Molecules that Modulate Apaf-1 Activity

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1. INTRODUCTION

Apoptosis is composed of highly regulated cellular pathways responsible for programmed cell death to remove DNA damaged, virally infected or otherwise unneeded cells. Several studies and review articles on the main features and complexity of the different cell death processes have been already reported.[1-14] Due to such complexity, it is not possible to cover the current understanding of cell death in one review. Therefore, here we would like to focus only on some detailed view points on apoptosis activation with particular interest on Apaf-1 (apoptotic protease-activating factor).

Diverse apoptotic stimuli, including activation of cell surface death receptors, anticancer agents, irradiation, lack of survival factors, and ischemia[15] induce signaling cascades that activate the caspase family of cysteine aspartyl proteases (Fig. 1). These caspases are essential to the apoptotic process as they are required for the initiation and execution of programmed cell death. Effector caspases (e.g., caspases-3 and -7) are responsible for the disassembly of cellular components[16] while initiator caspases (e.g., caspases-8, -9 and -10) are responsible for activation of the effector caspases. In particular, caspase-9 activates upon the release to the cytosol of proapoptotic proteins from the mitochondrial inter-membrane space into the cytosol when apoptosis-inducing signals, such as DNA damage or metabolic dysfunction are perceived by the cell.[17,18] The formation of the macromolecular complex named the apoptosome is a key event in this pathway, which has also been defined as the intrinsic apoptosis pathway. The apoptosome is a holoenzyme multiprotein complex formed by cytochrome *c*-activated Apaf-1 (apoptotic protease-activating factor), dATP and procaspase-9.[19] When cytochrome *c* is released from mitochondria, it binds to Apaf-1, causing Apaf-1 to hydrolyze the bound nucleotide and promotes the oligomerization of the Apaf-

1/cytochrome *c* complex.[18,20] dATP/ATP is exchanged for the hydrolyzed nucleotide, allowing the formation of a seven Apaf-1/cytochrome *c* based wheel-like apoptosome where the Apaf-1 CARD domain is now accessible to bind to the CARD domain of procaspase-9.[18,20,21] Apoptosome-bound procaspase-9 is activated and subsequently proteolyzes the downstream effector caspases leading to progression of cell death.

Defects in the regulation of apoptosis are at the root of a variety of diseases. When cells acquire resistance to induction and execution of apoptosis it frequently correlates with cancer or autoimmune diseases. In this sense, the development of new anti-cancer therapies importantly relies on inducing apoptosis.[22] In contrast, excessive apoptosis induces unwanted cell death and promotes several pathological conditions such as tissue infarctation, ischemia-reperfusion damage, degenerative diseases and AIDS.[7,23,24] To identify molecules that could ameliorate disease-associated excessive apoptosis, drug discovery efforts initially targeted the inhibition of caspase activity, particularly the effector caspase-3.[25-28] However, caspase-3 inhibitors have encountered problems in their pharmacological development. The active sites of all caspases have a requirement for an aspartyl functionality in the P1 amino acid and an electrophilic carbonyl necessary to engage the catalytic cysteine.[29] Peptidomimetic inhibitors bearing such requirements have been identified.[28,30-33] While these chemical features are needed for high affinity binding they are not compatible with achieving potent cell-based activity. The activity of most of the caspase inhibitors is greatly attenuated, and even in the presence of cell extracts the reduction in potency, in comparison to isolated caspases is, in most cases, up to two orders of magnitude.[34] Thus, there is a considerable need for more selective, stable, and cell permeable caspase inhibitors that could reduce pathology-related apoptosis. Alternatively, protein-protein interactions upstream of caspase activation can be also relevant points of intervention for the development of

modulators of apoptosis pathways. In particular, recent data propose the formation of the multiprotein complex apoptosome as an interesting target for the development of apoptotic modulators. Recently, multiprotein complexes have been shown to be important points of regulation in cellular signaling pathways[35-38] and have raised attention as target for the development of chemical modulators. In addition, cells have developed multiprotein complexes as signaling devices that control and mediate the regulation of different signaling pathways.[36,38-40] This also applies to apoptosis-related proteins and protein complexes.[39,41,42] On the other hand, it is now well recognized that protein and/or protein complexes originally described as members of the 'death machinery' are implicated in non-apoptotic functions.[43-50] Interestingly, Apaf-1, the central component of the apoptosome, has been also described to contribute to a specific intra-S-phase DNA damage checkpoint.[51]

Apaf-1 is a large protein (130 kDa) that contains an N-terminal caspase recruitment domain (CARD), a central nucleotide-binding oligomerization domain (NBD), and multiple WD40 repeats at the carboxy terminal half that are responsible for both, binding to cytochrome *c* and regulating Apaf-1 function (Fig. 2).[52,53] Apaf-1 is cytoplasmic by nature and it is found in most tissues with highest expression in adult spleen and peripheral blood leukocytes, fetal brain, kidney, and lung. Apaf-1 is alternatively spliced up to five forms that vary in length. The importance of Apaf-1 in the development of the central nervous system was clearly elucidated upon generation of Apaf-1-deficient mice.[54,55] These mice present embryonic lethality with severe craniofacial malformations, brain overgrowth, and other phenotypic features of severe apoptosis impairment. Anomalous apoptosome function contributes to carcinogenesis,[56] and has also been implicated in the inappropriate apoptosis described in different degenerative disorders, heart disease and ischemic-related pathologies.[57] As

such, the apoptosome is a relevant point of intervention for the development of modulators of apoptosis pathways. Although some of the features of apoptosome have previously been summarized in several recent reviews,[58-65] it is the scope of this review both to provide an overview summarizing the literature involving proteins and other natural molecules described to modulate Apaf-1 and consequently the apoptosome activity and to comment on the progress made in the discovery and development of synthetic modulators of Apaf-1.

