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Classification of mitocans, anti-cancer drugs acting on mitochondria

Jiri Neuzil\textsuperscript{a,b,*} Lan-Feng Dong,\textsuperscript{a} Jakub Rohlena,\textsuperscript{b} Jaroslav Truksa,\textsuperscript{b} Stephen J. Ralph\textsuperscript{a}

\textsuperscript{a}School of Medical Science, Griffith University, Southport, Qld, Australia
\textsuperscript{b}Institute of Biotechnology, Czech Academy of Sciences, Prague, Czech Republic

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Abbreviations: ANT, adenine nucleotide translocase; BH3, Bcl-2 homology-3; 3BP, 3-bromopyruvate; CI, complex I; DCA, dichloroacetate; 2DG, 2-deoxyglucose; ECs, endothelial cells; ETC, electron transport chain; G6P, glucose-6-phosphate; GSAO, 4-[N-(S-glutathionylacetyl)amino]phenylarsineoxide; HK, hexokinase; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; mtDNA, mitochondrial DNA; PDK, pyruvate dehydrogenase kinase; PEITC, phenylethyl isothiocyanate; PTPC, permeability transition pore complex; Q\textsubscript{d}, distal UbQ-binding site in CII; Q\textsubscript{i}, Q\textsubscript{o}, UbQ-binding sites in CIII; Q\textsubscript{p}, proximal UbQ-binding site in CII; SDH, succinate dehydrogenase; SDHA, succinate dehydrogenase subunit A; SQR, succinate quinone reductase; TCA, tricarboxylic acid cycle; \(\alpha\)-TOS, \(\alpha\)-tocopheryl succinate; TPP\*, triphenylphosphonium; UbQ, ubiquinone; UbQH\textsubscript{2}, ubiquinol; \(\Delta\Psi\mbox{m,i}\), mitochondrial inner trans-membrane potential; VDAC, voltage-dependent anionic channel; VE, vitamin E.

*Corresponding author: Jiri Neuzil, Apoptosis Research Group, School of Medical Science and the Griffith Health Institute, Griffith University, Southport, 4222 Qld, Australia; phone: +61-2-55529109; fax: +61-2-555-28444; e-mail: j.neuzil@griffith.edu.au.
Abstract

Mitochondria have recently emerged as an intriguing target for anti-cancer drugs, inherent to vast majority if not all types of tumours. Drugs that target mitochondria and exert anti-cancer activity have become a focus of recent research due to their great clinical potential (which has not been harnessed thus far). The exceptional potential of mitochondria as a target for anti-cancer agents has been reinforced by the discouraging finding that even tumours of the same type from individual patients differ in a number of mutations. This is consistent with the idea of personalised therapy, an elusive goal at this stage, in line with the notion that tumours are unlikely to be treated by agents that target only a single gene or a single pathway. This endows mitochondria, an invariant target present in all tumours, with an exceptional momentum. This train of thoughts inspired us to define a class of anti-cancer drugs acting by way of mitochondrial ‘destabilisation’, termed ‘mitocans’. In this communication, we define mitocans (many of which have been known for a long time) and classify them into several classes based on their molecular mode of action. We chose the targets that are of major importance from the point of view of their role in mitochondrial destabilisation by small compounds, some of which are now trialed as anti-cancer agents. The classification starts with targets at the surface of mitochondria and ending up with those in the mitochondrial matrix. The purpose of this review is to present in a concise manner the classification of compounds that hold a considerable promise as potential anti-cancer agents.

1. Introduction

In the post-genomic era of the third millennium biomedical research has witnessed resurgence of some of the ‘yonder-years’ scientific discoveries. It is now clear that some of the processes that were the focus of research decades ago, are now being exploited as potential targets in cancer treatment. Interestingly, the products of the same genes whose mutations can promote
malignant transformations are also the emerging targets for novel, thus far largely unexploited anti-cancer agents. For example, the mitochondrial complex II (CII) has recently been described as a new target for anti-cancer drugs (Albayrak et al., 2005; Dong et al., 2008, 2009, 2011a,b; Rohlena et al., 2011). Intriguingly, mutations in the genes coding for its four subunits have been classified as tumour suppressors, since mutation in these genes are positively correlated with the incidence of certain infrequent neoplasias, viz. pheochromocytoma and paraganglioma (Astuti et al., 2001; Gottlieb and Tomlinson 2005; Maxwell 2005; Schiavi et al., 2005; Gimenez-Roqueplo et al., 2010; Burnichon et al., 2010). Therefore, CII is an example of a target for anti-cancer drugs and, at the same time, due to mutations in the genes coding its subunits it is involved in the mutagenic switch.

