

Review

# Pharmacological inhibition of mitochondrial membrane permeabilization for neuroprotection

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ARTICLE INFO

Article history:

Received 1 December 2008

Revised 26 February 2009

Accepted 3 March 2009

Available online 18 March 2009

Keywords:

Apoptosis

Mitochondria membrane permeabilization

Caspase

AIF

Apaf-1

Cytochrome c

Retina

ABSTRACT

Recent data have provided important clues about the molecular mechanisms underlying certain neurodegenerative diseases. Most cell death in vertebrates proceeds via the mitochondrial pathway of apoptosis. Mitochondria contain proapoptotic factors such as cytochrome *c* and AIF in their intermembrane space. Furthermore, mitochondrial membrane permeabilization (MMP) is a critical event during apoptosis, representing the “point of no return” of the lethal process. Modern medicine is developing an increasing number of drugs for neurodegenerative disease, but no neuroprotective treatment has yet been established. While current treatments temporarily alleviate symptoms, they do not halt disease progression. This paper briefly reviews the pharmacological inhibition of mitochondrial membrane permeabilization for neuroprotection.

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Introduction

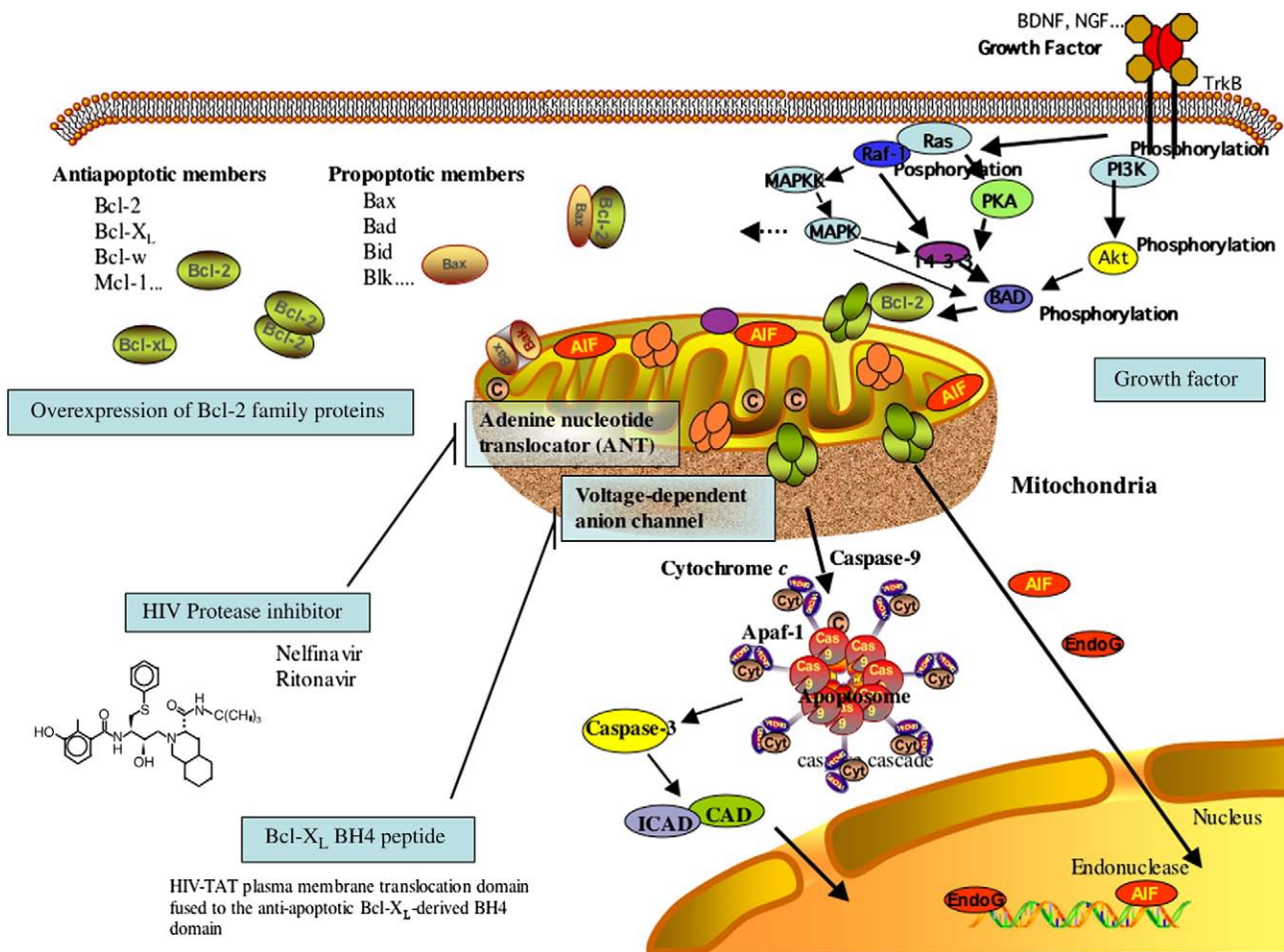
Many neurons die from necrosis and apoptosis through various mechanisms such as ischemia, mechanical stress, or degeneration. Therapeutic targeting of apoptosis (rather than necrosis) appears feasible because apoptosis is a delayed event and an energy-