2. PROTEIN-DEPENDENT REGULATION OF APAF-1

The apoptosome is regulated by a large number of proteins. In particular a series of proteins have been identified that bind to Apaf-1. From these proteins one can deduce potential apoptosis-related proteins, that act as an apoptosis self-regulatory system and non-classical apoptosis proteins, which will account for relationships between apoptosis and other cell signaling pathways (Fig. 2). The first characterized apoptosome components were identified in *Caenorhabditis elegans* (*C. elegans*) (Fig. 3) the inactive CED-3 zymogen (homologous to caspase-1) is activated by CED-4 (homologous to Apaf-1) that is retained in an inactive form in the mitochondrial membrane by CED-9 (homologous to Bcl-2). Upon induction of cell death, EGL-1 (a nematode 'BH3-only' protein) is up-regulated and binds to CED-9 releasing CED-4 that forms a 2:2 heterotetrameric complex with CED-4[66] that activates CED-3. It has been also suggested that upon its release from CED-9, CED-4 translocates to the nuclear envelope.[67] As mentioned above, Apaf-1 has been also described to contribute to a specific intra-S-phase DNA damage checkpoint in vertebrates[51] and to acquire a nuclear location when non-small cell lung carcinoma (NSCLC) cells were treated with different dosages of cisplatin.[68] In addition, several cell cycle-related proteins have been reported

as Apaf-1 interacting proteins. Nucling, has an increased expression shortly after apoptosis activation and influences both, the synthesis of apoptosome-related proteins and a putative translocation of the Apaf-1/procaspase-9 complex to the nucleus.[69,70] Also the histone H1.2 has been found to interact with Apaf-1.[71] PARCS (pro-apoptotic protein required for cell survival) was found to bind to a GST-Apaf-1 fusion protein containing the CARD and NBD domains.[72] PARCS induces cell cycle arrest in G1 phase[72-74] and has been implicated in the role of Apaf-1 in cell cycle.[51,75,76]

Probably influenced by the molecular mechanism of the cell death pathway in *C. elegans*, Bcl-$X_{L,}$ a member of the Bcl-2 family, was originally proposed to directly control Apaf-1 activity (Fig. 3).[77-79] However, recent studies have suggested that Apaf-1 does not bind to prosurvival members of Bcl-2 family of proteins.[80,81] Another Bcl-2 protein, Diva/Boo (death inducer binding to Bcl-2 and apoptosis-activating factor, Apaf-1), was reported as Apaf-1 interacting protein[82-84] and Aven (named after Aventine, a Roman stronghold – apoptosis caspase activation inhibitor) was identified as a new Bcl-X_L interacting protein and was also postulated to bind to Apaf-1 and prevent oligomerization.[85] Also, the apo form of cytochrome *c* (the apo form lacks of the heme group) is able to bind to Apaf-1 and compete with the holo form, but it is unable to form an active apoptosome.[86]

The heat-shock protein family (HSP) is induced in response to cellular stress to protect cells. The HSP proteins have different functions and it was shown that overexpression of Hsp70 protected cells from certain death stimulus through direct binding to Apaf-1 and inhibition of the formation of the apoptosome.[87-90] Furthermore, in small-cell lung carcinoma cells (SCLC) Hsp90 is a major inhibitor of apoptosis. In these cells selective inhibition of Hsp90 induces a release of Apaf-1 from an Apaf-1/Hsp90 complex that correlates with an increased association between Apaf-1 and

procaspase-9.[91] Interestingly, Hsp70, PHAPI (putative HLA-DR- associated proteins - see below) and cellular apoptosis susceptibility protein (CAS) were found to stimulate apoptosome formation by facilitating Apaf-1 conformational changes related to nucleotide exchange.[92] Another HSP protein, Hsp90β was shown to have affinity to bind to Apaf-1 WD40 domain and inhibit cytochrome *c*-mediated oligomerization of Apaf-1.[93,94] How can this dual role of HSP proteins be explained? Perhaps the different 'sensing' conditions of the cell and the relative intracellular protein concentration will provide the answer. In addition, it should be mentioned that early attempts to identify the composition of Apaf-1 apoptosomes in cell-free extracts activated with cytochrome c and dATP by immunopurification and mass spectrometry characterization[95,96] revealed that only Apaf-1, processed caspase-9, processed caspase-3 and XIAP were the major constituents. Cytochrome c was not stably associated to active apoptosome. Then, how and when do all the above reviewed and described as Apaf-1 binding proteins associate to Apaf-1 or to the apoptosome? Do these proteins have low affinity and therefore, are unable to withstand the immunopurification conditions? Do these proteins perform only transient interactions with Apaf-1? These and some other controversies related to the cellular role of Apaf-1 and Apaf-1-binding proteins present interesting questions to be investigated.

