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The adenine nucleotide translocator as a potential therapeutic target

Review Article

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Abbreviations: 4-hydroxynonenal, (HNE); 4-methylumbelliferone, (MU); 4-methylumbelliferyl phosphate, (MUP); adenine nucleotide translocator, (ANT); apoptosis inducing factor, (AIF); bongkrekic acid, (BA); cyclosporin A, (CsA); inner membrane, (IM); lonidamine, (LND); methyl-valine cyclosporin A, (m-val-CsA); mitochondrial membrane permeabilization, (MMP); mitochondrial transmembrane potential, ($D\Psi m$); nitric oxide, (NO); outer membrane, (OM); peripheral benzodiazepin receptor, (PBR); permeability transition pore complex, (PTPC); reactive oxygen species, (ROS); short chain fatty acids, (sCFA); tert-butylhydroperoxide, (t-BHP); viral mitochondria-localized inhibitor of apoptosis,vMIA); viral protein R(VpR); voltage-dependent anion channel);(VDAC)

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Summary

Identification of new targets for development of apoptosis-modulating drugs has become possible from the unraveling of the basic apoptosis mechanisms. Thus, mitochondrial membrane permeabilization has been recently recognized as a central rate-limiting step of apoptosis and its study has led to the identification of the adenine nucleotide translocator (ANT) as a potential therapeutic target. Three arguments support this possibility. First, ANT is a bi-functional protein, an ADP/ATP translocator and a non-specific pore, which contributes to apoptosis via its capacity to form a lethal pore under the control of the Bax/Bcl-2 family members. Second, the pore-forming activity of ANT is directly modulated by agents as diverse as proteins, lipids, ions, pro-oxidants or chemotherapeutic agents. Third, loss of ANT function is involved in several human pathologies, such as cardiomyopathy and aging, while reduced ANT expression or ANT mutation may lead to renal cancer and ophtalmoplegia. Hypothetically, ANT may thus constitute a new target for therapeutic intervention on apoptosis.

I. Introduction

Apoptosis is a regulated physiological process of cell death. It is devoted to the maintenance of cell number homeostasis and the elimination of unwanted, mutated or damaged cells during the whole life, from embryonic development to adult state (Thompson, 1995). Apoptosis deregulation can cause numerous pathologies, as various as cancer, autoimmune diseases, neurodegenerative diseases and AIDS, and resistance to therapeutic cell death induction. Recent advances in cell biology have led to a better understanding of the basic mechanisms of apoptosis (Kroemer and Reed, 2000) and to the emergence of apoptosis modulation as a promising therapeutic strategy to correct pathological apoptosis (Costantini et al, 2000; Huang and Oliff, 2001; Reed, 2001).

In many pathophysiological models, the apoptosis process is composed of three phases, induction (pre-mitochondrial), decision (mitochondrial) and degration (post-mitochondrial) (Kroemer et al, 1997). Heterogenous

induction pathways triggered by various stimuli such as radiation, receptor ligation or xenobiotic agents converge to the mitochondrion, which, in turn, behaves as a central rate-limiting integrator/coordinator of the cell decision to die (Brenner and Kroemer, 2000; Kroemer and Reed, 2000). Integration corresponds to mitochondrial membrane permeabilization (MMP), a process which may involve the opening of the permeability transition pore complex (PTPC) (Zamzami et al, 1995). As a result of MMP, harmfull intermembrane soluble proteins are released into the cytosol (Von Ahsen et al, 2000; Joza et al, 2001; Parrish et al, 2001). Proteins participating in the various phases can be classified as (i) death initiators such as TNF or Fas receptors, transcription factors (e.g. p53, fos, jun, myc) or phosphatases/kinases (e.g. calcineurin, AKT) which activate the induction phase, (ii) MMP modulators such as the Bax/Bcl-2 family members and the proteins from the PTPC, (iii) intermembrane proteins such as cytochrome *c* (a caspase activator) (Green and Reed, 1998), certain pro-caspases (Susin et al, 1999), Smac/DIABLO (an inhibitor of caspases inhibitors of the IAP family) (Verhagen et al, 2000), apoptosis inducing factor (AIF, a nuclease activator) (Joza et al, 2001), as well as endonuclease G (Parrish et al, 2001), which mediate the integration/coordination role of mitochondria and lead to caspase-dependent and -independent pathways, and (iv) post-mitochondrial hydrolases such as caspases 3 and 6, and caspase-activated DNAse **(Figure 1)**.

The adenine nucleotide translocator (ANT) is an inner membrane mitochondrial protein, which belongs to the polyprotein complex PTPC (Zoratti and Szabo, 1995). PTPC is located at the contact site between the outer membrane (OM) and the inner membrane (IM) and is composed of, at least, hexokinase (in the cytosol), the peripheral benzodiazepin receptor (PBR, in the OM), VDAC (voltage dependent anion channel or porin, in the OM), ANT and cyclophilin D (in the matrix) (Zoratti and Szabo, 1995; Bernardi, 1996; Crompton, 1999; Kroemer and Reed, 2000) **(Figure 2)**. Previously, PTPC has been shown to be a target for apoptosis regulation by Bcl-2-related proteins (Marzo et al, 1998a). As true for several apoptosis-associated proteins, ANT is a bifunctional protein endowed with vital and lethal characteristic depending on the cellular context. In physiological conditions, ANT catalyzes the stoechiometric exchange of ADP and ATP across the IM (Pfaff and Klingenberg, 1968; Pfaff et al, 1969). In contrast, during apoptosis, ANT forms a lethal non-specific pore in cooperation with the Bax-like proteins, and thus, contributes to MMP (Marzo et al, 1998a, 1998b; Brenner et al, 2000; Vieira et al, 2000). In this review, we will discuss the role of ANT in apoptosis, its potential as a therapeutic target, and its putative implication in various pathologies.

