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# **Targeting Mitochondria in Fighting Cancer**

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**Abstract:** During the last years, there have been a number of reports that prove involvement of mitochondria in the pathogenesis of variety of disorders including cancer and neurodegenerative diseases. Alteration of vital mitochondrial functions - production of ATP, calcium buffering capacity, abnormal production of reactive oxygen species, can be potentially responsible for pathogenesis of cancer and neurodegenerative diseases. Involvement of mitochondria in various types of cell death makes them attractive targets for tumor cell elimination. This review describes the role of mitochondria in tumor cells and how targeting of mitochondria can be used as a tool in fighting cancer.

Keywords: Cancer, apoptosis, necrosis, mitochondria, calcium, permeability transition, Bcl-2.

# INTRODUCTION

Observations made during the last decades revealed that variety of disorders including cancer and different neurodegenerative diseases such as Parkinson disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease have mitochondrial origin. Disturbance of mitochondrial vital functions, e.g., production of ATP, calcium buffering capacity, and excessive generation of reactive oxygen species (ROS), can be potentially involved in disease pathogenesis.

Mitochondrial dysfunction in tumors was first observed in 1926 by Otto Warburg. He found that cancer cells produce most of their ATP through glycolysis, even under aerobic conditions [1]. A comparison of various cancer cell lines revealed that they rely on glycolysis for ATP production to different extents, and typically, the more "glycolytic" tumor cells were found to be the most aggressive ones [2]. Warburg suggested that cancer is caused by damage of mitochondria. Even 40 years after his fundamental observation he stated in his lecture at the meeting of Nobel Laureates at Lindau, Lake Constance: "Cancer, above all other diseases, has countless secondary causes. But, even for cancer, there is only one prime cause. Summarized in a few words, the prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar" [3]. Warburg suggested that tumor cells might be eliminated through the inhibition of mitochondrial metabolism (for instance, by moderate doses of ionizing radiation), which would reduce the activity of these organelles below a threshold level critical for cell survival, whereas mitochondria in nonmalignant cells would still be able to produce enough ATP.

The impact of mitochondrial activities on cellular physiology is not restricted to ATP production for metabolic demands. Mitochondria produce ROS, which are involved in the regulation of many physiological processes, but which might also be harmful to the cell if produced excessively. Furthermore, mitochondria are involved in maintaining intracellular  $Ca^{2+}$  homeostasis, and, which is very important, they are key participants in the regulation of cell death pathways. Obviously, all these functions are of crucial importance for tumor cell physiology, growth and survival, and intervention with these metabolic pathways might make tumor cells more susceptible to anticancer treatment.

# TYPES OF CELL DEATH

The aim of anticancer treatment is tumor cell elimination. Apoptosis and necrosis are the main forms of cell death. Necrosis is usually regarded as a form of unnatural cell death. It is characterized by disruption of the plasma membrane and membranes of intracellular organelles, cell swelling, chromatin digestion, extensive DNA hydrolysis, and finally cell lysis [4]. One of the causes of necrosis is a collapse of mitochondrial functions followed by a drastic drop in ATP level. This in turn causes the disturbance of cytosolic Ca<sup>2+</sup> homeostasis, Ca<sup>2+</sup> overload in cytosol and stimulation of various Ca<sup>2+</sup>dependent catabolic enzymes – phospholipases, proteases, DNases. Necrosis is often associated with unwarranted cell loss in human pathologies and can lead to local inflammation, presumably through the release of factors from dead cells that alert the innate immune system [5]. Necrosis is known to play a prominent role in many pathological conditions, including ischemia (i.e., stroke and myocardial infarction), trauma, and possibly some forms of neurodegeneration.

In contrast to necrosis, apoptosis represents a regulated form of cell death. Apoptosis is an evolutionarily conserved and genetically regulated process of critical importance for embryonic development and maintenance of tissue homeostasis in the adult organism that plays a significant role in tumor cell biology [6]. Apoptosis may be involved in spontaneous elimination of potentially malignant cells and therapeutically-induced tumor regression, one of the unique features of tumor, whereas defects in apoptosis program may contribute to tumor progression and resistance to treatment. Apoptosis is characterized by a variety of morphological and biochemical features. Contrary to necrosis, apoptotic cell death machinery needs ATP; mitochondria in apoptosis remain relatively intact, although permeabilization of the OMM in some models of apoptosis leads to a release of cytochrome c and suppression of respiration.

Interestingly, inhibition of apoptosis in many cases does not completely prevent cell death, but rather causes a switch from apoptotic to regulated caspase-independent cell death with morphological features resembling necrosis. One of these modes of cell death called necroptosis has recently been described [7]. Necroptosis is initiated by death receptors, such as tumor necrosis factor receptor; it requires the kinase activity of receptor-interacting protein 1 (RIP1; also known as RIPK1) and RIP3 (also known as RIPK3), and its execution involves the active disintegration of mitochondrial, lysosomal and plasma membranes, reviewed in [8]. In the absence of intracellular apoptotic signaling, stimulation of the Fas/TNFR receptor family triggered necroptosis and activation of

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autophagy. The authors demonstrated that necroptosis was responsible for delayed ischemic brain injury in mice *in vivo* through a mechanism distinct from that of apoptosis. A specific and potent small-molecule inhibitor of necroptosis, necrostatin-1, was shown to block a critical step in necroptosis offering new possibilities for neuroprotection. In addition, other modes of cell death have been also described; in particular, caspase-independent programmed cell death with necrotic morphology [9], which might be dependent on ROS [10].

The interaction among different forms of cell death is complex and still a matter of debate. In fact, apoptosis and necrosis can occur simultaneously in tissues or cell cultures exposed to the same stimulus and often the intensity of the same initial insult determines the mode of cell death. Thus, intracellular energy levels and mitochondrial function are rapidly compromised in necrosis, but not in apoptosis of neuronal cells [11]. ATP generation either by glycolysis or by mitochondria is required for the active execution of the final phase of apoptosis. Indeed, during ischemic brain injury, glutamate accumulation leads to overstimulation of postsynaptic glutamate receptors with intracellular Ca<sup>2+</sup> overload and neuronal cell death either via necrosis or delayed apoptosis. During and shortly after exposure to glutamate, a subpopulation of neurons was shown to die by necrosis. If, however, neurons survived the early necrotic phase, recovered mitochondrial potential and energy levels, later they underwent apoptosis, with formation of apoptotic nuclei and chromatin degradation into high and low molecular weight fragments [11]. Hence, mitochondrial stability is of a critical importance and can determine the mode of neuronal death.

#### INVOLVEMENT OF MITOCHONDRIA IN CELL DEATH

Involvement of mitochondria in necrotic cell death was known for long time. Certain conditions, notably mitochondrial calcium accumulation and oxidative stress, can trigger the opening of a high-conductance pore in the inner mitochondrial membrane (IMM). This leads to a collapse of the electrochemical potential for  $H^+$ , thereby ceasing ATP production and triggering production of ROS. The mitochondrial collapse occurs in several forms of necrotic cell death, including oxidative stress, pH-dependent ischemia/reperfusion injury and Ca<sup>2+</sup>ionophore toxicity [12, 13].