Apoptosis has been connected to both acute and chronic phases of ischemia-related pathologies as heart failure, stroke and those derived from organ transplantation.[97,98] Hypoxia-inducible factor (HIF) is the principal transcription factor involved in the regulation of transcriptional responses to hypoxia[99] and some HIF-regulated proteins showed putative Apaf-1 binding properties.[100,101]

Apaf-1 has also been described as target for both protein kinases and phosphatases[102-106] and the phosphorylation state of Apaf-1 could have relevance on apoptosome activity regulation.

3. CHEMICAL REGULATION OF APAF-1/APOPTOSOME

Apoptosome formation, which depends on oligomerization of Apaf-1, is a crucial step in the apoptosis signaling. Thus, alterations in the function of proteins that form the apoptosome could be related to diseases.[107-114] For this reason, chemical modulation of the apoptosome components, such as Apaf-1, represent potential targets in the development of new therapeutic strategies for treating these diseases.

Apaf-1 has to be considered as an attractive target for the development of chemical modulators. However, small molecules have yet to be discovered that regulate Apaf-1. New methods that focus on the molecular mechanism of apoptosome formation need to be devised in order to uncover modulators (Fig. 4). Prior to mitochondrial-dependent apoptosis induction, Apaf-1 is monomeric, inactive and the caspase recruitment domain (CARD) is not accessible.[19] Only upon cytochrome *c* release, Apaf-1 activates by exchanging dATP/ATP by the hydrolyzed nucleotide and forms the apoptosome.[18,20] With these precedents, the pharmacological target Apaf-1 can be dually seen as a 'classical' ATP hydrolase or alternatively as a protein-protein interaction-based target. To the best of our knowledge, no successful attempts to inhibit the hydrolase activity with small molecules have been reported. The studies so far described aiming to identify chemical modulators of Apaf-1 have addressed the protein-protein interaction-based formation of the apoptosome. As Apaf-1 was a new target, the drug discovery process was based in the screening of large collections of compound libraries in a suitable high throughput screening (HTS) format.[115,116] The assay format

could be initially based both, in an *in vitro* apoptosome reconstitution with recombinant proteins or, alternatively, in cell extracts that can be stimulated to form the apoptosome and the modulatory activity of chemicals can be evaluated. Large scale production and purification of the essential components required for apoptosome reconstitution-based assays (Apaf-1, procaspase-9 and procaspase-3) is laborious and expensive. Thus, most of the initial assays searching for apoptosome modulators were developed using cell extracts. However, cell extracts-based HTS assays are indirect and potentially engage many different points in the pathway. Thus, follow-up assays, also referred to as 'secondary screens' or 'counter screens', should be planned. These assays will validate compounds targeting the intended biological interaction(s) and assist in the elimination of compounds that generate a positive signal via other mechanisms.

3.1 Identification of activators of the Apaf-1-mediated apoptosome assembly/activity

Two simultaneous studies appeared in 2003[117,118] with the interesting goal of identifying compounds that could induce a chemical activation of the apoptosis machinery. Cancer cells have developed mechanisms to inhibit cell apoptosis and early drug discovery efforts targeted inhibition of events upstream of cytochrome *c* release (inhibitors of the antiapoptotic members of the Bcl-2 family).[119-121] Studies that focus on small molecules as direct activators of apoptosis are less abundant, however, examples do exist. In their study, Nguyen and Wells[118] used a *in vitro* mimic of the *in vivo* activation of the apoptosome obtained when cytochrome *c* and dATP were added into a HeLa cell cytoplasmic extract.[122] This cell extract has the apoptosis machinery dormant and exogenous cytochrome *c* and millimolar concentrations of dATP/ATP will initiate an Apaf-1-dependent stepwise series of caspase activation events. Procaspase-9 will be activated to caspase-9 in the apoptosome and will induce the activation of effector

caspases like caspase-3 and caspase-7. The output of the assay was followed by providing a fluorogenic substrate for caspase-3/7 (DEVDase activity). A library of 3500 diverse compounds was screened. From this, 116 compounds that induced DEVDase activity (3.3% hit rate) were initially selected for further investigation. Secondary screens with the initial 116 hits started with the direct visualization of procaspase-3 processing by immunoblot in order to eliminate those hits that provided intrinsic fluorescence-based false positives. The number of hits was reduced to 20 (0.5% hit rate) and compound 1[118] (Table I) was selected for optimization. The biological activity of compound 1 was demonstrated to be linked to the dichlorobenzyl and carbamate moieties. A focused chemical library was synthesized and evaluated for activity allowing the identification of compound 2 and compound 3[118] (Table I) as more potent activators of the DEVDase activity than compound 1. Convincing experimental evaluation demonstrated that the active compounds do not directly activate the pro-forms of caspase-9 or caspase-3, in contrast they seemed to favor Apaf-1 oligomerization. Even more, partial silencing of Apaf-1 gene expression in Jurkat cells with small interfering RNA (siRNA) correlated with a decrease in activity of compound 2, suggesting that the biological activity of compound 2 is Apaf-1-dependent. In addition compound 2 showed selectivity as cytotoxic agent for tumor cells that died with hallmarks of apoptosis like caspase-3 activation, PARP (poly ADP ribose polymerase) cleavage, and DNA fragmentation.[118] The detailed molecular mechanism of action of compound 2 was not totally described; however it was reported that 2 required for bioactivity a decreased concentration of cytochrome *c* but it was inactive in its absence. Thus, these compounds seemed to enhance the ability of cytochrome *c* to activate the apoptosome.