dependent, regulated process. Mitochondria are considered to be the central regulators of apoptotic cell death. In various paradigms of cell death, mitochondrial membrane permeabilization (MMP) delimits the frontier between life and death (Fig. 1). Mitochondria control the intrinsic pathway of apoptosis, in which MMP ignites the activation of caspases and other catabolic enzymes, and mitochondria participate in the extrinsic pathway of apoptosis, in which they amplify the self-destructive process (Green and Reed, 1998; Hengartner, 2000; Kroemer et al., 1997). Irrespective of its initiation at the inner or outer mitochondrial membrane, MMP culminates in the functional and structural collapse of mitochondria. The functional loss of mitochondria is accompanied by the dissipation of the mitochondrial membrane potential, shutdown of ATP synthesis, and a redox imbalance. The structural disruption of mitochondria leads to the reorganization of cristae and to the release of toxic intermembrane space proteins into the cytosol. MMP has a profound impact on cellular metabolism, activates caspase-dependent and -independent executioner mechanisms, and finally results in the demise of the cell (Ferri and Kroemer, 2001; Galluzzi et al., 2008). The lethal consequences of MMP relate to the critical position occupied by mitochondria in cellular bioenergetics and the release of proapoptotic proteins into the cytosol and the nucleus. Proapoptotic proteins liberated as a consequence of MMP include activators of the caspase cascade (e.g. cytochrome *c*), as well as caspase-independent death effectors (e.g. apoptosis-inducing factor (AIF) and endonuclease G) (Garrido et al., 2006; Li et al., 2001). Indeed, mitochondrial membrane

permeabilization (MMP) is the main checkpoint of programmed cell death, and lethal pathways of signal transduction are often activated in neurodegenerative diseases. Hence, pharmacological agents that target mitochondria to subvert MMP are being evaluated as therapeutic approaches for the avoidance or treatment of neurodegenerative disorders. Here, we summarize the checkpoints of mitochondrion-dependent apoptosis and review current concepts on pharmacological targeting of mitochondria for neuroprotection.

### Mitochondrial outer membrane permeabilization

MMP may affect the outer membrane through at least two distinct mechanisms. First, the activation of proapoptotic proteins of the Bcl-2 family (e.g., Bax, Bak) can lead to the generation of multimeric channels, allowing for the release of intermembrane space proteins (Zamzami and Kroemer, 2001), or alternatively to the formation of lipidic pores due to the interaction between proapoptotic Bcl-2 family members (e.g., Bax, truncated Bid) and lipids contained in mitochondrial membranes (Galluzzi et al., 2008; Green and Kroemer, 2004; Kroemer et al., 2007). Second, outer membrane permeabilization can occur upon its physical rupture, be it induced accidentally or as part of a regulated process originating at the inner membrane (the so-called mitochondrial permeability transition). Irrespective of the precise molecular mechanisms, outer membrane permeabilization culminates in the release of proapoptotic intermembrane space proteins, which trigger the execution process of apoptotic cell death.



**Fig. 1.** The mitochondrial pathway to apoptosis and its inhibition for neuroprotection. The scheme summarizes the mechanisms of mitochondrial membrane permeabilization discussed in this review, as well as the post-mitochondrial effector mechanisms that are either caspase-dependent or caspase-independent. Different strategies for neuroprotection are also enumerated. For details see main text.

