Review

Mitochondria as targets for cancer therapy

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Mitochondria have recently emerged as intriguing targets for anticancer drugs. A variety of compounds have been now identified that act *via* mitochondria. These compounds, termed mitocans (an acronym for mitochondria and cancer), destabilise mitochondria and cause apoptosis, which is, at least in some cases, selective for cancer cells. Mitochondria are the powerhouse of the cell, providing it with energy, as well as the source of important mediators of apoptosis. Recent findings show that individual types of cancers are complex and can differ considerably in their array of DNA mutations, harbouring different sets of genetic causes. This indicates that it will be very unlikely to cure cancer by drugs targeting only a few gene products or single pathways that are essential for tumour survival. What is needed then is an invariant target, common to all cells, but which is predominantly only affected by drugs when delivered inside the cancer cells. Such targets appear to be mitochondria, with very rare mutations, and mitocans can be expected to be very efficient drugs of choice for a number of different types of the neoplastic disease.

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1 Introduction

Treatments that selectively target cancer cells represent the panacea for cancer. Gleevec treatment of chronic myelogenous leukaemia (CML) provided the first example that this is possible by targeting the Bcr-Abl tyrosine kinase specific to the survival of these cancer cells [1]. Together with the development of the mAb, Herceptin that inhibits the erbB2 receptor on breast cancer cells [2], Gleevec has confirmed that the age of molecularly targeted cancer therapeutics has finally arrived.

We are witnessing the emergence of compounds that selectively target mitochondria in cancer cells. Mitochondria, acting as the powerhouse producing energy, are potential 'powder kegs' which when ignited can culminate in the death of malignant cells. These novel drugs offer substantial promise as clinical treatments with minimal side effects compared to current chemotherapeutics. Although in the early phase of clinical trials, an in-depth analysis and understanding of how mitochondrial targeted anticancer drugs selectively kill cancer cells supports such claims and their future potential.

2 Targeting differences in energy metabolism between cancer and normal cell mitochondria to selectively destroy cancer cells

Understanding the molecular basis of metabolic changes that distinguish cancer from normal cells assists identification of sensitive targets with precise accuracy and provides

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Abbreviations: ALDH, aldehyde dehydrogenase; **ANT,** adenine nucleotide transporter; **ASK,** apoptosis signal-regulating kinase; **BH3,** Bcl-2 homology-3; **3BP,** 3-bromopyruvate; **CL,** cardiolipin; **ETC,** electron transport chain; **FH,** fumarate hydratase; **Grx,** glutaredoxin; **GST,** glutathione *S* transferase; **HIF-1,** hypoxia-inducible factor-1; **HK,** hexokinase; **LDH,** lactate dehydrogenase; **MIM,** mitochondrial inner membrane; **MOM,** mitochondrial outer membrane; **MPT(P),** mitochondrial permeability transition (pore); **NEM,** *N*-ethylmaleimide; **OXPHOS,** oxidative phosphorylation; **PDH,** pyruvate dehydrogenase; **PDK,** pyruvate dehydrogenase kinase; **PEITC,** phenyl ethyl isothiocyanate; **PHD,** prolyl hydroxylase; **Prx,** peroxiredoxin; **ROS,** reactive oxygen species; **SDH,** succinate dehydrogenase; **TCA,** tricarboxylic acid cycle; **α-TOS,** α-tocopheryl succinate; **TRX,** thioredoxin; **TM,** transmembrane; **TxR,** TRX reductase; **UbQ,** ubiquinone; **VDAC,** voltage-dependent anion channel; **VHL,** von Hippel Lindau

more effective therapies that selectively kill cancer cells. This review focuses on the mechanisms whereby metabolic changes affecting mitochondrial function inside cancer cells endow these cells with distinctive properties and survival advantages worthy of drug targeting. Particular focus relates to the emerging theme that mitochondria, as key sensors for activating the suicide signal, have this function suppressed in cancer cells. The more recent concept of suppression of autophagy and the theory that damaged mitochondria remain, promoting the onset of cell malignancy, is not covered. The chapter also reviews many different types of anticancer therapeutics currently under development that target mitochondrial function with the aim of inducing cancer cell death. To simplify the generic naming for this class of compounds, we have coined the acronym 'mitocans' for the verbose terminology 'mitochondrially targeted, apoptosis-inducing anticancer compounds' encompassing the expanding list of different subgroups. We describe the growing number of mitocans and their mechanisms for targeting mitochondria.

An essential aspect of mitocan therapies that targets cancer and not normal cells relates to the differences in properties of mitochondria from these cell types. The general metabolic state of cancer cells was reported early to differ from that of normal cells, with cancer cells using higher levels of glycolysis for their growth [3]. In general, respiration is reduced in cancer cells as they grow in a hypoxic environment during the development and progression of the tumour mass. Consequently, the tumour microenvironment differs from that of normal tissues, particularly in their vasculature and regions of hypoxia [4–6]. This results in metabolic modifications reflecting the growth of tumour cells under hypoxia including changes in signalling pathways and transcription factors that lead to significant alterations in gene expression. The major changes in energy metabolism affecting mitochondrial function in cancer cells are the subject of the following sections.

3 Hypoxia during cancer progression leads to hypoxia-inducible factor-1 (HIF-1)-induced changes in energy flow to mitochondria

Besides genetic mutation in cell growth regulatory genes, a significant contributing factor to cancer cell development is the dysregulation in the supply of oxygen available to a tumour. During the emergence of cancer, cells in the tumour microenvironment undergo intermittent periods of oxygen deprivation [4]. This occurs as a result of rapid cell growth shifting cells from a state of sufficient nutrient and oxygen supply from the nearby vasculature to one of competition, relative starvation and hypoxia. In response to the hypoxic stress, tumour cells undergo a selection process that involves the activation of a transcription factor called HIF-1.

HIF-1 is a master oxygen sensor and regulator of energy metabolism. When oxygen is available, the HIF-1α subunit is modified by oxygen-dependent hydroxylation mediated by prolyl hydroxylases (PHDs 1–3). Prolyl hydroxylation of HIF-1 acts as a marker for its ubiquitination by the von Hippel Lindau (VHL) E3 ligase leading to HIF-1 destruction [7, 8]. HIF-1 is not usually activated in cells under normoxic conditions. When cells become hypoxic, the α subunit is no longer turned over rapidly and binds the HIF-1β subunit to produce an active transcription factor complex [8] inducing expression of genes including key enzymes that increase the glycolytic pathway flux. HIF-1 also induces vascular endothelial growth factor (VEGF) expression, resulting in formation of new blood vessels within the tumour, which restores the supply of nutrients and oxygen [8].

Amongst genes induced by HIF-1 activation, some are particularly important to the altered state of energy metabolism in cancer cells. The first encodes hexokinase II (HKII) that is overexpressed and bound to the mitochondrial outer membrane (MOM) *via* the porin-like protein voltage-dependent anion channel (VDAC) protein (Fig. 1). With the adenine nucleotide transporter (ANT), VDAC forms a channel that transports ATP made by the mitochondrial inner membrane (MIM)-located ATP synthase directly to active sites on HKII. The higher levels of HK-II bind both ATP and incoming glucose, producing increased glucose-6-phosphate levels in the cytosol. A correlation between the growth rate of tumours and their mitochondrial-associated HK activity has been established [9]. Among 12 different tumours tested, only those with rapid growth rates showed mitochondrial associated HK activity. In addition, mitochondrial bound HK limits respiration when tumour cells are utilising glucose as their energy source (a situation known as the Crabtree effect) [10]. Even though large amounts of ADP are made in this situation, the continuous phosphorylation of glucose by ATP (through mitochondria-bound HK) also reduces phosphate levels available for oxidative phosphorylation (OXPHOS) and will prevent the tumour cell mitochondria from entering into the maximum rate of state 3 respiration [11]. HK binding to VDAC on the mitochondria appears to restrict channel conductance, including the entry of Ca^{2+} ions from the cytosol required to induce apoptosis. Hence, HK binding stabilises tumour cell mitochondria, suppressing apoptosis and promoting cancer cell survival [12].

