

## Exploiting BH3 only protein function for effective cancer therapy

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## 1. ABSTRACT

Failure to induce tumour selective, efficient cell killing is a major problem underlying the limitations of conventional cytotoxic chemotherapy. Greater understanding of the downstream death inducing signalling pathways and how they are regulated in the drug resistant setting is crucial for improvement of survival in most solid cancers. Here we review the role played by BH3 only proteins in mediating cell death through BAX and BAK, and how this knowledge has lead to a new generation of targeted agents with promising cancer cell killing efficacy.

## 2. APOPTOSIS RESISTANCE AS A HALLMARK OF CANCER

Evasion of programmed cell death or apoptosis is now recognized as a central hallmark of cancer, that contributes significantly to drug resistance (1). Apoptosis is a biochemically stereotyped, phylogenetically conserved process characterized by blebbing of the plasma membrane, cell shrinkage, nuclear condensation, internucleosomal fragmentation, and externalization of the aminophospholipid phosphatidylserine.

Historically, complete objective tumour response has been a recognized prerequisite, where cure is achievable. In a subset of relatively rare cancers (for example, leukaemia, lymphoma or seminoma), this equates to efficient neoplastic cell killing, a factor dependent in part on the intrinsic sensitivity to chemotherapy. For the majority of solid tumours however, complete remission is never achieved due to inherent drug resistance. Even in settings where high dose therapy has been employed, to maximize the killing of tumour cells, this has consistently failed to demonstrate clinical benefit in non-haematological, non-germ cell malignancies. It is now recognized that cancer cells exhibit genetic and phenotypic heterogeneity and are subject to Darwinian evolutionary processes leading to acquisition of resistance. This is most clearly evidenced in settings where oncogene addiction has prevailed initially, only to be followed by acquisition of resistance following initially successful treatment with targeted therapies as discussed later in this chapter. With respect to chemotherapy however, the majority of solid tumours exhibit *de novo* resistance to chemotherapy associated with single agent chemotherapy response rates in the order of 20%. This response rate is commonly even

lower due to acquired resistance, at the time of re-treatment in the salvage setting, when the disease has relapsed.

In recent years, delineation of biochemical mechanisms that underlie the death and survival pathways capable of conferring selective advantage to neoplastic cells during tumorigenesis have been identified. This knowledge has underpinned a new therapeutic objective in which overcoming constitutive suppression of apoptosis either through re-sensitization or direct activation is just beginning to be evaluated in the clinical setting. This review will discuss our current state of knowledge regarding the processes which initiate and regulate induction of apoptosis, how this is dysregulated in human cancer, and how novel therapies that target core apoptosis signalling machinery of the cell may be used to overcome drug resistance and improve clinical outcome.

### 3. MITOCHONDRIA: A CHECKPOINT FOR APOPTOSIS REGULATION

A biochemical feature of apoptosis is the induction of cellular demolition by a family of phylogenetically conserved aspartate specific cysteine proteases termed caspases. CED3, a known regulator of programmed cell death, was identified in the flatworm *C.elegans* gene, as an ortholog of human and mouse interleukin-1 beta converting enzyme (2). This finding led to the discovery that evolutionarily conserved cysteine proteases function in mammalian programmed cell death (3, 4). One of the key executioner caspases initially characterized as CPP32/Yama beta was identified as a *C.elegans* CED3 homolog in humans (5). Subsequently 14 caspases have been cloned and sequenced. The caspases cleave over 1000 substrates to manifest the apoptotic phenotype, recognizing the pentapeptide motif AQCXG where X is the amino acid D, Q or R (6). Caspases can also autoactivate as well as activating other relatives. In general, activation involves cleavage to yield a large and small subunit resulting in assembly of a heterotetrameric active enzyme (Figure 1A). The classification of caspases has led to the subdivision into two groups; the initiator and executioner caspases. Caspases 3 and 7 are key executioners whereas caspases 2,8,9, and 10 are classified as initiator caspases. Two relatively well defined pathways of initiator caspase activation have been defined; the intrinsic pathway, involving caspase 9 activation, and the extrinsic pathway involving caspase 8 and 10. The role of Caspase 2, one of first caspases to be identified (7) has been shown to initiate cell death in response to genotoxic stress (8). The intrinsic pathway of apoptosis plays a particularly important role in the response to chemotherapy and will be discussed next.