II. ANT in apoptosis

Four lines of evidence support the role of ANT in apoptosis. First, inhibitors of PTPC opening, such as bongkrekic acid (BA), an ANT ligand, cyclosporin A (CsA), a cyclophilin D ligand, and m-Val-CsA, a non-immunosuppressive derivative of CsA, protect cells from apoptosis in several in vivo models, such as ischemia/reperfusion (Cao et al, 2001), brain traumas (Sullivan et al, 1999), fulminant hepatocyte apoptosis induced by injection of anti-CD95 antibodies (Feldmann et al, 2000) or gluthathione depletion (Haouzi et al, 2001). Second, nuclear apoptosis is preceded by a loss of the mitochondrial transmembrane potential $\Delta\Psi m$) that can be prevented in vitro by the above-mentioned inhibitors. This applies to several cell types, including neurons, fibroblasts, B and T lymphocytes, pre-B cells, thymocytes, myelomonocytic cells, carcinoma cell lines and to various apoptosis inducers including growth factor withdrawal, tumor necrosis factor, ceramide, glucocorticoids, genotoxic stress, and hyperexpression of Bax (Kroemer et al, 1997). Third, numerous death inducers can act directly on isolated mitochondria to stimulate concomitantly a loss of the $\Delta\Psi m$, matrix swelling and release of cytochrome *c* and AIF (Kroemer and Reed, 2000). Fourth**,** when purified PTPC or ANT are reconstituted into liposomes or planar lipid bilayers, they exhibit strongly similar properties to the whole PTPC in mitochondria or cells and can form non-specific pores accounting for the diffusion of solutes of MM<1500Da (Marzo et al, 1998a, 1998b; Brenner et al, 2000). Opening of the PTPC pore is inhibited by Bcl-2, CsA and BA. Moreover, Bcl-2, BA, ATP and ADP prevent the formation of pore in proteoliposomes containing purified ANT. Altogether, these studies indicate that ANT has two opposed functions; it is an ADP/ATP translocator in physiological conditions and a lethal pore in apoptosis.

III ANT as a pharmacological target

To investigate whether ANT could be a pharmacological target for apoptosis induction/or prevention, we developped a screening assay to measure the capacity of an agent to stimulate ANT to convert into a non-specific pore. To this end, ANT was purified from rat heart mitochondria and reconstituted into phosphatidyl/cardiolipin liposomes. Then, various molecules, such as calcein, ^{3}H-glucose, ^{3}H -inulin, malate, or 4-methylumbelliferyl phosphate (MUP) were encapsulated into liposomes and their release was determined as a quantitative measure of ANT pore opening (Beutner et al, 1996; Marzo et al, 1998a, 1998b; Brenner et al, 2000·, 2000; Belzacq et al, 2001a) **(Figure 3)**. Alternatively, ANT was incorporated in planar lipid bilayers to determine its electrophysiological properties such as single channel activity or macroscopic conductance, opening frequency and ionic specificity (Brenner et al, 2000; Zamzami et al, 2000; Jacotot et al, 2001). In these experimental systems, a number of pro-apoptotic agents capable of inducing MMP in isolated mitochondria were found to elicit ANT-dependent non-specific channel formation **(Table 1)**. This applies to

endogenous molecules participating in the apoptosis activation cascade (proteins, lipids, ions such as $Ca^{2+)}$ as well as xenobiotic agents (pro-oxidants, reactive oxygen species (ROS) donors and chemotherapeutic agents) (Hortelano et al, 1997, Larochette et al, 1999, Ravagnan et al, 1999, Marchetti et al, 1999, Granville and Hunt, 2000) **(Figure 4)**.

A. The Bax/Bcl-2 family

The Bax/Bcl-2 family is composed of pro-apoptotic proteins (Bax, Bak, Bad, Bid...) acting as tumor suppressors and of anti-apoptotic proteins (Bcl-2, Bcl-X_L…) participating in oncogenesis. Several hypotheses have been proposed to explain their mode of action. They could regulate apoptosis via their capacity of homo- and hetero-oligomerization, via channel formation, and/or by increasing the level of cell tolerance to ROS damage (Harris and Thompson, 2000, Voehringer and Meyn, 2000). In many studies, it has been established that they regulate the apoptosis process at the mitochondrial level, by increasing MMP (the case of pro-apoptotic Bax/Bcl-2 family members) or by stabilizing the barrier function of mitochondrial membranes (the case of anti-apoptotic Bax/Bcl-2 family members) respectively. We found that after mitochondrial translocation, Bax interacts with ANT to form a non-specific lethal pore, this process being inhibited by BA and CsA, the two inhibitors of the PTPC (Marzo et al, 1998b). Immunodepletion of Bax from the PTPC or the use of PTPC purified from $Bax^{-/-}$ mice, revealed that the presence of Bax within PTPC is required for an optimal pore formation response to atractyloside, an ANT ligand classically used as MMP inducer (Marzo et al, 1998b). Direct physical interactions between ANT, Bax and Bcl-2 were demonstrated by co-immunoprecipitation in cancer cell lines and confirmed by means of the yeast two hybrid system (Marzo et al, 1998b).

