Targeting Apoptosis Pathways in Cancer Therapy

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ABSTRACT Apoptosis, or programmed cell death, is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. The understanding of apoptosis has provided the basis for novel targeted therapies that can induce death in cancer cells or sensitize them to established cytotoxic agents and radiation therapy. These novel agents include those targeting the extrinsic pathway such as tumor necrosis factor-related apoptosis-inducing ligand receptor 1, and those targeting the intrinsic Bcl-2 family pathway such as antisense bcl-2 oligonucleotides. Many pathways and proteins control the apoptosis machinery. Examples include p53, the nuclear factor kappa B, the phosphatidylinositol 3-Kinase (PI3K)/Akt leading to defects in apoptosis. This review will discuss the current knowledge about the pathways and new treatments that target them.
Materials and Methods

Original data for inclusion in this review were identified through a MEDLINE and PubMed search of the literature. All articles from 1966 through February 2004 were identified by use of the following search terms: apoptosis, cancer therapy, and signal transduction modulators. All original research and review articles related to apoptosis and therapeutic interventions relating to apoptotic signaling were identified. This search was supplemented by a manual search of the proceedings of the annual meetings of the American Association for Cancer Research, the American Society of Clinical Oncology, and the American Society of Hematology on New Anticancer Drugs.

The Apoptosis Pathways

There are two major mechanisms of cell death—necrosis and apoptosis. Cells that are damaged by external injury undergo necrosis, while cells that are induced to commit programmed suicide because of internal or external stimuli undergo apoptosis. Although understanding of the detailed signaling pathways that trigger apoptosis is incomplete, this process is controlled by a number of complex proteins, which are activated by various triggers and arranged in sequential signaling modules. Apoptosis occurs through two main pathways. The first, referred to as the extrinsic or cytoplasmic pathway, is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily. The second pathway is the intrinsic or mitochondrial pathway that when stimulated leads to the release of cytochrome-c from the mitochondria and activation of the death signal. Both pathways converge to a final common pathway involving the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell (Figure 1). The pathways are linked; thus, distinction between the two pathways is simplistic. Overexpression of Bcl-2 in the intrinsic pathway may lead to the inhibition of extrinsic mediated apoptosis; conversely, TNFα may increase the expression of NFκB and stimulate antiapoptotic members of the Bcl-2 family proteins.

The Extrinsic Pathway: Fas

This pathway comprises several protein members including the death receptors, the membrane-bound Fas ligand, the Fas complexes, the Fas-associated death domain, and caspases 8 and 10, which ultimately activate the rest of the downstream caspases leading to apoptosis (Figure 1). Activation of the extrinsic pathway is initiated with the ligation of cell surface receptors called death receptors (DRs). Fas is a member of the tumor necrosis factor receptor superfamily and is also called Apo-1 or CD95. Other TNF receptors include TNF R1, DR3 (Apo 2), DR4 (tumor necrosis factor-related apoptosis-inducing ligand receptor 1 [TRAIL R1]), DR5 (TRAIL R2), and DR6. Fas signaling plays an important role in immune surveillance of transformed or virus-infected cells and in the removal of self-reactive lymphocytes. Therefore, defects in this pathway have been implicated in many malignancies and autoimmune diseases.

The Fas ligand (FasL)-Fas system is mainly recognized for its death-related functions, but it is also involved in several proliferative and inflammatory signaling pathways that are not well defined. When a death stimulus triggers the pathway, the membrane-bound FasL interacts with the inactive Fas complexes and forms the death-inducing signaling complex. The Fas death-inducing signaling complex contains the adaptor protein Fas-associated death domain protein and caspases 8 and 10 and leads to activation of caspase 8, which in turn can activate the rest of the downstream caspases. In some cells, the activation of caspase 8 may be the only requirement to execute death, while in other cell types, caspase 8 interacts with the intrinsic apoptotic pathway by cleaving Bid (a proapoptotic member of the Bcl-2 family), leading to the subsequent release of cytochrome-c.

Several pathways and proteins regulate the activation of the extrinsic pathway. Dysregulation of these modulators may also lead to malignant transformation, as mutations or deletions of the
Fas gene have been found in some hematologic malignancies. Regulators of the pathway include transcription factors such as NFκB and activating protein 1 that regulate the FasL gene, because it is a transcriptionally inactive gene. Other inhibitors of the pathway include FAP-1, Fas–associated–death–domain–protein like interleukin–1β–converting enzyme–like inhibitory protein, and the soluble decoy receptors such as DcR3, TRAIL R-3/DcR1, and TRAIL R-4/DcR2. These decoy receptors antagonize the stimulation of Fas by FasL though competition with the ligand.

