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Chemotherapeutic Approaches for Targeting Cell Death Pathways

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Abstract

For several decades, apoptosis has taken center stage as the principal mechanism of programmed cell death in mammalian tissues. It also has been increasingly noted that conventional chemotherapeutic agents not only elicit apoptosis but other forms of nonapoptotic death such as necrosis, autophagy, mitotic catastrophe, and senescence. This review presents background on the signaling pathways involved in the different cell death outcomes. A re-examination of what we know about chemotherapy-induced death is vitally important in light of new understanding of nonapoptotic cell death signaling pathways. If we can precisely activate or inhibit molecules that mediate the diversity of cell death outcomes, perhaps we can succeed in more effective and less toxic chemotherapeutic regimens.

Keywords

Chemotherapy; Apoptosis; Necrosis; Autophagy; Senescence; Mitotic catastrophe

Introduction

Strategically targeted cancer therapies are emerging from enormous efforts spent investigating basic signaling mechanisms involved in cell growth and cell death pathways. Many of the novel small molecules and biological agents being developed target pathways involved in apoptosis. Uncovering the molecular events that control and mediate apoptotic death has been fascinating and encouraged by the wealth of reagents and assays that offer specificity in detection. Examination of other modes of cell death has lagged behind, in part because of the difficulty in their measurement. It is often stated as fact that chemotherapies induce death solely through apoptotic mechanisms. Accumulating evidence suggests that tumor cell response to chemotherapy is not confined to apoptosis but also includes other modes of death [1]. In the first section of this review, we present discussions of the current knowledge of mechanistically described cell death outcomes, with an emphasis on their role in tumorigenesis and response to chemotherapy. In the second section, we examine the status of novel chemotherapeutic agents that target molecules involved in signaling of different cell death pathways.

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Types of Cell Death

Four categories of dynamic cellular activities that lead to cell death have been described: apoptosis, autophagy, necrosis, and mitotic catastrophe [2]. Permanent growth arrest, known as senescence, is also considered a type of cell death in the context of cancer therapy [3]. These five cell death classifications are based on distinct biochemical and morphological characteristics present in the dying cell (Table 1). Two of these processes, apoptosis and autophagy, have been considered to be "programmed," which refers to their strict genetic control [4, 5]. Programmed cell death results in the disintegration of cellular components and their engulfment by surrounding cells. Tissue remolding events during normal development of multicellular eukaryotic organisms rely on programmed cell death to help form the adult species. They also operate in adult organisms to maintain normal cellular tissue. Necrosis and mitotic catastrophe are generally considered passive responses to massive cellular insult. However, new findings suggest that these forms of death may also be genetically controlled [6-8]. Senescence is an essential process of aging and occurs following a gene-directed program involving the erosion of telomeres and the activation of tumor suppression signaling pathways [9]. Dysregulation of the signaling pathways that control each of these forms of cell death has been implicated in tumorigenesis.

Other models of cell death have been described, including caspase-independent apoptosis, necroptosis, paraptosis, pyroptosis, and slow cell death, whose morphologic and biochemical characteristics vary from current definitions of the major cell death pathways described above [10-13]. In an attempt to simplify this discussion, only the five best-described cell death outcomes (apoptosis, necrosis, autophagy, mitotic catastrophe, and senescence) are presented herein. It is also worth noting that a recent effort put forth by the editors of *Cell Death and Differentiation* has proposed to characterize cell death strictly in the precise terms of the parameters used to measure it and not in general terms that describe the presumed cell death pathway involved [14].

Apoptosis

Though the phenomenon had been described for almost a century, in 1972, Kerr, Wyllie, and Currie first coined the term "apoptosis" in order to differentiate naturally occurring developmental cell death from necrotic cell death that results from acute tissue injury [15]. They also noted that apoptosis was responsible for maintaining tissue homeostasis by mediating the equilibrium between cell proliferation and death. Morphologic characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation, and nucleosomal fragmentation. Under normal circumstances, cells undergoing apoptosis are recognized by macrophages, or neighboring cells that consume the cells' fractionated carcasses. Apoptosis has been considered a major mechanism of chemotherapy-induced cell death, and pathways regulating apoptosis are the focus of many preclinical drug discovery investigations.

There are two distinct molecular signaling pathways that lead to apoptotic cell death: (a) the *intrinsic*, or mitochondria-mediated pathway, and (b) the *extrinsic*, or extracellular activated pathway [4, 16, 17]. The intrinsic pathway is usually activated in response to intracellular stress signals, which include DNA damage and high levels of reactive oxygen species (ROS), as well as by viral infection and activation of oncogenes. The extrinsic pathway is triggered by the binding of an extracellular ligand to a receptor on the plasma membrane. Both pathways activate proteolytic enzymes called caspases that mediate the rapid dismantling of cellular organelles and architecture. Caspases are a family of proteins containing a nucleophilic cysteine residue that participates in the cleavage of aspartic acid–containing motifs [18]. Caspases are expressed as inactive precursors that form active oligomers after initiating cleavage events. There are two groups of caspases, the initiator/

apical caspases (caspase-8, caspase-9, and caspase-10), and effector/executioner caspases (caspase-3, caspase-6, and caspase-7). Evidence suggests that initiator caspases are capable of autocatalytic activation while effector caspases need activation by initiator caspase cleavage.

The Intrinsic Pathway—Bcl-2 family members act by regulating the efflux of apoptogenic proteins from mitochondria. Bcl-2 proteins contain from one to four Bcl-2 homology (BH) domains. The number and combination of the BH domains dictate whether the proteins are proapoptotic or antiapoptotic. Antiapoptotic Bcl-2 members contain all four BH domains and include Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bf1-1/A1. Proapoptotic members lack the BH4 domain and are divided into two groups, the "BH3-only" members and the multidomain BH1-3 proapoptotic members Bax and Bak. In mouse cells, deletion of Bax and Bak is sufficient to prevent mitochondrial outer membrane permeabilization (MOMP) induced by upstream apoptotic events [19, 20]. Bax and Bak normally exist as inactive monomers. Bax resides in the cytosol or loosely attached to intracellular membranes [21], and Bak is bound by Mcl-1, Bcl-xL, or voltage-dependent anion channel protein 2 (VDAC-2) in the mitochondrial outer membrane [22, 23].

