PERSPECTIVE

Relevance and therapeutic potential of CypA targeting to block apoptosis inducing factor-mediated neuronal cell death

Programmed cell death (PCD) signaling pathways are important contributors to acute neurological insults such as hypoxic-ischemic brain damage, traumatic brain injury, stroke *etc*. The pathogenesis of all these diseases is closely linked with aberration of apoptotic cell death pathways.

Mitochondria play a crucial role during PCD, acting as both sensors of death signals, and as initiators of biochemical pathways, which cause cell death (Bras et al., 2005). Cytochrome c was the firstly identified apoptogenic factor released from mitochondria into the cytosol, where it induces apoptosome formation through the activation of caspases. Other proteins, such as apoptosis inducing factor (AIF), have been subsequently identified as mitochondrial released factors. AIF contributes to apoptotic nuclear DNA damage in a caspase-independent way (Bras et al., 2005).

AIF is a flavoprotein with NADH oxidase activity, anchored in the mitochondrial inter-membrane space (IMM). Its mature form contains two flavin adenine dinucleotide (FAD)-binding domains (residues 122–262 and 400–477), a nicotinamide adenine dinucleotide (NADH)-binding domain (residues 263–399) and a C-terminal domain (residues 478–613) (Sevrioukova, 2011).

In mammalian cells, AIF plays a crucial role in mitochondrial metabolism, while its absence causes a respiratory chain defect that is coupled to the post-transcriptional downregulation of protein subunits belonging to respiratory chain complexes I, III, and IV. Harlequin (Hq) mice, which feature a significant reduction of AIF expression, develop severe neuromuscular mitochondriopathies that lead to premature death (Sevrioukova, 2011).

Upon apoptotic stimuli, AIF is cleaved by calpains and/or cathepsins to yield the pro-apoptotic form AIF(Δ1-121), which relocates from mitochondria to the cytosol and nucleus, where it provokes chromatinolysis and large scale DNA fragmentation (~50 kb) in dying neurons (Candé et al., 2004). Hq mice show reduced brain damage after cerebral ischemia and their neurons are resistant to N-methyl-D-aspartic acid (NMDA) or glutamate neurotoxicity *in vitro* (Piao et al., 2012).

These findings have indicated that AIF plays a key role in PCD following neurological insults and is a novel therapeutic target to prevent the effects of neurodegenerative diseases. Strategies to block AIF lethal action include the delivery of Bcl-xL proteins, or the inhibition of Bid activation to prevent mitochondrial membrane permeabilization and AIF release (Culmsee and Plesnila, 2006). In alternative to these strategies, which imply the activation of indirect mechanism of protection, inhibition of the complex between AIF(Δ1-121) and cyclophilin A (CypA) has been suggested for the direct interference of the AIF-mediated cell death (Candé et al., 2004).

CypA is a member of the peptidyl-prolyl isomerase (PPIase) family, a group of proteins that catalyze *cis-trans* isomerization of peptidyl-prolyl bonds during protein folding and/or conformational changes (Handschumacher et al., 1984). CypA was identified as the primary intracellular target of the immunosuppressive drug cyclosporin A (CsA). CsA binds tightly into the catalytic pocket of CypA and the resulting complex inhibits the phosphatase activity of calcineurin (CaN), leading to T-cell inactivation (Handschumacher et al., 1984). Beyond the different roles played into several cellular contexts, CypA promotes the AIF-mediate neuronal cell death, by mediating its nuclear translocation and/or its DNase activity. During the first stage of apoptosis AIF and CypA co-immunolocalize in the nucleus. *In vitro*, recombinant AIF and CypA cooperate in the degradation of plasmid DNA, and induce DNA loss in purified nuclei. Noteworthy, AIF fails to induce apoptosis in CypA-knockout cells, but this is reversed by the reintroduction of the CypA gene into CypA-deficient cells (Candé et al., 2004). Overall, these findings demonstrate the key role of the AIF/CypA complex in neurodegeneration with a significant relevance in those diseases where PCD is prominent.

At the molecular level, two different models have been proposed to explain the AIF/CypA mediated neuronal loss. One proposes that the lethal translocation of AIF to the nucleus requires its direct interaction with CypA in the cytosol. In support of this hypothesis, it has been found that the nuclear translocation of AIF is significantly reduced in a model of perinatal hypoxia/ischemia in $CypA^{-/-}$ mice compared to wild type mice. Similarly, in Hq mice, CypA staining is reduced in the nucleus after injury, and this well correlates with the protective effects in models of cerebral ischemia (Zhu et al., 2007). A second model provided evidences according to an independent translocation of AIF and CypA in the nucleus, where then they regulate chromatinolysis and programmed necrosis by generating an active DNA-degrading complex involving other partners. Evidences in support of this idea are produced in N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced programmed necrosis, where CypA down-regulation does not arrest nuclear translocation of AIF but reduces DNA damage (Artus et al., 2010).