The Intrinsic Pathway

One of the most important regulators of this pathway is the Bcl-2 family of proteins. The bcl-2 gene was originally identified at the chromosomal breakpoint of the translocation of chromosome 18 to 14 in follicular non-Hodgkin lymphoma (NHL). The Bcl-2 family are key regulators of apoptosis and are overexpressed in many malignancies even without the presence of the t (14; 18) chromosomal translocations. Increased expression of Bcl-2 causes resistance to chemotherapeutic drugs and radiation therapy, while decreasing Bcl-2 expression may promote apoptotic responses to anticancer drugs. In addition, overexpression of Bcl-2 may result in accumulation of cells in the G0 phase of cell cycle division and contribute to chemoresistance.

The Bcl-2 family includes proapoptotic members such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk, and antiapoptotic members such as Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1. Antiapoptotic Bcl-2 members act as repressors of apoptosis by blocking the release of cytochrome-c, whereas proapoptotic members act as promoters. These effects are more dependent on the balance between Bcl-2 and Bax than on Bcl-2 quantity alone.
Following a death signal, proapoptotic proteins undergo posttranslational modifications that include dephosphorylation and cleavage resulting in their activation and translocation to the mitochondria leading to apoptosis. All BH3-only molecules require multidomain BH3 proteins (Bax, Bak) to exert their intrinsic proapoptotic activity. In response to apoptotic stimuli, the outer mitochondrial membrane becomes permeable, leading to the release of cytochrome-c and second mitochondria-derived activator of caspase (also called direct IAP-binding protein with low pi). Cytochrome-c, once released in the cytosol, interacts with Apaf-1, leading to the activation of caspase-9 proenzymes. Active caspase-9 then activates caspase-3, which subsequently activates the rest of the caspase cascade and leads to apoptosis. Activated caspases lead to the cleavage of nuclear lamins and breakdown of the nucleus through caspase-3

The Final Pathway: Caspases

The final pathway that leads to execution of the death signal is the activation of a series of proteases termed caspases. Not all caspases are involved in apoptosis. The caspases that have been well described are caspases-3, -6, -7, -8, and -9. The intrinsic and extrinsic apoptotic pathways converge to caspase-3, which cleaves the inhibitor of the caspase-activated deoxyribonuclease, and the caspase-activated deoxyribonuclease becomes active leading to nuclear apoptosis. The upstream caspases that converge to caspase-3 are caspases-9 and -8 in the intrinsic and extrinsic pathways, respectively. The downstream caspases induce cleavage of proteins such as cytoskeletal proteins, DNA repair proteins, inhibitory subunits of endonucleases (CIDE family), and finally, destruction of “housekeeping” cellular functions. Caspases also affect cytoskeletal structure, cell cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation and fragmentation, and membrane blebbing.

Regulation of the Apoptotic Proteins

The extrinsic and intrinsic apoptotic pathways are regulated by proteins such as the p53, NFκB, the ubiquitin proteosome system, and the PI3K pathway (Figure 2). These will be briefly described because of their relevance to the new drugs discussed in this review.

p53

p53 functions as a transcription factor regulating downstream genes important in cell cycle arrest, DNA repair, and apoptosis. The critical role that p53 plays is evident by the large number of tumors that bear a mutation in this gene. Loss of p53 in many cancers leads to genomic instability, impaired cell cycle regulation, and inhibition of apoptosis. After DNA damage, p53 holds the cell at a checkpoint until the damage is repaired. If the damage is irreversible, apoptosis is triggered. The mechanism by which p53 promotes apoptosis is still not fully understood.

NFκB

NFκB is a nuclear transcription factor that regulates expression of a large number of genes involved in the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and many autoimmune diseases. NFκB is activated by a variety of stimuli that include growth factors, cytokines, lymphokines, radiation, pharmacologic agents, and stress. In its inactive form, NFκB is sequestered in the cytoplasm, bound inhibitor proteins of the IκB family. The various stimuli that activate NFκB cause phosphorylation of IκB, which is followed by its degradation. This results in exposure of the nuclear localization signals on NFκB subunits and the subsequent translocation of the molecule to the nucleus. In the nucleus, NFκB binds with the consensus sequence of various genes and thus activates their transcription.

NFκB has been shown to have both anti- and proapoptotic functions that may be determined by the nature of the death stimulus rather than by the origin of the tissue. Under physiologic conditions, the activation of NFκB induces resistance to apoptotic stimuli through the activation of many complex proteins including TNF receptor-associated factor, IAP, and X-linked IAP. However, in response to certain stimuli, NFκB activation may lead to
induction of apoptosis. This may be explained by the activation of some proapoptotic proteins such as interferon-regulated factor-1, c-myc, p53, and caspases such as caspase 1. In some viral infections, induction of apoptosis by the virus is dependent on NFκB activation.

The Ubiquitin/Proteosome System

The ubiquitin/proteosome system is composed of a large proteinase complex that is responsible for the turnover of most intracellular proteins and consequently regulates cell growth and apoptosis. Protein degradation is a highly coordinated process that involves recognition of the protein by attaching it to multiple ubiquitin molecules and then its digestion by the 26S proteosome. Many cell cycle regulators and transcription factors such as p53, cyclins and cyclin-dependent kinase inhibitors, and NFκB are regulated by the ubiquitin/proteosome system.