The importance of mitochondria for apoptotic cell death became evident later. In one of the earliest publications, Bcl-2 protein, which is overexpressed as a result of a chromosomal translocation in B cell lymphomas, and known by its ability to suppress programmed cell death, was shown to be localized to the IMM, although the precise mechanism of its action was unclear [14]. Cells over-expressing Bcl-2 had a higher mitochondrial membrane potential than wild-type cells. The higher membrane potential was suggested to be responsible for the resistance of cells after TNF treatment [15]. The key role of mitochondria in apoptosis was further hypothesized by C. Richter [16]. According to his viewpoint, uncontrolled production of oxygen radicals, a common step in many models of apoptosis [17], stimulates Ca<sup>2+</sup> release from mitochondria, followed by Ca<sup>2+</sup> cycling across the IMM, dissipation of mitochondrial membrane potential, ATP depletion, massive disturbance of cellular Ca<sup>2+</sup> homeostasis, and a direct stimulation of Ca<sup>2+</sup>dependent endonuclease(s). To explain the protective functions of Bcl-2, a model was proposed in which Bcl-2 regulates an antioxidant pathway at sites of free radical generation [18], although the detailed investigation of the location of Bcl-2 revealed that it is localized not in the inner but in the outer mitochondrial membrane (OMM) [19]. Currently it is widely accepted that mitochondria play a key role in the regulation of apoptosis [20].

Of the two major apoptotic pathways known, the extrinsic (receptor-mediated) pathway engages initiator pro-caspase-8, which subsequently activates pro-caspase-3 and other effector caspases. By contrast, the intrinsic pathway involves permeabilization of the OMM followed by the release of cytochrome c and other proteins from the intermembrane space of mitochondria. Permeabilization of the OMM is a key event in cell death induction and is considered a "point of no return" in many models of apoptosis. Cytochrome *c*, once released, triggers the formation of the apoptosome complex in the cytosol and subsequent activation of the caspase cascade, cleavage of cellular proteins, which is primarily responsible for the biochemical and morphological characteristics of apoptosis. Release of apoptosis inducing factor (AIF) upon OMM permeabilization, triggers caspase-independent cell death pathway, which ends up in DNA fragmentation [20].

What are the mechanisms of OMM permeabilization?

#### MECHANISMS OF OMM PERMEABILIZATION

One of the main mechanisms of OMM permeabilizationin apoptosis involves pro-apoptotic members of the Bcl-2 family of proteins. The first indication that genes and proteins, which play a role in tumorogenesis, might be involved in thenegative regulation of cell death came from the study on Bcl-2 [21]. Overexpression of this protein was shown to inhibit cell death induced by different stimuli such as IL-3 deprivation, chemotherapeutic agents and heat shock, reviewed in [22].

Today in addition to Bcl-2 above 30 other members and related proteins have been identified. They can be divided into two groups: Bcl-2-like survival factors, or antiapoptotic proteins (Bcl-2, Bcl-X<sub>L</sub>,Bcl-w, Mcl-1, and others) all contain three or four characteristic regions of homology(BH1-4; Bcl-2 Homology domains), and proapoptotic proteins. According to their structureand biochemical function, the pro-apoptotic Bcl-2 family members can be divided into two subgroups. Bax, Bak, Bcl-X<sub>S</sub>, and some others, contain two or three BH domains, and a special group of proteins, such as Bad, Bid, Hrk/DP5, Bim, Noxa, Puma, which share with other members of the family onlythe short (9-16 amino acid) BH3 domain [23]. There is accumulating evidence supporting a view that anti-apoptotic members of the Bcl-2 family act as oncogenes [24]. Thus, transgenic overexpression of Bcl-X<sub>L</sub> induced lymphomagenesis, or development of pancreatic B-cell tumors, and overexpression of Mcl-1 resulted in B-cell lymphomas. Bcl-w, which is expressed in almost all murine myeloid cell lines analyzed and in a wide range of tissues, is frequently overexpressed in colorectal adenocarcinomas and appears to play a role in the progression from adenoma to adenocarcinoma in the colorectal epithelium. Bcl-w is also expressed in a majority of infiltrative gastric adenocarcinomas.

Permeabilization of the OMM was shown to require the oligomeric form of Bax or Bak (Fig. 1A). This process involves the truncated form of the BH3-only pro-apoptotic protein Bid (*t*Bid), which is most lycleaved by caspase-8. Cells deficient in both Bax and Bak, but not cells lacking only one of these proteins, demonstrate resistance to a variety of apoptotic stimuli acting through the mitochondrial pathway [25]. Anti-apoptotic proteins, e.g., Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, and Bcl-w, can bind to proapoptotic proteins, Bax or Bak, and prevents their oligomerization (Fig. 1B). The balance between pro- and antiapoptotic proteins in the OMM is a critical factor, which determines cell fate. In many tumors a disproportion between anti-apoptotic and pro-apoptotic mediators in favor of the formers suppresses mitochondrial pathway in apoptosis [26].

Another pathway, which can be engaged in both necrotic and apoptotic cell death, involves the induction of mitochondrial permeability transition (MPT) due to the opening of a non-specific pore in the IMM. This phenomenon was described some thirty years ago by Haworth and Hunter. They showed that  $Ca^{2+}$  uptake by mitochondria stimulates drastic changes in mitochondrial morphology and functional activity [27]. Pore opening is followed by osmotic swelling of the mitochondria, drop of the mitochondrial membrane potential, and the rupture of the OMM, causing the release of intermembrane space proteins, including cytochrome *c*. MPT induction is a  $Ca^{2+}$  dependent process and can be facilitated by a number of factors, such as inorganic phosphate, oxidation of pyridine nucleotides, ATP depletion, low pH, and ROS [28]. According to the traditional view on MPT pore machinery, it is a multimeric complex composed of voltage dependent anion channel (VDAC) located in the OMM, adenine nucleotide translocase (ANT), an integral protein of the IMM, and a matrix protein, cyclophilin D (CyPD) (Fig. **1C**). VDAC and ANT form contact sites between OMM and IMM. In addition, other proteins may bind to the pore

complex, in particular, kinases (e.g. hexokinase, creatine kinase) [29].

MPT pore plays a decisive role in various types of neurodegeneration. Axonal degeneration triggered by toxic insults or distinct mechanical was shown to be dependent on MPT stimulation. Targeting of cyclophilin D both pharmacologically and genetically, protected severed axons and vincristine-treated neurons from axonal degeneration in *ex vivo* and *in vitro* mouse and rat model systems [30]. It has been also shown that interaction of CyPD with mitochondrial amyloid-beta protein potentiates mitochondrial, neuronal and synaptic stress [31]. CyPD-deficient cortical mitochondria were resistant to amyloid-beta protein- and Ca<sup>2+</sup>-induced MPT and subsequent swelling. In addition, CyPD deficiency markedly improves learning and memory and synaptic function in an Alzheimer's disease mouse model. Thus, blockade of CyPD may be of benefit for Alzheimer disease treatment.

