Mechanisms of Therapy Resistance in Adrenocortical Carcinoma

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1 Abstract

Adrenocortical Carcinoma (ACC) is a rare and aggressive malignancy of the adrenal cortex characterized by fast progression and dismal prognosis. It commonly features excessive production of steroid hormones leading to Cushing's syndrome, virilization, feminization and, in rare cases, Conn's syndrome. Adrenocortical carcinoma mostly develops sporadically between the fourth and fifth decade of life. The only curative treatment known to date is the complete surgical resection of the tumor. However, recurrence after resection is reported in 40-60% of the patients. Mitotane is the only drug approved for ACC treatment. Its major mode of action is the inhibition of sterol-o-acyl transferase (a major regulator of intracellular free cholesterol) leading to accumulation of free cholesterol, endoplasmic reticulum stress and apoptosis. It is commonly used in combination with etoposide, doxorubicin and cisplatin in advanced or metastatic ACC and as adjuvant treatment after resection. The use of mitotane may prolong recurrence free survival but does not affect overall survival. Moreover, patients commonly do not respond or face recurrence during mitotane therapy, even when therapeutic blood levels are maintained. These observations suggest a mechanism of acquired resistance towards mitotane in ACC. The underlying mechanisms, however, have not been investigated. The present thesis therefore investigates the mechanisms underlying mitotane resistance in an *in vitro* model of mitotane resistant ACC.

Mitotane resistant clonal cell lines were established from long-term treated HAC-15 cells, and were subsequently characterized by *in vitro* studies, a gene expression microarray study with subsequent Gene Ontology enrichment analysis and a whole exome sequencing study. Finally, selected driver pathway candidates were investigated *in vitro* and intracellular content of several lipid species and mitotane was assessed by ESI-MS/MS and GCMS, respectively.

Mitotane resistance in the present *in vitro* model was not accompanied by doxorubicin resistance, and upregulation of the gene expression of common multidrug resistance transporters MDR1, MRP1 and BCRP was excluded in the microarray study. Further, the IC_{50} of mitotane was found to be positively correlated with medium content of HDL, LDL and cholesterol. Moreover, mitotane resistance was mitigated at low medium levels of HDL and LDL. Gene Ontology enrichment analysis demonstrated upregulation of pathways involved in Wnt signaling and cell growth and downregulation of pathways involved in biosynthesis and metabolism of steroid hormones, lipid transport as well as

lipoprotein binding and clearance in resistant cells. Exome sequencing revealed high genetic similarity among mitotane resistant clonal cell lines. Investigation of candidates (SCARB1, AGTR1, Wnt and DDIT4L/mTor) did not lead to identification of singular driver genes or pathways. Investigation of intracellular lipid content revealed resistant cells to be depleted of cholesteryl esters. Treatment with 50 µM mitotane caused a significant increase in intracellular free cholesterol, the major mediator of mitotane associated cytotoxic effects, as well as proapoptotic ceramides and lysophosphatidylcholines in nonresistant, but not in resistant cells. Also, sphingomyelins were significantly increased in nonresistant cells in comparison to resistant cells at all conditions, and treatment with 50 µM mitotane caused a significant increase in sphingomyelins in nonresistant, but not in resistant cells. Mitotane resistant cells showed a tendency towards reduced intracellular mitotane content, however no significant effect was found.

The data provided in the present thesis suggest that mitotane resistance in the present *in vitro* model is different from common multidrug resistance. According to the whole exome sequencing study, mitotane resistant clonal cell lines may derive from a single, mitotane resistant cell. *In vitro* experiments and the gene expression microarray study suggest a role of medium lipoprotein content and lipoprotein uptake in mitotane resistance in the present *in vitro* model. Discovery of the downregulation of genes implicated in pathways connected to metabolism and biosynthesis of steroid hormones may help explain the reduction of steroid hormone excess commonly observed in mitotane treated patients. Absence of mitotane dependent increase in intracellular free cholesterol suggests impaired sterol-o-acyltransferase inhibition in resistant cells.

1 Zusammenfassung

Das Nebennierenrindenkarzinom (engl.: adrenocortical carcinoma; ACC) ist eine seltene Tumorerkrankung der Nebennieren, die durch eine rasche Progression und eine schlechte Prognose gekennzeichnet ist. Dabei kommt es aufgrund einer Überproduktion an Steroidhormonen häufig zur Entstehung eines Cushing Syndroms, einer Überproduktion an Geschlechtshormonen und seltener eines Conn Syndromes. Das ACC entwickelt sich häufig sporadisch im Alter von 40-50 Jahren und kann momentan nur durch eine vollständige, chirurgische Entfernung des Tumors geheilt werden. Allerdings kommt es in 40-60% der Patienten zu Rezidiven. Mitotan ist momentan das einzige Medikament, das zur Behandlung des ACC zugelassen ist. Mitotan wirkt hauptsächlich, indem es das Enzym Sterol-o-acyltransferase, den wichtigsten Regulator von intrazellulären Cholesterinspiegeln, hemmt. Dies führt zu intrazellulären Cholesterinablagerungen, endoplasmatischem Retikulum Stress und Apoptose. Mitotan wird in der adjuvanten Therapie nach Operation und zur Behandlung von fortgeschrittenem und metastasiertem ACC eingesetzt, meist als Kombinationspräparat mit Doxorubicin, Etoposid und Cisplatin. Mitotan kann das rezidivfreie Überleben verbessern, scheint das Gesamtüberleben aber nicht zu beeinflussen. Darüber hinaus kommt es auch häufig unter therapeutisch wirksamen Blutspiegeln von Mitotan zu Rezidiven und Nonrespondern. Diese Beobachtungen legen nah, dass es unter Mitotantherapie zu einer erworbenen Resistenz kommt, die bislang noch nicht näher untersucht wurde. Ziel der vorliegenden Doktorarbeit war es daher, die der Mitotanresistenz zugrunde liegenden Mechanismen anhand eines geeigneten *in vitro* Modells näher zu untersuchen. Dazu wurden mitotanresistente, klonale Zelllinien aus langzeitbehandelten HAC-15 Zellen isoliert. Die resistenten Zelllinien wurden daraufhin anhand von *in vitro* Studien, einem Genexpressionsmicroarray und mittels Exomsequenzierung charakterisiert. Daraufhin wurden ausgewählte Kandidaten und Signalwege auf ihre Rolle als Driver hin untersucht. Des Weiteren wurde der intrazelluläre Gehalt von Mitotan und diversen Lipiden mittels Massenspektrometrie bestimmt.

Im vorliegenden *in vitro* Modell der Mitotanresistenz wurde keine Resistenz gegen Doxorubicin festgestellt. Auch wurde im Genexpressionsarray keine Veränderung der Genexpression der wichtigsten Multiresistenzgene MDR1, MRP1 und BCRP festgestellt. Es wurde eine positive Korrelation zwischen der Konzentration an HDL, LDL und Cholesterin im Medium und der IC_{50} von Mitotan gefunden. Darüber hinaus war die

Mitotanresistenz in Anwesenheit von geringen Lipoproteinkonzentration im Medium abgeschwächt. Eine Gene Ontology Enrichment Analyse identifizierte hochregulierte Signalwege mit einer Rolle in der Wnt Signalkaskade und dem Zellwachstum und runterregulierte Signalwege mit einer Rolle in der Biosynthese und dem Abbau von Steroidhormonen und Lipiden und in der Bindung und Clearance von Lipoproteinen in resistenten Zellen. In Untersuchungen von SCARB1, AGTR1, Wnt and DDIT4L/mTor als Kandidaten konnten keine singulären Driver der Resistenz identifiziert werden. Resistente Zellen wiesen einen signifikant stark verringerten Gehalt an Cholesterinestern auf. Der intrazelluläre Gehalt an freiem Cholesterin, dem hauptsächlichen Verursacher der Mitotan vermittelten Cytotoxizität, und an proapoptotischen Lysophosphatidylcholinen und Ceramiden war in nichtresistenten, mit 50 µM Mitotan behandelten Zellen signifikant erhöht, nicht aber in resistenten Zellen. Sphingomyeline waren in resistenten Zellen unter allen getesteten Bedingungen signifikant erniedrigt. Darüber hinaus führte eine Behandlung mit 50 µM Mitotan zu einer signifikanten Erhöhung von Sphingomyelinen in nichtresistenten, nicht aber in resistenten Zellen. Der intrazelluläre Mitotangehalt in resistenten Zellen war tendenziell niedriger als in nichtresistenten Zellen, allerdings wurde kein signifikanter Effekt festgestellt.

Die im Rahmen der vorliegenden Doktorarbeit erhobenen Daten legen die Schlussfolgerung nahe, dass sich die Mitotanresistenz in vorliegendem *in vitro* Model von der häufig beobachteten Multidrug-Resistenz unterscheidet. Die verringerte Expression von Genen, die eine Rolle in Biosynthese und Abbau von Steroidhormonen spielen, könnte einen Erklärungsansatz für die oft beobachtete Linderung der durch Steroidhormone verursachten Symptome in Mitotan behandelten ACC Patienten liefern. Die Resultate der Exomsequenzierungsstudie deuten darauf hin, dass alle resistenten Zelllinie von einer gemeinsamen Vorläuferzelle abstammen. Die durchgeführten *in vitro* Studien weisen, zusammen mit der Genexpressionsstudie, auf eine bedeutende Rolle des Lipoproteingehaltes des Medium und der Lipoproteinaufnahme im vorliegenden *in vitro* Modell hin. Das Ausbleiben einer Erhöhung des intrazellulären freien Cholesterins unter Mitotanbehandlung in resistenten Zellen deutet auf eine Störung der Inhibition der Sterol-o-acyltransferase durch Mitotan hin.

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5 List of Abbreviations

ABCA1	ATP-binding cassette subfamily A member 1
ABCG1	ATP-binding cassette subfamily G member 1
ACC	adrenocortical carcinoma
ACTH	corticotropin
AGTR1	angiotensin II receptor 1
Aldo	aldosterone
Andr	androgens
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
BP	biological process
BWS	Beckwith-Wiedemann syndrome
CCS	cosmic calf serum
CER	ceramides
CER	cholesteryl esters
CGH	comparative genomic hybridization
CM	cell membrane
CNV	copy number variant
Cort	cortisol
COX x	cytochrome c oxidase subunit x
cPD	cumulative population doublings
CRH	corticotroph releasing hormone
CSC	cancer stem cell
CT	computerized tomography
CYP11A1	cholesterol side-chain cleaving enzyme
CYP11B1	steroid-11β-hydroxylase
CYP11B2	aldosterone synthase
CYP17A1	steroid 17α-hydroxylase
CYP21A2	steroid 21-hydroxylase
DDIT3	DNA-damage-inducible transcript 3 (CHOP)
DDT	dichlorodiphenyltrichloroethane
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DMSO	dimethyl sulfoxide
ENaC	epithelial sodium channel
ENSAT	European Network for the Study of Adrenal Tumor
ERM	endoplasmic reticulum membrane
ESI-MS/MS	electrospray ionization tandem mass spectrometry
FCS	fetal calf serum
FDG PET	18F-fluorodeoxyglucose positron emission tomography
GCMS	gas chromatography mass spectrometry
GDF15	differentiation factor 15
GO	Gene Ontology
HDL	high density lipoprotein
HSD3B	3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase
HSL	hormone sensitive lipase
IC_{50}	half-maximal inhibitory concentration
IGF2	insulin-like growth factor 2
IST	insulin-transferrin-selenium
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LFS	Li-Fraumeni syndrome
LPC	lysophosphatidylcholines
MAM	mitochondria associated membranes
MC2R	melanocortin 2 receptor
MDR1	multidrug resistance protein 1
MF	molecular function
mitotane-EDP	mitotane, etoposide, doxorubicin and cisplatin
MM	mitochondrial membrane
MRI	magnetic resonance imaging
MRP1	multidrug resistance-associated protein 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NR5A1	cf. SF-1
NuS	Nu-Serum
o,p'-DDD	1,1-(dichlorodiphenyl)-2,2-dichloroethane
o,p′-DDE	1,1-(o,p′-dichlorodiphenyl)-2,2 dichloroethene
o,p′DDA	1,1-(o,p′-dichlorodiphenyl) acetic acid
PBS	Dulbecco's Phosphate Buffered Saline
PCx	principle component x
PLA2G12A	phospholipase 2 subfamiliy 12 group A
SCARB1	Cf. SR-BI
SCD	stearoyl-CoA desaturase
SDS	sodium dodecyl sulfate
SF-1	steroidogenic factor-1
SOAT1	sterol-O-acyl transferase
SPM	sphingomyelins
SR-BI	scavenger receptor 1B
SREBF1	sterol regulatory element binding transcription factor 1
StAR	steroidogenic acute regulatory protein
SULT2A1	sulfotransferase family 2A member 1
TF	transcription factor
VLDL	very low density lipoprotein

6 Introduction

6.1 The Adrenal Gland

6.1.1 Anatomy

The adrenal glands are a pair of endocrine organs that are essential for metabolism, blood pressure regulation as well as glucose and sodium homeostasis (1, 2). In fact, their importance in both, human health and disease has been recognized as early as 1855 (3). They consist of the adrenal cortex, which represents up to 90% of the adrenal weight and comprises the zonae glomerulosa, fasciculata and reticularis, as well as the adrenal medulla (1, 2). The adrenal cortex is a major production site of steroid hormones, while the adrenal medulla is a major site of catecholamine production (1, 4). The anatomy of the adrenal gland is shown in figure 1.

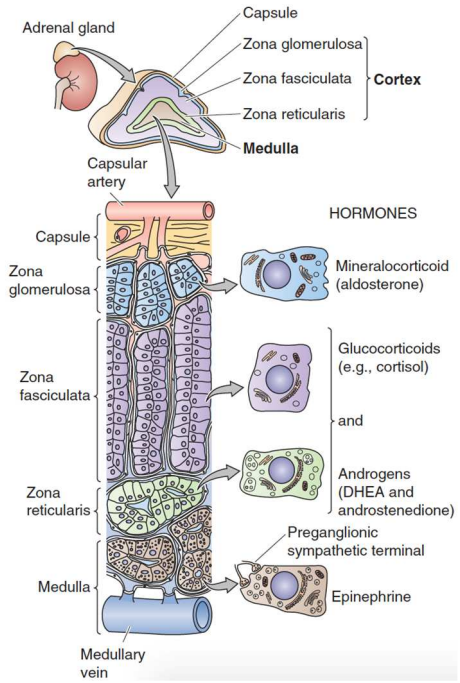


Figure 1: Anatomy of the adrenal glands. The adrenal glands are a pair of endocrine organs situated above the kidney. They are surrounded by a capsule and consist of the cortex and the medulla. The adrenal cortex comprises the zona glomerulosa, which secretes aldosterone, the zona fasciculata, which secretes cortisol and the zona reticularis, which secretes adrenal androgens (DHEA and androstenedione). The medulla, which secretes epinephrine and norepinephrine, contains chromaffin cells innervated by sympathetic nerve fibers. Adrenal blood supply enters the cortex from capsular arteries and flows through anastomotic capillary beds towards the medullary vein. Abbreviation: DHEA, dehydroepiandrosterone (2). Reprinted with permission of Elsevier Saunders[1].

[1] Reprinted from Medical Physiology, 3rd edition, Barrett EJ, Chapter 50: The Adrenal Gland, 1018-35. Copyright (2016), with permission from Elsevier Saunders.

6.1.2 Steroidogenesis

During steroidogenesis, all steroid hormones of the human adrenal cortex are synthesized from their common precursor cholesterol by mitochondrial and smooth endoplasmic reticulum enzymes (5). The major steroidogenic pathways of the three zones of the adrenal cortex are shown in figure 2.

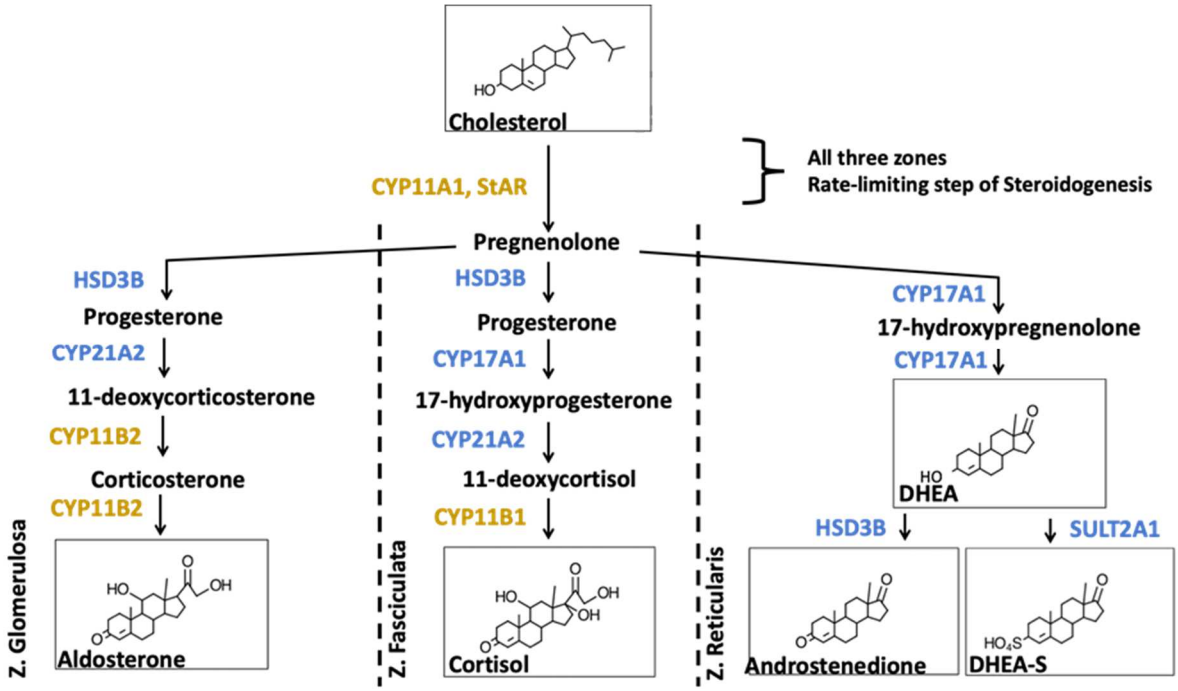


Figure 2: Steroidogenic pathways of the adrenal gland. Steroid hormones of the adrenal gland are synthesized from their common precursor cholesterol. The first step is the rate-limiting step of steroidogenesis and involves cholesterol transport into mitochondria by StAR and synthesis of pregnenolone by CYP11A1. Pregnenolone is then converted either to aldosterone in the zona glomerulosa, to cortisol in the zona fasciculata or to androstenedione and DHEA-S in the zona reticularis in multistep-reactions involving enzymes of the mitochondria (brown) and smooth endoplasmic reticulum (blue). Abbreviations: CYP11A1, cholesterol side-chain cleaving enzyme; StAR, steroidogenic acute regulatory protein; HSD3B, 3β-Hydroxysteroid dehydrogenase/Δ5-4 isomerase; CYP21A2, steroid 21-hydroxylase; CYP17A1, steroid 17α-hydroxylase; CYP11B2, aldosterone synthase; CYP11B1, steroid-11β-hydroxylase; SULT2A1, sulfotransferase family 2A member 1; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate. Adapted from (5) and (6).

For steroidogenesis, cholesterol may be provided either by uptake of plasma low density lipoprotein (LDL), mobilization of intracellular cholesterol storage or *de novo* synthesis from acetate (7). The human adrenal covers 80% of its cholesterol needs by uptake of plasma cholesterol (8), either mediated by the LDL receptor (LDLR), involving endocytosis and lysosomal degradation of LDL particles (9), or by selective uptake

of cholesterol from lipoproteins (LDL and high density lipoprotein, HDL) mediated by the scavenger receptor 1B (SR-BI) (10). In the adrenal cortex, excess free cholesterol is esterified by the sterol-O-acyltransferase (SOAT) and stored as cholesteryl esters in lipid droplets (11), while mobilization of cholesterol from storage is catalyzed by hormone sensitive lipase (12) under stimulation of adrenaline, glucagon and ACTH (13).

6.1.3 Histology and Functional Aspects of the Adrenal Cortex and Medulla

Histologically, the human adrenal cortex mostly consists of lipid-rich, epithelial cells (14). According to their arrangement and function, epithelial cells of the adrenal cortex are compartmentalized into three distinct zones (14, 15). In the outermost zone beneath the tissue capsule (zona glomerulosa) aldosterone is produced as part of the renin-angiotensin-aldosterone system, exerting an important role on regulation of blood pressure and electrolyte balance (16). Epithelial cells of the zona glomerulosa are clustered in irregular nests (17, 18). Expression of *CYP11B2,* and thereby aldosterone secretion, is mainly stimulated by angiotensin II as well as increased serum potassium, and, to less extent, by corticotropin (ACTH) and inhibited by atrial natriuretic peptide (16). Aldosterone binds to the mineralocorticoid receptor in the distal tubules and the collecting duct of the kidney and thereby increases the activity of several downstream effectors including epithelial sodium channel (ENaC) and sodium/potassium-ATPase, leading to retention of sodium and water as well as the excretion of potassium (19, 20).

In comparison to the zona glomerulosa, epithelial cells of the zona fasciculata are large, high in lipid content, contain pronounced smooth endoplasmic reticula and are rather organized in columns than in clusters (21, 22). The zona fasciculata comprises most of the adrenal cortex (22), and, as part of the hypothalamo-pituitary axis, is responsible for cortisol production (23). In response to stress, corticotroph releasing hormone (CRH) is released by the hypothalamic paraventricular nucleus, which then causes release of ACTH by the adenohypophysis (24). Corticotropin, in turn, binds to the melanocortin 2 receptor (MC2R) of fasciculata cells and causes cortisol production by translocation of cholesterol from lipid droplets to the mitochondria and increase of *CYP11B1* expression (25, 26). Cortisol, by binding to glucocorticoid receptors (27), influences numerous biological processes, including skeletal growth, immune response, glucose and lipid metabolism, cognition, and reproduction (28-30).

Epithelial cells of the zona reticularis are also organized in columns, but, in comparison to epithelial cells of the zona fasciculata, relatively small, low in lipid content and therefore appear to be more compact (17). Cells of the androgen producing zona reticularis are characterized by high expression of *CPY17A1* and *SULT2A1* and low expression of *HSD3B* (31), causing production of DHEA, DHEA-S and androstenedione (32, 33). Adrenal androgens serve as precursors for testosterone and dihydrotestosterone in peripheral tissues (34) and play an important role in human reproduction and pubertal development (35).

The adrenal medulla mainly consists of two distinct, chromaffin populations of cells (36), in which phenylalanine is either converted to adrenaline or noradrenaline (36, 37). During sympathetic fight-and-flight response, secretion of adrenaline and noradrenaline is stimulated by acetylcholine release at preganglionic neurons (2), which then binds to nicotinic acetylcholine receptors thereby triggering exocytosis of catecholamines into the bloodstream (38, 39). By binding to $\alpha_{1\text{-}2}$ and $\beta_{1\text{-}3}$ adrenergic receptors (40-43) catecholamines affect a broad range of tissues and thereby cause a variety of physiological effects, such as changes in blood flow, blood pressure, blood glucose and fat, coagulation, and gastrointestinal activity (44).

6.2 Tumors of the Adrenal Gland

Tumors of the adrenal glands are among the most common tumors in humans as they are found in at least 3% of persons older than 50 years of age (45). They are commonly discovered during radiological investigation of unrelated disorders, in which case they are also called adrenal incidentalomas (46), and represent a heterogeneous group of entities with respect to origin, malignancy and endocrine function. Approximately half of adrenal incidentalomas (40-60%) are reported to be nonfunctional adrenal adenomas (47-49), while between 15 and 29% show hypersecretion of adrenal hormones with a higher incidence in patients with bilateral tumors (50, 51). Further common causes of adrenal incidentalomas are catecholamine producing tumors arising from the adrenal medulla (pheochromocytoma, 7-23 % of the cases), carcinoma of the adrenal cortex (ACC) (4-12%) or distant metastases of primary tumors located elsewhere (2-7%) (51-54). Interestingly, adrenal metastases are present in approximately 30% of malignancies of epithelial origin and may arise from a broad range of primary epithelial tumors, while adrenocortical carcinoma is a rare disease in the general population (55).

In order to exclude functionality and malignancy of adrenal tumors that require further treatment, all patients with adrenal incidentalomas should undergo endocrine workup and computerized tomography (CT) scan (45) (for endocrine workup and CT scan, please refer to chapter 6.3.2).
Apart from adrenal tumors, adrenal hyperplasia may also have a significant prevalence in the general population as a study from one radiology apartment comprising 564 CT scans of the adrenal glands found incidentally discovered adrenal enlargement due to hyperplasia to be present in 11.3% of the cases (56).

6.3 Adrenocortical Carcinoma

6.3.1 Prevalence

Adrenocortical carcinoma is a very rare disease with an incidence of up to 2 cases per million people per year (57-60). The majority of patients are female (55-70%), and approximately half of the patients are diagnosed with advanced or metastatic disease (60-64). At diagnosis, most patients are between 40 and 50 years of age. In a study comprising 47 patients from Sao Paolo, Brazil, a bimodal age distribution with peak incidences in the first and fourth to fifth decade of life has been suggested, which has not been confirmed by studies of larger cohorts or comprehensive literature reviews (58, 64-68). Interestingly, the incidence of childhood ACC in the state of Parana (close to Sao Paolo) has been demonstrated to be 10-15 times higher than in the US, probably due to accumulation of an inherited, low penetrance germ-line variant of p53 causing Li-Fraumeni-Syndrome (LFS), suggesting that the overall incidence of childhood cases of ACC inferred on basis of such a cohort might be overestimated (for LFS, please refer to chapter 6.3.3) (69-71).

6.3.2 Symptoms and Diagnosis

In 30-55% of ACC patients, symptoms related to tumor size such as abdominal mass and pain are present (72-77). General symptoms such as weakness and weight loss are present in 6-38% of the patients, and fever is present in 2-20% (72, 73, 75, 76). Twenty-four to seventy percent of the patients present with symptoms related to overproduction of adrenal steroid hormones, most prominently hypercortisolism or Cushing's syndrome (16-40% of all patients), virilization (2-44%) and feminization (2-13%) (73-76, 78). Cushing's syndrome features a multitude of symptoms, including weight

gain, hypertension, diabetes mellitus, lethargy, acne, depression and hirsutism (79). In very rare cases, patients may also present with hyperaldosteronism or Conn's syndrome (80, 81). Conn's syndrome features hypertension and may also include hypokalemia, alkalosis and, in rare cases, hypernatremia (82).

Due to the high number of patients presenting with symptoms of hormone excess, endocrine work up is crucial to establishing the adrenal origin of the tumor and hence is recommended in evaluation of adrenal incidentaloma (83). Endocrine evaluation includes testing for glucocorticoid excess by dexamethasone suppression test, 24 h free urinary cortisol as well as basal cortisol and ACTH in serum and plasma, respectively (84). Assessment of sexual steroid hormones and steroid precursors should include measurement of serum DHEA-S, 17-hydroxy-progesterone, androstenedione and testosterone (85). In hypertensive patients, mineralocorticoid excess can be assessed by serum potassium and aldosterone to renin ratio (86). Additionally, pheochromocytoma should be excluded by 24 h urine catecholamines and plasma meta- and normetanephrines (84).

Malignancy can be ascertained using CT and magnetic resonance imaging (MRI) (85). Due to facilitated standardization and, in combination with a chest scan, convenient pre-operative evaluation of possible metastasis, CT scan is recommended (87). In these scans, malignancy may be predicted by tumor size, since the likelihood of malignant disease increases with tumor size (88), the median size of ACC is more than 11 cm (87). Further characteristics are a well-defined margin, low central attenuation and extension into the vena cava including thrombus (89). During the last years, 18F-fluorodeoxyglucose positron emission tomography (FDG PET) has become an increasingly popular alternative to MRI and CT scanning for staging and evaluation of treatment response (90-92).

For histological confirmation of the adrenocortical origin of a tumor and estimation of the prognosis of ACC patients, expression of steroidogenic factor-1 (SF-1), a transcription factor of key importance for the adrenal glands' endocrine function (93), is the most suitable immunohistochemical marker known to date (94).

For further confirmation of malignancy, the Weiss' scoring system for histopathological diagnosis of ACC can be applied. According to the Weiss' criteria, an adrenal tumor must meet three or more of the following criteria in order to be regarded as ACC (95): 1) high nuclear grade; 2) mitotic rate six or more per 50 high power field; 3) atypical mitosis; 4) less than 25% of the cells clear; 5) diffuse architecture pattern in more than

one third of the tumor; 6) confluent necrosis; 7) invasion of vein; 8) invasion of sinus; and 9) capsular invasion.

Further, risk of recurrence after resection of localized ACC (for resection, please refer to chapter 6.3.4) can be predicted using expression of Ki67 as an immunohistochemical marker for proliferation (96). Ki67 is a protein involved in ribosomal RNA transcription of cells that is highly expressed during proliferation (97) and is therefore used as a well-established, pathological marker for proliferation (98).

Precise staging of ACC is key to predicting disease-specific survival and disease-free survival during treatment (99) (for stage dependent survival of ACC, please refer to chapter 6.3.6). Therefore, the European Network for the Study of Adrenal Tumors (ENSAT) in 2008 proposed a comprehensive staging system based on tumor size, invasiveness, metastasis and involvement of lymph nodes (99). The ENSAT staging system is shown in figure 3.

Stage	ENSAT 2008
I	T1, N0, M0
II	T2, N0, M0
III	T1-T2, N1, M0
	T3-T4, N0-N1, M0
IV	T1-T4, N0-N1, M1

ENSAT indicates European Network for the Study of Adrenal Tumors; T1, tumor ≤5 cm; T2, tumor >5 cm; T3, tumor infiltration into surrounding tissue; T4, tumor invasion into adjacent organs or venous tumor thrombus in vena cava or renal vein; N0, no positive lymph nodes; N1, positive lymph node(s); M0, no distant metastases; M1, presence of distant metastasis.

Figure 3: Staging system for adrenocortical carcinoma. Current staging system as proposed by the European Network for the Study of Adrenal Tumors (ENSAT) (99). Tumors are staged according to size and invasiveness (T1-4), involvement of lymph nodes (N0-N1) and presence of distant metastasis (M0-M1). Reprinted with permission of John Wiley and Sons[2].

6.3.3 Genetic Causes

Adrenocortical carcinoma develops sporadically in most of the cases, but can also arise from hereditary syndromes, most importantly LFS and the Beckwith-Wiedemann syndrome (BWS) (100). First being described in 1969, LFS is a cancer predisposition disorder of autosomal inheritance, which leads to cancer development within the first

[2] Reprinted from Cancer 115(2), Fassnacht M, Johanssen S, Quinkler M, Bucsky P, Willenberg HS, Beuschlein F, Terzolo M, Mueller H, Hahner S and Allolio B, for the German Adrenocortical Carcinoma Registry Group and the European Network for the Study of Adrenal Tumors, Limited prognostic value of the 2004 International Union Against Cancer staging classification for adrenocortical carcinoma: Proposal for a Revised TNM Classification, 243-250. Copyright (2009), with permission from John Wiley and Sons.

four decades of live, most commonly breast cancers, soft tissue sarcomas, osteosarcomas, brain tumors, leukemias, and adrenocortical carcinomas (101-104). Despite being very rare in the general population, ACC is strongly associated with LFS and may occur in 2.5-4.7 % of all patients with LFS (101, 105). Two groups demonstrate germ-line loss of function mutations in the tumor suppressor gene *TP53* to cause LFS (106, 107). *TP53* exerts an important role on genome integrity by inducing cell cycle arrest and regulating apoptosis and is therefore also called “guardian of the genome” (108).The Beckwith-Wiedemann syndrome is inherited in a complex, heterogenous inheritance pattern (109), features infant overgrowth and malformations and is accompanied by and increased risk of fetal tumor development (110, 111). The Beckwith-Wiedemann syndrome is caused by abnormalities in an imprinted gene cluster on chromosome 11p15 containing *CDKN1C*, *IGF2* and *H19*, that may be caused by epigenetic errors leading to impaired methylation, a mutation in the *CDKN1C* gene or chromosomal alterations (112). *IGF2* encodes for a hormone that regulates fetal growth by influencing cell proliferation, growth, migration, differentiation and survival (113), *H19* encodes for a RNA that serves as enhancer for *IGF2* (114) and *CDKN1C* encodes for a cyclin dependent kinase inhibitor that exerts an important role in cell cycle control by inducing G1 arrest (115).

Unlike in hereditary cancer predisposition syndromes, the genetics underlying sporadic ACC are still not clearly understood. Adrenocortical carcinoma and adenoma show a monoclonal cell pattern, suggesting that they develop from single cells bearing somatic driver mutations (116). Transformation of adrenocortical neoplasms is accompanied by mitosis abnormalities, and malignant lesions are characterized by an accumulation of aneuploidy and polyploidy (117-119). Genomic instability is a hallmark of cancer as it allows for genomic variability to occur, which may lead to selective advantages of tumors cells and facilitates transformation of neoplasms towards malignancy (120). Studies using comparative genomic hybridization (CGH), a method used to detect changes in copy number of tumor DNA (121), reveal a high number of chromosomal aberrations connected to adrenocortical carcinoma, while chromosomal aberrations in adenoma are rather rare (122, 123). These studies report copy number gains on chromosomes 4, 5, 7, 8, 12, 15, 16, 19 and 20 and losses on chromosomes 1, 2, 3, 6, 7, 8, 9, 11, 13, 14, 15, 16 and 17. Interestingly, one study demonstrates that amplifications of 6q, 7q, 12q, and 19p and deletions within 3, 8, 10p, 16q, 17q, and 19q are connected to decreased survival (123). During the last two decades, the fulminant rise

of genetics (the study of a limited number of genes and their functions) and genomics (the study of entire genomes) has helped to shed further light on the complex genetics underlying sporadic ACC leading to the identification of driver genes and pathways involved in ACC development and progression (124-128).

In an early gene expression microarray study, Giordano et al. report 91 differentially expressed genes with significant, at least threefold differential expression in ACC compared to normal adrenals and adrenal adenoma samples (126). Most noteworthy, they report the expression of *IGF2,* a gene also involved in the pathogenesis of BWS, to be upregulated in 90.9% of the ACC samples. Another RNA-based microarray study from the same group reveals 2,875 differentially expressed genes in ACC (125). Again, they provide evidence for perturbation of the 11p15 locus, also involved in BWS. Furthermore, they report expression changes in genes involved in regulation of cell cycle and proliferation, such as *CCNB2*, *ASPM, RRM2, TOP2A*, and *CDKN3*.

The advent of exome sequencing techniques during the last decade has paved the way for major achievements in understanding the genetics underlying formation of benign adrenocortical tumors and led to identification of several somatic and germ-line mutations (129-133). The human exome represents ~1% of the human genome, yet it is estimated to harbor 85% of disease-causing mutations (134), suggesting that exome sequencing may also be a promising approach to identify new driver mutations in ACC. Assie et al. report a comprehensive genetic analysis of an initial cohort of 45 ACC cases and a follow up of 77 cases, including exome sequencing, SNP arrays, DNA methylation analysis, mRNA expression arrays and miRNA sequencing (124). They report the discovery of somatic alterations including mutations and homozygous deletions in genes belonging to the β-catenin pathway (*ZNRF3*, 21% of all tumors; *CTNNB1*, 16%; *APC*, 2%), p53/Rb signaling (*TP53*, 16% of all tumors; *CDKN2A*, 11%; *RB1*, 7%; *CDK4*, 2%; *MDM2*, 2%) and chromatin remodeling (*MEN1*, 7% of all tumors; *DAXX*, 6%; *ATRX*, 4%) by exome sequencing. Further, they present somatic mutations in *MED12* (5% of all tumors) and recurring focal amplifications of *TERT* (6%; encoding for telomerase). They also assign tumors to two different molecular groups according to their mRNA expression, DNA methylation and miRNA expression: C1A comprising 3 subtypes all connected to low overall survival and C1B comprising 2 subtypes connected to high overall survival.

In another exome sequencing study, Juhlin et al. screen 41 matched pairs of ACC and nontumor samples for somatic mutations and copy number alterations discovering a

total of 966 nonsynonymous single nucleotide variants (variants that cause changes in amino acid sequence) with 23.6 protein altering mutations per tumor (127). They confirm somatic alterations in genes also reported by Assie et al. (124), including single nucleotide variants in *TP53* (19.5% of all tumors), *CTNNB1* (9.8%), a somatic mutation and homozygous deletion *ZNRF3* (9.8%) as well as recurring amplifications at 5p15.33 including *TERT* (14.6%). Previously unreported findings include nonsynonymous somatic mutations in *CDC27, SCN7A, SDK1* (each in 7.3% of all tumors) and *NF2* (4.9%) as well as homozygous deletion in *KREMEN1* (7.3%). By further screening for mutations either previously reported in cancer or at positions with previously reported recurrent mutations using the COSMIC database (135), Juhlin et al. reveal potentially disease-causing mutations in *RB1* and *GNAS*. By gene ontology analysis (136), they identify alterations in genes associated with the Wnt pathway in 66% of all tumors suggesting a fundamental role for Wnt-signaling in the pathogenesis of ACC.

Zheng et al. present a comprehensive pan-genomic characterization of ACC in 91 patients, including whole exome sequencing, mRNA sequencing, miRNA sequencing, DNA copy number analysis, DNA methylation arrays and targeted proteome analysis (128). By exome and RNA sequencing, they identify 6664 nonsynonymous mutations including mutations in previously reported ACC driver genes *TP53*, *CTNNB1* and *MEN1* as well as in *PRKAR1A* and *RPL22,* genes unreported in ACC. Interestingly, they find decreased protein expression in mutant cases of *PRKAR1A* and increased expression of MEK and BRAF, suggesting a role of the RAF-MEK-ERK cascade in ACC. Additionally, by comparison of the mutated genes with Cancer Gene Census (137), they reveal *NF1* and *MLL4* as possible driver genes of ACC. They further confirm previously reported focal amplifications in *TERT* and homozygous deletions in *ZRNF3* and provide evidence for whole genome doublings as a marker for tumor progression in ACC.

Taken together, these studies suggest that genomic instability leading to chromosomal abnormalities and DNA copy number alterations is a crucial event in transformation of adrenocortical neoplasms towards malignancy. Focal amplification of the *TERT* gene is repeatedly reported and is consistent with significant telomerase activity reported in ACC (138). Comprehensive genome analyses of ACC suggest a fundamental role of the Wnt pathway in ACC pathogenesis, a major pathway in development that is also tightly linked to various cancers (139). Insights from hereditary LFS and comprehensive genome analyses also point to an important role of the tumor suppressor gene

TP53 and p53/Rb signaling. Further, the RAF-MEK-ERK cascade, *IGF2* and pathways of chromatin remodeling have been implicated to play a role in ACC pathogenesis. However, the exact genetic mechanisms underlying ACC pathogenesis are still unknown. Additional studies of large cohorts are needed in order to identify the hierarchy of genomic abnormalities leading to adrenocortical neoplasm transformation and decipher pathways underlying malignancy that might be exploited for novel treatment options in the future.

