Targeting mitochondria for cancer therapy

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Abstract | Mitochondria are the cells' powerhouse, but also their suicidal weapon store. Dozens of lethal signal transduction pathways converge on mitochondria to cause the permeabilization of the mitochondrial outer membrane, leading to the cytosolic release of pro-apoptotic proteins and to the impairment of the bioenergetic functions of mitochondria. The mitochondrial metabolism of cancer cells is deregulated owing to the use of glycolytic intermediates, which are normally destined for oxidative phosphorylation, in anabolic reactions. Activation of the cell death machinery in cancer cells by inhibiting tumour-specific alterations of the mitochondrial metabolism or by stimulating mitochondrial membrane permeabilization could therefore be promising therapeutic approaches.

Evasion of cell death is a hallmark of human cancers and a major cause of treatment failure[1,2]. The lack of efficacy of established therapeutic regimens is due, at least in part, to the oncogenic blockade of cell death pathways[3]. Thus, drugs designed to activate the cell death machinery may represent a more effective therapeutic option. This machinery is composed of catabolic hydrolases, mostly proteases and nucleases, which are held in check by specific inhibitors or by the sequestration of their activators. The permeabilization of the mitochondrial outer membrane is a potent way of unleashing such activators. Multiple apoptosis-inducing and necrosis-inducing biochemical cascades converge on mitochondria to cause their deregulation and permeabilization[4].

Mitochondria exert both vital and lethal functions in physiological and pathological scenarios[4,5]. On the one hand, mitochondria are indispensable for energy production and hence for the survival of eukaryotic cells. On the other hand, mitochondria are crucial regulators of the intrinsic pathway of apoptosis. Mitochondria control the activation of apoptotic effector mechanisms by regulating the translocation of pro-apoptotic proteins from the mitochondrial intermembrane space to the cytosol. Furthermore, mitochondria play a major part in multiple forms of non-apoptotic cell death and, in particular, in necroptosis (regulated necrosis)[6,7]. As mitochondria are key regulators of cell death and as mitochondrial functions are often altered in neoplasia[8], mitochondrially-targeted compounds represent a promising approach to eradicate chemotherapy-refractory cancer cells.

There is ample evidence of metabolic alterations affecting the capacity of malignant cells to engage in catabolic processes, including apoptosis, necrosis and autophagy[9]. Moreover, modifications in the levels of reactive oxygen species (ROS) have recently been linked to the intrinsic chemotherapy resistance of cancer stem cells[10]. These changes are intricately linked to the bioenergetic functions of mitochondria, making these organelles attractive drug targets[9,11].

Owing to their role in the regulation of fundamental cellular functions, it is not surprising that mitochondria have been implicated in multiple aspects of tumorigenesis and tumour progression[8]. For instance, mutations of the mitochondrial or nuclear DNA that affect components of the mitochondrial respiratory chain result in inefficient ATP production, ROS overproduction and oxidative damage to mitochondria and other macromolecules (including DNA, thereby favouring chromosomal instability and carcinogenesis)[12]. Furthermore, numerous polymorphisms and mutations of the mitochondrial DNA correlate with an increased risk of developing several malignancies including breast cancer, prostate cancer and thyroid cancer[13,14]. Accordingly, multiple hallmarks of cancer cells, including limitless proliferative potential, insensitivity to anti-growth signals, impaired apoptosis, enhanced anabolism and decreased autophagy, have been linked to mitochondrial dysfunctions[11,15].

Cancer cell mitochondria are structurally and functionally different from their normal counterparts[8,12]. Moreover, tumour cells exhibit an extensive metabolic reprogramming that renders them more susceptible to mitochondrial perturbations than non-immortalized cells[9,11]. Based on these premises, mitochondrially-targeted

Intrinsic pathway of apoptosis
Also known as mitochondrial apoptosis, it is triggered by intracellular stimuli such as Ca^{2+} overload and overproduction of reactive oxygen species. By contrast, extrinsic apoptosis is initiated at the plasma membrane by specific transmembrane receptors.

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agents emerge as a means to selectively target tumours. The correction of cancer-associated mitochondrial dysfunctions and the (re)activation of cell death programmes by pharmacological agents that induce or facilitate mitochondrial membrane permeabilization represent attractive strategies for cancer therapy. The rationale underlying this approach has been extensively discussed in a number of recent publications[16–18]. Here, we provide a comprehensive compendium on the mitochondrially-targeted compounds that show the greatest promise for the treatment of human malignancies. Moreover, we discuss the perspectives and future developments of this area of research.

Targeting mitochondrial permeability transition

Under physiological conditions, mitochondria harbour a robust mitochondrial transmembrane potential (represented as: $\Delta\psi_m$)[19], and the low-conductance state of the permeability transition pore complex (PTPC) might contribute to the exchange of small metabolites between the cytosol and the mitochondrial matrix, a process that is mainly controlled by mitochondrial solute carriers.

The PTPC is a highly dynamic supra-molecular complex for which the precise structural identity is poorly understood. This is probably because its constituents exist in multiple isoforms, and a number of distinct but functionally related proteins (such as solute carriers) can substitute for each other within the PTPC. Accordingly, while the prototypical PTPC would be composed of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocase (ANT) in the mitochondrial inner membrane and cyclophilin D (CYPD) in the mitochondrial matrix[4], mouse knockout studies revealed that both VDAC and ANT (but not CYPD) are dispensable for the lethal functions of the PTPC (see below)[20–23]. Additional PTPC-interacting proteins include the peripheral benzodiazepine receptor (PBR; also known as TSPO) in the outer membrane and hexokinase (HK), which makes contacts with the mitochondrial outer surface from the cytosol.

HK interacts with the PTPC in healthy cells, thereby inhibiting mitochondrial membrane permeabilization[24]. In response to pro-apoptotic stimuli, including ROS and Ca^{2+} overload, the PTPC assumes a high-conductance state that allows the deregulated entry of small solutes into the mitochondrial matrix along their electrochemical gradient[4]. This phenomenon, which is known as mitochondrial permeability transition (MPT), results in immediate dissipation of the mitochondrial membrane potential and osmotic swelling of the mitochondrial matrix. As the surface area of the inner membrane considerably exceeds that of the outer membrane, MPT eventually leads to mitochondrial outer membrane permeabilization (MOMP)[4] (FIG. 1).

Multiple compounds can act on the components of the PTPC to induce MPT and apoptosis. Indirect permeabilizing effects can be obtained by depleting endogenous inhibitors of PTPC opening such as glucose, ATP, creatine phosphate and glutathione. Similarly, MPT can be triggered by agents that increase cytosolic Ca^{2+} concentrations or stimulate ROS generation[4].

Compounds that act on PTPC constituents. Within the PTPC, different isoforms of ANT may have distinct functions. ANT1 and ANT3 are pro-apoptotic, whereas ANT2 (which is often overexpressed in proliferating cells) is anti-apoptotic[25–27]. ANT1 interacts with both anti-apoptotic and pro-apoptotic members of the B-cell lymphoma protein 2 (BCL-2) protein family such as BCL-2 itself and BCL-2-associated X protein (BAX), which act as allosteric activators and inhibitors, respectively, of the ANT1 ATP/ADP antiporter activity[28,29]. Intriguingly, the interaction between the *Caenorhabditis elegans* BCL-2 and ANT orthologues (CED-9 and WAN-1, respectively) is required for developmental and homeostatic cell death in nematodes[30], suggesting that the physical and functional interplay between the PTPC and BCL-2-like proteins is phylogenetically conserved[31].

Several ANT ligands have been reported to induce mitochondrial apoptosis and cell death (see below). However, none of these compounds has so far been described to specifically target one isoform of ANT. It remains to be explored whether isoform-specific ANT ligands (for example, binding to the pro-apoptotic variants ANT1 and ANT3), may be especially suitable for stimulating mitochondrial apoptosis in cancer cells.

4-(*N*-(*S*-glutathionylacetyl)amino) phenylarsenoxide (GSAO), a glutathione-coupled trivalent arsenical compound (FIG. 2), has been shown to cross-link critical cysteine residues of ANT (Cys160 and Cys257), resulting in inhibition of its ATP/ADP antiporter activity, ROS overproduction, cytosolic ATP depletion, mitochondrial depolarization and apoptosis[32] (TABLE 1). It has been suggested that GSAO would preferentially target proliferating cells owing to their high mitochondrial Ca^{2+} levels and elevated respiration rates, which would render them more susceptible to PTPC opening than normal cells[32]. The relative selectivity of GSAO for the endothelial component of tumours may be related to the fact that endothelial cells contain higher amounts of mitochondria than tumour cells and may have a reduced capacity to buffer the arsenical moiety of GSAO[32].

Lonidamine, an indazole carboxylate, is another putative ANT ligand that triggers mitochondrial apoptosis[33]. Lonidamine can permeabilize ANT-containing (but not ANT-free) proteoliposomes in a manner that can be blocked by the ANT ligand bongkrekic acid. In a Phase II clinical study with patients with recurrent glioblastoma multiforme, lonidamine (in combination with the PBR ligand diazepam, see below) was well tolerated and showed a cytostatic effect on tumour growth[34]. The addition of lonidamine to the anthracycline epirubicin increased the response rate of patients with solid tumours[35], but it had no additional effects when combined to an epirubicin/cyclophosphamide regimen[36] (see Supplementary information S1 (table) for the development status of lonidamine and other mitochondrially-targeted agents mentioned in the text).

Some bisphosphonates, such as the nitrogen-free compound clodronate, act as competitive ANT inhibitors, leading to the inhibition of mitochondrial oxygen consumption, dissipation of the mitochondrial membrane

Mitochondrial membrane permeabilization
The rupture of mitochondrial membranes leads to their functional impairment as well as to the release of toxic mitochondrial intermembrane space proteins into the cytosol.

Mitochondrial permeability transition
(MPT). Long-lasting openings of the permeability transition pore complex lead to an abrupt increase in the inner mitochondrial membrane permeability to ions and low molecular mass solutes, in turn provoking osmotic swelling of the mitochondrial matrix and mitochondrial membrane permeabilization.