### Mitochondrial inner membrane permeabilization

Mitochondrial membrane permeabilization may also start at the inner membrane. In contrast to the outer membrane, the inner membrane from healthy cells is nearly impermeable to small solutes and ions. When inner membrane impermeability is lost, for instance following the opening of the so-called permeability transition pore complex (PTPC), solutes enter the mitochondrial matrix, accompanied by a net influx of water. This process is known as mitochondrial permeability transition and can be inhibited by the knockout of cyclophilin D (a mitochondrial matrix protein) or by the use of cyclosporin A or other pharmacological inhibitors of cyclophilin D (Broekemeier et al., 1989; Marzo et al., 1998a,b; Vieira et al., 2000; Nakagawa et al., 2005; Baines et al., 2005).

### Importance of caspase-dependent pathways on neurodegenerative disease

Cystein aspartate-specific proteases or caspases are the central molecules involved in initiation and execution of apoptosis (Riedl and Shi, 2004). Caspases are processed through proteolytic cleavage at the sites containing aspartate residue. Mitochondria contain the entire cellular pool of cytochrome *c*, as well as a fraction of caspase-9, in the intermembrane space and both cytochrome *c* and caspase-9 are liberated into the cytoplasm after apoptotic insults. Cytochrome *c* triggers the formation of the apoptosome in the presence of ATP along with Apaf-1 and caspase-9, initiating the activation of the caspase cascade (Kroemer et al., 1997; Green and Reed, 1998; Mehta et al., 2007). The “forebrain over growth” (*fog*) mutation leads to an autosomal recessive neural tube closure defect due to the near-to-complete lack of Apaf-1 expression (Harris et al., 1997; Honarpour et al., 2001). However, a complete deficiency in *Apaf-1* usually results in perinatal lethality, while *fog/fog* mice readily survive into adulthood (Honarpour et al., 2001; Yoshida et al., 1998), allowing to assess the role of Apaf-1 and Apaf-1-dependent cytochrome *c*-mediated caspase activation in neuronal apoptosis. An antiapoptotic effect of the *fog* mutation mice was observed in staurosporine induced bone marrow cell death (Katoh et al., 2008). Similarly, Apaf-1 deficiency reduced the retinal detachment (RD)-induced neuronal photoreceptor apoptosis, as assessed by TUNEL staining (Hisatomi et al., 2008). These results suggest that pharmacological inhibition of Apaf-1 should be evaluated for their neuroprotective potential.

### Importance of AIF in caspase-independent pathways on neurodegenerative disease

AIF is a 67 kDa protein flavoprotein that is highly conserved among mammalian species (>95% amino acid identity between mouse and human) and bears a highly significant homology with flavoprotein oxidoreductases from all eukaryotic and prokaryotic kingdoms in its C-terminal portion (Lorenzo et al., 1999; Susin et al., 1999). AIF is normally confined to the mitochondrial intermembrane space. However, AIF translocates to the cytosol and to the nucleus after apoptotic insults (Susin et al., 1999; Lorenzo et al., 1999; Daugas et al., 2000; Hisatomi et al., 2001, 2002, 2003, 2008). Harlequin (*Hq*) mice exhibit an X chromosome-linked ataxia due to the progressive degeneration of terminally differentiated cerebellar neurons (Barber, 1971; Klein et al., 2002). The *Hq* mutation has been identified as a proviral insertion in the apoptosis-inducing factor (*Aif*) gene, also known as programmed cell death 8 (*pdcd8*), causing an approximate 80% reduction in AIF expression (Klein et al., 2002). In contrast to *Aif* knockout mice (which die in utero) (Joza et al., 2005), *Hq* mice are born at normal Mendelian ratios and are healthy until the age of 3 months. After the apoptotic insult mediated by RD, *Hq/Y* retinas exhibited significantly less TUNEL<sup>+</sup> apoptotic neural cells than wt controls, and exhibited a reduced cell loss in the retina (Hisatomi et al.,

2008). Similar neuroprotective effects were observed in brain ischemia in adult (Culmsee et al., 2005) and neonate (Zhu et al., 2007) mice, as well as in a model of traumatic brain injury (Slemmer et al., 2008). Hence, further research on AIF and caspase-independent pathways may reveal novel therapeutic target for neuroprotection.

