Inhibitor of Apoptosis Proteins and Their Potential in Cancer Therapy

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Inhibitor of apoptosis proteins (IAPs) are a family of endogenous, pro-survival molecules found within the cell. They prevent apoptosis, a form of programmed cell death, by interfering with apoptotic pathways and inhibiting caspase cascades. IAPs are over expressed in many forms of cancer and thus contribute to one of the key hallmarks of cancer- the evasion of apoptosis. As a result, IAPs represent a novel target for the specific treatment of a range of cancers, especially those resistant to standard treatments such as chemotherapy and radiotherapy. The use of IAP inhibitors, antagonists and antisense oligonucleotides to downregulate IAPs has been shown to produce potent anti-tumour activity. Furthermore, targeting IAPs has proven markedly effective when used as part of a combination treatment, such as with the pro-apoptotic cytokine TRAIL.

Introduction

Apoptosis is an energy dependent form of programmed cell death. It is critical during development, in the maintenance of homeostasis and as an immunological and anti-tumor defence mechanism. Dysregulation of apoptosis contributes to a wide variety of human conditions such as cancer, neurodegenerative diseases and autoimmune disorders (Favaloro *et al.*, 2012). Distinct morphological changes characterise apoptosis. These include blebbing, cell shrinkage, chromatin condensation and DNA fagmentation (Elmore, 2007). Initiation of cell death pathways triggers sophisticated caspase cascades leading to cell death. Caspases are a family of aspartic acid specific proteases that are endogenous in

the cytoplasm as single chain zymogens (Nicholson, 1999). Upon apoptotic signal initiation upstream initiator caspases, such as caspase 8, cleave effector caspases at internal aspartic acid residues, transforming them into their tetrameric active form (Thornberry and Lazebnik, 1998). These effector caspases, such as caspase 3, activate targeted cellular proteins to trigger apoptosis. Apoptosis can be initiated intracellularly or extracellularly resulting in two distinct pathways: the intrinsic and the extrinsic pathway (Figure 1).

The intrinsic or mitochondrial pathway involves diverse, non-receptor mediated stimuli such as genotoxic agents, cell stress or loss of cell survival factors. These apoptotic signals result in the formation of the mitochondrial permeability transition pore, loss of the mitochondrial transmembrane potential and release of pro-apoptotic proteins into the cytosol (Saelens *et al.*, 2004). Smac (Second Mitochondrial Activator of Caspases) binds to and neutralises X-linked IAP (XIAP) which releases and facilitates the activation of caspase 3, 7 and 9 (Schimmer, 2004). Similarly, the serine protease Omi/HtrA2 binds to and inhibits XIAP (Suzuki *et al.*, 2001a). Cytochrome c binds to and activates apoptotic protease factor 1 (Apaf-1) along with ATP and pro-caspase 9 forming the apoptosome (Chinnaiyan, 1999). Caspase 9 is activated, initiating the caspase cascade. It induces the cleavage of pro-caspase 3 releasing the active effector caspase 3, and ultimately resulting in programmed cell death. Many conventional cancer treatments, such as chemotherapy and radiotherapy, induce DNA damage thus targeting the intrinsic apoptotic pathway in a p53 dependent manner (Fulda and Debatin, 2006).

The extrinsic pathway is initiated when a transmembrane death receptor (DR) is bound by its corresponding death receptor ligand (DRL). A number of these DR and DRL pairs have been described including members of the TNF superfamily, such as TNF-α with TNFR1, FasL with FasR, and TRAIL with DR4 or DR5 (Guicciardi and Gores, 2009). Upon ligation, adaptor proteins with corresponding death domains are recruited, such as Fas-Associated protein with Death Domain (FADD) and TNF receptor associated factor (TRAF), which then associate with pro-caspase 8 forming the death inducible signalling complex (DISC) (Kischkel *et al.*, 1995). Pro-caspase 8 is auto cleaved to caspase 8 which goes on to activate caspase 3, triggering the proteolytic cascade and apoptosis. Caspase 8 may also feedback into the intrinsic pathway by processing and activating BH3 interacting domain death agonist (Bid). Bid provokes the release of cytochrome c, fuelling apoptosome mediated cell death (Roy and Nicholson, 2000).

