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Mitochondrial Permeability Transition as Target of Anticancer Drugs

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Abstract: Mitochondria are the cell powerhouses but also contain the mechanisms leading to cell death. Many signals converge on mitochondria to cause the permeabilization of mitochondrial membranes by the mitochondrial permeability transition (MPT) induction and the opening of transition pores (PTPs). These events cause loss of ionic homeostasis, matrix swelling, outer membrane rupture leading to pro-apoptotic factors release, and impairment of bioenergetics functions. The molecular mechanism underlying MPT induction is not completely elucidated however, a growing body of evidence supports the concept that pharmacological induction of PTPs in mitochondria of neoplastic cells is an effective and promising strategy for therapeutic approaches against cancer. The first part of this article presented as a review also evidences the main constituents of PTP and several compounds targeting them for inducing the phenomenon. The second part of the article regards the recent experimental development in the field, in particular, the effects of peniocerol (PEN), a sterol isolated from the root of *Myrtillocactus geometrizans*, at cellular and mitochondrial level. PEN exhibits a cytotoxic activity on some human tumor cell lines, whose mechanism is attributable to the oxidation of critical thiols located on adenine nucleotide translocase, the protein mainly involved in PTP. This event in the presence of Ca^{2+} induces the MPT with the release of the pro-apoptotic factors cytochrome c and apoptosis inducing factor. These observations evidence that PEN may trigger both the caspase-dependent and caspase-independent apoptotic pathways. This characteristic renders PEN a very interesting compound that could be developed to obtain more effective antiproliferative agents targeting mitochondria for anticancer therapy.

Keywords: Mitochondria, Permeability transition, Antiproliferative effect, Natural compounds, Peniocerol.

INTRODUCTION

Mitochondria play critical roles in energy transduction, metabolism, apoptosis and intracellular signaling. They have the ability to change their morphology, functions and numbers in response to pathophysiological conditions and stressors such as diet, exercise, temperature, hormones, and drugs. Proper mitochondrial function is crucial for maintenance of metabolic homeostasis and activation of appropriate cell responses. Changes in mitochondrial activity are involved in aging, diabetes, neurodegenerative diseases and cancer. As there is thus an important link between mitochondrial disfunction and human diseases, therapeutic intervention with compounds targeting mitochondria may produce unimaginable results against the above diseases. One of the main bioenergetic functions of mitochondria is to generate ATP by means of oxidative phosphorylation. The electrons deriving from glucose, amino acids or fatty acids which flow along the respiratory chain have their substantial energy used to pump protons from the matrix to the intermembrane space, generating the electrochemical membrane potential ($\Delta\mu_H^+$). The electrons reduce oxygen to form water, and the protons flow down their gradient through ATPsynthase, driving the production of ATP. Reactive oxygen species (ROS) are normal side-products of the respiration process. An increased $\Delta\mu_H^+$ caused by impaired electron flux or overabundant nutrients may in fact result in aberrant electron transport by the respiratory chain, causing oxidative damage due to elevated generation of ROS. Consequently cells have evolved several defense mechanisms against oxidative stress. Mitochondria are the primary site of ROS production within cells, and increased oxidative stress is proposed as one of the causes of mammalian aging and disease.

In recent years, mitochondria have been recognized as regulators of cell death in both apoptosis and necrosis, in addition to their essential role for cell survival. Cellular disfunctions induced by intra- or extra-cellular insults converge on mitochondria and induce a sudden increase in the permeability of the inner mitochondrial membrane, the so-called mitochondrial permeability transition (MPT). This is caused by the opening of permeability transition pores (PTPs) and, together with high levels of Ca^{2+}, ROS are the main agents responsible for the induction of the MPT. The opening of PTPs is followed by loss of ionic homeostasis, matrix swelling, outer membrane rupture, and the loss of proteins from the intermembrane space, the so-called pro-apoptotic factors. These events result in the triggering of the pro-apoptotic pathway mediated by the cascade of the caspases. The activity of these catabolic enzymes together with the bioenergetic collapse and redox catastrophe of mitochondria signaling, finally leads to cell death meaning that mitochondria coordinate the late stage of cellular demise. Thus MPT is the decisive event which defines the frontier between survival and death and the mitochondrial membranes constitute the battleground on which opposing signals combat to seal the fate of the cell. Pathological cell death induced by ischemia-reperfusion, intoxication due to xenobiotics, neurodegenerative diseases or viral infections also depend on the MPT as a critical event. The inhibition of the MPT constitutes an important strategy for the pharmaceutical prevention of unwarranted cell death.

Mitochondria are also involved in multiple aspects of tumorigenesis and tumor progression. Mutations of mitochondrial DNA (mtDNA) which affect components of the respiratory chain result in poor ATP synthesis, ROS overproduction and oxidative damage of macromolecules, including DNA, thereby favoring chromosomal instability and carcinogenesis. Mutations of mtDNA are also correlated with the risk of developing several types of malignancy. Multiple hallmarks of cancer cells, including limitless proliferation, insensitivity to anti-growth signals and impaired apoptosis, are linked to mitochondrial dysfunction. Several authors have analyzed the correlations between mtDNA oxidations and mutations and tumorigenesis. In particular, some reviews summarize the recent progress in the field [1, 2].

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Cancer cell mitochondria are structurally and functionally different from those of normal cells. Tumor cells also show extensive metabolic reprogramming, which renders them more susceptible to mitochondrial dysfunction than normal ones. The correction of cancer-associated mitochondrial perturbations and re-activation of cell death programs by pharmacological agents which can induce or facilitate MPT are also attractive strategies for cancer therapy [3, 4].

In conclusion, the pharmaceutical manipulation of MPT is to be considered as an essential focal point on which efforts for the therapeutic treatment of all the above-mentioned diseases should converge.

MITOCHONDRIAL PERMEABILITY TRANSITION AND PERMEABILITY TRANSITION PORE

A number of pro-apoptotic stimuli, including an increase in Ca^{2+} concentration and ROS generation, can induce a drastic change in mitochondria permeability conditions by promoting a phenomenon known as MPT. The MPT is a sudden increase of the inner mitochondrial membrane (IM) permeability to ions and solutes with molecular mass up to 1.5 kDa in response to noxious stimuli such as increased Ca^{2+} matrix concentration, oxidative stress and cytotoxic agents [5]. The prevailing idea is that this phenomenon is caused by the opening of a regulated protein channel, known as the PTP. The PTP is a highly dynamic supramolecular complex and it is widespread believed that PTP is formed at the contact sites between the inner and outer membranes and that it spans both membranes. Nevertheless, recent experiments on mitoplasts, i.e. in the absence of an intact outer mitochondrial membrane (OM), challenge the above statement because the MPT of mitoplasts retains the fundamental features of MPT in intact mitochondria, suggesting that it represents fundamentally an IM event. Interestingly, in this study for OM an important regulatory role on PTP modulation is strongly claimed [6]. Despite major efforts, the precise structural identity of PTP is still an unsolved riddle, nevertheless, there is a general consensus that its scaffold nature is based on the dynamic interaction between the voltage-dependent anion channel (VDAC) located in the OM, the adenine nucleotide translocase (ANT) in the IM, cyclophilin D (CypD) a soluble protein of the mitochondrial matrix and target of cyclosporin A (CsA) and other proteins, such as hexokinase (HK) in the OM and creatine kinase in the intermembrane space [4, 7-10].

VDAC is the most abundant protein of the OM and it physically interacts with the peripheral benzodiazepine receptor (PBR, also known as translocator protein 18 kDa, or TSPO) in the OM, as well as with several proteins involved in the regulation of energy metabolism such as HK. This glycolytic enzyme binds at OM contact sites with high affinity and interacts with VDAC by influencing its structure and conductance. The mitochondria-HK interaction occurs in healthy cells, thereby inhibiting MPT, and indeed the disruption of such interaction induces apoptosis [4, 11]. Moreover, it was demonstrated that HK-VDAC interaction is critical for preventing the induction of apoptosis in tumor-derived cells. Interestingly, in cells over-expressing HK-I or HK-II, such as cancer cells, the inhibition of the interaction between HK and VDAC1 by synthetic VDAC1-based peptides, provokes detachment of HK from mitochondria and cell death in several cancer cell lines, but not in normal cells [12].

The implication of VDAC in PTP was first proposed nearly 20 years ago on the basis of the similarities between the electrical conductance properties of VDAC with those of PTP [13, 14]. Nevertheless, more recently, the assumption that VDAC is an essential component of PTP becomes matter of debate. In particular, the occurrence of an intact MPT response both in isolated mouse mitochondria and cells essentially deficient for all three isoforms of VDAC, prompts to suggest that VDACs are dispensable for MPT and are not essential part of PTP [15]. Furthermore, it was demonstrated that the apoptosis induced by the dissociation of HK-II from mitochondria requires opening of PTP independently from VDAC [16].

ANT is the most abundant protein of IM in which it is inserted through six transmembrane helices and normally carries out the exchange of ATP and ADP between the matrix and the intermembrane space. In details, it catalyzes the ATP export from the mitochondrial matrix to the intermembrane space and conversely, the import of ADP from the intermembrane space to the matrix [17, 18]. In this connection, the antiport function of ANT plays a central role allowing the coupling of mitochondrial ATP synthesis to cytosolic delivery. Furthermore, ANT, having the ability to import protons across the IM in some circumstances, might mediate a mild uncoupling process defending in this way the cell by the ROS produced by the electron transport chain or under conditions of high oxidative damage [19]. Nevertheless, the discovery in 1996 that ANT also functions as a non-specific pore upon stimulation of a variety of stimuli opens a new scenario where ANT is switched from a vital function to a lethal one, corresponding to its PTP forming activity [8, 19]. The hypothesis that ANT constitutes a component of PTP, is still controversial, put forward from the observation that the opening of PTP was modulated by two specific ANT inhibitors, carboxyatractyloside and bongkrekic acid (BKA), as well as by ADP and ATP [5, 19]. Moreover, isolated and reconstituted ANT could be converted into an unusually large low cation selective channel whose opening requires Ca^{2+}. Furthermore, the channel is sensitive to ANT specific inhibitors and to the substrate ADP [20]. Otherwise, mitochondria isolated from mice knock-out for ANT1/2 showed a permeability transition response in the presence of Ca^{2+} [21].

PBR was first described as a high affinity binding site for diazepam in rat peripheral tissues [22]. This leads to the label "peripheral" benzodiazepine receptor in contrast with the "central" benzodiazepine receptor, which is localized solely to neuronal cells in the central nervous system. Later, regardless some facts, like i) many ligands such as cholesterol and protoporphyrin IX also bind to PBR, ii) not all benzodiazepines bind to it, iii) it is also expressed in glial and ependymal cells that are located in the central nervous system and iv) it is not a typical receptor, a renamed was proposed. In particular, based on insights into its structure and molecular function, TSPO (Translocator protein) was proposed, which regards the minimal functional binding subunit for all PBR ligands [23]. To date, both names are widely accepted by the scientific community. The predominant localization is mitochondrial, specifically in the OM, nevertheless, several studies demonstrated the presence also in other subcellular compartments, such as in the nuclear fraction and plasma membrane in heart, liver, adrenal and testis and on hemopoietic cells [24, 25]. Regarding the function of mitochondrial PBR on PTP, recent studies suggest a dual role for this protein. In particular, PBR can act both as regulatory protein when it binds its selective ligands, and as transport protein by transferring PTP-active compounds to their regulatory sites that can be located in the IM or in the matrix [6, 26].

CyPD is the mitochondrial isoform of cyclophilins family, ubiquitous proteins sharing a peptidyl-prolyl *cis-trans* isomerases activity. CyPD is a nuclear encoded protein endowed with a mitochondrial targeting sequence that is cleaved after import into the mitochondrial matrix, yielding two mature proteins of 17.5 and 18.6 kDa [27]. Mice null for the gene that encodes for CyPD ($Ppfi^{-/-}$) do not display a severe disease phenotype, suggesting that CyPD is dispensable for embryonic development and viability. Moreover, CyPD seems implicated in some diseases because knock-out mice score better with respect to wild-type littermates in mouse model for Alzheimer's disease and muscular dystrophy [28, 29] and the genetic ablation of CyPD or its inhibition by CsA rescues mitochondrial defects and prevents apoptosis in muscle of mice suffering from collagen VI myopathy [30, 31]. Nevertheless, notwith-

standing the clear involvement of CyPD in a variety of pathophysiological processes, its physiological role in mitochondria is not yet completely clarified. CyPD is the unique mitochondrial isoform of cyclophilins in mammals and its identification was prompted by the demonstration that CsA affects mitochondrial Ca^{2+} fluxes, an effect ascribable to the desensitization of PTP [32]. In particular, it has been widely discussed its role in PTP and studies on liver mitochondria from mice with genetic ablation of Ppfi gene demonstrated that CyPD is the unique target for CsA-mediated inhibition of PTP and that PTP can form and open in the absence of CyPD. Subsequent experiments wide the understanding in CyPD role toward PTP highlighting a modulatory activity. Indeed, it has been proposed that CyPD ablation or the treatment with CsA unmasks an inhibitory site for Pi, which could be the actually desensitizing agent [33]. More recently, based on the ability of CyPD to constitutively bind F_0-F_1 ATP synthase with consequent reduction in ATP synthesis and hydrolysis rate, and on the well-established fact that Pi is an inducer of the PTP, it is emerging that ANT may be regulating PTP opening through interaction with the phosphate carrier and perhaps the F_0-F_1 ATP synthase [34].

PRO-APOPTOTIC FACTORS RELEASED FROM MITOCHONDRIA

PTP can exhibit different opening states: a low conductance state that exhibits a very low permeability and a high conductance state in which the protein channel diameter is estimated of about 3 nm and allows the deregulated entry of ions and molecules up to 1.5 kDa into mitochondrial matrix along their electrochemical gradient [7, 26]. PTP opening is highly sensitive to Ca^{2+} and indeed requires the presence of Ca^{2+} in matrix as essential factor, along with an additional factor or inducer. Once PTP opens in high conductance state, the dissipation of the mitochondrial membrane potential occurs along with a massive entry of water in matrix favoring matrix osmotic swelling. Owing to the fact that the surface of IM considerably exceed that of OM, MPT eventually induces local ruptures of this latter leading to mitochondrial outer membrane permeabilization to large molecules [7]. As a consequence of these phenomena, some pro-apoptotic proteins that are normally confined into the mitochondrial intermembrane or intracristae space, such as cytochrome c (cyt c), apoptosis inducing factor (AIF), endonuclease G (EndoG), Smac/DIABLO and HtrA2/Omi are released into cytosol [35]. In this connection the MPT, with the opening of the PTP, is currently considered a point-of-no-return in the apoptotic cascade and as a consequence, an interesting and promising therapeutic target, as its drug-mediated induction can stimulate cell death under many circumstances.

Cyt c was originally identified for its essential role in ATP synthesis from oxidative phosphorylation in mitochondria. Indeed, it accepts a single electron from complex III and carries it to complex IV (cyt c oxidase). The obligate function of cyt c in electron transport is emphasized by the embryonic lethality of cyt c knock-out mice [36]. The essential requirement of cyt c for the activation of caspase cascade came from experiments on HeLa-cells S-100 depleted of cyt c by a monoclonal antibody. In these cells the activation of caspase 3 did not occur. Moreover, coimmunoprecipitation experiments confirmed the essential role of cyt c [37]. Nowadays it is well known that the cytosolic release of cyt c is one of the crucial steps into the caspase-dependent mitochondria-mediated apoptotic pathway. The MPT is mandatory for apoptotic cyt c release and indeed, following the occurrence of MPT, cyt c is released from intermembrane space to cytosol. In the cytosol it binds to dATP thus mediating the allosteric activation and hepta-oligomerization of the Adaptor molecule apoptosis-Protease Activating Factor 1 (APAF-1). This initiates the formation of a complex known as apoptosome that includes cyt c, APAF-1 and ATP/dATP. Upon formation of apoptosome, caspase 9 acquires the ability to activate proteolitically pro-caspase 3 thus triggering the downstream caspase cascade, which leads to chromatin condensation and DNA fragmentation and ultimately to cell death [35, 38]. The requirement for cyt c in the apoptotic process was demonstrated by generating a mouse model expressing a mutant form of cyt c that retained normal respiratory function, but lacked the ability to oligomerize APAF-1. Differently from cyt c null embrios, which die before the formation of most organs, in this "knock-in" model, few animals survived and showed defect in CNS, a neurological phenotype similar to that seen in APAF-1, caspase 9 and caspase 3 null mice and impairment in lymphocyte homeostasis. Interestingly, embryonic fibroblasts from these mice showed normal cyt c release into the cytoplasm upon apoptotic stimuli, nevertheless, neither procaspase 3 nor procaspase 9 activation occurred, rendering the fibroblast resistant to apoptotic stimuli, like staurosporine and UV irradiation [39].

AIF, identified in 1996, is a flavin adenine dinucleotide-containing protein and possesses various NAD(P)-dependent redox activity [40, 41]. It is a 62 kDa inner membrane-anchored protein whose N-terminus is exposed to mitochondrial matrix and C-terminus protrudes into intermembrane space [41, 42]. In this form AIF plays a vital role for the cell for optimal detoxification of ROS and for the assembly or maintenance of the respiratory chain complex I [43]. Accordingly, in HeLa cells the ablation of AIF gene leads to a diminished expression of complex I subunits and the associated 40-50% decrease in complex I activity resulted in a reduction in the rate of oxidative phosphorylation and consequent increase in a dependence on glicolysis [43]. Moreover, sensitivity to oxidative stress is increased in AIF-deficient neurons and cardiomiocytes but, the mechanism by which AIF ameliorates the damaging effects of oxidative stress is not still clarified [44, 45]. Upon an apoptotic insult, AIF undergoes a caspase-independent proteolytic cleavage that yields the truncated form with an apparent molecular weight of 57 kDa. The cleavage is mediated by two families of cysteine proteases: calpains (cytosolic and/or mitochondrial) and cathepsins. Calpains regulated the cleavage of AIF in a Ca^{2+}-dependent manner, while the AIF activation due to cathepsins occurs apart from calcium ion [46]. After initiation of MPT, the activated form of AIF is released from mitochondria to cytosol and translocates to the nucleus where induces chromatin condensation and DNA fragmentation in a caspase-independent manner. To induce these effects, AIF, through the C-terminal proline-rich module, associates with phosphorylated histone H2AX, cyclophilin A (CyPA) [47]. It has been suggested that this multi-protein complex takes place through a sequential interaction mode, because AIF interacts with H2AX even in the absence of CyPA, while the opposite does not occur [47]. Once in the cytoplasm, AIF can interact with cytoplasmic proteins acting as pro-survival or pro-death inducer. B-cell lymphoma 2 (Bcl-2) and heat shock proteins (Hsp) family members have been shown to interfere with AIF-mediated cell death by different means. Bcl-2 acts upstream of the AIF release, while Hsp70 prevents its nuclear import. Direct Hsp70-AIF association was demonstrated by *in vivo* experiments showing that cytoplasmic Hsp70 specifically interacts with exogenous and endogenous AIF and inhibits its relocation into the nucleus [40]. The most important cytoplasmic interaction for the apoptogenic role of AIF occurs with CyPA that also comigrates with AIF to the nucleus and mediates binding of AIF to DNA and nuclear components to promote chromatinolysis [47]. Because AIF does not possess any intrinsic endonuclease activity, it was proposed that the AIF-mediated DNA degradation depends on the recruitment of downstream nucleases or that AIF directly interact with DNA and disrupts or collapses chromatin structure by displacing chromatin-associated proteins [48, 49].