Mitochondrial outer membrane permeabilization
(MOMP). The pore-forming activity of pro-apoptotic BCL-2 family members like BAX and BAK results in the loss of the outer mitochondrial membrane impermeability to proteins.

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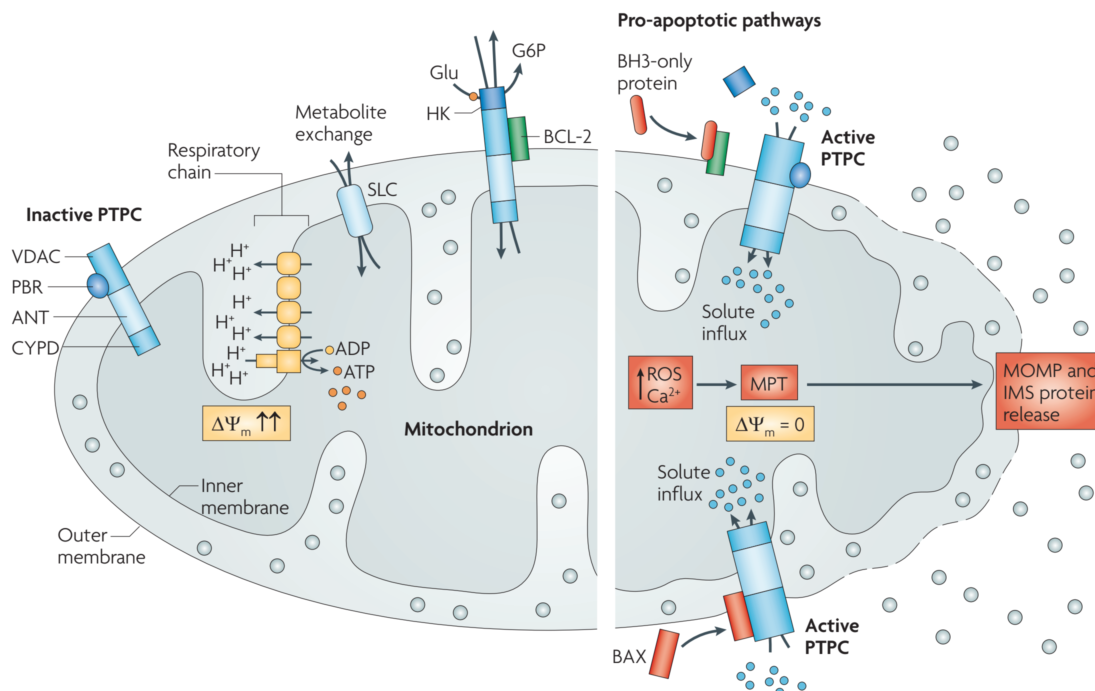

Figure 1 | **Mitochondrial permeability transition (MPT).** The so-called permeability transition pore complex (PTPC) is a highly dynamic supramolecular entity that can be constituted by the voltage-dependent anion channel (VDAC; embedded in the mitochondrial outer membrane), the adenine nucleotide translocase (ANT; residing in the mitochondrial inner membrane) and cyclophilin D (CYPD; a peptidyl-prolyl *cis-trans* isomerase of the mitochondrial matrix). In physiological conditions, mitochondria exhibit a high mitochondrial transmembrane potential ($\Delta\psi_m$), which is generated by the respiratory chain and exploited for ATP generation. It has been proposed that in these conditions the PTPC would exist in a low-conductance state (which would be favoured by its interaction with anti-apoptotic proteins from the B-cell lymphoma protein 2 (BCL-2) family), thereby contributing to the exchange of small metabolites between the cytosol and the mitochondrial matrix, a process that is predominantly mediated by mitochondrial solute carriers (SLCs). The PTPC has also been suggested to interact with the peripheral benzodiazepine receptor (PBR) and with hexokinase (HK), which uses mitochondrial ATP for catalysing the rate-limiting step of glycolysis; that is, the conversion of glucose (Glu) into glucose-6-phosphate (G6P). In response to some pro-apoptotic signals including the accumulation of reactive oxygen species (ROS) and Ca^{2+} overload, the PTPC assumes a high-conductance conformation that allows the deregulated entry of small solutes into the mitochondrial matrix driven by electrochemical forces. MPT can be favoured by pro-apoptotic proteins of the BCL-2 family such as BCL-2-associated X protein (BAX), which directly interact with the PTPC, as well as by BH3-only proteins, which may displace the PTPC from inhibitory interactions with BCL-2. MPT results in the immediate dissipation of the $\Delta\psi_m$ and in the osmotic swelling of the mitochondrial matrix, which eventually leads to mitochondrial outer membrane permeabilization (MOMP) — as the surface of the inner membrane largely exceeds that of the outer membrane — and to the release into the cytosol of cytotoxic proteins normally confined within the mitochondrial intermembrane space (IMS). Such cytotoxic proteins include caspase activators such as cytochrome *c* and DIABLO, as well as caspase-independent cell death effectors like apoptosis-inducing factor and endonuclease G.

potential and apoptosis[37]. Addition of oral clodronate to postoperative adjuvant breast cancer therapy has recently been shown to improve the overall survival of patients with primary breast cancer and bone-marrow micrometastases compared with patients who received postoperative adjuvant therapy alone[38]. However, the precise molecular mechanisms underlying the *in vivo* efficacy of clodronate remain uncertain[39,40].

Retinoid-related compounds such as CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid) (FIG. 2) and all-*trans*-retinoic acid are best known for their ability to stimulate the expression of retinoic acid receptor-responsive genes, leading to complete clinical remission in a high proportion of patients with acute promyelocytic leukaemia[41]. Interestingly, these retinoids also trigger ANT-dependent MPT and cell death independent from nuclear receptor binding, which suggests that another potential mechanism of action is involved[33,42,43] (TABLE 1). ST1926 ((*E*)-3-(4′-hydroxy-3′-adamantylbiphenyl-4-yl)acrylic acid) also causes mitochondrial perturbations independent from nuclear receptors, possibly by altering Ca^{2+} homeostasis[44]. Whether ST1926 directly targets ANT is unknown; however, ST1926 is currently under clinical investigation (Phase I) as monotherapy in patients with ovarian cancer[45] (TABLE 1).

PBR interacts with the PTPC through VDAC and is overexpressed in many cancers[4,46,47]. PBR has been implicated in the regulation of mitochondrial apoptosis as it blocks the anti-apoptotic effect of BCL-2 proteins including BCL-2 itself, BCL-X_L (also known as BCL2L1) and myeloid cell leukaemia sequence 1 (MCL1)[48,49]. PBR

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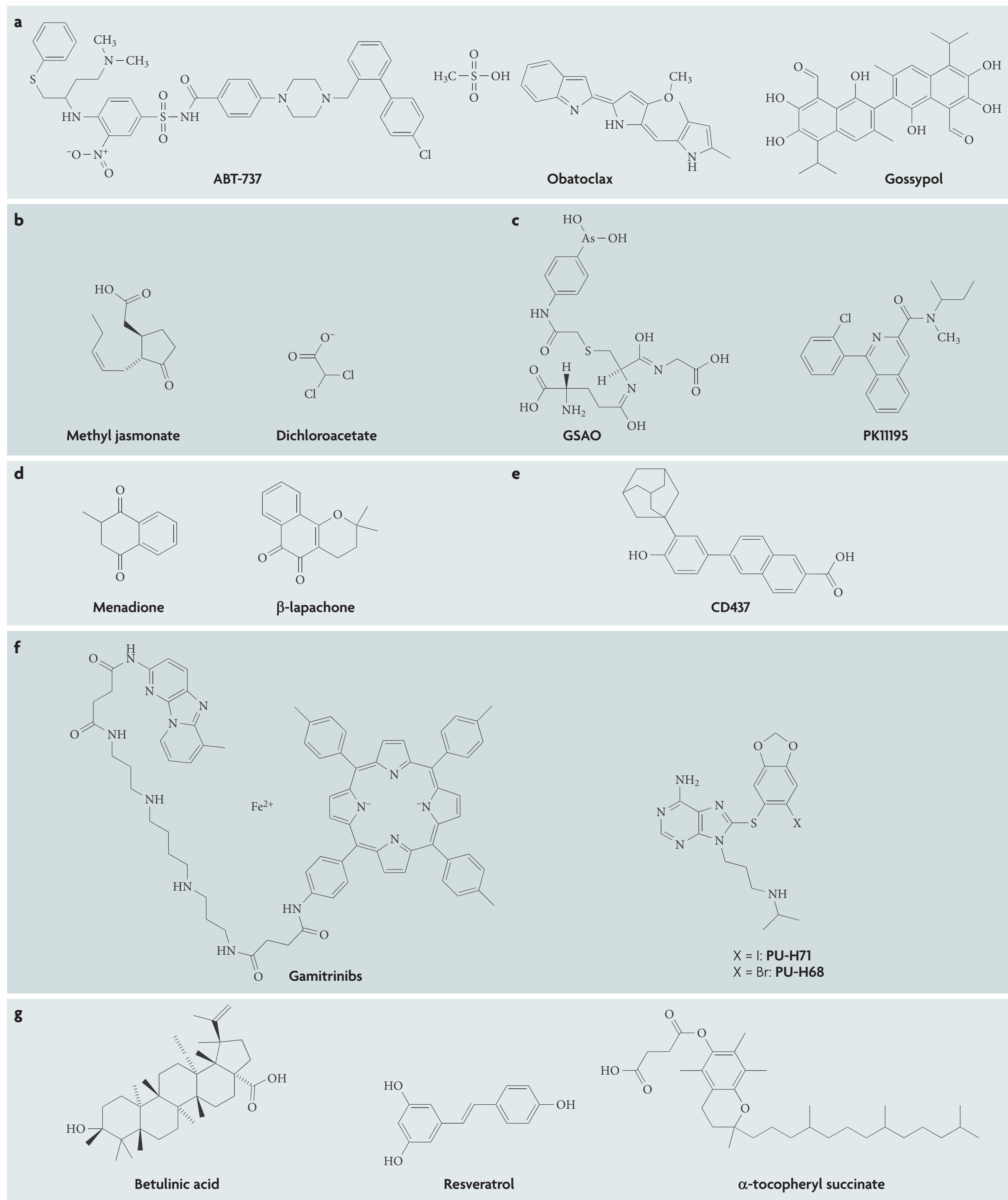

Figure 2 | **Chemical structures of selected mitochondrially-targeted anticancer agents. a** | Modulators of the B-cell lymphoma protein 2 protein family. **b** | Metabolic inhibitors. **c** | Agents targeting voltage-dependent anion channels and/or adenine nucleotide translocase. **d** | Regulators of reactive oxygen species generation. **e** | Retinoids. **f** | Inhibitors of heat-shock protein 90. **g** | Natural compounds and derivatives. CD437, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphtalene carboxylic acid; GSAO, 4-(*N*-(*S*-glutathionylacetyl)amino) phenylarsenoxide. For information related to the development status of these compounds see Supplementary information S1 (table).

