HDL) (Hu et al., 2003, Phelan et al., 2008, Huuskonen et al., 2004, Janowski et al., 1996, Fu et al., 2001).

LXRα is highly expressed in liver, innate immune cells and adrenal cortex (Cummins et al., 2006) and its sensitivity to intracellular cholesterol is effected through endogenous ligand molecules, oxysterols, generated from precursor cholesterol. The common oxysterol, 27-hydroxycholesterol (27HC) is catalysed by CYP27A1, highly expressed in adrenal cortex (Fedorova et al., 2015, Chen et al., 1998). Higher intracellular FC results in increased oxysterol production, activating LXRα dependent transcription of cholesterol efflux pumps. The cholesterol regulatory function of LXRα is conserved across cell types, mediating reverse cholesterol transport as well as providing cellular protection against toxic FC accumulation (Cignarella et al., 2005, Engel et al., 2007).

In the adrenal, oxysterols and LXRα also has a tissue-specific role whereby they increase steroidogenesis by upregulating Steroidogenic Acute Regulatory (StAR) protein (Cummins et al., 2006, Nilsson et al., 2007). In breast and prostate cancer, LXRα stimulation facilitates cancer cell survival which is partially explained by its role to enhance aerobic glycolysis, while also hypothesised to reduce FC-induced cytotoxicity (Nelson et al., 2013, Flaveny et al., 2015, Kim et al., 2018).

We hypothesised that inhibition of LXRα-modulated cholesterol efflux pump expression could potentiate mitotane-induced lipotoxic adrenocortical cell death at usually subtherapeutic mitotane
concentrations. We investigated the dose-dependent interactions of mitotane with pharmacological inhibition of LXRα, and dominant-negative (DN) interference of LXRα activity, in two human ACC cell lines (H295R and MUC-1). Using this strategy, we demonstrate an enhanced ability of mitotane in combination with LXRα inhibition to kill ACC cells at lower concentrations than for mitotane alone. We show that this effect is associated with higher intracellular FC accumulation.
METHODOLOGY

Cell Culture and Treatments

H295R Human primary ACC cells (NCI-H295R, CRL-2182; (American Type Culture Collection, Manassas, VA, USA))(Rainey et al., 1994) were maintained in DMEM/F12 (Thermofisher), containing 2.5% Nu Serum (Corning), 1% ITS+ (Corning), 1% Penicillin/Streptomycin. H295R cells were isolated from a primary adrenocortical tumour of a 48-year-old black female and are mitotane sensitive. MUC-1 Human metastatic ACC cells were established by Hantel, et al (Hantel et al., 2016) and maintained in DMEM Advanced (Thermofisher) containing 5% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. Originally isolated from a neck metastasis of a 24-year-old male, MUC-1 cells are an aggressive, mitotane resistant model of ACC. HepG2 Human Hepatocellular Carcinoma cells (HB-8065™️; (American Type Culture Collection, Manassas, VA, USA)) were utilised as a comparator cell line and maintained in Eagle’s MEM (Thermofisher), containing 5% FBS, 1% Penicillin/Streptomycin (Di et al., 2012, Bruschi et al., 2019). Cells were grown in a humidified atmosphere containing 5%CO₂ and 37°C, used between passage 3-10 (H295R/HepG2) or passage 21-26 (MUC1). H295Rs were obtained from ATCC (July 2017) and cells are routinely tested for steroid production, forskolin responsiveness and cell line characteristics.

Pharmacological Treatments

Cells were treated at the indicated concentrations of GSK2033 - LXRα antagonist (Bio-techne)(Griffett and Burris, 2016), SR9243 - LXRα inverse agonist (Flaveny et al., 2015), 27-Hydroxycholesterol - endogenous LXRα agonist (Enzo-Life Science), T0901317 - synthetic, non-steroidal LXRα agonist and mitotane (2,4'-DDD). Cells were cholesterol loaded by preparing supplemented media using 45 µg/ml water-soluble cholesterol methyl-β-cyclodextrin. All drugs were prepared in DMSO and the vehicle control remained below 0.1% at all times. H295R cells were pretreated with GSK2033(5µM), SR9243(1µM) or 27HC(5µM) for 18hrs followed by Mitotane(20, 40, 50µM) for 6hrs. MUC-1 cells were pretreated with SR9243(5µM) for 24hrs followed by mitotane(50, 100, 200µM) for 24hrs. Both cell lines were cholesterol loaded 1hr prior to mitotane treatment. Reagents were obtained from Sigma unless otherwise indicated.
Plasmids, Transfection & Luciferase Assay

H295R were transfected using Lipofectamine 3000 (Thermofisher) using either pCMX-hLXRα dominant negative (LXR-DN) or wild type (LXR-WT) expressing plasmids(Willy et al., 1995). Transfection efficiency was evaluated using green fluorescent protein co-expression (pmaxGFP, Lonza) using microscopy and flow cytometry (Supplementary figure 1I&J). H295R were transfected in antibiotic free media for 24hrs prior to drug treatments. Luciferase assay was carried out using firefly (pGL3-TK-LXRRE-Luc) and renilla luciferase vectors (10:1). Activity were measured following 24hrs stimulation with LXRα agonist T0901317 (1µM) using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's guidelines. Luminescence was recorded on the Synergy HT Multi-Detection Microplate Reader (BioTek), normalised for renilla luciferase activity and represented as relative light units (RLU).

Gene Expression

Following drug treatments, cells were washed with cold phosphate buffered saline (PBS) and total RNA was extracted from adherent cells using TRI Reagent®. Following spectrophotometric quantification, 1µg total RNA was reverse transcribed into cDNA using the Qiagen RT² first strand kit. Polymerase chain reaction (PCR) assay was carried out using Go Taq® Green Master Mix (Promega), according to manufacturer's guidelines. Amplified products were separated using a 4% agarose gel, visualized using SYBR Safe (Thermofisher) stain and imaged using the ChemiDoc™ XRS+ Imaging system (Bio-Rad Laboratories, Maryland, USA). Real-time quantitative PCR expression was assayed on Applied Biosystems StepOne Plus (Applied Biosystems, Carlsbad, California, USA) and carried out using SYBR green mastermix (Promega). Primer sequences are listed in Supplementary Table 1. Fold change was calculated using the ΔΔCT method. Data is represented as fold change of mRNA relative to vehicle control.

Western Blotting

Samples were harvested on ice and lysed using radio-immuno-precipitation (RIPA) buffer and 1X protease inhibitor cocktail (Calbiochem). Lysates were quantified by BCA assay, denatured at 95°C and separated
by SDS-Page using a 10% Tris-Glycine gel. Proteins were transferred to PVDF membrane, blocked using 5% Milk and incubated with relevant antibodies. Membranes were incubated with primary antibodies overnight at 4°C followed by relevant HRP-linked secondary antibodies (Supplementary Table 2). Imaging was carried out using the ChemiDoc™ XRS+ Imaging system (Bio-Rad Laboratories, Maryland, USA) with SuperSignal™ HRP substrate (Thermofisher) and Image Lab™ Software (Bio-rad, V5.2.1). Semi-quantitative densitometry was carried out using ImageJ and normalised to the loading control (β Actin).

**Cell death & Apoptosis**

H295R were grown as described above and stained with Calcein AM (Invitrogen) (1μM) and Propidium iodide (PI) (5μg/ml) to assess viability via microscopy, all images (10X) were taken using the EVOS® Cell Imaging System (Thermo Scientific). For quantitative analysis, cells were trypsinised and resuspended in FACS buffer (PBS, 1% FBS, 0.05% Sodium Azide) following treatment. AnnexinV:FITC (Bio-Rad) staining was carried out at 10μL/10⁶ cells for 30mins, followed by 5mins PI staining. All fluorochromes were excited using a 488nm laser followed by 530/30 (AnnexinV:FITC) and 585/35 (PI) bandpass filter. Compensated flow cytometry was performed on FACS CantoII® with FACS DiVa 6.0® acquisition software (BD Biosciences, Oxford, UK) and FlowJo® V10 analysis software (Tree Star Inc., Ashland, OR). Gating strategy is described Supplementary Figure 2. AnnexinV and PI positive cells are represented as % cells relative to vehicle control.