EndoG is a non-specific nuclease that cleaves single and double-stranded DNA and RNA in a Mg^{2+}/Mn^{2+}-dependent manner and is implicated in mitochondrial DNA replication and in apoptosis [50]. It is a nuclear-encoded protein that normally resides within mitochondria and some studies suggest its localization within the

intermembrane space and bound to the IM. The aminoterminal 48 aminoacids sequence of the encoded inactive propeptide targets the protein to mitochondria and is subsequently cleaved off when the protein is imported into the organelles. Upon the induction of MPT, the mature 27kDa EndoG is released from mitochondria and imported to the nucleus where can initiate oligonucleosomal DNA fragmentation [36, 40]. EndoG-induced apoptosis can occur independently from caspase activity but cooperation between AIF and EndoG in DNA degradation has not been demonstrated so far during apoptosis in mammalian cells [40].

Second mitochondrial-derived activator of caspase (Smac) and Direct IAP binding protein with low pI (DIABLO) were initially discovered independently by two groups and now recognized as a unique protein named Smac/DIABLO [51, 52]. Immature Smac/DIABLO is encoded within the nuclear genome and contains an N-terminal 55 amino acids sequence that constitutes a mitochondrial targeting signal peptide. The cleavage occurs after import into the mitochondria to generate the mature 23-kDa protein and is required to expose the IAP-binding domain [52]. Mature Smac/DIABLO resides within the intermembrane space and is released into cytosol upon induction of MPT. It has both monomeric and dimeric forms, this latter is the active form that promotes apoptosis by inhibiting various inhibitor of apoptosis proteins (IAPs) and then allowing the activation of apoptotic proteins. The release of Smac/DIABLO seems to be linked to that of cyt c during apoptotic stimuli. Indeed, it was reported that Smac/DIABLO is not released from the mitochondria of cells null for cyt c in response to staurosporine or etoposide. These results raised the interesting possibility that cyt c is required for the activation of a mechanism crucial for the Smac/DIABLO release or that the lack of cyt c abolishes a signaling pathway essential for the release [53]. The caspase-dependent apoptosis is regulated by a number of factors and between them, the members of the inhibitor of apoptosis protein (IAP) family. IAPs were first identified as baculoviral gene products able to inhibit the apoptotic response of insect cells toward infection, and subsequently identified also in both invertebrates and vertebrates [54-56]. Indeed, due to the presence of IAPs, cyt c alone cannot be sufficient to trigger apoptosis because Smac/DIABLO must bind to IAPs for releasing the active caspase to promote cell death. IAP family members, such as XIAP, c-IAP1, c-IAP2 and surviving, contain typically three baculoviral IAP repeat (BIR) each capable to inhibit caspase independently and to interact with Smac/DIABLO [57]. Monomeric Smac/DIABLO can bind to only one BIR domain, while dimeric form can bind two BIRs simultaneously [58]. In particular, Smac/DIABLO possesses some regions that are structurally related to the regions of caspases that mediate the binding to IAPs. Therefore, this proapoptotic factor can activate apoptosis by competitive binding to IAPs regions devoted to the interaction with caspases, thus removing the inhibitory effect [51].

The members of HtrA (high-temperature requirement) family are evolutionarily conserved proteins and act as oligomeric serine proteases. The human HtrA member, named HtrA2/Omi plays essential roles in mitochondria and contributes to apoptosis [59]. HtrA2/Omi is expressed as a 49 kDa proenzyme that includes a mitochondrial localized sequence at the N-terminal. The precursor protein is anchored into mitochondrial IM and it undergoes proteolytic maturation within the intermembrane space to expose an IAP-binding motif [60]. Mnd2 mice, which are homozygous for a naturally occurring Ser276Cys mutation in the HtrA2/Omi protease domain that greatly reduces the catalytic activity, show a striking Parkinsonian phenotype along with reduced body weight and organs, including heart, thymus and spleen [61]. Recent identification of HtrA2/Omi as a novel Parkinson's disease locus has further supported the role of mitochondrial disfunction in the pathogenesis of this disease [62]. Moreover, the stimulation of mitochondria isolated from mnd2 mouse embryonic fibroblasts revealed an increase in susceptibility to permeabilization in comparison with mitochondria isolated from the WT [61]. In contrast, the activity of the enzyme belonging to the electron transport chain were not reduced in omi-deficient cells [63].

Following mitochondrial membrane permeabilization, HtrA2/Omi is released into the cytoplasm where it contributes to apoptosis through both caspase-dependent and -independent mechanisms. Indeed, HtrA2/Omi competitively binds to the BIR domains of IAPs via the IAP-binding motif, so that the caspases are released and reactivated. HtrA2/Omi cleaves various IAPs *in vitro* and it was demonstrated that the cleavage of c-IAP1 is catalytic and irreversible and reduces its ability to inhibit and polyubiquitylate caspases [64]. In addition to antagonize IAPs, HtrA2/Omi contributes to apoptotic process also through mechanisms independent from caspases. In particular, the observation that HeLa cells pretreated with the pan-caspase inhibitor zVAD-fmk show an almost complete block of staurosporine-induced apoptosis by siRNA-mediated knockdown of HtrA2/Omi, while caspase inhibition alone was significantly less effective, suggested the occurrence of additional mechanism independent from caspase activation [65]. A comprehensive proteome-wide analysis was performed to identify further apoptotic targets of HtrA2/Omi [66]. The incubation of cell lysates with recombinant HtrA2/Omi or its catalytically inactive mutant, allowed to identify 15 potential HtrA2/Omi substrates. This group of potential targets includes the cytoskeleton-associated proteins actin, α- and β-tubulin and vimentin, two proteins involved in protein translation, eukaryotic translation initiation factor 4 gamma 1 (eIF-4GI) and elongation factor 1-alpha (EF-1a), and KIAA1967 and KIAA0251, two proteins associated with apoptosis. These results raises the possibility that HtrA2/Omi-mediated cleavage could affect cytoskeletal reorganization and contribute to the inhibition of the *de novo* protein synthesis during apoptosis [59, 66].

BCL-2 FAMILY PROTEINS AND PTP

The proteins belonging to the Bcl-2 family exert the greatest control over the mitochondrial pathway of apoptosis by regulating the permeabilization of the mitochondrial OM. The Bcl-2 family comprises both pro- and anti-apoptotic members that engage in a complex network of interactions to regulate apoptosis. Two multidomain proteins within this family, namely Bax (Bcl-2 Associated protein X) and Bak (Bcl-2 homologous antagonist/killer) are pro-apoptotic effectors and have been shown to be required for the activation of this pathway [67, 68]. Bax is generally more strongly expressed than Bak, and this latter is overexpressed and active when Bax has been previously inactivated [69]. In non-apoptotic cells Bax remains in a close conformation that renders it poorly able to interact with other proteins. It was suggested that a number of proteins could retain Bax in this inactive conformation, nevertheless it is not clear if this ability occurs in healthy cells or if it has to be considered the result of the overexpression of these proteins, linked to some pathological conditions such as cancer [70-72]. Apoptotic stimuli ultimately converge on Bax to promote permeabilization of mitochondrial OM, subsequent caspase activation and apoptotic cell death. Activated Bax can be sequestered by a number of multidomain anti-apoptotic proteins within the Bcl-2 family, which are localized to mitochondria and in the endoplasmic reticulum. Among these are Bcl-2 itself, Bcl-x_L (B-cell lymphoma-extra large) and others. The anti-apoptotic effect of these proteins can be antagonized by a third group of proteins belonging to Bcl-2 family, the BH3-only proteins [73].

The relationships between Bax and PTP formation is still an intriguing question. It has been reported that recombinant Bax and Bak induce mitochondrial changes typical of PTP, i.e. collapse of mitochondrial transmembrane potential, swelling and cyt c release. These effects depend on Ca^{2+}, are prevented by CsA and BKA and inhibited by recombinant Bcl-xL and transgene-derived Bcl-2. Interestingly, co-immunoprecipitation experiments demonstrated that Bax and Bak interact with the VDAC raising the possibility that

Bax/Bak induces opening of the PTP through a direct interaction [74]. Moreover, it was demonstrated that purified recombinant Bax can promote the opening of PTP in isolated mitochondria depending on concentration. At low concentration, Bax causes a transient, non-synchronous activation of the PTP, followed by a rapid recovery of mitochondrial integrity. No mitochondrial swelling or depolarization were observed, nevertheless, release of cyt c, adenylate kinase and of matrix-sequestered calcein occur and all these events are prevented by CsA. At high Ca^{2+} concentration the MPT is induced with mitochondrial swelling and depolarization, both prevented by CsA [75]. It was also proposed that PTP may upstream contribute to the OM permeabilization mediated by Bax. In particular, following the mitochondrial transmembrane potential collapse due to the PTP opening, cytosolic Bax undergoes a conformational change that provokes its recruitment on the OM and the oligomerization, which forms channels for cyt c release [76]. Accordingly, because a Bax mutant that constitutively localized to mitochondria circumvents the requirement for PTP opening to induce apoptosis, it was argue that in cerebellar granule neurons the major role of the opening of PTP is to trigger Bax translocation to mitochondria, thus leading to cyt c release and caspase activation [77]. A more recent study highlighted the hypothesis that Bax may also regulates the sensitivity of cells to MPT-induced cell death, as the absence of Bax protected against treatment with the Ca^{2+} ionophore ionomycin in mouse embryonic fibroblasts. Interestingly, sensitivity can be restored by reconstitution with Bax [78].

MITOCHONDRIAL PERMEABILITY TRANSITION AS TARGET OF ANTICANCER AGENTS

Many compounds can act on the components of the PTP to induce MPT and apoptosis. MPT can also be triggered by agents that stimulate ROS generation or increase cytosolic Ca^{2+} concentrations. Furthermore MPT may be induced by decreasing endogenous inhibitors of PTP opening as glucose, creatine phosphate, ATP and glutathione [7]. Some examples of the prominent members of these groups are discussed below.

ANT TARGETING AGENTS

Human ANT presents four isoforms, each of them with a specific expression. Several studies have revealed that ANT1 and ANT3 induce mitochondrial apoptosis whereas ANT2 and ANT4 are anti-apoptotic [79]. ANT2 is expressed mainly in proliferative, undifferentiated cells and may therefore contribute to carcinogenesis. Since the expression of ANT2 is closely linked to the mitochondrial bioenergetics of tumors, it should be taken into account for individualizing cancer treatments and for the development of anticancer strategies. Several compounds affecting ANT have been reported to induce mitochondrial apoptosis; however none of them has so far been described to specifically target one isoform of ANT [4, 79, 80].

GSAO (4-(N-(*S*-glutathionylacetyl)amino) phenylarsonous acid) and **PENAO** (4-(N-(*S*-penicillaminylacetyl) amino) phenylarsonous acid) (Fig. **1**) are tumor metabolism inhibitors that target ANT. Metabolism of GSAO is required to exert the antimitochondrial effect. At the cell surface GSAO is first transformed to GCAO (4-(N-(*S*-cysteinylglycylacetyl)amino) phenylarsonous acid), which enters in the cell via an organic ion transporter and is further processed to produce CAO (4-(N-(*S*-cysteinylacetyl)amino) phenylarsonous acid) (Fig. **1**) in the cytosol. Then, CAO enters the mitochondrial matrix and reacts with ANT [4, 81].

PENAO (Fig. **1**) has been designed to bypass the pro-drug processing and metabolism of GSAO. It is a cysteine mimetic of CAO characterized by a significative increased antiproliferative activity and several-fold increased antitumor efficacy in mice with respect to GSAO. Moreover, in contrast to GSAO, PENAO targets both proliferating endothelial and tumour cells.

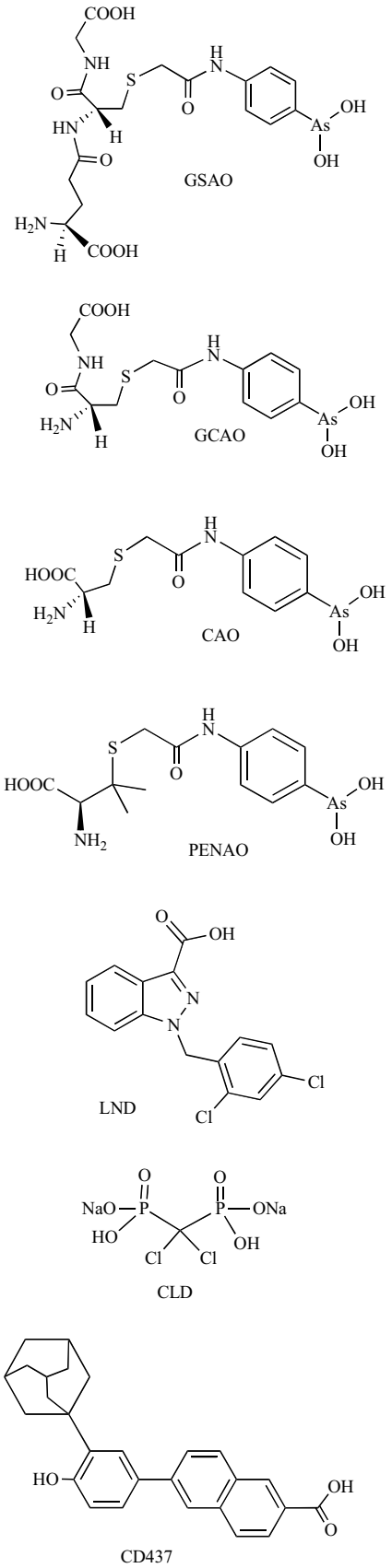


Fig. (1). Structure of ANT Targeting Agents.

It has been shown that these arsenic compounds cross link critical cysteine residues of ANT (Cys57 and Cys257), resulting in: inhibition of its ATP/ADP antiporter activity, ROS overproduction, cytosolic ATP depletion, mitochondrial depolarization and apoptosis. Both compounds, GSAO and PENAO, are currently being trialled in a Phase I/IIa escalation study in patients with solid tumors refractory to standard therapy [4, 81].

Lonidamine (LND), a derivative of indazole carboxilate (Fig. **1**), is an antineoplastic drug that is effective against a wide range of solid tumors [82]. Clinical phase II and phase III trials demonstrated the efficacy of this compound on different types of human cancers, even more, preclinical studies demonstrated the ability of LND to increase the response of tumor cells to several antineoplastic drugs such as cisplatin, paclitaxel, adriamycin and etoposide. LND also affects energy metabolism, and alters the plasma and mitochondrial membranes. It has been shown that LND triggers apoptosis independently of the p53 gene [83] and that Bcl-2 overexpression blocks LND-induced apoptosis [84]. LND was also demonstrated to trigger apoptosis through a direct Bcl-2 inhibitory effect on the PTP [85] and to favor mitochondrial membrane permeabilization through a direct effect on ANT [86]. In a cell-free system, LND induced the permeabilization of ANT proteoliposomes, but not ANT-free liposomes. The ANT-dependent membrane permeabilization was inhibited by the two ANT ligands ATP and ADP, as well as by recombinant Bcl-2 protein. LND added to synthetic planar lipid bilayers containing ANT, elicited ANT channel activities [86]. When added to isolated nuclei, LND fails to provoke DNA degradation unless mitochondria are added simultaneously. In isolated mitochondria, LND causes the dissipation of the mitochondrial inner transmembrane potential ($\Delta\Psi$) and the release of apoptogenic factors capable of inducing nuclear apoptosis *in vitro*. Thus, the mitochondrion seems to be the subcellular target of LND. Interestingly, all effects of LND on isolated mitochondria are counteracted by CsA [85].

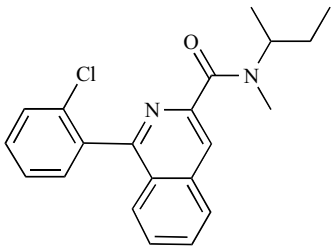
Clodronate (CLD), a biphosphonate that lacks nitrogen (Fig. **1**), is an important antiresorptive drug used for the treatment of diseases characterized by excessive bone resorption. This compound can be metabolized intracellularly to an analog of ATP (AppCCl2p) (Fig. **1**). CLD have been found to cause apoptosis of osteoclasts and macrophages *in vitro*. It has been suggested that the mechanism by which CLD causes osteoclast apoptosis, arises from the formation of the metabolite AppCCl2p (Fig. **1**). This latter, by inhibiting the mitochondrial ANT, causes mitochondrial membrane depolarization and subsequent events, such as cyt c release and caspase activation, leading to cell death [87]. However, the molecular mechanisms responsible for the *in vivo* efficacy of CLD remain uncertain. A second-generation of biphosphonates reduces skeletal tumor burden and exhibits antitumor activity in the bone and on visceral metastases [88, 89]. These newer biphosphonates have been found to block proliferation and induce apoptosis of various tumor cell lines [90, 91]. Alteration of Bcl-2 expression, activation of caspase-3 and the stimulation of mitochondrial cyt c release are discussed as mechanisms underlying the apoptotic effect of biphosphonates [92].

CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid) (Fig. **1**) and all-*trans*-retinoic acid are best known for their ability to stimulate the expression of retinoic acid receptor-responsive genes. Interestingly, this retinoid also triggers ANT-dependent MPT, as LND and arsenite. Indeed, in cell free systems, CD437 induces the permeabilization of ANT proteoliposomes and elicited ANT channel activities when is added to synthetic planar lipid bilayers containing ANT [86, 93, 94].

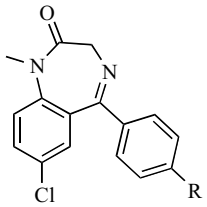
PBR TARGETING AGENTS

PBR is expressed in almost all tissues, although the level of expression varies, being particularly high in organs involved in steroidogenesis. It is well known that PBR is overexpressed in highly aggressive tumors, especially those of the breast [95, 96].

PK11195 (1-(2-chlorophenyl)- N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide), (Fig. **2**) is a ligand of PBR with a nanomolar binding affinity [97]. It has been shown that PK11195 enhanced apoptosis initiated by a number of different agents, such as etoposide, doxorubicin, dexamethasone, γ-irradiation, LND and ceramide [98]. Furthermore, in tumor cells showing high levels of PBR the treatment with PK11195 appears to decrease the proliferation, the invasion and the migration, causing an increase of apoptosis [95]. When PK11195 is added to isolated mitochondria, it facilitates dissipation of the $\Delta\Psi$ and release of both cyt c and AIF. Moreover, PK11195 abolishes the inhibition of apoptosis mediated by Bcl-2 via a direct effect on mitochondria [98, 99].



PK11195



R = H: Diazepam
R = Cl: RO5-4864

Fig. (2). Structure of PBR Targeting Agents.

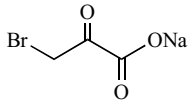
RO5-4864 and Diazepam (Fig. **2**) are also prototypic ligands of PBR. These compounds, like PK11195, have demonstrated antitumor effects *in vitro* and *in vivo*, both as single agents or combined with etoposide or ifosfamide [98]. A panel of human tumor cell lines was used to study the potential role of these three PBR ligands and the results showed that RO5-4864 can sensitize cells to apoptosis induction, both *in vitro* and *in vivo*. In particular RO5-4864 was characterized by an increased mitochondrial release of cyt c and Smac/DIABLO proteins and an enhanced activation of caspases 9 and 3. Irrespective of the exact molecular mechanism accounting for the chemosensitizing effect of RO5-4864 and diazepam, the authors speculated that the binding of the PBR ligand to its receptor induces a peculiar conformation of the PTP, which sensitizes the cell to an apoptotic message [98].

HEXOKINASE (HK) TARGETING AGENTS

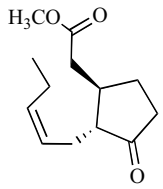
Cancer cells are characterized by a high-ATP demand, and in order to support growth and invasion of healthy tissues they display an altered energy metabolism, known as Warburg effect in which a highly glycolytic phenotype and a depressed respiration, either in normoxia or hypoxia conditions, are observed. Hexokinase type II (HK-II) is the key enzyme for maintaining increased glycolysis in cancer cells where it is overexpressed and more tightly bound to VDAC than in their normal counterparts.

3-Bromopyruvate (3-BrPA) (Fig. **3**) an inhibitor of HK-II, induces cell death in cancer cells. It was reported that HK-II plays

an important role in mediating the cytotoxic action of 3-BrPA [100, 101]. Treatment of tumor cells with 3-BrPA resulted in a preferential covalent modification of HK-II protein in a concentration- and time-dependent manner. This chemical modification was also demonstrated *in vitro* when protein extracts were incubated with 3-BrPA in a cell-free mixture [102]. Direct treatment of isolated mitochondria with 3-BrPA caused a dissociation of HK-II from the mitochondria and the subsequent release of AIF from the organelles. It was suggested that 3-BrPA may directly target HK-II and to cause cell death by two important mechanisms: depletion of ATP by inhibition of glycolysis and release of AIF by the dissociation of HK-II from mitochondria [102, 103]. Because the Warburg effect is frequently seen in cancers and HK-II is over-expressed in many cancer cells, targeting HK-II could be a logical strategy to effectively and selectively kill cancer cells [101, 102]. It has also been shown that ATP depletion-dependent necrosis and apoptosis and mitochondrial dysregulation due to ROS production, are involved in 3-BrPA-induced cell death in hepatoma cells [101]. Therefore these and other studies reveal the strong potential of 3-BrPA as an anticancer agent [104].



3-BrPA



Methyl jasmonate

Fig. (3). Structure of HK Targeting Agents.

Jasmonates as methyl jasmonate (Fig. **3**) are plant stress hormones that induce suppression of proliferation, membrane depolarization, cyt c release and death in intact human cancer cell lines [105, 106]. Jasmonates induce a rapid depletion of ATP in cancer cells, and this steep drop occurs when no signs of cell death are detectable yet [106]. Jasmonates induced cyt c release and swelling in mitochondria isolated from cancer cells but not from normal ones. Moreover, it has been reported that jasmonates bind to HK thereby detatching it from the mitochondria and from its mitochondrial anchor—the VDAC [107]. In conclusion, jasmonates seem to act directly on mitochondria in cancer cells in a PTP-mediated manner, and they therefore could bypass premitochondrial apoptotic blocks. In this connection, jasmonates can be considered promising candidates for the treatment of different types of cancer [105].

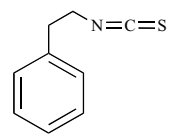
AGENTS CAUSING ROS OVERPRODUCTION

The generation of ROS by mitochondria is a physiological event and under aerobic conditions about 2% of the total mitochondrial oxygen consumption becomes superoxide anion ($O_2^{\bullet-}$). Despite the moderate chemical reactivity of $O_2^{\bullet-}$ in aqueous solutions, it can generate a highly oxidative and cytotoxic ROS, the hydroxyl radical ($HO^{\bullet}$) [108]. In mitochondria the antioxidant defense system is represented by enzymes such as superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, NAD(P)/NADH transhydrogenase, and by compounds such as glutathione (GSH), NADH, vitamins E and C [109, 110]. In physiological conditions the result of the above processes is a balance between the oxidant and antioxidant systems. However, in conditions in which an excess of ROS is generated and/or the antioxidant defense system is exhausted, a state of oxidative stress is created and many oxidative alterations of mitochondrial membrane components such as lipid peroxidation or protein thiol oxidation may occur, leading to mitochondrial permeabilization and, subsequently, PTP opening [108].

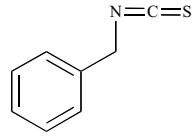
In this contest MPT may also be induced, in the presence of Ca^{2+}, by exogenous ROS-generating systems [5]. Even the oxidation of protein thiol groups buried in the lipid phase of mitochondrial membrane cause MPT and these thiols becomes also targets for ROS oxidation [108].

Arsenic trioxide (As_2O_3) is an effective anticancer drug used for the treatment of a wide range of solid tumours, and acute promyelocytic leukaemia. It was found to cause oxidative modification of thiol groups in ANT with the subsequent release of cyt c through MPT induction. However, at clinically achieved concentrations, the same drug stimulated cyt c release and apoptosis through a Bax/Bak dependent mechanism [111, 112]. It seems also that the oxidative stress provoked by the compound has been linked to irreversible inhibition of thioredoxin reductase [113].

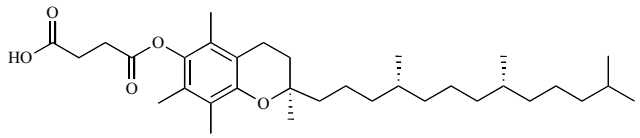
PEITC (phenylethylisothiocyanate) and **BITC** (benzylisothiocyanate) (Fig. **4**) are naturally occurring isothiocyanates present in cruciferous vegetables and capable of react with redox regulatory proteins and increase ROS levels in mitochondria [114, 115]. PEITC has been shown to inhibit the GSH antioxidant system by extruding GSH from the cell and by inhibiting glutatione peroxidase [115]. The apoptotic death induced by PEITC in cancer cells was associated also with ROS generation followed by disruption of $\Delta\Psi$ and release of apoptogenic molecules (cyt c, Smac/DIABLO, AIF and EndoG) from mitochondria. The apoptotic effect induced by PEITC was also correlated with the complex III inhibition, ATP depletion and with apoptotic DNA fragmentation [116, 117]. Moreover, the apoptosis induced by PEITC was found to be dependent of p66Shc, a lifespan-regulating protein, which it is known to increase ROS levels in the intermembrane space, through a high ROS production. Indeed, treatment of PC3 cells with PEITC resulted in translocation of p66(Shc) from cytosol to the mitochondria [118, 119] where it generates hydrogen peroxide using reducing equivalents of the mitochondrial electron transfer chain through the oxidation of cyt c [120]. In human breast cancer cells, the mitochondrial respiratory chain was target by BEITC that triggers ROS-dependent apoptosis [121].



PEITC



BITC



α- TOS

Fig. (4). Structure of Agents Causing ROS overproduction.

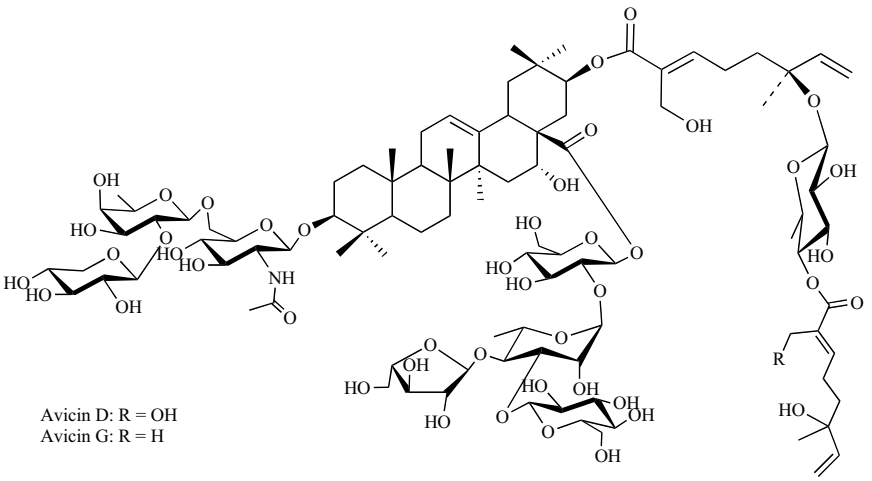
α-Tocopheryl succinate (α-TOS) (Fig. **4**) is a derivative of α-tocopherol and acts as a potent apoptogen and anticancer agent, nevertheless the mechanisms of α-TOS-mediated apoptosis are not understood in detail. Some studies suggest that the inhibitory effects of α-TOS on tumor cell growth are not a consequence of its antioxidant properties, but may, in fact, be due to one or more of its

other potential roles within the cells, such as the regulation of cellular enzyme activities involved in growth [122, 123]. In highly aggressive tumor cells it has been shown that α-TOS stimulated rapid entry of Ca^{2+} into the cytosol, compromised Ca^{2+} buffering capacity of the mitochondria and sensitized them towards MPT and subsequent apoptotic cell death [122]. Moreover, it was found that stabilization of mitochondria and the death of malignant cells induced by α-TOS is accompanied by a concomitant increase in the levels of free radicals and lipid peroxidation [123]. Another study provides evidence that mitochondria are critically involved in α-TOS-induced apoptosis because of induction of MPT and ensuing relocation of Bax and cyt c in human cancer cells. In particular, a crosstalk between death receptor- and mitochondria-related pathways via the signal-integrating protein Bid was suggested. These findings shed insights into the diverse caveats of the molecular mechanism of α-TOS-induced apoptosis [124].

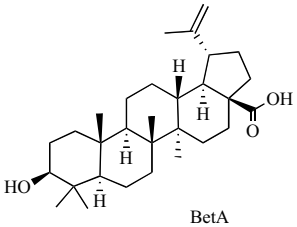
OTHER AGENTS

Avicins are triterpenoid saponins (Fig. **5**) from *Acacia victoriae*. The ability of avicins to induce apoptosis in transformed cells makes them potential anticancer agents, but the molecular mechanisms by which they inhibit tumor cell growth have not been fully defined, yet. Increasing evidences have shown that avicins may affect multiple cellular processes to suppress tumor cell growth or trigger cell death. These effects include activation of apoptosis and cellular stress response pathways, induction of autophagic cell death by regulation of the AMPK-tuberous sclerosis complex 2 (TSC2)-mammalian target of rapamycin (mTOR) pathway, as well as inhibition of growth factor signaling, inflammation and oxidative stress response [125]. In particular, it was hypothesized that they induce apoptosis by direct perturbation of mitochondria [126, 127]. Indeed some studies demonstrate that the avicins favor the closure of VDAC, resulting in suppression of nucleotide exchange and decrease in oxygen consumption [128].

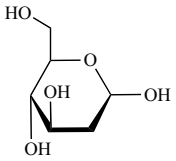
Betullinic acid (BetA) (3β, hydroxy-lup-20(29)-en-28-oic acid) (Fig. **5**) is a plant-derived pentacyclic triterpenoid of lupane-type found in various species throughout the plant kingdom. It exerts anti-inflammatory, anti-HIV, and antineoplastic activities both *in vitro* and *in vivo*. On account of its selective antimelanoma activity and its favorable therapeutic index, BetA is currently undergoing



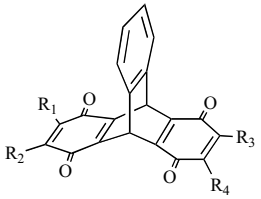
Avicin D: R = OH
Avicin G: R = H



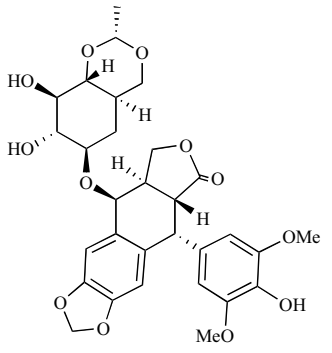
BetA



2DG



TT2: R_1= OMe, R_2 = R_3= R_4= H
TT13: R_1 = OMe, R_2 = Br, R_3 = R_4 = H
TT15: R_1 = Br, R_2 = NHMe, R_3 = R_4 = H
TT16: R_1 = OMe, R_2 = Br, R_3 = NMe_2, R_4 = H
and R_1 = OMe, R_2 = Br, R_3 = H, R_4= NMe_2



Etoposide

Fig. (5). Structure of various anticancer agents.

clinical trials. Nevertheless, the medical use in the pharmaceutical industry is limited because of its poor hydrosolubility and pharmacokinetic properties. To overcome these problems it has undertaken the synthesis of more water soluble BetA derivatives characterizated by polar moieties, such as amino acids and phthalates at C-3 and C-28 positions. In certain cases these substitutions enhance the hydrosolubility and anticancer activity of BetA [129, 130]. New semisynthetic derivatives of BetA (RS01, RS02 and RS03) showed improved cytotoxic activity, up to 45 times higher than that of BetA, suppressing the proliferation of malignant cells by inducing apoptosis through activation of caspases [131].

It was shown that BetA-induced apoptosis depends on the release of cyt c and on the formation of the apoptosome. Indeed, Bax/Bak deficient cells are as sensitive to BetA as their wild-type counterparts, suggesting that cyt c is released in a non classical fashion. It is proposed that BetA affects mitochondria and induces cyt c release directly via PTP. This release is only temporally prevented by the anti-apoptotic members of the Bcl-2 family, but independent of Bax and Bak. All these findings help to explain the efficacy of BetA against tumor cells of different origins and its effect in tumor cells resistant to other metabolites [132].

2-Deoxyglucose (2DG) (Fig. **5**) is a non metabolizable glucose analogue, which has been studied as selective inducers of apoptosis in cancer cells. This compound enhances the effectiveness of chemotherapy and radiotherapy in cell lines and animal models [133, 134]. Phase I/II clinical trials in solid tumors are ongoing, nevertheless it must be excluded a possible implication of the compound in the glycolytic metabolism of the brain and of the heart. Surprisingly, 2DG inhibited not only glycolytic pathways, but also endothelial cell angiogenesis *in vivo* and *in vitro* by different mechanism, rendering the compound very intriguingly for cancer therapy [135].

Although its mechanism of action in cancer therapy has not been fully elucidated, it was proposed its participation in different forms, being best known as an inhibitor of glucose metabolism. 2DG inside the cell is phosphorylated to 2DG-6P by HK, the first limiting enzyme in the glycolytic pathway. This product cannot be metabolized by the second enzyme of glycolysis and consequently is accumulated inside the cell. 2DG-6P inhibits competitively HK at the rate limiting step of glycolysis with an intracellular depletion of ATP [136, 137]. The inhibition of glycolysis by 2DG treatment has indirect effects on various signaling pathways, and also induces oxidative stress and disrupts thiol metabolism affecting the PTP [138-141].

Triptycene analogues (TTs) (Fig. **5**) are antitumor bisquinones derivatives that block nucleoside transport, macromolecule syntheses and DNA topoisomerase activities, induce cyt c release and apoptotic DNA fragmentation, inhibit the proliferation of drug-sensitive and -resistant tumor cells in the nM range *in vitro* and rapidly trigger the collapse of $\Delta\mu_H^+$ in cell and cell-free systems. TT interaction with isolated mitochondria rapidly causes a dose-dependent, large amplitude swelling and Ca^{2+} release in relation with their antiproliferative activities in some tumor cells *in vitro*. Thereby, antitumor TTs that trigger MPT in isolated mitochondria might interact with components of the PTP to boost its Ca^{2+}-sensitive transition from the closed to the open state [142].

Etoposide (Fig. **5**) is a derivative of the natural compound podophyllotoxin. Etoposide is widely used for the treatment of several forms of cancer by acting as a topoisomerase-II inhibitor. The response of some cells to etoposide is explained by the accumulation of DNA double strand breaks that are subsequently recognized by DNA-PK, a member of the PI3-kinase family, which phosphorylates and thereby activates p53. Upon activation, p53 causes an increase in the transcription of the pro-apoptotic protein Bax. Bax undergoes a conformational change and is able to translocate to the mitochondria. This movement of Bax to the mitochondria induces the MPT that results in the release of cyt c and culminates with loss of viability of the cells [143]. Nevertheless, the etoposide-induced apoptosis in Jurkat cells lacking p53 and Bax but containing p73 and Bid was explained through the translocation of full-length Bid to mitochondria [144]. The incubation of rat liver mitochondria with the drug results in a concentration-dependent induction of MPT, evidenced by an increased sensitivity to Ca^{2+}-induced swelling, depolarization of $\Delta\Psi$, Ca^{2+} release and stimulation of state 4 oxygen consumption. All these effects are prevented by preincubating the mitochondria with CsA by suggesting another possible mechanistic explanation about the antitumor effect induced by etoposide [145].

