Commentary

Mitochondrial Apoptosis and the Peripheral Benzodiazepine Receptor: A Novel Target for Viral and Pharmacological Manipulation

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Viruses mercilessly exploit their host cells to guarantee their own proliferation and propagation. To achieve this goal, many viruses suppress the apoptotic program, thereby avoiding premature death of the host cell. Indeed, apoptosis of infected cells may be considered as a pristine defense against infectious pathogens, as illustrated by a simple *Gedankenexperiment*: if all host cells died immediately after infection, then the virus could not replicate. It is only at late stages of the viral life cycle that some viruses actively induce apoptosis, either in their host cells or, via a variety of different strategies, in immunologically relevant cells, with the specific aim to subvert the host's innate or cognate immune response. As viruses have coevoluted with their host to adapt to particular ecological niches, they have "learned" to target strategic processes in their host cell's biology. One fascinating example is now provided by the poxvirus that causes myxomatosis, a lethal disease which has been eradicating entire populations of rabbits. Myxoma virus codes for a protein designated M11L, which, as shown in this issue (1), inhibits host cell apoptosis by acting on the peripheral-type benzodiazepine receptor (PBR).

M11L: An Antiapoptotic Virulence Factor Targeted to the Mitochondrial PBR. The 18-kD M11L protein is a major virulence factor for myxomatosis. In vivo, *M11L* knockout viruses provoke a greatly reduced mortality and induce more vigorous, presumably host-protective inflammatory reactions than pathogenic wild-type strains. In vitro, *M11L* knockout viruses cause accelerated apoptosis in infected rabbit lymphocytes or monocytes, as compared with wild-type controls (2, 3), suggesting that M11L acts as an inhibitor of apoptosis. Overexpression of M11L suffices to inhibit apoptosis induced via a variety of nonviral inducers suggesting that it acts as general rather than a virus- or signal-specific apoptosis inhibitor (1).

It is important to note that M11L is not the only antiapoptotic virulence factor encoded by myxoma virus. Additional antiapoptotic proteins include (a) Serp2, a putative caspase inhibitor, (b) T2, a TNF receptor homologue which neutralizes proapoptotic TNFα, (c) myxoma virus leukemia-associated protein, which down-regulates Fas/CD95 and class I molecules, and (d) T7, an interferon γ receptor homologue which inhibits proapoptotic interferon γ (4, 5). Myxoma virus relies on a complex strategy to intercept apoptotic and cytotoxic insults to virus-infected cells, because deletion of one single gene among these apoptosis inhibitors suffices to attenuate the virus.

The M11L protein targets mitochondria via a 25 amino acid long COOH-terminal targeting sequence that is similar to a unique transmembrane consensus sequence present in antiapoptotic Bcl-2 family members. Deletion of the COOH-terminal mitochondrial targeting sequence abolishes the antiapoptotic function of M11L (3) suggesting that M11L indeed acts on mitochondria to suppress apoptosis. Importantly, M11L homologues are encoded by other pathogenic poxviruses, namely rabbit fibroma virus (Gp011L), swinepox virus (C10), sheeppox virus (antiapoptotic virulence factor), lumpy skin disease virus (LSDV017), which infects cattle in Africa, and the virus responsible for Yaba-like disease (16L). Several among these proteins (e.g., Gp011L, C10) share the COOH-terminal mitochondrial localization motif with M11L, suggesting that M11L constitutes the prototype of a novel class of apoptosis regulators acting at the mitochondrial level.

Based on cross-linking studies, FRET analyses and functional tests, it appears that the M11L protein physically and functionally interacts with the PBR, the benzodiazepine receptor which is confined to mitochondria (1). The functional interaction between M11L and PBR is abolished by deletion of the COOH terminus, indicating that the mitochondrial localization of M11L is required for its action on PBR (1). As the interaction of M11L with the PBR is compatible with simultaneous binding of the synthetic PBR ligand FGIN-1–27 to its receptor (1), the capacity of M11L to inhibit apoptosis cannot be attributed to a mere PBR blockade. Thus, M11L acts as a functional (rather than competitive) PBR modulator. The question that remains to be answered is how does M11L then act?