6.3.4 Treatment

The only curative treatment of ACC known to date, if feasible, is the complete surgical resection of the tumor (140). While open surgery is considered as standard procedure for resectable ACC in stages I-III, laparoscopic surgery may provide a safe alternative for small tumors without preoperative signs of invasiveness (84, 141). Further, reoperation of recurring ACC, if feasible, may prolong survival in patients, if the time to first recurrence is 1 year or longer (142, 143). Mitotane (1,1-(dichlorodiphenyl)-2,2-dichloroethane, also o,p'-DDD), a congener of the insecticide DDT, due to its adrenolytic effects is used for treatment of Cushing's syndrome in dogs and humans as well as human ACC (144-146). In patients with ACC, treatment with mitotane may lead to transient tumor regression and reduction of steroid hormone excess (146). Mitotane is commonly used as adjuvant therapy after tumor resection (65, 68, 147). Adjuvant mitotane treatment prolongs recurrence free survival but does not affect overall survival (74, 148, 149). It is recommended in patients with high risk of recurrence (increased proliferative index; signs of residual disease after surgery) (150) while its efficacy in patients with low risk of recurrence is currently investigated within the ADIUVO trial (151, 152). In advanced or metastatic ACC, mitotane may be either used as high-dose monotherapy or in low-dose combination therapy alongside etoposide, doxorubicin and cisplatin (mitotane-EDP) or streptocozin (153). In combination therapy, mitotane-EDP seems to be superior to mitotane and streptozocin regarding recurrence free survival but not overall survival (154) and is therefore recommended as first-line cytotoxic treatment (155). Data on mitotane monotherapy in larger cohorts of patients with locally advanced or metastatic ACC is rare. Smaller studies show limited effects with response rates of 13-29% and rare cases of complete response (74, 146, 156). The largest study on mitotane monotherapy in advanced ACC known to date confirms these observations (response rate 20.5%; complete response in 2.4% of the patients)

(157). However, the authors conclude, that mitotane monotherapy might be better suited for patients with late diagnosis and low tumor burden than combination therapy. In recent years, insights from genomic analyses of ACC have led to the studies on novel therapeutic approaches. Insulin-like growth factor 2 (*IGF2*), a gene well established in pathogenesis of BWS (112), drives proliferation, migration, and metastasis in numerous cancers via the receptor tyrosine kinase IGF-1R (158), whose expression also is increased in pediatric ACC (159). However, selective inhibition of IGF-1R by linsitinib (160) within a phase III clinical trial shows no beneficial effect on overall survival in advanced or metastasized ACC (161). Combination therapy using mitotane and IFG-1R antibody cixutumumab is precluded from further trials as a phase II trial reveals low therapeutic efficacy and potentially fatal toxic effects in recurring and metastasizing ACC (162).

6.3.5 Pharmacodynamics and Pharmacokinetics of Mitotane

There are two isomers of mitotane, p,p'-DDD and m,p'-DDD, both of which show no significant adrenolytic effect (163). In the liver (164), mitotane is metabolized to 1,1-(o,p′-dichlorodiphenyl)-2,2 dichloroethene (o,p′-DDE) and 1,1-(o,p′-dichlorodiphenyl) acetic acid (o,p′DDA) by α- and β-oxidation, respectively (163). However, o,p′DDA lacks antitumor activity according to one *in vitro* study (165) and plasma levels of o,p′-DDE do not correlate with patients' response, suggesting o,p'-DDD as active form of mitotane (166). Mitotane is a lipophilic compound and hence tends to accumulate in circulating lipoprotein fractions including very low density lipoprotein (VLDL), LDL and HDL (167). The cytotoxic effects of mitotane, however, are mostly mediated by lipoprotein-free mitotane (167, 168).

The molecular mechanisms underlying the adrenolytic effects of mitotane have been extensively studied in human adrenocortical cancer cell lines H295R (169) and SW13 (170). Mitotane induces apoptosis in SW13 and H295R cells accompanied by dose-dependent mitochondrial impairment, including loss of integrity of the mitochondrial membrane, swelling and complete disruption (171). In H295R cells, mitotane treatment leads to downregulation of the expression of genes involved in steroidogenesis including *STAR*, *CYP11B1* and *CYP11B2,* accompanied by reduction of cortisol and 17-hydroxyprogesterone secretion by 70% (172). The same study demonstrates mitotane to induce a defect of the mitochondrial respiratory chain of H295R and SW13 cells by inhibiting cytochrome c oxidase activity and reducing gene expression of mitochondrial

genes encoding for subunits of cytochrome c oxidase (*COX2* and *COX4*). A recent study by Sbiera et al. demonstrates that mitotane inhibits the sterol-O-acyl-transferase 1 (SOAT1) in NCI-H295 cells (173). They also provide evidence that the inhibition of SOAT1 leads to accumulation of toxic lipids including free cholesterol and cholesterol precursors which, in turn, causes endoplasmic reticulum stress and apoptosis. Furthermore, this study shows that SOAT1 inhibitor Sandoz 58-035 mimics effects of mitotane on NCI-H295 cells. Sbiera et al further demonstrate that in patients receiving mitotane as palliative drug, tumors with high *SOAT1* expression showed significantly prolonged time to progression. They conclude that *SOAT1* expression is a prerequisite for mitotane efficacy.

Pharmacokinetics of mitotane is characterized by a large volume of distribution and slow elimination (174). After oral administration, mitotane shows moderate bioavailability and is excreted through urine and bile with a plasma half-life of up to 160 hours (164). Due to its lipophilic nature, mitotane accumulates in various tissues, including lung, liver, brain and adipose tissue (175-177). Hence, mitotane also affects extra adrenal steroid hormone metabolism (178, 179). Most noteworthy, the liver shows high uptake of the steroid hormone precursor cholesterol (180) and is known to play a pivotal role in cholesterol metabolism (181) and catabolism of adrenal steroid hormones (182). Mitotane treatment may lead to induction of hepatic CYP3A4 (183, 184) via activation of the nuclear steroid and xenobiotic receptor (185), members of the CPY3A family are involved in steroid hormone biotransformation (186). Reduction of steroid hormone excess (146, 187) and increase of serum cholesterol, HDL and LDL (188-191) is commonly observed during mitotane therapy in human ACC patients. These observations suggest a role of hepatic mitotane effects in the reduction of steroid hormone excess and increase of serum lipoprotein changes observed in human patients. However, studies on the hepatic effects of mitotane are rare. Induction of CYP3A4 also may lead to drug interactions with numerous drugs including macrolide antibiotics, dihydropyridine type calcium channel antagonists and HMG-CoA-reductase inhibitors (192).

Maintenance of mitotane blood levels >14 mg/L has been shown to be mandatory to achieve tumor response and significantly prolonged recurrence free survival in both, adjuvant therapy of resected and cytotoxic treatment of metastasizing ACC (166, 193), while mitotane blood levels may be subject to significant variation (194). Serious side effects are observed at blood levels >20 mg/L and include neurologic complications

and psychiatric abnormalities (195, 196). Hence precise monitoring of mitotane blood levels during treatment is considered as mandatory (87).

6.3.6 Prognosis and Therapy Resistance

Adrenocortical carcinoma is an aggressive malignancy with an overall five-year survival of 30-40%, prognosis drastically worsens with the stage of disease at diagnosis (60-63, 72, 197). Five-year survival decreases from approximately 60% at stages I and II, to 25-35% at stage III, while 5-year survival at stage IV is 0% (60, 198). Besides the stage of disease at diagnosis, two other major factors determining prognosis are resection status in localized ACC (199) and proliferative activity of the tumor (200). Additionally, patients diagnosed before 40 years of age occur to have a better overall prognosis, while sex and endocrine activity of the tumor seem to have no effect (76, 146).

Despite all efforts during the last decades in both, clinical management and basic research of ACC, prognosis has remained dismal, mostly due to failure of current treatment strategies. Recurrence of ACC after resection is reported in 40-60% of the patients (63, 201) and the outcome may, to some extent, be influenced by the center's expertise (202). Treatment strategies using mitotane, the only drug approved for treatment of ACC, fail to improve overall survival (74, 148, 149, 154). Patients commonly do not respond or face recurrence during mitotane therapy, even when therapeutic blood levels are maintained (193, 195). These observations suggest a mechanism of acquired resistance towards mitotane in ACC. Interestingly, normal adrenal tissue and adrenal tumors show high expression levels of the multidrug resistance gene *MDR1* (203, 204). *MDR1* encodes for P-glycoprotein, an ATP dependent drug transporter, that mediates export of xenobiotic compounds from the cytosol and thereby causes resistance to a multitude of drugs in a variety of cancers (205-207). In ACC patients however, mitotane therapy does not seem to be impeded by *MDR1* expression levels (208). Further, *in vitro* studies suggest, that mitotane may have positive effects on multidrug resistance as it counteracts P-glycoprotein activity and causes intracellular accumulation of doxorubicin (209, 210). These observations suggest a mechanism of mitotane resistance different from common multidrug resistance.

6.3.7 *In vitro* Models of Adrenocortical Carcinoma

Attempts to overcome difficulties such as tissue availability and quality in the use of primary adrenocortical cell cultures for research on adrenal steroid production have led to the establishment of several cell lines derived from human ACC, most of which lack steroidogenic potential (211).

The NCI-H295 cell line is derived from an invasive ACC of a 48 years old Bahamian woman with increased serum cortisol as well as increased urinary excretion of aldosterone and ketosteroids (212). NCI-H295 cells are a hypertriploid cell line, grow as floating clusters and show a relatively long population doubling time (212). They are responsive to potassium, angiotensin II and, to much less extent, ACTH and secrete a steroid profile characteristic of the adrenal cortex (212, 213). By selection for adherence, three substrains have been derived from NCI-H295 cells (H295R-S1-3) (214), that grow as adherent monolayers but show significant, medium dependent variation in growth characteristics and response to stimuli (211, 214). In a method similar to isolation of H295R strains, another substrain of NCI-H295, NCI-H295A, has been isolated (215), which also grows as adherent monolayer, but, unlike H295R, shows limited response to angiotensin II (211).

In an attempt to isolate a new, ACTH responsive human ACC cell line, the HAC-15 cell line was established (216). HAC-15 was originally reported to be derived from ACC of an 11 month old female presenting with hypertension and hirsutism but subsequent single nucleotide polymorphism arrays revealed, that this cell line is derived from H295R cells by cross-contamination (211). HAC-15 cells are responsive to angiotensin II, potassium and ACTH and show a similar steroidogenic profile in comparison to H295R cells (216).

Recently, the MUC-1 cell line was established from a mouse xenograft derived from a subcutaneous metastasis of a 24-year-old male presenting with a large adrenal tumor and abnormal profile of urinary steroids (217). The patient underwent adrenalectomy, nephrectomy and lymphadenectomy, but soon after developed metastases. Interestingly, MUC-1 cells seem to be resistant against treatment with mitotane-EDP.

6.4 Aims of Project and Research Questions

The overall aim of the project was to investigate the mechanisms contributing to mitotane resistance in an *in vitro* model of ACC. Although the molecular mechanisms of

mitotane have been extensively studied over the last decade and xenograft experiments using human tumor samples in mice have recently led to establishment of a mitotane-EDP resistant cell line, this issue has not been tackled so far.

The first target was to establish a mitotane resistant ACC cell line using the HAC-15 cell line as an *in vitro* model of ACC. Using mitotane resistant cells, the second target was to explore changes in gene expression and DNA integrity via gene expression microarray and exome sequencing techniques. Finally, the third target was to further investigate genes and pathways with a possible role in mitotane resistance in cell culture experiments.

Thereby, the present thesis aims at answering the following research questions:

1. Does mitotane treatment induce resistance *in vitro*?
2. Is mitotane resistance accompanied by genetic changes either concerning the transcription level or integrity of the DNA sequence?
3. Can molecular mechanisms of mitotane resistance be inferred from these genetic changes?
4. Are the molecular mechanisms of mitotane resistance consistent with observations in both, ACC patients and *in vitro* models?

7 Methods

7.1 Cell Culture of the HAC-15 Adrenocortical Carcinoma Cell Line

HAC-15 cells (a gift of William Rainey, University of Michigan, Ann Arbor, USA) were cultured in T75 Cell Culture Treated Flasks (Nunc by Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and 5% CO_2 in DMEM/Ham's F12 Medium+GlutaMAX (Gibco by Thermo Fisher Scientific) supplemented with 5% Cosmic-Calf Serum (CCS, Hyclone, South Logan, UT, USA), 1% Insulin-Transferrin-Selenium (ITS), 1% non-essential amino acids, 0.1% lipid mixture and 1% Penicillin/Streptomycin (all Gibco).

For passaging, cells were washed once with Dulbecco's Phosphate Buffered Saline (PBS; Merck-Biochrom, Berlin, Germany) and incubated with TRYPSIN/EDTA Solution (0,05%/0,02%; Merck-Biochrom) at 37 °C for approximately 3 min. Then, cells were detached by multiple pipetting using pre-warmed cell culture medium and pelleted by centrifugation for 10 min at 200 g. Afterwards, cells were resuspended in cell culture medium, counted using JuLI Br Live Cell Analyser (NanoEnTek, Seoul, Korea) and seeded as indicated.

For freezing, cells were resuspended in freezing medium (87.5% cell culture medium, 7.5% CCS, 5% dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA) at a density of 10^6 cells per mL. Aliquots of 1 mL were frozen in Nunc Cryotube Vials (Thermo Fisher Scientific) at -80 °C overnight using a MrFrosty freezing container (Nalgene by Thermo Fisher Scientific) and then transferred into a liquid nitrogen tank (-170 °C) for long term storage.

For thawing, frozen cell aliquots were thawed quickly at 37 °C and then added to 10 ml prewarmed cell culture medium. Cells were subsequently centrifuged for 10 min at 200 g, pellets were resuspended in 10 mL cell culture medium and seeded in T75 flasks. Approximately 24 h after thawing, medium was replaced.

7.2 Active Compounds

When cells were treated with active compounds dissolved in DMSO, it was ensured that the final concentration of DMSO did not exceed 1 ‰. If not indicated otherwise, stock solutions were stored at -20°C.

Mitotane (Sigma Aldrich) was dissolved in DMSO at a concentration of 100 mM.

Doxorubicin (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in DMEM/Ham's F12 Medium+GlutaMAX + 5% CCS + 1% ITS + 0.1% lipid mixture + 1% non-essential amino acids + 1% Penicillin/Streptomycin at a concentration of 1 µM.
Human HDL and LDL were purchased from Cedarlane (Burlington, Canada) and diluted to 10 mg/mL using a 15% solution of sucrose (Carl Roth) in DMEMF12+HEPES Medium (Gibco) in order to obtain stock solutions.
SCARB1 inhibitor BLT-1 (Sigma Aldrich) was dissolved in DMSO at a concentration of 200 mM.
AGTR1 inhibitor Losartan (Sigma Aldrich) was stored at 4°C. Prior to use, 30 mM solutions in PBS were freshly prepared.
MTOR inhibitor Rapamycin (Sigma Aldrich) was dissolved in DMSO at a concentration of 10 mM.
Wnt pathway inhibitor XAV939 (Sigma Aldrich) was dissolved in DMSO at a concentration of 10 mM.

7.3 Long-Term Mitotane Treatment of HAC-15 Cells

In order to induce mitotane resistance, HAC-15 cells were treated with 70 µM mitotane following a pulsed protocol (218). Medium was replaced every 3-4 days. At medium changes, cells alternatingly received either mitotane free medium or medium containing 70 µM mitotane or the corresponding amount of vehicle control (DMSO). At each passage, 10^7 cells were seeded and allowed to attach for 24 h before treatment was continued.

7.4 Clonal Selection of Long-Term Treated HAC-15 Cells

For selection of single clones, cells were plated on a 15 cm cell culture dish (VWR International) at a density of 56 cells/cm^2. No mitotane was added to the media. Clones were picked with cloning discs (Sigma Aldrich) when they had formed visible colonies. For picking, medium was removed and dishes were rinsed carefully with 5 mL PBS. Cloning discs were soaked in TRYPSIN/EDTA solution for at least 3 min and then placed on visible colonies. After approximately 1 min, cloning discs were transferred to 2 mL pre-warmed cell culture medium in a 24-well plate (Gibco) and subsequently expanded on 12-well plates (Gibco).

7.5 Growth Curves of Mitotane-Treated HAC-15 Cells

At each passage, cells were counted using JuLI Br Live Cell Imager (NanoEnTek) and cumulative population doublings (cPD) (219) were calculated from total cell numbers using equation 1. For growth curves, cPD was plotted against the time in culture (days).

$$cPD = cPD_L + \log_2\left[\frac{N_C}{N_S}\right]$$

Equation 1: Calculation of cumulative population doublings (cPD).
cPD is the cumulative populations doublings.
cPD_L is the cumulative population doublings after the last passage.
N_C is the number of cells counted at the current passage.
N_S is the number of cells seeded after the last passage.

7.6 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay

MTT-Assay was used to assess the cytotoxicity of various compounds (220). At approximately 80 % confluence, cells were harvested with TRYPSIN/EDTA solution, seeded in triplicates on a 96-well plate (Gibco) at a density of $4*10^4$ cells per well and allowed to attach at 37°C for 24 h. Then, cells were treated with increasing concentrations of cytotoxic compounds at 37°C for 72 h. Afterwards, a 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Aldrich) in PBS was added to each well yielding a final concentration of 0.45 mg/mL and plates were incubated at 37 °C for 2 h. Medium was then removed and violet MTT crystals were dissolved in 150 µL of a 10% solution of Triton X-100 (Sigma Aldrich) in 2-propanol (VWR International, Langenfeld, Germany) at pH 4.7 by shaking for 20 min and multiple pipetting. Absorption at 595 nm was recorded utilizing an EnSpire 2300 Multilabel Reader (PerkinElmer, Waltham, MA, USA). Concentrations of the compounds were $\log_{10}$-transformed and plotted against the averaged absorption at 595 nM. For calculation of IC_{50}, data were fitted to a four parameter dose-response curve with PRISM 7 software (GraphPad, La Jolla, CA, USA) according to equation 2.

$$Y = A + \left(\frac{(A-B)}{(1+10^{((logIC_{50}-X)*HS)})}\right)$$

Equation 2: Four parameter dose-response curve fit.
Y is the mitotane response (absorption at 595 nm).
A is the minimum asymptote or the maximally inhibited response.
B is the maximum asymptote or maximal response.

IC_{50} is the half-maximal inhibitory concentration.
X is the log_{10} transformed mitotane concentration.
HS is the Hill slope.

7.7 Measurement of the IC_{50} of Mitotane in Various Media and Correlation Analysis

Cells were detached using TRYPSIN/EDTA solution, counted and centrifuged as described above. Pellets were resuspended in DMEM/Ham's F12 Medium+GlutaMAX (Gibco) supplemented with 1% Penicillin/Streptomycin and 5% CCS (Hyclone), 2.5% CCS, 5% Nu-Serum (NuS, Corning), 2.5% NuS or 5% fetal calf serum (FCS, Merck-Biochrom) and cells were seeded on a 96 well plate at a density of $4*10^4$ cells per well. After 24 h, cells were treated with increasing concentrations of mitotane for 72 h and MTT assay was performed as described above. The serum concentrations of cholesterol, HDL, LDL and triglycerides in mg/L were determined by photometry by the Central Laboratory of the University Hospital Düsseldorf, Germany, and the final concentration of each of the compounds in the media was calculated. All groups were tested for normality using Shapiro-Wilk test. The concentration of the lipid species was plotted against their respective IC_{50}s, and correlation analysis according to Pearson was performed using PRISM 7 software.

7.8 Measurement of Intracellular Lipids by ESI-MS/MS

Intracellular amounts of Phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, PE based plasmalogens, phosphatidylserine, phosphatidylinositol, lysophosphatidylcholine, ceramide, cholesteryl ester and free cholesterol were determined in three nonresistant control clones and three mitotane resistant clones by Gerhard Liebisch from the Institute of Clinical Chemistry and Laboratory Medicine of the University Hospital Regensburg, Germany. Cells were seeded on a 6-well plate and allowed to attach for 24 h. Then, cells were treated with 10 µM mitotane and DMSO in cell culture medium containing 0% CCS and DMSO, 20 and 50 µM mitotane in cell culture medium containing 5% CCS for 72 h. Cells were then washed thrice with cold PBS and lysed in 1 mL water containing 0.1% sodium dodecyl sulfate (SDS, Biomol, Hamburg, Germany). Protein content was measured by BCA assay and lysates were frozen at -80 °C. The amount of lipids in nmol was measured by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and normalized to total protein quantity in mg (BCA

assay), as previously described (221). For statistics, groups were tested for normality using Shapiro-Wilk test. Groups passing the normality test were compared using a one-way ANOVA. As *post hoc* test for multiple comparisons Bonferroni's multiple comparisons test was used, when different conditions were compared within "nonresistant“ or “resistant“, and Fisher‘s LSD test was used when “nonresistant“ was compared to “resistant“ within one condition. Groups failing the normality test were compared using a Kruskal-Wallis test. As *post hoc* test for multiple comparisons Dunn’s test was used, when different conditions were compared within "nonresistant“ or “resistant“, and Dunn’s uncorrected test was used when “nonresistant“ was compared to “resistant“ within one condition.

7.9 Measurement of Mitotane by GCMS

Intracellular and medium mitotane concentration in three nonresistant control and three mitotane resistant clones was determined by Hans-Wolfgang Hoppe at the Medical Laboratory Bremen, Germany, using GCMS according to in-house protocols in accordance with European norms DIN EN ISO 17025 and 15189. Mitotane resistant and nonresistant control cells were seeded on 10 cm^2 ($5*10^5$ cells per plate). After 72 h, medium was replaced with medium containing 50 µM mitotane. After 48 h, medium was removed and stored at -80 °C. Cells were harvested using 4 mL PBS, centrifuged for 10 min at 200 g, resuspended in 400 µL ultrapure water, lysed at 30 Hz for 10 min using a Retsch Mixer Mill 400 (Verder Scientific, Haan, Germany), and samples were frozen at -80 °C.

One-hundred microliters lysate were treated with ultrasound for 5 min, diluted with 100 µL formic acid and mitotane was extracted using 1 mL isooctane. Finally, extracts were subjected to GCMS analysis. Mitotane concentration was normalized to protein concentration (BCA assay). For statistics, Mann-Whitney test used.

7.10 RNA Isolation

For RNA isolation, cells were washed twice with cold PBS (1 mL per well of a 6-well plate) and 700 µL QIAzol Lysis Reagent (Qiagen, Hilden, Germany) were added each well. Cells were incubated for approximately 5 min, harvested by scraping, and RNA was extracted from homogenates using the miRNeasy MiniKit and RNase-Free DNase

set (both from Qiagen) according to the manufacturer's instructions. Homogenates were incubated at rt for 5 min, and afterwards 140 µL chloroform were added followed by vigorous shaking for 15 s. Samples were incubated at rt for 3 min and then centrifuged at 12,000 g and 4 °C for 15 min. Afterwards, the aqueous phase was mixed with 530 µL 100% ethanol (VWR International) and pipetted on an RNeasy mini column followed by centrifugation at 8,000 g for 30 s. Then, 350 µL buffer RW1 was added to the column followed by centrifugation at 8,000 g for 30 s. For DNase digest, 70 µL buffer RDD were pre-mixed with 10 µL DNase and added to the column. After 15 min incubation at rt, 700 µL buffer RW1 were added and columns were centrifuged at 8,000 g for 30 s. Afterwards, columns were washed with two changes of buffer RPE (500 µL each) and centrifugation at 8,000 g for 30 s and 2 min, respectively. Then, columns were dried by centrifugation at full speed for 1 min. For elution of RNA, 30 µL water was added to the columns. After 1 min of incubation, elution was completed by centrifugation at 8,000 g for 1 min.

RNA concentration was measured using NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). RNA samples were kept at -80 °C until further use.

7.11 Complementary-DNA Synthesis and Real-Time PCR

For cDNA synthesis, 400 ng RNA were transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. For digest of genomic DNA, 2 µL gDNA Wipeout Buffer were added to 12 µL RNase-free water containing 400 ng RNA. Samples were incubated at 42°C for 3 min in a Mastercycler nexus SX1 (Eppendorf, Hamburg, Germany) and immediately placed on ice afterwards. Then, 1 µl Quantiscript Reverse Transcriptase, 4 µl 5x Quantiscript RT Buffer and 1 µL RT Primer Mix were added and samples were subsequently incubated at 42°C for 15 min and at 95°C for 3 min. CDNA was stored at -20 °C until further use.

Real-Time PCR was done using 2X Power SYBR Green PCR Master Mix (Applied Biosystems by Thermo Fisher Scientific). Real-time (rt) PCR was done using published primers (Eurofinns Genomics, Ebersberg, Germany) for *TBP* (222) and *AXIN2* (223), reconstituted with RNase free water (Qiagen) to 100 µM and diluted to 10 µM before use. Samples were prepared according to table 1.

Table 1: Sample preparation of real-time PCR.

Component	Volume [µL]	Final Concentration
Power SYBR Green (2x)	10	1X
Primer forward (10µM)	0.2	100 nM
Primer reverse (10µM)	0.2	100 nM
cDNA	1 µL	-
RNase free water	8.6	-

Reactions were run in 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the protocol shown in table 2.

Table 2: Real-time PCR protocol.

Step	Temperature [°C]	Time [mm:ss]	Comment
1	95	10:00	
2	95	00:15	
3	60	01:00	Go to step 2 (40x) data acquisition
4	95	00:15	
5	60	01:00	
6	95	00:15	
7	60	00:15	

Samples were run in triplicates. Relative gene expression was calculated from the averaged CT values according to the $2^{-\Delta\Delta CT}$ method as shown in equation 3 (224).

$$relative\ \ Expression = 2^{-\Delta\Delta CT} = 2^{-(CT_{XQ}-CT_{XR})-(CT_{KQ}-CT_{KR})}$$

Equation 3: Calculation of relative gene expression in any sample X compared to a reference sample K.
CT is threshold cycle.
Q is gene of interest.
R is reference gene.

7.12 Gene Expression Microarray Analysis

Six nonresistant control and six mitotane resistant HAC-15 clonal cell lines were seeded on a 6-well plate at a density of $1*10^6$ cells per well and allowed to attach for 24 h. Then, cells were treated with either vehicle control (DMSO) or 50 µM mitotane

for 18 h. Afterwards, RNA was isolated as described above and RNA integrity was confirmed using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Microarray procedure was performed at the Center for Applied Genomics at the Hospital for Sick Children (Toronto) using Affymetrix GeneChip PrimeView Human Gene Expression Array. For overall quality control, log_2 transformed raw intensities were plotted as box-plots and only perfect match probes were included into the analysis. Further quality control was done by examination of density plots of log_2 transformed raw intensities. Data was processed using the robust multi-array adjustment procedure (225) on raw intensities and included background correction, normalization and summarization of the raw intensity data.

7.13 Isolation of Genomic DNA from Cultured Cells

Genomic DNA from cells was isolated using DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol.

For DNA isolation, cells were grown on a six-well plate until approximately 80% confluency. Cells were rinsed with PBS once and harvested in PBS by scraping and centrifugation at 300 g for 5 min. Pellets were resuspended in 200 µL PBS. Then, 20 µL proteinase K and 200 µL buffer AL were added subsequently. Samples were vortexed for at least 20 s and incubated at 56°C for 10 min. Afterwards, 200 µL 100% ethanol were added followed by vortexing. The mixture was pipetted onto a DNeasy Mini spin column followed by centrifugation at 6000 g for 1 min. Columns were subsequently washed with 500 µL buffer AW1 and AW2 followed by centrifugation for 1 min at 6000 g and 3 min at 20000 g, respectively. For elution of DNA, 200 µL buffer AE were added to the column followed by incubation at rt for 1 min and centrifugation for 1 min at 6000 g. Concentration and purity of DNA were measured using NanoDrop 2000c Spectrophotometer.

7.14 Exome Sequencing

Exome sequencing was done at the Yale Center of Genome Analysis using the SeqCap EZ MedExome Kit (Roche NimbleGen) and 100-bp paired-end sequencing on the Illumina HiSeq platform, following the manufacturer's instructions. Discovery of somatic mutations was done in the working group of Murim Choi at the Seoul National

University, Seoul, Korea, as previously described (130). Sequences were mapped to the human genome (hg18) using Maq software (226). Aligned reads were further analyzed using Perl scripts. PCR duplicates were discarded using Samtools software (227) and novelty of variations was tested using dbSNP (228) and 1000 Genomes Database (229). Two-tailed Fisher's exact test was used to test for significance of differences in read distributions between founder cell line (HAC-15 cells at passage 3) and nonresistant control or mitotane resistant clonal cell lines.

7.15 Analysis of Gene Expression Microarray and Exome Sequencing Data

Comprehensive data analysis including discovery of differentially expressed genes, Gene Ontology enrichment analysis (136, 230, 231) and copy number variation (CNV) profiling was done by Clemens Messerschmidt and Benedikt Obermayer from the Core Unit Bioinformatics (CUBI) at the Berlin Institute of Health (BIH), Berlin, Germany. For CNV Profiling, each whole-exome data set was matched against genome reference GRCh37 using BWA-mem (232). Copy number alterations where analyzed in a tumor/normal paired fashion with the R package CopywriteR (233) with 50 kb bins and annotated with the CIViC database (234), where the nonresistant clone was treated as normal and mitotane resistant clones as tumor sample.

7.16 BCA Assay

BCA assay was done using Pierce BCA Protein Assay Kit (Pierce by Thermo Fisher Scientific) according to the manufacturer's protocol. As standards, bovine serum albumin (BSA) was used at 2000, 1500, 1000, 750, 500, 250, 125 and 25 µg/mL. Two-hundred microliters working solution (50:1, bicinchoninic acid solution "A" and Cu^{2+} solution "B") were added to 25 µL of each standard and sample, and samples were incubated for 30 min at 37°C. Afterwards, samples were kept at rt for approximately 10 min and absorption was then measured at 562 nm using EnSpire 2300 Multilabel Reader (PerkinElmer, Waltham, MA, USA). Samples were run in duplicates. For calculation of protein concentration, the mean was calculated, blank was subtracted, standard protein concentration was plotted against the absorbance using an Excel worksheet (Microsoft, Redmond, WA, USA) and data were fitted using a linear regression. Protein concentration was calculated according to equation 4.

$$C_P = \frac{A_{562\,nm} - b}{m}$$

Equation 4: Calculation of protein concentration from BCA assays.

Cp is the protein concentration in µg/mL

$A_{562\,nm}$ is the sample's absorption at 562 nm.

b is the y-intercept of the calibration curve.

m is the slope of the calibration curve.

8 Results

8.1 Development of an *In Vitro* Model of Mitotane Resistance

8.1.1 Generation of a Mitotane-Resistant Adrenocortical Carcinoma Cell Line

To assess the dose-dependency of the cytotoxic effects of mitotane on HAC-15 cells, dose-response curves using MTT assay as readout for cell viability were recorded, and the half-maximal inhibitory concentration (IC_{50}) was determined using a sigmoidal dose-response curve fit (figure 4).

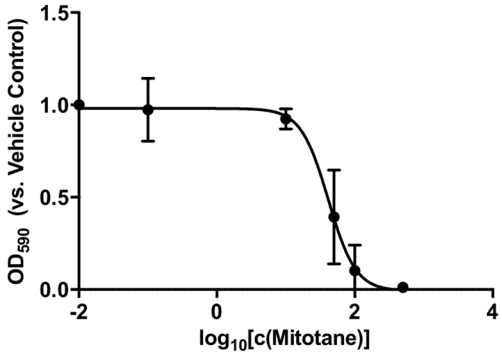


Figure 4: Dose-response curve of mitotane in HAC-15 cells. Cells were incubated with increasing doses of mitotane for 72 h and cell viability (OD_{590}) was measured by MTT-assay. Cell viability was normalized to vehicle control, and results are shown as mean percentage and SD of five independent experiments. Mitotane dose-dependently inhibits cell viability and proliferation in HAC-15 cells.

The IC_{50} of mitotane in HAC-15 cells was (47.4 ±15.0) µM. In order to induce mitotane resistance, HAC-15 cells were treated with 70 µM mitotane (1.47 times the IC_{50}) or the corresponding amount of vehicle control (DMSO) following a pulsed protocol (218). Growth curves of control and mitotane treated cells are shown in figure 5.

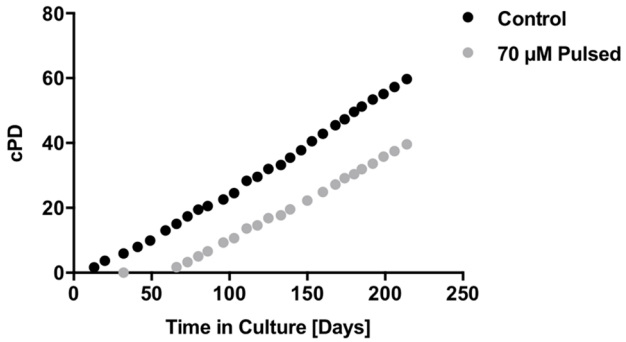


Figure 5: Growth curves of long-term mitotane treated HAC-15 cells. Every 3-4 days, cells were treated with 70 µM mitotane (70 µM Pulsed) or the corresponding amount of vehicle control (DMSO; Control) in on-off cycles. Cells were counted at each passage, and the total number of population doublings (cumulative population doublings;

cPD) was calculated. During long-term treatment the growth curve flattened drastically, but the population recovered after approximately 30 days.

At the beginning of the treatment, the growth curve of mitotane treated cells flattened drastically, but after approximately 30 days of treatment, the population of mitotane treated cells started to recover, and after approximately 70 days of treatment, the pace of the population growth in mitotane treated cells was comparable to vehicle control treated cells. The IC_{50} of mitotane was then measured in bulk cultures of vehicle control treated and mitotane treated cells (figure 6).

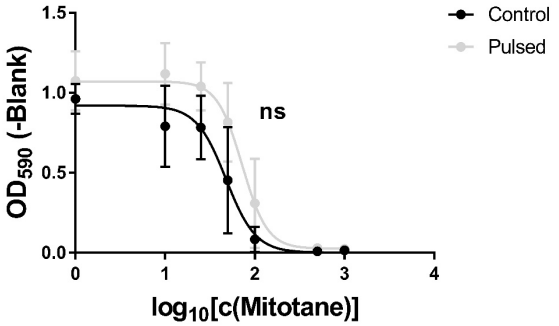


Figure 6: Dose-response curves of mitotane in long-term mitotane (70 µM Pulsed) versus vehicle control treated (Control) HAC-15 cells. The IC_{50} was measured by MTT-assay after 72h of incubation. Results are shown as mean±SD of three independent measurements. The dose-response curve of mitotane treated cells shows a shift to higher concentrations but differences in IC_{50} are not significant. For statistics, the Mann-Whitney-test was used. ns, $p>0.05$.

The IC_{50} of mitotane in vehicle control treated and mitotane treated cells was (45.9 ±25.5) µM and (76.7 ±24.5) µM, respectively. The dose-response curve of bulk cultures of mitotane treated cells showed a shift towards higher mitotane concentrations in comparison to the dose-response curve of vehicle control treated cells. While there was no significant difference in the IC_{50}s ($p=0.40$), this observation, together with the recovery of population growth observed in the growth curves, indicated that the sensitivity of long-term treated HAC-15 cells towards mitotane had decreased. In order to isolate clonal cell lines, mitotane treated and vehicle control treated cells were seeded out at low density after 72 days of treatment, and colonies were subsequently picked and expanded. To assess mitotane sensitivity of mitotane treated and vehicle control treated HAC-15 clonal cell lines, the IC_{50} of mitotane was again measured by MTT assay. The IC_{50}s of clonal mitotane and vehicle control treated cell lines are shown in figure 7.

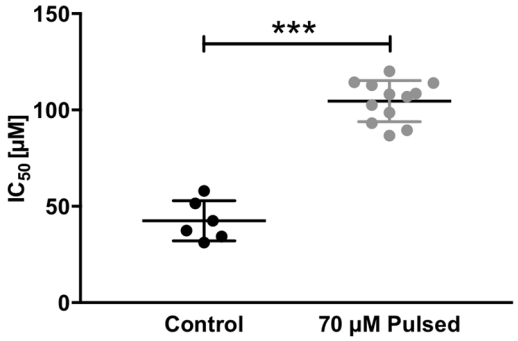


Figure 7: The IC_{50} of mitotane in clonally selected long-term mitotane (70 µM Pulsed, N=12) versus vehicle control treated (Control, N=6) HAC-15 cells. The IC_{50} was measured by MTT-assay after 72 h of incubation. Data of one representative experiment is shown. Results are shown in scatter dot plots with mean±SD. The IC_{50} of mitotane in clonal cell lines of long-term treated HAC-15 cells is significantly increased. For statistics, the Mann-Whitney-test was used. *** $p<0.001$.

The IC_{50} of long-term treated clonal cell lines was significantly increased ((102.2±7.3) µM versus (39.4±6.2) µM; p=0.0001), confirming 2.6 fold mitotane resistance (218) in long-term treated HAC15 cells.

8.1.2 Multidrug Resistance in Mitotane Resistant HAC-15 Clonal Cell Lines

Increased activity of P-glycoprotein is a common feature of multidrug resistant cancer cells (235). Doxorubicin is a substrate of P-glycoprotein (236) and therefore doxorubicin sensitivity has been used to assess P-glycoprotein activity in the NCI-H295 ACC cell line (210). In order to clarify whether long-term treatment with mitotane had induced P-glycoprotein activity and thereby common multidrug resistance, the IC_{50} of doxorubicin was measured by MTT assay in presence of various mitotane concentrations in nonresistant control and mitotane resistant clonal cell lines (figure 8 and table 3).

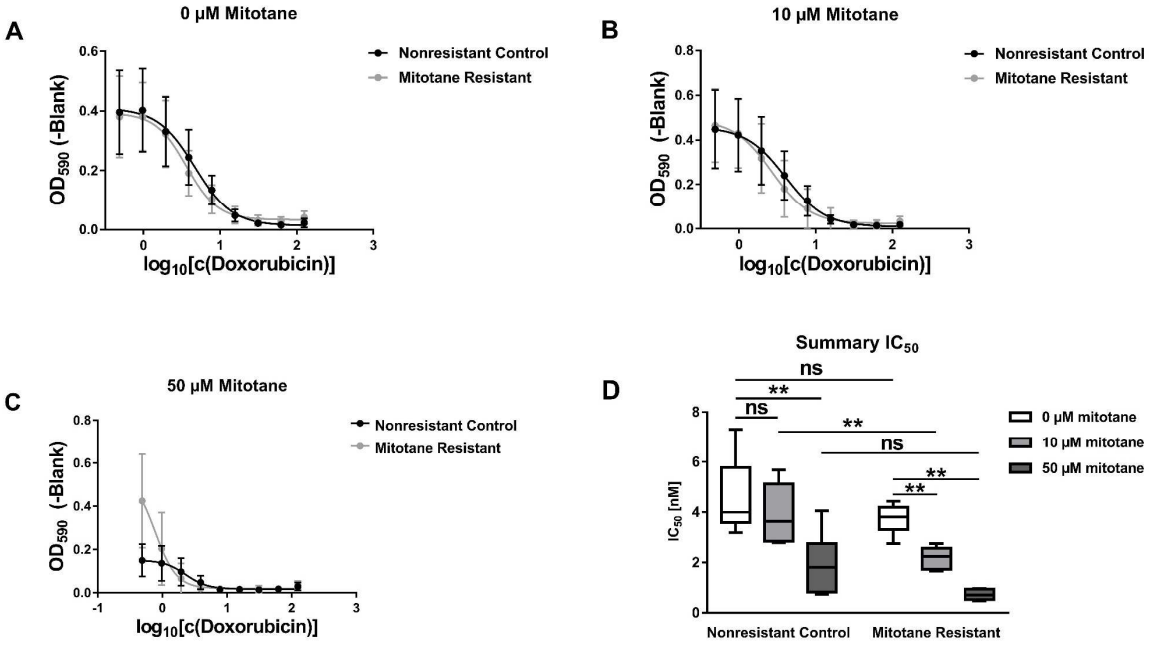


Figure 8 A-C: Dose-response curves of doxorubicin in mitotane resistant versus nonresistant control HAC-15 clones. Cells were incubated with increasing doses of doxorubicin for 72 h in presence of A) no mitotane, B) 10 µM mitotane or C) 50 µM mitotane, and cell viability (OD_{590}) was measured by MTT-assay. Blank was substracted, and results are shown as mean±SD of six mitotane resistant and nonresistant control clones. **D:** The IC_{50} of doxorubicin calculated from dose-response curves A-C using a four parameter dose-response curve fit. Results are shown in box-and-whiskers (min to max) plots. Boxes extend from 25th to 75th percentiles, the line is the median and whiskers go to the smallest (min) and to the largest value (max). Mitotane resistance does not lead to additional resistance to doxorubicin, while addition of mitotane leads to a significantly higher sensitivity towards doxorubicin in mitotane resistant cells compared to nonresistant control cells. For statistics, the Mann-Whitney test was used. *p<0.05; **p<0.01; ***p<0.001.

Table 3: The IC_{50} of doxorubicin in nonresistant control and mitotane resistant cells in absence and presence of two concentrations of mitotane. IC_{50}s were calculated from dose-response curves in figure 8 A-C (mean±SD), and p-values were determined by Mann-Whitney test.