A NOVEL ANTIPROLIFERATIVE NATURAL COMPOUND ACTING AS MPT INDUCER

Inside the research field focused on the development of novel anticancer drugs, mitochondria play undoubtedly an important role. Indeed, even if most of the current anticancer drugs do not directly target mitochondria but affect other molecular targets, their damage activates intracellular pathways that often converge on mitochondria to trigger the mitochondrial pathway of apoptosis. Moreover, for some clinically used antitumor drugs in addition to cytosolic and/or nuclear effects, a direct or indirect action on mitochondria was also demonstrated. The cardiotoxicity and the occurrence of multidrug resistance of anthracenediones and anthracyclines have been attributed to their accumulation in mitochondrial lipid membrane and subsequent redox activity of the quinone moiety [146-150]. Furthermore, etoposide and *m*-amsacrine, two well-known topoisomerase II targeting drugs, affect mitochondrial functions [144, 151, 152].

Interestingly, the ability to activate the apoptotic process by directly affecting mitochondria could represent a worthwhile property for some antiproliferative agents because of the reduction of genotoxic risk, an important side-effect correlated with the clinical use of many antitumor drugs targeting DNA and/or related nuclear processes. In addition, mitochondrial targeted agents could overcome the chemoresistance due to the lack of pro-apoptotic signals that converge to mitochondria, like p53 expression, or to the overexpression of antiapoptotic members of Bcl-2 family.

In our previous investigations, we extensively studied many synthetic and natural compounds characterized by appreciable cytotoxicity on human tumor cell lines and interestingly, the investigation on the mechanism of action suggested for some of them the induction of MPT as the molecular event responsible for the antiproliferative effect [153-155]. The investigation on the mechanism of action responsible for the cytotoxicity of the 2-amino-5*H*-pyrido[3',2'; 5,6]thiopyrano[4,3-*d*]pyrimidine demonstrated the occurrence of MPT via a pro-oxidant pathway. The capacity of the compound to reduce Fe^{3+} ions to Fe^{2+} and the lack of such ability for the 2-*p*-toluensulphonamido analogue permitted us to hypothesize that the *p*-amino derivative form imino radicals which, by interacting with molecular oxygen, could lead to the production of ROS. These latter, in turn, could react with the critical thiols responsible for PTP opening. The release of the pro-apoptotic factor cyt c accounts for the cell death and the preventive effect of CsA on cell growth inhibition correlates the antiproliferative activity with the MPT [153]. A significant cytotoxic effect along with the capacity to induce the opening of PTP, with the consequent release of the pro-apoptotic factors cyt c and AIF, was demonstrated also for the 1,4-dihydrobenzothiopyrano[4,3-*c*]pyrazole derivative. Cytofluorometric determinations in whole cells showed that the compound induces mitochondrial membrane depolarization and cell death through the apoptotic pathway. Interestingly, the correlation between the antiproliferative effect and MPT induction emerges from the ability of CsA to prevent the cytotoxic effect exerted by the derivative [154]. The 3α-hydroxymasticadienonic acid, a tirucallane-type triterpene isolated by *Amphipterygium adstringens*, an

endemic Mexican tree widely used in traditional medicine, shows antiproliferative activity on tumor cells and opposite dose-dependent effects on MPT in isolated rat liver mitochondria. At low concentration it behaves as an intrinsic pro-apoptotic agent promoting MPT and the release of cyt c and AIF. At high concentration it prevents MPT by blocking Ca^{2+} entry in mitochondria. In the first condition the PTP opening mediates cell death through the apoptotic pathway, in the second one an impairment of bioenergetic and Ca^{2+} dependent mitochondrial activities seems to be responsible for cell death [155].

As part of our research program assigned to the development of novel antiproliferative agents, we studied the mechanism of action of peniocerol (PEN, Fig. **6**), a sterol isolated from the root of *Myrtillocactus geometrizans*, a cactus endemic to Mexico [156]. Field observations highlighted that this cactus, commonly named "garambullo", is very resistant to environmentally stressed conditions and to insect attack [157, 158]. Actually, it was previously demonstrated that PEN shows insecticidal effects [156] and, more recently, it was highlighted the ability to induce a cytotoxic effect on some human tumor cell lines [157]. On the basis of these results, we extend the antiproliferative assays on more human tumor cell lines and investigate the mechanism of action responsible for cytotoxicity. In particular, the effects on mitochondrial bioenergetic functions and the induction of the MPT process in isolated rat liver mitochondria (RLM) was evaluated and discussed.

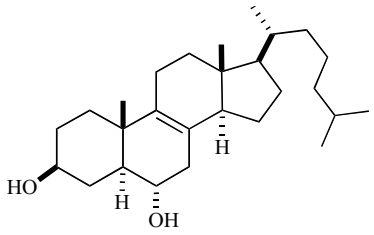


Fig. (6). Chemical structure of peniocerol (PEN).

EXPERIMENTAL PROCEDURES

Inhibition Growth Assay

HeLa (human cervix adenocarcinoma cells) were grown in Nutrient Mixture F-12 [HAM] (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen); A-431 (human epidermoid carcinoma) and HepG2 (human hepatocellular carcinoma) were grown in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% fetal bovine serum. 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Sigma Chemical Co.) were added to the media. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air. Cells ($4x10^4$) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of fresh medium, and various concentrations of the test agent were added. The cells were then incubated in standard conditions for a further 72 h. A trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as IC_{50} values, i.e., the concentration of the test agent resulting in 50% reduction in cell number compared with control cultures.

Mitochondria Isolation and Protein Determination

Twelve hours fasted male Wistar-derived rats (200-300 g) were sacrificed by cervical dislocation after stunning. The liver was promptly removed and immediately immersed in an ice cold 250 mM sucrose, and cut in small pieces. Then it was minced, thoroughly rinsed three time with ice cold 250 mM sucrose by eliminating blood and fat. Subsequently the liver was homogenized in 50 mL of isolation medium (250 mM sucrose, 5 mM Hepes, 0.5 mM EGTA, pH 7.4) using a Potter homogenizer with a Teflon pestle and subjected to centrifugation (900xg) for 5 min in a Beckman J2-21 refrigerated at 2°C for removing the cell debris, myofibrils, nuclei, and other heavy components. The pellet was discarded and the supernatant was centrifuged at 10,800xg for 10 min to precipitate the mitochondrial pellet. Then the supernatant was discarded and the pellet was carefully resuspended in isolation medium without EGTA and sedimented again at 15,800xg for 10 min. The resulting pellet was finally suspended in isolation medium without EGTA and the mitochondria protein content was measured by the biuret method. The absence of contaminant subcellular compartments in the preparation has been demonstrated as previously reported [159].

Standard Incubation Procedures

Incubations were carried out at 20°C in a water-jacketed cell with 1 mg of mitochondrial protein/mL and suspended in a standard medium containing 200 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 1.25 µM rotenone, and 1 mM phosphate, sodium salts were used. Rotenone was included in the medium in order to avoid the formation of oxalacetate, a strong competitive inhibitor of succinate dehydrogenase [160] and to obtain optimal succinate oxidation. When present 40 µM calcium chloride. Other additions are indicated in the figure legends. PEN was used in a range of 20-100 µM concentration, mainly at 40 µM in order to better evidence its effects. This concentration may be considered enough high if compared with other agents acting at cellular level. However other not reported results have shown qualitatively similar effects also at 5-10 µM concentrations. It is also to emphasize that mitochondria in the reported experiments are present at 1 mg protein/mL that is a very high density. Lower suspension densities of mitochondria require much more lower PEN concentrations than those mentioned above to exhibit its effect. The experiments were carried out at 20°C in order to compare the results with those obtained in other previous papers on MPT [*e.g.* see 161-163]. Whole RLM exhibit a reversible broad gel to liquid crystalline phase transition at 0°C [164] and at 20°C the membrane is in the sol-form. Under MPT conditions the fluidity of the membrane is greatly increased with respect to the control conditions [165] and further increases with increasing temperature. Therefore the choice of 20°C was made with the aim of minimizing alteration of the membrane, during the experiments regarding the MPT induction, due to excessive fluidity. Furthermore, it must be emphasized that at 30°C, the respiratory chain operates at high rate producing anaerobiosis, in suspension of isolated mitochondria, within a few minutes, particularly under MPT conditions.

Determination of Mitochondrial Functions

The respiratory control index (RCI) was calculated by determining the difference in rates of oxygen uptake between respiratory state 3 (presence of 300 µM ADP) and state 4 (absence of ADP) of RLM incubated in standard medium in the presence of the appropriate effectors.

Oxygen uptake was measured using a Clark electrode (Yellowspring Instruments Co. Inc.) in a closed, thermostatically controlled vessel with a magnetic stirrer, coupled to a Perkin Elmer 561 recorder [166].

The membrane potential ($\Delta\Psi$) was measured by assessing the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with an ion selective electrode specific for TPP^+ prepared according to Kamo *et al.* [167], with a calomeral reference electrode (Radiometer K401). The electrode potential is linear with respect to the logarithm of the TPP^+ activity, with a slope of 58 mV, according to the Nernst equation and the law of mass conservation. It was assumed an inner mitochondrial volume of 1 µl, calculated from the distribution of [^{14}C]sucrose and ^3H_2O according to Palmieri and Klingenberg [168]. TPP^+ was added at a concentration of 2 µM to allow accurate measurements while avoiding toxic effects on the H^+-ATPase [169] and Ca^{2+} movements [170]. $\Delta\Psi$ was calibrated using the equation $\Delta\Psi$=

$\Delta\Psi_{electrode}$-66.16/0.92 as proposed by Jensen *et al*. [171]. Mitochondrial swelling was qualitatively measured by monitoring the decrease in light scattering of the mitochondrial suspension at 540 nm using a Kontron- Uvikon-922 spectrophotometer equipped with a magnetic stirrer and thermostatic control. Mitochondria were suspended in standard medium and upon stabilization of the absorbance trace, swelling was assessed after additions of other compounds as described in the figure legends.

Measurement of the Redox Level of RLM

Determinations of protein sulfydryl groups and glutathione oxidation were carried out on mitochondrial suspensions from the various incubations used to determine mitochondrial swelling. In brief, at the end of incubations, the total suspension (1mg/mL) was placed in Eppendorf 4515c tubes and centrifuged for 1 min at 12,000xg; then the supernatant was discarded and the pellet used for both measurements. Sulfydryl group oxidation assay was performed after solubilization of the pellet with 1 mL of solubilization medium (10 mM EDTA, 0.2 M Tris, 1% SDS, pH 8.3), with 5,5'-dithio-bis-(2-nitrobenzoic acid) at 412 nm in a Kontron-Uvikon-922 spectrophotometer, according to Santos *et al*. [172].

Glutathione oxidation was assessed by deproteinization of the pellet with 3% metaphosphoric acid and subsequent centrifugation to separate the supernatant on which oxidized glutathione was determined by the method of Tietze [173].

The oxidation-reduction state of mitochondrial pyridine nucleotides was directly monitored fluorometrically on mitochondrial suspension with excitation at 352 nm and emission at 464 nm in an Aminco Bowman spectrofluorometer [174].

H_2O_2 production was determined fluorometrically [175]. Mitochondria at a concentration of 1 mg/mL were incubated in standard medium as above described, in the presence of horseradish peroxidase (9 U/mL), and 0.9 mM homovanillic acid. At intervals of time, aliquots of the incubation mixture were withdrawn and combined with 0.1 M NaOH. The fluorescence was then evaluated using a Perkin Elmer LS 50B spectrofluorometer with excitation at 324 nm and emission at 426 nm. Fluorometric calibration curves were prepared under the same experimental conditions using serial concentrations of commercial H_2O_2 in the absence or presence of 1 mg/mL mitochondrial protein. The presence of mitochondria did not modify the calibration curves, thus indicating that the mitochondrial suspensions used did not contain contaminating peroxisomal catalase that could interfere with the H_2O_2 assay. However, it should be noted that the measured amount of H_2O_2 refers to that diffusing out of mitochondria, as the enzyme for its determination (horseradish peroxidase) cannot enter mitochondria. This measurement gives a qualitative indication but cannot be considered for rigorous quantitative evaluations.

Detection of Cyt c and AIF Release

RLM were incubated in standard medium as above described with the appropriate additions. The reaction mixtures were then centrifuged at 12,000xg for 10 min at 4°C to obtain mitochondrial pellet. The supernatant fractions were further spun at 100,000xg for 15 min at 4°C to eliminate mitochondrial membrane fragments and concentrated five times by ultrafiltration through Centrikon 10 membranes (Amicon) at 4°C. Aliquots of 10 µl of the concentrated supernatants were subjected to 15% SDS-PAGE for cyt c and 10% for AIF and analyzed by Western blotting using mouse anti cyt-c antibody and rabbit anti-AIF antibody.

RESULTS

The effect of PEN on cell growth was evaluated on three human tumor cell lines, namely, HeLa (human cervix adenocarcinoma cells), HepG2 (hepatocellular carcinoma), and A-431 (epidermoid carcinoma). The results, expressed as IC_{50} values, are shown in Table **1**.

Table 1. Antiproliferative Activity of PEN against Three Human Cancer Cell Lines

Cytotoxicity (IC_{50} µM)[a]		
HeLa[b]	HepG2[c]	A-431[d]
40.1 ± 1.1	26.0 ± 0.7	34.6 ± 0.8

[a]Mean values ± S.D. of at least four experiments are reported. [b]Human cervix adenocarcinoma. [c]Human hepatocellular carcinoma. [d]Human epidermoid carcinoma.

Interestingly, PEN exerts an antiproliferative effect on all cell lines taken into consideration, showing IC_{50} values in the micromolar range. In particular, the compound appears more effective on hepatocellular carcinoma cells (HepG2) than on cervix adenocarcinoma (HeLa) and epidermoid carcinoma (A-431) cell lines.

The results reported in (Fig. **7**) show the effect of PEN on the RCI in RLM incubated in standard medium, in the absence (traces a, c, d) or presence (trace b, e) of Ca^{2+}. The calculation of mitochondrial RCI permits to evaluate the effectiveness of the organelles in performing their main bioenergetic process that is the oxidative phosphorylation, consisting in the coupling of ATP synthesis with the oxygen consumption. The control trace demonstrates a high phosphorylation efficiency of RLM (RCI=10.1, trace a), while in the presence of Ca^{2+} this value is lowered (RCI=7.5, trace b). However, the phosphorylation of ADP to form ATP in the presence of Ca^{2+} may be considered very high, yet.

In the presence of 40 µM or 100 µM PEN, mitochondria undergo a significant drop in the phosphorylation capacity (RCI=6.3 or 4.2, respectively, traces c, d), thus demonstrating a slight uncoupling effect but, however, maintaining the ability to synthesize ATP. If RLM are incubated with 40 µM PEN and Ca^{2+}, a complete collapse in ATP synthesis occurs (RCI=1.0, trace e).

Figure **8A** reports the effect of PEN on another important biochemical parameter, the $\Delta\Psi$, that permits to evaluate the intactness of the IM and the functionality of the mitochondrial respiratory chain. These properties are evidenced by the value of about 170 mV exhibited by the control trace. Changes in $\Delta\Psi$, instead, mean that the membrane has undergone some perturbations that affect its impermeability. In this regard, the presence of 40 µM or 100 µM PEN induces a drop in $\Delta\Psi$ of about 20 mV or 30 mV, respectively, while the addition of the immunosuppressant and MPT inhibitor CsA is ineffective in protecting $\Delta\Psi$ against the effect of 40 µM PEN.

The results of (Fig. **8A**) also evidence that the dose-dependent drop in $\Delta\Psi$ induced by PEN is not complete, but it is stabilized at an increased permeability level. This effect resembles that of a light protonophore and confirms the partial uncoupling activity suggested by the results of (Fig. **7**). The inefficiency of CsA in preventing the drop in $\Delta\Psi$ demonstrates that the increase in permeability is not due to the induction of the MPT, but instead to the opening of proton leaks. In the presence of Ca^{2+} (Fig. **8B**), $\Delta\Psi$ undergoes a drop from 170 mV to 150 mV, due to the establishment of Ca^{2+} cycling across the mitochondrial membrane that lowers the inner negative potential of mitochondria. The addition of 40 µM or 100 µM PEN causes, after a very low and transient $\Delta\Psi$ establishment, a complete collapse in $\Delta\Psi$. In the presence of CsA or ADP, $\Delta\Psi$ is maintained at high values, slightly lower than that observed with Ca^{2+} alone (Fig. **8B**). These observations suggest that PEN, besides to cause a change in the membrane permeability with the formation of proton leaks, it is also able to induce other alterations to the membrane, most likely of colloid-osmotic origin. The experiments of (Fig. **9**) account for this proposal. Indeed, PEN at concentrations ranging from 20 to 100 µM does not induce any change in the apparent absorbance of mitochondrial suspension at 540 nm, thus indicating that no osmotic changes occurred in this condition (Fig.