It was possible that the compounds identified in the Nguyen and Wells' study share some characteristics with those compounds identified by Xiadong Wang and co-workers.[117] Wang's group screened a library of 184000 compounds for caspase-3 activators with HeLa cell extracts (S-100 fraction) in the presence of 1 mM exogenous dATP. The percentage of hit rate from the screening was not reported but the molecule dubbed PETCM (α-(trichloromethyl)-4-pyridineethanol) was presented as the most potent positive hit from the screening campaign (Table I). It should be mentioned that Nguyen and Wells evaluated PETCM in their biological assay and suggested that PETCM promoted Apaf-1 oligomerization by a mechanism similar to that exerted by compound 2.[118] In fact, PETCM was able to induce Apaf-1 oligomerization in HeLa cell S-100 extracts even in the absence of exogenous dATP/ATP.[117] Wang's group further analyzed the molecular mechanism of action of PETCM through a series of cell extract-based biochemical fractionation experiments and mass spectrometry-based protein identification. Two new families of apoptosome regulatory proteins (putative HLA-DR- associated proteins, PHAPI and the oncoprotein prothymosin - ProT) were identified as members of the PETCM-related pathway. Further studies from Wang's group including the identification of CAS (cellular apoptosis susceptibility protein) and Hsp70 as members of the regulatory pathway of apoptosome activation[92] suggested a highly regulated event. Apoptosome formation is prevented by the oncoprotein ProT and such inhibition can be relieved by high dATP/ATP concentrations or other chemicals as PETCM (Fig. 2). It was demonstrated that siRNA-based removal of ProT sensitizes cells to apoptosis induction;[117] however the molecule (or biochemical events) responsible of *in vivo* removing ProT-based apoptosome inhibition has not been reported. In the molecular mechanism of apoptosome formation a reported key event for procaspase-9 activation is the role of dATP/ATP hydrolysis and hydrolyzed

nucleotide exchange. It is currently accepted that such hydrolysis-exchange cycle is regulated by a set of three proteins, PHAPI, CAS and Hsp70.[92] In contrast to CAS and Hsp70, PHAPI does not seem to interact with Apaf-1 directly. Nonetheless, one or more of these three proteins have been postulated to facilitate the nucleotide exchange of Apaf-1 during the oligomerization process. Whether these proteins act as the actual nucleotide exchanger or as scaffolds to help Apaf-1 to maintain the appropriate fold during such exchange[92] will need further experimentation.

Complex interactions within the intracellular environment and intrinsic gene products of the cell occur when apoptosis begins and, once activated, the apoptosis process continues without further extracellular signaling requirements. In such complex interactions an exquisite balance of cell signaling seems to converge on the apoptosome where Apaf-1 has a principal role. Hsp70 has been described both as inhibitor[87-90] and as activator[92,117] of Apaf-1. CAS is highly expressed in tumor cell lines and knocking-down its expression made cells resistant to apoptosis.[123,124] ProT is an oncoprotein[125] which one of its functions is to prevent apoptosome function. However, PHAP belong to a family of tumor suppressors that through inhibition of protein phosphatase 2A[126] and histone acetylase[127] inhibits cell growth. Furthermore, an overall control of the cell cycle has been attributed to Apaf-1.[51] How these biochemical functions are linked and integrated in the context of both non-apoptotic or under apoptotic cell signaling is not clear and in a near future we will probably learn more on the role of Apaf-1 as cell signaling integrator protein.

3.2 Use and utility of truncated versions of Apaf-1

The *in vitro* activity of Apaf-1 is also extremely complex. Within the cellular context the dATP levels are approximately 10 μM however, cellular extracts require at least 1

mM dATP/ATP for apoptosome activation and subsequent caspase 3/7-related DEVDase activity. Wang and colleagues suggested that this observation is linked to the presence of a ProT-inhibited Apaf-1 within cell extracts and the majority of exogenously added dATP/ATP is required to alleviate such inhibition. In fact, in the presence of the small molecule PETCM, the required level of dATP/ATP for activation is reduced to the micromolar range.[117] In *in vitro* experiments with an apoptosome reconstituted from recombinant proteins and using Apaf-1, the requirement for dATP/ATP is in the micromolar range.[21,128] However, the dATP/ATP concentration requirement for shortened versions of Apaf-1 is controversial.

