Targeting apoptosis in cancer therapy

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Abstract

Effective elimination of cancer cells by programmed cell death or apoptosis has been a mainstay and goal of clinical cancer therapy for over 3 decades. Clinical translation of effective proapoptotic agents includes drug discovery, bioavailability and stability, tumor penetration, acute or chronic normal tissue toxicity, drug interactions, off-target effects, tumor heterogeneity and evolution with emergence of resistant clones. The pathways of apoptosis include cell intrinsic and extrinsic processes triggered by cellular stress, DNA damage, and immune surveillance mechanisms. While tumor cell death can lead to therapy response, the selection, growth and dissemination of therapy-resistant cells makes tumors lethal, leading to patient mortality. We focus on apoptosis pathways, signaling pathways that impact on them as well as molecular targets, strategies, therapeutics in clinical translation and known resistance mechanisms.

Keywords

intrinsic pathway; BCL-2; BCL-XL; IAP; extrinsic pathway; TNF; TRAIL; death receptor; oncogene; growth factor; MCL1; mTOR; PI3K; EGFR

Introduction

The pathways of cell death are conserved with important insights coming from studying cell fate in lower organisms such as *C. elegans* work by Robert Horvitz that led to the 2002 Nobel Prize in Physiology or Medicine [1,2].

Early concepts of cancer development revolved around viral and cellular oncogenes [3], cellular proliferation and transformation. By the 1980's with discovery of DNA fragmentation in lymphoid cells after glucocorticoid exposure [4] and subsequently in tumors undergoing chemotherapy [5], the induction of apoptosis as a goal for cancer therapy became a logical target and realistic goal. Discovery of anti-apoptotic oncogenes such as BCL-2 [6] and later BCL-XL [7] provided insights into cell survival or the prevention of cell death [8,9] as a powerful mechanism for tumor growth [10] and drug resistance [11]. These eventually would become therapeutic targets, and would lead to knowledge of secondary resistance mechanisms [12].

During the 1990's and 2000's there was much learned about the mechanisms leading to cell death from within a cell as well as through the immune system [13–15]. In particular, features of apoptosis including the release of cytochrome c from the mitochondria became the focus of investigation for control of cell death [16]. The balance of controlling families of proteins such the proapoptotic versus anti-apoptotic members of the BCL-2 family would regulate cytochrome c release from the mitochondria [17,18]. Downstream of cytochrome c release would be formation of the apoptosome with caspase activation [19,20] that would be negatively regulated by the inhibitors of apoptosis (IAP) family of proteins [21]. An overview of the current understanding of the apoptosis signaling pathways is shown in Figure 1, along with intersection of the major relevant survival pathways that impact on the cellular phenotype, including drug resistance.

While progress over the last two decades has been made in targeted therapies for cancer through blockade of various kinases that promote tumor progression, in part through cell proliferation and survival, insights have been emerging regarding the connections between the cell death mechanisms and its contribution to efficacy of cancer targeted therapies [22,23]. Ultimately, tumor cell death is impacted by the balance of these pathways as well as the cell extrinsic immune anti-tumor mechanisms.

Molecular markers of apoptosis

Apoptosis is a cellular death catalyzed by the proteolytic cleavage of thousands of proteins through the enzymatic activity of effector caspases such as caspases 3, 6 and 7 [24]. Apoptotic death can also be activated by granzyme contained within cytotoxic granules in T-cells or Natural Killer (NK) cells and through perforin-mediated pore formation in target cells [25]. The apoptotic process involves nuclear membrane breakdown that occurs through lamin cleavage by caspase 6, membrane blebbing, and breakdown of genomic DNA into nucleosomal structures that classically was observed as a DNA ladder on agarose gels [26,27]. A frequently used cellular protein marker for caspase 3 activation is the PARP protein with PARP cleavage being observed in cells undergoing apoptosis [28]. M30 is a caspase 3 cleavage product of cytokeratin 18 detected in serum resulting from therapy-induced tumor apoptosis in patients [29,30]. On tissue sections cleaved caspases are detectable by western blot as is cleavage of DNA through the TUNEL assay [31]. Granzyme is detectable in cytolytic T-cells by immunohistochemistry [32]. In cultured cells, apoptosis is detectable by flow cytometry though sub-G1 DNA content, externalization of phosphatidylserine, or various cleaved caspases [33]. Caspase activation can also be seen by caspase enzymatic activity cell-based assays or even by imaging scans applied to humans [34,35]. Granzyme B has been detected by PET scanning in preclinical studies as a marker of immunotherapy response [36]. The detection of apoptosis has provided the tools for drug discovery as well as the experimental means for validation of drug action in clinical specimens. A nomenclature for cell death has been proposed to facilitate description of its various forms as well as use of appropriate assays for detection of the process in its various forms [37].

The core apoptotic signaling pathways in mammalian cells

Intrinsic cell death.

A final common pathway for cell-intrinsic apoptotic cell death involves mitochondrial outer membrane permeabilization (MOMP) [38] with the release of cytochrome c from the mitochondria, formation of the apoptosome with APAF1 and initiator caspase 9 followed by activation of executioner caspase 3 (Figure 1) [39]. In most mammalian cells, MOMP and release of cytochrome c into the cytoplasm are critical requirements to trigger apoptosis, and it has been considered the point of no return in cellular commitment to cell death that can occur independent of caspase activation [16,40,41]. Release of cytochrome c from the mitochondria is positively regulated by the proapoptotic BCL-2 family members such as BAX (BCL-2-associated X protein), BAK (BCL-2 antagonist killer 1), BIM, BID, PUMA and negatively regulated by the anti-apoptotic BCL-2 family members such as BCL-2, BCL-XL, Bcl-w, A1 and MCL1 [42–44]. BAX and BAK are central effectors of apoptosis as they form macropores in the mitochondria outer membrane causing MOMP . Following activation by proapoptotic proteins, BAX and BAK undergo conformational changes and oligomerization forming a pore in the mitochondrial membrane [45,46]. Apoptosis-inducing BCL-2 members containing only one BCL-2 homology (BH) domain (BH3-only) can be grouped as activators and sensitizers [47,48]. Activator proteins BIM, BID, and PUMA bind and directly activate BAX and/or BAK [49–57]. Sensitizer proteins (i.e., BAD, NOXA, BIK, BMF, HRK) do not activate BAX and/or BAK directly, but provide important regulation by inhibiting anti-apoptotic proteins (i.e., BCL-2, BCL-XL, MCL1) and/or displacing activators such as BIM or monomers of BAX or BAK that could ultimately lead to apoptosis [41,58,59]. Anti-apoptotic members have four BH domains forming a binding groove that sequesters proapoptotic activators or sensitizers BH3-only members as well as BAX and BAK [60]. BH3-only proteins such as PUMA, NOXA, BID, or BIM have a strong affinity to and inhibit MCL1 [47,60]. Whereas BAD has a stronger affinity to BCL-2, BCL-W, BCL-XL and a relatively weak affinity to MCL1 [47,61]. The levels of BCL-2 proteins and their interactions modulate sensitivity and resistance to apoptosis and can be assessed by functional assays such as BH3 cellular profiling, an important tool to predict response to apoptosis targeted agents incorporated in recent clinical trials [62]. For instance, cells with high levels of proapoptotic proteins are more susceptible to undergo apoptosis or "primed" as compared to cells with downregulation of BAX or BAK. BH3 profiling also reflects cellular dependence on specific anti-apoptotic proteins [63]. In addition to transcriptional regulation, BCL-2 proteins are functionally modulated by cytoplasmic localization and post-translational modifications such as phosphorylation, ubiquitination, and proteasomal degradation [54,64–70]. Cyclin-dependent kinases (CDK) and p53 mediate apoptosis by regulating transcription and phosphorylation of BCL-2 proteins [54]. For example, pan CDK inhibitor flavopiridol blocks cell cycle progression and induces apoptosis by inhibiting MCL1 transcription and upregulating BIM, NOXA, and Bik/NBK in multiple myeloma cells [71,72]. CDK5 regulates MCL1 through phosphorylation of its antagonist NOXA [73]. CDK5 inhibition decreased the levels of MCL1 in pancreatic cancer cells and showed synergism with BCL2 inhibitor navitoclax inducing apoptosis [74]. Following the release of cytochrome c from the mitochondria, caspase 3 activation can be negatively regulated by a family of Inhibitor of Apoptosis (IAP) proteins such as XIAP, c-IAP1 and c-IAP2 [75,76]. Peptides released from the

mitochondria along with cytochrome c (e.g., SMAC/Diablo) can block XIAP and allow caspase activity to kill the cell [75,77]. A number of drugs including small molecules [78–89] or stapled peptides [90–93] have been under various stages of development to block anti-apoptotic proteins such as BCL-2/BCL-XL, MCL1 or the IAP proteins, all of which can be overexpressed in human tumors. This includes SMAC mimetics [94,95]. The balance of BCL-2 family of anti-apoptotic proteins versus the proapoptotic family members has long been considered a cellular rheostat that controls the threshold of cell death in mammalian cells [64,96,97]. Proapoptotic members of the BCL-2 family such as BAX are classical targets for direct regulation by the p53 tumor suppressor protein [98]. As such their levels increase after exposure of wild-type p53-expressing cells to various cytotoxic or DNA damaging agents [99]. Increased expression of the proapoptotic BCL-2 family members can contribute to the cytotoxicity towards either tumor cells or normal cells. The molecular details of caspase processing and activation have been described [100].

Extrinsic cell death.

A second common pathway of cell death referred to as extrinsic cell death can initiate apoptosis from cell membrane proteins known as death receptors (DR). Proapoptotic death receptors include Fas, TNFR1, TNFR2, and the TRAIL receptors DR4 and DR5 [13,39,101–111]. Natural ligands such as FasL [112–116], TNF or TRAIL bind to Fas, the TNFR's or the TRAIL receptors DR4 and DR5, respectively [117–120]. The intracellular domains of the proapoptotic death receptors include a conserved protein-protein interaction domain referred to as the death domain [121,122]. Thus, upon ligand binding, the death receptors trimerize and aggregate within the cell membrane (a phenomenon known as capping) and this is followed by recruitment of adaptor proteins such as FADD (Fas or other death receptor associated death domain protein) and initiator caspase proteins such as caspase 8 or caspase 10 found in humans (mice have no caspase 10) [120,123–125]. Initiator caspase activation in the extrinsic pathway is negatively regulated by c-FLIP [126], while activation of caspases 8 and 10 can lead to activation of executioner caspases 3, 6, and 7 as well as BID cleavage. BID cleavage and myristoylation leads to its translocation to mitochondria and this can contribute to cytochrome c release [127]. BID is regulated by p53 [128]. BID is the link between the extrinsic cell death pathway and the mitochondrial pathway and in so called Type II cells serves to amplify the cell death signal through the mitochondria leading to more efficient cell death that ultimately requires the mitochondrial events for cell execution. Type I cells efficiently activate downstream caspases such as caspase 3 leading to cell death without the need to amplify the signal through the mitochondria [129,130].

