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Manipulating the apoptotic pathway: potential therapeutics for cancer patients

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This review summarizes the current state of scientific understanding of the apoptosis pathway, with a focus on the proteins involved in the pathway, their interactions and functions. This forms the rationale for detailing the preclinical and clinical pharmacology of drugs that modulate the pivotal proteins in this pathway, with emphasis on drugs that are furthest advanced in clinical development as anticancer agents. There is a focus on describing drugs that modulate three of the most promising targets in the apoptosis pathway, namely antibodies that bind and activate the death receptors, small molecules that inhibit the anti-apoptotic Bcl-2 family proteins, and small molecules and antisense oligonucleotides that inactivate the inhibitors of apoptosis, all of which drive the equilibrium of the apoptotic pathway towards apoptosis. These structurally different yet functionally related groups of drugs represent a promising novel approach to anticancer therapeutics whether used as monotherapy or in combination with either classical cytotoxic or other molecularly targeted anticancer agents.

Introduction

The scientific understanding of the biology of cancer has led to the recognition that it is the unusual properties of cancer cells plus the complementarity of the stroma which supports them that provides the optimal milieu for cancer cell proliferation [1, 2]. One of the unique properties of cancer cells is their ability to evade programmed cell death; this cell death pathway is termed apoptosis. Apoptosis is derived from the 5th Century BC Greek, meaning 'falling off', and is a term that was applied scientifically in the 1970s [3, 4].

Many chemotherapeutic drugs cause apoptosis indirectly, mostly via targets and pathways that ultimately modulate the intrinsic apoptotic pathway machinery. The primary objective of this review is to focus on the clinical pharmacology of drugs that directly target different steps in the apoptotic pathways, via direct activation of the death receptors, inactivation of anti-apoptotic Bcl-2 proteins or inactivation of inhibitor of apoptosis proteins (IAPs). This collection of therapeutics has the potential to overcome apoptosis resistance in cancers and enhance the rate of cancer cell death, thus providing a potential new approach to improve cancer treatment.

Overview of apoptosis

Apoptosis can be initiated by stress signals from within the cell or by external environmental stressors or toxins (Figure 1). This death signal then involves widespread proteolysis by caspases, nucleosomal fragmentation by endonucleases, and cell surface tagging for phagocyte engulfment [5]. Extrinsic apoptosis is regulated by members of the tumour necrosis factor (TNF) receptor protein family [death receptor (DR) family], which contain an extracellular ligand-binding domain and an intracellular death domain that signals for apoptosis. Intrinsic apoptosis is regulated at the mitochondrial membrane by members of the Bcl-2 protein family, in which the complex interaction between anti-apoptotic and pro-apoptotic members dictates whether apoptosis is triggered. Extrinsic apoptosis activates caspases 8 and 10 in the death-inducing signalling complex (DISC), while intrinsic apoptosis activates