Recently, the use of a WD-domain deleted Apaf-1 construct has been incorporated into both structural and biochemical studies. Riedl et al.[129] used an Apaf-1, residues 1-591 (Apaf-1 1-591) to determine the crystal structure and proposed the molecular mechanism by which Apaf-1 is in an inactive conformation prior to ATP binding. In this study, the optimal concentration of dATP for full procaspase-9 processing was above 1 μM, for ATP between 1 and 5 μM, while other non hydrolysable ATP analogs did not have effect on Apaf-1-mediated caspase-9 activation. Other studies have proposed that the molecular mechanism of bioactive deoxynucleoside analogs like cladribine (2-chlorodeoxyadenosine or 2CdA) or fludarabine (9-b-d-arabinofuranosyl-2-fluoradenine or F-Ara-A)[130,131] involves the direct binding of metabolites of these compounds to Apaf-1.[132] In agreement with this, the stimulatory effect on procaspase-9 activation of 2CdATP was demonstrated using an Apaf-1 1-591-based reconstituted apoptosome[133,134] (Table I). However, it has been also proposed that the WD-domain deleted Apaf-1 1-530 binds and processes procaspase-9 in the absence of dATP or cytochrome *c*.[53,135]

In the intrinsic, mitochondria-mediated apoptotic pathway, once cytochrome *c* is released, Apaf-1 assembles into the apoptosome and this multimeric protein complex recruits and activates caspase-9.[19,136] However, the molecular mechanism of apoptosome-mediated caspase-9 activation is controversial. The two currently accepted models are the induced proximity model[137-139] and the allosteric model.[140,141] Also, how does active caspase-9 activate caspase-3/7? Either Apaf-1 can release the activated caspase-9 from the complex and caspase-3/7 is activated in the cytosol, or it could bring procaspase-3/7 into the complex where it gets activated by bound caspase-9 and then is released into the cytosol. Recent results suggest the latter as the most possible option,[142-144] although it was also postulated that caspase-3 cleavage of caspase-9 is required for full activation of the apoptosome.[145] We were interested in these molecular mechanisms and wanted to explore long-term kinetic analysis of procaspase-3 and procaspase-7 activation in the presence of procaspase-9 and Apaf-1. To address this question, we reconstituted the apoptosome using a set of recombinant proteins (procaspase-3, procaspase-7, procaspase-9, procaspase-9 C285A (active site Cys was mutated to Ala), Apaf-1 1-591 and XIAP (X-linked inhibitor of apoptosis protein)). We adjusted the protein concentrations to obtain accurate assay response in the desired time frame (see Legend of Fig. 5). In these conditions, caspase-3 and caspase-7 were activated in the presence of Apaf-1 1-591 and procaspase-9 (Fig. 5). In addition, caspase-3 was activated in the presence of Apaf-1 1-591 and procaspase-9 C285A. This mutant procaspase-9 was incapable of activation in an *in vitro* enforced dimerization method routinely used by Salvesen *et al* [137] that we used as control. How can we explain the activation of procaspase-3 under these conditions? Can Apaf-1 in the absence of procaspase-9 induce procaspase-3 activation? When analyzing this possibility, we obtained a procaspase-3 activation profile similar to that obtained in the

presence of Apaf-1 1-591 and procaspase-9 C285A (Fig. 5A). Procaspase-3 activation was inhibited in the presence of XIAP (data not shown). Interestingly, although our preparation of procaspase-7 was more prone to self-activation (Fig. 5B), the Apaf-1 1-591- or Apaf-1 1-591 and procaspase-9 C285A-induced kinetic profile of activation of procaspase-7 was slower than that obtained for procaspase-3 in our experimental conditions (Fig. 5A and 5B). Overall these data suggest that both procaspase-9 and procaspase-3 can be recruited to the apoptosome[142,143] and partial Apaf-1-dependent activation of procaspase-3 can contribute to the massive Apaf-1-dependent activation of procaspase-9 and procaspase-3.

3.3. *Inhibitors of the Apaf-1-mediated apoptosome assembly/activity*

3.3.1. Ionic equilibrium concentrations in cell

The ionic equilibrium within a cell represents another modulation step for Apaf-1-mediated apoptosome assembly upon apoptotic stimulus. Compelling evidences indicate that K^+ efflux and intracellular K^+ depletion are key early steps in the regulation of apoptosis.[146-148] *In vitro* assays of apoptosome reconstitution with recombinant Apaf-1 and cytochrome *c* have shown that K^+ inhibits caspase activation by abrogating Apaf-1 oligomerization and apoptosome assembly.[149,150] Nevertheless, once assembled, the apoptosome remains insensitive to the effects of the ionic strength. The inhibitory effects of K^+ on apoptosome formation are antagonized in a concentration-dependent manner by cytochrome *c*. The necessary binding of cytochrome *c* to Apaf-1, that renders a competent Apaf-1 for apoptosome formation, is not accomplished in the presence of high concentrations of K^+ and in this way the physiological concentration of intracellular K^+ act as repressor of apoptotic effectors.

Classically, Ca^{2+} toxicity has been associated with necrosis, but several studies have shown that changes in the intracellular Ca^{2+} levels are associated with apoptotic processes.[151] In fact, the physiological concentration of Ca^{2+} has been shown to negatively affect the assembly of apoptosome. Preincubation of Apaf-1 with 1 mM calcium chloride prior to incubation with cytochrome *c* and dATP compromised apoptosome assembly.[152] Ca^{2+} binds to monomeric Apaf-1 with a dissociation constant in the micromolar range and induces an unproductive conformation that fails to oligomerize in the presence of cytochrome *c* and dATP. However, Ca^{2+} does not have any significant effect on a previously assembled apoptosome.