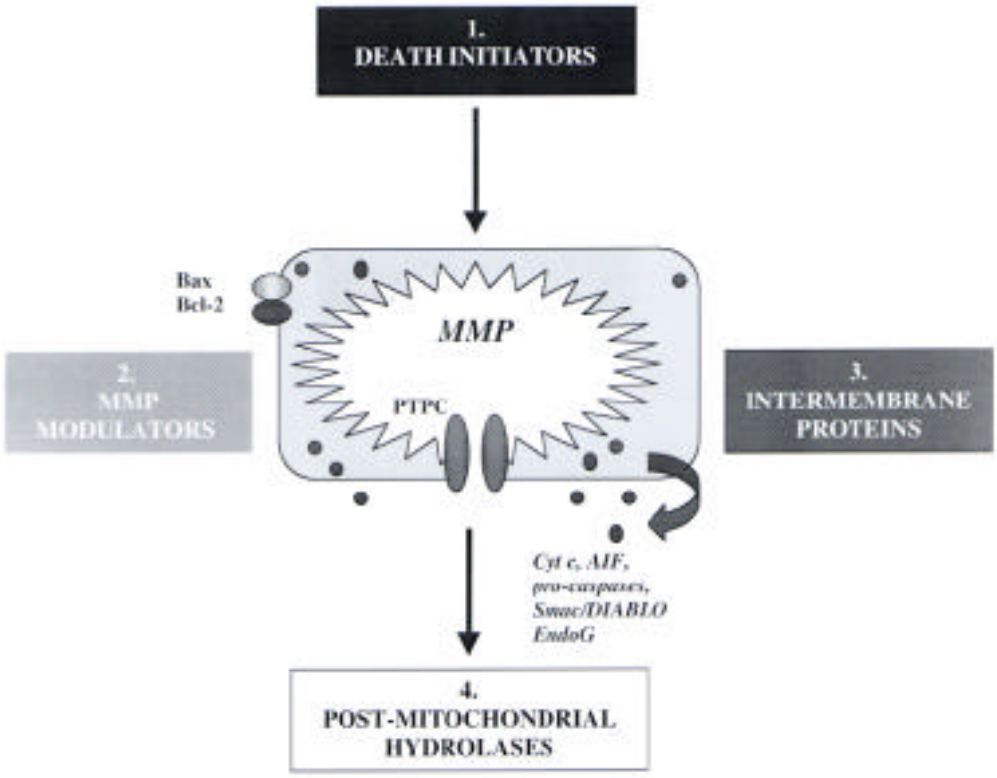

Figure 1. The four classes of proteins involved in the apoptotic process. The death initiators represent TNF or Fas receptors, transcription factors (e.g. p53, fos, jun, myc) or phosphatases/kinases (e.g. calcineurin, AKT) which activate the induction phase of apoptosis. The mitochondrial membrane permeabilization modulators are the Bax/Bcl-2 family members and the PTPC components. Intermembrane proteins consist in cytochrome *c*, apoptosis inducing factor (AIF), certains pro-caspases, Smac/DIABLO as well as endonuclease G. The postmitochondrial hydrolases are responsible for the degradation phase such as caspases and DNAses. ANT, adenine nucleotide translocator, Cyt *c*, cytoc14hrome *c*, MMP, mitochondrial membrane permeabilization.

Molecule	Effect	Dose	Inhibition
Bax	P	ratio 1:4	Bcl-2, ATP
Bax Δα5-6	-	ratio 1:4	ND
Bax ΔIGDE	-	ratio 1:4	ND
Bid	P	1-5μM	Bcl-2, ATP, ADP
Bcl-2	-	ratio 1:1	ND
Bcl-2 Δα5-6	-	ratio 1:1	ND
Bcl-2 G145A	-	ratio 1:1	ND
Atractyloside, Calcium	P	0.1-0.6mM	Bcl-2, ATP, ADP, BA
Diamide, t-BHP	P	0.1-0.3mM	Bcl-2, ATP, ADP
DTDP, BMH	P	5-20μM	Bcl-2, ATP, ADP
Vpr 52-96	P	1μM	Bcl-2, ATP, ADP
Verteporfin, Arsenite, LND	P	5- 50μM	Bcl-2, ATP, ADP
CD437	P	5-15μM	Bcl-2, ATP, ADP
NO, peroxynitrite, HNE	P	0.4-1.8mM	Bcl-2
Acetate	P	0.15-1mM	ATP, ADP
Propionate	P	0.15-1mM	ATP, ADP

"Dose" corresponds to the dose of a molecule inducing a permeabilization response. The dose is expressed as a molar ratio of protein: ANT, when proteins are co-reconstituted with ANT in artificial membranes, or as concentrations when molecules are incubated with ANT-containing liposomes. P, permeabilization response via ANT pore opening; -, no permeabilizing effect; ND, not determined.