### 4. BCL-2 FAMILY PROTEINS REGULATE OF MITOCHONDRIAL PERMEABILITY

#### 4.1. Mitochondrial Outer membrane permeabilization (MOMP)

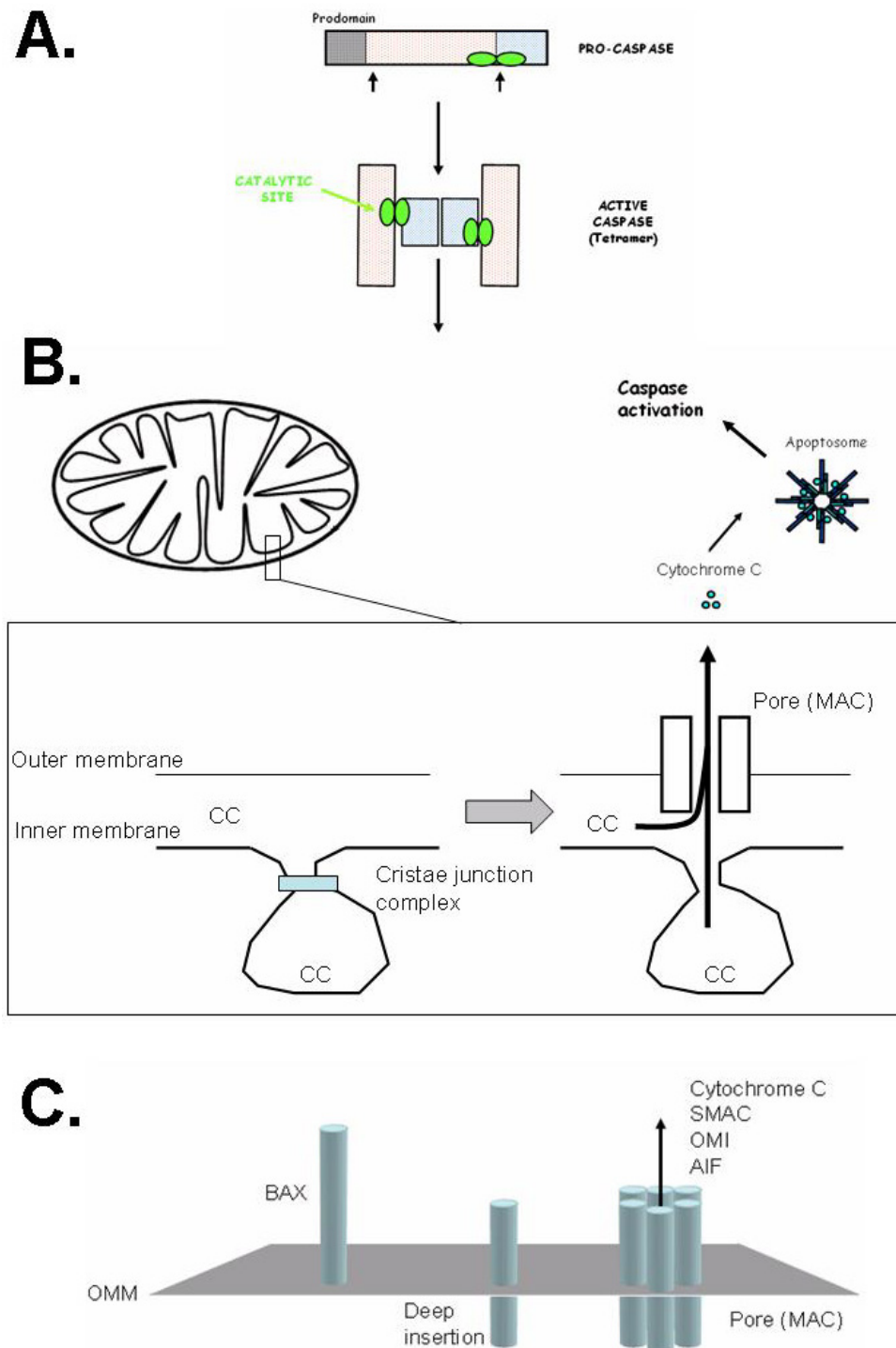
Mitochondria play a pivotal role in the regulation of apoptosis. These organelles are responsible in healthy cells for the cell's principle energy currency, ATP generated via the tricarboxylic acid cycle. However during