In addition, many of the Bcl-2 family members are substrates of the ubiquitin/proteosome. The induction of apoptosis by proteosome inhibitors leads to an initial accumulation of proteins such as p53, p27, proapoptotic Bad or Bax, or activation of the stress kinase, which leads to the release of cytochrome-c and the activation of the intrinsic apoptosis pathway. This critical link between the apoptotic machinery and the ubiquitin/proteosome system has led to an increased interest in the design of inhibitory agents that target these pathways.

PI3K

PI3K is a kinase that plays a central role in signaling pathways important to cell survival, proliferation, motility, and tissue neovascularization. PI3K is upregulated in many cancers. Cell-surface receptors induce the production of second messengers such as phosphatidylinositol 4,5-bisphosphate 3 and phosphatidylinositol 3,4,5-
trisphosphate, which convey signals to the cytoplasm from the cell surface. Phosphatidylinositol 4,5-bisphosphate 3 signals activate the kinase 3-phosphoinositide-dependent protein kinase-1, which in turn activates the kinase Akt. Akt activation leads to phosphorylation of certain proteins that lead to cell survival. For example, phosphorylation of IκB by Akt leads to activation of NFκB that promotes survival. Phosphorylation of Bad by Akt leads to its inactivation and blocking of the apoptotic signal. In addition, phosphorylation of caspase 9 blocks the induction of apoptosis. Akt also phosphorylates the protein Forkhead-related transcription factor 1. Several proteins important in human cancers can be dysregulated in the PI3K pathway. These are also important because of Food and Drug Administration (FDA)-approved agents targeting them. For example, epidermal growth factor stimulation activates Akt via PI3K, as does HER-2/neu activation. Loss of phosphatase and tensin homolog tumor suppressor gene also augments the activity of this pathway.

The mammalian target of rapamycin (mTOR) is downstream of Akt and is a target for novel therapies. The inhibition of mTOR blocks signals to two downstream pathways, the 40S ribosomal protein S6 kinase (p70S6k) and the 4E-binding protein-1 (4E-BP1). These control cell cycle progressions from G1 to S phase, and their inhibition leads to growth arrest. Abnormalities in cyclin D, p53, pRB, p16, and p27 can increase the PI3K activity in many cancer cells and make them more susceptible to mTOR inhibitors such as CCI779.

**THERAPEUTIC AGENTS**

We have described the regulation of apoptotic proteins and their pathways in a simplistic overview. Many of the proteins involved in apoptosis have redundant functions, and many pathways include signals that are involved in both the extrinsic and intrinsic pathways. Thus, selective blockers of these pathways may not be enough to induce apoptosis.

Several agents have directly or indirectly been implicated in the inhibition of apoptosis-related proteins. For example, epidermal growth factor inhibitors are not usually considered as proapoptotic agents. However, these agents control apoptosis indirectly through the PI3K/Akt pathway. In addition, CI-1,040, a mitogen-activated protein kinase inhibitor, can induce apoptosis through regulation of the downstream protein extracellular signal-regulated kinase that signals to the intrinsic apoptosis pathway (Table 2).

In the next section, we will discuss agents that primarily target the apoptotic machinery. Some of these therapeutic agents are in clinical use or in clinical trials, while others are still in preclinical stages. Table 1 summarizes many of these novel agents, their molecular targets, the pathway through which they control apoptosis, and their stage of development. These agents can be classified as those agents that target the extrinsic pathway, the intrinsic pathway, the common pathway (through caspases), or the proteins regulating apoptosis (Table 1 and Figure 3). Another approach is to classify the agents as either inducers of apoptosis or inhibitors of antiapoptotic proteins. We will describe the agents according to the former classification.

**Agents That Target the Extrinsic Pathway**

These include recombinant human TRAIL, monoclonal antibodies agonistic to Dr4 and Dr5, and all trans retinoic acid (ATRA).

**TRAIL**

Death receptors have been pursued as potential targets for cancer therapy for many years. TNF, Fas, and Fas L have extensive in vitro antitumor activity and have been utilized as potential therapeutic targets in vivo. Unfortunately, they were also found to activate nonspecific TNF receptors resulting in extensive ischemic and hemorrhagic lesions in several tissues leading to septic shock and fulminant hepatic failure in animal models.

TRAIL, or APO2L, has been introduced as an extrinsic pathway inducer that does not have the toxicities of Fas and TNF. TRAIL induces apoptosis in a variety of tumor cell types and suppresses the growth of colon and
breast xenografts. Synergistic antitumor effects are also seen when combined with chemotherapy or radiation.\textsuperscript{47,48} TRAIL was found to induce apoptosis in human hepatocytes\textsuperscript{51} and in human brain cells in vitro; however, it did not lead to apoptosis in the brain of animals in preclinical studies.\textsuperscript{47,48} Preclinical testing of TRAIL in combination with conventional chemotherapeutic agents such as doxorubicin has demonstrated significant inhibition of tumor growth in a prostate cancer in vivo model.\textsuperscript{52}