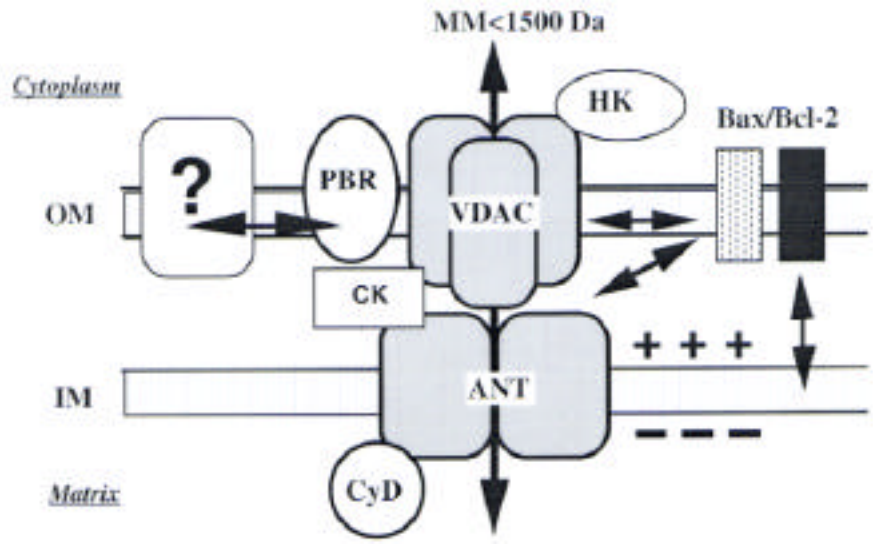

Figure 2. Scheme of the putative PTPC organization at the contact site of mitochondrial membranes. PTPC is a polyprotein complex located at the contact site of the mitochondrial membranes. ANT, adenine nucleotide translocator; VDAC, voltage dependent anion channel; PBR, peripheral benzodiazepin receptor; CyD, cyclophilin D. HK, hexokinase; CK, creatine kinase. OM, outer membrane; IM, inner membrane;?, unknown protein; -/+, ΔΨm

When ANT and Bax were reconstituted together into liposomes or planar lipid bilayers, we obtained a more efficient permeabilizing and pore-inducing effect of atractyloside than for each protein alone (Marzo et al, 1998b; Brenner et al, 2000). ANT-Bax channels (30 et 80pS) possessed an higher opening frequency than those formed by ANT (30pS) or by Bax alone (200pS). The selectivity of ANT-Bax channels was cationic, whereas Bax channel selectivity was anionic. Bcl-2, BA, ATP and ADP, the natural ligands of ANT, closed the atractyloside-elicited ANT-Bax channels (Marzo et al, 1998b; Brenner et al, 2000). Inactive mutants of Bax or Bcl-2, which reportedly have lost their apoptosis-modulatory function failed to affect the formation of channel by ANT (Marzo et al, 1998b; Brenner et al, 2000). It thus appears the ANT/Bax pair permeabilizes artificial lipid bilayers in response to an atractyloside-induced conformational change of ANT, whereas the ANT/Bcl-2 pair does not.

The endogenous signals (pH alteration, ATP loss, and/or oligomerization…), which render ANT sensitive to Bax regulation in vivo remain elusive. Altogether, these results suggest that Bax and Bcl-2 can cooperate with ANT to convert it as a non-specific pore and to regulate MMP.

B. Pro-oxidants

Costantini et al showed that a series of different thiol cross-linking agents (diazenedicarboxylic acid bis 5N, N-dimethylamide (diamide), dithiodipyridine (DTDP), bis-maleimido-hexane (BMH) and phenylarsine oxide) induced MMP and cell death irrespective of the expression level of Bcl-2 (Costantini et al, 2000). The same agents conferred a membrane permeabilization response when added to ANT-containing liposomes due to the oxidation of a critical cysteine residue (Cys 56) of ANT (Costantini et al, 2000). Concomitantly, recombinant Bcl-2 failed to prevent thiol modification of ANT. These data indicate that thiol cross-linkers cause a covalent modification of ANT which, beyond any control by Bcl-2, leads to ANT pore opening, MMP and cell death. In contrast, tert-butylhydroperoxide (t-BHP), a ROS donor, was found to induce MMP and apoptosis in a fashion that was inhibited by Bcl-2 (Costantini et al, 2000). t-BHP also permeabilizes ANT proteoliposomes without causing Cys 56 oxidation, in a Bcl-2 inhibitable fashion. Previously, nitric oxide (NO), peroxynitrite and 4-hydroxynonenal (HNE) have been shown to induce physiological or pathological apoptosis via various mechanisms such as ceramide formation, induction of surface receptors for lethal ligands and presumably, MMP (Kristal et al, 1996; Hortelano et al, 1997; Nicotera et al, 1999). Recently, we found that these three agents induced MMP when added