The generalized scheme of intrinsic pathway activation is the oligomerization of Bax and Bak in the mitochondrial outer membrane to activate MOMP, thus permitting release of apoptogenic factors such as cytochrome c, second mitochondria–derived activator of caspase/direct inhibitor of apoptosis (IAP) binding protein with low pI (Smac/DIA-BLO), and Omi stress-regulated endoprotease/high temperature requirement protein A2 (Omi/HtrA2). Once released, cytochrome c binds apoptotic protease-activating factor 1 (Apaf-1), which recruits pro-caspase-9, promoting its self-activation. Activated caspase-9 cleaves the downstream effectors caspase-3 and caspase-7, which rapidly cleave intracellular substrates. Proteins of the IAP family, including X-linked IAP (XIAP), c-IAP1, and c-IAP2, can bind and inhibit the active sites of caspase-3, caspase-7, and caspase-9. When released from mitochondria, Smac/DIA-BLO and Omi/HtrA2 can bind these IAPs and prevent their inhibition of the activated caspases [24-26].

Antiapoptotic Bcl-2 proteins block oligomerization of Bax and Bak, or their associations with BH3-only proteins, thus preventing MOMP [17]. The BH3-only proteins are universally proapoptotic, and each can act either to antagonize antiapoptotic members or activate proapoptotic members. Members of the BH3-only family include Bid, Bad, Bim, Puma, Noxa, Bmf, and several others. BH3-only proteins need to be activated in order to elicit their death signal. The proapoptotic activity of BH3-only proteins appears to be kept in check by either transcriptional control (mainly by p53) or post-translational events. For example, cellular stresses, such as ionizing radiation (IR) or chemotherapy, activate a DNA damage response that stabilizes the p53 tumor suppressor protein. p53 acts to either arrest the cell division cycle by transcriptionally activating the cyclin-dependent kinase inhibitor p21, giving the cell time to repair the damage, or else it helps to mediate apoptotic cell death. p53 also activates proapoptotic genes, including those encoding Bax and the BH3-only proteins Puma, Noxa, and Bid [27]. Another role for p53 has been identified showing that p53 acts directly to increase MOMP by binding Bcl-2 family members and helping mediate Bax and Bak dimerization [28, 29].

The Extrinsic Pathway—The extrinsic pathway is activated by members of two protein families, the tumor necrosis factor (TNF) family and the receptors for these ligands (TNFR) [30]. Most TNF family members bind receptors that activate signals involved in proinflammatory responses and do not signal cell death. The TNF ligands that can induce apoptosis are TNF-α, FasL (also known as CD95L), and TNF receptor apoptosis-inducing ligand (TRAIL; also known as Apo2L) [31, 32]. After extracellular ligand binding, the

cytoplasmic end of the TNFR recruits initiating caspases. TRAIL binding to its death-inducing receptors acts in a manner similar to FasL, while TNF-mediated signaling is more complex [33]. The ligand-bound Fas or TRAIL death receptors (DR4 and DR5) recruit the adapter protein Fas-associating death domain-containing protein (FADD) [34]. Bound FADD recruits initiator caspase-8 and caspase-10, and this assembly of proteins (receptor, FADD, and caspases) is termed the death-inducing signaling complex(DISC) [35]. Recruitment of caspase-8/10 to the DISC leads to their autoproteolytic cleavage [36, 37]. Caspase-8/10 activity can be blocked by a protein with which they share high homology, FLIP (FADD-like interleukin-1β-converting enzyme inhibitory protein). FLIP can oligomerize with caspase-8/10 but lacks critical residues in its caspase domain, including the catalytic cysteine, suggesting it to be a dominant-negative inhibitor. In some cells, named type I cells, activation of effector caspases by activated caspase-8/10 alone is sufficient to induce apoptosis [38]. In type II cells, activated caspase-8/10 stimulates the release of factors from mitochondria. The BH3-only protein Bid connects the extrinsic pathway to mitochondria. Bid is cleaved by caspase-8, resulting in its myristyolization of a newly-exposed glycine residue to form tBid. tBid is then targeted to membranes where it promotes Bax and Bak oligomerization [39, 40].

Apoptotic cell death is as a key element in maintaining immune homeostasis and preventing the emergence of lymphomas or the development of autoimmunity [41]. Cells derived from the hematopoietic progenitor cells ($CD8^+$ T cells, natural killer cells [NK], dendritic cells) have the capacity to mediate cell death through the use of the extrinsic pathway. For example, antigen stimulation of T cells causes the induction of FasL, TNF, and TRAIL that mediates contact-dependent destruction of their targets [42-46]. Cytotoxic immune cells can also induce apoptosis through exocytosis of specialized granules that contain perforin and caspase-like proteases, called granzymes [47]. Entry of these proteins into target cells activates apoptosis, through both caspase-dependent and independent mechanisms.

Necrosis

While apoptosis is increasingly well defined at the molecular level, necrosis has been lacking a molecular signature and has been referred to as a form of cell death that is uncontrolled and pathological. However, recent studies suggest that necrosis is a regulated event that may be involved in multiple developmental, physiological, and pathological scenarios [7, 48, 49]. The fundamental features of necrosis include cellular energy depletion, damage to membrane lipids, and loss of function of homeostatic ion pumps/channels. Unlike apoptosis, in which the Bcl-2 family of proteins and caspases play key roles, necrosis is induced by inhibition of cellular energy production, imbalance of intracellular calcium flux, generation of ROS, and activation of nonapoptotic proteases. These events often potentiate each other and synergize to cause necrosis.