Signaling through death receptors can be attenuated through decoy receptors at the cell surface that bind to death ligands and compete for them, thereby reducing activation of the proapoptotic death receptors. A decoy receptor for Fas ligand is known to be amplified and overexpressed in some colorectal and lung tumor cells [131], and two TRAIL receptor decoys are known (DcR1 or TRID and DcRII or TRUND) to be overexpressed in normal cells thereby eliminating toxicity of TRAIL to normal tissues [132]. Low caspase 8 expression occurs in normal cells, thereby also reducing toxicity of death ligands and receptors [133]. Caspase 8 can also be actively degraded in tumor cells [134]. The death receptors including to a large extent the TNF receptors and to a lesser extent the TRAIL receptors activate NFkB

[135]. Fas receptors do not activate NFkB, but their therapeutic activation leads to liver necrosis that was blocked by BID deletion in mice [136]. The TNF receptors strongly activate NFkB and this limited the therapeutic development of TNF in cancer therapy due to systemic inflammatory responses in patients who were treated such that TNF is only used for regional therapy of bulky tumors such as sarcomas and melanomas combined with chemotherapy in limb perfusion protocols [137]. While TNF does not have significant cytotoxic activity against tumor cells *in vitro*, its therapeutic benefit derives from the direct cytotoxicity on tumor vasculature promoting hemorrhagic necrosis, increased vascular permeability that enhances local accumulation of chemotherapy [138–140]. The TRAIL receptors generally have minimal NFkB activation and this has been a key characteristic that favored therapeutics activating TRAIL receptors (TRAIL or TRAIL receptor agonists). However, in TRAIL-resistant tumors, activation of NFkB in the absence of cell death could promote tumor progression and metastasis and foster a tumor-supportive immune microenvironment by inducing secretion of CCL2 and increased accumulation of myeloid-derived suppressor cells (MDSCs) and M2-like macrophages [141,142]. Furthermore, TRAIL activation promoted metastasis and invasion of KRAS-driven models of NSCLC and pancreatic adenocarcinoma via PI3K/Rac1, providing a biological rationale for these TRAIL-resistant tumors to overexpress TRAIL receptors [141]. These results suggested a therapeutic benefit from blocking TRAIL receptors in KRAS-driven models with overexpression of TRAIL receptor. The p53 tumor suppressor directly transcriptionally regulates death receptors such as Fas or DR5, and thus therapeutics that stabilize p53 can induce cell death through both the extrinsic and intrinsic cell death pathways [143]. Mice have only one proapoptotic TRAIL receptor that is regulated by p53, and the knockout mice have reduced apoptosis in vivo following exposure to whole-body irradiation [144,145].

Apoptosis is a stochastic process within a population of cells [146,147]. Thus, there is a fractional cell killing within a population that has been attributed to variations in cell death-relevant protein concentrations [148]. Recent evidence suggests the stochastic nature of apoptosis may be related to the cellular content of mitochondria and the distribution of BCL-2 family of proteins within the mitochondrial surface [149].

Extrinsic cell death can also occur through a process referred to as necroptosis [150] that involves loss of cell membrane integrity while caspase activity is inhibited. During the process of death receptor activation, RIPK1, cIAP1, and cIAP2 are recruited to the plasma membrane with subsequent activation of NFkB which together with its target c-FLIP inhibits apoptosis and caspase activation while allowing for plasma membrane disruption leading to the death known as necroptosis [150] that is RIPK1-dependent. The interplay between necroptosis and apoptosis, as well as other forms of regulated necrosis such as pyroptosis or ferroptosis are areas of active investigation [150], and ultimately effects of drugs on these interactions will be relevant to cancer therapy.

Survival pathways that impact on cell death.

In addition to the anti-apoptotic members of the BCL-2 family, the IAP proteins, and c-FLIP, a number of cellular pro-survival pathways impact on the cell death phenotype of cells treated with agents that activate the core apoptotic pathways [151–153]. The pro-survival

pathways are, in many if not all cases, oncogenic pathways and/or are otherwise activated by cellular oncogenes including growth factor receptors [22]. While many of the pro-survival pathways have become established drug targets in oncology, it is important to recognize that ultimately the drugs induce apoptosis through the core apoptotic pathways [154–156]. Examples include kinase signaling pathways involving AKT, ERK, Ras, Raf, MEK, or mTOR kinases [157–175]. Inhibition of growth factor receptors, including EGFR, Her2/Neu, other ERBB family members, c-MET, or NTRK, among others, also leads to apoptosis [176–196].

These pathways to which tumor cells become addicted have cross-talk to the mediators of apoptosis in core cell death pathways to inactivate cell death. Various therapeutic inhibitors of these pathways relieve the blockade on cell death, thereby unleashing the cell death machinery in tumor cells. For instance, the anti-tumor efficacy of EGFR inhibitors is dependent on their ability to modulate the expression of BCL-2 family members and induce apoptosis. Expression of proapoptotic BIM (BCL-2 interacting mediator of cell death) in lung cancer cell lines predicts the apoptotic response to EGFR tyrosine kinase inhibitor (TKI) gefitinib, and downregulation of BIM attenuates the anti-tumor response in vivo [197]. ERK1/2 signaling causes phosphorylation of BIM, leading to its proteasomal degradation and thereby promotes cell survival [198]. Similarly, AKT and Ras-MAPK activation induce phosphorylation of BAD (BCL-2 agonist of cell death; a proapoptotic BCL-2 family member) and inhibit apoptosis; providing another intersection between cell survival signals and active inhibition of the cell death machinery [199,200]. Intrinsic modulation of BCL-2 proteins has also been linked to the development of resistance to MEK and PI3K inhibitors providing a scientific rationale for combinations of BCL-2 family inhibitors and TKIs currently in clinical development [201,202].

Other forms of cells death.

Autophagy ('self-eating') is a regulated process that can promote cell survival (and tumor growth) when cells are deprived of nutrients, e.g. in the tumor microenvironment, but however, in its very late stages autophagy can cause cell death [203–206]. The blockade of autophagy, for example with chloroquine is a strategy that has moved to the clinic and holds some promise [207]. Current thinking is that the complex process of autophagy can involve other cellular functions including vesicle extrusion, cytokinesis, proliferation and autophagy-dependent cell death (ADCD) [208]. The ultimate interplay between autophagic signaling and apoptosis pathways, and modulation of that cross-talk by cancer therapeutic agents is an important area of investigation.

Ferroptosis represents another distinct form of cell death triggered by the accumulation of cytosolic and lipid iron-dependent reactive oxygen species (ROS) [209–211]. Glutathione peroxidase (GPX4) catalyzes the reduction of lipid peroxides utilizing reduced glutathione (GSH) and functions as a central switch to protect the cell against ferroptosis [212]. Various cancer cell types are susceptible to ferroptosis, including melanoma, lymphoma, gastric cancer, and neuroblastoma [213–216]. Dedifferentiation of melanoma cancer cells observed during the development of resistance to tyrosine kinase inhibitors occurs in tandem with increasing sensitivity to ferroptosis [213]. Mesenchymal cellular features were associated with

increased expression of ZEB1 (zinc finger E-box-binding homeobox 1) and changes in lipid metabolism creating a dependency on GPX4, which could be explored as a therapeutic vulnerability in treatment-resistant tumors and triggered to eradicate residual cancer cells and reduce recurrence [217]. The GPX4 dependency of this mesenchymal state has sparked interest in strategies to induce ferroptosis for cancer therapeutics using direct inhibitors of GPX4 (i.e withaferin A, ML210, RSL3) [212,214,218,219], modulators of glutathione levels (i.e., buthionine sulfoximine) [212] or inhibitors of cystine-glutamate antiporter (i.e., erastin, sulfasalazine, sorafenib) [209,210,220]. Naked nanoparticles or those carrying withaferin and other chemicals can induce ferroptosis [214,221,222]. P53 also regulates sensitivity to ferroptosis, which seems to contribute to its tumor suppression function [223–225]. P53 promotes ferroptosis in conditions of oxidative stress by downregulating SLC7A11 that reduces cystine cellular uptake [223]. In other contexts, restoring wild-type P53 protected cancer cells against ferroptosis [226]. Further progress in the understanding of molecular mechanisms of ferroptosis will promote novel therapeutic strategies targeting ferroptosis to control cancer growth [227].

Clinical translation of modulators of apoptosis

Monumental scientific efforts placed at translating the modulation of cell death mechanisms to therapeutic benefit of patients with cancer have led to FDA-approved BCL-2 targeting drugs with meaningful clinical benefits and spurred several strategies in clinical development.

Strategies targeting the intrinsic pathway

The discovery of the *BCL-2* gene in patients with follicular lymphoma as a result of the chromosomal translocation t(14;18) ignited the interest in targeting this family of proteins to control cancer growth by promoting apoptosis [6,228]. Initial efforts focused on identifying inhibitors of anti-apoptotic members of the family such as BCL-2. These approaches included oligonucleotides targeting BCL-2 expression and small molecules mimicking the binding of BH3-only proapoptotic members (BH3 mimetics) to anti-apoptotic BCL-2 members (Table 1).

BH3 mimetics

ABT-737 was the first small molecule designed to selectively bind hydrophobic pockets within BCL-2, BCL-XL, and BCL-w where proapoptotic BH3-only proteins interact [229–231]. ABT-737 demonstrated single-agent activity against lymphoma and small cell lung carcinoma cell lines as well as synergy with radiation and chemotherapy agents such as paclitaxel in other tumor types [229]. These results provided proof-of-principle for this anti-cancer therapeutic strategy and supported the development of 2nd generation navitoclax (ABT-263) [232]. Approximately 35% of patients with refractory chronic lymphocytic leukemia (CLL) achieved partial responses in the phase I trial with navitoclax [233]. The response rates increased to 70% when it was combined with rituximab in untreated CLL [234]. Development of navitoclax and dose escalation were hampered by (on-target) thrombocytopenia related to BCL-XL inhibition in platelets, but combinations of navitoclax

with MEK inhibitors or other tyrosine kinase inhibitors continue to be explored in advanced solid tumors [235–238]. BCL-2 selective inhibitor venetoclax (ABT-199) showed significant activity in preclinical models of CLL and non-Hodgkin lymphoma (NHL) overexpressing BCL2 without causing thrombocytopenia [239]. The phase I study of venetoclax in heavily pretreated patients with refractory CLL showed tumor responses in all dose levels with an overall response rate of 79% and complete responses in 20% of the patients [240]. Complete responses were also present among patients with chromosome 17p deletion – a group associated with poor outcomes. While thrombocytopenia was avoided by BCL-2 selectivity, the most relevant side effect observed with venetoclax was tumor lysis syndrome (TLS) related to rapid tumor responses, especially among patients with high tumor burden. This adverse event has been circumvented by an alternative dose schedule (5-week ramp-up) and preventative hydration and use of anti-hyperuricemic agents [241]. Other side effects included mild nausea, diarrhea, and neutropenia. Progression-free survival (PFS) at 15 months was 66%, and the duration of response was longer among those achieving complete response. Aggregate results of four venetoclax early-phase trials in refractory CLL (347 patients) showed that patients with partial response had a median duration of response (DoR) of 24 months while a median DoR among patients with complete response patients was not reached [242]. These results supported the first FDA approval for venetoclax for the treatment of patients with 17p deletion CLL [240,243]. Subsequent studies with venetoclax combinations expanded the benefits of venetoclax in both refractory and treatment naïve CLL settings. A randomized phase 3 study showed that venetoclax combined with rituximab was superior to bendamustine/rituximab in refractory CLL with higher rates of response (92% vs. 72%) and progression-free survival (PFS) (2-year PFS 84% vs. 36%; P<0.001) [244]. Venetoclax plus obinutuzumab (anti-CD20) was also incorporated to front line treatment of CLL, providing a chemotherapy-free option for these patients based its superior efficacy to chlorambucil/ obinutuzumab in another phase 3 trial [245]. The combination of venetoclax with ibrutinib - an inhibitor of Bruton tyrosine kinase - showed significant efficacy in the front line treatment of high-risk CLL representing another treatment option in this setting [246]. Venetoclax plus standard chemoimmunotherapy regimen R-CHOP for treatment of B-cell NHL was safe and achieved 87% response rates in a phase I trial with subsequent phase 2 expansion cohort ongoing [247]. In addition to transforming the treatment of lymphoid malignancies especially CLL, BCL-2 inhibition has had a significant impact on the treatment of elderly patients with acute myeloid leukemia (AML), a group of patients that frequently does not receive AML-directed treatment because of limited efficacy and toxicities with standard chemotherapy.