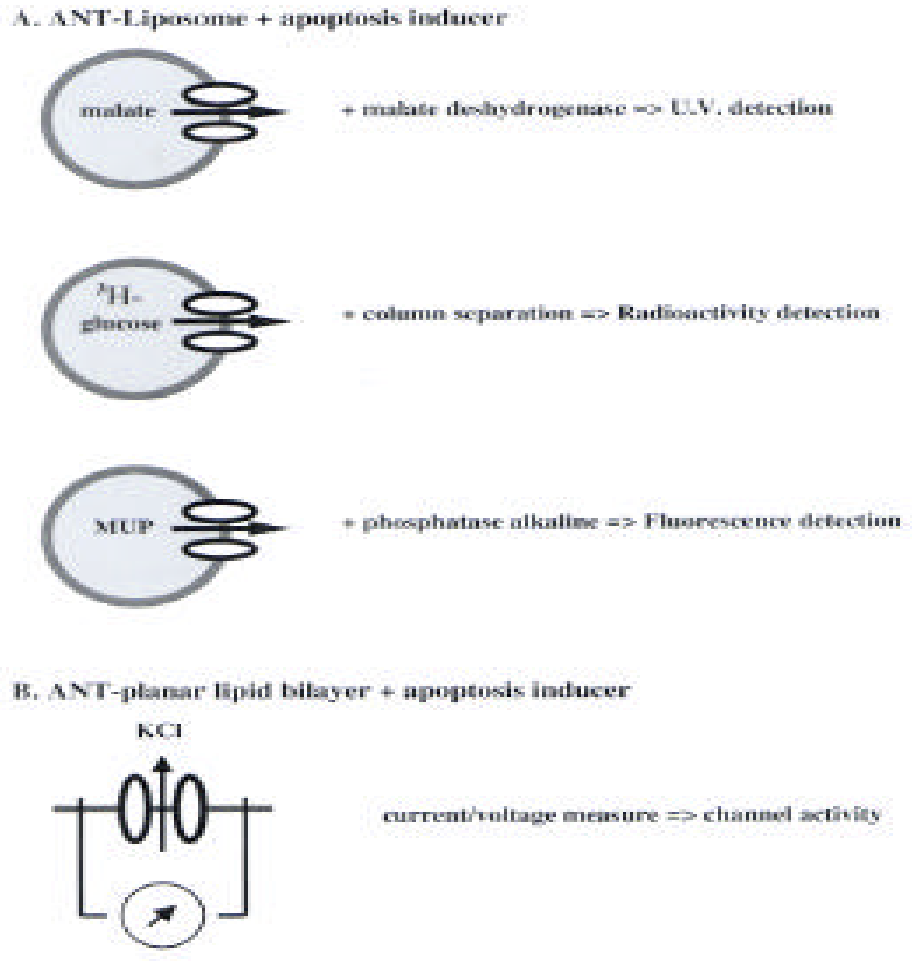

Figure 3. Experimental devices for the evaluation of the capacity of agents to convert ANT into a non-specific pore. ANT is purified from rat heart and reconstituted in phosphatidyl/cardiolipin liposomes (A) or planar lipid bilayers (B). Depending on the compound encapsulated in liposomes, the opening of ANT pore is detected by UV (the case of malate release), radioactivity (the case of radiolabelled compound release such as glucose) or fluorescence (the case of 4-methylumbelliferone, MU). The reconstitution of ANT in planar lipid bilayers allows the estimation of ANT channels activity by electrophysiology.

to mitochondria (Vieira et al, 2001). In intact cells, MMP was prevented by overexpression of Bcl-2, vMIA or pre-incubation with CsA (Vieira et al, 2001). Moreover, NO, peroxynitrite and HNE permeabilize ANT-containing liposomes. These effects are partially inhibited by Bcl-2 in proteoliposomes. Depending on the inducer, some carbonylation (the case of NO donors), tyrosyl-nitrosylation (the case of NO donors and peroxynitrite), thiol derivatization of ANT (tha case of NO donors, HNE and peroxynitrite) or lipid peroxidation (peroxynitrite) were detected. This indicates that ANT can be one of the targets of NO, HNE and peroxynitrite.

C. Viral proteins

Viral protein R (Vpr) is an apoptogenic accessory protein encoded by HIV-1. Vpr has been shown to induce MMP via a specific interaction with PTPC (Jacotot et al, 2000). A synthetic Vpr-derived peptide (Vpr 52-96), corresponding to the C-terminal moiety of the protein, uncouples the respiratory chain and induces a rapid inner MMP to protons and NADH. This inner MMP preceded cytochrome *c* release. In isolated mitochondria, Vpr52-96 induces matrix swelling and inner MMP, which both are prevented by pre-incubation of mitochondria with recombinant Bcl-2 protein (Jacotot et al, 2001). Recently, we observed that Vpr52-96 and purified ANT cooperatively form large conductance channels in artificial membranes (ANT-containing liposomes or planar lipid bilayers) and that Vpr 52-96 specifically binds to the intermembrane face of the ANT with an affinity in the nanomolar range (Jacotot et al, 2001). This cooperative channel formation relies on a direct protein-protein interaction since it is abolished by the addition of a peptide corresponding to the Vpr binding site of ANT (amino acids 104-116). Bcl-2 inhibits channel formation by the ANT-Vpr complex in synthetic membranes and reduces the ANT-Vpr interaction, as determined by affinity purification and plasmon resonance studies (Jacotot et al, 2001). Accordingly, Vpr modulates MMP through a direct structural and functional interaction with ANT.