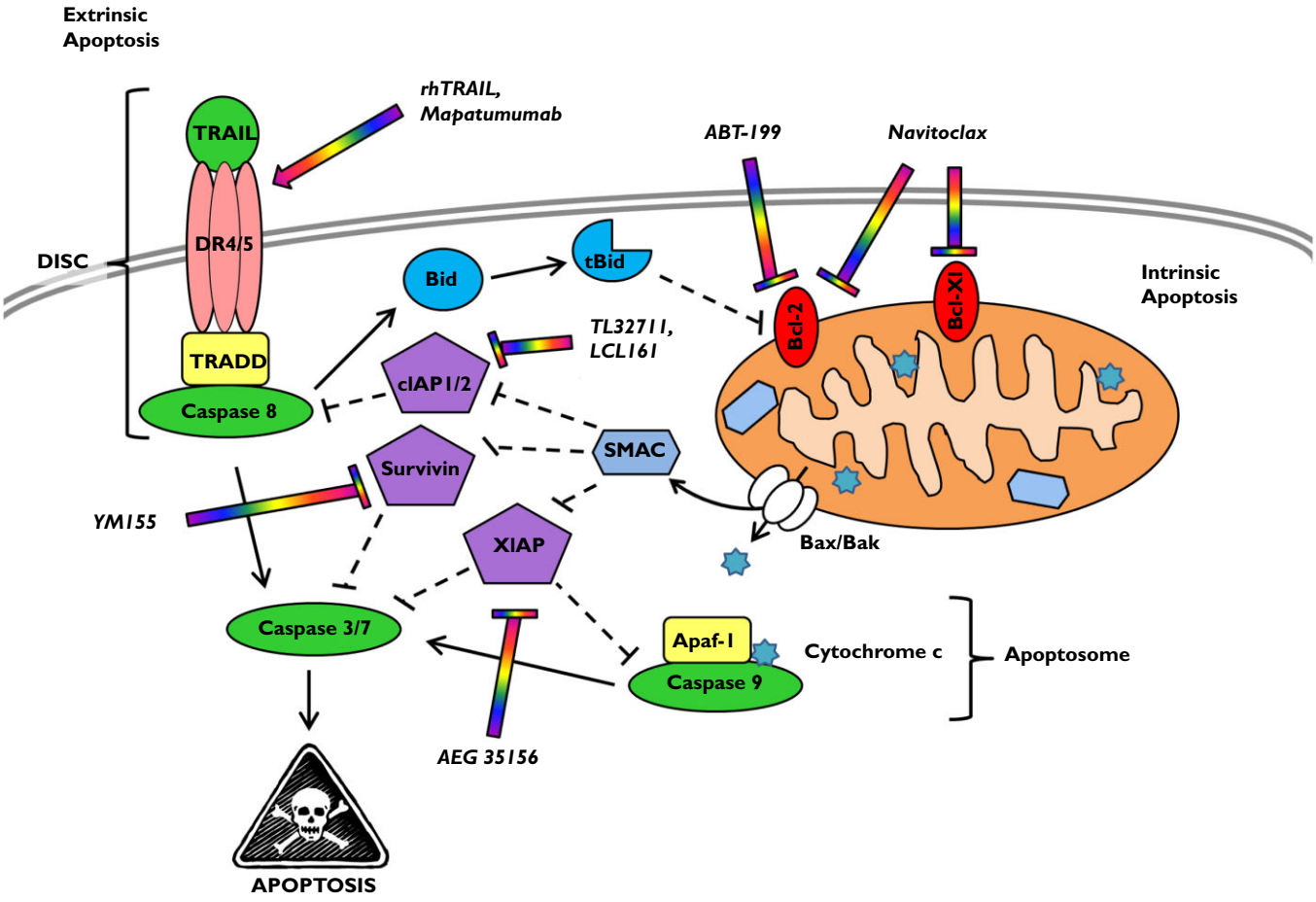

Figure 1

The extrinsic and intrinsic apoptotic pathways. The extrinsic apoptotic pathway entails death ligands such as tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) binding to and activating its cognate receptors (TRAIL-R1/DR4, TRAIL-R2/DR5). This complex then recruits tumour necrosis factor-associated death domain protein (TRADD) and the initiator caspase 8. In this death-inducing signalling complex (DISC), caspase 8 is auto-activated by proteolysis and released into the cytosol. This leads to activation of caspases 3/7 and subsequently apoptosis. The intrinsic apoptotic pathway can be triggered by internal stress signals (e.g. radiation/chemotherapy damage to DNA) or via death receptor activation and caspase 8-mediated cleavage of Bid. Cleaved Bid (tBid) and/or other pro-apoptotic Bcl-2 proteins induce translocation of Bax/Bak into the mitochondrial membrane, leading to cytosolic release of cytochrome *c* and the second mitochondria-derived activator of caspase (SMAC). Cytochrome *c* binds the adaptor proteins Apaf-1 and caspase 9 to form the apoptosome which activates caspase 9. This caspase further activates caspases 3/7, resulting in apoptosis. Bcl-2 and Bcl-XL can inhibit apoptosis by preventing release of cytochrome *c* from the mitochondria. The inhibitor of apoptosis (IAP) proteins (e.g. cIAP1/2, XIAP and survivin) block caspase activation further downstream. SMAC displaces these IAP proteins, thus promoting apoptosis. The lead clinical drugs for each target in the apoptotic pathway are shown (multicoloured)

caspase 9 within the apoptosome. These initiator caspases go on to activate the effector caspases 3 and 7 that amplify the proteolytic caspase cascade, committing the cells to die. However, this cascade can be blocked by inhibitors of apoptosis (IAPs), which bind active caspases and prevent further proteolysis.

One of the hallmarks of cancer cells is their ability to evade apoptosis. This can occur by upregulation of anti-apoptotic proteins, by downregulation or loss of pro-apoptotic proteins or by defective functioning of pro-apoptotic proteins [6]. Thus, the apoptotic machinery is a pivotal potential target for cancer therapeutics.

Role of the death receptor family in apoptosis

The TNF receptor superfamily [TNFR, Fas (CD95/Apo1), death receptor 4 (DR4/TRAIL-R1) and death receptor 5 (DR5/TRAIL-R2)] manages many functions, including cell death/survival, differentiation and immune regulation [7]. Upon binding their respective ligands, these death receptors are activated to form homotrimers, clustering the receptor death domains, leading to recruitment of intracellular adaptor molecules (e.g. TRADD and FADD). These adaptor molecules recruit caspase 8 or 10 to the DISC,

causing caspase self-cleavage and activation, which then goes on to activate the apoptotic caspase cascade [6]. Internalization of Fas and TNFR, but not DR4 or DR5, is required for DISC formation.

Death receptor-triggered apoptosis can be either dependent on or independent of the mitochondria, creating crossover between the extrinsic and the intrinsic apoptotic pathway (see Figure 1). Type 1 cells activate sufficient amounts of caspase 8 so that apoptosis occurs independent of the mitochondrial pathway. However, type 2 cells activate little caspase 8 and therefore require the activation of the mitochondrial apoptotic pathway, via caspase cleavage and activation of the pro-apoptotic protein Bid, in order to activate the full apoptotic caspase cascade. Additional intracellular control points in death receptor signalling include cellular FLICE (FADD-like interleukin-1β-converting enzyme)-inhibitory protein (c-FLIP), a catalytically inactive caspase 8/10 homologue that can bind and block signalling of FADD or caspase 8/10, and IAP family proteins which bind caspases, blocking their signalling.

Role of the Bcl-2 apoptotic protein family in apoptosis

Intrinsic apoptosis is regulated by the Bcl-2 family of proteins, which maintains the integrity of the mitochondrial membrane. The anti-apoptotic members of this protein family are Bcl-2, Bcl-Xl, Bcl-w, Bcl-B, Bfl-1 and Mcl-1, which contain four Bcl-2 homology domains (BH1–4) allowing them to lie within the outer mitochondrial membrane and bind/sequester pro-apoptotic proteins [8]. The pro-apoptotic family members include Bax and Bak, which contain domains BH1–3, and the BH3-only members Bad, Bid, Bim, Noxa, Puma, Bik, Bmf and Hrk. The BH3-only members can act as apoptosis sensitizers by binding to anti-apoptotic proteins and releasing Bax/Bak. Furthermore, Bid and Bim can operate as activators of Bax/Bak, stimulating Bax/Bak to oligomerize and form pores in the mitochondrial membrane.

To trigger apoptosis, the balance of anti-apoptotic and pro-apoptotic Bcl-2 proteins must be shifted so that there is an excess of pro-apoptotic proteins at the mitochondria and/or neutralization of anti-apoptotic proteins. The crucial step in triggering intrinsic apoptosis is mitochondrial outer membrane permeabilization by Bax/Bak, releasing pro-death factors (i.e. cytochrome *c*) that commit the cell to die. Thus, true intrinsic apoptosis is considered to be Bax/Bak dependent. Once released into the cytosol, cytochrome *c* forms the apoptosome with Apaf-1 and caspase 9, initiating the caspase cascade [9]. Mitochondrial outer membrane permeabilization also releases second mitochondria-derived activator of caspases (SMAC), which binds and inhibits IAPs. Furthermore, mitochondrial outer membrane permeabilization releases apoptosis-inducing factor and endonuclease G, which activate caspase-independent apoptosis, causing chromatin condensation and large-scale DNA fragmentation. Thus, even in the absence of caspase activity, mitochondrial outer membrane permeabilization can commit the cell to die via a back-up cell death programme [10].

Alterations in the expression of Bcl-2 family members contribute to neoplastic transformation and cancer cell chemoresistance, with the anti-apoptotic members serving as oncogenes. Initially, the *BCL-2* gene was identified in chromosomal translocations t(14;18), causing excessive Bcl-2 expression in follicular lymphoma [11]. A survey of 68 cancer cell lines revealed that Bcl-2 and Bfl-1 expression was highest in leukaemia, lymphoma and melanoma cell lines, while Mcl-1 expression was predominant in glioma, lung, prostate, breast, ovarian and renal cancers [12]. Clinically, Bcl-2 expression in B cells from acute myeloid leukaemia (AML)/acute lymphoblastic leukaemia (ALL) patients was high in comparison to normal B cells and yielded a survival advantage against chemotherapy [13, 14]. Furthermore, high expression levels of Bcl-2, Bcl-Xl and Mcl-1 have all been reported to protect a wide spectrum of malignancies, causing resistance to various chemotherapeutic drugs and making them strong candidates for drug intervention.