#### ROS FACILITATE OMM PERMEABILIZATION.

Mitochondria consume over 90% of the cellular oxygen and are considered the major sites of aerobic cellular ROS production [32]. Approximately 2% of the molecular oxygen consumed during respiration is converted into the superoxide anion radical, the precursor of most ROS. Normally, a four-electron reduction of O2, resulting in the production of two molecules of water, is catalyzed by complex IV of the mitochondrial respiratory chain. However, the electron transport chain contains several redox centers (e.g., in complex I and III) that can leak electrons to molecular oxygen, serving as the primary source of superoxide production. The oneelectron reduction of oxygen is thermodynamically favorable for most mitochondrial oxidoreductases. Superoxide-producing sites and enzymes were analyzed in detail in a comprehensive review [33]. ROS, if not detoxified, oxidize cellular proteins, lipids, and nucleic acids and, by doing so, cause cell dysfunction or death. A harmful ROS activity is controlled by a cascade of water and lipid soluble antioxidants and antioxidant enzymes. Oxidative stress, an imbalance that favors the production of ROS over antioxidant defenses, is implicated in a wide variety of pathologies, including malignant and neurodegenerative diseases. It should be mentioned that mitochondria themselves are sensitive target for the damaging effects of oxygen radicals. ROS produced by mitochondria can oxidize proteins and induce lipid peroxidation, compromising the barrier properties of biological membranes. One of the targets of ROS is mitochondrial DNA (mtDNA), which is especially susceptible to attack by ROS, owing to its close proximity to the electron transporting chain, the major locus for free-radical production, and the lack of protective histones. MtDNA encodes several proteins essential for the function of the mitochondrial respiratory chain and, hence, for ATP synthesis by oxidative phosphorylation. mtDNA, therefore, represents a crucial cellular target for oxidative damage, which might lead to lethal cell injury through the loss of electron transporting chain activity and ATP generation. For example, the level of oxidatively modified bases in mtDNA is 10- to 20-fold higher than that in nuclear DNA [34]. Oxidative damage induced by ROS is probably a major source of mitochondrial genomic instability leading to respiratory dysfunction.

Stimulation of ROS production contributes to both MPT- and Bax/Bak-dependent release of cytochrome c (Fig. **1D**). Oxidative stress can modify two thiol groups on the ANT and thus stimulate pore opening [35]. Oxidation of mitochondrial pyridine nucleotides by a variety of treatments also increased the sensitivity of MTP

opening to  $Ca^{2+}$  under conditions where glutathione was maintained in the reduced state [36]. Thus, mitochondria through excessive ROS generation and self-directed induction of MPT can regulate the release of proteins involved in apoptosis induction.

Since the bulk of cytochrome c is bound to the IMM, it appears that the electrostatic and hydrophobic interactions between cardiolipin and cytochrome c must be "breached" in order for cytochrome c to leave the mitochondria. Apparently, simple permeabilization of the OMM by oligomeric Bax in a low-ionic strength medium is insufficient for cytochrome c release from mitochondria [37]. It was early found that oxidation of cardiolipin decreases its binding affinity for cytochrome c and, more recently, that oxidative modification of cardiolipin facilitates cytochrome c mobilization from the IMM. Based on these results we hypothesized that cytochrome c release during apoptosis occurs by a two-step process, involving first the detachment of the hemoprotein from the IMM, followed by permeabilization of the OMM and the release of cytochrome c into the extramitochondrial milieu [37] (Fig. 1D). These findings indicate that cardiolipin plays an important role not only in mitochondrial energy metabolism, but also in the retention of cytochrome c within the intermembrane space.

Vogelstein's group reported that oxidative degradation of mitochondrial cardiolipin occurred during p53- mediated apoptosis [38]. Furthermore, a model of glutamate toxicity in neurons demonstrated that cytochrome c is released from mitochondria in a ROSdependent, and a burst of ROS in growth factor-deprived neurons was found to cause a profound loss of cardiolipin and mitochondrial damage [39]. Dependence of cytochrome c release mechanisms on ROS might provide a reasonable explanation for the anti-apoptotic effects reported for multiple mitochondrial antioxidant enzymes [40].

#### IMPORTANCE OF ROS IN CANCER

ROS are tumorigenic by their ability to stimulate cell proliferation and survival as well as cellular migration. They can also induce DNA damage, leading to genetic lesions that initiate tumorigenicity and sustain subsequent tumor progression [41]. A variety of cancer cells are characterized by elevated ROS levels. What are then the mechanisms of ROS formation in tumors and how are ROS involved in tumor physiology? The hypoxic environment created by rapidly proliferating tumor cells facilitates ROS production. Cellular hypoxia and re-oxygenation are two essential elements of ischemia-reperfusion injury. Massive production of ROS is normally observed during re-oxygenation of hypoxic tissue. However, ROS levels may also be increased by hypoxia, when electron transport complexes are in the reduced state [42]. Therefore, under hypoxic conditions and, especially after normalization of oxygen supply, production of ROS in tumor cells can be enhanced to an extent that might induce damage to vital cellular components, including mitochondrial DNA. This might trigger a vicious cycle: hypoxia -ROS production - mtDNA mutations - malfunction of the mitochondrial respiratory chain - further stimulation of ROS production, etc.

Although formation of ROS in cancer cells has been documented in numerous publications, there is still some uncertainty about the extent of this process in tumors *in situ*. A majority of malignant cells do indeed produce significant amounts of ROS when cultured under conditions, which can be regarded as hyperoxic (about 159 mmHg) [43]. The absence of antioxidants and the presence of iron in some media can contribute to excessive rates of ROS formation. On the other hand, elevated levels of ROS in tumors can also be a consequence of suppressed activity of antioxidant enzymes. Thus, the level of mitochondrial MnSOD, catalyzing the conversion of superoxide to hydrogen peroxide, seems to be lowered in certain cancer cells, and stimulated expression of MnSOD appears to suppress malignant phenotypes in some experimental models [44]. In some tumors, alterations of glutathione



#### Fig. (1). Main pathways of OMM permeabilization.

A – Formation of pores by Bax (Bak) mediated by truncated Bid (*t*Bid). B – Induction of mitochondrial permeability transition (MPT), followed by the entry of water and solutes into mitochondrial matrix and organelle swelling. C - Reactive oxygen species facilitate MPT induction, detachment of cardiolipin form the inner mitochondrial membrane, and activation of Bax. D – Antiapoptotic Bcl-2 family proteins bind to Bax (Bak) and prevent their interaction with *t*Bid.

S-transferases, have been reported [45], confirming an association between cancer incidence and various disorders of GSH-related enzyme functions. On the other hand, ROS production has been shown to be reversely correlated to the proliferation status of cells and dependent on the mode of ATP supply. Thus, in proliferating thymocytes, producing 86% of their ATP by glycolysis and only 14% by oxidative phosphorylation, PMA-induced ROS production was nearly abolished, in contrast to resting thymocytes, producing ATP mainly by oxidative glucose breakdown (88%). Similarly, no ROS formation was observed in proliferating human promyelocytic HL-60 cells, whereas differentiated, non-proliferating HL-60 cells responded by ROS production upon priming with PMA [46]. The observed reduction of ROS formation in resting thymocytes incubated with pyruvate suggested that pyruvate might function as a H<sub>2</sub>O<sub>2</sub> scavenger and control the level of ROS in tumor cells, when mitochondrial respiration is suppressed. Further, pyruvate has been reported to protect mitochondria from oxidative stress also in neuroblastoma cells [47], and to prevent DNA damage in hepatocellular carcinoma cells exposed to hypoxia and reoxygenation [48].

Stimulation of ROS production as a result of glucose deprivation can cause inhibition of cell proliferation [49]. Moreover, enhanced glycolysis was reported to protect cells from oxidative stress, which might be one possible mechanism of cellular immortalization (reviewed in [50]). Immortalized MEFs suffered less oxidative damage than control cells as estimated by cytoplasmic ROS staining, or quantification of 8-hydroxydeoxyguanosine, a hallmark of oxidative DNA damage. Therefore, extrapolation from cultured cells to tumor tissue *in vivo* must be done with caution. Certainly, tumor cells can respond to hypoxic conditions by production of ROS, but subsequent activation of glucose metabolism might rescue them from ROS-induced cell death and allow them to continue to proliferate. Although hypoxic conditions and oxidative stress can impair mitochondrial function and cause a shift to glycolytic ATP production, mitochondrial activity might be restored, at least partially, under favorable conditions. For example, normalization of oxygen tension through angiogenesis can significantly enhance glucose metabolism and, at the same time, optimize mitochondrial respiration, providing growth advantage and supporting tumor cell survival [51].

