

Review Article

Harnessing the cell death pathway for targeted cancer treatment

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Abstract: Genotoxic agents have long targeted apoptotic cell death as a primary means of treating cancer. However, the presence of cellular defects in many cancers has contributed to an acquired resistance to apoptotic cell death, lowering the effectiveness of chemo- and radiotherapies. The mechanisms by which cells achieve this resistance to treatment are still being investigated, but an alternative approach is the study of cell death pathways that are mechanistically distinct from apoptosis. These pathways, including autophagy and necrosis, have arisen as attractive targets for cancer therapy. This review will discuss apoptosis, autophagy, and necrosis in the context of tumorigenesis and drug resistance, as well as provide an up-to-date preclinical and clinical review of inhibitors targeting these cell death pathways for multiple cancer types. The goal of these studies is to identify molecular targets that will enhance the efficacy and specificity of current cancer therapies.

Keywords: Cancer, apoptosis, autophagy, necrosis, cancer stem cells

Introduction

The balance between survival and death is of such importance that the cell spends considerable energy maintaining it. Nowhere else is the maintenance of this balance more fraught with risk than in the cancer cell. The birth and death of a cancer cell hinges on the function of several ancient, highly conserved cell death pathways; apoptosis, necrosis, and autophagy. For cells to generate tumors, there are often accompanying defects in the progression of cell death that can eventually result in neoplasia. This is the basis for cancer therapies that attempt to rescind a cancer cell's longevity, and has been one of the most important goals of research. The approach has been to target the cell death pathways, particularly apoptosis, to render a cancer cell once again sensitive to regulated cell death.

To identify the best target for enhancing cell death in cancer cells, we must first understand

the defects in the cell death pathways that generate cancer cell tumorigenesis and treatment resistance. Many cancer therapies aim to induce cell death in order to curb tumor growth. However, the presence of genetic defects in cancer cells limits the clinical efficacy of these death-inducing agents. Therefore, more focused individualized therapy is needed to address these defects to improve clinical outcome.

Although much is known about apoptosis, other death pathways have only recently gained attention as potential targets of therapy. Because defects in cell death pathways are nearly ubiquitous among cancers, targeting the cell death components can be used as a comprehensive treatment strategy for a broad range of cancers. However, understanding the differences between the cell death pathway defects in each type of cancer can offer a more tailored approach to choosing treatments to enhance cell death that is specific and effective.

In this review, we will discuss what is known about the molecular pathways that lead to a cell's demise, and how this knowledge can be used to maximize a patient's response to their cancer treatment. First, we will present a summary of the cell death pathways apoptosis, autophagy, and necrosis. We will then explore the genetic components that participate in each of these processes, and the manner in which defects of these components may lead to tumorigenesis, treatment resistance, and disease relapse. We will review the drugs in development that attempt to restore a cancer cell's susceptibility to cell death, and the results of clinical trials that have evaluated a treatment's safety and efficacy. The complexity of cell death will be discussed with an emphasis on the master regulator of cell survival, p53. We will briefly review studies that suggest that cancer stem cells lead to disease relapse because of an acquired resistance to apoptosis that is likely due to the accumulation of mutations over time. Lastly, we will argue that targeted treatment strategies must take into consideration the type of cell death, the type of cancer, and the micro-environment, so that cell death resistance can be effectively lowered.

Apoptosis

Apoptosis is composed of two separate pathways: intrinsic and extrinsic (**Figure 1**). In the older, intrinsic pathway, DNA damage induces release of cytochrome C from the intermembrane space. The Apoptotic protease activating factor 1 (APAF-1) activates caspase-9 through cleavage, and caspase-9 generates a signaling cascade of caspase cleavage that results in direct DNA fragmentation [1-3]. The intrinsic pathway is antagonized by the mitochondrial anti-apoptotic proteins Bcl-2 and Bcl-xL, which inhibit the pro-apoptotic proteins Bax and Bak (**Figure 1**). The extrinsic pathway begins with ligand binding to the Fas or the TNF-related apoptosis-inducing ligand (TRAIL) death receptors, which causes formation of the death-inducing signaling complex (DISC). Once activated, caspase-8 activates effector caspases such as caspase-3. Inhibition of the extrinsic pathway occurs through cellular FLICE-inhibitory protein (cFLIP) function, which is a procaspase-8/-10 homolog that prevents caspase-8 recruitment to the DISC [4]. These two apoptotic pathways converge at the level of the effector caspases, leading to apoptotic death. Apoptotic cell death is regulated by the Inhibitors of apop-

osis proteins (IAPs). The most well-known member of this family is the x-linked IAP (XIAP), which inhibits caspase-3 and -9. XIAP is itself inhibited by the second mitochondria-derived activator of caspases (Smac), a pro-apoptotic gene (**Figure 1**).

Intrinsic pathway defects in apoptosis

Cancers limit an apoptotic response by overexpressing anti-apoptotic proteins or causing defects in pro-apoptotic proteins. Overexpression of the anti-apoptotic protein Bcl-2 is present in patients with follicular cell lymphoma [5]. Moreover, constitutively increased levels of Bcl-2 or Bcl-xL have been associated with more aggressive malignant phenotypes and/or drug resistance to various chemotherapeutic agents in acute lymphoblastic leukemia cell culture [6, 7], prostate cancer [8], and the NCI 60 human cancer cell lines [9]. Another anti-apoptotic protein, Survivin, has been shown to be the fourth most common transcribed protein in the genome of human cancers [10], and increased levels in primary neuroblastoma cell lines were associated with worsened prognosis [11].

Decreased expression of pro-apoptotic proteins also enhances tumorigenesis. Frameshift mutations in the Bcl-2-associated X protein (Bax) gene are present in some mismatch repair-deficient colon cancer cell lines and malignant hematopoietic cell lines [12, 13]. Cells from APAF-1 knockout mice are resistant to apoptosis induced by chemotherapeutic agents such as etoposide and dexamethasone [14].

Lung malignancies often have many defects in the intrinsic apoptotic pathway that affect both tumorigenesis and response to therapy. Lung tumor cell lines (e.g., non-small cell lung cancer) were found to express *in vitro* caspase-9S [15], a truncated form of pro-caspase-9, which binds to and inhibits APAF-1 [16]. The high expression of heat shock proteins (HSPs), which interfere with apoptotic signaling, has also been observed in many cancers [17]. Changes in the components of the intrinsic pathway, such as cytochrome C, have been observed in patients with acute myeloid leukemia and correlated with reduced patient sensitivity to induction chemotherapy [18].