apoptosis, they act to signal the activation of caspases. During the late 1990s cytochrome C was identified as a key mitochondrial signalling protein released from mitochondria, which with dATP and the CED-4 homolog APAF-1, activates the formation of a caspase 9 activating scaffold called the apoptosome (9-11). Genetic knockout studies subsequently confirmed the essential role of cytochrome C in apoptosis (12). Although the precise molecular mechanism of cytochrome C release from mitochondria has not been universally agreed, there is compelling evidence supporting the requirement of a novel pore formation early during apoptosis.

Multimeric proapoptotic proteins of the BCL-2 family BAX and BAK were identified as binding partners of the antiapoptotic protein BCL-2, the first member of this important apoptosis regulating protein family, and which was originally identified as the gene product of the t(14;18) translocation in follicular lymphoma (13-16). BAX and BAK are functionally redundant proteins. BAK resides constitutively in the outer mitochondrial membrane and endoplasmic reticulum, whereas BAX is predominantly cytosolic or loosely attached to mitochondrial, nuclear or endoplasmic reticulum membrane. Both proteins comprise BCL-2 homology (BH) domains 1, 2 and 3. The third BH domain, called BH3 was shown in isolated oligopeptide studies and by mutagenesis to be essential for the cell death activity of these proteins (17-19).

During apoptosis, BAX and BAK undergo deep membrane insertion and oligomerization, assembling into higher molecular weight clusters or heterooligomers (20) at the mitochondrial surface, resulting in the release of cytochrome C. In the case of BAX, exposure of its C terminus membrane addressing anchor and translocation to the outer mitochondrial membrane occurs (21-23). At this time, mitochondrial outer membrane permeabilization or MOMP occurs resulting in the release of cytochrome C. Genetic studies have suggested a model in which BAX and BAK are essential for cytochrome C release, although there is increasing evidence that this event can occur in their absence in some model systems (24). BAX and BAK exhibit other distinct functions including the regulation of mitochondrial morphology and calcium homeostasis

The mechanism by which cytochrome C is released across the outer mitochondrial membrane remains controversial. There is evidence supporting the formation of a novel conductance known as the mitochondrial apoptosis channel, that comprises BAX and BAK (25, 26). However it has been argued that this channel represents a late event *in vivo*. The release of cytochrome C has been shown to proceed through a two stage process of which BAX and BAK dependent MOMP is but one. Most cytochrome C is localized in the cristae compartment, and sequestered from the mitochondrial intermembrane space by cristae junctions. During release of cytochrome C, these junctions, which are stabilized by mitochondrial shaping proteins OPA-1 and PARL are opened, allowing the complete cytochrome C content to be available for release across the outer mitochondrial membrane (27-29), Figure 1B.



**Figure 1.** A. Caspase activation: - cleavage of a procaspase to form a large and small subunit resulting in the assembly of a heterotetrameric active caspase. B. The release of cytochrome c following mitochondrial outer membrane permeabilization (MOMP) enables the formation of the apoptosome resulting in the activation of caspase 9. BH3 only protein BID causes remodeling of mitochondrial ultrastructure, leading to opening of cristae junctions, which are kept closed (in a PARL and OPA-1 dependent manner). MOMP ensures release of all cytochrome C from the mitochondrion into the cytoplasm. C: Activation of Bax results in its translocation to the mitochondrial surface followed by deep insertion into the mitochondrial membrane. Activated Bax and Bak assemble into heteroligomers resulting in mitochondrial outer membrane permeabilization and the release of mitochondrial proteins including SMAC, OMI, AIF and cytochrome c.