**Monoclonal Antibodies**

Monoclonal antibodies (HGS-ETR1, HGS-ETR2, and HGS-TR2J) with agonist function...
at the Dr4 (TRAIL R1) and Dr5 (TRAIL R2) sites may also induce apoptosis and caspase activation and have shown tumor regression in colon tumor xenograft models. The potential advantage of these antibodies is their long half-life compared with Apo2L/TRAIL, which potentially may translate into a less frequent administration schedule.53 Phase I and Phase II trials in patients with advanced solid tumors, nonsmall cell lung cancer, colon cancer, and NHL are in progress. The antibodies are being studied as single agents and in combination with cytotoxic chemotherapy.54–58

**ATRA**

ATRA is one of the first examples of targeted therapy in human cancer. It is a liposomal form of tretinoin that is administered intravenously. In acute promyelocytic leukemia (APL), a dose of 90 mg/m² intravenously every other day has been given for induction, followed by maintenance with the same dose three times weekly for nine months. An intravenous dose of 120 mg/m² three times weekly has been administered in acquired immunodeficiency syndrome-related Kaposi sarcoma. ATRA induces differentiation in APL by modulation of the PML-RARα protein. It is also thought to induce apoptosis through the extrinsic pathway by inducing the paracrine release of membrane-associated TRAIL, which leads to apoptosis in ATRA-treated APL cells and in adjacent non-ATRA responsive and non-APL cells.59 One of the main side effects of ATRA is the ATRA syndrome that is characterized by respiratory distress, fever, pulmonary infiltrates, and pleural effusions, and it occurs in up to 26% of patients treated with the drug alone, especially in the presence of leukocytosis of more than 10,000 cells/μl. Treatment of APL with ATRA alone or in combination with chemotherapy
yields a complete remission rate as high as 85% to 95%.60,61

Agents That Target the Intrinsic Pathway

These include agents that act directly on the mitochondrial inner membrane, agents that antagonize the antiapoptotic members of the Bcl-2 protein family, and agents that enhance the activity of the proapoptotic members of the Bcl-2 family of proteins such as Bax.

Agents That Target the Mitochondrial Inner Membrane

Arsenic Trioxide

Arsenic trioxide targets the PML-RAR α protein. At higher concentrations, it induces apoptosis of the leukemic cells by disruption of the mitochondrial inner membrane potential, leading to inhibition of Bcl-2 expression and elevation of caspase-3. At lower concentrations, it triggers differentiation, and at both concentrations, it causes degradation of PML-RAR α protein.61 This drug is FDA approved for the treatment of APL.62 Phase II trial results for multiple myeloma, adult T-cell leukemia/lymphoma, and renal cell carcinoma have been published, and studies of other malignancies (including nonsmall cell lung cancer, gliomas, breast cancer, and esophageal cancer) are underway.63–65 The main side effects include QT prolongation, torsade de pointes, congestive heart failure, hypokalemia, hypomagnesemia, and leukocytosis.

Lonidamine

This investigational agent is a derivative of indazole-3-carboxylic acid that acts on the mitochondrial to induce apoptosis through the disruption of the intrinsic transmembrane potential. It has a potent antiproliferative effect on neoplastic cells by inhibiting oxygen consumption and interfering with the energy metabolism of neoplastic cells.66 This drug has been shown to potentiate the cytotoxic effects of chemotherapeutic agents, especially anthracyclines in human breast cancer cell lines. It also potentiates radiotherapy. Lonidamine is given orally as 450 mg/day. The most frequent toxicities are gastrointestinal and hematologic side effects. A Phase II trial of lonidamine in combination with epirubicin and cisplatin as a first line therapy for metastatic breast cancer has shown an overall response rate of 73% with 13% complete response for a median of 9.8 months.67 However, a multicenter prospective randomized trial of lonidamine with high-dose epirubicin and cyclophosphamide showed no improvement in the disease-free survival and overall survival in patients with metastatic breast cancer over epirubicin and cyclophosphamide alone.68 The combination of lonidamine and cytotoxic chemotherapy was active in nonrandomized Phase II trials against advanced nonsmall cell lung cancer and ovarian cancer.69,70 In the absence of cytotoxic drugs, lonidamine showed little activity against nonsmall cell lung cancer or glioblastoma multiforme.71,72

Agents That Target Bcl-2

The bcl-2 gene is overexpressed in most follicular B-cell lymphoma, CLL, and about 25% of B- large cell NHL.73 The Bcl-2 protein is overexpressed in many solid organ malignancies that do not harbor the t(14;18) translocation such as prostate cancer, breast cancer, nonsmall cell lung cancer, small cell lung cancer, and melanoma.74 Bcl-2 also plays a critical role in regulating response to chemotherapy, hormonal therapy, and irradiation.75 The clinical importance of this antiapoptotic protein has stimulated interest in using antisense therapy to modulate its expression. Agents that can affect the Bcl-2 protein include antisense oligonucleotides such as G3,139 (Genasense [Genta, Inc., Berkeley Heights, NJ], oblimersen sodium), small molecules that recognize the surface pocket of Bcl-2 or Bcl-xL, and antisense Bcl-xL, which is in preclinical development.