Drugs targeting the intrinsic pathway

Many drugs are currently in development that

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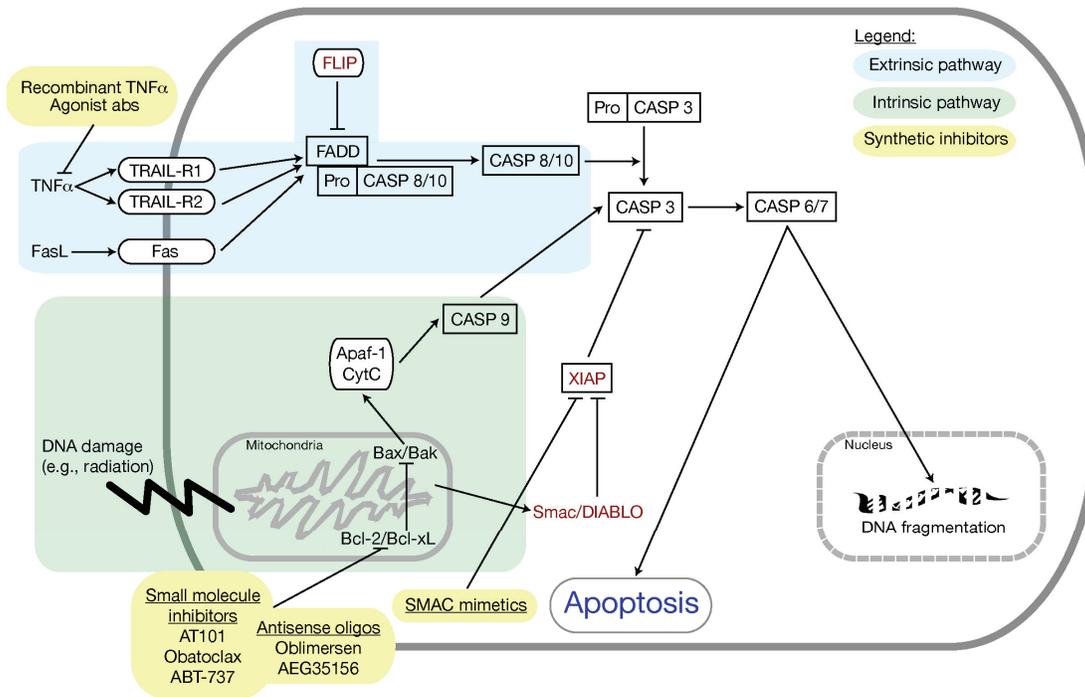


Figure 1. The intrinsic and extrinsic pathways of apoptosis. The extrinsic pathway is highlighted in blue and the intrinsic pathway is highlighted in green. Synthetic inhibitors are highlighted in yellow. Abbreviations: Apaf-1, apoptotic protease activating factor 1; Casp, caspase; BAK, BCL-2 homologous antagonist/killer; BAX, BCL-2-associated X protein; BCL-2, B-cell leukemia/lymphoma 2; BCL-XL, B-cell leukemia/lymphoma XL; CytC, cytochrome C; DIABLO, direct IAP binding protein with low PI; FADD, Fas-associated protein with death domain; FLIP, FLICE-like inhibitory protein; TNF α , tumor necrosis factor α ; TRAIL-R1, TNF-related apoptosis-inducing ligand receptor 1; TRAIL-R2, TNF-related apoptosis-inducing ligand receptor 2; SMAC, second mitochondria-derived activator of caspases; XIAP, X-linked inhibitor of apoptosis protein.

target and induce apoptosis at various stages of the intrinsic pathway (**Figure 1, Table 1**). The first class of drugs being developed is anti-sense oligonucleotides targeting anti-apoptotic genes. Clinical trials have tested the effect of oblimersen, a Bcl-2 inhibitor, in Waldenstrom's macroglobulinemia, Non-Hodgkin's lymphoma, multiple myeloma, breast, prostate, esophageal, and gastric cancer [19-27]. Treatment with oblimersen reduced Bcl-2 mRNA and protein levels in breast cancer cell lines, but could not decrease Bcl-2 levels and increase doxorubicin efficacy in phase I/II trials on breast cancer patients [28, 29]. Another phase II clinical trial showed that oblimersen in combination with carboplatin and etoposide did not confer a significant benefit when treating advanced-stage small-cell lung cancer [30]. Another anti-sense oligonucleotide that has been tested is AEG35156, which targets XIAP. Because pa-

tients with Acute myeloid leukemia (AML) were found to overexpress caspase-3 and -9, AEG35156 was tested for its therapeutic efficacy. In phase I/II clinical trials, 47% of patients had complete remission (bone marrow <5% myeloblasts with normal maturation, peripheral blood counts: Hgb \geq 11 g/dL; Plt \geq 100 \times 10⁹; Neutrophils \geq 1 \times 10⁹; Blasts 0%) with the highest doses of AEG35156 in combination with idarubicin and cytarabine (**Table 1**) [31]. Therefore, these results suggest that further testing is needed to clarify the roles of anti-sense oligonucleotides in the treatment of cancer, especially in view of the considerable toxicity that has been reported (**Table 1**).

The second class of drugs is small molecule inhibitors. Gossypol, called AT-101 in its oral tablet form, binds to the anti-apoptotic proteins Bcl-2, Bcl-x, and MCL1 and inhibits their binding

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Table 1. Summary of clinical trials for the intrinsic apoptotic pathway

Trial	Phase	Patient Characteristics	Treatment Regimen (Target), Route	Patients enrolled, n=	Results	Safety
Schimmer, et al. [34] O'Brien, et al. [33]	I	Leukemia, refractory	Obatoclox mesylate (Bcl-2), iv	70	4% had CR*; 21% had hematologic improvement	CNS effects (somnolence, dizziness, fatigue, euphoria), worsening cytopenia
Schwimmer, et al. [29]	I/II	AML, relapsed or refractory	AE35156(XIAP), iv; Idarubicin; Cytarabine	32	47% had CR with incomplete platelet count recovery	Peripheral neuropathy
Gertz, et al. [20]	I	Waldenstrom's macroglobulinemia	Oblimersen(Bcl-2), iv	9	Not available	Fever, low BP
Pro, et al. [22]	II	Non-Hodgkin's lymphoma, recurrent	Oblimersen(Bcl-2), iv	48	42% had objective responses; 28% had stable disease	Anemia, thrombocytopenia, neutropenia, fatigue, edema
Badros, et al. [18]	II	Multiple myeloma	Oblimersen(Bcl-2), iv	33	55% had objective responses; 6% had CR; 12% had a partial response	Inc Creatinine, thrombocytopenia, neutropenia, fatigue, anorexia
Chanan-Khan, et al. [19]	III	Multiple myeloma	Oblimersen(Bcl-2), iv; Dexamethasone	244	No significant difference of objective responses or TTP between the two groups	Fatigue, fever, nausea
Rom, et al. [24]	I	Breast CA	Oblimersen(Bcl-2), iv; Docetaxel; Adriamycin; Cyclophosphamide	13	28% had CR, 28% had partial remission Molecular readout: 15% had decreased Bcl-2 mRNA after 4 days of treatment	Not available
Modi, et al. [21]	I	HER2+ Breast Ca	Tanespimycin (Hsp90), iv; Trastuzumab	25	4% had a partial response; 16% had stable disease at 4 months	Emesis, fatigue, nausea, inc AST/ALT
Moulder, et al. [28]	I/II	Breast Ca, locally advanced	Oblimersen(Bcl-2), iv; Doxorubicin; Docetaxel	30	70% had a confirmed partial response	Neutropenic fever, infection, inc AST/ALT, neuropathy, diarrhea
Raab, et al. [23]	I	Esophageal Ca, Gastric Ca, advanced	Oblimersen(Bcl-2), iv; Cisplatin; 5-fluorouracil	15	20% had an objective response, median PFS=2.5 mo, median OS=7.5 mo	Neutropenia, hypokalemia, bacterial infection, mucositis, fatigue, dizziness
Shah, et al. [26]	II	Merkel cell carcinoma, advanced	Oblimersen(Bcl-2), iv	12	25% had stable disease, no objective responses observed	Grade 4 lymphopenia (8%), cytopenia, hypophosphatemia, pain, elevated Creatinine
Sternberg, et al. [25]	II	Prostate Ca, castration-resistant	Oblimersen(Bcl-2), iv; Docetaxel	111	Partial response by RECIST criteria in 24% of O+D vs. 18% of D Molecular readout: PSA response in 37% of O+D vs. 46% of D	Grade 3 fatigue, mucositis, thrombocytopenia