Many of the agents with anti-cancer activity that act on mitochondria, mitocans, hold a substantial promise to be developed into efficient anti-cancer drugs, based on their selectivity for cancer cells (Wallace et al., 2010; Fulda et al., 2010; Ralph et al., 2010a,b; Wang et al., 2010; Fulda and Kroemer, 2011; Kepp et al., 2011; Lemarie and Grimm, 2011; Shoshan-Barmatz and Ben-Hail, 2012). The importance of mitochondria as an emerging and perspective target for anti-cancer agents is corroborated by the recent findings that tumours differ in the level of expression of a high number of genes and mutations even amongst patients with the same type of tumour. This has been documented for pancreatic cancer and glioblastoma multiforme (Jones et al., 2008; Parsons et al., 2008), and, even worse, there are differences in mutations within the same tumour (Gerlinger et al., 2012). These findings are echoed in the somber-sounding editorial in Nature titled ‘Cancer complexity slows quest for cure’ (Hayden, 2008). This indicates that it will be unlikely to suppress cancer by targeting a single gene or a single pathway that may alter amongst cancer patients and that can be subject to mutations. Rather, it is imperative to search for a target that is invariant and whose
exploitation may present a general strategy for efficient treatment across the landscape of neoplastic pathologies.

It appears clear that such a target is represented by mitochondria that are, at least to some extent, functional in the vast majority if not all cancers (Ralph et al., 2010a,b). Mitochondria, while being the ‘powerhouse’ of the cell, are also reservoirs of a number of apoptosis-promoting proteins that are essential for apoptosis induction and its progression downstream of these organelles, in order for the cancer cell to go into the commitment phase and undergo the final demise (Kroemer et al., 2007; Galuzzi et al., 2010). It is also important to take into consideration the aberrant mitochondrial metabolism in malignant cells (Koppenol et al., 2011; Ward and Thompson). Thus, the recent decade or so has witnessed an unprecedented focus and discovery of novel agents that target mitochondria to induce cancer cell death. In some cases, ‘old’ compounds have been re-discovered for their propensity to destabilise mitochondria and kill cancer cells. Similarly and with an undisputable involvement in the molecular mechanism of the mitochondria-targeting anti-cancer agents, the Warburg’s hypothesis published in the 1920s (Warburg, 1956) has been recently experiencing a renaissance of sorts (Vander Heiden et al., 2009; Cairns et al., 2011; Koppenol et al., 2011; Hannahan and Weinberg, 2000, 2011).

The paramount importance of discovering novel and efficient anti-cancer agents is even more accentuated by the fact that neoplastic diseases are now the greatest threat of the Western society and are likely to increase in frequency (Jemal et al., 2011; Siegel et al., 2012; Simard et al., 2012). We believe that targeting mitochondria, for tumour treatment may lead to a potential future breakthrough in the management of malignancies. In this review, we propose the classification of anti-cancer agents that act via mitochondrial destabilisation (mitocans, an acronym for ‘mitochondria and cancer’) and provide several examples epitomising the individual classes of mitocans, in particular those that are perceived as
clinically relevant anti-cancer agents. The classification is based on the site of action of the individual agents from the surface of the mitochondrial outer membrane (MOM) to the mitochondrial matrix. The selection of the sites also stems from their importance as targets for the development of drugs that hold substantial promise to be utilised in the clinical practice.

2. Class 1 mitocans: Hexokinase inhibitors

This class of agents comprises compounds targeting hexokinase (HK), which is an enzyme whose main role is to phosphorylate glucose converting it to glucose-6-phosphate (G6P), a substrate for metabolic pathways ultimately coupled with ATP generation. HK has a very important function in cancer. Besides converting glucose to G6P, which can then enter the metabolic machinery to, ultimately, yield ATP, HK is associated with the cytosolic site of the porin-like voltage-dependent anionic channel (VDAC), a trans-membrane protein in the MOM (Pedersen, 2008). When expressed at higher levels, as in cancer cells, HK (type II) binds both ATP and glucose, resulting in the production of G6P. A direct correlation has been established between the growth of carcinomas and the levels of HK activity (Bustamante et al., 1981). According to Koobs (1972), mitochondrial-bound HK limits respiration when tumour cells are utilising glucose (known as the Crabtree effect), even though large amounts of ADP continue to be produced. The continuous phosphorylation of glucose by ATP (proceeding by the mitochondrial-bound HK) reduces the level of phosphate available for oxidative phosphorylation, and thereby prevents attaining maximal rates for state 3 respiration (Baggetto and Testa-Parussini, 1990). Hence, HKII via its mitochondrial localisation also helps to stabilise mitochondria, suppressing apoptotic death of cancer cells and promoting their survival (Mathupala et al., 2006).

Several hexokinase inhibitors have been found to suppress cancer growth and of these, considerable focus has been on 2-deoxyglucose (2DG) and 3-bromopyruvate (3BP). The basis
for the action of 2DG is to inhibit HK activity and, hence, glycolysis with the result that the binding of HK to VDAC is prevented, promoting the susceptibility of malignant cells to other forms of treatment (Simons et al., 2007). This finding as well as recent report that 2DG promotes cancer cell apoptosis when used in combination with another mitocan, the anti-diabetic drug, metformin, provides the basis for testing these compounds in the clinical setting (Ben Sahra et al., 2010). 3BP is an alkylating agent that inhibits both HK activity and the mitochondrial complex II (CII) and consequently is included in the mitocan class 2 and 5 (Figure 1). Recent data indicate that 3BP acts by binding covalently to HKII, causing its dissociation from VDAC (Chen et al., 2009). 3BP causes cancer cell death due to the rapid depletion of ATP and suppresses tumour growth in animal models. For these reasons, 3BP is another candidate for cancer clinical trials (Mathupala et al., 2006).