3.3.2. Natural molecules

Taurine (Table I) is a non-essential sulfur-containing amino acid reported to decrease the amount of Apaf-1-associated caspase 9. Taurine was known as an efficient antioxidant in cell protection and also conferring beneficial effects on cardiovascular functions.[153,154] Its role as putative apoptosome-interfering molecule was investigated in an ischemia model of neonatal cardiomyocytes[155] that showed resistance to apoptosis when cells were treated with taurine. However, the increased Akt activity observed after taurine treatment was suggested as the actual negative regulator of the Apaf-1/caspase-9 interaction.[155]

Nitric oxide (NO) has been shown to inhibit apoptosis in some experimental systems. The NO donor S-Nitroso-N-acetyl-penicillamine (SNAP- Table I) was shown to inhibit the correct assembly of Apaf-1 into an active apoptosome interfering the Apaf-1/procaspase-9 CARD/CARD interactions.[156]

As discussed above, it was early demonstrated that in the *in vitro* reconstituted apoptosome, Apaf-1 binds and hydrolyzes ATP or dATP to ADP or dADP,

respectively.[18] However, the high millimolar concentrations of nucleotides in the cell opened discussions on how such physiological nucleotide levels affect the apoptosome. A dose-dependent inhibitory effect of dATP on the cytochrome *c*-initiated caspase activation was observed.[157] It was shown that the nucleotides directly interact with some lysine residues of cytochrome *c* inhibit its association with Apaf-1 and therefore prevent apoptosome formation.

3.3.3. Synthetic inhibitors of the Apaf-1-mediated apoptosome assembly/activity

The first attempt to identify chemical inhibitors of the apoptosome was reported by Jäättelä and col.[158] From a diverse library of 5000 compounds the authors selected a representative collection of 392 compounds (the parameters of selection were not described) and identified in a cell-extract based assay 11 compounds (2.8% hit rate) that inhibited the cytochrome *c*- and dATP-induced activation of caspase-3/7 activity. After cell toxicity-based evaluation three diarylurea-based compounds (NS1764, NS1784 and NS3694, Table I) were further selected. Secondary screens started with the evaluation of the diarylurea compounds as direct inhibitors of the final enzyme of the signaling pathway, i.e., caspase-3. The compounds were incubated with active caspase-3 in the presence of a fluorogenic enzyme substrate. All three compounds failed to inhibit the enzyme. Further secondary screens included evaluation of direct caspase-9 activity, immunobloting employing antibodies specific for caspases and their cellular substrates, exclusion chromatography fractionation of treated and control cell extracts and co-immunoprecipitation with caspase-9 antibody. Overall, the authors concluded that the diarylurea compounds inhibited the formation of the active 700 kDa apoptosome complex.[158] However, neither the molecular mechanism of action nor follow up optimization studies on these interesting compounds were reported.

All three screening campaigns herein reviewed for the identification of Apaf-1/apoptosome modulators were cell extract-based assays that were interrogated with collections of small molecules. However, in our laboratory, we were more interested in a true and direct Apaf-1-mediated biological assay. We developed a medium-throughput assay with purified recombinant Apaf-1, cytochrome *c*, dATP and [^{35}S]-Met procaspase-9. The use the recombinant protein-based approach could facilitate the identification of molecules which act on Apaf-1 specifically. As a source of chemical diversity we used a positional scanning diversity-oriented library of alkylglycines trimers (peptoids) composed of 52 controlled mixtures and a total of 5120 compounds.[128,159,160] From the four discrete compounds derived from the library screening and deconvolution (hit rate 0.08%) that inhibited the *in vitro* apoptosome-dependent activation of procaspase-9 the most potent was peptoid 1 (Table I). Peptoid 1 contains two dichlorophenylethylamino moieties that were found to be important for activity.[128,161] Noticeably, the activators of the Apaf-1 mediated apoptosome assembly above discussed reported by Nguyen and Wells[118] also contain a dichlorobenzylamino moiety. Likewise, a halophenyl moiety, in this case bearing a trifluoromethyl group, is also present in the diarylurea derivatives identified by Lademann et al.[158] as inhibitors of the formation of the apoptosome complex. Then, it appears that lipophilic interactions promoted by a haloaryl moiety can exert an interaction with the apoptosome complex. However, those structural complementary features that confer an activation effect or an inhibitory activity on the formation of the apoptosome complex and /or switch on of the apoptosis machinery, remain still to be elucidated. Peptoid 1 was fused to two additional *N-alkylamine* residues at the *N-terminus* end, in order to improve its solubility (peptoid 1a - Table I). Fluorescence polarization assays revealed that a fluorescent derivative of peptoid 1a binds to Apaf-1 but not to cytochrome *c*, and

peptoid 1a failed to inhibit recombinant caspase 3.[128] Despite the *in vitro* activity of peptoid 1a, this compound exhibited low membrane permeability with modest efficiency in cell assays. In order to improve the cellular uptake, a new series of peptoid 1 analogues were synthesized. The conjugation of peptoid 1 to cell penetrating peptides (e.g., penetratin, HIV-1 Tat)[162] or to a water soluble polymeric carrier (see below),[163] resulted in improved cellular internalization and antipoptotic activity (Table I).