Oblimersen Sodium (G3,139, Genasense)

Genasense is a phosphothioate oligonucleotide consisting of 18 modified DNA bases. The single-stranded DNA molecule must first be in-
corporated into the cell by endocytosis and then target the mRNA by having a complementary sequence to it. Bcl-2 mRNA fragments are subsequently destroyed by ribonucleases. The effect of G3,139 is dose dependent and leads to degradation of bcl-2 mRNA and subsequent inhibition of Bcl-2 protein expression. In vitro and in vivo studies have demonstrated antitumor activity. Genasense also potentiates the effect of various chemotherapeutic agents including antimetabolites, alkylators, corticosteroids, radiation, and monoclonal antibodies. Waters et al described the first Phase I studies with single agent G3,139. Twenty-one patients with NHL received subcutaneous infusion of G3,139 with dose escalation from 4.6 mg/m²/day to 195.8 mg/m²/day. The maximum tolerated dose (MTD) was 5.3 mg/kg/day. Other Phase I studies in solid tumors and patients with CLL were subsequently performed. The most common toxicities from multiple studies included fatigue, transient liver function abnormalities, and thrombocytopenia. Interestingly, there have been discrepancies seen in the MTD between hematologic malignancies and solid tumors. Doses of 7 mg/kg/day that are well tolerated in solid tumor studies were poorly tolerated in a Phase I-II study of 14 patients with CLL. These effects may be secondary to the antilymphocytic effect of G3,139, leading to the release of cytokines and the development of tumor lysis syndrome. A large randomized Phase III trial was conducted in patients with melanoma who had previously received chemotherapy. The patients were randomized to receive dacarbazine plus G3,139 or dacarbazine alone. Four hundred and eighty patients were followed for approximately one year. The median overall survival for patients was 9.1 month for the G3,139 plus dacarbazine group versus 7.9 months for the dacarbazine arm (P = 0.18). For patients who were followed for more than one year, overall survival was superior for the G3,139/dacarbazine arm versus dacarbazine alone (10.1 months versus 7.9 months). Response to therapy (CR and PR) was achieved in 11.7% of patients treated with G3,139 compared with only 6.8% for those treated with dacarbazine alone. The average duration of progression-free survival was improved over 50% (78 days) in the G3,139 group compared with 49 days for the dacarbazine group. However, the FDA advisory committee did not recommend marketing approval of Genasense, and the company withdrew the application for new drug application in May 2004. Other Phase III trials have been conducted in CLL and multiple myeloma. A Phase III randomized trial of fludarabine/cyclophosphamide chemotherapy with or without G3,139 for patients with relapsed or refractory CLL was recently presented. Major responses occurred in 16% of the 124 patients in the G3,139 arm compared with 7% of the 124 patients in the control arm. However, progression of disease was worse in the G3,139 arm, with a median time to progression of 6.1 months compared with 8.9 months in the control arm. Another Phase III randomized trial of dexamethasone with or without G3,139 in patients with advanced multiple myeloma did not achieve statistical significance for the primary endpoint, which was time to development of progressive disease. Overall, G3,139 has demonstrated modest responses in melanoma and CLL but not in multiple myeloma. The drug has been denied FDA approval for malignant melanoma therapy.

**Antisense Bcl-xL**

Bcl-xL shares high sequence homology regions with Bcl-2 but exhibits a different biological role than that of Bcl-2. These two proteins can be coexpressed in many tumors. Antisense bcl-xL oligonucleotide and bispecific antisense bcl-2/bcl-xL oligonucleotide, targeting the mRNA homology region of both bcl-2 and bcl-xL, are in preclinical development. The bispecific antisense oligonucleotide simultaneously downregulates Bcl-2 and Bcl-xL expression, induces apoptosis, and inhibits growth of different tumor types in vitro and in vivo.

**Antisense Clusterin**

Clusterin is also known as testosterone-repressed prostate message 2. It is a glycoprotein that is expressed widely in various tissues, and its function has been linked with cell response to stress. It has also been implicated in many pathologic processes including preven-
tion of apoptosis. Clusterin exerts paradoxical pro and antiapoptotic functions, which may be related to two different isoforms of the protein, a cytoplasmic and a nuclear form. Because of its important role, the inhibition of clusterin using sequence-specific antisense oligonucleotides has been shown in both in vitro and in vivo models to enhance chemotherapy effects in hormone refractory prostate cancer.

Small Molecules That Recognize the Surface Pocket of Bcl-2 or Bcl-xL

X-ray crystallography and nuclear magnetic resonance spectroscopy have discovered a surface pocket on the antiapoptotic Bcl-XL protein that interacts with the BH3 domain of death agonists of the Bcl-2 family. There are natural products and synthetic organic peptides that recognize the surface pocket of Bcl-2 and Bcl-xL. The natural products are identified from random screening processes such as Antimycin-A. These products provide important templates for the development of specific inhibitors targeting Bcl-2 or other proteins in the intrinsic pathway. The synthetic BH3 organic peptides designed by computer-aided techniques have demonstrated in vitro activity in inducing apoptosis. Newer molecules with reduced molecular size for better cell membrane permeability and enhanced biological half-life are being designed. For example, HA14-1, which binds to a surface pocket of the Bcl-2 protein, is in preclinical development.

