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Apoptosis: from biology to therapeutic targeting*

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biology of apoptosis

Apoptosis, or programmed cell death, is a complex process by which cells, malignant and normal, undergo cell death to control growth or eliminate damaged cells. While the process is intrinsic to every cell in the body, malignant cells have co-opted the many pathways governing cell death, shifting the balance of the process toward survival and immortality. In the early 1970s, apoptosis was recognized as an intrinsic cellular process that was considered complementary to cell division and mitosis in regulating tissue homeostasis [1]. Over a decade later, the Bcl-2 gene was identified and found to play a critical role in influencing cell survival. Inhibition of programmed cell death was quickly recognized as a central step in tumor development. In fact, the coupling of both pro-survival (Bcl-2) and growth promoting genetic lesions was sufficient to induce transformation in *in vitro* models [2]. Subsequent research into the biology of apoptosis then established several key principle observations, including: (i) the marked evolutionary conservation of the apoptotic machinery; (ii) the central role of a class of cysteine proteases, later called caspases; (iii) the existence of pro- and anti-apoptotic relatives of Bcl-2 characterized by complex interactions; (iv) the existence of at least two distinct pathways to apoptosis in mammalian cells, one involving the mitochondria ('intrinsic' pathway) and the other involving the death receptors ('extrinsic' pathway) [3–7]. The extrinsic or cytoplasmic pathway is triggered through the Fas death receptor, which is a member of the tumor necrosis factor (TNF) superfamily. The intrinsic pathway, frequently referred to as the mitochondrial pathway, leads to release of cytochrome *c* from the mitochondria, with activation of the death signal. The intrinsic and extrinsic pathways converge in a final common pathway which leads to the activation of a family of intracellular proteases called caspases. Caspases cleave regulatory and structural proteins in the cytoplasm, which leads to programmed cell death.

These observations opened the door in our thinking regarding how this complex biology might be manipulated in a therapeutic fashion. Central to these observations was the fact that all Bcl-2 family members contain characteristic regions of homology termed BH (Bcl-2 homology) domains [8]. These family members are subdivided into three distinct groups based on their structure and functions. The anti-apoptotic (pro-survival) family includes familiar proteins such as Bcl-2, Bcl-X_L, Mcl-1, Bcl-w and A1, which each contain four BH domains. The second group, commonly referred to as the proapoptotic (pro-death) family members, primarily include Bax and Bak. These proteins typically contain multiple BH domains [8]. The third group of proteins are commonly referred to as BH3-only proteins. This group includes at least eight related family members, including Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa and PUMA, all of which display sequence homology with other Bcl-2 family members within the amphipathic and α-helical BH3 segments [9]. The relatively high abundance of BH3-only proteins is thought to provide an added level of control in regulating the threshold required to induce cell death [8]. The regulatory control is attributed to the interactions between the α-helical BH3 domains of proapoptotic proteins (Bax and Bak) which bind to the hydrophobic groove and neutralize the anti-apoptotic influence [10].

In healthy cells, basal levels of anti-apoptotic proteins like Bcl-2 and Bcl-X_L prevent Bax and Bak from being dissociated from the complex. Once the apoptosis machinery is stimulated, BH3-only proteins are activated and competitively bind to the hydrophobic grooves of anti-apoptotic proteins like Bcl-2 through the BH3 domains [11]. This high-affinity interaction displaces Bax and Bak from the anti-apoptotic proteins, allowing them to form multimers in the outer mitochondrial membrane, which then influences mitochondrial membrane permeabilization, leading to release of cytochrome *c*, and then induction of programmed cell death [12]. Most if not all apoptotic signals transmitted by BH3 domains converge through Bax and Bak [13]. Once a cell becomes committed to apoptosis, a cascade of downstream events is triggered which leads to cell death. This complex pathway involves near complete depolarization of the mitochondrial membrane potential (something that can be readily measured in the laboratory and used as a direct measure of apoptosis), release of death promoting mitochondrial proteins such as cytochrome *c*, SMAC/Diablo and AIF, and activation of caspases [14, 15].