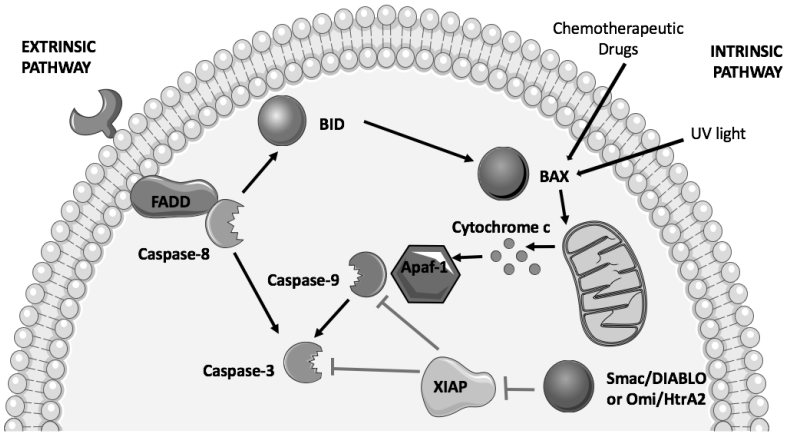

Figure 1. *The intrinsic and extrinsic cell death pathways Intrinsic signals trigger the release of pro-apoptotic proteins from the mitochondria. Smac and Omi/HtrA2 inhibit XIAP while cytochrome c, Apaf-1 and pro-caspase 9 form the apoptosome. Activated caspase 9 further activates caspase 3 thus triggering the caspase cascade resulting in apoptosis. Extracellular DRLs bind their DRs, adaptor proteins are recruited and caspase 8 is activated. This either goes on to activate caspase 3 and initiate the cascade or else activates Bid which promotes release of cytochrome c from the mitochondria. Adapted from Salvesen and Duckett (2002).*

Evasion of apoptosis is one of the key hallmarks of cancer and allows cells to divide and grow uncontrollably (Hanahan and Weinberg, 2011). Transformed cells employ a plethora of mechanisms in order to escape death. These include loss of p53, downregulation of Bid and importantly, upregulation of IAPs (Fernald and Kurokawa, 2013). Indeed, targeting IAPs represents a novel strategy for treating tumours. Because of this, many efforts have been made to develop antagonists, inhibitors and antisense oligonucleotides that downregulate IAPs in tumour cells, particularly in those that are already multi drug resistant. This review will discuss the molecular and cellular characteristics of the IAP family, their potential role in cancer therapy and the emerging importance of combination therapies.

Inhibitor of Apoptosis Proteins

IAPs are a family of endogenously expressed anti-apoptotic proteins and hence are promising targets in cancer drug discovery. There are eight members of the

IAP family in humans: cellular IAP1 (cIAP1), cIAP2, XIAP, melanoma IAP (ML-IAP), survivin, IAP-like protein 2 (ILP-2), neuronal apoptosis inhibiting protein (NAIP) and Apollon (de Almagro and Vucic, 2012). The activity of IAPs is regulated by endogenous antagonists such as Smac. Overexpression of IAPs has been found in many cancers and facilitate cancers in the evasion of apoptosis and ultimately contribute to tumorigenesis and metastasis (Sung *et al.*, 2009).

The Structure of IAPs

IAPs are characterised by protein domains in their tertiary structure (Figure 2). All IAPs have at least one Bacculoviral IAP Repeat (BIR) domain located at their N terminal (Fulda and Vucic, 2012). cIAP1, cIAP2, NAIP and XIAP each have three BIR domains, while ML-IAP, survivin, Apollon and ILP-2 have one. BIR domains consist of a highly conserved sequence of eighty amino acids with a characteristic arrangement of $CX_2CX_{16}HX_6C$, where C is cysteine, H is histidine and X can be any amino acid. Within the BIR fold, a zinc atom is tetrahedral coordinated by the histidine and the three cysteine residues to establish a hydrophobic environment (Hinds *et al.*, 1999). BIR domains mediate protein-protein interactions between IAPs and their targets. For example, XIAP binds caspases via its BIR2 or BIR3 domain, while cIAP1 and cIAP2 bind TRAFF via their BIR1 domain (Zheng *et al.*, 2010, Huang *et al.*, 2001).