Cells can generate ATP through oxidative phosphorylation or glycolysis. Glycolysis occurs in the cytosol, while oxidative phosphorylation occurs in the mitochondrial matrix. Since cells depend on so many ATP-dependent reactions, ATP levels can rapidly decline if the cell's ability to generate ATP is impaired. ATP depletion results in loss of cellular function and necrosis. Uncoupling the electron transport chain disrupts ATP production, resulting in the depolarization of the mitochondrial inner membrane, referred to as mPT [50, 51]. mPT leads to the loss of the proton gradient and shutdown of ATP generation through oxidative phosphorylation. Increased intracellular Ca^{2+}, inorganic phosphate, alkaline pH, and ROS also can cause mPT. Although mPT has been proposed to mediate apoptosis by inducing the release of mitochondrial apoptogenic factors, persistent opening of the PT pore leads to necrosis [52-54]. Cyclophilin D (CypD), a component of the PT pore, is required for mPT and subsequent necrosis [55-58]. Highly proliferative cells are dependent on glycolysis for energy production, and if glycolysis is inhibited, cellular ATP levels can dramatically

decline. One method for inhibiting glycolysis is hyperactivation of poly(ADP-ribose) polymerase (PARP) following DNA alkylating damage [59].

Cells in an aerobic environment are constantly generating ROS. While physiologic levels of ROS can serve as signaling molecules to regulate transcription, excessive production of ROS leads to oxidative stress, damage of intracellular molecules and organelles, and ultimately necrosis. ROS can damage DNA by causing cleavage of DNA strands, DNA-protein cross-linking, and oxidation of purines [60]. ROS also modify lipids at the multiple double bonds in polyunsaturated fatty acids. Lipid oxidation can lead to the loss of integrity of both the plasma membrane and intracellular membranes of organelles (lysosomes and the endoplasmic reticulum [ER]) that may lead to the influx of Ca^{2+}, or leak of noncaspase proteases, resulting in necrosis [61].

Intracellular Ca^{2+} overload is also an important necrosis inducer. Necrosis can occur following entry of extracellular Ca^{2+} or release of the ER Ca^{2+} into the cytosol. Cell death can be initiated as a result of the activation of Ca^{2+}-dependent proteases and mitochondrial Ca^{2+} overload. Ca^{2+}-mediated necrosis is, so far, the best example of “programmed necrosis.” In *Caenorhabditis elegans*, a gain-of-function (hyperactive) mutant of the DEG/ENaC (denegerin/epithelial Na^{+} channel) family members mechanosensory abnormal (MEC)-4 and MEC-10 (MEC-4(d) and MEC-10(d)) induces necrosis of touch neurons independent of apoptosis regulators [62-64]. In mammalian cells, intracellular Ca^{2+} entry activates Ca^{2+}-dependent proteases such as calpains that cleave the plasma membrane Ca^{2+} exchanger that is required for extrusion of Ca^{2+}, leading to sustained intracellular Ca^{2+} increase [65]. The prolonged cytosolic Ca^{2+} can trigger mitochondrial Ca^{2+} overload, resulting in mPT and ATP depletion, possibly by effecting CypD conformation and the activation of Ca^{2+}-dependent proteases.

Autophagy

Unlike apoptosis and necrosis, autophagy is not synonymous with cell death. Autophagy is evolutionarily conserved and occurs in all eukaryotic cells, from yeast to mammals [66]. Autophagy is activated in response to nutrient starvation, differentiation, and developmental triggers. It is an adaptive process responding to metabolic stresses that results in degradation of intracellular proteins and organelles [5, 67]. During autophagy, portions of the cytoplasm are encapsulated in a double-membrane structure referred to as an autophagosome. Autophagosomes then fuse with lysosomes where the contents are delivered, resulting in their degradation by lysosomal hydrolases. Under normal physiological conditions, autophagy occurs at basal levels in most tissues, contributing to the routine turnover of cytoplasmic components. It can promote cell adaptation and survival during stresses such as starvation, but under some conditions cells undergo death by excessive autophagy.

In yeast, a cassette of autophagy-related genes (referred to as *ATG)* have been identified that regulate autophagy induction, autophagosome formation and expansion, fusion with lysosomes, and the recycling of autophagosome contents [66]. Some of the mammalian orthologs to these genes have been identified. Studies involving *Beclin 1*, the mammalian ortholog of yeast *Atg6*, gave the first indications linking dysfunctional autophagy with tumorigenesis. *Beclin 1* is required for autophagosome formation and has been suggested to be a haploinsufficient tumor suppressor gene. *Beclin 1* +/− mice suffer from a high incidence of spontaneous tumors [68, 69], and *Beclin 1* is monoallelically deleted in a high percentage of sporadic human breast, ovarian, and prostate carcinomas [70].

Several lines of evidence have found that a cross-talk exists between autophagic and apoptotic pathways. Beclin 1 was originally identified through its interaction with Bcl-2 [71]. Recent findings have shown that Bcl-2 and Bcl-xL expression can sensitize cells to

autophagic death induced by etoposide [72], and that Bcl-2 inhibits Beclin 1-mediated autophagy in response to starvation [73]. These contradictory findings suggest that the outcome of the autophagic response may vary depending on the type of insult or cellular stress.