CR criteria (International Working Group criteria, [128]) must last >4 weeks: bone marrow <5% myeloblasts with normal maturation, peripheral blood counts (Hgb \geq 11 g/dL; Plt \geq 100 x 10⁹; Neutrophils \geq 1 x 10⁹; Blasts 0%). Abbreviations: AST, alanine aminotransferase; AST, aspartate aminotransferase; CR, complete remission; D, docetaxel; O, oblimerson; OS, overall survival; PFS, progression-free survival; PSA, prostate-specific antigen; RECIST, response evaluation criteria in solid tumors; TTP, time to progression.

to pro-apoptotic targets. Preclinical testing of *in vitro* and *in vivo* models of B-cell lymphoma showed that gossypol promotes apoptosis when used in combination with several different chemotherapies including carfilzomib, etoposide, and doxorubicin [32]. In a phase I/II study, when patients with prostate cancer were treated with escalating doses of AT-101, 2 of the enrolled patients with hormone-refractory prostate cancer had at least a 50% reduction in levels of prostate-specific antigen (n=23) [33]. Another small molecule inhibitor of the anti-

apoptotic Bcl-2 family proteins is obatoclox mesylate, which was cytotoxic to melanoma cell lines when used in combination with an ER stress inducer such as tunicamycin or thapsigargin [34]. In phase I trials, obatoclox had modest improvement in patients with advanced CLL and other myelodysplastic disorders [35, 36]. A third small molecule is the ABT-737, which binds to and inhibits function of Bcl-2 and Bcl-x by working in conjunction with doxorubicin to cause an apoptotic response in HL-60 leukemic cells [37]. Geldanamycin, a small molecule inhibi-

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Table 2. Summary of clinical trials for the extrinsic apoptotic pathway

Trial	Phase	Patient Characteristics	Treatment Regimen (Target), Route	Patients enrolled, n=	Results	Safety
Wakalee, et al. [54] Plummer, et al. [51]	I	Solid malignancies, relapsed or refractory	<u>Lexatumumab (TRAIL-R2), iv</u>	68	31% with stable disease, none with a partial response	Well tolerated, fatigue and nausea in >10% of patients, 1 patient with grade 3 hyperamylasemia
Mom, et al. [50]	I	Solid tumors, advanced	<u>Mapatumumab (TRAIL-R1), iv</u> ; Gemcitabine; Cisplatin	49	24% had a partial response, 51% had stable disease (median duration, 6 mo)	Grade 3 transaminitis, neutropenic fever, grade 4 thrombocytopenia
Leong, et al. [49]	I	Solid tumors, advanced	<u>Mapatumumab (TRAIL-R1), iv</u> ; Paclitaxel; Carboplatin	27	19% had a confirmed partial response; 12% had stable disease	Grade 3 hypersensitivity, neutropenic fever, alopecia, fatigue, nausea
Tolcher, et al. [52]	I	Solid tumors, advanced	<u>Mapatumumab (TRAIL-R1), iv</u>	49	39% had stable disease; no objective responses to treatment	Fatigue, fever, myalgia
Greco, et al. [55]	II	NSCLC, advanced	<u>Mapatumumab (TRAIL-R1), iv</u>	32	29% had stable disease, none had responses fulfilling RECIST criteria	Fatigue, cough, nausea, dyspnea, constipation
Trarbach, et al. [53]	II	Colorectal Ca, refractory	<u>Mapatumumab (TRAIL-R1), iv</u>	38	Median PFS=1.2 mo, 32% had stable disease, median duration 2.6 mo)	Fatigue, nausea, anorexia, abdominal pain

Abbreviations: NSCLC, non-small cell lung cancer; PFS, progression-free survival; RECIST, response evaluation criteria in solid tumors.

tor, inhibits HSPs, which can interfere with apoptosis and have anti-tumor effects [38]. Its less hepatotoxic analogue, tanesprimycin (17-AAG) plus trastuzumab in phase I clinical trials was well tolerated and demonstrated antitumor activity in patients with HER-2+ breast cancer [22]. These results suggest that small molecule inhibitors of anti-apoptotic proteins have potential in combination with other therapies for a wide range of cancers.

Other drugs that target the intrinsic apoptotic pathway include Smac mimetics, which inhibit XIAP and sensitize prostate cancer cell lines to apoptotic cell death *in vitro* [39]. In combination with multiple chemotherapeutic agents, Smac mimetics increased apoptotic responses in multiple tumor cell lines *in vitro* including melanoma, NSCLC, breast cancer, and neuroblastoma.

Extrinsic pathway defects in apoptosis

Because the death receptors are the gateway to the extrinsic apoptotic pathway, it is of little surprise that reduced ligand binding has been implicated as the mechanism of resistance to apoptotic cell death in multiple cancers. For example, the decreased expression of the Fas receptor resulted in increased resistance to the signaling effects of TRAIL and TNF-alpha in

U937 cell lines or human monocytic cell lines [40]. Loss of cell surface expression of TRAIL-R1 and TRAIL-R2 on breast cancer cell lines correlated with decreased sensitivity to TRAIL [41]. The overexpression of decoy TRAIL receptors is another means for cancers to bypass Fas - or TRAIL-induced apoptosis. Decoy receptors bind to Fas or TRAIL ligands, effectively decreasing signal transduction of the extrinsic pathway. For example, overexpression of decoy receptor 3 leads to decreased Fas-mediated apoptosis in B-cell lymphoma and diffuse large B-cell lymphoma cell lines [42].

In addition to abnormal expression of cell death and decoy receptors, defective signaling from the death receptor to caspase-8 can functionally block signal transduction at the receptor level (**Figure 1**). For instance, mutated variants of caspase-8 can prevent the recruitment of normal caspase-8 to the DISC, thus acting as dominant-negative forms [4]. Mutated caspase-8, while infrequent, has been linked to increased bladder cancer incidence, according to a hospital-based case control study [43]. Caspase-8 can also be functionally silenced by the hypermethylation of gene regulatory sequences, which has been noted in a number of biopsied tumor samples including neuroblastoma [44], Ewing's sarcoma [45], and glioblastoma multiforme [46].

