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Review article

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UNDERSTANDING MITOTANE MODE OF ACTION

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Adrenocortical carcinoma is a rare disease with poor prognosis. Mitotane is the most effective agent in post-operative treatment (or when inoperable). It selectively limits growth and bioactivity of adrenal tissue. Despite 60 years of use, the basis for its action has yet to be convincingly established. This review summarizes current knowledge of mitotane effects, based on studies on adrenal tissue and primary cell cultures, with emphasis on more recent studies of cell lines. We consider features of the adrenal cortex that might explain mitotane selectivity, and review effects on non-adrenal cells. Since the most clear-cut mitotane effects have been observed for mitochondria, this topic is the core of the review. Mitochondria present unique characteristics in steroidogenic tissue and are known to be important in malignancy development and apoptosis. We look at the evidence for mitotane activation within mitochondria, its impact on mitochondrial energy metabolism and other cellular processes as well as on downstream effects in the cell, such as apoptosis initiation. Further genomic and proteomic investigative studies are likely to yield useful results.

Key words: *adrenal cortex neoplasms, adrenocortical carcinoma, apoptosis, cell culture, proteome, proteome, mitochondria, mitotane, primary cell culture*

HISTORICAL BACKGROUND

Mitotane (o,p'DDD) is an adrenolytic drug recommended for treatment of primary and recurrent adrenocortical carcinoma (ACC) (1-4), and also in Cushing syndrome (5). Though it has had limited success, no pharmacological options of better efficacy have yet become available. Mitotane synthesis from the insecticide DDT and its recognition as an agent that selectively damages adrenocortical tissue originates in the 1940s. Since 1959, mitotane has been used for treatment of inoperable ACC (6), but its role in prolonging survival has been in question.

In 2002, the European Commission granted the drug orphan designation under the trade name Lysodren, based on the low prevalence of the disease. A large retrospective study by Terzolo *et al*., which investigated mitotane use as an adjuvant treatment (7), has led to its widespread adoption in this setting. It remains a question whether continuing mitotane use assists disease-free and overall survival in low-grade and radically operated ACC patients. This is being addressed in an ongoing international study (ADIUVO), which randomises patients to treatment or no treatment (8, 9). This initiative builds on ongoing highly successful collaboration established through the European Network for the study of Adrenal Tumours (ENS@T). Addition of chemotherapeutic agents to mitotane has been assessed in the international FIRM-ACT study, where the treatment aim was to prevent metastasis development or further growth of a tumour that had not been fully resected. Additional treatment with a combination of etoposide, deoxyrubicin and cisplatin was compared with streptozocin, with switching to the alternative if disease progression occurred. The first was associated with a better response rate and progression - free survival but there was no difference in effect on overall survival, which was still regarded as dismal, with medians of 14.8 and 12.0 months (10).

The treatment aim is to prevent metastasis development or further growth of a tumour that has not been fully resected.

Mitotane sensitivity differs widely between species, but there is a relationship between morphological and functional toxic effects. The dog, a particularly sensitive species, has been studied the most. Observations in the human are fragmentary and harder to interpret, but high mitotane doses do cause adrenal atrophy. In contrast, rats, mice, rabbits and monkeys are relatively insensitive (11). Administration of mitotane results in gross destruction of zona fasciculata and zona reticularis of the adrenal cortex, but not of zona glomerulosa, which correlates across species with loss of steroid secretion that is dependent on adrenocorticotrophic hormone (ACTH) (12). Observed by light microscopy, the orderly arrangement of cells into cords in the zona fasiculata is disrupted, the nuclei show heteropycnosis and loss of cellular integrity is visible. Under the electron microscope the mitochondria show stages of destruction, from swelling and loss of the close packing of the tubular cristae to dissolution of the internal architecture to complete collapse of the cristae. The smooth endoplasmic reticulum (ER) loses its regular pattern. The same gross and ultrastructural effects are seen in H295R and SW13 cell lines established from human ACC tissue and show a dose-response relationship in the therapeutic range (12).

Extensive investigations into the mechanisms of adrenocorticolytic activity, reviewed below, offer potential clues to the mode of action of mitotane; however, no entirely coherent understanding has yet emerged of the characteristics of the adrenal cortex cells that explains their sensitivity to mitotane.

MITOTANE EFFECTS IN ADRENOCORTICAL PRIMARY CULTURES AND CELL LINES

The NCI-H295 cell line was established from a human ACC with the aim of preserving their typical clinical, biochemical, and pathological properties. They show features of steroid-secreting cells, including a well-developed smooth ER and a Golgi apparatus characteristic of secretory activity, with mitochondrial cristae typical for adrenal tissue. They retain a number of features of the original cells, including large numbers of mitochondria, nuclei with prominent nucleoli and lipid droplets. Production of a large range of steroids, reflecting a full complement of steroidogenic enzyme, was established at the outset (14). This cell line has been the most important in uncovering mitotane mechanisms of action. The 3-dimensional structure of adrenocortical cristae has been clarified by use of electron microscopic tomography (15). *Fig. 1* represents an arrangement of tubular cristae that is likely in adrenocortical cells.

The cell line, SW13, was derived from a small cell carcinoma in the adrenal cortex, but however, it does not show any capacity for steroid production and is endothelial in appearance (10). It has, however, played a supporting role in studies of mitotane action, showing similar gross and ultrastructural damage to H295 cells and similar effects on mitochondrial respiratory chain enzyme activity (13) (17).

Fang-8 cells are another cell line derived from an ACC, which in this case was feminising. They retain the original capacity for oestrogen secretion, but do not secrete other classes of steroids. They have characteristics of zona reticularis cells, with elongated mitochondria with tubular cristae and lipid droplets (11).

The above adrenal cell cultures have provided the basis for the most productive studies. In one study of H295 cells, mitotane rapidly accumulated intracellularly in a dose- and time-dependent manner, in parallel with an impact on viability and proliferation within the therapeutic range of 30 – 50 µM. After 2 h of exposure to 50 µM mitotane, more than half of the cells had undergone apoptosis, with swelling and depolarisation of the mitochondrial membrane (17). Progressive dissolution of the inner matrix was visible by electron microscopy. In contrast, there was no significant effect on fibroblasts, used in the same study as a control. Immunocytochemistry using a COX-2 (a respiratory chain enzyme) antibody showed that this concentration caused drastic morphological changes to a more punctiform appearance during mitochondrial fragmentation. These findings support the proposal that the main role of mitotane is induction of oxidative stress within the adrenal tissue (18).

Exposure of Fang 8 cells to mitotane resulted in microscopically visible evidence of a toxic reaction, including air bubbles at the cell surface and cell shrinkage, followed by partial detachment from the culture surface. There were clear ultrastructural changes in mitochondria. Blockage of cell division, growth and function occurred in a dose-related manner with progressive loss of cell protein at concentrations above 84 µM. Interestingly, when surviving cells that remained attached were returned to mitotane-free medium, they recovered normal function with no sign of any residual toxic effect (11).