Mitochondrial Regulation of Apoptosis. Beyond their role as the cell's power house, mitochondria exert a major function as suicide organelles (6). In response to multiple different apoptosis-inducing stimuli, which may involve signals as

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different as Ca^{2+}, reactive oxygen species, ganglioside GD3, proapoptotic members of the Bcl-2 family, kinases, or specific proteases, mitochondrial membrane permeabilization (MMP) occurs. Antiapoptotic proteins of the Bcl-2 family have the property to reside in mitochondrial membranes and to locally inhibit MMP. Massive MMP is incompatible with further cell survival and thus indicates that the cell has trespassed the point-of-no-return of the apoptotic program. Partial permeabilization of the inner mitochondrial membrane leads to an abrupt collapse of the transmembrane potential ($\Delta\Psi_m$), a sign of MMP, while complete permeabilization of the outer membrane, linked to remodeling of mitochondrial ultrastructure, culminates in the release of potentially toxic intermembrane proteins into the rest of the cell. Such intermembrane proteins include caspase activators such as cytochrome c (which together with Apaf-1 and caspase-9 forms a caspase-3–activatory complex, the apoptosome), and Smac/DIABLO (an inhibitor of the caspase-inhibitory IAP proteins), as well as caspase-independent death effectors such as apoptosis-inducing factor (AIF) and endonuclease-G (7). Apparently, M11L does block MMP, as it prevents both the dissipation of $\Delta\Psi_m$ (1, 3) and the mitochondrial release of cytochrome c (1).

Permeabilized mitochondria also release endozepine (also called "acyl-CoA-binding protein" or "diazepam binding inhibitor"), the endogenous ligand of the PBR (8, 9). On theoretical grounds, endozepine released from mitochondria which have undergone MMP may act on the PBR of yet intact mitochondria and thus participate in a positive feedback loop which accelerates MMP induction throughout the cell (8, 9). The finding that M11L acts on PBR thus suggests that M11L acts on one of the neuralgic points of apoptotic control. Indeed, M11L prevents the induction of MMP by protoporphyrin IX, an apoptogenic PBR ligand, in permeabilized cells (1), suggesting a direct effect on the mitochondrial checkpoint of apoptosis.