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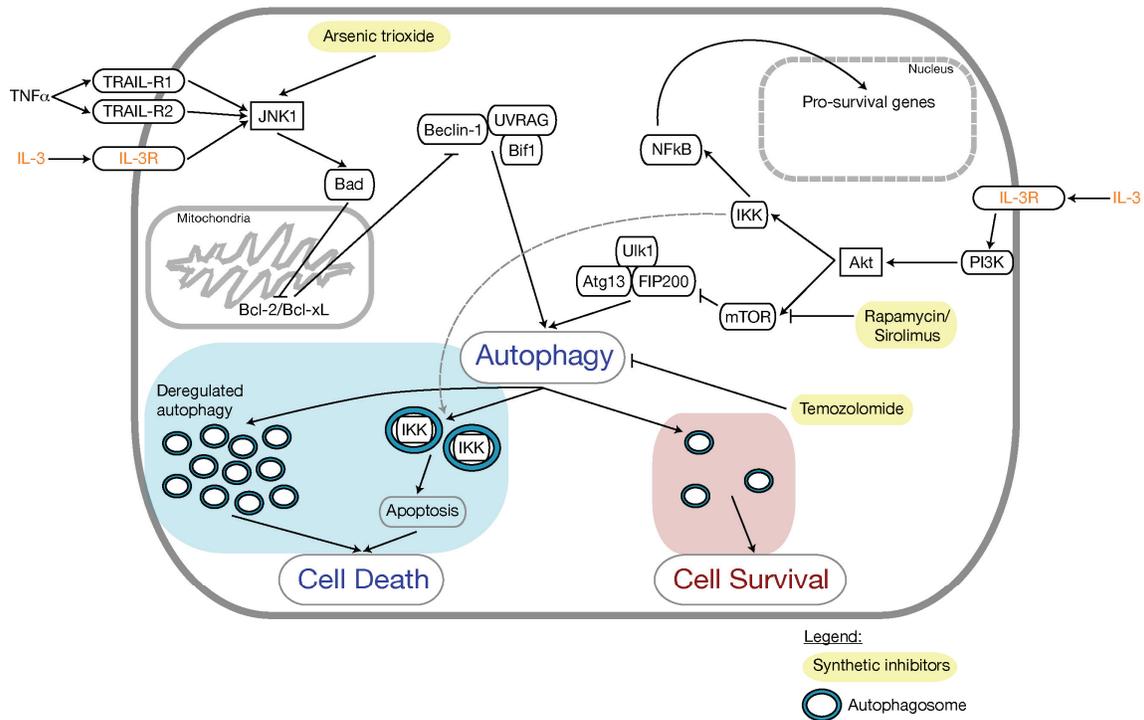


Figure 2. The molecular mechanism of autophagy and its role in cell death and survival. Autophagy is mediated through mTOR- and Beclin-1-dependent pathways. The effects of autophagy on cell death are highlighted in blue and the effect of autophagy on cell survival is highlighted in red. Synthetic inhibitors are highlighted in yellow. Autophagosomes are highlighted in blue. Abbreviations: AKT, protein kinase B; BAD, Bcl-xL/Bcl-2-associated death promoter; BCL-2, B-cell leukemia/lymphoma 2; BCL-XL, B-cell leukemia/lymphoma XL; BIF1, Endophilin B1; FIP200, focal adhesion kinase family-interacting protein of 200 kD; IL-3, interleukin 3; IL-3R, interleukin 3 receptor; IKK, I kappa beta kinase; JNK1, jun NH2-terminal kinase 1; mTOR, mammalian target of rapamycin; NFκB, nuclear factor kappa beta; PI3K, phosphoinositide 3-kinase; TNFα, tumor necrosis factor α; TRAIL-R1, TNF-related apoptosis-inducing ligand receptor 1; TRAIL-R2, TNF-related apoptosis-inducing ligand receptor 2; ULK1, unc-51-like kinase 1; UVRAG, UV radiation resistance-associated gene protein.

Drugs targeting the extrinsic pathway

Current therapeutic strategies targeting the extrinsic pathway are based on two primary approaches: TRAIL-receptor recombinant ligands and agonist antibodies (**Figure 1**). Recombinant human TRAIL (rhTRAIL) has been developed for clinical investigation as a soluble zinc-coordinated homotrimer. The mechanism of action remains unclear, but it can trigger apoptosis in a p53-independent manner in 50% of cancer cell lines and has little if any effect on non-malignant cells. Other recombinant ligands include recombinant human Fas, APO010, and golnervinogen pradenovec, a replication deficient adenovector that expresses tumor necrosis factor (TNF)α under a radiation-inducible

promoter [47, 48].

Monoclonal antibodies are available for both the TRAIL-R1 (mapatumumab) and TRAIL-R2 (lexatumumab, drozitumab) receptors. These antibodies have been tested *in vitro* and in Phase I and II clinical trials [49-55]. In ovarian cell cultures, it was shown that cells treated with paclitaxel and carboplatin in combination with mapatumumab enhanced cytotoxicity to treated tumor cells [56]. It has been shown in phase I clinical trials that mapatumumab in combination with paclitaxel and carboplatin demonstrated increased anticancer activity and clinical benefit for the majority of the patients enrolled on the trial [49]. Lexatumumab treatment in combination with radiation increased

long-term tumor control in a nude mouse model [57]. *In vitro*, drozitumab treatment induced apoptosis in a panel of breast cancer cell lines but was had no effect in normal human primary osteoblasts, fibroblasts, or mammary epithelial cells. Drozitumab treatment led to the complete regression of advanced mammary tumors in a murine model [58]. Lexatumumab has been tested in phase I clinical trials, but its efficacy has yet to be determined in phase II trials [54]. Although these antibodies were found to have substantial pro-apoptotic activity *in vitro*, few significant objective responses have been noted to date.

These results emphasize that identifying the specific location of the defects in a cell death pathway has a profound impact on treatment. For example, in malignancies with a known caspase-9 mutation that renders treatment resistance, targeting the intrinsic apoptotic pathway may show minimal benefit because the pathway itself converges on caspase-9. Therefore, further studies to understand the effects of cell death pathway drugs and the character of the cell death pathway defects are needed to design the most optimal treatment.

Autophagy

Autophagy is a cell death pathway that recycles cell components and degrades proteins (**Figure 2**). Autophagy is stimulated by starvation, cytokines, caspase inhibition (discussed in further detail below), and drug treatment (e.g., rapamycin/sirolimus). In yeast, autophagy is an adaptive mechanism to changes in the availability of energy sources. In mammals, however, the role of autophagy is much more varied, ranging from cell survival, cell death, immunity (pathogen clearance and antigen presentation), and to disease states such as cancer and neurodegeneration [59, 60]. There are multiple types of autophagy that are distinguished by bulk degradation or subcellular compartment-specific degradation. Bulk degradation occurs via macroautophagy, which is the classically described process that occurs following nutritional deprivation in yeast. Macroautophagy is characterized by the formation of double membrane vesicles that arise from an unknown source to engulf the cytosol [59, 60]. Following fusion with a lysosome to form an autophagolysosome, the contents of the vesicles are enzymatically degraded. Subcellular compartment-specific auto-

phagy includes chaperone-mediated autophagy (CMA), which occurs in response to serum withdrawal, cytoplasmic to vacuole yeast targeting (CVT) autophagy, pexophagy (autophagy of peroxisomes that occurs in yeast and mammals), and mitophagy. Mitophagy is the autophagy of damaged mitochondria that especially occurs in response to starvation. This process begins with the release of intermembrane mitochondrial proteins that activate the process, unless mitochondrial permeabilization is blocked (i.e., in response to cyclosporine A treatment) [60].