Mitotic Catastrophe

Mitotic catastrophe is a process involving aberrant mitosis resulting from improper segregation of chromosomes during sister chromatid separation. Generally, it is not considered a form of death, but rather an irreversible trigger for death [74]. Eukaryotic cells have complex surveillance mechanisms that monitor the structure of chromosomes and activate multiple signaling pathways after detecting DNA damage. This can result in inhibition of cell cycle progression and activation of DNA repair machinery (checkpoint). If the damage is severe, initiation of a permanent block to the cell cycle occurs (senescence) or the cell is eliminated through a death mechanism. Mitotic death is sometimes used to refer to the execution of a death pathway directly from mitosis. A recently proposed definition for mitotic catastrophe is death that occurs during mitosis, resulting from "a combination of deficient cell-cycle checkpoints (in particular the DNA structure checkpoints and the spindle assembly checkpoint) and cellular damage" [6]. DNA structure checkpoints refer to the activation of cell-cycle arrest in response to DNA damage or unreplicated DNA. These checkpoints occur following activation of kinases in the phosphatidylinositol 3′-kinase (PI3K) family, primarily the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) proteins. These proteins phosphorylate the Chk1 and Chk2 kinases, which phosphorylate a multitude of proteins involved in checkpoint signaling [75]. The spindle assembly checkpoint, also known as the mitotic checkpoint, prevents anaphase (separation of sister chromatids) until all chromosomes have obtained bipolar attachment. The mitotic checkpoint is responsible for the production of genetically identical daughter cells by ensuring accurate chromosome segregation. Chromosomes connect to spindle microtubules through their kinetochores, thereby attaching them to the mitotic spindle. Unattached kinetochores generate checkpoint signals that delay sister chromatid separation until each kinetochore is attached. The advance to anaphase is prevented by inhibiting activation of the anaphase-promoting complex (APC). The APC is an E3 ubiquitin ligase that targets mitotic substrates for proteosome-mediated destruction necessary for anaphase onset.

The mitotic checkpoint was first recognized in experiments using drugs that cause microtubule depolymerization and cause kinetochores to detach [76, 77]. The mis-segregation of many chromosomes results in rapid cell death. Morphologically, mitotic catastrophe is associated with the formation of giant cells with either many micronuclei or two nuclei. Micronucleated cells are nonviable and arise through the formation of nuclear envelopes around clusters of chromosomes or chromosome fragments during catastrophic mitosis. Mitotic DNA damage may also induce cytokinesis failure, leading to binucleation. Cells containing two nuclei are arrested in G_1 in a p53-dependent manner. Damage leading to mitotic catastrophe can be induced by chemotherapeutic drugs that act as microtubule poisons. Defects in genes that function to induce mitotic catastrophe can contribute to tumorigenesis. Since cancer cells are frequently deficient in cell cycle checkpoints, they may be particularly susceptible to the induction of mitotic catastrophe by such drugs.

Senescence

Replicative senescence was first described in the context of normal human cells explanted in culture that failed to divide beyond a finite number of population doublings [78]. One mechanism involved in the activation of replicative senescence is the DNA damage response activated by the shortening of telomeres. Telomeres are repetitive DNA sequences that

protect the ends of chromosomes [9]. After each cell division, telomeres in human cells progressively shorten because of the erosion of the telomeric repeats. Most human adult cells lack sufficient amounts of the enzyme telomerase that adds the telomeric repeats to chromosome ends. When the telomere shortens beyond a certain limit, a DNA damage response is triggered that results in cell cycle arrest [79, 80]. The DNA damage response triggered by other cellular stresses, like exposure to chemotherapeutic agents or oncogenic or mitogenic signals, also can induce senescence, often termed premature senescence, both in vitro and in vivo [81-83]. Therefore, it has been proposed that defects in senescence signaling contributes to tumorigenesis [3]. Indeed, the *p53* and retinoblastoma (*Rb*) tumor suppressor genes have been identified as two principal regulators of senescence [84].

Different classes of chemotherapeutic agents and IR induce senescence in human cancer cell lines in vitro and in mouse tumor xenografts. The molecular signals that play critical roles in mediating DNA damage–induced senescence include p53, Rb, $p16^{INK4A}$, p21, and Bcl-2. Knockout of p53 or p16 eliminates treatment-induced senescence in the *Eμ-myc* lymphoma mouse model [82]. Senescent cells in culture are large in size, appear flattened, and are often vacuolated. The most commonly used marker for senescence is senescence-associated β-galactosidase, which is detected by a colorimetric assay using 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) as a substrate at a pH of 6.0 [85]. This assay is limited in its application, however, because molecular mechanisms that define this activity are not understood. Accumulation of heterochromatic foci that are concentrated spots of transcriptionally silenced DNA have also been seen in senescent cells [86]. Staining of these foci and their microscopic determination is another detection method for senescence.

Targeted Approaches to Activating Cell Death

A basic tenet of cancer biology is that tumorigenesis occurs in part because of genetic changes to cell death–signaling pathways. A paradox of medical oncology is that chemotherapeutic agents work because they kill cancer cells. If cell death pathways aren't functional, how do these agents work? Normal (untransformed) cells respond to most conventional chemotherapeutic agents by undergoing cell-cycle arrest and DNA repair, or if the damage is too severe, death. This process principally depends upon intact p53 signaling. The majority of human cancers have mutations in p53 or defects in p53 signaling [87]. Mutations in the Bcl-2 family are also commonly found. The significance of p53 and the Bcl-2 family and other molecules that drive apoptosis have provided the most opportunities for pharmaceutical exploitation [88, 89]. Some of these efforts have resulted in novel agents now being tested in the clinic. These agents are discussed below as well as others designed to target molecules that can lead not only to apoptosis, but also to alternative cell death pathways (Table 2).

Activating Apoptosis

Bcl-2 Family—Overexpression of antiapoptotic Bcl-2 family members, such as Bcl-2, Bcl-xL, A1, or Mcl-1, is frequently observed in many tumor types and contributes to chemotherapeutic resistance. Several strategies are under investigation to target these antiapoptotic proteins. These include the use of: (a) interfering oligonucleotides to downregulate expression; (b) BH3-only peptides or controlled Bax expression to abrogate protection; and (c) small molecules that can inhibit protective interactions. One agent that is currently the most advanced in clinical trials is oblimersen (Genasense®; Genta Inc., Berekeley Heights, NJ), a nuclease-resistant antisense oligonucleotide targeting Bcl-2 mRNA. Oblimersen is in phase II and III clinical trials treating a wide variety of adult and childhood tumors [90]. Oblimersen was not approved for treatment of melanoma because results from phase III trials showed it did not extend survival [89]. But oblimersen showed a

favorable outcome when combined with docetaxel in patients with hormone-refractory prostate cancer [91].