Human cytomegalovirus (CMV) is a herpes virus that causes opportunistic infections in immunocompromised individuals. CMV inhibits apoptosis mediated by death receptors and encodes a viral mitochondria-localized inhibitor of apoptosis, namely vMIA, capable of suppressing apoptosis induced by diverse stimuli (Goldmacher et al, 1999). vMIA, inhibits Fas-mediated apoptosis at a point downstream of caspase-8 activation and Bid cleavage but upstream of cytochrome *c* release. vMIA is localized in mitochondria and associates with ANT. These functional properties resemble those ascribed to Bcl-2. However, the absence of sequence similarity to Bcl-2 or any other known cell death suppressors suggests that vMIA defines a previously undescribed class of anti-apoptotic proteins preventing cell death by a direct interaction with ANT.

D. Chemotherapeutic agents

An increasing number of experimental chemotherapeutic agents induce apoptosis by directly triggering MMP (Costantini et al, 2000). Indeed, both in intact cells and in isolated mitochondria, MMP is induced by lonidamine (LND), an agent used in Phase II clinical trials for breast, ovarian, lung and colon cancers), arsenic trioxide (arsenite, a therapeutic agent for acute promyelocytic leukemia), betulinic acid (an agent which kills neuroectodermal cells), CD437 (a retinoid derivative which kills various cancer cell lines) and the photosensitizer verteporfin (an agent studied in phase I/II of melanoma treatment) (Fulda et al, 1998a, 1998b; Larochette et al, 1999; Marchetti et al, 1999; Ravagnan et al, 1999; Belzacq et al, 2001a). Cells overexpressing the cmv-encoded protein vMIA or the oncoprotein Bcl-2 were strongly protected against the MMP-inducing and apoptogenic effects of the four chemotherapeutic drugs, LND, arsenite, CD437 and verteporfin (Belzacq et al, 2001b). In ANT-containing liposomes, they induce the membrane permeabilization via the conversion of ANT into a non-specific channel (Belzacq et al, 2001b). The ANT-dependent membrane permeabilization is inhibited by the two ANT ligands ATP and ADP, as well as by recombinant Bcl-2 protein. Although LND, arsenite, CD437 and verteporfin could interact with other endogenous targets, ANT pore function can be modulated by these anticancer agents to induce apoptosis.

E. Lipids

The genus *Propionibacterium* is composed of dairy and cutaneous bacteria which produce short-chain fatty acids (SCFA), mainly propionate and acetate, by fermentation. Recently, we showed that *P. acidipropionici* and *freudenreichii*, two species which can survive in the human intestine, can kill two human colorectal carcinoma cell lines by apoptosis (Jan et al, 2001). Propionate and acetate were identified as the major cytotoxic components secreted by these bacteria. Bacterial culture supernatants as well as pure SCFA induced typical signs of apoptosis including a loss of the $\Delta\Psi m$, generation of ROS, caspase-3 processing, and nuclear chromatin condensation. Bcl-2 and vMIA, both inhibited cell death induced by SCFA, suggesting that mitochondria and ANT are involved in the cell death pathway (Jan et al, 2001). Accordingly, propionate and acetate induce mitochondrial swelling when added to purified mitochondria in vitro. Moreover, they specifically permeabilize ANT-containing proteoliposomes, indicating that ANT can mediate SCFA-induced apoptosis.

IV. ANT-related pathologies

Circumstantial evidence has implicated ANT in several human pathologies.

A. Ophtalmoplegia

Human ANT exists as three isoforms, which are encoded by distinct genes **(Figure 5)**. The isoform 1 (ANT1) gene is located on the chromosome 4, band 4q35; the isoform 2 (ANT2) gene on chromosome X, band Xq24-q26 and the isoform 3 (ANT3) gene on chromosome X band Xp22.32. Kaukonen et al, (2000) identified a mutation of the ANT1 gene in which a transversion in exon 2, codon 114, produced an Ala→Pro substitution (Kaukonen et al, 2000). The associated disease was found to be an autosomal dominant progressive ophtalmoplegia, characterized by exercise intolerance mimicking mitochondrial myopathy, proliferation of mitochondria and reduced rates of mitochondrial ADP-stimulated repiration. Despite the absence of genealogical confirmation, this study suggested a founder mutation and common ancestry (Kaukonen et al, 2000). All these symptoms were previously observed in mice with targeted inactivation of ANT1 which, in addition, manifest an hypertrophic cardiomyopathy and multiple deletions of mitochondrial DNA (Graham et al, 1997).