**Metabolic Activity Assay**

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out in 96 well plates, cells were seeded at 1x10³ cells per well and grown for 3, 7, 10 and 14 days. Drug treatments GSK2033 (5μM) and SR9243 (1μM) were given at the indicated time points (Day 0, 3, 7, 10) for 24hrs followed by mitotane (50μM) for 24hrs. Following incubation, MTT reagent (0.5mg/ml) was added. The assay was terminated by solubilising MTT using DMSO and absorbance was read at 570nm as described above. Experimental conditions were carried out in replicates of 6 and the experiment was repeated four times. Data is represented as % metabolic activity adjusted to matched vehicle control (100%) for each individual time point.
Cholesterol Analysis

Filipin complex III (Sigma) from *Streptomyces filipinensis* was used to assess free cholesterol (Hassall and Graham, 1995). Filipin is a fluorescent marker of FC that is excited by a 405nm laser followed by a 450/50 bandpass filter. Cells were treated as indicated, harvested via trypsinisation and fixed using fixation buffer (Biolegend) according to manufacturer’s guidelines. Staining was carried out at 1mg/ml for 1 hour at 4°C in the dark and analysed by microscopy and flow cytometry. CholEsteryl BODIPY™ FL C12 (CholEsteryl 4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Dodecanoate) (Invitrogen) was used to label cholesterol esters (Hsieh et al., 2012). Cells were grown to 60% confluence and media was supplemented with 5µM CholEsteryl BODIPY™ FL C12 overnight. Cells were harvested via trypsinisation and analysed by flow cytometry on the BD FACSCantoll (BD Bioscience). Sytox blue (Invitrogen) (1µM) was used as a viability dye. Fluorescence was recorded using a 488nm excitation laser followed by a 530/30 bandpass filter (CholEsteryl BODIPY™ FL C12) and a 405 laser followed by 450/50 bandpass filter (Sytox Blue).

Single, live cells were gated according to Supplementary Figure 6 and the resolution metric was calculated as described (Brando and Sommaruga, 1993). Additionally, cells were analysed via imaging flow cytometry using the Amnis® ImageStream®Mk II (Luminex Corporation, Seattle WA). High sensitivity mode (60X) was used to record 3500 events per sample. Analysis was carried out using IDEAS® software (V6.2), briefly, focused cells were gated according to Gradient RMS_M01_Ch01 (<50). Single cells were gated using Area_M01 vs Aspect Ratio_M01 (size vs circularity). Live cells were gated by using Sytox Blue (Invitrogen) viability dye according to Intensity_MC_Ch07. The population of live, single, focused cells was used for analysis of CholEsteryl BODIPY™ FL C12 according to Intensity_MC_Ch02. Image Display Properties were standardised as follows: X Range [37, 563], Midpoint [300, 127], X Axis Scale [32, 568].

Data Analysis

Drug synergism was evaluated using CompuSyn (download from www.compusyn.com) according to the Chou-Talalay method (Chou, 2008). Data was input as single agent and combined (non-constant ratio) agents as mean ± standard error of the mean (SEM). Pharmacological synergism was interpreted according to the combination index (CI) model. CI plots and data tables are available in Supplementary
Linear regression and statistical analyses were performed using GraphPad® Prism V 8.0 (GraphPad Software, San Diego, CA, USA). Data is represented as mean ± SEM unless stated otherwise. Paired sample analyses were performed using a two-sided Student’s t-test. Multiple-group comparisons were carried out using an analysis of variance (ANOVA) followed by Tukey’s post-hoc test. Statistical significance for two-tailed analyses (p-value) was assigned for values <0.05.
RESULTS

LXRα inhibition combined with mitotane enhances ACC cell death as well as significantly reducing ACC cell metabolic activity, when compared to mitotane alone.

Mitotane produced dose-dependent cytotoxicity in H295R in line with previous findings [15, 27, 28] (Figure 1A; Supplementary Figure 1A) with maximally observed adrenocortical cell death occurring at the therapeutic concentration of 50μM (16mg/L) (Figure 1A). For the mitotane-resistant ACC cell line MUC-1, significant cell death was observed only at the supratherapeutic concentration of 200μM mitotane (64mg/L) (Figure 1C; Supplementary Figure 1D). Consistent with previous findings, cell death in MUC-1 (49.1%, Mitotane 200μM) was significantly lower than that observed for H295R (66.2%, Mitotane 50μM) (Hantel et al., 2016).

Co-treatment of H295R with LXRα inhibitors [GSK2033 (5μM) or SR9243 (1μM)] and mitotane significantly increased cell death at subtherapeutic mitotane concentration of 20μM [16% (Mitotane) versus 39% (GSK2033+Mitotane), p<0.0001; 27% (SR9243+Mitotane), p=0.0308]. This effect was also present for co-treatment of either agent with 40μM mitotane [32% (Mitotane) versus 52% (GSK2033+Mitotane), p<0.0001; versus 47% (SR9243+Mitotane) p=0.0006] and with 50μM mitotane co-treated with SR9243 [66% (Mitotane), 76% (SR9243+Mitotane); p=0.0194] (Figure 1A&B). For MUC-1, LXRα inhibition with SR9243 only increased ACC cell death at mitotane 200μM [46% (Mitotane) versus 69% (SR9243+Mitotane), p<0.0001] (Figure 1C).

A time-course experiment demonstrated significantly reduced cell metabolic activity of H295R and MUC-1 in the presence of 50μM mitotane alone or in combination with LXRα inhibitors (Figure 1D&E) (Supplementary Table 3). This effect was greater in H295R and reached significance at day 3 in H295R [18% (Mitotane), p<0.0001; 15% (GSK2033+Mitotane), p<0.0001; 9% (SR9243+Mitotane) p<0.0001] and at day 7 in MUC-1 [72% (Mitotane), p<0.0001; 58% (GSK2033+Mitotane), p<0.0016; 60% (SR9243+Mitotane), p<0.0089].
We validated these findings of pharmacological LXRα inhibition by transfecting H295R cells with a LXRα dominant-negative construct (LXRα-DN, transfection efficiency: 40%) that blocks LXRα-wildtype (WT) functioning (Supplementary Figure 1G-J). LXRα-DN-expressing cells reached significantly higher rates of mitotane-induced cytotoxicity for all mitotane concentrations, compared to LXRα-WT [11%(WT:Mitotane 20μM) versus 24%(DN:Mitotane 20μM), p=0.0044; 27%(WT:Mitotane 40μM) versus 53% (DN:Mitotane 40μM), p<0.001; 55%(WT:Mitotane 50μM) versus 67%(DN:Mitotane 50μM), p=0.0089] (Figure 1F). These findings suggest an LXRα-mediated mechanism of enhanced mitotane-induced cytotoxicity. We validated these results by stimulating LXRα activity using the endogenous ligand oxysterol 27HC. 27HC reduced mitotane-induced cytotoxicity in H295R at concentrations between 10 and 40μM [32%(Mitotane) versus 27%(Mitotane+27HC 10μM), p=0.265; versus 15%(Mitotane+27HC 20μM), p=0.0002; versus 8%(Mitotane+27HC 40μM), p<0.0001] (Figure 1G).

Pharmacological inhibition of LXRα alone with GSK2033 or SR9243 reduced cell metabolic activity in both cell lines and this effect was similar across all timepoints (Figure 1D&E). LXRα inhibition using GSK2033 but not SR9243 had a moderately induced dose-dependent cell death in H295R but not MUC-1 (Supplementary Figure 1B,C,E,F). We calculated the combination index (CI) using CompuSyn to investigate cytotoxic mechanistic synergy between mitotane and LXRα inhibition. SR9243 demonstrated CI values <1.0 indicative of modest synergy with mitotane for its cytotoxic effects in H295R across all doses [0.7 (SR9243+Mitotane 20μM); 0.95 (SR9243+Mitotane 40μM); 0.64 (SR9243+Mitotane 50μM); 0.59 (GSK2033+Mitotane 20μM); 0.86 (GSK2033+Mitotane 40μM); 0.84 (GSK2033+Mitotane 50μM)] and for MUC-1 at mitotane 200μM only [0.81 (SR9243+Mitotane 200μM)] (Supplementary Figure 1K-M).