Intracellular stress signals can activate BH3-only proteins to antagonize antiapoptotic Bcl-2 family members. An attempt to mimic the BH3-only action was the development of BH3 peptides containing the exposed BH3 domains using a chemical strategy termed hydrocarbon stapling. These peptides, named stabilized α-helix of Bcl-2 domains (SAHBs) proved to be protease-resistant and cell-permeable molecules that bind with high affinity to multidomain Bcl-2 member pockets [92]. A SAHB of the BH3 domain from Bid was effective in inhibiting growth of human leukemia xenografts in vivo in short-term assays. A recently described small molecule BH3 mimetic, ABT-737, was identified using a structure-based combinatorial chemical approach to target Bcl-xL, and binds Bcl-xL, Bcl-2, and Bcl-w with very high affinity (50% inhibitory concentration ≤1 nM) [93]. ABT-737 synergized with paclitaxel and the activated BH3-only protein tBid to cause apoptotic cell death. Because overexpression of Bcl-2 and Bcl-xL is the key to many cancers' resistance to apoptotic stimuli, Bid SAHBs or ABT-737 will very likely synergize with other chemotherapeutic agents. Two other compounds that exhibit broad-spectrum inhibition of Bcl-2 family members are currently in phase I trials: GX15-070 and gossypol. Little information is publicly available about GX15-070, a compound developed by GeminX, Inc. (Montreal, Canada) Gossypol is a natural product found in cottonseed oil that historically was investigated for its male contraceptive properties ascribed to its inhibition of adenylate cyclase [94]. It also has been used in phase I/II clinical trials against metastatic breast cancer [95] but showed negligible efficacy and some toxicity. Attempts are underway to improve on gossypol's structure to make a more tolerable compound [96].

Caspases—Another promising approach for targeting apoptosis is to disrupt IAP binding to caspases by developing Smac/DIABLO mimics. Peptides containing the Smac/DIABLO IAP binding motif have been developed, but their usefulness as a therapy is hindered by their rapid degradation in vivo. A clever approach to overcome this obstacle was to create a peptidomimetic composed of non-natural amino acid replacements [97]. One peptidomimetic (named compound 3) has a high affinity for all three IAP proteins. Compound 3 was shown to act synergistically with TRAIL to induce apoptosis in glioblastoma cells in vitro. Design of small molecules that mimic the Smac/DIABLO binding domain have been identified and show significant preclinical promise [98, 99]. These agents described above were designed to mimic Smac binding to the BIR3 domain of XIAP. A different approach focused instead on disrupting XIAP inhibition of caspase 3 and 7 through its BIR2 domain [100]. A chemical screen identified several unique polyphenylureas that succeeded in activating apoptosis, even in cells with Bcl-2/xL overexpression or *bax/bak* deficiency [101]. A third approach, antisense to XIAP (AEG35156/GEM640), recently began Phase I testing as a single agent and in combination with docetaxel (trials ongoing in Canada and the UK).

Directly activating caspases has also been explored for cancer therapy. Adenoviral vectors have been designed to mediate caspase activity in the targeted cell. One example is a genetically modified caspase-9 (iCaspase9) put under the control of a prostate-specific, androgen-responsive promoter and mechanically targeted to tumor xenografts [102]. Administration of an activating drug results in caspase-9 autoproteolysis followed by apoptosis. A similar approach was taken to specifically target the vasculature using a vascular endothelial growth factor receptor 2 promoter driving iCaspase9 in endothelial cells [103]. Drug administration resulted in the specific killing of the tumor vasculature.

TNF—Ever since the discovery of TNF, great attention has been focused on the TNF ligands as mediators of cancer cell death [104-106]. Despite the ability of TNF and FasL to

induce apoptosis in cancer cells, severe toxic side effects preclude both ligands from use in systemic anticancer therapy. Systemic administration of TNF caused an inflammatory response resembling septic shock in humans [107]. FasL or agonistic anti-Fas antibody caused lethal liver injuries in preclinical models [108]. Nevertheless, recombinant TNF was approved for isolated limb perfusion therapy against sarcomas in Europe in 1998 [109]. TNF combined with chemotherapeutic agents such as melphalan shows specificity toward destruction of tumor vasculature and is very effective when used for localized treatment of sarcomas and melanomas [110, 111]. TNF plus melphalan is awaiting approval following phase III clinical trials for use in the U.S.

TRAIL—In contrast to TNF or FasL, recombinant human TRAIL showed no toxicity when systemically administered in rodents and nonhuman primates [112-114]. Recombinant human TRAIL has apoptosis-inducing capacity in a variety of tumor cells in culture and in tumor implants in severe combined immunodeficient mice [115]. Recombinant TRAIL and activating DR4 and DR5 antibodies are currently in phase I/II trials [115-117]. Like most normal cells, many cancer cells are resistant to TRAIL-induced apoptosis. However, many conventional and novel agents can act synergistically when combined with TRAIL. Chemotherapy or irradiation sensitized resistant cells to TRAIL in vitro and in vivo [118-122]. Many cytotoxic chemotherapeutic agents result in DNA damage and other cellular stresses that cause stabilization of the p53 tumor suppressor protein. p53 transcriptionally activates DR5 and other proapoptotic proteins that synergize with TRAIL. Therefore, combining TRAIL with such agents should prove to be a useful therapeutic strategy in tumors harboring functional p53.

An interesting facet of death receptor signaling is that it can occur in the absence of functional p53. Inhibitors of histone deacetylases (HDACIs) can induce apoptosis in cancer cells and are currently in clinical trials. One action of HDA-CIs is the increased expression of TRAIL in acute myeloid leukemia, resulting in selective apoptosis of these cells [123]. HDACIs enhance synthesis of several proteins involved in TRAIL signaling, including DR5, and when combined with TRAIL show the ability to sensitize TRAIL-resistant cells [124, 125]. Both glucocorticoids and IFN-γ also increase DR5 expression, which may enhance TRAIL activity [126]. There are no published reports investigating oblimersen in combination with TRAIL preclinically, but it was shown to sensitize a Fas- and INF-γ–resistant renal cancer cell line to INF-γ combined with a Fas-activating antibody [127].