3. Class 2 mitocans: Compounds targeting Bcl-2 family proteins

This class of mitocans includes compounds acting as mimetics of the Bcl-2 homology-3 (BH3) domains, integral parts of Bcl-2 family of proteins. When the levels of expression of the pro-apoptotic members of the family is greater, the cell will undergo the demise, whereas higher levels of expression of the anti-apoptotic Bcl-2 family proteins will provide a pro-survival ‘environment’. Although recent findings revealed novel functions for the Bcl-2 family members, including a role in the biogenesis of mitochondria (Suen et al., 2008), we will only briefly discuss these proteins as targets for class 2 mitocans. The basis for the action of this mitocan class stems from the finding that the anti-apoptotic and pro-apoptotic Bcl-2 family proteins interact via their BH3 domains thereby preventing the BH3 domain-containing proteins Bak and/or Bax from forming large channels or pores in the MOM (Youle and Strasser, 2008). Since the MOM channel is made of oligomers of the pro-apoptotic Bax or Bak proteins, increased expression of the anti-apoptotic BH3-interacting proteins Bcl-2, Bcl-
\(x_L\) or Mcl-1 will protect cancer cells from apoptosis, and anti-apoptotic Bcl-2 family proteins are often over-expressed in cancer cells (Lessene et al., 2008). Therefore, small molecules or BH3 mimetics, targeting the interaction between the anti- and pro-apoptotic Bcl-2 protein members, are of clinical importance (Kang and Reynolds, 2009; Zeitlin et al., 2008).

The BH3 mimetics include the natural, polyphenolic compound gossypol. This agent has been shown to interact with BH3 binding domains, thereby interfering with the interaction between Bcl-2, Bcl-\(x_L\) or Mcl-1 and the pro-apoptotic proteins, Bax or Bak. The result is the oligomerisation of Bax or Bak to form channels and activation of the post-mitochondrial apoptotic signalling (Oliver et al., 2005). Gossypol has served as a lead compound for developing more efficient BH3 mimetics, such as the highly intriguing agent ABT-737 (van Delft et al., 2006). ABT-737 as well as its orally applicable version, ABT-263 (Tse et al., 2008) are now being tested in clinical trials. ABT-263 (Navitoclax) has been successfully tested in phase 1 clinical trial of lymphoid tumour and chronic lymphocytic leukemia, resulting in the design of phase 2 study (Wilson et al., 2010; Roberts et al., 2012). It has also been tested on solid tumours. Phase 1 clinical trial with small-cell lung cancer (SCLC) or pulmonary carcinoid, patients resulted in good outcome, prompting a phase 2 study (Roberts et al., 2012).

The apoptogenic compound \(\alpha\)-tocopheryl succinate (\(\alpha\)-TOS) has been reported to interact with the BH3-binding domain of Bcl-2 and Bcl-\(x_L\), which suppresses their interaction with the pro-apoptotic protein Bak, arresting proliferation of prostate cancer cells and inducing their death by apoptosis (Shiau et al., 2006). This activity of \(\alpha\)-TOS is interesting in that it complements its second apoptogenic activity due to its ability to interact directly with the ubiquinone (UbQ) sites of the mitochondrial CII (see below for details). In addition, the mitochondrial CIII \(Q_i\) site inhibitor, antimycin A, also acts as a BH3 mimetic (Tzung et al., 2001), suggesting that on a more general level, compounds that interact with UbQ-binding
sites in the mitochondrial electron transport chain (ETC) may have a tendency to be BH3 mimetics as well, consistent with the possibility that the BH3-binding site in Bcl-2 family members might also present a UbQ-binding site (Neuzil et al., 2007).

4. Classes 3 and 4 of mitocans: Thiol redox inhibitors plus VDAC/ANT targeting drugs

Classes 3 and 4 comprise thiol redox inhibitors and the VDAC/ANT targeting drugs, and their activity is linked to the redox environment of cancer cells, which is distinct from that of normal cells in that cancer cells show higher intrinsic levels of ROS. As a result, it makes cancer cells more vulnerable to agents that induce further elevations in oxidative stress, since their anti-oxidant capacity is relatively inferior (Huang et al., 2000; Szatrowski and Nathan, 1991). Therefore, compounds that oxidise thiol group and/or deplete the mitochondrial GSH pool will cause substantial apoptosis of cancer cells (Fulda et al., 2010; Trachootham et al., 2009). Agents like arsenic trioxide (Miller, 2002; Pelicano et al., 2003) or isothiocyanates, represented by phenylethyl isothiocyanate (PEITC) (Trachootham et al., 2006; Xu and Thornalley, 2001) have been shown to possess relative selectivity in killing cancer cells by upsetting the normal homeostasis in the cellular redox environment. Intriguingly, PEITC has been reported to efficiently kill resistant leukemia cells (Trachootham et al., 2008).