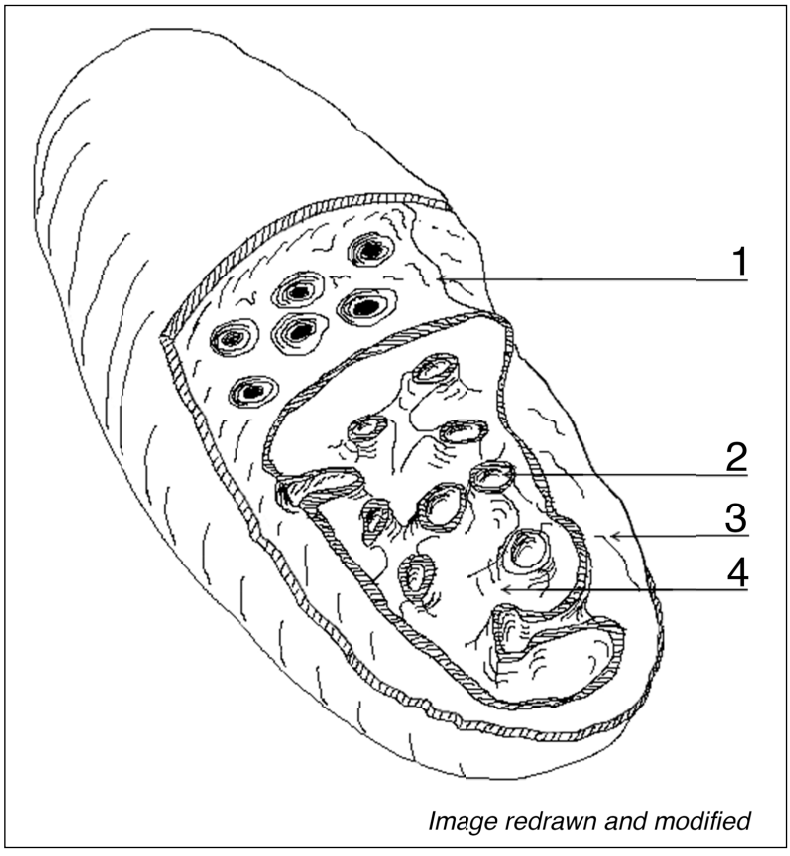

Fig. 1. Mitochondria with tubular cristae characteristic of steroidogenic tissue. Matrix and the outer membrane surround the organelle. Internal features are: inner membrane [1], tubular cristae [2], intracristal space [3] and matrix [4]. Adapted from ref. (15), (77).

Primary adrenal cell cultures from dogs have been used to demonstrate loss of steroidogenesis and cell death in response to mitotane, among other toxicants (19). *Table 1* summarises major studies of mitotane effects on adrenal tissue, primary cell cultures and cell lines.

MITOTANE EFFECTS ON THE STEROIDOME

Short term incubation of adrenal cells derived from human adrenal tumours exposed or not to mitotane for 2 h with radiolabelled steroid precursors showed rates of conversion of 11-deoxycortisol to cortisol and cortisone to be much lower in five treated patients (mean 1.8; 0.6% respectively) than in two controls (mean 11; 11%) or two untreated patients (mean 34; 64%) (20). Conversion rates of corticosterone to aldosterone were lower for 5 patients (mean 0.14%) than for one control (0.32%) (20). These may be explained by inhibition of the mitochondrial enzymes CYP11B1 and CYP11B2 respectively (*Fig. 2*). Stigliano and colleagues incubated the H295R cell line with 10 µM mitotane, a dose that minimally inhibits cell proliferation (18). After 72 h, percent inhibition of production of four targeted steroids was: progesterone - 68, testosterone - 55, cortisol - 70 and aldosterone - 49. This indicates additional inhibition of conversion of cholesterol to pregnenolone, the other mitochondrial step in the steroid biosynthetic pathways by CYP11A1 and/or the related StAR protein (see *Fig. 2*). In contrast, it has not been conclusively shown that the steps catalysed by microsomal enzymes were affected. Hescot and colleagues investigated H295R cells exposed to 50 µM mitotane for 48 h and found over 80% suppression of cortisol and 17-hydroxyprogesterone secretion (17).

Gene expression analyses using microarray and qPCR techniques have given results that are variable between different authors, some suggesting that suppression of activity of the steroidogenic mitochondrial enzymes by mitotane is not accompanied by decreased expression of their genes while others indicate that different genes are suppressed. These include the microsomal steroidogenic enzymes *HSD3B1, HSD3B2* and *CYP21A2* (21), *CYP11A1* and *CYP17A* (22), and *STAR, CYP11A1, HSD3B2,* and *CYP11B2* in H295R cells (17). These findings are difficult to reconcile, but do offer the possibility that the observed steroid inhibitory effects are not only mediated by inhibition of CYP11s or by adrenolytic activity, but by a direct effect on other steroidogenic enzymes levels (21).

It would be surprising if the observed decreases of 11β-hydroxylation and of cholesterol to pregnenolone conversion were not also due to deleterious effects on the mitochondria rather than on gene expression. Direct competitive inhibition is also possible, but, as described below, mitotane action is not attenuated by the specific 11-hydroxylase blocking agents metyrapone and etomidate (23).

An additional mitotane mode of action on steroidogenesis may be through mimicking steroids *via* binding to steroid receptors or duplicating their non-genomic effects. As small organic diphenolic compounds, they share features with other well-known endocrine disruptors, including natural compounds, such as lignans, equol and synthetic mimics, such as diethylstilbestrol (21, 24).

MITOTANE EFFECTS ON THE PROTEOME

Mitotane and its active metabolites produce irreversibly bound protein adducts on incubation with H295R cells (25). Modulation of the proteome in the H295R cell line seems to most closely relate to processes of stress response and energy metabolism (26). Important further insights have been provided by studies using these cells by Stigliano *et al.* (18) who used two-dimensional electrophoresis and MALDI-TOF to identify