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ligands such as PK11195 (FIG. 2), RO5-4864 and diazepam have demonstrated antitumour effects *in vitro* and *in vivo*; both as single agents or combined with the chemotherapeutic agents etoposide or ifosfamide[49]. Such chemosensitizing effects were observed irrespective of high BCL-2 expression levels, suggesting that PBR could be exploited to bypass BCL-2-imposed chemoresistance[49]. The pro-apoptotic activity of PBR ligands has been also attributed to PBR-independent mechanisms. For example, PK11195 and RO5-4864 effectively potentiated starvation-induced cell death in tumour cells depleted of PBR by RNA interference (RNAi)[50]. Similarly, PK11195 sensitized leukaemia and myeloma cells to chemotherapy by directly modulating P-glycoprotein-mediated drug efflux irrespective of PBR expression[51]. PK11195 and RO5-4864 have entered clinical trials, and promising results were obtained in patients with recurrent glioblastoma treated with diazepam plus lonidamine[34]. The individual contribution of diazepam and lonidamine, as well the therapeutic efficacy of this regimen against other malignancies, remain to be fully elucidated.

Compounds that induce the overproduction of ROS. Menadione (2-methyl-1,4-naphthoquinone) (FIG. 2), which undergoes futile redox cycles on the respiratory chain, and thiol cross-linking agents such as diamide (diazenedicarboxylic acid bis 5*N*,*N*-dimethylamide), bismaleimido-hexane and dithiodipyridine, all cause ANT thiol oxidation and can bypass BCL-2-mediated cytoprotection[52–54]. Early Phase I studies in patients with advanced cancer revealed mild toxicities at high menadione doses but no objective (partial or complete) responses[55]. However, a more recent clinical trial has demonstrated that this compound can induce objective clinical responses in patients with advanced hepatocellular carcinoma[56].

The aromatic macrocycle motexafin gadolinium (gadolinium texaphyrin) displays an elevated oxidizing potential, thereby triggering excess generation of ROS and inhibiting antioxidant systems[57]. Motexafin gadolinium has been shown to preferentially accumulate in cancer cells, perhaps due to their metabolic perturbations, and to enhance the *in vivo* response to radiation and chemotherapy of xenotransplanted tumours[57]. A Phase III study based on motexafin gadolinium plus brain radiotherapy for the treatment of patients with lung cancer and brain metastasis documented a motexafin gadolinium-derived prolongation in the time for neurological progression[58]. The same regimen proved to be well tolerated in paediatric glioblastoma patients[59], indicating that the combination of motexafin gadolinium and radiotherapy should be further investigated.

β-lapachone (ARQ 501) (FIG. 2) reportedly undergoes futile redox cycles that are catalysed by NAD(P)H:quinone oxidoreductase 1 (NQO1), thereby inducing the overproduction of ROS, poly(ADP-ribose) polymerase 1 (PARP1) hyperactivation and cell death[60]. β-lapachone is currently under clinical investigation as monotherapy or in combination with gemcitabine in patients with pancreatic cancer as well as in patients with head and neck cancer.

The inhibition of antioxidant systems provides an alternative mechanism that leads to ROS accumulation. For instance, buthionine sulphoximine elevates ROS levels by inhibiting the synthesis of reduced glutathione (GSH)[61], whereas imexon depletes the GSH pool due to its thiol-binding activity[62]. The association between buthionine sulphoximine and the alkylating agent melphalan is currently being evaluated in Phase I clinical trials in patients with neuroblastoma and melanoma. In a Phase I trial with patients affected by breast, prostate or lung cancer, treatment with imexon alone or in combination with docetaxel demonstrated some efficacy[62,63].

Isothiocyanates such as the dietary β-phenylethyl isothiocyanates (PEITCs) are thiol modifiers that react with redox regulatory proteins[64,65]. PEITCs have been shown to inhibit the GSH antioxidant system by extruding GSH from the cell and by inhibiting glutathione peroxidase[64,65]. This leads to ROS overproduction, oxidative damage of mitochondria, MOMP and apoptosis preferentially in cancer cells, presumably due to their increased constitutive ROS levels[64,65]. Clinical studies with PEITCs are currently being initiated.

Mangafodipir is a superoxide dismutase (SOD) mimic with catalase and glutathione reductase activities[66]. It acts as an antioxidant in normal cells, but in cancer cells mangafodipir has been shown to increase H_2O_2 levels and to potentiate the antitumour activity of paclitaxel against xenotransplanted colon cancer in mice[66]. Mangafodipir (in association with chemotherapy) is being tested in a Phase II trial in patients with colon cancer.

Some oestrogen derivatives such as 2-methoxyoestradiol selectively kill human leukaemia cells (but not normal lymphocytes) by inhibiting SOD and hence causing superoxide accumulation[67,68]. Similar effects are triggered by the intracellular copper-chelating agent tetrathiomolybdate (ATN-224)[69]. Arsenic trioxide, an effective chemotherapeutic drug used for the treatment of acute promyelocytic leukaemia and a wide range of solid tumours, is thought to exert its antitumour activity mainly through oxidative stress, which has recently been linked to irreversible inhibition of thioredoxin reductase[70]. The anticancer effects of the small-molecule elesclomol (STA-4783) have also been ascribed to its pro-oxidant activity[71]. Several Phase I/II studies testing 2-methoxyoestradiol in patients with solid malignancies or with multiple myeloma demonstrated that 2-methoxyoestradiol is well tolerated and causes disease stabilization[72–75]. Although elesclomol (alone or in combination with paclitaxel) yielded promising results in Phase I/II clinical trials in patients with refractory solid tumours[76,77], a recent Phase III study conducted in patients with melanoma has been discontinued due to safety concerns.

Several classes of compounds with distinct mechanisms of action can stimulate MPT and mitochondrial apoptosis in cancer cells, pointing to some functional redundancy and implying that alternative biochemical cascades leading to mitochondrial membrane permeabilization are likely to exist.

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Table 1 | **Examples of mitochondrially-targeted compounds**

Class	Compound	Target or mode of action	Refs
Modulators of the BCL-2 protein family	A-385358	BCL-X_L	117
	ABT-263, ABT-737	BCL-2, BCL-X_L, BCL-W	94
	AT-101	BCL-2, BCL-X_L, BCL-W, MCL1	238
	GX15-070 (obatoclax)	BCL-2, BCL-X_L, BCL-W, MCL1	122
	HA14-1	BCL-2	129
	Oblimersen	BCL-2 mRNA antisense	154
Metabolic inhibitors	2-Deoxy-D-glucose	HK	135
	3-Bromopyruvate	HK2–VDAC interaction	142
	Dichloroacetate	PDK inhibitor	143
	HK2 peptide	HK2–VDAC interaction	137
	LDH-A shRNA	LDH-A	144
	Methyl jasmonate	HK2–VDAC interaction	139
	Orlistat	Fatty acid synthase	148
	SB-204990	ATP citrate lyase	145
	Soraphen A	Acetyl-CoA carboxylase inhibitor	147
VDAC-targeting and/or ANT-targeting agents	Arsenite trioxide	ANT ligand, ROS production	33
	Clodronate	ANT inhibitor	37
	GSAO	ANT cross linker	32
	Lonidamine	ANT ligand	34
	PK11195	PBR ligand	49
ROS regulators	2-Methoxyestradiol	SOD inhibition	67,68
	ATN-224	SOD inhibition	69
	β-lapachone	ROS production	60
	Buthionine sulphoximine	GSH synthesis inhibitor	61
	Imexon	GSH depletion	62
	Mangafodipir	SOD mimic	66
	Menadione	ROS production	54
	Motexafin gadolinium	ROS production	57
	PEITCs	GSH depletion, GPX inhibition	64
	STA-4783	ROS production	71,239
Retinoids	All-*trans*-retinoic acid	ANT ligand	43
	CD437	Permeability transition pore complex	33,42
	ST1926	Perturbation of Ca^{2+} homeostasis	44
HSP90 inhibitors	Gamitrinibs	Mitochondrial HSP90 ATPase inhibitors	154
	PU24FCl, PU-H58, PU-H71	HSP90 inhibitors	156
	Shepherdin	Inhibitor of the HSP90–survivin interaction	152
Natural compounds and derivatives	α- tocopheryl succinate	Ubiquinone-binding sites in respiratory complex II	187
	Betulinic acid	Permeability transition pore complex	160
	DMAPT	ROS production	196
	Parthenolide	ROS production	197
	Resveratrol	F_1-ATPase	174

ANT, adenine nucleotide translocase; ATN-224, tetrathiomolybdate; BCL-2, B-cell lymphoma protein 2; BCL-W, also known as BCL2-like protein 2 (BCL2L2); BCL-X_L, also known as BCL2-like protein 1 (BCL2L1); CD437, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; DMAPT, dimethylamino-parthenolide; GSAO, 4-(*N*-(*S*-glutathionylacetyl)amino) phenylarsenoxide; HA14-1, 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate; GPX, glutathione peroxidase; GSH, reduced glutathione; HK, hexokinase; HSP90, heat-shock protein, 90 kDa; LDH-A, lactate dehydrogenase A; MCL1, myeloid cell leukaemia sequence 1; PBR, peripheral benzodiazepine receptor; PDK, pyruvate dehydrogenase kinase; PEITCs, phenyl ethyl isothiocyanates; PU24FCl, 8-(2-chloro-3,4,5-trimethoxybenzyl)-2-fluoro-9-(pent-4-ynyl)-9*H*-purin-6-amine; PU-H58 (8-(6-bromobenzo[d][1,3]dioxol-5-ylthio)-9-(pent-4-ynyl)-9*H*-purin-6-amine; PU-H71, 8-(6-iodobenzo[d][1,3]dioxol-5-ylthio)-9-(3-(isopropylamino)propyl)-9*H*-purin-6-amine; ROS, reactive oxygen species; shRNA, short hairpin RNA; SOD, superoxide dismutase; ST1926, (*E*)-3-(4′-hydroxy-3′-adamantylbiphenyl-4-yl)acrylic acid; STA-4783, elesclomol; VDAC, voltage-dependent anion channel.