The permeability transition pore complex (PTPC) forms as a superchannel comprising the VDAC/ANT system of proteins embedded in the MOM and MIM, respectively, interconnecting the mitochondrial matrix with the cytosol, and serves as a mode of transport for a variety of solutes and small molecules including ATP and ADP (Zhivotovsky et al., 2009). Deregulation of the VDAC/ANT complex results in apoptosis induction in cancer cells. Compounds that modulate the PTPC include lonidamine, arsenites and steroid analogues (represented by CD437) (Belzacq et al., 2001). Interestingly, an arsenite analogue 4-[N-(S-glutathionylacetyl)amino]phenylarsineoxide (GSAO) was shown to inhibit the
function of ANT by crosslinking its cysteine residues. This was followed by the generation of oxidative stress and induction of apoptosis, which was selective for proliferating, angiogenic endothelial cells (ECs) while being non-toxic to growth-arrested ECs (Don et al., 2003). These results indicate that GSAO can selectively kill ECs in the context of a growing tumour, acting in an anti-angiogenic manner. Similar findings were reported for one of the mitocans, α-TOS (see more on its molecular mechanism below).

5. Class 5 mitocans: Electron redox chain targeting drugs

Class 5 mitocans comprises a large group of different compounds that target the mitochondrial complexes, a part of the ETC (Scheffler, 2008). Some of them were discussed in recent reviews (Fulda et al., 2010; Galluzzi et al., 2007; Gogvadze et al., 2009; Ralph and Neuzil, 2009; Wang et al., 2010).

The production of energy is achieved by transporting electrons in a coordinated manner from NADH or FADH$_2$ (generated from substrates in the TCA cycle) to the final acceptor, molecular oxygen, to produce water (Figure 1). The five macromolecular protein complexes of the mitochondrial ETC are embedded in the mitochondrial inner membrane (MIM), and, by passing electrons from CI and CII via CIII to CIV with the help of electron carriers, UbQ and cytochrome c, generate energy that is maintained as an electrochemical proton gradient across the MIM. CV, the F$_1$F$_0$-ATPase, then uses the energy from the proton gradient to generate ATP from ADP and inorganic phosphate, which provides the cell with its fundamental energy substrate. The ETC is the major source of mitochondrial reactive oxygen species (ROS) due to the large electron flows, with CI and CIII identified as prime superoxide-generating sites, although CII are also implicated in the process (Saraste 1999, Adam-Vizi and Chinopoulos 2006; Ralph et al., 2011). The superoxide by-product is not harmful when ROS production is controlled within the constraints of the cellular redox system, as the cell must maintain
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Moderate ROS levels necessary to sustain the cellular signalling processes. However, deregulation leading to permanently higher levels of ROS may predispose, over time, to carcinogenesis (Murphy, 2009). By contrast, a sudden and substantial increase in ROS levels may have a much more immediate effect and commit the cell to undergo apoptosis (Fruehauf and Meyskens, 2007; Kadenbach et al., 2010).

Figure 1 and Table I show several examples of mitocans targeting the mitochondrial complexes. Due to lack of space, we will only focus here on several representatives of these compounds. Of high interest is tamoxifen, a routinely used drug against estrogen-positive breast cancer (Higgins and Stearns, 2011), that has recently been reported to induce apoptosis via the mitochondrial CI, most likely by interfering with the FAD-binding site (Moreira et al., 2006). We have been pursuing for a while the anti-cancer agent from the family of VE analogues, \( \alpha \)-TOS, that selectively kills cancer cells (Neuzil et al., 2001a,b; Weber et al., 2002, 2003). The mitochondrial CI (dos Santos et al., 2012) and, in particular CII have been proposed as targets of \( \alpha \)-TOS (Dong et al., 2008, 2009). We have documented that \( \alpha \)-TOS interferes with the CII’s proximal and distal UbQ-binding sites (\( Q_p \) and \( Q_d \), respectively) that have been documented recently in the crystallographic study of mammalian CII (Sun et al., 2005).