Other common domains include the Really Interesting New Gene (RING) domain which provides E3 ubiquitin ligase activity, the Ubiquitin-Associated (UBA) domain which allows IAPs to bind ubiquitin chains and the Caspase-Activating And Recruitment Domain (CARD domain) which functions as a protein-protein interacting domain and mediates protein oligomerization (Vaux and Silke, 2005) (Gyrd-Hansen *et al.*, 2008).

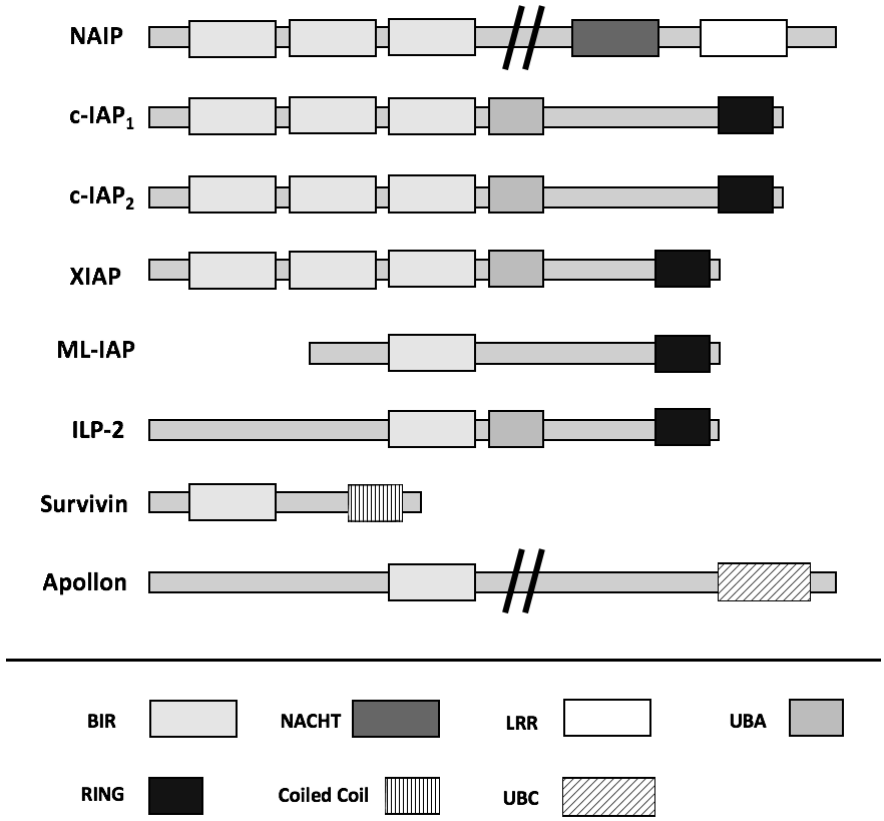

Figure 2. *The protein domains of IAPs. BIR (Bacculoviral IAP Repeat): protein-protein interactions. RING (really interesting new gene): E3 ubiquitin ligase activity. Coiled coil: super-secondary protein structure of coiled alpha helices. NAHCT (Neuronal Apoptosis Inhibitor Protein): NTPase activity. CARD (Caspase-Activating and Recruitment Domain): protein oligomerization. UBA (Ubiquitin-Associated Domain): polyubiquitin binding. UBC (Ubiquitin-Conjugating Enzyme): addition of ubiquitin. LRR (Leucine-Rich Repeat): secondary protein structure of an α/β horseshoe fold (Nishikawa and Scheraga, 1976, Koonin and Aravind, 2000, Gyrd-Hansen et al., 2008, Sekine et al., 2005, Enkhbayar et al., 2004). Adapted from Fulda and Vucic (2012).*