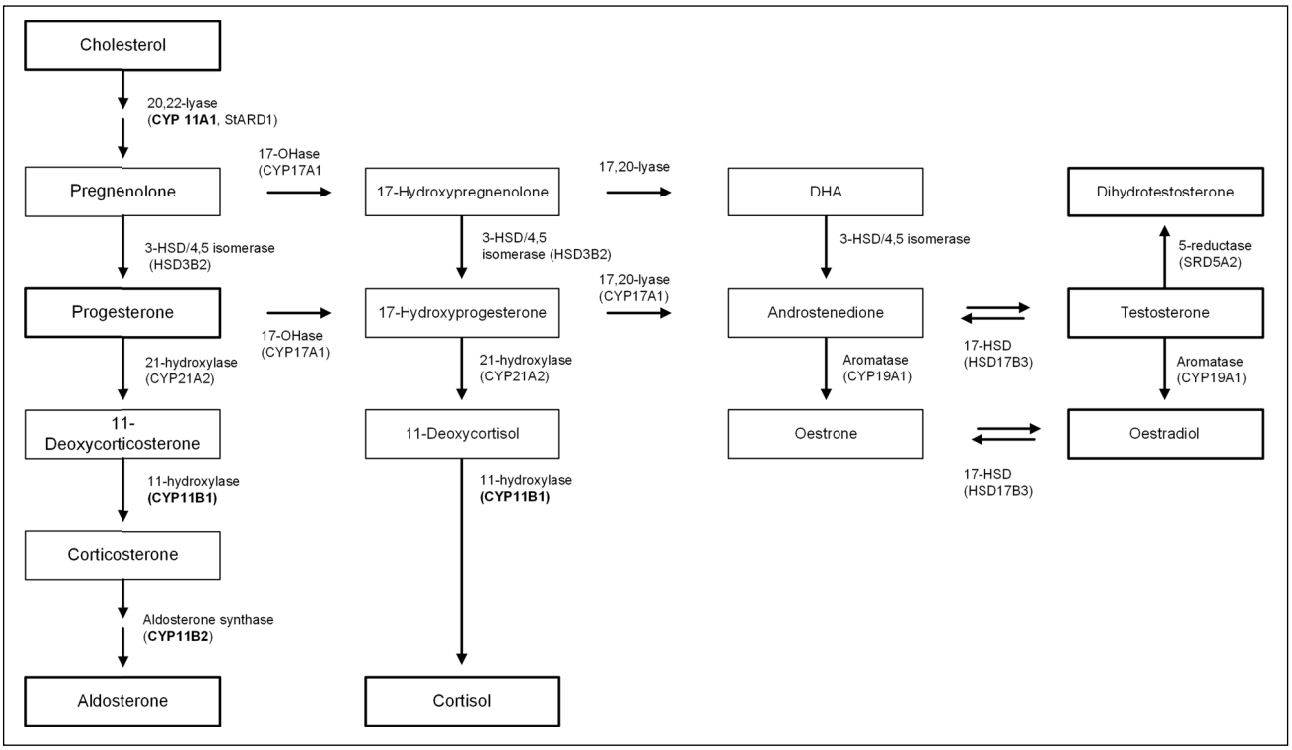

Fig. 2. Pathways of steroid hormone synthesis. Mitochondrial enzymes are shown in bold, (based on ref. (75), (78), (79)).

Table 1. Described effects of mitotane in adrenal (H295R, SW13, Fang-8) and non-adrenal cell lines, primary cultures, and animal adrenal tissue in the preclinical studies.

Mitotane effects on:	**Cellular material**	**References**
Gross morphology and cell growth		
Inhibition of cell growth and proliferation	H295R	(26), (28), (62), (82)
Blockage of cell division, growth and function in a dose-related, reversible manner	H295R	(11)*, (26)
G2 arrest	H295R	(22), (62)
Cytotoxicity, with more than half cells undergoing apoptosis in the therapeutic range	H295R	(11)*, (13)
Cell death	Adrenal primary culture	(19)
Higher toxicity for adrenal cell lines than other cell lines	H295R	(13), (28)
Higher toxicity for adrenal cell lines when combined with IGF-1R antagonists	H295R mouse xenograft	(83)
Apoptosis	H295R	(11)*, (13), (18), (22), (26), (42), (83), (84),
Caspase cascade-mediated apoptosis	H295R	(22)
Apoptosis not acting by the P53 c-Myc pathway	H295R	(22), (62)
Suppression of tumour necrosis factor alpha (TNF)-α-induced apoptosis	ER-positive MCF-7 human breast carcinoma cells	(85)
Combined stronger inhibition of cell growth with sirolimus	H295R	(82)
Combined stronger inhibition of cell growth with gemcitabine	SW13	(34)
Combined weaker inhibition of cell growth with gemcitabine	H295R	(34)
Adrenal damage observed by light microscopy, including haemorrhage and necrosis	Animal tissue preps	(1), (26), (31), (34), (40)
Similarity of effects across animal species	Animal tissue preps	(33), (34), (40)
Mitochondrial structure		
Visible evidence of cytotoxicity on adrenal cells on electron microscopy	H295R	(11)*, (13), (26), (87)
Visible evidence of a toxic reaction on electron microscopy	Animal tissue preps	(12)
Swelling or defragmentation of mitochondria with dissolution of the inner matrix.	H295R	(11)*, (13), (17)
Swelling or defragmentation of mitochondria	Animal tissue preps	(12), (31), (34), (40)
Mitochondrial function		
Enhancement of mitochondrial biogenesis	H295R	(42)
Mitochondrial membrane depolarisation and permeability	H295R	(13), (17), (18)
Decrease of net oxygen consumption	H295R	(13)
Effects on mitochondrial respiratory chain enzyme activity, oxidative stress, ROS production	H295R	(13), (17), (18), (26), (42)

Note: * reference 11 described Fang-8 cells.

proteins that undergo changes. These included proteins involved in energy metabolism, stress response, cytoskeletal structure and tumourigenesis. Sampling at 15 min, 1, 5, 24 and 48 h after mitotane exposure showed both increased and decreased expression, with opposite changes at different time points for some proteins.

Proteins modulated by mitotane included those involved in energy metabolism such as: D-3 phosphoglycerate dehydrogenase isoforms, and nucleotide diphosphate kinase (which play a role in cholesterol trafficking to the inner mitochondrial membrane) and the glycolytic enzymes triose phosphate isomerase and enolase. Proteins involved in stress response include peroxiredoxins 2 and 6 (with antioxidant functions), heat shock proteins B1 (HSP 27) and the 70 kDa protein 1A, together with heat shock cognate 71 kDa protein. These stress response proteins play roles in protection of cells against oxidative stress and cytotoxic effects and possibly conferring drug resistance. The next group of proteins affected by mitotane is related to the cytoskeleton and comprises profilin, which is involved in actin filament polymerization, and tubulin, which is an intrinsic component of mitochondrial membranes. This mitotane effect would interfere with

Mitotane effects on:	Cellular material	References
Mitochondria associated ER membranes		
ER stress caused by mitotane with key role of inhibition of SOAT1 enzyme complex, resulting in reduction of cholesterol ester and increase of free cholesterol, transducing signal through transciptional factors, with final CHOP triggering intrinsic pathway by interaction with Bax and Bcl-2. Action mimics Sandoz 58-035 (SOAT1 inhbitor)	H295R	(59)
FATE1 expression decreases apoptosis on exposure to mitotane; FATE1 knockdown increases it	Dox - treated H295RTR SF-1	(55)
Mitotane mechanism of action is similarto ATR-101 (SOAT1 inhibitor) (23), inducing caspase-dependent apoptosis	H295R	(86)
Cell biochemistry - interaction with steroidogenesis		
Loss of steroidogenesis, steroid inhibitory effects	H295R	(17), (18), (21), (26), (42)
Loss of steroidogenesis, steroid inhibitory effects	Adrenal primary culture	(19), (20)
Non effectiveness of unchanged mitotane; activation involves cytochrome P450(s)	Adrenal primary culture	(20), (80)
Activation *via* cytochrome P450(s)	Animal tissue preps	(26), (31), (33), (34), (40)
Steroid inhibitory effects or loss of steroidogenesis	Animal tissue preps	(1), (32)
Androgen receptor antagonism	Animal tissue preps	(1)
Proteome		
Intracellular formation and irreversible binding of protein adducts	H295R	(13), (26)
Modulation of proteins playing roles in: tumourigenesis, respiratory chain enzyme activity, oxidative stress, cytoskeletal structure, growth, ageing, transcription, RNA splicing	H295R	(18)
Mitochondrial protein macromolecule formation	Animal tissue preps	(26)
Irreversible formation and binding of protein adducts and binding to phospholipids	Animal tissue preps	(34), (40)
Genome		
Suppression of complex IX (COX) gene expression but not complex II or III	H295R	(17)
Expression changes in 567/1967 genes: of 30 most downregulated, 8 involve in lipid metabolism and steroidogenesis; of 30 most upregulated, 6 relate to apoptosis; activation of 22/53 ER stress response genes	H295R	(58)
Suppression of genes coding for steroidogenic enzymes	H295R	(17), (21), (22), (36), (87)
Oestrogenic effect via induction of gene expression for SHBG and CBG	Human hepatoma cells	(29)

Note: *reference 11 describes Fang-8 cells

mitochondrial membrane permeability and thus cholesterol trafficking (18).

