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Review

Therapeutic peptides: Targeting the mitochondrion to modulate apoptosis

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Abstract

For many years, medical drug discovery has extensively exploited peptides as lead compounds. Currently, novel structures of therapeutic peptides are derived from active pre-existing peptides or from high-throughput screening, and optimized following a rational drug design approach. Molecules of interest may prove their ability to influence the disease outcome in animal models and must respond to a set of criteria based on toxicity studies, ease of administration, the cost of their synthesis, and logistic for clinical use to validate it as a good candidate in a therapeutic perspective. This applies to the potential use of peptides to target one central intracellular organelle, the mitochondrion, to modulate (i.e. activate or prevent) apoptosis. Putative mitochondrial protein targets and the strategies already elaborated to correct the defects linked to these proteins (overexpression, inactivation, mutation…, etc.) are described, and recent advances that led or may lead to the conception of therapeutic peptides via a specific action on these mitochondrial targets in the future are discussed.
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1. Introduction

For many years, some natural peptides and synthetic derivatives are successfully used as therapeutic drugs to treat chronic human pathologies [1]. For instance, this includes peptides as diverse as GnRH for prostate cancer, insulin and glucagon for diabetes type I and II, β-amyloid peptide for Alzheimer's disease, T-20 (pentafuside) a synthetic 36-mer peptide for HIV cell entrance inhibition, calcitonin for osteoporosis, as well as vaso-intestinal peptide (VIP) for the treatment of erectile dysfunction. The current approach to design therapeutic peptides is to derive novel structures from active pre-existing peptides or from high-throughput screening, to modify it until optimization for a particular pathology following the so-called rational drug design approach [1]. Thus, independently of its origin or its mode of conception, the molecule of interest should prove its ability to influence the disease outcome in animal models and respond to a set of criteria based on toxicity studies, ease of administration, the cost of their synthesis, and the cost in clinical use to validate it as a good candidate in a pharmacological perspective.

This review focuses on the potential use of peptides to target one central intracellular organelle, the mitochondrion, to modulate (i.e. activate or prevent) apoptosis [2], a form of cell death that has been shown to play a critical role in most human diseases [3]. Thus, it is generally admitted that cells die by several mechanisms, two main of which being necrosis or apoptosis, that can be accompanied by autophagy. In necrosis, the application of a toxic stimulus onto a cell overwhelms its homeostatic balance, committing the cell in an uncontrolled

Abbreviations: ANT, adenine nucleotide translocator; Cyt *c*, cytochrome *c*; $\Delta\Psi m$, mitochondrial transmembrane potential; HK, hexokinase; MMP, mitochondrial membrane permeabilization; MUP, 4-methylumbelliferyl phosphate; IM, inner membrane; OM, outer membrane; ROS, reactive oxygen species; PTPC, permeability transition pore complex; PBR, peripheral benzodiazepine receptor; VDAC, voltage-dependent anion channel; Vpr, Viral protein R

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death [4]. Autophagy is a highly conserved mechanism for the degradation of long-lived proteins and the cytoplasmic organelles by the cell's own lysosomal system [5]. Genetic or pharmacological deregulation of the autophagic process can lead to cell death [6–8] and the term "autophagic cell death" describes a form of programmed cell death morphologically distinct from apoptosis and presumed to result from excessive levels of cellular autophagy [9–12]. Apoptosis, is the best-understood mechanism of controlled cell death, which is regulated by an evolutionarily conserved cellular pathway that consists in the caspase family, the Bcl-2 family, and the adaptor protein Apaf-1 [13]. Apoptosis is a strictly regulated removal of unwanted, damaged or mutated cells without a deleterious inflammatory response of the organism [2]. In some settings, autophagy and apoptosis may be interconnected and mitochondria may be central organelles integrating the two types of cell death [14,15]. Thus, a cell can be provoked into apoptosis through a disruption of the balance between the receipt of positive and negative signals, acting on the interplay between endogenous inhibitors and activators of the apoptosis pathway.

To illustrate the interest of targeting the mitochondrion for apoptosis modulation, we will overview some of the putative mitochondrial proteic targets and the strategies already elaborated to correct the defects linked to these proteins (over-expression, inactivation, mutation…, etc.). Finally, we will discuss recent advances that led or may lead to the conception of therapeutic peptides via a specific action on these mitochondrial targets.

2. The modulation of apoptosis via the mitochondrion

The idea of a mitochondrial basis for killing tumor cells has emerged from Warburg hypothesis [16], that is built-up on the general differences in metabolic control occurring between cancer and normal cells. Subsequently, deregulation of programmed cell death or apoptosis has been related to the development of cancer [17], and mitochondria have been identified as gatekeepers or integrators in many apoptotic-signaling pathways [18–20] establishing a link between "cancer/metabolism/apoptosis/mitochondrion" [21]. This opened the path to a promising future for innovative apoptosis-based therapies of cancer [22,23]. In numerous experimental systems, the apoptosis process can be divided into three different stages (for review, see [24]. The induction phase corresponds to the convergence onto the mitochondrion of heterogeneous induction pathways triggered by various stimuli (radiation, receptor ligation, genotoxic stress, hypoxia, oncogene activation and chemotherapy…). The following decision phase is controlled by oncogenes and anti-oncogenes of the Bax/Bcl-2 family and manifests as a change in mitochondrial membrane permeability (MMP), which in turn is responsible for the last degradation phase of apoptosis and cell dismantling [24,25]. MMP may occur via several mechanisms (Fig. 1), which include Bax or Bak oligomerization in the outer mitochondrial membrane to form a pore that allows the release of cytochrome c (Cyt *c*), the opening of (poly)protein complexes, such as the ATP-dependent potassium channel (mtK-ATP [26,27] and the permeability transition pore complex (PTPC; [28,29]). In various pathways of apoptosis, MMP involves the permeabilization of the OM via the formation of channels composed of resident mitochondrial proteins (VDAC, Bak, MAC…) and/or cytosolic proteins that have translocated to mitochondrial membranes (Bax, Bid, Bad …) in a complex interplay with resident proteins such as Bak and Bcl-2 [30]. Under such MMP mechanism, one class of BH3-only proteins (for instance Bid and Bim) are required to activate Bax and Bak pore-forming activity in the OM, and another class of BH3-only proteins (for instance Bad and Bik) may bind Bcl-2 displacing sequestered Bid [30–32]. Such selective binding by BH3 only proteins for particular multidomain prosurvival or proapoptotic BH protein suggest that highly specific drugs can be found. The extensive study of the MMP process revealed that mitochondria contain several therapeutic targets (for reviews, see [33–35]. Indeed, MMP (i) is rate limiting for the induction of cell death irrespective of the primary site or organelle affected (plasma membrane, nucleus, endoplasmic reticulum and/or lysosome). This suggests that mitochondria are potent integrators of diverse signaling pathways of apoptosis via gene expression deregulation (p53, bcl-2, bax, myc…), metabolic changes (e.g. intracellular ATP content, ROS and calcium imbalance…), exogenous stimuli (nutrient deprivation, radiations, xenobiotic…); (ii) is a checkpoint in the apoptosis process and its induction is irreversible. In most conditions, MMP is induced and amplified by a positive amplification loop by Ca^{2+}-induced Ca^{2+} release (CiCR) [36], reactive oxygen species (ROS) generation [37], and caspase activation [38,39], and (iii) coordinates the degradation phase of apoptosis by releasing soluble pro-apoptotic factors (Cyt *c*, AIF, Smac/diablo, EndoG, Omi/Htra2, pro-caspases…).