The Functions of IAPs

The members of the IAP family each have different functions within the cell. cIAP1/ cIAP2 serve to promote cell survival signals induced by members of the TNF superfamily. Upon ligation, they are recruited to the TNF receptor via TRAFF1/2

(Benetatos *et al*., 2014). Here they function as ubiquitin ligases by catalysing the addition of ubiquitin chains using their RING domain, for example to NFκB-inducing kinase. These chains can then serve as a scaffold, as signal transducers or as signals to target specific proteins for degradation (Walczak, 2011).

ML-IAP, also known as livin, has pro- and anti-apoptotic activity. Primarily, it binds Smac and promotes XIAP activity and caspase 9 degradation. It also promotes degradation of caspases via its E_3 ligase activity (Vucic *et al*., 2005). In contrast, ML-IAP can be cleaved at aspartate-52 by caspases forming a truncated form with pro-apoptotic activity (Nachmias *et al*., 2003). Thus, ML-IAP overexpression has been associated with drug resistance in melanoma, as well as leukaemia, bladder, cervical, colon, lung, breast and colorectal cancer (Vucic *et al*., 2000, Li *et al*., 2013, Tamm *et al*., 2000).

Survivin is structurally unique in that it is the smallest IAP in the family and only contains one protein domain, a BIR domain (Ambrosini *et al*., 1997). It has roles in the cellular stress response, in development and in mitosis (Altieri, 2010). Survivin has been shown to interact with heat shock proteins *in vivo*, which may help in its subcellular localisation or in the preservation of survivin stability (Fortugno *et al*., 2003, Yano *et al*., 2003). Within the cell survivin binds to and enhances the stability and activity of XIAP via their BIR domains (Dohi *et al*., 2004). During mitosis it has been reported to regulate chromosomal alignment, spindle assembly and stabilisation and cytokinesis (Lens *et al*., 2006).

XIAP is the best characterised member of the family and has the greatest anti-apoptotic activity. Two domains in XIAP are capable of binding and inhibiting nascent active caspases. The BIR3 domain binds directly to the C terminal of caspase 9. The BIR2 domain does not actually bind substrate but serves as a regulatory element for caspase binding and Smac neutralisation. Instead, a small segment directly at the amino terminal side of BIR2 is required for binding of caspase 3 and 7 (Stennicke *et al*., 2002). The sequence of this segment, known as 'the linker', binds the caspases with high affinity in the opposite orientation to their substrates (Huang *et al*., 2001). The BIR1 domain of XIAP induces NFκB and MAP kinase activation via TGF-beta activated kinase 1 (TAB1), an interaction that is subject to inhibition by Smac (Lu *et al*., 2007). Other functions of XIAP include regulation of the cell cycle (Levkau *et al*., 2001) and ubiquitination via its E3 ligase activity (Suzuki *et al*., 2001b)

IAPs in Cancer

The evasion of apoptosis allows cells to divide and grow uncontrollably thus contributing to tumorigenesis and metastasis. Upregulation of IAPs is believed to be a crucial mechanism by which tumours achieve this (de Almagro and Vucic, 2012). IAPs not only prevent cell death but also promote cell survival via NFκB and MAP kinase activation. Tamm *et al.* (2000) conducted an analysis of the expression of all of the IAPs in 60 human tumour cell lines and the expression

of XIAP in 78 untreated patients. Unsurprisingly, XIAP and $cIAP_1$ were overexpressed in most of the cell lines and $cIAP_2$ was overexpressed in more than half.

Each of the IAPs have different associations with particular cancers. For example, cIAP is associated with cervical cancer (Imoto *et al.*, 2002), survin with and livin with neuroblastoma (Islam *et al.*, 2000) and XIAP with pediatric leukemia (Sung *et al.*, 2009). The overexpression of IAPs in cancer is frequently an unfavourable prognostic parameter associated with poor treatment response and reduced relapse free survival. Notably, it seems that mRNA levels are often higher than protein levels, hinting at post-translational or post-transcriptional regulatory mechanisms (Tamm *et al.*, 2000, Hundsdoerfer *et al.*, 2010).