Cyclization of peptoid 1 was also contemplated. Admittedly, peptoids could hardly be considered as lead candidates due to the risks of interaction with undesired targets as consequence of their great conformational freedom. Fortunately, peptoids are molecules simple enough to design second generation analogues addressed to restrain such conformational freedom. Among those possibilities, the generation of heterocyclic derivatives in which the ring could play the scaffold role and the bioactive chemical diversity from the peptoid could be inserted, constitutes an attractive strategy. In addition, it is reasonable to think that such conformational restraints would play a role in important physicochemical features related to bioavailability and cellular permeability of the original peptoids. Actually, these properties could be maintained or even improved, thus leading to compounds with a better pharmacological profile. Confirming this hypothesis the conformationally restrained mimetic (2 in[161], QM31 in[75], now SVT016426 – Table I) elicited improved antiapoptotic activity in the different studied cellular models.[161] Furthermore, SVT016426 was shown to inhibit the release of cytochrome c from mitochondria and suppressed the Apaf-1-dependent intra-S-phase DNA damage checkpoint[75]. The molecular mechanism involved in the antiapoptotic activity of SVT016326 is currently under study. Nevertheless, experimental evidences suggest that, as previously demonstrated in vitro,[128] in the presence of Apaf-1 inhibitors, doxorubicin-induced apoptotic cells showed a decreased apoptosome-dependent

procaspase-9 processing (to be published elsewhere). Additional modifications of the structure that are currently being explored to complete the structure activity relationship studies will provide the clues for the achievement of an optimized antiapoptotic drug.

3.3.4. Nanomedicines as inhibitors of the Apaf-1-mediated apoptosome assembly/activity

Apart from the identification of new drugs for established pharmacological targets, such as Apaf-1, current pharmaceutical development also demands advancements in macromolecular analogues. Such analogues will be able to improve the therapeutic capabilities of existing drugs by enhancing their biological activity and specificity. To accomplish the full therapeutic potential of a bioactive agent a specific molecular delivery is required. It is crucial to target therapeutics to the diseased cells and once there, promote their efficient delivery to the required intracellular compartment ensuring availability in an appropriate time window. Nanoscience and nanotechnology are the bases of innovative delivery techniques and offer great potential benefits to patients and new markets to pharmaceutical industry.[164,165] Polymer therapeutics are nanosized hybrid constructs that covalently combine a drug, protein or antibody with a polymer; they can rightly be viewed as the first polymeric nanomedicines (see [166-170] for more information on this field). The successful clinical application of polymer–protein conjugates (PEGylated enzymes and cytokines)[171,172] and the promising results arising from clinical trials with polymer-bound chemotherapeutics[167] have established the potential of polymer therapeutics as anticancer therapy. Furthermore, such advances are setting the basis for the development of more sophisticated second-generation constructs.[168] However, many challenges and opportunities still lay ahead providing scope to further develop this technological platform. Delivery of new anticancer agents

focusing on novel molecular targets and their combination, development of both new and exciting polymeric materials with defined architectures and treatment of diseases other than cancer (e.g. rheumatoid arthritis, diabetes or ischemia) are the most exciting and promising areas.[170] The later, is one of the most interesting advances in the field of polymer-drug conjugates and in particular the design of macromolecular systems to promote tissue regeneration.[173,174]

Within this context, and in particular tissue regeneration and ischaemic diseases, the research carried out considering the above-mentioned Apaf-1 inhibitors as bioactive agents could be enclosed. Poly-L-glutamic acid (PGA)-based conjugates were suggested as adequate platforms for the delivery of this first-in-class family of apoptosis inhibitors.[163] The conjugation of the poorly soluble peptoid 1 compound to a hydrophilic polymeric carrier, such as PGA, offered a marked solubility enhancement and a more specific intracellular trafficking that coupled to an efficient lysosomotropic drug release on the cytosol[166] highly enhanced the antiapoptotic activity of peptoid 1. PGA-peptoid conjugates were obtained by a linker-mediated attachment of peptoid 1 to the PGA polymer obtaining by this way the first family of antiapoptotic nanomedicines[161,163] (Fig. 6). The first conjugate in this series was the so called QM56 (compound 5 in [163]) where a diglycyl sequence was used as linker between the peptoid 1 moiety and the carrier PGA. QM56 proved to be highly effective inhibiting caspase 3 activity in different cell lines even after long-incubation times. In the cellular environment, the PGA-based conjugates are substrates of the enzyme cathepsin B.[175] Additionally, peptide-based linkers containing hydrophobic residues at P1 and P2 positions have been also described as appropriate substrates for this enzyme.[176] Even more, for some applications the drug release rate for compounds comprising a Gly residue at P1 could be considered too slow to achieve an effective concentration in the

desired time range; therefore, in order to enhance drug release kinetics different possibilities for linker optimization on QM56 have been explored (Fig. 6).