Proteins modulated by mitotane that are involved in tumourigenesis play roles in growth, ageing, transcription and RNA splicing. This may be effective in reversing the changes to cell function that permit the growth of an ACC. Histidine triad nucleotide binding protein (Hint) is a hydrolytic enzyme that may function as a tumour suppressor, being involved in apoptosis by inhibition of TCF-β-catenin-mediated transcription, an activity that is constitutive in H295R cells. The prohibitin (PHB) protein level was initially diminished and then increased. Consistent overexpression of its isoforms PHB1p and PHB2p has been observed in neoplastic tissue from a wide range of anatomical sites. Although PHB proteins were originally considered to be putative negative regulators of the cell cycle, recent studies indicate that they act as chaperones in the assembly of subunits of mitochondrial respiratory chain complexes, binding directly to newly synthesised mitochondrial translation products and stabilising them against degradation. They may also play roles as structural scaffolds. Their overexpression in tumours may lead to reduction of oxidative stress and tumour insensitivity to this condition (27). Diminution of PHB expression by mitotane again suggests that mitochondrial injury and oxidative damage are the main sites of mitotane action. There was a decrease of heterogeneous nuclear ribonucleoprotein (hnRNP) isoforms A2/B1, proteins acting as multifunctional transcription and translation factors in human adrenal tissue. Importantly, B1 expression is boosted in various adrenal hormone-secreting tumours, with a negative correlation between B1 expression and steroidogenesis. Increase of cathepsin D is also a significant factor, which is activated by hnRNP (18).

MITOTANE EFFECTS ON NON-ADRENAL CELLS

While most literature reports have concentrated on the action of mitotane on adrenal tissue, it exerts toxic effect in a variety of

cell types at higher threshold concentrations. Our group showed that 10-µM mitotane (incubation for 24 h) exerts higher toxicity in H295R than in other human cancer cell lines from colon, lung and breast (40 µM for 48 h) (28).

There is a considerable body of evidence that non-adrenal effects of mitotane are widespread. For example, it has an estrogenic effect on sex hormone-binding and corticosteroid-binding globulins in hepatoma cell line transfected with human oestrogen receptor-α (29).

Concerns have also been raised about effects of mitotane residues and related chlorinated pesticides in the environment, especially on reproductive function of fish and other wildlife. Most *in vitro* studies on the effects of mitotane and related compounds on non-adrenal cells show that they do not involve the mitochondria and mimic one of the earliest events in the non-genomic action of steroids, which bind G-protein-coupled receptors to induce the formation of inositol triphosphate, leading to rapid influx of Ca^{2+} *via* voltage-gated Ca^{2+} channels or *via* activation of the ER Ca^{2+} pump, an intracellular messaging system that controls many cellular processes. Examples of these actions, reviewed by Wu *et al*. (30), include mimicry of androstenedione on luteinizing human granulosa cells, competing for the nuclear androgen receptor with the synthetic androgen compound R1881 in the PALM prostatic cell line, and mimicry of oestradiol action in the SKBR3 breast cancer cells. Mitotane binds to the plasma membrane receptor for progestogens in the ovaries of spotted sea trout; o,p'DDE blocks the progesterone-induced stimulation of sperm motility in Atlantic croaker. The changes in Ca^{2+} flux caused by pesticide exposure may affect calcium-binding proteins and gene expression, as shown in trophoblast cells, inhibiting cell proliferation and inducing apoptosis *via* several trophoblast differentiation genes. Mitotane and related compounds also appear to exert steroid receptor-mediated effects at high concentrations, for example, acting as an androgen antagonist at concentrations above 10 µM in a human hepatoma cell line transiently transfected with the human androgen receptor. The mitochondria are also responsive to Ca^{2+} elevations, with ATP formation, loss of cytochrome c (cyt c) and onset of apoptosis, but these occur at high concentrations, so may be relevant only *in vitro* (30).

MECHANISMS OF ADRENOCORTICAL CELL DEATH AND STASIS IN RESPONSE TO MITOTANE

Many observations suggest that there is a variety of different processes by which mitotane prevents adrenocortical cell proliferation and promotes cell death. These may include both direct toxic effects and those caused by toxic products uniquely produced in the adrenal glands that then interfere with general or unique features of adrenal biochemistry, leading to cell cycle arrest and apoptosis.

ACTIVATION OF MITOTANE

The specificity of mitotane towards adrenal cortex may derive from metabolic transformation of the drug to an active product *via* an enzyme system that is unique to this tissue. It was early established that extensive transformation of radiolabelled mitotane occurs *in vivo*; these products undergo covalent binding, with close correlation between rates of transformation, binding and bioactivity across species (31). Addition of mitotane to a cytochrome P450 fraction from bovine adrenals gave rise to a light absorption difference spectrum similar to that caused by steroid binding (32). Formation of adducts with electrophoretic mobility corresponding to P450scc, but not adrenodoxin, in canine, bovine and human adrenocortical homogenates has been demonstrated (33). Mitotane metabolism and covalent binding are closely correlated across species (34).

The hypothesis that unchanged mitotane is not an effective agent has been supported by a lack of a direct effect on primary

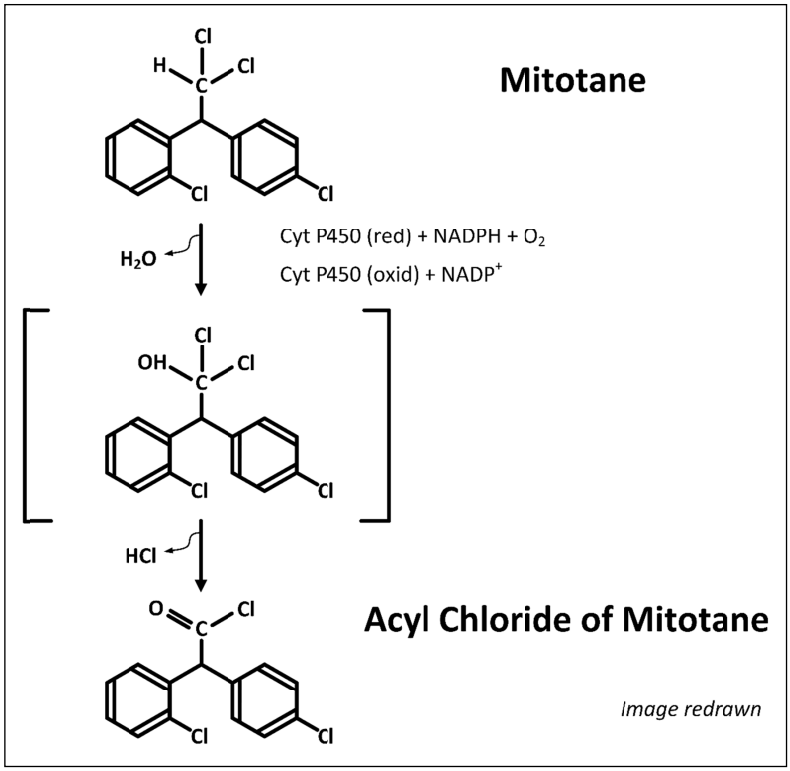

Fig. 3. Mitotane transformation to an active form. Adapted from reference (80).

culture of adrenal homogenates (20). Comparing toxic activity of mitotane analogues on Fang-8 cells has shown a common requirement for a dichloro- or trichlorethylene structure (11). The essential dichloromethyl moiety undergoes P450-catalysed β-hydroxylation followed by rapid dechlorination to generate an acyl chloride (*Fig. 3*).