The complex and tightly regulated stoichiometric balance of the various Bcl-2 family members is what sets the threshold required to induce cell death, or to maintain homeostasis [16]. Because the different BH3-only proteins have different binding specificities for Bcl-2 and its related family members, the different BH3 proteins are known to have markedly different capabilities for inducing apoptosis in different cell types [17–19]. For example, the BH3 domains of PUMA and Bim can bind to all five anti-apoptotic proteins,

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while in contrast, Bad and Bmf preferentially interact with Bcl-2, Bcl-X_L, Bcl-w, but not with Mcl-1 or A1.

The so called 'extrinsic' pathway of apoptosis is centered on the role of TNF-related apoptosis-inducing ligand (TRAIL) and its receptors. TRAIL induces programmed cell death by directly binding to the death receptor (DR)4 and DR5 receptors. This binding interaction then induces polymerization of cellular death domains of these receptors which leads to recruitment of FADD and activation of caspase 8, caspase 3 and caspase 7. Activation of caspase 8 additionally amplifies the death signal by activating the intrinsic apoptosis pathway through cleavage of the BID. This proteolytic event leads to binding of Bax and Bak, inducing release of cytochrome *c* and SMAC from the mitochrondria. This results in the activation of caspase 9 and, subsequently, other downstream caspases [20].

Clearly, our very detailed understanding of these complex interactions, though by no means complete, has created innumerable opportunities to modulate this biology in a fashion that has enormous therapeutic potential. Below are some of the emerging examples of how the pathway can be pharmacologically manipulated.

pharmacologic modulation of apoptotic pathways

small-molecule inhibitors of anti-apoptotic proteins

Over the past several years, many small molecules have been generated that have the potential to intercept the binding interactions between the various Bcl-2 family members. Strategies designed to develop BH3-only mimetics offer an opportunity to disrupt the binding interaction between anti-apoptotic family members such as Bax and Bak, leading to the liberation of free proapoptotic proteins. Similarly, proteaosme inhibitors, which have the potential to unfavorably affect the balance of various family members, have been shown to induce accumulation of the BH3-only proteins, which has the advantage of shifting the stoichiometry of these proteins in a way that leads to liberation of free Bax and Bak. Furthermore, proteasome inhibitors have been shown to increase the amount of Bax and Bak, which in and of itself is highly proapototic. Examples of these therapeutic applications are discussed below.

gossypol derivatives: AT-101. Gossypol is an orally available compound found in cotton seed extracts. The oil extracts from the seeds were originally used as an herbal medicine in China [21]. The (–)-enantiomer [(–)-gossypol or AT-101], binds to the BH3-binding grooves of Bcl-2, Bcl-X_L and Mcl-1, displacing BH3 peptides with a submicromolar inhibitory concentration of 50% (IC_{50}) [22]. AT-101 promotes an allosteric conformational change in Bcl-2 and loss of mitochondrial membrane potential in a Bax/Bak-independent fashion [23]. AT-101-induced apoptosis involves cytochrome *c* release from mitochondria and activation of several caspases [24, 25]. Recently, it has been shown that AT-101 can improve the efficacy of cyclophosphamide–adriamycin–vincristine–prednisone (CHOP) and cyclophosphamide–rituximab-based regimens in lymphoma xenograft models [24, 26]. While studies in human lymphoid malignancies are ongoing, several phase II studies in patients with non-small-cell lung cancer and small-cell lung cancer have been reported with AT-101 being given in combination with conventional chemotherapy regimens. While many of these studies are now oriented toward randomized studies looking at the combination of conventional chemotherapy plus or minus AT-101, it is clear that most developmental strategies for AT-101, and other anti-apoptotic small molecules for that matter, will be oriented more towards combination strategies.