	IC_{50} Nonresistant Control [nM]	IC_{50} Mitotane Resistant [nM]	p-Value
0 µM Mitotane	4.6±1.4	3.7±0.5	0.4848
10 µM Mitotane	3.9±1.0	2.2±0.4	0.0022
50 µM Mitotane	1.9±1.1	0.7±0.2	0.0649

In absence of mitotane and presence of 50 µM mitotane, no significant difference between the IC_{50} of doxorubicin in nonresistant control and mitotane resistant clonal cell lines was found. In presence of 10 µM mitotane, the IC_{50} of doxorubicin was significantly lower in mitotane resistant compared to nonresistant control clonal cell lines. The lack of doxorubicin resistance in mitotane resistant cells suggests, that the

underlying mechanism of mitotane resistance in this *in vitro* model of mitotane resistant ACC is different from common multidrug resistance. In mitotane resistant and nonresistant control cells, simultaneous treatment with 50 µM mitotane significantly decreased the IC_{50} of doxorubicin (p(resistant)=0.0022; p(nonresistant)=0.015), while 10 µM mitotane only had a significant effect on the IC_{50} of doxorubicin in mitotane resistant cells (p(resistant)=0.0022; p(nonresistant)=0.48).

8.1.3 The Influence of Lipoprotein Species on Mitotane Resistance

It was noticed during long-term cell culture of HAC-15 cells, that the IC_{50}s of mitotane in HAC-15 cells cultured in media containing different amounts of Nu-Serum (NuS), cosmic calf serum (CCS) and fetal calf serum (FCS) differed remarkably. Circulating lipoprotein species have been shown to influence the cytotoxicity of mitotane *in vitro* (167, 168). In order to investigate a possible correlation between the IC_{50} of mitotane and the concentrations of HDL, LDL, cholesterol and triglycerides in media containing different amounts of NuS, CCS and FCS, the concentration of the lipoprotein species was determined in each of the sera, and final concentrations in the media were calculated. Then, the concentration of HDL, LDL, cholesterol and triglycerides was plotted against the respective IC_{50}, and Pearson correlation coefficients were computed. Dose-response curves in various media and a plot of the IC_{50}s versus the concentrations of HDL, LDL, cholesterol and triglycerides are shown in figure 9, correlation coefficients and p-values are shown in table 4.

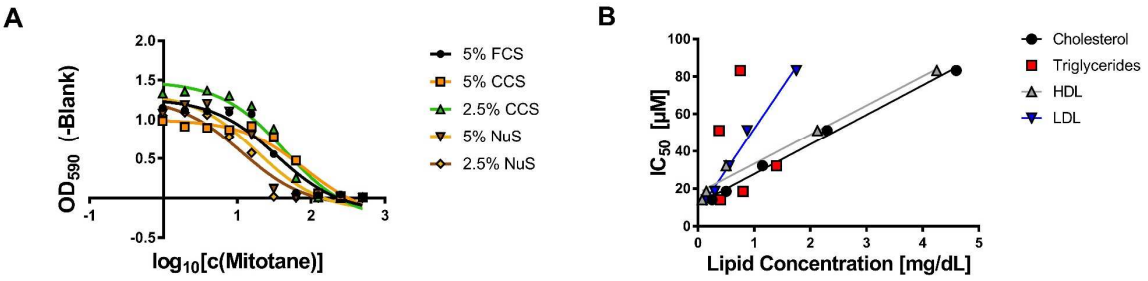


Figure 9 A and B: The IC_{50} of mitotane in different media and correlation analysis. **A:** Dose-response curves of mitotane in HAC-15 cells cultivated in media containing different amounts of Nu-Serum (NuS), cosmic calf serum (CCS) and fetal calf serum (FCS). Cells were incubated with increasing doses of mitotane for 72 h, and cell viability (OD_{590}) was measured by MTT-assay. **B:** The IC_{50} of mitotane in HAC-15 cells plotted against the concentration of cholesterol, triglycerides, HDL and LDL in the cell culture medium with linear regression. The concentration of cholesterol, triglycerides, HDL and LDL in these sera were measured by photometry, and the amount of each of

the compounds in the media was calculated. All data passed the Shapiro-Wilk test for normality and correlation analysis revealed a significant positive correlation between IC_{50} and the concentration of cholesterol, HDL, LDL, but not triglycerides.

Table 4: Pearson correlation coefficients and p values for correlations between the IC_{50} of mitotane and the concentration of cholesterol, HDL, LDL and triglycerides in the medium.

	IC_{50} vs. Cholesterol	IC_{50} vs. Triglycerides	IC_{50} vs. HDL	IC_{50} vs. LDL
r (Pearson)	0.9969	-0.02693	0.9888	0.9949
p-Value	0.0002	0.9657	0.0004	0.0014

A strongly significant, positive correlation was found between the IC_{50} of mitotane and the concentration of HDL, LDL and free cholesterol but not triglycerides in the medium. In order to clarify whether these compounds also play a role in mitotane resistance, the IC_{50} of mitotane in nonresistant control and mitotane resistant cells in presence of different concentrations of HDL and LDL was measured (figure 10 and table 5).

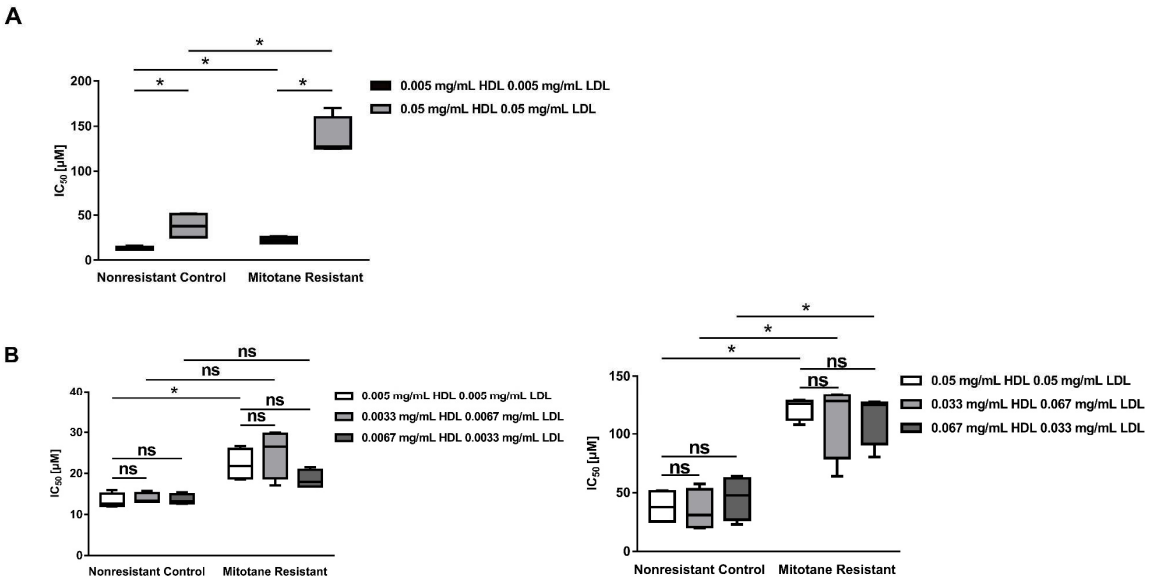


Figure 10 A and B: The influence of HDL and LDL on mitotane cytotoxicity and resistance. **A:** The IC_{50} of mitotane in four mitotane resistant versus four nonresistant control HAC-15 clones in presence of different amounts of HDL and LDL, determined by MTT-assay after 72h of incubation. Results are shown in box-and-whiskers (min to max) plots. Boxes extend from 25th to 75th percentiles, the line is the median and whiskers go to the smallest (min) and to the largest value (max). Mitotane resistance is dependent on HDL and LDL concentration in cell culture media. **B:** The IC_{50} of mitotane of mitotane in four mitotane resistant versus four nonresistant control HAC-15 clones in presence of different HDL to LDL ratios, determined by MTT-assay after 72h of incubation. Results are shown in box-and-whiskers (min to max) plots. Mitotane resistance depends on total cholesterol concentration in cell culture medium, but not the HDL to LDL ratio. For statistics, the Mann-Whitney-test was used. ns, $p>0.05$; *$p<0.05$.

Table 5: The IC_{50} of mitotane in nonresistant control and mitotane resistant cells in presence of different amounts of HDL and LDL in the medium (mean±SD) and p-values (Mann-Whitney test).

Concentration [mg/mL]	IC_{50} Nonresistant Control [µM]	IC_{50} Mitotane Resistant [µM]	p-Value
0.05 (HDL); 0.05 (LDL)	38.1±14.4	137.5±21.7	0.029
0.005 (HDL); 0.005 (LDL)	13.3±1.8	22.2±3.8	0.029
0.033 (HDL); 0.067 (LDL)	34.8±17.2	113.5±33.2	0.029
0.067 (HDL); 0.033 (LDL)	45.6±18.4	114.1±22.6	0.029
0.0033 (HDL); 0.0067 (LDL)	13.8±1.3	22.9±9.8	0.343
0.0067 (HDL); 0.0033 (LDL)	13.6±1.3	16.5±5.5	0.343

At all conditions except for 0.0033 mg/mL HDL; 0.0067 mg/mL LDL and 0.0067 mg/mL HDL; 0.0033 mg/mL LDL, the IC_{50} of mitotane in mitotane resistant cells was significantly increased in comparison to nonresistant control cells. At 0.005 mg/mL HDL and LDL, the IC_{50} of both, nonresistant control and mitotane resistant cells was significantly lower than at 0.05 mg/mL HDL and LDL. Interestingly, the fold resistance was decreased in media with reduced lipoprotein content (3.6 at 0.05 mg/mL HDL and LDL versus 1.7 at 0.005 mg/mL HDL and LDL), while varying the ratio of HDL and LDL did not have a prominent influence on fold resistance (for p-values, please refer to table 6).

Table 6: P-values (Mann-Whitney test) for comparisons of IC_{50}s of mitotane resistant and nonresistant control cells in presence of different HDL to LDL ratios (figure 10).

Comparison (Concentrations in mg/mL)	Condition	p-Value
0.05 (HDL); 0.05 (LDL) vs. 0.005 (HDL); 0.005 (LDL)	Resistant	0.029
0.05 (HDL); 0.05 (LDL) vs. 0.033 (HDL); 0.067 (LDL)	Resistant	0.890
0.05 (HDL); 0.05 (LDL) vs. 0.067 (HDL); 0.033 (LDL)	Resistant	0.086
0.005 (HDL); 0.005 (LDL) vs. 0.0033 (HDL); 0.0067 (LDL)	Resistant	0.686
0.005 (HDL); 0.005 (LDL) vs. 0.0067 (HDL); 0.0033 (LDL)	Resistant	0.200
0.05 (HDL); 0.05 (LDL) vs. 0.005 (HDL); 0.005 (LDL)	Nonresistant	0.029
0.05 (HDL); 0.05 (LDL) vs. 0.033 (HDL); 0.067 (LDL)	Nonresistant	0.686
0.05 (HDL); 0.05 (LDL) vs. 0.067 (HDL); 0.033 (LDL)	Nonresistant	0.686
0.005 (HDL); 0.005 (LDL) vs. 0.0033 (HDL); 0.0067 (LDL)	Nonresistant	0.343
0.005 (HDL); 0.005 (LDL) vs. 0.0067 (HDL); 0.0033 (LDL)	Nonresistant	0.686

These observations suggest that mitotane resistance in this *in vitro* model of mitotane resistant ACC is influenced by the overall amount of HDL and LDL in the cell culture medium.

8.2 Investigation of Gene Expression and DNA Integrity in Mitotane Resistant and Nonresistant Control Cells

8.2.1 Gene Expression Microarray Analysis

In order to investigate the underlying mechanisms of mitotane resistance in the present *in vitro* model, gene expression microarray analysis was performed in six mitotane resistant versus six nonresistant control clones treated with either vehicle control (DMSO) or 50 µM mitotane.

Principle component analysis of gene expression data sets (figure 11) revealed two distinct clusters of mitotane resistant and nonresistant control clonal cell lines.

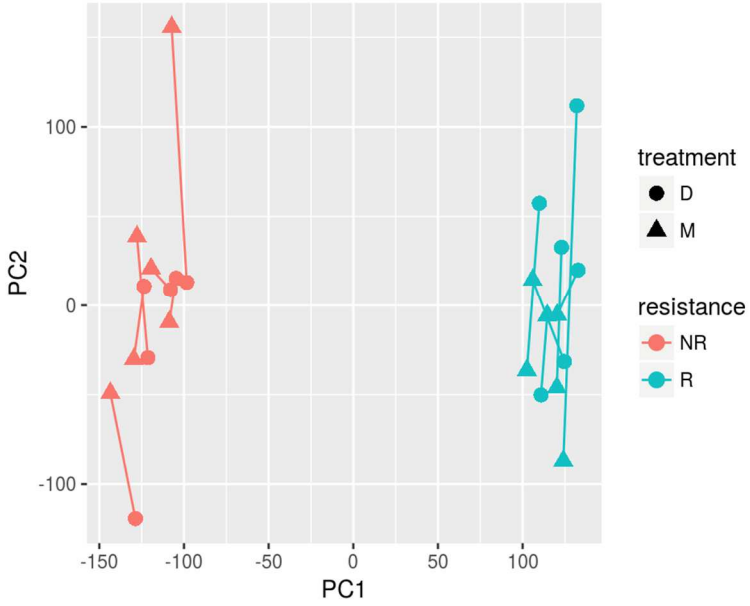


Figure 11: Principle component analysis of gene expression array data sets. Mitotane resistant (R) and nonresistant control clonal cell lines (NR) cluster in two distinct clusters. Abbreviations: D, DMSO; M, Mitotane; NR, nonresistant control; R, mitotane resistant; PC1, principle component 1; PC2, principle component 2.

Preceding data suggested a role of lipoproteins in mitotane resistance in the present *in vitro* model. Therefore, as a first approach to gene expression data analysis, only genes annotated for pathways related to „cholesterol“ and „steroids“ according to the Gene Ontology (GO) Consortium (136, 230) were included in the data analysis. Multiple t-test discovery analysis at a threshold of q-Value<0.01 yielded 72 differentially

expressed genes between mitotane resistant and nonresistant control cells. A heatmap is shown in figure 12. Differentially expressed genes that were downregulated included *STAR* (encoding for steroidogenic acute regulatory protein), *CYP11A1* (encoding for cholesterol side-chain cleaving enzyme) and *CYP11B2* (encoding for aldosterone synthase) in mitotane resistant cells. Both, *STAR* and *CYP11A1* catalyze the first, rate-limiting step of steroidogenesis (237), while *CYP11B2* is only expressed in the zona glomerulosa and the most important regulator of aldosterone secretion (238). Further, *SOAT1* (encoding for sterol-O-acyl-transferase 1), a major intracellular target of mitotane (173), was found to be downregulated in mitotane resistant cells, alongside with several genes implicated in transmembrane traffic of cholesterol including very low-density lipoprotein receptor (*VLDLR*) (239), ATP-binding cassette, sub-family A member 1 (*ABCA1)* (240), *LDLR* and *SCARB1* (7).

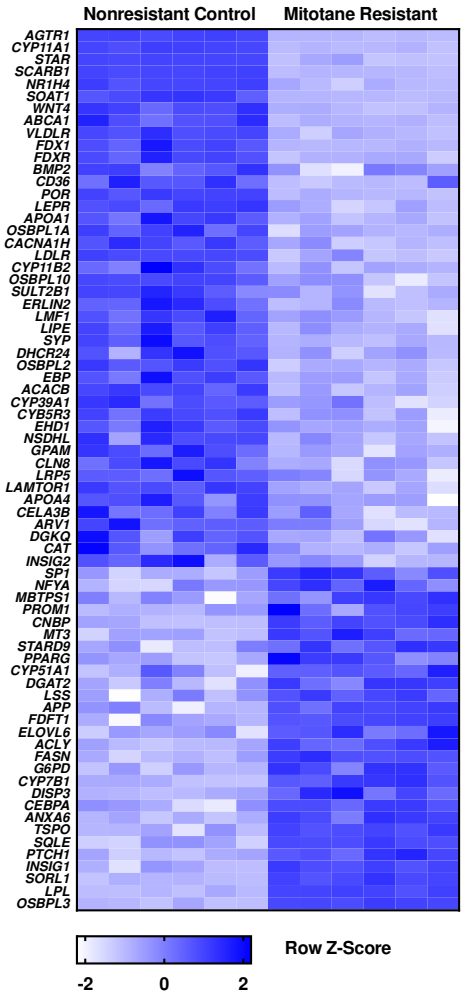


Figure 12: Heat map of significantly regulated genes annotated for GO pathways related to „cholesterol“ and „steroids“ in mitotane resistant compared to nonresistant HAC-15 clones. Genes are sorted according to their $\log_2$ fold change. GO pathways were found with AmiGO2. Genes were selected from an Affymetrix PrimeView RNA

array comprising 49495 probes and tested for significance by multiple t-test discoveries (threshold for discovery: q-Value<0.01).

As a second, more unbiased approach comparing gene expression microarray data of nonresistant control and mitotane resistant clonal cell lines, all probes with the highest average expression within a probe set were included into the analysis. Introducing cut-offs (5% false discovery rate, $|\log_2$ fold change$|> 0.5$ and average expression> 5) yielded a total of 1581 differentially regulated genes comparing vehicle control (DMSO) treated nonresistant and mitotane resistant clonal cell lines, 60 differentially expressed genes comparing DMSO treated nonresistant to mitotane treated nonresistant clonal cell lines and 1636 differentially expressed genes comparing mitotane treated mitotane resistant to mitotane treated nonresistant clonal cell lines. Interestingly, applying the same analytical procedure on microarray comparison of mitotane treated versus DMSO treated mitotane resistant clones yielded no differentially expressed genes. A heatmap of the 150 most significantly regulated genes between DMSO treated nonresistant and mitotane resistant clonal cell lines is shown in figure 13. Unbiased analysis confirmed significant downregulation of *LDLR*, *SCARB1*, *STAR* and *CYP11A1* previously discovered during biased analysis, and further discovered significant downregulation of *NR4A1* (encoding for NGFIB), *NR5A1* (encoding for SF-1), *HSD3B2, CPY17A1, CYP19A1* and *CYP21A2*. Further, unbiased analysis revealed no significant changes in gene expression of *MDR1, ABCC1* and *ABCG2,* encoding for P-glycoprotein, MRP1 and BCRP, the three most common ABC transporters involved in multidrug resistance (235).

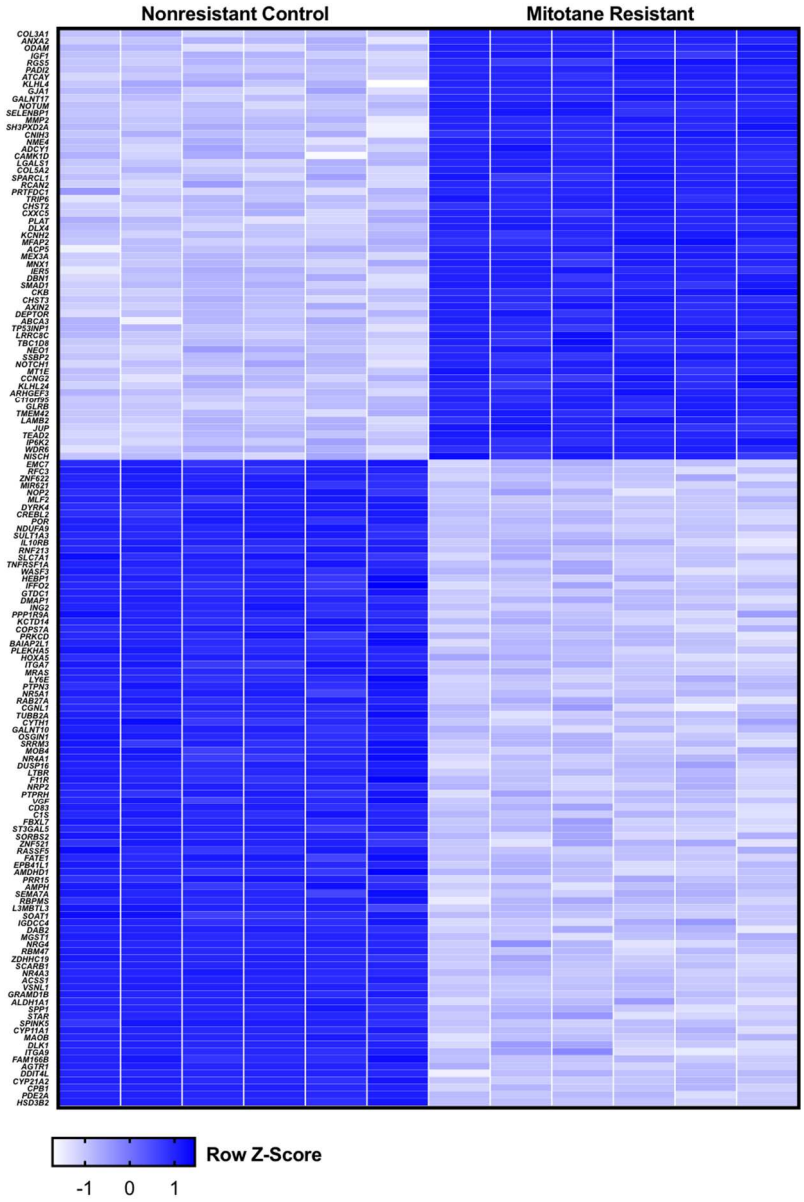


Figure 13: Heat map of the 150 most significantly regulated genes in mitotane resistant cells. Genes were discovered by Affymetrix PrimeView RNA array of six vehicle control (DMSO) treated mitotane resistant versus six DMSO treated nonresistant control HAC-15 clones, sorted according to log2 fold change. Only probe sets with the highest expression were included into the analysis, and cutoffs at 5% false discovery rate, |log2 fold change |> 0.5 and average expression > 5 were introduced leading to discovery of a total of 1581 differentially regulated genes.

Gene Ontology enrichment analysis (136, 230, 231) of upregulated genes comparing DMSO treated mitotane resistant to nonresistant control clonal cell lines revealed significantly enriched terms for multiple biological processes and molecular functions implicated in the Wnt signaling pathway and cell growth. Applying GO enrichment analysis on downregulated genes between DMSO treated mitotane resistant and nonresistant control clonal cell lines revealed significantly enriched terms for multiple biological processes implicated in cellular response to toxic and xenobiotic substance, biosynthesis and metabolism of adrenal steroid hormones, lipid transport (including

cholesterol) as well as lipoprotein clearance and metabolism. Also, significantly enriched terms implicated in biological processes and molecular functions connected to transcription factor binding were discovered for upregulated genes.
Selected results of the GO enrichment analysis are listed in table 7 and complete results are shown in appendix 1.

Table 7: Selected biological processes (BP) and molecular functions (MF) of a Gene Ontology enrichment analysis of differentially expressed genes comparing DMSO treated mitotane resistant to nonresistant control clonal cell lines. For complete results, please refer to appendix 1.

Term	Note	GO.ID	P Value	Regulation	Genes
Positive regulation of programmed cell death	BP	GO:0043068	0.00010	Up	50
Cell growth	BP	GO:0016049	0.00017	Up	41
Transcription, DNA-templated	BP	GO:0006351	0.00021	Up	216
Canonical Wnt signaling pathway	BP	GO:0060070	0.00023	Up	30
Regulation of Wnt signaling pathway	BP	GO:0030111	0.00028	Up	31
Nucleic acid-templated transcription	BP	GO:0097659	0.00030	Up	216
Positive regulation of transcription by RNA polymerase II	BP	GO:0006366	0.00049	Up	137
Positive regulation of transcription from RNA polymerase II	BP	GO:0045944	0.00151	Up	70
Coreceptor activity involved in Wnt signaling pathway	MF	GO:1904929	0.00019	Up	4
Transcription factor binding	MF	GO:0008134	0.00087	Up	49
Wnt-activated receptor activity	MF	GO:0042813	0.00586	Up	4
Growth factor activity	MF	GO:0008083	0.00795	Up	8
Steroid metabolic process	BP	GO:0008202	0.00011	Down	28
Glucocorticoid biosynthetic process	BP	GO:0006704	0.00015	Down	5
Glucocorticoid metabolic process	BP	GO:0008211	0.00016	Down	6
Cellular lipid metabolic process	BP	GO:0044255	0.00049	Down	69
Mineralocorticoid biosynthetic process	BP	GO:0006705	0.00055	Down	4
Mineralocorticoid metabolic process	BP	GO:0008212	0.00055	Down	4
C21-steroid hormone metabolic process	BP	GO:0008207	0.00091	Down	7
C21-steroid hormone biosynthetic process	BP	GO:0006700	0.00106	Down	6
Androgen metabolic process	BP	GO:0008209	0.00106	Down	6
Positive regulation of lipid biosynthetic process	BP	GO:0046889	0.00114	Down	9
Regulation of plasma lipoprotein particle levels	BP	GO:0097006	0.00148	Down	10
Cellular response to xenobiotic stimulus	BP	GO:0071466	0.00180	Down	10
Cellular response to toxic substance	BP	GO:0097237	0.00260	Down	12
Plasma lipoprotein particle clearance	BP	GO:0034381	0.00455	Down	7
Cholesterol transport	BP	GO:0030301	0.00471	Down	8
Steroid biosynthetic process	BP	GO:0006694	0.00559	Down	18
Lipid transport	BP	GO:0006869	0.00596	Down	21
Chylomicron remnant clearance	BP	GO:0034382	0.00648	Down	2
Triglyceride-rich lipoprotein particle clearance	BP	GO:0071830	0.00648	Down	2
Positive regulation of estradiol secretion	BP	GO:2000866	0.00648	Down	2
Positive regulation of lipid metabolic processes	BP	GO:0045834	0.00768	Down	11
Sterol import	BP	GO:0035376	0.00866	Down	3
High-density lipoprotein particle binding	MF	GO:0008035	0.00049	Down	3
Apolipoprotein binding	MF	GO:0034185	0.00051	Down	4
Lipoprotein particle receptor activity	MF	GO:0030228	0.00350	Down	4
Low-density lipoprotein particle binding	MF	GO:0030169	0.00433	Down	3

Gene Ontology enrichment analysis of upregulated genes between mitotane treated and DMSO treated nonresistant control clonal cell lines revealed significantly enriched terms for multiple biological processes connected to apoptosis as well as endoplasmic reticulum stress response and regulation, while GO enrichment analysis of downregulated genes revealed significantly enriched terms for biological processes connected to lipid transport, biosynthesis and homeostasis. Also, molecular functions implicated in regulatory DNA binding were discovered for upregulated genes. Selected results of the GO enrichment analysis are listed in table 8 and complete results are shown in appendix 1.

Table 8: Selected biological processes (BP) and molecular functions (MF) of a Gene Ontology enrichment analysis of differentially expressed genes comparing mitotane treated to DMSO treated nonresistant control clonal cell lines. For complete results, please refer to appendix 1.

Term	Note	GO.ID	P Value	Regulation	Genes
Cell death	BP	GO:0008219	0.00168	Up	14
Apoptotic signaling pathway	BP	GO:0097190	0.00300	Up	7
Response to endoplasmic reticulum stress	BP	GO:0034976	0.00455	Up	5
Regulation of cell death	BP	GO:0010941	0.00539	Up	11
Regulation of cellular response to stress	BP	GO:0080135	0.00666	Up	7
Apoptotic process	BP	GO:0006915	0.00671	Up	12
Regulation of endoplasmic reticulum stress	BP	GO:1902235	0.00710	Up	2
Programmed cell death	BP	GO:0012501	0.00827	Up	12
Regulation of apoptotic process	BP	GO:0042981	0.00877	Up	10
Regulation of programmed cell death	BP	GO:0043067	0.00943	Up	10
Positive regulation of response to ER stress	BP	GO:1905898	0.00949	Up	2
transcription regulatory region DNA binding	MF	GO:0044212	0.00960	Up	7
regulatory region DNA binding	MF	GO:0000975	0.00971	Up	7
regulatory region nucleic acid binding	MF	GO:0001067	0.00982	Up	7
Unsaturated fatty acid biosynthetic process	BP	GO:0006636	0.00011	Down	2
Intracellular lipid transport	BP	GO:0032365	0.00017	Down	2
Cellular response to fatty acid	BP	GO:0071398	0.00028	Down	2
Cholesterol homeostasis	BP	GO:0042632	0.00030	Down	2
Sterol homeostasis	BP	GO:0055092	0.00030	Down	2
Glycerolipid biosynthetic process	BP	GO:0045017	0.00037	Down	3
Cholesterol transport	BP	GO:0030301	0.00042	Down	2
Sterol transport	BP	GO:0015918	0.00058	Down	2
Response to fatty acid	BP	GO:0070542	0.00064	Down	2
Regulation of plasma lipoprotein particle levels	BP	GO:0097006	0.00064	Down	2
Unsaturated fatty acid metabolic process	BP	GO:0033559	0.00073	Down	2
Lipid homeostasis	BP	GO:0055088	0.00098	Down	2
Regulation of fatty acid metabolic process	BP	GO:0019217	0.00109	Down	2
Response to lipid	BP	GO:0033993	0.00623	Down	3
Lipid transport	BP	GO:0006869	0.00737	Down	2
Lipid modification	BP	GO:0030258	0.00818	Down	2
Lipid localization	BP	GO:0010876	0.00892	Down	2

8.2.2 Whole Exome Sequencing Analysis

In order to investigate possible DNA level changes involved in mitotane resistance, exome sequencing was performed in HAC-15 cells at passage 3 (founder), one non-resistant control clone and six mitotane resistant clones. Discovery of somatic mutations in nonresistant control and mitotane resistant clones versus the founder cell lines was done as previously described (130) and revealed a much higher number of somatic mutations in mitotane resistant clonal cell lines. Copy number variations were analyzed in mitotane resistant versus nonresistant control clonal cell lines using CopywriteR (233). A profile of copy number changes for each of the mitotane resistant clonal cell lines discovered during exome sequencing is shown in figure 14.

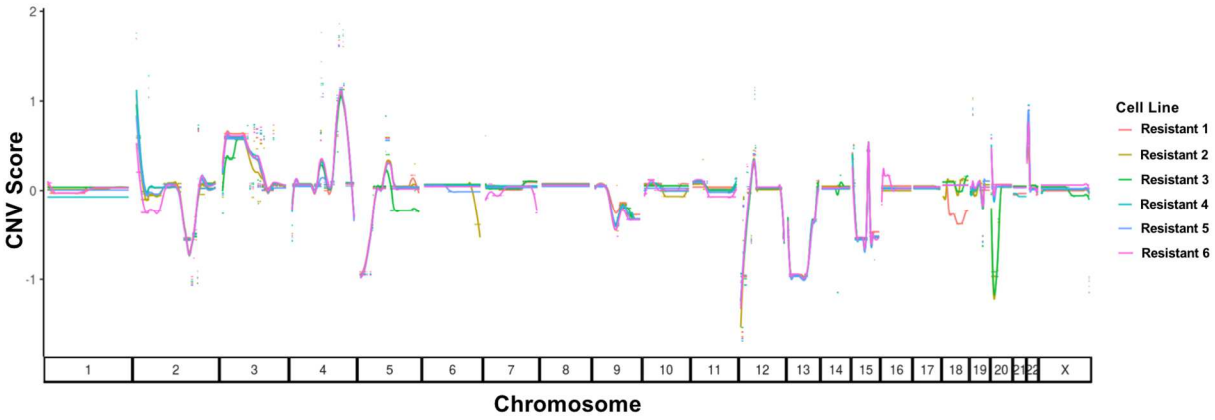


Figure 14: Copy number variation (CNV) profiles of mitotane resistant clonal cell lines. Copy number profiles comparing mitotane resistant to nonresistant control clonal cell lines were obtained using CopywriteR. Copy number profiles of mitotane resistant cell lines show high similarity.

Copy number profiles showed high similarity among all mitotane resistant clonal cell lines and included a loss on chromosome 9, profound losses on chromosomes 13 and 15 as well as large gains on chromosomes 3 and 4. Cell line specific copy number variations (CNVs) included one gain on chromosome 5 and a loss on chromosome 18 specific for mitotane resistant clonal cell line 1 as well as a loss on chromosome 20 specific for mitotane resistant clonal cell line 3. High genetic similarity among mitotane resistant clonal cell lines suggests, that all clonal cell lines derive from a single, mitotane resistant cell or a subset of genetically similar cells during long-term treatment.

8.3 Functional Analyses

Building on the results of *in vitro* experiments, the gene expression microarray study and whole exome sequencing, functional analyses were performed in order to further unravel the mechanisms underlying mitotane resistance in the present *in vitro* model. Functional analyses included cell culture validation of driver pathway candidates as well as measurement of intracellular lipids and intracellular mitotane concentration.

8.3.1 Cell Culture Validation of Possible Pathways of Mitotane Resistance

In order to identify possible drivers of mitotane resistance among the multitude of genes and pathways discovered during the gene expression microarray study, several candidate pathways were investigated in *in vitro* experiments. Genes and pathways were selected according to a previously established role in cancer and adrenal cortex homeostasis as well as results from previous *in vitro* studies presented in this thesis.
During both, biased and unbiased analysis of gene expression microarray data, *SCARB1,* encoding for scavenger receptor 1B (SR-BI), was discovered to be significantly and strongly downregulated in mitotane resistant clonal cell lines. According to GO enrichment analysis comparing DMSO treated mitotane resistant to nonresistant control clonal cell lines, *SCARB1* was annotated for numerous biological processes connected to lipid metabolism and transport. Due to the well-established role of SR-BI in selective uptake of cholesterol from lipoproteins (LDL and HDL) (241) and previous data suggesting a role of HDL and LDL in mitotane resistance in the present *in vitro* model, the effect of SR-BI inhibition on the cytotoxic effects of mitotane in HAC-15 cells was investigated. Results are shown in figure 15 and p-values are shown in table 9.

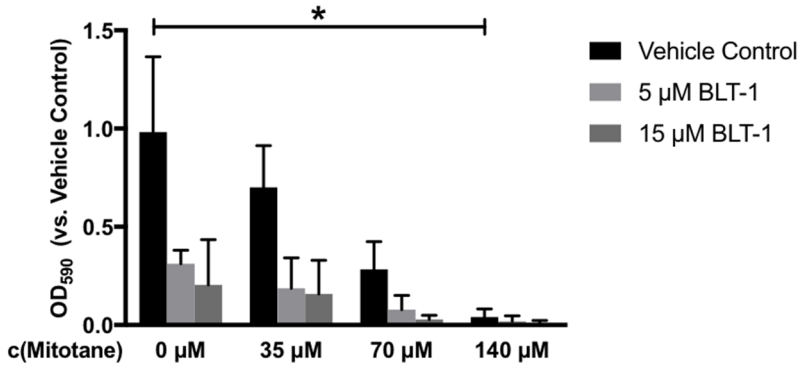


Figure 15: The cell viability of HAC-15 cells in presence of different concentrations of mitotane and SR-BI inhibitor BLT-1. Cell viability was measured by MTT assay after 72 h of incubation. Data is shown in mean+SD of two measurements. Inhibition of SR-BI does not mitigate the cytotoxic effects of mitotane in HAC-15 cells. Groups were compared using a Kruskal-Wallis test. As post hoc test Dunn's uncorrected test was used. *p<0.05.

Table 9: P-values (Kruskal-Wallis test and Dunn's uncorrected *post hoc* test) for comparisons of cell viability in absence and presence of different concentrations of mitotane and SR-BI inhibitor BLT-1 (figure 15)

Comparison	P-Value
0 µM mitotane vs. 35µM mitotane	0.89
0 µM mitotane vs. 70µM mitotane	0.40
0 µM mitotane vs. 140µM Mitotane	0.024
0 µM mitotane l vs. 0µM mitotane 5µM BLT-1	0.36
0 µM mitotane vs. 0µM mitotane 15µM BLT-1	0.20
0µM mitotane 5µM BLT-1 vs. 35µM mitotane 15µM BLT-1	0.48
0µM mitotane 5µM BLT-1 vs. 70µM mitotane 15µM BLT-1	0.18
0µM mitotane 5µM BLT-1 vs. 140µM mitotane 15µM BLT-1	0.056
0µM mitotane 15µM BLT-1 vs. 35µM mitotane 15µM BLT-1	0.72
0µM mitotane 15µM BLT-1 vs. 70µM mitotane 15µM BLT-1	0.32
0µM mitotane 15µM BLT-1 vs. 140µM mitotane 15µM BLT-1	0.12

As expected, mitotane decreased cell viability in HAC-15 cells. However, mitotane effects on cell viability were only significant using 140 µM mitotane, probably due to the low number of repeated measurements (N=2). Incubation with SR-BI inhibitor BLT-1 using 5 µM, a concentration that has been used in H295R cells (168), and 15 µM showed a tendency towards the reduction of cell viability in HAC-15 cells in absence or presence of different concentrations of mitotane. This tendency was contrary to the expectation that inhibition of SR-BI may counteract mitotane cytotoxicity, and experiments were discontinued after two measurements.

Another gene discovered to be consistently downregulated in mitotane resistant clonal cell lines was *AGTR1,* encoding for angiotensin II receptor 1. According to GO enrichment analysis comparing DMSO treated mitotane resistant to nonresistant control clonal cell lines, *AGTR1* was annotated for numerous biological processes connected to steroid metabolism and for molecular functions connected to transmembrane signaling, consistent with its well-established role in aldosterone production in the adrenal zona glomerulosa (242). In order to investigate a possible role in mitotane resistance, the effect of angiotensin receptor 1 inhibition on cytotoxic effects of mitotane in HAC-15 cells was investigated. Results are shown in figure 16 and p-values are listed in table 10.

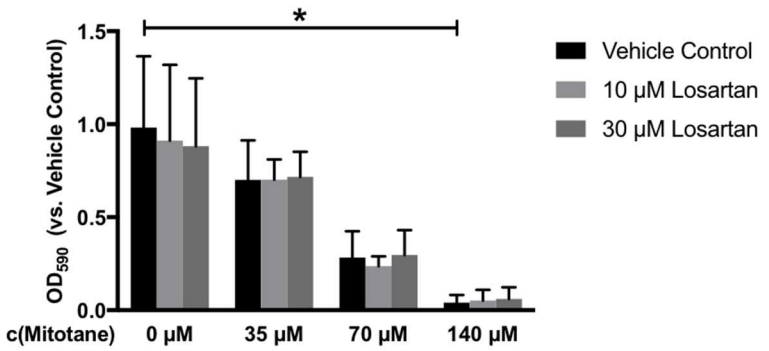


Figure 16: The cell viability of HAC-15 cells in presence of different concentrations of mitotane and AGTR1 inhibitor losartan. Cell viability was measured by MTT assay after 72 h of incubation. Data is shown in mean+SD of two measurements. Vehicle control treated samples were identical to figure 15. Inhibition of angiotensin II receptor 1 does not mitigate the cytotoxic effects of mitotane in HAC-15 cells. Groups were compared using a Kruskal-Wallis test. As *post hoc* test Dunn's uncorrected test was used. *p<0.05.

Table 10: P-values (Kruskal-Wallis test and Dunn's uncorrected *post hoc* test) for comparisons of cell viability in absence and presence of different concentrations of mitotane and angiotensin II receptor 1 inhibitor losartan (figure 16).

Comparison	p-Value
0 µM mitotane vs. 35µM mitotane	0.89
0 µM mitotane vs. 70µM mitotane	0.40
0 µM mitotane vs. 140µM mitotane	0.024
0 µM mitotane vs. 0µM mitotane 10 µM losartan	0.36
0 µM mitotane vs. 0µM mitotane 30 µM losartan	0.20
0µM mitotane 10 µM losartan vs. 35µM mitotane 30 µM losartan	0.78
0µM mitotane 10 µM losartan vs. 70µM mitotane 30 µM losartan	0.32
0µM mitotane 10 µM losartan vs. 140µM mitotane 30 µM losartan	0.10
0µM mitotane 30 µM losartan vs. 35µM mitotane 30 µM losartan	0.72
0µM mitotane 30 µM losartan vs. 70µM mitotane 30 µM losartan	0.32
0µM mitotane 30 µM losartan vs. 140µM mitotane 30 µM losartan	0.12

Vehicle control treated samples were identical to figure 16. Inhibition of angiotensin receptor 1 showed no tendency to reduce or increase cell viability in absence or presence of various concentrations of mitotane. Hence, experiments were discontinued after two measurements.

Another significantly and strongly downregulated gene in mitotane resistant clonal cell lines was *DDIT4L,* encoding for REDD2, an inhibitor of mTOR signaling (243), a pathway connected to proliferation and survival that is also linked to cancer (244). In order to clarify, whether inhibition of mTOR mitigates mitotane resistance in the present *in vitro* model, the IC_{50} of mitotane in nonresistant control and mitotane resistant clonal cell lines in presence of mTOR inhibitor rapamycin was determined. Results are shown in figure 17.