The second HIF-1-activated gene encodes a distinct lactate dehydrogenase isoform (LDH-M) whilst the third gene encodes pyruvate dehydrogenase kinase (PDK-1). These two genes are important because they cause significant changes to metabolism of pyruvate following glycolysis. In nonmalignant cells metabolising under normoxia, much of the pyruvate is transported into the mitochondria for PKD production of acetyl-CoA for entry into the tricarboxylic acid (TCA) cycle. In cancer cells, after HIF-1 activation,

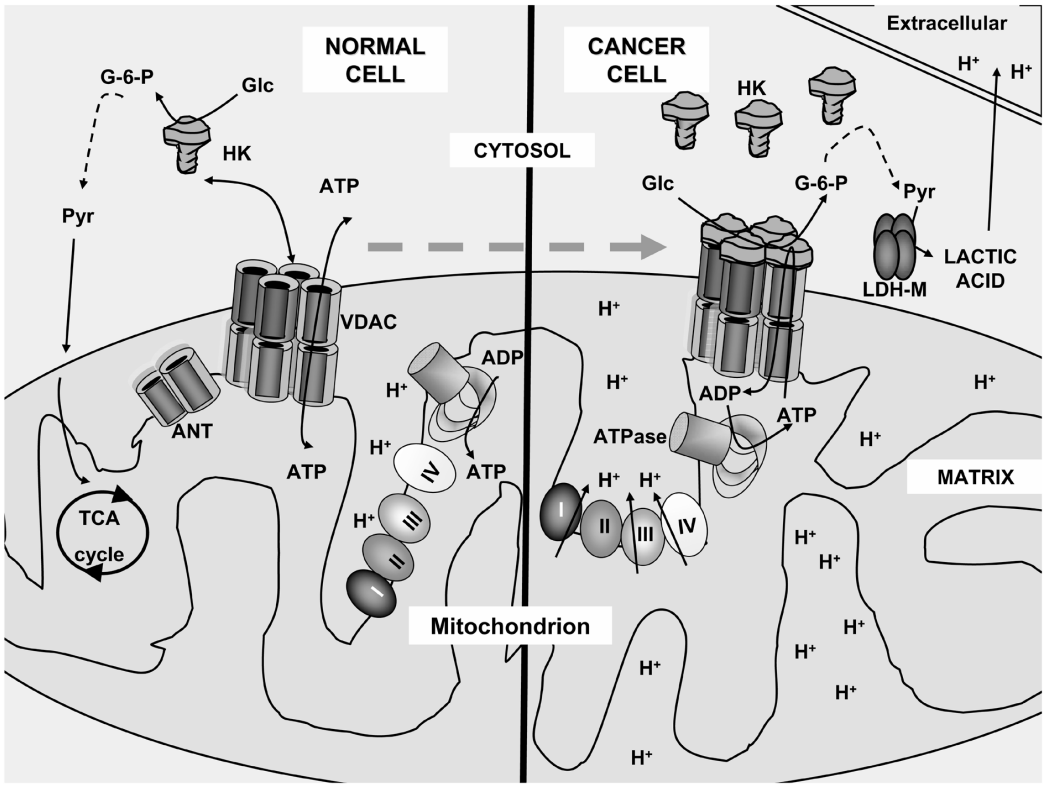

Figure 1. Comparison of mitochondrial metabolism between a normal *versus* a cancer cell. In normal cells, pyruvate formed from glycolysis is transported into the mitochondria to be converted for entry into the TCA cycle. In cancer cells, much greater levels of HK exist bound to mitochondria, promoting greater glycolysis and preventing ion channel flow *via* VDAC. In cancer cells, pyruvate does not enter the mitochondria as it mostly converts to lactic acid in the cytosol that in turn, ends up acidifying the extracellular milieu. Cancer mitochondria show a greater TM potential across the MIM, reduced OXPHOS and flux along the ETC.

the situation is very different because LDH-M and PDK-1 expression and activity become greatly elevated and these enzymes control the flow of pyruvate from glycolysis by effectively restricting pyruvate from entering into mitochondria, instead re-directing pyruvate in the cytosol to produce high levels of lactic acid.

LDH exists in two different isoforms, each encoded by separate genes; LDH-L is a low-activity isoform and LDH-M is the more active isoform that catalyses the reduction of pyruvate with NADH to produce lactic acid and NAD^+ [13, 14]. The LDH enzyme exists as tetramer containing different ratios of the two isoforms and in cancer cells the predominant species is a tetramer of LDH-M with the highest catalytic activity. The LDH-M tetramer (also known as LDH5 or LDHA) and the Keq of pyruvate to lactate reaction greatly enhance lactic acid production, particularly with increase in glycolysis and high levels of NADH [15, 16]. In head and neck carcinomas and many other human cancers, excessive tumour lactate levels are significantly correlated with the likelihood of metastases and poor clinical outcome. Furthermore, high levels of the LDH5 enzyme isoform have also been associated with advanced and aggressive forms of many different types of cancers [14, 17].

PDK-1 directly modifies cancer cell pyruvate metabolism by phosphorylating PDH, inhibiting its activity, with the net effect that pyruvate becomes more available in the cytosol as it is no longer metabolised by PDH to acetyl coA in mitochondria [7, 18, 19]. The result of inhibiting PDH and pyruvate metabolism *via* the TCA cycle, favouring pyruvate conversion to lactic acid, is that there is a significant reduction in the mitochondrial production and availability of substrates like NADH and succinate required for the respiratory pathway.

The major modifications induced by HIF-1, acting to increase PDK and LDH activity and their effects on pyruvate metabolism, as well as the shift to greater glycolysis, are also associated with other metabolic changes. Thus, cancer cells exhibit a shift in aldehyde dehydrogenase (ALDH) isoforms to those with reduced affinity for the substrate acetaldehyde, leading to increased acetaldehyde [4]. The increase in acetaldehyde turns the PDH complex into a nonoxidative decarboxylase converting pyruvate to acetaldehyde, a highly reactive species. Within the PDH complex,

acetaldehyde also reacts with decarboxylated pyruvate (as a hydroxyethyl–thiamine–pyrophosphate–enzyme complex) to form acetoin, a neutral compound not normally present in mammalian cells [20]. At this point, with its ensuing build-up, acetoin becomes a competitive inhibitor, also preventing the PDH complex from using pyruvate in tumours. In addition, acetoin build-up in tumour cells appears to directly inhibit succinate oxidation *via* complex II of the respiratory chain, further adding to the inhibition of mitochondrial respiratory function in tumour cells [21]. The low residual pyruvate (5–15%) [4] not converted to lactic acid, is most probably transported into the mitochondria to produce acetoin. Nevertheless, many different factors contribute to prevent pyruvate entering into the TCA cycle in mitochondria of cancer cells. The net outcomes from HIF-1 activation and acetoin production are that mitochondrial oxygen consumption and ATP production are inhibited and cancer cells mainly survive on glycolysis.

4 Pseudohypoxia

Studies of several human neuronal malignancies and renal-cell carcinomas have provided insight into one protective device whereby cancer cells promote their own survival. This process, known as ‚pseudohypoxia', is distinct from the hypoxic development described above. These cancers contain mutations that make the cells behave as if they were hypoxic at normal levels of oxygen. In pseudohypoxic cancers, somatic mutations occur in either the succinate dehydrogenase (SDH) or fumarate hydratase (FH or fumarase), enzymes that are part of the TCA cycle linking glucose metabolism in the cytosol and OXPHOS in mitochondria. The *SDHA* gene encodes the enzymatic subunit SDHA, located on the matrix side of MIM, associated with three other subunits, SDHB, SDHC and SDHD of the ubiquinone (UbQ) oxidoreductase complex II. Inherited or somatic mutations in *SDHB*, *SDHC* or *SDHD* are associated with phaeochromocytomas or paragangliomas, whereas those in the *FH* gene are associated with leiomyomas, leiomyosarcomas or renal cell cancers [22].

As a consequence of mutations in *SDH* or *FH*, a build-up in the levels of succinate or fumarate, intermediates of the TCA cycle, increases. The two metabolites feed back to inhibit PHD3/ENGL3 [23–28]. In this event, the transcription factor HIF-1α is no longer modified by PHDs to facilitate its ubiquitination and degradation. This situation resembles the same state found in hypoxia when levels of activated HIF-1α become elevated, despite the continued presence of oxygen. The effect of higher HIF-1α is promotion of malignancy of the cells, similar as for the hypoxic situation, *i. e.* high levels of aerobic glycolysis and reduced mitochondrial respiration.

Other situations also occur in cancers where a pseudohypoxic condition is established. One example is the VHL mutation where the VHL tumour suppressor protein is absent. As this gene encodes the E3 ligase required for ubiquitinating HIF-1α, when it is missing, HIF-1α becomes stabilised even in nonhypoxic cells, promoting the relevant metabolic changes supporting the malignant phenotype [7].

The 2-oxoacids, pyruvate and oxaloacetate, can also feed back to inhibit PHD 1 and 2 (HPH or PHD 1,2) [29]. However, these metabolites work by a different mechanism to that of succinate and fumarate, possibly by targeting distinct PHD isoforms [30]. Even in the presence of oxygen, most cancer cells accumulate high levels of pyruvate and lactate. Accumulation of pyruvate and oxaloacetate as a result of increased glycolysis could contribute to cancer cell development by providing a feedback pathway promoting increased activation of HIF-1, independent of the levels of oxygenation.