It has been shown that the loss of p53 function in normal cells results in an enhanced intracellular level of ROS, which can cause DNA damage, and an increased mutation rate [52]. The inability to activate p53-mediated apoptosis in response to these oncogenic alterations might ultimately result in the development of cancer. However, functional wild type p53 might fulfill both pro- and antioxidant functions. In the absence of stress, or after mild stress, a low level of p53 might trigger the transcription of several antioxidant genes, which can inhibit ROS accumulation and protect cells from DNA damage [52]. On the other hand, strong activation of p53 by severe stress leads to the induction of pro-oxidant genes and results in an elevated ROS level and cell death. Although the precise mechanisms by which the p53-dependent modulation of the ROS level occurs remain unclear, both types of responses might contribute to tumor suppression.

Apoptosis can be triggered by a variety of signals and pathophysiological conditions, including oxidative stress [53]. Many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism [54]. The anti-cancer effect of a number of conventional treatments (radiation, etoposide, arsenates vinblastine, cisplatin, mitomycin C, doxorubicin, camptothecin, inostamycin, neocarzinostatin and several others,) is based on their ability to stimulate ROS production. Normally, antioxidant defense systems can cope with increased ROS production, but it is well known that several antioxidant enzymes, including catalase and SOD, are down-regulated in most solid tumors [55].

Thus, modulation of the cellular redox balance *via* pharmacological stimulation of intracellular ROS production and/or depletion of protective reducing metabolites (such as glutathione and NADPH) may lead to oxidative stress and induction of apoptosis [56]. Under such circumstances, a shift in the balance towards ROS formation can be expected to destabilize mitochondria and facilitate permeabilization of the OMM with release of cytochrome c and other intermembrane space proteins involved in apoptotic cell death.

# TARGETING CELLULAR ENERGY PRODUCING PATH-WAYS —A TOOL FOR TUMOR CELL ELIMINATION

#### 1. Inhibition of Glycolysis in Tumor Cells

Traditionally apoptosis and cancer are regarded as antagonistic processes in cell physiology. Apoptosis may be involved in spontaneous regression of tumors, whereas defects in apoptosis programs may contribute to tumor progression and resistance to treatment. As it has been mentioned above, most tumor cells are characterized by predominant glycolytic production of ATP. An important consequence of the glycolytic shift in tumor cells is their acquired resistance to apoptotic cell death and, hence, resistance to treatment. Multiple observations support the opinion that the glycolytic shift makes tumor mitochondria less susceptible to OMM permeabilization and, hence, more resistant to activation of the mitochondrial pathway of apoptosis.

The dependence of tumor cells on glycolysis for ATP generation offers a rationale for therapeutic strategies aimed at selective inhibition of the glycolytic pathway. Thus glucose deprivation promoted death receptor-mediated apoptosis in human tumors [57]. Administration of 2-deoxyglucose (Fig. 2A), a non-metabolizable glucose analogue, to human histiocytic lymphoma cells had an effect similar to that of reduction of the extracellular glucose concentration and enhanced apoptosis caused by TNF, whereas low concentrations of 2-deoxyglucose alone showed minimal cytotoxicity [58]. The suppression of the intracellular ATP level by 2deoxyglucose, alone or in combination with glucose-free medium, potentiated Fas-induced phosphatidylserine exposure, suggesting that intracellular ATP can influence the externalization of PS during apoptosis [59]. Experiments performed with nude mice xenograft models of human osteosarcoma and non-small lung cancer cells showed that combination treatment with 2-deoxyglucose and conventional drugs - adriamycin or paclitaxel resulted in a significant reduction in tumor growth compared with either agent [60]. 2-Deoxyglucose triggeredfour types of response in human malignant cells of various origins (ovarian, squamous, cerebral, hepatic, colonic and mesothelial): suppression of proliferation; proliferation arrest without signs of apoptosis; strong cell cycle arrest accompanied by moderate apoptosis induction; massive apoptosis [61].

Similar to the effects of inhibiting key steps in the glycolytic pathway, suppression of glucose uptake might also sensitize tumor cells to anticancer agents. Thus, phloretin (Fig. **2A**), a glucose transporter inhibitor, markedly enhanced effects of daunorubicin, a conventionally used anticancer drug [62]. Overall, these results provide a rationale for clinical trials using glycolytic inhibitors in combination with chemotherapeutic agents to increase their therapeutic effectiveness. Surprisingly, 2-deoxyglucose inhibited not only glycolytic pathway, but also endothelial cell angiogenesis *in vitro* and *in vivo*, at concentrations below those affecting tumor cells directly [63]. These new findings highlight the importance of glucose metabolism on neovascularization, and demonstrate a novel approach for anti-angiogenic strategies.

Presently, several glycolytic inhibitors are in preclinical and clinical development. Thus, cardiac drug digitoxin was shown to inhibit the growth and induce apoptosis in cancer cells at concentrations commonly found in the plasma of cardiac patients treated with this drug [64]. Digitalis (mainly digitoxin and digoxin used clinically, extracts from *Digitalis purpurea* and *Digitalis lanata*, respectively) has been used as cardiac drug for more than 200 years [65]. The main known pharmacological effect of therapeutic doses of

digoxin and digitoxin is Na+/K+ ATPase inhibition. Digitalis in non-toxic concentrations has been shown to induce apoptosis in different malignant cell lines. Apparently, the apoptosis-inducing capability is explained by mechanisms other than just Na<sup>+</sup>/K<sup>+</sup> AT-Paseinhibition. Digitalis was shown to stimulate Ca<sup>2+</sup> entry into the cell across the plasma membrane, which might be responsible for apoptotic changes, reviewed in [65]. First generation of glycosidebased anticancer drugs is currently in clinical trials.

A synthetic brominated derivative of pyruvic acid, 3bromopyruvate (3-BrPA) (Fig. 2A), has been found to selectively kill hepatocellular carcinoma cells in vitro, leaving normal hepatocytes unharmed. Moreover, systemic delivery of 3-BrPA suppressed "metastatic" lung tumors with no apparent harm to other organs or to the animals [66]. Analysis of potential cellular targets for 3-BrPA revealed that this compound markedly (by 80%) suppressed the glycolytic capacity of tumor, abolished the activity of mitochondrially-bound hexokinase, and, in addition, totally inhibited respiration of isolated mitochondria. A dramatic decrease in the level of ATP was regarded as the main cause of cell death (reviewed in [67]), however, recently, it has been demonstrated that 3-BrPA can stimulate the production of ROS and cause mitochondrial deregulation [68]. It should be also mentioned, that one of the possible consequences of ATP depletion in tumor cells could be a rapid dephosphorylation of the pro-apoptotic protein Bad, migration of Bax to the mitochondria, permeabilization of the OMM and subsequent massive cell death [69].