The generation of the acyl chloride almost certainly is mediated by a mitochondrial cytochrome P450 (13), which does not seem to be CYP11B1, since mitotane shows equal toxicity towards the ACC cell lines expressing (H295R) and not expressing (SW13) this enzyme (17). However, transfection of the gene for *CYP11B1*, but not for *CYP11A1* or *CYP11B2*, into monkey kidney COS cells caused them to take up a mitotane analogue and generate metabolites (35). Other evidence against CYP11B1 is that it not subject to competition by the specific inhibitors etomidate and metyrapone. The less specific agent ketoconazole, which inhibits among others CYP11A1, CYP17 and CYP11B2, is a competitor, pointing to an alternative mitochondrial P450, possibly active in xenobiotic metabolism in the adrenal cortex (23, 36). Binding between mitotane and a cytochrome P450 has been directly observed using an antibody to CYP11A1. This offered a plausible candidate, given that it mediates a metabolic transformation inhibited by mitotane (below), but this was not competed for by its substrate, cholesterol, nor the CYP11A1 inhibitor, aminoglutethimide (37). Another candidate is CYP2W1. This is highly expressed in fetal life and in some cancers, most notably colon cancer, and whilst a clear physiological role has not been established, it shows catalytic activity towards a range of substrates including some procarcinogens. Ronchi *et al*. (38) reported CYP2W1 immunoreactivity in normal and malignant adrenocortical tissue, with increased expression in steroid-secreting compared with non steroid-secreting tumours and a modest positive effect of immunoreactivity on disease progression and survival time during mitotane treatment. However, Nole *et al*. (39), using an antibody claimed to be more specific for CYP2W1, did not find expression in either normal adrenocortical tissue or in adrenocortical tumours.

The acyl chloride is a highly reactive molecule, which can bind to particular binucleophiles in target cells to exert an adrenolytic effect *via* induced oxygen activation. Although not conclusively proven, metabolic transformation and oxidative damage through production of free radicals are generally accepted as the mechanisms that mediate this cytotoxic effect. It mostly binds to proteins that contain one to six phospholipids (40, 41). Addition of water causes generation of the acetic acid derivative o,p'DDA, which is extensively excreted in urine and has recently been shown to be inactive, with no antitumour properties in adrenal cells, being unable neither to activate oxidative stress and apoptosis in H295R cells, nor cause the down-regulation of the genes involved in steroidogenesis (42). Touitou *et al*. reported lack of effect on steroidogenesis in fresh homogenates of normal adrenals incubated for 2 h with two unsaturated metabolites (o,p'DDE or p,p'DDMU) at a concentration of 10 mM, or with p,p'DDOH at 2.1 mM (20).

THE MITOCHONDRION AS A TARGET FOR MITOTANE ACTIONS THAT LEAD TO APOPTOSIS

Apoptosis, or programmed cell death, is an orderly process of cell removal that results in generation of cell fragments that can undergo phagocytosis. The major routes of apoptosis are shown in *Fig. 4*. Mitotane is likely to act on the intrinsic pathway, also called the mitochondrial pathway, which is initiated from within the cell in response to signals resulting from DNA damage, loss of cell-survival factors, or other cell stressors, such as anoxia and toxins. There is now a consensus that these can cause accumulation of unfolded proteins (the unfolded protein response, UPR) within the endoplasmic reticulum (ER), resulting in release of ER calcium stores and transmission of calcium into the mitochondrial lumen, primarily *via* voltage-gated anion channels (VDAC), as a major initiator of the apoptotic cascade (43). Transmission is likely to take place at points of close contact between ER and the mitochondria, which can be fractionated as mitochondrial-associated membranes (MAM). Conversely, antiapoptotic members of the Bcl-2 family of proteins downregulate calcium flux through the plasma membrane and limit calcium release from the ER. While calcium is essential for mitochondrial function, being an activator of three Kreb's cycle enzymes, excess is deleterious. The described effects on mitochondrial morphology of calcium loading closely resemble those summarised above for effects of mitotane. There is also ample evidence for direct actions of mitotane on the mitochondria (43, 44).

Activation of Bax and Bak within the mitochondrial membrane causes opening of megachannels (permeability transition pores) *via* association of the pore protein mt-PTP with the proapoptotic protein Bax. This results in dissipation of the transmembrane potential and increase in reactive oxygen species (ROS), which intensifies efflux of cyt c and collapse of ATP generation (45). Decrease of the Bcl-2/Bax ratio appears to be crucial for apoptosis induction (46). The cyt c associates with the adaptor Apaf1 to recruit procaspase 9 to form the apoptosome. In the presence of ATP, caspase 9 is activated. This in turn activates effector (executioner) caspases 3, 6 and 7, which leads to the degradation of cell components (47, 48). The mitochondrial permeability transition pore can be activated by decreased membrane potential, high-energy phosphates (such as ATP), a more oxidised redox status, increased ROS production and increased Ca^{2+} level (49).

Promotion of apoptosis and cell death by mitotane has been shown in H295R and SW13 cells by Poli *et al*. (13), who used cytofluorimentric separation. They reported increase of both living and dead apoptotic cells, detected by tagging for caspase 3/7 activity, within the range 30 – 50 µM mitotane, accompanied by loss of membrane potential and a sharp reduction in oxygen consumption. These events coincided with damage to the mitochondrial cristae. Lehman, Wrzesinski and Jagodzinski (22) directly demonstrated increase of caspase 3/7 activity in the medium of H295R cells exposed to 62.5 µM and 100 µM mitotane. The lysosomal protease, cathepsin D, is increased by mitotane (18), so that direct damage to mitochondrial cristae is also possible.

The role of ROS in both promotion and prevention of apoptosis has been reviewed (50). They are products of oxidative phosphorylation and are increased by oxidative stress, such as in reperfusion injury (51). Their precursors are transported through VDAC. Mitochondria produce ROS by direct donation of excess electrons, primarily from complex I to O_2, giving rise to superoxide anions that generate H_2O_2 by catalysis by Mn superoxide dismutase, which is mostly found in complex III. In the presence of reduced transition metals, H_2O_2 can be the most reactive of hydroxyl radicals (52). Since the effects of mitotane on the respiratory chain complexes appear to be confined to inhibition of complexes I and IV, this may not be a point at which mitotane acts (17).