Autophagy is largely regulated by mammalian target of rapamycin (mTOR) and Beclin-1. Autophagosome formation is governed by the Atg13/Ulk1/FIP200 complex [61]. mTOR is an important regulator of protein synthesis, ribosome biogenesis, and autophagy in mammalian cells [62, 63]. Activation of the PI3K/Akt/mTOR pathway inhibits the Atg13/Ulk1/FIP200 complex through phosphorylation of the Autophagy-related protein 13 (Atg13), which destabilizes the complex. Beclin-1 is a tumor suppressor that activates autophagy when bound to the UV irradiation resistance-associated gene (UVRAG) and endophilin B1 (Bif1) (**Figure 2**).

Autophagy was initially characterized as a pro-survival factor due to its ability to maintain ATP levels during nutrient deprivation due to cellular component recycling (**Figure 2**). In addition, cells from Bak/Bax-deficient mice, which have reduced apoptosis, require autophagy induction to sustain their energetic requirements following IL-3 withdrawal [64]. In addition, it is speculated that mitophagy, which is a subtype of autophagy, enhances survival of the cell by degrading damaged mitochondria before the release of pro-apoptotic factors from the mitochondria and the accumulation of ROS lead to DNA damage [65].

Although early studies have described autophagy in dying cells, it was unclear whether this was a part of the cell death mechanism or a terminal survival attempt by the cell. However, recent studies confirm that autophagy also plays a role in cell death [66, 67]. There are multiple models for the mechanism of autophagy-dependent cell death. The first is that overabundant autophagy results in cell death [60] (**Figure 2**). The second model is that autophagy activates apoptosis, which was described in *Drosophila* and during HIV infection following

engagement of the HIV glycoprotein Env and the CXCR4 receptor. In the third model, autophagy selectively degrades survival factors to reduce cell viability [60]. An example of this was shown in an elegant study using mouse L929 cells. When treated with a caspase inhibitor, these cells showed increased autophagic cell death, which required Beclin-1 and Atg7 and was mediated by JNK and RIP signaling. In this form of autophagy, catalase was selectively degraded by autophagy, leading to ROS accumulation, cell membrane damage, and cell death [67]. These results suggested that autophagy could lead to cell death via a caspase-independent mechanism. Another survival factor, IKK, which facilitates nuclear translocation of NF κ B, can be selectively degraded via autophagy *in vitro* [60]. These results suggest that autophagy plays an important role in regulated cell death as well as cell survival.

Autophagy defects in cancer

Interestingly, the level of autophagy in cancer cells depends on the context of disease. Advanced tumors have increased autophagy in areas of high metabolic stress, likely due to the increased energetic requirements and adaptability required in this environment [61]. However, early data suggested that autophagy-related genes were tumor suppressors. Monoallelic deletions of the Beclin-1 locus were frequently observed in human breast, ovarian, and prostate cancer cell lines [68-70]. Heterozygous gene disruption of Beclin-1 in mice resulted in decreased autophagy and increased cellular proliferation of breast and hepatocellular carcinoma, suggesting that Beclin-1 is a haploinsufficient tumor suppressor [5, 71]. Beclin-binding molecules such as UVRAG and Bif-1 are also associated with tumorigenesis. UVRAG localizes to the chromosome region 11q13. Mutations in this region were associated with the development of gastric and colon cancer through analysis of patient biopsies [72-75]. In addition, reduced Bif-1 expression was noted in tissues samples from patients with gastric carcinoma [76], invasive urinary bladder, and gallbladder cancer [77]. A homozygous deletion of the Bif-1 gene was also identified in patients with mantle cell lymphoma [78]. These data implicate components of autophagy in the suppression of tumorigenesis.

When Beclin-1 is bound by Bcl-2, autophagy is

inhibited. Furthermore, increased autophagy was accompanied by increased cell death when the Bcl-2-binding domain was deleted [71]. Although the pro-survival properties of Bcl-2 were previously attributed solely to apoptotic pathway inhibition, downregulation of Bcl-2 also results in caspase-independent cell death in human leukemic HL60 cell lines [79]. Furthermore, the silencing of Bcl-2 in breast cancer cell lines with RNA-interference has been shown to promote autophagic cell death [80]. These data demonstrate the cross-talk that exists between the cell death pathways.

Because Bcl-2 functions as a direct inhibitor of autophagy, promoting the dissociation of Beclin-1 from Bcl-2 represents one of the novel therapeutic to enhancing autophagic cell death in cancer patients. Recently, it was found that c-Jun N-terminal kinase (JNK) phosphorylates Bcl-2 to release Beclin-1 from the inhibitory effects of Bcl-2 [81]. Intriguingly, only the endoplasmic reticulum-localized Bcl-2 featured a JNK-dependent regulation of Beclin-1 [81, 82]. Therefore, the spatial dependence of autophagy regulation by Bcl-2 has become an attractive topic in the research on autophagy and cancer. Targeting Bcl-2 to increase autophagy levels in cancer cells might therefore be achieved by the activation of JNK or the competitive antagonism of the Bcl-2-binding/BH3 domain of Beclin-1 by BH3-mimetics. Use of these strategies may prevent the inhibition of Beclin-1 and activate autophagy in cancer cells.

While pro-survival factors including mTOR, PI3K/Akt, and Bcl-2 inhibit autophagy, tumor suppressors including p53 and LKB1 stimulate autophagy [83]. Specifically, low nutrient levels inhibit mTOR and activate autophagy via LKB1/AMP-activated protein kinase (AMPK) [71, 84, 85]. Factors involved in this signaling pathway appear to be closely linked to tumorigenesis. For instance, mutated LKB1 was recently found to enhance tumor metastasis and aggression in lung cancer cell line and lung cancer tumor specimen biopsied from patients [86]. LKB1 is somatically mutated in approximately one third of the NSCLC patient population. Genetically-engineered mouse models of NSCLC with a LKB1 mutation gained metastatic potential and exhibited histologic changes corresponding to adenocarcinoma, and squamous cell carcinoma *in vivo* [87]. Therefore, multiple upstream regulators of autophagy perform tumor-suppressing

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Table 3. Summary of clinical trials for the autophagy pathway