The early apoptosis marker AnnexinV increased dose-dependent for all mitotane concentrations in the presence and absence of GSK2033 or SR9243 in H295R (Figure 2A). The combination of mitotane with GSK2033 resulted in higher AnnexinV expression versus 40μM mitotane alone [32% (Mitotane 40μM) versus 50% (GSK2033+Mitotane 40μM), p=0.0004]. Combination with SR9243 demonstrated higher AnnexinV expression versus all mitotane alone [10% (Mitotane 20μM) versus 26% (SR9243+Mitotane 20μM), p=0.0008; 32% (Mitotane 40μM) versus 53% (SR9243+Mitotane 40μM), p<0.0001; 52% (Mitotane...
50μM) versus 67% (SR9243+Mitotane 50μM), p=0.0008]. The late apoptosis marker, cleaved caspase 3 was also increased for mitotane at doses of 40μM and 50μM, reflective of the higher cell death at these mitotane concentrations. Higher cleaved-caspase 3 expression was present for the combination with either LXRα inhibitor versus 40μM or 50μM mitotane alone (Figure 2B; Supplementary Figure 3A&B).

There was also higher expression of the ER stress markers CHOP and XBP-1S/U in mitotane-treated H295R across all concentrations, which increased in combination with either LXRα inhibitor (Figure 2C).

For MUC-1, higher cell death at 200μM mitotane was also reflected in higher expression of AnnexinV, which in turn was significantly higher with LXRα inhibition with SR9234 [3% (VehicleControl) versus 22.6% (Mitotane 50μM), p<0.0001; 22.6% (Mitotane 50μM) versus 39% (SR9243 Mitotane 50μM), p<0.0001)] (Figure 2D).

**LXRα inhibition reduces expression of cholesterol efflux pumps in H295R and MUC-1 ACC cells.**

In the H295R and MUC-1 cell lines, LXRα expression at baseline (Figure 3A&B) was equivalent to expression in the positive control HepG2 hepatocellular carcinoma cells and similar, high expression levels, of the oxysterol synthetic enzyme CYP27A1 were detected (Figure 3A&B). Transfection with a luciferase construct under the control of an LXRα-responsive element (TK-LXRRE-Luc) showed significant constitutive baseline activation of LXRα, which was increased by stimulation with the LXRα agonist T0901317 (1μM) (Figure 3C). These data indicate high activity of the LXRα/oxysterol pathway in adrenal cells, equivalent to that of hepatic tissue.

Expression of LXRα target genes, the FC efflux pumps ABCA1 and ABCG1, were reduced by each LXRα inhibitor in H295R and MUC-1 respectively (Figure 3D-G; Supplementary figure 4A-D). In line with these findings, expression of ABCA1 and ABCG1 increased in both cell lines when stimulated using the non-steroidal LXRα agonist T0901317 (Figure 3D-G). Expression for ABCA1 and ABCG1 also decreased in the presence of the LXRα-DN construct (Figure 3H) confirming direct involvement of LXRα in their expression.

The responses of ABCA1 and ABCG1 in the presence of increasing concentrations of mitotane did not significantly change (Figure 3I&J). However, the efficacy of GSK2033 or SR9243 to reduce ABCA1 and...
ABCG1 expression were attenuated with mitotane treatment in a dose-dependent manner, which is unlikely to reflect a direct effect of mitotane on cholesterol efflux pumps. However, this may be explained by increasing intracellular FC accumulation at higher mitotane doses. We next investigated intracellular cholesterol within this model.

**LXRα inhibition enhances mitotane-induced toxic accumulation of free cholesterol in H295R ACC cells.**

Mitotane exposure resulted in a dose-dependent increase in intracellular FC in H295R, reaching significance at all mitotane concentrations (Figure 4A) [15, 16]. The inhibition of LXRα with GSK2033 and SR9243 significantly increased intracellular FC in H295R at baseline and in combination with mitotane, with higher levels for each dose of mitotane versus mitotane alone (Figure 4A; Supplementary Figure 5A). For MUC-1, only the cytotoxic 200μΜ mitotane concentration resulted in a significant increase in intracellular FC, increased further with SR9243 (Figure 4B). Cell death and intracellular cholesterol correlated for both cell lines: H295R (r²=0.5210, p=0.008) and MUC-1 (r²=0.9299; p=0.0001) (Supplementary Figure 5B&C). While intracellular FC differed in response to mitotane and LXRα inhibition, baseline levels were similar for H295R and MUC-1(Figure 4C&D). Interestingly, intracellular lipid droplet storage of CE was undetectable in MUC-1, when compared to H295R (Figure 4E-G; Supplementary Figure 6 A&B).

We next evaluated mitotane induced cytotoxicity, in the presence and absence of LXRα inhibition, for each cell line under cholesterol loaded cell culture conditions. Cholesterol-loading alone induced a moderate but significant increase in percentage cell death for both H295R [2% (Baseline) versus 15% (Cholesterol Loaded)] and MUC-1 [3% (Baseline) versus 20% (Cholesterol Loaded), p=0.0068] when compared to non-loaded cell culture conditions. Treatment with each LXRα inhibitor resulted in a 2-fold increase in H295R cell death in cholesterol-loaded cells. When combined with mitotane, both pharmacological LXRα inhibitors demonstrated significantly higher cell death for sub-therapeutic doses of mitotane, with maximally observed cell death at 40μΜ (12.4 mg/L) (Figure 4H). Cholesterol loading of MUC-1 resulted in an increased cell death at 100μΜ and 200μΜ mitotane [7%...
(Baseline Mitotane100µM) versus 19% (Cholesterol Loaded Mitotane100µM), \( p<0.011 \) and 200µM (46% (Baseline Mitotane200µM) versus 62% (Cholesterol Loaded Mitotane200µM), \( p<0.003 \)), The LXR\( \alpha \) inverse agonist SR9243 only significantly increased cell death when combined with mitotane at 200µM (Figure 4I).

SOAT-1 expression was significantly reduced by mitotane at doses <40µM in H295R (Supplementary Figure 5D). However, SOAT-1 expression was unaffected by LXR\( \alpha \) inhibition alone in both cell lines (Figure 5E&F), suggesting that intracellular FC is increasing due to the alternative mechanism of reduced efflux pump expression (Figure 3).
Adrenocortical Carcinoma (ACC) has demonstrated resistance to most cytotoxic chemotherapeutic approaches, with mitotane representing the single best pharmacotherapeutic for disease control, however with a limited therapeutic window. Recent work demonstrated the putative adrenotoxic mechanism of mitotane through inhibition of the enzyme SOAT-1, resulting in intracellular FC accumulation and suggests SOAT-1-specific inhibitors as stand-alone therapy or adjuncts to mitotane in the management of ACC (Sbiera et al., 2015, LaPensee et al., 2016). In the study presented herein, we explored LXRα/cholesterol efflux pathway and proposed this as a complementary mechanism to enhance toxic intracellular FC accumulation in ACC. We investigated these effects on two validated in vitro models of ACC: H295R cells which are mitotane-sensitive and MUC-1 a metastatic, mitotane-insensitive cell line (Hantel et al., 2016).

Transcellular cholesterol flux in steroidogenic cells, such as ACC, is central to cellular function and cholesterol provides the principle substrate for steroidogenesis. In steroidogenic cells intracellular cholesterol is (i) stored within lipid droplets following conversion to cholesteryl esters by SOAT-1 (Cignarella et al., 2005, Dove et al., 2006); (ii) provides a substrate for steroidogenesis following transfer to the mitochondrion via the STAR protein (Stocco and Clark, 1996) or (iii) removed from the cells by the cholesterol efflux pumps, ABCA1 or ABCG1 to circulating ApoA1 and HDL, respectively (Kraemer, 2007).

Cholesterol efflux is tightly regulated by the oxysterol/LXRα system through conversion of surplus FC to oxysterols through the action of enzymes such as CYP27A1. In the adrenal the role of LXRα and its ligand oxysterols are of key relevance in all of these processes. This was highlighted by Cummins et al who demonstrated the contribution of oxysterol/LXRα to (i) regulating steroidogenesis, (ii) controlling adrenocortical cholesterol metabolism and (iii) acting as a “safety valve” through the ABCA1/ABCG1-mediated efflux pathway, to provide a basal protective mechanism in preventing free-cholesterol induced adrenal lipotoxicity (Cummins, 2006).
Our data shows that pharmacological inhibition or functional blockade of LXRα significantly reduced cholesterol efflux pump expression (ABCA1 and ABCG1) and is accompanied by higher intracellular FC concentrations, ER stress, apoptosis and cell death markers. Using the combined therapeutic approach, the mitotane-sensitive H295R underwent apoptosis and cell death at subtherapeutic mitotane concentrations. However, MUC-1, while combined mitotane/LXRα inhibition considerably enhanced the cytotoxic effect of mitotane alone, they retained their resistance to pharmacologically acceptable mitotane concentrations below 200µM. In both cell lines, higher intracellular FC levels were associated with increased cell death and when challenged with a cholesterol-loaded extracellular environment, the cytotoxic effect of mitotane alone and in combination with LXRα inhibition was further enhanced. These findings support our overarching hypothesis, and are also in line with the findings of other groups (Sbiera and La Pensee) who have investigated SOAT-1 inhibition (Sbiera et al., 2015, LaPensee et al., 2016). While H295R were very sensitive to combined LXRα/mitotane treatment and MUC-1 demonstrated enhanced sensitivity to the combination at high mitotane doses, MUC-1 gave disappointing results in terms of a therapeutic strategy. However, when taken together, we believe our data convincingly support a role for LXRα and cholesterol storage/efflux in ACC cell adaptation and survival.