Inhibition of glycolysis might be most useful in cells with mitochondrial defects, or under hypoxic conditions when glycolysis is the predominant source of ATP and the mitochondrial contribution to cellular bioenergetics is minimal. Under such circumstances inhibition of glycolysis would severely deplete ATP [70]. However, in cancer cells with functionally competent mitochondria, this approach might be insufficient, because mitochondrial ATP synthesis would compensate for the inhibited glycolysis, and the ultimate fate of the cancer cell will be dependent on how efficiently mitochondrial oxidative phosphorylation can substitute for the inhibited glycolysis in providing the ATP needed for cellular demands. Therefore, under these circumstances, supplementary suppression of mitochondrial activity might be needed to kill cancer cells. Indeed, non-toxic doses of apoptolidin, an inhibitor of mitochondrial ATP synthase, were found to trigger cell death in malignant cell lines when applied together with the lactate dehydrogenase (LDH) inhibitor oxamate [71]. Similar results were obtained when 2deoxyglucose was used instead of oxamate to inhibit glycolysis. Thus, combined strategies involving manipulation of both the glycolytic and the mitochondrial pathways might be useful tools in the elimination of cancer cells that would otherwise survive due to mitochondrial ATP production.

#### 2. Stimulation of Mitochondrial Activity in Tumors

Increasing body of evidence shows that mitochondria could be a promising targets in tumor cells eradication [72]. Considering mitochondria a possible target, two different therapeutic approaches can be suggested [73]: a) to stimulate mitochondrial activity, in order to restore metabolic pathways characteristic for nonmalignant cells, and b) to stimulate mitochondrial deterioration causing OMM permeabilization and stimulation of mitochondriadependent cell death pathways.

Recent observations suggest that, in some experimental systems, stimulation rather than suppression of mitochondrial activity might be used as a tool against tumor growth. The fate of the end product of glycolysis pyruvate is controlled by the relative activities of two enzymes, pyruvate dehydrogenase (PDH) and LDH. In cancer cells, PDH activity is controlled by PDH kinase (PDK1)mediated phosphorylation. Upregulation of PDK1 suppresses activity of PDH and more pyruvate is converted into lactate instead of being oxidized in mitochondria. Several attempts have been made to redirect pyruvate towards oxidation in the mitochondria. Thus, inhibition of PDK1 by dichloroacetate might stimulate the activity of PDH and, hence, direct pyruvate to the mitochondria (Fig. **2B**). A similar effect can be achieved by inhibition of LDH by oxamate. Overall, suppression of PDK1 and/or LDH activities will stimulate mitochondrial activity and might be lethal to tumor cells, even if these inhibitors are used at non-toxic doses. As a result, such treatment decreased mitochondrial membrane potential and increased mitochondrial production of ROS in cancer cells, but not in normal cells [74].

Similarly, overexpression of frataxin, a protein associated with Friedreich ataxia, stimulated oxidative metabolism and elevated mitochondrial membrane potential and ATP content in several colon cancer cell lines [75]. Friedreich ataxia is an inherited neurodegenerative disorder caused by the reduced expression of frataxin. It leads to impaired ATP synthesis and caused premature death due to cardiac failure. Frataxin was shown to promote mitochondrial oxidative metabolism, most probably by stimulation of the synthesis of Fe/S clusters in the mitochondria. In addition to stimulation of mitochondrial activity, frataxin inhibited colony formation and markedly suppressed tumor formation when injected into nude mice. These results are in accordance with those described above and demonstrate that stimulation of mitochondrial metabolism in cancer cells can actually suppress tumor growth.

#### 3. Neutralization of Anti-apoptotic Bcl-2 Family Proteins

Pro-apoptotic proteins  $Bcl-X_L$  and Bcl-2 are overexpressed in many cancers and contribute to tumor initiation, progression and resistance to therapy. Localization of these proteins in the OMM stabilizes the membrane and prevents the release of cytochrome *c*  and other pro-apoptotic proteins upon treatment with anticancer agents. Synthetic peptides corresponding to the BH3 domain of Bak can bind to Bcl-X<sub>L</sub> and antagonize its anti-apoptotic function. This caused a rapid apoptosis induction when delivered into intact cells *via* fusion to the Antennapedia homeoprotein internalization domain. Treatment of HeLa cells with the Antennapedia-BH3 fusion peptide resulted in peptide internalization and induction of apoptosis within 2-3 h [76]. A point mutation within the BH3 peptide that blocks its ability to bind to Bcl-X<sub>L</sub> abolished its apoptotic activity, suggesting that interaction of the BH3 peptide with Bcl-2-related death suppressors, such as Bcl-xL, may be critical for its activity in cells.

Recently, ABT-737, a small molecule was synthesized, which can bind to the anti-apoptotic proteins Bcl-2, Bcl-X<sub>L</sub> and Bcl-w, and disrupts their interaction with antiapoptotic Bcl-2 family proteins. This causes the release of Bax from the complex with Bcl-2 or Bcl-X<sub>L</sub> with subsequent formation of pores in OMM and cytochrome c release (Fig. 1E). Mechanistic studies revealed that ABT-737 can enhance the effects of death signals and displays synergistic cytotoxicity with chemotherapeutic drugs and radiation [77]. ABT-737 was found to facilitate TRAIL-induced cell killing by releasing Bim and Bak from their binding sites and to enhance Bax conformational changes induced by TRAIL in human pancreatic cancer cells [78]. Further, ABT-737 stimulated the activity of various anticancer drugs, such as, vincristine, L-ASP, and dexamethasone in lymphoblastic leukemia in vitro and in vivo [79]. In addition, ABT-737 was shown to induce apoptosis in chronic myeloid leukemia cells with diverse drug-resistance mechanisms [80], lymphomas, small-cell lung carcinoma lines, as well as in primary patient-derived cells and animal models [81]. ABT-737 was also



#### Fig. (2). Inhibition of glycolysis and stimulation of mitochondria activity as a tool for cell sensitization to death.

Suppression of glucose transport by phloretin, using non-metabolizable analog of glucose – 2-deoxyglucose, inhibition of hexokinase by 3-bromopyruvate, inhibits glycolysis and decreases the level of ATP in the cell ( $\mathbf{A}$ ). This sensitizes cells to conventional anticancer drugs. The fate of pyruvate is controlled by the relative activities of two enzymes, pyruvate dehydrogenase (PDH) and lactate dehydrogenase (LDH). In cancer cells, PDH activity is suppressed by PDH kinase (PDK1)-mediated phosphorylation, and, therefore pyruvate is mostly converted into lactate. Inhibition of PDK1 by dichloroacetate (DCA) can stimulate the activity of PDH and, hence, direct pyruvate to the mitochondria ( $\mathbf{B}$ ). A similar effect can be achieved by inhibition of LDH by oxamate. Overall, suppression of PDK1 and LDH activities will stimulate mitochondrial ATP production and might be lethal to tumor cells, even if these inhibitors are used at non-toxic doses.

shown to improve survival and initiate regression of established tumors in a high percentage of tumor-bearing mice [77].