New-generation gossypol derivatives are now also moving toward the clinic, including one particular compound referred to as apogossypol. This compound has been shown to have superior efficacy and less toxicity in transgenic mouse models resembling low-grade follicular lymphoma in humans [27] .

obatoclax (GX015-070). Obatoclax is an indole derivative and a broad-spectrum inhibitor of pro-survival Bcl-2 family proteins [28]. The compound activates the mitochondrial apoptotic pathway by displacing Bak from Mcl-1 and Bcl-X_L, upregulating Bim and inducing Bax and Bak conformational changes, mitochondrial depolarization and caspase activation [29, 30]. Interestingly, the drug potently synergizes with the proteasome inhibitor bortezomib in mantle cell lymphoma (MCL) cell lines without any significant cytotoxicity to peripheral blood mononuclear cells from healthy donors [29]. A phase I study of obatoclax using a 1-h infusion identified neuropsychiatric toxicity as the dose-limiting toxicity, with an established a maximum tolerated dose of 1.25 mg/m^2. A 3-h infusion of obatoclax administered once weekly to patients with both solid tumors and lymphoma established an improved safety profile, with dose-limiting toxicity of 20 mg/m^2. Interestingly, one patient with relapsed non-Hodgkin's lymphoma experienced a partial remission lasting 2 months. A phase II study in patients with myelofibrosis explored a fixed dose of 60 mg as a 24-h infusion once every 2 weeks. This study, however, did not demonstrate any significant activity of the drug in this setting [31]. As with the other BH3-only mimetics, future studies are primarily being directed toward combination studies, with the notion that the addition of the apoptosis-influencing drug will lower the threshold required for induction of cell death, leading to improved outcomes in the presence of proapoptotic inducing chemotherapy.

navitoclax (ABT-737). Navitoclax is a synthetic small-molecule compound, and probably the most potent and specific Bcl-2/Bcl-X_L inhibitor described to date [32]. Navitoclax has extremely high affinity for the anti-apoptotic proteins Bcl-X_L, Bcl-2 and Bcl-w, with a dissociation constant (K_i) of <1 nM for each of them. Unlike AT-101, which is known to bind with relatively much higher affinity for MCL-1, naviroclax binds poorly to Mcl-1 and A1. Mechanistic studies have shown that navitoclax is similar to the BH3 domain of Bad. The drug induces cell death by disrupting the complex of Bax and Bcl-2, which triggers conformational alteration of Bax. In addition, navitoclax also displaces the BH3-only protein Bim from its binding partners. Interestingly, the effects of ABT-737 are completely abrogated in Bax and Bak deficient cells [33]. This strict dependence on Bax and Bak distinguishes navitoclax from other small-molecule Bcl-2/Bcl-X_L inhibitors and suggests its function as an authentic BH3 mimetic. As a single agent,

navitoclax is most efficacious against small-cell lung carcinoma and several lymphoid malignancies including follicular lymphoma, diffuse large B-cell lymphoma, chronic lymphocytic leukemia, acute lymphocytic leukemia and acute myeloid leukemia with IC_{50}s in the nanomolar range [32, 34–36]. Navitoclax alone is also active in multiple myeloma in the micromolar range [37–39]. Navitoclax exhibits striking synergy when combined with γ-irradiation as well as a variety of anticancer agents including etoposide, doxorubicin, cisplatin, melphalan, araC, paclitaxel, vincristine, dexamethasone, thalidomide and bortezomib [32, 33, 37, 39, 40]. It has also been shown to enhance the anticancer effects of several investigational agents, such as cyclin-dependent kinase (CDK) inhibitor roscovitine and MDM2 inhibitor Nutlin-3a [37, 41].

Recently, Wilson et al. [42] have reported on a phase I study of navitoclax inpatients with lymphoid malignancies. This study demonstrated that navitoclax exhibited a novel mechanism of peripheral thrombocytopenia, and T-cell lymphopenia, which was attributable to high-affinity inhibition of Bcl-X_L and Bcl-2, respectively. The phase I experience established a 150-mg 7-day lead in dose followed by a 325-mg dose administered on a continuous 21/21-day schedule. Future studies will clearly explore the single-agent activity in lymphoid diseases, and will focus on combination strategies as discussed above with the other Bcl-2-targeted drugs.