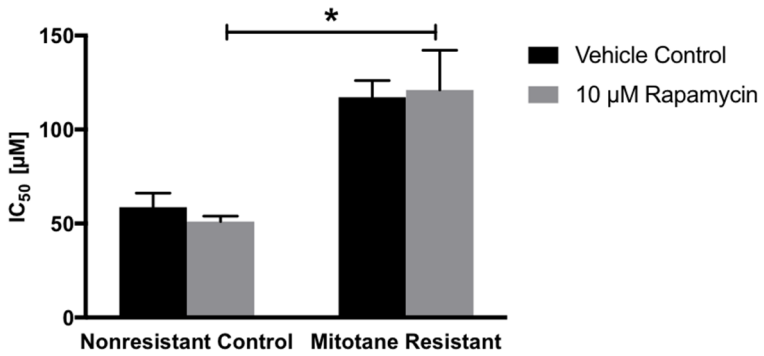


Figure 17: The IC_{50} of mitotane in mitotane resistant and nonresistant control clonal cell lines in presence of vehicle control and mTOR-pathway inhibitor rapamycin. The IC_{50} was measured by MTT assay after 72 h of incubation. Results are shown as mean+SD of three different clones. Simultaneous treatment with rapamycin does not influence the IC_{50} of mitotane in nonresistant control and mitotane resistant clonal cell lines. Groups were compared using a Kruskal-Wallis test. As *post hoc* test Dunn's uncorrected test was used. *p<0.05.

Treatment with rapamycin did not cause changes in mitotane tolerance in mitotane resistant or nonresistant control clonal cell lines (p=0.013), although a rapamycin concentration was used (10 µM versus 25-100 nM) in comparison to a previous study in H295R cells (245).

GO enrichment analysis also suggested an upregulation of pathways implicated Wnt signaling in mitotane resistant cells. *AXIN2* exerts an important role on Wnt signaling by regulating β-catenin stability (246). Elevated *AXIN2* expression is considered a marker for increased Wnt activity (247) and has been used to assess Wnt signaling activity in murine adrenal glands (248). Increased *AXIN2* expression was confirmed by rt-PCR (p=0.0022; figure 18).

In order to investigate a possible role of Wnt signaling in mitotane resistance, the IC_{50} of mitotane was measured in mitotane resistant clonal cell lines in presence of Wnt pathway inhibitor XAV939. Results are shown in figure 19. Simultaneous treatment with 10 µM XAV939, a concentration that has also been used to inhibit the Wnt pathway in H295R and SW13 cells (249), did not cause changes in the IC_{50} of mitotane resistant clonal cell lines (p=0.70).

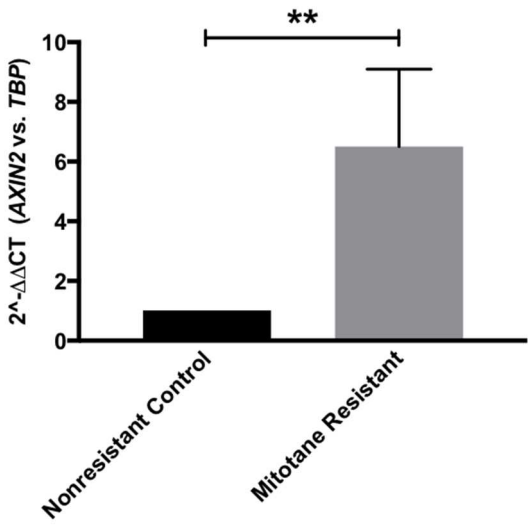


Figure 18: The relative expression of AXIN2 in mitotane resistant versus nonresistant control cells. Gene expression was measured by rt-PCR, normalized to expression of *TBP* and results are shown as mean+SD of six clones. Expression of *AXIN2* is significantly increased in mitotane resistant clonal cell lines. For statistics, Mann-Whitney test was used. **p<0.01.

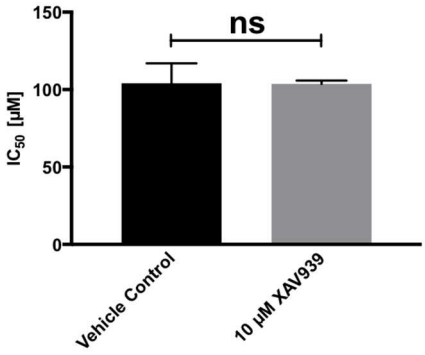


Figure 19: The IC_{50} of mitotane in mitotane resistant clonal cell lines in presence 10 µM Wnt pathway inhibitor XAV939. The IC_{50} was measured by MTT assay after 72 h of incubation. Results are shown as mean+SD of three different clones. Inhibition of Wnt signaling does not influence the IC_{50} of mitotane in mitotane resistant clonal cell lines. For statistics, the Mann-Whitney test was used. ns, p>0.05.

8.3.2 Intracellular Measurement of Various Lipid Species

In order to further explore the possible role of cholesterol in mitotane resistance suggested by cell culture experiments and subsequent microarray gene expression analysis, the concentration of various lipid species was determined using ESI-MS/MS in lysates of three nonresistant control and three mitotane resistant clones cultured in presence of different concentrations of mitotane and different amounts of CCS. A heatmap of summarized results is shown in figure 20.

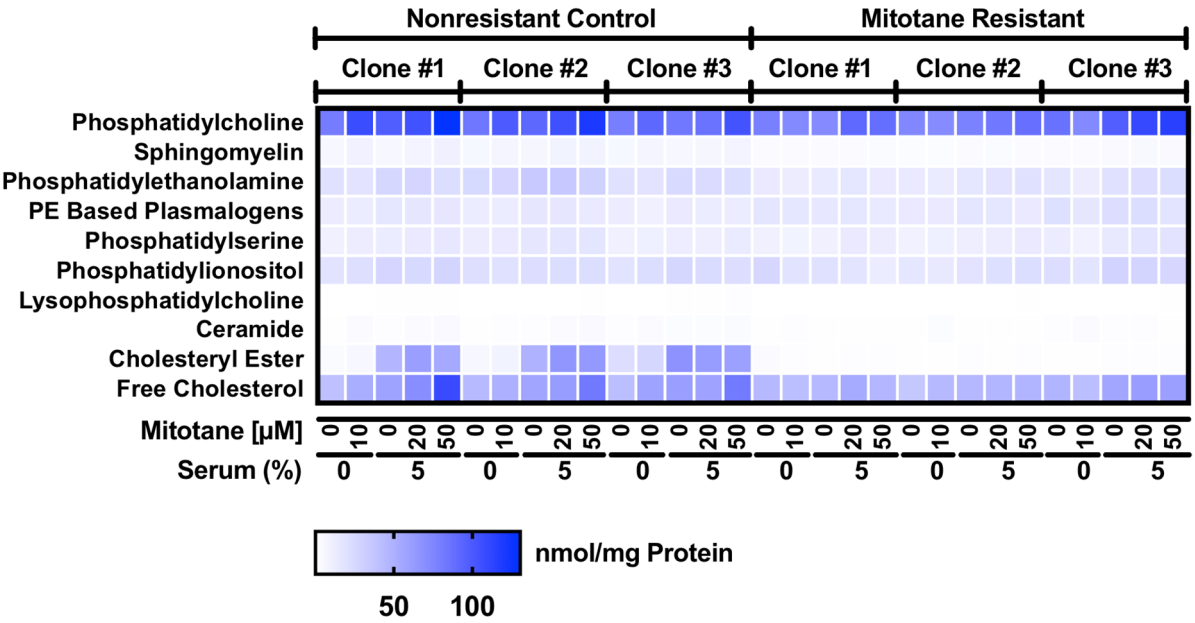


Figure 20: Intracellular content of various lipid species. The amount of phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, PE based plasmalogens, phosphatidylserine, phosphatidylinositol, lysophosphatidylcholine, ceramide, cholesteryl ester and free cholesterol in three nonresistant control clones versus three mitotane resistant clones. Cells were treated with increasing concentrations of mitotane in presence of different concentrations of serum for 72 h. The amount of the indicated compounds in nmol was measured by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and normalized to total protein quantity in mg (BCA assay).

Of all lipid species investigated, the intracellular concentrations of lysophosphatidylcholines (LPC), ceramides (CER), sphingomyelins (SPM), cholesteryl esters (CE) and free cholesterol differed consistently and systematically between either nonresistant and mitotane resistant cells or different treatment conditions (for p-values, please refer to table 11 and appendix 2). Heatmaps comprising all species of these lipids analyzed are shown in figure 21.

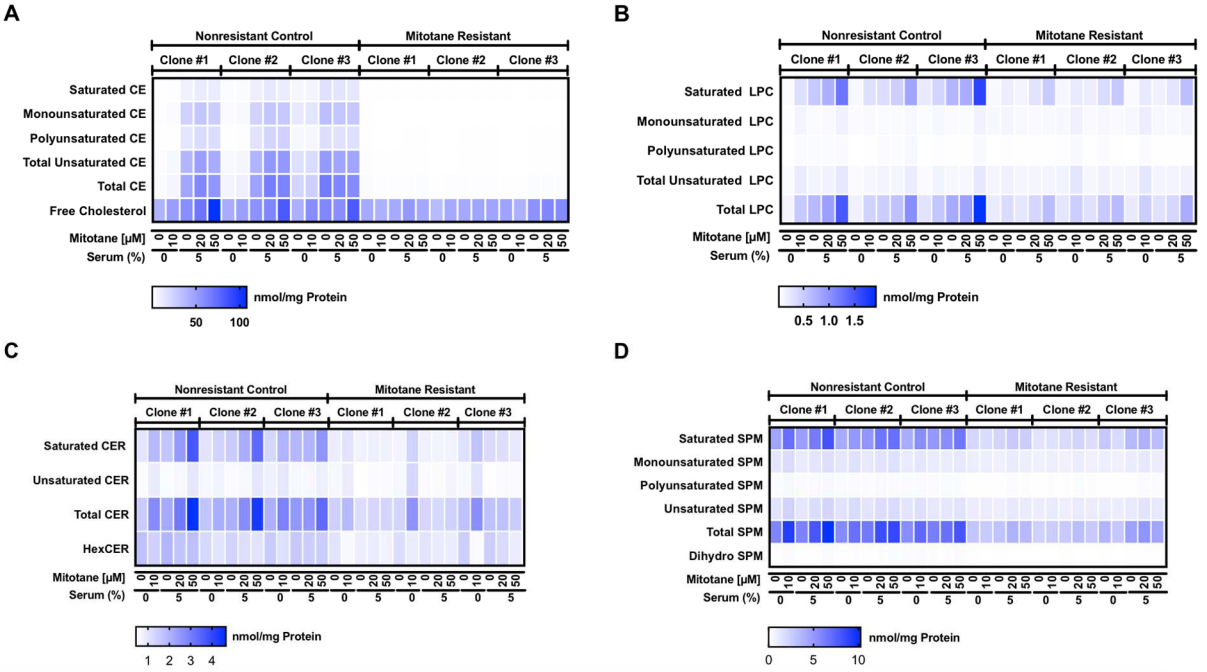


Figure 21 A-D: All species of cholesterol, lysophosphatidylcholine, ceramide and sphingomyelin analyzed by ESI-MS/MS. Cells were treated with increasing concentrations of mitotane in presence of different concentrations of serum for 72 h. The amount of the lipid species in nmol was measured by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and normalized to total protein quantity in mg (BCA assay). **A)** The amount of saturated, monounsaturated, polyunsaturated, total unsaturated and total cholesteryl ester (CE) as well as free cholesterol in three nonresistant control clones versus three mitotane resistant clones. **B)** The amount of saturated, monounsaturated, polyunsaturated, total unsaturated and total lysophosphatidylcholine (LPC) in three nonresistant control clones versus three mitotane resistant clones. **C)** The amount of saturated, unsaturated, total ceramides (CER) and hexylceramides (HexCer) in three nonresistant control clones versus three mitotane resistant clones. **D)** The amount of saturated, monounsaturated, polyunsaturated, total unsaturated, total and dihydro sphingomyelin (SPM) in three nonresistant control clones versus three mitotane resistant clones. For statistical analysis, all groups were tested for normality using Shapiro-Wilk test. Groups passing the normality test were compared using a one-way ANOVA. As *post hoc* test for multiple comparisons Bonferroni's multiple comparisons test was used, when different conditions were compared within "nonresistant“ or “resistant“, and Fisher‘s LSD test was used when “nonresistant“ was compared to “resistant“ within one condition. Groups failing the normality test were compared using a Kruskal-Wallis test. As *post hoc* test for multiple comparisons Dunn’s test was used, when different conditions were compared within "nonresistant“ or “resistant“, and Dunn’s uncorrected test was used when “nonresistant“ was compared to “resistant“ within one condition. For p-values, please refer to table 11 and appendix 2. *p<0.05; **p<0.01; ***p<0.001; **** p<0.0001.

Table 11: P-values (Kruskal-Wallis test and Dunn's uncorrected *post hoc* test or one-way ANOVA and Fisher's LSD test) for comparisons of intracellular lipids in absence and presence of different concentrations of mitotane and CCS (figure 20 and 21). For a complete list of p-values, please refer to appendix 2.

Lipid Species	CCS	Comparison	p-Value
free cholesterol	5%	0 µM vs. 50 µM mitotane (nonresistant)	0.00060
	5%	0 µM vs. 50 µM mitotane (resistant)	>0.99
	0%	0 µM vs. 10 µM mitotane (nonresistant)	>0.99
	0%	0 µM vs. 10 µM mitotane (resistant)	>0.99
cholesteryl ester	0%	resistant vs. nonresistant (0 µM mitotane)	0.11
	0%	resistant vs. nonresistant (10 µM mitotane)	0.046
	5%	resistant vs. nonresistant (0 µM mitotane)	0.023
	5%	resistant vs. nonresistant (20 µM mitotane)	0.012
	5%	resistant vs. nonresistant (50 µM mitotane)	0.047
lysophosphatidylcholine	0%	resistant vs. nonresistant (0 µM mitotane)	0.81
	0%	resistant vs. nonresistant (10 µM mitotane)	0.62
	0%	0 µM vs. 10 µM mitotane (nonresistant)	>0.99
	0%	0 µM vs. 10 µM mitotane (resistant)	>0.99
	5%	resistant vs. nonresistant (0 µM mitotane)	0.013
	5%	resistant vs. nonresistant (20 µM mitotane)	0.018
	5%	resistant vs. nonresistant (50 µM mitotane)	<0.0001
	5%	0 µM vs. 50 µM mitotane (nonresistant)	0.0002
	5%	0 µM vs. 50 µM mitotane (resistant)	0.33
ceramide	0%	resistant vs. nonresistant (0 µM mitotane)	0.40
	0%	resistant vs. nonresistant (10 µM mitotane)	0.52
	0%	0 µM vs. 10 µM mitotane (nonresistant)	0.36
	0%	0 µM vs. 10 µM mitotane (resistant)	0.21
	5%	resistant vs. nonresistant (0 µM mitotane)	0.0077
	5%	resistant vs. nonresistant (20 µM mitotane)	0.0001
	5%	resistant vs. nonresistant (50 µM mitotane)	<0.0001
	5%	0 µM vs. 50 µM mitotane (nonresistant)	0.0001
	5%	0 µM vs. 50 µM mitotane (resistant)	>0.99
sphingomyelin	0%	resistant vs. nonresistant (0 µM mitotane)	0.0009
	0%	resistant vs. nonresistant (10 µM mitotane)	<0.0001
	0%	0 µM vs. 10 µM mitotane (nonresistant)	0.34
	0%	0 µM vs. 10 µM mitotane (resistant)	>0.99
	5%	resistant vs. nonresistant (0 µM mitotane)	0.0006
	5%	resistant vs. nonresistant (20 µM mitotane)	<0.0001
	5%	resistant vs. nonresistant (50 µM mitotane)	<0.0001
	5%	0 µM vs. 20 µM mitotane (nonresistant)	>0.99
	5%	0 µM vs. 20 µM mitotane (resistant)	>0.99
	5%	0 µM vs. 50 µM mitotane (nonresistant)	0.033
	5%	0 µM vs. 50 µM mitotane (resistant)	>0.99

Treatment with 50 µM mitotane in presence of 5% CCS caused a significant increase of free cholesterol in nonresistant cells but not mitotane resistant cells, while treatment

with 10 µM mitotane in presence of 0% CCS had no significant effect. Further, at all conditions except for 0% CCS and 0 µM mitotane, total CE were significantly increased in nonresistant cells compared to mitotane resistant cells. In presence of 5% CCS and 0, 20 and 50 µM mitotane, total LPCs were significantly increased in nonresistant control in comparison to mitotane resistant cells. Also, treatment with 50 µM mitotane in presence of 5% CCS caused a significant increase in total LPCs in nonresistant control cells, but not in mitotane resistant cells. In presence of 0% CCS, no significant changes in LPC levels were found. Total CER were significantly increased in nonresistant control in comparison to mitotane resistant clonal cell lines in presence of 5% CCS and 0, 20 and 50 µM mitotane. Also, treatment with 50 µM mitotane in presence of 5% CCS caused a significant increase in total CER in nonresistant control cells, but not in mitotane resistant cells. In presence of 0% CCS, no significant changes in total CER levels were found. Total SPMs were significantly increased in nonresistant control compared to mitotane resistant cells at all conditions. Further, treatment with 50 µM mitotane significantly increased sphingomyelins in nonresistant control, but not in mitotane resistant cells, while no significant effect was observed during treatment with 10 µM and 20 µM in presence of 0% and 5% CCS, respectively.

8.3.3 Mitotane Measurement by GCMS

In order to clarify whether intracellular mitotane levels differed between mitotane resistant and nonresistant control cells, intracellular and medium mitotane concentration was determined in lysates and medium of three nonresistant control and three mitotane resistant clones (figure 22).

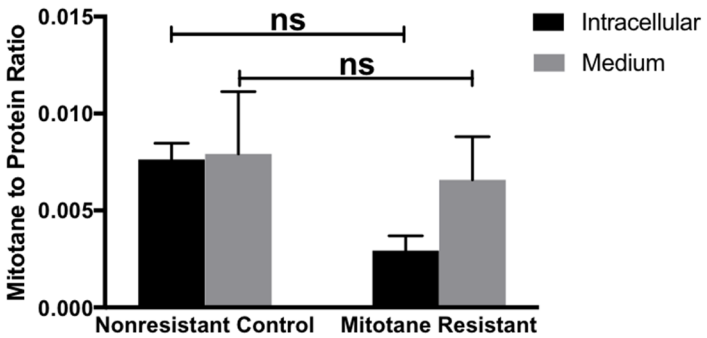


Figure 22: The concentration of mitotane in supernatant and lysates of nonresistant control and mitotane resistant clonal cell lines. Mitotane concentrations in mg/mL were determined by GCMS and normalized to protein concentration in mg/mL (BCA). Results are shown as mean+SD of three different clones. Intracellular and medium mitotane concentration was unchanged in mitotane resistant clonal cell lines. For statistics, the Mann-Whitney test was used. ns, $p>0.05$.

No significant change in intracellular ($p=0.10$) and medium mitotane ($p=0.70$) between nonresistant control and mitotane resistant cells was found. However, mitotane resistant cells showed a tendency towards reduction in intracellular mitotane content, suggesting that the number of repeated measurements ($N=3$) was too low in order to reach significance.

9 Discussion

9.1 Generation of a Mitotane Resistant Adrenocortical Carcinoma Cell Line

9.1.1 Mitotane Long-Term Treatment Induces Resistance in HAC-15 Cells

Resistance of neoplasms towards cytotoxic treatment has already been recognized in early trials using mustard gas (250). It may occur due to a plethora of mechanisms including decreased uptake, increased efflux, increased DNA repair, alterations in drug metabolism or cellular targets and inhibition of apoptosis (251). Relapse during cytotoxic treatment of ACC using mitotane poses a significant challenge in contemporary clinical management. Therefore, the present thesis aims at investigating mechanisms contributing to mitotane resistance in an *in vitro* model of mitotane resistant ACC. In order to obtain chemotherapy resistant cell lines, cells may be exposed to long-term treatment following either a pulsed or a continuous treatment protocol (218). During continuous treatment cells are continuously exposed to a small, slowly increasing drug dose. During pulsed treatment, cells are exposed to high drug concentrations (IC_{50} or higher) thereby selecting for a small percentage of cells with comparably high tolerance towards the drug. Selected cells are subsequently allowed to recover in drug free medium and subjected to further selection cycles (218). A series of mouse xenograft experiments using two different clones of NCIH295, the progenitor cell line of H295R and HAC-15, reports tumors with remarkable differences in histology and response to mitotane-EDP (217), suggesting the selection-driven, pulsed treatment (218) as suitable approach in order to induce mitotane resistance in HAC-15 cells. The initial IC_{50} of mitotane in HAC-15 cells was (47.4±15.0) µM, or (15.3±4.8) mg/L, corresponding to the recommended blood levels in human ACC patients (>14 mg/L) (166, 193). HAC-15 cells were subjected to long-term treatment with mitotane following a pulsed protocol using approximately 1.5 times the IC_{50} (70 µM or 22.4 mg/L). Hence, the drug dose used for long-term treatment of HAC-15 cells exceeded the therapeutic window of mitotane in human patients as mitotane blood levels >20 mg/L are associated with serious side effects (195).

During long-term treatment, the population of mitotane treated cells was initially diminished, likely due to the well-established cytotoxic effects of mitotane on ACC cells (171-173). Full recovery of the population after approximately 70 days and a slight yet not significant right-shift of the dose-response curve of mitotane in bulk cultures of long-term mitotane treated cells suggested that mitotane resistance had developed. After

isolation of clonal cell lines, 2.6fold resistance in mitotane treated clonal cell lines was confirmed by MTT assay. Chemotherapy resistant cancer cell lines derived from patients of other tumor entities commonly show a 2-5fold resistance (218), indicating that mitotane resistance in this *in vitro* was within a clinically relevant range.

9.1.2 Mitotane Resistance is Different from Multidrug Resistance in Long-Term Treated HAC-15 Cells

Multidrug resistance in cancer is most prominently caused by ABC transporters P-glycoprotein, MRP1 and BCRP (encoded by *MDR1, ABCC1* and *ABCG2*) (235). *MDR1* is highly expressed in normal adrenal tissue and adrenal tumors (203, 204). By measuring doxorubicin sensitivity, increased activity of P-glycoprotein was excluded in the present *in vitro* model of mitotane resistant ACC. Interestingly, simultaneous treatment with mitotane increased doxorubicin sensitivity in mitotane resistant and nonresistant control cells, while doxorubicin sensitivity in mitotane resistant cells was significantly lower in comparison to nonresistant control cells. These observations are consistent with previous studies showing that mitotane increases intracellular accumulation of various cytotoxic compounds including doxorubicin in *in vitro models* of colon carcinoma (209) and the NCI-H295 ACC cell line (210), likely due to impairment of P-glycoprotein function (210). Of note, doxorubicin is also a substrate of MRP1 (252) and transfection of BCRP into the MCF-7 breast cancer cell line confers resistance to doxorubicin (253). Further, increased expression of *MDR1, ABCC1* and *ABCG2* in mitotane resistant clonal cell lines was excluded by subsequent gene expression microarray analysis. These observations suggested a mechanism of mitotane resistance different from common multidrug resistance in the present *in vitro* model.

9.1.3 Lipoproteins and Cholesterol Are Correlated with Mitotane Cytotoxicity, and Mitotane Resistance is Influenced by Medium HDL and LDL

Insights from a patient with ACC suggest that approximately 90% of serum mitotane is associated with lipoproteins including VLDL, LDL and HDL (167), while cytotoxic effects of mitotane appear to be mediated by lipoprotein free mitotane (167, 168). In accordance with these findings, IC_{50} of mitotane was found to be positively correlated with the amount of HDL, LDL and cholesterol in the medium. Also, the overall amount of HDL and LDL (but not the HDL to LDL ratio) mitigated mitotane resistance from 3.6fold at 0.05 mg/mL HDL and LDL to 1.7fold resistance at 0.005 mg/mL HDL and

LDL. Interestingly, multiple studies report an increase of serum cholesterol, HDL and LDL in ACC patients during mitotane therapy (188-191). This implies a possible, direct link between *in vivo* side effects of mitotane treatment and *in vitro* mitotane resistance. Mitotane induced hyperlipidemia could cause increased association of active, lipoprotein free mitotane with lipoproteins, thereby decreasing antitumoral effects. Accordingly, administration of statins during mitotane therapy of metastatic ACC leads to a significantly higher number of patients with stable disease or partial response after 6 months (168).

9.2 Gene Expression Microarray Analysis

9.2.1 Unbiased and Biased Analysis of Gene Expression Microarray Data Reveal Profound Gene Expression Changes in Mitotane Resistant Cells

Apart from upregulation of *ABC* transporter gene expression (235), chemotherapy resistance may also be connected to alterations in pathways implicated in tumor suppression, cell growth and DNA repair (254). In order to investigate gene expression changes in the present *in vitro* model of mitotane resistant ACC, Affymetrix GeneChip PrimeView Human Gene Expression Array was performed in six mitotane resistant and six nonresistant control clonal cell lines treated with either mitotane or vehicle control (DMSO) for 18 h. In a first, biased approach only genes annotated for pathways related to ”cholesterol“ and “steroids“ according to the Gene Ontology (GO) Consortium were included into microarray data analysis. As a second, unbiased approach all probes with the highest average expression within a probe set were included into the analysis and multiple cutoffs were introduced (5% false discovery rate, $|\log_2$ fold change$|> 0.5$ and average expression> 5). Unbiased data analysis comparing mitotane and DMSO treated nonresistant control clonal cell lines revealed 60 differentially expressed genes, suggesting that cellular effects of mitotane treatment are, to some extent, conferred by changes in gene expression. Two studies using NCI-H295 cells report gene expression changes during mitotane treatment (173, 255). Sbiera et al. report a microarray study in NCI-H295 cells treated with 50 and 100 µM mitotane for 6 hours (173). While no list of significantly regulated genes is provided in their publication, they present Gene Ontology pathway analysis of the 30 most up- and downregulated genes. They report downregulation of pathways implicated in lipid metabolism and steroidogenesis. These

include enriched genes *LDLR*, stearoyl-CoA desaturase (*SCD*), sterol regulatory element binding transcription factor 1 (*SREBF1*), and ATP-binding cassette subfamily G member 1 (*ABCG1*). Further, they report upregulation of pathways implicated in apoptosis. These include enriched genes growth differentiation factor 15 (*GDF15*) and DNA-damage-inducible transcript 3 (*DDIT3*(CHOP)). In accordance with their findings, unbiased gene expression microarray data analysis presented in this thesis discovered downregulation of *LDLR*, *SCD* and *SREBF1* and upregulation of *DDIT3* and *GDF15*. Zsippai et al report a microarray study in NCI-H295 cells treated with mitotane for 48 and 72 hours (255). Combining gene expression data of 72 and 48 hours, they identify upregulated expression of *GDF15,* also discovered by Sbiera et al (173) and the unbiased microarray data analysis presented in this thesis. Interestingly, they also report downregulation of the expression of steroidogenic enzymes *HSD3B2, CYP21A2* and *CYP19A1* after 72 hours of mitotane exposure. Downregulation of the expression of steroidogenic enzymes is reported by an *in vitro* study using H295R and SW13 cells exposed to mitotane for 48 hours (172) but was not discovered during the gene expression microarray analysis of mitotane treated nonresistant cells presented in this thesis. Mitotane exposure in the present thesis was shorter than in both studies reporting (18 h vs. 48 and 72 h). Hence, gene expression of steroidogenic enzymes may change in response to cytotoxic effects of mitotane occurring as early as 6 hours after exposure (173). Unlike in nonresistant cells, mitotane exposure of resistant cells, according to the unbiased analysis, did not lead to changes in gene expression. This observation confirms mitotane resistance on gene expression level in the present *in vitro* model of mitotane resistant ACC.

Biased gene expression microarray data analysis comparing DMSO treated mitotane resistant and nonresistant control clonal cell lines led to the discovery of multiple, significantly regulated genes. Genes that were downregulated in resistant cells included *STAR, CYP11A1* and *CYP11B2*. Unbiased analysis confirmed significant downregulation of STAR and *CYP11A1,* and further discovered significant downregulation of *HSD3B2, CPY17A1, CYP19A1* and *CYP21A2* in mitotane resistant cells. Downregulation of key steroidogenic enzymes by long-term mitotane treatment is consistent with a previous *in vitro* study using H295R and SW13 cells (172), and may also explain the reduction of steroid hormone excess that is observed in mitotane treated ACC patients (146, 187). However, the present thesis solely focuses on HAC-15 cells. Hence, a possible influence of other organs on steroid hormone levels are neglected. The liver

plays an important role in catabolism of adrenal steroid hormones (182) and changes in urinary steroid profiles may also be caused by hepatic action of mitotane. Mitotane treatment may lead to changes in hepatic parameters including increases in serum levels of alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and gamma glutamyl transferase activity (146) and may induce hepatic CYP3A4 (183, 184), but data on hepatic action of mitotane is rare. Expression of *CYP11B2* in H295R cells is regulated by binding of angiotensin II the angiotensin II receptor 1 (*AGTR1*) via induction of transcription factors NGFIB and NURR1 (256). Significant downregulation of *AGTR1* was discovered during biased and unbiased analysis of gene expression microarray data. Unbiased analysis also discovered significant downregulation of *NR4A1*, encoding for NGFIB. Downregulation of *AGTR1* was profound, while downregulation of *NR4A1* was less pronounced (log_2fold change of -4.2 and -1.5, respectively). Loss of steroidogenic potential may further be explained by downregulation of *NR5A1,* encoding for steroidogenic factor-1 (SF-1), discovered during the unbiased gene expression array data analysis. Steroidogenic factor-1 is a transcription factor of key importance for the adrenal glands' endocrine function (93), and silencing endogenous *NR5A1* expression in H295R cells leads to repression of major steroidogenic enzymes including CYP11B1, CYP11B2, CYP11A1, HSD3B2, CYP21, CYP17, and StAR (257). During biased analysis, expression of *VLDLR* was discovered to be significantly downregulated in mitotane resistant clonal cell lines. Expression of *VLDLR* on RNA level is predominantly found in human heart, skeletal muscle, ovaries and kidney (258), but on protein level, abundant amounts of VLDLR are present in the adrenal glands (259, 260). Interestingly, there is evidence, that VLDL increases aldosterone production in the bovine zona glomerulosa and H295R cells by a short-term increase in *STAR* and *CYP11B2* expression (261), while the role of VLDLR in adrenal cholesterol homeostasis remains largely unknown. Thus, downregulation of *VLDLR* may, to a small extend, explain loss of steroidogenic potential in long-term mitotane treated HAC-15 cells.

Biased analysis furthermore revealed expression of *LDLR, SCARB1* and *ABCA1* to be significantly downregulated in mitotane resistant clonal cell lines. Unbiased analysis confirmed significant downregulation of *LDLR* and *SCARB1*. The role of both, *LDLR* and *SCARB1* in adrenal cholesterol uptake is well established (7). Originally, *ABCA1* is believed to exert a key role on cholesterol efflux and HDL biogenesis under the control of Liver X Receptor (262). However, one study reports the adrenal glands of

ABCA1-KO mice to be depleted of lipids and free cholesterol, suggesting that the role of *ABCA1* in adrenal cholesterol balance is far more complex (263). There is evidence that cytotoxic effects of mitotane are mediated by intracellular accumulation of toxic lipids leading to endoplasmic reticulum stress and apoptosis (173). Taken together with previous data concerning the influence of medium lipoprotein content on mitotane resistance in this *in vitro* model of mitotane resistant ACC, downregulation of the expression of genes implicated in lipoprotein uptake poses a possible mechanism of mitotane resistance. Impaired cholesterol uptake may have led to increased lipoprotein concentration in the medium which, in turn, was positively correlated with the IC_{50} of mitotane. Also, decreased lipoprotein uptake may have led to cholesterol depletion in mitotane resistant cells which may have counteracted cytotoxic effects of mitotane. Consistently, a study in SR-BI KO mice reports adrenal cholesterol depletion and increased plasma HDL (264). There is evidence that the commonly observed increase of serum cholesterol, HDL and LDL in ACC patients during mitotane therapy (188-191) occurs due to increased hepatic cholesterol synthesis (265). However, impaired adrenal cholesterol uptake may, to very a small extent, contribute to these serum cholesterol changes. Additionally, gene expression of *SOAT1,* encoding for sterol O-acyltransferase, was found to be significantly downregulated in mitotane resistant clonal cell lines during both, biased and unbiased gene expression array analysis. Sterol O-acyltransferase regulates levels of free intracellular cholesterol by esterification (11). Inhibition of *SOAT1* by mitotane leads to accumulation of toxic lipids including cholesterol, endoplasmic reticulum stress as well as apoptosis, and *SOAT1* expression is a prerequisite for mitotane efficacy (173). However, *SOAT1* expression was not completely silenced (fold change= -2.0), challenging a possible role of *SOAT1* expression in mitotane resistance in the present *in vitro* model.

9.2.2 Pathways involved in Wnt Signaling, Lipid Transport and Lipoprotein Clearance Are Changed in Mitotane Resistant Cells

Gene Ontology enrichment analysis of differentially expressed genes between mitotane and DMSO treated nonresistant control clonal cell lines discovered upregulation of pathways connected to apoptosis and endoplasmic reticulum stress response and regulation as well as downregulation of pathways connected to metabolism and transport of lipids, confirming the previous results of Sbiera et al. in NCI-H295 cells (173). Gene Ontology enrichment analysis of differentially expressed genes between

DMSO treated mitotane resistant and nonresistant control clonal cell lines revealed upregulation of pathways connected to Wnt signaling, cell growth, developmental growth and development of multiple organs and downregulation of pathways connected to metabolism and transport of steroid hormones and lipids (including cholesterol). Also, downregulated genes were significantly enriched in molecular functions connected to extracellular lipoprotein binding and clearance. Findings of the GO enrichment analysis regarding downregulation of steroid hormone transport and metabolism were consistent with the antisteroidogenic effects of mitotane observed *in vitro* (172) and *in vivo* (146, 187). Discovery of biological processes and molecular functions implicated in cholesterol transport as well as binding and clearance of lipoproteins further supported a possible role of medium lipoproteins in the present *in vitro* model of mitotane resistance. The Wnt signaling pathway exerts a fundamental role on human organ homeostasis and aberrant Wnt signaling is also linked to cancer (266). In the adrenal gland, Wnt signaling exerts an important role on stem and progenitor cells by maintaining an undifferentiated state through induction of *DAX1* and *INHA* (267). Multiple genetic analyses of ACC report mutations in *CTNNB1*, encoding for β-catenin, (124, 127, 128), a major constituent of the Wnt signaling pathway also mutated in a variety of other cancers (268). Of note, H295R, the progenitor cell line of HAC-15, harbors an activating S45P mutation in *CTNNB1* (269, 270). In a review article published in Nature in 2001, Reya et al. propose the existence of a side population of cells in solid tumors, also called cancer stem cells (CSCs), that possess stem cell like properties of extensive proliferation and tumor formation upon transplantation (271). Corresponding to its role in healthy organs, Wnt signaling also may play a role in survival of CSCs (272). Moreover, *in vitro* studies link increased Wnt signaling activity to a multidrug resistant phenotype in neuroblastoma (273) as well as in side populations of colon cancer (274), and head and neck squamous cell carcinoma (275). Cancer stem cells are characterized by rapid growth (276-278). However, growth curves of long-term treated HAC-15 cells drastically flattened at the beginning of the treatment and the population did not start to recover for approximately 30 days. Further, the pace of the growth curves of mitotane treated cells was comparable to vehicle control treated cells after approximately 70 days of treatment. Starting with the assumption, that HAC-15 cells contained a significant, treatment resistant side population with rapid growth properties at the beginning of long-term treatment, one would expect this population to expand early during mitotane treatment. Given the

rapid growth of CSCs, the pace of population growth of long-term mitotane treated cells should have been comparable to vehicle control treated cells earlier than at day 70. Hence, the mitotane resistant clonal cell lines established in this thesis were unlikely derived from HAC-15 cells via expansion of a treatment resistant subpopulation.

9.3 Whole Exome Sequencing

9.3.1 Mitotane Resistant Clonal Cell Lines Show High Genetic Similarity

In 2011, Hanahan and Weinberg published an updated version of their previous review on the hallmarks of cancer (279), providing evidence-based fundamentals of human neoplasm development (120). According to this work, cancer progression can be seen as a series of clonal expansions driven by a selective advantage for a subset of cells. Genomic instability leading to changes in DNA integrity including copy number variations (CNVs) contributes to genomic variability. Genomic variability may lead to an advantageous phenotype and hence is considered a hallmark of cancer development. In order to investigate DNA level changes connected to mitotane resistance, whole exome sequencing was performed in six mitotane resistant clones, one nonresistant control clone and HAC-15 cells at passage 3 using the Illumina platform. Copy number variation profiling is a versatile tool that may be used to investigate stepwise progression (280), evolutionary dynamics (281) and clonal origin (282) of a tumor. Mitotane resistant clones showed a high similarity in their copy number profiles, while stem-cell like populations in glioblastoma are genetically diverse (283). Hence, high genetic similarity among mitotane resistant clonal cell lines further refuted the hypothesis, that mitotane resistant cell lines were derived via expansion of a treatment resistant side population. Moreover, genetic similarity between mitotane resistant clones suggested that selection driven, pulsed treatment (218) had led to expansion of a single, mitotane resistant cell or a subset of resistant cells of high genetic similarity. This cell, prior to expansion, may have acquired an advantageous, mitotane resistant phenotype through a genetic event. This genetic event may have been one (or multiple) nonsynonymous somatic mutation(s). Interestingly, mitotane resistant clonal cell lines showed very high number of somatic mutations in comparison to the nonresistant control clonal cell line and a higher mutational burden has also been reported in relapsed ovarian cancer (284).

9.4 Functional Analyses

Profound gene expression changes between nonresistant and mitotane resistant clonal cell lines were discovered during gene expression microarrays presented in this thesis. Further, subsequent GO enrichment analysis revealed a multitude of pathways with a possible role in mitotane resistance in the present in vitro model of mitotane resistant ACC. In accordance with these findings, chemotherapy resistance is connected to profound gene expression changes in both, other tumor entities (241, 242) and multiple cancer cell lines (243). These insights suggest, that chemotherapy resistance may be caused by a plethora of changes in gene expression and resultant cellular functions. In order to further unravel the cellular mechanisms contributing to mitotane resistance, functional analyses were performed including cell culture validation of driver pathway candidates, measurement of intracellular lipids and intracellular mitotane concentration.

9.4.1 Mitotane Resistance is Likely not Caused by a Single Driver Pathway

SCARB1 (Scavenger Receptor B1)

Based on the insights from both, cell culture experiments and subsequent genetic analysis, a role of possible driver genes and pathways was further validated cell culture studies.

Gene expression of *SCARB1* encoding SR-BI, which plays a key role in adrenal cholesterol uptake (7), was found to be strongly and significantly downregulated in mitotane resistant cells. Further, the IC_{50} of mitotane was shown to be positively correlated with the concentration of HDL, LDL and cholesterol in the medium. In order to investigate a possible role of SR-BI impairment in this context, cell viability of HAC-15 cells was measured in presence of mitotane and SR-BI inhibitor BLT-1 (285). Simultaneous treatment with mitotane and BLT-1 did improve cell viability of HAC-15 cells. In absence and presence of mitotane, BLT-1 showed a tendency to reduce HAC-15 cell viability and experiments were discontinued after two measurements. These observations are consistent with a similar experiment in H295R cells (168). Gene expression microarray data analyses presented in this thesis also discovered downregulation of two other major cholesterol transporters, *LDLR* and *ABCA1*. Among all three transporters, downregulation of *SCARB1* gene expression was most pronounced. However, BLT-1 does not affect *ABCA1* mediated cholesterol traffic (286) and effects on *LDLR*

dependent cholesterol transport a not known. Hence, a role of cholesterol transport in the present *in vitro* model of mitotane resistant ACC cannot be excluded on basis of experiments using BLT-1. A possible, mitotane independent cytotoxic effect of SR-BI inhibition might have occurred due to the fact that the human adrenal covers 80% of its cholesterol needs by uptake of plasma cholesterol (8) and thus inhibition of a major cholesterol transporter may have induced senescence. However, a study reports the adrenal glands of *SCARB1* KO mice in comparison to wild type mice to be smaller and cholesterol depleted yet otherwise normal (264).