IAP Inhibition and Antagonism

Over the past decade, various strategies for targeting and inhibiting IAPs have been designed. These include IAP inhibitors and antagonists and antisense oligonucleotides (Fulda and Vucic, 2012). Antisense oligonucleotides are single stranded short pieces of synthetic DNA normally containing 12-30 oligonucleotides. These are designed to downregulate target protein expression by binding to complimentary stretches of mRNA and initiating its degradation (Jansen and Zangemeister-Wittke, 2002). AEG35156 is an example of an antisense oligonucleotide that targets XIAP expression (LaCasse *et al.*, 2006). It has displayed potent anti-tumour activity in mouse models of prostate, colon, ovarian and lung cancer (Fulda and Vucic, 2012). It has also been shown to sensitize cancer patients to cytotoxic agents such as chemotherapy and thus has completed Phase I/II of clinical trials (Holt *et al.*, 2011).

Smac mimetics are IAP inhibitors that mimic the N terminal portion of the endogenous protein Smac. This portion comprises just four hydrophobic amino acids in a conserved sequence: Ala-Val-Pro-Ille. The chain is essential for interacting with the hydrophobic environment of the BIR2 and BIR3 domains of the IAP proteins (Liu *et al.*, 2000). Monovalent as well as bivalent Smac mimetics have been designed (Figure 3). Dispute remains as to which is more favourable – bivalent mimetics seem to work more efficiently yet require intravenous administration while monovalent mimetics have less severe side effects and can be taken orally (Fulda, 2015).

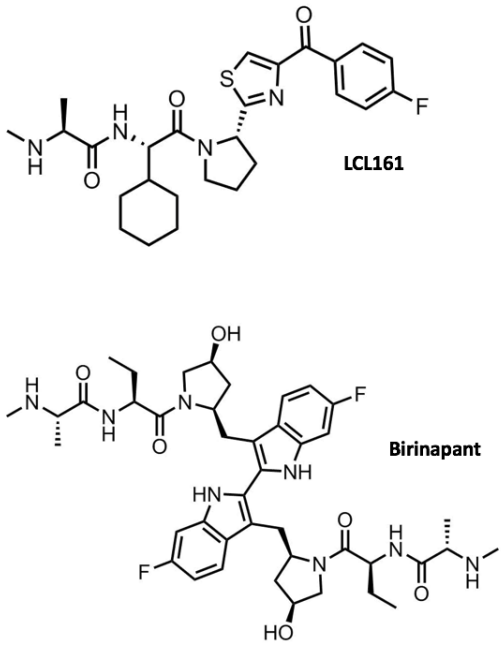

Figure 3. *The chemical structure of Smac mimetics. Examples of a monovalent (LCL161) and bivalent (Birinapant) Smac mimetic that are in the early stages of clinical trials. Adapted from Fulda (2015).*

Smac mimetics bind to several IAPs such as XIAP and cIAP to release XIAP from its inhibitory function and promote activation of activator caspases 3, 7 and 9 (Fulda and Vucic, 2012). They also induce cIAP1/2 proteasomal degradation by stimulating the auto-ubiquitination of cIAP1/2 via its E3 ubiquitin ligase activity (Guicciardi *et al.*, 2011). Furthermore, the reduction in cIAP1/2 results in the activation of the NFκB signalling pathway, resulting in the production of pro-inflammatory cytokines such as TNFα and interferon-γ, both of which have anti-tumour properties (Zarnegar *et al.*, 2008).