Recently it has been demonstrated that in addition to apoptosis induction, ABT-737 can induce cancer cell senescence [82]. Several solid tumor cell lines did not undergo apoptosis when incubated with this agent. ABT-737-induced cellular senescence was shown to be p53-dependent. Induction of senescence occurred as a result of ROS elevation followed by low-level activation of the caspase cascade, insufficient to induce apoptosis, but sufficient to lead to minor DNA damage and increases in p53, p21, IL-6 and 8 proteins. In addition it has been demonstrated that ABT-737 not only disrupts pro- and anti-apoptotic Bcl-2 protein interaction, but also induces IMM permeabilization, resulting in mitochondrial matrix swelling and rupture of the OMM, thereby permitting a rapid efflux of cytochrome c from the mitochondrial intermembrane space [81]. This observation can be viewed as an additional support for a cooperation between Bcl-2 family proteins and MPT induction [83]. ABT-737 and a related derivative ABT-263 bind with high affinity to Bcl-2, Bcl-X<sub>L</sub> and Bcl-w, and both promise to be useful tools for mechanistic studies. ABT-263 is in early clinical trials in lymphomas, small-cell lung cancer and chronic lymphocytic leukemia [84]. Another BH3-mimetic, 072RB, is localized to mitochondria and can cause cell death in various cultured leukemic cells, as well as in cells derived from acute myeloid leukemia patients [85]. Intravenous administration of 072RB to xenografts of human acute myeloid leukemiacells in NOD/SCID mice significantly delayed leukemic cell growth with no evidence of toxicity to normal tissue. Thus, BH3 mimetics can be used as anticancer agents, both in mono- and combinatorial therapy.

Similar beneficial effects were achieved using non-peptide inhibitors of Bcl-2. HA14-1 (ethyl 2-amino-6-bromo-4-(1-cyano-2ethoxy-2-oxoethyl)-4H-chromene-3- carboxylate) was apparently the first small molecular Bcl-2 inhibitor shown to induce apoptosis in several tumor cell lines [86]. An inhibitor of mitochondrial complex III, antimycin A and a BH3 peptide were shown to bind competitively to recombinant Bcl-2 and Bcl-X<sub>L</sub> [87]. Antimycin A and BH3 peptide both induced mitochondrial swelling and loss of the mitochondrial membrane potential upon addition to mitochondria from Bcl-X<sub>I</sub> expressing cells. Interestingly, the 2-methoxy derivative of antimycin A, which does not block mitochondrial respiration, still retained toxicity for Bcl-X<sub>L</sub> overexpressing cells and mitochondria isolated from these cells. Furthermore, antimycin A prevented the pore-forming activity of Bcl-X<sub>L</sub> in liposomes, demonstrating that a small non-peptide ligand can directly inhibit the function of Bcl-2-related proteins [87].

ABT-737 and similar agents compete with proapoptotic members of the Bcl-2 protein family for binding in the hydrophobic groove formed by the BH1-BH3 domains of Bcl-2. It has been shown that another region of Bcl-2, BH4 domain mediates interaction of Bcl-2 with the inositol 1,4,5-trisphosphate (IP3) receptor, an IP3-gated Ca<sup>2+</sup> channel on the endoplasmic reticulum. BH4 peptide binds to the regulatory and coupling domain of the IP3 receptor and inhibits IP3-dependent channel opening, release of Ca<sup>2+</sup> from the endoplasmic reticulum, and Ca<sup>2+</sup>-mediated apoptosis [88]. A novel synthetic peptide has been reported recently, which binds to the BH4 domain of Bcl-2 and disrupts the Bcl-2-IP3 receptor interaction [89]. The peptide induced release of  $Ca^{2+}$  from endoplasmic reticulum followed by induction of apoptosis in chronic lymphocytic leukemia cells, but not in normal peripheral blood lymphocytes, suggesting a new target for therapeutic intervention. Another pan-Bcl-2 inhibitor, GX15-070, was shown to promote the release of cytochrome c from isolated leukemia cell mitochondria, and induced apoptosis in B cells from nine of the eleven chronic lymphocytic leukemia samples studied [90].

Despite encouraging results of *in vitro* experiments with compounds targeting Bcl-2 family proteins, effects of these compounds *in vivo* needs to be further investigated.

## 4. Induction of MPT

As mentioned above, overexpression of anti-apoptotic Bcl-2 family proteins in tumors might inhibit OMM permeabilization by Bax/Bak-mediated pore formation. If so, induction of MPT, as a more severe destabilizing agent, should overcome the resistance of OMM to permeabilization due to overexpression of antiapoptotic Bcl-2 family proteins.

MPT was originally believed to be the predominant mechanism underlying cytochrome c release in response to different apoptosis inducing stimuli [91, 92]. Later, this notion was challenged by the discovery of the pro-apoptotic Bcl-2 family proteins, which are now regarded as physiologically more significant. MPT can occur under normal physiological conditions, especially in mitochondria located in a close proximity to calcium "hot spots," i.e., microdomains in which the local concentration of ionized calcium far exceeds the average concentration measured in the cytosol. [93]. This local Ca<sup>24</sup> concentration might be high enough to induce Ca2+ overload and subsequent pore opening. Several factors, including ceramide metabolites [94] or palmitate [95], were shown to sensitize mitochondria toward MPT. Under such circumstances, the release of Ca<sup>2+</sup> from the endoplasmic reticulum, or the uptake of extracellular  $\mbox{Ca}^{2+}$ and its subsequent accumulation by mitochondria, might be sufficient for MPT induction and initiation of cell death [96].

The effect of various anticancer drugs and treatments (e.g., ionizing radiation, etoposide, and arsenates) is based on their ability to produce ROS - a factor facilitating MPT induction. MPT induced by various anticancer drugs may thus result from ROSmediated modification of components of the MPT pore. For example, at higher doses the chemotherapeutic agent, arsenic trioxide, was found to cause oxidative modification of thiol groups in ANT and subsequent release of cytochrome c through MPT induction. However, at clinically achievable concentrations the same drug stimulated cytochrome c release and apoptosis through a Bax/Bakdependent mechanism [97, 98]. Distinct pathways of cytochrome c release were also shown for the DNA-damaging anticancer drug etoposide. This drug was found to stimulate MPT induction and cytochrome c release [99], however at low therapeutic doses it affected mitochondria through activation of caspase-2. Apparently, both pathways might be relevant for its clinical effects. Analysis of ANT isoform expression in several transformed human cell lines demonstrated predominant expression of ANT2 [100], an isoform lacking the apoptotic activity of ANT1 [101]. Transient overexpression of ANT3, or ANT1 [102] or invalidation of anti-apoptotic isoform of ANT2 [103] was shown sensitize cells to apoptosis.

Another component of the MPT pore is VDAC responsible for most of the metabolite fluxes between the cytosol and the mitochondria [104]. Binding of VDAC to hexokinase II, a key glycolytic enzyme that is usually upregulated in tumor cells, facilitates glucose phosphorylation by hexokinase II, at the expense of mitochondrially produced ATP. In addition, hexokinase keeps VDAC in the open state, preventing its closure. Closure of VDAC, for example, upon growth factor withdrawal can induce apoptosis by inhibition of ADP-ATP exchange and a resultant decrease in metabolite fluxes over the mitochondrial membranes, which can cause mitochondrial deterioration and cytochrome c release [105]. Another consequence of hexokinase-VDAC interaction is that it prevents binding of pro-apoptotic proteins to VDAC and thereby the induction of apoptosis [106]. A variety of compounds stimulate cell death via interaction with VDAC. Thus, avicins, pro-apoptotic, antiinflammatory molecules with antioxidant effects both in vitro and in vivo, perturb mitochondrial functions and initiate apoptosis in tumor cells. Biophysical studies using lipid bilayers revealed that avicins target and close VDAC [107]. In contrast, the interaction of hexokinase-I with VDAC might be regarded as a mechanism that protects the mitochondria from MPT induction. Thus, binding of hexokinase-I to VDAC was shown to inhibit cytochrome c release and protect against apoptotic cell death [108]. A recent genetic

study indicated that mitochondrial VDAC is dispensable for MPT induction and apoptotic cell death [109]. It appears that the role of VDAC in permeabilization of OMM during apoptosis requires further study.