Knowing the target for \( \alpha \)-TOS, we modified the agent by tagging it with the cationic triphenylphosphonium (TPP\(^+\)) group (see Figure 2A for structures), in analogy with the earlier work of Murphy and Smith on UbQ (Murphy and Smith, 2007). This mitochondrially targeted VE succinate (MitoVES) was found to associate almost exclusively with mitochondria (Figure 2B) and kill a variety of cancer cell lines in a selective manner more efficiently by the untargeted parental compound by 1-2 orders of magnitude (Dong et al., 2011a,b). Importantly, MitoVES showed very high anti-cancer activity in clinically relevant mouse models of HER2-high breast cancer and colorectal cancer (Dong et al., 2011a,b) (Figure 2C). More detailed
analysis of the molecular mechanism of the interaction of MitoVES with CII revealed that MitoVES inhibited the SDH activity of CII (associated with the SDHA subunit of CII) with the IC$_{50}$ of $\sim$70 $\mu$M, while it suppressed the succinate quinone reductase (SQR) activity with the IC$_{50}$ of $\sim$1.5 $\mu$M (Figure 3). This indicates that the molecular target of MitoVES is ideally placed, such that the SDH activity is only mildly depressed, allowing for the Krebs cycle to proceed, i.e. for succinate to be converted to fumarate with the two electrons channeled via the SDHB group’s [Fe-S] clusters to the MIM part of CII (Figure 3). Since MitoVES displacesUbQ bound between SDHC and SDHD, electrons cannot be intercepted and form superoxide inducing apoptosis in the cells by way of activating the Mst1 kinase that, in turn, phosphorylates the transcription factor FoxO1, which causes upregulation of the BH3-only protein Noxa. This protein then provokes the formation of the Bak and/or Bax channel in the MOM (Prochazka et al., 2010; Valis et al., 2012; Dong et al., 2011a,b) (Figure 3).

Similar to GSAO (see Chapter 3), $\alpha$-TOS as well as MitoVES kill proliferating ECs while being non-toxic to their arrested counterparts. The mechanism for the resistance for the confluent ECs to the two agents involves their lack in ROS generation when challenged with the compounds. Moreover, we also found that MitoVES cannot efficiently enter arrested ECs due to their lower level of $\Delta\Psi_{m,i}$; due to the cationic nature of MitoVES due to the presence of the TPP$^+$ group, this agent associates better with mitochondria when the $\Delta\Psi_{m,i}$ is higher (see below) (Dong et al., 2007; Rohlena et al., 2011).

Addition of the cationic TPP group to mitocans with targets within the mitochondrial complexes appears a new paradigm of efficient cancer treatment approach, since by tagging these agents with TPP, directly send them to the proximity of their targets, therefore increasing considerably their concentration in the compartment where ‘they matter’. We are now attempting to tag with TPP$^+$ also mitocans that target other mitochondrial complexes than
CI. It is worth mentioning that TPP$^+$ tagging has been tested for polyphenolic compounds, some of which induce apoptosis (Biasutto et al., 2010; Smith et al., 2011; Sassi et al., 2012).

6. Class 6 mitocans: Lipophilic cations targeting the inner membrane

The molecular target of lipophilic cations acting on the MIM is given by the relatively high trans-membrane potential, $\Delta \Psi_{m,i}$ that exists across the MIM. It has been documented that cancer cells have a considerably higher $\Delta \Psi_{m,i}$ than in non-malignant cells due to altered mitochondrial bioenergetics (Modica-Napolitano and Aprille, 1997). This feature will dictate the intracellular targeting of lipophilic cations that as a result of the increased $\Delta \Psi_{m,i}$ in cancer cells will make these mitocans relatively more selective for malignant cells. This follows from the Nernst law defining that with each increase of $\Delta \Psi_{m,i}$ by -60 mV, a corresponding 10-fold higher accumulation of cationic compounds in the MIM occurs (Modica-Napolitano and Aprille, 2001, Wang et al., 2010).

A prime example of this mitocan class targeting the MIM is rhodamine-123, which was reported to accumulate in mitochondria of src-transformed cells in the 1980s (Johnson et al. 1980), showing selectivity for cancer cells (Lampidis et al., 1983). Following from this, the selective toxicity of a number of delocalised lipophilic cationic agents, including the peptide (KLAKKLAK)$_2$ towards cancer cells was found (Ellerby et al., 1999). A compound termed F16, with a delocalised positive charge, was identified by high-throughput screening and was found to be selective and effective against breast carcinomas with high level of HER2 expression (Fantin et al., 2002).

7. Class 7 mitocans: Drugs targeting the tricarboxylic acid cycle

The tricarboxylic acid (TCA) cycle, also referred to as the citric acid cycle or Kreb’s cycle, is a source of electrons that are fed into the ETC and that are used to drive the electrochemical
proton gradient required for the generation of ATP and is the target of class 7 mitocans. The TCA cycle is based on the addition of acetyl-CoA (formed in the mitochondrial matrix by the conversion of pyruvate (catalysed by pyruvate dehydrogenase) to oxaloacetate to form citrate. Citric acid is then in a series of reactions converted to oxaloacetate, which again adds a molecule of acetyl-CoA. During this process, electrons are released to drive the proton gradient, which is coupled to the generation of ATP. An interesting step in the TCA cycle involves SDH (or CII). 3BP, mentioned earlier as an inhibitor of HK, is also an inhibitor of SDH (see Chapter 4) (Sun et al., 2005). Therefore, 3BP acts at the crossroads of the TCA cycle and the ETC. It suppresses conversion of succinate to fumarate, thereby slowing down the TCA cycle and, consequently, CI, while also inhibiting the generation of electrons that are fed into CIII via CII (see Chapter 4). From this point of view, 3BP as an inhibitor of CII may not be advantageous, while of more importance making it a potential anti-cancer agent may be its inhibitory activity on HK (Pedersen, 2012).