Initial release of ROS by mitochondria may be mediated by the adaptor protein p66shc, which translocates to the mitochondria and acts to strip electrons from the coenzyme respiratory chain (43). ROS could directly or indirectly increase the gating potential of the permeability transition pore, so that mitochondria would be both source and target of ROS. Mitochondrial hydroxylations are also known to be associated

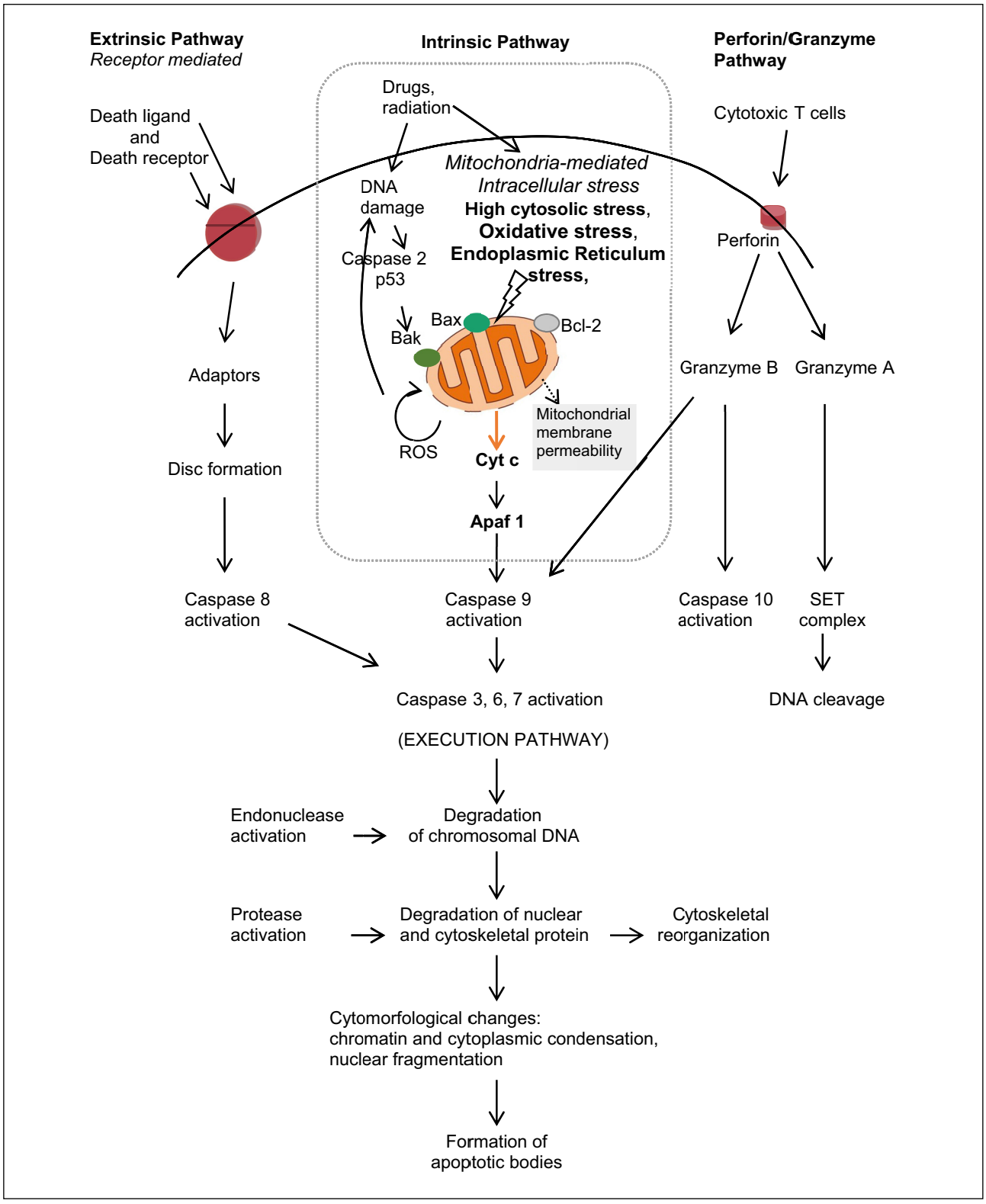

Fig. 4. Major apoptotic pathways (redrawn from Bali *et al*., (59), with additions from Beesoo *et al*., (81) and Ashkenazi *et al*., (47). A dotted-line box marks the mitochondrial pathway that is initially induced by mitotane.

with ROS generation, so that the process of activation of mitotane might itself be contributory to mitochondrial damage (41, 53).

Superoxide can act as a messenger between mitochondria, leading to a wave of mitochondrial membrane depolarisation and further ROS release. This change is reversible until a critical threshold is reached, leading to global depolarisation (54). Generation of ROS following exposure to mitotane is likely to be highly significant. A variable capacity for ROS production in adrenal tumours may offer an explanation for their variable sensitivity to mitotane (41).

Active forms of mitotane may react with specific targets. Covalent linkage with proteins may result in adducts that then accumulate in the intermembrane space. One plausible mechanism is formation of disulphides, damaging accumulation of which is associated with ROS production (33).

Calcium flux at the MAM appears to be a critical control point for signalling between ER and mitochondria, providing opportunities for cancers to limit apoptosis by diminishing passage and one at which mitotane can act to promote calcium influx and thus apoptosis. The cancer-testis antigen FATE1 is localised to the MAM and decreases calcium flux by widening the ER-mitochondrial distance and is overexpressed in some cancers. Doghman-Boughuerra (55) developed H295/TR SF-1 cells, in which overexpression of SF-1 was deoxycycline dependent and was found to induce high FATE1 expression. Using this model, they demonstrated a decreased apoptotic response to mitotane, but when FATE1 was knocked down, it was increased. Patients with adrenocortical tumours and receiving mitotane who had relatively high tumour expression of FATE1 had worse outcomes than those in whom it was low (55). Another factor regulating calcium flux at the MAM is the thioredoxin TMX1, offering another potential drug target (56).

Lipid signalling is also critical to MAM function. Drugs designed to inhibit esterification of cholesterol by sterol-O-acyl transferase 1 (SOAT1) were found to be toxic towards adrenocortical tissue. The SOAT inhibitor ATR-101 has been shown to activate a caspase response in H295 cells concomitant with increase of free cholesterol and ER stress responses (57). Mitotane has recently been shown to also inhibit this enzyme and mimic many of the drug actions on signalling systems that enhance apoptosis. In a very comprehensive series of studies in H295 cells, Sbiera *et al*. (58) have directly shown mitotane inhibition of this enzyme and an association with increase of free cholesterol and numerous other lipids, while genes involved in lipid metabolism and steroidogenesis were downregulated. Many genes associated with ER stress were upregulated, including CHOP activated by eIF2A, important elements of the signalling cascade that results in calcium loading of the mitochondria and triggers proapoptotic mitochondrial Bax and suppresses antiapoptotic Bcl-2. These effects were not seen in other cell types, which the authors ascribed to lower baseline expression of SOAT1 (58).

High-energy ATP or other phosphates can contribute to apoptosis induction. Interestingly, when all mitochondria in cells are ruptured after cyt c release and caspase activation, this results in necrosis. Necrotic cells can cause further damage, such as by triggering immune responses. Over-abundant apoptosis also causes tissue atrophy (59). When some mitochondria remain functional and produce enough ATP, apoptotic cell death occurs (54). While it seems likely that ATP generation overall is diminished by mitotane, local increases may trigger this process.