The role of CyPD, a putative component of the MPT pore, in apoptotic cell death is still controversial and needs further clarification. Cyclophilins represent a group of peptidyl-prolylcistransisomerases (PPIase) with highly conserved protein sequences, which are important for protein folding [110]. For many years, CyPD was considered critical for the opening of the MPT pore. This view was based on the observation that Cyclosporin A (CsA),a potent inhibitor of some forms of necrosis and mitochondriallymediated apoptosis [110], blocks the opening of the MPT pore at concentrations similar to those needed to inhibit the enzymatic activity of CyPD. Based on these properties CyPD was thought to promote the opening of the MPT pore and thereby to facilitate cell death. Indeed, CyPD overexpression sensitizes the ANT to agents (Ca<sup>2+</sup> and oxidants) that transform it into the MPT pore. CyPD was shown to activate the formation of permeability transition pores from purified ANT in black lipid membranes [111]. Overexpression of CyPD was found to promote MPT pore formation in both stressed and unstressed B50 cells, as well as in isolated mitochondria [112]. In addition, it has been reported that CypD-deficient mitochondria do not undergo MPT. However, CypD-deficient cells died normally in response to various apoptotic stimuli, but showed resistance to necrotic cell death induced by ROS and Ca<sup>2+</sup> overload [113]. Surprisingly, overexpression of CyPD was found to augment the resistance of HEK293 and rat glioma C6 cells to apoptotic stimuli [114]. Protection from apoptosis required PPIase activity, whereas CyPD binding to ANT was not affected by the loss of enzyme activity. Thus, it seems unlikely that the protective effect of CyPD on apoptotic cell death is due to binding of CyPD to ANT. More recently, CyPD was shown to be specifically upregulated in human tumors of the breast, ovary, and uterus [115]. The authors suggested that CyPD is a new type of apoptosis inhibitor, which is effective at a functional level, different from that of the previously known inhibitors of the Bcl-2 family.

Suppression of apoptosis by CyPD was shown to be dependent on the amount of hexokinase II bound to mitochondria [116]. Mitochondrial binding of hexokinase II was essential for apoptosis suppression by CyPD, since its anti-apoptotic effect was lost upon detachment of hexokinase II from the mitochondria. Thus, inactivation of endogenous CyPD by small interference RNA, or by a CyPD inhibitor, caused detachment of hexokinase II from the mitochondria and stimulation of Bax-mediated apoptosis. Furthermore, CypD dysfunction appears to abrogate hexokinase II-mediated apoptosis suppression. Recent studies confirmed that the antiapoptotic effect of CypD is obviously MPT-independent, but requires the interaction with some key apoptosis regulator, such as Bcl-2 [117]. The authors suggest that although Bcl-2 resides in the OMM and is, therefore, separated from CypD, which is located in the matrix, the presence of Bcl-2 at mitochondrial contact sites might make such interaction possible, considering that MPT complexes, including CypD, are also localized to the mitochondrial contact sites. Based on their data, the authors propose that targeting CypD to disrupt its interaction with Bcl-2 might increase the sensitivity of cells to apoptosis. Apparently, overexpression of CyPD is important for tumor cell protection against apoptosis, although the molecular mechanism of this protection remains elusive.

Recently it has been demonstrated that the molecular chaperone heat shock protein 60 (Hsp60) can be directly associated with CypD. Remarkably, this interaction occurs in a multichaperone complex which consists of Hsp60, Hsp90, and tumor necrosis factor receptor-associated protein-1. Formation of such complex is observed in tumor but not in normal mitochondria. Silencing of Hsp60 by siRNA caused CypD-dependent MPT and caspase-dependent apoptosis. Apparently, Hsp60 can be regarded as a novel regulator of MPT, antagonizing CypD-dependent cell death in tumors [118].

To avoid unwanted interaction of CsA with non-mitochondrial cyclophilins, Crompton and colleagues have targeted CsA to mitochondria and made it selective for CyPD by conjugating the inhibitor to the triphenylphosphonium, a lipophilic cation, which can be accumulated in mitochondria electrophoretically driven by the mitochondrial membrane potential. Such targeting to mitochondria markedly enhanced the capacity of CsA to prevent cell necrosis brought about by oxygen and glucose deprivation. Interestingly, targeting to mitochondria prominently abolished the capacity of CsA to prevent glutamate-induced cell death. The authors concluded that cytoprotection against glutamate excitotoxicity primarily reflects CsA interaction with extramitochondrial CyPs and calcineurin, whereas mitochondrial CyPD is mainly responsible for cell damage caused by energy failure [119].

The importance of mitochondria as targets for anti-cancer treatment has also been documented in experiments with all-trans retinoic acid (ATRA), a natural derivative of vitamin A, which is used successfully in the treatment of acute promyelocytic leukemia [120]. ATRA perturbed mitochondrial functions in the myeloid cell line P39 long before any signs of apoptosis were detected. It suppressed mitochondrial respiration, decreased the mitochondrial membrane potential and triggered opening of MPT pores. ATRA-induced mitochondrial dysfunction and activation of caspases were abolished by nifedipine, a calcium channel blocker, indicating the involvement of Ca<sup>2+</sup> in mitochondrial deterioration.

Cell death induced by inhibitors of the mitochondrial respiratory chain is often mediated by ROS. Some of naturally occurring isothiocyanates such as phenethylisothiocyanate (PEITC), benzyl isothiocyanate (BITC) and sulforaphane are effective inhibitors of cancer induction in rodents treated with carcinogens [121]. Oral administration of PEITC significantly retarded growth of PC-3 xenografts in athymic mice [122]. The PEITC-induced cell death in PC-3 cells was associated with generation of ROS followed by disruption of the mitochondrial membrane potential and release of cytochrome c and Smac/DIABLO from mitochondria. These changes were successfully blocked by mimetics of superoxide dismutase and catalase. The apoptotic effect of PEITC correlated with inhibition of complex III activity, suppression of oxidative phosphorylation, and ATP depletion (Fig. 3). The Rho-0 variants of PC-3 cells were more resistant to PEITC-mediated ROS generation, apoptotic DNA fragmentation, and collapse of mitochondrial membrane potential compared with respective wild-type cells [123]. PEITC-induced apoptosis was found to be dependent on p66Shc,a lifespan-regulating protein contributing to mitochondrial ROS metabolism and regulating the mitochondrial apoptosis pathway. p66Shc responds to a variety of proapoptotic stimuli by increasing ROS levels in the mitochondrial intermembrane space through an inherent ROS-producing activity. This ROS formation might trigger initiation of the mitochondrial apoptosis pathway [124]. Mouse embryonic fibroblasts derived from p66(Shc) knockout mice were significantly more resistant to PEITC-mediated growth inhibition, cytoplasmic histone-associated apoptotic DNA fragmentation, and caspase-3 activation compared with wild-type fibroblasts. Treatment of PC-3 cells with PEITC resulted in translocation of p66(Shc) to the mitochondria [125], where it generated hydrogen peroxide using reducing equivalents of the mitochondrial electron transfer chain through the oxidation of cytochrome c [126].