Trial	Phase	Patient Characteristics	Treatment Regimen (Target), Route	Patients enrolled, n=	PFS*	Overall Survival	Safety
Clark, et al. [88]	II	Melanoma with brain metastasis	Temozolomide, po; Thalidomide	64	6 mo PFS=15%	1 year=35%	Abdominal pain, anemia, anorexia, cardiac ischemia/infarction, CNS ischemia, confusion, constipation
Zustovich, et al. [89]	II	Malignant glioma, temozolomide-refractory	Temozolomide, po; cisplatin	24	Median PFS=3.8 mo; 6 mo PFS=28%	Median OS=7 mo	Granulocytopenia, anemia, thrombocytopenia, anorexia, nausea, vomiting
National Cancer Institute of Canada Clinical Trials Group, [90]	III	Glioblastoma multiforme	Temozolomide, po; Radiation therapy	573	2 yr PFS=11% in the T+R group vs. 2% in the R group; 5 yr PFS=4% in the T+R group vs. 1% in the R group	2 yr OS=27% in the T+R group vs. 1.9% in the R group; 5 yr OS=9.8% in the T+R group vs. 1.9% in the R group	Myelosuppression
Reardon, et al. [92]	I	Malignant glioma	Sirolimus(mTOR), po; Gefitinib	34	Median PFS=8.2 wks, 6 mo PFS=23.5%	Not available	Mucositis, thrombocytopenia, rash, diarrhea
Ghobrial, et al. [93]	II	Waldenstrom's macroglobulinemia, relapsed or refractory	Everolimus(mTOR), po	50	6 mo PFS=75%, 12 mo PFS=62%	Not available	Pancytopenia, pneumonia, upper airway infection, hypercholesterolemia, hyperglycemia
Hess, et al. [94]	III	Mantle cell lymphoma	Temsirolimus (mTOR), iv vs. Investigator's choice therapy	162	Median PFS=4.8 mo in the T group vs. 1.9 mo in the I group (p=0.0009)	Not available	Thrombocytopenia, anemia, neutropenia, asthenia

Abbreviations: mTOR, mammalian target of rapamycin; OS, overall survival; PFS, progression-free survival; R, Radiation therapy; T, Temozolomide.

functions that may be of interest in the therapeutic elevation of autophagy in cancer cells.

Drugs targeting autophagy

Temozolomide is a drug that has been shown to induce autophagic cell death (Table 3). The cytotoxicity of temozolomide is mediated by the formation of O6 methylguanine in DNA, leading to thymine mispairing in the subsequent replication cycle and resultant cell cycle arrest in G2/M, as demonstrated in glioblastoma cell lines [88]. Phase II trials of temozolomide in combination with thalidomide or cisplatin for patients with melanoma or malignant glioma showed a modest 6 month progression-free survival (PFS) (15% and 28%, respectively) [89, 90]. However, Phase III trials demonstrated that temozolomide with radiation therapy offered significant benefit to glioblastoma patients [91]. Adding temozolomide to radiotherapy increased the median survival and the two-year survival in newly diagnosed patients with glioblastoma (11% vs. 2% in patients treated with radiation alone) (Table 3) [91].

Rapamycin and rapalogs (RAD001, CCI-779, AP23573) are mTOR inhibitors (Table 3). Silencing of mTOR with a siRNA stimulates autophagy and thereby reduces tumor cell viability. Targeting the autophagy gene Beclin-1 with siRNA suppressed the cytotoxicity of rapamycin in rapamycin-sensitive malignant glioma cell lines, implicating autophagic cell death as the primary mediator of the antitumor effects [92]. Phase I studies showed that of 34 patients enrolled with malignant glioma with prior chemotherapy and radiation therapy, 2 patients had a partial response and 13 patients achieved stable disease with the mTOR inhibitors and gefitinib treatment regimen [93, 94]. A phase III clinical trial showed that mTOR inhibitors significantly improved the clinical outcomes of patients with mantle cell lymphoma (4.8 mo PFS vs. 1.9 mo in the control) [95].

Arsenic trioxide (AT) could effectively treat multiple malignancies *in vitro* including multiple myeloma, lymphoma, leukemia, and neuroblastoma cell lines [96-99]. Furthermore, AT induced both apoptosis and autophagy *in vitro* in human cell

Targeting cell death in cancer

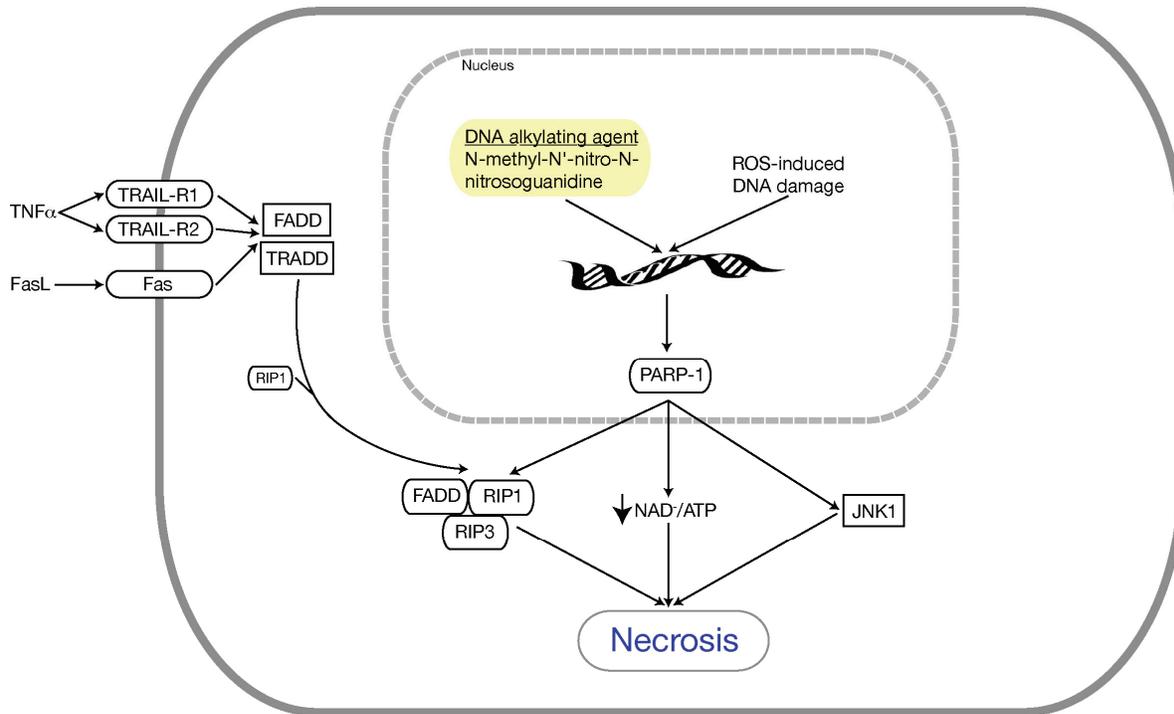


Figure 3. The molecular mechanism of necrosis. Drug treatment that results in DNA damage is highlighted in yellow. Abbreviations: ATP, adenosine triphosphate; FADD, Fas-associated protein with death domain; JNK1, jun NH2-terminal kinase 1; NAD, nicotinamide adenine dinucleotide; PARP-1, poly(ADP-ribose) polymerase 1; RIP1, receptor interacting protein 1; RIP3, receptor interacting protein 3; TNF α , tumor necrosis factor α ; TRADD, tumor necrosis factor receptor type 1-associated death domain protein; TRAIL-R1, TNF-related apoptosis-inducing ligand receptor 1; TRAIL-R2, TNF-related apoptosis-inducing ligand receptor 1.

lines of T-lymphocytic leukemia and myelodysplastic syndrome. The addition of an autophagy inhibitor (3-methyl adenine) to malignant leukemia cells overexpressing Beclin-1 also suppressed the increased autophagic death in treated cells [100]. AT is especially noteworthy because it can induce autophagic cell death in malignant gliomas, which are resistant to many commonly used therapies [88]. In addition, it has a low side effect profile. A Phase II trial arsenic trioxide, interferon alpha, and zidovudine treatment regimen showed a 100% response in patients with T-cell leukemia/lymphoma (ATL) (n=10) [101], suggesting that combination therapies with AT have significantly improved clinical outcomes in comparison with prior treatment regimens.