Embelin is a unique Smac mimetic in that it is a naturally occurring, non-peptidic compound. It effectively neutralises XIAP by binding to its BIR3 domain (Nikolovska-Coleska *et al.*, 2004). It was discovered via computational structure-based screening of a library of Japanese traditional herbal medicines. Nikolovska-Coleska *et al.* showed that it binds XIAP with an affinity similar to that of endogenous Smac and to crucial residues within the BIR3 domain that Smac and caspase 9 bind to. Embelin promotes apoptosis by relieving caspase 9 inhibition and inhibiting NFκB signalling (Park *et al.*, 2013). Indeed, it has been shown to be effective in the treatment of many cancers such as glioma, where it blocks cell proliferation and induces apoptosis via inhibition of NFκB, and also prostate

cancer, where it was shown to increase mitochondrial apoptosis and reduce Akt and β-catenin signalling (Park *et al*., 2013, Park *et al*., 2015).

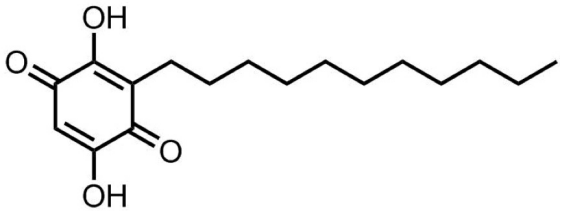

Figure 4. *The chemical structure of Embelin. Embelin is an alkyl substituted hydroxyl benzoquinone. The alkyl chain is crucial for XIAP binding; truncation to an ethyl group abolishes affinity. Taken from Nikolovska-Coleska et al. (2004).*

Combination Therapies

Due to the heterogeneous nature of tumours and their ability to rapidly develop resistance to treatment, IAP inhibitors and antagonists often have low efficacy when used as single agents and therefore research is now focused on combination therapies. These combination therapies make use of a wide range of cytotoxic stimuli, such as chemotherapy, radiation, DR agonists, signal transduction modulators and immune stimuli (Fulda, 2015). These have been shown to treat a wide variety of solid and haematological cancers (Table 1).

Of these stimuli, TRAIL has shown exceptional potential as a combination treatment for IAP inhibitors. TRAIL is a pro-apoptotic cytokine expressed by many tissues and immune cells (Wiley *et al*., 1995). Unlike many of the other stimuli, TRAIL exhibits cytotoxic selectivity towards cancer cells whilst sparing healthy cells (Walczak *et al*., 1999).

Table 1. *Combination Smac mimetic therapy in the treatment of cancer. Various Smac mimetics have shown potent anti-tumour activity when used in combination with treatments such as chemotherapy and radiotherapy. This has been shown in vitro and in vivo, in solid as well as haematological malignancies. CLL; Chronic lymphocytic leukemia, ALL; acute lymphoblastic leukemia, AML; acute myeloid leukemia.*

Combination Treatment	Smac Mimetic	Stimulus	Cancer Type	Reference
Chemotherapy	JP1400	Cisplatin	Lung	(Probst *et al.*, 2010)
	BV6	Glucocorticoids/ Cytarabine	ALL	(Belz *et al.*, 2014, Chromik *et al.*, 2014)
Death Receptor Agonists	Compound 3	TNF α	Solid tumours	(Wang *et al.*, 2008)
	IDN	TRAIL	Pancreatic carcinoma/ CLL/ ALL	(Vogler *et al.*, 2009, Stadel *et al.*, 2010, Loeder *et al.*, 2009, Fakler *et al.*, 2009)
Radiation	IDN	γ irradiation	Pancreatic carcinoma	(Giagkousiklidis *et al.*, 2007)
	BV6/ LBW242/ IDN	γ irradiation	Glioblastoma	(Berger *et al.*, 2011, Ziegler *et al.*, 2011, Vellanki *et al.*, 2009)
Signal transduction inhibitor	LBW242	Imatinib	Glioblastoma	(Ziegler *et al.*, 2008)
	BV6/ Birinapant	5-Aza, DAC	AML	(Steinhart *et al.*, 2013, Carter *et al.*, 2014)
Immune Stimuli	LCL161	Oncolytic virus/ poly(I:C)/ CpG oligonucleotides	Solid tumours	(Beug *et al.*)
	BV6	IFN α	AML	(Bake *et al.*, 2014)