More recently, we analyzed the role of AIF/CypA complex in HT-22 neuronal cell, an *in vitro* model to study glutamate cytotoxicity, a characteristic of many neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Doti et al., 2014). These cells lack caspase-3 and only AIF translocation occurs during oxidative stress. In these cells, the down regulation of CypA prevents AIF(Δ1-121) translocation to the nucleus despite the glutamate challenge and preserves mitochondrial bioenergetics (Doti et al., 2014). In particular, the first responses to oxidative stress is the rapid accumulation of CypA in the nucleus some hours before AIF(Δ1-121) mitochondrial release, suggesting that CypA can work as both a nuclear carrier of AIF and as a signaling player that controls cell death on glutamate exposure. Also, a massive condensation of mitochondria around the nucleus during the apoptotic process is observed. These data, altogether, support the hypothesis of a mechanism by which oxidative insults induce first the nuclear translocation of CypA and, subsequently, a fast release of AIF, which is shuttled from cytosol to the nucleus by CypA. The two proteins then together catalyze DNA fragmentation and induce apoptosis (Doti et al., 2014). These findings further demonstrate the crucial role of the complex in PCD paradigms, though the molecular events of caspase-independent cell death depend on both cell type and cellular stress.

The proof-of-concept of this mechanism came few years ago. Indeed, we provided evidence that a peptide targeting the CypA-binding site on AIF (region 370–394) is able to block the AIF/CypA axis and to induce neuroprotection in cultured HT-22 cells treated with glutamate. The delivery of the peptide blocks the nuclear translocation of both AIF and CypA in a way similar to that observed following siRNA-mediated CypA downregulation and drastically reduces the sensitivity to glutamate-induced oxidative stress in cells. Importantly, like CypA siRNA, the peptide induces mitoprotection without direct effects on mitochondria, suggesting alternative mechanisms of mitochondrial function recovery and a possible interference with the CypA-dependent mechanisms in the cytosol and in the nucleus. Such a new activity broadens the protective effects of CypA targeting and its strong neuroprotective potential, as the inhibition of AIF release through the protection at level of mitochondria is much more efficient than that deriving from the inhibition of AIF nuclear entry alone (Doti et al., 2014). Moreover, due to the dual role of AIF in both mitochondria respiratory chain and apoptosis, the pharmacological use of AIF inhibitors should be able to attenuate the pro-apoptotic role of AIF, without interfering with its vital functions. With this aim, targeting of CypA to inhibit the AIF-mediated neuronal loss might represent a valuable strategy. This interaction does not impair the enzymatic activity of AIF, and furthermore, unlike Hq mice, CypA$^{-/-}$ animals are phenotypically normal. However, the lack of interference with enzymatic and immune-suppressive activity of CypA is a necessary condition for the clinical use of such CypA-targeting compounds.

In order to obtain new insights on the molecular recognition mechanism underlying this interaction, which is crucial for the design of new inhibitors, we have undertaken a NMR structural and biophysical characterization of the AIF(Δ1–121)/CypA and AIF(370–394)/CypA complexes (Farina et al., 2017). In substantial agreement with a previous molecular model of the complex, we mapped the binding surface of AIF(Δ1–121) on CypA, on loops α1–β3 (G47, S51), β4–β5 (F67, R69, T73, G74, K76, I78, K82, E86, G96) and β5–β6 (A101, N102, S110, Q111). The structure-based model shows that AIF(Δ1–121) binds CypA on a hydrophilic region very close to the catalytic and CsA binding site that includes residues G42–C52 and G65–S99. By this model, CypA residue R69 is crucial for AIF recognition, establishing hydrogen bond and Van der Waals interactions with V374 and S375 of AIF(Δ1–121) and is one of the most important hot spots for structure-based drug design. Remarkably, the CypA binding surface suggested for AIF(Δ1–121), accommodates very well residues included in AIF(370–394), but the peptide appears to bind also the crucial catalytic site R55 of CypA in a manner closely resembling that of CsA. However, most importantly, like AIF(Δ1–121), the peptide has no effects on the catalytic activity of CypA. The non-suppressive character of the peptide compared to CsA, derived from its reduced affinity for the CypA catalytic residue. Indeed, AIF(370–394) recognition is substantially retained in the R55A mutated CypA and peptide binding is prevented in the presence of CsA. These results propose AIF(370–394) as a lead compound for developing novel drugs for therapeutic applications and as a chemical tool to further elucidate the role of the complex *in vitro* and *in vivo* models of neurodegeneration.

Further studies are underway to identify the crucial peptide residues that interact with CypA and that are critical for the rational design of new selective inhibitors of the AIF/CypA complex.

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Open peer review reports:
Reviewer 1: Gopi Margabandhu, University of Madras, India.
Comments to authors: The authors described and well explained about the effect of CypA. Furthermore, more reference article coated and supported for AIF/CypA complex neuroprotection against like AD, PD. They demonstrated positive effects of this complex on oxidative stress and also structure based molecular interaction well explained using drug designing. Finally say that this complex new selective inhibitor and therapeutic application of neurodegeneration model.
Reviewer 2: Cyrus David Mintz, Johns Hopkins School of Medicine, USA.
Comments to authors: This is a logical, well constructed brief review of AIF as a critical component of apoptosis mechanisms and the possibility of using CypA as an anti-apoptotic target.

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