A number of compounds exist that target the TCA cycle as well as the reaction converting pyruvate to acetyl-CoA, a prerequisite of pyruvate to enter the mitochondria and the TCA cycle. The enzyme pyruvate dehydrogenase that catalyses the reaction is regulated by phosphorylation via the pyruvate dehydrogenase kinase (PDK). The inhibition of PDK results in increased activity of pyruvate dehydrogenase and higher activity of the TCA cycle. Dichloroacetate (DCA), a relatively basic compound, is selective for killing cancer cells by suppressing the activity of PDK (Bonnet et al., 2007). By promoting the activity of pyruvate dehydrogenase, DCA causes a shift from anaerobic glycolysis to oxidative glycolytic metabolism accompanied by a decrease in the $\Delta \Psi_{\text{m,i}}$, ROS generation and activation of the K$^+$ channel, events that are selective for cancer cells (Bonnet et al., 2007). DCA is already in clinical use to treat patients with mitochondrial deficiencies and, therefore, its development as
an anti-cancer drug could be less complicated than when dealing with a completely novel agent.

8. Class 8 mitocans: Drugs targeting mtDNA

Group 8 of mitocans comprises agents targeting mitochondrial DNA (mtDNA). Mitochondria are unique organelles because they carry their own genetic information encoded on a small circular genome, referred to as mitochondrial DNA (mtDNA). The mammalian mitochondrial genome has the size of over 16 kB, and encodes 13 subunits of the mitochondrial complexes I, III, IV and V, 24 tRNAs, 12S and 16S rRNA, and also contains a region called the D-loop sequence, which is important in the regulation of mtDNA replication (Anderson et al., 1981).

To date, several compounds have been reported that interfere with the function and stability of mtDNA and other drugs that affect the activity of the mitochondrial DNA polymerase-γ. For example, vitamin K3 (menadione) targets mtDNA by inhibiting the activity of DNA polymerase γ that is specific for replication of mtDNA, with ensuing induction of apoptosis (Sasaki et al., 2008). Similar effects were reported for fialuridine, which induces mitochondrial structural defects (Lewis et al., 1996). The Parkinsonian toxin 1-methyl-4-phenylpyridinium causes a reduction in the copy number of mtDNA by destabilising the structure of the mitochondrial D-loop (Miyako et al., 1997; Umeda et al., 2000).

We have been studying VE analogues as anti-cancer drugs, epitomised by the redox-silent α-TOS (see Chapters 4 for details on the apoptogenic signalling induced by α-TOS). The agent was made more efficient by tagging with the TPP group (Dong et al., 2008, 2011a,b), as has been done previously for a variety of antioxidants (Murphy and Smith, 2007) (Figure 2). While the mitochondrially targeted VE analogues are superior to the untargeted α-TOS in its apoptogenic activity, we found another very intriguing feature of MitoVES. It was observed that the agent modulates mtDNA and more specifically, suppresses the D-loop
transcript levels at sub-apoptotic doses in a range of cancer cells, a phenomenon that was not observed for \( \alpha \)-TOS. This was accompanied by the inhibition of cancer cell proliferation (Truksa et al., 2012). It is not clear at this stage, whether modulation of mtDNA by MitoVES is a direct effect of the drug on the mitochondrial genome; it appears that this is mediated by the fast dissipation of \( \Delta \Psi_{m,i} \) in response to MitoVES (Figure 4) and rapid ROS generation (Truksa et al., 2012). Nevertheless, MitoVES provides an intriguing possibility for interfering with tumour progression by means of suppressing the proliferation of cancer cells without necessarily inducing apoptosis.

9. Conclusions and future perspectives: Clinical relevance of mitocans

Mitocans are, in quite a few cases, selective for cancer cells, which is a prerequisite for a potentially clinically relevant anti-cancer agent. Except for a few compounds, mitocans have not been employed in the clinical setting. One of these exceptions is tamoxifen, one of the most frequently used drugs against breast cancer, which is now also used as a preventive agent, albeit thus far largely due to its effect on the ER, competing with its activating ligand estradiol (Higgins and Stearns, 2011; Cuzick et al., 2011). However, the recent findings that tamoxifen also acts as a class 5 mitocan via targeting the mitochondrial CI (Moreira et al., 2006) endows it with a novel translational spin, such that its modification whereby it will localise primarily to mitochondria will give it an additional, thus far unprecedented bioactivity. We are currently working on this highly intriguing possibility (Neuzil et al., unpublished data).