BCL-2 promotes survival of leukemic blasts, mediates resistance to chemotherapy, and its inhibition has significant activity in preclinical models of AML [248–250]. Supporting the dependence on BCL-2 in AML cells, ABT-737 and venetoclax induced apoptosis of both AML myeloblasts and AML stem cells and demonstrated significant anti-tumor activity in AML xenograft models [248–250]. Single-agent venetoclax was well tolerated with responses observed in 19% of patients with high risk and refractory AML but limited duration of responses fostered combination studies [251]. BH3 profiling performed on pretreatment bone marrow biopsies and peripheral blood specimens showed the correlation between myeloblast dependence on BCL-XL or MCL-1 and shorter duration of therapy with venetoclax. Venetoclax also improved the clinical outcomes of elderly patients (> 75 years old) with

AML when combined with low-dose cytarabine with 54% complete response rates and median overall survival (OS) of 10 months [252]. These results were superior to low-dose cytarabine monotherapy (11–19% complete responses and OS 5.5 months), and compared favorably to intensive chemotherapy (45–57% remission rates and OS 5–12months) [253,254]. Supported by synergistic effect between hypomethylating 5-azacytidine and BCL-2 inhibitors in preclinical models [231,255], a phase I trial showed that combination of venetoclax with azacytidine or decitabine displayed a favorable adverse profile with 60–70% complete remission rates and median OS of 17 months, a remarkable result for elderly patients with AML that led to FDA approval [256]. The successful clinical translation and significant impact of the first BCL-2 inhibitor for the treatment of hematological malignancies fostered preclinical and clinical development of other selective and dual BCL-2 inhibitors [228,257–266].

Selective BCL-XL inhibitors have also been developed, and their clinical track has focused in solid tumors. A first-in-class BCL-XL inhibitor antibody-drug conjugate (ABBV-155) was recently disclosed and has reached the clinic in a phase I study investigating its safety and preliminary activity in refractory solid tumors as monotherapy or combined with taxanes [267,268]. A BCL-XL-based vaccine is also being evaluated in a phase I trial enrolling patients with prostate cancer [269] (Table 1). Other compounds have demonstrated significant activity in preclinical models [270–277].

The success of BH3 mimetics has occurred in parallel to the advancement of strategies to target protein-protein interactions using stapled peptides, synthetic proteins "locked" in their α-helix secondary structure by a chemical "staple" [278,279]. Stapled peptides carry the promise of targeting critical intracellular protein-protein interactions that have been considered undruggable and display chemical stability and resistance to proteolysis, increased cellular penetration and higher affinity to their target compared to small-molecules or biologics [278,279]. The proof of concept and potential therapeutic application of hydrocarbon-stapled peptides was first demonstrated with peptides that mimic the α-helical BH3 segment of proapoptotic BID called SAHB$_A$ (stabilized alpha-helix of BCL-2 domains) [280]. SAHB$_A$ penetrated leukemia cells, bound to BCL-XL, induced apoptosis and demonstrated anti-tumor activity in xenograft mice models of leukemia [280]. Stapled-peptides targeting the p53-MDM2 interaction surface have been developed as an attempt to reactivate p53 and related compounds have transitioned to clinical trials and others are targeting MCL1, anti-apoptotic BFL-1 among other biological switches such as β-catenin [93,281–286].

Small molecules targeting a regulatory site at serine 184 of BAX which regulates its subcellular localization and ability to insert into the mitochondrial membrane have been shown to induce apoptosis in lung cancer models [287]. The small molecule BAX agonists (SMBA1–3) selectively bind to BAX, inhibit S184 phosphorylation, promote oligomerization that leads to cytochrome c release, and trigger apoptosis. Significant activity of lead compound SMBA1 in models of lung cancer which is characterized by increased BAX expression fostered the development of optimized compounds with activity also in breast cancer cell lines such as CYD-4–61 and GL0385 [288,289]. Other BAX-activating compounds such as BAM-7 and BTSA1 have shown promising preclinical activity in glioblastoma and AML cell lines [290–292]. Identification of small-molecule inhibitors of BAX

called BAIs (BAX Activation Inhibitors) revealed a new binding site on inactive BAX and a mechanism for allosteric inhibition with significant therapeutic potential [293].

The proapoptotic protein BIM that preferentially activates BAX exemplifies the regulatory link between the intrinsic pathway and other kinases such as ERK1/2, PKB, and JNK-c-Jun pathways [198,294–297]. The ERK1/2 and MAPK1 pathways promote cell survival in part by phosphorylating BIM that leads to its proteasomal degradation [198], an important mechanism for survival in kinase-driven tumors such as non-small cell lung cancer (NCSLC) and chronic myeloid leukemia (CML) [177,298]. A germline deletion polymorphism in *BCL2L11* encoding BIM without the BH3 domain results in resistance to EGFR TKIs *in vitro* and identifies patients with NSCLC that have inferior clinical outcomes when treated with gefitinib or crizotinib [299–301]. These results coupled with the upregulation of BIM mediating chemotherapy-induced apoptosis and synergy between kinase inhibitors (i.e., cerdulatinib-SYK/JAK inhibitor) and venetoclax is fostering an interest in BIM as a biomarker and promoting strategies to modulate its expression for therapeutic applications [302,303].

MCL1 inhibitors

Significant efforts to develop inhibitors of anti-apoptotic MCL1 have reached an important milestone with novel compounds arriving in the clinic in first-in-human trials (Table 1). The therapeutic potential of these inhibitors is highlighted by the involvement of MCL1 in the pathogenesis of several malignancies, frequent amplification, and its association with resistance to chemotherapy or inhibitors of BCL-2/BCL-XL [304,305]. Small molecule AM-8621, a novel class of spiromacrocyclic MCL1 inhibitor, binds to an MCL1 groove displacing BIM and inducing apoptosis of several cell lines with multiple myeloma, AML and lymphoma cells displaying the highest levels of sensitivity. AMG 176, a derivative of AM-8621 with a more favorable pharmacokinetic profile, showed synergy with chemotherapy and venetoclax paving the way to ongoing phase I trials of AMG 176 as monotherapy or combined with venetoclax in patients with refractory AML, lymphoma or multiple myeloma [85,306,307]. AZD5991, another macrocyclic specific MCL1 inhibitor, is being investigated in patients with advanced hematologic malignancies [308,309]. The MCL1 inhibitors VU661013 and S63845 showed synergy with venetoclax in preclinical models of hematological malignancies including venetoclax-resistant xenograft models of AML supporting the potential of combinatorial strategies to circumvent venetoclax resistance that might be relevant to other anti-BCL-2 compounds [310–316]. The clinical investigation of these combinations will also inform the value of sequential treatments in settings where the temporal upregulation of these proteins determines response to treatment. BH3 profiling of cells and expression levels of BCL-2 could reveal anti-apoptotic addiction to BCL-2, BCL-XL, or MCL1 and help predict response to this growing number of apoptosis-inducing drugs [63,248,317–323].

B-cell lymphoma 2-related protein A1 (BCL2A1) also called BCL-2 related gene expressed in fetal liver (BFL-1) represents another anti-apoptotic BCL-2 protein overexpressed in hematological malignancies and solid tumors with specific inhibitors in development including stapled peptides [284,324–326]. These molecules not only have significant therapeutic potential for BCL2A1/BFL-1 dependent malignancies (i.e., B cell malignancies) but recent

results show that intrinsic resistance to inhibition of BCL-2, BCL-XL and MCL1 is mediated by BFL-1 and Bcl-w highlighting the importance of developing specific inhibitors of these relatively understudied members [318].

IAP inhibitors to target apoptosis

The inhibitor of apoptosis (IAP) proteins are overexpressed in several malignancies with a negative impact on prognosis through their promotion of cell survival by the prevention of caspase activation [327]. Inhibitors of IAPs have long been sought as tools and potential cancer therapeutics that could induce cell death. Among the eight proteins in humans, the most relevant with anti-apoptotic activity are X-chromosome-linked IAP (XIAP), cellular IAP1 and IAP2 (c-IAP1 and c-IAP2), and melanoma IAP (ML-IAP). Second mitochondria-derived activator of caspase (SMAC) as well as Omi/HtrA2 are released from the mitochondria and can independently inhibit XIAP and prevent its negative regulation of the apoptosome [328]. Cytochrome c released from the mitochondria together with cytoplasmic apoptotic protease-activating factor 1 (APAF1) and procaspase 9 form the apoptosome to drive caspase 9 activation [329]. SMAC plays a central role binding to XIAP, c-IAP1, c-IAP2 and especially preventing XIAP from directly inhibiting caspases 3, 7 and 9. c-IAP1 and c-IAP2 are not potent inhibitors of caspases, but they can promote apoptosis by binding to SMAC and preventing its association with XIAP and subsequent inhibition of caspases [330,331]. IAP proteins regulate cell survival at multiple levels and significant efforts have been devoted to develop inhibitors of IAP and SMAC mimetics as cancer therapeutics [21]. Several IAP antagonists have transitioned to clinical trials including small molecules and oligonucleotides, but none has been FDA-approved thus far. Safety concerns have been raised with documented cytokine release syndrome and increased adverse events when combining the IAP antagonist LCL161 with chemotherapy [332,333]. Interestingly, LCL161 displayed anti-tumor activity in patients with refractory multiple myeloma by upregulating IFN signaling and promoting an inflammatory response highlighting the potential therapeutic benefits independent of inducing cell death in this disease setting [334]. LCL161 also displayed radiosensitization properties in head and neck squamous carcinoma models [335]. Another compound birinapant (TL32711) which did not show significant activity as a single-agent is being evaluated in combination with anti-PD1 pembrolizumab in solid tumors and is being combined with radiation therapy in patients with head and neck cancer [336–339]. Despite the challenges of these drugs with several trials without meaningful clinical results, the potential to enhance an anti-tumor immune response by combining SMAC mimetics with checkpoint inhibitors or radiation therapy remains high with ongoing clinical trials in advanced solid tumors including lung and colon cancers [340–343].