It is unclear why MUC-1 are insensitive to LXRα/mitotane treatment. MUC-1 were derived from a metastatic neck lesion in a young, mitotane resistant, patient and for whom four cycles of combination chemotherapy were unsuccessful in limiting the extent disease spread. When we evaluated intracellular cholesterol in MUC-1 we demonstrated an almost complete absence of stored cholesteryl esters (CE) at baseline and even under cholesterol-loaded conditions (data not shown). In contrast, mitotane-sensitive H295R supported a rich CE store within their intracellular lipid droplets under baseline and cholesterol-loaded culture conditions. Yet in spite of markedly different intracellular CE stores, intracellular FC was similar for both cell lines. The difference in intracellular cholesterol handling between MUC-1 and H295R is interesting particularly in the context of the putative mechanism of mitotane activity through SOAT-1. We did not perform a SOAT-1 activity assay however, the reduced storage of CE in MUC-1 in the presence of similar FC suggests low baseline SOAT-1 activity, irrespective of the expression of this enzyme. This also suggests that, in contrast to H295R, MUC-1 favour alternative mechanism(s) of regulation and clearance of
intracellular cholesterol, providing a possible escape mechanism from mitotane-induced lipotoxicity. Seidel et al have described similar findings in relation to FC and CE handling in a strain of HAC15 with induced resistance to mitotane (Seidel et al., 2020). Detailed evaluation of lipid/cholesterol metabolism in ACC, using the full range of cell lines and banked tumour samples is necessary to evaluate the significance for development of future therapeutic strategies.

In the presence of increasing FC accumulation, higher mitotane doses attenuated the inhibitory effect of LXRα blockade on cholesterol efflux pump expression in H295R. In our study mitotane alone had no significant effect on either ABCA1 or ABCG1 expression. Sbeira et al previously demonstrated a short-lived decrease in ABCG1 which quickly increased back to baseline with prolonged therapy. It is therefore unlikely that this is a direct pharmacodynamic effect of mitotane. It is possible a secondary effect of intracellular FC accumulation which is a substrate for catalysis to 27HC by CYP27A1 and will compete with the LXRα antagonists. In line with this observation, we have also demonstrated that stimulating LXRα with 27HC, reduces the therapeutic efficacy of mitotane. Whether or not this represents a mechanism whereby ACC cells can overcome long-term susceptibility to mitotane needs more detailed investigation.

The role of LXRα in supporting the cancer microenvironment and promoting cancer cell survival is established in other human cancer models (Kim et al., 2018; Wang et al., 2019). Higher tumour grade and enhanced metastasis in breast cancer has been associated with higher LXRα expression and activation (Nelson et al., 2013). Work by Flaveny et al also demonstrated that the LXRα inverse agonist SR9243 induced apoptosis across models of lung, colon and prostate cancer (Flaveny et al., 2015). Other work has demonstrated the potential for LXRα inhibition to induce lipotoxicity, both through FC accumulation (Schuster et al., 2002; Bradley et al., 2007).

We propose that, based on our date, the use of LXRα inhibitors can be translated into a therapeutic strategy for mitotane-sensitive ACC by adopting a complementary approach to one of mitotane’s known mechanisms of action: intracellular FC induced lipotoxicity. We show that enhanced FC accumulation within H295R, in response to LXRα blockade increased the cytotoxicity of mitotane at half the usual minimum therapeutic dose. The main limitation to mitotane’s clinical use is (i) no significant...
improvements in progression or recurrence-free survival of disease below 14mg/L (Terzolo et al., 2013, Berruti et al., 2017) and (ii) poor patient tolerability, with significantly impact on quality of life or indeed their ability to continue therapy above 20mg/mL (Haak et al., 1994). Recent data have suggested that adjunctive mitotane should continue indefinitely even in individuals with Ro resection without tumour recurrence (Berruti et al., 2017). Therefore, the development of strategies to facilitate the longterm tolerability of this drug is an important clinical consideration. In relation to mitotane-resistant disease, we demonstrated increased cell death for MUC-1 in response to combined mitotane and \textit{LXR\alpha} inhibition, but show that mitotane-resistance was not overcome. Therefore, while adopting this combination strategy offers promise in mitotane-sensitive disease and to enhance adjuvant mitotane, it is unlikely to be of benefit in disease that is mitotane resistant. However, the differences in cholesterol handling between mitotane-sensitive and mitotane-resistant ACC cells demonstrates the merits of better understanding the adaptation of ACC cells to facilitate metastasis and cell survival. Finally, we propose that the selective induction of high cholesterol uptake or selective delivery of cholesterol to ACC cells as a combination therapy represents an interesting challenge for future translational research.

In summary, two cell lines representing some of the best validated human cell culture models of ACC, the mitotane-resistant metastatic MUC-1 cell line and the mitotane-sensitive H295R, representing the standard for early mechanistic/therapeutic investigation, were used to investigate \textit{LXR\alpha} inhibitors and mitotane’s effect to induce adrenal cytotoxicity (Rainey et al., 1994, Bird et al., 1995, Wang and Rainey, 2012, Hantel et al., 2016). Our data suggest a complementary pathway to enhance mitotane-induced cytotoxicity by preventing FC efflux from ACC cells mediated through blockade of the \textit{LXR\alpha} pathway and enhanced by cholesterol loading. Therefore, it is reasonable to conclude that the mitotane-\textit{LXR\alpha} inhibition combined therapeutic approach exploits separate but complementary and synergistic mechanisms to accumulate toxic levels of FC and to induce ACC cytotoxicity. The findings provide valuable mechanistic insights and an important backdrop to support further animal and pre-clinical studies of the effects of \textit{LXR\alpha} inhibition as a therapeutic target for ACC in mitotane-sensitive disease. Of the two pharmacological inhibitors chosen for investigation, the inverse agonist SR9243 is well tolerated in animals and has a favourable toxicity profile (Flaveny et al., 2015, Wu et al., 2019). This drug therefore presents good
translational feasibility in future \textit{in vivo} studies. We also highlight the ongoing challenge of approaching effective strategies for drug-resistant metastatic disease, and highlight the importance of better understanding cholesterol metabolism in ACC cells in this regard.
**FIGURE LEGENDS**

**Figure 1**

(A) Graphical representation of percentage cell death using pharmacological inhibitors of LXRα, GSK2033 (5μM) and SR9243 (1μM) to potentiate mitotane killing in H295R as quantified by flow cytometric analysis and (B) microscopy. (C) Graphical representation of percentage cell death using SR9243 (5μM) to potentiate mitotane killing in MUC-1 as quantified by flow cytometric analysis. Graphical representation of percentage metabolic activity of (D) H295R and (E) MUC-1 following GSK2033 (5μM) and SR9243 (5μM) alone and in combination with 50μM mitotane at day 3, 7, 10 and 14 as quantified by MTT assay and adjusted to matched vehicle control. (F) Graphical representation of percentage cell death following transfection with LXRα dominant negative (LXR-DN) versus LXRα wild type (LXR-WT) in combination with mitotane in H295R cells. (G) Graphical representation of percentage dead cells following 27HC treatment in combination with mitotane in H295R.