Cytochrome C is not the only protein to be released during apoptosis. Other proapoptotic proteins such as SMAC, OMI and apoptosis inducing factor and several other proteins, have been shown to be released from mitochondria (30-33). The kinetics of their coordinate release suggests a common pathway (34)

BAX and BAK are oligomerized in response to activating BCL-2 family proteins (Figure 1C); a subgroup which share proapoptotic BH3 domains (Figure 2A), called BH3 only proteins. This growing list of proteins include BID, BIM, PUMA, BIK, BAD, NOXA, BNIP3, BMF, MULE/ARF-1, and beclin-1. BH3 only proteins promote BAX and BAK and act as death signals from different cellular compartments. It was originally proposed that certain BH3 domains can directly oligomerize BAX and BAK; namely, BID, BIM and PUMA (35-37). Recent reports however have suggested that BH3s act not to directly activate BAX and BAK, but to indirectly activate them through disruption of binding to antiapoptotic BCL-2 proteins (38, 39), as discussed more fully in the next section. It should be noted that BAX and BAK may also be activated in the absence of BH3 only proteins by environmental stimuli such as heat (40). The tumour suppressor, p53 is one of the most commonly mutated genes in cancer. Following DNA damage, p53 signals to either repair the damage or irreversibly commit the cell to death. The mechanism of tumour suppression has been shown *in vivo* to involve induction of apoptosis via BAX and BAK (41). Recent evidence has shown that p53 can directly activate BAX at the mitochondrial surface and induce its expression (42, 43).

Genetic studies have established a critical role for BAX and BAK in the induction of cell death arising from a wide range of toxic stimuli including chemotherapy and are required for BH3 only protein induced cell death (44, 45) BAX and BAK do not only regulate permeabilization of the outer mitochondrial membrane, but have been identified as regulators of calcium homeostasis (44, 46, 47), and mitochondrial morphogenesis (48) both of which are implicated in apoptotic death.

BAX and BAK are inhibited by antiapoptotic proteins of the BCL-2 family that include BCL-2, BCL-XL, MCL-1, BCL-W, A1 and BCL-B. These proteins prevent BAX and BAK oligomerization and cytochrome C release through heterodimerisation (14, 49). In addition, these proteins can sequester BH3 only proteins, acting as a sink to neutralize them (50, 51).

#### **4.2. Suppression of MOMP by Prosurvival BCL-2 family proteins**

Antiapoptotic BCL-2 family members are overexpressed in several cancers and may contribute to clinically relevant apoptosis resistance by preventing BAX and BAK oligomerization. Structural analysis has shown that antiapoptotic BCL-2 family proteins exhibit a shallow groove on their surface with which proapoptotic BH3 domains can interact (52, 53). This BH3 “receptor” has been exploited for the rational development of small molecule antiapoptotic BCL-2 family antagonists that are

now entering the clinic, as discussed further on. A further function of BCL-XL is to bind to and hold open the mitochondrial voltage dependent anion channel, VDAC1 (54, 55). This beta-barrelled pore, analogous to bacterial porin regulates pyridine nucleotide exchange across the outer mitochondrial membrane in conjunction with the adenine nucleotide transporter (ANT).

High rates of ADP/ATP trafficking contribute to apoptotic resistance and high glycolytic rates observed in cancer cells. Since cell survival is now known to be associated with open VDAC1, previous reports that open VDAC1 was a cytochrome C release channel are now believed to be incorrect (56). Recently, a new class of small molecules have been identified that target and require VDAC for activity, called erastins, that can selectively kill ras mutated cells (57). This suggests that although physiologically, VDAC does not appear to be required component of the cell death pathway, its role in mitochondrial homeostasis in cancer, may still make it a potential target for therapeutic targeting in the future.

#### **4.3. BH3 only proteins regulate BAX/BAK oligomerization**

The presence of a shallow groove on the surface of antiapoptotic BCL-2 proteins provides a “receptor” for sequestration of both multidomain and BH3 only proteins (53). Mitochondrial BAK is sequestered by BCL-XL and MCL-1 both of which appear to be required to be dissociated before BAK can undergo full activation. Furthermore, in its open conformation, BAK oligomerization can be arrested by BCL-2 in a manner mimicked by the adenoviral oncogenic 19K E1B protein (58). VDAC2 has also been shown to suppress BAK through a BH3 only protein inhibitable, and direct interaction. BAK therefore can be restrained through multiple interactions, which require disruption in order to enable its full activation. Dissociator BH3 mimetics are capable of disrupting these interactions, and therefore, in the context of cancers where there is multiple antiapoptotic BCL-2 expression, exogenous BH3 mimetics have the potential to enhance apoptosis signalling via derepression of multidomain.