Mechanisms of resistance to BCL-2 targeting therapies

Several mechanisms of resistance to strategies targeting the BCL-2 family of proteins have been described including modulation of BCL-2 family members expression levels, mutations altering their binding site and cellular localization as well as modifications in mitochondrial function. Point mutations in the BCL-2 causing resistance to venetoclax were identified both in vitro and among patients with CLL progressing on venetoclax treatment [344,345]. Continued exposure of lymphoma cell lines to venetoclax led to the acquisition of BCL-2

mutations in the BH3-binding groove interfering with drug binding [344]. This in vitro result was supported by the identification of a novel mutation (Gly101Val) in the BCL-2 binding groove described using genomic analyses of paired specimens collected from patients with CLL treated with venetoclax and having progression of disease [345]. This mutation was not detected in venetoclax naïve patients or cancer (COSMIC) or population databases (gnomAD). It reduced significantly BCL-2 binding affinity to venetoclax (~180-fold compared to wild type BCL-2) without altering the affinity to BAX and BIM, which allows the mutant protein to retain its pro-survival activities. Highly sensitive digital droplet PCR detected this mutation as early as 25 months prior to overt CLL clinical progression, potentially serving as an earlier biomarker of progression. While Gly101Val was not detected in other B-cell malignancies, another acquired *BCL-2* mutation (Phe104Ile) also interfering with venetoclax binding was described in a patient with follicular lymphoma (FL) with secondary resistance to venetoclax [346]. The crystal structures of venetoclax bound to wild type BCL-2 and mutants (G101V and F104L) were described, and restorage of venetoclax affinity was demonstrated with another *BCL-2* mutation E152 in experimental models [347]. Mutations in *BTG1* and homozygous *CDKN2A/B* (p16^{Ink4a}/p14^{Arf}) deletions were also identified in patients with CLL and resistance to venetoclax [348]. Other mechanisms of resistance have been described among the growing number of hematologic malignancies receiving treatment with inhibitors of BCL-2 family members.

Increased expression of BCL-XL induced by NFkB signaling was associated with venetoclax resistance in samples from patients with mantle cell lymphoma (MCL) and cell lines models [349,350]. Furthermore, patients with MCL with primary resistance to the combination of venetoclax and ibrutinib [351] displayed loss of chromosome 9p and mutations in the SWI-SNF chromatin-remodeling complex that resulted in upregulation of BCL-XL [352]. Resistance of AML cell lines and patient samples to venetoclax was also associated with overexpression of BCL-XL and lower levels of BCL-2 [248]. Genome-wide CRISPR/Cas 9 screen in human AML cell lines showed that inactivation of *TP53*, *BAX* and *PMAIP1* genes leads to venetoclax resistance [353]. The *TP53* knockout cells had upregulation of BCL-XL and decreased expression of BCL-2 and MCL-1 making them sensitive to BCL-2/BCL-XL inhibitor AZD-4320 and less sensitive to MCL-1 inhibitor; *BAX* knockout cells did not have decreased levels of MCL-1 and were more sensitive to MCL-1 inhibitor. Venetoclax-resistant cells had distinctive changes in mitochondria metabolic profile characterized by increased anaerobic glycolysis and relative protection against mitochondria membrane depolarization in response to various stimuli. Another CRISPR/Cas 9 screen in AML cells uncovered the role of OPA1 (optic atrophy 1) protein and structural changes in mitochondria cristae in resistance [354]. Mitochondria from venetoclax-resistant AML cells had tighter and increased number of cristae (inner membrane lamellae) compared to cells sensitive to venetoclax. These morphological changes were associated with increased expression of OPA1, which controls mitochondria fusion and protects the cell from apoptosis by regulating mitochondria cristae shape and cytochrome c release from the cristae to the intermembrane space, a crucial step prior to cytochrome c release into the cytosol [355–357]. Venetoclax-resistance in AML cells was also mediated by CLPB protein (a mitochondrial AAA+ ATPase chaperonin) as demonstrated by its increased expression in resistant cells and resensitization following CLPB depletion using CRISPR sgRNA. This study expanded the

known functions of CLPB beyond a protein chaperone by describing its anti-apoptotic function through direct interaction with proteins HAX1 and OPA1 modulating mitochondrial morphology [358–360]. These results highlight the importance of molecular regulators of intrinsic mitochondrial function and structure as mediators of resistance to BCL-2-based therapeutic strategies.

While mutations in the proapoptotic BAX have well documented in various malignancies [361–363], a unique mutation in the C-terminal transmembrane domain of BAX (G179E) compromised its anchoring to mitochondria outer membrane causing resistance of lymphoma cell lines to venetoclax [344]. Considering the importance of mitochondria size and membrane lipid composition to BAX-induced membrane permeabilization and sensitivity to apoptosis [364,365], it is conceivable that lipid composition might influence resistance to venetoclax. Further delineation of resistance mechanisms to BCL-2 inhibitors will be critical to guide patient selection, inform combinatorial treatment strategies and sequence of therapies.

Strategies targeting the extrinsic pathway

Death receptor agonists

Activation of the extrinsic pathway is triggered by extracellular signals activating the proapoptotic death receptors (DRs), transmembrane proteins members of the TNF receptor superfamily (TNFR) [14,27,39]. The following DRs have been identified: TNFR1, Fas (CD95, APO-1), DR3, DR4 (TRAILR1), DR5 (TRAILR2) and DR6. Upon binding of their respective ligands, Fas, TNFR1, TNFR2, DR4 and DR5 recruit adaptor proteins such as FADD (Fas-associated death domain) to assemble the DISC (death-inducing signaling complex) that activates caspase-8 and −10 and the downstream apoptosis cascade. Cellular FLICE-inhibitory protein (c-FLIP) can also become recruited to the DISC and in some cellular settings competes with caspase-8 and −10 and inhibits initiator caspase activation and apoptosis (Figure 1).

Apo2L/TRAIL (TNF-Related Apoptosis-Inducing Ligand), the extracellular ligand of DR4 and DR5, is a transmembrane trimeric glycoprotein that be cleaved by metalloproteases and released as a soluble ligand [111,132]. TRAIL is expressed on the surface of immune cells, including natural killer cells and cytotoxic T cells and regulates an innate immune response [366,367]. The immune cells can also induce apoptosis in target cells using the granzyme-perforin pathway (Figure 1). TRAIL also binds to decoy receptors (DcR1, DcR2, osteoprotegerin) without intracellular caspase activation. Direct activation of the extrinsic pathway with agonists of DR4 and DR5 has provided a compelling rationale to induce apoptosis of cancer cells with therapeutic intent [368,369]. While severe liver toxicity was observed with Fas ligands (CD95 agonists) in preclinical models and pro-inflammatory effects limited the application of TNF in the clinic, DR4 and DR5 agonists showed selective activity towards cancer cells without damage to normal tissues in preclinical models. Therapeutic approaches targeting DR4 and DR5 were brought to the clinic in the early 2000's: recombinant human (rh)Apo2L/TRAIL (activating DR4 and DR5) and agonistic monoclonal antibodies against DR4 or DR5 (Figure 2) [370–373].

The recombinant APO2L/TRAIL (dulanermin) was extensively investigated in clinical trials as a single-agent and in combination with chemotherapy or rituximab in solid tumors and hematologic malignancies but clinically meaningful activity was not observed [374–377]. These results have been attributed to its short half-life (30min), limited capacity to induce receptor clustering, binding to decoy DRs (i.e., DcR1, DcR2, osteoprotegerin) and lack of sufficient relevant biomarkers to inform sensitivity [369]. Other potential mechanisms of resistance that have been proposed include epigenetic silencing of DR4, post-translational modifications of DR such as O-glycosylation, decreased expression and/or density of receptors on the cellular surface [378–383]. Intracellular levels of BCL-2 family members such as BAX and MCL1 and other regulators of the intrinsic pathway (e.g., XIAP) can also mediate the resistance to TRAIL agonists in type II cells that depend on mitochondria for the induction of apoptosis [384–386]. In colon cancer cell lines, the efficiency of BID cleavage by caspase influenced sensitivity to TRAIL [130]. Targeting XIAP overcame TRAIL-resistance in cell lines deficient in BAX/BAK and those overexpressing BCL-2 [385,387]. Furthermore, the limited activity of soluble TRAIL can also be associated with its reduced ability to induce receptor clustering as suggested by higher responses with membrane-bound or oligomerized forms of TRAIL [388–390]. Agonist monoclonal antibodies against DR4 and DR5 were promising given their longer half-lives, significant preclinical activity and ability to induce receptor clustering, but they also have shown limited clinical efficacy in various clinical settings particularly as single-agents. Mapatumumab, a DR4 agonist antibody, was well tolerated in clinical trials but showed limited clinical benefit when administered with chemotherapy in patients with NSCLC, colorectal cancer, and other solid tumors [391–394]. Patients with untreated advanced NSCLC (stage IIIB or IV; 52% adenocarcinoma) were randomized to receive carboplatin and paclitaxel, or carboplatin, paclitaxel, and mapatumumab (10 mg/kg or 30 mg/kg) combination. Grade 3/4 lymphopenia occurred more frequently in the mapatumumab arms (22 and 19% vs. 3%), but there were no other significant toxicities attributed to mapatumumab and overall dose intensity was comparable among treatment arms. The addition of mapatumumab did not improve response rates, progression-free survival, or overall survival in this population. Lack of benefit was observed in all histological subgroups, and variable TRAIL-R1 receptor staining was not associated with response [391]. Thirty-eight patients with colorectal cancer refractory to 5-fluorouracil (5-FU), oxaliplatin and irinotecan-based regimens were treated with single-agent mapatumumab which was relatively well tolerated without hepatotoxicity. There were no tumor responses and median PFS for this heavily pretreated population was only 1.2 months [393]. Mapatumumab was also combined with gemcitabine in a phase I trial with 49 patients with advanced solid tumors (majority of patients with pancreatic cancer) [393]. The combination was well tolerated with transient elevation of LFTs and 12 patients had confirmed partial responses that were associated with TRAIL-R1 staining. Single-agent mapatumumab showed encouraging activity in patients with refractory non-Hodgkin's lymphoma, including 2 complete responses among 40 patients [395]. It was also administered in combination with tyrosine kinase inhibitor sorafenib to patients with hepatocellular carcinoma without clinical benefit [396]. The DR5 agonist lexatumumab was investigated as single-agent, and combined with chemotherapy in advanced solid tumors with some patients achieving durable stable disease including pediatric osteosarcoma and adult sarcomas [397–399]. Thirty-seven patients with advanced solid tumors (32% soft tissue sarcoma, 19% osteogenic sarcoma, and 11%

colorectal cancer) were treated with the most common adverse effects being constipation, fatigue, and mild nausea [397]. Dose-limiting toxicities included grade 4 transaminitis. There were no confirmed responses, but 32% of patients had stable disease with 3 patients receiving > 10 treatment cycles. A second study evaluating lexatumumab every 2 weeks documented stable disease in 29% of patients and a mixed response in a patient with Hodgkin's disease [400]. Other DR5 agonists investigated in clinical trials included conatumumab (AMG655) [370,401–404], tigatuzumab [405,406], LBY135 [407], drozitumab (PRO95780) [408,409]. Approximately 190 treatment naïve patients with metastatic colorectal cancer were randomized to receive mFOLFOX6/bevacizumab, mFOLFOX6/bevacizumab with conatumumab or placebo. The combination was relatively well tolerated, but there were no significant differences in tumor responses, PFS or OS across the arms. Patients with high-affinity forms of the FCGR3A receptor (immunoglobulin gamma Fc region receptor III-A) had a non-significant improvement of PFS supporting previous results suggesting the importance of high affinity to promote receptor crosslinking [403]. Recent efforts have explored combinations with drugs capable of sensitizing cells to TRAIL-induced apoptosis either by modulating the expression of TRAIL or inhibiting other anti-apoptotic mechanisms. A CDK9 inhibitor reversed the resistance of non-small cell lung cancer cell lines to TRAIL [410]. Inhibition of MCL1 sensitized breast cancer cells to TRAIL through the upregulation of DR4 [411]. The combination of conatumumab and Apo2L/TRAIL enhanced the antitumor activity by promoting receptor crosslinking, oligomerization, and increasing the formation of death-inducing signaling complex (DISC) [412]. This approach suggested that DR5 agonist-induced receptor crosslinking facilitated and enhanced the signaling of soluble Apo2L/TRAIL that could overcome the therapeutic limitations of monotherapy.