*Viability data represented as percentage dead cells relative to vehicle control. Metabolic activity data represented as percentage cells relative to vehicle control (adjusted to 100% at each respective time point). All data shown as mean ± SEM, n=4. Statistical significance is denoted as *p<0.05, **p<0.01, ***p<0.01, ****p<0.001 relative to vehicle control or as otherwise indicated.*

**Figure 2**

(A) Graphical representation of percentage AnnexinV positive cells showing an increase in apoptosis following GSK2033 (5μM) or SR9243 (1μM) alone and in combination with mitotane treatment in H295R as quantified by flow cytometric analysis. (B) Western blotting image represents pro-caspase-3, cleaved caspase-3 and β actin expression following GSK2033 (5μM) or SR9243 (1μM) alone and in combination with mitotane treatment in H295R. (C) Electrophoresis gel image representing XPB1 unspliced, XBP1 spliced, CHOP and β actin mRNA expression following GSK2033 (5μM) or SR9243 (1μM) alone and in combination with mitotane treatment in H295R. (D) Graphical representation of percentage Annexin V positive cells showing an increase in apoptosis in SR9243 (5μM) versus mitotane alone in MUC-1 as quantified by flow cytometric analysis.
Western blot image is representative of 3 independent experiments. Data are represented as mean ± S.E.M. (n=4), statistical comparisons were performed using two-way ANOVA followed by Tukey's post-hoc analysis. Statistical significance is denoted as *p<0.05, **p<0.01, ***p<0.01, ****p<0.001 relative to vehicle control or as otherwise indicated.

**Figure 3**

(A) Western blot images demonstrating protein expression of LXRc and (B) CYP27A1 in H295R, MUC-1 and HepG2 cell lines relative to β-actin. (C) Graphical representation of luciferase activity in relative luciferase units (firefly/renilla) at baseline and following LXRα agonist T0901317 (1μM) stimulation in transient transfected H295R cells with TK-LXRRE-Luc as reporter assay and a renilla luciferase control plasmid. (D) Graphical representation of ABCA1 and ABCG1 mRNA expression as fold change relative to vehicle control following GSK2033, SR9243 and T0901317 stimulation in H295R and (F&G) MUC-1. (H) Graphical representation of ABCA1 and ABCG1 mRNA expression as fold change relative to wild type (WT) transfected versus LXRα dominant negative (DN) transfected H295R. (I) Graphical representation of ABCA1 and (J) ABCG1 mRNA expression as fold change relative to vehicle control (VC) following GSK2033 (5μM) and SR9234 (1μM) treatment alone and combination with mitotane in H295R.

Western blot image is representative of 3 independent experiments. Data are represented as mean ± S.E.M. (n=4), statistical comparisons were performed using a two-tailed t-test or a two-way ANOVA followed by Tukey’s post-hoc analysis. Statistical significance is denoted as *p<0.05, **p<0.01, ***p<0.01, ****p<0.001 relative to vehicle control or as otherwise indicated.

**Figure 4**

(A) Graphical representation of FC (filipin fluorescence), quantified by flow cytometry following combination treatments in H295R and (B) MUC-1. (C) Flow cytometry histograms demonstrating baseline FC (filipin fluorescence) levels in H295R and MUC-1 relative to respective unstained control. (D) Graphical representation of ABCA1 and ABCG1 mRNA expression as fold change relative to vehicle control (VC) following GSK2033 (5μM) and SR9234 (1μM) treatment alone and combination with mitotane in H295R.
representation of FC resolution metric in H295R and MUC-1. (E) Flow cytometry histograms demonstrating baseline cholesteryl ester fluorescence in H295R and MUC-1 relative to respective unstained control. (F) Graphical representation of cholesteryl ester resolution metric in H295R and MUC-1 cells. (G) Representative images (60X) of H295R and MUC-1 of brightfield and cholesteryl ester fluorescence captured via Amnis ImageStream® Imaging Flow Cytometry. (H) Graphical representation of percentage dead cells following combination treatments in the presence of cholesterol (45μg/ml), inset graph indicating percentage death in the presence and absence of cholesterol in H295R and (I) in MUC-1.

Data are represented as mean ± S.E.M. (n=3), statistical comparisons were performed using a two-tailed t-test or a two-way ANOVA followed by Tukey's post-hoc analysis. Statistical significance is denoted as *p<0.05, **p<0.01, ***p<0.001 relative to vehicle control or as otherwise indicated. Images are representative of approx. 2000 focused, single, live cells.

Supplementary Figure 1

Graphical representation of percentage cell death in a dose dependent manner following treatment mitotane, GSK2033 and SR9243 (A-C) in H295R and (D-F) MUC-1. (G) pCMX-hLXRα dominant negative (LXR-DN) versus wild type (LXR-WT) validation using T0901319 stimulation evaluated via luciferase assay. (H) LXR domain structure and sequence (I) Graphical representation of percentage GFP positive cells following transfection versus lipofectamine only control and corresponding GFP (J) Microscopy (10X). (K-L) Compusyn synergism analysis demonstrates combination index plots (CI>1 = Synergism) following (K) GSK2033 (5μM) and mitotane (20, 40, 50μM) in H295R, (L) SR9243 (1μM) and Mitotane (20, 40, 50μM) in H295R and (M) SR9243 (5μM) and Mitotane (50, 100, 200μM) in MUC-1 with corresponding data tables.
Data are represented as mean ± S.E.M. (n=4), statistical comparisons were performed using two-way ANOVA followed by Tukey’s post-hoc analysis. Statistical significance is denoted as *p<0.05, **p<0.01, ***p<0.001 relative to vehicle control or as otherwise indicated.

Supplementary Figure 2

(A) Flow cytometry gating strategy for H295R and MUC-1. (B) AnnexinV/PI dot plots for representative viability assays for H295R and (C) MUC-1.

Supplementary Figure 3

(A) Additional western blots representing procaspase-3, cleaved caspase-3 and beta actin expression following combination treatments in H295R. (B) Semi-quantified analysis carried out using ImageJ software plotted in corresponding graph. Data were normalised based on β actin loading control.

Data are represented as mean ± S.E.M. statistical comparisons were performed statistical comparisons were performed using two-way ANOVA followed by Tukey’s post-hoc analysis. Statistical significance is denoted as ****p<0.001 relative to vehicle control.

Supplementary Figure 4

(A) Graphical representation of ABCA1 and ABCG1 mRNA expression following 24 hours treatment of GSK2033 (1, 5, 10μM) and (B) SR9243 (0.1, 1μM) relative to vehicle control in H295R (C) Graphical representation of ABCA1 and ABCG1 mRNA expression following 24 hours treatment of GSK2033 (1, 5μM) and (B) SR9243 (1, 5, 10μM) relative to vehicle control in MUC-1.

Data are represented as mean ± S.E.M. Statistical comparisons were performed using two-way ANOVA followed by Tukey’s post-hoc analysis. Statistical significance is denoted as ****p<0.001 relative to vehicle control.
Supplementary Figure 5

(A) Representative fluorescent microscopy pictures showing FC accumulation with flipin staining (blue) following treatment with LXRα inhibitors (GSK2033 and SR9243) and mitotane in H295R and MUC-1. (B) Linear regression analysis evaluating cell death and FC correlation. Data represented as mean values and 95% confidence interval accompanied by the correlation coefficient $r^2$ and $p$ value in H295R and (C) MUC-1. (D) Graphical representation of SOAT-1 mRNA expression as fold change relative to vehicle control following mitotane treatment in a dose dependent manner in H295R. (E) Graphical representation of SOAT-1 mRNA expression as fold change relative to vehicle control following GSK2033 (5μM) and SR9243 (1μM) in H295R and (F) MUC-1.

Data are represented as mean ± S.E.M or mean ± 95% confidence intervals (5B&C). Statistical comparisons were performed using two-way ANOVA followed by Tukey's post-hoc analysis. Linear regression analysis was performed to evaluate correlation coefficient.

Supplementary Figure 6

ImageStream gating strategy (A) H295R and (B) MUC-1. Cells were evaluated on a Gradient RMS_M01_Ch01 histogram to gate focused cells (approx<50), Area_M01 vs Aspect Ratio Intensity_M01_Ch01 was used to identify single cells followed by Live/Dead discrimination using SytoxBlue (Intensity_MC_Ch07). BODIPY Cholesteryl Ester fluorescence (Intensity_MC_Ch02) is represented via histogram and a selection (in order of acquisition) of brightfield (Ch01) and Ch02 images.

Supplementary Table 1

Primer sequences for gene expression analysis.

Supplementary Table 2
Details of antibodies used in the study.