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Targeting MOMP

MOMP can cause MPT to ensue, but MPT can also result from events that originate at the outer membrane. First, MOMP can be mediated by the pore-forming activity of pro-apoptotic members of the BCL-2 family such as BAX and BCL-2 homologous antagonist/killer (BAK)[78]. In healthy cells, BAX is cytosolic whereas BAK is an integral outer membrane protein. In response to pro-apoptotic triggers, BAX translocates to the outer membrane and BAK locally undergoes conformational changes[78]. These two processes lead to the assembly of homo-oligimers and/or hetero-oligomers that form protein-permeable conduits through which toxic intermembrane space proteins are released into the cytosol[78]. Activated BAX and BAK can be sequestered by their counterparts BCL-2 and BCL-X_L, which therefore act as strong cytoprotectors[4]. In this context, small proteins such as BH3-only proteins can exert pro-apoptotic functions either by directly stimulating BAX and/or BAK or by displacing them from inhibitory interactions with anti-apoptotic BCL-2 family members[79]. BCL-2 and BCL-X_L can also interact with activated BH3-only proteins, thereby blocking the pro-apoptotic cascade before BAX activation[80] (FIG. 3).

The outer membrane actively supports protein-to-protein interactions between BCL-2-like proteins. Accordingly, upon an apoptotic insult, truncated BH3-interacting domain death agonist (tBID) rapidly binds to the outer membrane and interacts with BAX, thereby triggering BAX insertion into the outer membrane, oligomerization and MOMP[80]. Cytoplasmic p53 has also been suggested to directly activate BAX or to release BAX from inhibitory interactions with anti-apoptotic BCL-2 family members such as BCL-2 itself, BCL-X_L and MCL1 (REFS 81,82). The binding groove of BAX that mediates its heterodimerization with anti-apoptotic BCL-2 proteins was identified many years ago, but the site for direct activation by pro-apoptotic BH3-only proteins from the BCL-2 family has only recently been identified by nuclear magnetic resonance analysis[83]. This represents a new target for the therapeutic induction of BAX-mediated apoptosis. A core component of the outer membrane protein translocation pore, TOM22, has been indicated as the mitochondrial receptor for BAX[84], although recent results suggest that BAX and BAK oligomerize independently of TOM22 (REF. 85). Other BAX-interacting proteins such endophilin B1 have also been reported to mediate BAX-dependent MOMP[86].

Pro-apoptotic stimuli can also initiate MOMP by destabilizing mitochondrial lipids, leading to the formation of transient gaps in the outer membrane that allow for intermembrane space protein leakage. Activated BH3-only proteins like tBID have been shown to interact with BAX and the phospholipid cardiolipin to initiate supramolecular openings in the outer membrane that allow the passage of large intermembrane space proteins to the cytosol during apoptosis[87]. Notably, BAX insertion into the outer membrane and oligomerization require cardiolipin, yet are inhibited by phosphatidylethanolamine[88], which supports the concept that distinct lipids differentially contribute to MOMP regulation.

Inner membrane proteases also regulate the release of cytochrome *c* from mitochondria. Presenilin-associated rhomboid-like (PARL) is an integral protease of the inner membrane that modulates cytochrome *c* release by cleaving the dynamin-related protein optic atrophy 1 (OPA1), thereby controlling the remodelling of cristae independently of mitochondrial fusion[89,90]. Accordingly, PARL-deficient cells displayed reduced levels of OPA1 in the intermembrane space and were more susceptible to intrinsic apoptosis triggers[89]. Inhibition of PARL might be used as a strategy to stimulate MOMP and cell death.

There is now ample evidence demonstrating that the ratio of pro-apoptotic versus anti-apoptotic BCL-2 proteins determines the susceptibility of cancer cells to undergo apoptosis. Thus, shifting the balance of the so-called BCL-2 rheostat towards pro-apoptotic members, for example, BH3-only proteins, provides a powerful means to initiate MOMP-dependent apoptosis.

BH3 mimetics are small molecules that have close structural or functional similarity to BH3-only proteins. Most BH3 mimetics that are currently under preclinical and clinical development bind to and antagonize pro-survival members of the BCL-2 family of proteins (TABLE 1). BH3 mimetics that activate pro-apoptotic BCL-2-like proteins are still in preclinical stages of development[91]. It has recently been shown that several BH3 mimetics (with the notable exceptions of ABT-737 and ABT-263, see below) also bind to cellular targets that are unrelated to BCL-2, which might compromise further development due to potential toxicity issues[92,93].

One of the most advanced and best-characterized BH3 mimetics is ABT-737 (FIG. 2), which predominately binds to BCL-2, BCL-X_L and BCL-W (also known as BCL2L2), thereby displaying a binding profile similar to that of the BH3-only protein BCL-2 antagonist of cell death (BAD)[94]. ABT-737 mostly induces cell death through the intrinsic pathway of apoptosis, as it is unable to kill cells that lack both BAX and BAK[95].

The susceptibility of cancer cells to ABT-737 as a single treatment depends on the expression profile of the BCL-2 family proteins. Tumour cells harbouring high endogenous levels of BCL-2 (including cells derived from small-cell lung carcinoma (SCLC) and from different types of leukaemia and lymphoma) are especially sensitive to ABT-737 (REFS 94,96,97). Notably, BH3-only proteins released from interactions with BCL-2, BCL-X_L and BCL-W by ABT-737 can still interact with another anti-apoptotic protein from the BCL-2 family that is not antagonized by ABT-737, namely MCL1 (REF. 98). Thus, the expression levels of MCL1 may represent a key factor for the resistance of some cancer cell types to ABT-737 (REFS 96,98).

ABT-737 has been shown to cooperate with conventional chemotherapy and radiotherapy against haematological malignancies and solid tumours[97–102]. Furthermore, ABT-737 reversed the chemoresistance of cancer cells against conventional anticancer agents, and, vice versa, ABT-737 resistance could be overcome in the presence of classical cytotoxic drugs[100,101]. ABT-737 has also been demonstrated to act in concert with inhibitors of oncogenic

BH3-only proteins
A subset of proteins from the BCL-2 family that share significant homology only within the BCL-2 homology 3 (BH3) domain and act as intracellular stress sensors.

Mitochondrial fusion
In physiological conditions, the mitochondrial network is constantly remodelled by fusion and fission events, which allow mitochondria to adapt to the metabolic needs of the cell.

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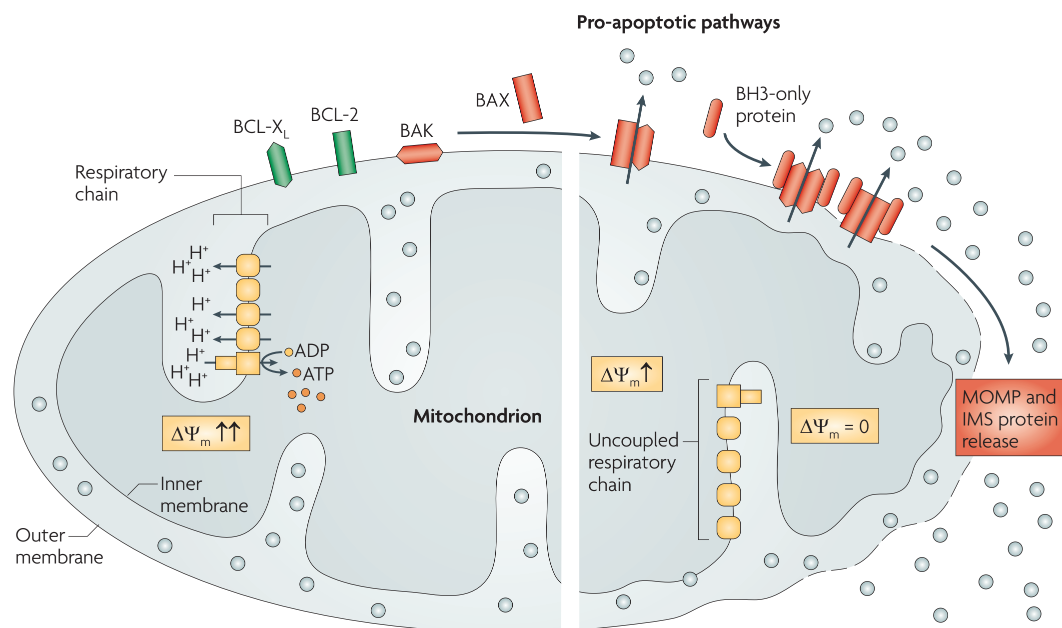