5 Proton flux regulation and potential difference across the MIM of cancer cells

A commonly observed difference in cancer cell compared to normal cell mitochondria is the greater mitochondrial inner TM potential ($\Delta\Psi_{m,i}$) in the former. As a result of the metabolic changes, the $\Delta\Psi_{m,i}$ is increased to greater negative values (−150 to −170 mV) in carcinoma cells [31–35], with ∼60 mV difference across MIM. Many proposals have attampted to explain the reasons for this difference, and include differences in mitochondrial respiratory enzyme complexes, electron carriers, ATPase, ANT and/or changes in membrane lipid metabolism. Other proposals for the increased mitochondrial membrane potential in cancer cells include altered electron transfer activity, proton translocation and utilisation or conductance. Mitochondria isolated from hepatocellular carcinomas display reduced uncoupler-stimulated ATP hydrolysis, decreased rates of respiration-linked ATP synthesis and reduced phosphorylating capacity compared with normal liver cells [36–39].

Marked changes in enzyme function, particularly in ATPase, have been shown to occur in cancer cell mitochondria. ATPase isolated from carcinomas features reduced maximal velocity, decreased levels of the β subunit of the F1 component and/or overexpression of the ATPase inhibitor protein (IF1) [36–40]. A reduced ability to use the proton gradient to make ATP, with a resulting build-up in protons within MIM would account for the greater $\Delta\Psi_{m,i}$ in tumour mitochondria.

Another possibility that may account for greater $\Delta\Psi_{m,i}$ in cancer cells is that acetoin undergoes an ATP-dependent reaction, almost doubling the reaction rate to produce citrate in tumour cells [21], which is then exported *via* the tricarboxylate or citrate carrier (CIC) to the cytosol where it is cleaved to oxaloacetate and acetyl-coA. The net effect is the provision of a cytoplasmic acetyl-coA precursor for sterol biosynthesis, promoting cancer cell production of

cholesterol [21]. The resulting build-up of cholesterol in MIM reduces by several-fold their passive proton permeability, helping to establish the greater TM potential in cancer cells [21, 41].

6 Proton flux across the plasma membrane makes cancer cells selective to anticancer drugs

The enhanced glycolytic activity due to very high energetic demand increases cytoplasmic levels of lactic acid in cancer cells. To maintain neutral cytosolic pH, cells activate plasma membrane proton pumps causing extracellular acidification. Typically, the pH of the tumour interstitium is 6.2 – 6.5, while the pH of normal tissue interstitium is neutral [42, 43]. The class V ATPase that is the major type of proton pump involved in the process [44] has relatively low activity in nonmalignant cells, while its activity is increased in cancer cells [45, 46]. This led to the development of a novel anticancer strategy based on inhibiting the activity of the ATPase, causing acidification of the cancer cell cytosol that results in the demise of the cell. For example, chondropsin compounds [47] and siRNA targeting the ATPase subunit ATP6L [30] have been successfully used to kill cancer cells. Other important regulators of cytosolic pH are the Na^+/H^+ antiporter [48], the H^+/lactate symporter [49], and the Na^+-dependent Cl^-/HCO_3^- exchanger [50]. Similarly as for the V-class ATPase, these transporters have been proposed as anticancer drug targets [45, 49, 51].

The difference in pH gradient across the plasma membrane of cancer cells has been used to design drugs that are weak acids, with pK_a values of <6, and which are deprotonated at neutral pH but accept a proton at the pH of the tumour interstitium [42]. A prototypic example of such a drug is chlorambucil [52]. It was reported that the relatively selective uptake and anticancer efficacy of chlorambucil were enhanced by injection of glucose into mice with tumours, thereby futher promoting glycolytic activity of tumour cells by lowering the tumour interstitium pH while not affecting the pH of the cytosol [53].

We have observed similar effects for the mitocan α-tocopheryl succinate (α-TOS), a compound with a pK_a of ~5.6, of which ~98% is deprotonated at neutral pH and with 10 – 15-fold higher percentage in the protonated form at the pH of 6.2 – 6.4 [54]. Since there are no known transporters for compounds like α-TOS, it most likely crosses the plasma membrane by free diffusion. Accordingly, we found that when the pH of the tissue culture medium was more acidic (pH ~6.2), it resulted in ~3 times greater apoptogenic efficacy of α-TOS against T lymphoma cells compared to neutral media. The most likely reason is faster uptake of the compound at lower pH [54]. The pH differential across the tumour plasma membrane may be an important paradigm for targeted delivery whereby certain anticancer agents exert selectivity for malignant tissues.

7 ROS, HIF-1 activation and changes in cytochrome *c* oxidase (COX) activity

Oxygen utilisation in cells occurs in mitochondria at the endpoint of the respiratory chain where it acts as a substrate for the COX that constitutes complex IV. During OXPHOS, the respiratory chain transfers electrons through a series of coupled acceptor systems in a relay that culminates in a reaction with oxygen. The electron acceptors contain redox centres (Fe – S complexes, heme groups, FMN or FAD) in complexes I – IV that also work as proton pumps in order to generate the proton gradient across MIM (Fig. 2). Energy stored in the proton gradient is then used to drive ATP synthase to make ATP. However, the electron transport chain (ETC), as a highly charged system, is not without its containment problems, particularly when coping with overloads or blockages [55, 56]. The single-electron chemistry involved in the ETC predisposes to the generation of reactive oxygen species (ROS) because the redox centres along the respiratory chain can leak electrons to molecular oxygen producing the superoxide anion radical ($O_2^{\bullet -}$).

Studies with specific inhibitors of each of the complexes I – IV or with cells containing mutations affecting their activity have revealed that either of the complexes, when blocked, is capable of leaking electrons and producing significant levels of $O_2^{\bullet -}$ [57 – 61]. The evidence indicates that the source of $O_2^{\bullet -}$ production occurring along the respiratory chain depends on the type of energy substrate that is being used and the extent of energy/electron flow. Enhanced ROS production occurs when electron carriers in the respiratory chain are kept in the reduced state as a result of either inhibition of OXPHOS or because of excess energy substrate availability for use by the respiratory complexes. The unpaired electron of ubisemiquinone bound to the CoQ sites of complexes I – III is capable of reacting directly with O_2 to produce $O_2^{\bullet -}$ as a toxic by-product.

In hyperpolarised cancer cell mitochondria, the ETC stalls because ADP levels drop due to the demands of high glycolysis and conversion to ATP, inhibiting ATP synthase by limiting ADP availability. Together with reduced passive proton permeability caused by increased sterol production, ATP synthase inhibition greatly restricts the proton flow back to the matrix either across the membrane or *via* the ATP synthase proton channel. The respiratory chain continues to use energy substrates and pump protons across MIM into the matrix increasing the $\Delta\Psi_{m,i}$ until it becomes energetically unfavourable, and further proton flow becomes limited. At this point, the chain stalls and the electron carriers become reduced [62]. UbQ molecules become converted to the ubisemiquinone radical anions that then favour

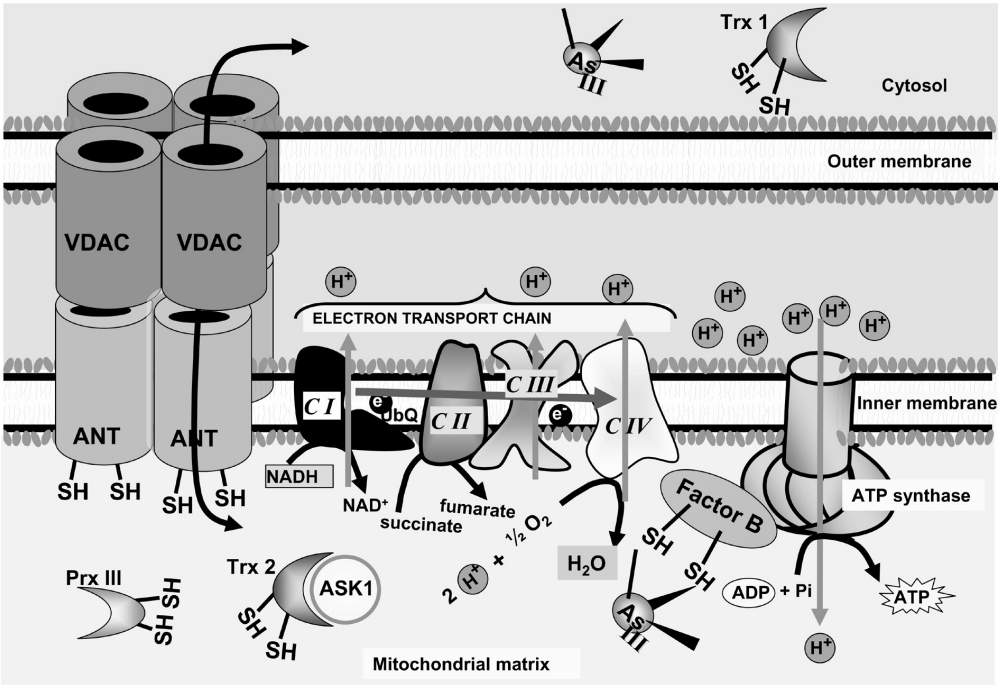

Figure 2. Many vicinal thiol containing proteins important to REDOX control in the mitochondria are mitocan targets. Arsenite and particularly organic arsenites will disrupt the normal redox systems functioning in the mitochondrial matrix and intermembrane space by targeting vicinal thiols in proteins and enzymes that regulate these systems. Such enzymes include Prx III and TRX2. In addition, several key mitochondrial functions are affected, including Factor B regulating the ATP synthetase activity, and ANT, amongst others. See text for further detail.