**Supplementary Table 3**

Results tabulated from metabolic activity study for H295R and MUC-1. The following drug treatments were evaluated at day 3, 7, 10 and 14: vehicle control, GSK2033 (5μM), SR9243 (5μM), mitotane (50μM), GSK2033 + mitotane and SR9243 + mitotane. Data is represented as % metabolic activity (Data adjusted using vehicle control (100%) at each respective time point) ± SEM and statistical output generated using GraphPad Prism.
Conflict of Interest

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
Bibliography

BERRUTI, A., GRISANTI, S., PULZER, A., CLAPS, M., DAFFARA, F., LOLI, P., MANNELLI, M., BOSCARO, M., ARVAT, E., TIBERIO, G., et al. 2017. Long-Term Outcomes of Adjuvant Mitotane Therapy in Patients With Radically Resected Adrenocortical Carcinoma. *J Clin Endocrinol Metab*, 102, 1358-1365.

BIRD, I. M., MATHIS, J. M., MASON, J. I. & RAINEY, W. E. 1995. Ca(2+)-regulated expression of steroid hydroxylases in H295R human adrenocortical cells. *Endocrinology*, 136, 5677-84.

BRADLEY, M. N., HONG, C., CHEN, M., JOSEPH, S. B., WILPITZ, D. C., WANG, X., LUSIS, A. J., COLLINS, A., HSEUH, W. A., COLLINS, J. et al. 2007. Ligand activation of LXR beta reverses atherosclerosis and cellular cholesterol overload in mice lacking LXR alpha and apoE. *J Clin Invest*, 117, 2337-46.

BRANDO, B. & SOMMARUGA, E. 1993. Nationwide quality control trial on lymphocyte immunophenotyping and flow cytometer performance in Italy. *Cytometry*, 14, 294-306.

BRUSCHI, F. V., CLAUDELI, T., TARDELLI, M., STARLINGER, P., MARRA, F. & TRAUNER, M. 2019. PNPLA3 I148M Variant Impairs Liver X Receptor Signalling and Cholesterol Homeostasis in Human Hepatic Stellate Cells. *Hepatol Commun*, 3, 1191-1204.

CHEN, L. D., KUSHWAHA, R. S., MCGILL, H. C., JR., RICE, K. S. & CAREY, K. D. 1998. Effect of naturally reduced ovarian function on plasma lipoprotein and 27-hydroxycholesterol levels in baboons (Papio sp.). *Atherosclerosis*, 136, 89-98.

CHOU, T. C. 2008. Preclinical versus clinical drug combination studies. *Leuk Lymphoma*, 49, 2059-80.

CIGNARELLA, A., ENGEL, T., VON ECKARDSTEIN, A., KRATZ, M., LORKOWSKI, S., LUEKEN, A., ASSMANN, G. & CULLEN, P. 2005. Pharmacological regulation of cholesterol efflux in human monocyte-derived macrophages in the absence of exogenous cholesterol acceptors. *Atherosclerosis*, 179, 229-36.

COSTET, P., LUO, Y., WANG, N. & TALL, A. R. 2000. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem*, 275, 28240-5.

CUMMINS, C. L., VOLLE, D. H., ZHANG, Y., MCDONALD, J. G., SION, B., LEFRANCOIS-MARTINEZ, A. M., CAIRA, F., VEYSSIERE, G., MANGELSDORF, D. J. & LOBACCARO, J. M. 2006. Liver X receptors regulate adrenal cholesterol balance. *J Clin Invest*, 116, 1902-12.

DE REYNIES, A., ASSIE, G., RICKMAN, D. S., TISSIER, F., GROUSSIN, L., RENE-CORAIL, F., DOUSSET, B., BERTAGNA, X., CLAUSER, E. & BERTHERAT, J. 2009. Gene expression profiling reveals a new classification of adrenocortical tumors and identifies molecular predictors of malignancy and survival. *J Clin Oncol*, 27, 1108-15.

DI, D., WANG, Z., LIU, Y., LUO, G., SHI, Y., BERGGREN-SODERLUND, M., NILSSON-EHLE, P., ZHANG, X. & XU, N. 2012. ABCA1 upregulating apolipoprotein M expression mediates via the RXR/LXR pathway in HepG2 cells. *Biochem Biophys Res Commun*, 421, 152-6.

DOVE, D. E., SU, Y. R., SWIFT, L. L., LINTON, M. F. & FAZIO, S. 2006. ACAT1 deficiency increases cholesterol synthesis in mouse peritoneal macrophages. *Atherosclerosis*, 186, 267-74.

ENGEL, T., KANNENBERG, F., FOBKER, M., NOFER, J. R., BODE, G., LUEKEN, A., ASSMANN, G. & SEEDORF, U. 2007. Expression of ATP binding cassette-transporter ABCG1 prevents cell death by transporting cytotoxic 7beta-hydroxycholesterol. *FEBS Lett*, 581, 1673-80.

FASSNACHT, M., DEKKERS, O., ELSE, T., BAUDIN, E., BERRUTI, A., DE KRIJGER, R. R., HAAK, H. R., MIHAI, R., ASSIE, G. & TERZOLO, M. 2018. European Society of Endocrinology Clinical Practice Guidelines on the Management of Adrenocortical
Carcinoma in Adults, in collaboration with the European Network for the Study of Adrenal Tumors. *Eur J Endocrinol*.

FASSNACHT, M., KROISS, M. & ALLOLIO, B. 2013. Update in adrenocortical carcinoma. *Clin Endocrinol Metab*, 98, 4551-64.

FEDOROVA, O. V., ZERNETKINA, V. I., SHILOVA, V. Y., GRIGOROVA, Y. N., JUHASZ, O., WEI, W., MARSHALL, C. A., LAKATTA, E. G. & BAGROV, A. Y. 2015. Synthesis of an Endogenous Steroidal Na Pump Inhibitor Marinobufagenin, Implicated in Human Cardiovascular Diseases, Is Initiated by CYP27A1 via Bile Acid Pathway. *Circ Cardiovasc Genet*, 8, 736-45.

FLAVENY, C. A., GRIFFETT, K., EL-GENDY BEL, D., KAZANTZIS, M., SENGUPTA, M., AMELIO, A. L., CHATTERJEE, A., WALKER, J., SOLT, L. A., KAMENECKA, T. M. et al. 2015. Broad Anti-tumor Activity of a Small Molecule that Selectively Targets the Warburg Effect and Lipogenesis. *Cancer Cell*, 28, 42-56.

FU, X., MENKE, J. G., CHEN, Y., ZHOU, G., MACNAUL, K. L., WRIGHT, S. D., SPARROW, C. P. & LUND, E. G. 2001. 27-hydroxycholesterol is an endogenous ligand for liver X receptor in cholesterol-loaded cells. *J Biol Chem*, 276, 38378-87.

GRIFFETT, K. & BURRIS, T. P. 2016. Promiscuous activity of the LXR antagonist GSK2033 in a mouse model of fatty liver disease. *Biochem Biophys Res Commun*, 479, 424-428.

HAAK, H. R., HERMANS, J., VAN DE VELDE, C. J., LENTJES, E. G., GOSLINGS, B. M., FLEUREN, G. J. & KRANS, H. M. 1994. Optimal treatment of adrenocortical carcinoma with mitotane: results in a consecutive series of 96 patients. *Br J Cancer*, 69, 947-51.

HANTEL, C., SHAPIRO, I., POLI, G., CHIAPPONI, C., BIDLINGMAIER, M., REINCKE, M., LUCONI, M., JUNG, S. & BEUSCHLEIN, F. 2016. Targeting heterogeneity of adrenocortical carcinoma: Evaluation and extension of preclinical tumor models to improve clinical translation. *Oncotarget*, 7, 79292-79304.

HASSALL, D. G. & GRAHAM, A. 1995. Changes in free cholesterol content, measured by filipin fluorescence and flow cytometry, correlate with changes in cholesterol biosynthesis in THP-1 macrophages. *Cytometry*, 21, 352-62.

HERMSEN, I. G., FASSNACHT, M., TERZOLO, M., HOUTERMAN, S., DEN HARTIGH, J., LEBOUILLEX, S., DAFFARA, F., BERRUTI, A., CHADAREVIAN, R., SCHLUMBERGER, M., et al. 2011. Plasma concentrations of o,p'DDD, o,p'DDA, and o,p'DDE as predictors of tumor response to mitotane in adrenocortical carcinoma: results of a retrospective ENS@T multicenter study. *J Clin Endocrinol Metab*, 96, 1844-51.