Figure 3 | **Mitochondrial outer membrane permeabilization (MOMP).** In healthy cells, the mitochondrial outer membrane hosts multiple anti-apoptotic proteins from the B-cell lymphoma protein 2 (BCL-2) family including BCL-2 itself and BCL-X_L (also known as BCL2L2), as well as their pro-apoptotic counterpart BCL-2 homologous antagonist/killer (BAK) (in an inactive conformation). The same does not apply to BCL-2-associated X protein (BAX), which is cytosolic when inactive, nor to BH3-only proteins. In response to apoptotic triggers, BAX and BAK undergo conformational modifications that allow them to translocate to and/or fully insert into the outer membrane. Active BAX and BAK can be intercepted by BCL-2 and BCL-X_L (not shown) or they can assemble into homomeric or heteromeric pores that mediate MOMP. In this context, BH3-only proteins can exert pro-apoptotic effects either by directly binding to (and hence activating) BAX and BAK or by displacing them from inhibitory interactions with BCL-2 or BCL-X_L (not shown). Upon MOMP, cytotoxic proteins that normally reside within the mitochondrial intermembrane space (IMS), including cytochrome *c*, DIABLO, apoptosis-inducing factor and endonuclease G, are released into the cytosol. Of note, while the high mitochondrial transmembrane potential ($\Delta\psi_m$) is immediately dissipated by the opening of the permeability transition pore complex (see also FIG. 1) during BAX/BAK-mediated MOMP, the loss of $\Delta\psi_m$ is secondary to the depletion of soluble components of the respiratory chain such as cytochrome *c*.

kinases; for example, inhibitors of BCR–ABL, fms-related tyrosine kinase 3 (FLT3), epidermal growth factor receptor (EGFR) and MAPK/ERK kinase 1 (MEK1)/MEK2 (REFS 103–108), proteasome inhibitors[109,110], inhibitors of histone deacetylases[111] and the death receptor ligand tumour necrosis factor-related apoptosis inducing ligand (TRAIL)[112,113]. The *in vivo* antitumour activity of ABT-737 has been demonstrated in several preclinical models of human malignancies, including SCLC and acute leukaemia[96,97,101,114], both as monotherapy[96,97] and in combination regimens[101,114].

To enhance the clinical potential of ABT-737, an orally available derivative, ABT-263, has been generated. ABT-263 mimics the mode of action of ABT-737 and has shown antitumour properties in multiple preclinical models, including a SCLC xenograft model with acquired resistance to chemotherapy[96,115–117]. ABT-263 is currently under clinical evaluation in Phase I/II trials for chronic lymphocytic leukaemia, lymphoma and SCLC, as monotherapy or in combination with chemotherapeutics or with monoclonal antibodies depending on the tumour type[115].

A-385358 is a small molecule that binds more avidly to BCL-X_L than to BCL-2 (REF. 117). A-385358 potently enhanced cell death induced by various chemotherapeutic agents including paclitaxel, etoposide, cisplatin and doxorubicin in cancer cell lines and in a xenograft model of non-SCLC[117]. Clinical trials with A-385358 have not yet been initiated.

Gossypol (AT-101) is a natural phenolic compound found in cotton plants[118] that simultaneously inhibits BCL-2, BCL-X_L, BLC-W and MCL1 (REF. 119) (FIG. 2). Gossypol demonstrated clinical activity in a Phase I trial against prostate cancer[120] and is currently under evaluation as monotherapy or in combination with topotecan or temozolomide in patients with chronic lymphocytic leukaemia, SCLC or advanced B-cell malignancies. Its derivative apogossypol has been reported to exhibit superior antitumour activity and reduced toxicity[121].

Obatoclax (GX15-070) is a small-molecule indole bipyrrole compound that — similarly to gossypol — antagonizes BCL-2, BCL-X_L, BCL-W and MCL1 (REFS 122–124) (FIG. 2). Unlike ABT-737, obatoclax efficiently disrupts the interaction between BAK and MCL1, thereby overcoming the MCL1-dependent resistance to ABT-737 and to the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals), the gold standard treatment for patients with multiple myeloma[122]. Furthermore, obatoclax has been shown to synergize with ABT-737 and AraC

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to induce apoptosis in leukaemic cell lines as well as in primary acute myeloid leukaemia samples[125]. In a Phase I clinical study in patients with advanced chronic lymphocytic leukaemia, obatoclax demonstrated modest activity as a single agent[126], but it is currently under clinical evaluation (Phase I/II trials), alone or in combination regimens, for the treatment of haematological malignancies and solid tumours[127].

HA14-1 (2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate) is an organic compound that was identified as a BCL-2 interactor with specific BCL-2 inhibitory functions[128,129]. HA14-1 enhances the sensitivity of cultured glioblastoma cells to chemotherapy or radiotherapy[129]. The development of HA14-1 is still in preclinical phase.

Oblimersen (G3139) is a phosphorothioated oligonucleotide that anneals to the first six codons of the BCL-2 mRNA, thereby inhibiting BCL-2 biosynthesis. Oblimersen has been clinically evaluated in various cancer types, most frequently in combination with chemotherapeutic agents including doxorubicin, docetaxel, fludarabine and cyclophosphamide[130–133]. In a Phase III trial with patients with relapsed or refractory chronic lymphocytic leukaemia, the addition of oblimersen to fludarabine plus cyclophosphamide significantly increased both the response rate and its duration[131]. However, a Phase III clinical trial in patients with multiple myeloma revealed no significant differences between the objective response rate of the group treated with dexamethasone plus oblimersen and that of the group that received dexamethasone alone[134].

Targeting mitochondrial metabolism

The mechanisms underlying the characteristic alterations of mitochondrial function in cancer cells could reveal new anticancer drug targets (BOX 1). Approaches that concomitantly reverse the hyperglycolytic state of cancer cells and prime them to the induction of death may lead to the development of selective antitumour therapies.

Inhibition of glycolysis by 2-deoxy-D-glucose (2DG) significantly increased the cytotoxicity of cisplatin in human head and neck cancer cells by enhancing oxidative stress[135]. Phase I/II clinical trials in patients with advanced solid tumours or prostate cancer are ongoing. However, there are concerns that 2DG might compromise the glycolytic metabolism of the brain and of the heart, and it remains to be seen whether the therapeutic window of 2DG is broad enough to justify its clinical development. To address this issue, it may be interesting to develop specific inhibitors of some glucose transporter isoforms that are frequently upregulated in cancer.

The HK–VDAC interaction offers another intriguing target to selectively trigger cancer cell death. HK is frequently overexpressed in human tumours and HK binds to VDAC more tightly in cancer cells than in their normal counterparts[136] (BOX 1). Strategies aimed at disrupting the interaction between HK and VDAC at the outer membrane have been shown to preferentially kill tumour cells, both *in vitro* and *in vivo*, by promoting PTPC opening and MPT[137–141] (TABLE 1). This has been demonstrated for a short peptide derived from the HK2 amino terminus[137], for the HK inhibitor 3-bromopyruvate[142], as well as for the plant hormone methyl jasmonate[139] (FIG. 2). 3-Bromopyruvate (alone or in combination with the heat-shock protein, 90 kDa (HSP90) inhibitor geldanamycin, see below) has been shown to exert pronounced antitumour effects against hepatic and pancreatic cancer *in vivo*[140,141]. Methyl jasmonate binds to HK, thereby detaching it from mitochondria and initiating apoptotic cell death[139]. As this effect is obtained at relatively high concentrations (around 1 mM), it remains to be seen whether methyl jasmonate may serve as a useful lead compound for the development of specific agents that disrupt the HK–VDAC interaction.

Inhibition of mitochondrial pyruvate dehydrogenase kinase (PDK) by dichloroacetate (FIG. 2) may be exploited to reverse the abnormal metabolism of cancer cells from glycolysis to glucose oxidation (BOX 1). As PDK negatively regulates pyruvate dehydrogenase, dichloroacetate indirectly stimulates the pyruvate to acetyl-CoA conversion. Dichloroacetate has been shown to downregulate the aberrantly high mitochondrial membrane potential of cancer cells, increase mitochondrial ROS generation and activate K^+ channels in malignant, but not in normal cells[143]. Dichloroacetate also upregulated the expression of the K^+ channel Kv1.5, which is often underexpressed by tumour cells, through the transcription factor nuclear factor of activated T cells 1 (NFAT1)[143]. Dichloroacetate-normalized mitochondrial functions were accompanied by reduced proliferation, increased apoptosis and suppressed tumour growth without apparent toxicity[143], suggesting that the mitochondria–NFAT–Kv axis and PDK represent promising anticancer drug targets. Dichloroacetate as monotherapy is currently being tested in a Phase I study in patients with advanced solid tumours.

Inhibition of lactate dehydrogenase A (LDHA) is an alternative strategy to target aerobic glycolysis in cancer cells[144]. Knockdown of LDHA by short hairpin RNAs (shRNAs) led to increased mitochondrial respiration, decreased mitochondrial membrane potential, reduced proliferation and impaired tumorigenicity, suggesting that LDHA plays an important part in tumour maintenance[144] (TABLE 1). These results underscore the need to develop isoform-specific LDH inhibitors.

Frequently, cancer cells redirect pyruvate towards lipid synthesis[145], as this is instrumental to support the increased demand for membrane generation in highly proliferating cells. Accordingly, ATP citrate lyase (ACL), the key enzyme that links glucose metabolism to lipid synthesis by catalysing the conversion of citrate to cytosolic acetyl-CoA (BOX 1), represents a potential drug target[145]. Inhibition of ACL by RNAi or a pharmacological inhibitor (SB-204990) restrained the proliferation of tumour cells *in vitro* and suppressed tumour growth (while inducing differentiation) in mice bearing xenotransplanted human tumours[145]. Recently, it has been demonstrated that ACL is required to provide sufficient amounts of acetyl-CoA for histone acetylation and hence affects gene expression[146]. This suggests that ACL inhibition might not only normalize

Heat-shock proteins
(HSPs). A family of evolutionarily conserved proteins that contribute to the proper folding of native polypeptides and prevent the aggregation of denatured proteins. The expression of HSPs is increased in response to elevated temperatures and other types of stress.