Role of the inhibitors of apoptosis protein family in apoptosis

The IAP family contains eight members, including XIAP, cIAP1, cIAP2 and survivin. All IAPs have baculoviral IAP repeat (BIR) domains that allow them to bind active caspases directly and either suppress or target the IAP–caspase complex for degradation [15], serving as brakes of the final common pathway for intrinsically or extrinsically triggered apoptosis. However, IAPs can be regulated negatively by XAF1, HTRA2 and SMAC to release the apoptotic brakes. XIAP is considered to be the most potent of the IAPs, with a K_i (inhibition constant) in the high picomolar range [16, 17], showing the greatest protection from apoptotic events [18, 19]. Expression of XIAP in tissues is ubiquitous, and the loss of XIAP is thought to be a prerequisite for cell death and results in immune rejection and tumour regression more so than other apoptosis modulators [20, 21]. Overexpression of IAPs has been reported to contribute to initiation of haematological malignancy, chemoresistance in both solid and haematopoietic tumours, and poor clinical prognosis [22–24].

Agents that target and activate the death receptors

Pro-apoptotic receptor agonists (PARAs)

Pro-apoptotic receptor agonists are proteins or small molecules that target the death receptors DR4, DR5, Fas or TNFR.

Table 1

Clinical studies of pro-apoptotic (death) receptor agonists (PARAs)

Clinical drug	Drug type	Target	Phase	Treatment	Patients	Status*
Mapatumumab	mAb	DR4	I/II	+Cisplatin and radiotherapy	Advanced cervical cancer	Active
			I	+Sorafenib	Advanced hepatocellular carcinoma	Completed
			II	+Sorafenib	Advanced hepatocellular carcinoma	Closed
			II	+Bortezomib	Relapsed or refractory MM	Completed
			II	+Carboplatin and paclitaxel	NSCLC	Completed
			II	Monotherapy	Relapsed or refractory NHL	Completed
			II	Monotherapy	Relapsed or refractory NSCLC	Completed
Lexatumumab	mAb	DR5	I	±Interferon γ	Paediatric relapsed or refractory solid tumours and lymphomas	Completed
Conatumumab	mAb	DR5	I/II	+Capecitabine, radiotherapy and gemcitabine	Locally advanced pancreatic cancer	Terminated
			I	+Bortezomib or vorinostat	Lymphoma	Suspended
			I/II	+Carboplatin and paclitaxel	Untreated NSCLC	Completed
			I/II	+mFOLFOX6 and bevacizumab	Untreated metastatic CRC	Closed
			I/II	+Doxorubicin	Untreated unresectable soft tissue sarcoma	Closed
			I/II	+Gemcitabine	Metastatic pancreatic cancer	Completed
			I/II	+Panitumumab	Metastatic CRC	Closed
			I/II	+AMG479	Advanced refractory solid tumours	Terminated
			II	+FOLFIRI	Metastatic KRas mutant CRC	Closed
			II	±FOLFOX6, ganitumab, bevacizumab	Advanced tumours	Closed
Tigatuzumab	mAb	DR5	I	Monotherapy	Metastatic CRC	Active
			I	Monotherapy	Advanced solid tumours and lymphomas	Completed
			I	+FOLFIRI	Metastatic CRC	Closed
			II	+Abraxane	Metastatic triple negative breast cancer	Closed
			II	+Sorafenib	Advanced liver cancer	Closed
			II	+Carboplatin and paclitaxel	Metastatic or unresectable NSCLC	Completed
			II	+Carboplatin and paclitaxel	Locally advanced or metastatic ovarian cancer	Completed
			II	Monotherapy	Untreated, unresectable pancreatic cancer	Completed
Apomab	mAb	DR5	I	Monotherapy	Advanced recurrent or metastatic disease	Completed
			I	+Irinotecan and cetuximab or +FOLFIRI	Metastatic CRC	Completed
			I	+FOLFOX and bevacizumab	Untreated and metastatic CRC	Completed
			II	+Rituximab	NHL	Completed
			II	+Paclitaxel, carboplatin and bevacizumab	Untreated or recurrent NSCLC	Completed
			II	Monotherapy	Advanced chondrosarcoma	Terminated
LBY-135	mAb	DR5	I	±Capecitabine	Advanced solid tumours	Completed
Dulanermin	rhTRAIL	DR4, DR5	I	+Camptosar and erbitux or +FOLFIRI ±bevacizumab	Metastatic CRC	Closed
			I	+FOLFOX and bevacizumab	Untreated, locally advanced, recurrent or metastatic CRC	Closed
			II	+Carboplatin and paclitaxel ±bevacizumab	Untreated NSCLC	Completed
			I/II	±Rituximab	NHL	Terminated
APO010	Recombinant protein	FasR	I	Monotherapy	Solid tumours	Completed

Abbreviations are as follows: CRC, colorectal cancer; DR4, death receptor 4; DR5, death receptor 5; FasR, Fas receptor; FOLFIRI, folinic acid, 5-fluorouracil, irinotecan regimen; FOLFOX, folinic acid, 5-fluorouracil, oxaliplatin regimen; mAb, monoclonal antibody; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma; NSCLC, nonsmall cell lung cancer; rhTRAIL, recombinant human TRAIL. *Status according to http://www.clinicaltrials.gov as of 3 January 2013: closed = active, not recruiting; and suspended = temporarily halted patient recruitment.

The PARAs work from outside the cell and directly trigger apoptosis via ligand-mediated receptor agonism. TNFR signalling is complex because it can activate caspases but can also stimulate pro-inflammatory pathways, which lead to induction of nuclear factor-κB and expression of anti-apoptotic genes (e.g. cIAP2, c-FLIP, Bcl-Xl and Bfl-1), thus driving cell survival and proliferation. Tumour necrosis factor signalling in normal tissues has greatly impeded its clinical development due to hepatic and cardiovascular toxicity [15]. However, TNFR has been successfully targeted to treat autoimmune diseases with antagonists such as anti-TNFR antibodies and soft tissue sarcomas of the extremities by isolated limb perfusion using nanoparticles tagged with TNF. Fas receptor targeting results in massive hepatocyte apoptosis and lethal liver damage in animal models [25], and is generally unsuitable for clinical development. However, FasL fusion proteins based on a collagen trimerization platform (including APO010) may be better tolerated and are in early clinical safety evaluation. The lead clinical PARAs are discussed below, while a detailed list of current clinical trials for all death receptor agonists can be found in Table 1.