HESCOT, S., SLAMA, A., LOMBES, A., PACI, A., REMY, H., LEBOUILLEX, S., CHADAREVIAN, R., TRABADO, S., AMAZIT, L., YOUNG, J., et al. 2013. Mitotane alters mitochondrial respiratory chain activity by inducing cytochrome c oxidase defect in human adrenocortical cells. *Endocr Relat Cancer*, 20, 371-81.

HSIEH, K., LEE, Y. K., LONDOS, C., RAAKA, B. M., DALEN, K. T. & KIMMEL, A. R. 2012. Perilipin family members preferentially sequester to either triacylglycerol-specific or cholesteryl-ester-specific intracellular lipid storage droplets. *J Cell Sci*, 125, 4067-76.

HU, X., LI, S., WU, J., XIA, C. & LALA, D. S. 2003. Liver X receptors interact with corepressors to regulate gene expression. *Mol Endocrinol*, 17, 1019-26.

HUUSKONEN, J., FIELDING, P. E. & FIELDING, C. J. 2004. Role of p160 coactivator complex in the activation of liver X receptor. *Arterioscler Thromb Vasc Biol*, 24, 703-8.

JANOWSKI, B. A., WILLY, P. J., DEVI, T. R., FALCK, J. R. & MANGELSDORF, D. J. 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*, 383, 728-31.

KENNEDY, M. A., BARRERA, G. C., NAKAMURA, K., BALDAN, A., TARR, P., FISHEB, M. C., FRANK, J., FRANCON, O. L. & EDWARDS, P. A. 2005. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab*, 1, 121-31.
Kerkhofs, T. M., Baudin, E., Terzolo, M., Allolio, B., Chadarevian, R., Muller, H. H., Skogseid, B., Leboullieux, S., Mantero, F., Haak, H. R. et al. 2013. Comparison of two mitotane starting dose regimens in patients with advanced adrenocortical carcinoma. J Clin Endocrinol Metab, 98, 4759-67.

Kim, S., Lee, M., Dhanasekaran, D. N. & Song, Y. S. 2018. Activation of LXRa/beta by cholesterol in malignant ascites promotes chemoresistance in ovarian cancer. BMC Cancer, 18, 1232.

Kraemer, F. B. 2007. Adrenal cholesterol utilization. Mol Cell Endocrinol, 265-266, 42-5.

Lapensee, C. R., Mann, J. E., Rainey, W. E., Cruzo, V., Hunt, S. W., 3rd & Hammer, G. D. 2016. ATR-101, a Selective and Potent Inhibitor of Acyl-CoA Acyltransferase 1, Induces Apoptosis in H295R Adrenocortical Cells and in the Adrenal Cortex of Dogs. Endocrinology, 157, 1775-88.

Lehmann, T. P., Wrzesinski, T. & Jagodzinski, P. P. 2013. The effect of mitotane on viability, steroidogenesis and gene expression in NCI-H295R adrenocortical cells. Mol Med Rep, 7, 893-900.

Libe, R. 2019. Clinical and molecular prognostic factors in adrenocortical carcinoma. Minerva Endocrinol, 44, 58-69.

Montgomery, D. A. & Welbourn, R. B. 1965. Adrenocortical Carcinoma Treated with O,P'-Ddd. Br Med J, 1, 1356-8.

Nelson, E. R., Warren, S. E., Jasper, J. S., Park, S., Suchindran, S., Howe, M. K., Carver, N. J., Pillai, R. V., Sullivan, P. M., Sondhi, V., et al. 2013. Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. Science, 342, 1094-8.

Nilsson, M., Stulin, T. M., Lin, C. Y., Yeo, A. L., Nowotny, P., Liu, E. T. & Steffenken, K. R. 2007. Liver X receptors regulate adrenal steroidogenesis and hypothalamic-pituitary-adrenal feedback. Mol Endocrinol, 21, 126-37.

Phealan, C. A., Weaver, J. M., Steger, D. J., Joshi, S., Maslany, J. T., Collins, J. L., Zuercher, W. J., Willson, T. M., Walker, M., Jaye, M. et al. 2008. Selective partial agonism of liver X receptor alpha is related to differential corepressor recruitment. Mol Endocrinol, 22, 2241-9.

Pommier, R. F. & Brennan, M. F. 1992. An eleven-year experience with adrenocortical carcinoma. Surgery, 112, 963-70; discussion 970-1.

Rainey, W. E., Bird, I. M. & Mason, J. I. 1994. The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. Mol Cell Endocrinol, 100, 45-50.

Sbiera, S., Leich, E., Liebisch, G., Sbiera, I., Schirbel, A., Wiemer, L., Matysik, S., Eckhardt, C., Gardill, F., Gehl, et al. 2015. Mitotane Inhibits Sterol-O-Acyl Transferase 1 Triggering Lipid-Mediated Endoplasmic Reticulum Stress and Apoptosis in Adrenocortical Carcinoma Cells. Endocrinology, 156, 3895-908.

Schuster, G. U., Parini, P., Wang, L., Alberti, S., Steffenken, K. R., Hansson, G. K., Angelin, B. & Gustafsson, J. A. 2002. Accumulation of foam cells in liver X receptor-deficient mice. Circulation, 106, 1147-53.

Seidel, E., Wala, C., Messerschmidt, C., Obermayer, B., Peitzsch, M., Wallace, P. W., Bahethi, R., Yoo, T., Choi, M., Schrade, P., et al. 2020. Generation and characterization of a mitotane-resistant adrenocortical cell line. Endocrinology, 156, 3895-908.
al. 2007. Adjuvant mitotane treatment for adrenocortical carcinoma. N Engl J Med, 356, 2372-80.

TERZOLO, M., BAUDIN, A. E., ARDITO, A., KROISS, M., LEOULLEUX, S., DAFFARA, F., PEROTTI, P., FEELDERS, R. A., DEVRIES, J. H., ZAGGIA, B., et al. 2013. Mitotane levels predict the outcome of patients with adrenocortical carcinoma treated adjuvantly following radical resection. Eur J Endocrinol, 169, 263-70.

WANG, K., XU, T., RUAN, H., XIAO, H., LIU, J., SONG, Z., CAO, Q., BAO, L., LIU, D., WANG, C., et al. 2019. LXRalpha promotes cell metastasis by regulating the NLRP3 inflammasome in renal cell carcinoma. Cell Death Dis, 10, 159.

WANG, T. & RAINEY, W. E. 2012. Human adrenocortical carcinoma cell lines. Mol Cell Endocrinol, 351, 58-65.

WANG, X., COLLINS, H. L., RANALLETTA, M., FUKI, I. V., BILLHEIMER, J. T., ROTHBLAT, G. H., TALL, A. R. & RADER, D. J. 2007. Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. J Clin Invest, 117, 2216-24.

WILLY, P. J., UMESONO, K., ONG, E. S., EVANS, R. M., HEYMAN, R. A. & MANGELSDORF, D. J. 1995. LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev, 9, 1033-45.

WU, G., WANG, Q., XU, Y., LI, J., ZHANG, H., QI, G. & XIA, Q. 2019. Targeting the transcription factor receptor LXR to treat clear cell renal cell carcinoma: agonist or inverse agonist? Cell Death Dis, 10, 416.
Figure 2

A

H295R

% Apoptotic cells (AnnexinV positive)

Mitotane (μM)

0 20 40 50

B

Mitotane

+ GSK2033

+ SR9243

Procaspe 3

Cleaved Caspase 3

B Actin

C

Vehicle

GSK2033

Mitotane

+ GSK2033

+ SR9243

D

MUC-1

% Apoptotic cells (AnnexinV Positive)

Mitotane (μM)

0 50 100 200

XBP1 unspliced

XBP1 spliced

CHOP

B Actin

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Supplementary Figure 2

A Flow Cytometry Gating Strategy

B H295R

Vehicle

GSK2033 5 μm

Propidium Iodide

SR9243 1 μm

AnnexinV-FITC

C MUC1

Vehicle

Propidium Iodide

SR9243 5 μm

AnnexinV-FITC
Supplementary Figure 3

A

Procaspe 3
Cleaved Caspe 3
B Actin

Procaspe 3
Cleaved Caspe 3
B Actin

B

Relative Expression (AU)

Mitotane (µM)

Vehicle
GSK2033
SR9243

Cleaved Caspase-3

****

*
Supplementary Figure 4

A

H295R

mRNA Fold change vs vehicle treated control

ABCA1  ABCG1

0 1 5 10  0 1 5 10

GSK2033 (µM)