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Box 1 | **Mitochondrial metabolism and metabolic reprogramming**

Mitochondrial metabolism

Under normal oxygen tension conditions, non-malignant cells mainly rely on oxidative phosphorylation for ATP production, whereas cancer cells exhibit enhanced glycolysis despite high oxygen tension[207,208]. Accordingly, the electron flow through the respiratory chain is substantially lower in malignant cells than in their normal counterparts. ATP production by aerobic glycolysis (which directly results in increased generation of lactate) is advantageous to cancers cells because it allows them to better survive under conditions of varying oxygen tension[11]. Both hexokinase (HK) isoforms (HK1 and HK2) are more tightly bound to the voltage-dependent anion channel (VDAC), a component of the permeability transition pore complex (PTPC), at the mitochondrial outer membrane in cancer cells than in non-malignant cells, thereby coupling residual ATP production and export from mitochondria to the rate-limiting step of glycolysis (that is, the conversion of glucose (Glu) into glucose-6-phosphate (G6P))[11]. The PTPC also comprises adenine nucleotide translocase (ANT) and cyclophilin D (CYPD). Moreover, whereas in normal cells pyruvate (the end product of glycolysis) is imported into mitochondria and enters the tricarboxylic acid (TCA) cycle, in cancer cells it is preferentially converted to lactate by lactate dehydrogenase (LDH) in the cytosol leading to acidification (see the figure)[11].

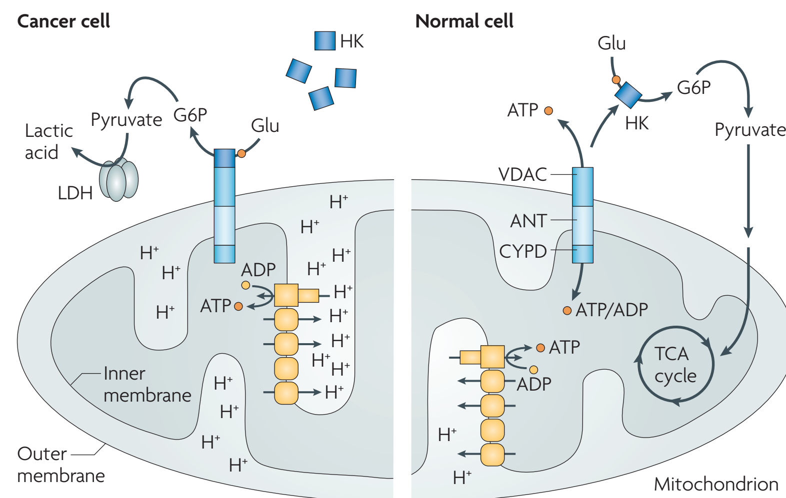
Metabolic reprogramming

The overall increase in anabolism that characterizes malignant cells is, at least in part, caused by their extensive metabolic reprogramming[11]. Elevated oncogenic kinase signalling favours the binding of HK to VDAC by AKT-dependent phosphorylation of HK2 (REF. 209) as well as by AKT-mediated inhibition of VDAC phosphorylation by glycogen synthase kinase 3β, an event that reportedly blocks the interaction between VDAC and HK2 (REF. 136). Oncogenic kinase signalling results in increased fatty acid biosynthesis and steroidogenesis via fatty acid synthase and ATP citrate lyase activation[210,211], and deviates the elevated glycolytic flux towards anabolism by suppressing the activity of the embryonic M2 isoform of pyruvate kinase, an enzyme that catalyses dephosphorylation of phosphoenolpyruvate to pyruvate (the last step of glycolysis)[212].

Cancer-associated loss of p53 function contributes to increased glycolysis via defective transactivation of TIGAR, an isoform of 6-phosphofructo-2-kinase that inhibits glycolysis and generation of reactive oxygen species[213], as well as of SCO2, a mitochondrial protein that is required for proper assembly of cytochrome *c* oxidase and hence for efficient mitochondrial respiration[214].

The transcription factor hypoxia-inducible factor 1 (HIF1) transactivates many genes involved in aerobic metabolism[215], for example, pyruvate dehydrogenase kinase that negatively regulates the conversion of pyruvate to acetyl-CoA by inhibiting pyruvate dehydrogenase[216], thereby impairing oxidative phosphorylation[217]. Germline mutations of of the TCA cycle enzymes fumarate hydratase and succinate dehydrogenase result in HIF1 induction via accumulation of intermediate metabolites that suppress HIF1 degradation[218].

Possible therapeutic strategies to target the aberrant mitochondrial metabolism of cancer cells include inhibition of glycolysis, disruption of the HK–VDAC interaction and inhibition of LDH.



tumour metabolism at the level of lipid synthesis but also plays a part in the epigenetic reprogramming of cancer cells.

Cancer-associated metabolic alterations can also be targeted at the level of fatty acid synthesis (BOX 1). For example, inhibition of acetyl-CoA carboxylases, which generate malonyl-CoA, the substrate for fatty acid synthesis, by soraphen A, an antifungal polyketide from myxobacteria, has been shown to preferentially kill malignant cells[147]. By reducing malonyl-CoA, soraphen A inhibits fatty acid synthesis and stimulates fatty acid oxidation, resulting in reduced phospholipid content, growth inhibition and enhanced cell death[147] This cytotoxic response preferentially developed in prostate cancer cells rather than in premalignant BPH-1 cells[147]. Thus, targeting the dependence of tumour cells on the continuous supply of fatty acids may provide a valuable means to induce cell death. Intriguingly, another inhibitor of fatty acid synthesis, orlistat, also demonstrated antitumour activity in mice xenotransplanted with human melanoma cells[148].

These examples illustrate attempts to interfere with cancer-specific metabolic programmes that are executed (at least in part) within mitochondria. Future refinement of these strategies should lead to the development of agents that combine relative cancer specificity with an acceptable toxicological profile.

Other ways to target mitochondria in cancer

HSP90 inhibitors. HSP90 is contained in mitochondria of cancer cells but not in their normal counterparts[149,150]. Although some oncogenes such as *RAS* and *AKT* have been suggested to favour the mitochondrial import of HSP90 (REFS 150,151), the molecular basis for the preferential localization of HSP90 within the mitochondria of malignant cells is still elusive. In mitochondria, HSP90 forms a complex with tumour necrosis factor receptor-associated protein 1 (TRAP1), an HSP90-like chaperone, and the PTPC component CYPD[150]. The HSP90–TRAP1 complex reportedly controls CYPD-regulated MPT via protein folding mechanisms[150]. Mitochondrially-targeted HSP90 antagonists might therefore be exploited to interfere with signalling networks in specific subcellular compartments of tumour cells (FIG. 4).

Shepherdin was developed as a peptidomimetic that inhibits the interaction between HSP90 and its client protein survivin[152]. Membrane-permeant variants of shepherdin were generated by fusing its N terminus to either helix III of the *Antennapedia* homeodomain protein or the HIV TAT sequence[152]. Cell-permeant shepherdin reportedly accumulates in the mitochondrial compartment and rapidly triggers CYPD-mediated (and hence cyclosporine A inhibitable) MPT and cell death, independently of both p53 and BCL-2 expression levels[150,152,153]. Shepherdin-mediated cell death correlates with its physical binding to HSP90 and TRAP1 within mitochondria[150]. In preclinical xenograft models of multiple human cancers, the systemic administration of shepherdin was safe and resulted in tumour growth inhibition[152,153].

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Gamitrinibs (GA mitochondrial matrix inhibitors) (FIG. 2) have been developed by combinatorial chemistry as a class of small molecules that antagonize the ATPase activity of HSP90 in cancer cell mitochondria[154]. Mitochondrial targeting was achieved by coupling the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) to lipophilic cationic moieties, for instance 1–4 tandem repeats of cyclic guanidinium or triphenylphosphonium[16]. Gamitrinibs have demonstrated consistent mitochondrial toxic effects that spare non-malignant cells but cause cancer cell death and suppression of tumour growth *in vivo*[154]. Thus, the pathway-oriented development of compartmentalized drugs may open new perspectives for tumour-selective cytotoxicity.

HSP90 inhibitors of the PU class (FIG. 2) specifically bind to the HSP90 N-terminal regulatory pocket and exhibit favourable pharmacological features, including negligible microenvironmental inactivation and escape from P-glycoprotein-mediated export[155]. Representatives of this class include PU24FCl (8-(2-chloro-3,4,5-trimethoxybenzyl)-2-fluoro-9-(pent-4-ynyl)-9*H*-purin-6-amine); PU-H58 (8-(6-bromobenzo[d][1,3]dioxol-5-ylthio)-9-(pent-4-ynyl)-9*H*-purin-6-amine); and PU-H71 (8-(6-iodobenzo[d][1,3]dioxol-5-ylthio)-9-(3-(isopropylamino)propyl)-9*H*-purin-6-amine)[155]. PU-H71 has been reported to exert potent and durable antitumour effects in breast cancers that lack the expression of oestrogen, progesterone and HER2 (also known as ERBB2) receptors[156]. Proteomics analysis revealed that PU-H71 causes the downregulation of various HSP90 client proteins including components of the RAS–RAF–MAPK pathway, cell-cycle regulators, anti-apoptotic factors and AKT[156]. Intriguingly, SCLC turned out to be particularly susceptible to HSP90 small-molecule inhibitors, including PU-H71, PU24FCI and PU-H58 (REF. 155). The relative contribution of the inhibition of mitochondrial versus cytosolic HSP90 to these effects remains to be determined.

Natural compounds. Betulinic acid (3b, hydroxy-lup-20(29)-en-28-oic acid) is a natural pentacyclic triterpenoid of the lupane class that is contained in various species throughout the plant kingdom[157–159] (FIG. 2). Betulinic acid triggers mitochondrial apoptosis preferentially in cancer cells and exhibits potent antitumour activities[160,161].