As demonstrated in cell-free assays, several cytotoxic drugs acting directly on MMP have previously been described (for review, see: [24]). They can be classified as alpha-helicoidal amphipathic cationic peptides (e.g. Vpr-derived peptides, $(KLAKKLAK)_2$), steroid-derived-molecules (e.g. resveratrol, CD437, betulinic acid), BH3-mimetics, PBR-ligands (e.g. PK11195), VDAC ligands and ANT-ligands (e.g. Verteporfin, lonidamine, arsenite, CD437, Clodronate, MT21), pro-oxidant molecules (superoxide, HNE), cationic lipophilic agents (F16, FTY720), Bcl2 ligand (HA14-1), mtK-ATP openers (diazoxide, nicorandil, BMS 191095), mtK-ATP blockers (glibenclamide, 5-hydroxydecanoate, MCC-134).

Depending on their structure, some MMP inducers exhibit anti-tumoral activity *in vitro*, in animal and/or in human supporting the idea of targeting the mitochondria for the modulation of apoptosis as a seductive therapeutic strategy to treat a wide range of diseases [40].

3. Potential mitochondrial targets

As explained above, the mitochondrion contains numerous regulatory molecules involved in the control of the energetic metabolism and intrinsic phase of apoptosis, which constitute potential therapeutic targets [40]. This applies to key apoptotic