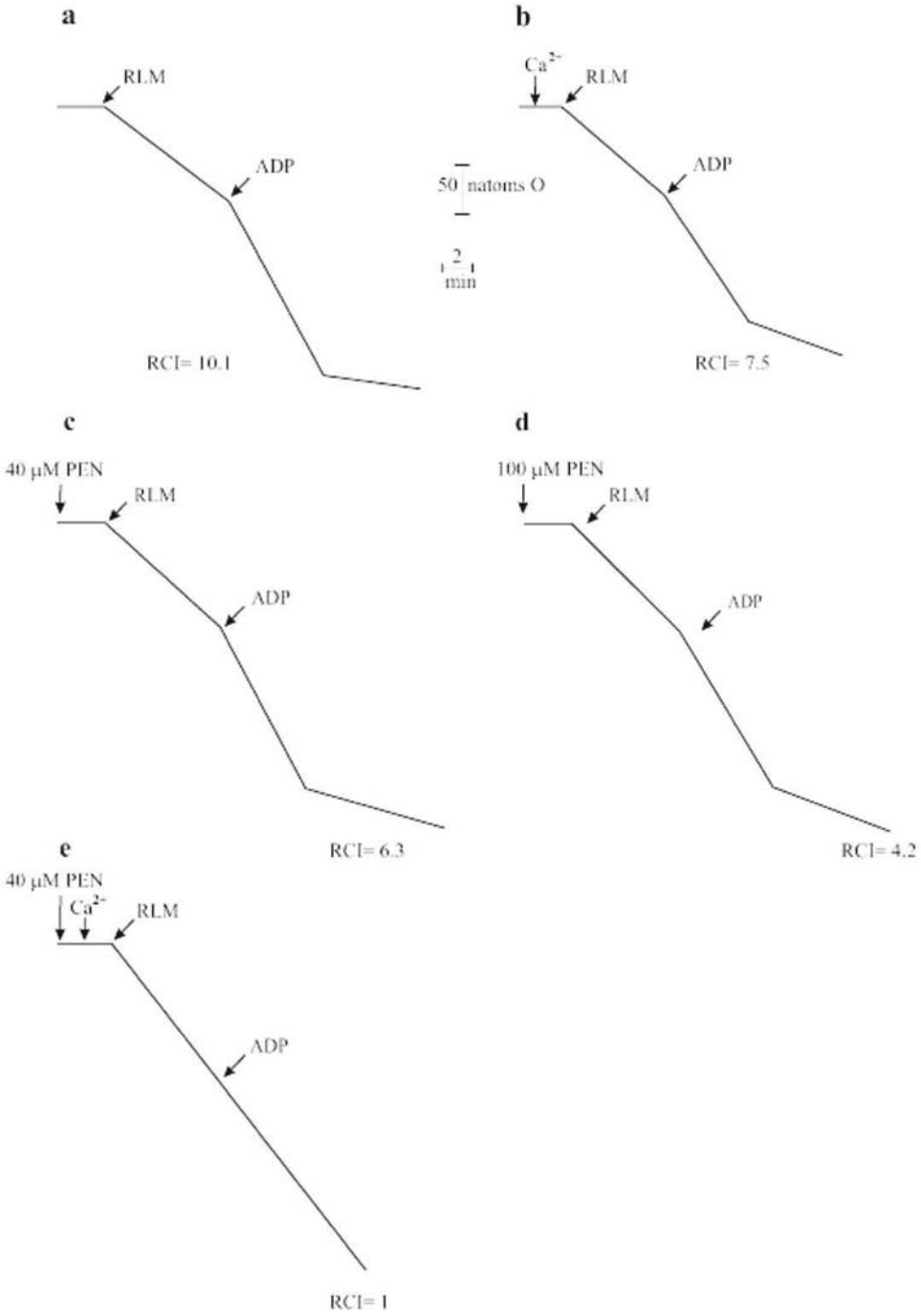


Fig. (7). Concentration dependent decrease in mitochondrial RCI value induced by PEN in the absence or presence of Ca^{2+}.
RLM were incubated in standard medium in the conditions described in Experimental Procedures. ADP was added at 300 μM concentration. When present in the medium 40 μM Ca^{2+}, 40 μM or 100 μM PEN as indicated in the figure. RCI values are indicated at side of the traces. The figure reports a typical experiment. Other five experiments gave almost identical results. The mean values of RCI of these experiments was 10.1 ± 0.2 (**a**), 7.5 ± 0.3 (**b**), 6.3 ± 0.1 (**c**), 4.2 ± 0.4 (**d**), 1 ± 0.1 (**e**).

9A). This result suggests that, although PEN is able to increase the proton permeability of the membrane (see Fig. **8**), the ingress of other solutes is not allowed. If Ca^{2+} is also present, 40 μM or 100 μM PEN induces a very strong dose-dependent decrease in the suspension absorbance, indicative of the occurrence of a large amplitude colloid-osmotic matrix swelling (Fig. **9B**). Interestingly, the mitochondrial swelling induced by 40 μM PEN in the presence of Ca^{2+}, is almost completely abolished by some typical MPT inhibitors, such as CsA, ADP and the adenine translocase inhibitor, BKA (Fig. **10A**) and by the antioxidant agents agents dithiothreitol (DTE), N-ethylmaleimide (NEM), spermine (SPM) (Fig. **10B**). These results demonstrate that PEN is able, in the presence of Ca^{2+}, to induce the phenomenon of the MPT and that this latter is related to an oxidative stress.

Fig. **11** shows a dose-dependent effect of PEN (20-100 μM), on the redox state of mitochondrial sulfydryl groups, and indeed by

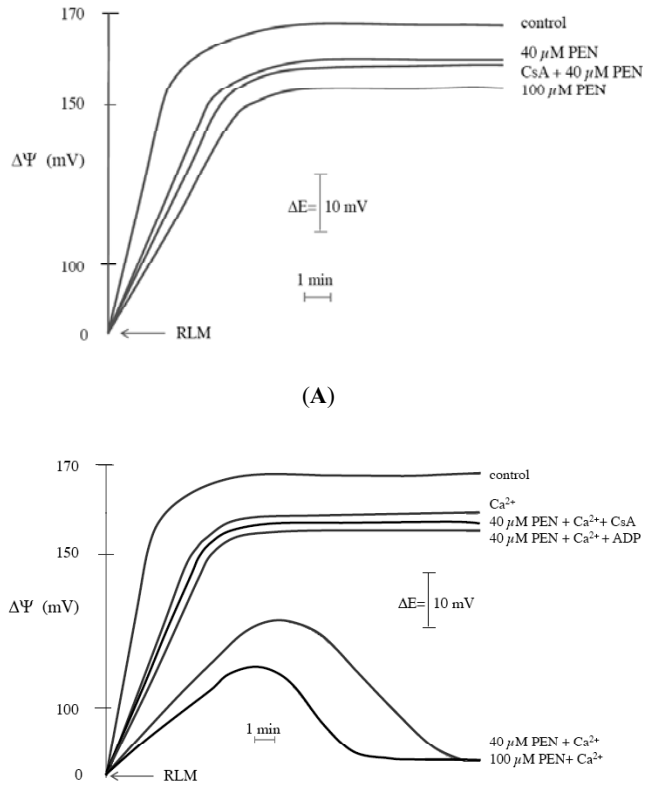


Fig. (8). Drop in mitochondrial ΔΨ values induced by PEN in the absence (**A**) or presence (**B**) of Ca^{2+}. Partial protection by CsA and ADP.
RLM were incubated in standard medium in the presence of 2 μM TPP^{+}, in the conditions described in Experimental Procedures. Where indicated 40 μM or 100 μM PEN, 1 μM CsA, 500 μM ADP, 40 μM Ca^{2+}, were present. A typical experiment is reported, seven other experiments showed very similar results. ΔE = electrode potential.

increasing PEN concentration, the percentage of SH groups gradually diminishes, demonstrating that the compound causes the oxidation of thiols with the formation of disulphides. The addition of NEM, DTE, or SPM induces a significant protection against SH oxidation (Fig. **12**). In the presence of Ca^{2+} the oxidation of SH groups is significantly higher (about 45%) with respect to that obtained for both PEN (15%) or Ca^{2+} (10%) (Fig. **13**). Thus, as regard the SH oxidation effect, PEN and Ca^{2+} seem to exert a significant apparent synergic effect that is strongly prevented by DTE, NEM or SPM (Fig. **13**). The pro-oxidant effect of PEN is also confirmed by experiments performed on pyridine nucleotide. Also in this case a dose-dependent effect similar to that reported for thiol groups occurs (compare Fig. **11** and Fig. **14**), significantly prevented by CsA and the anti-oxidant agents (Fig. **15**). It is to emphasize that the mitochondrial redox homeostasis is regulated by glutathione and by the activity of its related enzymes, namely glutathione peroxidase and glutathione reductase, which also regulate the NAD(P)H /NAD(P) ratio. In this connection, all the agents that affect the ratio GSH/GSSG may have significant effects on mitochondrial functionality and, consequently, on cellular activity. The above effects exhibited by PEN on sulfydryl groups and pyridine nucleotides suggest that also glutathione may be oxidized by the sterol. Fig. **16** shows that PEN and Ca^{2+} oxidize gluthatione, for about 15% and 10%, respectively. The concomitant presence of both the sterol and the cation induces a strong, synergic oxidation of glutathione (about 50%) that is strongly prevented, as in previous experiments (Figs. **13** and **15**), by NEM, DTE and SPM. The utilization of different

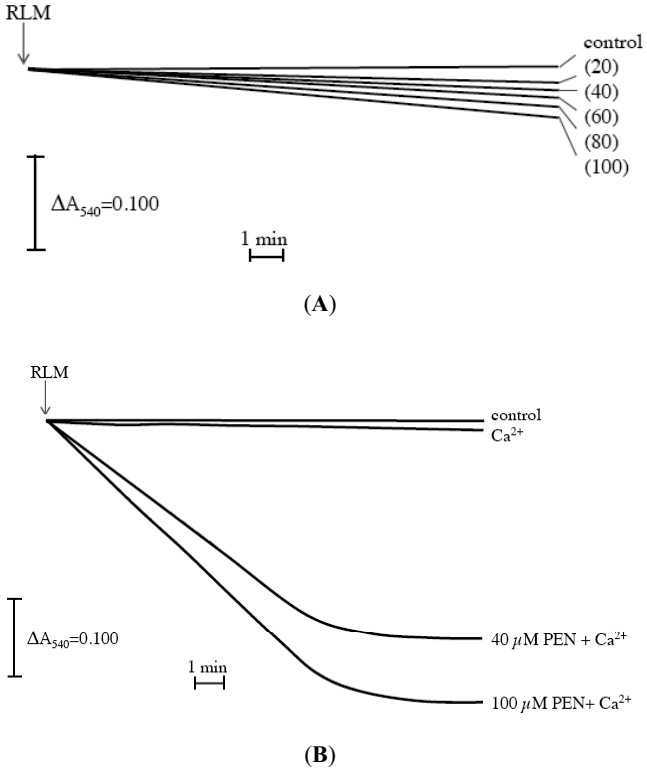


Fig. (9). Mitochondrial swelling induced by PEN in the absence (**A**) or in the presence of Ca^{2+} (**B**).
RLM were incubated in the same conditions as in (Fig. **8**). PEN was present at the indicated μM concentrations. Where present Ca^{2+} was 40 μM. A downward deflection indicates absorbance decrease. The assays were performed five times with comparable results.

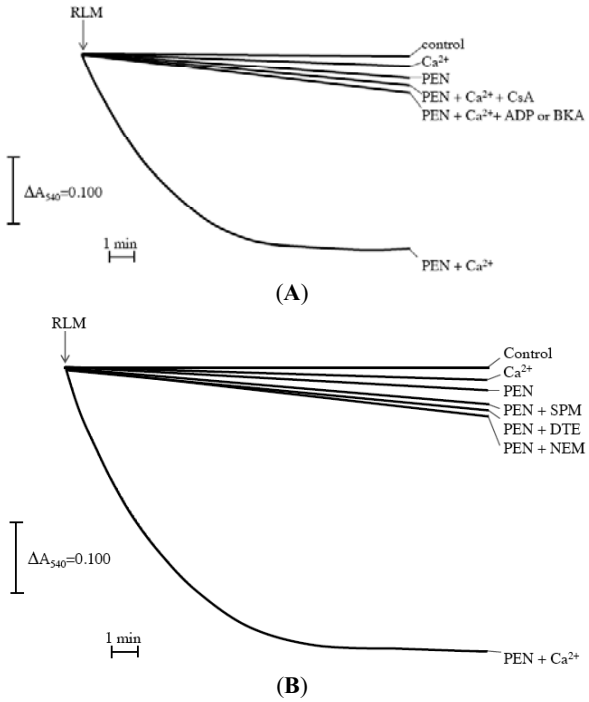


Fig. (10). Prevention of mitochondrial swelling induced by PEN and Ca^{2+}, by typical MPT inhibitors (**A**) and antioxidant agents (**B**).
RLM were incubated in the same conditions as in (Fig. **8**). PEN and Ca^{2+} were present at 40 μM concentration. When added to the medium: 1 μM Csa, 500 μM ADP, 5 μM BKA, 10 μM NEM, 5 mM DTE, 100 μM spermine (SPM). The assays were performed five times with comparable results.

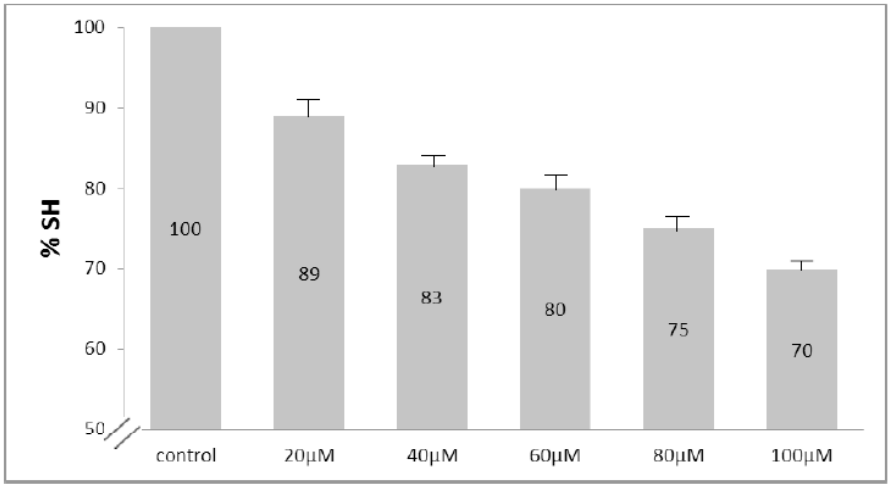


Fig. (11). Dose-dependent sulfydryl group oxidation induced by PEN.
RLM were incubated for 15 min in standard medium in the conditions described in Experimental Procedures. PEN was present at the indicated μM concentrations. Data are expressed as percentage of thiol reduction and represent average ± mean SD from five independent experiments. The histograms report the percentage value of SH.

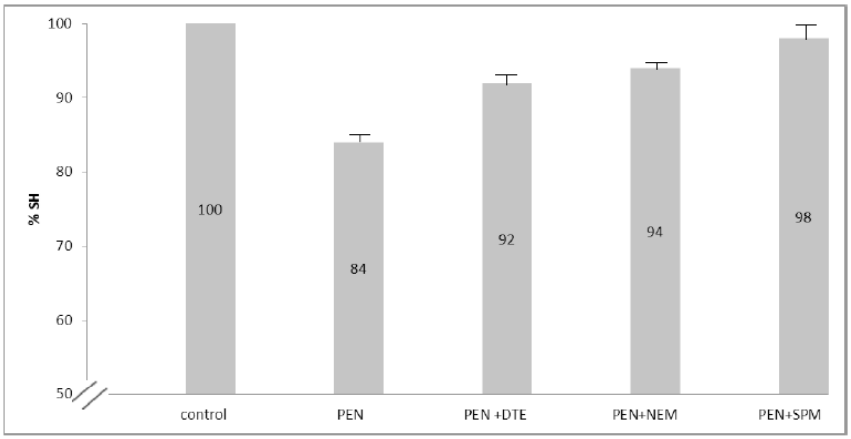


Fig. (12). Protection by antioxidant agents on sulfydryl group oxidation induced by PEN.
RLM incubation, data expression and statistics as in (Fig. **11**). PEN was present at 40 μM concentration. When added to the incubation medium: 10 μM NEM, 5 mM DTE, 100 μM SPM.

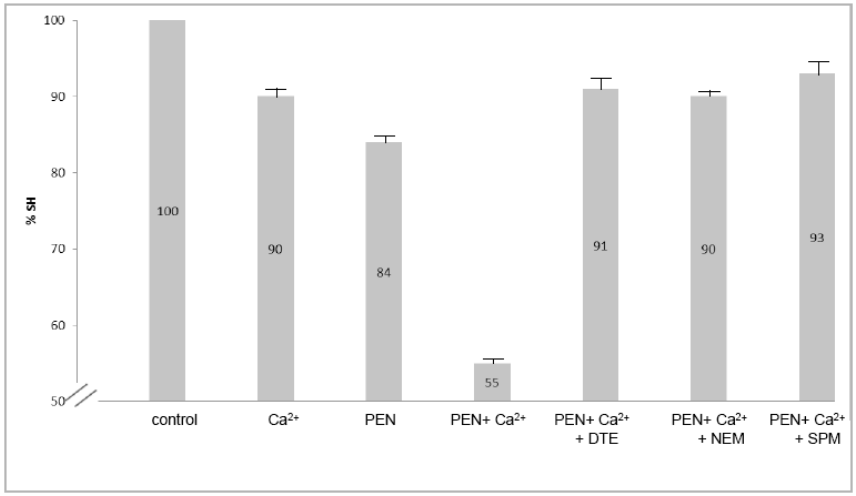


Fig. (13). Synergic action of PEN and Ca^{2+} in inducing sulfydryl group oxidation. Prevention by antioxidant agents.
RLM incubation, data expression and statistics as in (Fig. **11**). PEN and Ca^{2+} were present at 40 μM concentrations. When added to the incubation medium: 5 mM DTE, 10 μM NEM, 100 μM SPM.

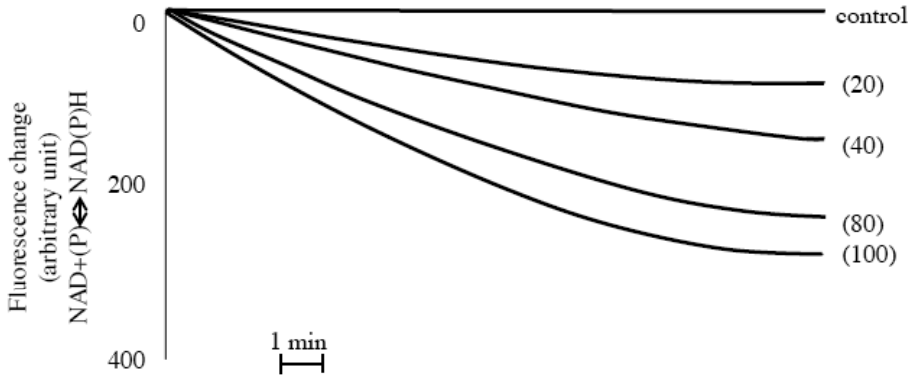


Fig. (14). Dose-dependent pyridine nucleotide oxidation induced by PEN.
RLM were incubated as in (Fig. **11**). PEN was present at the indicated μM concentrations. Five additional experiments exhibited same trend in fluorescence change.