B. Cardiomyopathies

In several long-term investigations, Shultheiss and Dörner, found autoantibodies against the ANT in sera of patients with myocarditis and dilated cardiomyopathy (Schultheiss et al, 1996; Dorner and Schultheiss, 2000). To elucidate the pathophysiological importance of these antibodies, they studied the function and the expression of ANT in the heart muscle tissue of these patients and observed a strongly lowered ADP/ATP transport capacity of the translocator accompanied by an elevation in total ANT protein content. The alteration in ANT protein amount resulted from an ANT isoform expression modification, i.e. an increase in ANT 1 isoform protein associated with a decrease in ANT 2 isoform and an unchanged ANT 3 content. Since it is known from enzymatic studies in yeast that ANT2 exchange rate is higher whose of ANT1 and ANT3, the isoform shift may explain the lowered capacity of the carrier expressed in the myocardial tissue of patients with dilated cardiomyopathy. This isoform shift was not a progressive process during the disease period but occurred early in the illness and became permanent. In contrast, ANT implication was not observed in patients suffering from ischemic or valvular heart diseases (Schultheiss et al, 1996; Dorner and Schultheiss, 2000). However, no clear explanation of the mechanism by which an antibody might affect the function of an integral membrane protein located in the mitochondrial IM has been proposed limiting the relevance of these observations.

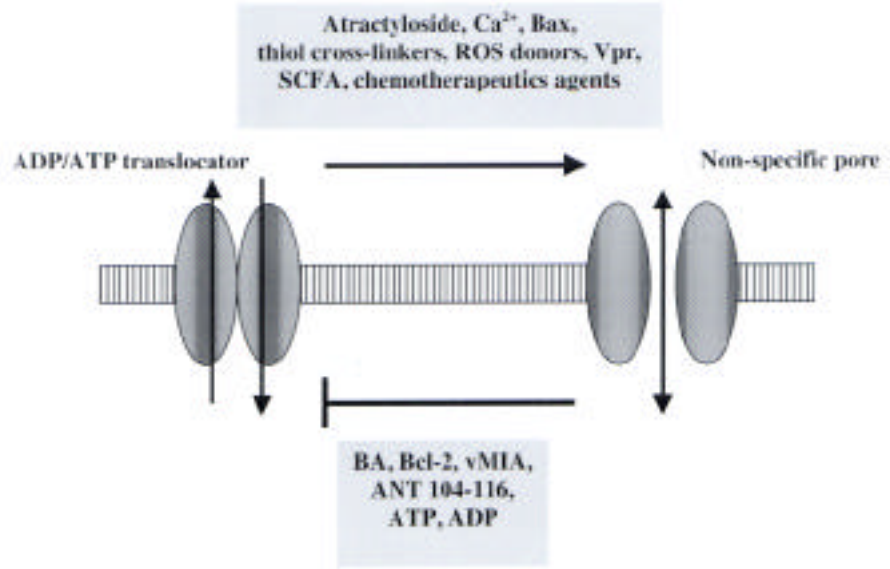

Figure 4. Regulation of ANT functions. ANT is a bifunctional protein, a physiologic ADP/ATP translocator and a pro-apoptotic pore. Atractyloside, Ca^{2+}, Bax, thiol cross-linkers, reactive oxygen donors (ROS), Vpr from HIV-1, short-chain fatty acids, or chemotherapeutic agents such as lonidamine, arsenic trioxide, CD437, or verteporfin can convert ANT into a non-specific pore. In contrast, bongkrekic acid (BA), Bcl-2, vMIA, a cytomegalovirus-encoded protein, the peptide ANT104-116, ATP and ADP, inhibit the pore formation.

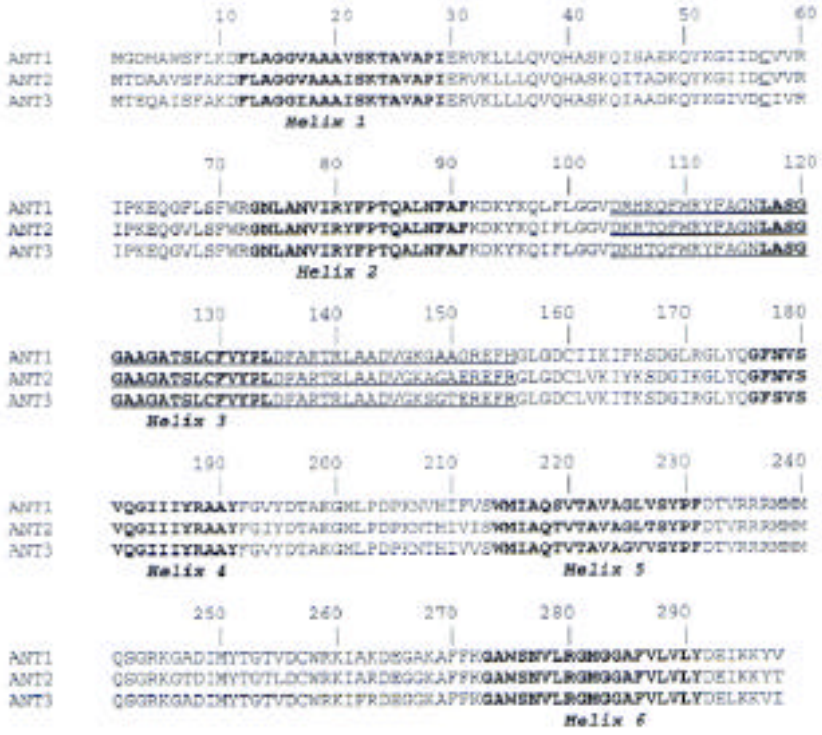

Figure 5. Alignement of the three human isoforms of ANT. The primary sequences alignement of the three isoforms of ANT has been obtained using the software CLUSTAL W 1.74 multiple sequence alignment. Amino acids 105-156, which correspond to the binding site of Bax and Bcl-2 to ANT are underlined. Similarly, Cys 56 which is cross-linked by prooxidants such as diamide, BMH and DTDP is underlined. The localization of the six putative transmembrane helices of ANT is indicated in bold.