monoclonal antibodies targeting TRAIL. The death receptors of the TNF superfamily represent potential targets for promoting apoptosis in cancer. Death receptor-mediated apoptosis is thought to be independent of p53 status, thus, cancers with inactivating p53 mutations might be particularly vulnerable to activation of TRAIL. Agonistic antibodies directed against DR4 [43] and DR5 [44, 45] have been reported and studied in both the preclinical and clinical settings. These antibodies have been shown to induce cell death almost exclusively in cancer cells and not in normal cells. In addition, they also reduce the growth of tumors in xenograft tumor models with essentially no systemic toxicity. A phase II study with mapatumumab in patients with relapsed or refractory non-Hodgkin's lymphoma reported three responses in 14 patients with follicular lymphoma including one complete response [46]. Once again, is it very likely that the single-agent activity of any of these targeted agents will be limited, and that by combining them with other drugs targeting distinctly different aspects of the apoptotoic machinery, or with conventional chemotherapy, these drugs will exhibit markedly improved activity in a host of different types of cancer.

proteasome inhibitors. Proteasome inhibitors may not be among the first class of drugs one thinks about when discussing the pharmacomodulation of Bcl-2 family members. However, a number of studies have clearly demonstrated that the combination of proteasome inhibition and Bcl-2-directed modulation represents a very synergistic platform. The proteasome degrades ubiquitylated proteins, including inhibitor of κBα (NF-κBIα), which inhibits NF-κB and modulates several pathways that influence survival. The treatment of Jurkat (T-cell tumor) and Namala (B-cell tumor) cell lines with the proteasome inhibitor lactacysin resulted in a marked increase in the proapoptotic Bcl-2 family member Bik, an effect mediated through impaired proteolytic degradation of Bik.

In MCL Mcl-1 is known to play a major prosurvival role. Some reports have suggested that proteasome inhibition can increase the level of MCL-1, theoretically antagonizing the effects of other proapoptotic influences [47, 48]. Irrespective of MCL-1 increase with proteasome inhibition, it is well established that the proteasome inhibitor bortezomib exhibits marked activity in patients with relapsed or refractory MCL, sufficient to warrant FDA approval for the indication [49]. It turns out that this anti-apoptotic effect is mitigated in part through the modulation of various BH3-only proteins important in MCL. Nencioni et al. [50] have shown that proteasome inhibitors induced cell death despite augmented Mcl-1 accumulation. Others have clearly demonstrated that the apoptosis was mediated by Bak liberation from Mcl-1 and Bcl-X_L, which was regulated through the accumulation of Noxa [51]. When both Mcl-1 and Bcl- X_L are inactivated by BH3-only proteins, proapoptotic family members become liberated, inducing apoptosis. Noxa was shown to not only affect levels of free Bak, but also to promote the proteasome-dependent degradation of Mcl-1. This concept was also validated in part by Perez-Galan et al. [29, 52] who established that bortezomib potently induces apoptosis, mitochondrial depolarization, Bax and Bak conformational changes and caspase activation in MCL lines [29, 52]. Bortezomib also resulted in accumulation of Noxa independent of p53 status. These authors demonstrated that the Bcl-2-targeted drug GX15-070 synergized with bortezomib by enhancing Noxa-mediated activation of Bak.

future directions

It has become very clear that the ability to modulate the many different members of the Bcl-2 family has emerged as one of the most promising new strategies for the treatment of cancer. The notion that one could modulate the distinct arms of the cell death machinery has opened an almost endless number of opportunities to think about how best to use these drugs to improve the care of patients with almost any malignancy. Moving forward, it will be necessary to further understand the pharmacologic ramifications of using these drugs in combination, and to determine the optimal strategies for scheduling them in the clinic. It is also possible, given how sophisticated our understanding of this biology has become, that combination of drugs targeting the discrete arms of this biology may be associated with therapeutic advantage. This is in direct contradiction to the oncologic dogma that drugs of similar mechanism should not be combined. However, the tailored modulation of those aspects of the apoptotic pathway with discrete inhibitors, may provide a new venue for thinking about how best to personalize specific therapies and combinations to specific molecular contexts.

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