The tumour suppressor p53 stabilized by c-Myc appears not to be involved in mitotane-induced apoptosis: transcript levels for c-Myc are unchanged by mitotane in H295R cells (22). c-Myc guards the S phase of the cell cycle, whereas mitotane treatment has been shown to delay the G2 phase (18). However, at the G2/M checkpoint, p53 blocks cyclin-dependent kinase and the cyclin B complex, by inducing Gadd45, p21, and 14-3-3 sigma protein. Cells arrested in this pathways are in G2 phase (60). Gadd45 was found to be upregulated specifically in H295R, but not in three other cancer cell lines (breast, lung, colon) (28). This factor can trigger JNK stress-activated kinase-dependent apoptosis, suggesting that it might be one mediator of mitotane action (61). Comparison of the effects of ionizing radiation, mitotane and their combination on the cell cycle in H295R and SW13 cell lines showed an inhibition of cell growth in both cell types by radiation with and without mitotane. Cells recovered spontaneously after radiation alone but remained arrested in the G2 phase after combination treatment. In the latter, cyclin B1 in the form of a complex with Cdc2 proteins was increased, as was Cdk2 kinase activity. Sequence analysis of p53 showed a large deletion of exons 8 and 9. The same arrest by combination therapy occurred in H295R cells with restored wild-type p53, suggesting that this mechanism is not mediated by the p53, c-Myc pathway (62).

POTENTIAL GENOMIC EFFECTS OF MITOTANE RELATING TO MITOCHONDRIAL FUNCTION

Given the extensive above-mentioned evidence, mitochondria are the structures within the adrenal cells that play a major role in mitotane susceptibility. The major genetic signals important for mitochondrial integrity and function are described below. These are potential targets for mitotane action.

The mitochondria are responsible for efficient energy accumulation and management within the cell and thus have intimate connections with most biosynthetic pathways. The process of oxidative phosphorylation produces cellular energy, regulates mitochondrial and cellular redox status, generates most of the ROS and regulates Ca^{2+} concentration (63). As semi-autonomous bodies within the cells, mitochondria influence nuclear gene expression via a messaging system termed retrograde mitochondrial signalling. On the other hand, mitochondria require a nuclear contribution to produce many of their structural and functional proteins, since most of the required genes are located in nuclear DNA, comprising those responsible for mitochondrial structural elements, glycolysis and oxidative metabolism, components of the transcriptional machinery needed for expression of the mitochondrial genome as well as those required for nuclear genes, as reviewed by Whelan and Zuckerbraun (54). Proteins coded by nuclear genes destined for the mitochondria are tagged with a 20-35-mer peptide presequence and maintained in an unfolded state by chaperone proteins to enable passage across the mitochondrial membrane (64).

IMPORTANCE OF MITOCHONDRIA IN CANCER CELLS

Mitochondrial function is indispensable for cancer cells. Mutations of genes associated with mitochondrial structure and metabolism are known to enhance cell survival or promote cell proliferation and invasion (52). Mitochondrial defects have been associated with many cancer types. Warburg classically described how most cancer cells predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by a comparatively low-rate of glycolysis followed by pyruvate oxidation in mitochondria, as for the majority of normal cells (65). This phenomenon, commonly known as the ‘Warburg effect’ led to the assumption that mitochondrial function was unimportant in cancer cells, but measurements of absolute rates of mitochondrial oxidative metabolism often show that they are not lower than in normal cells and levels of ATP production are not impaired (66).

An important factor related to mitochondria is the well-known tumour suppressor gene coding p53 (*TP53*). Usually in the case of DNA damage or metabolic stresses, such as hypoxia, it acts with other cytokines to mediate growth arrest and induce apoptosis (67). However, in response to energy limitation it becomes phosphorylated by AMP-activated protein kinase, which activates cell cycle checkpoints and can stimulate tumourigenesis. Additionally, the activity of p53 supports ATP production by oxidative phosphorylation and decreased cellular

ROS production, due to an action on glycolysis and apoptosis regulation. It is responsible for negative regulation of hypoxia by transferring carbon flow away from glycolysis into the pentose phosphate pathway, which increases NADPH production. Also, inhibition of glycolysis and enhancement of oxidative phosphorylation in complex IV occurs as a result of negative regulation of phosphoglycerase mutase and serine/threonine kinase Akt. Inactivation of p53 could increase hypoxia, decreasing oxidative phosphorylation and inhibiting apoptosis. However, under other circumstances, p53 can activate processes that result in reduction of mitochondrial function and impairment of their ability to divide (52).

The transcription factor HIF1 is one of the most important regulatory agents. Under low oxygen conditions, HIF1 induces glycolysis, stimulating expression of genes for glucose transporters, glycolytic proteins and angiogenesis factors (VEGF and erythropoietin). HIF1 inhibits mitochondria and can activate mitophagy in a number of ways, the most important of which is down-regulation of mitochondrial metabolism by blocking expression of proteins involved in iron-sulphur centre synthesis and genes for subunits of complexes I, II and IV. Mitochondria regulate ROS production in complex III, which stabilises HIF1α, mediated by sirtuin-3 protein. Additional factors that stabilise HIF1α include cyt c interaction with complex IV and mitochondrial disulphide proteins (52).

Mitochondrial activity is strongly regulated by calcium influx, as noted above. The calcium uniporter, which is energised by an electrochemical gradient, is located in the mitochondria-associated ER membrane. Inactivation of this protein may reduce apoptosis induction and thus promote neoplasms (52). It is emerging that a balance between cell survival and apoptosis, which may be considered a feature of every living cell and primarily operates via calcium movements at the MAM, depends on a multitude of factors, many of which are beyond the scope of this review. Cancer may promote survival by limiting calcium influx, while mitotane and other agents that are adrenolytic may enhance the toxic insult to the cell and thus overcome this restraint. It is striking that expression of the two factors described, FATE1 and SOAT1 should be associated with mitotane response, even in small study groups, but variations in expression of other as yet unknown factors may well prove to be equally important. Mitotane-treated adrenocortical carcinoma patients had shorter time to progression if they had lower tumour SOAT1 expression, both for those on palliative and adjuvant treatment (55, 58).

When mitochondrial ROS are overproduced, they can induce apoptosis or necrosis. However, when apoptosis is inhibited in cancer, they can become potential mitogens and thus mitochondrial ROS production may contribute to transformation towards cancer (52).

Just as control factors such as p53 and HIF1 exert ambivalent functions, either promoting tumours, or acting as a suppressor, other oncogenes, such as FOXO and c-Myc, can enhance or decrease mitochondrial biogenesis (52).

MITOTANE INFLUENCE ON MITOCHONDRIAL ENERGY METABOLISM

As for many types of cancer, ACC cells appear to exhibit the Warburg effect, since PET scanning shows they preferentially take up 18-fluorodeoxyglucose. This is enhanced by mitotane and has been suggested as a means to directly monitor response to treatment. Uptake may also be enhanced in the contralateral adrenal, supporting a deleterious effect of mitotane on oxidative phosphorylation, although the effect of enhanced ACTH stimulation secondary to diminution of serum cortisol could not be excluded (68).

There is a major requirement in the adrenals for molecular oxygen for steroid metabolism. This may be a factor in the tendency to necrosis in larger adrenocortical tumours, so intact tumour tissue may be already close to the limit of its oxygen requirements. One possible mode of action of mitotane might thus be to promote tissue destruction by enhancement of mitochondrial oxygen utilisation. Oxidative stress is undoubtedly an important factor in mitotane - mediated apoptosis (43). Stress due to generalised toxic effects of mitotane may also activate the hypothalamo-pituitary adrenal axis, a process mediated by interleukin-1β (69).

Proteomic studies of Stigliano *et al.* (18) have shown that mitotane disrupts activity of D-3-PGDH isoforms, enzymes involved in a key process of maintaining redox potential, the electron transfer from ferredoxin, which in sequence gives electrons to P450s. This results in changed electron flow in CYP11A1 and CYP11B1.