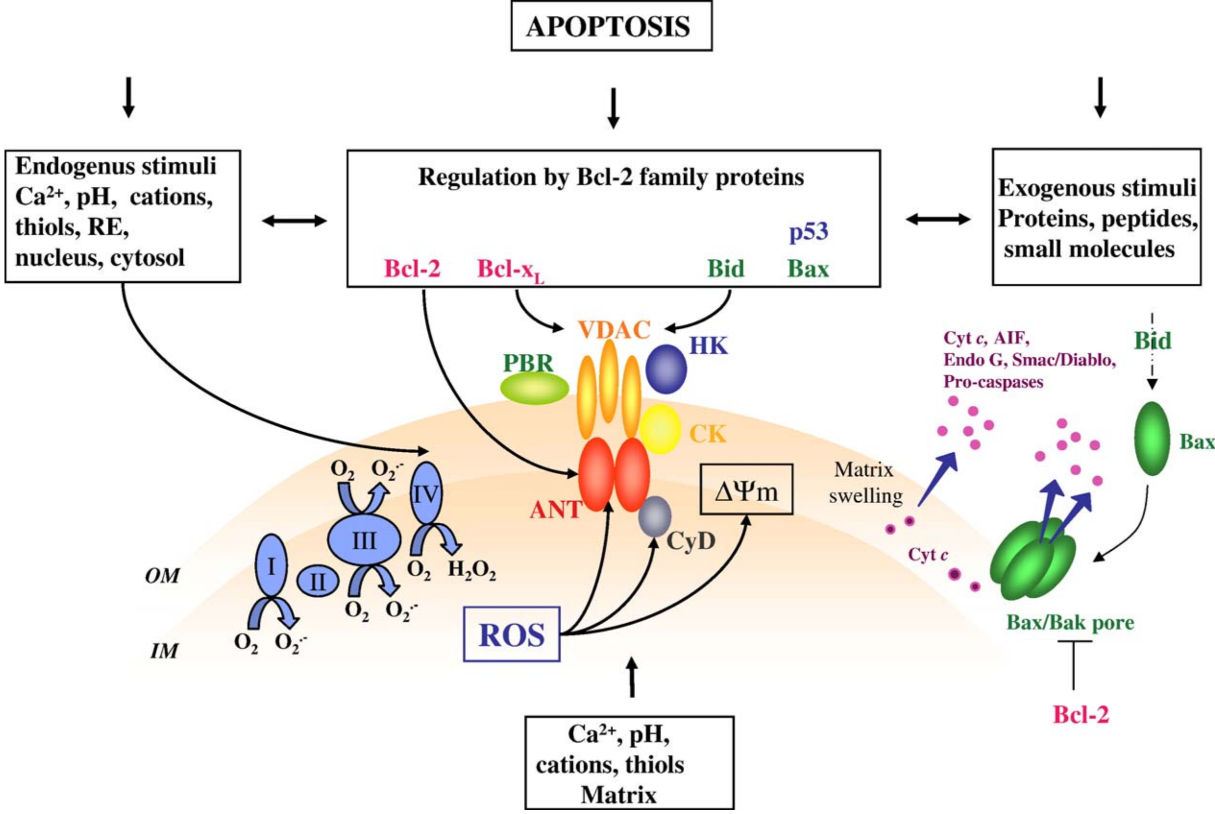

Fig. 1. A model of the mitochondrial membrane permeabilization process and its potential pharmacological targets. Apoptosis can be induced by exogenous stimuli as well as by endogenous stress signals. The process of membrane permeabilization can be activated directly (by Ca^{2+}, changes of the redox state, the ionic concentration or the pH) and/or regulated by Bax/Bcl-2 family members. The opening of the permeability transition pore results from the targeting of its various components, and manifests as a dissipation of the transmembrane inner membrane potential ($\Delta\Psi m$) and the release of intermembrane soluble pro-apoptotic factors like cytochrome c. Apoptotic stimuli can also activate the BH3 only proteins (for instance Bid), triggering Bax or Bak oligomerization at the OM where it forms a pore for the release of pro-apoptotic factors in the cytosol. The various components of the respiratory chain are represented in blue. HK, hexokinase; PBR, peripheral benzodiazepine receptor; VDAC, voltage-dependent anion channel; CK, creatine kinase; ANT, adenine nucleotide translocator; CypD, cyclophilin D; ROS, reactive oxygen species; IM, inner membrane; and OM, outer membrane.

executioners (e.g. Bax homologs, constitutive mitochondrial proteins from the oxidative phosphorylation, the import machinery as well as from the PTPC...), co-factors of cytoplasmic or nuclear executioners (pro-caspases, Cyt c, AIF, EndoG), and to inhibitors of the apoptotic program (e.g. Bcl-2 homologues, smac/DIABLO, IAPs, heat shock proteins). Via a putative role in the process of mitochondrial matrix swelling, the recently-identified aquaporin-8 water channel could also constitute an attractive target [41]. In this review, we will overview three groups of proteins, Bax/Bcl-2 family members, proteins from the PTPC and proteins from the respiratory chain to describe the state of the art of the identification of pharmacological targets for the conception of potential therapeutic molecules.

3.1. Bax/Bcl-2 family members: pro- and anti- apoptotic proteins

3.1.1. Anti-apoptotic members

The overexpression of Bcl-2 was first implicated in the progression of follicular lymphoma as a consequence of a chromosomal translocation [42]. Subsequently, the overexpression of other anti-apoptotic members of this protein family has also been implicated in a variety of human cancers in correlation with their capacity to inhibit apoptosis at the level of the mitochondrion [43]. Moreover, numerous cellular stresses, including DNA damage, hypoxia, UV radiations, and chemotherapy can be integrated by mitochondria, leading to irreversible alterations and apoptosis. Thus, the Bcl-2 homologues, localized to mitochondrial membranes, appear to be able to prevent the hallmarks of the mitochondrial dysfunction, i.e. the loss of $\Delta\Psi m$, swelling of the matrix, release of intermembrane space proteins (Cyt *c*, AIF, Smac/DIABLO, Endo G..., etc.), generation of ROS, and activation of executioner caspases such as caspases-3, -7 and -9. Many hypothesis have been launched to explain their potent inhibitory activity such as oligomerization [43], pore formation [44], regulation of channel such as VDAC [45] and/or ANT [46], an increase of cell resistance towards oxidative stress [47], Ca^{2+} flux regulation at the endoplasmic reticulum and the mitochondrial membranes [48–50] and an effect on the apoptosome formation, a cytosolic complex constituted by APAF-1, Cyt c, dATP and caspase-9 [51].

3.1.2. Pro-apoptotic proteins

In contrast with anti-apoptotic members (e.g. Bcl-2 and Bcl-X_L), pro-apoptotic proteins of the Bax/Bcl-2 family (e.g. Bax,

Bak and Bid) are dedicated to the activation of the apoptotic machinery. A wide variety of apoptotic stimuli (e.g. p53-dependent apoptosis, nuclear stress, redox pro-oxidant stress, chemotherapy) request the participation of the BH3 family proteins (listed in Fig. 2). As a result of apoptosis induction, they become "activated", and propagate the cell death stimulus to the core apoptosis machinery [11]. Activation refers generally to events of dimerization (e.g. Bax), post-translational modification (e.g. Bad), translocation from cytosol to mitochondria and endoplasmic reticulum (e.g. Bax, Bid, Bim), homo and heterooligomerization (e.g. Bak, Bad) [52], but triggering factors of this activation process as well as the hierarchy of the associated events remain to be elucidated.

3.1.3. Potential therapeutic molecules targeting Bax/Bcl-2 family members

Because they constitute central actors of the apoptotic machinery, Bax/Bcl-2 family members appeared rapidly as attractive candidates for the design of therapeutic molecules. Several leading laboratories and/or companies developed strategies to target Bax/Bcl-2 family members either directly with small molecules, antisense oligonucleotides or molecules interfering within critical protein–protein interactions.

Thus, Bax/Bcl-2- or Bak/Bcl-X_L- heterodimerizing capacities have been extensively used for high-throughput screening of chemical libraries to identify pro-apoptotic as well as anti-apoptotic molecules (e.g. [53,54]). For instance, to identify Bcl-X_L antagonists, two high-throughput screens were generated on the basis of the binding of fluorescein-labeled peptide the BH3 domain of BAD protein to Bcl-X_L and on an affinity selection/mass spectrometry (ASMS) assay. Utilizing a screening format of 384-well plate with mixtures of 10 drugs per well, 370,400 compounds were screened in duplicate and 425 inhibitors with an IC(50) below 100 μM were identified with the first screening assay. With the second assay, 263,382 compounds were screened and 29 ligands with affinities below 100 μM were identified. Thus, two novel classes of Bcl-X_L inhibitors were identified by both methods and confirmed to bind $^{(13)}$C-labeled Bcl-X_L using heteronuclear magnetic resonance spectroscopy [54].

Reed JC et al. developed antisepses oligodeoxynucleotides specific for sequences in mRNAs from the bcl-2 gene to inhibit the growth in culture of human leukemia cell lines [55]. The study led to the cell death through sequence-specific mechanisms, with reductions in cellular viability generally lagging the inhibitory effects on cellular growth by about 2 days, indicating that these oligodeoxynucleotides can be sequence-specific inhibitors of leukemic cell growth and survival [55]. Fifteen years later, *bcl-2* antisenses are evaluated in phase III clinical trials in lymphocytic leukaemia, non-small-cell lung cancer, advanced malignant melanoma, and multiple myeloma.