alternative reactions with molecular oxygen producing $O_2^{\bullet -}$. Ubisemiquinone anions play an important role in electron transfer to oxygen from complex I [63], complex II [64] and complex III [65, 66]. The importance of ubisemiquinones as the source of $O_2^{\bullet -}$ production was first recognised by Turrens [57] and confirmed more recently [66].

The COX activity in complex IV is regulated to enable cells to cope with the demands made by varying oxygen levels. Such changes can cause electron flux problems within the chain components, resulting in aberrant levels of ROS production. Intriguingly, HIF-1 activation requires ROS production by the ETC [67, 68]. However, this induces changes in the regulatory subunit of complex IV, causing a switch in the catalytic subunit to a different isoform. The net effect is an increase in the oxidase activity, optimising the flow down the respiratory chain, ATP production and O_2 consumption, which helps to prevent ROS production during hypoxic conditions [69, 70]. Nevertheless, despite this homeostatic mechanism to avoid excessive ROS production, cancer cells maintain their high $\Delta\Psi_{m,\,i}$ and low OXPHOS, and hence have a significant ability to generate ROS. This is likely to be further exacerbated by the accumulation of mtDNA mutations and the effects of oncogenes in enhancing HIF-1 expression and glycolysis, while reducing OXPHOS [62].

The dependence of ROS generation on $\Delta\Psi_{m,\,i}$ and on the reduced state of pyridine nucleotides indicates that under conditions where the ATP synthase is nearly inactive and not bypassed by uncouplers, the high $\Delta\Psi_{m,\,i}$ and the relatively high NADH/NAD^+ ratio will all contribute to promote ROS production [71].

8 Importance of ROS in tumour cell development and progression and the residual respiratory function of cancer cells

Previous sections emphasise the importance of ROS for cells to undergo genetic changes leading to transformation and to changes in energy metabolism and mitochondrial function. $O_2^{\bullet -}$ may also play another role, since it has recently been found to directly inhibit the mitochondrial form of ALDH2, which is involved in metabolising the carcinogenic acetaldehyde into acetic acid [72]. In support of the significance of the role played by acetaldehyde in cancer, subjects with a defective *ALDH2* allele who consume alcohol are highly predisposed to squamous cell carcinomas of the upper digestive tract [72].

A common feature of cancer cells is that they are selected to survive large transitions in the redox state resulting from

switching/cycling between intermittent periods of normoxia and ischemia. Mutations resulting in altered mitochondrial function and increased expression of prosurvival proteins also enable the cells to overcome stressful growth conditions, forcing a greater dependency on glycolysis. Despite this ability of cancer cells to survive and grow under conditions of low or reduced oxygen levels, they still maintain a residual capacity for aerobic respiration [73].

Cancer cells are very sensitive to mitochondrial respiratory poisons. For example, a study with leukaemia cells [74] outlined a strategy to hinder mitochondrial electron transport and increase $O_2^{\bullet-}$ generation inside these cells as a novel and selective means for enhancing apoptosis induced by anticancer agents. This strategy initially established the proof-of-principle by using rotenone, an inhibitor of the NADH oxidoreductase in complex I. The resulting partial inhibition of mitochondrial respiration increased electron leakage from the transport chain, leading to increased $O_2^{\bullet-}$ generation. The net effect was sensitisation of the cells to anticancer agents whose action involves free radical generation. As_2O_3, a clinically useful antileukemia agent that inhibits mitochondrial respiratory function, also increased free radical generation and enhanced the activity of other $O_2^{\bullet-}$-generating agents also against primary leukemia cells isolated from patients [74].

An additional impact of defects in mitochondrial respiration operates *via* the cytosolic Akt (protein kinase B) pathway that promotes cancer cell growth and survival [75]. Disruption of the mitochondrial function causes an increase in the cellular NADH/NAD^+ ratios. This leads to activation of the Akt pathway *via* NADH-mediated inactivation of the phosphatase PTEN. This situation was simulated with respiration-deficient (ρ^0) cells lacking mtDNA that were dependent on glycolysis. These cells produced increased levels of NADH that activated Akt, resulting in drug resistance and survival advantages in hypoxia [75]. Pharmacological inhibitors of mitochondrial respiration or conditions of hypoxia activate Akt in a similar manner: Studies revealed that with cells lacking mitochondrial respiration (ρ^0 cells) or chemically treated to inhibit the ETC (*i.e.* pseudohypoxia), or where normal cells were exposed to hypoxia, all resulted in significant Akt activation [75]. The evidence revealed that the cellular NADH/NADPH ratio increased abnormally when mitochondrial respiration was suppressed, in parallel with a decrease in plasma membrane-associated PTEN. Addition of exogenous NADH led to inactivation of PTEN and activation of Akt *in vitro*. Inactivation of PTEN results from redox modulation when NADH competes with NADPH binding to thioredoxin (TRX) reductase (TxR), decreasing the enzyme activity [76]. As a result, oxidised PTEN (inactive) is unable to dephosphorylate Akt and inhibit its kinase activity. These results are consistent with previous studies showing inactivation of PTEN by hydrogen peroxide [77 – 79]. Cells lacking functional PTEN did not respond to respiratory inhibition or hypoxia and exhibited no increase in Akt activation, confirming the important role of PTEN in Akt regulation [75].

NADH produced by glycolysis and the TCA cycle drives OXPHOS *via* complex I, as does NADPH produced *via* the pentose phosphate pathway. We have described above how mitochondrial defects in OXPHOS make cancer cells dependent on glycolysis. When OXPHOS is restricted as in cancer cells, the NADH produced from the TCA cycle is no longer used to drive complex I. Increased NADH is available for alternative processes such as conversion of pyruvate to lactate. Overall, NADH accumulates while the levels of NADPH decline because of the increased utilisation of glucose. It has been observed that the NADH/NADPH ratio increased in ρ^0 cells [75]. These authors also showed that the antioxidant *N*-acetyl cysteine (NAC) suppressed Akt activation induced by H_2O_2, but did not decrease rotenone-induced Akt phosphorylation. They proposed that the redox-sensitive PTEN was inactivated when the ratio of NADH/NADPH was significantly increased and that the elevated ratio could not be modulated by NAC when cells were treated with rotenone. In contrast, NAC effectively decreased H_2O_2 and its direct effect on PTEN.

The phosphoinositol-3 kinase (PI3K)-Akt pathway is critical for cell survival [80 – 82]. Activation of PI3K results in generation of PIP3, which leads to activation of phosphoinositide dependent kinase-1 and phosphorylation of Akt. In contrast, the phosphatase PTEN dephosphorylates PIP3, acting as a negative regulator of Akt. Loss of PTEN leads to Akt activation in cancer cells [83]. It is likely that oxidation of PTEN suppresses its phosphatase activity leading to Akt activation. The demonstration that respiration defects lead to activation of the Akt pathway in response to the accumulation of NADH and inactivation of PTEN reveals an important adaptive mechanism enabling cancer cell survival under respiration-compromised conditions. These findings also provide a novel mechanistic insight into the Warburg effect and how metabolic changes in cancer cells reduce sensitivity to therapeutic agents.