### Pharmacological targeting of mitochondria for preventing release of proapoptotic molecules via MMP inhibition

Mitochondrial outer membrane permeabilization (MOMP) is a critical event during apoptosis, representing the “point of no return” of the lethal process. Cytochrome *c* is released from mitochondria upon MOMP and binds to cytosolic apoptotic protease activating factor-1 (Apaf-1) to induce its dimerization and a conformational change (Bao et al., 2005). Apaf-1 then oligomerizes into apoptosomes that recruit and activate caspase-9 followed by serial activation of caspase-3 and other apoptosis-execution molecules (Acehan et al., 2002; Bao and Shi, 2007). However, MOMP may cause cell death even if caspases are inhibited (Bouchier-Hayes et al., 2005) and a broad caspase inhibitor, Z-VAD-fmk, fails to inhibit neuronal apoptosis (Hisatomi et al., 2001; Murakami et al., 2008). AIF is a caspase-independent apoptogenic factor and is normally confined to the mitochondrial intermembrane space (Susin et al., 1999). During apoptosis, AIF translocates to the cytosol and then to the nucleus where it triggers peripheral chromatin condensation and interacts with cyclophilin A to generate a DNase complex that is responsible for the so-called “large-scale” DNA degradation to fragments of approximately 50 kbp (Cande et al., 2004; Susin et al., 1999). AIF translocation has been reported for mammalian neural cells in numerous cases, for instance for photoreceptors upon retinal detachment (RD) (Hisatomi et al., 2001), dopaminergic neurons in models of Parkinson's disease (Wang et al., 2003) including phenylpyridinium toxicity (Chu et al., 2005) and photoreceptor cells in retinitis pigmentosa (Sanges et al., 2006). Pharmacological targeting of MMP inhibition may limit the release of these proapoptotic intermembrane space proteins into the cytosol, as discussed below.

### Growth factor dependent survival of neuronal cells

An increasing number of growth factors and nerve growth factors are being shown to support cell survival after various apoptotic insults in neuronal cells (Chaum, 2003). Such neuroprotective effects have been reported for proteins from the nerve growth factor family (NGF, BDNF, etc.), basic fibroblast growth factor (bFGF), and vascular permeability factor (VEGF). BDNF and bFGF confer substantial neuroprotective effects in neuronal apoptosis induced by retinal detachment (Hisatomi et al., 2001), brain ischemia (Lee et al., 2008; Tsukahara et al., 1994), and Parkinson disease (Altar et al., 1992; Frim et al., 1994). These growth factors activate their counter receptors (i.e., NGF receptors) and stimulate cellular intrinsic pathways (i.e., activation of PI3K/Akt signaling pathways and mitogen-activated protein kinase (MAPK) pathways, which leads to the upregulation of cytoprotective proteins (Bogaerts et al., 2008). Recently, pigment epithelium-derived factor (PEDF) has also been reported to be neuroprotective in retinal degeneration in retinitis pigmentosa. While the receptor of PEDF remains unclear, lentivirus-mediated retinal gene transfer of PEDF inhibited MMP, as indicated by an intact mitochondrial transmembrane potential, and inhibited the AIF translocation from mitochondria to the nuclei of neural cell in the retina (Murakami et al., 2008).

### Overexpression of Bcl-2 family proteins for inhibiting MOMP for neuroprotection

Bcl-2 family proteins are essential regulators of apoptosis, and its over 30 family members share homology in Bcl-2 homology regions

(BH1 to BH4) (Borner, 2003; Mehta et al., 2007). These can be grouped into Bcl-2 like survival factors (antiapoptotic) (Boise et al., 1993; Gibson et al., 1996) and Bcl-2 like death factors (proapoptotic) (Boise et al., 1993; Oltvai et al., 1993; Inohara et al., 1997). The Bcl-2 like survival factors possess all four BH domains (BH1 to BH4) domains that mediate their antiapoptotic function and gate the release of apoptotic proteins by preventing the MMP. The proapoptotic members of Bcl-2 family protein either possess three BH domains (BH1 to 3) or just one BH domain (BH3) and promote MMP via perturbation of mitochondrial membrane integrity, the interactions with other proapoptotic and antiapoptotic factors, including with MMP-related proteins such as adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC). The ratio of antiapoptotic and proapoptotic proteins may determine the fate of neurons (Kroemer and Reed, 2000; Green and Kroemer, 2004). The overexpression of antiapoptotic members of Bcl-2 family protein has a major neuroprotective effect (Mehta et al., 2007). Bcl-2 overexpression protects against neuron loss induced by brain ischemia, through prevention of the mitochondrial release of cytochrome *c* accumulation and the subsequent activation of caspase-3 (Zhao et al., 2003). Moreover Bcl-2 transfection via a recombinant herpes simplex virus blocks AIF translocation from mitochondria to nucleus (Zhao et al., 2004), supporting the idea that Bcl-2 proteins constitute potentially useful targets for neuroprotection.