Antimycin A has been predicted to interact with the Bcl-2 homology domain 3 (BH3)-binding hydrophobic groove of Bcl-X_L by computational molecular docking analysis [56]. Thus, antimycin A binds competitively to recombinant Bcl-2, induces mitochondrial swelling and loss of $\Delta\Psi$m on addition to mitochondria expressing Bcl-X_L After chemical modification, the 2-methoxy derivative of antimycin A3 does not inhibit cellular respiration, but still retains toxicity for Bcl-X_L-overexpressing cells, mitochondria and Bcl-X_L-containing proteoliposomes [56].

Altogether, these studies validate the interest of novel therapeutic agents derived from Bcl-2/Bax family proteins to treat pathologies resulting from an excess or a defect in apoptosis and support the various clinical studies of phase I and II currently undertaken.

Bax (63-71)	L	K	R	I	G	D	E	L	D
Bak (78-86)	L	A	I	I	G	D	D	I	N
Bid (90-98)	L	A	Q	V	G	D	S	M	D
Bad (114-122)	L	R	R	M	S	D	E	F	V
Puma (41-49)	L	R	R	M	A	D	D	L	N
Noxa (29-37)	L	R	R	F	G	D	K	L	N
Bim (120-128)	L	R	R	I	G	D	E	F	N
Bik (61-69)	L	A	C	I	G	D	E	M	D
Hrk (37-45)	L	K	A	I	G	D	E	L	H
Bmf (137-145)	L	Q	C	I	A	D	Q	F	H

Fig. 2. BH3 domain of pro-apoptotic members of Bax/ Bcl-2 family. The BH3 proteins are all pro-apoptotic and share sequence homology within the amphipatic alpha-helical BH3 region as shown in the sequences alignment. Are presented the BH3 domains of the human proteins: Bax, Bcl-2 associated X protein; Bad, Bcl-2 antagonist of cell death; Bak, Bcl-2 homologous antagonist/ killer; Bid, BH3-interacting domain; Puma, P53 up-regulated modulator of apoptosis; Noxa, identified (as a mediator of p53-dependent apoptosis) in cells exposed to noxious stresses; Bim, Bcl-2 interacting mediator of cell death; Bik, Bcl-2 interacting killer; Hrk, activator of apoptosis harakiri; Bmf, Bcl-2 modifying factor. BH3 peptides could serve as prototypes for pro-apoptotic molecules as they act directly on isolated mitochondria to induce (outer) mitochondrial membrane permeabilization.

3.1.3.1. The permeability transition pore. The permeability transition pore, PTPC, is a mitochondrial polyprotein complex (MW ≈ 600 kDa) involved in the regulation of mitochondrial matrix homeostasis It is built-up at the mitochondrial contact sites, requiring an association with IM and OM lipids such as cardiolipin and cholesterol to function properly. Its opening as a large unspecific channel leads to the mitochondrial transition permeability that corresponds to an increase in the permeability of the IM, permitting the influx or efflux of any molecule of MW ≤ 1500 Da [57]. In physiological conditions, the PTPC pore is regulated by endogenous signals as diverse as: the $\Delta\Psi$m, matrix pH, the concentration of divalent cations (Ca^{2+}, Mg^{2+}), the redox equilibrium (ROS, thiols), and the matrix volume [58]. As measured by electrophysiology, mammalian PTPC exhibits multiple conductance states, suggesting that the channel is composed of several cooperating subunits [59].

However, in stress conditions, mitochondrial transition permeability can be deleterious to the mitochondrial physiology by the initiation of the mitochondrial phase of apoptosis, necrosis or autophagy [60]. Thus, as a consequence of the long lasting opening of PTPC, the mitochondrial matrix swells, the $\Delta\Psi$m dissipates and the outer membrane (OM) ruptures locally triggering the release of apoptogenic proteins into the cytosol [61]. Upon apoptosis initiation, PTPC opening can precede Bax

translocation from cytosol to mitochondria [62] or can occur concomitantly with pro-apoptotic Bax/Bcl-2 family members (Bax, Bid…) translocation, but usually before release of Cyst *c* and AIF from mitochondria (for review: [63]). Thus, irrespective to the hierarchy of events, a new class of channels can be formed via cooperation between Bax and ANT, and lead to MMP induction and cell death [64].

The composition of PTPC is dynamic and may evolve in response to the energetic demand of the cell and to apoptosis. This may result from the remodeling of mitochondrial cristae and of contact sites during the process of apoptosis induction [65]. From biochemical and functional studies using purified proteins reconstituted in artificial biomembranes (e.g. liposomes or black lipid membranes), two models of structure/function relationship of PTPC emerged. One model proposes that three proteins constitute PTPC: the adenine nucleotide translocator (ANT, in the IM), voltage-dependent anion channel (VDAC, in the OM) and cyclophilin D (CypD, in the matrix) [66–68], and alternative models suggest a variable complex composition depending on tissue, differentiation, metabolic status and development state [69,70]. This is notably the case of the composition of ATP synthasome and various complexes involving ANT, VDAC and CypD that varies upon the energetic activity of the mitochondrion [69,70]. The core of PTPC would be constituted by ANT, VDAC and CypD and regulated by, at least, the peripheral benzodiazepine receptor (PBR, in the OM), hexokinase (HK, in the cytosol), and creatine kinase (CK, in the intermembrane space) [71] and GST [72].