Recombinant human TNF-related apoptosis-inducing ligand (rhTRAIL) – dulanermin

Preclinical studies Early preclinical studies to activate the TRAIL receptors DR4 and DR5 used recombinant soluble

fragments of murine TRAIL and showed antitumour activity in xenograft models. Soluble TRAIL was optimized for better stability and solubility, while eliminating early concerns of hepatotoxicity [26–28]. These studies ultimately led to the development of recombinant human TRAIL (rhTRAIL) which is composed of amino acids 114–281 of the natural ligand and activates both DR4 and DR5. Clinical grade soluble rhTRAIL showed antitumour activity *in vitro* in 16 of 39 cancer cell lines but not in several cell lines from normal tissues [26]. Recombinant human TRAIL showed promising antitumour efficacy in mouse xenografts of human cancers [colon [29], lung [30], pancreas [31], multiple myeloma (MM) [32], non-Hodgkin's lymphoma (NHL) [33] and glioma [34, 35]]. Combinations of rhTRAIL with proteasome inhibitors [36–38], HDAC inhibitors [39], the anti-CD20 antibody rituximab [33], antimetabolites, topoisomerase inhibitors, DNA-damaging agents or microtubule-targeting agents have shown additive or synergistic antitumour effects in preclinical models (reviewed in [40]). Preclinical studies of rhTRAIL included safety assessments in cynomolgus monkeys and chimpanzees, and revealed no liver or other major organ/tissue toxicity, but a limited half-life of approximately 25 min [26].

Clinical studies In phase I and II studies, patients received rhTRAIL (dulanermin) doses up to 15 mg kg^{-1} intravenously for 5 days consecutively. The serum half-life was approximately 36 min at 8 mg kg^{-1}, and rhTRAIL was well tolerated at this dose, with partial responses seen in two chondrosarcoma patients [41]. However, the antitumour benefit of rhTRAIL as part of combination therapy in phase II studies in solid tumours (e.g. colorectal cancer and nonsmall cell lung cancer) has not fulfilled its apparent early potential.

Monoclonal antibodies against the TRAIL receptors

Preclinical studies Mapatumumab is a fully human IgG1 antibody that activates DR4 and has antitumour effects *ex vivo* and *in vivo* as a single agent in colon, nonsmall cell lung cancer (NSCLC) and renal cancer murine models. Mapatumumab also showed enhanced antitumour effects in combination with 5-fluorouracil, irinotecan, topotecan or irradiation in colon xenograft models [42, 43]. Of the numerous anti-DR5 antibodies in development, lexatumumab and conatumumab are fully human IgG1 antibodies, while tigatuzumab is a humanized IgG1. Lexatumumab showed preclinical efficacy in renal cell carcinoma and colorectal xenografts [43, 44]. Conatumumab was effective as a single agent and in combinations with gemcitabine and CPT-11 in colorectal, lung and pancreatic carcinoma models [45]. Likewise, tigatuzumab demonstrated preclinical antitumour activity in NSCLC, colorectal and pancreatic cancer *in vitro* and *in vivo* [46].

Clinical studies Phase I studies have established that mapatumunab is well tolerated at doses between 10 and 30 mg kg^{-1} administered every 21 days. The pharmacokinetics showed a mean terminal elimination half-life of 18.8 days, which was dose independent and justified dosing every 2–3 weeks. Toxicities were all mild and included fatigue, fever and myalgias [47]. A randomized, controlled phase II trial to evaluate mapatumumab in combination with carboplatin (C) and paclitaxel (P) as first-line therapy in advanced NSCLC was conducted. Patients were randomly assigned to Arm A, paclitaxel 200 mg m^{-2} + carboplatin area under the curve (AUC) 6.0 (PC); Arm B, PC + mapatumumab 10 mg kg^{-1}; or Arm C, PC + mapatumumab 30 mg kg^{-1}. One hundred and eleven patients entered the study; no benefit from the addition of mapatumumab to PC was found. Adverse events were generally balanced across treatment groups; there was no evidence that mapatumumab exacerbated toxicities associated with PC. Further evaluation of mapatumumab in combination with PC in patients with advanced NSCLC was not supported by these study data [48]. The results of ongoing combination studies (see Table 1) are awaited.

The preliminary disappointing efficacy of these monoclonal antibodies targeting DR4 or DR5 has been attributed to a number of potential issues, namely inadequate tissue penetration to compete for binding with the endogenous ligand, lack of tumour expression of DR4 or DR5 (or mutations of these receptors), and the possibility that the scheduling of combinations with cytotoxic agents is suboptimal. Other possible reasons for failure include overexpression of decoy receptors, requirement of *O*-glycosylation for full activation, and alterations in the downstream pathway, such as high c-FLIP, IAP or Bcl-2 proteins, that block activation/amplification [7].

A number of future approaches for death receptor targeting are being pursued preclinically. These include fusion proteins of TRAIL, recombinant soluble TRAIL genetically linked to a receptor-selective antibody fragment, and designed PARAs with higher affinity binding [49].

Agents that target and inhibit anti-apoptotic Bcl-2 proteins

Many inhibitors of Bcl-2 family proteins have reached the clinic. An overview of the preclinical and clinical results for the lead clinical Bcl-2 inhibitors is discussed below, with a more detailed list of later phase trials shown in Table 2.