QM56 was reported as an efficient compound inhibiting hypoxia-induced apoptosis in cardiomyocytes in an *ex vivo* model of myocardial infarction[161]. More recently, QM56 has been demonstrated to provide protection from cytokine-induced injury on mesothelial cells obtained from effluents of stable peritoneal dialysis patients and from omentum of non-dialysis patients allowing an effective wound healing and long-term recovery.[177] New derivatives are currently being developed in order to further improve nanoconjugate therapeutic output.

4. CONCLUSIONS

The significant advances in cell death research and the establishment of appropriate cellular and animal models have enabled the discovery of the molecular mechanism that controls the process and has also established direct links with diseases. As such, proposals for the development of new therapeutic approaches aimed at alleviating apoptosis-dependent disorders have come into sight. Putative drug targets derived from cell death studies have permitted the advancement through different clinical phases of pharmacological candidates.[23] In fact, most of the key players in the apoptotic signaling pathway have been explored as drug targets: death receptors that control apoptosis induction from the cell surface,[178] caspases that are the executioners of apoptosis,[26,27] endogenous caspase inhibitors as IAP[179] and Bcl-2 protein family members.[120] However, the apoptosome, and its primary scaffolding protein Apaf-1, have been less tractable as drug targets. However, the strategic position of Apaf-1 upstream of caspase 3 and downstream of mitochondria makes it an interesting target for the development of chemical modulators. One of the most important achievements in cell death research is

the elucidation of how apoptosis signaling events are linked to a variety of other non-apoptotic signaling pathways. A distinctive feature of cell biology is how the whole system, in an exquisite equilibrium, modulates cellular signaling. It is still a complex system, requiring the understanding of many pathways that can influence cell survival and death. As we have attempted to review here, the apoptosome and in particular Apaf-1, actively participate in such networking strategies through both, protein-protein interactions and sensitivity to the levels of different metabolites. This reinforces the attractiveness of Apaf-1 as drug target. We are inclined to speculate that by inhibiting Apaf-1, important signals for cell dismantling can be inhibited. This will offer advantages to Apaf-1 inhibitors over other apoptosis inhibitory strategies focused later down in the process and such inhibitors can be considered as drug-lead compounds for the development of a new class of cytoprotective antiapoptotic agents. In that sense, selective inhibition of the apoptosome can report some advantages as it was early discussed and inhibiting apoptosis through Apaf-1 inhibition can find applications in acute diseases and surgery interventions as organ transplantation. However, concerns about apoptosis inhibition raised as such treatments would decrease the body defense system repertoire to fight against tumor growth events although recent reports suggest that apoptosis inhibition can help in solid tumor sensitization to radiation.[180,181] Nevertheless, promising new concepts and potential applications are emerging from these studies and we are beginning to understand the entire process in the whole cellular environment.

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Figure Legends

Figure 1. The apoptotic machinery (see main text for details).

Figure 2. Apaf-1 has three main protein domains (CARD, NBD and WD40 – see main text for details). Apaf-1 interacting proteins. Inhibitory proteins are shown in black and Apaf-1 activator proteins in white. Proteins with an unknown function are represented as grey boxes. The different proteins are located according to the Apaf-1 domain of interaction.

Figure 3. Apoptotic pathway in *C. elegans.* After a cell death stimuli Egl-1 (BH3 mammals homolog) blocks the mitochondrial antiapoptotic protein CED-9 (Bcl-2 mammal homolog) and releases CED-4 (Apaf-1 mammal homolog) that binds to CED-9 as a dimer. Once in the cytosol, two CED-4 dimers oligomerize into a tetramer and recruits inactive CED-3 (caspase mammal homolog). The subsequent cleavage of CED-3 activates the protein and the cell dismantling process (apoptosis) is triggered.

Figure 4. Schematic apoptosome assembly. Apaf-1 remains inactive in the cytosol until its interaction with cytochrome *c*. The subsequent exchange of ADP for dATP/ATP in the NBD domain induces a conformational change of Apaf-1 into its active isoform and its oligomerization into the apoptosome. Once assembled, procaspase 9 is recruited to the apoptosome.

Figure 5. Apaf-1 1-591-mediated activation of procaspase-3 and procaspase-7. Reaction progress curves of caspase-3/7 tetrapeptide substrate Ac-DEVD-AFC (excitation and emission wavelengths were 405 nm and 508 nm, respectively) cleavage

by **(A)** procaspase-3, **(B)** procaspase-7, in the absence (white square) or in the presence of Apaf-1 1-591 (2.2 μM) and procaspase-9 (0.1 μM) (black square); Apaf-1 1-591 and procaspase-9 C285A (black triangle); Apaf-1 alone (grey square). All measurements were performed in 100 μl reactions in 20 mM Hepes pH 7.5, 100 μM ATP, 5 mM $MgCl_2$, 100 mM KCl, 5 mM DTT buffer at 37ºC. The protein concentrations in the assay were: procaspase-3, procaspase-7, procaspase-9 and procaspase-9 C285A at 0.1 μM and Apaf-1 1-591 at 2.2 μM.

Figure 6. Nanoconjugates inhibitors of apoptosome assembly: first described antiapoptotic nanomedicines. **(A)** General chemical structure for PGA-peptoid (QM56) derivatives, **(B)** Influence of polymer-drug linker on drug release kinetics for QM56 derivatives in presence of lysosomal thiol protease cathepsin B.[150]