***

B

H295R

mRNA Fold change vs vehicle treated control

ABCA1  ABCG1

0.1  1 0.1  1

SR9243 (µM)

***

****

C

MUC1

mRNA Fold change vs vehicle treated control

ABCA1  ABCG1

0 1 5 0 1 5

GSK2033 (µM)

*

*

D

MUC1

mRNA Fold change vs vehicle treated control

ABCA1  ABCG1

0 1 5 10  0 1 5 10

SR9243 (µM)

*
Supplementary Figure 5

A

H295R

Vehicle

20 μM Mitotane

40 μM Mitotane

60 μM Mitotane

GSK2033

6 μm

SR9243

1 μm

MUC1

Vehicle

80 μM Mitotane

100 μM Mitotane

200 μM Mitotane

SR9243

6 μm

B

H295R

Free Cholesterol (Filipin Staining Metric)

% Death

r² = 0.5210
p = 0.0080

C

MUC1

Free Cholesterol (Filipin Staining Metric)

% Death

r² = 0.9299
p = 0.0001

D

H295R

SOAT1 mRNA Expression

Mitotane (μM)

E

H295R

SOAT1 mRNA Expression

Vehicle GSK2033 SR9243

F

MUC1

SOAT1 mRNA Expression

Vehicle GSK2033 SR9243
Supplementary Figure 6

ImageStream Gating Strategy

A H295R

Focused → Single Cells → Live Cells

B MUC1

Focused → Single Cells → Live Cells

Image Display Properties: X Range (27, 663), Midpoint (300, 127), X Axis Scale (32, 568)
**Supplementary Table 1**

| GENE     | GenBank® Accession No. | Direction  | Primer Sequence       |
|----------|------------------------|------------|-----------------------|
| ABCA1    | NM_013454.3            | Forward    | GTGTTTCTGGATGAACCC    |
|          |                        | Reverse    | TTCCATTGACCATGATTGC   |
| ABCG1    | NM_004915.3            | Reverse    | AGGGAGATGAAGAAATCCC   |
| SOAT1    | NM_001252511.1         | Reverse    | TTCAAGGACAGTGCTGACG   |
| DDIT3 (CHOP) | NM_004083  | Reverse    | GATTCCTTCCTCTCTTTCCAG |
|          |                        | Forward    | GGGAATGAAGTGAGGCCAG   |
| XBP1     | NM_005080.3            | Reverse    | TGAAGAGTCAATACCGCCA   |
### Supplementary Table 2

| Antigen                  | UniProt® Accession No. | Source & Type | Dilution | Company                        |
|--------------------------|------------------------|---------------|----------|--------------------------------|
| Pro Caspase 3            | P42574                 | Rabbit IgG    | 1:1000   | Cell Signalling Technology     |
| Cleaved Caspase 3        | P42574                 | Rabbit IgG    | 1:1000   | Cell Signalling Technology     |
| Liver X Receptor         | Q13133                 | Mouse IgG2b   | 1:1000   | Santa Cruz Biotechnology       |
| CYP27A1                  | Q02318                 | Rabbit IgG    | 1:1000   | Abcam                          |
| B Actin                  | P60709                 | Mouse IgG2a   | 1:5000   | Sigma                          |
| Cat No.  |
|---------|
| 9662    |
| 9661s   |
| 271064  |
| 64889   |
| A5316   |
### Supplementary Table 3

| Treatment                  | Day | % Metabolic Activity | 95.00% CI of diff. | Adjusted p Value | % Metabolic Activity | 95.00% CI of diff. | Adjusted p Value |
|----------------------------|-----|----------------------|--------------------|------------------|----------------------|--------------------|------------------|
| H295R                      |     |                      |                    |                  | MUC-1                |                    |                  |
| Vehicle Control            | 3   | 100.02 ± 1.98        | -                  | -                | 99.38 ± 4.13         | -                  | -                |
| GSK2033                    | 3   | 96.54 ± 3.36         | -2.208 to 12.01    | ns 0.2758        | 86.53 ± 2.67         | 3.514 to 22.17     | ** 0.0031        |
| SR9243                     | 3   | 100.45 ± 3.47        | -6.232 to 7.989    | ns 0.9979        | 86.48 ± 4.94         | 3.564 to 22.22     | ** 0.003         |
| Mitotane                   | 3   | 17.76 ± 1.86         | 75.34 to 89.56     | **** <0.0001     | 97.45 ± 3.97         | -7.403 to 11.25    | ns 0.9783        |
| Mitotane & GSK2033         | 3   | 15.19 ± 1.13         | 77.65 to 91.87     | **** <0.0001     | 82.77 ± 5.52         | 7.280 to 25.94     | **** <0.0001     |
| Mitotane & SR9243          | 3   | 9.08 ± 1.38          | 84.86 to 99.08     | **** <0.0001     | 84.65 ± 3.76         | 5.397 to 24.05     | 0.0006           |
| Vehicle Control            | 7   | 100.00 ± 6.45        | -                  | -                | 99.71 ± 0.89         | -                  | -                |
| GSK2033                    | 7   | 86.02 ± 5.04         | 8.156 to 22.38     | **** <0.0001     | 85.09 ± 1.27         | 5.697 to 24.35     | *** 0.0004       |
| SR9243                     | 7   | 81.19 ± 2.78         | 14.11 to 28.33     | **** <0.0001     | 81.4 ± 2.41          | 9.380 to 28.04     | **** <0.0001     |
| Mitotane                   | 7   | 0.00 ± 0.00          | 94.49 to 108.7     | **** <0.0001     | 72.72 ± 2.41         | 18.06 to 36.72     | **** <0.0001     |
| Mitotane & GSK2033         | 7   | 0.00 ± 0.00          | 94.49 to 108.7     | **** <0.0001     | 58.23 ± 0.99         | 32.55 to 51.21     | **** <0.0001     |
| Mitotane & SR9243          | 7   | 0.00 ± 0.00          | 94.49 to 108.7     | **** <0.0001     | 60.01 ± 2.43         | 30.77 to 49.43     | **** <0.0001     |
| Vehicle Control            | 10  | 99.69 ± 1.22         | -                  | -                | 99.91 ± 0.89         | -                  | -                |
| GSK2033                    | 10  | 70.93 ± 2.47         | 21.57 to 35.79     | **** <0.0001     | 79.02 ± 1.26         | 11.56 to 30.22     | **** <0.0001     |
| SR9243                     | 10  | 66.16 ± 5.22         | 27.62 to 41.84     | **** <0.0001     | 70.77 ± 2.88         | 19.81 to 38.46     | **** <0.0001     |
| Mitotane                   | 10  | 0.00 ± 0.00          | 92.67 to 106.9     | **** <0.0001     | 67.24 ± 1.61         | 23.34 to 41.99     | **** <0.0001     |
| Mitotane & GSK2033         | 10  | 0.00 ± 0.00          | 92.67 to 106.9     | **** <0.0001     | 53.08 ± 0.91         | 37.49 to 56.15     | **** <0.0001     |
| Mitotane & SR9243          | 10  | 0.00 ± 0.00          | 92.67 to 106.9     | **** <0.0001     | 57.17 ± 1.43         | 33.41 to 52.06     | **** <0.0001     |
| Vehicle Control            | 14  | 104.57 ± 4.19        | -                  | -                | 98.82 ± 1.73         | -                  | -                |
| GSK2033                    | 14  | 58.16 ± 2.40         | 39.98 to 54.20     | **** <0.0001     | 55.36 ± 1.54         | 34.12 to 52.78     | **** <0.0001     |
| SR9243                     | 14  | 61.87 ± 2.93         | 37.71 to 51.94     | **** <0.0001     | 55.34 ± 0.86         | 34.15 to 52.80     | **** <0.0001     |
| Mitotane                   | 14  | 0.00 ± 0.00          | 98.40 to 112.6     | **** <0.0001     | 49.14 ± 2.52         | 40.35 to 59.01     | **** <0.0001     |
| Mitotane & GSK2033         | 14  | 0.00 ± 0.00          | 98.40 to 112.6     | **** <0.0001     | 34.23 ± 1.13         | 55.26 to 73.92     | **** <0.0001     |
| Mitotane & SR9243          | 14  | 0.00 ± 0.00          | 98.40 to 112.6     | **** <0.0001     | 38.07 ± 0.72         | 51.42 to 70.08     | **** <0.0001     |