FLIP—Whether a tumor cell is sensitive to death ligand-induced apoptosis depends on both receptor cell surface expression and an intact apoptotic pathway. FLIP is an important regulator of death receptor signaling, and a compound was recently discovered that reduces FLIP expression. The synthetic oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) was reported to have potent differentiating, antiproliferative, and anti-inflammatory properties and reduce tumor growth in vivo [128, 129]. CDDO activates a pathway resulting in FLIP degradation and caspase-8 cleavage [130-132]. CDDO can cause apoptosis and cell death in a number of different human cancers, but it has shown potent synergy when used in combination with TNF or TRAIL [133-136].

p53—Restoring wild-type p53 signaling to cancer cells is a major therapeutic strategy currently in clinical trials. One attempt is being made to activate an immune response against tumor cells expressing mutant forms of p53. Many trials are also assessing delivery of adenoviral vectors containing wild-type p53 directly to tumors, alone or in combination with IR or conventional DNA-damaging agents. These efforts have been described elsewhere [137]. Pharmaceutical strategies have been devised to reactivate mutant p53 through small molecules or peptides that can restore DNA binding function. Mutant p53 is often expressed at high levels in cancers, unlike wild-type p53, because they often do not

bind to regulators such as the human homolog of mouse double minute 2 (MDM2). It is unclear whether the first small molecules identified to restore DNA binding to p53 mutants, CP-31398 and Prima-1, will enter clinical trials [138, 139]. Another approach has been activation of wild-type p53 function by disrupting p53 interactions with proteins that mediate its degradation. Several small molecules were identified (called Nutlins) that prevent p53 degradation by MDM2, thereby activating p53 function [140].

Activating Necrosis

Noting the fact that many cancers have defective apoptosis machinery, it is reasonable to consider whether activating other death pathways, such as necrosis, may be an effective rationale for cancer therapy. Approaches reported to be able to induce necrotic death in cancer cells include photodynamic treatment (PDT) and alkylating DNA damaging agents. Several other chemicals or drugs, such as β-lapachone, apoptolidin, and honokiol, have been demonstrated to induce cancer cell death through necrosis [141-144]. Some of these approaches were not necessarily designed in a mechanism-based fashion but only later were found to induce necrotic features in the dying cells.

PDT was developed to selectively target abnormal cells while preserving normal surrounding tissues. This is achieved by the administration of exogenous photosensitizing molecules such as porphyrins, chorines, or phthalocyanines to the target cells [145]. The preferential accumulation of certain photosensitizing compounds in tumor cells and the ability to treat only the defined tumor area make PDT a promising therapeutic approach. PDT can induce necrosis at several levels. Upon excitation, the photosensitizers generate ROS (primarily singlet oxygen) that can lead to cell death [146, 147]. When photosensitization compounds localize to the plasma membrane, cells die by necrosis resulting from the loss of plasma membrane integrity [148, 149]. Activation of photosensitizers on lysosomes may disrupt the lysosomal membrane and result in the release of lysosomal proteases leading to necrosis [150]. However, the majority of the PDT lethality appears to result from loss of mitochondrial inner membrane potential [151]. The mPT inhibitor cyclosporine A showed a protective effect during PDT treatment [152, 153].

DNA-damaging agents are the most widely used and effective chemotherapeutic approach to cancer treatment [154]. One important molecule in the DNA damage response is PARP. PARP is activated by DNA strand breaks and facilitates DNA repair enzymes access to damaged DNA. While inhibition of PARP may lead to tumor development by inducing genomic instability [155, 156] or contribute to cell death as an antitumor strategy in cells lacking other components of DNA repair [155, 157, 158], hyperactivation of PARP depletes cytosolic NAD and induces necrosis [59]. This may result in tumor-selective cell death because highly proliferating tumor cells are dependent on cellular NAD to generate energy through aerobic glycolysis [159].

An important feature of necrosis is that unlike apoptosis, necrosis elicits a proinflammatory response. This inflammatory response may help recruit cytotoxic immune cells to the tumor site, thereby increasing the efficacy of the chemotherapeutic drugs. Conversely, the inflammatory response may damage normal tissue or induce the production of mitogenic or prosurvival cytokines, such as high-mobility group box 1 protein (HMGB1) and hepatoma-derived growth factor (HDGF). These molecules can function to activate signaling pathways that promote cell outgrowth in the damaged area and also induce cell migration and associated tumor cell metastasis [160, 161]. This may explain why bad prognoses are often associated with tumors harboring necrotic areas.

Disabling the Mitotic Checkpoint

The mitotic checkpoint was first recognized in experiments using drugs that cause microtubule depolymerization and cause kinetochores to detach [76, 77]. Damage leading to mitotic catastrophe can be induced by chemotherapeutic drugs that act as microtubule-hyperpolymerizing agents (taxanes such as paclitaxel), by microtubule-depolymerizing agents (vinca alkaloids such as vinblastine and vincristine), and by IR. However, since microtubules are essential in other aspects of cellular function, these microtubule poisons disrupt normal function of nondividing cells, resulting in significant side effects such as peripheral neuropathy. A novel targeted approach to activating mitotic catastrophe has focused on proteins that drive mitotic machinery. For example, kinesin spindle protein (KSP)/Eg5 is a member of the kinesin family of microtubule-dependent motor proteins and is required for spindle-pole separation [74]. Several KSP inhibitors have been identified, one of which (SB-715992; ispinesib) is currently in multiple clinical trials alone and in combination with other chemotherapies [162-164]. It is hoped that since these inhibitors only function during mitosis, they may reduce the off-target toxicity of microtubule poisons.