3BP, a class 1, 5 and 7 mitocan, has been pursued for its possible clinical relevance. A whole special issue of the Journal of Bioengineering and Biomembranes has been devoted to the compound (Pedersen, 2012). From the clinical point of view, of great interest is a recent case report documenting that 3BP extended considerably the survival of a patient with
fibrolamellar hepatocellular carcinoma with relatively low side effects (Ko et al., 2012). The patient did succumb to the disease after two years of 3BP therapy, possibly due to the ‘poisoning’ by dead cancer cells that could not be detoxified by the apparently only partially functional liver. Whether this toxicity is possibly also due to ‘too many’ targets that 3BP acts on (Shoshan, 2012) is not clear. Notwithstanding the ultimate demise of the patient, 3BP can be considered as one of the mitocans that can be developed into efficient anti-cancer drugs, perhaps in combination with other agents.

We tested the class 5 mitocan α-TOS in a single case clinical situation. A female mesothelioma patient was allowed to be transdermally treated with the agent. Daily administration of α-TOS suspended in a cream with a transcutaneous enhancer helped remove the pain associated with mesothelioma and resulted in the shrinkage of the tumour. However, the tumour eventually started to progress again and the patient deceased. In spite of the ultimately fatal outcome, the life of the patient, being of good quality, was extended by several years, i.e. considerably more than expected. In a very recent in vitro study we have found that long-term exposure of cancer cells to α-TOS renders them resistant to the agent due to upregulation of a member of the ABC class of transporters. We also discovered that these resistant cells were, quite surprisingly, considerably susceptible to MitoVES (Neuzil et al., unpublished observation), possibly due to the altered respiration of the resistant cells, a condition likely favouring the killing of the cells by the mitochondrially targeted VE analogue. This is a neat example how modification of mitocans whereby they directly interact with their target may be utilised for suppression of tumours that are otherwise resistant, and also indicate the possibility of a prospective intermittent therapy.

While, quite obviously, more needs to be done, it is undisputable that mitocans hold a substantial promise as future anti-cancer agents, in particular due to mitochondria being a rather invariant target across the landscape of the variety of types of neoplastic pathologies.
The few examples above also document the fact that basic mitocans can be further modified to accumulate in the mitochondrial compartment, where their presence ‘matters’, as exemplified by tagging them with the TPP\(^+\) cationic group that targets them to the interphase of the MIM and the mitochondrial matrix. It is proposed that carefully planned clinical trials with selected mitocans are highly warranted to shift the odds of cancer patients toward their survival and complete cure. Given the unrelenting increase in the number of cancer patients, this may appear as a highly daunting task. Notwithstanding this grim perspective, the emerging knowledge of the importance of mitochondria as clinically relevant targets for anti-cancer drugs seems to provide the ‘light at the end of the tunnel’.

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Table I. Classification of mitocans and examples of compounds in individual classes.

<table>
<thead>
<tr>
<th>Class number</th>
<th>Type</th>
<th>Examples</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexokinase inhibitors</td>
<td>3BP, 2DG</td>
<td>Chen et al., 2009; Ko et al., 2004; Mathupala et al., 2006; Ben Sahra et al., 2010; Simons et al., 2007</td>
</tr>
<tr>
<td>2</td>
<td>Compounds targeting Bcl-2 family proteins</td>
<td>gossypol, ABT-737, antimycin A, α-TOS</td>
<td>Kang et al., 2009; van Delf et al., 2006; Tzung et al., 2001; Shiau et al., 2006; Pelicano et al., 2003</td>
</tr>
<tr>
<td>3</td>
<td>Thiol redox inhibitors</td>
<td>Isothiocyanates, arsenic trioxide</td>
<td>Trachootham et al., 2007; Xu and Thornalley, 2001; Millet, 2002; Pelicano et al., 2003</td>
</tr>
<tr>
<td>4</td>
<td>VDAC/ANT targeting drugs</td>
<td>lonidamine, arsenites, steroid analogues like CD437</td>
<td>Belzacq et al., 2001; Don et al., 2003</td>
</tr>
<tr>
<td>5</td>
<td>Electron redox chain targeting drugs</td>
<td>α-TOS, MitoVES, tamoxifen, adaphostin, 3BP</td>
<td>Dong et al., 2008, 2009, 2011a,b; Rohlena et al., 2011; Moreira et al., 2006; Le et al., 2007; Pereira da Silva et al., 2009</td>
</tr>
<tr>
<td>6</td>
<td>Lipophilic cations targeting inner membrane</td>
<td>rhodamine-123, F16. (KLAKKLAK)_2</td>
<td>Bernal et al., 1983; Johnson et al., 1980; Lampidis et al., 1983; Fantin et al., 2002; Ellerby et al., 1999</td>
</tr>
<tr>
<td>7</td>
<td>Drugs targeting the tricarboxylic acid cycle</td>
<td>DCA, 3BP</td>
<td>Bonnet et al., 2007; Dell’Antone, 2011; Pereira da Silva et al., 2009</td>
</tr>
<tr>
<td>8</td>
<td>Drugs targeting mtDNA</td>
<td>vitamin K3, fialuridine, 1-methyl-4-phenylpyridinium, MitoVES</td>
<td>Lewis et al., 1996; Sasaki et al., 2008; Miyako et al., 1997; Umeda et al., 2000; Truksa et al., 2012</td>
</tr>
<tr>
<td>9</td>
<td>Drugs targeting other (unknown) sites</td>
<td>betulinic acid</td>
<td>Fulda et al., 1998</td>
</tr>
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Legend to Figures