The key feature of TRAIL cell signalling is the involvement of four diverse transmembrane receptors that each bind the cytokine. Two of them, TRAIL-R1 (DR4) and TRAIL-R2 (DR5/ Apo2L), transmit pro-apoptotic signals into the cell. Binding of TRAIL, which naturally occurs as a trimer, to one of these induces receptor trimerisation, recruitment of FADD, formation of the DISC and ultimately apoptosis (Kischkel *et al*., 2000). The two other receptors, TRAIL-R3 (decoy receptor 1 (DcR1)) and TRAIL-R4 (DcR2), lack functional intracellular death domains and therefore do not transmit pro-apoptotic signals (Sheridan *et al*., 1997). DcR1 is a glycosyl-phosphatidyl-inositol-anchored receptor lacking any intracellular domain. DcR2 contains a truncated, non-functional death domain which has been reported to form inactive hetero-complexes with DR5, and trigger cell survival signalling pathways such as NFκB and PKB/Akt (Degli-Esposti *et al*., 1997, Lalaoui *et al*., 2011).

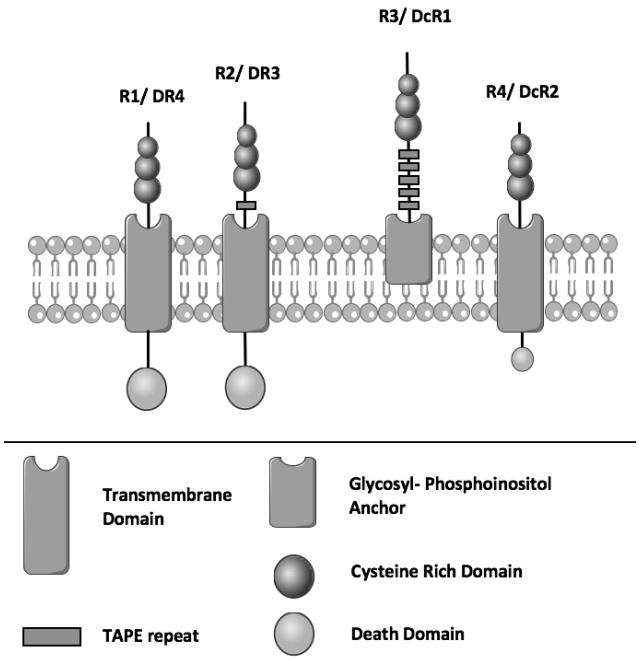

Figure 5. *The TRAIL death and decoy receptors. TRAIL has two death receptors, DR4 and DR5, which are capable of transmitting pro-apoptotic signals. Adapted from Lemke et al. (2014).*

Indeed, it is the preferential expression of decoy receptors in healthy cells and pro-apoptotic receptors in cancer cells that makes TRAIL signalling selective. This unique feature results in less toxic side effects in patients (Kruyt, 2008). Since this profound discovery, TRAIL receptor agonists and human recombinant TRAIL have been developed. Numerous Smac mimetics have exhibited promising broad clinical activity when combined with TRAIL *in vitro* and *in vivo* (Fulda *et al*., 2002, Li *et al*., 2004). The Smac mimetic Birinapant in combination with the TRAIL DR5 agonist Conatumumab has recently successfully completed its Phase 1 trial (clinicaltrials.gov).

Conclusion

IAPs, which are recurrently found upregulated in cancer, not only control cell death but also influence signal transduction pathways and progression of the cell cycle. With the recognition of evasion of apoptosis as a fundamental hallmark of cancer, the targeting of IAP proteins is now acknowledged as an incredibly promising and novel treatment strategy. Indeed, many aspect of IAP molecular biology and their contribution to cancer remains to be discovered and so they are being heavily researched. Future work must focus on implementing protocols to determine what patients will benefit the most from this novel modality of cancer therapy, in particular patients with tumour profiles specifically overexpressing IAPs. In addition, further trials need to be conducted in order to elucidate the combinations of therapies that maximise efficacy for specific patient cohorts.

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