When betulinic acid is added to isolated mitochondria in a cell-free system, it directly triggers MOMP in association with mitochondrial membrane potential dissipation and cytochrome *c* release[161]. The cytotoxicity of betulinic acid could not be blocked by the pan-caspase inhibitor zVAD.fmk (although zVAD.fmk abrogated the morphological signs of betulinic acid-triggered apoptosis), yet it was reduced by bongkrekic acid[162,163], as well as by BCL-2 and BCL-X_L overexpression[160]. This suggests that the anticancer effects of betulinic acid are mediated by the MPT, which may be initiated by ROS overproduction[160,164,165]. As betulinic acid triggers apoptosis in doxorubicin-resistant neuroblastoma cells[162], it could help circumvent some forms of chemotherapy resistance.

Betulinic acid modulates the expression levels of BCL-2 family proteins in a context-dependent manner, including upregulation of pro-apoptotic members such as BAX and BCL-X_S (also known as BCL2L1)[160]. In some cancer cell types including melanoma cells, increased expression of the anti-apoptotic BCL-2 family member MCL1 has been reported in response to betulinic acid, whereas no changes in MCL1 levels were detected in squamous cell carcinoma cells[166–168]. Notably, betulinic acid-mediated apoptosis was not associated with p53 accumulation and occurred in a p53-independent manner (as evaluated in p53-deficient cells and in cells harbouring mutated p53)[160,164,166,169–172].

Resveratrol (FIG. 2) — a polyphenolic compound from grapes and wine — has recently been shown to improve mitochondrial function by stimulating the sirtuin 1 (SIRT1)-dependent deacetylation of the transcriptional co-activator peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α)[173]. Under basal conditions acetylated PGC1α is inactive, but exercise or fasting induces the deacetylation-dependent activation of PGC1α through increased intracellular concentration of NAD^+, which in turn stimulates the SIRT1 axis[173]. Deacetylated PGC1α acts as a co-activator for nuclear respiratory factor 1 (NRF1), which transactivates genes involved in oxidative phosphorylation and mitochondrial biogenesis[173]. The resveratrol-induced amelioration of mitochondrial functions has been shown to promote longevity and to improve glucose homeostasis[173]. It is unclear whether this property of resveratrol is also linked to its chemopreventing and antitumour activities.

Structural biology studies revealed that resveratrol inhibits the synthetic and hydrolytic activities of F_1-ATPase by binding to a hydrophobic pocket that is located between the carboxy-terminal tip of the γ subunit and the β subunit[174]. Thus, resveratrol inhibits mitochondrial ATP synthesis, which eventually contributes to cell death induction[174]. Resveratrol has also been shown to trigger MOMP in isolated mitochondria[175]. Whether this effect is related to the binding of resveratrol to F_1-ATPase (which interacts with ANT and the inorganic phosphate carrier to form the 'ATP synthasome')[176] remains an open question.

The mitochondrial targeting of resveratrol has been achieved by coupling it to the membrane-permeant lipophilic triphenylphosphonium cation[177]. Such resveratrol derivatives including 4-triphenylphosphoniumbutyl-4′-*O*-resveratrol iodide and its bis-acetylated variant have been shown to efficiently accumulate in mitochondria and may therefore provide a tool to directly interfere with mitochondrial redox functions[177]. Four resveratrol analogues (HS-1784, HS-1792, HS-1791 and HS-1793) exert improved antitumour activity compared with the parental compound[178]. In particular, HS-1793 has been shown to circumvent BCL-2-mediated apoptosis resistance in U937 leukaemia cells, possibly by downregulating 14-3-3 at the post-transcriptional level[178]. The 14-3-3 protein family includes cytosolic multifunctional phosphoserine binding proteins that can interact with (and inhibit) multiple clients, including the pro-apoptotic factors BAX and BAD[179]. Thus, resveratrol-mediated cell death may involve the increased availability of unbound

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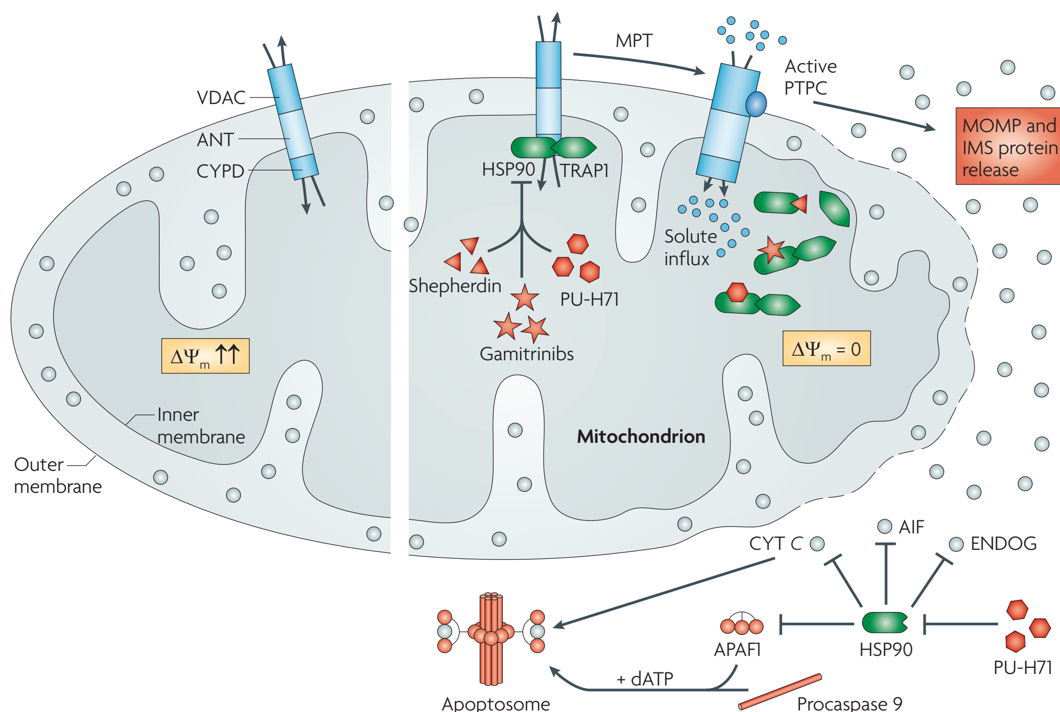

Figure 4 | **The heat-shock protein, 90 kDa (HSP90) system in cancer cells.** In healthy cells, the permeability transition pore complex (PTPC) exhibits a low-conductance conformation that might contribute to the exchange of metabolites between the mitochondrial matrix and the cytosol. In response to multiple signals of stress, the PTPC assumes a high-conductance state that mediates mitochondrial permeability transition (MPT), resulting in the immediate dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$) and eventually in mitochondrial outer membrane permeabilization (MOMP) and the cytosolic spillage of mitochondrial intermembrane space (IMS) proteins. These proteins include cytochrome *c* (CYT C), apoptosis-inducing factor (AIF) and endonuclease G (ENDOG). Unlike their normal counterparts, mitochondria from cancer cells contain a large fraction of the intracellular pool of HSP90. Within mitochondria, HSP90 interacts with tumour necrosis factor receptor-associated protein 1 (TRAP1) and cyclophilin D (CYPD), thereby exerting anti-apoptotic functions. In the cytosol, CYT C recruits the adaptor protein apoptotic peptidase activating factor 1 (APAF1), deoxyATP (dATP) and procaspase 9 to assemble the apoptosome, a platform for the activation of the pro-apoptotic caspase cascade. By contrast, AIF and ENDOG exert caspase-independent apoptogenic effects (not shown). HSP90 also functions as a pro-survival factor in the cytosol by inhibiting APAF1 (and hence preventing the assembly of the apoptosome), by preventing AIF mitochondrial-cytosolic translocation and by inhibiting the nucleolytic activities of both AIF and ENDOG. Thus, mitochondrially-targeted HSP90 inhibitors like shepherdin and gamitrinibs display tumour-selective cytotoxic properties. It remains unclear what the relative contribution of mitochondrial versus extra-mitochondrial effects are to the anticancer effects of non-targeted inhibitors like PU-H71. ANT, adenine nucleotide translocase; VDAC, voltage-dependent anion channel.

BH3-contaning proteins that can translocate to mitochondria and trigger MOMP[180]. Besides being tested for cancer chemoprevention[181], resveratrol is currently under early clinical evaluation (as monotherapy or combined with bortezomib) for the treatment of colon cancer or multiple myeloma.

Vitamin E analogues — with α-tocopheryl succinate (α-TOS) as a prototype compound (FIG. 2) — have been shown to selectively trigger mitochondrial apoptosis in tumour cells[182]. α-TOS is a derivative of α-tocopherol (α-TOH) in which the hydroxyl group at position C_6 of the chromanol ring (which is responsible for α-TOH redox activity), has been substituted for by succinic acid[183]. In addition to α-TOS, a series of other vitamin E analogues have been developed, including a non-hydrolysable ether-linked acetic acid derivative of α-TOH (that is, α-TEA), which demonstrated improved antitumour activity in some cancer types and which can be administered orally[184–186]. Additional vitamin E analogues with anticancer activity comprise α-tocopheryl maleyl amide, α-tocopheryl oxalate and α-tocopheryl malonate, α-tocopheryloxybutyric acid and tocotrienols[183].

Biochemical, genetic and molecular modelling studies aimed at understanding the mechanisms underlying the anticancer activity of α-TOS revealed that α-TOS is targeted to mitochondria due to interactions with the proximal and distal ubiquinone-binding sites (Q_P and Q_D, respectively) of respiratory complex II[187]. This results in the displacement of ubiquinone from complex II, followed by recombination of succinate dehydrogenase-generated electrons with molecular oxygen and ROS generation[187]. Genetic evidence for the key role of respiratory complex II as a target of α-TOS-mediated *in vivo* anticancer activity was obtained by experiments with tumours derived from *H-Ras*-transformed Chinese hamster lung fibroblasts that harboured functional, dysfunctional or

Apoptosome
A supramolecular complex comprising cytochrome *c*, apoptotic peptidase activating factor 1 and deoxyATP that is required for the autocatalytic activation of procaspase 9.

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Box 2 | **Drug delivery to mitochondria**

The mitochondrial transmembrane potential ($\Delta\psi_m$), the electrochemical gradient built across the inner membrane by the respiratory chain complexes, constitutes a distinguishing feature of mitochondria that can be exploited for targeting of drugs to this organelle.