Agents That Modulate the Proapoptotic Proteins Bax and Bcl-xs

The activation of the proapoptotic Bax protein can be induced by gene therapy through the delivery of Bax vectors. However, because of the proapoptotic property of the gene, adenoviral vectors expressing the Bax gene are difficult to construct. Recently, with different molecular techniques, vectors have been constructed, and this approach has been successful in inducing apoptosis in cancer cell lines. The use of Bcl-xs gene therapy can be promising in inducing tumor regression.

Agents That Target the Common Pathway: Caspases Activators

These include synthetic activators of caspases, Apoptin, and IAP targets such as survivin.

Synthetic Activation of Caspases

Synthetically engineered caspase-activating agents, named death switches, can be produced by the fusion of one or more chemically inducible dimerization domains. These drugs lead to protein aggregation inside the cell that induces caspase activation and downstream apoptosis. The activation of caspases 1 and 3 may be sufficient to induce death in the cells. These agents are in preclinical development.

Apoptin

Another caspase-inducing agent is Apoptin. This is a protein derived from chicken anemia virus that can result in selective apoptosis in malignant cells and not in normal cells. This selectivity may be due to the nuclear localization of the protein in tumor cells that is necessary for its activation. In normal cells, the protein localizes in the cytoplasm. This drug is still in preclinical testing.

Targeting IAP, Survivin

Survivin is a member of the IAP family that plays a dual role in suppressing apoptosis and regulating cell division. The precise role of survivin remains to be defined, but it may prevent apoptosis through the inhibition of caspases by direct or indirect binding. Survivin contains a baculovirus inhibitor of apoptosis repeat protein domain that is the active domain for inhibition of apoptosis. Interestingly, survivin is expressed in embryonic tissues as well as in the majority of human cancers and some premalignant tissues, but it is not expressed in most normal adult tissues. Moreover, the protein may be involved in cell resistance to chemotherapeutic agents and ionizing radiation. The selective expression of survivin as well as its important antiapoptotic function have led to further studies of this
molecule as a useful diagnostic marker of cancer and a potential target for cancer treatment.\textsuperscript{92–94} In vitro and in vivo studies have shown survivin to induce apoptosis, reduce tumor growth potential, and sensitize tumor cells to chemotherapeutic drugs. This agent is showing promising results in preclinical testing and will enter clinical testing in the near future.\textsuperscript{90}

**Agents That Target Modulators of the Apoptosis Pathways**

These include proteosome inhibitors, mTOR inhibitors, and p53 inhibitors (Table 1, Figure 3).

**Proteosome Inhibitors**

The ubiquitin/proteosome system is important in the turnover of regulatory proteins and serves as an important regulator of cell proliferation and apoptosis.\textsuperscript{95} Proteosome inhibitors have been shown to potently induce apoptosis in cell lines in vitro, even chemotherapy-resistant cell lines, show in vivo antitumor activity, and sensitize cells to apoptosis. They have also been shown to inhibit angiogenesis and metastasis in vivo.\textsuperscript{29,95}

The effects of these agents may be selective to cancer cells because they induce apoptosis in proliferating or transformed cells. For example, high expression of the c-myc oncogene makes cancer cells more susceptible to proteosome inhibitor-induced apoptosis. Actively dividing cells are also more sensitive to proteosome inhibition than are nonproliferating cells. This effect is not simply secondary to the high replication rate of malignant cells. When disruption in cell turnover occurs by the proteosome inhibitors, normal cells arrest cell division using checkpoint mechanisms and resume proliferation after the proteosome activity is restored. However, malignant cells that have dysfunctional checkpoint mechanisms are more susceptible to the proteosome inhibitor-induced apoptosis.\textsuperscript{95} Proteosome inhibitors include natural products such as lactacystin, peptide aldehydes such as MG132, ALLN, and MG115, which are in the preclinical stages, and the boronic acid inhibitors, such as bortezomib (formerly PS341),\textsuperscript{96} which is in clinical trials and has been FDA approved for the therapy of refractory multiple myeloma in the United States.