AGTR1 (Angiotensin II Receptor Type 1)

Another gene discovered to be significantly and strongly downregulated during unbiased and biased gene expression array analysis was *AGTR1,* encoding for angiotensin II receptor type 1. In the adrenal gland, *AGTR1* is predominantly expressed in the zona glomerulosa and plays an important role in aldosterone production (242) but may also be linked to cancer. In invasive ovarian adenocarcinoma, *AGTR1* expression is connected to tumor invasion, angiogenesis, and peritoneal dissemination (287). In order to investigate a possible role of *AGTR1* in mitotane resistance in HAC-15 cells, cell viability of HAC-15 cells was measured in presence of mitotane and *AGTR1* inhibitor losartan. Simultaneous treatment with losartan did not affect mitotane cytotoxicity.

DDIT4L (REDD2) and mTOR Pathway

Another pathway investigated that is also linked to cancer (244) was mTOR signaling. While not discovered during GO enrichment analysis, mTOR inhibitor *DDIT4L* (243)*,* encoding for REDD2, was found to be strongly and significantly downregulated during unbiased gene expression microarray analysis. There is evidence that mTOR signaling may be activated in aldosterone producing adenoma and that inhibition of mTOR signaling using rapamycin in H295R cells decreases aldosterone production and proliferation (245). Simultaneous treatment with mTOR inhibitor rapamycin did not cause significant changes in the IC_{50} of mitotane resistant and nonresistant control clonal cell lines. However, this does not completely exclude a role of mTOR signaling in mitotane resistance in the present *in vitro* model. MTOR signaling is conveyed by two functionally distinct complexes, mTORC1, which drives anabolic cell growth through increases in nucleotide, lipid and protein synthesis as well as glycolysis and mTORC2, which is connected to cytoskeleton organization and cell survival (288). In cardiomyocytes,

DDTI4L inhibits mTORC1 and activates mTORC2 (289) while the exact role of *DDIT4L* on mTOR signaling in the adrenal gland remains unknown. Acute exposure with rapamycin inhibits mTORC1, while chronic exposure may also inhibit mTORC2 (288). Thus, only effects of mTORC1 on mitotane cytotoxicity were investigated in the present thesis, while there is evidence for oncogenic activity of mTORC2 in hepatocellular carcinoma (290).

Wnt Signaling Pathway

Finally, a possible role for Wnt signaling as driver pathway of mitotane resistance in the present *in vitro* model was assessed. Pathways implicated in Wnt signaling were discovered during GO enrichment analysis of upregulated genes among mitotane resistant and nonresistant control clonal cell lines. Increased Wnt activity was confirmed by *AXIN2* rt-PCR as previously described (223). Due to its fundamental role in adrenal development (267) and well-established role in cancer (139), the effect of Wnt inhibition using XAV939 (291) on the IC_{50} of mitotane in mitotane resistant clonal cell lines was assessed. Simultaneous treatment with XAV939 did not change the IC_{50} of mitotane in mitotane resistant cells. Using XAV939 in H295R cells at the same concentration used in the present thesis reportedly decreases cell viability by 30% (249).

9.4.2 Mitotane Resistance is Accompanied by Profound Changes in Intracellular Lipids

Studies suggest a pivotal role of lipids including cholesterol in the mitotane mode of action. It is assumed that sterol-o-acyltransferase 1 inhibition leading to accumulation of intracellular free cholesterol is a major mechanism of mitotane mediated cell death (173). Further, one study provides evidence that mitotane may also directly interact with phosphatidyl ethanolamine rich lipid bilayer membranes due to its lipophilic nature, thereby disturbing membrane bilayer structure (292). The authors argue, that mitochondria associated endoplasmic reticulum membranes are phosphatidyl ethanolamine rich (293) and that mitotane, by associating with these membranes, may cause impairment of sterol-o-acyltransferase 1 activity as well as mitochondrial cholesterol uptake. Also, evidence for mitotane induced dysfunction of mitochondria associated endoplasmic reticulum membranes in H295R cells is provided by another study (294). If provable, these assumptions may pose an explanation for mitotane mediated cyto-

toxicity as well as impairment of adrenal steroid hormone production. Moreover, evidence for a possible role of medium lipoprotein content and lipid transport and metabolism in mitotane resistance was provided by gene expression microarray studies and *in vitro* experiments presented in this thesis. In order to further clarify a possible role of cholesterol and other lipids in mitotane resistance in the present *in vitro* model, the amount of various lipid species was determined using ESI-MS/MS in lysates of three nonresistant control and three mitotane resistant clones cultured in presence of different concentrations of mitotane and different amounts of CCS in the medium. In line with Sbiera et al. (173), treatment with 50 µM mitotane in presence of 5% CCS significantly increased intracellular free cholesterol in nonresistant control cells. Interestingly, mitotane treatment did not affect free cholesterol levels in mitotane resistant cells. Intracellular accumulation of free cholesterol in response to sterol-o-acyltransferase 1 inhibition is a major mediator of the cytotoxic effects of mitotane in NCI-H295 cells (173). The absence of intracellular cholesterol accumulation in response to mitotane treatment in resistant cells therefore poses a possible explanation for reduced cytotoxicity of mitotane observed during MTT assays and the absence of differentially expressed genes in mitotane treated mitotane resistant clonal cell lines. Further, it suggests that mitotane mediated SOAT1 inhibition may be impaired in mitotane resistant cells. In presence of 5% CCS, intracellular CE were significantly reduced in mitotane resistant cells by approximately 30fold. Depletion of intracellular cholesterol storage is consistent with the downregulation of the expression of *SOAT1*, a major regulator of intracellular cholesterol storage (11), and cholesterol transporters *LDLR, ABCA1* and *SCARB1* discovered during gene expression microarray study. Interestingly, mitotane treatment did not cause significant changes in CE content of mitotane resistant and nonresistant cells. This finding suggests not intracellular CE stores but rather cholesterol uptake from lipoproteins and *de novo* synthesis to be the primary source of free cholesterol in the context of mitotane mediated cytotoxicity.

Treatment with 10 µM mitotane in presence of 0% CCS did not lead to significant changes of free cholesterol in nonresistant and mitotane resistant cells, while *in vitro* experiments presented in this thesis have demonstrated cytotoxic effects of mitotane in presence of low concentrations of lipoproteins. Starting with the assumption that in patient's serum approximately 10% of mitotane is lipoprotein free (active) (167), treatment with 50 µM mitotane, corresponding to 16.0 mg/mL and thereby within therapeutic blood levels in human ACC patients (>14 mg/L) (166, 193), used in this series may

have led to exposure with 5 µM active mitotane. Exposure to 10 µM lipoprotein free mitotane may consequently have led to massive apoptosis. Since upregulation of apoptotic pathways is already present 6 hours after mitotane exposure (173), incubation for 72 hours as used in the present thesis may have been too long in order to observe changes in free cholesterol related to mitotane mode of action. Also, absence of changes in free cholesterol in nonresistant control cells may be explained by a another, cholesterol independent mechanism of intracellular mitotane cytotoxicity. This may include loss of lipid bilayer membrane integrity due to hydrophobic interaction (292). A cholesterol independent mode of mitotane action may also explain the mitigation of mitotane resistance in media with reduced lipoprotein content observed in *in vitro* experiments present in this thesis.

Further, intracellular LPCs were significantly increased in nonresistant control cells in presence of 5% CCS and 0, 20 and 50 µM mitotane, and treatment with 50 µM mitotane caused a significant increase in LPCs in nonresistant, but not mitotane resistant cells. In presence of 0% CCS, no significant differences in intracellular LPCs were observed.

Lysophosphatidylcholines are produced during oxidation of LDL by phospholipase A2 (295) and exert cytotoxic effects on human endothelial cells (296). In a mouse model of cholesterol driven nonalcoholic fatty liver disease, hepatic LPC production is increased (297), and LPC is connected to lipoapoptosis in human hepatocytes (298). Interestingly, expression of *PLA2G12A,* encoding for phospholipase 2 group 12 subfamily A, was found to be downregulated in mitotane resistant cells. Gene Ontology enrichment analysis discovered *PLA2G12A* to be significantly enriched in pathways connected to lipid metabolism and transport. However, its function in the adrenal gland remains largely obscure.

Also, intracellular CERs were significantly increased in nonresistant control cells in presence of 5% CCS and 0, 20 and 50 µM mitotane, and treatment with 50 µM mitotane caused a significant increase in CERs in nonresistant, but not mitotane resistant cells. In presence of 0% CCS, no significant differences in intracellular CERs were observed.

The role of CER production in apoptosis is well established and includes stimulation by TNFα receptor and BAX dependent activation of the caspase cascade (299-301).

Intracellular SPMs were significantly higher in nonresistant cells at all conditions tested, and treatment with 50 µM mitotane in presence of 5% CCS significantly increased intracellular SPMs by approximately 1.5fold in nonresistant cells, but not in mitotane resistant cells. Interestingly, treatment with 10 µM mitotane (0% CCS) and 20 µM mitotane (5% CCS) also showed a tendency to increase SPM, while in mitotane resistant cells SPM remained at baseline value. However, no significant effects were found, probably due to low sample size (N=3). In absence of CCS and mitotane no significant differences in intracellular SPMs were observed. In macrophages, SOAT1 activity is regulated by uptake of LDL (302). Interestingly, the threshold at which SOAT1 is activated seems to increase with intracellular SPM content (303). Hence, increases in SPMs during mitotane treatment in nonresistant cells may increase SOAT1 threshold, thereby inhibiting SOAT1 activity. On the other hand, sustaining low levels of SPM during mitotane treatment in mitotane resistant cells may lead to persistent SOAT1 activity and prevent excessive accumulation of free cholesterol. However, the role of SPMs in regulation of the SOAT1 activation threshold in HAC-15 cells and the adrenal gland is still not known.

9.4.3 Intracellular Mitotane Concentration is not Changed in Mitotane Resistant Cells

To investigate whether mitotane resistant cells showed decreased intracellular drug levels, mitotane concentration in supernatant and lysates of three nonresistant control and mitotane resistant clones was determined. No significant difference in intracellular and medium mitotane was found. However, resistant cells showed a tendency towards reduced intracellular mitotane levels. Intestinal absorption of mitotane might involve chylomicron binding (167). The exact molecular mechanism of cellular mitotane uptake is still unknown. It can be speculated that cellular uptake, due to the lipophilic character of mitotane, may be mediated by uptake of mitotane-rich LDL and HDL particles. However, mitotane uptake in H295R cells is increased in lipoprotein free medium (168), and lipoprotein free mitotane seems to mediate the cytotoxic effects of mitotane (167, 168). In one study, Hescot et al. quantify mitotane and its inactive metabolites o,p'DDA and o,p'DDE in samples of human ACC and a normal human adrenal gland collected after surgery of a mitotane-treated ectopic Cushing patient (165). They show that mitotane content in the normal adrenal gland is approximately tenfold higher than average mitotane content of ACC samples. The authors conclude from these insights that mitotane uptake may involve active transport, which may be impaired in ACC. Further

studies on the mechanism of mitotane uptake are needed in order to prove this assumption.

10 Limitations of the Study

The current thesis describes a genetic and *in vitro* study on mitotane resistance in ACC using mitotane resistant HAC-15 cells as an *in vitro* model. This study has certain limitations and hence any conclusions should be drawn with caution.

In order to investigate the underlying mechanisms of mitotane resistance, mitotane resistant clonal cell lines were generated using the HAC-15 cell line. While the IC_{50} of mitotane in HAC-15 cells and the extent of resistance in generated cell lines were within clinically relevant range, it is known that multidrug resistant cell lines of several other tumor entities do not sufficiently reflect gene expression profiles observed in human patients (304). Also, conventional two-dimensional cell culture used in the present thesis does not reflect the three-dimensional architecture of a tumor and thus may influence cell shape, growth, motility, differentiation and even gene expression in comparison to three-dimensional *in vitro* models (305). Further, two-dimensional cell culture does not mimic the tumor microenvironment, which has also been implicated in chemotherapy resistance (306). A possible role of the tumor microenvironment in mitotane resistance may be assessed using a mouse xenograft model (307). Finally, no studies in human ACC patients were conducted in the present thesis. Studies on gene expression profile, lipoprotein homeostasis and intracellular lipid composition in human patients with recurring ACC may further substantiate findings concerning mitotane resistance in ACC. Also, the cell culture model used in the present thesis does not adequately reproduce the complexity of the human organism. During long-term treatment, HAC-15 cells were treated with mitotane for 72-96 hours, while mitotane has a half-life of up to 160 hours in human plasma (164). Also, mitotane is metabolized to its inactive metabolites o,p'DDA and o,p'DDE in the liver and excreted through urine and bile (164). Increased excretion and metabolic inactivation may be involved in failure of mitotane treatment in human ACC but were completely neglected by the cell culture studies conducted in the present thesis.

11 Clinical Relevance

Adrenocortical carcinoma is an aggressive malignancy with a dismal prognosis (60-63, 72, 197). Treatment with mitotane, the only drug approved for ACC treatment, fails to increase overall patient survival (74, 148, 149, 154). Furthermore, the low incidence of ACC (57-60) and poor prognosis drastically limit cohort size and complicate clinical studies on this disease. Despite the limitations discussed above, HAC-15 cells secrete the full spectrum of adrenocortical hormones (216) and thus pose an easily accessible model to investigate therapy resistance in adrenocortical carcinoma on a cellular and molecular level. Mitotane resistance generated in the current thesis was found to be within clinically relevant range for other tumor entities (218). Insights on changes in gene expression and intracellular lipid content during mitotane treatment of non-resistant cells confirmed and expanded the findings of Sbiera et al. (173). Findings regarding a possible role of lipoproteins in mediating mitotane resistance may help to explain the positive influence on simultaneous statin treatment on outcome of mitotane treated patients previously reported (168). Building on these findings, a clinical study comparing mitotane monotherapy and combination therapy using statins and mitotane may further clarify a role of serum lipoproteins in mitotane therapy outcome and hence could pave the way for improved disease management.

12 Summary, Conclusions and Outlook

The current thesis presents a genetic analysis as well as functional studies in an *in vitro* model of mitotane resistant ACC. Mitotane resistant clonal cell lines were generated using the HAC-15 ACC cell line. In order to investigate changes in gene expression and DNA integrity in mitotane resistant and nonresistant control cells, whole exome sequencing and a gene expression microarray study were performed. In subsequent functional studies, possible driver genes and pathways as well as changes in cellular lipid and mitotane content were assessed in mitotane resistant and nonresistant clonal cell lines.

12.1 Does mitotane treatment induce resistance *in vitro*?

In the current thesis, mitotane resistance in HAC-15 cells was induced using a selection driven, pulsed treatment approach followed by clonal selection. Mitotane resistance was found to be in clinically relevant range and different from common multidrug resistance. Further, evidence was provided that the IC_{50} of mitotane is positively correlated with the concentration of HDL, LDL and cholesterol in the cell culture medium. Furthermore, mitotane resistance was mitigated in presence of low concentrations of HDL and LDL, while the HDL to LDL ratio did not influence mitotane resistance. These findings suggest that mitotane resistance in the present *in vitro* model of mitotane resistant ACC is dependent on cholesterol and lipoprotein medium content. In order to investigate a possible involvement of serum lipoproteins in mitotane therapy relapse in human patients, further studies on human patients are needed. Interestingly, a mitotane-EDP resistant cell line has recently been established (217), suggesting that, unlike in the present *in vitro* model, mitotane resistant may also be accompanied by common multidrug resistance. Comparison of these two *in vitro* models may corroborate the findings provided in the present thesis and could shed further light on the genetic and molecular mechanisms contributing to mitotane resistance.

12.2 Is mitotane resistance accompanied by genetic changes either concerning the transcription level or integrity of the DNA sequence?

The gene expression microarray study confirmed mitotane resistance on gene expression level. Gene expression data analysis revealed no differentially expressed genes

in mitotane treated versus vehicle control treated mitotane resistant clonal cell lines. Gene ontology enrichment analysis of differentially expressed genes between mitotane and vehicle control treated nonresistant cells revealed upregulation of endoplasmic reticulum stress response, proapoptotic pathways as well as downregulation of pathways implicated in cholesterol and sterol homeostasis, localization, transport and metabolism.

Further, the gene expression microarray study revealed profound changes in gene expression in mitotane resistant clonal cell lines. During principle component analysis, mitotane resistant and nonresistant control clonal cell lines clustered in two distinct clusters. Gene ontology enrichment analysis of differentially expressed genes between mitotane resistant and nonresistant clonal cell lines revealed upregulation of pathways implicated in Wnt signaling, cell growth and development and downregulation of pathways implicated in biosynthesis and metabolism of adrenal steroid hormones as well as lipoprotein binding and clearance. Biased data analysis, focusing on genes annotated for “cholesterol” and “steroid”, also discovered downregulation of the expression of intracellular mitotane target *SOAT1* as well as cholesterol transporters *SCARB1, LDLR* and *ABCA1*. Whole exome sequencing revealed highly similar CNV profiles between all mitotane resistant clonal cell lines.

Taken together, the genetic analyses provide evidence that mitotane resistance in the present *in vitro* model may occur due to a clonal expansion of a single, or several genetically very similar, progenitor cell(s), driven by selective pressure of mitotane treatment. Clonal expansion may occur due to a genetic event during long-term treatment of HAC-15 cells leading to an advantageous, mitotane resistant phenotype. The mitotane resistant phenotype of resistant clonal cell lines could be substantiated further by a series of mouse xenograft experiments. The gene expression in the current *in vitro* model is unaffected by mitotane treatment. Furthermore, gene expression analysis, consistent with *in vitro* experiments, suggests an involvement of lipoprotein transport in mitotane resistance. Building on these insights, investigations using the mitotane-EDP resistant cell line established by Hantel et al. (217) as well as human patients may further clarify a role of lipoproteins in mitotane resistance.

12.3 Can molecular mechanisms of mitotane resistance be inferred from these genetic changes?

In order to identify possible driver genes and pathways implicated in mitotane resistance, functional studies on several selected genes or pathways were performed in HAC-15 cells. Inhibition of SR-BI, angiotensin II receptor type I, mTOR signaling and Wnt signaling did not affect mitotane cytotoxicity. These findings suggest that a mitotane resistant phenotype is driven by a plethora of genetic changes rather than a single driver pathway in the current *in vitro* model of mitotane resistant ACC. However, a revised study of possible driver genes comprising adjusted concentrations and incubation times, different inhibitors and combinations of several inhibitors may still lead to discovery of driver pathways in the present *in vitro* model.

Building on the possible role of cholesterol transport in mitotane resistance and genetic changes in lipid metabolism and transport observed in gene expression microarrays of mitotane resistant clonal cell lines, intracellular lipid content was investigated. Nonresistant cells upon treatment with 50 µM mitotane in presence of 5% CCS showed increases in free cholesterol, SPM as well as apoptotic lipids LPC and CER, while all four lipid species remained unchanged in mitotane resistant cells. Furthermore, CE were strongly and significantly reduced in mitotane resistant cells.

Taken together, absence of increase in free cholesterol, LPC and CER further supports a role of lipid and cholesterol metabolism and transport in mitotane resistance in the current *in vitro* model. Absence of increases in intracellular free cholesterol upon mitotane treatment suggests impaired SOAT1 inhibition by mitotane. As discussed above, increased SPM content could help to explain mitotane resistance. In analogy to macrophages, increased cellular SPM content at both, basal conditions and during mitotane treatment could increase sterol-o-acyltransferase activation threshold in nonresistant cells. At baseline, free cholesterol content did not differ among resistant and nonresistant cells. However, mitotane resistant cells were depleted in CE, probably due to downregulation of *SOAT1, ABCA1, SCARB1* and *LDLR* gene expression. Mitotane dependent increase in SPM might then elevate sterol-O-acyltransferase threshold in nonresistant cells, while the threshold remains unchanged in resistant cells. Consequently, mitotane resistant cells maintain normal yet decreased intracellular lipid metabolism while impaired cholesterol esterification in nonresistant cells may lead to accumulation of intracellular free cholesterol, endoplasmic reticulum stress and apoptosis. However, SPM dependent regulation of sterol-o-acyltransferase activity has only

been shown in macrophages, and the exact lipid composition in ACC cells as well as normal adrenal glands has not yet been investigated. Further studies on these subjects are needed in order to prove or falsify this hypothesis. Alternatively, downregulation of *ABCA1, SCARB1* and *LDLR* gene expression may have led to increased medium concentrations of HDL and LDL. Medium content of HDL, LDL and cholesterol was found to be positively correlated with the IC_{50} of mitotane. In order to further prove this hypothesis, levels of HDL and LDL should be quantified in supernatants of mitotane resistant cells. Also studies with radioactively labeled cholesterol may further prove impaired lipoprotein uptake in mitotane resistant cells. Interestingly, increased levels of serum cholesterol, HDL and LDL during mitotane therapy are commonly observed in human ACC patients (188-191) and may occur due to increased hepatic cholesterol synthesis (265). In presence of 0% CCS, intracellular LPC, CER and free cholesterol remained unchanged. As discussed above, this could be due to stronger mitotane effects in lipoprotein depleted medium. Alternatively, this may also suggest a second, cholesterol independent mode of mitotane action. An alternative mode of action may also help to explain mitigation of mitotane resistance observed in media with reduced HDL and LDL content.

Finally, intracellular content of mitotane in nonresistant and mitotane resistant cells was assessed. No difference in intracellular mitotane concentration was found, probably due to low sample size. Sample size should be increased in order increase statistical power. The exact mechanism of cellular mitotane uptake and release is not known. In order to further clarify a possible role of impaired mitotane uptake and release in the present *in vitro* model, further studies investigating the kinetics of mitotane uptake in resistant cells are needed.

12.4 Are the molecular mechanisms of mitotane resistance consistent with observations in both, ACC patients and *in vitro* models?

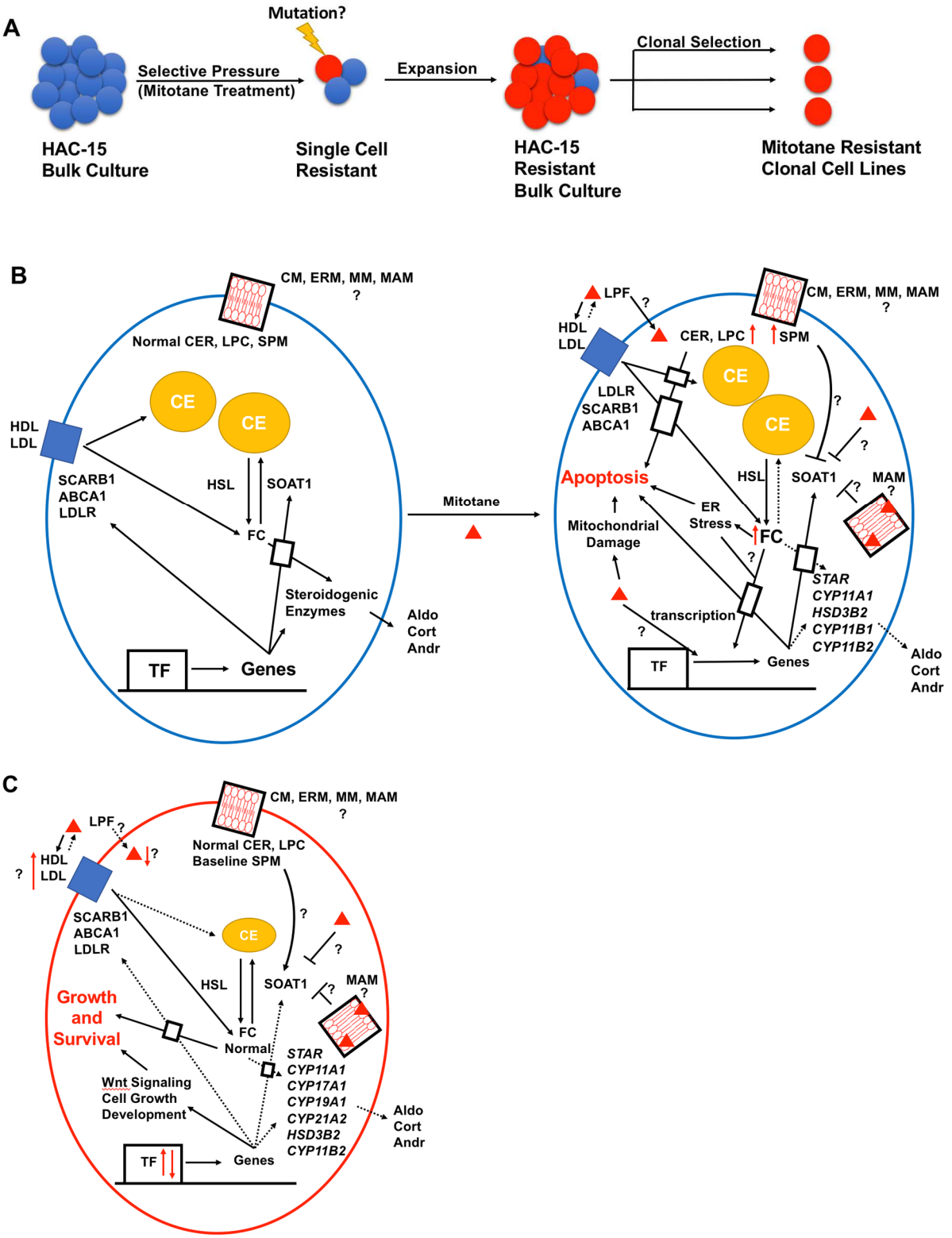
The current thesis has helped to confirm previous studies on mitotane action and expand the knowledge on the mechanisms underlying mitotane resistance. Results are summarized in figure 23.

During long-term treatment, mitotane treated cells fully recovered after approximately 70 days (2.3 months) while median progression free survival during mitotane monotherapy is 4.1 months (157). Unlike MUC-1 cells, the other mitotane resistant cell line

available (217), the present *in vitro* model lacked signs of common multidrug resistance. Doxorubicin sensitizing effects of both, short- and long-term mitotane treatment observed is consistent with a previous report using NCI-H295 cells (210). The influence of medium lipoproteins on mitotane cytotoxicity confirmed previous studies (167, 168). However, cholesterol dependency of mitotane resistance has not been reported so far and may help to explain improved disease management in patients treated with statins and mitotane (168). Finally, gene expression microarray analysis and measurement of intracellular lipids of mitotane treated nonresistant cells confirmed and expanded insights from a previous study (173).

Figure 23 A-C: Summary of findings from the present thesis and the literature concerning mitotane mode of action and mitotane resistance. **A)** Generation of mitotane resistant clonal cell lines. Mitotane long-term treatment drastically reduces cell growth in mitotane sensitive HAC-15 cells (blue). By a certain event, e.g., a genetic mutation, a single cell may acquire an advantageous, mitotane resistant phenotype and may expand during long-term mitotane treatment. Clonal cell lines can subsequently be isolated through clonal selection. **B)** Mitotane mode of action in nonresistant HAC-15 cells. In nonresistant cells, multiple transcription factors (TF) including SF-1, NURR1 and NGFIB control expression of numerous genes including steroidogenic enzymes, cholesterol transporters including *SCARB1, ABCA1*, *LDLR* and *SOAT1* (256, 257). Cholesterol and cholesteryl esters (CE) are taken up by transporters including *SCARB1, ABCA1* and *LDLR*. Free cholesterol is esterified by *SOAT1*, stored in lipid bodies and may be released from CE storage by hormone-sensitive lipase (HSL). Steroidogenic enzymes produce cortisol (Cort), aldosterone (Aldo) and adrenal androgens (Andr). The intracellular concentration of ceramides (CER), lysophosphatidylcholine (LPC) and sphingomyelin (SPM) in nonresistant HAC-15 cells is at baseline levels. However, distribution of these lipids between the cell membrane (CM), endoplasmic reticulum membrane (ERM), mitochondrial membrane (MM) and mitochondria associated membranes (MAM) has not been investigated so far. A large fraction of mitotane is associated with lipoproteins in the cell culture medium, while lipoprotein free mitotane (LPF) mediates the cytotoxic effects (167, 168). The mechanism of cellular mitotane uptake is not known. Intracellular mitotane causes impairment of mitochondrial electron transport chain leading to apoptosis (172). Also, mitotane inhibits *SOAT1*, which may involve direct inhibition (173), association with MAMs (292) or increase of *SOAT1* activation threshold by increases in intracellular SPMs (303). Impairment of SOAT1 activity leads to intracellular accumulation of free cholesterol causing endoplasmic reticulum (ER) stress (173). In response to either free cholesterol accumulation or ER pathways, ER stress response genes and proapoptotic genes are increasingly expressed. Also, mitotane treatment increases intracellular content of proapoptotic lipids CER and LPC. Treatment with mitotane for 48 h decreases expression of steroidogenic enzymes *STAR, CYP11A1, HSD3B2, CYP11B1* and *CYP11B2* (172). **C)** Intracellular mechanisms of mitotane resistance. Mitotane resistant cells show profound changes in gene expression including up- and downregulation of transcription factor activity. They are characterized by increased expression of pathways connected to cell growth, Wnt signaling and development as well as decreased expression of numerous steroidogenic enzymes, *SCARB1, ABCA1*, *LDLR* and *SOAT1.* Downregulation of cholesterol transporters may lead to increase in medium HDL and LDL and thus decrease of lipoprotein free (active) mitotane. Further, mitotane uptake may be impaired in mitotane resistant cells. Also, mitotane resistant cells are depleted of CE, while free cholesterol is not changed. During mitotane treatment, intracellular levels of CER and LPC remain unchanged in mitotane resistant cells. Baseline free cholesterol in resistant cells is comparable to nonresistant cells, however no increase of free cholesterol is observed during mitotane treatment. This may be

explained by baseline SPM levels during mitotane treatment, since SPM levels regulate SOAT1 activation threshold in macrophages (303).



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15 Appendix

Appendix 1: Complete Results of the Gene Ontology Enrichment Analysis

Biological Processes for Upregulated Genes Nonresistant DMSO vs. Mitotane

GO.ID	Term	Annotated	Significant	Expected	classicFisher	GO.ID	Term	Annotated	Significant	Expected	classicFisher
48514	blood vessel morphogenesis	233	36	18,77	0.00010	60745	mammary gland branching involved in preg...	5	3	0,4	0.00460
8202	steroid metabolic process	164	28	13,21	0.00011	71635	negative regulation of transforming grow...	5	3	0,4	0.00460
70372	regulation of ERK1 and ERK2 cascade	102	20	8,22	0.00015	1902337	regulation of apoptotic process involved...	5	3	0,4	0.00460
6704	glucocorticoid biosynthetic process	8	5	0,64	0.00015	1904748	regulation of apoptotic process involved...	5	3	0,4	0.00460
8211	glucocorticoid metabolic process	12	6	0,97	0.00016	6909	phagocytosis	115	18	9,27	0.00465
50896	response to stimulus	4190	383	337,61	0.00019	42326	negative regulation of phosphorylation	276	35	22,24	0.00466
9605	response to external stimulus	914	103	73,65	0.00019	10466	negative regulation of peptidase activit...	89	15	7,17	0.00466
32501	multicellular organismal process	3139	297	252,93	0.00019	51480	regulation of cytosolic calcium ion conc...	89	15	7,17	0.00466
36150	phosphatidylserine acyl-chain remodeling	5	4	0,4	0.00020	30301	cholesterol transport	34	8	2,74	0.00471
2920	regulation of humoral immune response	17	7	1,37	0.00020	52547	regulation of peptidase activity	189	26	15,23	0.00481
30855	epithelial cell differentiation	287	41	23,13	0.00021	7015	actin filament organization	199	27	16,03	0.00497
1525	angiogenesis	189	30	15,23	0.00024	48638	regulation of developmental growth	152	22	12,25	0.00499
43277	apoptotic cell clearance	18	7	1,45	0.00031	71363	cellular response to growth factor stimu...	358	43	28,85	0.00501
32101	regulation of response to external stimu...	274	39	22,08	0.00031	45055	regulated exocytosis	389	46	31,34	0.00505
9410	response to xenobiotic stimulus	48	12	3,87	0.00032	50678	regulation of epithelial cell proliferat...	143	21	11,52	0.00507
9719	response to endogenous stimulus	810	92	65,27	0.00033	72659	protein localization to plasma membrane	143	21	11,52	0.00507
71495	cellular response to endogenous stimulus	706	82	56,89	0.00035	71634	regulation of transforming growth factor...	15	5	1,21	0.00508
6509	membrane protein ectodomain proteolysis	24	8	1,93	0.00039	6936	muscle contraction	134	20	10,8	0.00512
6812	cation transport	462	58	37,23	0.00040	30148	sphingolipid biosynthetic process	57	11	4,59	0.00515
48646	anatomical structure formation involved ...	462	58	37,23	0.00040	72006	nephron development	57	11	4,59	0.00515
60326	cell chemotaxis	78	16	6,28	0.00040	23051	regulation of signaling	1750	168	141,01	0.00518
1902904	negative regulation of supramolecular fi...	71	15	5,72	0.00043	35556	intracellular signal transduction	1474	144	118,77	0.00540
71310	cellular response to organic substance	1320	138	106,36	0.00045	50728	negative regulation of inflammatory resp...	42	9	3,38	0.00540
32940	secretion by cell	680	79	54,79	0.00045	55074	calcium ion homeostasis	144	21	11,6	0.00550
2526	acute inflammatory response	43	11	3,46	0.00045	51240	positive regulation of multicellular org...	678	73	54,63	0.00557
44255	cellular lipid metabolic process	578	69	46,57	0.00049	6875	cellular metal ion homeostasis	220	29	17,73	0.00557
60541	respiratory system development	95	18	7,65	0.00050	6694	steroid biosynthetic process	117	18	9,43	0.00559
30001	metal ion transport	338	45	27,23	0.00051	33619	membrane protein proteolysis	35	8	2,82	0.00568
6072	glycerol-3-phosphate metabolic process	3	3	0,24	0.00052	8643	carbohydrate transport	74	13	5,96	0.00571
48732	gland development	225	33	18,13	0.00052	55080	cation homeostasis	290	36	23,37	0.00584
6705	mineralocorticoid biosynthetic process	6	4	0,48	0.00055	6688	glycosphingolipid biosynthetic process	10	4	0,81	0.00592
8212	mineralocorticoid metabolic process	6	4	0,48	0.00055	10743	regulation of macrophage derived foam ce...	10	4	0,81	0.00592
71447	cellular response to hydroperoxide	6	4	0,48	0.00055	34105	positive regulation of tissue remodeling	10	4	0,81	0.00592
3012	muscle system process	183	28	14,75	0.00069	72576	liver morphogenesis	10	4	0,81	0.00592
51716	cellular response to stimulus	3546	326	285,72	0.00072	1990000	amyloid fibril formation	10	4	0,81	0.00592
50673	epithelial cell proliferation	166	26	13,38	0.00074	9617	response to bacterium	173	24	13,94	0.00594
6805	xenobiotic metabolic process	39	10	3,14	0.00080	6869	lipid transport	145	21	11,68	0.00596
9888	tissue development	876	96	70,58	0.00084	70661	leukocyte proliferation	100	16	8,06	0.00596
8207	C21-steroid hormone metabolic process	21	7	1,69	0.00091	6469	negative regulation of protein kinase ac...	155	22	12,49	0.00630
32963	collagen metabolic process	47	11	3,79	0.00102	22617	extracellular matrix disassembly	43	9	3,46	0.00636
6700	C21-steroid hormone biosynthetic process	16	6	1,29	0.00106	2000379	positive regulation of reactive oxygen s...	43	9	3,46	0.00636
8209	androgen metabolic process	16	6	1,29	0.00106	10646	regulation of cell communication	1724	165	138,91	0.00638
1901615	organic hydroxy compound metabolic proce...	244	34	19,66	0.00110	1867	complement activation, lectin pathway	2	2	0,16	0.00648
46889	positive regulation of lipid biosyntheti...	34	9	2,74	0.00114	2577	regulation of antigen processing and pre...	2	2	0,16	0.00648
1901342	regulation of vasculature development	110	19	8,86	0.00115	2578	negative regulation of antigen processin...	2	2	0,16	0.00648
2921	negative regulation of humoral immune re...	7	4	0,56	0.00120	2583	regulation of antigen processing and pre...	2	2	0,16	0.00648
30837	negative regulation of actin filament po...	28	8	2,26	0.00124	2584	negative regulation of antigen processin...	2	2	0,16	0.00648
6873	cellular ion homeostasis	265	36	21,35	0.00126	2589	regulation of antigen processing and pre...	2	2	0,16	0.00648
8406	gonad development	111	19	8,94	0.00128	2590	negative regulation of antigen processin...	2	2	0,16	0.00648
6887	exocytosis	464	56	37,39	0.00130	2677	negative regulation of chronic inflammat...	2	2	0,16	0.00648
6935	chemotaxis	219	31	17,65	0.00138	3310	pancreatic A cell differentiation	2	2	0,16	0.00648
42330	taxis	219	31	17,65	0.00138	5988	lactose metabolic process	2	2	0,16	0.00648
52548	regulation of endopeptidase activity	173	26	13,94	0.00138	5989	lactose biosynthetic process	2	2	0,16	0.00648
97006	regulation of plasma lipoprotein particl...	42	10	3,38	0.00148	7341	penetration of zona pellucida	2	2	0,16	0.00648
1901136	carbohydrate derivative catabolic proces...	104	18	8,38	0.00150	7354	zygotic determination of anterior/poster...	2	2	0,16	0.00648
7548	sex differentiation	139	22	11,2	0.00161	9812	flavonoid metabolic process	2	2	0,16	0.00648
22612	gland morphogenesis	57	12	4,59	0.00164	10899	regulation of phosphatidylcholine catabo...	2	2	0,16	0.00648
32613	interleukin-10 production	12	5	0,97	0.00164	21984	adenohypophysis development	2	2	0,16	0.00648
44259	multicellular organismal macromolecule m...	50	11	4,03	0.00175	30299	intestinal cholesterol absorption	2	2	0,16	0.00648
2683	negative regulation of immune system pro...	185	27	14,91	0.00176	32455	nerve growth factor processing	2	2	0,16	0.00648