9 The relationship between thiol redox exchange, ROS and induction of apoptosis

Studies with a variety of arsenical compounds have implicated protein thiols and thiol-reactive systems such as TRX, TxR, glutathione and glutathione reductase in regulating apoptosis and ROS availability in cells. Many of these arsenical compounds react with mono- and dithiol groups, particularly the latter when two thiols are in close proximity. The different arsenical compounds are excellent agents for probing the thiol redox system in cancer cells. In the previous section it was pointed out how mitochondria and the ETC are the main source of ROS in cells [84] and may be the key point of thiol – redox exchange affecting cancer

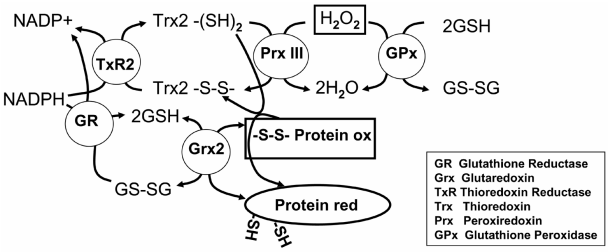

Figure 3. Mitochondrial redox systems regulating ROS levels *via* thiol-disulphide exchange/coupling reactions. The mitochondrial form of TRX2 is likely to play the major role in reducing disulphides formed by vicinal thiols in both the mitochondrial Prx III and other proteins. Prx III is one of the main ways by which cancer cells can reduce the build-up of H_2O_2 as a result of overload from electron transport flow. The glutathione redox system comprising GSH/GSSG, glutathione reductase, Grx and glutathione peroxidase, although present in the mitochondria, are more likely to only become of major importance during the more extreme conditions of oxidative stress. Both of these systems are targets for inhibition by arsenic-containing compounds and other mitocans. See text for further detail.

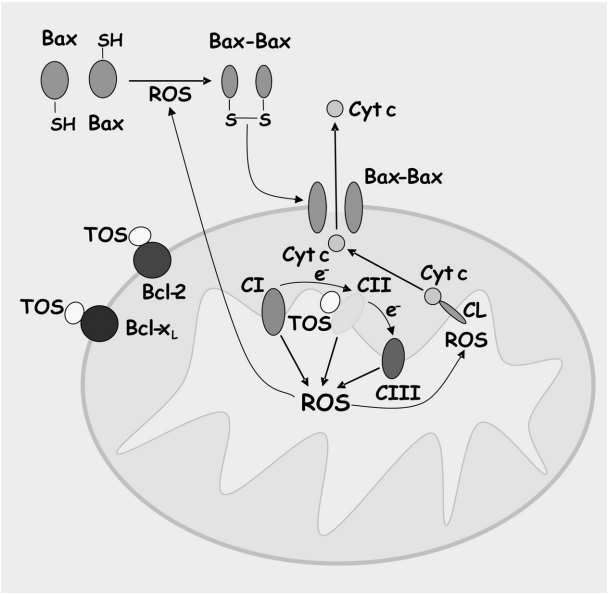

Figure 4. Universal model for the molecular mechanism of cancer cell apoptosis initiated by mitocans *via* ROS production as exemplified by α-TOS. According to this model, there are at least two roles for mitocan mediated cell death: the major role involves the drug inhibiting oxidative respiration at the level of electron transport complexes, and an ancilliary role, which involves binding and blocking Bcl-2 and Bcl-x_L function to allow Bax to form mitochondrial membrane channels. Thus, for example, α-TOS, as shown in the right hand panel, upon inhibiting the activity of complex II, impairs electron transfer flowing along the redox chain. This leads to generation of ROS such as $O_2^{\bullet -}$. ROS then contribute to the ensuing events leading to cell death. The cysteine residues on Bax monomers are oxidised to form disulphide bridges, whereby the protein dimerises. This changes the conformation of Bax, so that the mitochondrial-docking motif is exposed and the dimers merge in the MOM, forming multimeric channels. ROS also oxidise CL within the MIM. This leads to dissociation of cytochrome *c* (Cyt *c*) from the intermembrane face of CL. Free Cyt *c* then escapes *via* the Bax channels in the MOM to the cytoplasm. α-TOS, occupying the BH3 domains of Bcl-2 and Bcl-x_L, prevents Bax from forming inactive oligomers with Bcl-2 and Bcl-x_L, thereby increasing the pool of available Bax dimers that form MOM channels promoting induction of apoptosis.

cell death. The TRX system regulates many cellular functions including redox signalling, transcriptional changes, the intracellular redox environment, cell growth, defence against oxidative stress and apoptosis [85], and is very sensitive to arsenic-based drugs and this may be the basis for their mechanism of action in inducing cancer cell death. The TRX system operates as a thiol–disulphide exchange reaction (see Figs. 2, 3). TRX1 and TRX2 are key regulatory isozymes that catalyse the reduction of protein disulphide bonds. They are cofactors of the apoptosis signal-regulating kinase 1 (ASK1) that mediates TNF- and oxidative stress-induced apoptosis *via* the mitochondrial dependent pathway [86].

In their reduced forms, cytosolic TRX1 and mitochondrial TRX2 each contain vicinal thiol groups in their active sites, binding to Cys250 and Cys30, respectively, in the regulatory N-terminal domain of ASK1 to maintain the enzyme in an inhibited state. Activation by TNF results in increased production of ROS which oxidises the TRX dithiol group to a disulphide, and inhibits binding of TRX to ASK1. Loss of TRX2 binding to the mitochondrial-located ASK1 leads to apoptosis in a JNK-independent manner. Upon loss of TRX1 binding, cytosolic ASK1 activates as a MAPKKK, causing JNK activation, Bid cleavage and translocation of Bax to mitochondria [87]. Since it is known that TRXs are major targets of arsenic-containing compounds [88], it can be predicted that arsenical-mediated oxidative binding to TRX will have a similar outcome as TNF signalling, leading to the release and activation of ASK1 and induction of apoptosis.

Another TRX-associated protein important in thiol mediated redox regulation in mitochondria is peroxiredoxin III (PrxIII), which is abundantly expressed in the mitochondria of cancer cells, protecting them from oxidative stress [89, 90]. PrxIII is an important antioxidant that acts in conjunction with TRX2/TXR (Fig. 3) in mitochondria to remove peroxides such as H_2O_2. PrxIII contains three Cys residues, two of which act as redox-active sites in the formation of a stable inter-subunit disulphide-bonded dimer, which is reduced by TRX to the monomer. PrxIII is a more abundantly expressed arsenic-binding protein in arsenic resistant cells compared to normal cells [91]. Hence, PrxIII is another protein whose function is likely to be inhibited by arsenic-containing compounds, promoting apoptosis.

It is apparent that mitochondrial dithiol-containing redox proteins act as sensors to changes in the redox state. Many

redox-active proteins contain one or more vicinal pairs of reactive thiol groups [92, 93] and many of these proteins can bind arsenic-containing compounds. The most important dithiol-containing redox proteins are located in mitochondria, providing high sensitivity to arsenic-containing compounds whose actions culminate in triggering apoptotic pathways in cancer cells *via* the induction of ROS (Fig. 4). Two members of the glutaredoxin (Grx) family, including Grx2 located primarily in the mitochondria, catalyse GSH-dependent TRX-disulphide redox and protein thiol–disulphide redox reactions, in particular the reversible glutathionylation of protein SH [94]. Human Grx1 and Grx2 contain Cys-comprising active sites as well as additional structural Cys residues. They are also likely to react with arsenic-containing compounds.

Another mitochondrial protein targeted by arsenic-containing compounds is the regulatory protein 'Factor B'. Addition of recombinant Factor B back to bovine submitochondrial particles depleted of this protein restored the energy-coupling activity. Reverse electron transfer from succinate to complex I, enabling NAD^+ reduction and ATP synthesis, was increased [95]. The F0-F1 ATPase activity requires Factor B coupled to it for full activity (Fig. 2). However, Factor B contains six thiols, and Cys 92 and Cys 94 in the bovine protein were shown to bind phenylarsine oxide [96], and phenylarsine oxide or arsenite inhibited the Factor B-coupling activity [97]. From all of these studies, it is becoming clear that redox changes to vicinal thiols affect regulation of the mitochondrial function and that these thiols also provide major targets for inhibition by thiol-reactive mitocans including arsenic-containing compounds.

10 ANT critical thiol groups as targets of arsenic-containing compounds in the mitochondria

A channel formed by the association of two proteins, VDAC in MOM and ANT in MIM (Fig. 2) is a complex involved in the induction of apoptosis activated *via* the mitochondrial pathway. The two components of this complex form part of the mitochondrial permeability transition pore (MPTP), a channel mediating release of molecules from mitochondria that activate apoptosis. The rapidly increasing permeability of MIM proceeds to apoptosis that is mediated by the MPTP.