However, as isolated liver mitochondria from ANT1−/− ANT2−/− mice still undergo PT for high doses of calcium, it has been proposed from genetic studies that, at least, ANT1 and ANT2 isoform knockout could be compensated by another IM protein (e.g. ANT4, another mitochondrial carrier protein, an unknown channel) [73], and for critical commentary [74]). Moreover, *three* genetic studies confirmed that cyclophilin D participates to the PTPC function during necrosis and ischemia–reperfusion [75–77]. It is important to note that, in both structure/function models, some proteins are anti-apoptotic (HK, CK, CypD), whereas others are pro-apoptotic (ANT, VDAC, PBR) favoring the closed or the open state of PTPC, respectively, and thus enabling a tight regulation of the MMP process. In addition, PTPC function could be modulated by physical interactions of some of its members with onco- and anti-oncoproteins from the Bax/Bcl-2 family. ANT, VDAC and HK (glucokinase in liver) were identified as ligands of Bax, Bcl-2, Bid, Bcl-X_L, Bad and Bak [45,46,78–81]. Among these proteins, some members of Bax/Bcl-2 family were shown to be regulators of channel activity of ANT and VDAC, as well as modulators of the ADP/ATP translocase activity of ANT [45,64,82]. Recently, Machida et al. have found that inactivation of endogenous CypD by small interference RNA or cyclophilin inhibitor releases hexokinase-II from mitochondria and enhances Bax-mediated apoptosis [83]. These authors further proposed that mitochondrial bound hexokinase II plays an essential role in the CypD mediated anti-apoptotic effect.

Recently, extensive study of the role and function of ANT in MMP led to the proposal, that ANT could be a therapeutic target [84]. To mimic membrane insertion and mitochondrial compartimentation, several functional assays were designed and based on the reconstitution of rat heart purified ANT into artificial bilayers such as liposomes. Various substrates, such as calcein, ^{3}H glucose, ^{3}H inulin, malate, or 4-methylumbelliferyl phosphate (MUP), were encapsulated in ANT-containing proteoliposomes and their release studied as a quantitative measure of ANT pore opening [85,86]. This methodology allowed the evaluation of the capacity of various agents known to induce MMP, to permeabilize selectively ANT-containing liposomes (e.g. proteins, peptides, lipids, pro-oxidants and chemotherapeutics agents). Thus, the effects of endogenous components involved in the activation cascade of apoptosis and/or xenobiotics were characterized and their inhibitory profile determined. These compounds include pro-apoptotic members (Bax, Bid) of the Bax/Bcl-2 family [46,87], pro-oxidants agents (diazenedicarboxylic-acid-bis-5N, N-dimethylamide (diamide), dithiodipyridine (DTDP), or bis-maleimido-hexane (BMH), tert-butylhydroperoxide (t-BHP), nitric oxide (NO) [88,89], viral protein R (Vpr from HIV-1) and derived peptides [90,91], a viral mitochondria-localized inhibitor of apoptosis (vMIA) from cytomegalovirus [92], short chain fatty acids [93], as well as chemotherapeutic agents (arsenite, CD437, lonidamine and Verteporfin) [94,95]. Although we do not exclude that these molecules can have additional intracellular target, they can act directly on ANT to convert it into a non-specific pore. This assumption is supported by the fact that ATP and ADP (endogenous ligands of ANT), bongkrekic acid, cyclosporin A and m-val-cyclosporin A, and Bcl-2 prevent the permeabilizing effects of these molecules in ANT-proteoliposomes and in intact cells. Altogether, the diversity of the molecules suggested that ANT could be a potential target for apoptosis modulation, what has been confirmed in three acute pathologies in mice [96], but awaits *in vivo* confirmation in relevant human physio-pathological models. Interestingly, the structure of ANT in complex with carboxyatractyloside has been resolved recently [97], what should help the future conception of molecules able to bind ANT within its key domains (sulfhydril amino acids, hydrophilic loops, inhibitors binding pockets…, etc.).

Despite the structure of the PTPC channel remains elusive, VDAC is currently considered to be the protein constituting the OM channel of the complex. *In vitro*, it has been shown that VDAC pro-apoptotic function can be modulated by various stimuli, including the anion superoxide, Bax, Bcl-X_L, and a small molecule, Ro 68–3400 (for review: [98]). Other PTPC members, such as PBR, HK, CypD and CK being rather regulators than true components of the PTPC channel, should also be considered in a therapeutic perspective. However, all these proteins are not ubiquitously expressed and are usually involved in specific pathologies, such as Lupus erythematosus (PBR), some cancers (HK) and mitochondrial diseases (CK), limiting their application domains to specific pathologies.

3.2. The respiratory chain proteins

The respiratory chain is composed of four proteic complexes (Fig. 3). These complexes are embedded within the inner mitochondrial membrane and associated with two cofactors, coenzyme Q or ubiquinone and cytochrome c, that allow the interface between all the complexes. The function of the respiratory chain is to collect the energy from the oxidation of NADH and $FADH_2$ to generate a proton gradient, an electron flux, and ATP synthesis. The use of several natural or chemical functional inhibitors of the various complexes (rotenone, KCN, oligomycin…) has allowed the understanding of the coupled function of these complexes and the quantification of energy generated for the general cell metabolism.

Mitochondrial defects have been implicated in a wide variety of degenerative diseases, in aging, and in cancer [76]. Recently, clinical studies revealed an interplay between mutations in the mitochondrial and nuclear genomes, and a role of mitochondrial respiratory chain in cellular energy production, of ROS generation, and of the induction of apoptosis. The importance and relationship of these functions are now studied in mouse models of mitochondrial disease [99]. These models may allow to identify key actors (genes and/or proteins) and to dissect essential regulatory mechanisms.

Several approaches have linked the cellular respiratory activity to apoptosis and notably, to the choice between apoptosis and necrosis via the level of intracellular ATP [100]. Moreover, using human myelogenous leukemia cells, it has been demonstrated that respiratory function is essential for tumor necrosis factor-induced Cyt *c* release. In a cell free system using mitochondrial fraction from the same cells, initiation of respiration by substrates for complexes I, II, and III, but not IV, released Cyt *c*, suggesting that reduction of coenzyme Q or complex III is essential for Cyt *c* release [101].

Nevertheless, one key issue for the identification of pertinent therapeutic targets would be to discriminate direct effects on respiratory chain's functions from indirect effects altering other key mitochondrial structures, such as the import machinery or the PTPC.