1568	blood vessel development	280	37	22,56	0.00180
71466	cellular response to xenobiotic stimulus	43	10	3,46	0.00180
30324	lung development	81	15	6,53	0.00181
43269	regulation of ion transport	223	31	17,97	0.00186
51239	regulation of multicellular organismal p...	1322	134	106,52	0.00193
19934	cGMP-mediated signaling	4	3	0,32	0.00196
32911	negative regulation of transforming grow...	4	3	0,32	0.00196
34367	macromolecular complex remodeling	4	3	0,32	0.00196
34368	protein-lipid complex remodeling	4	3	0,32	0.00196
34369	plasma lipoprotein particle remodeling	4	3	0,32	0.00196
52646	alditol phosphate metabolic process	4	3	0,32	0.00196
60696	regulation of phospholipid catabolic pro...	4	3	0,32	0.00196
61101	neuroendocrine cell differentiation	4	3	0,32	0.00196
61370	testosterone biosynthetic process	4	3	0,32	0.00196
19722	calcium-mediated signaling	66	13	5,32	0.00202
72359	circulatory system development	463	55	37,31	0.00205
10033	response to organic substance	1632	161	131,5	0.00205
9653	anatomical structure morphogenesis	1234	126	99,43	0.00206
1944	vasculature development	292	38	23,53	0.00207
48286	lung alveolus development	18	6	1,45	0.00214
60042	retina morphogenesis in camera-type eye	18	6	1,45	0.00214
8585	female gonad development	44	10	3,55	0.00216
45137	development of primary sexual characteri...	116	19	9,35	0.00218
6702	androgen biosynthetic process	8	4	0,64	0.00225
19852	L-ascorbic acid metabolic process	8	4	0,64	0.00225
7369	gastrulation	108	18	8,7	0.00232
19932	second-messenger-mediated signaling	108	18	8,7	0.00232
50801	ion homeostasis	324	41	26,11	0.00238
30325	adrenal gland development	13	5	1,05	0.00249
30449	regulation of complement activation	13	5	1,05	0.00249
86010	membrane depolarization during action po...	13	5	1,05	0.00249
2000257	regulation of protein activation cascade	13	5	1,05	0.00249
46718	viral entry into host cell	60	12	4,83	0.00260
97237	cellular response to toxic substance	60	12	4,83	0.00260
30323	respiratory tube development	84	15	6,77	0.00263
30155	regulation of cell adhesion	296	38	23,85	0.00264
72358	cardiovascular system development	296	38	23,85	0.00264
98771	inorganic ion homeostasis	296	38	23,85	0.00264
32272	negative regulation of protein polymeriz...	38	9	3,06	0.00264
46434	organophosphate catabolic process	76	14	6,12	0.00268
1990778	protein localization to cell periphery	163	24	13,13	0.00275
22603	regulation of anatomical structure morph...	502	58	40,45	0.00295
30003	cellular cation homeostasis	259	34	20,87	0.00302
51494	negative regulation of cytoskeleton orga...	77	14	6,2	0.00304
7492	endoderm development	46	10	3,71	0.00307
40011	locomotion	750	81	60,43	0.00326
48878	chemical homeostasis	494	57	39,8	0.00329
32502	developmental process	2938	270	236,73	0.00332
46660	female sex differentiation	54	11	4,35	0.00333
71901	negative regulation of protein serine/th...	78	14	6,28	0.00344
48583	regulation of response to stimulus	2001	191	161,23	0.00345
1818	negative regulation of cytokine producti...	112	18	9,02	0.00349
9311	oligosaccharide metabolic process	26	7	2,09	0.00362
9880	embryonic pattern specification	26	7	2,09	0.00362
10742	macrophage derived foam cell differentia...	14	5	1,13	0.00362
90077	foam cell differentiation	14	5	1,13	0.00362
45321	leukocyte activation	549	62	44,24	0.00365
55082	cellular chemical homeostasis	342	42	27,56	0.00370
48870	cell motility	635	70	51,17	0.00376
51674	localization of cell	635	70	51,17	0.00376
6952	defense response	603	67	48,59	0.00377
33673	negative regulation of kinase activity	167	24	13,46	0.00378
6067	ethanol metabolic process	9	4	0,73	0.00379
32490	detection of molecule of bacterial origi...	2	2	0,16	0.00648
32808	lacrimal gland development	2	2	0,16	0.00648
32902	nerve growth factor production	2	2	0,16	0.00648
34196	acylglycerol transport	2	2	0,16	0.00648
34197	triglyceride transport	2	2	0,16	0.00648
34382	chylomicron remnant clearance	2	2	0,16	0.00648
42078	germ-line stem cell division	2	2	0,16	0.00648
43435	response to corticotropin-releasing horm...	2	2	0,16	0.00648
46069	cGMP catabolic process	2	2	0,16	0.00648
46351	disaccharide biosynthetic process	2	2	0,16	0.00648
46618	drug export	2	2	0,16	0.00648
48133	male germ-line stem cell asymmetric divi...	2	2	0,16	0.00648
50910	detection of mechanical stimulus involve...	2	2	0,16	0.00648
51643	endoplasmic reticulum localization	2	2	0,16	0.00648
51694	pointed-end actin filament capping	2	2	0,16	0.00648
60535	trachea cartilage morphogenesis	2	2	0,16	0.00648
61669	spontaneous neurotransmitter secretion	2	2	0,16	0.00648
71376	cellular response to corticotropin-relea...	2	2	0,16	0.00648
71596	ubiquitin-dependent protein catabolic pr...	2	2	0,16	0.00648
71830	triglyceride-rich lipoprotein particle c...	2	2	0,16	0.00648
86097	phospholipase C-activating angiotensin-a...	2	2	0,16	0.00648
90272	negative regulation of fibroblast growth...	2	2	0,16	0.00648
98581	detection of external biotic stimulus	2	2	0,16	0.00648
98728	germline stem cell asymmetric division	2	2	0,16	0.00648
98856	intestinal lipid absorption	2	2	0,16	0.00648
98912	membrane depolarization during atrial ca...	2	2	0,16	0.00648
2000866	positive regulation of estradiol secreti...	2	2	0,16	0.00648
2673	regulation of acute inflammatory respons...	22	6	1,77	0.00652
60563	neuroepithelial cell differentiation	22	6	1,77	0.00652
72376	protein activation cascade	22	6	1,77	0.00652
10631	epithelial cell migration	128	19	10,31	0.00665
90132	epithelium migration	128	19	10,31	0.00665
97435	supramolecular fiber organization	323	39	26,03	0.00672
6749	glutathione metabolic process	36	8	2,9	0.00680
6956	complement activation	16	5	1,29	0.00691
7157	heterophilic cell-cell adhesion via plas...	16	5	1,29	0.00691
71604	transforming growth factor beta producti...	16	5	1,29	0.00691
48869	cellular developmental process	1986	187	160,02	0.00705
31032	actomyosin structure organization	93	15	7,49	0.00708
7159	leukocyte cell-cell adhesion	138	20	11,12	0.00712
90130	tissue migration	129	19	10,39	0.00723
2274	myeloid leukocyte activation	315	38	25,38	0.00751
45834	positive regulation of lipid metabolic p...	60	11	4,83	0.00768
50777	negative regulation of immune response	52	10	4,19	0.00769
34765	regulation of ion transmembrane transpor...	158	22	12,73	0.00789
72503	cellular divalent inorganic cation homeo...	158	22	12,73	0.00789
70373	negative regulation of ERK1 and ERK2 cas...	37	8	2,98	0.00808
61028	establishment of endothelial barrier	23	6	1,85	0.00823
1775	cell activation	613	66	49,39	0.00825
2000377	regulation of reactive oxygen species me...	86	14	6,93	0.00839
1704	formation of primary germ layer	69	12	5,56	0.00842
1706	endoderm formation	30	7	2,42	0.00845
70848	response to growth factor	369	43	29,73	0.00850
34220	ion transmembrane transport	453	51	36,5	0.00853
72009	nephron epithelium development	45	9	3,63	0.00865
30207	chondroitin sulfate catabolic process	6	3	0,48	0.00866
32905	transforming growth factor beta1 product...	6	3	0,48	0.00866
32908	regulation of transforming growth factor...	6	3	0,48	0.00866
33630	positive regulation of cell adhesion med...	6	3	0,48	0.00866
35376	sterol import	6	3	0,48	0.00866
35428	hexose transmembrane transport	6	3	0,48	0.00866
48671	negative regulation of collateral sprout...	6	3	0,48	0.00866
51004	regulation of lipoprotein lipase activit...	6	3	0,48	0.00866

32703	negative regulation of interleukin-2 pro...	9	4	0,73	0.00379
32733	positive regulation of interleukin-10 pr...	9	4	0,73	0.00379
36151	phosphatidylcholine acyl-chain remodelin...	9	4	0,73	0.00379
72574	hepatocyte proliferation	9	4	0,73	0.00379
72575	epithelial cell proliferation involved i...	9	4	0,73	0.00379
90036	regulation of protein kinase C signaling	9	4	0,73	0.00379
15918	sterol transport	40	9	3,22	0.00383
15850	organic hydroxy compound transport	79	14	6,37	0.00388
6654	phosphatidic acid biosynthetic process	20	6	1,61	0.00389
46473	phosphatidic acid metabolic process	20	6	1,61	0.00389
32879	regulation of localization	1268	127	102,17	0.00396
10951	negative regulation of endopeptidase act...	88	15	7,09	0.00418
46545	development of primary female sexual cha...	48	10	3,87	0.00425
6874	cellular calcium ion homeostasis	141	21	11,36	0.00429
44236	multicellular organism metabolic process	56	11	4,51	0.00447
5996	monosaccharide metabolic process	160	23	12,89	0.00452
10876	lipid localization	160	23	12,89	0.00452
9948	anterior/posterior axis specification	27	7	2,18	0.00455
34381	plasma lipoprotein particle clearance	27	7	2,18	0.00455
1845	phagolysosome assembly	5	3	0,4	0.00460

60046	regulation of acrosome reaction	6	3	0,48	0.00866
60484	lung-associated mesenchyme development	6	3	0,48	0.00866
61081	positive regulation of myeloid leukocyte...	6	3	0,48	0.00866
70508	cholesterol import	6	3	0,48	0.00866
1904659	glucose transmembrane transport	6	3	0,48	0.00866
1905950	monosaccharide transmembrane transport	6	3	0,48	0.00866
6012	galactose metabolic process	11	4	0,89	0.00871
7340	acrosome reaction	11	4	0,89	0.00871
32653	regulation of interleukin-10 production	11	4	0,89	0.00871
45940	positive regulation of steroid metabolic...	11	4	0,89	0.00871
50858	negative regulation of antigen receptor-...	11	4	0,89	0.00871
51043	regulation of membrane protein ectodomai...	11	4	0,89	0.00871
1902532	negative regulation of intracellular sig...	308	37	24,82	0.00884
2697	regulation of immune effector process	141	20	11,36	0.00901
6066	alcohol metabolic process	179	24	14,42	0.00903
61458	reproductive system development	218	28	17,57	0.00905
16042	lipid catabolic process	160	22	12,89	0.00911
578	embryonic axis specification	17	5	1,37	0.00915
2455	humoral immune response mediated by circ...	17	5	1,37	0.00915
1816	cytokine production	299	36	24,09	0.00940
6575	cellular modified amino acid metabolic p...	105	16	8,46	0.00953
10634	positive regulation of epithelial cell m...	62	11	5	0.00984
15749	monosaccharide transport	62	11	5	0.00984
9887	animal organ morphogenesis	478	53	38,52	0.00993
50900	leukocyte migration	133	19	10,72	0.00998

Biological Processes for Downregulated Genes Nonresistant DMSO vs. Mitotane

GO.ID	Term	Annotated	Significant	Expected	classicFisher
6636	unsaturated fatty acid biosynthetic proc...	18	2	0,02	0.00011
43436	oxoacid metabolic process	671	5	0,62	0.00013
6082	organic acid metabolic process	674	5	0,63	0.00013
32365	intracellular lipid transport	22	2	0,02	0.00017
71398	cellular response to fatty acid	28	2	0,03	0.00028
42632	cholesterol homeostasis	29	2	0,03	0.00030
55092	sterol homeostasis	29	2	0,03	0.00030
15914	phospholipid transport	31	2	0,03	0.00035
42472	inner ear morphogenesis	31	2	0,03	0.00035
45017	glycerolipid biosynthetic process	166	3	0,15	0.00037
16053	organic acid biosynthetic process	169	3	0,16	0.00039
46394	carboxylic acid biosynthetic process	169	3	0,16	0.00039
30301	cholesterol transport	34	2	0,03	0.00042
15918	sterol transport	40	2	0,04	0.00058
70542	response to fatty acid	42	2	0,04	0.00064
97006	regulation of plasma lipoprotein particl...	42	2	0,04	0.00064
42471	ear morphogenesis	44	2	0,04	0.00070
33559	unsaturated fatty acid metabolic process	45	2	0,04	0.00073
15748	organophosphate ester transport	47	2	0,04	0.00080
55088	lipid homeostasis	52	2	0,05	0.00098
19217	regulation of fatty acid metabolic proce...	55	2	0,05	0.00109
1901576	organic substance biosynthetic process	3735	8	3,47	0.00125
9058	biosynthetic process	3770	8	3,5	0.00134

GO.ID	Term	Annotated	Significant	Expected	classicFisher
48839	inner ear development	68	2	0,06	0.00167
45596	negative regulation of cell differentiat...	309	3	0,29	0.00224
15850	organic hydroxy compound transport	79	2	0,07	0.00224
30324	lung development	81	2	0,08	0.00236
30323	respiratory tube development	84	2	0,08	0.00253
43583	ear development	85	2	0,08	0.00259
60541	respiratory system development	95	2	0,09	0.00323
46907	intracellular transport	1339	5	1,24	0.00334
71229	cellular response to acid chemical	105	2	0,1	0.00393
9719	response to endogenous stimulus	810	4	0,75	0.00399
45444	fat cell differentiation	106	2	0,1	0.00400
90596	sensory organ morphogenesis	106	2	0,1	0.00400
10565	regulation of cellular ketone metabolic ...	115	2	0,11	0.00470
51093	negative regulation of developmental pro...	404	3	0,38	0.00481
45595	regulation of cell differentiation	860	4	0,8	0.00497
51649	establishment of localization in cell	1471	5	1,37	0.00512
51049	regulation of transport	881	4	0,82	0.00543
48562	embryonic organ morphogenesis	126	2	0,12	0.00561
33993	response to lipid	443	3	0,41	0.00623
6869	lipid transport	145	2	0,13	0.00737
42180	cellular ketone metabolic process	152	2	0,14	0.00808
30258	lipid modification	153	2	0,14	0.00818
10876	lipid localization	160	2	0,15	0.00892
9891	positive regulation of biosynthetic proc...	1037	4	0,96	0.00980
9889	regulation of biosynthetic process	2523	6	2,34	0.00991
10243	response to organonitrogen compound	524	3	0,49	0.00996

Go Terms (Molecular Functions) for Upregulated Genes Nonresistant DMSO vs. Mitotane

GO.ID	Term	Annotated	Significant	Expected	classicFisher
5342	organic acid transmembrane transporter a...	52	4	0,27	0.00014
46943	carboxylic acid transmembrane transporte...	52	4	0,27	0.00014
15179	L-amino acid transmembrane transporter a...	25	3	0,13	0.00028
19842	vitamin binding	71	4	0,37	0.00048
8514	organic anion transmembrane transporter ...	74	4	0,38	0.00056
15291	secondary active transmembrane transport...	79	4	0,41	0.00072
8483	transaminase activity	10	2	0,05	0.00115
16769	transferase activity, transferring nitro...	11	2	0,06	0.00141
16646	oxidoreductase activity, acting on the C...	13	2	0,07	0.00198
140101	catalytic activity, acting on a tRNA	104	4	0,54	0.00199
16645	oxidoreductase activity, acting on the C...	14	2	0,07	0.00230
1047	core promoter binding	112	4	0,58	0.00261

GO.ID	Term	Annotated	Significant	Expected	classicFisher
8509	anion transmembrane transporter activity	113	4	0,59	0.00270
16829	lyase activity	114	4	0,59	0.00278
16874	ligase activity	130	4	0,67	0.00446
42803	protein homodimerization activity	437	7	2,27	0.00676
46983	protein dimerization activity	675	9	3,5	0.00692
99516	ion antiporter activity	25	2	0,13	0.00732
22804	active transmembrane transporter activit...	150	4	0,78	0.00739
42802	identical protein binding	946	11	4,91	0.00761
16597	amino acid binding	27	2	0,14	0.00851
44212	transcription regulatory region DNA bind...	467	7	2,42	0.00960
975	regulatory region DNA binding	468	7	2,43	0.00971
1067	regulatory region nucleic acid binding	469	7	2,43	0.00982

Go Terms (Molecular Functions) for Downregulatedregulted Genes Nonresistant DMSO vs. Mitotane

GO.ID	Term	Annotated	Significant	Expected	classicFisher
5506	iron ion binding	67	2	0,06	0.00160
16491	oxidoreductase activity	425	3	0,39	0.00544

Go Terms (Biological Processes) for Upregulated Genes Nonresistant vs Resistant DMSO

GO.ID	Term	Annotated	Significant	Expected	classicFisher	GO.ID	Term	Annotated	Significant	Expected	classicFisher
43068	positive regulation of programmed cell d...	361	50	29,21	0.00010	7045	cell-substrate adherens junction assembl...	47	10	3,8	0.00374
45778	positive regulation of ossification	43	12	3,48	0.00011	46425	regulation of JAK-STAT cascade	47	10	3,8	0.00374
30048	actin filament-based movement	56	14	4,53	0.00011	48041	focal adhesion assembly	47	10	3,8	0.00374
48608	reproductive structure development	215	34	17,4	0.00011	48660	regulation of smooth muscle cell prolife...	47	10	3,8	0.00374
2000027	regulation of organ morphogenesis	147	26	11,9	0.00011	1904892	regulation of STAT cascade	47	10	3,8	0.00374
	cardiac ventricle development	63	15	5,1	0.00011	9893	positive regulation of metabolic process	1891	182	153,03	0.00375
10976	positive regulation of neuron projection...	139	25	11,25	0.00011	60828	regulation of canonical Wnt signaling pa...	157	23	12,71	0.00375
2521	leukocyte differentiation	198	32	16,02	0.00011	65009	regulation of molecular function	1718	167	139,03	0.00381
51271	negative regulation of cellular componen...	131	24	10,6	0.00011	32970	regulation of actin filament-based proce...	185	26	14,97	0.00382
7405	neuroblast proliferation	26	9	2,1	0.00013	43269	regulation of ion transport	223	30	18,05	0.00384
55117	regulation of cardiac muscle contraction	26	9	2,1	0.00013	1710	mesodermal cell fate commitment	9	4	0,73	0.00385
60412	ventricular septum morphogenesis	26	9	2,1	0.00013	33151	V(D)J recombination	9	4	0,73	0.00385
7369	gastrulation	108	21	8,74	0.00013	60253	negative regulation of glial cell prolif...	9	4	0,73	0.00385
61138	morphogenesis of a branching epithelium	78	17	6,31	0.00013	60441	epithelial tube branching involved in lu...	9	4	0,73	0.00385
8038	neuron recognition	16	7	1,29	0.00013	1900103	positive regulation of endoplasmic retic...	9	4	0,73	0.00385
86004	regulation of cardiac muscle cell contra...	16	7	1,29	0.00013	1905331	negative regulation of morphogenesis of ...	9	4	0,73	0.00385
1903115	regulation of actin filament-based movem...	16	7	1,29	0.00013	19438	aromatic compound biosynthetic process	2515	235	203,52	0.00387
51172	negative regulation of nitrogen compound...	1423	151	115,16	0.00013	90066	regulation of anatomical structure size	233	31	18,86	0.00393
21987	cerebral cortex development	71	16	5,75	0.00013	2011	morphogenesis of an epithelial sheet	33	8	2,67	0.00397
14066	regulation of phosphatidylinositol 3-kin...	44	12	3,56	0.00013	43266	regulation of potassium ion transport	33	8	2,67	0.00397
42471	ear morphogenesis	44	12	3,56	0.00013	43627	response to estrogen	33	8	2,67	0.00397
51049	regulation of transport	881	101	71,29	0.00013	768	syncytium formation by plasma membrane f...	20	6	1,62	0.00397
31103	axon regeneration	21	8	1,7	0.00014	46676	negative regulation of insulin secretion	20	6	1,62	0.00397
31670	cellular response to nutrient	21	8	1,7	0.00014	90278	negative regulation of peptide hormone s...	20	6	1,62	0.00397
21543	pallium development	101	20	8,17	0.00014	1903053	regulation of extracellular matrix organ...	20	6	1,62	0.00397
40013	negative regulation of locomotion	141	25	11,41	0.00014	36293	response to decreased oxygen levels	214	29	17,32	0.00399
61458	reproductive system development	218	34	17,64	0.00014	7044	cell-substrate junction assembly	55	11	4,45	0.00400
8217	regulation of blood pressure	51	13	4,13	0.00015	30203	glycosaminoglycan metabolic process	79	14	6,39	0.00403
15850	organic hydroxy compound transport	79	17	6,39	0.00015	30260	entry into host cell	71	13	5,75	0.00411
10810	regulation of cell-substrate adhesion	94	19	7,61	0.00015	44409	entry into host	71	13	5,75	0.00411
3177	pulmonary valve development	8	5	0,65	0.00016	51806	entry into cell of other organism involv...	71	13	5,75	0.00411
3184	pulmonary valve morphogenesis	8	5	0,65	0.00016	51828	entry into other organism involved in sy...	71	13	5,75	0.00411
51953	negative regulation of amine transport	8	5	0,65	0.00016	43549	regulation of kinase activity	487	56	39,41	0.00421
14065	phosphatidylinositol 3-kinase signaling	58	14	4,69	0.00016	2573	myeloid leukocyte differentiation	88	15	7,12	0.00435
6936	muscle contraction	134	24	10,84	0.00016	10629	negative regulation of gene expression	1142	116	92,42	0.00436
1902105	regulation of leukocyte differentiation	110	21	8,9	0.00017	51153	regulation of striated muscle cell diffe...	48	10	3,88	0.00439
16049	cell growth	283	41	22,9	0.00017	34654	nucleobase-containing compound biosynthe...	2474	231	200,21	0.00444
60512	prostate gland morphogenesis	12	6	0,97	0.00017	45637	regulation of myeloid cell differentiati...	123	19	9,95	0.00450
60740	prostate gland epithelium morphogenesis	12	6	0,97	0.00017	44093	positive regulation of molecular functio...	1010	104	81,73	0.00464
85029	extracellular matrix assembly	12	6	0,97	0.00017	3337	mesenchymal to epithelial transition inv...	5	3	0,4	0.00466
2001224	positive regulation of neuron migration	12	6	0,97	0.00017	7442	hindgut morphogenesis	5	3	0,4	0.00466
30098	lymphocyte differentiation	126	23	10,2	0.00017	16198	axon choice point recognition	5	3	0,4	0.00466
9612	response to mechanical stimulus	95	19	7,69	0.00018	30638	polyketide metabolic process	5	3	0,4	0.00466
3205	cardiac chamber development	80	17	6,47	0.00018	30647	aminoglycoside antibiotic metabolic proc...	5	3	0,4	0.00466
42060	wound healing	248	37	20,07	0.00019	33689	negative regulation of osteoblast prolif...	5	3	0,4	0.00466
72073	kidney epithelium development	59	14	4,77	0.00019	35413	positive regulation of catenin import in...	5	3	0,4	0.00466
3150	muscular septum morphogenesis	5	4	0,4	0.00020	38092	nodal signaling pathway	5	3	0,4	0.00466
21520	spinal cord motor neuron cell fate speci...	5	4	0,4	0.00020	44597	daunorubicin metabolic process	5	3	0,4	0.00466
45741	positive regulation of epidermal growth ...	5	4	0,4	0.00020	44598	doxorubicin metabolic process	5	3	0,4	0.00466
71880	adenylate cyclase-activating adrenergic ...	5	4	0,4	0.00020	45907	positive regulation of vasoconstriction	5	3	0,4	0.00466
45661	regulation of myoblast differentiation	22	8	1,78	0.00020	48243	norepinephrine secretion	5	3	0,4	0.00466
6351	transcription, DNA-templated	2174	216	175,93	0.00021	48617	embryonic foregut morphogenesis	5	3	0,4	0.00466
45620	negative regulation of lymphocyte differ...	17	7	1,38	0.00021	60601	lateral sprouting from an epithelium	5	3	0,4	0.00466
1903524	positive regulation of blood circulation	17	7	1,38	0.00021	61525	hindgut development	5	3	0,4	0.00466
61448	connective tissue development	112	21	9,06	0.00022	72283	metanephric renal vesicle morphogenesis	5	3	0,4	0.00466
22407	regulation of cell-cell adhesion	162	27	13,11	0.00023	9948	anterior/posterior axis specification	27	7	2,18	0.00466
60070	canonical Wnt signaling pathway	188	30	15,21	0.00023	10830	regulation of myotube differentiation	27	7	2,18	0.00466
45682	regulation of epidermis development	40	11	3,24	0.00024	97530	granulocyte migration	27	7	2,18	0.00466
45765	regulation of angiogenesis	97	19	7,85	0.00024	2000273	positive regulation of receptor activity	27	7	2,18	0.00466
71496	cellular response to external stimulus	171	28	13,84	0.00024	51384	response to glucocorticoid	64	12	5,18	0.00470

90287	regulation of cellular response to growt...	129	23	10,44	0.00024
60537	muscle tissue development	181	29	14,65	0.00027
48754	branching morphogenesis of an epithelial...	68	15	5,5	0.00028
30111	regulation of Wnt signaling pathway	199	31	16,1	0.00028
35909	aorta morphogenesis	13	6	1,05	0.00029
42249	establishment of planar polarity of embr...	13	6	1,05	0.00029
50432	catecholamine secretion	13	6	1,05	0.00029
1932	regulation of protein phosphorylation	709	83	57,38	0.00029
14706	striated muscle tissue development	173	28	14	0.00029
97659	nucleic acid-templated transcription	2187	216	176,98	0.00030
7188	adenylate cyclase-modulating G-protein c...	41	11	3,32	0.00030
42733	embryonic digit morphogenesis	41	11	3,32	0.00030
10001	glial cell differentiation	91	18	7,36	0.00030
16358	dendrite development	123	22	9,95	0.00031
7160	cell-matrix adhesion	99	19	8,01	0.00031
51966	regulation of synaptic transmission, glu...	18	7	1,46	0.00032
31326	regulation of cellular biosynthetic proc...	2481	241	200,77	0.00032
3151	outflow tract morphogenesis	35	10	2,83	0.00032
60976	coronary vasculature development	29	9	2,35	0.00032
36445	neuronal stem cell division	9	5	0,73	0.00033
51968	positive regulation of synaptic transmis...	9	5	0,73	0.00033
55057	neuroblast division	9	5	0,73	0.00033
90103	cochlea morphogenesis	9	5	0,73	0.00033
1901201	regulation of extracellular matrix assem...	9	5	0,73	0.00033
1704	formation of primary germ layer	69	15	5,58	0.00033
42307	positive regulation of protein import in...	48	12	3,88	0.00033
44087	regulation of cellular component biogene...	527	65	42,65	0.00033
1763	morphogenesis of a branching structure	84	17	6,8	0.00033
90101	negative regulation of transmembrane rec...	62	14	5,02	0.00034
60048	cardiac muscle contraction	55	13	4,45	0.00034
32774	RNA biosynthetic process	2194	216	177,55	0.00036
31346	positive regulation of cell projection o...	193	30	15,62	0.00037
10562	positive regulation of phosphorus metabo...	519	64	42	0.00037
45937	positive regulation of phosphate metabol...	519	64	42	0.00037
48534	hematopoietic or lymphoid organ developm...	439	56	35,53	0.00037
1902533	positive regulation of intracellular sig...	469	59	37,95	0.00038
48640	negative regulation of developmental gro...	42	11	3,4	0.00038
45936	negative regulation of phosphate metabol...	332	45	26,87	0.00038
50900	leukocyte migration	133	23	10,76	0.00038
6793	phosphorus metabolic process	1885	189	152,54	0.00038
42326	negative regulation of phosphorylation	276	39	22,34	0.00040
60395	SMAD protein signal transduction	24	8	1,94	0.00040
10563	negative regulation of phosphorus metabo...	333	45	26,95	0.00040
1904591	positive regulation of protein import	49	12	3,97	0.00040
21761	limbic system development	56	13	4,53	0.00041
44236	multicellular organism metabolic process	56	13	4,53	0.00041
98742	cell-cell adhesion via plasma-membrane a...	56	13	4,53	0.00041
51171	regulation of nitrogen compound metaboli...	3265	306	264,22	0.00042
6942	regulation of striated muscle contractio...	30	9	2,43	0.00043
10812	negative regulation of cell-substrate ad...	30	9	2,43	0.00043
1901699	cellular response to nitrogen compound	363	48	29,38	0.00043
31345	negative regulation of cell projection o...	86	17	6,96	0.00044
6941	striated muscle contraction	71	15	5,75	0.00045
90288	negative regulation of cellular response...	71	15	5,75	0.00045
60317	cardiac epithelial to mesenchymal transi...	19	7	1,54	0.00047
3382	epithelial cell morphogenesis	14	6	1,13	0.00047
60561	apoptotic process involved in morphogene...	14	6	1,13	0.00047
61036	positive regulation of cartilage develop...	14	6	1,13	0.00047
48638	regulation of developmental growth	152	25	12,3	0.00047
23056	positive regulation of signaling	837	94	67,73	0.00049
6366	transcription from RNA polymerase II pro...	1306	137	105,69	0.00049
30099	myeloid cell differentiation	207	28	16,75	0.00474
33674	positive regulation of kinase activity	305	38	24,68	0.00475
10171	body morphogenesis	34	8	2,75	0.00483
14855	striated muscle cell proliferation	34	8	2,75	0.00483
51148	negative regulation of muscle cell diffe...	34	8	2,75	0.00483
60415	muscle tissue morphogenesis	34	8	2,75	0.00483
10556	regulation of macromolecule biosynthetic...	2407	225	194,78	0.00484
32386	regulation of intracellular transport	366	44	29,62	0.00484
10959	regulation of metal ion transport	142	21	11,49	0.00490
45860	positive regulation of protein kinase ac...	286	36	23,14	0.00500
6022	aminoglycan metabolic process	81	14	6,55	0.00508
30324	lung development	81	14	6,55	0.00508
50796	regulation of insulin secretion	81	14	6,55	0.00508
2761	regulation of myeloid leukocyte differen...	49	10	3,97	0.00512
6638	neutral lipid metabolic process	49	10	3,97	0.00512
48659	smooth muscle cell proliferation	49	10	3,97	0.00512
1901888	regulation of cell junction assembly	49	10	3,97	0.00512
71345	cellular response to cytokine stimulus	429	50	34,72	0.00514
7520	myoblast fusion	15	5	1,21	0.00518
21801	cerebral cortex radial glia guided migra...	15	5	1,21	0.00518
22030	telencephalon glial cell migration	15	5	1,21	0.00518
33687	osteoblast proliferation	15	5	1,21	0.00518
42310	vasoconstriction	15	5	1,21	0.00518
43268	positive regulation of potassium ion tra...	15	5	1,21	0.00518
45601	regulation of endothelial cell different...	15	5	1,21	0.00518
45920	negative regulation of exocytosis	15	5	1,21	0.00518
46189	phenol-containing compound biosynthetic ...	15	5	1,21	0.00518
50873	brown fat cell differentiation	15	5	1,21	0.00518
51150	regulation of smooth muscle cell differe...	15	5	1,21	0.00518
86009	membrane repolarization	15	5	1,21	0.00518
2762	negative regulation of myeloid leukocyte...	21	6	1,7	0.00519
6949	syncytium formation	21	6	1,7	0.00519
19748	secondary metabolic process	21	6	1,7	0.00519
60425	lung morphogenesis	21	6	1,7	0.00519
98900	regulation of action potential	21	6	1,7	0.00519
32409	regulation of transporter activity	107	17	8,66	0.00525
34644	cellular response to UV	57	11	4,61	0.00532
46824	positive regulation of nucleocytoplasmic...	65	12	5,26	0.00535
22409	positive regulation of cell-cell adhesio...	90	15	7,28	0.00540
30217	T cell differentiation	90	15	7,28	0.00540
43542	endothelial cell migration	90	15	7,28	0.00540
30001	metal ion transport	338	41	27,35	0.00550
30512	negative regulation of transforming grow...	42	9	3,4	0.00556
10770	positive regulation of cell morphogenesi...	82	14	6,64	0.00569
48588	developmental cell growth	108	17	8,74	0.00577
6027	glycosaminoglycan catabolic process	28	7	2,27	0.00579
22029	telencephalon cell migration	28	7	2,27	0.00579
71695	anatomical structure maturation	74	13	5,99	0.00592
61041	regulation of wound healing	50	10	4,05	0.00595
7413	axonal fasciculation	10	4	0,81	0.00601
19229	regulation of vasoconstriction	10	4	0,81	0.00601
32793	positive regulation of CREB transcriptio...	10	4	0,81	0.00601
48670	regulation of collateral sprouting	10	4	0,81	0.00601
48730	epidermis morphogenesis	10	4	0,81	0.00601
50433	regulation of catecholamine secretion	10	4	0,81	0.00601
60231	mesenchymal to epithelial transition	10	4	0,81	0.00601
70884	regulation of calcineurin-NFAT signaling...	10	4	0,81	0.00601
86005	ventricular cardiac muscle cell action p...	10	4	0,81	0.00601
106030	neuron projection fasciculation	10	4	0,81	0.00601
106056	regulation of calcineurin-mediated signa...	10	4	0,81	0.00601
2000479	regulation of cAMP-dependent protein kin...	10	4	0,81	0.00601

44259	multicellular organismal macromolecule m...	50	12	4,05	0.00049
72006	nephron development	57	13	4,61	0.00050
6796	phosphate-containing compound metabolic ...	1826	183	147,77	0.00051
60541	respiratory system development	95	18	7,69	0.00052
1934	positive regulation of protein phosphory...	455	57	36,82	0.00052
48644	muscle organ morphogenesis	37	10	2,99	0.00053
2064	epithelial cell development	103	19	8,34	0.00053
8544	epidermis development	136	23	11,01	0.00053
14807	regulation of somitogenesis	3	3	0,24	0.00053
71798	response to prostaglandin D	3	3	0,24	0.00053
71799	cellular response to prostaglandin D sti...	3	3	0,24	0.00053
1990834	response to odorant	3	3	0,24	0.00053
1900182	positive regulation of protein localizat...	72	15	5,83	0.00053
48844	artery morphogenesis	25	8	2,02	0.00055
71396	cellular response to lipid	309	42	25,01	0.00055
34330	cell junction organization	145	24	11,73	0.00056
3188	heart valve formation	6	4	0,49	0.00056
7440	foregut morphogenesis	6	4	0,49	0.00056
15874	norepinephrine transport	6	4	0,49	0.00056
30205	dermatan sulfate metabolic process	6	4	0,49	0.00056
30208	dermatan sulfate biosynthetic process	6	4	0,49	0.00056
48665	neuron fate specification	6	4	0,49	0.00056
60363	cranial suture morphogenesis	6	4	0,49	0.00056
42472	inner ear morphogenesis	31	9	2,51	0.00056
50773	regulation of dendrite development	80	16	6,47	0.00057
51216	cartilage development	80	16	6,47	0.00057
30097	hemopoiesis	417	53	33,75	0.00058
10647	positive regulation of cell communicatio...	831	93	67,25	0.00059
48593	camera-type eye morphogenesis	51	12	4,13	0.00060
21819	layer formation in cerebral cortex	10	5	0,81	0.00061
60413	atrial septum morphogenesis	10	5	0,81	0.00061
71875	adrenergic receptor signaling pathway	10	5	0,81	0.00061
90179	planar cell polarity pathway involved in...	10	5	0,81	0.00061
97094	craniofacial suture morphogenesis	10	5	0,81	0.00061
90130	tissue migration	129	22	10,44	0.00061
12501	programmed cell death	1114	119	90,15	0.00062
48015	phosphatidylinositol-mediated signaling	73	15	5,91	0.00062
1933	negative regulation of protein phosphory...	254	36	20,55	0.00062
42692	muscle cell differentiation	190	29	15,38	0.00062
31669	cellular response to nutrient levels	121	21	9,79	0.00064
48584	positive regulation of response to stimu...	1039	112	84,08	0.00066
60993	kidney morphogenesis	38	10	3,08	0.00066
1903531	negative regulation of secretion by cell	66	14	5,34	0.00067
32924	activin receptor signaling pathway	20	7	1,62	0.00067
1990266	neutrophil migration	20	7	1,62	0.00067
42306	regulation of protein import into nucleu...	105	19	8,5	0.00067
9889	regulation of biosynthetic process	2523	242	204,17	0.00069
19220	regulation of phosphate metabolic proces...	889	98	71,94	0.00070
6915	apoptotic process	1085	116	87,8	0.00071
55017	cardiac muscle tissue growth	45	11	3,64	0.00071
48017	inositol lipid-mediated signaling	74	15	5,99	0.00072
2376	immune system process	1329	138	107,55	0.00072
3401	axis elongation	15	6	1,21	0.00073
30857	negative regulation of epithelial cell d...	15	6	1,21	0.00073
48339	paraxial mesoderm development	15	6	1,21	0.00073
61098	positive regulation of protein tyrosine ...	15	6	1,21	0.00073
1902895	positive regulation of pri-miRNA transcr...	15	6	1,21	0.00073
42110	T cell activation	183	28	14,81	0.00074
21872	forebrain generation of neurons	26	8	2,1	0.00074
46888	negative regulation of hormone secretion	26	8	2,1	0.00074
45444	fat cell differentiation	106	19	8,58	0.00076
51174	regulation of phosphorus metabolic proce...	902	99	72,99	0.00076
2682	regulation of immune system process	647	75	52,36	0.00077
1901654	response to ketone	98	18	7,93	0.00077
45785	positive regulation of cell adhesion	157	25	12,71	0.00078
8219	cell death	1187	125	96,06	0.00078
1901362	organic cyclic compound biosynthetic pro...	2608	241	211,05	0.00605
34097	response to cytokine	485	55	39,25	0.00606
6606	protein import into nucleus	182	25	14,73	0.00606
50731	positive regulation of peptidyl-tyrosine...	58	11	4,69	0.00610
31667	response to nutrient levels	240	31	19,42	0.00610
1657	ureteric bud development	43	9	3,48	0.00654
3014	renal system process	43	9	3,48	0.00654
30799	regulation of cyclic nucleotide metaboli...	43	9	3,48	0.00654
3253	cardiac neural crest cell migration invo...	2	2	0,16	0.00654
3383	apical constriction	2	2	0,16	0.00654
6642	triglyceride mobilization	2	2	0,16	0.00654
7418	ventral midline development	2	2	0,16	0.00654
9753	response to jasmonic acid	2	2	0,16	0.00654
10643	cell communication by chemical coupling	2	2	0,16	0.00654
10868	negative regulation of triglyceride bios...	2	2	0,16	0.00654
14060	regulation of epinephrine secretion	2	2	0,16	0.00654
14900	muscle hyperplasia	2	2	0,16	0.00654
21936	regulation of cerebellar granule cell pr...	2	2	0,16	0.00654
21937	cerebellar Purkinje cell-granule cell pr...	2	2	0,16	0.00654
21938	smoothened signaling pathway involved in...	2	2	0,16	0.00654
21940	positive regulation of cerebellar granul...	2	2	0,16	0.00654
31444	slow-twitch skeletal muscle fiber contra...	2	2	0,16	0.00654
31652	positive regulation of heat generation	2	2	0,16	0.00654
32764	negative regulation of mast cell cytokin...	2	2	0,16	0.00654
32811	negative regulation of epinephrine secre...	2	2	0,16	0.00654
33634	positive regulation of cell-cell adhesio...	2	2	0,16	0.00654
35425	autocrine signaling	2	2	0,16	0.00654
38026	reelin-mediated signaling pathway	2	2	0,16	0.00654
45162	clustering of voltage-gated sodium chann...	2	2	0,16	0.00654
45578	negative regulation of B cell differenti...	2	2	0,16	0.00654
48241	epinephrine transport	2	2	0,16	0.00654
48242	epinephrine secretion	2	2	0,16	0.00654
60994	regulation of transcription from RNA pol...	2	2	0,16	0.00654
71386	cellular response to corticosterone stim...	2	2	0,16	0.00654
71395	cellular response to jasmonic acid stimu...	2	2	0,16	0.00654
71492	cellular response to UV-A	2	2	0,16	0.00654
72007	mesangial cell differentiation	2	2	0,16	0.00654
72008	glomerular mesangial cell differentiatio...	2	2	0,16	0.00654
72143	mesangial cell development	2	2	0,16	0.00654
72144	glomerular mesangial cell development	2	2	0,16	0.00654
90209	negative regulation of triglyceride meta...	2	2	0,16	0.00654
97114	NMDA glutamate receptor clustering	2	2	0,16	0.00654
98957	anterograde axonal transport of mitochon...	2	2	0,16	0.00654
1900747	negative regulation of vascular endothel...	2	2	0,16	0.00654
1901078	negative regulation of relaxation of mus...	2	2	0,16	0.00654
1901898	negative regulation of relaxation of car...	2	2	0,16	0.00654
1902548	negative regulation of cellular response...	2	2	0,16	0.00654
1902951	negative regulation of dendritic spine m...	2	2	0,16	0.00654
1902957	negative regulation of mitochondrial ele...	2	2	0,16	0.00654
1903225	negative regulation of endodermal cell d...	2	2	0,16	0.00654
1904238	pericyte cell differentiation	2	2	0,16	0.00654
1904761	negative regulation of myofibroblast dif...	2	2	0,16	0.00654
1905064	negative regulation of vascular smooth m...	2	2	0,16	0.00654
1905447	negative regulation of mitochondrial ATP...	2	2	0,16	0.00654
1905664	regulation of calcium ion import across ...	2	2	0,16	0.00654
1990164	histone H2A phosphorylation	2	2	0,16	0.00654
2000969	positive regulation of AMPA receptor act...	2	2	0,16	0.00654
18130	heterocycle biosynthetic process	2517	233	203,69	0.00659
6816	calcium ion transport	155	22	12,54	0.00662
31111	negative regulation of microtubule polym...	22	6	1,78	0.00666
48747	muscle fiber development	22	6	1,78	0.00666
61045	negative regulation of wound healing	22	6	1,78	0.00666
51149	positive regulation of muscle cell diffe...	51	10	4,13	0.00688
1901698	response to nitrogen compound	595	65	48,15	0.00698
1903426	regulation of reactive oxygen species bi...	36	8	2,91	0.00698
1904888	cranial skeletal system development	36	8	2,91	0.00698