Recent evidence has suggested that endogenous activator BH3s (BID/BIM) are present in the mitochondrial outer membrane of some cancers, neutralized through constitutive interactions with antiapoptotic BCL-2 proteins. This state has been termed ‘priming for death’ (59, 60). The origin of these death signals may be secondary to oncogene activation. For example, C-myc can induce death signals including activation of BID, through the activation of CD95. It has been suggested that some cancers, such as small cell lung cancer and some leukaemias require antiapoptotic BCL-2 family proteins to protect against these death signals and are therefore addicted to overexpression of antiapoptotic BCL-2 proteins. This potential apoptotic Achilles heel now presents a promising opportunity for therapy.

### 5. EXPLOITATION OF BH3 ONLY PROTEINS FOR ANTI-CANCER THERAPY

#### 5.1. Small Molecule BH3 peptidomimetics

Based on structure based modelling of the BH3 receptor in BCL-2 using NMR, the first drugable high affinity small molecule BH3 peptidomimetic was reported in 2005 (61) (Figure 2B). Although a number of other BH3 mimetics have been reported previously, robust studies employing BAX and BAK double knockout cells, have confirmed that many of these do not exhibit bone fide BH3 peptidomimetic pharmacology. The efficacy of BH3 peptidomimetics depends on the affinity for specific antiapoptotic BCL-2s. Thus, ABT-737 has a BAD like binding profile showing preferred interactions with BCL-2, BCL-XL. And BCL-W. Consequently, MCL-1 expression has been demonstrated to be an important resistance marker of ABT-737 (62-64). In cancers such as small cell lung cancer, where MCL-1 is not highly expressed, ABT-737 (and BAD) can induce single agent toxicity *in vivo* (61). This is consistent with this cancer being primed for death by BH3s. An oral formulation of this compound (ABT-263) has been produced that is now in phase I clinical trial in small cell lung cancer and leukaemia. Other BH3 peptidomimetics including obatoclax (GX15-070) (65, 66) (Figure 2B) and AT-101 (- gossypol) (67) which are reported to interact with MCL-1, are also in clinical trial, both as single agents and in combinations. The results of early efficacy studies should provide evidence of activity.

A completely different approach for targeting antiapoptotic BCL-2 family proteins, is the downregulation using Antisense oligonucleotides. The use of a phosphorothioate, G3139 has been shown to promote apoptosis in the preclinical and clinical setting. A recent phase III study confirmed that this agent was capable of improving the response rates of patients with chronic lymphocytic leukaemia (68). The pharmacodynamics of phosphorothioates has been controversial however, in part due to the ability of this chemistry to mediate several off target effects such as binding to cell surface proteins (69, 70). Recently, G3139 has been shown to interact with the mitochondrial protein VDAC (71); in so doing, G3139 can modulate pyridine nucleotide exchange mitochondrial homeostasis in a manner antagonistic to BCL-XL. This unexpected activity may therefore contribute significantly to the proapoptotic activity of this compound.

#### 5.2. Modulation of BCL-2 family by 20S proteasome inhibition

Bortezomib is an inhibitor of the 20S proteasome licenced for use in myeloma, involved in the turnover of proteins following their ubiquitination. Recent evidence has shown that bortezomib requires BAX and BAK for induction of apoptosis (72), and furthermore, the activation of the BH3 proteins BIM, NOXA and BIK are required for activity (70, 73, 74). Although MCL-1 exhibits rapid turnover by the proteasome, it appears that the induction of NOXA (which interacts with MCL-1) may be sufficient to prevent the antiapoptotic effects of its increase in level. Indirectly therefore, bortezomib can activate the intrinsic pathway. Since taxane chemotherapy causes the increase in

BIM, it has been proposed that bortezomib and taxol presents a rational strategy for maximizing synergy. Evidence *in vivo* however has not borne this hypothesis out in one phase II trial in lung cancer possibly due to other resistance factors.