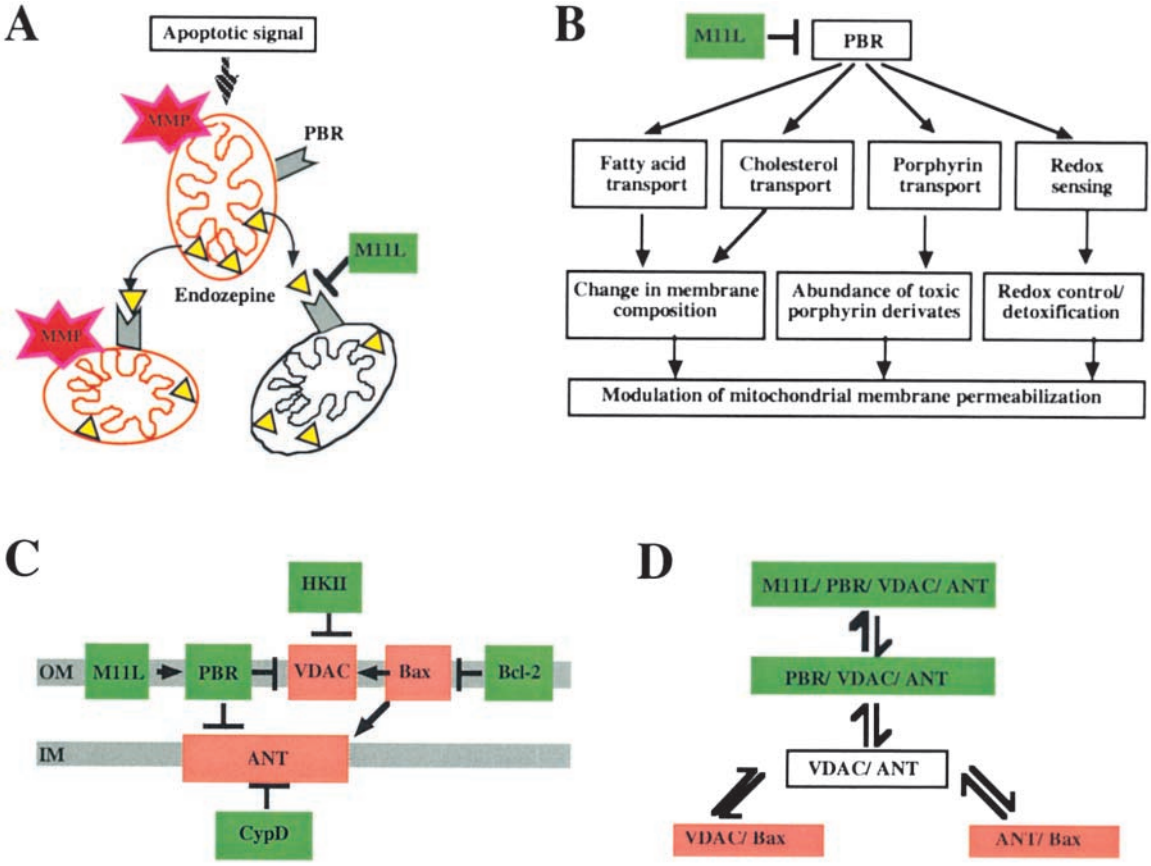

Figure 1. Four alternative (and nonexclusive) hypotheses to explain the apoptosis-inhibitory mode of action of M11L. (A) M11L-mediated blockade of PBR responses to an endogenous proapoptotic ligand. One of the ligands of PBR, endozepine, is known to be released from mitochondria when they undergo the apoptotic permeabilization reaction. If endozepine binds to PBR of yet intact mitochondria, thereby participating in the permeabilization reaction, the function neutralization of PBR would have an antiapoptotic effect by blocking an amplification loop. (B) M11L-mediated alterations of PBR function having indirect effects on mitochondrial metabolism and apoptotic control. PBR is known to participate in the regulation of mitochondrial lipid metabolism, porphyrin transport, and redox sensing. Changes in the function of PBR induced by its interaction with M11L thus could indirectly modulate apoptosis. (C) M11L-mediated alterations in the function of pore-forming proteins within the PTPC. In this scenario, PBR would impinge on the conformation of its interacting partners VDAC and ANT, thereby preventing them to convert into nonspecific pores. Hexokinase II (HKII) and cyclophilin (CypD) may have similar effects. (D) M11L-induced shifts in the interactions among PTPC proteins. Hypothetically, PBR would, via complexing ANT and VDAC, prevent the pore-forming interaction with other proteins such as Bax. M11L binding would stimulate this function of PBR. Proapoptotic proteins, protein complexes, or processes are depicted in red and antiapoptotic ones in green.

Whether M11L interrupts a hypothetical endozepine-mediated, PBR-dependent MMP process (Fig. 1 A), remains, however, a subject of speculation, and other possible modes of action of M11L are conceivable.

PBR: A Regulator of Mitochondrial Apoptosis. PBR is a widely expressed, evolutionarily conserved 18-kD outer mitochondrial membrane protein. It is likely to possess a five-transmembrane structure, and it is endowed with several functions including cholesterol transport from the outer to the inner mitochondrial membrane, regulation of steroidogenesis, porphyrin transport, sensing of reactive oxygen species, and regulation of apoptosis (10). Transfection-enforced overexpression of PBR attenuates apoptosis induced by oxygen radicals or ultraviolet light (10). In line with this predominantly apoptosis-inhibitory function, PBR overexpression also enhances the antiapoptotic effect of M11L (1). The benzodiazepine Ro5–5864 and the isoquinoline carboxamide PK11195 exhibit nanomolar affinity for the PBR, and are the archetypic pharmacological tools for detecting and exploring the receptor (Table I). Saturating (micromolar) doses of PK11195 or Ro5–4864 can sensitize cancer cells to the induction of apoptosis, either in vitro or in vivo (11).