1900180	regulation of protein localization to nu...	140	23	11,33	0.00080
8589	regulation of smoothened signaling pathw...	39	10	3,16	0.00083
45445	myoblast differentiation	39	10	3,16	0.00083
46718	viral entry into host cell	60	13	4,86	0.00083
30178	negative regulation of Wnt signaling pat...	132	22	10,68	0.00085
1902531	regulation of intracellular signal trans...	981	106	79,39	0.00086
1904589	regulation of protein import	107	19	8,66	0.00086
1935	endothelial cell proliferation	53	12	4,29	0.00087
48144	fibroblast proliferation	53	12	4,29	0.00087
48145	regulation of fibroblast proliferation	53	12	4,29	0.00087
50807	regulation of synapse organization	53	12	4,29	0.00087
35296	regulation of tube diameter	33	9	2,67	0.00093
45669	positive regulation of osteoblast differ...	33	9	2,67	0.00093
97746	regulation of blood vessel diameter	33	9	2,67	0.00093
90102	cochlea development	21	7	1,7	0.00094
1902893	regulation of pri-miRNA transcription fr...	21	7	1,7	0.00094
9725	response to hormone	476	58	38,52	0.00094
31668	cellular response to extracellular stimu...	133	22	10,76	0.00094
2520	immune system development	466	57	37,71	0.00094
34332	adherens junction organization	76	15	6,15	0.00096
6939	smooth muscle contraction	27	8	2,18	0.00098
21532	neural tube patterning	27	8	2,18	0.00098
51051	negative regulation of transport	223	32	18,05	0.00099
71417	cellular response to organonitrogen comp...	299	40	24,2	0.00102
7416	synapse assembly	54	12	4,37	0.00103
42493	response to drug	196	29	15,86	0.00104
3283	atrial septum development	11	5	0,89	0.00105
45662	negative regulation of myoblast differen...	11	5	0,89	0.00105
90177	establishment of planar polarity involve...	11	5	0,89	0.00105
90178	regulation of establishment of planar po...	11	5	0,89	0.00105
21766	hippocampus development	47	11	3,8	0.00105
32963	collagen metabolic process	47	11	3,8	0.00105
31960	response to corticosteroid	69	14	5,58	0.00106
3209	cardiac atrium morphogenesis	16	6	1,29	0.00109
30593	neutrophil chemotaxis	16	6	1,29	0.00109
35137	hindlimb morphogenesis	16	6	1,29	0.00109
35412	regulation of catenin import into nucleu...	16	6	1,29	0.00109
51937	catecholamine transport	16	6	1,29	0.00109
1902742	apoptotic process involved in developmen...	16	6	1,29	0.00109
2000826	regulation of heart morphogenesis	16	6	1,29	0.00109
71407	cellular response to organic cyclic comp...	339	44	27,43	0.00109
45665	negative regulation of neuron differenti...	101	18	8,17	0.00111
1903844	regulation of cellular response to trans...	62	13	5,02	0.00115
32102	negative regulation of response to exter...	110	19	8,9	0.00121
42327	positive regulation of phosphorylation	481	58	38,92	0.00121
31323	regulation of cellular metabolic process	3456	318	279,67	0.00121
16339	calcium-dependent cell-cell adhesion via...	7	4	0,57	0.00122
45932	negative regulation of muscle contractio...	7	4	0,57	0.00122
70986	left/right axis specification	7	4	0,57	0.00122
34333	adherens junction assembly	55	12	4,45	0.00123
30902	hindbrain development	70	14	5,66	0.00123
51048	negative regulation of secretion	70	14	5,66	0.00123
1101	response to acid chemical	171	26	13,84	0.00124
45833	negative regulation of lipid metabolic p...	41	10	3,32	0.00126
60419	heart growth	48	11	3,88	0.00127
42325	regulation of phosphorylation	797	88	64,5	0.00127
30574	collagen catabolic process	28	8	2,27	0.00128
35249	synaptic transmission, glutamatergic	28	8	2,27	0.00128
48645	animal organ formation	28	8	2,27	0.00128
60350	endochondral bone morphogenesis	28	8	2,27	0.00128
86001	cardiac muscle cell action potential	28	8	2,27	0.00128
31099	regeneration	94	17	7,61	0.00128
7193	adenylate cyclase-inhibiting G-protein c...	22	7	1,78	0.00128
10464	regulation of mesenchymal cell prolifera...	22	7	1,78	0.00128
21700	developmental maturation	119	18	9,63	0.00699
45879	negative regulation of smoothened signal...	16	5	1,29	0.00703
2262	myeloid cell homeostasis	84	14	6,8	0.00707
30323	respiratory tube development	84	14	6,8	0.00707
7156	homophilic cell adhesion via plasma memb...	29	7	2,35	0.00711
21885	forebrain cell migration	29	7	2,35	0.00711
35329	hippo signaling	29	7	2,35	0.00711
9991	response to extracellular stimulus	253	32	20,47	0.00734
7159	leukocyte cell-cell adhesion	138	20	11,17	0.00746
2683	negative regulation of immune system pro...	185	25	14,97	0.00746
43588	skin development	120	18	9,71	0.00762
33273	response to vitamin	44	9	3,56	0.00764
72163	mesonephric epithelium development	44	9	3,56	0.00764
72164	mesonephric tubule development	44	9	3,56	0.00764
45185	maintenance of protein location	68	12	5,5	0.00774
45859	regulation of protein kinase activity	449	51	36,33	0.00782
34762	regulation of transmembrane transport	167	23	13,51	0.00802
10468	regulation of gene expression	2599	239	210,32	0.00811
30814	regulation of cAMP metabolic process	37	8	2,99	0.00829
1818	negative regulation of cytokine producti...	112	17	9,06	0.00832
43123	positive regulation of I-kappaB kinase/N...	112	17	9,06	0.00832
32069	regulation of nuclease activity	23	6	1,86	0.00840
71621	granulocyte chemotaxis	23	6	1,86	0.00840
86065	cell communication involved in cardiac c...	23	6	1,86	0.00840
1905207	regulation of cardiocyte differentiation	23	6	1,86	0.00840
48545	response to steroid hormone	216	28	17,48	0.00847
10604	positive regulation of macromolecule met...	1776	169	143,72	0.00856
1901214	regulation of neuron death	168	23	13,6	0.00861
1706	endoderm formation	30	7	2,43	0.00864
6026	aminoglycan catabolic process	30	7	2,43	0.00864
72091	regulation of stem cell proliferation	30	7	2,43	0.00864
2000377	regulation of reactive oxygen species me...	86	14	6,96	0.00870
1840	neural plate development	6	3	0,49	0.00876
3413	chondrocyte differentiation involved in ...	6	3	0,49	0.00876
7016	cytoskeletal anchoring at plasma membran...	6	3	0,49	0.00876
21513	spinal cord dorsal/ventral patterning	6	3	0,49	0.00876
21910	smoothened signaling pathway involved in...	6	3	0,49	0.00876
32926	negative regulation of activin receptor ...	6	3	0,49	0.00876
33630	positive regulation of cell adhesion med...	6	3	0,49	0.00876
35358	regulation of peroxisome proliferator ac...	6	3	0,49	0.00876
42416	dopamine biosynthetic process	6	3	0,49	0.00876
43497	regulation of protein heterodimerization...	6	3	0,49	0.00876
48368	lateral mesoderm development	6	3	0,49	0.00876
48671	negative regulation of collateral sprout...	6	3	0,49	0.00876
60028	convergent extension involved in axis el...	6	3	0,49	0.00876
60442	branching involved in prostate gland mor...	6	3	0,49	0.00876
60525	prostate glandular acinus development	6	3	0,49	0.00876
60713	labyrinthine layer morphogenesis	6	3	0,49	0.00876
71679	commissural neuron axon guidance	6	3	0,49	0.00876
72015	glomerular visceral epithelial cell deve...	6	3	0,49	0.00876
72109	glomerular mesangium development	6	3	0,49	0.00876
72310	glomerular epithelial cell development	6	3	0,49	0.00876
72578	neurotransmitter-gated ion channel clust...	6	3	0,49	0.00876
98722	asymmetric stem cell division	6	3	0,49	0.00876
1900746	regulation of vascular endothelial growt...	6	3	0,49	0.00876
1902547	regulation of cellular response to vascu...	6	3	0,49	0.00876
1903896	positive regulation of IRE1-mediated unf...	6	3	0,49	0.00876
2000271	positive regulation of fibroblast apopto...	6	3	0,49	0.00876
10837	regulation of keratinocyte proliferation	11	4	0,89	0.00885
21846	cell proliferation in forebrain	11	4	0,89	0.00885
35357	peroxisome proliferator activated recept...	11	4	0,89	0.00885
45671	negative regulation of osteoclast differ...	11	4	0,89	0.00885
48333	mesodermal cell differentiation	11	4	0,89	0.00885
50819	negative regulation of coagulation	11	4	0,89	0.00885

35924	cellular response to vascular endothelia...	22	7	1,78	0.00128
60563	neuroepithelial cell differentiation	22	7	1,78	0.00128
61614	pri-miRNA transcription from RNA polymer...	22	7	1,78	0.00128
80090	regulation of primary metabolic process	3362	310	272,07	0.00129
30036	actin cytoskeleton organization	332	43	26,87	0.00130
9892	negative regulation of metabolic process	1711	170	138,46	0.00130
51050	positive regulation of transport	473	57	38,28	0.00135
10631	epithelial cell migration	128	21	10,36	0.00135
90132	epithelium migration	128	21	10,36	0.00135
9967	positive regulation of signal transducti...	767	85	62,07	0.00138
10469	regulation of receptor activity	146	23	11,81	0.00144
1952	regulation of cell-matrix adhesion	56	12	4,53	0.00145
30856	regulation of epithelial cell differenti...	56	12	4,53	0.00145
31214	biomineral tissue development	56	12	4,53	0.00145
50803	regulation of synapse structure or activ...	56	12	4,53	0.00145
35265	organ growth	79	15	6,39	0.00146
45944	positive regulation of transcription fro...	610	70	49,36	0.00151
46058	cAMP metabolic process	42	10	3,4	0.00153
3230	cardiac atrium development	17	6	1,38	0.00156
33173	calcineurin-NFAT signaling cascade	17	6	1,38	0.00156
97756	negative regulation of blood vessel diam...	17	6	1,38	0.00156
35637	multicellular organismal signaling	64	13	5,18	0.00157
60038	cardiac muscle cell proliferation	29	8	2,35	0.00164
43408	regulation of MAPK cascade	336	43	27,19	0.00164
43433	negative regulation of DNA binding trans...	80	15	6,47	0.00166
60972	left/right pattern formation	12	5	0,97	0.00167
1905276	regulation of epithelial tube formation	12	5	0,97	0.00167
23014	signal transduction by protein phosphory...	457	55	36,98	0.00168
14068	positive regulation of phosphatidylinosi...	23	7	1,86	0.00171
42490	mechanoreceptor differentiation	23	7	1,86	0.00171
48147	negative regulation of fibroblast prolif...	23	7	1,86	0.00171
60113	inner ear receptor cell differentiation	23	7	1,86	0.00171
72132	mesenchyme morphogenesis	23	7	1,86	0.00171
71229	cellular response to acid chemical	105	18	8,5	0.00176
61337	cardiac conduction	50	11	4,05	0.00181
2040	sprouting angiogenesis	36	9	2,91	0.00182
45619	regulation of lymphocyte differentiation	65	13	5,26	0.00182
43523	regulation of neuron apoptotic process	114	19	9,23	0.00187
7010	cytoskeleton organization	712	79	57,62	0.00198
3176	aortic valve development	4	3	0,32	0.00198
3180	aortic valve morphogenesis	4	3	0,32	0.00198
3190	atrioventricular valve formation	4	3	0,32	0.00198
14012	peripheral nervous system axon regenerat...	4	3	0,32	0.00198
21514	ventral spinal cord interneuron differen...	4	3	0,32	0.00198
33604	negative regulation of catecholamine sec...	4	3	0,32	0.00198
35905	ascending aorta development	4	3	0,32	0.00198
35910	ascending aorta morphogenesis	4	3	0,32	0.00198
45986	negative regulation of smooth muscle con...	4	3	0,32	0.00198
55059	asymmetric neuroblast division	4	3	0,32	0.00198
60710	chorio-allantoic fusion	4	3	0,32	0.00198
60973	cell migration involved in heart develop...	4	3	0,32	0.00198
72203	cell proliferation involved in metanephr...	4	3	0,32	0.00198
165	MAPK cascade	440	53	35,61	0.00199
3206	cardiac chamber morphogenesis	58	12	4,69	0.00199
6023	aminoglycan biosynthetic process	58	12	4,69	0.00199
6024	glycosaminoglycan biosynthetic process	58	12	4,69	0.00199
44243	multicellular organismal catabolic proce...	30	8	2,43	0.00208
48661	positive regulation of smooth muscle cel...	30	8	2,43	0.00208
61035	regulation of cartilage development	30	8	2,43	0.00208
6954	inflammatory response	224	31	18,13	0.00213
9187	cyclic nucleotide metabolic process	51	11	4,13	0.00214
45216	cell-cell junction organization	124	20	10,03	0.00215
72593	reactive oxygen species metabolic proces...	124	20	10,03	0.00215
60074	synapse maturation	11	4	0,89	0.00885
71379	cellular response to prostaglandin stimu...	11	4	0,89	0.00885
90075	relaxation of muscle	11	4	0,89	0.00885
97529	myeloid leukocyte migration	45	9	3,64	0.00889
7259	JAK-STAT cascade	61	11	4,94	0.00898
97696	STAT cascade	61	11	4,94	0.00898
31325	positive regulation of cellular metaboli...	1755	167	142,02	0.00900
1904062	regulation of cation transmembrane trans...	122	18	9,87	0.00902
90090	negative regulation of canonical Wnt sig...	113	17	9,14	0.00908
8584	male gonad development	78	13	6,31	0.00926
7189	adenylate cyclase-activating G-protein c...	17	5	1,38	0.00931
30225	macrophage differentiation	17	5	1,38	0.00931
1901890	positive regulation of cell junction ass...	17	5	1,38	0.00931
46822	regulation of nucleocytoplasmic transpor...	141	20	11,41	0.00943
43069	negative regulation of programmed cell d...	475	53	38,44	0.00956
90276	regulation of peptide hormone secretion	87	14	7,04	0.00963
70482	response to oxygen levels	228	29	18,45	0.00964
6641	triglyceride metabolic process	38	8	3,08	0.00977
8593	regulation of Notch signaling pathway	38	8	3,08	0.00977
60079	excitatory postsynaptic potential	38	8	3,08	0.00977
31444	slow-twitch skeletal muscle fiber contra...	2	2	0,16	0.00654
31652	positive regulation of heat generation	2	2	0,16	0.00654
32764	negative regulation of mast cell cytokin...	2	2	0,16	0.00654
32811	negative regulation of epinephrine secre...	2	2	0,16	0.00654
33634	positive regulation of cell-cell adhesio...	2	2	0,16	0.00654
35425	autocrine signaling	2	2	0,16	0.00654
38026	reelin-mediated signaling pathway	2	2	0,16	0.00654
45162	clustering of voltage-gated sodium chann...	2	2	0,16	0.00654
45578	negative regulation of B cell differenti...	2	2	0,16	0.00654
48241	epinephrine transport	2	2	0,16	0.00654
48242	epinephrine secretion	2	2	0,16	0.00654
60994	regulation of transcription from RNA pol...	2	2	0,16	0.00654
71386	cellular response to corticosterone stim...	2	2	0,16	0.00654
71395	cellular response to jasmonic acid stimu...	2	2	0,16	0.00654
71492	cellular response to UV-A	2	2	0,16	0.00654
72007	mesangial cell differentiation	2	2	0,16	0.00654
72008	glomerular mesangial cell differentiatio...	2	2	0,16	0.00654
72143	mesangial cell development	2	2	0,16	0.00654
72144	glomerular mesangial cell development	2	2	0,16	0.00654
90209	negative regulation of triglyceride meta...	2	2	0,16	0.00654
97114	NMDA glutamate receptor clustering	2	2	0,16	0.00654
98957	anterograde axonal transport of mitochon...	2	2	0,16	0.00654
1900747	negative regulation of vascular endothel...	2	2	0,16	0.00654
1901078	negative regulation of relaxation of mus...	2	2	0,16	0.00654
1901898	negative regulation of relaxation of car...	2	2	0,16	0.00654
1902548	negative regulation of cellular response...	2	2	0,16	0.00654
1902951	negative regulation of dendritic spine m...	2	2	0,16	0.00654
1902957	negative regulation of mitochondrial ele...	2	2	0,16	0.00654
1903225	negative regulation of endodermal cell d...	2	2	0,16	0.00654
1904238	pericyte cell differentiation	2	2	0,16	0.00654
1904761	negative regulation of myofibroblast dif...	2	2	0,16	0.00654
1905064	negative regulation of vascular smooth m...	2	2	0,16	0.00654
1905447	negative regulation of mitochondrial ATP...	2	2	0,16	0.00654
1905664	regulation of calcium ion import across ...	2	2	0,16	0.00654
1990164	histone H2A phosphorylation	2	2	0,16	0.00654
2000969	positive regulation of AMPA receptor act...	2	2	0,16	0.00654
18130	heterocycle biosynthetic process	2517	233	203,69	0.00659
6816	calcium ion transport	155	22	12,54	0.00662
31111	negative regulation of microtubule polym...	22	6	1,78	0.00666
48747	muscle fiber development	22	6	1,78	0.00666
61045	negative regulation of wound healing	22	6	1,78	0.00666
51149	positive regulation of muscle cell diffe...	51	10	4,13	0.00688
1901698	response to nitrogen compound	595	65	48,15	0.00698

1953	negative regulation of cell-matrix adhes...	18	6	1,46	0.00219
10470	regulation of gastrulation	18	6	1,46	0.00219
14014	negative regulation of gliogenesis	18	6	1,46	0.00219
15844	monoamine transport	18	6	1,46	0.00219
21904	dorsal/ventral neural tube patterning	18	6	1,46	0.00219
30199	collagen fibril organization	18	6	1,46	0.00219
35411	catenin import into nucleus	18	6	1,46	0.00219
1903706	regulation of hemopoiesis	234	32	18,94	0.00221
71214	cellular response to abiotic stimulus	178	26	14,4	0.00223
104004	cellular response to environmental stimu...	178	26	14,4	0.00223
35150	regulation of tube size	37	9	2,99	0.00223
50880	regulation of blood vessel size	37	9	2,99	0.00223
2062	chondrocyte differentiation	44	10	3,56	0.00223
10771	negative regulation of cell morphogenesi...	44	10	3,56	0.00223
30510	regulation of BMP signaling pathway	44	10	3,56	0.00223
50772	positive regulation of axonogenesis	44	10	3,56	0.00223
1903845	negative regulation of cellular response...	44	10	3,56	0.00223
10463	mesenchymal cell proliferation	24	7	1,94	0.00225
30514	negative regulation of BMP signaling pat...	24	7	1,94	0.00225
32835	glomerulus development	24	7	1,94	0.00225
46649	lymphocyte activation	263	35	21,28	0.00225
3174	mitral valve development	8	4	0,65	0.00228
3183	mitral valve morphogenesis	8	4	0,65	0.00228
3215	cardiac right ventricle morphogenesis	8	4	0,65	0.00228
21511	spinal cord patterning	8	4	0,65	0.00228
50651	dermatan sulfate proteoglycan biosynthet...	8	4	0,65	0.00228
50655	dermatan sulfate proteoglycan metabolic ...	8	4	0,65	0.00228
61179	negative regulation of insulin secretion...	8	4	0,65	0.00228
72111	cell proliferation involved in kidney de...	8	4	0,65	0.00228
72202	cell differentiation involved in metanep...	8	4	0,65	0.00228
86011	membrane repolarization during action po...	8	4	0,65	0.00228
2000112	regulation of cellular macromolecule bio...	2362	224	191,14	0.00233
97191	extrinsic apoptotic signaling pathway	134	21	10,84	0.00243
32101	regulation of response to external stimu...	274	36	22,17	0.00245
3156	regulation of animal organ formation	13	5	1,05	0.00254
38084	vascular endothelial growth factor signa...	13	5	1,05	0.00254
43616	keratinocyte proliferation	13	5	1,05	0.00254
98901	regulation of cardiac muscle cell action...	13	5	1,05	0.00254
1903779	regulation of cardiac conduction	31	8	2,51	0.00261
2000177	regulation of neural precursor cell prol...	31	8	2,51	0.00261
1666	response to hypoxia	208	29	16,83	0.00262
1667	ameboidal-type cell migration	171	25	13,84	0.00265
1823	mesonephros development	45	10	3,64	0.00267
1936	regulation of endothelial cell prolifera...	45	10	3,64	0.00267
72009	nephron epithelium development	45	10	3,64	0.00267
17015	regulation of transforming growth factor...	60	12	4,86	0.00270
1903034	regulation of response to wounding	60	12	4,86	0.00270
9953	dorsal/ventral pattern formation	38	9	3,08	0.00272
1901343	negative regulation of vasculature devel...	38	9	3,08	0.00272
34329	cell junction assembly	118	19	9,55	0.00280
21536	diencephalon development	25	7	2,02	0.00291
21795	cerebral cortex cell migration	25	7	2,02	0.00291
45980	negative regulation of nucleotide metabo...	25	7	2,02	0.00291
1900543	negative regulation of purine nucleotide...	25	7	2,02	0.00291
51129	negative regulation of cellular componen...	397	48	32,13	0.00297
21879	forebrain neuron differentiation	19	6	1,54	0.00298
45746	negative regulation of Notch signaling p...	19	6	1,54	0.00298
48016	inositol phosphate-mediated signaling	19	6	1,54	0.00298
51057	positive regulation of small GTPase medi...	19	6	1,54	0.00298
86002	cardiac muscle cell action potential inv...	19	6	1,54	0.00298
97720	calcineurin-mediated signaling	19	6	1,54	0.00298
8360	regulation of cell shape	77	14	6,23	0.00316
1903426	regulation of reactive oxygen species bi...	36	8	2,91	0.00698
1904888	cranial skeletal system development	36	8	2,91	0.00698
21700	developmental maturation	119	18	9,63	0.00699
45879	negative regulation of smoothened signal...	16	5	1,29	0.00703
2262	myeloid cell homeostasis	84	14	6,8	0.00707
30323	respiratory tube development	84	14	6,8	0.00707
7156	homophilic cell adhesion via plasma memb...	29	7	2,35	0.00711
21885	forebrain cell migration	29	7	2,35	0.00711
35329	hippo signaling	29	7	2,35	0.00711
9991	response to extracellular stimulus	253	32	20,47	0.00734
7159	leukocyte cell-cell adhesion	138	20	11,17	0.00746
2683	negative regulation of immune system pro...	185	25	14,97	0.00746
43588	skin development	120	18	9,71	0.00762
33273	response to vitamin	44	9	3,56	0.00764
72163	mesonephric epithelium development	44	9	3,56	0.00764
72164	mesonephric tubule development	44	9	3,56	0.00764
45185	maintenance of protein location	68	12	5,5	0.00774
45859	regulation of protein kinase activity	449	51	36,33	0.00782
34762	regulation of transmembrane transport	167	23	13,51	0.00802
10468	regulation of gene expression	2599	239	210,32	0.00811
30814	regulation of cAMP metabolic process	37	8	2,99	0.00829
1818	negative regulation of cytokine producti...	112	17	9,06	0.00832
43123	positive regulation of I-kappaB kinase/N...	112	17	9,06	0.00832
32069	regulation of nuclease activity	23	6	1,86	0.00840
71621	granulocyte chemotaxis	23	6	1,86	0.00840
86065	cell communication involved in cardiac c...	23	6	1,86	0.00840
1905207	regulation of cardiocyte differentiation	23	6	1,86	0.00840
48545	response to steroid hormone	216	28	17,48	0.00847
10604	positive regulation of macromolecule met...	1776	169	143,72	0.00856
1901214	regulation of neuron death	168	23	13,6	0.00861
1706	endoderm formation	30	7	2,43	0.00864
6026	aminoglycan catabolic process	30	7	2,43	0.00864
72091	regulation of stem cell proliferation	30	7	2,43	0.00864
2000377	regulation of reactive oxygen species me...	86	14	6,96	0.00870
1840	neural plate development	6	3	0,49	0.00876
3413	chondrocyte differentiation involved in ...	6	3	0,49	0.00876
7016	cytoskeletal anchoring at plasma membran...	6	3	0,49	0.00876
21513	spinal cord dorsal/ventral patterning	6	3	0,49	0.00876
21910	smoothened signaling pathway involved in...	6	3	0,49	0.00876
32926	negative regulation of activin receptor ...	6	3	0,49	0.00876
33630	positive regulation of cell adhesion med...	6	3	0,49	0.00876
35358	regulation of peroxisome proliferator ac...	6	3	0,49	0.00876
42416	dopamine biosynthetic process	6	3	0,49	0.00876
43497	regulation of protein heterodimerization...	6	3	0,49	0.00876
48368	lateral mesoderm development	6	3	0,49	0.00876
48671	negative regulation of collateral sprout...	6	3	0,49	0.00876
60028	convergent extension involved in axis el...	6	3	0,49	0.00876
60442	branching involved in prostate gland mor...	6	3	0,49	0.00876
60525	prostate glandular acinus development	6	3	0,49	0.00876
60713	labyrinthine layer morphogenesis	6	3	0,49	0.00876
71679	commissural neuron axon guidance	6	3	0,49	0.00876
72015	glomerular visceral epithelial cell deve...	6	3	0,49	0.00876
72109	glomerular mesangium development	6	3	0,49	0.00876
72310	glomerular epithelial cell development	6	3	0,49	0.00876
72578	neurotransmitter-gated ion channel clust...	6	3	0,49	0.00876
98722	asymmetric stem cell division	6	3	0,49	0.00876
1900746	regulation of vascular endothelial growt...	6	3	0,49	0.00876
1902547	regulation of cellular response to vascu...	6	3	0,49	0.00876
1903896	positive regulation of IRE1-mediated unf...	6	3	0,49	0.00876
2000271	positive regulation of fibroblast apopto...	6	3	0,49	0.00876
10837	regulation of keratinocyte proliferation	11	4	0,89	0.00885
21846	cell proliferation in forebrain	11	4	0,89	0.00885

30183	B cell differentiation	46	10	3,72	0.00317	35357	peroxisome proliferator activated recept...	11	4	0,89	0.00885
30282	bone mineralization	46	10	3,72	0.00317	45671	negative regulation of osteoclast differ...	11	4	0,89	0.00885
45638	negative regulation of myeloid cell diff...	46	10	3,72	0.00317	48333	mesodermal cell differentiation	11	4	0,89	0.00885
50877	nervous system process	308	39	24,92	0.00321	50819	negative regulation of coagulation	11	4	0,89	0.00885
51130	positive regulation of cellular componen...	714	78	57,78	0.00322	60074	synapse maturation	11	4	0,89	0.00885
1903725	regulation of phospholipid metabolic pro...	32	8	2,59	0.00323	71379	cellular response to prostaglandin stimu...	11	4	0,89	0.00885
60548	negative regulation of cell death	523	60	42,32	0.00326	90075	relaxation of muscle	11	4	0,89	0.00885
16310	phosphorylation	1313	132	106,25	0.00336	97529	myeloid leukocyte migration	45	9	3,64	0.00889
61387	regulation of extent of cell growth	54	11	4,37	0.00344	7259	JAK-STAT cascade	61	11	4,94	0.00898
51402	neuron apoptotic process	129	20	10,44	0.00346	97696	STAT cascade	61	11	4,94	0.00898
60326	cell chemotaxis	78	14	6,31	0.00357	31325	positive regulation of cellular metaboli...	1755	167	142,02	0.00900
30879	mammary gland development	70	13	5,66	0.00362	1904062	regulation of cation transmembrane trans...	122	18	9,87	0.00902
6940	regulation of smooth muscle contraction	14	5	1,13	0.00369	90090	negative regulation of canonical Wnt sig...	113	17	9,14	0.00908
14904	myotube cell development	14	5	1,13	0.00369	8584	male gonad development	78	13	6,31	0.00926
35116	embryonic hindlimb morphogenesis	14	5	1,13	0.00369	7189	adenylate cyclase-activating G-protein c...	17	5	1,38	0.00931
45581	negative regulation of T cell differenti...	14	5	1,13	0.00369	30225	macrophage differentiation	17	5	1,38	0.00931
45992	negative regulation of embryonic develop...	14	5	1,13	0.00369	1901890	positive regulation of cell junction ass...	17	5	1,38	0.00931
9880	embryonic pattern specification	26	7	2,1	0.00371	46822	regulation of nucleocytoplasmic transpor...	141	20	11,41	0.00943
35987	endodermal cell differentiation	26	7	2,1	0.00371	43069	negative regulation of programmed cell d...	475	53	38,44	0.00956
60443	mammary gland morphogenesis	26	7	2,1	0.00371	90276	regulation of peptide hormone secretion	87	14	7,04	0.00963
61383	trabecula morphogenesis	26	7	2,1	0.00371	70482	response to oxygen levels	228	29	18,45	0.00964
1903035	negative regulation of response to wound...	26	7	2,1	0.00371	6641	triglyceride metabolic process	38	8	3,08	0.00977
1508	action potential	47	10	3,8	0.00374	8593	regulation of Notch signaling pathway	38	8	3,08	0.00977
						60079	excitatory postsynaptic potential	38	8	3,08	0.00977

Go Terms (Biological Processes) for Downregulated Genes Resistant vs Nonresistant DMSO

GO.ID	Term	Annotated	Significant	Expected	classicFisher	GO.ID	Term	Annotated	Significant	Expected	classicFisher
48514	blood vessel morphogenesis	233	36	18,77	0.00010	6874	cellular calcium ion homeostasis	141	21	11,36	0.00429
8202	steroid metabolic process	164	28	13,21	0.00011	44236	multicellular organism metabolic process	56	11	4,51	0.00447
70372	regulation of ERK1 and ERK2 cascade	102	20	8,22	0.00015	5996	monosaccharide metabolic process	160	23	12,89	0.00452
6704	glucocorticoid biosynthetic process	8	5	0,64	0.00015	10876	lipid localization	160	23	12,89	0.00452
8211	glucocorticoid metabolic process	12	6	0,97	0.00016	9948	anterior/posterior axis specification	27	7	2,18	0.00455
50896	response to stimulus	4190	383	337,61	0.00019	34381	plasma lipoprotein particle clearance	27	7	2,18	0.00455
9605	response to external stimulus	914	103	73,65	0.00019	1845	phagolysosome assembly	5	3	0,4	0.00460
32501	multicellular organismal process	3139	297	252,93	0.00019	60745	mammary gland branching involved in preg...	5	3	0,4	0.00460
36150	phosphatidylserine acyl-chain remodeling	5	4	0,4	0.00020	71635	negative regulation of transforming grow...	5	3	0,4	0.00460
2920	regulation of humoral immune response	17	7	1,37	0.00020	1902337	regulation of apoptotic process involved...	5	3	0,4	0.00460
30855	epithelial cell differentiation	287	41	23,13	0.00021	1904748	regulation of apoptotic process involved...	5	3	0,4	0.00460
1525	angiogenesis	189	30	15,23	0.00024	6909	phagocytosis	115	18	9,27	0.00465
43277	apoptotic cell clearance	18	7	1,45	0.00031	42326	negative regulation of phosphorylation	276	35	22,24	0.00466
32101	regulation of response to external stimu...	274	39	22,08	0.00031	10466	negative regulation of peptidase activit...	89	15	7,17	0.00466
9410	response to xenobiotic stimulus	48	12	3,87	0.00032	51480	regulation of cytosolic calcium ion conc...	89	15	7,17	0.00466
9719	response to endogenous stimulus	810	92	65,27	0.00033	30301	cholesterol transport	34	8	2,74	0.00471
71495	cellular response to endogenous stimulus	706	82	56,89	0.00035	52547	regulation of peptidase activity	189	26	15,23	0.00481
6509	membrane protein ectodomain proteolysis	24	8	1,93	0.00039	7015	actin filament organization	199	27	16,03	0.00497
6812	cation transport	462	58	37,23	0.00040	48638	regulation of developmental growth	152	22	12,25	0.00499
48646	anatomical structure formation involved ...	462	58	37,23	0.00040	71363	cellular response to growth factor stimu...	358	43	28,85	0.00501
60326	cell chemotaxis	78	16	6,28	0.00040	45055	regulated exocytosis	389	46	31,34	0.00505
1902904	negative regulation of supramolecular fi...	71	15	5,72	0.00043	50678	regulation of epithelial cell proliferat...	143	21	11,52	0.00507
71310	cellular response to organic substance	1320	138	106,36	0.00045	72659	protein localization to plasma membrane	143	21	11,52	0.00507
32940	secretion by cell	680	79	54,79	0.00045	71634	regulation of transforming growth factor...	15	5	1,21	0.00508
2526	acute inflammatory response	43	11	3,46	0.00045	6936	muscle contraction	134	20	10,8	0.00512
44255	cellular lipid metabolic process	578	69	46,57	0.00049	30148	sphingolipid biosynthetic process	57	11	4,59	0.00515
60541	respiratory system development	95	18	7,65	0.00050	72006	nephron development	57	11	4,59	0.00515
30001	metal ion transport	338	45	27,23	0.00051	23051	regulation of signaling	1750	168	141,01	0.00518
6072	glycerol-3-phosphate metabolic process	3	3	0,24	0.00052	35556	intracellular signal transduction	1474	144	118,77	0.00540
48732	gland development	225	33	18,13	0.00052	50728	negative regulation of inflammatory resp...	42	9	3,38	0.00540
6705	mineralocorticoid biosynthetic process	6	4	0,48	0.00055	55074	calcium ion homeostasis	144	21	11,6	0.00550
8212	mineralocorticoid metabolic process	6	4	0,48	0.00055	51240	positive regulation of multicellular org...	678	73	54,63	0.00557
71447	cellular response to hydroperoxide	6	4	0,48	0.00055	6875	cellular metal ion homeostasis	220	29	17,73	0.00557
3012	muscle system process	183	28	14,75	0.00069	6694	steroid biosynthetic process	117	18	9,43	0.00559
51716	cellular response to stimulus	3546	326	285,72	0.00072	33619	membrane protein proteolysis	35	8	2,82	0.00568
50673	epithelial cell proliferation	166	26	13,38	0.00074	8643	carbohydrate transport	74	13	5,96	0.00571
6805	xenobiotic metabolic process	39	10	3,14	0.00080	55080	cation homeostasis	290	36	23,37	0.00584
9888	tissue development	876	96	70,58	0.00084	6688	glycosphingolipid biosynthetic process	10	4	0,81	0.00592
8207	C21-steroid hormone metabolic process	21	7	1,69	0.00091	10743	regulation of macrophage derived foam ce...	10	4	0,81	0.00592
32963	collagen metabolic process	47	11	3,79	0.00102	34105	positive regulation of tissue remodeling	10	4	0,81	0.00592
6700	C21-steroid hormone biosynthetic process	16	6	1,29	0.00106	72576	liver morphogenesis	10	4	0,81	0.00592

8209	androgen metabolic process	16	6	1,29	0.00106
1901615	organic hydroxy compound metabolic proce...	244	34	19,66	0.00110
46889	positive regulation of lipid biosyntheti...	34	9	2,74	0.00114
1901342	regulation of vasculature development	110	19	8,86	0.00115
2921	negative regulation of humoral immune re...	7	4	0,56	0.00120
30837	negative regulation of actin filament po...	28	8	2,26	0.00124
6873	cellular ion homeostasis	265	36	21,35	0.00126
8406	gonad development	111	19	8,94	0.00128
6887	exocytosis	464	56	37,39	0.00130
6935	chemotaxis	219	31	17,65	0.00138
42330	taxis	219	31	17,65	0.00138
52548	regulation of endopeptidase activity	173	26	13,94	0.00138
97006	regulation of plasma lipoprotein particl...	42	10	3,38	0.00148
1901136	carbohydrate derivative catabolic proces...	104	18	8,38	0.00150
7548	sex differentiation	139	22	11,2	0.00161
22612	gland morphogenesis	57	12	4,59	0.00164
32613	interleukin-10 production	12	5	0,97	0.00164
44259	multicellular organismal macromolecule m...	50	11	4,03	0.00175
2683	negative regulation of immune system pro...	185	27	14,91	0.00176
1568	blood vessel development	280	37	22,56	0.00180
71466	cellular response to xenobiotic stimulus	43	10	3,46	0.00180
30324	lung development	81	15	6,53	0.00181
43269	regulation of ion transport	223	31	17,97	0.00186
51239	regulation of multicellular organismal p...	1322	134	106,52	0.00193
19934	cGMP-mediated signaling	4	3	0,32	0.00196
32911	negative regulation of transforming grow...	4	3	0,32	0.00196
34367	macromolecular complex remodeling	4	3	0,32	0.00196
34368	protein-lipid complex remodeling	4	3	0,32	0.00196
34369	plasma lipoprotein particle remodeling	4	3	0,32	0.00196
52646	alditol phosphate metabolic process	4	3	0,32	0.00196
60696	regulation of phospholipid catabolic pro...	4	3	0,32	0.00196
61101	neuroendocrine cell differentiation	4	3	0,32	0.00196
61370	testosterone biosynthetic process	4	3	0,32	0.00196
19722	calcium-mediated signaling	66	13	5,32	0.00202
72359	circulatory system development	463	55	37,31	0.00205
10033	response to organic substance	1632	161	131,5	0.00205
9653	anatomical structure morphogenesis	1234	126	99,43	0.00206
1944	vasculature development	292	38	23,53	0.00207
48286	lung alveolus development	18	6	1,45	0.00214
60042	retina morphogenesis in camera-type eye	18	6	1,45	0.00214
8585	female gonad development	44	10	3,55	0.00216
45137	development of primary sexual characteri...	116	19	9,35	0.00218
6702	androgen biosynthetic process	8	4	0,64	0.00225
19852	L-ascorbic acid metabolic process	8	4	0,64	0.00225
7369	gastrulation	108	18	8,7	0.00232
19932	second-messenger-mediated signaling	108	18	8,7	0.00232
50801	ion homeostasis	324	41	26,11	0.00238
30325	adrenal gland development	13	5	1,05	0.00249
30449	regulation of complement activation	13	5	1,05	0.00249
86010	membrane depolarization during action po...	13	5	1,05	0.00249
2000257	regulation of protein activation cascade	13	5	1,05	0.00249
46718	viral entry into host cell	60	12	4,83	0.00260
97237	cellular response to toxic substance	60	12	4,83	0.00260
30323	respiratory tube development	84	15	6,77	0.00263
30155	regulation of cell adhesion	296	38	23,85	0.00264
72358	cardiovascular system development	296	38	23,85	0.00264
98771	inorganic ion homeostasis	296	38	23,85	0.00264
32272	negative regulation of protein polymeriz...	38	9	3,06	0.00264
46434	organophosphate catabolic process	76	14	6,12	0.00268
1990778	protein localization to cell periphery	163	24	13,13	0.00275
22603	regulation of anatomical structure morph...	502	58	40,45	0.00295
1990000	amyloid fibril formation	10	4	0,81	0.00592
9617	response to bacterium	173	24	13,94	0.00594
6869	lipid transport	145	21	11,68	0.00596
70661	leukocyte proliferation	100	16	8,06	0.00596
6469	negative regulation of protein kinase ac...	155	22	12,49	0.00630
22617	extracellular matrix disassembly	43	9	3,46	0.00636
2000379	positive regulation of reactive oxygen s...	43	9	3,46	0.00636
10646	regulation of cell communication	1724	165	138,91	0.00638
1867	complement activation, lectin pathway	2	2	0,16	0.00648
2577	regulation of antigen processing and pre...	2	2	0,16	0.00648
2578	negative regulation of antigen processin...	2	2	0,16	0.00648
2583	regulation of antigen processing and pre...	2	2	0,16	0.00648
2584	negative regulation of antigen processin...	2	2	0,16	0.00648
2589	regulation of antigen processing and pre...	2	2	0,16	0.00648
2590	negative regulation of antigen processin...	2	2	0,16	0.00648
2677	negative regulation of chronic inflammat...	2	2	0,16	0.00648
3310	pancreatic A cell differentiation	2	2	0,16	0.00648
5988	lactose metabolic process	2	2	0,16	0.00648
5989	lactose biosynthetic process	2	2	0,16	0.00648
7341	penetration of zona pellucida	2	2	0,16	0.00648
7354	zygotic determination of anterior/poster...	2	2	0,16	0.00648
9812	flavonoid metabolic process	2	2	0,16	0.00648
10899	regulation of phosphatidylcholine catabo...	2	2	0,16	0.00648
21984	adenohypophysis development	2	2	0,16	0.00648
30299	intestinal cholesterol absorption	2	2	0,16	0.00648
32455	nerve growth factor processing	2	2	0,16	0.00648
32490	detection of molecule of bacterial origi...	2	2	0,16	0.00648
32808	lacrimal gland development	2	2	0,16	0.00648
32902	nerve growth factor production	2	2	0,16	0.00648
34196	acylglycerol transport	2	2	0,16	0.00648
34197	triglyceride transport	2	2	0,16	0.00648
34382	chylomicron remnant clearance	2	2	0,16	0.00648
42078	germ-line stem cell division	2	2	0,16	0.00648
43435	response to corticotropin-releasing horm...	2	2	0,16	0.00648
46069	cGMP catabolic process	2	2	0,16	0.00648
46351	disaccharide biosynthetic process	2	2	0,16	0.00648
46618	drug export	2	2	0,16	0.00648
48133	male germ-line stem cell asymmetric divi...	2	2	0,16	0.00648
50910	detection of mechanical stimulus involve...	2	2	0,16	0.00648
51643	endoplasmic reticulum localization	2	2	0,16	0.00648
51694	pointed-end actin filament capping	2	2	0,16	0.00648
60535	trachea cartilage morphogenesis	2	2	0,16	0.00648
61669	spontaneous neurotransmitter secretion	2	2	0,16	0.00648
71376	cellular response to corticotropin-relea...	2	2	0,16	0.00648
71596	ubiquitin-dependent protein catabolic pr...	2	2	0,16	0.00648
71830	triglyceride-rich lipoprotein particle c...	2	2	0,16	0.00648
86097	phospholipase C-activating angiotensin-a...	2	2	0,16	0.00648
90272	negative regulation of fibroblast growth...	2	2	0,16	0.00648
98581	detection of external biotic stimulus	2	2	0,16	0.00648
98728	germline stem cell asymmetric division	2	2	0,16	0.00648
98856	intestinal lipid absorption	2	2	0,16	0.00648
98912	membrane depolarization during atrial ca...	2	2	0,16	0.00648
2000866	positive regulation of estradiol secreti...	2	2	0,16	0.00648
2673	regulation of acute inflammatory respons...	22	6	1,77	0.00652
60563	neuroepithelial cell differentiation	22	6	1,77	0.00652
72376	protein activation cascade	22	6	1,77	0.00652
10631	epithelial cell migration	128	19	10,31	0.00665
90132	epithelium migration	128	19	10,31	0.00665
97435	supramolecular fiber organization	323	39	26,03	0.00672
6749	glutathione metabolic process	36	8	2,9	0.00680
6956	complement activation	16	5	1,29	0.00691