*A cell-permeable peptide corresponding to the BH-4 domain of Bcl-X<sub>L</sub> inhibits neuronal apoptosis via blocking MOMP*

The BH4 domain is specific for the antiapoptotic proteins of the Bcl-2 family and is responsible for the binding of Bcl-2 or Bcl-X<sub>L</sub> to VDAC (Shimizu et al., 2000). Recently a cell-permeable MOMP-inhibitory recombinant fusion protein, HIV-TAT BH4, composed by the HIV-TAT plasma membrane translocation domain and the antiapoptotic Bcl-X<sub>L</sub>-derived BH4 domain, has been reported to inhibit neuronal apoptosis (Asoh et al., 2002; Dietz et al., 2002; Hisatomi et al., 2008; Yin et al., 2006). Cell-penetrating peptide constructs comprising the HIV-1 TAT basic domain or related peptides have been developed to deliver bioactive peptides into cells (Shimizu et al., 2000; Cao et al., 2002; Schwarze et al., 1999; Dietz et al., 2002; Asoh et al., 2002; Sugioka et al., 2003; Yin et al., 2006; Hotchkiss et al., 2006). Rapid and receptor-independent uptake of TAT-conjugated peptides has been demonstrated to occur *in vitro* and *in vivo* (Dietz et al., 2002). Indeed, the intraperitoneal injection of HIV-TAT protein led to its distribution into neuronal cells (*in vivo*), exactly as this was observed after addition of HIV-TAT BH4 to primary neuronal cell cultures (*in vitro*) (Hisatomi et al., 2008). Moreover, the HIV-TAT BH4 peptide efficiently inhibited caspase-3 activation in ischemic neurons (Cao et al., 2002), prevented AIF translocation in neonatal brain damage (Yin et al., 2006), and suppressed AIF and cytochrome *c* translocation in retinal cells (Hisatomi et al., 2008). Based on these results, peptidomimetics should be designed to act like the HIV-TAT BH4 peptide and then evaluated for their neuroprotective effects. By analogy to the BH3-mimetics, which mediate proapoptotic effects on cancer cells (Adams and Cory, 2007), such components could be referred to as “BH4 mimetics”.

*A novel mechanism of HIV protease inhibitors for neuroprotection through inhibition of mitochondrial apoptosis*

HIV protease inhibitors (PIs) have originally been designed to block the formation of HIV viral proteins by viral proteases, and are currently administered to millions of patients with HIV worldwide (Staszewski et al., 1999). Importantly, PIs may have an “off-target” effect and inhibit apoptosis through a direct action on host cells. PIs do not only inhibit virus replication but also suppress CD4<sup>+</sup> T lymphocyte apoptosis at concentrations similar to those that are achieved in the

plasma of PI-treated patients (Phenix et al., 2002). In several cases, HIV infected individuals recovered normal levels of circulating CD4<sup>+</sup> T cells upon PI treatment although the therapy had no effects on the viral titers, suggesting that PIs might inhibit apoptosis of CD4<sup>+</sup> T cells *in vivo*, independently from their effect on HIV replication (Badley, 2005; Deeks and Grant, 1999; Staszewski et al., 1999). Several groups investigated the mechanisms by which PIs inhibit apoptosis. Altered transcriptional regulation of regulatory proteins (Estaquier et al., 2002), as well as direct inhibition of caspase-1 (Sloand et al., 1999) or calpain (Ghibelli et al., 2003) have been reported. However, these proposed mechanisms may not explain the ability of PIs to block cell death induced by a wide range of apoptotic insults (Phenix et al., 2002) and are not compatible with other studies reporting poor effects of PIs on effector caspases (Phenix et al., 2001) or on the net synthesis of apoptosis regulators (Phenix et al., 2001). However, the protective effects of PIs contrast with the observation that pharmacological caspase inhibitors largely fail to inhibit cell death (Green and Kroemer, 2005).