In addition to PK11195 and Ro5–5864, several apoptosis-inducing or apoptosis-inhibitory compounds act on the PBR. Thus, the neuroprotective (antiapoptotic) drug rasagiline prevents PK11195 binding to the PBR (12). Conversely, the platelet-activating factor (PAF), a phospholipid involved in neuronal excitotoxicity, induces cytochrome c release from isolated mitochondria in a fashion that is inhibited by PK11195 and Ro5–4864 (13). Photosensitizing porphyrins, as well as their precursors and derivatives, may also induce mitochondrial membrane permeabilization and apoptosis through an effect on the PBR (14).

Although these pharmacological data do not elucidate the mode of action of PBR, they illustrate the importance of PBR as an endogenous modulator of mitochondrial apoptosis and as a prospective drug target. How PBR modulates apoptosis is not clear. PBR could regulate apoptosis through indirect effects on mitochondrial metabolism (Fig. 1 B), by serving as a receptor for other apoptosis-relevant proteins (such as endozepine or perhaps PRAX-1, another

Table I. *Endogenous Apoptosis Regulatory Proteins Acting at the Mitochondrial Level*

Target protein	Endogenous modulators	Viral modulators or analogs	Pharmacological modulators (examples)
PBR	Endozepine Platelet-activating factor	**M11L (myxoma virus)**	PK11195, XK469, FGIN-1-27, 4′-chlorodiazepam, Ro5-4864, BBL22, protoporphyrin IX, **rasagiline**?
VDAC	**NADH** **Hexokinase II** **Bcl-2**, Bax	HVB-X protein[a]	**BH4 domain peptides**
ANT	**ATP, ADP** **Bcl-2**, Bax Oxidative stress	Vpr (HIV-1) **vMIA (cytomegalovirus)**	Atractyloside, **bongkrekate**, lonidamine, CD437, MT-21
Cyclophilin D	Misfolded proteins?	Unknown	**Cyclosporin A** **N-methyl-4-valine-cyclosporin** **N-methyl-4-isoleucine-cyclosporin**
Bcl-2/Bax	BH3-only proteins Kinases + phosphatases Proteases	**BORFB2F, BALF1** **5-HL.A179L, HVS-Bcl-2** **M11**	Peptides containing BH3 motifs HA-14-1, BH3I-1, BH3I-2 Antimycin A
Hexokinase II	**Glucose**, Glucose-6-phosphate	Unknown	Unknown

Underline, apoptosis inducers; bold, apoptosis inhibitors.
[a]HVB-X has also been described to inhibit apoptosis.

PBR-binding protein), or by influencing the overall function of a complex of proteins interacting with PBR.

The Permeability Transition Pore Complex, a PBR-associated Polyprotein Complex Targeted by Viral Proteins. PBR interacts with several resident mitochondrial proteins, in particular with the voltage-dependent anion channel (VDAC) in the outer membrane and the adenine nucleotide translocase (ANT) in the inner membrane, both of which form the backbone of a polyprotein complex designated as the permeability transition pore complex (PTPC). The PTPC involves additional proteins, in particular hexokinase II (which binds to the cytosolic face of VDAC), the proteins from the Bcl-2/Bax family (which interact with VDAC and ANT), and cyclophilin D (which binds to the matrix side of ANT). Although the exact stoichiometry and molecular architecture of the PTPC remains elusive, it has been extensively documented that Bax-like proteins, VDAC, and ANT can mediate the permeabilization of model membranes in vitro (6). Thus, PBR could modulate apoptosis through direct molecular interactions with pore-forming PTPC components (Fig. 1 C). Alternatively, PBR could influence the molecular dynamics of intra-PTPC protein–protein interactions in an indirect fashion (Fig. 1 D), for instance by removing VDAC or ANT from their proapoptotic interaction with cyclophilin D or Bax-like proteins.