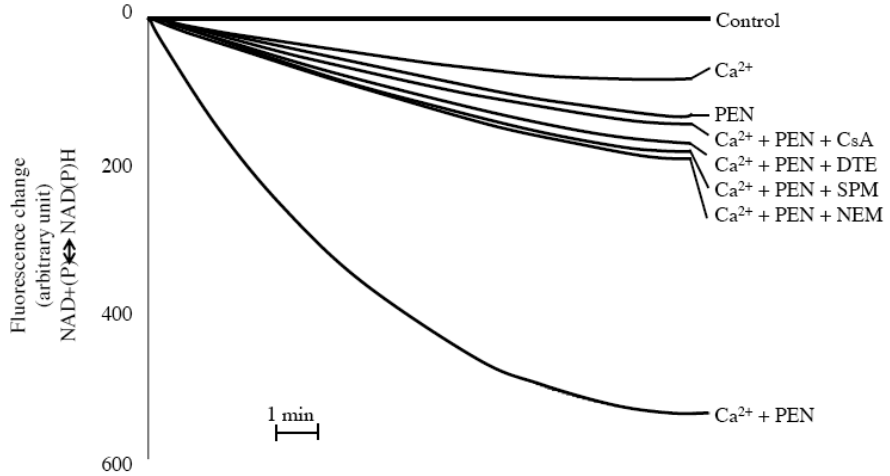


Fig. (15). Prevention by antioxidant agents and CsA on pyridine nucleotide oxidation induced by PEN in the presence of Ca^{2+}.
RLM were incubated as in (Fig. **11**). PEN and Ca^{2+} were present at 40 μM concentrations. When added to the incubation medium: 5 mM DTE, 5 μM NEM, 100 μM SPM. Five additional experiments exhibited same trend in fluorescence change.

concentrations of the antioxidant agents (10 μM NEM, 5mM DTE, 100 μM spermine) has been done in order to obtain their optimal effects in maintaining the reduced level in mitochondria. Thus, 10 μM NEM alkylates most of SH groups by preventing the oxidation without damaging mitochondrial level [176]. 5 mM DTE completely reduces all the sulfydryl groups [177]. 100 μM spermine is able to scavenge the reactive oxygen species by preventing the oxidative stress [178]. Overall, the results showed in (Figs. **11-16**) demonstrate that the ΔΨ collapse and the colloid-osmotic effects induced by PEN (Figs. **8** and **9**, respectively) are the result of a general oxidative stress, enhanced in the presence of Ca^{2+}. This statement is further confirmed by the dose-dependent production of hydrogen peroxide caused by 20-80 μM PEN (Fig. **17A**). The remarkable production of H_2O_2 observed in these conditions can account for the establishment of the oxidative stress. In the presence of Ca^{2+} the oxidative stress is further increased as in 10 min of incubation 40 μM PEN produces about 1.2 nmol/mg prot of H_2O_2, (Fig. **17B**), instead of about 0.35 nmol/mg prot produced by PEN itself (Fig. **17A**). Moreover, DTE, NEM, and CsA inhibit H_2O_2 generation, although only partially (Fig. **17B**). The finding that PEN plus Ca^{2+} causes ΔΨ collapse and large amplitude mitochondrial swelling by means of a general mitochondrial oxidation strongly supports the possibility of a rupture of the outer membrane with the resulting release of critical proteic pro-apoptotic factors. Actually, (Fig. **18A**) shows the release of cyt c induced by 40 μM PEN (lane 2), by Ca^{2+} (lane 3) and by both agents (lane 4). In this latter experimental condition, the release is significantly higher than that induced by Ca^{2+} or PEN by itself and it is almost completely prevented by CsA (lane 5). A comparable behavior is observed if the AIF release is determined in the same experimental conditions (Fig. **18B**).

DISCUSSION

Due to the important role played by mitochondria inside the activation of the apoptotic process, we investigate the ability of PEN to interfere with the physiological functions of these organelles, in order to elucidate the possible intracellular mechanism responsible for the observed cytotoxicity. In particular, because of the significant antiproliferative effect of PEN on HepG2, a human tumor cell line derived from a hepatocellular carcinoma, the investigation was performed on mitochondria isolated from rat liver.

Mitochondria play a role in physiological and pathophysiological mechanisms such as the process of oxidative phosphorylation and MPT. In the aerobic cells, phosphorylating respiration is responsible for production of about 95% of the total amount of ATP. Most of the formed ATP molecules is exported by ANT from mitochondria to the cytoplasm where it is hydrolyzed to support the energy consuming processes in the cell. Only small amount of ATP synthesized in mitochondria is consumed by these organelles. Thus, mitochondria are considered the "powerhouses" of the cell.

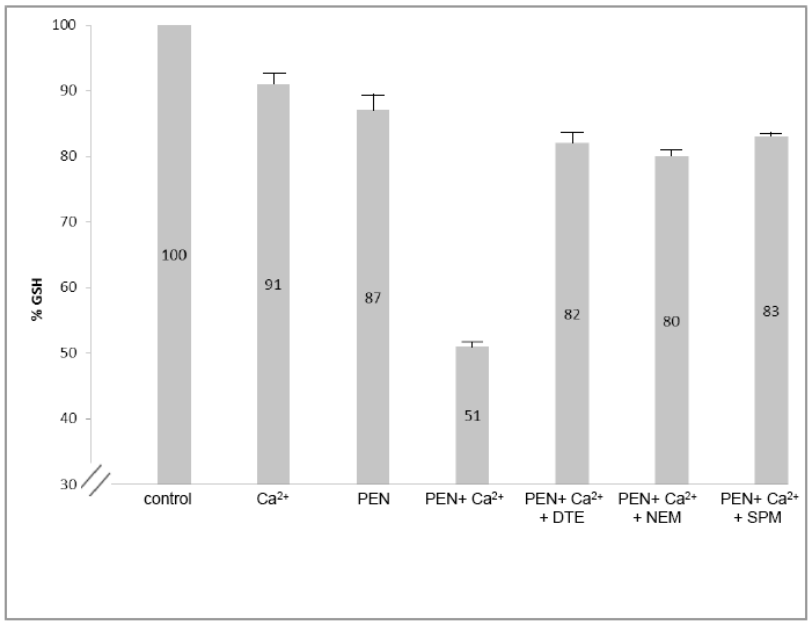
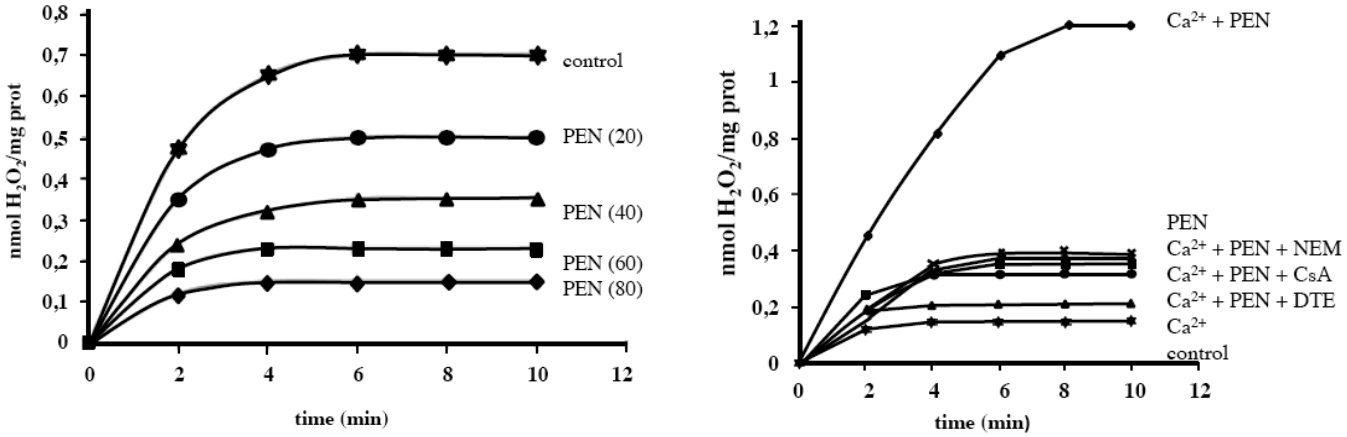


Fig. (16). Synergic action of PEN and Ca^{2+} in inducing GSH oxidation. Prevention by antioxidant agents.
RLM incubation, data expression and statistics as in (Fig. **11**). PEN and Ca^{2+} were present at 40 μM concentrations. When added to the incubation medium: 5 mM DTE, 5 μM NEM, 100 μM SPM.



(**A**) (**B**)

Fig. (17). Induction by PEN of a dose-dependent production of hydrogen peroxide (**A**). Synergic effect by Ca^{2+} on this production and partial prevention by the antioxidant agents and CsA (**B**).
RLM were incubated for 10 min in the conditions described in Experimental Procedures. Panel **A**: PEN was present at the indicated μM concentrations. Panel **B**: PEN and Ca^{2+} were present at 40 μM concentrations. When added to the incubation medium: 5 mM DTE, 5 μM NEM, 1 μM CsA. Assays performed four times with almost identical results.

The experimental results reported in this study show that the sterol PEN causes a dose-dependent gradual loss of the capacity of RLM to synthesize ATP (evaluated as RCI decrease). Nevertheless, also at a very high PEN concentration (100 μM) RLM maintain an RCI value sufficiently high to perform an efficient ATP synthesis (Fig. **7**). Otherwise, in the presence of Ca^{2+} a complete bioenergetics collapse is observable (RCI = 1), but in this case it is to consider the occurrence of the phenomenon of MPT induction (see below). The observed gradual but not complete loss in bioenergetic capacity, induced by PEN, is confirmed by the effects observable on the insulating membrane properties (Fig. **8A**). The concentration-dependent reduction in ΔΨ values demonstrates a change in the membrane permeability, most likely due to the opening of proton leaks. However, the constant trend of the PEN-induced ΔΨ values, although to a lower levels, means that the membrane has not undergone crucial structural damages. This is further supported by the inability of PEN to cause mitochondrial swelling (Fig. **9A**) by which, most likely, it just causes an increased proton permeability.

The loss of bioenergetics capacity caused in mitochondria by PEN might be accounted for the toxicity on human tumor cell lines (see Table **1**), but the partial effect on RCI measurements and the effects induced by PEN in the presence of Ca^{2+} on RCI, ΔΨ collapse, and mitochondrial swelling (see below), suggest that the drop in ATP synthesis might contribute just partially to cytotoxicity.

The full collapse in ΔΨ paralleled to the induction of a colloid-osmotic swelling and the inhibition of these events by typical inhibitors of MPT, such as CsA, ADP, and BKA (Figs. **8**-**10**), unequivocally demonstrate that, in the presence of Ca^{2+}, PEN behaves like a typical MPT inducer. Moreover, the inhibitory effect exerted by NEM, DTE and SPM supports the hypothesis that PEN-induced MPT may be the result of an oxidative stress. This is demonstrated

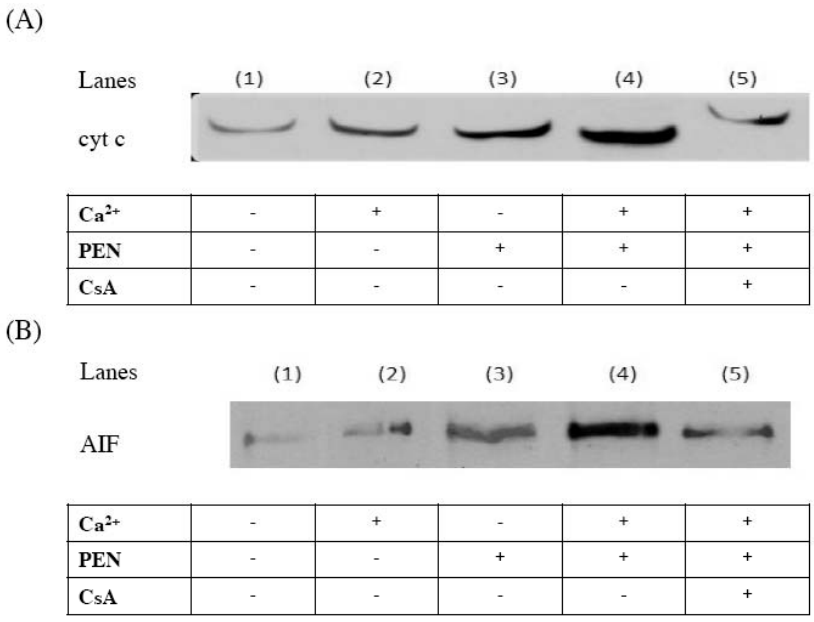


Fig. (18). Release of cyt c (**A**) and AIF (**B**) induced by PEN in the absence or presence of Ca^{2+}. Prevention by CsA.
Results of Western blotting on supernatant of mitochondrial incubations. RLM were incubated for 15 min in standard medium as described in Experimental Procedures. Lane 1: control (-Ca^{2+}); lane 2: 40 μM Ca^{2+}; lane 3: 40 μM PEN; lane 4: 40 μM PEN + 40 μM Ca^{2+}; lane 5: 40 μM PEN + 40 μM Ca^{2+} + 1 μM CsA. Assays performed four times with almost identical results.

by the ability of the sterol to induce oxidation of sulfydryl groups, pyridine nucleotides and glutathione and by the antioxidant effect of NEM, DTE and SPM (Figs. **11**-**16**). It should be recalled that to open the PTP (for reviews see [5, 7]) it is necessary that two main events take place: (i) Ca^{2+}, at supraphysiological concentration (40 μM in this case), must interact at the level of critical sites located on ANT in order to favor the subsequent interaction of CyPD with this protein [179]; (ii) two critical thiol groups, belonging to two cysteins located on ANT, have to be oxidized to form the corresponding disulphide bridge [180]. If only one of these events takes place, the pore remains closed. Ca^{2+}, besides interacting with ANT, also oxidizes 10% of SH (Fig. **13**) by a mechanism explained below. However, the pore is closed (see control curves with Ca^{2+} alone showed in (Figs. **8B**, **9B** and **10**)) because, most likely, the critical cysteins remain reduced. As regard PEN, at 40 μM concentration, it oxidizes 17% of SH (Fig. **11**), but also in this case the pore is closed (see control curves of (Figs. **8A**, **9A** and **10**)) since, even if the critical cysteins are oxidized, the absence of Ca^{2+} does not permit the interaction of CyPD with ANT. The pore opens when Ca^{2+} and PEN are both present (Figs. **8B**, **9B** and **10**), that is when the above mentioned events take place. In this case a very strong oxidation of thiol groups, of about 45%, occurs. This apparent synergism in SH oxidation is due to the opening of PTP that in turn, causes the collapse of the electrochemical gradient with an increased oxygen consumption and production of ROS, responsible of a further increase of oxidative stress. The observed oxidation of pyridine nucleotides and glutathione induced by PEN itself (Figs. **14**-**16**) accounts for the loss of the safety systems of mitochondria and for the involvement of hydrogen peroxide (Fig. **17**) and/or its derivatives in PTP opening. The enhancement of the extent of these oxidations in the presence of Ca^{2+} is related, as above mentioned for SH groups, to the opening of the PTP and all these conditions are called "redox catastrophe" [181]. The oxidation of thiols, pyridine nucleotides and glutathione by Ca^{2+} alone is, most probably, attributable to its interaction with membrane phospholipids, which should lead to a disorganization of membrane, to an alteration of ubiquinone mobility and of electron flux, resulting in ROS production [108]. However, this generation of ROS is not involved in the oxidation of critical SH groups responsible for PTP opening, presumably because these ROS are produced away from the critical thiols.

In conclusion, in the presence of Ca^{2+}, PEN acts as a typical MPT inducer via an interaction with the mitochondrial membrane that results into the generation of ROS and leads to a general oxidative stress. The mechanism of action of PEN is still under investigation. Nevertheless, preliminary results suggest that PEN acts at the level of electron transfer on bc_1 complex (the respiratory complex III) with the generation of H_2O_2 (for the reaction see [182]), as shown by the results of (Fig. **17**). Subsequently, part of H_2O_2, by interacting with Fe^{2+} of the respiratory complexes, may be transformed by means of the Fenton reaction in the high reactive hydroxyl radical, $OH^{\bullet}$. Hydroxyl radical is, most likely, the main responsible of the oxidative stress caused by PEN. Thus, the pathway of ROS generation should be addressed to reaching the critical thiol groups responsible for PTP opening. The opening of the PTP and the involvement of hydroxyl radical is supported by the protective effect of SPM on the observed oxidations (Figs. **13**, **15**, **16**) and on MPT induction (Fig. **10B**). Indeed, SPM is a specific scavenger of $OH^{\bullet}$ ineffective toward the other ROS [178]. The opening of the PTP and the consequent mitochondrial swelling, generally provokes the breakdown of outer membrane, resulting in the release of pro-apoptotic factors. Actually, the results of (Fig. **18**) demonstrate that PEN, in the presence of Ca^{2+}, causes the loss of the pro-apoptotic factors cyt c and AIF. The release of these factors accounts for the activation of two intrinsic apoptotic pathways: caspase-dependent for cyt c and caspase-independent for AIF. In this regard, the results of (Fig. **7**) showing a gradual loss of RCI induced by PEN suggest that mitochondria *in vivo* can maintain the capacity to synthesize ATP thus favoring a cell death by apoptosis.