Product	Swelling DE_{50} (μM)	ΔΨm DE_{50} (μM)
$_D$Vpr (67-82[C76S])	0,03	0,05
$_L$Vpr (67-82[C76S])	0,24	0,29
$_L$Vpr (67-79[C76S])	0,73	0,64
Vpr52-96	0,38	0,45
hBidBH3(84-99)	84,42	23,31
hBaxBH3(57-72)	> 200	> 200
hBakBH3	> 200	> 200
$_D$(KLAKLAK)$_2$	0,44	0,4
HA-14	4,4	5,8
CD437	3	ND
F-16	ND	29,2
Lonidamine	> 200	181,2
PK11195	> 200	106,2

Fig. 3. Comparison of the half-maximal effects (ED50) of different peptides and small molecules in mitochondrial swelling and Δ*Ψ*m loss assays. Liver mitochondria were isolated from 3 to 4 weeks old BALB/c mice as previously described [124]. Freshly isolated mitochondria were distributed in 96-well plates in a medium containing 200 mM sucrose, 5 mM succinate, 10 mM Tris–MOPS (pH 7.4), 1 mM H3PO4, 2 μM Rotenone, and 10 μM EGTA, supplemented with 1 μM rhodamine 123 (Rh123; Molecular Probes™), followed by the addition of serial dilutions of compounds. Then, absorbance at 545 nm and Rh123 fluorescence (excitation 485 nm, emission 535 nm) are recorded during 30 cycles of 1 min using a fluorescence multi-well plate reader (Genios, Tecan®, Männedorf, Switzerland). CaCl2 (30 μM) and mClCCP (20 μM) treatments were considered as 100% baseline for the swelling and Δ*Ψ*m loss, respectively. ED50 on mitochondrial swelling and Δ*Ψ*m were extrapolated from the dose–response curves (log 2 dilutions). Results are the mean of three independent experiments (SD were negligible). Note: compounds were added to mitochondria without any calcium pre-pulse.

3.2.1. Complexes I and II as direct functional targets in the pro-apoptotic cascade

Two complexes of the respiratory chain, I and II, constitute direct functional targets of the executioner caspase-3, a pro-apoptotic protease. Thus, the dysfunction of these two complexes leads to the dissipation of Δ*Ψ*m, the generation of ROS and contributes to the dismantling of the cell via a retro-amplification loop following the cytosolic Cyt *c* release [39]. Indeed, caspase-3 was inefficient to induce mitochondrial alterations when added directly onto intact isolated mitochondria requiring a permeabilizing event (e.g. Bid addition) to access to the IM. Thus, in apoptotic HeLa or Jurkat cells, caspase-3 disrupts oxygen consumption induced by complex I and II substrates, but not that induced by electron transfer to complex IV. In addition, complex III activity measured by Cyt *c* reduction remains intact after caspase-3 treatment [39]. These data are compatible with previous studies showing that in Fas-induced or TNF-induced apoptosis a reduction of complex I and II activity can be measured ([102,103], but these studies did not reveal the proteolytic substrate of caspase 3 that is responsible for this pro-apoptotic effect.

3.2.2. Complex I as an indirect regulator of MMP

Rat skeletal muscle mitochondria can undergo a mitochondrial permeability transition following Ca^{2+} uptake in the presence of Pi. However, in this case, this permeability transition can be dramatically modified by the substrates used for energization and the rate of electron flow through complex I [104]. This increased sensitivity of PTPC opening does not depend on differences in membrane potential, matrix pH, Ca^{2+} uptake, oxidation–reduction status of pyridine nucleotides or production of H_2O_2 In addition, ubiquinone 0 at concentrations which partially inhibit respiration and do not depolarize the IM, inhibits the PTPC opening [105,106], suggesting a site of regulation of the PTPC by complex I, and drawing perspectives

for its pharmacological modulation in living cells. These convincing studies were led with isolated mitochondria, waiting now for a molecular identification of the target site within PTPC.

In summary, as respiration is a crucial mitochondrial function, it is intuitively a potential mechanism to target for apoptosis modulation, notably via the modulation of ROS generation, and will undoubtedly furnish valuable therapeutic tools as soon as key components will be identified and characterized. One major issue will be the specificity of the pharmacological agent that should kill only some cells, and not all respiring cells.

4. Peptides targeting the mitochondrion to induce apoptosis

As our knowledge of apoptosis deregulation increases, the possibilities for pro and anti-apoptotic therapeutics seems not only promising, but a realistic adjunct to current treatments. Some peptides, for instance BH3-derived peptides, have reached the proof of principle step and can be either used as a basis for large screening using small molecule libraries or alternatively directly optimized as a backbone for the design of new therapeutically functional pseudopeptides. This later drug development strategy has strongly evolved during the last two decades and is now proved to be efficient, for instance in the field of opioid peptide-based analgesics, or HIV-1 host–cell attachment and entry) Indeed, structures of peptidic leads can be optimized in term of structure–efficacy relationships, conformation and stability in biological fluids and ease of synthesis, keeping in mind future scale-up requirement (Table 1).

Three examples of pro-apoptotic peptides have been selected to illustrate the potential use of peptides to modulate apoptosis via an effect on the mitochondrion. These peptides can be easily obtained by chemical synthesis and are in one case non-natural and in the other cases, derived from endogenous proteins (Bax/Bcl-2) and a viral protein, Vpr.