Oblimersen (Genasense)

Preclinical studies The antisense oligonucleotide for Bcl-2, oblimersen (Genasense), targets the first six codons of Bcl-2 mRNA and is active in preclinical models of prostate cancer, melanoma, NHL, NSCLC, AML, B cell malignancies and several other malignancies [15, 50]. Preclinical studies of oblimersen showed enhanced antitumour activity of

Table 2
Clinical studies of Bcl-2 family inhibitors

Clinical drug	Drug type	Target	Phase*	Treatment	Patients	Status†
Genasense	Antisense oligonucleotide	Bcl-2	I	+Cytarabine and daunorubicin	AML	Closed
			I	+Abraxane and temodar	Melanoma	Closed
			II	+Carboplatin and paclitaxel	Melanoma	Closed
			III	+Dacarbazine	Melanoma	Completed
			III	+Cytarabine and daunorubicin	Untreated AML	Completed
			III	+Dexamethasone	Relapsed or refractory MM	Completed
			III	+Fludarabine and cyclophosphamide	Relapsed or refractory CLL	Completed
			II/III	+Docetaxel	NSCLC	Unknown
			II/III	Monotherapy	Advanced melanoma	Terminated
			III	+Rituximab and fludarabine	Untreated CLL	Withdrawn
Gossypol/AT-101	Polyphenolic aldehyde	Pan-Bcl-2, ER stress	II	+Docetaxel	Laryngeal cancer	Active
			II	+Docetaxel	Squamous cell carcinoma of the head and neck	Active
Obatoclax (GX-15–070)	BH3 mimetic	Pan-Bcl-2, Kinases	I	+Bortezomib	Aggressive relapsed or recurrent NHL	Active
			I	+Vincristine, doxorubicin and dexrazoxane	Relapsed or refractory solid tumours, lymphoma or leukaemia	Closed
			I/II	+Topotecan	Relapsed or refractory SCLC or advanced tumours	Closed
			I/II	+Bortezomib	Relapsed or refractory melanoma	Closed
			III	+Carboplatin and etoposide	Chemotherapy-naïve SCLC	Withdrawn
Navitoclax (ABT-263)	BH3 mimetic	Bcl-2, Bcl-Xl	I	+Fludarabine/cyclophosphamide/rituximab or +bendamustine/rituximab	Relapsed or refractory CLL	Closed
			I	+Rituximab	Lymphoid cancers	Closed
			I	±Erlotinib or irinotecan	Solid tumours	Closed
			I/II	Monotherapy	Relapsed or refractory CLL	Closed
			II	Monotherapy	Relapsed or refractory lymphoid malignancies	Closed
			II	Monotherapy	CLL	Closed
ABT-199	BH3 mimetic	Bcl-2	I	Monotherapy	Relapsed or refractory CLL and NHL	Active
			I	+Rituximab and bendamustine	Relapsed or refractory NHL	Active
			I	+Rituximab and bendamustine	Relapsed or refractory CLL	Active
			I	+Obinutuzumab	CLL	Suspended
			I	+Rituximab and bendamustine	CLL	Suspended

Abbreviations are as follows: AML, acute myeloid leukaemia; CLL, chronic lymphocytic leukaemia; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma; NSCLC, nonsmall cell lung cancer; SCLC, small cell lung cancer. *Only active or late phase clinical trials are shown. A more comprehensive list of Bcl-2 drug targeting trials can be found at http://www.clinicaltrials.gov. †Status according to http://www.clinicaltrials.gov as of 3 January 2013: closed = active, not recruiting; and suspended = temporarily halted patient recruitment.

several chemotherapeutics, e.g. taxanes, vinca alkaloids, anthracyclines, alkylators, platinum-containing agents [6], endocrine therapy [51] and radiation [52].

Clinical studies Studies with oblimersen (as a single agent or in combination) in many solid and haematological malignancies have unfortunately not confirmed the preclinical promise of this agent. An example of this is in melanoma. Early work controversially suggested that Bcl-2 was a viable target in melanoma [53]. Using the recommended phase II dose of oblimersen, 7 mg kg^{-1} day^{-1}, as a continuous infusion for 5 days, followed by 1000 mg m^{-2} darcabazine (DTIC), a small phase II trial showed efficacy [54]. Therefore, a nonblinded, randomized trial in 775 patients comparing oblimersen + DTIC *vs*. DTIC alone was undertaken. Patients were prestratified by baseline lactate dehydrogenase (LDH). A continuous improvement in overall survival was observed in the patients receiving oblimersen as a function of baseline LDH. Patients with LDH ≤0.8 times the upper limit of normal showed the greatest benefit in overall survival (OS), and those with LDH ≥1.1 times the upper limit of normal showed no difference in OS [55]. On the basis of these results, a randomized, phase III trial of oblimersen + DTIC *vs*. DTIC alone was performed in 300 patients with LDH ≤0.8 times the upper limit of normal. This study revealed no difference in OS. Thus ended clinical trials of oblimersen, which had also failed in myeloma and whose development in chronic lymphocytic leukaemia (CLL) was halted [56]. Other bispecific antisense oligonucleotides targeting Bcl-2 and Bcl-Xl have been designed [57], but currently none has yet reached the clinic.

BH3 mimetics

Numerous BH3 mimetics have been developed to target the anti-apoptotic Bcl-2 proteins. These moieties are

designed to bind the BH3 hydrophobic groove of these anti-apoptotic proteins, displacing pro-apoptotic proteins, which then trigger intrinsic apoptosis. Most of these BH3 mimetics are reported as pan-Bcl-2 inhibitors (i.e. bind Bcl-2, Bcl-Xl and Mcl-1). The problems with many of these designed mimetics (terphenyl-based structures, HA14-1, compound 6, antimycin A, S1, chelerythrine and stabilized BH3 peptides) include poor pharmacological properties, weak binding affinities and induction of Bax/Bak-independent cell death [58–62]. Thus, most do not appear to function as BH3 mimetics.

Gossypol and AT-101

Preclinical studies Several natural compounds have been reported to bind the anti-apoptotic Bcl-2 proteins, including gossypol (cotton seeds), epigallecatechin gallate (green tea), theaflavins (black tea), chelerythrine (tropical plants) and antimycin A (bacterial metabolites), many of which are unstable *in vivo* and degrade to an inactive form, with the pan-Bcl-2 inhibitor gossypol showing the most promise as a potential therapeutic agent [15]. The measured binding affinities of gossypol for the anti-apoptotic proteins have varied due to its poor solubility [58]. AT-101 is the racemically purified version of gossypol that is being used for clinical trials due to increased potency in preclinical studies [63, 64].

There are reports of AT-101 activity as a single agent and in combination with rituximab in animal models of MM, B cell lymphoma and primary CLL (reviewed in [58]). Gossypol has been modified to apogossypol to make a more stable, effective form of the compound, but this compound has not yet reached clinical trials. Another gossypol derivative, apogossypolone, inhibited growth of follicular lymphoma cell lines and primary mantle cell lymphoma and CLL tumours. Xenograft models showed apogossypolone protection from tumour-cell bone marrow infiltration and extended survival times (review [58]). Whether gossypol is a BH3 mimetic has been questioned, because it does not appear to function as such *in vitro* [62, 65].