Importantly, Bax-like proteins, VDAC, and ANT have been identified as targets of pathogenic viral proteins (Table I). Thus, the genomes of several viruses code for death-inhibitory Bcl-2 analogs which preferentially localize to mitochondria and may interact with proapoptotic Bax homologues: Epstein-Barr virus (proteins: BORFB2F and BALF1), African swine fever virus (protein 5-HL/A179L), herpesvirus saimiri (HVS-Bcl-2), Kaposi sarcoma-associated herpes virus 8 (KSBcl-2), bovine herpesvirus 4 (BHRF-1), and murine gammaherpesvirus-68 (M11; reference 15). The apoptosis-modulatory hepatitis B virus X protein (HBV-X) interacts with the human VDAC3 isoform and stimulates local ROS production (16). The protein vMIA (viral mitochondrial inhibitor of apoptosis) encoded by cytomegalovirus specifically interacts with ANT. Mutations of vMIA which abolish its mitochondrial localization also curtail its antiapoptotic function, suggesting that vMIA indeed acts on mitochondria to inhibit apoptosis (17).

ANT is also one of the targets of Vpr, a small (96 amino acids) accessory protein of human immunodeficiency virus-1 (HIV-1), which interacts with ANT through an α-helical dodecapeptide domain (residues 71–82) and can form a composite ion channel with ANT (18). Intriguingly, mutations which abolish the apoptogenic Vpr/ANT interaction, either due to stop codons (19) or due to a selective point mutation (R77Q; reference 20), can be found among long-term nonprogressors, correlating with a decrease in apoptosis induction by the corresponding HIV-1 isolates (19). This strongly suggests that Vpr is an important virulence factor. These examples illustrate that a variety of different viruses, in addition to M11L, target the PTPC.

The PTPC: A Therapeutic Target? Until now the notion that PBR controls apoptosis has been based on the non-physiological overexpression of PBR (10) and on the use of pharmacological PBR ligands (21), which, especially at high concentrations, may have additional, PBR-unrelated effects (22). The finding that a pathogenic virus targets PBR to inhibit apoptosis (1), thus provides an important confirmation that PBR does participate in the regulation of cellular demise. This discovery thus relaunches PBR as a putative drug target for apoptotic regulation. When combined with cytotoxic agents, PBR ligands reportedly have therapeutic effects in preclinical animal models of tumor chemotherapy (11, 23). This applies to diazepam, which cooperates with lonidamine to eradicate human glioblastoma (23), and to Ro5–4864 which cooperates with etoposide and ifosfamide to inhibit the growth of human small cell lung cancers xenografted into nude mice (11). Importantly, it appears that PBR ligation can overcome apoptosis resistance conferred by Bcl-2 in a number of models and that PBR is overexpressed in some tumors (1, 11, 21).

PBR-associated proteins within the PTPC may also serve as targets for the therapeutic manipulation of apoptosis (Table I). Thus, several prospective anti-cancer agents such as lonidamine and MT21 may target ANT (21). Similarly, small molecules binding to the BH3 peptide-binding groove of antiapoptotic Bcl-2 family proteins promote cancer cell apoptosis (24). The pore forming function of VDAC can be inhibited by a peptide containing residues 4–23 of Bcl-x(L), the so-called BH4 domain, conjugated to the protein transduction domain of HIV TAT. Such a peptide (TAT-BH4) can reduce infarct size in a model of heart ischemia (25). Attempts are also on the way to create non-immunosuppressive ligands of cyclophilin D which inhibit the PTPC and have cytoprotective effects in vitro (26) and cardioprotective effects in vivo (27).

Another fascinating possibility that emerges from the finding that pathogen-encoded proteins act on PTPC concerns the treatment of viral disease. For instance, if M11L binding to PBR is required for the lethal development of myxomatosis, then drugs which inhibit this interaction or neutralize its functional consequences should cure myxomatosis. A similar approach may be envisaged for neutralizing viral PTPC-targeted proteins relevant for human diseases such as heptatitis B (HBV-X), cytomegalovirus infection (mVIA), or AIDS (Vpr).

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