Another isothiocyanate, benzyl isothiocyanate, was also shown to target the mitochondrial respiratory chain to trigger ROSdependent apoptosis in human breast cancer cells [127]. ROS production and apoptosis were inhibited by overexpression of catalase and Cu, Zn-superoxide dismutase, as well as by the inhibition of the mitochondrial respiratory chain. Similarly, cells lacking mitochondrial DNA were resistant to benzyl isothiocyanate-mediated ROS



# Fig. (3). Potential mitochondrial targets that can be exploited for cancer therapy (see details in the text).

ABT-737 and similar compounds disrupt interaction of anti- and proapoptotic proteins, facilitating oligomerization of Bax (Bak) followed by OMM permeabilization. PEITC induces apoptosis *via* inhibition of complex III activity, stimulation of ROS production, suppression of oxidative phosphorylation, and ATP depletion.  $\alpha$ -TOS stimulates ROS production *via* interaction with the coenzyme Q binding site in complex II of the mitochondrial respiratory chain. It induces OMM permeabilization as a result of translocation of Bax and Bak from the cytosol to the mitochondria and alsovia Ca<sup>2+</sup> mediated MPT.As a result of OMM permeabilization, cytochrome *c*triggers the formation of the apoptosome complex in the cytosol and subsequent activation of the caspase cascade, cleavage of cellular proteins, which is primarily responsible for the biochemical and morphological characteristics of apoptosis.

generation and apoptosis. Thus, alteration of the mitochondrial respiratory chain and subsequent ROS formation is a powerful tool in fighting cancer.

Recently a range of compounds named mitocans (an abbreviation formed from MITOchondria and CANcer), were shown to cause cell death via targeting mitochondria [128]. One of these compounds is a derivative of  $\alpha$ -tocopferol-  $\alpha$ -tocopheryl succinate (a -TOS). In 1982, Prasad and Edwards-Prasad reported for the first time that, this redox-silent analogue of vitamin E, induced morphological changes and growth inhibition in mouse melanoma cells [129]. a-TOS was shown to inhibit the proliferation of avian reticuloendotheliosis virus-transformed lymphoblastoid cells in a dosedependent manner, block the cells in the G2/M cell cycle phase, and induce apoptosis [130]. a-TOS was also found to destabilize mitochondria, stimulate the production of ROS and kill malignant cells at concentrations non-toxic to normal cells and tissues [131]. Although α-tocopherol is known as an important chain breaking antioxidant in cells, its derivative,  $\alpha$ -TOS, is apparently unable to act as an antioxidant unless the succinate component is cleaved off. It has been reported that some non-malignant cells have the ability to hydrolyze a-TOS, gradually releasing a-tocopherol to prevent membrane oxidative damage [132], whereas the hydrolysis of  $\alpha$ -TOS in malignant cells is suppressed due to a lower activity of such esterases [131, 133].

It has recently been reported that  $\alpha$ -TOS stimulates ROS production *via* interaction with the coenzyme Q binding site in complex II of the mitochondrial respiratory chain [134] (Fig. 3). Moreover, investigation of xenografts derived from Chinese hamster lung fibroblasts with functional, dysfunctional and reconstituted Complex II revealed that growth of Complex II-functional and Complex II-reconstituted tumors was strongly suppressed by the  $\alpha$ -TOS, and this was accompanied by high level of apoptosis induction in the tumor cells [135]. Although the precise mechanisms of  $\alpha$ -TOS action remain to be elucidated, the results obtained so far makes it an attractive candidate for antitumor therapy.

α-TOS was shown to induce OMM permeabilization as a result of translocation of Bax and Bak from the cytosol to the mitochondria [136]. Recently we found that in addition to Bax/Bak-mediated OMM pemeabilization,  $\alpha$ -TOS causes release of cytochrome c via MPT.  $\alpha$ -TOS triggered Ca<sup>2+</sup> influx into the neuroblastoma cells and its subsequent accumulation in the mitochondria that destabilized mitochondria and facilitated MPT. The mitochondrial Ca<sup>2+</sup> loading was important for apoptosis progression, as inhibition of mitochondrial Ca<sup>2+</sup> uptake by Ru360 significantly mitigated the apoptotic response [137]. Recently a-TOS was generated, which can be targeted to mitochondria (mito-VES). Such targeting should increase its apoptotic activity. This construction of Mito-VES was based on the work from the group of Murphy and Smith, reviewed in [138], who prepared a series of mitochondrially targeted antioxidants by tagging them with the positively charged triphenylphosphonium group (TPP<sup>+</sup>). Using TPP<sup>+</sup> group might be advantageous for the cancer cell since in cancer cell mitochondrial membrane potential is higher than in normal cells [139]

Several reports describe a beneficial effect of  $\alpha$ -TOS treatment *in vivo*. Combination treatment of  $\alpha$ -TOS and paclitaxel showed promising anticancer effects in terms of inhibiting bladder cancer cell growth and viability *in vitro* and *in vivo* [140]. The anticancer effect of  $\alpha$ -TOS was also examined on JHU-022 solid tumor xenograft growth in immunodeficient mice [141]. A prominent antitumor effect was demonstrated by another vitamin E analogue  $\alpha$ -tocopheryl oxyacetic acid ( $\alpha$ -TEA). Dietary  $\alpha$ -TEA delivery significantly inhibited primary tumor growth and dramatically reduced spontaneous metastatic process [142].

It should be mentioned, however, that in murine model of mesothelioma analysis of toxicity of  $\alpha$ -TOS revealed that  $\alpha$ -TOS was not only ineffective at inhibiting established tumor development at the published doses, but resulted in severe side effects characterized by both behavioural changes, intra-peritoneal abnormalities and the destruction of T cells [143]. The authors conclude that the translation of animal studies to clinical treatment with  $\alpha$ -TOS requires careful consideration.

Tagging to lipophilic cations seems to be a promising approach to deliver drugs to mitochondria [144]. Recently new mitochondrially targeted compounds, cationic plastoquinone derivatives (SkQs), containing positively charged phosphonium or rhodamine moieties connected to plastoquinone have been developed. SkQs easily penetrate through planar, mitochondrial, and outer cell membranes, and possess strong antioxidant activity in aqueous solution, BLM, lipid micelles, liposomes, isolated mitochondria, and cells at low (nanomolar) concentrations. In an *in vitro* experiments SkQ1 prevented oxidation of cardiolipin, which is especially sensitive to attack by ROS. In cell cultures, SkQ1 and its analog plastoquinonyldecyl rhodamine (SkQR1) suppressed hydrogen peroxide-induced apoptosis [145]. SkQ1 was shown to prolong lifespan and prevent development of traits of senescence [146], to inhibit tumor development from p53-deficient cells [147], and to be beneficial in treatment of some ROS- and age-related diseases, such as heart arrhythmia, heart infarctions, kidney ischemia, and stroke [148].

### CONCLUDING REMARKS

The primary strategic problem in cancer therapy is how to selectively activate apoptosis in transformed cells. Extensive studies are being performed to find efficient mechanisms of cell death induction. Despite the heterogeneity of tumors, which dictates an individual approach to anticancer treatment, almost all tumor cells demonstrate enhanced uptake and utilization of glucose, a phenomenon known as the Warburg effect. One of the consequences of the upregulation of glycolysis in tumors is stabilization of the mitochondria and increased resistance to OMM permeabilization and apoptotic cell death. Successful elimination of cancer cells is therefore based on the ability of anti-cancer treatment to activate apoptotic pathways, which are suppressed in tumor cells. Targeting mitochondria might be a promising strategy to increase the sensitivity of tumor cells to apoptotic stimuli. A successful outcome of this effort must include modulation of cellular energy metabolism to sensitize tumor cells to otherwise dormant apoptotic mechanisms. Targeting mitochondria is an essential component of this approach and may result in the development of new and more efficient therapeutic strategies aimed at killing cancer cells and suppressing tumor growth.

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#### DISCLOSURE

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