Clinical studies Phase I studies of AT-101 undertaken in prostate cancer patients established the recommended monotherapy phase II dose as 30 mg day^{-1} for 21 days out of 28. However, during phase II studies, better tolerability was achieved with 20 mg day^{-1} for 21 out of 28 days. The major adverse effects were gastrointestinal disturbances, anorexia, fatigue and bone and back pain [66]. Other Phase I studies of combination treatments of 1–5 days have established a maximal tolerated dose of AT-101 of 40 mg twice daily. Pharmacokinetic studies revealed a half-life of 3–5 h, and food produced a 45% increase in AUC [67]. Phase II randomized studies of AT-101 plus docetaxel *vs.* docetaxel alone in chemonaïve prostate cancer patients (n = 221) did not show improved overall survival, although the study suggests that patients with high-risk disease may benefit [68].

Obatoclax (GX15-070)

Preclinical studies The reported pan-Bcl-2 inhibitor obatoclax is a synthetic prodiginine derivative with single agent activity in multiple cancer cells lines, with IC_{50} values of 0.26–15 μM. Synergy with several anticancer molecules has also been reported; however, obatoclax can kill cells independently of Bax and Bak, causing S–G2 cell cycle arrest [58, 60, 69]. These studies suggest that obatoclax sensitizes cells independent of the intrinsic apoptosis family and has off-target effects.

Clinical studies Infusion of obatoclax mesylate was studied in phase I and phase II studies in haematological cancers. Unfortunately, it was found that the concentrations needed for preclinical anticancer efficacy could not be sustained clinically because of dose-limiting neurotoxicity (ataxia, mood alteration, somnolence and cognitive dysfunction) [70–72].

Navitoclax and its congeners (e.g. ABT-199)

The BH3 mimetics, such as ABT-737, ABT-263 and ABT-199, directly bind the BH3 groove of anti-apoptotic proteins, leading to Bax/Bak activation and true intrinsic apoptosis [9]. ABT-737 and ABT-263 bind to Bcl-2, Bcl-Xl and Bcl-w, while ABT-199 binds more selectively to Bcl-2. Navitoclax (formerly ABT-263) is an analogue of ABT-737 that is orally bioavailable.

Preclinical studies ABT-737 showed *in vitro* toxicity as a monotherapy in numerous malignant cell lines and primary leukaemia and lymphoma cells (reviewed in [73]). Navitoclax (ABT-263) showed synergy with etoposide and carboplatin that was most likely to be due to p53 activation and upregulation of pro-apoptotic proteins Puma, Noxa and Bim by the DNA-damaging agents. A mechanism of resistance against navitoclax and ABT-737 is the expression of Mcl-1. Rationalized targeting of Mcl-1, a short-lived protein, with cyclin-dependent kinase inhibitors that block global transcription in combination with ABT-737 can sensitize leukaemia cells [74]. When navitoclax was combined with rituximab, R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) or bortezomib in mouse models of large B cell lymphoma and mantle cell lymphoma, it demonstrated enhanced antitumour effects (reviewed in [58]). However, navitoclax absorption is limited by its dissolution, with a half-life of 4.6–8.4 h in the mouse, rat, dog and monkey (reviewed in [58]), leading to a daily dosing regimen in human clinical trials.

ABT-199, the most recent navitoclax congener, was effective alone or in combination with rituximab ± bendamustine in several leukaemia and lymphoma animal models. ABT-199 was more potent at killing peripheral blood CLL cells while sparing circulating platelets in comparison to navitoclax, suggesting that it may be more successful clinically [75].

Clinical studies A phase I trial of oral navitoclax was conducted in 29 patients with relapsed or refractory CLL. Navitoclax was given daily for 14 days (10, 110, 200 or 250 mg day^{-1}; n = 15) or 21 days (125, 200, 250 or 300 mg day^{-1}; n = 14) of each 21 day cycle. Lymphocytosis was reduced by more than 50% in 19 of 21 patients with baseline lymphocytosis. Among 26 patients treated with navitoclax ≥110 mg day^{-1}, nine (35%) achieved a partial response and seven maintained stable disease for >6 months. Dose-related thrombocytopenia was the major dose-limiting toxicity, with some milder gastrointestinal adverse effects. Navitoclax had a mean half-life of 17 h with first-order elimination, and could not be detected in urine, indicating negligible navitoclax renal clearance. A navitoclax dose of 150–250 mg day^{-1} in solid tumours or haematological malignancies in a continuous dosing schedule was defined as optimal for phase II studies [76–78]. Navitoclax has little activity as monotherapy for NSCLC [79]. However, several phase II studies with navitoclax monotherapy in lymphoid malignancies are nearing completion, as are combination studies with bendamustine and rituximab.

The newest drug in the BH3-mimetic pipeline, ABT-199, has already entered the clinic for patients with NHL. Data from an ongoing phase I study revealed that ABT-199 is tolerated at oral doses ranging from 50 to 600 mg daily, with a time to maximum concentration (T_{max}) at 7 h and a mean half-life of approximately 15 h. Its AUC is increased threefold when ingested with food. The most common adverse effects were gastrointestinal disturbances (nausea, dyspepsia and diarrhoea) and fatigue, as well as grade 3 anaemia and grade 4 neutropenia. The maximal tolerated dose has not yet been determined. It caused little or no thrombocytopenia, with encouraging antitumour efficacy in eight of 15 patients and four of five with follicular lymphoma [80].

Agents that target the inhibitors of apoptosis proteins

The lead clinical IAP inhibitors are discussed below, and a detailed list of current clinical trials for all IAP inhibitors can be found in Table 3.

IAP antisense oligonucleotides

Preclinical studies There are a number of antisense oligonucleotides in development that target IAPs. Of these, AEG35156, which targets XIAP, has made the most clinical progress. AEG35156 reduces XIAP mRNA and protein in the nanomolar range in cell lines, enhancing sensitivity to TRAIL in NSCLC and pancreatic cancer lines. In paediatric tumour cell lines, AEG35156 demonstrated XIAP downregulation and antitumour efficacy in combination with doxorubicin or etoposide [81]. Tumour growth delay was observed in colon and prostate cancer xenografts with AEG35156 used as a single agent, which was enhanced in combination with docetaxel [82].

Clinical studies Early clinical studies with AEG35156 revealed several problematic pharmacological properties, including poor oral bioavailability, requiring continuous intravenous infusions over hours or days [21]. The drug caused chills and lethargy and accumulated in the liver, causing transaminitis and liver toxicities. Tumour lysis syndrome, hypophosphataemia and QTc prolongation (with one death) were all observed dose-limiting toxicities. Shorter infusions were studied in combination with chemotherapy but caused neuropathy, and altering the dosing schedule to make AEG35156 safer limited its effectiveness (reviewed in [21]). Even with these problems, XIAP targeting is considered a better approach than SMAC mimetics that target cIAP1 and cIAP2, even though the latter may be tumour suppressors in NHL, Hodgkin's disease, small lymphocytic leukaemia, ALL, CLL, MM or Waldenstrom's macroglobulinaemia [21]. AEG35156 development seems to be halted, but antisense oligonucleotides for IAPs are still attracting considerable interest as potential anticancer therapeutics.