**Bortezomib**

Bortezomib is a boronic acid inhibitor that selectively and potently inhibits chymotryptic threonine protease activity, the rate-limiting proteolytic step in the proteosome. In vitro and mouse xenograft studies of bortezomib have shown antitumor activity in a broad range of tumor types including myeloma, CLL, prostate cancer, pancreatic cancer, and colon cancer.\textsuperscript{96–98} It has shown activity as a single agent and in combination with chemotherapy or radiation, and it has shown specificity to transformed and rapidly proliferating cells.\textsuperscript{99} Inhibition of the proteosome is dose dependent and reversible. Another important property of this drug is the rare occurrence of resistance to its effect even in the presence of known drug resistance factors including the absence of p53 or the overexpression of Bcl-2.\textsuperscript{28} Based on its promising preclinical data and clinical activity in Phase I studies in myeloma, a Phase II multicenter trial in refractory and relapsed multiple myeloma patients was performed.\textsuperscript{100} Bortezomib was given at a dose of 1.3 mg/m\textsuperscript{2} on Days 1, 4, 8, and 11 in a 21-day cycle for a maximum of eight cycles. Of 193 patients who could be evaluated, 92\% had received three or more previous therapies, and 91\% were refractory to the most recent treatment. The responses to bortezomib were encouraging, with 27\% of patients achieving a partial response and 4\% a complete response with a median duration of response of 12 months. The success of this trial led to the accelerated FDA approval of bortezomib in patients with relapsed or refractory multiple myeloma.\textsuperscript{100,101} Toxicities include low-grade fever, fatigue, thrombocytopenia, peripheral neuropathy, and low-grade diarrhea.\textsuperscript{99–101}

Another Phase II trial was performed using 1.0 or 1.3 mg/m\textsuperscript{2} in patients who had relapsed after initial induction and consolidation therapy.\textsuperscript{101} Responses occurred in 33\% of those receiving 1.0 mg/m\textsuperscript{2} and in 50\% of those receiving 1.3 mg/m\textsuperscript{2}. Initial trials allowed a maximum use of eight cycles of bortezomib. However, recent data suggest that it is safe to
administer at least an additional five or six cycles of therapy without undue toxic effects. Recently, a Phase III trial in patients with multiple myeloma comparing bortezomib with pulsed dexamethasone therapy was closed early because of a longer time to disease progression in patients receiving bortezomib. These results demonstrate the efficacy of single-agent bortezomib in multiple myeloma. Studies using combination of bortezomib with other agents are currently being performed.

NFκB Inhibitor (IkB Kinase Inhibitor, PS-I, 145)

The IkB kinase inhibitor PS-1,145 (Millennium Pharmaceuticals, Cambridge, MA) is a specific inhibitor of IKKβ, an important regulator of IkB. Preclinical studies using PS-1,145 have demonstrated that this agent inhibits phosphorylation of IkB in multiple myeloma cells and modestly directly inhibits their growth. PS-1,145 abrogates adhesion of multiple myeloma cells and the upregulation of adhesion molecules induced by NFκB. In addition, PS-1,145 blocks the protective effect of IL-6 against apoptosis induced by both conventional (dexamethasone) and novel (immunomodulatory derivative of thalidomide, IMiD3) therapies. These promising preclinical studies suggest the potential utility of specific NFκB inhibitors in the treatment of multiple myeloma and other malignancies with constitutively activated NFκB pathway. In addition, they suggest the combined use of thalidomide/IMiDs with PS-1,145 as a potential clinical strategy to overcome drug resistance in multiple myeloma.

mTOR Inhibitors (CCI-779 and RAD001)

The mammalian target of rapamycin, mTOR, is a downstream component of the PI3K/Akt pathway. Tumors that depend on the activation of the PI3K/Akt pathway or that harbor mutations that cause constitutive activation of this pathway may be more prone to the effect of this targeted therapy. For example, deletion or inactivation of the phosphatase and tensin homolog may lead to upregulation of this pathway and has been shown to increase sensitivity to the effect of CCI-779. Other checkpoint regulators such as p53, RB, p16, p27, and cyclin D can also influence the effectiveness of mTOR inhibitors on tumor cells.35

CCI-779

Rapamycin is an mTOR inhibitor. Rapamycin is a natural product that has antimicrobial, immunosuppressive, and anticancer properties. It is approved as an immunosuppressive drug for renal transplant patients (sirolimus, Rapamune [Wyeth-Ayerst Laboratories, Madison, NJ]). It was found to have antiproliferative activity against a variety of B-lymphoproliferative as well as solid tumor cell lines when it was evaluated by the National Cancer Institute. CCI-779 is a soluble ester of rapamycin and has been shown to be an effective in vitro and in vivo cytostatic agent. CCI-779 is in an intravenous formulation and is currently being tested in several clinical trials for cancer therapy. In xenograft models, CCI-779 induces tumor growth inhibition and not regression, and therefore, it may be an important agent in delaying disease progression or may be used in combination with other anticancer agents. Two Phase I studies have tested increasing doses and different schedules ranging from 0.75 to 24 mg/m²/day in the first study and 7.5 to 220 mg/m²/week in the second study. The MTD of the first study was 19.1 mg/m²/day, and the recommended Phase II dose for heavily pretreated patients was 15 mg/m²/day. In the second study, the MTD was not defined at the time of trial report. The most common toxicities were skin reactions, stomatitis, hyperlipidemia, and thrombocytopenia. These toxicities are similar to what is seen with rapamycin. Pneumonitis that is seen with rapamycin has not been observed yet with CCI-779. Monitoring for unusual infections is also important because laboratory studies have shown increased viral protein synthesis with the use of these agents.