Direct studies of the respiratory complexes using spectrophotometry (17) have shown that mitotane exerts inhibitory activity, but in a selective manner. At a concentration of 50 μM after 48 h, a dose-dependent inhibition of complex IV was observed, with an IC_{50} of 67 μM. Transcription of mRNA for complex I and complex IV as well as expression of proteins for the whole COX complex was strongly decreased after treatment in H295R and non-secreting SW13 cell lines, whereas for complex II and III no such changes were observed. There was also increased expression of mitochondrial DNA content and peroxisome proliferator-activated receptor alpha (PGC1α) in response to mitotane. In our work (publication in preparation), use of a mitochondrial energy metabolism gene expression panel has demonstrated changed expression of many genes in H295R cells and three cell lines derived from other cancer types (28). Moreover, mitochondria are quantitatively the most significant source of intracellular reactive oxygen species and leakage from the respiratory chain is the main entry point (43).

A MITOCHONDRIAL BASIS FOR MITOTANE SPECIFICITY

Comparing studies describing mitotane actions on different tissue types, it is clear that, although mitotane exerts toxic effects in many of them, adrenocortical tissue is the much most sensitive. One recently identified element of this is the high expression of SOAT1, reflecting the quantitatively important management of cholesterol for steroid synthesis. Sbiera *et al.* (59), suggest that this is the main determinant of mitotane sensitivity, but given the near ubiquitous occurrence of this enzyme in mammalian cells, it is difficult to accept that this argumentis the main determin. Specificity appears to be more closely related to the particular configuration of the mitochondria, with expression of at least three mitochondrial cytochrome P450s required for steroid synthesis. There is no evidence that it acts in this way in other tissues, although there is some clinical evidence, supported by studies of the mouse pituitary cell line TalphaT1, for a deleterious effect on pituitary thyrotrophes, and induction of apoptosis in the mouse cell line TαT1. Mitotane influenced cell survival and function of ACTH-secreting cells derived from human pituitary adenomas in primary culture of and in the mouse pituitary cell line AtT20/D16v-F2 (70, 71). Since the gonads are also sites of steroidogenesis, they may also be sensitive to mitotane, but we know of no studies of gross or ultrastructual effects in this tissue or in isolated cells. Some features of mitochondrial ultrastructure, such as presence of lamellar cristae, are common to adrenal and gonadal tissue (72). A number of case reports show response of testicular tumours to mitotane (73), but women

receiving mitotane continue to cycle and may achieve pregnancy (74). Both men and women show marked decreases in urinary androgen metabolite excretion during mitotane (71, 75). Only one of the identified mitochondrial steroidogenic cytochrome P450s, CYP11A1, functions in this tissue, but as suggested above for adrenal tissue, an alternative cytochrome that is responsible for activation of mitotane may be present.

Adrenal mitochondria employ adrenal ferredoxin, also called ferredoxin-1, to transfer electrons from NADPH to cytochrome P450. This was thought initially to be a source of mitotane specificity, but this same ferredoxin has also been detected in mitochondria in a number of other tissues, including the testes, ovaries, placenta, kidney, liver and brain, being involved in P450-dependent hydroxylation (76).

Studies of mitotane effects on expression of both mitochondrial and nuclear genes, show that these are extensive (28, 58). There are clearly opportunities to develop this field and to further characterise the proteomic profile of this sensitive tissue. Recent developments in the understanding of the epigenetic control of nuclear DNA gene expression may point to secondary mechanisms that are initiated by mitotane. The processes of gene expression are compound-dependent, controlled by a set of nuclear-encoded transcription factors. For example, NRF-1 has specific binding sites in the promoters of the genes for the electron transport chain and cyt c. Further, these nuclear factors are coordinated by coactivators, which provide a link between the products of mitochondrial function or malfunction and subsequent adjustments of nuclear gene expression. Mitochondrial dysfunction correlates with alterations in nuclear gene expression. Further, there are other crucial factors that control nuclear gene expression, such as the ATP/ADP concentration: each mitochondrial promoter has been shown to have a unique sensitivity to the mitochondrial ATP level (54).

CONCLUSIONS

Developing a more comprehensive understanding of mitotane mode of action remains an important objective, holding promise for the development of specific selective agents effective in inhibiting growth or reducing proliferation of adrenocortical carcinoma. Recent work adds important insights, especially on interaction between ER and mitochondria. Mitotane activation by adrenal mitochondria is likely to be key to its specificity, but many details remain elusive, such as the specific cytochrome P450 involved in this process. Inhibition of SOAT1 may also confer specificity. Among many described deleterious effects of mitotane, generation of oxidative stress appears to be central, but it is not yet possible to define one critical mechanism. There may be multiple points of action that jointly overcome the survival mechanisms of the cell. Described effects on the proteome and gene expression support this hypothesis, but their relative importance cannot be judged. Current technologies enable the quantification of changes in expression in large panels of genes, but interpretation of the generated data remains challenging. Increase or decrease of gene expression may reflect the response of cell signalling mechanisms, involvement of an undefined signalling pathway or a secondary consequence of a toxic effect that has yet to be recognised. Preliminary findings have shown that there are changed patterns of energy metabolism in response to mitotane, which does support the interpretation that these are organised responses rather than only nonspecific toxic effects. There is clearly an opportunity to exploit these techniques further. Given that current understanding of mitochondrial signalling is mostly based on study of unicellular organisms (54) any description of these processes in the human remains at an early stage.

Abbreviations: ACC, adrenocortical carcinoma; ACTH, adrenocorticotrophic hormone; ADIUVO, efficiency of adjuvant mitotane treatment; Apaf1, apoptotic protease activating factor 1; Bak, Bcl-2 antagonist/killer protein; Bax, Bcl-2-associated x protein; BCL-2, B-cell lymphoma 2; COX-2, cyclooxygenase-2; cyt c, cytochrome c; Cdk2, cyclin dependent kinase; Cdc2, cyclin B; CHOP, C/ebp homologous protein; CYPs, cytochrome P450s; DHA, dehydroepiandrosterone; ENS@T, European Network for the Study of Adrenal Tumours; eIF2A, eukaryotic translation initiation factor 2A; ER, endoplasmic reticulum; FATE1, fetal and adult testis expressed 1; FIRM-ACT, first international randomized trial in locally advanced and metastatic adrenocortical carcinoma treatment; FOXO, forkhead transcription factor; HIF1, hypoxia-inducible factor 1-alpha; Hint, histidine triad nucleotide binding protein; hnRNP, heterogeneous nuclear RiboNucleoProtein; JNK, c-Jun N-terminal kinase; MAM, mitochondrial-associated membranes; MALDI-TOF, matrix assisted laser desorption - ionization time of flight mass spectrometry; NRF1, nuclear respiratory factor 1; p53 (TP53), tumour protein 53 or transformation-related protein 53; PET, positron emission tomography; PGC1α, peroxisome proliferator-activated receptor alpha; PHB, prohibitin; qPCR, quantitative polymerase chain reaction; SF-1, steroidogenic-tissue enriched factor; SOAT 1, sterol-O-acyl transferase 1; TR, containing a tetracycline response system; TMX, thioredoxin; ROS, reactive oxygen species, UPR, the unfolded protein response; VDAC, voltage-gated anion channels; VEGF, vascular endothelial growth factor

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