4.1. Anti-cancer activity of targeted pro-apoptotic peptides

Tumor development is largely dependent of oxygen and nutrient supply, which is provided by blood vessels irrigating the tumoral tissue. Thus, cancer growth requires continuous colonization by neo-vessels, a process which is called tumor angiogenesis. Selective inhibition of tumor angiogenesis thus presents many attractive features for oncology: as it is a very general process, and relies on normal untransformed host cells (mainly endothelial cells, which are the forerunners of tissue colonization during angiogenesis), it could be applied to many types of cancers, and avoid many hurdles of standard chemotherapies, such as the diversity of tumorisation processes, heterogeneity within a given tumor, genetic or chromosomal instability, and drug resistance. Antiangiogenesis is expected to be particularly efficient against metastasis development. Thus, this strategy raised an enormous interest, with various scientific strategies [107]. It is worth to note that among products in clinical or preclinical development for antiangiogenesis, most are so called " vasculostatics", i.e. inhibiting vessel growth, such as tyrosine kinase inhibitors, metalloproteinases inhibitors, antisenses, or endothelial cell surface receptors (e.g. integrin) blockers. Vasculotoxic compounds would be more interesting, as able to disrupt established vessels, and thus destroying tumors areas depending on their blood supply. However, most of these are in the early preclinical stages, mostly being of the targeted toxin type. In this context, Ellerby et al. [108] elaborated a computational approach to design short peptides composed of two functional domains, one a tumor blood vessel 'homing' motif and the other a programmed cell death-inducing sequence and synthesized them by chemistry [108]. One example of such peptide is the sequence CNGRC-GG-$_D$(KLAKLAK)$_2$. The 'homing' domain (CNGRC) was chosen to render the peptide specific of targeted cells and to allow its internalization. In addition, the pro-apoptotic domain ($_D$(KLAKLAK)$_2$) was designed to be toxic only when internalized into targeted cells by the disruption of mitochondrial membranes. Thus, the prototypes (only 21 and 26 residues) were selectively toxic to angiogenic endothelial cells, permeabilized mitochondrial membrane, and activated caspases to induce cell death by apoptosis [108]. Interestingly, these peptides showed anticancer activity in mice. Inoculation of the targeted pro-apoptotic peptide and a control non-targeted 'mimic' CARAC-GG$_D$(KLAKLAK)$_2$ peptide *in vivo*, in nude mice bearing human MDA-MD-435 breast carcinoma xenografts revealed that the pro-apoptotic peptides inhibited tumor growth and metastasis by inducing apoptosis and necrosis, without detectable toxicity and immunogenicity [108]. Those data, and subsequent studies demonstrating selective destruction of prostate carcinoma through similar strategy [109], constitute a proof of principle for the development of bi-functional peptides that trigger specifically neo-endothelia's mitochondrial membrane permeabilization and apoptosis.

Future will tell if such compounds can be sufficiently optimized to combine strong efficacy, with acceptable safety and ADME criteria, together with successful process development for large-scale production.

Table 1
Approaches for optimization of peptide-based drugs

- Structural analysis (RMN, RX) and computer-assisted molecular modelization
- Rationale and systematic ("alanine scanning" or "multi-scan" approaches) amino-acid substituting
- Insertion of amino acids analogs (N-methyl amino-acids, D-amino-acids, non-naturals residues), blocking of N-ter and C-ter ends (acylation, amidation), or cyclization (for instance S–S, CO–NH or C–C bonds)
- Improve linker structure of bifunctional/chimeric peptides through amino-acids changes or replacement by cleavable bonds (disulfide-linked conjugates)
- Peptide backbone modifications including: carbon–carbon double bonds (oleofinic constraints), rings (benzene, carbohydrates, lactams and azoles), bio-isosteric peptide bond replacements (for instance retro-inverso, methylene amino, or tetrazole isostere), and foldameres (β-peptides, γ-peptides, and isosteric backbones)

4.2. Homology domains of Bax/Bcl-2 family members

Bax/Bcl-2 family members sequences can be composed of 1 to 4 homology domains (BH1 to BH4) [110]. The various BH3

domains are recognized as necessary for the pro-apoptotic activity of the members, whereas the BH4 domains, as present only in anti-apoptotic proteins, would be associated to the inhibition of cell death.

Thus, BH3 peptides from pro-apoptotic members have been extensively studied as inducers of apoptosis *in vitro* but their precise mechanisms of action are still debated [30]. The proof of concept that BH3 mimetics can be designed that initiate apoptosis at definable points in the genetic pathway was published by Letai et al. [30]. This work described synthetic BH3 peptides that initiated cell death either by activating proapoptotic members or by counteracting anti-apoptotic members, displacing BH3 domains from their pockets. In a fusion strategy, the BH3 domain of Bak has been linked to a viral protein VP22 [111]. The BH3-VP22 protein was associated into oligonucleotides containing particles that entered cells and remained stable in the cytoplasm without toxicity. Via light activation, the fusion protein entered into cell, localized in the cytoplasm and the nucleus and induced the subsequent cell death by apoptosis. In control experiments, particles containing a mutant BH3 peptide, although indistinguishable in cell uptake and regulated release, showed no apoptotic effect. However, other studies utilizing chimeric cell permeant BH3 peptides have suggested that some of the cytotoxic effects were independent of direct interaction with Bcl-2 family members [112,113]. BH3 peptides are cationic amphipatic α-helices and consequently may act directly on mitochondrial membranes either through interaction(s) with sessile membrane proteins (for instance VDAC or ANT) or through lipid–peptide interactions leading to membrane disruption. Taking into account these « secondary » mechanisms of action, two opposite drug development strategies can be proposed : (1) use peptides, pseudo-peptides or small molecules selected to bind selectively Bcl-XL/Bcl-2, with considerable attention to have low toxicity onto mitochondrial membranes, (2) or, in contrast, to select peptides or pseudo-peptides with a very strong capacity to disrupt mitochondrial membranes and to combine them with a targeting strategy, as discussed in the previous section ($[KLAKKLAK]_2$ peptide) and in the next section (Vpr derived peptides). In line with the first, Bcl-2/ Bcl-X_L-based strategy, many groups have tried to improve peptide function by stabilizing the α-helical conformation of BH3 peptides (for review: [114]). An interesting example was obtained by Walensky et al. by the use of 'hydrocarbon stapling' to stabilize the alpha-helix of a Bid mimetic peptide and obtain a cell-permeable molecule that kills leukemia cells *in vitro* and *in vivo* [115]. This modification enhanced affinity for Bcl-2, protease resistance, and leukemia cell line toxicity *in vitro*. The compound was able to induce Cyt *c* release from isolated mitochondria in a Bak-dependent fashion. Moreover, mice bearing leukemia cell line xenografts had statistically significant survival improvement of 6 days. Although this observed efficacy with this compound remains insufficient to reach the clinical step, future optimized BH3-based compounds, possibly combined with conventional therapy, may provide a therapeutic benefit.