Antisurvivin agents

Preclinical studies The role of survivin as an IAP protein is controversial, because it contains only one BIR domain, leading to poor caspase binding. However, survivin association with other proteins (e.g. XIAP and hepatitis B X-interacting protein) enables caspase binding and inhibition. Furthermore, survivin has a critical role in cell division, making it an attractive target for cancer therapy [83].

The preclinical studies of survivin inhibitors EZN3042/SPC3042 showed reduced tumour weight when combined with paclitaxel in prostate cancer xenografts [84]. A survivin antisense oligonucleotide, LY2181308, showed inhibition of survivin protein in glioblastoma and melanoma xenografts with tumour growth delay as a single agent. LY2181308 combined with gemcitabine or paclitaxel increased caspase 3 activity and delayed tumour growth by 2–5 days [85].

EZN-3042 is a locked nucleic acid oligonucleotide that effectively targets survivin in several ALL cell lines. Apoptosis initiated by EZN-3042 was enhanced by several individual chemotherapeutic drugs, with ALL xenografts showing reduced blast counts [86].

YM155 (now known as sepantropium bromide) is an imidazolium-based small molecule that exclusively downregulates survivin according to a promoter activity assay. Preclinical studies show that YM155 reduces survivin protein in hormone-refractory prostate cancer PC-3 cells, accompanied by increased caspase 3 activity and decreased cell viability. Furthermore, several human tumour xenograft models (hormone-refractory prostate cancer, NSCLC, breast, bladder and melanoma) showed reduced survivin protein in the tumour and tumour regression in response to YM155 [87, 88].

Table 3

Clinical studies of inactivators of inhibitors of apoptosis (IAP) proteins

Clinical drug	Drug type	Target	Phase	Treatment	Patients	Status*
YM155	Small molecule	Survivin	I	Monotherapy	Advanced cancers	Completed
			II	Monotherapy	HRPC	Completed
			II	Monotherapy	NSCLC	Completed
			II	Monotherapy	Melanoma	Completed
			II	Monotherapy	Diffuse large B cell lymphoma	Terminated
			II	+Docetaxel ± prednisone	HRPC, solid tumours	Completed
			II	+Docetaxel	Refractory melanoma	Completed
			II	+Docetaxel	Untreated Her2-negative breast cancer	Closed
			II	+Rituximab	NHL	Closed
			I/II	+Paclitaxel and carboplatin	Solid tumours, NSCLC	Closed
AEG35156	Antisense oligonucleotide	XIAP	I	Monotherapy	Advanced cancers	Terminated
			I	+Docetaxel	Solid tumours	Completed
			II	+Cytarabine and idarubicin	AML	Terminated
			I /II	Monotherapy	CLL, B cell lymphoma	Terminated
			I/II	+Carboplatin and paclitaxel	Advanced NSCLC	Terminated
			I/II	+Sorafenib	Advanced hepatocellular carcinoma	Completed
			I/II	+Gemcitabine	Pancreatic cancer	Terminated
			I/II	+Paclitaxel	Advanced breast cancer	Terminated
AT-406	Small molecule	IAPs	I	Monotherapy	Solid tumours, lymphoma	Active
			I	+Daunorubicin and cytarabine	AML	Active
LCL161	SMAC mimetic	IAPs	I	Monotherapy	Advanced solid tumours	Completed
			I	+Paclitaxel	Advanced solid tumours	Active
			II	+Paclitaxel	Triple-negative breast cancer	Active
HGS1029	Small molecule	IAPs	I	Monotherapy	Advanced solid tumours	Completed
			I	Monotherapy	Lymphoid malignancies	Terminated
GDC-0917	SMAC mimetic	IAPs	I	Monotherapy	Solid tumours	Completed
GDC-0152	SMAC mimetic	IAPs	I	Monotherapy	Solid cancers	Completed
TL32711	SMAC mimetic	IAPs	I	Monotherapy	Refractory solid tumours, lymphoma	Closed
			I	+Gemcitabine	Advanced solid tumours	Active
			I/II	Monotherapy	AML	Active
			I/II	+Carboplatin and paclitaxel, +irinotecan, +docetaxel, +gemcitabine or +liposomal doxorubicin	Adult cancers	Closed
			II	Monotherapy	Advanced ovarian, Fallopian tube and peritoneal cancer	Active

Abbreviations are as follows: AML, acute myeloid leukaemia; CLL, chronic lymphocytic leukaemia; HRPC, hormone-refractory prostate cancer; NHL, non-Hodgkin's lymphoma; NSCLC, nonsmall cell lung cancer. *Status according to http://www.clinicaltrials.gov as of 3 January 2013: closed = active, not recruiting.

Clinical studies

LY2181308 (antisense oligonucleotide against survivin) LY2181308 has been studied as single agent therapy as a 3 h intravenous infusion of 750 mg for 3 days consecutively, then once weekly thereafter in refractory solid tumours in Japanese patients (n = 12) and in relapsed/refractory AML in US patients (n = 16). LY2181308 was reasonably well tolerated, with reversible and low-grade toxicities of flu-like syndrome, prolonged prothrombin time, thrombocytopenia and fatigue. A dose-limiting toxicity of reversible grade 3 elevation of aspartate or alanine aminotransferase/gamma-glutamyl transpeptidase was reported in one patient. The systemic disposition LY2181308 revealed a long terminal elimination half-life of 21 days with a large apparent volume of distribution, indicating tissue accumulation. There was reduced survivin expression in tumours of patients with high survivin expression. One of the 12 Japanese patients in this study was observed to have stable disease [89]. The US study initially treated a patient cohort (n = 8) with LY2181308 as monotherapy (dosed as described above). In a subsequent cohort (n = 16), LY2181308 was combined with cytarabine (1.5 g m^{-2} 4 h intravenous infusion) and idarubicin (12 mg m^{-2}, 30 min intravenous infusion) on days 3, 4 and 5 and then again on days 1, 2 and 3 of subsequent 28 day cycles. The combination produced four of 16 complete responses, one of 16 incomplete response, and four of 16 AML patients showed cytoreduction. Six of eight patients who received LY2181308 monotherapy died, and three of 16 who received combination therapy died [90].