30003	cellular cation homeostasis	259	34	20,87	0.00302
51494	negative regulation of cytoskeleton orga...	77	14	6,2	0.00304
7492	endoderm development	46	10	3,71	0.00307
40011	locomotion	750	81	60,43	0.00326
48878	chemical homeostasis	494	57	39,8	0.00329
32502	developmental process	2938	270	236,73	0.00332
46660	female sex differentiation	54	11	4,35	0.00333
71901	negative regulation of protein serine/th...	78	14	6,28	0.00344
48583	regulation of response to stimulus	2001	191	161,23	0.00345
1818	negative regulation of cytokine producti...	112	18	9,02	0.00349
9311	oligosaccharide metabolic process	26	7	2,09	0.00362
9880	embryonic pattern specification	26	7	2,09	0.00362
10742	macrophage derived foam cell differentia...	14	5	1,13	0.00362
90077	foam cell differentiation	14	5	1,13	0.00362
45321	leukocyte activation	549	62	44,24	0.00365
55082	cellular chemical homeostasis	342	42	27,56	0.00370
48870	cell motility	635	70	51,17	0.00376
51674	localization of cell	635	70	51,17	0.00376
6952	defense response	603	67	48,59	0.00377
33673	negative regulation of kinase activity	167	24	13,46	0.00378
6067	ethanol metabolic process	9	4	0,73	0.00379
32703	negative regulation of interleukin-2 pro...	9	4	0,73	0.00379
32733	positive regulation of interleukin-10 pr...	9	4	0,73	0.00379
36151	phosphatidylcholine acyl-chain remodelin...	9	4	0,73	0.00379
72574	hepatocyte proliferation	9	4	0,73	0.00379
72575	epithelial cell proliferation involved i...	9	4	0,73	0.00379
90036	regulation of protein kinase C signaling	9	4	0,73	0.00379
15918	sterol transport	40	9	3,22	0.00383
15850	organic hydroxy compound transport	79	14	6,37	0.00388
6654	phosphatidic acid biosynthetic process	20	6	1,61	0.00389
46473	phosphatidic acid metabolic process	20	6	1,61	0.00389
32879	regulation of localization	1268	127	102,17	0.00396
10951	negative regulation of endopeptidase act...	88	15	7,09	0.00418
46545	development of primary female sexual cha...	48	10	3,87	0.00425
7157	heterophilic cell-cell adhesion via plas...	16	5	1,29	0.00691
71604	transforming growth factor beta producti...	16	5	1,29	0.00691
48869	cellular developmental process	1986	187	160,02	0.00705
31032	actomyosin structure organization	93	15	7,49	0.00708
7159	leukocyte cell-cell adhesion	138	20	11,12	0.00712
90130	tissue migration	129	19	10,39	0.00723
2274	myeloid leukocyte activation	315	38	25,38	0.00751
45834	positive regulation of lipid metabolic p...	60	11	4,83	0.00768
50777	negative regulation of immune response	52	10	4,19	0.00769
34765	regulation of ion transmembrane transpor...	158	22	12,73	0.00789
72503	cellular divalent inorganic cation homeo...	158	22	12,73	0.00789
70373	negative regulation of ERK1 and ERK2 cas...	37	8	2,98	0.00808
61028	establishment of endothelial barrier	23	6	1,85	0.00823
1775	cell activation	613	66	49,39	0.00825
2000377	regulation of reactive oxygen species me...	86	14	6,93	0.00839
1704	formation of primary germ layer	69	12	5,56	0.00842
1706	endoderm formation	30	7	2,42	0.00845
70848	response to growth factor	369	43	29,73	0.00850
34220	ion transmembrane transport	453	51	36,5	0.00853
72009	nephron epithelium development	45	9	3,63	0.00865
30207	chondroitin sulfate catabolic process	6	3	0,48	0.00866
32905	transforming growth factor beta1 product...	6	3	0,48	0.00866
32908	regulation of transforming growth factor...	6	3	0,48	0.00866
33630	positive regulation of cell adhesion med...	6	3	0,48	0.00866
35376	sterol import	6	3	0,48	0.00866
35428	hexose transmembrane transport	6	3	0,48	0.00866
48671	negative regulation of collateral sprout...	6	3	0,48	0.00866
51004	regulation of lipoprotein lipase activit...	6	3	0,48	0.00866
60046	regulation of acrosome reaction	6	3	0,48	0.00866
60484	lung-associated mesenchyme development	6	3	0,48	0.00866
61081	positive regulation of myeloid leukocyte...	6	3	0,48	0.00866
70508	cholesterol import	6	3	0,48	0.00866
1904659	glucose transmembrane transport	6	3	0,48	0.00866
1905950	monosaccharide transmembrane transport	6	3	0,48	0.00866
6012	galactose metabolic process	11	4	0,89	0.00871
7340	acrosome reaction	11	4	0,89	0.00871
32653	regulation of interleukin-10 production	11	4	0,89	0.00871
45940	positive regulation of steroid metabolic...	11	4	0,89	0.00871
50858	negative regulation of antigen receptor-...	11	4	0,89	0.00871
51043	regulation of membrane protein ectodomai...	11	4	0,89	0.00871
1902532	negative regulation of intracellular sig...	308	37	24,82	0.00884
2697	regulation of immune effector process	141	20	11,36	0.00901
6066	alcohol metabolic process	179	24	14,42	0.00903
61458	reproductive system development	218	28	17,57	0.00905
16042	lipid catabolic process	160	22	12,89	0.00911
578	embryonic axis specification	17	5	1,37	0.00915
2455	humoral immune response mediated by circ...	17	5	1,37	0.00915
1816	cytokine production	299	36	24,09	0.00940
6575	cellular modified amino acid metabolic p...	105	16	8,46	0.00953
10634	positive regulation of epithelial cell m...	62	11	5	0.00984
15749	monosaccharide transport	62	11	5	0.00984
9887	animal organ morphogenesis	478	53	38,52	0.00993
50900	leukocyte migration	133	19	10,72	0.00998

Go Terms (Molecular Functions) for Upregulated Genes Resistant vs Nonresistant DMSO

GO.ID	Term	Annotated	Significant	Expected	classicFisher
1990837	sequence-specific double-stranded DNA bi...	402	54	32,3	0.00011
987	core promoter proximal region sequence-s...	199	32	15,99	0.00011
43395	heparan sulfate proteoglycan binding	8	5	0,64	0.00015
43169	cation binding	2133	212	171,4	0.00016
978	RNA polymerase II core promoter proximal...	194	31	15,59	0.00016
31406	carboxylic acid binding	80	17	6,43	0.00016
43177	organic acid binding	81	17	6,51	0.00019
1904929	coreceptor activity involved in Wnt sign...	5	4	0,4	0.00019
46983	protein dimerization activity	675	80	54,24	0.00020
3690	double-stranded DNA binding	461	58	37,04	0.00035
4930	G-protein coupled receptor activity	70	15	5,62	0.00036
46872	metal ion binding	2101	206	168,83	0.00045
5126	cytokine receptor binding	79	16	6,35	0.00045
4647	phosphoserine phosphatase activity	3	3	0,24	0.00052
30552	cAMP binding	10	5	0,8	0.00059
8134	transcription factor binding	387	49	31,1	0.00087
1618	virus receptor activity	33	9	2,65	0.00088
104005	hijacked molecular function	33	9	2,65	0.00088
1228	transcriptional activator activity, RNA ...	196	29	15,75	0.00093
30551	cyclic nucleotide binding	11	5	0,88	0.00101
48018	receptor ligand activity	76	14	6,11	0.00261
43167	ion binding	3230	294	259,55	0.00292
3677	DNA binding	1330	132	106,87	0.00415
8201	heparin binding	49	10	3,94	0.00487
15026	coreceptor activity	15	5	1,21	0.00502
46332	SMAD binding	50	10	4,02	0.00567
42813	Wnt-activated receptor activity	10	4	0,8	0.00586
43236	laminin binding	10	4	0,8	0.00586
4351	glutamate decarboxylase activity	2	2	0,16	0.00645
4935	adrenergic receptor activity	2	2	0,16	0.00645
4936	alpha-adrenergic receptor activity	2	2	0,16	0.00645
4938	alpha2-adrenergic receptor activity	2	2	0,16	0.00645
8158	hedgehog receptor activity	2	2	0,16	0.00645
8597	calcium-dependent protein serine/threoni...	2	2	0,16	0.00645
18636	phenanthrene 9,10-monooxygenase activity	2	2	0,16	0.00645
30160	GKAP/Homer scaffold activity	2	2	0,16	0.00645
31014	troponin T binding	2	2	0,16	0.00645
31696	alpha-2C adrenergic receptor binding	2	2	0,16	0.00645
33691	sialic acid binding	2	2	0,16	0.00645
47086	ketosteroid monooxygenase activity	2	2	0,16	0.00645

98632	cell-cell adhesion mediator activity	16	6	1,29	0.00105
977	RNA polymerase II regulatory region sequ...	321	42	25,79	0.00105
1012	RNA polymerase II regulatory region DNA ...	322	42	25,87	0.00112
70410	co-SMAD binding	7	4	0,56	0.00119
71936	coreceptor activity involved in Wnt sign...	7	4	0,56	0.00119
31690	adrenergic receptor binding	12	5	0,96	0.00162
5113	patched binding	4	3	0,32	0.00194
8430	selenium binding	4	3	0,32	0.00194
32052	bile acid binding	4	3	0,32	0.00194
35374	chondroitin sulfate binding	4	3	0,32	0.00194
48407	platelet-derived growth factor binding	4	3	0,32	0.00194
50786	RAGE receptor binding	4	3	0,32	0.00194
86080	protein binding involved in heterotypic ...	4	3	0,32	0.00194
5178	integrin binding	51	11	4,1	0.00202
5160	transforming growth factor beta receptor...	18	6	1,45	0.00211
8146	sulfotransferase activity	18	6	1,45	0.00211
70888	E-box binding	18	6	1,45	0.00211
98631	cell adhesion mediator activity	18	6	1,45	0.00211
8092	cytoskeletal protein binding	518	60	41,62	0.00223
47115	trans-1,2-dihydrobenzene-1,2-diol dehydr...	2	2	0,16	0.00645
51379	epinephrine binding	2	2	0,16	0.00645
80023	3R-hydroxyacyl-CoA dehydratase activity	2	2	0,16	0.00645
86008	voltage-gated potassium channel activity...	2	2	0,16	0.00645
1902282	voltage-gated potassium channel activity...	2	2	0,16	0.00645
1078	transcriptional repressor activity, RNA ...	67	12	5,38	0.00650
8013	beta-catenin binding	51	10	4,1	0.00655
30545	receptor regulator activity	85	14	6,83	0.00739
8083	growth factor activity	37	8	2,97	0.00795
5072	transforming growth factor beta receptor...	6	3	0,48	0.00859
16775	phosphotransferase activity, nitrogenous...	6	3	0,48	0.00859
35014	phosphatidylinositol 3-kinase regulator ...	6	3	0,48	0.00859
30165	PDZ domain binding	53	10	4,26	0.00865
5109	frizzled binding	17	5	1,37	0.00904
43425	bHLH transcription factor binding	17	5	1,37	0.00904
16773	phosphotransferase activity, alcohol gro...	447	50	35,92	0.00982

Go Terms (Molecular Functions) for Downregulated Genes Resistant vs Nonresistant DMSO

GO.ID	Term	Annotated	Significant	Expected	classicFisher
4714	transmembrane receptor protein tyrosine ...	22	8	1,73	0.00017
8239	dipeptidyl-peptidase activity	5	4	0,39	0.00018
8201	heparin binding	49	12	3,86	0.00032
4888	transmembrane signaling receptor activit...	179	28	14,12	0.00034
61135	endopeptidase regulator activity	50	12	3,94	0.00039
5007	fibroblast growth factor-activated recep...	3	3	0,24	0.00049
8035	high-density lipoprotein particle bindin...	3	3	0,24	0.00049
8331	high voltage-gated calcium channel activ...	3	3	0,24	0.00049
34185	apolipoprotein binding	6	4	0,47	0.00051
8236	serine-type peptidase activity	66	14	5,2	0.00051
5262	calcium channel activity	38	10	3	0.00054
5520	insulin-like growth factor binding	10	5	0,79	0.00054
8395	steroid hydroxylase activity	10	5	0,79	0.00054
1901681	sulfur compound binding	98	18	7,73	0.00057
5244	voltage-gated ion channel activity	39	10	3,08	0.00068
22832	voltage-gated channel activity	39	10	3,08	0.00068
4866	endopeptidase inhibitor activity	46	11	3,63	0.00070
30414	peptidase inhibitor activity	46	11	3,63	0.00070
1618	virus receptor activity	33	9	2,6	0.00077
3707	steroid hormone receptor activity	33	9	2,6	0.00077
104005	hijacked molecular function	33	9	2,6	0.00077
50840	extracellular matrix binding	21	7	1,66	0.00080
99600	transmembrane receptor activity	189	28	14,9	0.00083
5215	transporter activity	591	68	46,6	0.00083
5245	voltage-gated calcium channel activity	11	5	0,87	0.00093
19199	transmembrane receptor protein kinase ac...	34	9	2,68	0.00097
3779	actin binding	219	31	17,27	0.00098
4871	signal transducer activity	428	52	33,75	0.00103
38024	cargo receptor activity	22	7	1,73	0.00110
8194	UDP-glycosyltransferase activity	64	13	5,05	0.00124
4879	nuclear receptor activity	29	8	2,29	0.00139
98531	transcription factor activity, direct li...	29	8	2,29	0.00139
15075	ion transmembrane transporter activity	334	42	26,34	0.00159
15269	calcium-activated potassium channel acti...	4	3	0,32	0.00184
86007	voltage-gated calcium channel activity i...	4	3	0,32	0.00184
4867	serine-type endopeptidase inhibitor acti...	18	6	1,42	0.00192
8307	structural constituent of muscle	18	6	1,42	0.00192
22891	substrate-specific transmembrane transpo...	369	45	29,1	0.00205
22857	transmembrane transporter activity	400	48	31,54	0.00206
15145	monosaccharide transmembrane transporter...	8	4	0,63	0.00207
5539	glycosaminoglycan binding	60	12	4,73	0.00217
8092	cytoskeletal protein binding	518	59	40,85	0.00231
46873	metal ion transmembrane transporter acti...	137	21	10,8	0.00234
22892	substrate-specific transporter activity	487	56	38,4	0.00241
30545	receptor regulator activity	85	15	6,7	0.00241
4713	protein tyrosine kinase activity	78	14	6,15	0.00282
61134	peptidase regulator activity	70	13	5,52	0.00289
4114	3',5'-cyclic-nucleotide phosphodiesteras...	9	4	0,71	0.00350
17017	MAP kinase tyrosine/serine/threonine pho...	9	4	0,71	0.00350
30228	lipoprotein particle receptor activity	9	4	0,71	0.00350
30169	low-density lipoprotein particle binding	5	3	0,39	0.00433
31994	insulin-like growth factor I binding	5	3	0,39	0.00433
47184	1-acylglycerophosphocholine O-acyltransf...	5	3	0,39	0.00433
8233	peptidase activity	291	36	22,95	0.00438
5267	potassium channel activity	15	5	1,18	0.00463
16757	transferase activity, transferring glyco...	145	21	11,43	0.00466
4112	cyclic-nucleotide phosphodiesterase acti...	10	4	0,79	0.00548
33549	MAP kinase phosphatase activity	10	4	0,79	0.00548
15085	calcium ion transmembrane transporter ac...	51	10	4,02	0.00575
8081	phosphoric diester hydrolase activity	36	8	2,84	0.00598
48018	receptor ligand activity	76	13	5,99	0.00602
1730	2'-5'-oligoadenylate synthetase activity	2	2	0,16	0.00621
4062	aryl sulfotransferase activity	2	2	0,16	0.00621
4064	arylesterase activity	2	2	0,16	0.00621
4577	N-acetylglucosaminyldiphosphodolichol N-...	2	2	0,16	0.00621
4771	sterol esterase activity	2	2	0,16	0.00621
8131	primary amine oxidase activity	2	2	0,16	0.00621
34186	apolipoprotein A-I binding	2	2	0,16	0.00621
47555	3',5'-cyclic-GMP phosphodiesterase activ...	2	2	0,16	0.00621
98772	molecular function regulator	787	81	62,06	0.00657
5496	steroid binding	45	9	3,55	0.00754
16765	transferase activity, transferring alkyl...	45	9	3,55	0.00754
5355	glucose transmembrane transporter activi...	6	3	0,47	0.00815
97493	structural molecule activity conferring ...	6	3	0,47	0.00815
5201	extracellular matrix structural constitu...	17	5	1,34	0.00836
72509	divalent inorganic cation transmembrane ...	80	13	6,31	0.00929

Appendix 2: Complete Statistical Analysis of Intracellular Lipid Content

Free Cholesterol

Bonferroni's multiple comparisons test

Comparison	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-33,05 to 10,09	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-22,28 to 20,86	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-38,46 to 4,683	No	ns	0,3342
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-28,46 to 14,68	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-43,07 to 0,0771	No	ns	0,0516
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-33,85 to 9,293	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-70,72 to -27,57	Yes	****	<0,0001
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-30,87 to 12,28	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-31,93 to 11,22	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	-21,16 to 21,99	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-37,34 to 5,809	No	ns	0,5201
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-27,34 to 15,81	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-41,94 to 1,203	No	ns	0,0819
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-32,73 to 10,42	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-69,59 to -26,45	Yes	****	<0,0001
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-29,74 to 13,4	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-26,98 to 16,16	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-16,98 to 26,16	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-31,59 to 11,56	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-22,37 to 20,77	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-59,24 to -16,09	Yes	****	<0,0001
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-19,39 to 23,76	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-37,75 to 5,395	No	ns	0,4424
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-27,75 to 15,39	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-42,36 to 0,7891	No	ns	0,0692
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-33,14 to 10	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-70,01 to -26,86	Yes	****	<0,0001
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-30,16 to 12,99	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-26,18 to 16,97	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-16,96 to 26,18	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-53,83 to -10,68	Yes	***	0,0006
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-13,98 to 29,17	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-36,18 to 6,968	No	ns	0,8125
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-26,96 to 16,18	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-63,83 to -20,68	Yes	****	<0,0001
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-23,98 to 19,17	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-49,22 to -6,077	Yes	**	0,0041
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-9,372 to 33,77	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-58,44 to -15,29	Yes	***	0,0001
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-18,59 to 24,56	No	ns	>0,9999

Uncorrected Fisher's LSD

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	-12,95 to 10,7	No	ns	0,844605
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-1,06 to 22,6	No	ns	0,072086
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-1,83 to 21,83	No	ns	0,093123
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-2,613 to 21,04	No	ns	0,119769
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	28,02 to 51,68	Yes	****	<0,000001

Total Cholesteryl Ester

Dunn's multiple comparisons test

Comparison	Mean rank diff,	Significant?	Summary	Adjusted P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-1	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	13,33	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-7	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	9,333	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-9,333	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	8,667	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-7,667	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	7,333	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-12,33	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	2	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-18,33	No	ns	0,484
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-2	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-20,67	No	ns	0,1817
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-2,667	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-19	No	ns	0,3694
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-4	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-6	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	10,33	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-8,333	No	ns	>0,9999

0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	9,667	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-6,667	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	8,333	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-20,33	No	ns	0,2102
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-4	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-22,67	No	ns	0,0726
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-4,667	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-21	No	ns	0,1567
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-6	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-2,333	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	15,67	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-0,6667	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	14,33	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-18,67	No	ns	0,4233
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,6667	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-17	No	ns	0,8112
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-2	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	1,667	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	16,67	No	ns	0,9185
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-16,33	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-1,333	No	ns	>0,9999

Uncorrected Dunn's test

Comparison	Mean rank diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	11,33	No	ns	0,1149
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	14,33	Yes	*	0,0461
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	16,33	Yes	*	0,0231
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	18	Yes	*	0,0123
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	15	Yes	*	0,0369

Total Lysophosphatidylcholine

Bonferroni's multiple comparisons test

Comparison	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-0,8256 to 0,2063	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-0,7563 to 0,2756	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-0,9692 to 0,06269	No	ns	0,1461
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-0,6014 to 0,4305	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-1,11 to -0,07766	Yes	*	0,0131
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,7605 to 0,2714	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-1,801 to -0,7688	Yes	****	<0,0001
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-1,006 to 0,02572	No	ns	0,0778
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-0,7919 to 0,2401	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	-0,7226 to 0,3093	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-0,9355 to 0,09643	No	ns	0,2579
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-0,5677 to 0,4643	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-1,076 to -0,04392	Yes	*	0,0235
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,7268 to 0,3051	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-1,767 to -0,7351	Yes	****	<0,0001
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,9725 to 0,05946	No	ns	0,1383
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-0,6596 to 0,3723	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-0,2918 to 0,7401	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-0,8 to 0,232	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,4509 to 0,581	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-1,491 to -0,4592	Yes	****	<0,0001
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,6966 to 0,3354	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-0,7289 to 0,3031	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-0,3611 to 0,6709	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-0,8692 to 0,1627	No	ns	0,7629
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,5202 to 0,5118	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-1,56 to -0,5284	Yes	****	<0,0001
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,7659 to 0,2661	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-0,6563 to 0,3756	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,3073 to 0,7247	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-1,347 to -0,3155	Yes	***	0,0002
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,5529 to 0,479	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-1,024 to 0,007793	No	ns	0,0572
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,6751 to 0,3568	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-1,715 to -0,6833	Yes	****	<0,0001
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,9208 to 0,1112	No	ns	0,3296

5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-1,207 to -0,1752	Yes	**	0,0025
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,4126 to 0,6193	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-1,556 to -0,5242	Yes	****	<0,0001
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,7616 to 0,2703	No	ns	>0,9999

Uncorrected Fisher's LSD

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	-0,3167 to 0,2492	No	ns	0,806
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-0,2136 to 0,3522	No	ns	0,6151
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	0,08492 to 0,6507	Yes	*	0,0134
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	0,06615 to 0,632	Yes	*	0,0181
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	0,5116 to 1,077	Yes	****	<0,0001

Total Ceramide

Bonferroni's multiple comparisons test

Comparison	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-1,947 to 0,2487	No	ns	0,3621
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-1,761 to 0,4346	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-1,61 to 0,5859	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-0,7548 to 1,441	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-2,169 to 0,02678	No	ns	0,0621
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,7778 to 1,418	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-3,478 to -1,282	Yes	****	<0,0001
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,768 to 1,428	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-2,2 to -0,003882	Yes	*	0,0485
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	-2,014 to 0,1821	No	ns	0,2149
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-1,863 to 0,3333	No	ns	0,6924
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-1,007 to 1,189	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-2,422 to -0,2258	Yes	**	0,008
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-1,03 to 1,166	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-3,73 to -1,534	Yes	****	<0,0001
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-1,021 to 1,175	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-0,7607 to 1,435	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	0,09444 to 2,29	Yes	*	0,0233
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-1,32 to 0,8761	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	0,07149 to 2,267	Yes	*	0,028
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-2,628 to -0,4325	Yes	**	0,0015
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	0,08126 to 2,277	Yes	*	0,0259
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-0,9467 to 1,249	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-0,09149 to 2,104	No	ns	0,1045
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-1,506 to 0,6901	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,1144 to 2,081	No	ns	0,1256
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-2,814 to -0,6184	Yes	***	0,0004
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,1047 to 2,091	No	ns	0,1161
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-1,657 to 0,5388	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,2657 to 1,93	No	ns	0,4133
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-2,966 to -0,7697	Yes	***	0,0001
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,256 to 1,94	No	ns	0,3832
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-2,512 to -0,3163	Yes	**	0,0039
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-1,121 to 1,075	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-3,821 to -1,625	Yes	****	<0,0001
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-1,111 to 1,085	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-2,406 to -0,2106	Yes	**	0,0091
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	0,3031 to 2,499	Yes	**	0,0043
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-3,798 to -1,602	Yes	****	<0,0001
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-1,088 to 1,108	No	ns	>0,9999

Uncorrected Fisher's LSD

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	-0,3495 to 0,8546	No	ns	0,3919
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-0,4161 to 0,788	No	ns	0,5267
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	0,2532 to 1,457	Yes	**	0,0077
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	0,7893 to 1,993	Yes	***	0,0001
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	2,108 to 3,312	Yes	****	<0,0001

Total Sphingomyelin

Bonferroni's multiple comparisons test

Comparison	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-4,621 to 0,5708	No	ns	0,3423
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	0,391 to 5,583	Yes	*	0,0131
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-3,167 to 2,024	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-0,3677 to 4,824	No	ns	0,1742
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-4,642 to 0,5498	No	ns	0,3194
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-1,044 to 4,148	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-5,904 to -0,7124	Yes	**	0,0044
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,4463 to 4,745	No	ns	0,2266
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-7,29 to -2,098	Yes	****	<0,0001
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	-2,278 to 2,914	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-5,836 to -0,6446	Yes	**	0,0055
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-3,037 to 2,155	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-7,311 to -2,119	Yes	****	<0,0001
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-3,713 to 1,479	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-8,573 to -3,381	Yes	****	<0,0001
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-3,115 to 2,076	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-1,142 to 4,049	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	1,657 to 6,849	Yes	***	0,0002
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-2,617 to 2,575	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	0,981 to 6,173	Yes	**	0,0018
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-3,879 to 1,313	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	1,579 to 6,77	Yes	***	0,0003
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-6,154 to -0,9626	Yes	**	0,0019
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-3,355 to 1,837	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-7,629 to -2,437	Yes	****	<0,0001
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-4,031 to 1,161	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-8,891 to -3,699	Yes	****	<0,0001
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-3,433 to 1,758	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-4,07 to 1,121	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-0,4724 to 4,719	No	ns	0,2472
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-5,333 to -0,1409	Yes	*	0,0309
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	0,1252 to 5,317	Yes	*	0,0326
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-6,87 to -1,678	Yes	***	0,0002
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-3,272 to 1,92	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-8,132 to -2,941	Yes	****	<0,0001
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-2,674 to 2,517	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-3,858 to 1,334	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	1,6 to 6,791	Yes	***	0,0002
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-7,456 to -2,264	Yes	****	<0,0001
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-1,998 to 3,193	No	ns	>0,9999

Uncorrected Fisher's LSD

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	1,246 to 4,092	Yes	***	0,0009
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	3,589 to 6,435	Yes	****	<0,0001
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	1,376 to 4,223	Yes	***	0,0006
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	2,175 to 5,021	Yes	****	<0,0001
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	4,034 to 6,881	Yes	****	<0,0001

Total Phosphatidylcholine

Bonferroni's multiple comparisons test

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-35,77 to -0,8703	-50,15 to 13,51	No	ns
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-8,529 to 26,37	-22,91 to 40,75	No	ns
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-26,89 to 8,018	-41,26 to 22,39	No	ns
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-18,31 to 16,59	-32,69 to 30,97	No	ns
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-34,76 to 0,1427	-49,14 to 14,52	No	ns
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-30,71 to 4,195	-45,08 to 18,57	No	ns
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-53,79 to -18,89	-68,17 to -4,51	Yes	*
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-33,28 to 1,625	-47,65 to 16	No	ns
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-37,52 to -2,613	-51,89 to 11,76	No	ns
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	-10,27 to 24,63	-24,65 to 39,01	No	ns
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-28,63 to 6,276	-43 to 20,65	No	ns
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-20,05 to 14,85	-34,43 to 29,23	No	ns
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-36,5 to -1,6	-50,88 to 12,78	No	ns
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-32,45 to 2,453	-46,83 to 16,83	No	ns
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-55,53 to -20,63	-69,91 to -6,252	Yes	**
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-35,02 to -0,1174	-49,4 to 14,26	No	ns
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-8,563 to 26,34	-22,94 to 40,72	No	ns
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	0,01238 to 34,92	-14,36 to 49,29	No	ns
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-16,44 to 18,46	-30,81 to 32,84	No	ns
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-12,39 to 22,52	-26,76 to 36,89	No	ns
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-35,47 to -0,5646	-49,84 to 13,81	No	ns
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-14,96 to 19,95	-29,33 to 34,32	No	ns

0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-35,81 to -0,9047	-50,18 to 13,47	No	ns
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-27,23 to 7,671	-41,61 to 22,05	No	ns
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-43,68 to -8,78	-58,06 to 5,597	No	ns
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-39,63 to -4,728	-54,01 to 9,649	No	ns
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-62,71 to -27,81	-77,09 to -13,43	Yes	**
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-42,2 to -7,298	-56,58 to 7,079	No	ns
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-25,33 to 9,576	-39,7 to 23,95	No	ns
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-21,27 to 13,63	-35,65 to 28	No	ns
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-44,36 to -9,453	-58,73 to 4,924	No	ns
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-23,84 to 11,06	-38,22 to 25,43	No	ns
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-33,9 to 1,001	-48,28 to 15,38	No	ns
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-29,85 to 5,053	-44,23 to 19,43	No	ns
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-52,93 to -18,03	-67,31 to -3,652	Yes	*
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-32,42 to 2,483	-46,8 to 16,86	No	ns
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-36,48 to -1,578	-50,86 to 12,8	No	ns
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-15,97 to 18,93	-30,35 to 33,31	No	ns
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-40,53 to -5,63	-54,91 to 8,747	No	ns
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-20,02 to 14,88	-34,4 to 29,26	No	ns

Uncorrected Fisher's LSD

Comparison	Mean Diff,	95,00% CI of diff,	Significant?	Summary
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	1,742	-15,71 to 19,19	No	ns
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	27,24	9,793 to 44,7	Yes	**
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	8,576	-8,876 to 26,03	No	ns
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	4,052	-13,4 to 21,5	No	ns
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	20,51	3,06 to 37,96	Yes	*

Total Phosphatidylethanolamine

Bonferroni's multiple comparisons test

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-12,25 to 12,93	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-3,549 to 21,63	No	ns	0,5784
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-19,32 to 5,854	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-8,016 to 17,16	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-19,72 to 5,454	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-10,13 to 15,05	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-16,05 to 9,124	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-9,595 to 15,58	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-18,78 to 6,4	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	-10,08 to 15,1	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-25,85 to -0,6747	Yes	*	0,031
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-14,54 to 10,63	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-26,25 to -1,075	Yes	*	0,0234
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-16,66 to 8,519	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-22,58 to 2,595	No	ns	0,3044
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-3,89 to 21,29	No	ns	0,7242
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-19,66 to 5,513	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-8,357 to 16,82	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-20,06 to 5,113	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-10,47 to 14,71	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-16,39 to 8,783	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-9,936 to 15,24	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-28,36 to -3,185	Yes	**	0,0053
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-17,05 to 8,12	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-28,76 to -3,585	Yes	**	0,004
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-19,17 to 6,01	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-25,09 to 0,08536	No	ns	0,0531
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-18,63 to 6,542	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-12,99 to 12,19	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-3,393 to 21,78	No	ns	0,5214
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-9,318 to 15,86	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-2,861 to 22,31	No	ns	0,3646
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-24,29 to 0,8824	No	ns	0,093
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-14,7 to 10,48	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-20,62 to 4,552	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-14,17 to 11,01	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-8,917 to 16,26	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-2,461 to 22,71	No	ns	0,2777
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-18,51 to 6,663	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-12,06 to 13,12	No	ns	>0,9999

Uncorrected Fisher's LSD

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	-0,3733 to 13,43	No	ns	0,0625
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	1,796 to 15,6	Yes	*	0,0161
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	4,403 to 18,21	Yes	**	0,0027
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	2,692 to 16,5	Yes	**	0,0089
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,4454 to 13,36	No	ns	0,0652

Total PE Based Plasmalogens

Dunn's multiple comparisons test

Comparisosns	Mean rank diff,	Significant?	Summary	Adjusted P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	0	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-13	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-13,33	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-19,33	No	ns	0,3218
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-10,67	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-20,33	No	ns	0,2102
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-6	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-11,33	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	17,67	No	ns	0,629
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	4,667	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	4,333	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-1,667	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	7	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-2,667	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	11,67	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	6,333	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-13,33	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-19,33	No	ns	0,3218
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-10,67	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-20,33	No	ns	0,2102
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-6	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-11,33	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-0,3333	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-6,333	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	2,333	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-7,333	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	7	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	1,667	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	2,667	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-7	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	7,333	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	2	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	8,667	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-1	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	13,33	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	8	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	4,667	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-0,6667	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	14,33	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	9	No	ns	>0,9999

Uncorrected Fisher's LSD

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	-9,028 to -1,048	Yes	*	0,0159
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-7,805 to 0,1742	No	ns	0,0599
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-6,975 to 1,004	No	ns	0,1342
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-7,458 to 0,5217	No	ns	0,0848
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-5,166 to 2,813	No	ns	0,5453

Total Phosphatidylserine

Bonferroni's multiple comparisons test

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-8,824 to 5,286	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-6,02 to 8,09	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-9,926 to 4,184	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-8,673 to 5,437	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-10,68 to 3,429	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-11,2 to 2,908	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-11,83 to 2,283	No	ns	0,8162
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-11,42 to 2,692	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-8,865 to 5,244	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	-6,062 to 8,048	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-9,967 to 4,143	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-8,714 to 5,395	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-10,72 to 3,388	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-11,24 to 2,866	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-11,87 to 2,241	No	ns	0,7778
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-11,46 to 2,65	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-8,157 to 5,953	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-6,904 to 7,206	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-8,912 to 5,198	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-9,433 to 4,677	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-10,06 to 4,052	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-9,649 to 4,461	No	ns	>0,9999

0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-10,96 to 3,149	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-9,708 to 4,402	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-11,72 to 2,394	No	ns	0,9289
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-12,24 to 1,873	No	ns	0,5039
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-12,86 to 1,248	No	ns	0,2365
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-12,45 to 1,657	No	ns	0,389
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-7,81 to 6,3	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-8,331 to 5,779	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-8,956 to 5,153	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-8,547 to 5,563	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-9,063 to 5,047	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-9,584 to 4,526	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-10,21 to 3,901	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-9,8 to 4,31	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-8,201 to 5,908	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-7,792 to 6,318	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-7,68 to 6,43	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-7,271 to 6,839	No	ns	>0,9999

Uncorrected Fisher's LSD

Comparison	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	-3,827 to 3,91	No	ns	0,9824
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-1,065 to 6,672	No	ns	0,1462
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-2,615 to 5,121	No	ns	0,507
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-4,389 to 3,347	No	ns	0,7815
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-3,459 to 4,277	No	ns	0,8276

Total Phosphatidylionositol

Bonferroni's multiple comparisons test

Comparison	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-13,41 to 10,31	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-9,524 to 14,19	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-17,7 to 6,012	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-14,31 to 9,407	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-15,88 to 7,838	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-14,29 to 9,43	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-16,54 to 7,175	No	ns	>0,9999
0 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-12,76 to 10,96	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Nonresistant	-11,14 to 12,58	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 0 CCS; 10 µM Mitotane Resistant	-7,257 to 16,46	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-15,44 to 8,279	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-12,04 to 11,67	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-13,61 to 10,1	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-12,02 to 11,7	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-14,28 to 9,442	No	ns	>0,9999
0 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-10,49 to 13,22	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-16,16 to 7,56	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-12,76 to 10,95	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-14,33 to 9,385	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-12,74 to 10,98	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-14,99 to 8,722	No	ns	>0,9999
0 CCS; 10 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-11,21 to 12,5	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Nonresistant	-20,04 to 3,678	No	ns	0,731
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 0 µM Mitotane Resistant	-16,64 to 7,072	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-18,21 to 5,503	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-16,62 to 7,096	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-18,88 to 4,84	No	ns	>0,9999
0 CCS; 10 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-15,09 to 8,622	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-10,03 to 13,68	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-8,44 to 15,28	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-10,7 to 13,02	No	ns	>0,9999
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-6,914 to 16,8	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Nonresistant	-13,43 to 10,29	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 20 µM Mitotane Resistant	-11,83 to 11,88	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-14,09 to 9,626	No	ns	>0,9999
5 CCS; 0 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-10,31 to 13,41	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-12,52 to 11,2	No	ns	>0,9999
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-8,74 to 14,98	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Nonresistant	-14,11 to 9,603	No	ns	>0,9999
5 CCS; 20 µM Mitotane Resistant vs. 5 CCS; 50 µM Mitotane Resistant	-10,33 to 13,38	No	ns	>0,9999

Uncorrected Fisher's LSD	95,00% CI of diff,	Significant?	Summary	Individual P Value
0 CCS; 0 µM Mitotane Nonresistant vs. 0 CCS; 0 µM Mitotane Resistant	-8,769 to 4,235	No	ns	0,4755
0 CCS; 10 µM Mitotane Nonresistant vs. 0 CCS; 10 µM Mitotane Resistant	-2,62 to 10,38	No	ns	0,2274
5 CCS; 0 µM Mitotane Nonresistant vs. 5 CCS; 0 µM Mitotane Resistant	-3,107 to 9,897	No	ns	0,2891
5 CCS; 20 µM Mitotane Nonresistant vs. 5 CCS; 20 µM Mitotane Resistant	-4,91 to 8,094	No	ns	0,615
5 CCS; 50 µM Mitotane Nonresistant vs. 5 CCS; 50 µM Mitotane Resistant	-2,72 to 10,28	No	ns	0,2392

16 Eidesstattliche Versicherung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.
Außerdem versichere ich, dass ich diese Dissertation an keiner anderen Fakultät eingereicht habe. Ich habe bisher keinen anderen erfolgreichen oder erfolglosen Promotionsversuch unternommen.

Berlin,

Eric Seidel