Arsenite induces apoptosis by a direct effect on the MPTP [98, 99], and VDAC has been shown to play a role in opening of the permeability pore and cytochrome *c* (Cyt *c*) release induced by arsenic trioxide, which also causes VDAC to homodimerise [100]. In addition, the thiol-reactive compound 4,4k-diisothiocyanostilbene-2,2′-disulphonate (DIDS) has been shown to block the VDAC channel [101] and inhibit ROS-mediated Cyt *c* release [102]. This suggests that VDAC may contain critical Cys residues that can form intermolecular crosslinks following reaction with arsenical compounds. Analysis of ion channel activity of purified ANT-containing lipid bilayers also revealed that arsenic trioxide altered the ANT channel electrophysiological properties [103]. Interestingly, GSH depletion leading to increased ROS may play an important role in the action of arsenic trioxide [99]. Whereas in both normal and cancer cells, glutathione *S* transferase (GST) is found to interact with ANT, during the induction phase of apoptosis GST dissociates from ANT suggesting that GST/GSH may act as a repressor of MPTP and ANT pore-opening [99, 104]. This possibility is supported by the observation that increasing the expression of GSTP1 in Jurkat and Raji cells renders them more resistant to arsenic trioxide-induced apoptosis at clinically relevant levels. GSTP1 expression in these cells was also accompanied by decreased production of H_2O_2 [104].

Facing inside the mitochondrial matrix, ANT has three exposed loops containing a conserved repeat structure with one Cys residue in each loop. These residues are important to the process of ANT dimerisation, but it is not clear how this operates and whether the Cys residues form intermolecular disulphide bonds [105], although dimerisation of ANT by intermolecular cross-linking of Cys56 (in the first matrix loop) were reported [106]. Phenylarsine oxide, eosin 5-maleimide and diamide formed intramolecular cross-links between Cys160 and Cys257 on the other two matrix loops, restricting ANT in the open conformation and promoting MPT [106]. Arsenic trioxide is much weaker than phenylarsine oxide at binding to the ANT Cys residues [107, 108], and this may explain the greater sensitivity exhibited by acute promyelocytic leukemia cells to phenylarsine oxide than to As_2O_3 [109].

Single thiol-interacting compounds such as *N*-ethylmaleimide (NEM), can inhibit MPT. This could occur either as a result of direct interaction with the key Cys residues on the matrix loops of ANT, or indirectly *via* reaction with GSH preventing its oxidation that would catalyse disulphide bridging between the adjacent thiol groups in the ANT loops [106]. NEM or monobromobimane preferentially react with GSH, leading to its modification in mitochondria and thereby preventing GSH from oxidation. As a result, NEM inhibits MPT activation by the thiol-reactive compounds [107, 108].

11 The importance of ROS production by mitocans in triggering apoptosis: A general model

Since many mitocans rely on their ability to act as pro-oxidants in mitochondria of malignant cells, production of ROS by these organelles is very likely to be an important trigger leading to the activation of cancer cell apoptotic signalling pathway. The general mechanism whereby ROS

activate the mitochondrial apoptotic pathway has not yet been resolved. We have recently proposed a plausible model to explain the process [110]. Although $O_2^{\bullet-}$ is the immediate product from the respiratory chain leakage of electrons, it is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). In particular, $O_2^{\bullet-}$ released from ubisemiquinone on the MIM face of the respiratory chain [111, 112] reacts with MnSOD resulting in production of H_2O_2 [113, 114], which is then released across MOM into the cytosol [115]. The MnSOD activity can be inhibited by TXR located in MIM [116].

ROS probably have two major roles in apoptotis induction, including the mitochondrial translocation of Bax and cytosolic mobilisation of Cyt *c*. In a healthy cell, monomeric Bax is located predominantly in the cytoplasm, with the C-terminal mitochondrial-docking motif hidden due to its conformation. Upon initiation of apoptosis, Bax conformation changes, exposing the docking motif and provoking dimerisation of Bax, which then moves to MOM where Bax aggregates to form transmembrane (TM) channels [117]. Several domains within the Bax molecule are critical for Bax oligomerisation and movement to MOM. These include the Bcl-2 homology-3 (BH3) domain close to the N-terminus essential for dimerisation of Bax [118], and the C-terminal TM or mitochondrial-docking domain important for mitochondrial recognition of Bax homodimers [119]. It also appears that the Bax BH3 and TM domains interact with each other during apoptosis signalling. Both dimerisation and exposure of the TM domain occur before translocation of Bax to mitochondria [120], consistent with these events occurring early during apoptogenic signalling.

Other studies have reported that Bax dimers formed in the cytoplasm move to MOM where they associate into tetramers and octamers that allow larger molecules, such as Cyt *c*, to pass through, and that these 'megachannels' do not include VDAC [120, 121]. It has been shown that Bax dimerisation and mitochondrial translocation proceeds prior to mitochondrial events [122, 123] and that the chaperone HSC70, that maintains proteins in an unfolded state, prevents Bax translocating to the cytosolic face of MOM [124]. Recent evidence suggests that ROS production causes dimerisation of Bax in the cytosol by generating disulphide bridges between critical Cys residues [125]. The ROS originates from the initial mitochondrial events in the intrinsic apoptogenic signalling, both in whole cells and in mitochondria-free cytosolic fractions. Computer simulations have shown that these disulphide bridges change the conformation of Bax, so that it can translocate to MOM [125]. Bax contains two Cys residues that are accessible to oxidants [126]. Molecular modelling has suggested that the most favourable disulphide bridges in Bax dimers are formed by linking Cys162–Cys162 or Cys162–Cys62. The latter combination is more likely since Cys62 is within the BH3 domain of Bax that is essential for Bax dimerisation and conformational change, as well as for the interaction of Bax with Bcl-2 and Bcl-x_L proteins [127, 128]. Further, experimental validation of this proposal is still required.

Another central player in mitochondrial-dependent apoptosis is Cyt *c* [129]. This mitochondrial inter-membrane protein is anchored to MIM due to its affinity for the mitochondria-specific phospholipid cardiolipin (CL), and the binding is disrupted upon oxidation of CL by ROS derived from the OXPHOS complexes [130, 131]. Oxidation of CL and subsequent release of Cyt *c* are both prevented by endogenous SOD [130] or exogenous cell-permeable antioxidants [132]. Cyt *c* alone appears to have an oxidase activity that results in modification of CL and release of the protein from CL. Using oxidative lipidomics, it has been shown that CL was the only phospholipid in mitochondria oxidised in the early stages of apoptosis initiated by staurosporine or actinomycin D, and that it was triggered by mitochondria-dependent generation of ROS [133]. Cells deficient in complex I generate ROS, leading to CL oxidation and subsequent Cyt *c* detachment from the phospholipid [134], followed by cytoplasmic relocalisation of Cyt *c* *via* Bax channels [127]. These results link mitochondrial ROS production, ROS-dependent Bax dimerisation and formation of channels in MOM and release of Cyt *c* into the cytoplasm.

Mitochondrial proteins Bcl-2 and Bcl-x_L exert their antiapoptotic activity by diverting Bax and preventing it from forming MOM channels. These antiapoptotic Bcl-2 family members contain BH3-binding domains in the form of a hydrophobic groove exposed on the cytosolic face of MOM, and they both bind Bax *via* the Bax BH3 domain [134, 135]. Since heterodimers between Bax and Bcl-2/Bcl-x_L are favoured over heterotetramers [87], it is possible that Bcl-2 and Bcl-x_L capture Bax monomers before they dimerise to become 'activated', thereby preventing Bax from forming channels in MOM [136]. A novel function of one of the mitocans, α-TOS, has been reported in that it associates with the same hydrophobic groove on Bcl-2 or Bcl-x_L [137]. α-TOS binding prevented capture of the Bax-homologue Bak by the two proteins thereby increasing the pool of Bak required for the formation of MOM channels. Since Bak also binds to the BH3 domains of Bcl-2/Bcl-x_L, it is possible that α-TOS will, in an analogous manner, release Bax to form MOM channels. α-TOS-induced ROS production would facilitate ROS-dependent dimerisation of Bax, otherwise monomeric Bax would be captured *via* the hydrophobic pocket on the cytosolic side of unoccupied Bcl-2 or Bcl-x_L [87, 136].