Necrosis

Necrosis is regulated by poly(ADP-ribose) polymerase 1 (PARP-1), a DNA repair protein (**Figure**

3). Hyperactivation of PARP-1 by DNA damage through alkylating agents such as doxorubicin or ROS accumulation can deplete cytosolic nicotinamide adenine dinucleotide (NAD) and trigger necrosis. Receptor-interacting serine/threonine kinases 1 and 3 (RIP1, RIP3) have also been found to regulate necrosis by forming a complex with FADD that was found to be caspase-independent. Recent evidence also showed that RIP1 activation led to apoptosis in the absence of RIP3, suggesting that RIP3 is required to activate necrotic cell death [102-104].

Necrotic defects in cancer

Traditionally, necrotic cell death is inhibited in cancer patients to sensitize tumors to chemotherapy. One way that this occurs is via PARP inhibition, which potentiates DNA-damaging chemotherapy in mouse models [105]. Because PARP functions in DNA repair, PARP inhibitors, combined with standard chemother-

apy, are thought to promote apoptotic cell death while inhibiting necrotic cell death. Because hyperactivation of PARP-1 induces necrosis, PARP-1 function may be desirable in the cellular context of other defective cell death pathways [106]. *In vitro* studies have shown that PARP-1 activation induced JNK signaling through RIP1 and TRAF2, which led to mitochondrial depolarization and permeabilization and finally, caspase-independent cell death [107, 108]. Interestingly, patients with inactivated Retinoblastoma protein do not benefit from PARP inhibition, as the basis of chemotherapy in this context is increased necrotic cell death [106]. This research suggests that the activity of PARP-1 signaling impacts greatly upon the sensitivity of cells to induced death. In the future, cancer patients may benefit from screening for PARP mutations, as this might influence the patient's response to treatment.

Stimulation of death receptors with TNF or other agonists also induced necrosis through the kinase activity of RIP1. Although RIP1 is essential for programmed necrosis, it can also influence NF- κ B, as well as apoptotic pathways [109]. Its interaction with RIP3 appears to determine which cell death pathway is utilized. Very recent findings have shown RIP3 overexpression led to necrosis, whereas a lack of RIP3 triggers RIP1-mediated apoptosis [102]. Moreover, *in vivo* studies in mice confirmed that RIP3 promotes programmed necrosis with viral infection, suggesting a role for RIP3 in cancer treatment [110]. Therefore, a better understanding of the interaction and the outcomes of RIP1 and RIP3 mutations may be the key to harnessing necrosis as an additional therapeutic force in cancers, using increased in necrotic cell death to aid in treatment.

Drugs targeting necrosis

DNA-alkylating agents (**Figure 3**) cause necrotic cell death with equal efficacy in cells with or without functional apoptosis, a process that is likely mediated by PARP-1 activation [111]. Necrosis is also activated by photodynamic treatment (PDT); PDT selectively targets abnormal cells while preserving normal surrounding tissues. The preferential accumulation of certain photosensitizing compounds in tumor cells and the ability to treat only the defined tumor make PDT a promising therapeutic approach. *In vitro* studies have shown that measuring the size of

DNA fragments or screening the different forms of cytokeratin-18 (caspase-cleaved versus non-cleaved) in plasma samples discriminates between apoptotic and necrotic/non-apoptotic cell death, respectively [112, 113]. Increased necrotic death in breast cancer patients is associated with better survival [114], and patients with endometrial tumors predominantly express the non-cleaved form of cytokeratin-18 after treatment with chemotherapy [115]. These data suggest that there is a therapeutic basis for enhancing necrosis in certain cancer types, such as breast cancer.

Cross-talk and regulation of the cell death pathways

p53 is a critical regulator of cell cycle checkpoints, senescence, and apoptosis. Therefore, it is no surprise that nearly 50% of all human cancers harbor mutated or deleted p53. However, p53 may also positively or negatively regulate autophagy, suggesting a more complex role of p53 in cancer than previously suspected [116]. The fundamental regulation of autophagy by p53 lies in its localization: nuclear p53 leads to autophagy and autophagic cell death, while cytoplasmic p53 hinders it.

The critical role of p53 in the treatment of cancer was also demonstrated with the surprising finding that p53 expression levels were a prognostic indicator in a subset of patients with glioblastoma multiforme that were treated with radiation treatment and temozolomide chemotherapy [117]. Patients were sorted to include those with decreased expression of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT), which has been associated with increased sensitivity to temozolomide treatment [118]. Interestingly, patients that had both low MGMT expression and low p53 expression showed significantly improved PFS ($p=0.015$) and overall survival (OS) ($p=.047$) when compared to those with high p53 expression [117]. A study in mice with intracranial glioblastoma multiforme xenografts revealed that the best treatment responses were obtained with concurrent temozolomide and radiation treatment, rather than sequential treatment [119]. These results suggest that the level of p53 expression has a major impact on the susceptibility of cancer cells to treatment and is an additional factor to consider when designing a treatment protocol.

These data emphasize that the complex network involving autophagy, apoptosis, and p53 is still unclear and needs further research. p53 is thus a prime example of the growing need for awareness of cross talk between cell death pathways as well as careful understanding of the cellular environment, stress, cancer staging, and other factors that must be considered to prescribe the best therapy.

Apoptosis resistance in cancer stem cells

Cancer stem cells (CSCs) are a self-renewing population that have unlimited proliferation potential. Once cells disseminate from the primary tumor, they can persist prior to causing cancer metastasis, a stage called cancer dormancy [120, 121]. Studies suggest that CSCs may be the chemotherapy- and radiation treatment-resistant cells that represent the nidus for relapsed or refractory disease, as well as metastasis [2, 120]. CSCs are likely derived from early progenitor cells or stem cells and were first identified in hematological malignancies such as AML, but have since been demonstrated in a number of cancer types. Therefore, this population is a possible Holy Grail of cancer therapy and intense investigation is currently underway to identify markers for the targeted eradication of these cells.