Inhibiting the checkpoint kinase Chk1 also has been gaining momentum as a method for chemosensitization. Because p53 mutations lead to G_1 DNA damage checkpoint defects, they are thought to contribute to increases in cell division and the accumulation of mutations that drive tumor progression. G_1 checkpoint–deficient cells are dependent upon their G_2 checkpoint following DNA damage. Therefore, direct inhibition of the G_2 checkpoint in conjunction with DNA damage provides a possible therapeutic rationale [165]. Although Chk2 contributes to the G_2-M arrest, Chk1 is mainly responsible for the DNA damage response, and inhibitors of Chk1 have been demonstrated to be sufficient to disrupt the mitotic checkpoint and sensitize p53-deficient cells to DNA damaging agents. siRNA studies confirmed the requirement for Chk1 in the G_2-M checkpoint in response to IR and some DNA damaging drugs [166, 167]. UCN-01 (7-hydroxystaurosporine), originally identified and tested in clinical trials as a protein kinase C inhibitor, was also found to inhibit Chk1 [168]. It is currently being tested in several phase I/II trials, alone and in combination with different chemotherapies. Several other Chk1 inhibitors are also in preclinical development [169-172].

Targeting Senescence

Activating senescence is an important therapeutic effect of IR and many commonly used chemotherapies. Little direct attention has been given to targeted therapies that activate a senescence response. Different classes of chemotherapeutic agents and IR induce senescence in human cancer cell lines in vitro and in mouse tumor xenografts. A senescence response in cancer cells following chemotherapy appears dependent upon functioning p53 and p16INK4A pathways [82]. Recent evidence suggests that methylation of histone H2 lysine-9 by the enzyme Suv39h1 also is important in this response [173].

Increased telomerase activity contributes to a cancer cell's loss of senescence controls. Several therapeutic strategies have tried to exploit the fact that tumor cells have high levels of telomerase activity. Telomerase-based therapies include using gene promoters of the various components of telomerase for gene-therapy "suicide" strategies and using telomerase peptides, proteins, or RNA as vaccines for immunotherapy [174]. Other approaches have been examined to inhibit telomerase activity directly. Although tumor cells express telomerase, they typically have short but stable telomere lengths, whereas normal cells do not express telomerase and have long, slowly shortening telomeres. This difference significantly contributes to making cancer cells more sensitive to telomerase inhibitors and may allow for a substantial therapeutic window for telomerase inhibition. One such inhibitor is GRN163L, a lipidated 13-mer oligonucleotide complementary to the RNA template

region of human telomerase RNA (hTR) [175, 176]. Its mechanism of action is not that of antisense-mediated RNase H catalyzed hydrolysis but that it acts as a template antagonist, thus inhibiting telomerase activity. A study showed that mice treated with GRN163L for 3 weeks following tail vein injection of human non-small cell lung cancer cell line A549 had virtually no metastases, while control mice had many [177].

Targeting Autophagy—To Activate or Inhibit?

The role of autophagy in cancer and in the response to chemotherapeutic agents is slowly gaining attention. A number of studies have reported that autophagy, or autophagic cell death, is activated in cancer cells derived from a variety of tissues in response to various anticancer therapies [178, 179]. For example, tamoxifen induces autophagic cell death in cultured breast cancer cells, in part, through a down-regulation of Akt [180]. Autophagy also plays a role in protecting some cells from chemotherapy-induced death. Bafilomycin A1 can inhibit autophagy by preventing the fusion of autophagosomes and lysosomes [181]. It is a V-type ATPase inhibitor that prevents acidification of lysosomal-based compartments, including autophagosomes [182]. Bafilomycin A1 inhibited γ-irradiation–induced autophagy in cancer cell lines derived from different tissues, and increased the amount of IR-induced death [183]. When apoptosis was inhibited in mouse fibroblasts by a caspase-8 inhibitor, autophagic cell death, dependent on Atg7 and Beclin 1 activity, was induced, and autophagy inhibitors decreased the amount of cell death [184]. One agent currently used clinically that can specifically target the autophagy pathway is rapamycin. Rapamycin inhibits mammalian target of rapamycin (mTOR), a kinase found to suppress autophagy. mTOR inhibitors are being evaluated in a large number of clinical trials. mTOR functions downstream of PI3K and Akt in growth factor signaling. When activated, mTOR phosphorylates targets that prevent autophagy induction and permits active mRNA translation [5]. mTOR is involved in many cellular processes, including apoptosis, and it is unclear what role autophagy plays in mediating inhibition of mTOR's effects.

Conclusions

Many novel strategically designed chemotherapeutic agents are now entering clinical trials with many more to follow. They were designed to target molecules that play critical roles in the execution of one or more cell death pathways. These new agents will provide a myriad of more choices for rationally based chemotherapeutic combinations that have the potential to kill even the most deadly tumors. But in order for novel combinations to succeed, a deeper understanding of how a given chemotherapy affects all of the signaling pathways involved in cell death is needed. Because the different death pathways involve some of the same signaling molecules, care must be taken when designing combinations of targeted therapies (Fig. 1). Attempts must be made to accurately predict whether drug actions will be cooperative or interfering based on the status of the molecules and the pathways being targeted.

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Learning Objectives

After completing this course, the reader will be able to:

1. List the multiple cell death pathways that are activated in response to chemotherapeutic agents.
2. Identify signaling molecules involved and morphological changes that occur in the different types of cell death pathways.
3. Describe mechanisms targeted by novel chemotherapeutic agents.