**Figure 1. Schematic illustration of the molecular targets of individual classes of mitocans.** The classes of mitocans comprise the following, as enumerated from the outside of the mitochondria towards the matrix. Class 1: hexokinase inhibitors; Class 2: BH3 mimetics and related agents that impair the function of the anti-apoptotic Bcl-2 family proteins; Class 3: thiol redox inhibitors; Class 4: agents targeting VDAC and ANT; Class 5: compounds targeting the mitochondrial electron transport chain; Class 6: hydrophobic cations targeting the MIM; Class 7: compounds that affect the TCA; and Class 8: agents that interfere with mtDNA. Class 9 (not shown) includes agents acting on mitochondria, whose molecular target has not been thus far described.

**Figure 2. Targeting of vitamin E succinate to MIM enhances its anti-cancer activity.**

A) Structure of α-tocopheryl succinate (α-TOS) and mitochondrially targeted vitamin E succinate (MitoVES) with 11-C aliphatic chain spanning the tocopheryl head-group and the TPP⁺ moiety. B) MitoVES associates primarily with mitochondria. Mouse breast cancer cells NeuTL were incubated with MitoTracker Red, fluorescently tagged MitoVES (MitoVES-F) or α-TOS (α-TOS-F), and the nuclei were stained using Hoechst 33342, and were inspected using confocal microscopy. The overlays document mitochondrial localisation of MitoVES while α-TOS localises to mitochondria as well as to other sites. C) MitoVES is superior in tumour suppression to α-TOS. The transgenic FVB/N202 c-neu mice with spontaneous formation of HER2-high breast carcinomas (left) and nude mice with human colorectal HCT116 cell line-derived xenografts (right) were treated with α-TOS at 15 or MitoVES at 1-2 μmol per animal per injection every 3-4 days. The tumors were regularly visualised and their
volume quantified using an ultrasound imaging instrument (Vevo 770 from VisualSonics) equipped with a 30-μm resolution scan head.

**Figure 3. Molecular mechanism for the generation of ROS and the induction of apoptosis by vitamin E analogues via targeting of CII.** In the absence of a VE analogue, the electrons generated during conversion of succinate to fumarate by SDH at the SDHA (or Fp) subunit are relayed to the [Fe-S] clusters in the SDHB (or Ip) subunit, which direct the electrons to the Qp (and/or, possibly, Qd) site made up by residues from the SDHC (or CybL) and SDHD (or CybS) subunits. This results in a two electron reduction of the oxidised form of UbQ to UbQH2, which has low affinity for its binding site(s) in CII. UbQH2 is then released from CII to bind to CIII, where it gives up the two electrons to CIII at the Qi site to be transferred to UbQ at the Qo site. Hence, the incoming UbQH2 is re-oxidised and released to return as UbQ to CII (A). This ‘electron shuttle’ activity of UbQ becomes disrupted in the presence of agents like the VE analogues, epitomised in this cartoon by MitoVES (B). In this case, MitoVES interferes with the function of UbQ, most likely by displacing it. Therefore, the electrons generated during conversion of succinate to fumarate cannot be intercepted by their natural acceptor. As a consequence, they interact with molecular oxygen to yield superoxide. The IC50 values for inhibition of the SDH activity and the CII→CIII electron transfer (represented by the SQR activity), i.e. ~70 and ~1.5 μM, respectively, are also indicated. The superoxide generated as indicated then causes (auto)phosphorylation of the Mst1 kinase, which phosphorylates the transcription factor FoxO1. The pFoxO1 protein translocates to the nucleus, where it triggers expression of the NOXA gene. The resulting increased level of the Noxa protein then causes the formation of the Bak and/or Bax channel in the MIM, ultimately resulting in the entry of the cell into the commitment phase of apoptosis.
Figure 4. Mitochondrial targeting of vitamin E succinate causes its fast uptake and dissipation of $\Delta \Psi_{m,i}$. MCF7 cells were incubated with fluorescently tagged MitoVES (green fluorescence) and labelled for mitochondrial potential using TMRM. The cells were placed on the platform of a confocal microscope with an incubator-style housing (37°C, 5% CO$_2$) and images taken at indicated time points. Nuclei were counterstained with DAPI. The graph in the right bottom corner indicates the intensity of green and red fluorescence at individual time points taken as an average relative intensity in several fields, each comprising 5-10 cells.
Figure 2
Figure 3

![Diagram showing the classification of mitocans](image)