The first-in-class small molecule ONC201 was originally discovered as a TRAIL-inducing compound (TIC10) [413]. TRAIL expression in numerous (but not all) cancer cell lines contributes to its potent anti-tumor activity, including against cancer stem cells [414] that has led to several ongoing clinical trials. The ONC201 which has a three-ring imipridone structure has been found to bind to dopamine receptors DRD2 and DRD3 [415] as well as the mitochondrial caseinolytic protease P (ClpP) leading to activation of integrated stress response protein ATF4 [416,417]. ATF4 activation by ONC201 leads to CHOP-dependent DR5 upregulation and cell death [418,419]. The anti-tumor activity of ONC201 was significantly enhanced by TRAIL or DR5 agonist in preclinical models of endometrial carcinoma and breast cancer [420,421]. This appears to be due to priming of tumor cells treated with ONC201 to TRAIL-induced cell death, and represents one promising avenue for combination therapy targeting tumor cell death. ONC201 has immune stimulatory effects that trigger cell death of tumor cells [422,423] and this is currently being further tested in combination with immune checkpoint therapy [424]. ONC201 has clinical activity against H3K27M-mutated midline gliomas such as DIPG with a reported 47% response rate [425,426]. There is data to support the hypothesis that the interplay of dopamine receptor family members, as could be influenced by epigenetic alterations such as H3K27M mutation, may impact sensitivity of tumors to ONC201 [427]. The relationship between ClpP and the other pathways engaged by ONC201 remains under study although the binding appears to be upstream of ATF4 activation (Lanlan Zhou and W.S.E-D., unpublished observations). There is a literature that ER stress blockade [428] may induce apoptosis as a cancer therapeutic strategy including blockade of ATF4 in c-

Myc-driven tumors [429]. It remains unclear how or why some tumors respond and undergo apoptosis following ATF4 activation while others to ATF4 blockade. The HRI- and PKR-dependent activation of CHOP and DR5 by drugs such as ONC201 may be involved when ATF4 is therapeutically activated [418] while uncoupling of translation from bioenergetic demands may be involved in settings where PERK- and GCN2-dependent ATF4 activation is blocked [429].

A novel platform utilizing a mixture of two antibodies targeting distinct epitopes on DR5 using technology that promotes the formation of hexamers due to E430G mutations in the Fc domains of both antibodies and enhance antibody-dependent cellular cytotoxicity (ADCC) have also transitioned to phase I clinical trial in solid tumors [430–432] (GEN1029). Another TRAIL agonist fused with Fc-domain of a human immunoglobulin G1 (ABBV-621) that assembles to maximize receptor clustering is also undergoing a phase I trial in solid tumors and hematological malignancies with cohorts in colorectal and pancreatic cancers receiving treatment in combination with cytotoxic chemotherapy, and cohorts with AML and DLBCL treated with single-agent or combination with venetoclax [433–436]. A fusion protein of IgG1 with single chain TRAIL has shown significant preclinical activity, particularly in PDX models of sarcoma combined with docetaxel leading to complete responses (MM-201) [437]. Pegylated formulations of recombinant TRAIL with prolonged half-life (e.g., subcutaneously administered TLY012) and stability is undergoing development of treatment of liver fibrosis with significant potential to be explored in cancer treatment [438]. These efforts have reinvigorated the interest and therapeutic potential of TRAIL-based strategies as a feasible anti-cancer therapy strategy. Combinatorial approaches utilizing TRAIL, DR agonists, and other modulators of apoptosis response such as BH3 mimetics have the potential to overcome the limitations of previous strategies.

Novel combinations and approaches

Targeting growth factor and oncogenic survival pathways to induce apoptosis

The therapeutic potential of targeting the interface between kinase signaling pathways and the apoptosis machinery with combinations of kinase inhibitors and inhibitors of the BCL-2 family has been demonstrated in preclinical models and is transitioning to clinical trials. Despite the upregulation of the MEK pathway in several malignancies including those with activating KRAS mutations, MEK inhibitors have limited activity as single-agents in part due to adaptive cell responses that include kinome reprogramming and upregulation of BH3 protein BIM [439–442]. However, MEK inhibition upregulation of proapoptotic BIM primes cancer cells to respond to BCL-2/BCL-XL inhibitors. Xenograft models of lung cancers with KRAS mutations were exquisitely sensitive to the combination of MEK inhibitor and navitoclax (ABT-263; dual BCL-2/BCL-XL inhibitor) [443]. The same combination also showed significant anti-tumor synergy in PDX models of serous ovarian cancer, and this response correlated with the degree of upregulation of BIM by MEK inhibitors [444]. An ongoing phase Ib/II trial is investigating the combination of trametinib (MEK inhibitor) and navitoclax in advanced solid tumors with activating KRAS or NRAS mutations [235]. A detailed analysis of KRAS-driven NSCLC cell lines showed that sensitivity to MEK inhibitors combined with BH3 mimetics (navitoclax, venetoclax and novel MCL1 inhibitors)

is complex and dependent upon cancer cell dependency on MCL1or BCL-XL to neutralize upregulated BIM [202]. Significant tumor regression of PDX models of KRAS-mutant lung adenocarcinoma was observed with trametinib plus AM-4907 (MCL1 inhibitor), but no activity was detected with trametinib plus navitoclax in these tumors where BIM was preferentially bound to MCL1. Nevertheless, pre-treatment with navitoclax shifted the subcellular localization of BIM to binding MCL1 and increased sensitivity to MEK plus MCL1 inhibitors. These results highlight that apoptotic responses of cancer cell lines have specific dependencies on BCL-2 proteins that could be challenging to prospectively identify in the clinic especially given the limitations of serial biopsies in patients with solid tumors. However, sequential treatment with BH3 mimetics offers the possibility to optimize the induction of apoptosis by targeted therapy combinations, an important strategy to be tested in the clinic as novel inhibitors enter clinical trials.

Ras and NFkB targeting to induce apoptosis—Intense effort has continued over three decades to target the Ras family of proteins, major oncogenic drivers, growth and pro-survival promoting proteins in multiple tumor types. Blockade of this signaling can lead to cell death, including apoptosis. Targeting farnesyl transferase activity was not an effective strategy in the clinic. In addition, targeting downstream effects such as through MEK inhibitor monotherapy has not led to sustained patient benefit in the clinic. An exciting development involves a specific KRAS mutant (G12C)-targeting drug that is now in clinical trials with recently reported promising early results in NSCLC and CRC [445].

NFkB activation leads to cell survival and pro-inflammatory signals that promote tumor progression and resistance to cancer therapeutics. There has been interest for many years in the therapeutic targeting of NFkB [446]. Proteasome inhibitors have been developed for some hematological malignancies, and they may, in part, act through inhibition of NFkB [447]. Much effort remains to target NFkB as a cancer therapeutic strategy that can lead to apoptotic cell death [448,449]. Moreover, there is also much interest and effort to target downstream mediators of NFkB, such as IL6, as a cancer therapeutic strategy through cytotoxic and proapoptotic immune responses against cancer cells [450]. IL-6 is regulated by another pro-survival pathway, STAT3, which is a cancer therapy target [451].

EGFR, Her2, c-MET, NTRK, BRAF, FLT3, BCR selective targeting induces apoptosis—Despite their dramatic clinical impact in EGFR-driven lung adenocarcinoma, resistance to EGFR tyrosine kinase inhibitors mediated by secondary EGFR mutations or MET gene amplification, among other mechanisms, have limited their benefit [452]. Third-generation EGFR TKIs osimertinib can overcome EGFR T790M but succumb to modulation of BIM and MCL1 levels demonstrating the critical importance of apoptosis machinery to the efficacy of these drugs [453]. Inhibitors of the c-Met/hepatocyte growth factor receptor (HGFR) such as crizotinib exert their anti-tumor activity by inducing apoptosis [191,192,454].

Tumors driven by NTRK-containing fusion proteins display constitutive activation of downstream pathways such as MAPK, PI3K, and STAT3 that can be effectively targeted with TRK inhibitors such as larotrectinib and entrectinib [194]. Trk inhibition triggers apoptosis in several cancer models [455–457].

Targeting tumor suppressor pathways to induce apoptosis

A number of promising approaches targeting tumor suppressor pathways have been under development as anti-tumor agents. Blocking the unregulated oncogenic downstream effectors (PI3K, Akt, beta-catenin, Myc, mTOR, CDKs, VEGF) of mutated tumor suppressors has been an area with significant progress [458,459].

Multiple efforts have been directed at the p53 pathway, a major target for inactivation in human cancer [460–463]. Among the most advanced, MDM2 (mouse double minute 2 protein) or MDMX inhibitors have been in clinical trials for several years [464,465]. These therapeutics block the major pathway of wild-type p53 degradation without inducing DNA damage. Early results are promising, but there are on-target hematological toxicities [466,467]. Numerous compounds targeting MDM2 have been developed since the nutlins, the first-in-class MDM2 inhibitors, including the nutlin-3a derivative RG7388 (idasanutlin) currently under investigation in trials for advanced hematologic malignancies and solid tumors as a single-agent or combined with chemotherapy, venetoclax or checkpoint inhibitors (atezolizumab) [468–473]. Other MDM2 antagonists in clinical trials include AMG-232, HDM201 [474], APG-115 (ongoing studies as single-agent and combination with pembrolizumab) [475], DS-3032b [476], BI 907828 [477] and the stapled peptide ALRN-6924 [478–486]. Significant efforts have also been directed to combine MDM2 antagonists with other targeted agents (i.e., MEK 1/2, BCL-2/BCL-XL, PI3K, BRAF inhibitors among others) or developing bifunctional molecules such as PROTAC (Proteolysis Targeting Chimeric) compounds that unify a E3 ubiquitination ligase with a small molecule targeting MDM2, a compelling strategy especially when considering the reported emergence of p53 mutations during treatment with these agents and in vitro models describing the mechanisms of resistance [487–491].