12 Types of mitocans and their targets

We have catalogued the mitocans into seven different classes depending on their particular target(s) associated with mitochondrial function. Each class of mitocans is cov-

Table 1. Compilation of the different classes of mitocans based on their particular mitochondrial target sites that exist in, or are associated with, cancer cell mitochondria and their distinctive properties

Mitocan class	Site of action	Drug names	References
(I) HK inhibitors	HK-1 or HK-II	3-Bromopyruvate, 2-deoxyglucaose	[12]
(II) BH3 mimetics	Bcl-2, Bcl-x_L	Gossypol, EGCG, antimycin, α-TOS, HA14-1	[137, 144 – 147]
(III) Thiol redox inhibitors	VDAC, ANT, GST, TRX	Isothiocyanates, PEITCs, phenylarseneoxides, arsenites, Cu-phenanthrolines	[101, 102, 106, 108, 109, 151 – 155]
(IV) VDAC/ANT targeting (not *via* SH)	VDAC, ANT	Lonidamine, bisphosphonates, MT21, steroid analogues like CD437, ATRA	[103, 157, 158]
(V) ETC-targeting drugs	Complex I, II, III or IV	α-TOS, 4-OH retinamide, tamoxifen, resveratrol, dicumarol	[160 – 162, 166 – 170]
(VI) Lipophilic cations targeting the MIM	MIM	Rhodamine 123, MKT-077, dequalinium, F16, (KLAKKLAK)2 peptide	[172 – 176,181, 182, 198]
(VII) Drugs targeting mtDNA	DNA polymerase γ	Menadione	[183, 184]
(VIII) Drugs targeting; other sites	Unknown	Betulinic acid, phenoxodiol, sesquiterpene lactones (parthenolides)	[185, 186, 189, 190, 193, 197]

ered separately, exploring the mechanism of action. Numerous drugs have been described that target mitochondria and induce apoptosis of cancer cells. However, only those whose site of action has been clearly identified are discussed in the present review. The different classes of mitocans encompass a growing number of drugs, whose examples are listed in Table 1. Classification relates to the different metabolic reactions associated with the mitochondrial organelle whose function is central to cell survival. Grouping the mitocans into different classes helps to rationalise the variety of mitocans and is based on the order and level from outside to inside mitochondria and their site of drug action. If one of these reactions is inhibited inside the cancer cell, then continued survival becomes compromised. The basis for the specificity of these drugs for cancer cells is examined in the following sections where examples from each class are discussed.

12.1 Class I: HK inhibitors

3-Bromopyruvate (3BP) and 2-deoxyglucose (2DG) belong to a class of mitocans inhibiting HKs that catalyse conversion of glucose to glucose-6-phosphate accompanied by ATP hydrolysis. HK-I and HK-II are found in cancer cells associated with the outer mitochondrial surface [12], where they help protect the mitochondrial TM channel formed between VDAC1 and ANT [138, 139]. HK association with VDAC prevents pro-apoptotic molecules like Bax from binding to the outer face of VDAC and activating the MOM pore. 3BP and 2DG are classed as mitocans because they reduce the stability of the HK – VDAC association, thereby increasing the propensity for pro-apoptotic molecules like Bax to bind to VDAC and facilitate formation of the MOM pore [140].

Inactivation of cyclophilin D with siRNAs or a cyclophilin inhibitor was found to release HK-II from mitochondria and enhance Bax-mediated apoptosis [141]. The anti-apoptotic effects of cyclophilin D were negated by the detachment of HK-II from mitochondria, demonstrating that mitochondrial binding of HK-II is essential to the suppression of apoptosis by cyclophilin D, whose dysfunction appears to abrogate HK-II-mediated suppression of apoptosis [141].

Not only does 3BP inhibit HKs [12], but also it inhibits the activity of many other glycolytic pathway enzymes and the TCA cycle/electron transport component, SDH [142], so that it is also included in class V of mitocans (see below).

12.2 Class II: BH3 mimetics

This group of mitocans is diverse, including drugs like the compound ABT-737 [143], the polyphenolic drug gossypol, the green tea constituent epigalocatechingallate (EGCG) and peptides based on the BH3 sequence [144, 145]. BH3 mimetics act by preventing Bcl-2 and Bcl-x_L from binding to Bax and Bak and blocking these pro-apoptotic proteins from forming pores in MOM. In the presence of excess BH3 mimetic, when Bax and Bak are activated by apoptotic signals, they become free to form the MOM pore and induce apoptosis [146, 147]. Although Bax and Bak have been shown to form pores either alone, or in complex with VDAC, the precise mechanism for this pore-forming process is not yet well defined [148]. An intriguing BH3 mimetic is α-TOS, which has been shown to disrupt interaction of Bak with Bcl-2 and Bcl-x_L [137]. Since this drug also induces apoptosis by modulating the electron redox chain causing generation of ROS [110, 149], the vitamin E analogue belongs to both mitocan class II and V.

12.3 Class III and IV: Thiol redox inhibitors/VDAC- and ANT-targeting drugs

The class III mitocans act by modifying redox-active thiols of key molecules that regulate the MPT function and the mitochondrial TM channel proteins. Both VDAC and ANT

contain two or more Cys residues that provide reactive thiol groups whose modification affects their function and ANT dimerises to form the nucleotide transporter channel [150]. It was proposed that phenylarsine oxide, eosin 5-maleimide or diamide form intramolecular cross-links between Cys160 and Cys257, restricting ANT in a state known as the C-confirmation, promoting mitochondrial permeability transition [106]. However, phenylarsine oxide, a highly toxic compound *in vivo*, is nonselective for cancer cells [151].

The glutathionyl peptide trivalent arsenical compound 4-(*N*-(*S*-glutathionylacetyl)amino) phenylarsenoxide (GSAO) inactivates ANT-mediated ATP/ADP transport and triggers influx of Ca^{2+} upon crosslinking Cys160 and 257 of ANT. This leads to increased cellular ROS, ATP depletion, mitochondrial depolarisation and apoptosis in angiogenic endothelial cells and inhibited tumour growth in mice with no apparent side effects [152]. Interestingly, the para form of GSAO revealed no apparent toxicity in animals undergoing treatment and inhibited tumour growth leading to phase I clinical trials as an anticancer agent [152], while the ortho form was toxic, possibly due to the loss of multidrug resistance-mediated efflux of the drug [153].

Single thiol-interacting compounds like NEM can inhibit MPT and this could be either the result of direct interaction with key Cys residues on ANT or indirectly *via* reaction with GSH, preventing it from being oxidised and catalysing disulphide bridging between adjacent thiol groups in ANT [106]. NEM or monobromobimane preferentially react with GSH, leading to its modification in mitochondria, thereby preventing oxidation of GSH. Thus, NEM inhibits MPT activation by thiol-reactive compounds, implying a role for GSSG in the action of these agents on MPT as a result of disulphide crosslinking [107]. Arsenites may have a similar action, modifying and inhibiting the role of GSH in redox control such that glutathione-based enzymes are unable to function, increasing ROS production.

Another group of compounds in the third class of mitocans are isothiocyanates, particularly the dietary phenyl ethyl isothiocyanates (PEITCs) [154, 155]. Again, these compounds are thiol modifiers, forming adducts with thiol groups in important redox regulators. PEITCs effectively inhibit the GSH antioxidant system, producing excessive ROS accumulation in transformed cells. The excessive ROS output by cancer cells promotes oxidative mitochondrial damage, inactivation of redox-sensitive molecules leading to massive cell death. In animal cancer models, PEITC was shown to exhibit therapeutic activity and to prolong animal survival.

Mitocans in class IV, like those in class III, also affect the function of the ANT ion channels and transporters of nucleotides, but this occurs by directly binding and modifying the protein subunit structure. Oligomeric structures comprising VDAC in MOM and ANT in MIM exist in a coupled state forming a channel spanning across both mitochondrial membranes [156]. Class IV mitocans that bind ANT include lonidamine, an indazole carboxylate [103], bisphosphonates that form cytotoxic ATP analogue-type metabolites [157], as well as retinoid-like structures (*e.g.* CD 437) and all-trans retinoic acid [158]. Reports of VDAC-binding modifiers acting as mitocans could not be found. Cations such as ruthenium red and La^{3+} binding to Ca^{2+} sites on VDAC block channel activity and appear to prevent activation of the MOM pore [159]. This may be because Ca^{2+} influx *via* VDAC into mitochondria is required for the induction of apoptosis and hence, drugs which block Ca^{2+} entry would help prevent apoptosis.

12.4 Class V: Electron transport chain-targeting drugs

Class V of mitocans comprises a large number of different drugs that target components of the electron redox chain, leading to ROS production and activation of apoptosis in cancer cells. These drugs include α-TOS, the main interest of the authors, as well as related drugs such as *N*-(4-hydroxyphenyl)retinamide (fenretinide). The pro-oxidant mechanism of 4-HPR activity has not been clearly identified, although it is likely to act in the micromolar range as an inhibitor of at least one of the complexes along the ETC, requiring mitochondrial respiration for its apoptotic activity [160]. Tamoxifen was shown to induce apoptosis in MCF-7 breast cancer cells at low levels by affecting the mitochondrial function and increasing ROS accumulation [161]. The target of tamoxifen action was later identified as the flavin mononucleotide site of complex I leading to H_2O_2 production.