In another approach, Rosenberg, Fesik and co-workers used a high-throughput screening combined with iterative modulation of chemical structure based on NMR to find small molecules that bind Bcl-X_L. Two low-affinity ligands, a substituted biphenyl and a tetrahydronaphthol, were found to bind in adjacent pockets of the Bcl-X_L groove. Cycles of medicinal chemistry improved the affinity by connecting the two sites through a sulfonamide linker and introducing functional groups that reduced binding to human serum albumin. The resultant compound, ABT-737 (Abbott Laboratories), displace BH3 domains from Bcl-2, Bcl-X_L, and Bcl-w with an IC50 of no more than 1 nM. ABT-737 requires Bax and Bak for cell killing, and assays showed that ABT-737 enhanced radiation and traditional cytotoxic chemotherapeutics killing of several tumor cell lines. ABT-737 induced apoptosis in a primary culture of cells derived from two kinds of hematological malignancies known to express high levels of Bcl-2-follicular lymphoma and chronic lymphocytic leukemia—with the dose at which 50% cell killing occurs being in the 10 nM range. When administered daily for 3 weeks (intraperitoneally at 75–100 mg/kg), ABT-737 have significant activity in several mouse xenograft models of lung cancer and lymphoma [116]. Most impressive is the complete regression of tumor observed in small-cell lung cancer (SCLC)-xenografted mice. Although side effects including lymphopenia and thrombocytopenia were noted in mice this small BH3 mimetic may be useful for the treatment of lymphoma and SCLC as monotherapy and a wide variety of cancers when given in combination with chemotherapy or radiation.

Bcl-2 homology domains have been also used to inhibit apoptosis. For instance, a pioneering study form the group of Tsujimoto showed that the BH4 domain of Bcl-X_L fused to the protein transduction domain of HIV Tat efficiently prevented apoptotic cell death, possibly through VDAC closure [117]. *In vitro* studies extended the anti-apoptotic potential of BH4 peptides to various human tissues including islet [118] and coronary endothelial cells [119]. Finally, in rat models of cardiac ischemia–reperfusion *in vivo*, X-ray small intestine induced apoptosis or Fas-induced fulminant hepatitis, Tat-BH4 peptides proved to be potent apoptosis inhibitors, and to suppress heart failure and (partially) hepatic damages [120], suggesting a potential interest of this molecule for further (pre-) clinical studies.

4.3. Peptides derived from viral protein R (Vpr)

Viral protein R (Vpr, 96 aa) is a small accessory protein of HIV-1 (for review see: [121]). Taking advantage of the basic study of Vpr-induced apoptosis mechanisms, it has been possible to identify the mitochondrio-toxic domain of Vpr as located within the C-terminal part of protein (aa71–82), and to derive several pro-apoptotic peptides from this sequence as well as inactive mutant. This showed that when Vpr is added to intact various type of cells (not only lymphoid cells) or purified mitochondria, it induces hallmarks of MMP: a rapid dissipation of $\Delta\Psi$m and the mitochondrial release of apoptogenic proteins such as Cyt *c* or AIF via a direct

interaction with the PTPC [91]; Jacotot, 2001 #3792]. In artificial bilayers containing the purified PTPC or ANT, Vpr favors the appearance of a conductance indicating the opening of a large channel in the presence of Bax. The c-terminal moiety of Vpr (Vpr52–96) binds to purified ANT, as well as a molecular complex containing ANT and VDAC. Interestingly, yeast strains lacking ANT or VDAC are less susceptible to Vpr-induced killing than control cells yet recover Vpr sensitivity when retransfected with yeast ANT or human VDAC [91]. Initial surface plasmon resonance studies identified a domain of ANT able to interact with Vpr52–96 as the 104–116 aminoacids sequence, i.e. a hydrophilic loop facing the intermembrane space [91,122]. To specify this interaction, Sabbah et al., synthesized fragments of both proteins demonstrated that *in vitro*, the [27–51] and [71–82] Vpr peptides bind to a region encompassing the first ANT intermembrane space loop and part of its second and third transmembrane helices [123]. These authors also constructed a three-dimensional model of the Vpr–ANT complex in which the N-terminus of Vpr plunges in the ANT cavity whereas the Vpr C-terminal extremity is located at the surface of the ANT allowing possible interactions with a third partner. These results could be used to design molecules acting as pro-apoptotic Vpr analogs or as apoptosis inhibitors preventing the Vpr–ANT interaction. Key features of Vpr toxicity appeared to reside within its conformation in amphipatic helix and within positively charged residues, as shown by the abolishment of mitochondriotoxicity by replacement of any of the arginines (R73, R77, R80) by alanine residues, or by insertion of D-proline to impose non helical conformation [67,68]. Our recent SAR study indicates that a modified D version of the cysteine-devoid peptide Vpr67–82 ($_D$Vpr67–82[C76S]) is a very potent inducer of the mitochondrial swelling and $\Delta\Psi$m loss (Fig. 3). *In vivo* studies are ongoing, using Vpr-derived peptides linked with cell-penetrating sequences or homing sequences to determine the capacity of such molecules to influence the outcome of tumor growth.

5. Conclusion

Apoptosis is a physiological mechanism that can be altered in a wide range of human diseases. Thus, a slight deregulation of apoptosis can lead to an accumulation of mutated or unwanted cells as well as an excessive depletion of a particular cell type. During apoptosis program, mitochondria play a decisive role acting as an integrator of multiple intracellular signaling pathways and a potent coordinator of the final degradation phase [24]. Thus, targeting the mitochondria to modulate cell death has focused the interest of the pharmaceutical research leading to the investigation of diverse strategies such as anti-sense oligonucleotides or small molecules. As many effectors of apoptosis are proteins and as they are in dynamic interactions with each other, one alternative possibility is to exploit the potential of chemistry to derive peptides from existing molecules (e.g. Bax/Bcl-2 members, Vpr) or to synthesize novel sequences from computational methods. Since peptides and pseudo-peptides cost of synthesis are decreasing and their toxicity is generally limited, once their *in vivo* stability and bio-disponibility will be optimized, this type of therapeutic molecules should be promising in terms of selectivity, specificity and efficiency. We anticipate that major advances in the mitochondrial apoptosis field will be made in the future and will help to define new molecules for the treatment of challenging human diseases.

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