A Phase II study in relapsed and refractory mantle cell lymphoma recently demonstrated a 38% response rate. In another Phase II trial, 7% of patients with advanced refractory renal
cell carcinoma had a complete or partial response, and 26% had minor responses.\textsuperscript{110} Phase III trials for renal cell carcinoma are ongoing. CCI-779 may potentially be useful for the treatment of cancers of various origins, including renal, breast, cervical, uterine, head and neck, lung, prostate, pancreatic, ovarian, colon, lymphoma, and melanoma.\textsuperscript{111}

**RAD001**

RAD001 (everolimus) is another rapamycin analog that is available in oral formulation. A Phase I trial of RAD001 demonstrated that the oral drug given at four dose levels—5, 10, 20, and 30 mg once a week—was well tolerated. Side effects included Grade 1 and 2 anorexia, fatigue, rash, mucositis, headache, hyperlipidemia, and gastrointestinal disturbance. Stable disease occurred in four patients, and one patient showed response at three weeks of therapy. Weekly administration of 20 mg of RAD001 gives the same pharmacokinetic and pharmacodynamic effects that correlate with antitumor effects in rodents treated with this schedule.\textsuperscript{112} Phase II clinical trials using RAD001 in hematologic malignancies and solid tumors are ongoing. Weekly dosing from 20 mg to 70 mg and daily dosing of 10 mg are being evaluated.

**P53 Inhibitors**

Trials to target p53 as cancer therapy include gene therapy, such as ONYX-015 and INGN201, and antisense therapy to the protein that controls p53 activity (MDM2).

**Gene Therapy**

Phase I p53-based gene therapy trials that lead to the reintroduction of wild type p53 in the cells have shown effective induction of apoptosis in tumor cells and in surrounding cells. However, gene therapy technology runs through the obstacle of the requirement to efficiently infect all, or nearly all, cancer cells. In addition, in some animal studies, untransformed thymocytes undergo apoptosis in response to p53 induction, which leads to the need for targeted delivery of transgenes.\textsuperscript{113,114} A large number of adenoviral agents are being developed, including replication-incompetent (eg, rAd.p53, SCH5,8500) and replication-selective (ONYX-015; CG7,870) oncolytic adenoviruses. The agents are introduced by intravascular infusion or intratumoral or epiru- moral injections. Phase I and II studies have demonstrated an acceptable toxicity profile of these agents with low grade fevers, dose-limiting cardiac output suppression (rAd.p53), and mild to moderate transaminitis in some patients receiving hepatic arterial infusions or intravenous infusions. More than 100 cancer patients have been treated with intravascular adenovirus constructs to date. Evidence of p53 gene expression or viral replication was demonstrated in some patients.\textsuperscript{115}

**ONYX-015**

ONYX-015 is a replication-competent virus genetically engineered to selectively replicate in and lyse p53-deficient cancer cells. ONYX-015 has no effect on normal cells. It is given as intratumoral and peritumoral injections.\textsuperscript{116} Phase I and II trials have been conducted in multiple tumor types including advanced head and neck cancer and pancreatic cancer. The development of this compound is currently suspended.

**INGN 201**

INGN 201 is an incompetent adenovirus that delivers a p53 expression. Preclinical studies in human cell lines and animals with head and neck cancers have shown that the p53 gene is efficiently transcribed and translated into p53 protein.\textsuperscript{117} Several Phase I and II clinical trials have been conducted, including a Phase II trial in 19 patients with nonmetastatic nonsmall cell lung cancer who were not eligible for chemo-radiation or surgery.\textsuperscript{118} Patients received three intratumoral injections of INGN 201 in conjunction with radiation therapy 60 Gy over six weeks. The most common side effects were Grade 1 and 2 fevers and chills. Complete responses occurred in 5% of patients; 58% achieved a partial response, 16% stable disease,
11% progressive disease, and 11% were not able to be evaluated. This trial demonstrated that intratumoral injection of INGN 201 in combination with radiation therapy was well tolerated and demonstrated evidence of tumor regression at the primary injected tumor. In a Phase I trial of intraperitoneal INGN 201 administration, 12% of patients with platinum- and paclitaxel-resistant ovarian cancer had mixed responses.\footnote{119}

**SUMMARY**

Increased understanding of the molecular genetic defects and the regulation of the complex signaling pathways in tumors, especially the regulation of apoptosis, can result in rationally designed anticancer strategies. Our knowledge of the intrinsic and extrinsic apoptotic pathways and the other signaling modulators such as the p53, proteosome/ubiquitin system, NFkB, and the PI3K/Akt pathways have led to the discovery of many novel agents that are showing effectiveness whether as single agents or in combination with conventional cytotoxic therapy or radiation. Further understanding of the different signaling pathways that control apoptosis in the different tumor types will help with the discovery of novel targeted agents and the design of clinical trials that are based on the molecular defects specific to the targeted tumor.

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