Approaches targeting restoration of the p53 tumor suppressor pathway in tumors with mutant p53 are best exemplified by APR-246, which has been in multiple clinical trials [492]. Boosting wild-type p53 or restoring the p53 signaling pathway in tumors with mutated p53 allows the endogenous growth arrest and apoptosis signaling in tumor cells to control tumor growth [493,494]. Preliminary results of combination of APR-246 and azacytidine in patients with p53 mutant myelodysplastic syndromes (MDS) and AML showed a favorable toxicity profile and responses in all evaluable patients including complete responses. Pathway analysis after APR-246 treatment also showed transcriptional activation of p53 targets [495].

CDK4/6 inhibitors such as palbociclib, currently approved for treatment of breast cancer, induce cancer cell death by sensitizing cells to TRAIL-induced apoptosis and can overcome hypoxia-induced resistance in colon cancer cell lines and promotes apoptosis in other cell types by other mechanisms including inhibition of DNA repair following radiation therapy [496–498]. Palbociclib also induces cell cycle arrest and senescence in models of glioblastoma and multiple myeloma [499,500]. Palbociclib in combination with MEK inhibitor trametinib overcame resistance of KRAS-mutant lung adenocarcinoma cell lines to MEK inhibitor by inducing apoptosis [501]. A preclinical approach involves micro-RNA targeting of CDK4/6 as an alternative strategy for therapeutic targeting of these kinases to induce tumor cell apoptosis [502].

Epigenetic approaches to targeting apoptosis

Epigenetic modulators such as histone deacetylases inhibitors (HDACi) and BET inhibitors (BETi) exert their anticancer activity through apoptosis with potential synergy with BCL-2 targeting agents. Combination BET inhibitor ABBV-075 with venetoclax has shown promising preclinical efficacy in models of AML, including in cell lines resistant to venetoclax and PDX models [503]. This synergy seems to be mediated by decreased protein levels of MCL1, BCL-XL, BCL-2 in the AML cell lines resulting from BETi modulation of chromatin and gene expression. Similar results were also observed in patient-derived CD34+ blast progenitor cells. Synergy between BETi and venetoclax was also observed in patient-derived cutaneous T cell lymphoma (CTCL) cells [504].

HDAC inhibitors alter chromatin remodeling and induce apoptosis by several mechanisms including upregulation of death receptors and ligands TRAIL or Fas, increased expression of BH3-only proteins (i.e., Bid, BIM, Bmf and BAK), decreased expression of BCL-2, BCL-XL, BCL-W, MCL1, and elevation of reactive oxygen species that contribute to intrinsic pathway activation and provide rationale for combinations with BCL-2 family targeting agents [505–508]. HDAC inhibitors prime rhabdomyosarcoma cells to venetoclax-induced apoptosis by increasing the expression of BIM [509]. Similarly, panobinostat induces NOXA and decreases MCL1 in diffuse large B cell lymphoma (DLBCL) cell lines enhancing the sensitivity to BCL-2 inhibitor [510]. HDAC inhibitors also potentiate the activity of MEK inhibitors and venetoclax in models of multiple myeloma [511]. Clinical studies are investigating the combination of venetoclax and fimepinostat in refractory lymphoma [512,513]. Hypomethylating agent azacytidine is also showing potent synergy with venetoclax and ABT-737. A clinical trial combining venetoclax with decitabine or azacytidine in patients with AML showed meaningful clinical efficacy with 67% of patients achieving complete remission including patients with high risk AML as described previously [231,248,255,256].

Targeting chaperones to induce apoptosis

Various strategies targeting cell stress including proteotoxic stress and ultimately leading to apoptosis continue to be explored in cancer therapy. Chaperones such as members of the heat shock protein family including HSP90 stabilize newly formed proteins which is very important in rapidly growing and dividing tumor cells that generate significant protein synthesis. HSP90 inhibitors including geldanamycin, ganatespib, onalespib, XL888, TAS116 and others have been under development [514–519]. Endoplasmic reticulum (ER) stress caused by several stimuli lead to apoptosis mediated by activation of PERK (Protein Kinase R (PKR)-like Endoplasmic Reticulum Kinase or pancreatic EIF-2-alpha kinase), FADD, caspase 8 as well as upregulation of ER chaperone GRP78 in the cell surface which serves as a receptor of proapoptotic protein Par-4 (prostate apoptosis response-4) [520–523]. Several lines of evidence highlight the crosstalk between ER stress response, ferroptosis, autophagy, and immune regulation of tumor microenvironment that collectively represent an active area of investigation [428,524,525]. Further understanding of ER stress and the role of GRP78 in cancer pathogenesis coupled with the development of molecules inhibiting GRP78 are helping validate this chaperone as a relevant therapeutic target in various malignancies including colorectal and pancreatic cancers [526–528].

Intersection between programmed cell death and anti-tumor immune response

In contrast to apoptosis which is considered an immunogenically silent process, other forms of programmed cell death activate pro-inflammatory signaling and promote anti-tumor immune responses including pyroptosis, necroptosis, and caspase-independent cell death (CICD) [529]. CICD triggered by mitochondrial outer membrane permeabilization (MOMP) [530] in the setting of caspase inhibition leads to the production of pro-inflammatory cytokines, immune cell tumor infiltration, and potent anti-tumor responses in vivo models [531]. In cells with blocked mitochondria-dependent caspase activity (APAF-1 knockdown or caspase-9 CRISPR/Cas9 deletion), MOMP induced NF-kB activation and TNF production through degradation of IAP and upregulation of NF-kB inducing kinase (NIK). MOMP coupled with caspase inhibition allows translocation of mitochondrial DNA (mtDNA) through BAK/BAX macropores to the cytosol where it activates cGAS/STING, promotes type I interferon (IFN) production, and triggers an innate immune response [532–534]. This mtDNA-driven immune response during cell death supports the role of caspases in suppressing the immune response during apoptosis. The mechanisms of these immunogenic forms of cell death could be explored therapeutically at intersection points to overcome apoptosis resistance and in conjunction with strategies in the clinic promoting an anti-tumor immune response such as with checkpoint inhibitors. In fact, the recent description that gasdermin E, an established executioner of pyroptosis cleaved by inflammatory caspases, can form pores in the mitochondria, activate caspases 3/7 and promote apoptosis, exemplifies the bridges between these pathways [535]. Caspase 8 cleavage of gasdermin D and induction of pro-inflammatory lytic cell death provides another example of cross-talk between apoptosis and pyroptosis [536–538]. One could envision interventions able to promote a switch between forms of cell death (i.e., apoptosis to CIDC) to enhance anti-tumor immune responses and/or overcome cell death resistance mechanisms.

Apoptotic priming as a road map to inform therapeutic index and predictive biomarkers

The sensitivity of healthy tissues to apoptosis varies according to histology and developmental stage, and is driven by the relationship between mitochondria and balance among BCl-2 family members. C-Myc-driven transcription of BCL-2 family members in healthy tissues during development plays a central role in the regulation of apoptosis priming [539]. Tissues with high proliferation rates such as the developing brain display high levels of proapoptotic proteins such as BAX, BIM and BID making it highly sensitive to proapoptotic stimuli that might contribute to its vulnerability to toxins and drugs. This pattern changes over time with the downregulation of proapoptotic proteins in the post-natal period [540,541]. In fact, the expression of BCL-2 family members is markedly reduced in adult mouse brain tissue [539]. A similar state of apoptotic priming can be observed in the hematopoietic system, and other tissues such as the gastrointestinal tract have been described as *unprimed* given the cellular heterogeneity in terms of apoptosis sensitivity [47]. These differences in tissue-specific patterns of apoptosis priming have been proposed as contributors of the enhanced susceptibility of pediatric cancer patients to chemotherapy and

radiation toxicities as compared to adults [539]. Functional assessment of apoptosis priming using BH3 profiling can also help predict the sensitivity of cancer cells (i.e. multiple myeloma, AML, CLL, ALL, and ovarian cancer) to chemotherapy drugs as well as to BH3 mimetics [542]. Relevant differences in BH3 profiling between AML myeloblasts with greater BCL-2 dependency as compared to hematopoietic stem cells provided the therapeutic index of venetoclax that was successfully explored in the clinical trials [249,251,543]. Distinct dependencies to BCL-2 proteins between cancer cells and normal counterparts have also been demonstrated in T cell development with early progenitor T cells (dependent on BCL-XL) and more mature pre-thymic T cell (dependent on BCL-2) [544]. A change in overall dependency of T cell ALL on BCL-XL to BCL-2 in the early T cell progenitor ALL subtype identified the susceptibility of this aggressive subtype of ALL to venetoclax [542]. While the BH3-profile and dependencies to specific BCL-2 family members has been well described in hematological malignancies and propelled the development of BCL-2 inhibitors, emerging results suggest strategies to optimize the efficacy of BH3 mimetics in solid tumors. A large screen of solid tumor cell lines revealed that approximately 50% of the cell lines tested displayed co-dependency on BCL-XL and MCL-1, and a marked synergistic efficacy of BCL-XL + MCL-1 inhibitors was observed [318]. Relatively rare dependencies to BCL-XL in tumor types such as bladder cancer and pancreatic adenocarcinoma were associated with significant sensitivity to BCL-XL inhibition. Investigation of pathways associated with sensitivity patterns showed that epithelial signatures correlated with co-dependency to BCL-XL and MCL-1, and an EMT signature was associated with BCL-XL dependency in alignment with other reports [443,545]. Some tumor types were resistant to all combinations of BCL-2, BCL-XL and MCL-1 inhibitors, but they were sensitive to BFL-1 and BCL-w inhibition. These results highlight potential therapeutic vulnerabilities in solid tumors that can inform the development of tissue-specific combinations of BH3 mimetics, and they demonstrate the value of expression profiling to determine BCL-2 gene dependencies. As novel BH3 mimetics enter the clinic, BH3 profiling assays and/or expression analyses will be critical to define not only cancer cell dependency and predict response, but also to guide the sensitivity of normal tissues to these agents [546]. Further development of biomarkers refining the efficacy and safety of novel therapies promoting apoptosis need to incorporate the analysis of apoptosis priming state of healthy tissues for assessment of potential toxicities.

Prospects for advancing the field of apoptosis-inducing agents in cancer therapy

Targeting apoptotic pathways in tumor cells is a potent anti-cancer strategy as dead tumor cells contribute to clinical responses and have no chance to contribute to tumor relapse. While some drugs that directly target the intrinsic apoptosis pathway are approved such as venetoclax, most approved therapeutics that cause cell death target the pathways indirectly. Examples include inhibitors of growth factor signaling pathways, kinase inhibitors, mTOR inhibitors, proteasome inhibitors, or HDAC inhibitors. More direct targeting of the apoptotic pathways is under development with some promising agents having tumor selectivity.

The development of effective therapies targeting cell death pathways is exceedingly complex given the number of therapeutic targets, resistance mechanisms, different standard treatments for different tumor types, and toxicities of single agents or combinations. Some

on-target or off-target toxicities are only discovered in clinical trials, and may necessitate development of secondary agents to avoid cardiotoxicity, or thrombocytopenia, by example. Additionally, it is important to bring agents into specific tumor types keeping in mind how those tumors are treated and which patients would be eligible and at what stage. It is typical for novel agents to be tested as salvage therapy for patients with advanced disease who are heavily pre-treated and then advanced in combinations to more front-line settings. Some agents that are promising in phase I trials are brought into phase II trials as combination therapies with an abbreviated phase I portion. A bias in the field has been that if certain agents are inactive in a particular tumor type, clinicians are somewhat reluctant to bring in promising combinations involving such agents that show preclinical synergy into therapeutic trials for that tumor type. Another bias is that some are reluctant to partner standard chemotherapy in novel combinations if the standard agent was used in a prior line of therapy. However, a rational approach would involve overcoming such biases and being more open to try, even on a small scale, promising therapeutic combinations as guided by available preclinical and clinical data.