The possibility of inducing cell death by apoptosis through two different pathways downstream mitochondria renders PEN a very interesting compound that could be further developed to obtain antiproliferative agents targeting mitochondrial functions to be utilized for more effective anticancer therapies.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

$\Delta\mu_H+$	=	Electrochemical membrane potential
ROS	=	Reactive oxygen species
MPT	=	Mitochondrial permeability transition
PTP	=	Permeability transition pore
VDAC	=	Voltage-dependent anion channel
ANT	=	Adenine nucleotide translocase
CypD	=	Cyclophilin D
CsA	=	Cyclosporine A
HK	=	Hexokinase
PBR	=	Peripheral benzodiazepine receptor
cyt c	=	Cytochrome c
AIF	=	Apoptosis inducing factor
EndoG	=	Endonuclease G
Bcl-2	=	B-cell lymphoma 2
OM	=	Outer mitochondrial membrane
IM	=	Inner mitochondrial membrane
HtrA	=	High-temperature requirement A
GSAO	=	4-(N-(S-glutathionylacetyl)amino phenylarsonous acid
PENAO	=	4-(N-(S-penicillaminylacetyl)amino) phenylarsonous acid
LND	=	Lonidamine
CLD	=	Clodronate
CD437	=	6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid
PK11195	=	1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide
3-BrPA	=	3-bromopyruvate
PEITC	=	Phenylethylisothiocyanate
BITC	=	Benzylisothiocyanate
α-TOS	=	α-tocopheryl succinate
BetA	=	3β, hydroxy-lup-20(29)-en-28-oic acid, betullinic acid
2DG	=	2-deoxyglucose
TTs	=	Triptycene analogues
PEN	=	Peniocerol
RCI	=	Respiratory control index
$\Delta\Psi$	=	Membrane potential
TPP^+	=	Tetraphenylphosphonium
RLM	=	Rat liver mitochondria
BKA	=	bongkrekic acid
SPM	=	spermine
DTE	=	dithiothreitol
NEM	=	N-ethylmaleimide

REFERENCES

[1] Li H, Hong ZH. Mitochondrial DNA mutations in human tumor cells. Oncol Lett 2012; 4: 868-72.

[2] Wallace DC. Mitochondria and cancer. Nat Rev Cancer 2012; 12: 685-98.

[3] Toninello A, Salvi M, Mondovì B. Interaction of biologically active amines with mitochondria and their role in the mitochondrial-mediated pathway of apoptosis. Curr Med Chem 2004; 11: 2349-74.

[4] Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. Nat Rev Drug Discov 2010; 9: 447-64.

[5] Zoratti M, Szabo I. The mitochondrial permeability transition. Biochim Biophys Acta 1995; 1241: 139-76.

[6] Sileikyte J, Petronilli V, Zulian A, *et al*. Regulation of the inner membrane mitochondrial permeability transition by the outer membrane translocator protein (peripheral benzodiazepine receptor). J Biol Chem 2011; 286: 1046-53.

[7] Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev 2007; 87: 99-163.

[8] Halestrap AP, Brenner C. The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. Curr Med Chem 2003; 10: 1507-25.

[9] McCommis KS, Baines CP. The role of VDAC in cell death: Friend or foe? Biochim Biophys Acta 2012; 1818: 1444-50.

[10] Crompton M, Virji S, Ward JM. Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. Eur J Biochem 1998; 258: 729-35.

[11] Majewski N, Nogueira V, Bhaskar P, *et al*. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. Mol Cell 2004; 16: 819-30.

[12] Arzoine L, Zilberberg N, Ben-Romano R, Shoshan-Barmatz V. Voltage-dependent anion channel 1-based peptides interact with hexokinase to prevent its anti-apoptotic activity. J Biol Chem 2009; 284: 3946-55.

[13] Szabó I, Zoratti M. The mitochondrial permeability transition pore may comprise VDAC molecules. I. Binary structure and voltage dependence of the pore. FEBS Lett 1993; 330: 201-5.

[14] Szabó I, De Pinto V, Zoratti M. The mitochondrial permeability transition pore may comprise VDAC molecules. II. The electrophysiological properties of VDAC are compatible with those of the mitochondrial megachannel. FEBS Lett 1993; 330: 206-10.

[15] Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. Nat Cell Biol 2007; 9: 550-5.

[16] Chiara F, Castellaro D, Marin O, *et al*. Hexokinase II detachment from mitochondria triggers apoptosis through the permeability transition pore independent of voltage-dependent anion channels. PLoS One 2008; 3: e1852.

[17] Pfaff E, Klingenberg M. Adenine nucleotide translocation of mitochondria. 1. Specificity and control. Eur J Biochem 1968; 6: 66-79.

[18] Pfaff E, Heldt HW, Klingenberg M. Adenine nucleotide translocation of mitochondria. Kinetics of the adenine nucleotide exchange. Eur J Biochem 1969; 10: 484-93.

[19] Brenner C, Subramaniam K, Pertuiset C, Pervaiz S. Adenine nucleotide translocase family: four isoforms for apoptosis modulation in cancer. Oncogene 2011; 30: 883-95.

[20] Brustovetsky N, Klingenberg M. Mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by Ca^{2+}. Biochemistry 1996; 35: 8483-8.

[21] Rasola A, Sciacovelli M, Pantic B, Bernardi P. Signal transduction to the permeability transition pore. FEBS Lett 2010; 584: 1989-96.

[22] Braestrup C, Squires RF. Specific benzodiazepine receptors in rat brain characterized by high-affinity (3H)diazepam binding. Proc Natl Acad Sci USA 1977; 74: 3805-09.

[23] Papadopoulos V, Baraldi M, Guilarte TR, *et al*. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci 2006; 27: 402-9.

[24] Panda V, Khambat P, Patil S. Mitocans as novel agents for anticancer therapy: an overview. Int J Clin Med 2011; 2: 515-29.

[25] Woods MJ, Williams DC. Multiple forms and locations for the peripheral-type benzodiazepine receptor. Biochem Pharmacol 1996; 52: 1805-14.

[26] Ricchelli F, Sileikytė J, Bernardi P. Shedding light on the mitochondrial permeability transition. Biochim Biophys Acta 2011; 1807: 482-90.

[27] Waldmeier PC, Zimmermann K, Qian T, Tintelnot-Blomley M, Lemasters JJ. Cyclophilin D as a drug target. Curr Med Chem 2003; 10: 1485-506.

[28] Du H, Guo L, Fang F, *et al*. Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease. Nat Med 2008; 14: 1097-105.

[29] Millay DP, Sargent MA, Osinska H, *et al*. Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy. Nat Med 2008; 14: 442-47.

[30] Palma E, Tiepolo T, Angelin A, *et al*. Genetic ablation of cyclophilin D rescues mitochondrial defects and prevents muscle apoptosis in collagen VI myopathic mice. Hum Mol Genet 2009; 18: 2024-31.

[31] Irwin WA, Bergamin N, Sabatelli P, *et al*. Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. Nat Genet 2003; 35: 367-71.

[32] Fournier N, Ducet G, Crevat A. Action of cyclosporine on mitochondrial calcium fluxes. J Bioenerg Biomembr 1987; 19: 297-303.

[33] Basso E, Petronilli V, Forte MA, Bernardi P. Phosphate is essential for inhibition of the mitochondrial permeability transition pore by cyclosporin A and by cyclophilin D ablation. J Biol Chem 2008; 283: 26307-11.

[34] Chinopoulos C, Adam-Vizi V. Modulation of the mitochondrial permeability transition by cyclophilin D: moving closer to F_0-F_1 ATP synthase? Mitochondrion 2012; 12: 41-5.

[35] Kilbride SM, Prehn JH. Central roles of apoptotic proteins in mitochondrial function. Oncogene 2013; 32: 2703-11.

[36] Li K, Li Y, Shelton JM, *et al*. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. Cell 2000; 101: 389-99.

[37] Li P, Nijhawan D, Budihardjo I, *et al*. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997; 91: 479-89.

[38] Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G. Mechanisms of cytochrome c release from mitochondria. Cell Death Differ 2006; 13: 1423-33.

[39] Hao Z, Duncan GS, Chang CC, *et al*. Specific ablation of the apoptotic functions of cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. Cell 2005; 121: 579-91.

[40] Sevrioukova IF. Apoptosis-inducing factor: structure, function, and redox regulation. Antioxid Redox Signal 2011; 14: 2545-79.

[41] Susin SA, Zamzami N, Castedo M, *et al*. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J Exp Med 1996; 184: 1331-41.

[42] Otera H, Ohsakaya S, Nagaura Z, Ishihara N, Mihara K. Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. EMBO J 2005; 24: 1375-86.

[43] Vahsen N, Candé C, Brière JJ, *et al*. AIF deficiency compromises oxidative phosphorylation. EMBO J 2004; 23: 4679-89.

[44] Klein JA, Longo-Guess CM, Rossmann MP, *et al*. The harlequin mouse mutation downregulates apoptosis-inducing factor. Nature 2002; 419: 367-74.

[45] van Empel VP, Bertrand AT, van der Nagel R, *et al*. Downregulation of apoptosis-inducing factor in harlequin mutant mice sensitizes the myocardium to oxidative stress-related cell death and pressure overload-induced decompensation. Circ Res 2005; 96: e92-e101.

[46] Delavallée L, Cabon L, Galán-Malo P, Lorenzo HK, Susin SA. AIF-mediated caspase-independent necroptosis: a new chance for targeted therapeutics. IUBMB Life 2011; 63: 221-32.

[47] Artus C, Boujrad H, Bouharrour A, *et al*. AIF promotes chromatinolysis and caspase-independent programmed necrosis by interacting with histone H2AX. EMBO J 2010; 29: 1585-99.

[48] Susin SA, Daugas E, Ravagnan L, *et al*. Two distinct pathways leading to nuclear apoptosis. J Exp Med 2000; 192: 571-80.

[49] Susin SA, Lorenzo HK, Zamzami N, *et al*. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999; 397: 441-6.

[50] Widlak P, Garrard WT. Discovery, regulation, and action of the major apoptotic nucleases DFF40/CAD and endonuclease G. J Cell Biochem 2005; 94: 1078-87.

[51] Qin S, Yang C, Li S, Xu C, Zhao Y, Ren H. Smac: Its role in apoptosis induction and use in lung cancer diagnosis and treatment. Cancer Lett 2012; 318: 9-13.

[52] Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000; 102: 33-42.

[53] Hansen TM, Smith DJ, Nagley P. Smac/DIABLO is not released from mitochondria during apoptotic signalling in cells deficient in cytochrome c. Cell Death Differ 2006; 13: 1181-90.

[54] Crook NE, Clem RJ, Miller LK. An apoptosis inhibiting baculovirus gene with a zinc finger like motif. J Virol 1993; 67: 2168-74.

[55] Liston P, Roy N, Tamai K, *et al*. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 1996; 379: 349-53.

[56] Uren AG, Pakusch M, Hawkins CJ, Puls KL, Vaux DL. Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. Proc Natl Acad Sci USA 1996; 93: 4974-8.

[57] Deveraux QL, Reed TC. IAP family proteins-suppressors of apoptosis. Genes Dev 1999; 13: 239-52.

[58] Verhagen AM, Vaux DL. Cell death regulation by the mammalian IAP antagonist Diablo/Smac. Apoptosis 2002; 7: 163-6.

[59] Vande Walle L, Lamkanfi M, Vandenabeele P. The mitochondrial serine protease HtrA2/Omi: an overview. Cell Death Differ 2008; 15: 453-60.

[60] Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell 2001; 8: 613-21.

[61] Jones JM, Datta P, Srinivasula SM, *et al*. Loss of Omi mitochondrial protease activity causes the neuromuscular disorder of mnd2 mutant mice. Nature 2003; 425: 721-7.

[62] Strauss KM, Martins LM, Plun-Favreau H, *et al*. Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. Hum Mol Genet 2005; 14: 2099-111.

[63] Martins LM, Morrison A, Klupsch K, *et al*. Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. Mol Cell Biol 2004; 24: 9848-62.

[64] Yang QH, Church-Hajduk R, Ren J, Newton ML, Du C. Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. Genes Dev 2003; 17: 1487-96.

[65] Kuninaka S, Nomura M, Hirota T, *et al*. The tumor suppressor WARTS activates the Omi/HtrA2-dependent pathway of cell death. Oncogene 2005; 24: 5287-98.

[66] Vande Walle L, Van Damme P, Lamkanfi M, *et al*. Proteome-wide identification of HtrA2/Omi substrates. J Proteome Res 2007; 6: 1006-15.

[67] Wei MC, Zong WX, Cheng EH, *et al*. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001; 292: 727-30.

[68] Farrow SN, White JH, Martinou I, *et al*. Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. Nature 1995; 374: 731-3.

[69] Cartron PF, Juin P, Oliver L, Martin S, Meflah K, Vallette FM. Nonredundant role of Bax and Bak in Bid-mediated apoptosis. Mol Cell Biol 2003; 23: 4701-12.

[70] Amsel AD, Rathaus M, Kronman N, Cohen HY. Regulation of the proapoptotic factor Bax by Ku70-dependent deubiquitylation. Proc Natl Acad Sci USA 2008; 105: 5117-22.

[71] Guo B, Zhai D, Cabezas E, *et al*. Humanin peptide suppresses apoptosis by interfering with Bax activation. Nature 2003; 423: 456-61.

[72] Nomura M, Shimizu S, Sugiyama T, *et al*. 14-3-3 Interacts directly with and negatively regulates pro-apoptotic Bax. J Biol Chem 2003; 278: 2058-65.

[73] Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. J Cell Sci 2009; 122: 437-41.

[74] Narita M, Shimizu S, Ito T, *et al*. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci USA 1998; 95: 14681-6.

[75] Pastorino JG, Tafani M, Rothman RJ, Marcinkeviciute A, Hoek JB, Farber JL. Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. J Biol Chem 1999; 274: 31734-9.

[76] De Giorgi F, Lartigue L, Bauer MK, *et al*. The permeability transition pore signals apoptosis by directing Bax translocation and multimerization. FASEB J 2002; 16: 607-9.

[77] Precht TA, Phelps RA, Linseman DA, *et al*. The permeability transition pore triggers Bax translocation to mitochondria during neuronal apoptosis. Cell Death Differ 2005; 12: 255-65.

[78] Whelan RS, Konstantinidis K, Wei AC, *et al*. Bax regulates primary necrosis through mitochondrial dynamics. Proc Natl Acad Sci USA 2012; 109: 6566-71.

[79] Gallerne C, Touat Z, Chen ZX, *et al*. The fourth isoform of the adenine nucleotide translocator inhibits mitochondrial apoptosis in cancer cells. Int J Biochem Cell Biol 2010; 42: 623-9.

[80] Chevrollier A, Loiseau D, Reynier P, Stepien G. Adenine nucleotide translocase 2 is a key mitochondrial protein in cancer metabolism. Biochim Biophys Acta 2011; 1807: 562-7.

[81] Park D, Chiu J, Perrone GG, Dilda PJ, Hogg PJ. The tumour metabolism inhibitors GSAO and PENAO react with cysteines 57 and 257 of mitochondrial adenine nucleotide translocase. Cancer Cell Int 2012; 12: 11.

[82] Del Bufalo D, Trisciuoglio D, Scarsella M, *et al*. Lonidamine causes inhibition of angiogenesis-related endothelial cell functions. Neoplasia 2004; 6: 513-22.

[83] Del Bufalo D, Biroccio A, Soddu S, *et al*. Lonidamine induces apoptosis in drug-resistant cells independently of the p53 gene. J Clin Invest 1996; 98: 1165-73.

[84] Biroccio A, Del Bufalo D, Fanciulli M, Bruno T, Zupi G, and Floridi A. Bcl-2 inhibits mitochondrial metabolism and lonidamine-induced apoptosis in adriamycin-resistant MCF7 cells. Int J Cancer 1999; 82: 125-30.

[85] Ravagnan L, Marzo I, Costantini P, *et al*. Lonidamine triggers apoptosis via a direct, Bcl-2 -inhibited effect on the mitochondrial permeability transition pore. Oncogene 1999; 18: 2537-46.

[86] Belzacq AS, El Hamel C, Vieira HL, *et al*. Adenine nucleotide translocator mediates the mitochondrial membrane permeabilization induced by lonidamine, arsenite and CD437. Oncogene 2001; 20: 7579-87.

[87] Lehenkari PP, Kellinsalmi M, Näpänkangas JP, *et al*. Further insight into mechanism of action of clodronate: inhibition of mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing metabolite. Mol Pharmacol 2002; 61: 1255-62.

[88] Green JR, Clezardin P. Mechanisms of bisphosphonate effects on osteoclasts, tumor cell growth, and metastasis. Am J Clin Oncol 2002; 25: S3-S9.

[89] Montague R, Hart CA, George NJ, Ramani VA, Brown MD, Clarke NW. Differential inhibition of invasion and proliferation by bisphosphonates: anti-metastatic potential of zoledronic acid in prostate cancer. Eur Urol 2004; 46: 389-401.

[90] Lee MV, Fong EM, Singer FR, Guenette RS. Bisphosphonate treatment inhibits the growth of prostate cancer cells. Cancer Res 2001; 61: 2602-8.

[91] Sato M, Grasser W, Endo N, *et al*. Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. J Clin Invest 1991; 88: 2095-105.

[92] Senaratne SG, Mansi JL, Colston KW. The bisphosphonate zoledronic acid impairs Ras membrane localisation and induces cytochrome c release in breast cancer cells. Br J Cancer 2002; 86: 1479-86.

[93] Marchetti P, Zamzami N, Joseph B, *et al.* The novel retinoid 6-[3-(1-adamantyl)- 4-hydroxyphenyl]-2-naphtalene carboxylic acid can trigger apoptosis through a mitochondrial pathway independent of the nucleus. Cancer Res 1999, 59: 6257-66.

[94] Notario, B, Zamora, M, Vinas O, Mampel T. All-trans-retinoic acid binds to and inhibits adenine nucleotide translocase and induces mitochondrial permeability transition. Mol Pharmacol 2003; 63: 224-31.

[95] Mukhopadhyay S, Guillory B, Mukherjee S, Das SK. Antiproliferative effect of peripheral benzodiazepine receptor antagonist PK11195 in rat mammary tumor cells. Mol Cell Biochem 2010; 340: 203-13.

[96] Azarashvili T, Grachev D, Krestinina O, *et al*. The peripheral-type benzodiazepine receptor is involved in control of Ca^{2+}-induced permeability transition pore opening in rat brain mitochondria. Cell Calcium 2007; 42: 27-39.

[97] Gonzalez-Polo RA, Carvalho G, Braun T, *et al.* PK11195 potently sensitizes to apoptosis induction independently from the peripheral benzodiazepin receptor. Oncogene 2005; 24: 7503-13.