Recently, PIs were shown to inhibit the MOMP-dependent release of cytochrome *c* (Matarrese et al., 2003; Phenix et al., 2001) via direct binding to and inhibition of the adenine nucleotide translocator, a protein from the inner mitochondrial membrane that can form pores and mediate MOMP (Weaver et al., 2005). Hence, PIs may simultaneously block caspase-dependent (activation of caspase-9, -3) and caspase-independent cell death pathways (AIF translocation) via blocking MOMP, presumably by inhibiting the adenine nucleotide translocator (Hisatomi et al., 2008). PIs have been reported to be neuroprotective in neuronal injury triggered by 4-hydroxynonenal, a lipid-soluble aldehydic product of membrane peroxidation *in vitro* (Wan and DePetrillo, 2002), and middle cerebral artery occlusion-induced stroke *in vivo* (Weaver et al., 2005). In our studies, PI had substantial antiapoptotic and neuroprotective effect on retinal photoreceptors after RD (Hisatomi et al., 2008). Paradoxically, PIs may also induce apoptosis, particularly of transformed cells, when used at higher doses (Estaquier et al., 2002; Gaedicke et al., 2002). Further studies must explore the mechanisms through which PIs inhibit neuronal apoptosis and explore their possible clinical application for neuroprotection.

## Conclusions

MMP is a pivotal event in the pathogenesis of acute and chronic neurodegenerative disorders. Thus, various neurodegenerative disorders that involve apoptosis could be amenable to drug- and gene-based therapies that target MMP. Multiple redundant cell death pathways with overlapping and cross-talking molecular mechanisms can come into action, suggesting that neuroprotective agents should optimally be directed at multiple and/or comprehensive targets. In our studies, neither the *Hq/Y* genotype (a hypomorphic mutation of the *aif* gene) nor the *fog/fog* genotype (a hypomorphic mutation of *apaf-1*) led to complete protection against RD-induced photoreceptor apoptosis (Hisatomi et al., 2008). There are two hypotheses that may explain this phenomenon. First, it is possible that residual amounts of AIF and Apaf-1 allow some apoptosis to occur in these hypomorphic mutants. As an alternative (and non-exclusive) possibility, molecular interactions between the different pathways might compensate for each other during the apoptotic process. AIF induces purified mitochondria to release cytochrome *c* and caspase-9, suggesting that AIF, once released from mitochondria, accelerates membrane permeabilization in a positive feed forward loop (Susin et al., 1999). While released cytochrome *c* binds Apaf1 that in turn activates caspase-9, caspases can also directly affect mitochondrial function and/or disrupt mitochondrial membrane permeability (Susin et al., 1997; Marzo et al., 1998a,b). We hypothesize that both pathways are activated after MOMP and simultaneously orchestrate complementary pathways culminating in cellular demise in the neurodegenerative

model of RD. This hypothesis is supported by the fact that antiapoptotic HIV-TAT BH4 protein, that inhibits MOMP and hence blocks the release of both cytochrome c and AIF (Cao et al., 2002; Shimizu et al., 2000), successfully reduced photoreceptor apoptosis after RD. Furthermore, administration of HIV-TAT BH4 protein had additive protective effects on *Hq/Y* mouse where it decreased the activation of caspase-9.

In conclusion, we propose the concept to target mitochondria, especially MMP, to block the release of multiple proapoptotic proteins and simultaneously protect energy metabolism for optimal neuroprotection. Furthermore, we believe that it is worthwhile to assess combination therapies in which growth factors, Bcl-2 family protein overexpression, BH4 mimetics, and HIV protease inhibitors are evaluated together for their neuroprotective action.

## Acknowledgments

GK is supported by the Ligue Nationale contre le Cancer (Equipe labellisée), European Commission (Active p53, Apo-Sys, RIGHT, TransDeath, ChemoRes, DeathTrain), Cancéropôle Ile-de-France, Fondation de France, and Fondation pour la Recherche Médicale.

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