It is essential to not miss the opportunity to learn from every patient in every clinical trial, and that includes biopsies of tissue, and liquid biopsies before treatment, during response and at progression. The history of clinical trials with apoptosis-inducing agents has missed opportunities to understand why certain agents appeared to fail even though the pathways (e.g., extrinsic cell death) are powerful tumor suppressor mechanisms. It is very important to incorporate candidate biomarkers, including BH3 profiling, as well as some open-ended molecular analysis (genomics, RNA-seq, proteomics, etc) of potential sensitivity and resistance mechanisms in order to continue to make progress. Analysis of tumor heterogeneity and evolution including at the single cell level has evolved to illuminate drug effects as well as the impact of the tumor microenvironment. Novel ways of targeting apoptosis and cell survival mechanisms (e.g. autophagy) in the hypoxic, low pH, nutrient-deprived tumor microenvironment are needed. These strategies must also unravel and target immune escape mechanisms within the tumor microenvironment. Additionally, a rational approach to novel therapy combination would go back to foundational concepts in oncology including using agents with largely non-cross-reacting mechanisms and non-overlapping toxicities. And to best help patients, clinicians need to be armed with back-up therapeutic strategies that exploit the common resistance mechanisms to cellular apoptosis, as well as patient tumor-specific changes that evolve with therapy. Monitoring tumor evolution and its heterogeneity using the latest available technologies such as liquid biopsy, single cell approaches, and imaging is expected to guide and improve cancer therapy targeting apoptotic pathways.

The future development of drugs that directly target apoptotic pathways could lead to tumor regression in difficult to treat tumors, and these drugs would be expected to be used in combination with agents targeting oncogenic survival pathways, immunotherapy, and classical chemotherapy and radiotherapy. Targeting apoptotic pathways remains a promising strategy for cancer therapy that will continue to evolve in future clinical practice.

Acknowledgements

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Key Points

- There are numerous ways of inducing apoptosis of cancer cells, some more direct than others, exploiting the various entry points into the final common pathway involving caspase-dependent proteolysis of 1000's of cellular proteins, membrane blebbing and DNAase-dependent endonucleolytic cleavage of chromosomal DNA.

- Few FDA-approved anti-cancer drugs act directly on the apoptotic pathway, near the point of execution, and these include small molecules that block anti-apoptotic BCL-2 family members to unleash the effect of proapoptotic BCL-2 family members on mitochondrial membrane permeability and cytochrome c release.

- There remain many promising strategies for engaging cell death activation in cancer therapy including strategies that trigger the extrinsic cell death pathway, target tumor suppressor pathways, target the tumor microenvironment including stromal fibroblasts and combinatorial drug therapies.

- Various FDA-approved drugs targeting cell survival and cell proliferation pathways in cells, including kinase inhibitors, growth factor targeting antibodies and small molecules such as MAPK pathway inhibitors such as MEK or BRAF inhibitors, mTOR inhibitors, proteasome and HDAC inhibitors, and anti-angiogenic agents, among others, impact less directly on cell death signaling pathways to achieve anti-tumor efficacy.

- Immunotherapy whether cell-mediated or antibody-mediated induces apoptosis of cancer cells through granzyme or extrinsic cell death and is currently being combined with numerous other targeted therapies as well as classical chemotherapy or radiation.
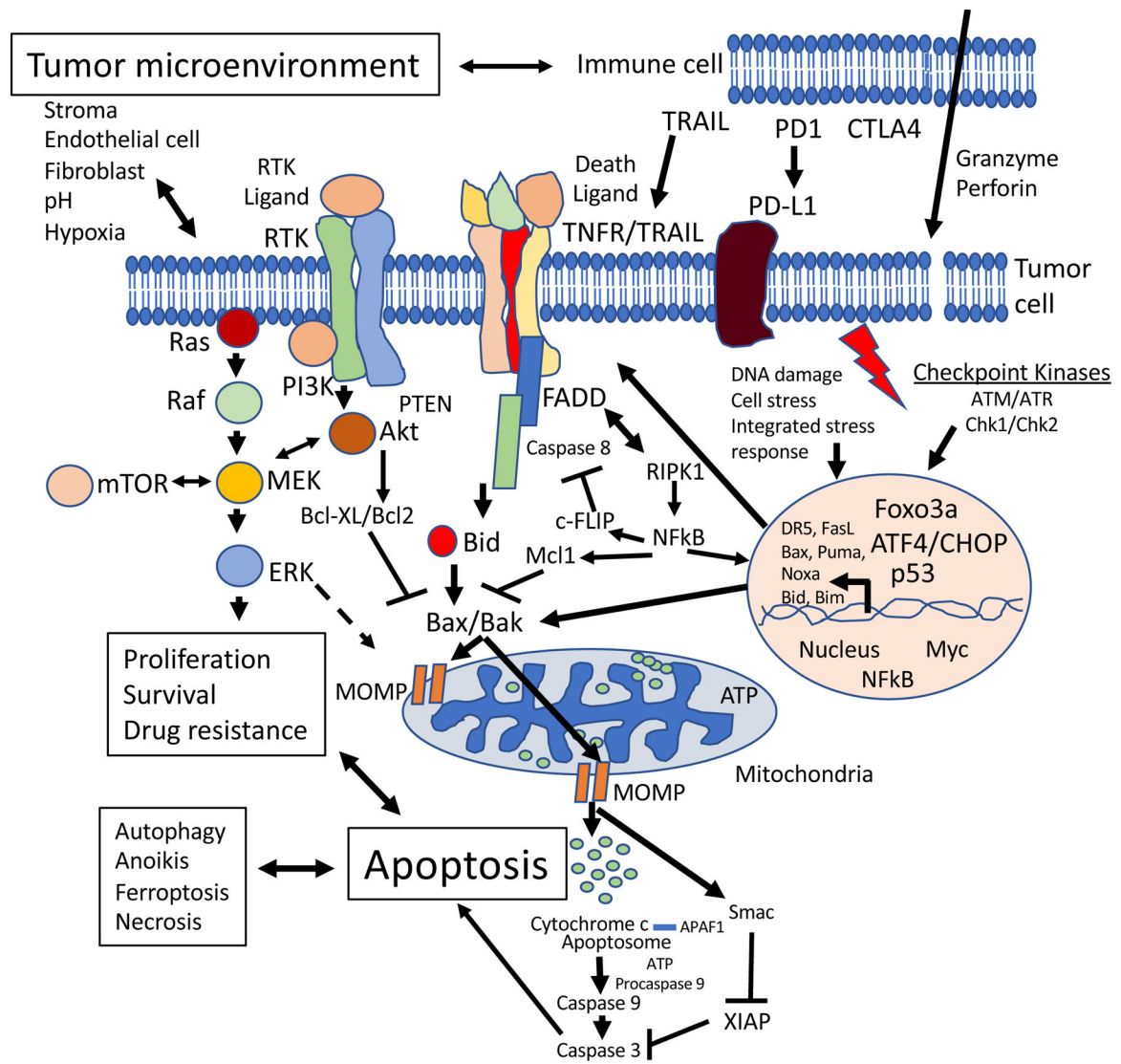
Figure 1. Overview of apoptosis signaling pathways and pro-survival, immune, or tumor microenvironment influences that impact on them.

Intrinsic mitochondrial apoptosis involves proapoptotic BCL-2 family members (e.g. BAX or BAK) located in the mitochondrial outer membrane promoting mitochondrial outer membrane permeability (MOMP), cytochrome c release, caspase activation and apoptosis. Cytochrome c binds APAF1, and in an ATP-dependent process, the multimeric apoptosome is formed. Procaspase 9 binds to the apoptosome, gets cleaved and activated, and then activates caspase 3 [20]. Members of the IAP family including XIAP negatively regulate caspase activation and they can be inactivated by SMAC. Anti-apoptotic members of the BCL-2 family (e.g. BCL-2, BCL-XL, MCL1, Bcl-w, A1) inhibit the proapoptotic BCL-2 family members (e.g. BAX and BAK). BH3-only proteins such as BIM, BAD, BID, PUMA and NOXA can inhibit MCL1. It is important to note that within the intrinsic apoptosis pathway, mitochondrial permeabilization represents the point of commitment to cell death (irrespective of caspase activity). The extrinsic apoptosis pathway is depicted at the cell membrane as a trimeric TNF family receptor (Fas-R, TRAIL-R1, TRAIL-R2, TNF-R1,