Because of the resistance of CSCs to treatment, it is hypothesized that they are also resistant to regulated cell death. Studies suggest that this occurs through defects in the cell death pathways as well as through regulation of extrinsic factors such as cytokine, chemokine, and adhesion signaling, which controls renewal and differentiation. CD34+ hematopoietic stem cells had decreased sensitivity to the extrinsic apoptotic pathway and showed decreased levels of caspase-8 mRNA in favor of a splice variant that could not activate the downstream effector caspase cascade [122]. Jurkat lymphoma and MCF7 breast cancer cell lines that were positive for CD133, a stem cell-associated marker, had decreased TRAIL-induced sensitivity to apoptosis that could be recovered by reducing FLIP expression [123]. Lastly, two studies in glioblastoma patient-derived stem cells expressed CD133 and had increased resistance to chemotherapy and TRAIL-induced apoptosis, likely due to promoter hypermethylation of caspase-8 [124]. In addition, expression of the anti-apoptotic genes Bcl-2, Bcl-xL, IAP, and FLIP

were increased. Resistance to radiation treatment was also observed in primary glioblastoma cells harvested by surgical biopsy, but sensitivity to treatment could be recovered with activation of the caspase cascade because of XIAP inhibition [125]. These results emphasize that it is not enough to target cell death pathways in cancer cells, but that it is also important to identify the target sub-population in which treatment will have the most potent outcome.

Discussion and Implications

We have discussed the primary modes of cell death and defects that are prevalent in various cancers. Understanding these defects represents the next big step for cancer research. It is important to keep in mind that although the majority of cancers harbor defects in the apoptotic pathway, the severity and scope of these defects can greatly influence the treatment outcomes. Because cancer cells rapidly mutate, there is a high possibility that multiple mutations exist in the same apoptotic pathway, particularly in treatment-resistant CSCs. Screening for these mutations can determine whether or not a targeted therapy should be implemented. For instance, in cases of multiple defects in the apoptosis pathway, a shift in treatment focus to alternative death pathways such as autophagy or necrosis may prove effective.

Because there is a growing appreciation for the complexity of cross-talk between the death pathways, cell death components must be examined in a broader context. For example, p53 mutations are extremely prevalent in cancers and commonly associated with its role in apoptosis, but there is growing evidence for the role of p53 in autophagy and necrosis as well. Many other proteins, from Bcl-2 to the death-associated protein kinase (DAPK) are becoming important players in other death pathways, and as we gain better insight into these connections, our therapies will improve as well. As the catalog of defects in these pathways grows, we will have greater tools to fight cancer, not only with new targets for therapy, but also with more personalized therapy. However, we must also be more aware of the tremendous complexity involved in cellular death.

To incorporate this complexity, efforts have been made not only to increase the number of molecular targets, but also to more effectively

use the cancer treatments available. One strategy, called cyclotherapy, attempts to circumvent the toxicity of current chemotherapy regimens to normal cells by using low doses of kinase inhibitors first to reversibly arrest growth in normal cells while restoring apoptosis to cancer cells. Chemotherapeutic agents are then used [63]. In addition, the development of simple and complex computational models to design dosing schedules for cell phase-specific chemotherapy drugs also minimizes toxicity and optimizes the molecular targets of the therapy [126]. This is especially important when multiple drugs are being administered, one of which arrests cells in a cell cycle phase that enhances the efficacy of action of the second drug [63]. For example, the protein kinase inhibitor UCN-01 causes three times as much G1-S-mediated apoptosis when it is administered before fluorouracil than after it [126, 127]. In addition, models that incorporate drug resistance that is both genetically based and cell cycle-mediated enhance the individualization of cancer treatment dosing regimens.

Although individualized therapy is not a novel concept, only recently has this model been applied to the field of oncology. The Food and Drug Administration approval of several drugs targeting specific molecules required for pathogenesis (notably trastuzumab, cetuximab, erlotinib and bevacizumab) has given credibility to individualized cancer therapy. Trastuzumab targets the human epidermal growth factor receptor 2 (HER2) in breast cancer and is effective only in the context of HER2 receptor overexpression. Similarly, epidermal growth factor (EGF) inhibitors erlotinib and cetuximab are most effective on tumors with EGF receptor (EGFR) mutations and amplification. Bevacizumab blocks vascular endothelial growth factor (VEGF), thereby restricting angiogenesis and metastasis, and thus has limited utility in non-metastatic settings. In treating malignancies today, it has become imperative to understand the individual patient's cancer in order to determine an optimal therapy. The power of this paradigm shift is most apparent in the current protocol to treat breast cancer, which prioritizes a patient's HER2 and ER/PR status because of the predictive value of these factors in disease management and prognosis. This approach applied to all disease is the future of medicine, and is the goal that we must align ourselves with to have the surest chance of advancing disease therapy.

Conclusion

Inducing apoptosis has long been a central goal of chemotherapy and radiation treatment. However, the rise of molecular targets in autophagy and necrosis allow for potentially greater flexibility when approaching cancers. The future of cancer therapy requires an understanding of molecular and genetic defects that lower the efficacy of current therapeutics to enhance cell death. We have discussed recent advances made on the contribution of cell death pathway defects to cancer resistance, as well as current drugs and clinical targeting of these defects to recover cell death. The key to sustainable and efficacious cancer therapy lies in a personalized approach, one that maximizes the specificity and efficacy of treatment for each type of cancer. We believe that continually refining our understanding of the nexus between cell death and cancer further tailors the ability to manage and predict the course of disease, constituting the future of personalized medicine.

List of Abbreviations: AIF: Apoptosis-inducing factor; AMPK: AMP-activated protein kinase; APAF-1: Apoptotic protease activating factor 1; AT: Arsenic trioxide; ATL: T-cell leukemia/lymphoma; BH3: Bcl-2 homology 3; Bif1: Endophilin B1; cFLIP: Cellular FLICE-inhibitory protein; CMA: Chaperone-mediated autophagy; CSC: Cancer stem cell; CVT: Cytoplasmic to vacuole yeast targeting; DAPK: Death-associated protein kinase; DISC: Death-inducing signaling complex; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; FADD: Fas-associated protein with death domain; FLICE: FADD-like IL-1 β -converting enzyme; FLIP: FLICE-inhibitory protein; HER2: Human epidermal growth factor receptor 2; HSP: Heat shock proteins; IAP: Inhibitors of apoptosis protein; JNK: c-Jun N-terminal kinase; MDR: Multidrug-resistant; MGMT: O⁶-methylguanine-DNA methyltransferase; mTOR: Mammalian target of rapamycin; NAD: nicotinamide adenine dinucleotide; NSCLC: Non-small cell lung cancer; PARP-1: poly(ADP-ribose) polymerase 1; PDT: Photodynamic treatment; PFS: Progression-free survival; rhTRAIL: Recombinant human TNF-related apoptosis-inducing ligand; RIP: Receptor-interacting serine/threonine kinases; Smac: Second mitochondria-derived activator of caspases; TNF α : Tumor necrosis factor α ; TRAIL: TNF-related apoptosis-inducing ligand; TRAIL-R: TNF-related apoptosis-inducing ligand receptor; UVRAG: UV irradiation resistance-associated gene; VEGF: Vascular endothelial growth factor; XIAP: X-linked inhibitors of apoptosis protein.

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