Delocalized lipophilic cations (DLCs)

These are attracted by the negatively charged mitochondrial matrix and can readily cross mitochondrial membranes, hence they efficiently accumulate within mitochondria[219]. Fluorescent DLCs including chloromethyl-X-rosamine (MitoTracker red), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) and triphenylphosphonium have been extensively used as probes for visualizing mitochondria and for studying their functions[220,221]. In addition, DLCs can be used for delivering small compounds or larger molecules to the mitochondrial matrix, but with some limitations[226]. These mainly concern the intrinsic mitochondrial toxicity of DLCs at high concentrations and of DLCs coupled to large polar molecules[222,223].

Mitochondrial targeting sequence (MTS)-containing polypeptides

Nuclear-encoded mitochondrial proteins harbour a MTS of 20–40 amino acids that is recognized by receptors at the mitochondrial surface. Various translocases of the outer membrane and the inner membrane mediate the import and intramitochondrial sorting of MTS-containing polypeptides, which is driven by ATP or by the mitochondrial transmembrane potential[224,225]. Multiple MTSs have been successfully used for the mitochondrial delivery of chemically different cargos, including proteins, catalytically proficient enzymes and nucleic acids[226–228]. The major pitfalls of this approach are linked to the considerable molecular size of the MTSs, their solubility and their intrinsically poor membrane permeability[221,224].

Synthetic peptides and amino-acid-based transporters

Recent work has shown that the mitochondrial localization of synthetic carriers can be controlled by altering lipophilicity and charge, which allows for the rational design of efficient transporters for drug delivery to mitochondria[221,229,230].

Vesicle-based carriers

These have been used for mitochondrial delivery of large or otherwise impermeable cargos. This approach is exemplified by the MITO-Porter system, which is based on liposomes that carry octaarginine surface modifications to stimulate their entry into cells as intact vesicles (via macropinocytosis)[231], and by DQAsomes, which are vesicles formed by the sonication of the dicationic amphiphile compound dequalinium[232].

reconstituted respiratory complex II[188]. In this context, α-TOS was indeed able to trigger ROS generation and apoptosis only when respiratory complex II was functional[188].

α-TOS also targets endothelial cells, thereby suppressing angiogenesis, as demonstrated both *in vitro* and *in vivo*, in a mouse model of breast cancer[189]. α-TOS preferentially triggered apoptosis in proliferating, but not cell-cycle-arrested, endothelial cells by causing ROS accumulation and activating the intrinsic pathway of apoptosis[189]. Mitochondrial-DNA-depleted endothelial cells were refractory to α-TOS, underscoring the crucial contribution of mitochondria to the anti-angiogenic activity of α-TOS[189]. The tumour selectivity of α-TOS has been attributed to its ester structure[190], which is responsible for an enhanced hydrolysis of α-TOS to α-TOH in normal cells but not in their malignant counterparts (harbouring lower levels of esterases)[191]. In a chemoprevention trial, daily dietary supplementation with α-TOS showed no effect on the incidence of upper aerodigestive tract cancers[192].

The sesquiterpene lactone parthenolide has been shown to exert tumour-selective cytotoxic effects in multiple human cell lines including chronic lymphocytic leukaemia, colorectal cancer and cholangiocarcinoma cells[193–195]. Parthenolide cytotoxicity reportedly involves ROS overproduction, nuclear factor-κB inhibition as well as the activation of p53 and pro-apoptotic BCL-2 family members[195–198] (TABLE 1). Recently developed parthenolide analogues such as dimethylamino-parthenolide (DMAPT) exhibit *in vivo* bioactivity and improved pharmacokinetic properties compared with the parental compound[196] (TABLE 1). It has been suggested that parthenolide may selectively target the cancer stem cell population while sparing normal non-transformed progenitors[197]. This might be particularly interesting as cancer stem cells are thought to have the capacity to repopulate the tumour and are refractory to conventional therapeutic approaches[199]. Similar to normal stem cells (in which stemness has been associated with reduced amounts of ROS, possibly in correlation with the hypoxic niches where these cells would reside)[200–206], breast cancer stem cells have been found to contain lower concentrations of ROS and higher levels of antioxidants than their non-tumorigenic progeny[10]. It will be interesting to determine whether parthenolide, its derivatives or other pro-oxidants may lead to tumour eradication by targeting the cancer stem cell population *in vivo*.

Outlook and future challenges

As mitochondria are the most prominent source of intracellular ROS and low levels of ROS have been implicated in cancer cell stemness[10], selective targeting of cancer stem cells with mitochondrially-targeted agents is likely to attract great interest. Furthermore, as cancer stem cells exhibit unique properties that make them vulnerable to certain classes of mitochondria-targeting drugs, including natural compounds such as parthenolide, this approach presents a promising avenue for further research.

A better understanding of the key pathophysiological differences between mitochondria in cancer cells and their counterparts in non-malignant cells will undoubtedly be instrumental for increasing the level of selectivity of mitochondrially-targeted anticancer agents.

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Box 3 | Strategies for the discovery of mitochondrially-targeted anticancer agents

Cell-based assays

Cell-cultured cells transiently or stably transfected with fluorescently-tagged mitochondrial intermembrane space proteins — such as green fluorescent protein (GFP)–cytochrome *c* or GFP– apoptosis-inducing factor (AIF) — can be used to screen for mitochondrial outer membrane permeabilization (MOMP)-inducing agents. These types of agent induce fluorescence redistribution from a mitochondrial (punctate) to a non-mitochondrial (diffuse in the case of GFP–cytochrome *c* or nuclear in the case of GFP–AIF) pattern[19]. Alternatively, cells can be stained with cationic lipophilic fluorochromes such as tetramethyl rhodamine methyl ester, which accumulate into the mitochondrial matrix[220]. Mitochondrial membrane permeabilization (MMP)-inducing agents lead to dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$) before the appearance of morphological signs of apoptosis, implying that mitochondrial transmembrane potential-sensitive fluorochromes can be used for the quantification of cells in the early stage of the apoptotic cascade[233].

Assays with isolated mitochondria

Mitochondria purified from cell cultures (or murine liver) can be exposed to experimental drugs, and MMP can be monitored by measuring:

- The presence of intermembrane space proteins in the supernatant
- Large amplitude swelling (which leads to a decrease in the absorbance of the mitochondrial suspension)
- The de-quenching of pre-loaded rhodamine 123 (whereby increased fluorescence indicates $\Delta\psi_m$ dissipation)
- Ca^{2+} release (which can result from inner membrane permeabilization and/or mitochondrial transmembrane potential loss)
- Mitochondrial uncoupling (which can be measured by respirometric techniques)[220]

The capacity of a cytotoxic agent to affect mitochondrial functions and/or integrity in a cell-free system is usually ascribed to a direct interaction between the candidate chemical and mitochondria. In this context, it is possible to compare mitochondrial preparations from distinct origins (for instance from normal versus neoplastic cells) or to perform the experiments in the presence of cytosolic extracts from different sources. The presence of contaminant membranes from the endoplasmic reticulum may yield heavily biased results and should therefore be carefully prevented (or experimentally controlled)[234].

Reconstituted proteoliposomes

The permeabilization of liposomes containing mitochondrial proteins from the permeability transition pore complex (PTPC) or purified pro-apoptotic proteins from the B-cell lymphoma protein 2 (BCL-2) family exposed to candidate compounds can be monitored by various methods including:

- The release of soluble macromolecules (such as proteins or fluorescent polysaccharides of defined size)
- The de-quenching of fluorochromes
- The release of chromogenic substrates that had previously been encapsulated into the proteoliposomes[235]

As the lipid component of the proteoliposomes can be modulated, this approach can lead to the discovery of permeabilizing agents with a precise protein- or lipid-targeting profile. Hypothetically, it is possible to generate proteoliposomes that contain one MMP-promoting factor — for example, BCL-2 homologous antagonist/killer (BAK) — together with one of its inhibitors — for example, voltage-dependent anion channel 2 (VDAC2), myeloid cell leukaemia sequence 1 (MCL1) — and to use these preparations to search for agents that abrogate such inhibitory interactions, thereby triggering proteoliposome permeabilization.

Chemical design

Experimental agents that have been discovered for their capacity to induce the permeabilization of isolated mitochondria or proteoliposomes can be specifically targeted to cancer cells by fusing them with peptides (or peptidomimetics) that either recognize cancer-cell-specific surface receptors (that are internalized upon binding) or allow the biologically active molecule to be translocated across the plasma membrane[236]. Furthermore, the bioavailability of pharmacological agents at (or in the proximity of) the inner membrane can be ameliorated by coupling them to lipophilic cationic moieties[237].

This will presumably lead to the generation of highly specific molecular tools that trigger mitochondrial cell death exclusively in malignant cells. Further research into the possibilities of targeting devices to mitochondria is also expected to speed up the transfer of this therapeutic principle into clinical practice.

Anticancer drugs that directly target mitochondria have the potential to bypass the resistance mechanisms that have evolved towards conventional chemotherapeutics. Most classical anticancer agents engage signalling pathways that lie upstream of mitochondria and converge on mitochondria due to their role as integrators of pro-death and pro-survival signals[4]. In this scenario, MOMP occurs as a consequence of upstream signalling events (for example, p53 activation), which are frequently deregulated in human cancers and which become resistant to a number of conventional therapeutic strategies that target upstream regulators of MOMP. Thus, drugs that directly target mitochondria (BOX 2) may provide a unique tool to circumvent the necessity of engaging such upstream processes, and may therefore be effective in otherwise resistant forms of cancer.

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A final important point for future drug discovery resides in the fact that many of the known agents that target mitochondria (TABLE 1) are derived from natural compounds and have been identified by serendipity rather than by systematic screening methods (BOX 3). This implies that a systematic global screening approach aimed at specifically identifying mitochondria-targeting drugs from large libraries of natural substances will most likely present a treasure trove for anticancer drug discovery.

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