C. Cancer

The investigation of the gene regulation encoding for the proteins involved in energy metabolism in cancer cells (a cell carcinoma, an oncocytoma, and urothelial tumors at two different stages) showed that different transcript patterns of ANT were observed in each of the tumoral and transformed cell lines. According to authors hypothesis, this could explain the difference in metabolism between the different tumors and the tumoral or transformed cell lines (Faure-Vigny et al, 1996, Heddi et al, 1996). In particular, a high transcript level for the ANT2 gene, which is usually not expressed in differentiated cells, was detected in oncocytoma and malignant urothelial renal tumor. This phenomenon was also shown in renal carcinoma cell lines and transformed cells. These data argued for the involvement of the ANT2 protein in glycolytic ATP uptake in cancer cell mitochondria. Subsequently, the growth-dependence expression of the ANT2 gene in mouse embryo fibroblasts was demonstrated to be regulated at the level of transcription and proposed as a marker of cell proliferation (Barath et al, 1999a, 1999b). If confirmed by additional studies, these results may open the way to an ANT2 antisense strategy for cancer therapy.

D. Aging

It is well known that mitochondria are main targets for aging-associated oxidative damage resulting in significant function loss (Salvioli et al, 2001). Indeed, during progressive aging, alterations accumulate at organism and cellular levels, molecular impairments affecting notably oxidative energy metabolism, i.e. the oxidative phosphorylation and the ADP/ATP translocation. Thus, Nohl et al observed that rat heart mitochondria from 30-month-old animals are 40% less active in translocating adenine nucleotides across the inner membrane than 3-month-old rats although the number of sites available for binding the specific ligands to the adenine nucleotide carrier were unchanged during aging (Nohl and Kramer, 1980, Nohl, 1982). In addition, the endogenous pool of the adenine nucleotides exhibited an age-dependent fall by more than 25%, essentially at the expense of ATP. Furthermore, Yan and Sohal identified an increase in carbonyl content of ANT after exposure of housefly flight muscles to 100% oxygen or during aging (Yan and Sohal, 1998). The oxygen-related damage appeared to be selective of ANT within mitochondrial membrane proteins and accompanied by loss of functional activity suggesting that ANT was altered by the aging process, at least, in the house fly. More recently, Rottenberg et al found that aging increases the

susceptibility to calcium-dependent cell death in the brain, liver, and possibly other murine tissues via a facilitated activation of PTPC opening (Mather and Rottenberg, 2000). However, these observations still await for a confirmation in human models to identify the human ANT as an aging target.

V. Conclusion and perspectives

Identification of new targets for drug development of molecules having the capacity to modulate apoptosis has become possible due to the unraveling of basic apoptosis mechanisms. Thus, MMP has been recently recognized as a central rate-limiting step of apoptosis and its study has led to the identification of ANT as a potential therapeutic target (Kroemer and Reed, 2000, Vieira et al, 2000). Indeed, ANT is a bi-functional protein, which can trigger MMP by forming a non-specific lethal pore under the control of the Bax/Bcl-2 family members (Vieira et al, 2000). The opening of the ANT pore causes water and ion movements across the IM, loss of the $\Delta\Psi m$, swelling of the mitochondrial matrix and release of intermembrane proteins through the OM. These events activate the coordination of downstream degradation pathways culminating in cell.

In vitro, ANT can respond to multiple stimuli and can be a direct target for agents affecting its pore forming activity, as diverse as proteins, lipids, ions, or chemotherapeutic agents (Brenner et al, 2000, Costantini et al, 2000, Belzacq et al, 2001a, 2001b; Jan et al, 2001; Vieira et al, 2001). This suggests that various molecules may modulate ANT pore function in a therapeutic perspective. Therefore, the success of ANT-based drug discovery will require the identification of molecules capable of converting ANT into a non-specific pore, but without affecting the physiological function of ANT, i.e. the ATP/ADP translocation.

ANT has been involved in various human pathologies, due to mutations, deficient expression or acquired loss-of-function. This suggests that ANT could be a candidate for gene therapy to correct defects at the transcription level or to re-introduce a functional gene to preserve cells. Various therapeutic strategies based on proteins belonging to the apoptosis core machinery (Bcl-2, TNF-family death ligand TRAIL, caspases …etc) are already in preclinical phases studies (Huang and Oliff, 2001; Reed, 2001). Next advances will determine whether ANT can serve to create a new class of drugs such as small molecules, peptidomimetics or anti-sense oligonucleotides, modulating apoptosis via an action on the ANT pore function.

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