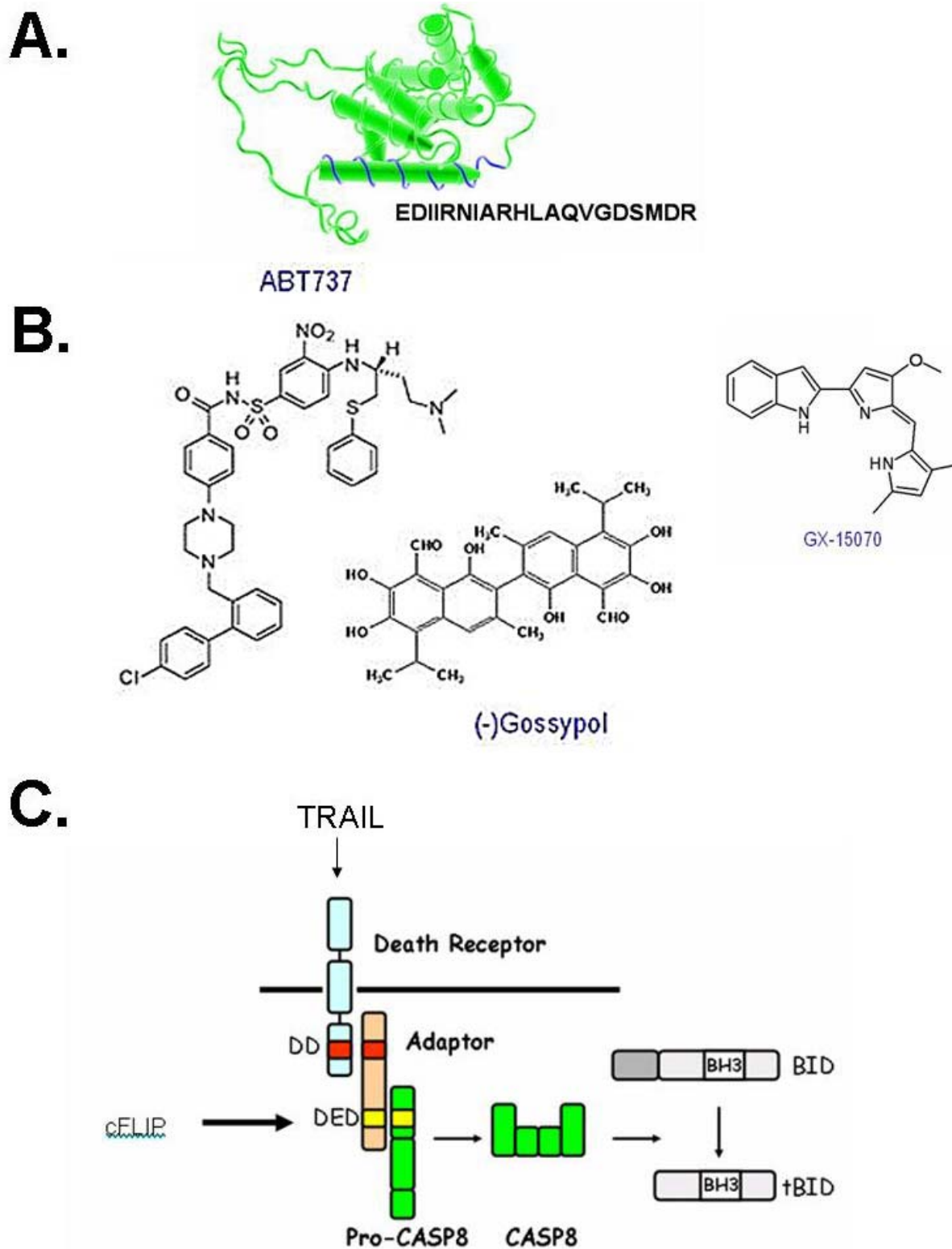
#### 5.3. Extrinsic pathway activation

Caspases can be directly activated via the ligation of cell surface death receptors. The first of these receptors to be identified was FAS (CD95) (75, 76). FAS plays a critical role during development of the immune system and is involved in the elimination of autoreactive immune cells (77). This receptor interacts with an adaptor protein via homotypic death domains (78), which in turn, recruits initiator caspases 8 or 10 (79) via homotypic death effector domains (80). Together these three proteins constitute the death inducing signalling complex or DISC. Caspase 8 cleaves BID upon FAS receptor ligation by FAS ligand, leads to BID cleavage to truncated BID (p15 tBID), resulting in the mitochondrial translocation and activation of BAX and BAK (81). FAS receptor is a member of the tumour necrosis factor (TNF) receptor family that includes receptors for TNF related apoptosis inducing ligand (TRAIL), DR4 and D5 (as well as decoy receptors) (82, 83). FADD interacts with proteins known as FLICE inhibitory proteins (FLIPs) and their viral homologues, blocking access to caspase 8 (84) (Figure 3C). FLIP (in particular the long isoform) is therefore antiapoptotic and appears to contribute to chemoresistance (85, 86). FLIP is degraded by the proteasome pathway promoted by activation of the stress kinase pathway (87). TRAIL can selectively induce toxicity in cancer but not healthy cells, and is currently entering the clinic as a therapeutic approach for activating cell death as discussed later.