TNF-R2) that is bound by the respective ligand with proteins FADD and caspase 8 recruited to form the death-inducing signaling complex (DISC). Caspase 8 or 10 activation at the DISC results in Bid cleavage which provides cross-talk to the mitochondria in Type II cells which amplifies the death signal at the level of cytochrome c leading to apoptosis. c-FLIP binds to caspase 8 and prevents its activation. Prototypical growth factor activated receptor tyrosine kinases (RTKs) are shown in the cell membrane as dimeric proteins (e.g. members of the ERBB family such as EGFR, Her2, ERBB3) with downstream events involving PI3K and Akt activation. The RAS-MAPK pathway is also depicted. These pro-survival pathways also promote cell proliferation and negatively impact on the intrinsic cell death pathway, in part, through phosphorylation of BCL-2 family members. TNF and TRAIL receptors can activate NFkB which transcriptionally activates anti-apoptotic genes in the nucleus thereby attenuating cell death and promoting cell survival. Immune mechanisms can activate cell death through the extrinsic pathway, e.g. through TRAIL produced by Natural Killer (NK) cells in response to Interferon action, or through the blockade of cell death ligands, e.g. PD-L1. Immune cells such as cytotoxic T-cells or NK cells also produce granzyme which is a serine protease that induces apoptosis in target cells such as cancer cells. A protein called perforin forms pores in target cells to mediate granzyme-induced cell death. Various other components of the tumor microenvironment including stroma, fibroblasts and physical factors can impact cell death and cell survival pathways in ways that impact on drug responses. Drugs targeting endothelial cells can deprive tumor cells of nutrients leading to cell death. DNA damage response (e.g. those involving checkpoint kinases such as ATM/ATR, Chk1/Chk2) and cell stress response pathways (e.g. the integrated tress response leading to ATF4 and CHOP activation) ultimately activate proapoptotic genes that induce cell death, in addition to p53. Among the transcribed genes following p53 activation, Bax, Puma, Noxa, DR5, and FasL are noteworthy, but it should also be noted that many kinase inhibitors ultimately signal transcriptional activation of Bim. Additional cellular processes (e.g. autophagy, anoikis, ferroptosis, regulated necrosis) can cross-talk with apoptosis signaling pathways and are impacted by cancer therapeutics including those used in combinations with proapoptotic agents.
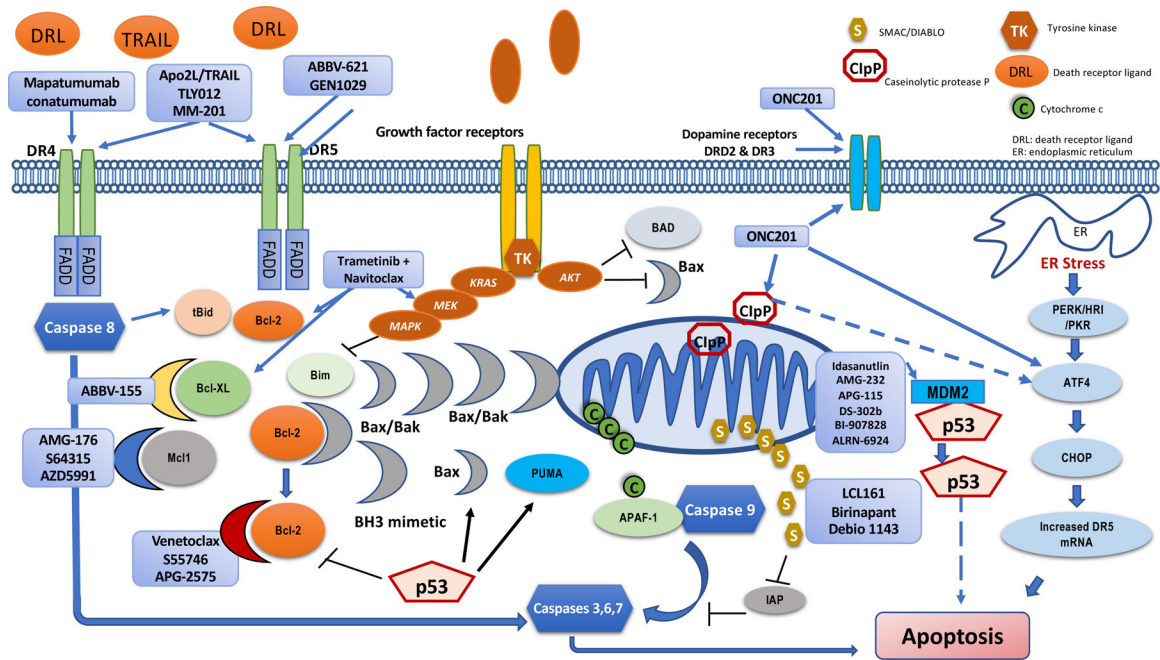
Figure 2. Cancer therapeutic approaches targeting apoptosis pathways.

Death receptors 4 and 5 (DR4, DR5) can be activated by agonist antibodies such as mapatumumab, conatumumab, Apo2L/TRAIL, ABBV-621 and GEN1029, fusion protein of recombinant TRAIL with IgG1 (MM-201) or pegylated recombinant TRAIL (TLY012). BH3 mimetics bind to anti-apoptosis BCL-2 family members (BCL-2, BCL-XL, MCL1) leading to activation of BAX and BAK that form pores in the outer mitochondrial membrane resulting in release of cytochrome C. Selective inhibitors of BCL-2 (venetoclax, S55746, APG-2575), BCL-XL (ABBV-155) and MCL1 (AMG-176, S64315, AZD5991) have reached clinical trials. Modulation of growth signaling pathways also induces apoptosis and combinations of MEK inhibitors with navitoclax (BCL-2/BCL-XL inhibitor) are being investigated in clinical trials. Pro-survival pathways such as AKT and MEK inhibit apoptosis by phosphorylation of proapoptotic proteins such as BAD (BCL-2-associated agonist of cell death) and BIM (BCL-2-interacting mediator of cell death). Strategies targeting the p53 pathway to induce apoptosis include MDM2 inhibitors (i.e., idasanutlin, AMG-232, APG-115, DS-302b, BI-907828, ALRN-6924). The ONC201 which has a three-ring imipridone structure has been found to bind to dopamine receptors DRD2 and DRD3 [415] as well as the mitochondrial caseinolytic protease P (CIpP) leading to activation of integrated stress response protein ATF4 [416,417]. ATF4 activation by ONC201 leads to CHOP-dependent DR5 upregulation and cell death [418,419]. The relationship between ClpP and the other pathways engaged by ONC201 remains under study although the binding appears to be upstream of ATF4 activation (Lanlan Zhou and W.S.E-D., unpublished observations). SMAC (second mitochondria-derived activator of caspase) is released from mitochondria together with cytochrome c. Cytochrome s forms the apoptosome complex with caspase 9 and APAF-1 (apoptotic protease-activating factor 1). Inhibitors of apoptosis proteins (IAP) prevent caspase activation and this is blocked by SMAC or Omi/HtrA2 which is also released from mitochondria. SMAC mimetics and inhibitors of IAP (i.e., LCL161, birinapant, Debio 1143) have transitioned to clinical trials. tBid: truncated BH-3 interacting

domain death agonist; BCL-XL: B cell lymphoma extra-large; MCL1: myeloid cell leukemia 1; BAX: BCL-2 associated X protein; BAK: BCL-2 antagonist/killer; MDM2: mouse double minute 2 protein; DRL: death receptor ligand; ER: endoplasmic reticulum. PERK: PKR-like ER kinase; ATF4: Activating transcription factor 4; CHOP: CCAAT-enhancer-binding protein homologous protein.

Table 1.

Therapeutic approaches targeting the apoptosis intrinsic pathway

Target	Active clinical trials	Phase	Histology focus	Clinicaltrial.gov ID
BH3 mimetics				
Dual BCL-2 and BCL-XL inhibitors				
Navitoclax	Yes	Phase I/II	CLL Advanced solid tumors Melanoma NSCLC	NCT02079740 NCT02520778 NCT01989585 NCT02143401
APG-1252	Yes	Phase I/II	SCLC Advanced solid tumors	NCT03387332
AZD4320	No		ALL NHL	
S44563	No		Melanoma SCLC	
BCL2–32	No		ALL NHL	
BM-1197	No		Colorectal cancer	
Selective BCL-2 inhibitors				
Venetoclax [#]	Yes	Phase I-III	CLL NHL AML	
S55746 (BCL201)	Yes	Phase I	CLL NHL Multiple myeloma	NCT02920697 NCT02603445
APG-2575	Yes	Phase I	CLL NHL	NCT03913949 NCT03537482
BCL-XL inhibitors				
ABBV-155 [*]	Yes	Phase I	Solid tumors	NCT03595059
WEHI-539	No		Breast cancer Ovarian cancer Chondrosarcoma Osteosarcoma	
A-1155463	No		AML	
A-1331852	No		Soft-tissue sarcoma	
MCL1 inhibitors				
AMG 176 ([**])	Yes	Phase I	AML NHL Multiple myeloma	NCT03797261 NCT02675452
MIK665 (S64315) ([**])	Yes	Phase I	AML NHL Multiple myeloma	NCT02992483 NCT02979366 NCT03672695
AZD5991	Yes	Phase I	NHL AML ALL Multiple myeloma	NCT03218683
S63845	No		NHL AML	
UMI-77	No		Pancreatic cancer	
A-1210477	No		Esophageal carcinoma Head and neck carcinoma	

Target	Active clinical trials	Phase	Histology focus	Clinicaltrial.gov ID
			AML NHL Triple negative breast cancer	
VU661013	No		AML	
Inhibitor of apoptosis (IAP) proteins and SMAC mimetics				
LCL161	Yes	Phase I/II	Advanced solid tumors Breast cancer Colorectal NSCLC SCLC Multiple myeloma Polycythemia Vera Myelofibrosis	NCT02649673 NCT03111992 NCT02098161
Birinapant (TL32711)	Yes	Phase I/II	Advanced solid tumors NHL	NCT02587962 NCT03803774

(#)Venetoclax has been approved by the FDA for treatment of acute myeloid leukemia in combination with azacytidine, or decitabine, or low-dose cytarabine for elderly adults (> 75 years old) or adults with comorbidities that prevent the use of standard chemotherapy. Venetoclax was also approved for treatment of chronic lymphocytic leukemia (CCL) or small lymphocytic lymphoma (SLL).

(*)antibody drug conjugate (ADC) based strategy

(**)Clinical trials evaluating monotherapy and combinations with venetoclax

(***)Administered as monotherapy or combined with radiation therapy or checkpoint inhibitors)

NSCLC: non-small cell lung carcinoma; SCLC: Small cell lung carcinoma; NHL: Non Hodgkin lymphoma; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; CLL: chronic lymphocytic leukemia

Table 2.

Therapeutic approaches targeting the apoptosis extrinsic pathway and other cell death mechanisms

Target	Active clinical trials	Phase	Histology focus	Clinicaltrials.gov ID
Death receptor agonists (DR4/5)				
GEN1029	Yes	Phase I	Colorectal cancer NSCLC Triple negative breast cancer Renal carcinoma Gastric cancer Pancreatic cancer Urothelial carcinoma	NCT03576131
ABBV-621	Yes	Phase I	AML NHL Pancreas cancer	NCT03082209
MM-201	No		Sarcoma	
TLY012	No		Fibrosis	
Strategies targeting p53				
MDM2 inhibitors				
Idasanutlin (RG73882) *	Yes	Phase I	AML Multiple myeloma Breast cancer NHL	NCT03850535 NCT02670044 NCT02545283 NCT02633059 NCT03566485 NCT03135262
AMG-232	Yes	Phase I/II	AML Sarcoma Multiple myeloma	NCT03041688 NCT03217266 NCT03031730
HDM201 **	Yes	Phase I	AML	NCT03940352
APG-115	Yes	Phase I	Advanced solid tumors Melanoma NHL Salivary gland	NCT02935907 NCT03611868 NCT03781986
DS-3032b	Yes	Phase I	AML NHL Advanced solid tumors	NCT03634228 NCT02319369 NCT01877382
BI 907828	Yes	Phase I	Advanced solid tumors	NCT03449381
ALRN-6924 (stapled peptide)	Yes	Phase I	Advanced solid tumors	NCT03725436
Restoring wild type activity of mutant p53				
APR246	Yes	Phase I	AML Ovarian cancer Esophageal carcinoma Melanoma	NCT02999893 NCT02098343 NCT03588078 NCT03391050 NCT03391050
Other cell death mechanisms				
ONC201 (binds to dopamine receptors DRD2/DRD3 as well as the mitochondrial caseinolytic protease P (CIpP) leading to activation of integrated stress response protein ATF4 and cell death)	Yes	Phase I/II	Solid tumors Breast cancer Endometrial cancer Colorectal cancer H3K27M Gliomas Multiple myeloma NHL AML	NCT03099499 NCT02863991 NCT03416530 NCT02420795 NCT03394027 NCT03295396 NCT03791398 NCT02392572
Epigenetic modulators favoring activation of intrinsic pathway of apoptosis				

Target	Active clinical trials	Phase	Histology focus	Clinicaltrials.gov ID
Fimepinostat +venetoclax	Yes	Phase I/II	NHL	NCT01742988
Azacytidine or decitabine + venetoclax	Yes	Phase I-III	AML	NCT03404193 NCT03941964

(*)Administered as monotherapy or combined with checkpoint inhibitors)

(**)Administered in combination with venetoclax