[98] Decaudin D, Castedo M, Nemati F, *et al.* Peripheral benzodiazepine receptor ligands reverse apoptosis resistance of cancer cells *in vitro* and *in vivo*. Cancer Res 2002; 62: 1388-93.

[99] Hirsch T, Decaudin, D, Susin, SA, *et al*. PK11195, a ligand of the mitochondrial benzodiazepine receptor, facilitates the induction of apoptosis and reverses Bcl-2-mediated cytoprotection. Exp Cell Res 1998; 241: 426-34.

[100] Rodrigues-Ferreira C, Pereira da Silva AP, Galina A. Effect of the antitumoral alkylating agent 3-bromopyruvate on mitochondrial respiration: role of mitochondrially bound hexokinase. J Bioenerg Biomembr 2012; 44: 39-49.

[101] Kim JS, Ahn KJ, Kim JA, *et al.* Role of reactive oxygen species-mediated mitochondrial dysregulation in 3-bromopyruvate induced cell death in hepatoma cells. J Bioenerg Biomembr 2008; 40: 607-18.

[102] Chen Z, Xu R, Achanta G, Zhang H and Huang P. Critical role of hexokinase II in 3-Bromopyruvate-induced cell death: A potential target for cancer therapeutics. Proc Am Assoc Canc Res 2006; 47.

[103] Chen Z, Zhang H, Lu W, Huang P. Role of mitochondria-associated hexokinase II in cancer cell death induced by 3-bromopyruvate. Biochim Biophys Acta 2009; 1787: 553-60.

[104] Ganapathy-Kanniappan S, Vali M, Kunjithapatham R, *et al.* 3-bromopyruvate: a new targeted antiglycolytic agent and a promise for cancer therapy. Curr Pharm Biotechnol 2010; 11: 510-7.

[105] Rotem R, Heyfets A, Fingrut O, *et al.* Novel anticancer agents acting directly and selectively on human cancer cell mitochondria. Cancer Res 2005; 65: 1984-93.

[106] Goldin N, Heyfets A, Reischer D, Flescher E. Mitochondria-mediated ATP depletion by anti-cancer agents of the jasmonate family. J Bioenerg Biomembr 2007; 39: 51-7.

[107] Goldin N, Arzoine L, Heyfets A, *et al.* Methyl jasmonate binds to and detaches mitochondria-bound hexokinase. Oncogene 2008; 27: 4636-43.

[108] Vercesi AE, Kowaltowski AJ, Grijalba MT, Meinicke AR, Castilho RF. The Role of Reactive Oxygen Species in Mitochondrial Permeability Transition. Bioscience Rep 1997; 17: 43-52.

[109] Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. 2nd ed. Halliwell and Gutteridge: Oxford Press 1989.

[110] Vercesi AE, Hoffman ME. Methods in Toxicology. Mitochondrial Dysfunction. Jones and Lash: Academic Press 1993.

[111] Larochette N, Decaudin D, Jacotot E, *et al*. Arsenite induces apoptosis via a direct effect on the mitochondrial permeability transition pore. Exp Cell Res 1999; 249: 413-21.

[112] Nutt LK, Gogvadze V, Uthaisang W, Mirnikjoo B, McConkey DJ, Orrenius S. Indirect effects of Bax and Bak initiate the mitochondrial alterations that lead to cytochrome c release during arsenic trioxide-induced apoptosis. Cancer Biol Ther 2005; 4: 459-67.

[113] Lu J, Chew EH, Holmgren A. Targeting thioredoxin reductase is a basis for cancer therapy by arsenic trioxide. Proc Natl Acad Sci USA 2007; 104: 12288-93.

[114] Xiao D, Lew KL, Zeng Y, *et al*. Phenethyl isothiocyanate-induced apoptosis in PC-3 human prostate cancer cells is mediated by reactive oxygen species-dependent disruption of the mitochondrial membrane potential. Carcinogenesis 2006; 27: 2223-34.

[115] Trachootham D, Zhou Y, Zhang H, *et al*. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by b-phenylethyl isothiocyanate. Cancer Cell 2006; 10: 241-52.

[116] Xiao D, Powolny AA, Moura MB, *et al*. Phenethyl Isothiocyanate Inhibits Oxidative Phosphorylation to Trigger Reactive Oxygen Species-mediated Death of Human Prostate Cancer Cells. J Biol Chem 2010; 285: 26558-69.

[117] Chen PY, Lin KC, Lin JP. Phenethyl Isothiocyanate (PEITC) inhibits the growth of human oral squamous carcinoma HSC-3 cells through G0/G1 phase arrest and mitochondria-mediated apoptotic cell death. Evid-Based Compl Alt Med 2012; 2012: 718320.

[118] Gertz M, Steegborn C. The Lifespan-regulator p66Shc in mitochondria: redox enzyme or redox sensor? Antioxid Redox Sign 2010; 13: 1417-28.

[119] Xiao D, Singh SV. p66Shc is indispensable for phenethyl isothiocyanate-induced apoptosis in human prostate cancer cells. Cancer Res 2010; 70: 3150-8.

[120] Giorgio M, Migliaccio E, Orsini F, *et al*. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell 2005; 122: 221-33.

[121] Xiao D, Powolny AA, Singh SV. Benzyl isothiocyanate targets mitochondrial respiratory chain to trigger reactive oxygen species dependent apoptosis in human breast cancer cells. J Biol Chem 2008; 283: 30151-63.

[122] Kruspig B, Nilchian A, Bejarano I, Orrenius S, Zhivotovsky B, Gogvadze V. Targeting mitochondria by α-tocopheryl succinate kills neuroblastoma cells irrespective of MycN oncogene expression. Cell Mol Life Sci 2012; 69: 2091-9.

[123] Ottino P, Duncan JR. Effect of α-tocopherol succinate on free radical and lipid peroxidation levels in BL6 melanoma cells. Free Radic Biol Med 1997; 22: 1145-51.

[124] Zhao Y, Li R, Xia W. Bid integrates intrinsic and extrinsic signaling in apoptosis induced by a-tocopheryl succinate in human gastric carcinoma cells. Cancer Lett 2010; 288: 42-9.

[125] Wang H, Haridas V, Gutterman JU, Xu ZX. Natural triterpenoid avicins selectively induce tumor cell death. Commun Integr Biol 2010; 3: 205-8.

[126] Haridas V, Higuchi M, Jayatilake GS, *et al.* Avicins: triterpenoid saponins from *Acacia victoriae* (Bentham) induce apoptosis by mitochondrial perturbation. Proc Natl Acad Sci USA 2001; 98: 5821-6.

[127] Mujoo K, Haridas V, Hoffmann JJ, *et al.* Triterpenoid saponins from *Acacia victoriae* (Bentham) decrease tumor cell proliferation and induce apoptosis. Cancer Res 2001; 61: 5486-90.

[128] Haridas V, Li XX, Higuchi M, Colombini M, and Gutterman JU, unpublished data in Li XX, Davis B, Haridas V, Gutterman JU, Colombini M. Proapoptotic Triterpene Electrophiles (Avicins) Form Channels in Membranes: Cholesterol Dependence. Biophys J 2005; 88: 2577-84.

[129] Petronelli A, Pannitteri G, Testa U. Triterpenoids as new promising anticancer drugs. Anticancer Drugs 2009; 20: 880-92.

[130] Thibeault D, Gauthier C, Legault J, Bouchard J, Dufour P, Pichette A. Synthesis and structure-activity relationship study of cytotoxic germanicane- and lupane-type 3β-O-monodesmosidic saponins starting from betulin. Bioorg Med Chem 2007; 15: 6144-57.

[131] Santos RC, Salvador JA, Cortés R, Pachón G, Marín S, Cascante M. New betulinic acid derivatives induce potent and selective antiproliferative activity through cell cycle arrest at the S phase and caspase dependent apoptosis in human cancer cells. Biochimie 2011; 93: 1065-75.

[132] Mullauer FB, Kessler JH, Medema JP. Betulinic acid induces cytochrome c release and apoptosis in a Bax/Bak-independent, permeability transition pore dependent fashion. Apoptosis 2009; 14: 191-202.

[133] Maschek G, Savaraj N, Priebe W, *et al*. 2-Deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers *in vivo*. Cancer Res 2004; 64: 31-4.

[134] Stein M, Lin H, Jeyamohan C. Targeting tumor metabolism with 2-deoxyglucose in patients with castrate-resistant prostate cancer and advanced malignancies. Prostate 2010; 70: 1388-94.

[135] Merchan JR, Kovács K, Railsback JW, *et al.* Antiangiogenic activity of 2-deoxy-D-glucose. PLoS ONE 2010; 5: e13699

[136] Mc Combf RB, Yushok WD. Metabolism of ascites tumor cells IV. Enzymatic reactions involved in adenosinetriphosphate degradation induced by 2-deoxyglucose. Cancer Res 1964; 24: 198-205.

[137] Karczmar GS, Arbeit JM, Toy BJ, Speder A, Weiner MW. Selective depletion of tumor ATP by 2-deoxyglucose and insulin, detected by ^{31}P magnetic resonance spectroscopy. Cancer Res 1992; b52: 71-6.

[138] Ahmad IM, Aykin-Burns N, Sim JE, *et al*. Mitochondrial $O_2^{\bullet-}$ and H_2O_2 mediate glucose deprivation-induced cytotoxicity and oxidative stress in human cancer cells. J Biol Chem 2005; 280: 4254-63.

[139] Lin X, Zhang F, Bradbury CM, *et al*. 2-Deoxy-D-glucose-induced cytotoxicity and radiosensitization in tumor cells is mediated via disruptions in thiol metabolism. Cancer Res 2003; 63: 3413-7.

[140] Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281: 1309-12.

[141] Zhang XD, Deslandes E, Villedieu M, *et al*. Effect of 2-Deoxy-D-glucose on various malignant cell lines *in vitro*. Anticancer Res 2006; 26: 3561-6.

[141] Perchellet EM, Wang Y, Lou K, *et al*. Antitumor triptycene analogs directly interact with isolated mitochondria to rapidly trigger markers of permeability transition. Anticancer Res 2007; 27: 3259-72.

[143] Karpinich NO, Tafani M, Rothman RJ, Russo MA, Farber JL. The course of etoposide-induced apoptosis from damage to DNA and p53 activation to mitochondrial release of cytochrome c. J Biol Chem 2002; 277: 16547-52.

[144] Karpinich NO, Tafani M, Schneider T, Russo MA, Farber JL. The course of etoposide-induced apoptosis in Jurkat cells lacking p53 and Bax. J Cell Physiol 2006; 208: 55-63.

[145] Custòdio JBA, Cardoso CMP, Madeira VMC, Almeida LM. Mitochondrial permeability transition induced by the anticancer drug etoposide. Toxicol *in vitro* 2001; 15: 265-70.

[146] Jung K, Reszka R. Mitochondria as subcellular targets for clinically useful anthracyclines. Adv Drug Deliv Rev 2001; 49: 87-105.

[147] Huigsloot M, Tijdens IB, Mulder GI, van de Water B. Differential regulation of doxorubicin-induced mitochondrial dysfunction and apoptosis by Bcl-2 in mammary adenocarcinoma (MTLn3) cells. J Biol Chem 2002; 277: 35869-79.

[148] Tao Z, Withers HG, Penefsky HS, Goodisman J, Souid A. Inhibition of cellular respiration by doxorubicin. Chem Res Toxicol 2006; 19: 1051-8.

[149] Munteanu E, Verdier M, Grandjean-Forestier F, *et al*. Mitochondrial localization and activity of P-glycoprotein in doxorubicin-resistant K562 cells. Biochem Pharmacol 2006; 71: 1162-74.

[150] Gong Y, Wang Y, Chen F, *et al*. Identification of the subcellular localization of daunorubicin in multidrug-resistant K562 cell line. Leukocyte Res 2000; 24: 769-74.

[151] Lin JH, Castora FJ. DNA topoisomerase II from mammalian mitochondria is inhibited by the antitumor drugs, m-AMSA and VM-26. Biochem Biophys Res Commun 1991; 176: 690-7

[152] Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Distinct pathways for stimulation of cytochrome c release by etoposide. J Biol Chem 2000; 275: 32438-43

[153] Dalla Via L, Marini AM, Salerno S, Toninello A. Mitochondrial permeability transition induced by novel pyridothiopyranopyrimidine derivatives: potential new antimitochondrial antitumour agents. Biochem Pharmacol 2006; 72: 1657-67.

[154] Dalla Via L, Marini AM, Salerno S, *et al*. Synthesis and biological activity of 1,4-dihydrobenzothiopyrano[4,3-*c*]pyrazole derivatives, novel pro-apoptotic mitochondrial targeted agents. Bioorg Med Chem 2009; 17: 326-36.

[155] Dalla Via L, Braga A, García-Argáez AN, Martínez-Vázquez M, Toninello A. 3α-Hydroxymasticadienonic acid as an antiproliferative agent that impairs mitochondrial functions. J Nat Prod 2012; 75: 557-62.

[156] Céspedes CL, Salazar JR, Martínez M, Aranda E. Insect growth regulatory effects of some extracts and sterols from *Myrtillocactus geometrizans* (Cactaceae) against *Spodoptera frugiperda* and *Tenebrio molitor*. Phytochemistry 2005; 66: 2481-93.

[157] Salazar JR, Martínez-Vazquez M, Cespedes CL, *et al*. Anti-inflammatory and cytotoxic activities of chichipegenin, peniocerol, and macdougallin isolated from *Myrtillocactus geometrizans* (Mart. ex Pfeiff.) Con. Z Naturforsch C 2011; 66: 24-30.

[158] Arias S, Gamma S, Guzmán L. 1997 Flora del Valle de Tehuacán-Cuicatlán. Fascículo 14. Cactacee A.L. Juss. Instituto de Biología, UNAM, México D.F., México.

[159] Salvi M, Brunati A M, Bordin L, La Rocca N, Clari G, Toninello A. Characterization and location of Src-dependent tyrosine phosphorylation in rat brain mitochondria. Biochim Biophys Acta 2002; 1589: 181-95.

[160] Pardee AB, Potter R. Inhibition of succinic dehydrogenase by oxalacetate. J Biol Chem 1948; 176: 1085-94.

[161] Salvi M, Battaglia V, Brunati AM, *et al*. Catalase takes part in rat liver mitochondria oxidative stress defense. J Biol Chem 2007; 282: 24407-15.

[162] Grancara S, Battaglia V, Martinis P, *et al*. Mitochondrial oxidative stress induced by Ca^{2+} and monoamines: different behaviour of liver and brain mitochondria in undergoing permeability transition. Amino Acids 2012; 42: 751-9.

[163] Salvi M, Battaglia V, Mancon M, *et al*. Agmatine is transported into liver mitochondria by a specific electrophoretic mechanism. Biochem J 2006; 396: 337-45.

[164] Blazyk JF, Steim JM. Phase transition in mammalian membranes. Biochim Biophys Acta 1972; 266: 737-41.

[165] Ricchelli F, Gobbo S, Moreno G, Salet C. Changes of the fluidity of mitochondrial membranes induced by the permeability transition. Biochemistry 1999; 38: 9295-300.

[166] Estabrook RW. Mitochondrial respiratory control and the polarographic measurement of ADP/O ratio. Meth Enzymol 1967; 10: 41-7.

[167] Kamo N, Muratsugu M, Hongoh R, Kobatake Y. Membrane potential of mitochondria measured with an electrode sensitive to tetra-

phenylphosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. J Membr Biol 1979; 49: 105-21.

[168] Palmieri F, Klingenberg M. Direct methods for measuring metabolite transport and distribution in mitochondria. Meth Enzymol 1979; 55: 279-301.

[169] Jensen BD, Gunter TE. The use of tetraphenylphosphonium (TPP^+) to measure membrane potentials in mitochondria: membrane binding and respiratory effects. Biophys J 1984; 45: 92 (abstr.).

[170] Karadyov JS, Kudzina L, Yu Zinchenko VP. TPP^+ inhibits Na^+-stimulated Ca^{2+} efflux from brain mitochondria. Cell Calcium 1986; 7: 115-9.

[171] Jensen BD, Gunter KK, Gunter TE. The efficiencies of the component steps of oxidative phosphorylation. II. Experimental determination of the efficiencies in mitochondria and examination of the equivalence of membrane potential and pH gradient in phosphorylation. Arch Biochem Biophys 1986; 248: 305-23.

[172] Santos AC, Uyemura SA, Lopes JL, Bazon JN, Mingatto FE, Curti C. Effect of naturally occurring flavonoids on lipid peroxidation and membrane permeability transition in mitochondria. Free Radic Biol Med 1998; 24: 1455-61.

[173] Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Anal Biochem 1969; 27: 502-22.

[174] Moore G, Rossi L, Nicotera P, Orrenius S, O'Brien P. Quinone toxicity in hepatocytes: studies on mitochondrial Ca^{2+} release induced by benzoquinone derivatives. Arch Biochem Biophys 1987; 259: 283-95.

[175] Matsumoto T, Furuta T, Nimura Y, Suzuki O. Increased sensitivity of the fluorometric method of Snyder and Hendley for oxidase assays. Biochem Pharmacol 1982; 31: 2207-9.

[176] Battaglia V, Salvi M, Toninello A. Oxidative stress is responsible for mitochondrial permeability transition induction by salicylate in liver mitochondria. J Biol Chem 2005; 280: 33864-72.

[177] Martinis P, Zago L, Maritati M, *et al.* Interactions of melatonin with mammalian mitochondria. Reducer of energy capacity and amplifier of permeability transition. Amino Acids 2012; 42: 1827-37.

[178] Sava IG, Battaglia V, Rossi CA, Salvi M, Toninello A. Free radical scavenging action of the natural polyamine spermine in rat liver mitochondria. Free Radic Biol Med 2006; 41: 1272-81.

[179] Halestrap AP, Kerr PM, Javadov S, Woodfield KY. Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. Biochim Biophys Acta 1998; 1366: 79-94.

[180] McStay GP, Clarke SJ, Halestrap AP. Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. Biochem J 2002; 367: 541-8.

[181] Susin SA, Zamzami N, Kroemer G. Mitochondria as regulators of apoptosis: doubt no more. Biochim Biophys Acta 1998; 1366: 151-65.

[182] Salvi M, Brunati AM, Clari G, Toninello A. Interaction of genistein with the mitochondrial electron transport chain results in opening of the membrane transition pore. Biochim Biophys Acta 2002; 1556: 187-96.

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