#### 5.4. Targeting Death Receptors for Cancer therapy

*In vivo* data suggests that hepatotoxicity following FAS receptor activation is unacceptably high to warrant clinical evaluation, however activation of TRAIL receptors appears to be an acceptable strategy that is being currently evaluated in the clinic. Thus, Apo2 ligand exhibits a homotrimeric structure, and upon docking with its receptor, causes homotrimerization (88). TRAIL ligand has been shown *in vivo* to exhibit significant toxicity against tumour cells, and increases in survival preclinically (89), whilst having little effect on normal cells (90, 91), presenting an attractive therapeutic index. Mutational screening has shown that the DR5 TRAIL appears to contribute more significantly to toxicity compared with DR4 receptor (92). Even in the absence of caspase 8, TRAIL can mediate toxicity by recruitment of the initiator caspase 10 to the DISC (79). TRAIL requires BAX for induction of apoptosis, reflecting essential crosstalk between death receptor activation, post-translational processing of BID and its signalling at the mitochondrial surface (93). Currently both trail ligand and trail agonistic antibodies (94) are being evaluated in early phase clinical trials, and exhibit potential to enhance conventional chemotherapy.

Cellular FLIP is expressed in a number of cancers and like viral FLIP can inhibit death receptor signalling



**Figure 2.** A. Highlight of the alpha-helical BH3 domain of the BH3 only proapoptotic Bcl-2 family member Bid and its peptide sequence. B: Structures of BH3 peptidomimetics, ABT737, GX-15070 and (-)Gossypol, currently in clinical trial. C. Simplified schematic representation of death receptor signaling through the DISC leading to caspase 8 recruitment and activation via homotypic DED interactions. FLIP inhibits recruitment by competing via the DED. TRAIL is an endogenous ligand of death receptors DR4 and DR5.

(85). Knockdown of the long isoform of FLIP has been shown to promote death receptor induced apoptosis (86). There is growing evidence that a number of tumours exhibit addiction to FLIP *in vivo*, such that knockdown of FLIP using siRNA alone is sufficient to efficiently induce cell death. Because FLIP interacts with the DISC through homotypic death effector domain interactions, it directly competes with caspase 8 and 10. consequently, strategies to antagonize FLIP interaction at the DED are likely to also affect caspase 8 activation, making this a particularly challenging antiapoptotic protein to target. Decoy receptors for TRAIL present another potential mechanism of antagonism to TRAIL that in theory, should be surmountable (82).

## 6. SUMMARY

Recently, there has been a considerable growth in knowledge related to the core apoptosis pathways in cancer and their role in promoting cell survival and drug resistance. In some cases, new pathways of cellular addiction have been discovered, and in some cases, the remarkable efficacy associated with targeting these pathways has been demonstrated in the clinic. The paradigm has emerged that cancer, to evolve successfully, must acquire mutations in protooncogenes that are inevitably proapoptotic, and critically, dependent on key survival pathways to enable tumorigenesis. We are now just beginning to understand and target these key pathways in the clinic. Already, the genomic plasticity evident in newly identified resistance mechanisms that develop to therapies targeting addiction pathways suggests that single cure may still be elusive for most solid tumours, however, the possibility to much more effective system therapy capable significantly improving the quality of life and survival from cancer remains a real possibility.

## 7. ACKNOWLEDGEMENTS

DF and AC are supported by Cancer Research UK and Action Cancer. DF is a recipient of a CR-UK Clinician Scientist Fellowship.

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**Key Words:** Cancer, Apoptosis, Drug Resistance, BAX, BAK, MOMP, BH3, BID, BIM, Review

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