YM155 (sepantropium bromide) YM155 has been studied as single agent therapy given as a continuous intravenous infusion for 168 h every 21 days in two phase I studies in patients with advanced solid tumours. The maximal tolerated dose in US patients (n = 41) was

4.8 mg m^{-2} day^{-1}, while in Japanese patients ($n = 33$) using a hydration regimen it was 8 mg m^{-2} day^{-1}. The dose-limiting toxicity in both studies was unexpected reversible acute tubular necrosis. Other milder toxicities included fever, arthralgia, nausea and diarrhoea. YM155 pharmacokinetics revealed an elimination half-life of 20.7 h. In the US study, responses were observed in refractory NHL (three of five; one complete) and in refractory prostate cancer (two of nine; both partial responses) and a minor response in one of two NSCLC patients. In the Japanese patients, a pretreatment hydration and hydration regimen supplemented the 168 h infusion of YM155. The dose-limiting toxicity was nephrotoxicity; additional lesser toxicities were microalbuminuria (suggesting glomerular effects), fever, anaemia, lymphopenia and infusion-site reactions. Pharmacokinetics in the Japanese study were similar to those found in the US study, with dose linearity and a similar terminal elimination half-life. The fractional dose excreted in the urine varied from 25 to 42% and was not related to dose. Stable disease in nine of 33 patients was observed on tumour burden assessment [91, 92]. YM155 pharmacokinetics were further studied, and a 30% increase in YM155 exposure (AUC) in patients with moderate (but not mild) renal dysfunction was observed, suggesting that dose modification would be beneficial in such patients [93].

Further clinical development of YM155 included phase II monotherapy studies in hormone-refractory prostate cancer, diffuse B cell lymphoma and NSCLC. Clinical data from these studies showed limited single agent YM155 activity [94–97]. However, strong preclinical data suggested beneficial effects of YM155 when combined with approved cytotoxic agents; thus, several phase II studies of combinations of YM155 are ongoing (see Table 3).

IAP antagonists

Preclinical studies An alternative means to target IAP proteins is by using mimetics of their natural antagonists (e.g. SMAC). Many small molecule IAP antagonists have been designed, including AT-406, LCL161, HGS1029, GDC-0917, GDC-0152 and TL32711. AT-406 is an orally active antagonist of multiple IAP proteins and has antitumour activity as single agent and in combination with carboplatin in ovarian cancer cells and in xenografts [98]. This activity may rely on its ability to downregulate XIAP. LCL161 has shown limited *in vitro* and *in vivo* activity as a single agent against paediatric cancer preclinical models and is under investigation as combination therapy [99]. GDC-0152 demonstrated K_i values of 17–43 nM for XIAP, cIAP1 and cIAP2 and showed remarkable tumour growth inhibition in a breast cancer xenograft model [100]. TL32711 is reported as a SMAC mimetic, whose preclinical data suggests that in combination with a DR4/5 agonist it may be effective against follicular and germinal cell lymphomas [101].

Clinical studies IAP antagonists currently reported in phase I trials include AT-406, LCL161, HGS1029, GDC-0917 and GDC-0152, with TL32711 in phase II trials [102] (see Table 3). Early clinical reports of HGS1029 suggest that it is well tolerated, with dose-limiting toxicities including severe fatigue, elevated amylase and lipase levels. Tumour regression was seen in one colon cancer patient and stable disease in two patients (NSCLC and adrenocortical carcinoma) in response to HGS1029 treatment [103]. TL32711 is currently under investigation under the name of birinapant, with early clinical results reporting that this SMAC mimetic is well tolerated and results in rapid and sustained cIAP1 suppression in tumour biopsies [101, 104].

Rationale for combining pro-apoptotic agents

The extrinsic and intrinsic apoptotic pathways can be stimulated independently of one another, although cross-talk can occur via caspase 8 activation of Bid. For example, FADD–/– and caspase 8–/– fibroblasts are resistant to death receptor stimulation but sensitive to cytotoxic drugs (activation of the intrinsic pathway), and caspase 9–/– embryonic stem cells and Apaf1–/– thymocytes are sensitive to death receptor agonists but resistant to cytotoxic drugs (activation of the extrinsic pathway) [6].

Malignancies are notorious for having defects in one or both apoptotic pathways and cannot be successfully targeted by single agent therapies. Thus, combination therapies are more likely to be successful. Multistep targeting of the apoptotic cascade by combining rhTRAIL with Bcl-2 antagonists or IAP-blocking mimetics enhances apoptosis rates in malignant cells *in vitro* [105–108]. Certain chemotherapeutic drugs cause endogenous expression and/or translocation of DR4/5 to the extracellular membrane, which could enhance their combined use with other pro-apoptotic agents. Furthermore, the expression of anti-apoptotic and pro-apoptotic proteins can be transcriptionally regulated by nuclear factor-κB and p53, respectively, in response to chemotherapeutic drugs. It should be emphasized that combining targeted pro-apoptotic agents with classical cytotoxic chemotherapy has an indirect effect on enhancing the pro-apoptotic equilibrium in cells compared with the use of the targeted pro-apoptotic agent alone.

Conclusions and future directions

Targeting apoptosis is an exciting paradigm in cancer drug development. Antibodies and recombinant TRAIL that target the death receptors are in phase II clinical trials against a range of solid tumours, but the maturing clinical data on their antitumour efficacy are disappointing. Bcl-2 family and IAP antagonists with preclinical efficacy are

showing promising antitumour efficacy in early clinical trials against certain tumours, especially haematological malignancies. Preclinical data will be important in identifying the genetic bases for the development of tumour resistance to this class of compounds by comparing the DNA, RNA and proteins in resistant and sensitive cancers. Tissue can then be collected in subsequent clinical trials to address these hypotheses in cancer patients. One aspect of further clinical trials with these agents should be an improved understanding of the systemic drug exposure (i.e. drug dose and schedule) relationship to drug molecular target tumour pharmacodynamics, emphasizing the importance of obtaining tumour tissue or developing a validated biomarker for the antitumour effects of such targeted drugs. Effective treatment for some tumours will probably require that pro-apoptotic agents are administered with other established anticancer drugs in scientifically guided combinations.

Competing Interests

D.J.P.B. has no competing interests to declare. L.D.L. has received research grants from Abbott Pharmaceuticals and Astellas for early clinical development studies of navitoclax (ABT-263) and sepantronium bromide (YM155), respectively.

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