The vitamin E analogue, α-TOS, selectively induces killing of cancer cells by a process involving mitochondria and accumulation of ROS. The molecular target of α-TOS was not known until recently when we showed that α-TOS works by inhibiting the SDH activity of complex II in the mitochondrial ETC [162]. The drug interacts with the UbQ-binding sites on complex II, based on biochemical analyses and computer-assisted molecular modelling [149]. Genetic modification of cancer cells to mutate the SDHC subunit of complex II rendered them resistant to killing by α-TOS and ROS accumulation. By reconstituting a functional complex II, the cells became susceptible again to α-TOS. Similar resistance to the drug was observed in cancer cells where expression of SDHC was reduced by siRNA. We propose that α-TOS acts on cancer cells by displacing UbQ from binding to complex II in cancer cell mitochondria. As a result, electrons generated by the action of SDH recombine with molecular oxygen to produce high ROS levels. Our data, as well as preclinical studies with breast cancer models highlight α-TOS targeting of complex II as a potent and selective cancer therapy, with additional anticancer effects due to its anti-angiogenic activity [163].

Resveratrol acts in the micromolar range at several different sites from complexes I to III, probably competing

with UbQ, and also inhibits the F1 ATPase [164, 165]. Although resveratrol is considered an antioxidant, it can also act as a pro-oxidant by enhancing ROS production in cells, inducing apoptosis *via* the mitochondrial pathway [166]. A methoxy derivative of resveratrol showed even more potent activity in inducing apoptosis in transformed cells [167]. Two additional reports with different cancer cell types further support the ability of resveratrol to act as a mitocan [168, 169].

3BP, already mentioned as a class I mitocan, also belongs to class V. It causes a rapid decrease in cellular production of ATP, as a result of its inhibition of SDH at the level of the catalytic subunit of complex II, SDHA [170]. A related drug targeting SDH is 3-nitropropionic acid and this inhibition is associated with a large increase in $O_2^{\bullet -}$, also resulting as a by-product of the ETC inhibition [171].

12.5 Class VI: Lipophilic cations targeting the mitochondrial inner membrane

This class of mitocans includes molecules that are delocalised lipophilic cations which accumulate at much greater concentrations in the mitochondrial matrix than in the cytoplasm of cells. These agents selectively accumulate in the mitochondrial matrix of cancer cells because of their greater potentials across the plasma membrane as well as their more polarised mitochondria with a much greater $\Delta\Psi_{m,\,i}$ than that in their nonmalignant counterparts [138, 172, 173]. The target for the lipophilic cation-based mitocans may be one of the inhibitory binding sites on ATPase [165]. One of the earliest members of this class identified for its anticancer activity was rhodamine-123 [174, 175]. It entered phase I trial for prostate cancer and revealed minimal side effects and safe administration at monthly intervals without detectable drug accumulation in the serum of patients [176]. It is likely that a related compound, Rose Bengal, works in a similar fashion to rhodamine 123, and Rose Bengal is likewise currently in clinical trials as a therapy for metastatic melanoma and recurrent breast cancer, causing complete remissions in some patients (Provectus PV-10-MM-01, www.ClinicalTrials.gov).

The drug F16 is a mechanistically more characterised example of this mitocan class and was shown to increase ROS production, depolarise mitochondria as a weak protonophore and collapse $\Delta\Psi_{m,\,i}$, leading to MPT and selective apoptosis of cancer cells in the micromolar range [176]. F16 was also reported in this study to inhibit the growth of mammary tumours in mice. MKT-077, a rhodocyanine dye analogue, is another example of this type of mitocan that entered phase I clinical trials, although these were terminated due to renal toxicity [177].

This raises the issue of toxicity with many of the class VI mitocans. A cautionary example of the potential for toxicity that must be carefully evaluated is the production of Parkinson's-like effects by the drug MPTP caused by the selective destruction of the nigrostriatal dopaminergic neurons. This toxicity was due to the selective uptake by the dopamine transporters on these cells as well as metabolites formed by the action of the enzyme, monoamine oxidase B that is highly expressed in the dopaminergic neurons. As a result of these two unique properties of dopaminergic neurons, the mitochondriotoxic drug MPP^+ is produced, which acts as an inhibitor of mitochondrial respiration by blocking the NADH-UbQ oxidoreductase site of complex I. It is likely that MPP+ is also a protonophore [178, 179] that would collapses the $\Delta\Psi_{m,\,i}$ leading to cell destruction. Together with the nonselective cell toxicity associated with another lipophilic cation and known mitochondrial poison, dequalinium chloride, this raises the importance of identifying class VI mitocans that are cancer cell-specific in terms of their uptake and toxicity, as recently described in a predictive model based on their structures [180].

The amphipathic and positively charged α-helical pro-apoptotic peptide $(KLAKLAK)_2$ has also been included in this class as a delocalised lipophilic cation. The peptide must first be coupled to a targeted delivery system for receptor binding and uptake by cancer cells [181, 182]. As with the other members of this class, the peptide has been shown to dissipate $\Delta\Psi_{m,\,i}$ leading to apoptosis, and it efficiently reduced tumour burdens in animal models [181].

12.6 ClassVII: Drugs targeting mtDNA

This is a recently identified group of mitocans. Thus far, the only member of this class is the redox cycler menadione (vitamin K3). The agent inhibits the activity of the mitochondria-specific DNA polymerase γ, but neither DNA polymerase α, β, δ or ε, which are all associated with nuclear DNA [182]. Consequently, menadione causes impaired replication and repair of mtDNA in a p53-independent manner. Interestingly, inhibition of DNA polymerase γ did not result in increase ROS generation, and suppressed proliferation of cancer cells. This effect was not observed with vitamin K1 or vitamin K2. In a recent paper, inhibition of angiogenesis was observed with vitamin K3, which inhibited DNA polymerase γ in proliferating endothelial cells [183].

12.7 Class VIII: Drugs targeting other sites

The last class of mitocans includes agents whose target site and mechanism of action on mitochondria is not well characterised or unclear at present. It includes drugs that bind to the peripheral benzodiazepine receptor such as PK-11195 and Ro5-4864 [185, 186]. Peripheral benzodiazepine receptor is also known as the MOM translocator protein [187], an 18kDa MOM-localised protein. It is important for porphyrin transport and heme synthesis, apoptosis, cell proliferation, anion transport, regulation of mitochondrial functions and immunomodulation. This protein is often overexpressed in cancer cells [188, 189] and is a potential target

for anticancer drugs that bind to it, but this area of research has not been well defined.

The other drugs included in this class are the natural product derived pentacyclic triterpenoids such as betulinic acid. Betulinic acid and its derivatives have been shown to be specifically cytotoxic to tumour cell lines [189, 190], causing rapid increase in ROS production and a concomitant dissipation of $\Delta\Psi_{m,\,i}$ in a dose- and time-dependent manner, resulting in apoptosis [191]. Betulinic acid was found to be nontoxic up to 500 mg/kg body weight in mice [192]. The target in mitochondria for betulinic acid-related compounds has not been identified and their mechanism for initiating mitochondrial dysfunction leading to apoptosis remains unknown.

The sesquiterpene lactones, such as parthenolides, are another group within the last class of mitocans encompassing a number of different naturally occurring structures that induce oxidative stress-mediated apoptosis in cancer cells acting in the micromolar range [193] via loss of $\Delta\Psi_{m,\,i}$ and induction of ROS accumulation [194 – 196]. They are also believed to target tumour stem cells whilst not affecting normal cells although their mechanism is presently unclear [197].

13 Conclusions and further perspectives

As a greater understanding arises of the key differences between mitochondria from normal *versus* malignant cells, we will gain in the ability to selectively target and kill tumours by activating apoptosis. Mitocans are providing a renewed impetus in the fight against cancer with their reduced level of side effects due to their greater target selectivity. It is still too early in the clinical testing phases to decide whether mitocans will prove to be effective anticancer agents, although preliminary data indicates a strong likelihood of success. As a general rule, mitocans act by disrupting mitochondrial function in cancer cells, particularly of the OXPHOS system and the ETC, causing overload reactions, ROS by-products, modification of thiol groups on key REDOX proteins and inhibition of prosurvival functions. The net effect is to activate apoptotic signal pathways in cancer cells. As anticancer drugs, the mitocans offer opportunities for additive or synergistic effects when used in combination with existing chemotherapeutic approaches, improving clinical outcomes. In addition, mitocans are providing approaches for directly attacking tumour stem cells. The goal is now in sight when we will be able to induce the clonal depletion and exhaustion of the progenitor cell populations leading to cancer cures with mitocan therapy.

The authors have declared no conflict of interest.

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