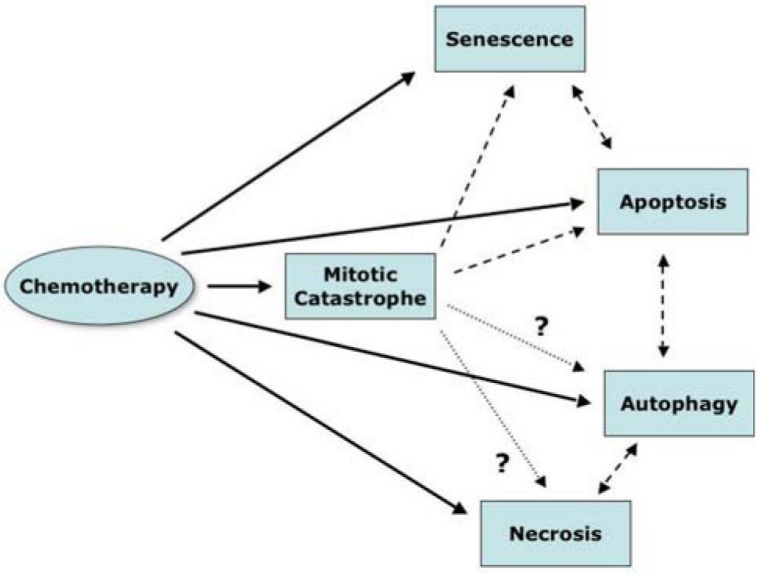

Figure 1.
Chemotherapies activate multiple signaling pathways that can lead to different cell death outcomes. Understanding how these pathways cooperate and interfere is essential for the design of rationally-based chemotherapeutic combinations.

Table 1

Cell death pathway characteristics

	Apoptosis	Autophagy	Necrosis	Senescence	Mitotic catastrophe
Morphologic changes					
Cell membrane	Blebbing, membrane integrity maintained	Blebbing	Loss of membrane integrity	Flattening; increase in cell size	?
Nucleus	Chromatin condensation, DNA laddering, nuclear fragmentation	Partial chromatin condensation, no DNA laddering	Random DNA degradation	Accumulation of heterochromatin foci	Mis-segregation of chromosomes during cytokinesis; micronuclei
Cytoplasm	Condensed membrane-bound cellular fragments; depolymerization of cytoskeleton	Increased number of autophagic vesicles, degradation of Golgi, polyribosomes, and the ER	Swelling of cellular organelles	Granularity	?
Detection methods	Annexin V staining, DNA fragmentation assays, caspase activation	LC3 localization	Early permeability to vital dyes, release of intracellular contents	Senescence-associated β-galactosidase activity	Visualization of multinucleated cells
Release of cellular contents	Lysophosphatidylcholine	?	HMGB1, S100 molecules, purine metabolites, heat-shock proteins, uric acid, HDGF	?	?
Immunologic response	Suppressive, engulfment of cell carcass	?	Stimulatory, initiation of cell growth and tissue repair	?	?

Abbreviations: ER, endoplasmic reticulum; HDGF, hepatoma-derived growth factor; HMGB1, high-mobility group box 1 protein; LC3, microtubule-associated protein 1, light chain 3.

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Table 2

Selective chemotherapeutic agents targeting cell death pathways

Death pathway	Protein classification	Chemotherapeutic agent	Target	Type of compound	Sponsoring organization	Stage
Apoptosis	Bcl-2	Oblimersen	Bcl-2	Antisense oligonucleotide	Genta/Aventis	Phase II/III
		SAHBs	Bcl-2 family	Peptidomimetic	Harvard University	Preclinical
		ABT-737	Bcl-2/Bcl-xL	Small compound	Abbott	Preclinical
		Gossypol	Bcl-2	Small compound	NCI/Ascenta	Phase I
	IAP	Compound 3	IAPs	Small compound	University of Texas Southwestern	Preclinical
		AEG 35156/ GEM640	XIAP	Antisense oligonucleotide	Aegera/Hybridon	Phase I (outside U.S.)
		Polyphenylureas	XIAP/caspase-3, -7	Small compound	The Burnham Institute	Preclinical
	p53	Nutlins	p53/MDM2	Small compound	Hoffman-La Roche	Preclinical
	Death receptors	TRAIL	DR4/DR5	Recombinant protein	Genentech/Amgen	Phase I
		HGS-ETR1	DR4	Agonistic mAb	Human Genome Sciences	Phase II
		HGS-ETR2	DR5	Agonistic mAb	Human Genome Sciences	Phase II
		TNF-α	TNFR	Recombinant protein		FDA approved, limb perfusion
		CDDO	FLIP	Small compound	Dartmouth College/Reata	Preclinical
Necrosis		PDT	Metabolism, ROS, Ca^{2+}			Clinical
	PARP	DNA alkylating agents	DNA damage, metabolism			Clinical
		β-Lapachone	Metabolism, ROS, Ca^{2+}	Natural compound		Preclinical
Mitotic catastrophe	Mitosis	SB-715992	KSP/Eg5	Small compound	Cytokinetics/GSK	Phase II
		UCN-01	Chk1	Small compound	NCI	Phase I/II
		CEP-3891	Chk1	Small compound	Cephalon	Preclinical
		SB-218078	Chk1	Small compound	GSK	Preclinical

Death pathway	Protein classification	Chemotherapeu-tic agent	Target	Type of compound	Sponsoring organization	Stage
		A-641397	Chk1	Small compound	Abbott	Preclinical
Senescence	Telomerase	GRN163L	hTR	Antisense oligonucleotide		Preclinical

Abbreviations: CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; DR, death receptor; FLIP, FADD-like interleukin-1β-convert enzyme inhibitory protein; GSK, GlaxoSmithKline; hTR, human telomerase RNA; IAP, inhibitor of apoptosis; KSP/Eg5; mAb, monoclonal antibody; MDM2, mouse double minute 2; mTOR, mammalian target of rapamycin; NCI, National Cancer Institute; PARP, poly(ADP-ribose)-polymerase; PDT, photodynamic treatment; ROS, reactive oxygen species; SAHBs, stabilized α-helix of Bcl-2 domains; TNF-α, tumor necrosis factor alpha; TNFR, TNF receptor; TRAIL, TNF receptor apoptosis-inducing ligand; XIAP, X-linked IAP.